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Optogenetic dissection of the dopaminergic circuitry involved in memory consolidation

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Abstract

The 'synaptic tagging-and-capture' (STC) theory of cellular memory consolidation holds that memory persistence can be altered by prior or subsequent patterns of neural activity (Redondo & Morris 2011). The aim of this thesis was to develop a realistic model of everyday memory for mice and use the optogenetic toolbox to investigate the neuromodulatory circuitry that modulates persistence of everyday spatial memories.

The task involved learning a win-stay rule with the daily goal of finding the location of food in the event arena. Using the developed task, it was confirmed that unrelated novel experiences can facilitate the persistence of spatial memory in a manner sensitive to pharmacological blockade of hippocampal dopamine D_1/D_5 receptors. Further analysis focused on identifying the specific neuromodulatory systems that mediate this effect. An influential model called the 'hippocampus-VTA loop' (Lisman & Grace 2005) points to the critical role of dopaminergic neurons in the ventral tegmental area (VTA), but recent evidence also implicates locus coeruleus (LC) as a potential source of dopamine in the hippocampus (Smith & Greene 2012).

In order to identify the dopaminergic structure(s) that may mediate the novelty effect on memory persistence, single unit activity of optogenetically identified catecholaminergic (CAergic) neurons in mouse VTA and LC was recorded in a novelty exploration paradigm. Using tyrosine hydroxylase-Cre knock-in mice and a Cre-dependent adeno-associated viral vectors, CAergic neurons in VTA and LC were selectively tagged with channelrhodopsin-2 (ChR2). Conditional ChR2 expression made it possible to reliably identify CAergic neurons during unit recording sessions in freely moving animals. The main conclusion of the study is that that CAergic neurons in both VTA and LC selectively increase their firing rates in novel environments, relative to both a familiar environment and a home cage baseline. When normalised to their average baseline firing rates, LC neurons are more strongly activated by novelty than VTA neurons.

In the final experiment outlined in this thesis, another cohort of Th-Cre mice, in which ChR2 was expressed in CAergic neurons of both VTA and LC using a Cre-dependent adeno-associated virus, was trained on the everyday appetitive spatial memory task. ChR2-mediated photoactivation of CAergic neurons in LC but not in VTA 30 min after encoding, substituting for novelty, was successful in enhancing the persistence of memory. Paradoxically, the effect of optogenetic LC activation was blocked by hippocampal microinfusion of dopamine D_1/D_5 receptor antagonist but not β-adrenergic receptor antagonist.

Results of experiments described in this thesis support the principle of STC theory and collectively indicate that dopamine released from hippocampal terminals of LC neurons mediates the novelty effect on memory persistence. Importantly, they also point to a more general of role of LC in gating of entry to long-term memory.

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Declaration

I declare that this thesis was composed by myself, that the work contained herein is my own except where explicitly stated otherwise in the text, and that this work has not been submitted for any other degree or professional qualification except as specified.

Adrian Jacek Duszkiewicz

This thesis is dedicated to my mother,

Krystyna Duszkiewicz

For her endless love, support and encouragement.

Abbreviations

AAV	adeno-associated virus
CA1	*Cornu Ammonis* 1
CA3	*Cornu Ammonis* 3
CAergic	Catecholaminergic
ChR2	channelrhodopsin-2
DA	dopamine
Dbh	dopamine beta-hydroxylase
DAT	dopamine transporter
DG	dentate gyrus
DMP	delayed matching-to-place
eYFP	enhanced yellow fluorescent protein
GABA	gamma-aminobutyric acid
LC	locus coeruleus
L-DOPA	L-3,4-dihydroxyphenylalanine
LTD	long-term depression
LTP	long-term potentiation
NA	noradrenaline (norepinephrine)
NET	noradrenaline (norepinephrine) transporter
NMDA	N-methyl-D-aspartate

PI	performance index
PD	Parkinson's disease
PP	perforant path
PT	probe test
SEM	standard error of the mean
SNc	substantia nigra pars compacta
STC	synaptic tagging and capture
TH	tyrosine hydroxylase
VTA	ventral tegmental area

Table of Contents

Chapter 1: Overview **1**

Chapter 2: Introduction **5**

2.1 Memory and the hippocampus 5

2.1.1 Overview of hippocampal anatomy 5

2.1.2 Synaptic plasticity and memory 9

2.1.3 Synaptic tagging and capture theory 11

2.2 Dopaminergic circuitry **16**

1.2.1 Dopamine-releasing neurons in the brain 17

2.2.2 Connectivity of dopaminergic systems 19

2.2.3 Dopamine receptors 29

2.2.4 Dopamine and hippocampal plasticity 32

2.2.5 Dopamine co-release from hippocampal LC terminals 39

2.3 Dopaminergic control of learning and memory **41**

2.3.1 Cognitive consequences of dopamine deficiency 41

2.3.2 Information content of dopamine signals 42

2.3.3 Dopamine and hippocampal memory 47

2.3.4 Behavioural tagging 56

2.3.5 NeoHebbian view of hippocampal memory – a systems perspective 64

2.4 Optogenetic dissection of dopaminergic circuits **71**

2.4.1 Overview of the optogenetic toolbox 71

2.4.2 Functional relationships between dopamine neurons and cognition 72

2.4.3 Optogenetic identification of dopaminergic neurons *in-vivo* 74

2.5 Aims of this thesis **77**

Chapter 3: Novelty enhances persistence of everyday memory in mice 82

3.1 Introduction 82

3.2 Methods 85

3.2.1 Subjects 85

3.2.2 Behavioural apparatus 85

3.2.3 Training protocol 89

3.2.4 Data collection and analysis 93

3.2.5 Surgery 94

3.2.6 Microinfusions and drugs 95

3.2.7 Experimental design 95

3.2.8 Perfusion and histology 97

3.3 Results 98

3.3.1 Task acquisition 98

3.3.2 Stable performance over months of training 104

3.3.3 Chance performance in non-encoding trials 104

3.3.4 Persistence of memory depends on the number of encoding trials 107

3.3.5 Novelty exploration enhances memory persistence 110

3.3.6 The novelty effect requires activation of hippocampal D_1/D_5 receptors 113

3.3.7 Meta-analysis of the novelty effect on memory persistence 118

3.4 Discussion 121

3.4.1 Everyday appetitive spatial memory protocol for mice 121

3.4.2 Novelty promotes memory consolidation in the hippocampus 123

3.4.3 Relevance of the novelty effect to the STC theory 124

3.4.4 Alternative explanations of the novelty effect 125

3.4.5 Noradrenaline and memory persistence 126

3.4.6 Possible sources of dopamine released during novelty exploration 127

Chapter 4: Catecholaminergic neurons in mouse ventral tegmental area and locus coeruleus are activated by novelty **129**

4.1 Introduction **129**

4.2 Methods **131**

4.2.1 Subjects 131

4.2.2 Viral vectors 131

4.2.3 Optetrode microdrive 131

4.2.4 Surgery 135

4.2.5 Acute *in vivo* optrode recording 136

4.2.6 Behavioural apparatus 137

4.2.7 *In vivo* single unit recording and optogenetic stimulation in behaving animals 137

4.2.8 Novelty exploration procedure 140

4.2.9 Data analysis 141

4.2.10 Perfusion and histology 142

4.3 Results **143**

4.3.1 Acute optrode recordings in VTA and LC 143

4.3.2 Responses of VTA-DA neurons to novelty 143

4.3.3 Responses of LC-NA neurons to novelty 162

4.3.4.Comparison of novelty response magnitude in VTA-DA and LC-NA neurons 175

4.4 Technical challenges **178**

4.5 Discussion 180

4.5.1 Mouse VTA-DA neurons are modestly activated by novelty 180

4.5.2 Mouse LC-NA neurons are strongly activated by novelty 183

4.5.3 Light-induced spikes and light stimulation frequency 184

4.5.4 LC-NA neurons are likely mediators of the novelty effect 184

Chapter 5: Optogenetic activation of locus coeruleus enhances persistence of everyday memory **186**

5.1 Introduction **186**

5.2 Methods **188**

5.2.1 Subjects 188

5.2.2 Viral vectors 188

3.2.3 Behavioural apparatus 188

3.2.4 Training protocol 188

5.2.5 Surgery 188

5.2.6 In vivo optogenetic stimulation 190

5.2.7 Microinfusions and drugs 193

5.2.8 Experimental design 193

3.2.9 Data collection and analysis 195

3.2.10 Perfusion and histology 196

5.3 Results **197**

5.3.1 Task acquisition 197

5.3.2 Stable performance over months of training 200

5.3.3.The 'weak memory' condition 200

5.3.4 Optogenetic stimulation of LC-NA neurons enhances memory persistence 203

5.3.5 Effect of LC-NA stimulation depends on hippocampal D_1/D_5 receptors 206

5.3.6 Meta-analysis of the optogenetic LC-NA stimulation effect 211

5.4 Discussion **214**

3.4.1 Animal performance 214

5.4.2 Optogenetic stimulation of LC-NA neurons promotes memory consolidation 215

5.4.3 LC-NA neurons enhance memory persistence through D_1/D_5 receptors 216

5.4.4 Non-specific effect of light delivery into VTA 218

5.4.5 Technical considerations 219

Chapter 6: Conclusions and General Discussion 221

Future directions 223

Bibliography 225

Chapter 1

Overview

The 'seven sins' of memory, as described by Daniel Schacter (Schacter 2001) are the attributes of human memory that are treated by many as 'design flaws' of the memory system. Indeed, some of the 'sins' included on the list, like suggestibility, are inherently detrimental. However, two of them, **transience** and **persistence**, stand out as manifestations of the two important features of an efficient memory system with a limited storage capacity. What is more, transient and persistent memories influence each other on a neurobiological level, and this thesis aims to explore the biological mechanisms behind this interplay.

The sins of transience and persistence

Most of the experiences in our daily lives, like the food we ate for lunch or the online article we skimmed through on our lunch break, although remembered for a short time, cannot be recalled in great detail even the following day. This apparent transience of everyday memories is not necessarily a sign of the shortcomings of neural mnemonic circuitry. It is likely that memories of these automatically encoded everyday experiences become largely expendable as the time passes and therefore are discarded in order to prevent saturation of the memory circuits.

On the other hand, memories of some unexpected, novel and/or emotionally stimulating events sometimes persist in memory for as long as a life-time in an exceptionally vivid and detailed form. This is illustrated by the phenomenon of a 'flashbulb memory', whereby a dramatic event (for example, hearing the news about the 9/11 attacks) results in a long-term memory of not only that very moment, but also some of the apparently irrelevant events that surrounded it (Brown & Kulik 1977). This interplay between memories of events encoded at different times is due to the *late-associativity* property of memory. Unexpected events like the ones that trigger flashbulb

memories create an aura of enhanced memory consolidation which extends not only forwards but also backwards in time, boosting retention of memories that would normally be quickly forgotten. One can imagine that this is part of the reason why information that is seemingly unimportant (at the time) is stored in the brain for a certain 'grace period'.

Flashbulb memories in rodents

In order to understand the neural mechanisms that behind late-associativity of memory traces, Wang and colleagues (Wang et al 2010) used a behavioural paradigm for rats that provides the background of everyday experience upon which the impact of novel and unexpected events can be tested. This paradigm, based on earlier rat studies (Bast et al 2005), provides rats with a daily opportunity to incidentally encode new spatial information (location of food reward), and can be continued for many months with the same cohort of animals. The transient spatial memories formed by rats during training are undetectable after 24 hours, paralleling everyday memory in humans.

On rare occasions, rats were placed in an open field with objects they had never encountered before, like mesh wire or cotton pads, shortly after but separately from the encoding session. When tested after 24 hours, rats did not only remember the novel event itself, but also the location of food reward they visited before novelty exploration. Importantly, when dopamine D_1/D_5 receptors in the hippocampus were pharmacologically blocked while rats explored the novel box, novelty failed to boost memory retention. This implies that dopamine, released in the hippocampus in response to novel and/or unexpected events, creates a flashbulb memory-like aura. In order to better understand the neural circuitry behind this phenomenon, it is imperative to zero in on where this dopamine signal comes from.

A systems perspective on cellular consolidation

The grand aim of this thesis is to pinpoint the source of the dopaminergic consolidation signal released in the hippocampus during novelty exploration. An influential theory of cellular memory consolidation called the 'hippocampal-VTA loop' (Lisman et al 2011, Lisman & Grace 2005), suggests that dopaminergic neurons in the ventral tegmental area (VTA), are the anatomical substrate that controls hippocampal cellular consolidation at the systems level. While this is certainly possible, a recent study in the hippocampal slice concluded that the bulk of hippocampal dopamine does not come from VTA, but is instead co-released with noradrenaline from the abundant hippocampal projections of locus coeruleus (LC) neurons (Smith & Greene 2012). The

role of these two nuclei in novelty-induced enhancement of memory persistence is explored in the experiments to be described.

Lighting up the dopamine circuits

The optogenetic toolbox is ideally suited for investigation of the brain neuromodulatory systems. Neuromodulatory neurons are usually contained in small brain nuclei and are genetically distinct due to the expression of genes involved in the synthesis of the neuromodulator. Excitatory and inhibitory opsins can be selectively expressed in these neurons using the Cre/lox technology of conditional gene expression and localised injections of viral vectors, and their activity can be controlled in a behavioural setting via light delivery through implanted optic fibres. This makes optogenetics an ideal tool to achieve the main aim of this study. If dopamine-releasing neurons in VTA or LC (henceforth referred to as VTA-DA and LC-NA neurons, respectively) indeed mediate the novelty effect, then activating these neurons in place of novelty should produce a memory-boosting effect that would also be dependent on hippocampal D_1/D_5 receptors. A test of this prediction is described in the final experimental chapter of this thesis (chapter 5).

This 'mimicry' experiment, if successful, would indicate that activated VTA-DA and/or LC-NA neurons are *capable* of enhancing memory through hippocampal dopamine release, but would not on its own constitute a proof that they do so during novelty exploration. Therefore, it is crucial to examine their activity patterns during novelty exploration and see whether dopamine-releasing neurons in either of these nuclei are activated in such circumstances. Single unit recordings of a particular neuronal subtype are challenging, the reason being that the electrophysiological criteria for identification of a particular neuron type are often unclear (Ungless & Grace 2012). However, optogenetic technology enables identification of opsin-expressing neurons during electrophysiological recordings based on their responses to light delivery. This technique, applied recently in a study of VTA neurons (Cohen et al 2012), was employed in the experiment described in chapter 4 that aims to investigate the activity of VTA-DA and LC-NA neurons during exploration of novel environments.

The experiments that guided the aims of this thesis were performed in rats. However, due to the need for conditional opsin expression in a particular set of neurons, it was necessary to conduct the present experiments in the mouse. Tyrosine hydroxylase (TH) is an enzyme expressed in dopaminergic and noradrenergic neurons, and the Th-Cre mouse line is ideal for comparative studies of VTA-DA and LC-NA neurons. This, in turn, necessitated creation of an everyday

spatial memory paradigm for mice. The paradigm, as well as demonstration of the novelty effect on memory persistence in this species, is described in chapter 3.

The experiments in these thesis, outlined above in reverse order for the sake of clarity of the rationale behind them, shed more light on the relative contribution of VTA-DA and LC-NA neurons to the phenomenon of novelty-induced memory enhancement. The following review chapter explores the structure of dopaminergic circuitry in the central nervous system and highlights the pivotal role of dopamine in hippocampal plasticity and memory.

Chapter 2

Introduction

2.1 Memory and the hippocampus

Memory processes have been traditionally classified in terms of dichotomies (Figure 2.1 a). In the temporal dimension, the line is often drawn between transient short-term memory and durable long-term memory. Lasting memories can either be consciously recalled (declarative memory) or retrieved without conscious awareness (non-declarative or procedural memory). Declarative memory is a concept that includes general knowledge in the form of semantic memory, but also, notably, the autobiographical narrative of one's life events, or episodes, with all the surrounding contextual details. Scientific debate regarding this fascinating form of 'mental time travel' revolves around the brain area called the hippocampus.

2.1.1 Overview of hippocampal anatomy

The hippocampal formation, situated deep within the medial temporal lobe, consists of the dentate gyrus (DG), the CA1, CA2 and CA3 fields of the hippocampus proper, as well as the subiculum (figure 2.1 b). Although in primates its size is overshadowed by the expanded neocortex, it rodents it stands out as one of the most prominent and recognisable structures in the central nervous system. Transverse section through the hippocampus exposes its highly ordered structure with narrow and sharply defined principal cell layers that contrast with the morphology of the overlaying cortex. Below and above the cell layers are lamina populated mostly by interneurons and dense neuropil.

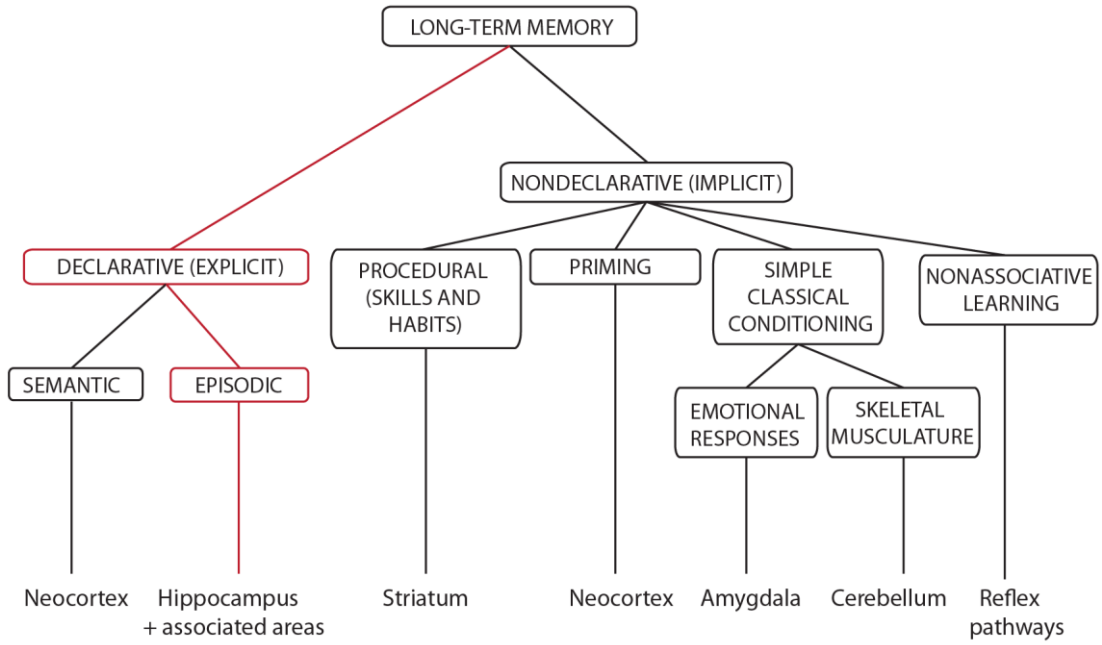
Figure 2.1: Taxonomy of the long-term memory systems and hippocampal anatomy.

(a) Episodic memory is a declarative memory system. The hippocampal formation is central to the processing of episodic memories.

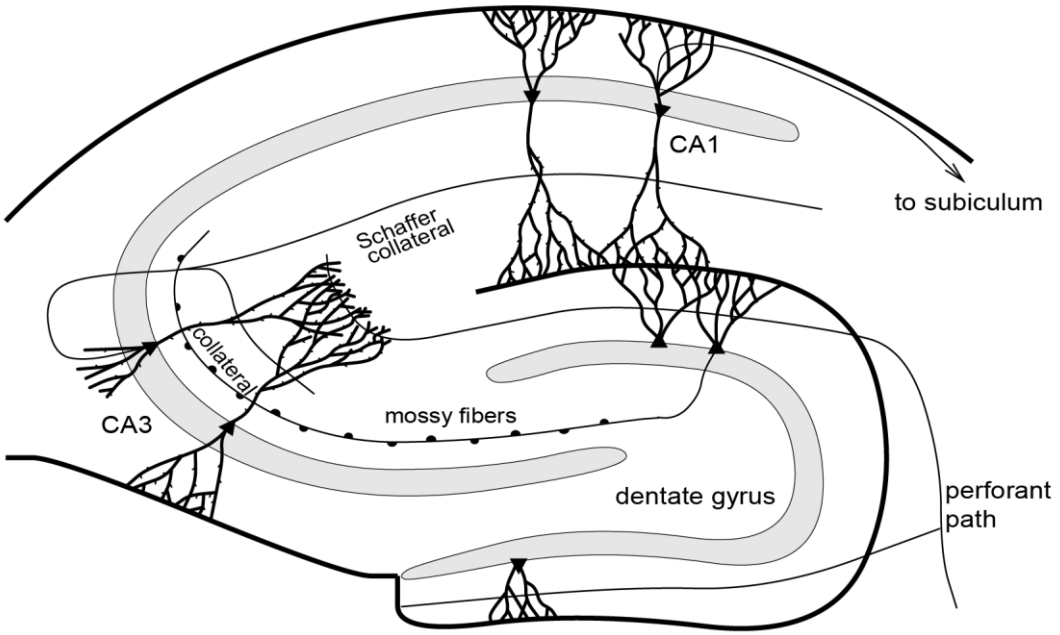
(b) The canonical tri-synaptic circuit in the hippocampus.

Figure (a) adapted from Squire & Zola 1996; figure (b) adapted from Schultz et al 1998.

a) Taxonomy of long-term memory systems



b) Anatomy of the hippocampal formation



Hippocampal excitatory connectivity is almost exclusively unidirectional. The main excitatory input to the hippocampus comes from the superficial layers of the entorhinal cortex. Entorhinal axons enter the hippocampus via a prominent fibre tract called the performant path and then synapse onto the dendrites of DG granule cells and to a smaller extent onto CA3 pyramidal cells. Granule cells project to the CA3 pyramidal cells directly via mossy fibres. Pyramidal cells in CA3 are unique in terms of their strong recurrent connectivity and form a dense auto-associative network. In addition, CA3 pyramidal neurons send extensive axon collaterals (Schaffer collaterals) as well as commissural fibres to the adjacent area CA1. CA1 pyramidal neurons, in contrast to CA3 cells, do not form associational connections and instead project to the subiculum and back to the deep layers of the entorhinal cortex.

A detailed description of the hippocampal anatomy as well as its intrinsic and extrinsic connectivity can be found in *The Hippocampus Book* (Andersen et al 2007).

Early clues to the hippocampal function

The conceptual connection between hippocampus and episodic memory processes is supported by over half a century of neurobiological research that began with the study of one of the most famous neurological patients, HM (Scoville & Milner 1957). In a desperate effort to reduce his uncontrollable epileptic seizures, HM underwent bilateral removal of the hippocampus and associated medial temporal lobe structures. The surgery left his personality and intelligence unchanged, but hippocampal excisions unexpectedly produced severe anterograde amnesia, accurately illustrated by the quote from the original research article by Scoville and Milner:

> "Ten months ago the family moved from their old house to the new one a few blocks away on the same street; he still has not learned the new address, though remembering the old one perfectly, nor can he be trusted to find his way home alone (…). This patient has even eaten louncheon in front of one of us (B. M.) without being able to name, a mere half-hour later, a single item of food he had eaten; in fact, he could not remember having eaten louncheon at all. Yet to a casual observer this man seems like a relatively normal individual, since his understanding and reasoning are undiminished." (Scoville and Milner, 1957)

Although HM's remote autobiographical memory was largely intact, his ability to form new episodic memories was severely impaired. He could, however, acquire new procedural skills, although he had no recollection of having learned any of them (Eichenbaum 2013). Based on this

landmark discovery, the hippocampus was hypothesised to be selectively involved in processing of autobiographiacal (episodic) memory.

Hippocampus as a cognitive map

The pivotal role of the hippocampus in various forms of declarative memory is supported by animal lesion studies as well as experiments that utilised reversible pharmacological inactivation techniques. For example, bilateral hippocampal lesions impair spatial learning in the Morris water navigation task (the 'watermaze') (Morris et al 1982), as does chronic inhibition of hippocampal excitatory transmission (Riedel et al 1999). What is more, hippocampal network activity is necessary for encoding of one-trial memories in tasks designed to probe the animal equivalent of human episodic memory (Bast et al 2005, Day et al 2003, Steele & Morris 1999).

These studies, supported by the discovery and subsequent detailed characterisation of hippocampal 'place cells' and their characteristic spatially-tuned activity patterns (O'Keefe & Dostrovsky 1971), reinforced the notion of the hippocampus as the module that forms high-level contextual representations of the environment and automatically encodes animal's attended experience in the form of episodic-like memories (for reviews, see Battaglia et al 2011, Buzsaki & Moser 2013, McNaughton & Morris 1987, O'Keefe & Nadel 1978, Wang & Morris 2010).

2.1.2 Synaptic plasticity and memory

But what exactly is a 'memory'? The question of how the brain stores information dates back to Santiago Ramón y Cajal, who pondered the significance of specific neural connectivity patterns in the brain and recognised the existence and importance of the synapse (Jones 1994). The idea that synaptic connections are modifiable by learning was first proposed by the visionary theoretical work of a Polish neurophysiologist Jerzy Konorski (Konorski 1948) and refined on the other side of the Iron Curtain by a Canadian psychologist Donald Hebb (Hebb 1949). The Hebb-Konorski postulate can be summarised in a colloquial slogan "*cells that fire together wire together*", and was supported a few decades later by evidence from the habituation and sensitisation studies in the mollusc *Aplysia* (Kandel 1978), as well as by the ground-breaking discovery of long-term potentiation (LTP) of synaptic transmission in the mammalian hippocampus by Bliss and Lømo (Bliss & Lømo 1973).

Synaptic plasticity and memory hypothesis

Importantly, duration of LTP observed by Bliss and Lømo at the synapses made by neurons the entorhinal cortex onto granule cells in the dentate gyrus was long enough for the authors to ponder

whether they might have indeed observed the mechanism that is used to encode information in the brain (Bliss & Lømo 1973). Since then, artificially-induced LTP (along with its mirror phenomenon, long-term depression, or LTD) has become a universally used paradigm for probing the molecular and cellular mechanisms of learning and memory (Bliss & Collingridge 1993, Malenka & Bear 2004). Indeed, there is strong evidence that LTP and LTD-like mechanisms are the neural substrates of not only simple stimulus-response associations but also complex associative and episodic-like representations (Martin et al 2000, Martin & Morris 2002, Takeuchi et al 2014). Induction of LTP in the hippocampus requires activation of glutamatergic N-methyl-D-aspartate (NMDA) receptors (Collingridge et al 1983), which parallels the requirement for hippocampal NMDA receptor activation in encoding of spatial and episodic-like memories (Bast et al 2005, Day et al 2003, Morris et al 1986, Steele & Morris 1999) Moreover, LTP at CA3-CA1 synapses has been observed in behaving animals following learning (Whitlock et al 2006), further indicating that long-lasting changes in synaptic strength are indeed the mechanism by which hippocampal memories are encoded.

Mechanisms of memory consolidation

Initial studies of memory formation and storage confirmed existence of transformation processes that convert recently encoded memories into a durable and long-lasting form. The mechanisms that govern consolidation of memory traces can be conceptualized on two levels of analysis: the level of individual neurons and synapses and the brain-wide systems level (Dudai 2004, Dudai 2012, Dudai & Morris 2000, Wang & Morris 2010).

Cellular (or synaptic) consolidation refers to the post-encoding intracellular processes that proceed in the time frame of minutes to hours and convert synaptic plasticity into its more long-lasting form. They do so through activation of signalling cascades that in turn initiate changes in gene expression. This process is reflected in the dynamics of LTP, which can be divided into its early and late phases (E-LTP and L-LTP, respectively) based on selective sensitivity of L-LTP to, for example, protein synthesis inhibitors (Frey et al 1988, Kandel 2001). Systems consolidation, on the other hand, refers to the process of reorganisation of long-term memory over distributed brain circuits. For hippocampal memories, this involves hippocampal-neocortical interactions that result in a once hippocampal-dependent memory trace becoming fully stored in the neocortex. The process often lasts days to years (depending on the species and task), but can occur very rapidly in presence of a previously acquired associative framework of information (a 'schema') that is congruent with information being encoded (Tse et al 2007, Wang & Morris 2010).

Although widely used, the conceptual division between fast intracellular synaptic consolidation and considerably slower network-level systems consolidation is arguably somewhat misleading. First of all, systems consolidation of hippocampal memory traces into pre-existing neocortical schemas requires NMDA receptor dependent plasticity in the medial prefrontal cortex (Tse et al 2011), which highlights the importance of intracellular processes in systems-level interactions. Second, induction of LTP at hippocampal synapses leads to a reorganisation of neocortical networks, as evidenced by functional magnetic resonance imaging (fMRI) in rats (Alvarez-Salvado et al 2014, Canals et al 2009), pointing to possible systems-level consequences of intrahippocampal plasticity. Finally, cellular consolidation in the hippocampus is gated by neuromodulatory inputs, which are thought to originate from catecholaminergic nuclei in midbrain and/or brainstem (Lisman et al 2011). Cellular consolidation in the hippocampus therefore relies on the action of systems-level mechanisms.

It is thus evident that 'cellular' and 'systems' consolidation processes both have intracellular and systems components.

2.1.3 Synaptic tagging and capture theory

This thesis focuses on the mechanisms that mediate the initial process of cellular memory consolidation in the hippocampus, taking place in the timeframe of several hours after memory encoding, which is consistent with the time course of L-LTP. Although experiments included in this thesis do not directly probe the mechanisms of synaptic plasticity, the rationale behind them stems from the phenomena initially observed in the hippocampal slice. Importantly, persistence of hippocampal LTP has been shown to depend on recent history of neural activity as well as immediate future activity. This feature of LTP was first observed in a set of *in vitro* electrophysiological experiments by Frey and Morris (Frey & Morris 1997, Frey & Morris 1998b) and was formalised as the Synaptic Tagging and Capture (STC) theory of memory consolidation (Frey & Morris 1998a, Redondo & Morris 2011).

Late-associativity of hippocampal LTP

Pyramidal cells in CA3 synapse onto CA1 pyramidal neurons ipsilaterally via axonal projections called the Schaffer collaterals and contralaterally via the commissural fibre tracts. Their connectivity is largely preserved in the transverse hippocampal slice, so that stimulation of CA3 axons reliably induces field excitatory post-synaptic potentials (fEPSPs) in CA1 stratum radiatum, where Schaffer collateral/commissural axons synapse onto the dendrites of CA1 pyramidal cells.

Figure 2.2: Mechanisms of synaptic tagging and capture.

(a) Weak tetanisation of a set of CA3 - CA1 synapses induces synaptic potentiation as well as formation of a synapse-specific tag. In absence of PRPs synaptic potentiation decays to baseline and the tag is reset.

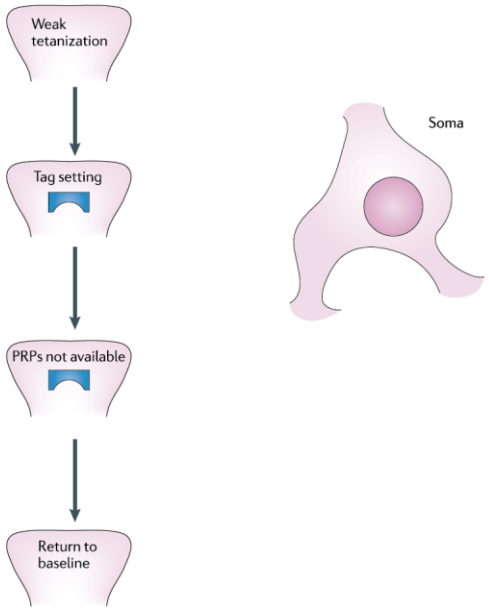
(b) Strong tetanisation, in addition to tag setting and synaptic potentiation, initiates synthesis of PRPs in the soma. PRPs are then distributed throughout the neuron and captured by tagged synapses, resulting in persistent L-LTP at the strongly tetanised input.

(c) PRPs synthesised in the soma in response to strong tetanus are captured by all synapses expressing synaptic tags, including weakly tetanised synapses onto the same neuron.

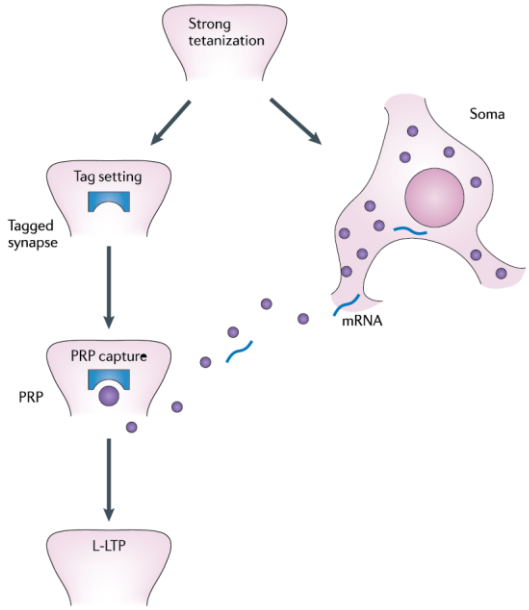
(d) Late-associativity of synaptic inputs is determined by longevity of the synaptic tag as well as availability of PRPs. In the diagram, only PRP-inducing events marked 1, 2 and 3 would result in L-LTP at the tagged synapse. Note that event number 2 occurs before the synaptic tag is set.

Figures (a), (b) and (c) adapted from Redondo & Morris 2011; figure (d) adapted from Reymann & Frey 2007.

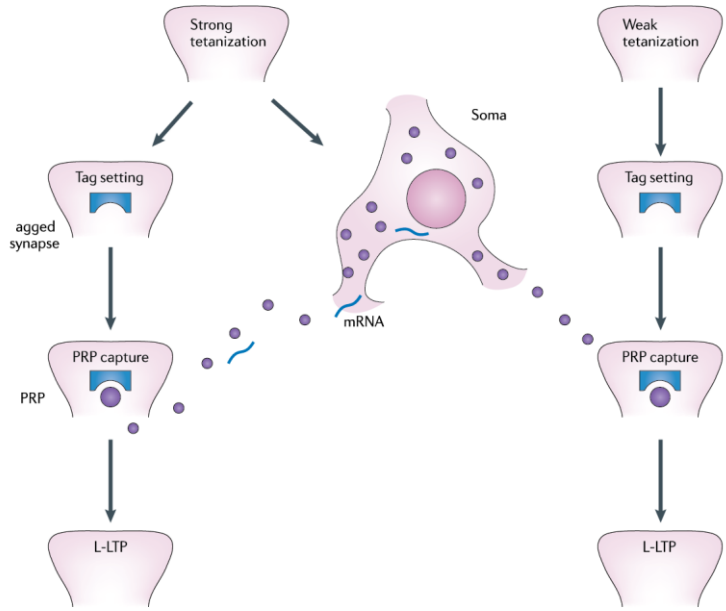
a) Weak tetanization induces E-LTP



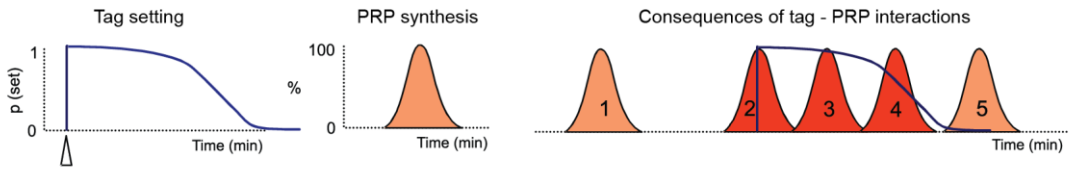
b) Strong tetanization induces L-LTP



c) 'Strong before weak' or 'weak before strong' induces L-LTP at both inputs



d) Heterosynaptic requirements for L-LTP



The geometry and sheer size of the CA3 to CA1 projection enables stimulation of independent sets of CA3 pathways onto the same population of CA1 cells and thus investigation of cross-synaptic interactions resulting from synaptic potentiation. Administration of a single burst of high frequency tetanic stimulation (weak tetanus) to CA3 axons reliably induces NMDA receptor-dependent E-LTP at CA3 - CA1 synapses that decays baseline several hours after tetanisation (figure 2.2a). In contrast, repeated tetanic stimulation (strong tetanus) of CA3 - CA1 projection leads to a long lasting and protein synthesis-dependent L-LTP that does not show signs of decay for the duration of the experiment (figure 2.2b). Frey and Morris (Frey & Morris 1997, Frey & Morris 1998b) were the first to observe that when LTP induced by weak tetanus is preceded by a strong, L-LTP-inducing tetanus delivered to the independent but convergent set of CA3 - CA1 inputs, both pathways exhibit robust L-LTP (figure 2.2c). This late-associativity of LTP, demonstrated by what later came to be known as 'weak-before-strong' paradigm, can only happen if the two pathways are tetanised within a critical time window (< 2.5 h) and does not occur if strong tetanisation is delivered in presence of a protein synthesis inhibitor anisomycin or in presence of a dopamine D_1/D_5 receptor blocker SCH23390 (Sajikumar & Frey 2004). Importantly, late-associativity is symmetrical, i.e. it is independent of the temporal order of strong and weak stimulations.

The STC interpretation of this phenomenon is that E-LTP induction with a weak tetanus leads to setting of a synaptic 'tag' that selectively marks recently potentiated synapses (fig 2.2d, left). On the other hand, induction of L-LTP, in addition to tag setting, induces *de novo* synthesis of plasticity-related proteins (PRPs)(figure 2.2, middle). These PRPs are distributed along the dendritic tree and 'captured' by tagged synapses, resulting in synapse-specific cellular consolidation (figure 2.2d, right) (Frey & Morris 1997, Redondo & Morris 2011). Importantly, this interpretation has significant consequences for the mechanisms of memory formation. If episodic-like memories are encoded through synaptic changes equivalent to LTP, then hippocampal memory should show the same kind of late-associativity as demonstrated in the *in vitro* STC experiments. In other words, an event that leads to PRP upregulation in the hippocampus should enhance persistence of unrelated transient episodic memories encoded within a window of opportunity defined by the availability of PRPs and longevity of the synaptic tags set during memory encoding.

Dopamine and late-associativity

But how are the PRPs induced? In another set of 'weak before strong' experiments conducted in hippocampal slice, bath application of a dopamine receptor blocker during strong tetanisation

mimicked the effect of anisomycin, not only preventing L-LTP at the strongly tetanized synapses, but also blocking the rescue of the E-LTP at the weakly tetanized pathway (Sajikumar & Frey 2004, Wang et al 2010). Therefore, it is conceivable that strong tetanisation promotes dopamine release from neuromodulatory afferents to the hippocampus, which in turn initiates PRP synthesis in CA1 pyramidal cells through activation of dopamine receptors. What is still unclear, however, is the identity and origin of these neuromodulatory fibres, as well as possible implications of their activation for late-associativity of hippocampal memory. In the following section I will review the dopaminergic circuitry that might supply dopamine to the hippocampus, as well as expand on the role that dopaminergic modulation plays in different forms of hippocampal plasticity.

2.2 Dopaminergic circuitry

Dopamine, the fourth major monoamine neuromodulator discovered (after 5-HT, adrenaline and noradrenaline), was first found in the brain by Kathleen Montagu (Montagu 1957) while investigating seasonal variations in concentrations of adrenaline (also called epinephrine) and noradrenaline (also called norepinephrine), two other catecholamines that share the same biosynthesis pathway, in rat tissues. Dopamine is synthesised from its precursor L-3,4-dihydroxyphenylalanine (L-DOPA) by the enzyme tyrosine hydroxylase (TH). The biosynthesis pathway stops here in dopaminergic cells, but in noradrenergic neurons dopamine is further hydroxylated to noradrenaline by the enzyme dopamine β-hydroxylase (Dbh) present in synaptic vesicles.

Dopamine was first thought to be only a precursor to noradrenaline, but the discovery of markedly different distributions of these two catecholamines within central nervous system by Avrid Carlsson, including an abundance of dopamine despite the absence of noradrenaline in the striatum, pointed to an active role of dopamine beyond that of merely a noradrenaline precursor (Carlsson 1959). Further evidence for the active functional role of dopamine emerged during investigation of peripheral effects of the antipsychotic drug reserpine known to induce rapid catecholamine depletion as well as Parkinson's Disease (PD)-like motor impairment (Utley & Carlsson 1965). These PD-like symptoms could be reversed by L-DOPA administration – an observation which led to an incredible breakthrough in treatment of PD in humans (Fehling 1966). PD patients administered with L-DOPA showed remarkable 'awakenings', with all of their motor impairments temporarily alleviated for a short time. Only later was it confirmed that PD symptoms are a direct consequence of a progressive degeneration of dopaminergic neurons and that L-DOPA administration provides a short-acting boost in dopamine synthesis that in turn leads to transient reversal of PD symptoms. At that time the concept of ubiquitous chemical transmission in the brain was still far from being universally accepted (Carlsson 2001), but Carlsson and colleagues found that L-DOPA treatment resulted in a dramatic elevation of dopamine levels in the basal ganglia without an appreciable rise in noradrenaline concentration in the same region, pointing to dopamine as an important neurotransmitter in its own right (Bertler & Rosengren 1959, Carlsson 1987).

Avril Carlsson was awarded the Nobel Prize in Physiology or Medicine for identification of dopamine as a major neurotransmitter in the central nervous system. Since the initial demonstration of dopamine as a critical neurotransmitter in the extrapyramidal motor circuit, the

focus of dopamine research shifted to its role in many cognitive processes, including reward processing, motivation, addiction and memory.

1.2.1 Dopamine-releasing neurons in the brain

Catecholamine-containing neurons in the mammalian brain were first observed by Carlsson's group (Carlsson et al 1962). Using the very laborious technique of histofluorescence, Dahlström and Fluxe then went on to describe midbrain and brainstem nuclei abundant in monoamine-containing neurons. They also observed high density of monoamine-containing axonal terminals in the forebrain and hypothalamus, as well more sparse axonal projections in the brainstem (Dahlstroem & Fuxe 1964, Fuxe 1965). Of these monoamine nuclei, originally labelled as A1 – A16 by Dahlström and Fluxe, areas A1 to A7 are known to contain noradrenergic, and areas A9 – A17 dopaminergic cells (Bjorklund & Dunnett 2007).

Thanks to various novel approaches in neuroanatomy, such as advanced immunohistochemistry and viral vector-based tract tracing, we now have a much more comprehensive view of the complex dopaminergic circuitry in the mammalian brain. Dopaminergic neurons in mammals are mainly concentrated in the ventral midbrain, although there are sizable populations of spinal cord-projecting dopamine neurons in the hypothalamus (Skagerberg & Lindvall 1985), dopaminergic interneurons in the olfactory bulb (Betarbet et al 1996), as well as, perhaps the most surprisingly, a dopaminergic sub-population of amacrine cells in the retina (Witkovsky 2004). In the midbrain, dopamine cells are grouped in three major nuclei: retrorubral field (RRF, A8), substantia nigra pars compacta (SNc, A9) and the ventral tegmental area (VTA, A10). In addition, recent evidence suggests that neurons in the locus coeruleus (LC, A7), the main source of noradrenaline in the brain, co-release dopamine, a direct precursor of noradrenaline, from their hippocampal terminals (Smith & Greene 2012). Due to the possibility of dopamine co-release from LC, as well as the important role of this nucleus in many cognitive processes, its anatomy and physiology will be reviewed alongside the aforementioned 'textbook' dopaminergic nuclei in the midbrain.

Ventral tegmental area

VTA, located in the ventral tegmentum, is the largest dopaminergic nucleus in the brain, containing 12 000 to 18 000 dopaminergic neurons in the mouse (figure 2.3a) (Zaborszky & Vadasz 2001). Although, overall, majority of the cells in VTA (approximately 60 – 75%) are positive for TH and thus considered dopaminergic (Margolis et al 2012, Nair-Roberts et al 2008), they are mixed with a sizable proportion of GABAergic neurons (20 – 35%) and a recently discovered small population of glutamatergic cells (~5%) (Kawano et al 2006, Yamaguchi et al

2007). Very little overlap exists between the three neural populations, with only 0.58% of cells in VTA expressing both dopaminergic and GABAergic markers, and 2% and 0.2% of glutamatergic cells co-expressing GABAergic markers and dopaminergic markers respectively (Nair-Roberts et al 2008). These different neuronal populations form local circuits: dopaminergic neurons in the VTA express $GABA_A$ and $GABA_B$ receptors (Ciccarelli et al 2012), and GABAergic VTA neurons are known to directly inhibit the firing of dopaminergic cells (Tan et al 2012).

VTA can be further divided into four sub-nuclei: parainterfasicular nucleus (PIF) located caudally, paranigral nucleus (PN) located medially, parabrachial pigmental area (PBP) located laterally and the rostral VTA (VTAR), located at the anterior end. Interestingly, these four nuclei contain varying ratios of dopaminergic to GABAergic cells, with IFN being almost exclusively dopaminergic (ratio 7.5), whereas at the other end of the spectrum, PIF, having an almost equal proportion of the two neural populations (ratio 1.3) (Nair-Roberts et al 2008). Additionally, a recently described area rostral to classically defined VTA but continuous with its neural architecture, named the tail of VTA (tVTA) was found to be made up almost exclusively of GABArgic cells (Bourdy & Barrot 2012, Perrotti et al 2005). Differential composition of VTA sub-nuclei likely reflects different functional modules within this highly heterogeneous structure.

Substantia nigra pars compacta

Lateral to VTA lies the substantia nigra, composed of two cellular bands so distinct in their morphology they were once likened by 19th century Italian neurologist Giovanni Mingazzini to the structure of the cerebral cortex: ventral pars reticulata (SNr) and dorsal pars compacta (SNc) (Bentivoglio & Morelli 2005). SNr is composed of sparsely packed GABAergic cells, while SNc forms a thin strip of densely packed neurons that are almost exclusively dopaminergic. This elongated layer of dopaminergic cells forms a two-tier (dorsal / ventral) continuum with VTA (Fallon & Moore 1978).

Locus coeruleus

The bulk of noradrenaline released in the central nervous system comes from a small nucleus in the brainstem, the locus coeruleus (LC) (figure 2.3b), located just below the fourth ventricle in the dorsal tegmentum of the rostral area of the pons (Samuels & Szabadi 2008a). It contains around 1500 neurons in the mouse (and a few thousand more in the rat), which is almost 50% of all noradrenergic neurons in the mouse nervous system (Sara 2009, Schwarz et al 2015, Szabadi 2013). The neural population in LC is very homogenous in terms of neurotransmitter content, with almost 100% neurons staining positive for TH and noradrenaline transporter (NAT) (Szabadi

2013). Because of this, throughout this thesis the phrase 'LC neuron' is used in reference to noradrenergic LC neurons. Early anatomical studies using the Golgi technique characterised four different morphological types of cells in LC: Large multipolar cells in the anterior and ventromedial parts of LC, fusiform cells at the dorsal edge, posterior pole cells in the caudal part, and core cells concentrated in the main body of LC (Loughlin et al 1986a). Those populations are not fully anatomically segregated, which complicates delineation of different LC sub regions.

2.2.2 Connectivity of dopaminergic systems

The catecholaminergic nuclei in the midbrain and brainstem described above, despite their relatively small size, supply the bulk of dopamine and noradrenaline in the central nervous system. Their widespread projections reflect their central role in catecholaminergic neuromodulation.

Afferents to VTA and SNc

Projections to the midbrain dopaminergic nuclei have recently been mapped using rabies virus-based whole-brain retrograde tracing (Watabe-Uchida et al 2012). Cell type-specific targeting of this technique is based on the Cre/loxP conditional gene expression system (Gong et al 2007, Tsien et al 1996a). Using a mouse line that expresses Cre recombinase in dopamine transporter (DAT)-positive neurons and Cre-dependent 'helper viruses' it was possible to selectively transfect DAT-positive (putative dopaminergic) neurons of VTA or SNc with the modified rabies virus. Rabies virus is transported retrogradely from the postsynaptic to the presynaptic terminal and was able infect cells that synapse into DAT-positive neurons. Importantly, rabies virus particles could not be transported further due to absence of helper viruses in afferent neurons.

This study of afferents to midbrain dopaminergic neurons paints a very clear picture of similarities and differences in connectivity between VTA and SNc. Both of these structures receive huge projections from the basal ganglia, with dorsal striatum neurons connecting mainly to SNc and nucleus accumbens neurons synapsing preferentially onto VTA cells. There are also dense projections from various midbrain nuclei (superior colliculus, reticular formation) that terminate in both VTA and SNc, and a large projection from the dorsal raphae nucleus that targets mainly VTA neurons.

Of interest is the complete absence of direct projections from the hippocampus and extremely sparse afferents from the cortex (except for prominent projection from the motor and somatosensory cortices to SNc). This implies that cortical and hippocampal signals reach

midbrain dopaminergic neurons through relays in other brain areas, though results of this study are corroborated by the fact that some VTA-DA neurons express very low levels of DAT (Lammel et al 2008), which may interfere with expression of Cre recombinase using a DAT promoter. Another intriguing possibility is that of indirect control of dopaminergic neuron activity through synapses onto local GABAergic neurons, but comprehensive connectivity mapping of this neural population has not yet been conducted.

Efferent projections from VTA and SNc

Dopaminergic neurons in the midbrain dopaminergic system send prominent projections to the basal forebrain and well as many cortical areas (figure 2.3c). They show a degree of laminar organisation according to their projection targets. Lammel and colleagues (Lammel et al 2008) injected fluorescently labelled latex beads into several mouse brain areas with known dopaminergic projections. Thanks to retrograde absorption of these beads, coupled with TH immunohistochemistry, the efferent connectivity of the VTA could be characterised with high precision and accuracy. Dopamine cells projecting to the prefrontal cortex and amygdala were found exclusively in medial parts of VTA, including PN and medial portion of PBP. Neurons projecting to the nucleus accumbens core and medial shell were localised mostly in the ventral tier of VTA (including both PN and PBP), while those afferent to the lateral shell were found in the ventral tier of both SNc and VTA (almost exclusively in PBP). Unsurprisingly, dopamine cells connecting to the dorsal striatum were found mostly in SNc, confirming results of previous anatomical studies (Bentivoglio & Morelli 2005). Interestingly, cells projecting to different brain regions showed marked differences in expression of mRNAs coding for two dopamine-related proteins: TH and DAT. Neurons projecting to the prefrontal cortex, nucleus accumbens core and the amygdala expressed markedly low levels of DAT, indicating less efficient dopamine reuptake in these regions. Unfortunately, despite thorough examination of cortical and limbic VTA projections, Lammel et al (2008) did not characterise dopaminergic afferents to the hippocampal formation. Their study does, however, highlight considerable anatomical and biochemical heterogeneity among midbrain dopamine neurons with different projection targets.

Connectivity between midbrain dopaminergic cells and the hippocampus (the mesohippocampal projection) has been extensively studied by Gasbarri and colleagues (Gasbarri et al 1991, Gasbarri et al 1994a, Gasbarri et al 1996a, Gasbarri et al 1997). In a series of anatomical studies in rats, retrogradely transported fluorescent tracer was injected into different areas within the hippocampal formation. When the tracer was injected into dorsal hippocampus, it resulted in substantial labelling of neurons in ventral part of PN as well as dorsal part of PBP, but no

fluorescent signal was observed in SNc (Gasbarri et al 1994a). When stained for TH, ~ 10% of VTA neurons projecting to the dorsal hippocampus showed positive immunohistochemical signal and therefore could be classified as dopaminergic. In contrast, dopaminergic afferents to the ventral hippocampus originated from SNc (~ 18% of all SNc neurons projecting to the hippocampus) as well as from all VTA sub-nuclei. Results of a complementary series of anterograde tracing experiments (with fluorescent tracer injected into VTA or SNc), indicated that afferents from the midbrain dopaminergic nuclei terminate mainly in subiculum and stratum oriens of CA1, with only a few axons observed in other hippocampal fields. Two important conclusions can be drawn from this study. First of all, VTA and SNc differ in the topology of their hippocampal projections: while SNc projections are weaker than those from VTA and cover mainly the ventral hippocampus, VTA projections, although also sparse, span the whole septo-temporal axis. Second, both VTA and SNc projections are mainly non-dopaminergic, with only a small proportion of neurons (10 – 18%) staining positive for TH. A sizable dopaminergic projection in the hippocampus has also been described in primates using differential double staining for TH and Dbh, which allows for differentiation between dopaminergic and noradrenergic fibres (Samson et al 1990). In monkeys, dopaminergic fibres seem to innervate mostly the dentate gyrus with much sparser axon terminals in CA1 and CA3.

Sparsity of the dopaminergic projection from VTA to the hippocampus was recently confirmed by another study that used a viral recombination technique in DAT-Cre mouse line (McNamara et al 2014). McNamara and colleagues described a sparse TH-positive projection from VTA to dorsal CA1, terminating in stratum radiatum and stratum oriens.

Afferents to the LC

LC is the main source of noradrenaline in the central nervous system and the sole noradrenergic nucleus projecting to the forebrain. Therefore, it is not surprising that, similarly to midbrain dopaminergic neurons, it receives prominent direct projections from many cortical and subcortical areas (Szabadi 2013). Several studies identified prominent projections to LC from the neocortex (Luppi et al 1995, Schwarz et al 2015), with the strongest excitatory input coming from the prefrontal regions (Jodo & Aston-Jones 1997). Central nucleus of the amygdala is the only limbic area that sends direct projections to LC (Van Bockstaele et al 1998), and LC does not receive any projections from the hippocampus and associated structures. Additionally, LC receives substantial input from the cerebellum as well as from several hypothalamic and brainstem nuclei involved in control of sleep and wakefulness (Szabadi 2013).

Figure 2.3: Anatomy and connectivity of dopaminergic circuits

(a) Anatomical location of the midbrain dopaminergic nuclei.

(b) Anatomical location of locus coeruleus.

(c) Main projections of the midbrain dopaminergic nuclei. Axons of VTA and SNc dopaminergic neurons terminate mainly in the basal ganglia as well as in prefrontal cortical areas.

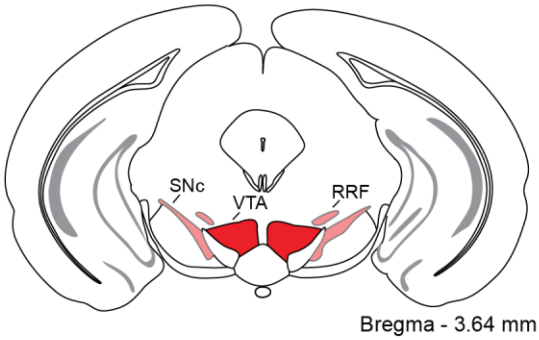
(d) Main projections of LC-NA neurons. Axons of LC-NA neurons send collaterals to many brain areas around the central nervous system, including the hippocampal formation and most of the neocortex.

(e) Two hypothetical extreme connectivity patterns of a brain area B receiving projections from A regions and sending axons to C regions. Connectivity of VTA-DA neurons is more consistent with the 'discrete pathways' model while connectivity of LC-NA neurons is more consistent with the 'integration + broadcast' model.

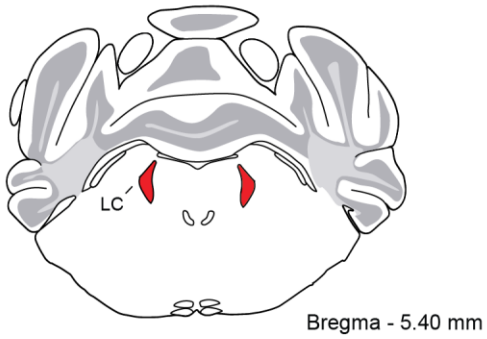
A, amygdala; ACC, anterior cingulate cortex; AON, anterior olfactory nucleus; AP-VAB, ansa peduncularis–ventral amygdaloid bundle system; BS, brainstem nuclei; C, cingulum; CC, corpus callosum; CER, cerebellum; CTT, central tegmental tract; CTX, cortex; DB, dorsal bundle; DPS, dorsal periventricular system; EC, entorhinal cortex; F, fornix; FC, frontal cortex; FR, fasiculus retroflexus; H, hypothalamus; HF, hippocampal formation; ML, medial lemiscus; MT, mamillothalamic tract; OB, olfactory bulb; OT, olfactory tract; pc, pars compacta; PC, piriform cortex; PRC, perirhinal cortex; PT, pretectal area; RF, reticular formation; S, septum; SC, spinal cord; ST, stria terminalis; SN, substantia nigra; T, tectum;TH, thalamus; VTA, ventral tegmental area.

Figures (c) and (d) adapted from Sara 2009; figure (e) adapted from Schwarz et al 2015.

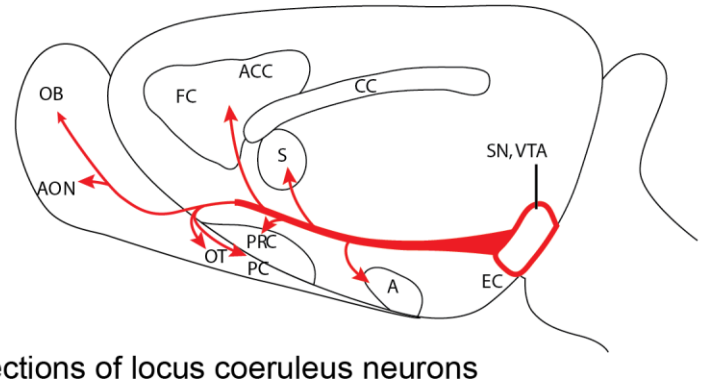
a) Midbrain dopaminergic nuclei



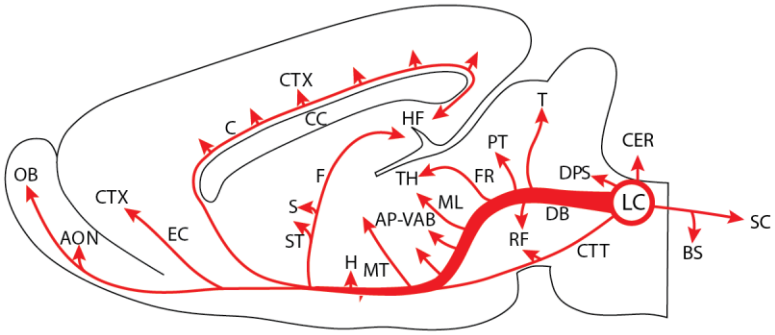
b) Locus coeruleus



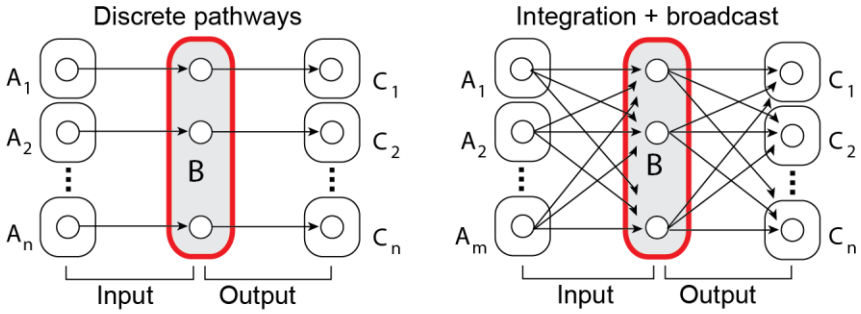
c) Main projections of midbrain dopaminergic neurons



d) Main projections of locus coeruleus neurons



e) Two hypothetical extreme connection patterns



Efferent projections from the LC

LC sends prominent projections to the vast majority of cortical and subcortical brain areas, with the notable exception of basal ganglia (Samuels & Szabadi 2008a, Sara 2009, Szabadi 2013) (figure 2.3d). Efferent projections of LC neurons can be broadly divided into three fibre tracts: the ascending pathway, the cerebellar pathway, and the descending pathway. The ascending pathway gives rise to the LC innervation of the forebrain, including the neocortex, thalamus and the medial temporal structures, including the amygdala, hippocampus and the parahippocampal areas. The cerebellar pathway, as the name implies, densely innervates the cerebellar cortex and associated cerebellar nuclei. Finally, the descending pathway connects to the motor nuclei in the brainstem as well as to the columns of the spinal cord (Jones & Yang 1985, Szabadi 2013).

Hippocampal projections originating from LC are extensive in rodents as well as in primates, covering the whole septotemporal axis of the hippocampus (Jones & Yang 1985, Pasquier & Reinoso-Suarez 1978, Samson et al 1990). Early tract tracing studies in rats described the highest axon density in stratum radiatum and lacunosum-moleculare of CA1 and in the hilus of the dentate gyrus, with a moderately-sized projection terminating in stratum radiatum and stratum oriens of CA3 (Jones & Moore 1977). More recent reports, based on Dbh immunostaining specific to noradrenergic neurons, indicate an ever larger extent of noradrenergic (presumably LC) axon arbours in the hippocampus, spanning virtually all hippocampal regions (Xu et al 1998b).

Early anatomical studies of LC neuron projection patterns done by Loughlin and colleagues (Loughlin et al 1986a, Loughlin et al 1982, Loughlin et al 1986b) described extensive collateralisation of LC axonal arbours throughout the nervous system (Loughlin et al 1982). They did, however, observe marked differences in preferred projection targets among different cell types (Loughlin et al 1986b). While the axons of core cells were found to project to the forebrain, cerebellum, hypothalamus and spinal cord, fusiform cells projected mainly to the forebrain, with only a small proportion sending collaterals to the hypothalamus and cerebellum. The two remaining cell types showed even greater specificity: the axons of multipolar cells were found preferentially in the cerebellum and spinal cord, whereas cells in the posterior pole projected almost exclusively to the hippocampus.

Discrepancies in the literature regarding the details of the output of LC neurons were recently settled using a powerful viral-genetic technique that made it possible to trace output divergence of genetically defined neural populations based on projections to a single output site (Schwarz et al 2015). Specificity for LC neurons was archived by using a Dbh-Cre mouse strain, with efficient

axonal transfection at LC projection sites achieved using the canine adenovirus (CAV). With this setup, the CAV injection into one LC projection site caused selective labelling of the whole axonal arbour of LC neurons projecting to that brain area. Injections of CAV were performed in four major LC projection sites: hippocampus, olfactory bulb, auditory cortex and medulla. Authors then quantified labelled axon arbours in cingulate cortex, cerebellum, hypothalamus and somatosensory cortex in addition to the injected areas. In a stark contrast to previous anatomical reports, axons of virally-labelled LC neurons projecting to each injected area collateralised into all investigated areas. Thus, thanks to the use of modern viral-genetic tract tracing technology it was possible to conclude that axons of individual LC neurons really do collateralise around many regions of the central nervous system.

Comparison of VTA/SNc and LC neuron connectivity

Differences in connectivity patterns between dopamine-releasing neurons in midbrain (VTA/SNc) and brainstem (LC) highlight possible functional differences between dopaminergic modules. While midbrain dopamine neurons often project to a single brain area, anatomy of LC neurons suggests high degree of input-output integration, with individual neurons receiving inputs from, as well as projecting to many different cortical and subcortical areas (Schwarz et al 2015). This contrasting morphology likely translates into different functional roles of the two systems. Midbrain dopamine cells seem to be ideally suited for localised dopamine release at individual projection sites, consistent with a connectivity model with discrete pathways (figure 2.3d, left). On the other hand, extensive collateralisation and diverse inputs observed in LC neurons implies that they integrate information from many brain areas and, once activated, broadcast the noradrenaline/dopamine signal around large regions of the brain (figure 2.3d, right).

Hippocampal projections from midbrain dopaminergic neurons seem to be much sparser than those from LC. While a typical hippocampal section contains no more than a few dopaminergic axons, noradrenergic terminals are much more abundant and widespread. What is more, it seems that dopaminergic SNc neurons are not functionally coupled to the dorsal hippocampus as most of the sparse dopaminergic axons in that area originate from VTA.

Although no comparative studies of VTA and LC connectivity have been published to date, unpublished experiments from our laboratory using anterograde viral-genetic tracing and retrogradely transported fluorescent beads largely confirm that hippocampal LC projections are much more abundant than those from VTA (Miwako Yamasaki and Tomonori Takeuchi, unpublished). Anterograde tracing using a Cre-dependent viral vector and Th-Cre mice indicates

that while LC axons span all hippocampal fields, dopaminergic VTA axons terminate almost exclusively in CA3 (figure 2.4a). What is more, injection of retrogradely transported fluorescent beads into the hippocampus results in cell body labelling in LC but not in VTA (figure 2.4b).

Anatomical and functional connectivity between VTA and LC

Anatomical and biochemical evidence presented so far seems to imply that there are two main catecholamine systems coupled to the hippocampus: sparse dopaminergic projection from VTA and dense noradrenergic/dopaminergic projection from LC. The question now arises whether they are fully independent or is there a degree of communication between the two. Early anterograde tracing studies described a moderate-sized projection from LC to VTA (Jones & Yang 1985), as well as detected dopamine-rich axon terminals in LC (Maeda et al 1994, Ornstein et al 1987), although the majority of VTA neurons projecting to LC seems to be non-dopaminergic (Swanson 1982). Results of a recent viral-genetic whole brain analysis of LC inputs is largely consistent with the early anatomical data, showing a moderate projection from midbrain dopaminergic neurons (SNc > VTA) (Schwarz et al 2015). In contrast, similar analysis of inputs to midbrain dopaminergic neurons did not detect a direct projection from LC to VTA or SNc dopaminergic cells (Watabe-Uchida et al 2012), which suggests that the LC axons in VTA might synapse exclusively onto GABAergic neurons. Thus, it appears that the majority of projections from VTA to LC are from non-dopaminergic (presumably GABAergic) neurons, which may in turn receive reciprocal connections from LC neurons.

These reciprocal projections likely mediate the functional coupling between the two nuclei. Chemical excitation of VTA with kianic acid injections causes considerable elevation of cortical noradrenaline levels that can be prevented by a 6-hydroxydopamine (6-OH-DA) neurotoxic lesion of VTA dopamine neurons (Deutch et al 1986). On the other hand, 6-OH-DA lesion of VTA dopaminergic neurons on its own causes an increase in the average firing rates of LC neurons (Guiard et al 2008b). Taken together, we have a paradoxical situation whereby both acute excitation and chronic lesioning of VTA dopamine cells leads to activation of LC. Nonetheless, as local application of dopamine into LC causes robust inhibition of LC neuron activity (Elam et al 1986, Guiard et al 2008a), it is likely that an excitatory effect of VTA stimulation on noradrenaline release could be mediated by an indirect mechanism or local interaction between dopaminergic and noradrenergic axon terminals at projection sites.

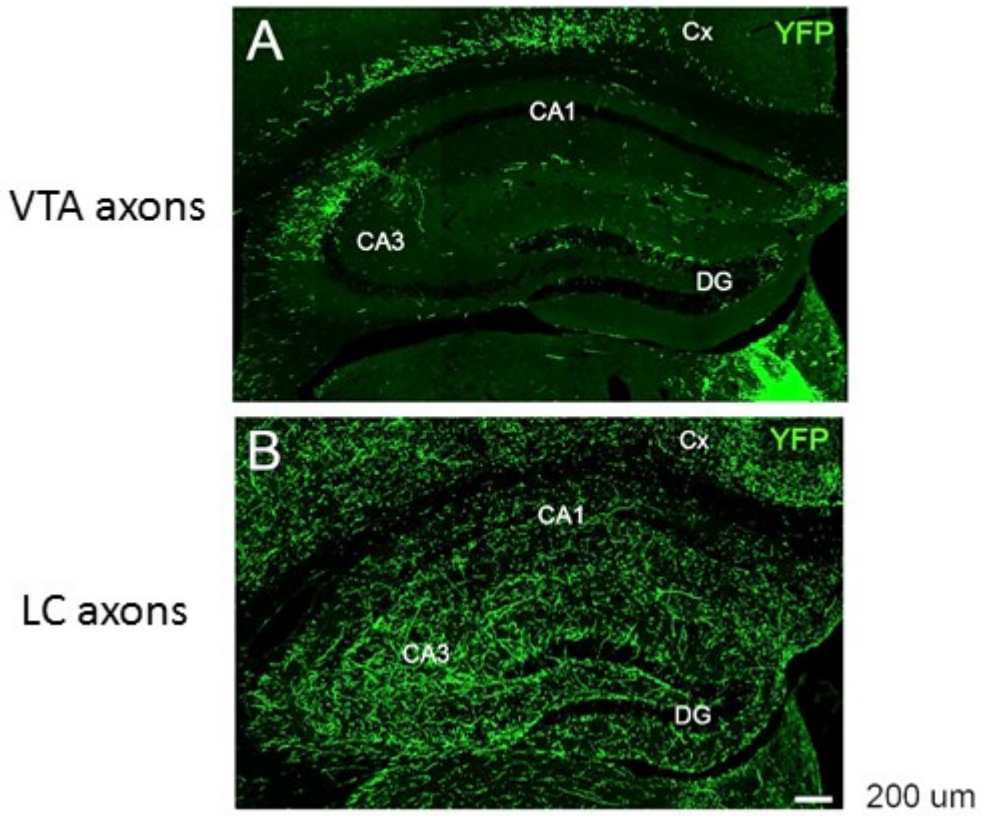
Figure 2.4: Hippocampal projections of VTA and LC neurons.

(a) Anterograde tracing of VTA-DA (top) and LC-NA (bottom) projections to the hippocampus. Hippocampal axons of VTA-DA neurons are sparse, terminating mainly in CA3. In contrast, hippocampal projections of LC-NA neurons are dense and span all hippocampal fields. Cell-type specificity was achieved by injecting a Cre-dependent viral vector with a eYFP coding sequence into VTA or LC of Th-Cre mice. Green: eYFP.

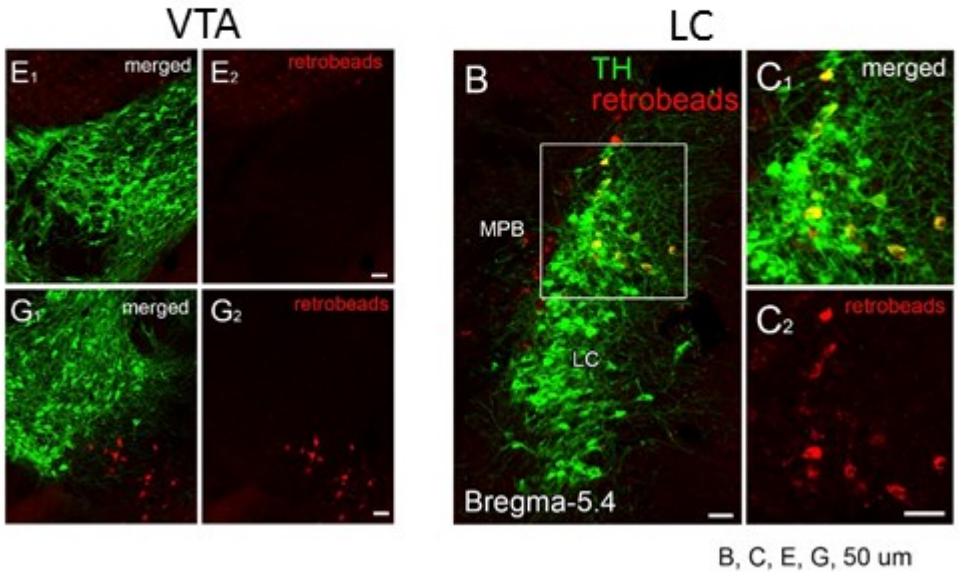
(b) Retrograde tracing of VTA (left) and LC (right) neurons projecting to the hippocampus using fluorescent latex beads injected into DG, CA1 and CA3. No fluorescent dye was detected in VTA, although significant fluorescence was observed in the surrounding areas. In contrast, dye-labelled cell bodies were detected in LC. Green: TH, Red: retrograde tracer dye.

Miwako Yamasaki and Tomonori Takeuchi, unpublished.

a) Anterograde tracing of TH+ VTA and LC projections to the hippocampus



b) Retrograde tracing of hippocampus-projecting VTA and LC neurons



Effects of LC stimulation on VTA dopamine neuron activity show a biphasic profile. Delivery of low frequency (0.5 Hz) electrical stimulation into LC leads to transient increase in VTA dopaminergic neuron firing rate, followed by a more long-lasting inhibition (Grenhoff et al 1993). In contrast, high frequency burst stimulation (20 pulses at 20 Hz every 10 seconds) leads to a prolonged inhibition of VTA dopaminergic neurons. Consistent with the mainly inhibitory effect of LC on VTA, local application of noradrenaline or dopamine into the VTA causes considerable attenuation of spiking activity (Guiard et al 2008a) and a selective lesion of LC neurons with local 6-OH-DA injection leads to a net increase in firing rates of VTA dopaminergic neurons (Guiard et al 2008b).

Catecholaminergic systems originating from VTA and LC seem, therefore, to engage in a complex interaction which, at least after prolonged activation of the presynaptic partner, is mainly inhibitory. It is therefore conceivable that rather than working in concert, they function in a mutually exclusive manner, providing either local (VTA) or distributed (LC) catecholamine release.

2.2.3 Dopamine receptors

The diversity of roles that dopamine plays in the central nervous system is maintained not only by the highly heterogeneous projection patterns of dopamine-releasing neurons, but also by the diversity of dopamine receptor classes and their distribution throughout the brain.

Classification of dopamine receptors

Dopamine exerts its function mainly via slow modulation of fast synaptic transmission and regulation of intracellular signalling cascades. Dopamine receptors are all G protein-coupled receptors, and are designated with numbers from 1 to 5 (D_1 – D_5). These five receptor types are segregated into two subfamilies, based on their biochemical and pharmacological properties: D_1-like (D_1 and D_5) and D_2-like (D_2, D_3 and D_4) (Spano et al 1978, Strange 1993, Vallone et al 2000). Historically, the division was based on the differential ability to stimulate synthesis of cyclic adenosine monophosphate (cAMP) (Kebabian & Calne 1979), but it is now supported by high degree of amino acid sequence homology within each subgroup as well as coupling to other downstream signalling cascades (Beaulieu & Gainetdinov 2011).

Distribution of dopamine receptors in the central nervous system

Dopamine receptors are expressed ubiquitously throughout the brain (main expression sites summarised in table 2.1). Although their expression levels vary greatly from region to region,

their expression pattern, with few exceptions, generally matches the topology of midbrain dopamine projections (Beaulieu & Gainetdinov 2011). Consistent with the central role of dopamine in the basal ganglia circuit, D_1 and D_2-like receptors are expressed at very high levels in the striatum and nucleus accumbens.

	Str	NAc	OT	SN	VTA	Ctx	Amg	HF	Tha	Hyp	Cer	OB
D_1	***	***		***		***	***	***	**	**	***	***
D_2	***	***		**	**	**	**	*		**		
D_3	*	**	**	*	*	*		*				
D_4	*			*		*	*	**	*			
D_5	*	*		**		**		***		**		

Table 2.1. Expression of dopamine receptor mRNA in the brain. Str, striatum; NAc, nucleus accumbens; OT, olfactory tubercle; SN, substantia nigra; VTA, ventral tegmental area; Ctx, cerebral cortex; Amg, amygdala; HF, hippocampal formation; Tha, thalamus; Hyp, hypothalamus; Cer, cerebellum; OB, olfactory bulb. Adapted from Beaulieu & Gainetdinov 2011.

Several dopamine receptor subtypes are expressed in the hippocampal formation, which, as discussed above, receives relatively sparse dopaminergic innervation. Expression of D_5 receptors spans principal cell layers of all hippocampal fields. In contrast, D_1 receptors seem to be expressed mainly along the granule cell layer of dentate gyrus (Sarinana et al 2014). D_4 receptors are the only D_2-like receptors expressed at substantial density in the hippocampus. They are expressed on cell bodies of pyramidal cells of CA1, as well as in granule cells of the dentate gyrus, with negligible expression in CA3 (Khan et al 1998). Both D_2 and D_3 receptors in the hippocampus are mainly localised to the neuropil strata and their overall hippocampal expression is low, with the exception being relatively strong expression of D_2 receptors on mossy fibers in the hilus and CA3. Because of the sparsity of their expression in the hippocampus, as well as lack of strong evidence

for their role in hippocampal plasticity and memory, detailed characterisation of D_2-like receptors is beyond the scope of this review.

D_1/D_5 receptors and their downstream signalling cascades

The D_5 receptor has a 10 to 100 times higher affinity for dopamine than the D_1 receptor (Sunahara et al 1991, Tiberi & Caron 1994), but there is a high degree of overlap between their molecular structures, as well as similar affinity to exogenous agonists (notably SKF-83556) and antagonists (notably SCH23390) (Sunahara et al 1991). Importantly, D_1-like receptors share many downstream signalling cascades. They are both coupled to the $G\alpha_{s/oli}$ class of G proteins that activate adenylyl cyclase (AC) and thus stimulate production of the second messenger cAMP. Increase in cAMP levels leads to amplification of protein kinase A (PKA) signalling. This, in turn, causes phosphorylation of a critical molecule involved in dopamine signalling – dopamine and cAMP-regulated phosphoprotein (DARPP-32), often labelled as the Rosetta Stone for understanding the mechanism of action of dopamine (Greengard 2001). Phosphorylated DARPP-32 (pDARPP-32) inhibits another crucial molecule involved in dopamine signalling – protein phosphatase 1 (PP1), which is the hub of downstream effects of D_1/D_5 receptor activation. PP1 normally keeps GluR1 subunits of AMPA receptors in a low-conductance dephosphorylated state, but activation of the cAMP - PKA - pDARPP-23 - PP1 cascade induces rapid phosphorylation of GluR1 and thus increases the sensitivity of glutamatergic synapses to glutamate (Greengard et al 1991). Similarly, pDARPP increases NMDA receptor currents through antagonism of PP-1, with possible implications for induction of synaptic plasticity (Flores-Hernandez et al 2002). In addition to PKA, D_1-like dopamine receptors also activate mitogen-activated protein kinase (MAPK), which in turn is dependent on activation of glutamate receptors and may serve as a coincidence detector for the activation of two neurotransmitter systems (Valjent et al 2005). Crucially, both PKA and MAPK are activators of the cAMP-response element binding protein (CREB), which a transcription factor and arguably the main regulator of plasticity-associated genes (Waltereit & Weller 2003). It is through the CREB pathway that D_1-like receptors are thought to promote persistence of synaptic plasticity and memory (Carlezon et al 2005, Kandel 2012).

In addition to their role in activation of the cAMP-PKA-CREB pathway, D_5 (but not D_1) receptors couple to another important signalling cascade, activating phospholipase C (PLC) through $G\alpha_q$ class of G proteins (So et al 2009). Activated PLC breaks down the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) into two active second messengers: diacyl glycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). IP_3 is a potent initiator of calcium release from

intracellular stores, which can in turn activate an array of calcium-dependent kinases, among them Ca2+/calmodulin-dependent protein kinase II (CaMKII) – a protein famously involved in induction and maintenance of long term potentiation in the hippocampus (Fink & Meyer 2002, Lisman et al 2012). DAG, on the other hand, activates the conventional form of protein kinase C (PKC), which, interestingly, is involved in potentiation of glutamatergic synapses onto midbrain dopaminergic neurons (Luu & Malenka 2008).

Dopamine D_1 and D_5 receptors are expressed in the hippocampus and couple to intracellular downstream cascades that control cell excitability and synaptic plasticity. Therefore, it is to be expected that dopamine and dopamine receptors play an important role in modulation of information processing in the hippocampus.

2.2.4 Dopamine and hippocampal plasticity

As discussed in section 2.1.2, NMDA receptor-dependent long-term potentiation (LTP) and long-term depression (LTD) in the hippocampus are well-established physiological models of neural plasticity, as well as plausible substrates for information storage in that brain region (Martin et al 2000, Martin & Morris 2002, Takeuchi et al 2014). Furthermore, initial studies of LTP persistence highlighted sensitivity of the late phase of LTP at CA3-CA1 synapses to D_1/D_5 receptor blockade (Frey et al 1991). Since then, a large body of evidence, accumulated over 25 years and reviewed in this section, has identified the dopaminergic system as one of the most potent gating mechanisms controlling different stages of hippocampal plasticity (Jay 2003, Lisman et al 2011).

Dopamine and long-term potentiation at CA3 - CA1

First evidence for the critical role of dopamine receptors in persistence of LTP came from work of Frey and colleagues, who studied the effects of dopamine receptor blockers on tetanus-induced LTP at CA3-CA1 synapses (Frey et al 1991, Frey et al 1990). Repeated tetanic stimulation (3 trains of 100 impulses at 100 Hz, with 10 min intervals) of Shaffer collateral inputs to CA1 pyramidal cells *in vitro* (electrode placement illustrated on figure 2.5a) normally produced long lasting potentiation of synaptic strength, which did not show any signs of decay for the duration of the experiment. Bath application of a non-specific dopamine receptor antagonist domperidone during tetanic stimulation had absolutely no effect on either basal synaptic transmission or the induction of LTP. It did, however, completely abolish the late phase of LTP (L-LTP), with synaptic strength decaying to baseline over 4-8 h post-tetanisation (Frey et al 1990). When the same experiment was repeated in presence of a selective D_1/D_5 receptor blocker SCH23390, the result was even more dramatic – the field excitatory post-synaptic potential (EPSP) evoked by

stimulation of Shaffer collaterals started decaying 2 h after LTP induction and was reduced to baseline after 3 h (see figure 2.5b for a similar experiment) (Frey et al 1991). The robust blockade of L-LTP by SCH23390 in hippocampal slices was replicated by several other laboratories (Huang & Kandel 1995, O'Carroll & Morris 2004, Swanson-Park et al 1999, Wang et al 2010). Results of these studies imply that strong tetanic stimulation causes release of dopamine from dopaminergic terminals in the hippocampus. This was demonstrated in the original study by Frey et al using radiolabelled [^{14}C]-dopamine preloaded into dopamine terminals during slice incubation (Frey et al 1990).

Dependence of L-LTP on D_1/D_5 receptors was also demonstrated in awake rats using chronically-implanted electrodes and systemic drug injection (Swanson-Park et al 1999). Curiously, a recent study in urethane-anaesthetised rats found L-LTP in CA1 to be insensitive to local SCH23390 infusion despite using a relatively high concentration of the drug (5 μg / μl) (Shires et al 2012). The possible reason for the discrepancy between the two experiments may lie in the different placement of stimulating electrodes. Swanson-Park et al. (1999) stimulated CA3 axons in CA1, while Shires et al. (2012) utilised a complex experimental design where they stimulated cell bodies in CA3 and recorded field EPSPs in both ipsilateral and contralateral CA1, one of which served as a control pathway. Drugs were infused into CA1 contralateral to the stimulation site, and because of the sparsity of the dopaminergic axons in the hippocampus, it is unlikely that dopamine was released contralaterally following tetanic stimulation. Still, it is clear that the stimulation protocol used by Shires et al. (2012) induced DA-independent L-LTP, illustrating that dopamine receptor activation is by no means the only mechanism behind long-lasting LTP in CA1 pyramidal neurons.

Results of early studies indicated that D_1/D_5 receptor activation is not only necessary but also sufficient to induce L-LTP in CA1 (Huang & Kandel 1995). Huang and Kandel observed that bath application of a D_1/D_5 receptor agonist SKF38393 induced a gradual but long-lasting potentiation of synaptic strength in the absence of tetanic stimulation (figure 2.5c). As shown over a decade later by another group (Navakkode et al 2007), this is only partially true. While SKF38393 induces L-LTP in presence of test stimulation, applied every minute in order to measure the field EPSP, this potentiation does not occur if the test stimulation is absent for the period of 3 h from the start of infusion. What is more, the effect is also blocked if test stimulation is performed in presence of an NMDA receptor blocker AP-5. This demonstrates that even though D_1/D_5 receptor activation can induce L-LTP in absence of initial NMDA-dependent early LTP (E-LTP), activation of NMDA receptors (achieved, in this case, through test stimulation), is still

Figure 2.5: Dopamine D_1/D_5 receptors and hippocampal LTP at CA3 – CA1 synapses.

(a) Typical placement of electrodes during CA3 – CA1 LTP experiments in hippocampal slices. Stimulating electrode is placed at the Schaffer collateral/commissural input to CA1, recording electrode is placed in CA1 stratum radiatum, where Schaffer collaterals make synaptic connections onto dendrites of CA1 pyramidal cells.

(b) Presence of D_1/D_5 antagonist SCH23390 during strong tetanic stimulation abolishes L-LTP but preserves E-LTP.

(c) Bath application of D_1/D_5 agonist SKF38393 induces slow and persistent potentiation in absence of tetanic stimulation.

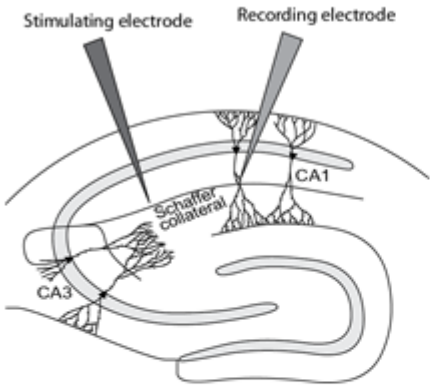
(d) D_1/D_5 agonist (+)Bromo-APB causes mild enhancement of LTP magnitude.

(e) D_1 receptor knockout mice are deficient in L-LTP at CA3 – CA1 synapses.

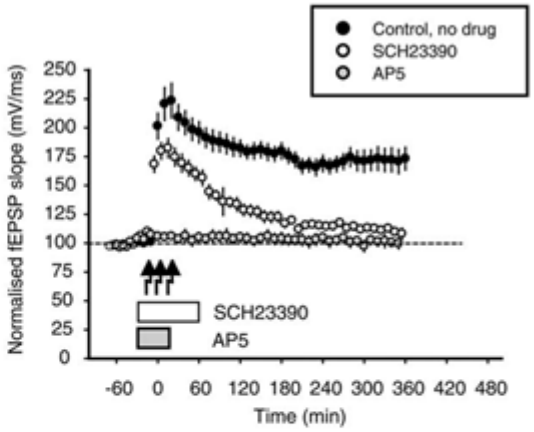
(f) E-LTP magnitude is decreased in D_1 receptor knockout mice.

Figure (a) adapted from Schultz et al 1998; figure (b) adapted from O'Carroll & Morris 2004; figure (c) adapted from Huang & Kandel 1995; figure (d) adapted from Otmakhova & Lisman 1996; figures (e) and (f) adapted from Granado et al 2008.

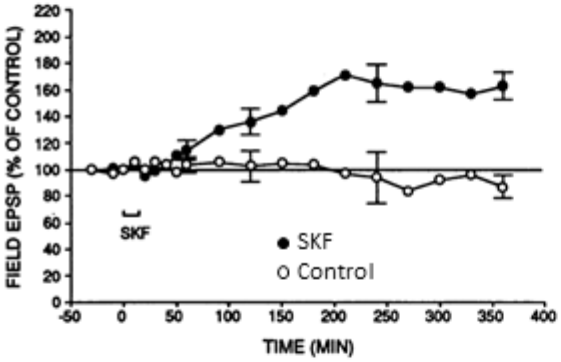
a) Electrode placement



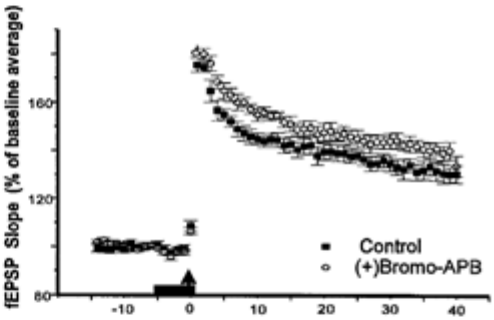
b) L-LTP blockade by a D_1/D_5 antagonist



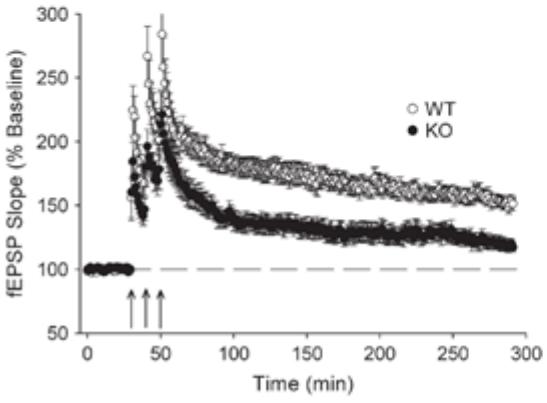
c) Slow potentiation by a D_1/D_5 agonist



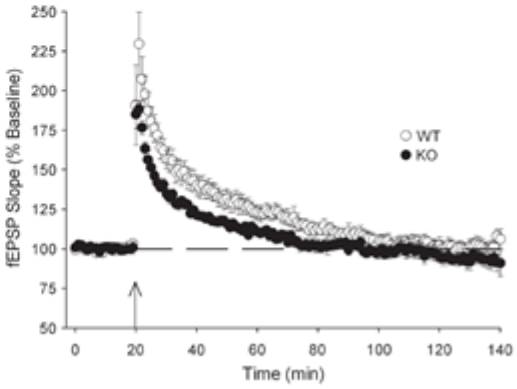
d) Enhancement of LTP magnitude by a D_1/D_5 agonist



e) LTP persistence is reduced in D_1 knockout mice



e) E-LTP magnitude is reduced in D_1 knockout mice



necessary for L-LTP to occur. This synergistic requirement for glutamatergic and dopaminergic activation presumably preserves the synapse-specificity aspect of LTP, as one can imagine that only synapses with sufficiently activated NMDA receptors would be potentiated following D_1/D_5 receptor activation.

Most of the studies of D_1-like dopamine receptors and CA3-CA1 LTP were performed using two pharmacological agents: SKF38393 (agonist) and SCH23390 (antagonist). Each of these drugs has similar affinitiy for D_1 and D_5 receptors, and although both receptors activate cAMP-PKA pathway, they differentially couple to several other downstream signalling cascades. Studies of mice with genetic ablation or downregulation of dopamine receptors have shed more light on specific roles of D_1 and D_5 receptors in CA3-CA1 LTP. The first LTP study in D_1-knockout mice highlighted their deficits in late but not early LTP in vitro (Matthies et al 1997). Importance of D_1 but not D_5 receptors was further proven by Granado et al. (Granado et al 2008) who not only demonstrated impaired CA3 – CA1 LTP in D_1-knockout mice (figure 2.5e), but showed that L-LTP deficit cannot be exacerbated by bath infusion of SCH23390. Unexpectedly, D_1-knockout mice in that study also showed a marked reduction in amplitude of E-LTP (figure 2.5f), which contrasts with lack of effect of SCH23390 on early phase of LTP (Granado et al 2008). The same deficit in early and late LTP was observed *in vivo* in D_1-knockouts, as well as in wild-type animals with hippocampal D_1 receptors downregulated in adulthood using locally injected small interfering RNAs (siRNAs) (Ortiz et al 2010). The effect of siRNA-mediated knockdown of D_1 receptors is particularly important for the interpretation of LTP deficits, as constitutive deletion of dopamine receptors may lead to abnormal development of dopaminergic terminals in the hippocampus (Rocchetti et al 2015). Nevertheless, the importance of D_1 but not D_5 receptors in CA3 – CA1 LTP is intriguing, given that D_1 expression in CA1 (Sarinana et al 2014) as well as its affinity for dopamine (Sunahara et al 1991, Tiberi & Caron 1994) are much lower than that of the D_5 receptor.

While most experiments with artificially-induced LTP at CA3 – CA1 synapses report lack of effects of D_1/D_5 antagonism on E-LTP, genetic ablation studies of these receptors described above highlight deficits in both E-LTP and L-LTP. One possible explanation is that D_1 receptor has a role in E-LTP that is independent of its activation, perhaps by maintaining structural integrity of the NMDA receptor complexes at synapses. Interestingly, several reports observed that D_1-class agonists (SKF38393 or (+)Bromo-APB), when applied during LTP induction, modestly increase the amplitude of E-LTP (Otmakhova & Lisman 1996, Stramiello & Wagner 2008) (figure 2.5d). What is more, LTP facilitated behaviourally appears to be sensitive to D_1/D_5 antagonism even at

its early stages. In their landmark study, Li and colleagues (Li et al 2003) demonstrated that exploration of a novel environment lowers threshold for LTP induction at CA3 – CA1 synapses. Authors chose a weak tetanic stimulation protocol that failed to induce LTP through a set of chronically implanted electrodes when rats were exploring a familiar environment. They then let rats explore a novel open field a few minutes before the weak tetanus was delivered, and observed robust and long-lasting (< 3 h) potentiation of fEPSP slope after tetanic stimulation. This novelty-facilitated LTP was blocked by infusion of SCH23390 prior to novelty exploration, pointing to the role of D_1-like receptors. Indeed, novelty exploration has been shown to transiently increase dopamine levels in the hippocampus (Ihalainen et al 1999), which leaves open the possibility that novelty may induce dopamine-dependent enhancement of not only hippocampal plasticity, but also hippocampal-dependent memory.

Taken together with previously discussed evidence for the central role of the cAMP-PKA-CREB pathway in long term plasticity at CA3 - CA1 synapses (Kandel 2012), as well as anatomical data on midbrain dopamine projections in CA1, the prevailing dogma is that dopamine released from sparse VTA/SNc axons in CA1 activates D_1 receptors on CA1 pyramidal cells. D_1 activation then leads to activation of the CREB gene expression mechanism and synthesis of proteins that promote stabilisation of synaptic modifications. Although the bulk of the argument is based on pharmacological or genetic manipulations, there is considerable evidence that certain behavioural manipulations (e.g. novelty exploration) modulate LTP at CA3 – CA1 synapses via dopamine release (Ihalainen et al 1999, Li et al 2003).

Other forms of hippocampal plasticity

L-LTP at CA3-CA1 synapses is the most thoroughly researched form of dopamine-dependent plasticity mechanism in the hippocampus, but there is limited evidence for the role of dopamine receptors in LTP in the dentate gyrus (DG), subiculum, as well as in LTD in the CA1 region.

The main line of evidence for the dopaminergic modulation of plasticity at the performant path (PP) – DG synapses comes from a study in freely moving rats (Yanagihashi & Ishikawa 1992). Systemic infusion of D_1 agonist SKF38393 before tetanic stimulation of PP had no effect on potentiation of the field EPSP in DG but completely abolished potentiation of the population spike, suggesting a decrease in excitability of DG granule cells. This effect was antagonised by SCH23390 in dose-dependent manner. Curiously, other laboratories failed to replicate this effect, with one study reporting potentiation of field EPSP (but not population spike) by SKF38393 (Kusuki et al 1997), and another reporting no effect of dopaminergic drugs but robust blockade

of LTP induction by β-adrenergic antagonists in rats (Swanson-Park et al 1999). Adding to the confusion, a recent thorough study involving forebrain-restricted D_1 knockout mice reported that these receptors are necessary for late phase of LTP at PP-DG synapses (Sarinana et al 2014). Overall, it seems that LTP at PP-DG synapses is modulated by activation of D_1/D_5 receptors under some circumstances (possibly influenced by stimulation protocols), but dopaminergic modulation of plasticity at these synapses is not nearly as robust as in CA1. On the other hand, complete loss of D_1 receptors in DG leads to a more pronounced LTP deficit at these synapses, which might stem from structural or developmental abnormalities in the circuit.

Subiculum is the main output region of the hippocampal formation, and subicular pyramidal cells show NMDA receptor-dependent plasticity that, in contrast to CA1 pyramidal cells, is mainly presynaptic (Behr et al 2009). While the effects of dopamine receptor activation or blockade on L-LTP have not been reported, there is considerable evidence that D_1-like receptors play a facilitatory role in E-LTP at CA1-Sub synapses *in vitro* (Roggenhofer et al 2010, Roggenhofer et al 2013). When the strength of LTP induction protocol is lowered just below the LTP induction threshold under normal conditions, bath application of SKF38393 during tetanic stimulation results in robust LTP at these synapses. Therefore, in addition to its significant role in plasticity within the hippocampal fields, dopaminergic activation may facilitate the output of information to the parahippocampal structures.

Once thought to be merely a mechanism for reducing signal-to-noise ratio at hippocampal synapses, homosynaptic LTD is now increasingly accepted as a major contributor to information storage (Kemp & Manahan-Vaughan 2007). Induction of LTD at CA3-CA1 synapses by low frequency stimulation in hippocampal slices is blocked by D_1/D_5 antagonists, and, surprisingly, also by D_2-like agonists, suggesting a receptor type-dependent control of synaptic depression by dopamine (Chen et al 1996). Sensitivity to D_1-like receptor modulation was also confirmed *in vivo* - D_1/D_5 antagonists block, and D_1/D_5 agonists potentiate induction of CA3-CA1 LTD in behaving animals (Lemon & Manahan-Vaughan 2006). Thus, the implication of the above studies is that dopamine may have significant influence not only on persistence of memory, but also on encoding of new information.

Overall, the central role of D_1/D_5 receptors in persistent forms of hippocampal plasticity is supported by many published replications of the original LTP experiments at CA3 – CA1 synapses as well as data from mouse knockout and knockdown studies. While pharmacological studies cannot differentiate between D_1 and D_5 receptors due the lack of specific agonists and

antagonists of each of these receptors, genetic studies suggest that dopamine promotes L-LTP through D_1 but not D_5 receptors. Although the role of dopamine receptors in CA1 is the most robustly characterised, several lines of evidence indicate that LTP and LTD in several hippocampal fields is modulated by dopaminergic transmission. What is more, in addition to its role in L-LTP, dopamine, acting through D_1/D_5 receptors, may also influence the dynamics of E-LTP, although results of pharmacological studies are consistent with a facilitatory rather than gating mechanism.

Homosynaptic, NMDA receptor-dependent synaptic plasticity in the hippocampus is the likely substrate for many forms of spatial, associative and episodic-like memory (Martin et al 2000, Martin & Morris 2002, Takeuchi et al 2014), and given the ubiquitous role of the dopaminergic system in promoting early and late forms of LTP, it is to be expected that hippocampal dopamine is a major modulator of different stages of memory formation.

2.2.5 Dopamine co-release from hippocampal LC terminals

Hippocampus receives sparse dopaminergic innervation from VTA and an even less prominent projection from SNc. At the same time, D_1-like receptors are densely expressed in the hippocampus and their activation is a pre-requisite for many forms of persistent hippocampal plasticity. This baffling mismatch caused a considerable amount of speculation as to the origin of hippocampal dopamine (Devoto & Flore 2006). Although dopamine is also synthesised in noradrenergic neurons and is actively concentrated into synaptic vesicles, it is rapidly converted to noradrenaline by vesicular Dbh. Still, there is circumstantial evidence from microdialysis studies that dopamine is present in cortical areas that receive little or no dopaminergic innervation (Devoto et al 2001, Devoto et al 2004, Devoto et al 2005). Additionally, even after selective 6-hydroxydopamine (6-OH-DA) lesions of VTA dopaminergic neurons dopamine can be detected at high concentrations in several cortical areas that receive noradrenergic input but not in areas with strong dopaminergic but no noradrenergic projections, such as the striatum (Devoto & Flore 2006). It is thus conceivable that, under certain conditions (e.g. Dbh saturation), dopamine may be released, along with noradrenaline, from the terminals of LC neurons.

More direct evidence of dopamine release from LC terminals came from a set of *in vitro* hippocampal slice experiments carried out by Smith and Greene (Smith & Greene 2012). They used the previously described (Huang & Kandel 1995, Navakkode et al 2007) D_1/D_5 receptor dependent potentiation of synaptic transmission at CA3 – CA1 synapses as a proxy for dopaminergic release. This slow potentiation could be achieved by application of a potent

monoamine release agent amphetamine (AMPH) but not by noradrenaline, establishing that a monoamine other than noradrenaline, if released in sufficiently large quantities, could have a robust effect on synaptic transmission. The implication was that this was dopamine. If the dopamine apparently released by AMPH application came from VTA dopaminergic neurons, Smith and Greene (2012) argued that prior blockade of catecholamine synthesis in VTA should abolish the amphetamine effect. On the contrary, they observed that prior knockdown of TH in VTA neurons with siRNAs did not affect the AMPH-induced potentiation. However, TH loss LC did abolish the AMPH-induced potentiation completely, indicating that dopamine released in the hippocampus by AMPH likely came from LC terminals. The same slow potentiation effect could be archived by pharmacological blockade of NET, adding further weight to the argument that the monoamine released by AMPH acting on noradrenergic fibres was dopamine.

The above experiments illustrate that, under certain biochemical conditions, noradrenergic fibres in the hippocampus are capable of dopamine release. However, the phenomenon may not occur under normal physiological conditions. Specifically, AMPH may induce a pathological state in LC terminals that does not reflect their physiology during normal patterns of LC activity. Still, preliminary evidence suggests that optogenetic stimulation of LC but not VTA terminals *in vitro* promotes persistence of LTP in the hippocampus in a manner sensitive to SCH23390 (R. Greene, personal communication). Nevertheless, remains to be established whether LC neurons co-release dopamine in the intact brain, and it is not known whether dopamine release from LC terminals has appreciable effects on animal cognition.

2.3 Dopaminergic control of learning and memory

As discussed in the previous chapter, midbrain dopaminergic neurons project to several brain areas known to serve important cognitive functions. Dopamine receptors are also expressed throughout the brain, and are critical mediators of many forms of synaptic plasticity, including late LTP at hippocampal CA3-CA1 synapses. It is therefore to be expected that dopamine is involved in many cognitive processes and may serve as a potent learning signal in different behavioural situations.

Considerable proportion of dopamine research in the last several decades focused on its role in addiction (Blum et al 2012, Nutt et al 2015), as well as on medical conditions characterised by dysregulation or degeneration of the dopamine system, including Parkinson's disease (Blesa & Przedborski 2014, Calabresi et al 2014, Dragicevic et al 2015, Schapira & Jenner 2011) and schizophrenia (Abi-Dargham 2014, Sigurdsson 2015). This extensive and important body of research is beyond the focus of this chapter, which instead zeroes in onto the role of dopamine signalling in non-pathological cognitive processing. Still, early pioneering work on the cognitive role of dopaminergic transmission often focused on consequences of dopamine loss, and these studies will be reviewed in the next section.

2.3.1 Cognitive consequences of dopamine deficiency

Dopamine is a powerful modulator of ongoing behaviour as well as an important learning signal. The prominent mesolimbic projection of midbrain dopamine neurons, connecting SNc and VTA with dorsal striatum and nucleus accumbens, is critical for maintaining motivation and plays a pivotal role in strengthening stimulus-response associations (Le Moal & Simon 1991, Wise 2004). First insights into the cognitive function of dopamine came from lesion studies performed using a neurotoxic dopamine analogue, 6-hydroxydopamine (6-OH-DA), selectively taken up by dopaminergic neurons (Beninger 1983). Complete degradation of nigrostriatal dopaminergic pathway in rats produced severe and long lasting aphagia and adipsia – lesioned animals were uninterested in obtaining any food or water, even after long periods of food- and water-deprivation (Ungerstedt 1971). They were also markedly hypoactive, and lost interest in exploration of their home cages as well as novel environments. Although hypoactivity of 6-OH-DA-treated animals was initially attributed to motor dysfunction, it was soon established that these rats can effectively perform complex motor sequences and avoidance behaviours when confronted with unpleasant or potentially dangerous situations, like being placed in cold water or among cats (Marshall et al

1976). Such a dramatic reversal of this hypokinetic phenotype suggests that dopamine depletion causes a deficit in *motivation* to perform a particular task, not the ability to perform it.

These effects of 6-OH-DA lesions were later confirmed by studies of a dopamine-deficient strain of mice (Zhou & Palmiter 1995). The mice were generated by disrupting the gene coding for tyrosine hydroxylase (TH), which resulted in total loss of catecholamine synthesis in all tissues, including dopaminergic and noradrenergic neurons. Normal function of the noradrenergic system (as well as adrenal glands) was then restored by expressing a TH coding sequence using a dopamine-β-hydroxylase (Dbh) promoter, the enzyme that synthesises noradrenaline from dopamine. These selectively dopamine-deficient mice became hypoactive shortly after birth and stopped feeding completely by the first month despite the anatomical connectivity of their dopamine system appearing completely normal. Motor deficits were apparent on the rotarod test, but the authors did not investigate whether activity of DA-deficient mice could be stimulated if placed in a more unpleasant on dangerous environment. They did, however, report that if the mice were kept alive by introduction of liquid diet directly in the mouth, they sometimes exhibited locomotor activity that was comparable to their wild-type littermates (Szczypka et al 1999). When administered with L-dihydroxyphenylalanine (L-DOPA), the product of TH-catalysed synthesis and direct precursor of dopamine, their adipsia and aphagia were reversed for a period of a few hours, but returned as soon as all of administered L-DOPA was converted to dopamine and released in the brain. Interestingly, L-DOPA-treated mice often exhibited a second wave of activity 24 – 48 hours after L-DOPA injection, during which they explored and investigated their food, consuming only very little. This finding indicated that dopamine-deficient mice are capable of actively seeking and ingesting food but are not interested in maintaining levels of consumption that are high enough to assure survival.

2.3.2 Information content of dopamine signals

Neurotoxic and genetic lesion studies of the dopamine system provided valuable insights into the motivational aspects of dopaminergic signalling but because of the apparent motor impairment, it has proved challenging to use such rodents to characterise the functions of dopamine neurons in animal cognition. In my view, a breakthrough in deciphering the dopaminergic neural code came from electrophysiological studies of spiking patterns of individual dopamine neurons in animals performing various cognitive tasks.

Midbrain dopamine neurons signal reward prediction errors

The role of dopamine in reward learning was initially hypothesised based on self-stimulation studies in rodents (Wise & Rompre 1989). However, the first direct evidence that dopaminergic cells encode reward-related signals came from pioneering studies of behavioural dopamine signals performed by Schultz and colleagues (Schultz 1998, Schultz 2007, Schultz 2010). These experiments involved single unit recording in midbrain dopaminergic areas (SNc, VTA, RRF) of awake monkeys performing reward learning tasks.

In a typical task, water- or food-deprived monkey was trained to press a lever in order to obtain water or food reward, and the start of each trial was signalled by a visual, auditory or somatosensory cue (Ljungberg et al 1991, Ljungberg et al 1992, Schultz et al 1993). Dopamine neurons maintain a low, tonic firing rate (1 – 3 Hz) in absence of external stimuli. During the task, majority of putative dopaminergic cells responded to reward delivery (unconditional stimulus, US) with short (~ 50 - 100 ms) and fast (20 – 30 Hz) bursts of action potentials, but a similar proportion of dopamine neurons also showed the same pattern of burst activation in response to the reward-predicting cue (conditional stimulus, CS) (Ljungberg et al 1991). The proportion of cells that responded to reward and reward-predicting stimuli was also observed to differ between the initial and final stages of learning. Specifically, and an important discovery, when the CS-US association was learned, many DA neurons ceased responding to the reward itself and shifted their phasic activation to coincide with the CS delivery (Schultz et al 1993), that is, to the time when reward was predicted.

Further studies established that dopamine cells also respond readily to an unexpected reward and are transiently inhibited when the expected reward is omitted (Fiorillo et al 2003, Hollerman & Schultz 1998). These findings led to the establishment of arguably the most prevalent theory of dopaminergic function, which postulates that dopaminergic signals encode errors in the prediction of occurrence of reward, as well as salient reward-predicting cues (Glimcher 2011, Redgrave et al 2008, Schultz 2007). This reward prediction error (RPE) is ideally suited to convey learning signals that reinforce stimulus-stimulus associations that are beneficial to the animal. This signal is distinct from a reward value signal, as it is absent when reward delivery can be predicted with high certainty based on already acquired information. It is argued that transient phasic activation of dopamine neurons in response to reward-predicting cues facilitates the cue – reward association, and that transient dopamine neuron inhibition during reward omission promotes extinction of the previously rewarded behaviour (Glimcher 2011, Redgrave et al 2008, Schultz 2007).

Beyond the RPE - do midbrain dopamine neurons respond to other types of stimuli?

While dopaminergic activity in primates is generally consistent with a reward prediction error signal, a growing body of evidence suggests that a considerable proportion of dopaminergic neurons is activated by stimuli not directly related to reward learning.

In the original study of dopaminergic neuron activity during acquisition of a reward learning task in primates two putative dopaminergic cells (the only cells recorded at this stage) showed phasic activation in response to novel stimuli in absence of reward (Ljungberg et al 1992). The stimulus in this case was the remote opening of a box that in later trials was used to deliver food reward. Perhaps the dopaminergic response was conditional on the novelty aspect of the stimulus as the neurons did not increase their firing rates in response to the same stimulus after the animal experienced it several times. Although the firing patterns of the two dopaminergic cells recorded during habituation were both modulated by stimulus novelty, such a small sample size makes it difficult to draw more general conclusions. In contrast, another study in awake cats exposed to strong visual and auditory stimuli reported that the phasic dopaminergic response did not diminish after repeated stimulus presentation (Horvitz et al 1997), which argues against the possibility that some dopaminergic neurons are specifically activated by the novel features of the stimulus. Interestingly, when dopaminergic neurons were recorded during heightened arousal states (e.g. paw immersion in cold water, feeding, grooming), visual and auditory stimuli failed to show a typical excitatory response (Strecker & Jacobs 1985), which implies that dopamine neuron firing can be dependent on animal's arousal level. Evidence of dopamine neurons conveying a 'novelty signal' is actually rather scarce, and more thorough studies are needed in order to unequivocally establish whether dopaminergic cells are indeed activated by novelty.

Initial reports of dopamine neuron responses to aversive stimuli concluded that although majority of dopamine neurons are activated only by stimuli of positive valence, a substantial minority (11 – 29%) shows moderate activation following non-noxious aversive stimuli (Guarraci & Kapp 1999, Mirenowicz & Schultz 1996, Schultz 2010). These findings presented a major challenge for the reward hypothesis of dopamine function, but they all relied on identification of recorded dopamine cells based on their waveform shape – an approach that was later criticized as ineffective (Margolis et al 2006, Ungless & Grace 2012). What is more, they were corroborated by more recent studies that utilised more powerful (and challenging) techniques of dopamine neuron identification. Using juxtacellular labelling to mark recorded neurons with a dye injected through glass recording electrode, Ungless and colleagues (Ungless et al 2004) concluded that dopaminergic neurons in rat VTA are uniformly inhibited by a noxious stimulus - a tail pinch.

Inhibition of VTA dopaminergic neurons by aversive stimuli was later investigated in a landmark study done by Cohen and colleagues (Cohen et al 2012). Most optogenetically identified dopaminergic cells in their study were inhibited in response to air puffs, but the authors also observed a small subpopulation of dopaminergic neurons that was weakly activated by the aversive stimulus. Thus, it seems that a small proportion of dopamine cells in VTA does indeed convey signals about both positive and negative stimuli – a pattern consistent with coding for stimulus salience. It is currently unknown whether these dopamine cells have a unique projection target, but dopaminergic neurons in SNc projecting to the dorsolateral but not dorsomedial striatum were recently shown to be uniquely activated by stimuli of both positive and negative valence, highlighting the possibility that different functional dopaminergic modules exist within the VTA as well.

Overall, while the activation pattern of dopaminergic neurons is largely consistent with RPE coding, it also shows a considerable heterogeneity with a subpopulation of neurons responding to all stimuli that are potentially important to the animal, including those that are rewarding, aversive or merely strong enough to catch animal's attention. Although evidence for dopaminergic activation by novelty is rather limited, there is also a sub-population of dopaminergic cells in SNc (and perhaps also in VTA) that seem to code for stimulus salience. Animals are naturally drawn to novel objects and environments (Schomaker & Meeter 2015), and it is tempting to speculate that firing rate of some dopamine neurons may also be modulated by this salient feature.

Locus coeruleus neurons signal salience and are related to attention

Until recently, locus coeruleus (LC) neurons have been thought to be solely noradrenergic in nature. For this reason, effects of pharmacological blockade of noradrenergic receptors, either local or systemic, were used as the main proxy tool for probing LC function. The role of noradrenaline in hippocampal memory consolidation is beyond the scope of this review, but due to recent evidence of dopamine co-release from noradrenergic terminals (Smith & Greene 2012) activity patterns of LC neurons in different behavioural paradigms are discussed in more detail.

Pioneering studies by Aston-Jones and others have revealed a clear relationship between LC neuron firing and animal's sleep-wake cycle. In rats, LC neurons fire tonically at around 2 Hz when the animal is awake, but their activity dampens to around 0.5 Hz during slow-wave sleep (Aston-Jones & Bloom 1981a). Interestingly, LC neurons are virtually silent during the rapid eye movement (REM) phase. These findings gave rise to the most prevalent theory of LC function - the promotion of arousal and wakefulness (Berridge 2008, Berridge et al 2012).

In awake animals, the tonic firing of LC neurons persists in absence of salient stimuli, but their firing mode is extremely sensitive to any stimulus that increases animal's arousal level (Aston-Jones & Bloom 1981b). When rats implanted with electrodes directed at LC were presented with a sensory stimulus (a light flash, a tone, or touch), these LC neurons fired synchronous and transient bursts at around 10 Hz. The magnitude of bursts was markedly decreased when the animal was engaged in automatic activities like grooming or water consumption at the time of stimulus delivery, suggesting that stimulus-evoked activity of LC neurons is dampened when the animal does not attend to the delivered stimulus. Indeed, LC neurons recorded in cats only responded when the animal exhibited an orienting response to the presented stimulus (Rasmussen et al 1986). Neurons in LC are also known to respond to acute noxious stimuli, such as corneal air puffs (Grant et al 1988), and prolonged stressors such as restraint or repeated, loud white noise (Abercrombie & Jacobs 1987). The latter paper reported habituation of neural responses to prolong stressors in cats, with LC neuron firing rate returning to baseline after 15 minutes of restraint or white noise delivery.

Even faster habituation of LC response was described in rats by Vankov and colleagues in a novelty exploration paradigm (Vankov et al 1995). Rats were allowed to explore an open field that contained two holes: an empty one and one with a novel object inside. During habituation sessions (with both holes empty), first visits to empty holes elicited a modest LC response, but LC cells did not increase their firing rates when the rat visited the holes again. When an object was inserted into one of the holes, LC neurons fired a short burst of spikes on the first visit to that hole, but not when the rat returned to the hole for the second time during the same session. Thus, it is apparent that, at least in rats, LC neurons show rapid habituation to neutral stimuli that are no longer novel.

Overall, observations discussed above suggest that LC neurons are activated by stimuli that are salient enough to attract animal's attention. However, studies in learning situations suggest that cells in LC habituate their responses even when the animal is actively attending to the stimulus. In a Pavlovian conditioning experiment in freely moving rats, Sara and colleagues (Sara et al 1991, Sara et al 1994) examined firing patterns of single units in LC during habituation, acquisition, reversal and extinction phases of associative learning. During habituation, rats were presented with a tone and, predictably, LC neurons fired bursts of action potentials in response to the auditory stimulus. The burst response waned after several presentations of the tone, but re-appeared when the tone (conditional stimulus, CS) was paired with a foot shock (unconditional stimulus, US). As the animal acquired the CS – US association, the burst response attenuated

again, but came back during the extinction phase, when the tone was still presented but the foot shock was absent. Similar pattern was observed using appetitive conditioning. This time, two different tones were presented, but only one (CS+) was associated with water reward, while the other was unrewarded (CS-). Initially LC neurons fired bursts in response to both CS+ and CS-, but they gradually shifted their response to CS+ only. When the CS contingencies were swapped (reversal), both tones elicited the burst response on the first few trials, after which LC neurons again developed selective response to CS+. The critical observation from this study is that selectivity of LC neuron response developed well before the rat expressed the acquired association behaviourally, and disappeared as soon as the rat learned to approach the reward location only after CS+ was delivered.

Overall, it seems that while tonic LC activity has a causal role in maintaining arousal, phasic LC neuron activation constitutes a signal which is correlated with changes in significance of the presented stimulus (Sara et al 1994). Even if the animal attends to the stimulus (like in case of the tone predicting reward), LC neurons will not respond unless the stimulus is either novel or its significance has somehow changed.

2.3.3 Dopamine and hippocampal memory

Activation of dopamine neurons by salient stimuli that are crucial for animal's survival (e.g. reward, pain, perhaps also novelty), as well as their pattern of projections, which includes many brain areas linked with memory formation and storage, place them in an ideal position to modulate and stabilise learning-induced changes in the brain. As discussed in section 2.2.4, dopamine receptors are important for induction and maintenance of hippocampal long-term potentiation (LTP), which is thought to be the substrate for associative and autobiographical memory (Malenka & Bear 2004, Martin et al 2000, Martin & Morris 2002, Takeuchi et al 2014). It is therefore to be expected that interfering with dopaminergic neurotransmission will have profound effects on hippocampus-dependent memory, as well as goal-directed behaviour in general.

Spatial reference memory

It was previously described that mice lacking dopamine receptors are deficient in various forms of hippocampal plasticity – the same forms that were shown to be crucial for successful acquisition of many spatial tasks, including the Morris water navigation task (the 'watermaze') (Morris et al 1986, Steele & Morris 1999, Tsien et al 1996b). As expected, global D_1 knockout mice (but not D_5 knockout mice) show a marked deficit in latency to the hidden platform in a standard reference memory version of the watermaze protocol (El-Ghundi et al 1999, Granado et

al 2008) despite apparently normal sensorimotor reflexes and locomotor activity. An early study of D_1 receptor knockouts reported a complete lack of learning of the hidden platform location even after many trials (Smith et al 1998), although in this particular report animals were also impaired in a cued version of the watermaze task that does not require spatial learning, which indicates that the deficit observed was not purely spatial. In fact, there is a considerable variability in motor phenotypes of different global D_1 knockout mouse strains, with reports ranging from mild impairment (Smith et al 1998) to normal motor function (El-Ghundi et al 1999, Granado et al 2008) and even hyperactivity (Xu et al 1994a, Xu et al 1994b). Nevertheless, all watermaze studies report chance occupation of the target quadrant during probe tests without the platform – a measure that is arguably less influenced by motor abnormalities than latency to the platform (El-Ghundi et al 1999, Granado et al 2008, Smith et al 1998).

Although spatial memory in the watermaze critically depends on the hippocampus (Morris et al 1982), some studies observed deficits in spatial search strategies following damage to the nucleus accumbens, a basal ganglia region very rich in D_1 receptors (Annett et al 1989), which may be a factor in the learning deficit observed in global knockout mice. The same argument can be used to interpret the observed impairment in spatial memory caused by systemic injection of D_1/D_5 receptor antagonist SCH23390 (Stuchlik et al 2007). Arguing in favour of the central role of hippocampal dopamine in spatial learning, Gasbarri and colleagues (Gasbarri et al 1996b) found a deficit in watermaze acquisition after 6-hydroxydopamine-induced (6-OH-DA) lesions of the mesohippocampal system (but see (Hagan et al 1983) for the apparent lack of memory impairment after more extensive lesions). Although 6-OH-DA injected into the hippocampus exerts a toxic effect on dopaminergic cells specifically projecting to that area, 6-OH-DA is also a potent neurotoxin of noradrenergic neurons, which form very dense hippocampal projections (Jones & Moore 1977, Schwarz et al 2015) and are also important for watermaze learning (Khakpour-Taleghani et al 2009). Therefore, the role of hippocampal dopamine in acquisition of spatial reference memory in the watermaze is still unclear.

The watermaze is still the most widely used spatial reference memory task, but the dry-land Barnes maze is another popular paradigm used to assess spatial memory (Barnes 1979). Based on the innate tendency of rodents to avoid well-lit spaces, Barnes maze is a circular arena with radially placed holes that the animal can explore. One of the holes leads to a dark compartment, and the animal gradually learns to head straight for the hiding place while ignoring the decoy holes. Although, in contrast with the watermaze, animals trained on Barnes maze frequently lack the incentive to reach the target location (especially after several exposures), the Barnes maze is

often more suited for exposure of milder spatial deficits (Patil et al 2009). Consistent with this, while on day 1 D_1 knockout mice took the same amount of time as controls to reach the black escape box, they did not improve their latencies even after 14 days of training, taking 4 times as much time as controls to find the escape box (Ortiz et al 2010). Still, although authors report similar immobility times in both groups on day 1, differences in movement speed or incentive to find the escape box could account for the reported deficit.

One-trial delayed matching-to-place

In addition to spatial reference memory, which involves training animals to repeatedly find (or avoid) the same spatial location over several days, hippocampus is actively recruited in several other learning paradigms with a significant spatial or contextual component. A defining feature of many of these hippocampus-dependent memory tasks is rapid (or 'automatic') encoding of information, with animals displaying long-term memory even after a single encoding trial (Morris 2006). The time point of memory encoding is also well defined, which makes it easier to determine the precise onset of consolidation. It is perhaps due to this procedural feature that the role of hippocampal dopamine system in such one-trial, episodic-like learning is quite thoroughly established.

The watermaze itself can be used in a way that induces rapid place learning (Steele & Morris 1999). In the delayed matching-to-place (DMP) protocol the position of hidden platform is changed every day, and animals that are given 4 daily trials with the same platform location show robust savings in the escape latency already after the first trial. Delay between the first and second trial can then be altered in order to look at persistence of such one-trial place memory. Intrahippocampal infusion of D_1/D_5 receptor blocker SCH23390 before trial 1 impaired the memory of platform location when tested (during trial 2) at the interval of 6 hours but not 20 minutes after encoding (O'Carroll et al 2006). This timescale matches very well with the duration of hippocampal LTP induced by weak and strong protocols (Frey et al 1991), and the effect of SCH23390 on hippocampal memory could be explained by D_1/D_5-dependent blockade of L-LTP at CA3-CA1 synapses. Infusion of the same pharmacological agent right after trial 1 had no effect on memory retention, which implies that the pulse of dopamine that promoted initial consolidation would have had to be induced at some point during the watermaze trial, either by the place encoding event or by the swimming experience itself, which is likely quite stressful and thus salient. Interestingly, a recent report utilising a similar DMP protocol in the watermaze found a significant effect of the same dose of SCH23390 (5 μg) on 30 min memory (Pezze & Bast 2012), indicating that hippocampal D_1-class receptor blockade may interfere with not only persistence

but also encoding of new information. This is in line with the postulated role of dopamine in modulation of both magnitude and induction threshold of E-LTP, based mostly on pharmacological D_1-like receptor agonist and genetic D_1 receptor knockout studies (Granado et al 2008, Ortiz et al 2010, Otmakhova & Lisman 1996, Stramiello & Wagner 2008). Still, most electrophysiological studies of the effects of D_1/D_5 antagonists on hippocampal plasticity did not report any deficits in E-LTP following D_1/D_5 blockade. This discrepancy may as well be due to different (perhaps too strong) LTP induction protocols, and will hopefully be clarified in further electrophysiological experiments. Nevertheless, the fact that blockade of D_1-like receptors affected memory encoding in the study by Pezze and Bast (2012) but not in the study by O'Caroll and colleagues (2006) begs further investigation, as it might be the case that subtle differences in the experimental protocol determine whether encoding of a one trial memory in a watermaze DMP task is D_1-like receptor dependent.

One-trial fear memory

Memories of events or stimuli associated with fear are often rapidly encoded and are known to persist for a very long time (LeDoux 2000). Therefore, many types of one-trial learning tasks include a strong noxious stimulus, most commonly a foot shock. Two paradigms commonly used to study fear memory in animal research, inhibitory avoidance and fear conditioning, rely respectively on active and reactive responses to stimuli associated with fear.

Fear conditioning is based on forming an association between a normally neutral conditional stimulus (CS) and an unpleasant, fear-inducing unconditional stimulus (US). Animal that is exposed to the CS again often shows increased 'freezing' behaviour, which serves as a readout of fear memory recall. The fear association is thought to form in the amygdala (Nabavi et al 2014), but the hippocampus is the site of formation and storage of the contextual representation of the environment the animal is in when it receives the foot shock (Liu et al 2012). This contextual aspect of fear conditioning (i.e. animal's environment as the CS) can be used to probe hippocampal function as the learned association between the fear-inducing stimulus and the context that the stimulus is delivered in is hippocampus-dependent (Phillips & LeDoux 1992, Sanders et al 2003). Thus, contextual fear conditioning (CFC) paradigms are widely used in hippocampal memory research.

Rats systemically injected with SCH23390 before encoding of the contextual fear memory freeze much less than control animals when re-exposed to the same context 24 h later (Inoue et al 2000). In contrast, the first report of behavioural deficits in global D_1 knockout mice failed to see any

impairment in 24h CFC memory (El-Ghundi et al 1999), although more recent reports which used a milder foot shock regime showed a moderate deficit in 24 h but not 5 min CFC memory (Ortiz et al 2010, Sarinana 2006). D_1 knockout mice in the study by Ortiz and colleagues (2010), though impaired in comparison with controls, nonetheless showed elevated freezing times after 24 h in comparison with pre-shock baseline. Then, when exposed to a new box, control mice decreased their freezing levels to baseline, while freezing times in D_1 knockouts were still elevated. This suggests that these mice are impaired in pattern separation – function thought to be performed by the dentate gyrus (DG) (McHugh et al 2007).

Recently, Tonegawa laboratory generated mice with conditional deletion of either D_1 or D_5 receptors in DG and tested them using the contextual fear conditioning paradigm (Sarinana et al 2014). Mice lacking D_5 receptors in DG showed increased freezing when re-exposed to the same context after 24h delay, on par with control animals. In contrast, mice that lacked D_1 receptors in DG froze much less than their control littermates, indicating that they did not remember the US-CS association. Unfortunately, although the authors reported an experiment where DG D_1 knockout mice were tested in two different contexts, low baseline levels of freezing in knockout animals prevented analysis of possible changes in pattern separation. Persistence of CFC memory in wild-type mice as well as in global D_1 knockouts can be enhanced by increasing the number of foot shocks (Ortiz et al 2010), and it would be of interest to see if DG D_1 knockout mice that acquired a long-term CFC memory have trouble differentiating between contexts. Nonetheless, the results reported in Sarinana et al's study, together with the PP-DG LTP experiments in DG-specific D_1 knockouts described in the previous sections, established the selective role of D_1 but not D_5 receptors in hippocampal long-term memory and identified the DG as a critical site of plasticity mediated by D_1 receptors. Despite the overwhelming evidence for the importance of D_1 and/or D_5 receptors in L-LTP at CA3-CA1 synapses, behavioural deficits in selective CA1 knockouts of these receptors have not yet been characterised.

Inhibitory avoidance (IA) learning in rodents makes use of their innate preference for dark compartments, as well as their ability to rapidly memorize fear-inducing stimuli. In a typical IA paradigm, the animal is placed in a rectangular box with a bright side and a dark side, with both compartments separated by a step or a doorway. When the animal steps into the dark compartment, a foot shock is delivered, which causes the animal to avoid returning to the side of the box associated with fear and pain for as long as it remembers the association between that place and the foot shock. Persistence of memory induced by the foot shock can be modulated by fine-tuning the strength of the shock itself, making it a practical paradigm for investigation of

memory consolidation mechanisms (although performance tends to be associated with very large inter-animal variability). It has long been established (e.g. (Izquierdo et al 2006)) that long-term memory of the foot shock-place association depends on the basolateral amygdala (the conditioning of fear), but also, importantly, on the hippocampus (context representation).

In a landmark pharmacological analysis of IA memory consolidation processes, Rosatto and colleagues (Rossato et al 2009) established that hippocampal D_1/D_5 receptor activation is necessary and sufficient for generation of IA long-term memory. By using two different levels of foot shock current, they were able to obtain IA memories with two different retention spans: short-term memory, induced by weak foot shock and detectable after 2 days but not after 7 days, and a long-term memory induced by a strong foot shock that was still detectable 14 days after encoding. Intrahippocampal infusion of a D_1/D_5 dopamine receptor antagonist SCH23390 as long as 12h after training blocked consolidation of the strong foot shock memory, markedly decreasing latency to step from the safe platform to the 'danger' zone after 7 days. Surprisingly, SCH23390 infusion either immediately or 9 hours after encoding had no effect on the long-term memory. The opposite effect was obtained by infusion of D_1/D_5 receptor agonist SKF38393 12 hours after weak foot shock encoding: rats were still taking considerable time to step from the platform even after 14 days – reflecting enhanced memory persistence. SKF38393 had no effect either immediately or 9 h after encoding, as had occurred with SCH23390. The lack of effect of immediate post-encoding D_1/D_5 modulation is surprising, but may be explained by the fact that the foot shock itself, being a noxious stimulus, causes sufficient dopamine release in the hippocampus to convert E-LTP to L-LTP, and thus the late action of these dopamine receptor ligands may be on systems consolidation. Unfortunately, effects of pre-encoding infusion of SCH23390 or SKF38393 on memory retention were not investigated, but the 2 day retention interval of the weak foot shock memory supports the hypothesis that initial cellular consolidation has already taken place. A series of other pharmacological interventions in the same study established that the D_1/D_5 –dependent consolidation of IA is mediated by the cAMP-PKA pathway that culminates in brain-derived neurotrophic factor (BNDF) release in CA1 (Rossato et al 2009). This argues against a systems consolidation interpretation, but indicates that there may be several determinants of enhanced persistence within the hippocampus.

In order to zero in on the origin of hippocampal dopamine that mediates this delayed consolidation, Rossato and colleagues (2009) bidirectionally modulated VTA activity by microinfusions of either a sodium channel blocker lidocaine (which blocks action potentials), NMDA receptor blocker AP-5 (which blocks VTA dopamine neuron burst firing) or NMDA itself

(which stimulates dopamine neuron burst firing). Inactivation of VTA or inhibition of its NMDA receptors blocked memory consolidation, while activation of VTA NMDA receptors promoted it. Interestingly, these manipulations were effective both immediately and 12 h post encoding, but not 9 h after the animal received a foot shock, indicating that there might be two waves of dopamine-mediated consolidation. In additional to its role in L-LTP, hippocampal dopamine promotes sharp wave-ripple (SWR) – associated replay of recently established hippocampal cognitive maps (McNamara et al 2014), which is a phenomenon associated with memory consolidation (Girardeau et al 2009, Girardeau & Zugaro 2011). This off-line replay may be the mechanism responsible for the second window for memory consolidation, and activation of hippocampal D_1/D_5 receptors 12 h post-encoding could influence occurrence of SWRs and/or stabilise synaptic modifications induced by SWR oscillations with a mechanism analogous to L-LTP.

Pavlovian conditioning over many trials

The central role of hippocampal dopamine in formation of long term memories is further supported by studies that utilised other hippocampal-dependent tasks. Pavlovian eyeblink conditioning is dependent on the hippocampus if a sufficiently long 'trace' period is introduced between delivery of the CS (e.g. a tone) and the US (corneal air puff) (Weiss et al 1999a, Weiss et al 1999b). Predictably, while wild-type mice learned the CS – US association in 10 training sessions (each consisting of 60 CS – US presentations), exhibiting a conditioned response on over 75% of trials by session 10, mice with hippocampal D_1 receptors knocked down in adulthood with siRNA failed to learn the association between CS and US, reaching asymptotic performance of circa 35% (against a pre-training baseline of 25%) in session 2 (Ortiz et al 2010). Learning curves in both animal groups were paralleled by dynamics of evoked CA3 – CA1 field excitatory postsynaptic potentials (fEPSPs) recorded during trace conditioning training with chronically implanted electrodes. In wild-type mice fEPSP slope increased proportionally to the percentage of successful conditioned responses, reaching 150% of the initial value at the end of training. At the same time, in mice with D_1 knockdown fEPSPs were potentiated by no more than 120%. Overall, these data not only constitute a very elegant demonstration of learning-induced synaptic changes in CA1, but also provide a direct link between physiological and behavioural effects of hippocampal D_1 ablation.

Paired-associate learning

Learning of paired associates, either with a spatial component (Day et al 2003, Tse et al 2007) or without it (Bunsey & Eichenbaum 1996), requires intact hippocampal circuitry, and is often used

to examine episodic memory in humans (Tulving 1983). Although initial associative learning of pairs of stimuli requires many trials, once a 'schema' is acquired (where a 'schema' may be considered a relational framework of several congruent associations) assimilation of new paired associates into the schema can be done in just one trial (Bethus et al 2010, Tse et al 2007). Rats were trained to associate different food flavours with different locations of food reward hidden in sand-filled wells in the open field referred to as the 'event arena'. When cued with a particular flavour in the start box, e.g. banana, rats often went directly to the sandwell where banana-flavoured food was placed. Rats acquired six such flavour-place associations over many days, but once they acquired the schema, they were able to learn two new flavour-place associations after a single training trial. Infusion of SCH23390 into dorsal hippocampus right before the encoding of new paired associates impaired memory retrieval 24h later (Bethus et al 2010). Importantly, retrieval of new paired associates 30 min after encoding was unaffected even by a relatively high dose of the drug (5 µl), pointing to the specific role of D_1/D_5 receptors in consolidation but not in encoding of paired associate memories.

Novelty recognition memory

Recognition memory in animals is often tested using various object recognition paradigms, which are based on the tendency of adult rodents to explore novel objects. In a basic version of the protocol, the animal is first allowed to explore two identical objects in an open field (sample phase). During a memory retrieval phase later on, one of these now familiar objects is replaced with a novel one, and a further period of exploration is scheduled. The animals are now confronted by a novel and familiar object and the usual finding, at least after short memory delay, is that rodents will preferentially explore the novel object (Dere et al 2007). The task can be modified to include higher level associations (e.g. 'object – place', 'object – context', 'object – place – context'). For example, in the 'object – context' paradigm, two sample phases in different boxes (different contexts) are conducted, each with two identical objects distinct for each context. The retrieval phase takes place in one of the boxes, with one object from the same context and the other object previously only encountered in the other context. Rodents tend to spot the object that seems out of place and explore it more.

The issue of hippocampus dependency of object recognition tasks is a complex one. While the general consensus is that the simplest version of the task (presenting the animal with a familiar object in familiar position, as well as a novel object) can be performed without the hippocampus, and depends instead on the integrity of the perirhinal cortex (Brown & Aggleton 2001), some laboratories report considerable (but not complete) impairment in object recognition in animals

with extensive hippocampal lesions at longer delays or with stronger encoding regimes (Ainge et al 2006, Broadbent et al 2004). Therefore, it appears that involvement of the hippocampus in novel object recognition is influenced by the training protocol. Still, there is convincing evidence that at least the 'object – place – context' version of object recognition is always dependent on the hippocampus even at a short, 2 min delay between encoding and retrieval (Langston & Wood 2010).

A pharmacological study of short term (90 min) and long term (24 h) non-associative object recognition memory found no effects of intrahippocampal SCH23390 or SKF38393 infusion at either delay (Balderas et al 2013), which is not surprising, as involvement of the hippocampus in non-associative object recognition is only partial at best (Dere et al 2007).

Lemon and Manahan-Vaughan (Lemon & Manahan-Vaughan 2006) , on the other hand, observed bidirectional modulation of long-term memory in their version of 'object – place' recognition memory by prior infusion of SCH23390 into the lateral ventricles (adjacent to the hippocampus, but arguably allowing also diffusion into the perirhinal cortex). Rats were placed into a square open field with four holes containing novel objects positioned in its corners. On the first day, they readily explored the objects (quantified as the number of head-dippings into the holes). On the second trial, conducted 24 hours later, the objects were no longer novel and the number of dips decreased substantially, suggesting memory of trial 1. On the third and final trial the spatial configuration of objects was changed, with the result that the number of dips into these holes decreased again. The decrease in head dipping was interpreted as detection of the new object configuration. Infusion of SCH23390 shortly before day 1 exploration session prevented the decrease in number of dips on day 2, which the authors interpreted as blockade of long-term recognition memory. The study went on to make another interesting observation. When the rats were exploring a novel object configuration on day 3, weak low frequency stimulation was delivered to the Schaffer collaterals and the field EPSP in CA1 underwent robust and long-lasting long-term depression (LTD). This novelty-facilitated L-LTD did not occur when rats were presented with a familiar object configuration, and could be blocked by prior intracerebroventricular infusion of SCH23390. Although it is not possible to conclude that encoding or detection of novelty requires activation of D_1-like receptors in the hippocampus *per se*, it is evident that novelty induces dopamine-dependent changes in hippocampal plasticity that may aid in the formation and persistence of new memories.

Overall, the importance of dopamine in hippocampal long-term memory is very well established. Pharmacological as well as gene knockout studies report deficits in memory consolidation that are largely irrespective of the chosen hippocampal-dependent task, implicating that activation of D_1/D_5 receptors in the hippocampus is a universal mechanism for gating of entry to long term memory. The bulk of the studies so far investigated the effect of either permanent dopamine receptor ablation or their pharmacological blockade during memory encoding. But does D_1/D_5 receptor activation need to *coincide* with memory encoding in order to result in consolidation of that memory trace? Evidence provided in the next section indicates that these two events may indeed occur separately in time.

2.3.4 Behavioural tagging

Dopamine, acting through D_1 receptors in the hippocampus, induces the consolidation of hippocampal memory traces in a way that is analogous to its function in promoting persistent forms of synaptic plasticity, once again making a clear connection between the molecular mechanisms that govern the dynamics of hippocampal LTP and those in control of hippocampal memory. One of the features of hippocampal LTP, stemming from the landmark Synaptic Tagging and Capture (STC) experiments (Frey & Morris 1997, Frey & Morris 1998a), is that stabilisation of this form of synaptic plasticity is not only dependent on its induction, but also on the recent history of neural activity and immediate future activity (Redondo & Morris 2011). For example, in what has come to be called the 'weak-before-strong' paradigm, both pathways show persistent potentiation when an L-LTP-inducing strong tetanic strong stimulation is delivered up to a few hours *after* E-LTP-inducing weak stimulation to separate but convergent CA1 inputs. In other words, strong stimulation of a set of inputs 'rescues' the normally fast-decaying E-LTP at the other set of synapses onto the same neural population. This phenomenon is symmetrical, so that the alternative 'strong-before-weak' order of tetanic stimulations also results in L-LTP at both pathways. Importantly, if a D_1-like antagonist is present at the time of strong tetanic stimulation, it not only blocks L-LTP at the strongly tetanised pathway, but also prevents the rescue of E-LTP at the weakly tetanised set of synapses.

This observation of dopamine-dependent late associativity of hippocampal LTP leads on to an important behavioural prediction: just like a strong tetanus delivered within the critical window of opportunity can rescue the weakly tetanised pathway, a salient event should not only result in persistent memory of the event itself, but also create a penumbra that promotes consolidation of

transient memories encoded around the time of the strong event. As we shall see, this prediction is critical to the design of the experiments of this thesis.

Flashbulb memories

The idea that surprising and/or emotional events are remembered for longer and with exceptional vividness has been formally explored by experimental psychologists since early 20th century (Stratton 1929). Brown and Kulik coined the term 'flashbulb memory' to describe a vivid memory for an event of a "very surprising, consequential or emotionally arousing" nature, like, for example, hearing the news of John F. Kennedy's assassination (Brown & Kulik 1977). The authors themselves described their own vivid memories of the event:

> "I was seated in a sixth-grade music class, and over the intercom I was told that the president had been shot. At first, everyone just looked at each other. Then the class started yelling, and the music teacher tried to calm everyone down. About ten minutes later I heard over the intercom that Kennedy had died and that everyone should return to their homeroom. I remember that when I got to my homeroom my teacher was crying and everyone was standing in a state of shock. They told us to go home." (Brown & Kulik 1977)

This detailed description involves not only the moment of hearing about the assassination, but also the details of the surrounding episodes. Brown and Kulik went on to suggest that there must be a neural mechanism, possibly innate, that is responsible for the aura of enhanced consolidation surrounding such dramatic events. The topic was widely debated in the field of experimental psychology, and various attempts were made to uncover the underlying psychological correlates of enhanced persistence and vividness of flashbulb memories. Although studies of the consistency of recollection of tragic events (e.g. 9/11 attacks or the Challenger disaster) indicate that flashbulb memories may not be as accurate as previously thought (Neisser & Harsch 1992, Talarico & Rubin 2007), they are consistently rated by participants as exceptionally vivid (Talarico & Rubin 2003).

The concept of a flashbulb memory is itself reminiscent of the behavioural predictions of the STC theory. When a novel, emotional or surprising event is experienced, small details of the scene and episodes surrounding the event are recalled with exceptional clarity. While the accuracy of recollection of such flashbulb events is difficult to assess due to the inherent variability of individual experiences and reactions, and that such national events cannot be 'planned'

experimentally, recent experiments in more controlled settings revealed that memory in school children can be enhanced by an unrelated novel and unexpected experience (Ballarini et al 2013).

Enhancement of memory persistence by novelty

On a mechanistic level, any experience that results in the release of dopamine in the hippocampus should induce opportunistic consolidation of hippocampal memory traces encoded within a few-hour 'window of opportunity'. Exploration of novel environments is known to increase dopamine levels in the hippocampus (Ihalainen et al 1999) and enhance hippocampal plasticity in D_1/D_5 receptor-dependent manner (Li et al 2003), so it is conceivable that spatial novelty could serve as the behavioural equivalent of strong tetanic stimulation.

The Viola laboratory (Moncada et al 2011, Moncada & Viola 2007) were the first to report experiments that tested the behavioural predictions of the STC theory. By carefully titrating stimulation parameters in an inhibitory avoidance (IA) paradigm, they were able to induce a transient IA memory that was readily detectable after 60 minutes but decayed over a 24 hour period. This weak training protocol can be thought of as equivalent to the weak tetanus in a typical STC experiment *in vitro*. Thus, according to the STC theory, it should result in transient synaptic potentiation (E-LTP) and the co-occurrent setting of synaptic tags, but would not induce *de novo* synthesis of plasticity-related proteins (PRPs) in hippocampal neurons. Remarkably, when the rats were allowed to explore a novel open field 1 hour before the IA training (a 'strong-before-weak' protocol), they showed robust memory of IA after 24 hours. In other words, prior novelty exploration enhanced persistence of an unrelated weak IA memory. The effect of novelty was also symmetrical, so that a weak-before-strong protocol (encoding before novelty) also induced long-term IA memory. Memory enhancement was dependent on the novelty of the stimulus, as exploration of a familiar open field had no effect on 24 h memory. What is more, the novelty effect could be blocked by prior systemic injection of D_1/D_5 receptor antagonist SCH23390 or a protein synthesis inhibitor anisomycin into CA1, mirroring pharmacological sensitivity of late-associativity in *in vitro* STC experiments. Importantly, the same effects were achieved through blockade of β-adrenergic receptors in the dentate gyrus (DG) with propranolol, implying that the novelty effect is mediated by concomitant release of dopamine and noradrenaline. Overall, it seems that induction of PRP synthesis in both CA1 and DG is required to rescue weak IA memory and that, by extension, long-term IA memory formation requires plasticity in both of these regions. Therefore, it is conceivable that novelty acts as a behavioural 'PRP donor', inducing *de novo* synthesis of proteins that are then captured then captured by synaptic tags set up in CA1 and DG neurons by prior or subsequent learning.

Novelty enhancement in an everyday spatial memory task

One trial fear learning, such as inhibitory avoidance, is often used to investigate factors affecting different stages of memory formation as it requires only a short habituation period and provides a robust memory readout. However, exposure to intense noxious stimuli creates a plethora of extreme physiological responses in the body, such as overdrive of the hypothalamic-pituitary-adrenal (HPA) axis (Cordero et al 1998), that are rarely observed in a natural setting. Fear learning in itself constitutes a traumatic episode, and it is often used as a model for post-traumatic stress disorder (Amstadter et al 2009, Mahan & Ressler 2012). Thus, fear learning paradigms may not be adequate for investigation of 'everyday' episodic memory. Moreover, animals subjected to such one-trial learning (and majority of other learning paradigms) are often experimentally naïve, while in humans events that induce flashbulb memories are encoded against a rich background of past 'everyday' experiences.

All these considerations led to development of a more realistic everyday memory paradigm, where transient episodic-like memories are encoded regularly over successive days of training, providing a background of past experience that is a closer analogy to human everyday experience (Bast et al 2005). The delayed match-to-place (DMP) paradigm involved the apparatus called an 'event arena', previously described by Day and colleagues (Day et al 2003). The event arena is a large square open field with a 7 x 7 grid of possible locations of sandwells and four start boxes located at the sides, as well as intra- and extramaze cues. Rats were trained to dig in sandwells to find food reward and, once they find it, to return to the start box to consume it. Subsequently, rats were trained according to a daily win-stay rule. During the encoding phase, the start box door opened and the animal explored the arena to retrieve food from a single sandwell at a new location. After a short delay, the retrieval phase was conducted with food available at the same location, but now with four additional nonrewarded sandwells at random locations on the arena. As the location of food reward changed every day, the protocol could be continued for several weeks, providing the animal with the everyday episodes in many ways similar to human episodic experience (e.g. where did I park my car today?). Memory retention was tested during unrewarded probe tests (with five empty sandwells), during which digging time in the correct location was scored. Probe tests at different intervals established that this one-trial spatial memory reliably decays over the period of several hours (Bast et al 2005). The sharp gradient of memory persistence in this task is consistent with the transient nature of many episodic-like memories.

The decay dynamics of one-trial appetitive spatial memory in the above paradigm render it ideally suited for testing of the behavioural predictions of STC theory. In a heroic follow-up study (Wang

et al 2010), a single cohort of rats underwent daily DMP training for six months, maintaining stable performance levels for the duration of the experiment. In parallel, a series of long-duration (> 10 h) *in vitro* LTP experiments was conducted for the purpose of comparison with the behavioural data. Once the rats acquired the task, a series of unrewarded probe tests, interspersed with regular training trials, was conducted. As reported previously (Bast et al 2005), one encoding trial (retrieval of 1 pellet from the sandwell) resulted in weak encoding, with robust 1 hour memory but no preference for the correct sandwell location after 24 h. If the number of encoding trials was increased to three, rats preferentially dug in the correct location even during the 24 hour probe test. Difference in memory decay dynamics in weak and strong memory conditions was paralleled by the dynamics of E-LTP and L-LTP induced by weakly and strongly tetanised CA3 – CA1 synapses, respectively. Additionally, 24 h retention of strong memory could be blocked by prior infusion of a D_1-like blocker SCH23390, confirming the importance of D_1-like receptor activation for long term memory formation.

In a manifestation of one of the critical STC phenomena, E-LTP could be prevented by prior ('strong-before-weak') or subsequent ('weak-before-strong') strong tetanisation of a convergent synaptic pathway onto CA1 neurons unless strong tetanus was delivered in presence of SCH23390 (Sajikumar & Frey 2004, Wang et al 2010). Similarly, exploration of a novel open field 30 min after the weak encoding trial converted weak memory into strong memory detectable after 24 hours (figure 2.6a). Just like the effect of strong tetanisation, effect of novelty could be blocked by prior hippocampal infusion of SCH23390 (figure 2.6b), and could only be observed if the novel was explored within a relatively narrow time window (< 6h). In the hippocampal slice, blockade of L-LTP by SCH23390 at one pathway can be rescued by delivery of another strong tetanus to the second pathway following drug washout. In the behavioural equivalent of this 'strong-before-strong' paradigm, strong encoding in presence of SCH23390 (which normally blocks memory consolidation) was preceded by novelty exploration. As predicted, novelty exploration cancelled out the effect of SCH23390 and rescued the strong memory, presumably by providing PRPs necessary for stabilisation of synapses in advance of encoding and subsequent tag-setting. Taken together, these findings showed that hippocampal memories follow the principles outlined in the STC hypothesis in a way that mirrors the tagging and capture dynamics of hippocampal LTP.

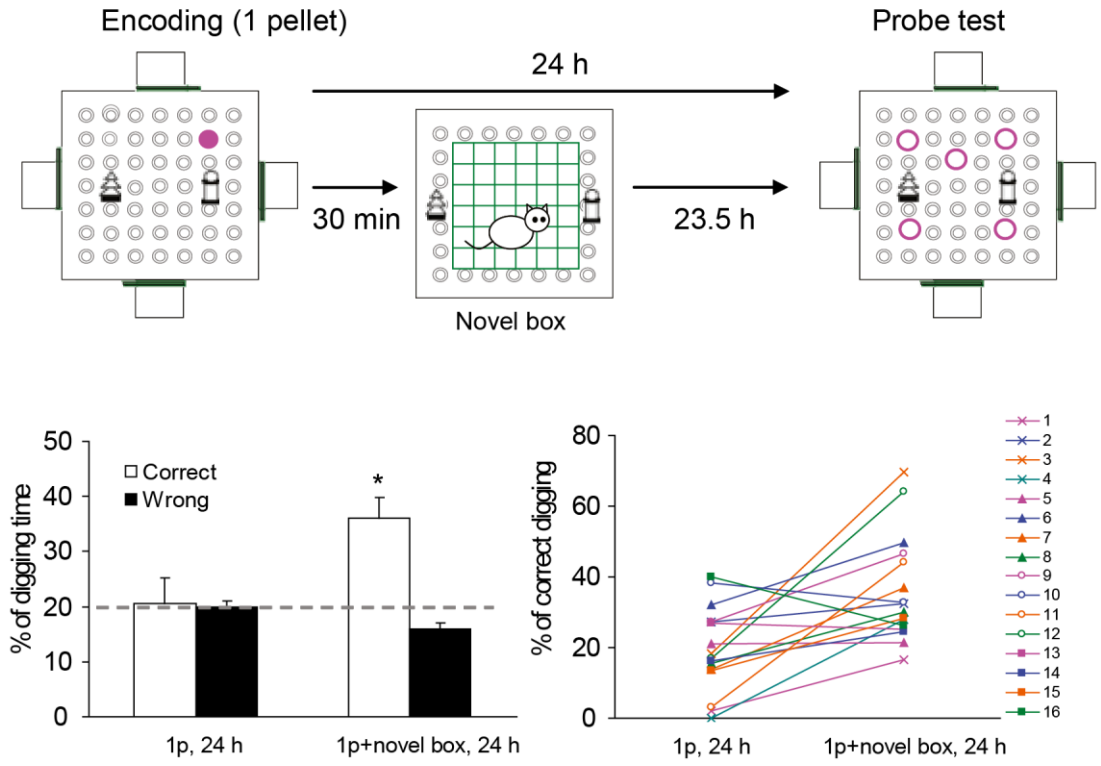
Figure 2.6: Behavioural tagging on the event arena in rats.

(a) Weak encoding was followed by exploration of a novel open field and then by an unrewarded probe test 24 hours later (top). During the probe test rats that did not explore the novel box after encoding spent equal amount of time digging in all five sandwells. In contrast, rats who explored the novel box spent significantly more time digging in the correct location (bottom left). Longitudal, within-subject design made it possible to compare each rat's performance to its own baseline. Majority of animals showed an increase in digging preference after novelty exploration.

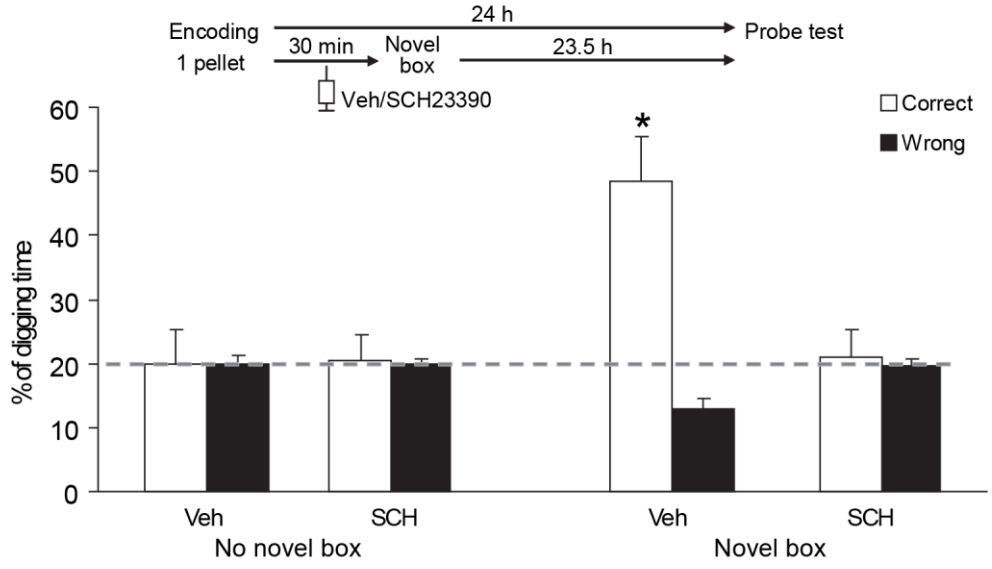
(b) Microinfusion of SCH23390 into the hippocampus after encoding but prior to novelty exploration blocked the effect of novelty on memory persistence.

Figures (a) and (b) adapted from Wang et al 2010.

a) Novelty exploration enhances memory persistence in rats



b) Novelty effect on memory is dependent on activation of D_1/D_5 receptors



Behavioural tagging is a general memory mechanism

Based on the experiments described above, it is evident that novel experiences can boost retention of unrelated spatial memories encoded within a critical window of opportunity through a STC-like mechanism referred to as 'behavioural tagging'. However, the question that remains is whether behavioural tagging is a phenomenon specific to these tasks or is it a general mnemonic mechanism that allows for late-associativity of two memory traces encoded separately in time.

Addressing this issue, another set of thorough studies conducted by the Viola laboratory established that novelty promotes persistence of memory in a variety of tasks that engage the hippocampus, and might therefore be a manifestation of a general mechanism of long term memory formation (Ballarini et al 2009, Moncada & Viola 2007). Their battery of behavioural tests included weak training protocols in spatial object recognition (also known as object displacement recognition), inhibitory avoidance and contextual fear conditioning. In all cases, exploration of a novel open field around the time of memory encoding resulted in detectable memory after 24 hour delay and thus in rescue of the transient memory trace. Interestingly, exploration of a familiar open field had no effect on persistence of weak memory, pointing to the role of 'novelty' as a critical feature of a stimulus that induces PRP synthesis.

In addition, the group tested one more crucial prediction of the STC theory, namely that in order for the late-associativity to happen, both tag-setting and PRP synthesis have to happen in overlapping populations of neurons. Using a conditioned taste aversion paradigm, crucially dependent on the insular cortex but independent of the hippocampus due to the lack of spatial or contextual component (Berman & Dudai 2001, Bermudez-Rattoni 2004), they established that an unrelated novel flavour, but not novel spatial environment, can enhance persistence of memory for a flavour associated with malaise (Ballarini et al 2009). Infusion of a protein synthesis inhibitor anisomycin into the insular cortex before exposure to the novel flavour abolished the effect, giving indirect evidence to the idea that in order for late-associativity to take place, synthesis of PRPs must happen in the population of neurons that store the weak memory trace.

Possible molecular mechanisms of the novelty effect

The novelty effect on memory persistence shows the same pharmacological profile as the *in vitro* LTP 'rescue' experiments in hippocampal slices: sensitivity to D_1-like antagonism and inhibition of *de novo* protein synthesis. This can be explained by novelty-induced release of dopamine in the hippocampus, which then induces synthesis of PRPs via D_1 receptor-mediated activation of the cAMP-PKA-CREB pathway. However, anisomycin was recently shown to have many

adverse effects on the hippocampal circuitry, including several-fold increase in neuromodulator levels (Qi & Gold 2009), as well as near-complete and long-lasting (> 6h) silencing of spontaneous neural activity (Sharma et al 2012). Both of these effects could contribute to the recently reported detrimental effects of anisomycin on behavioural performance (Dubue et al 2015). Therefore, the anisomycin-induced block of the novelty effect on its own does not prove that novelty induces synthesis of PRPs in hippocampal neurons.

Still, novelty has been shown to enhance plasticity mechanisms in the hippocampus. Exploration of novel environments promotes hippocampal LTP and LTD in CA1 through D_1-like receptors (Lemon & Manahan-Vaughan 2006, Li et al 2003) and in the dentate gyrus through β-adrenergic receptors (Davis et al 2004, Straube et al 2003a, Straube et al 2003b). Additionally, novel environments promote phosphorylation of hippocampal CREB (Winograd & Viola 2004) as well as increase expression of an array of plasticity-related genes, including c-fos, arg3.1 and zif268 (Guzowski et al 1999, Hall et al 2000, Pace et al 2005, Papa et al 1993). Although evidence for the precise molecular mechanisms behind memory-promoting effect of novelty is still lacking, phosphorylation of CREB or expression of immediate early genes is necessary for long-term memory formation (Fleischmann et al 2003, Jones et al 2001, Plath et al 2006). Thus, it is conceivable that novelty acts through the same long-term memory-promoting mechanisms. As the nature of PRPs captured by tagged synapses is being unravelled, new candidate molecular targets that are likely to mediate the novelty effect should emerge. In particular, PRP synthesis and/or availability has been shown to depend on activity of CaMK kinase (KaMKK) in an *in vitro* STC protocol (Redondo et al 2010) and it would be of interest to determine whether the novelty effect is mediated by the same intracellular mechanism.

2.3.5 NeoHebbian view of hippocampal memory – a systems perspective

At the heart of the classical Hebbian view of synaptic plasticity is a notion that activity patterns in the pre- and post-synaptic neuron are the main determinants of the synaptic strength between the two neurons. In the hippocampus, this principle is beautifully illustrated by input-specific, associative NMDA receptor-dependent LTP (McNaughton & Morris 1987), which requires two factors: glutamate release from the presynaptic terminal and concomitant depolarisation of the postsynaptic neuron (Malenka & Bear 2004). Although the central role of this Hebbian molecular learning mechanism in encoding of hippocampal memory is thoroughly established (Martin et al 2000, Martin & Morris 2002, Takeuchi et al 2014), evidence presented in previous sections indicates that synaptic modifications formed through the Hebbian mechanism are transient

without concomitant activation of the hippocampal dopaminergic system. Similarly, on a behavioural level, hippocampal memories are short-lived if underlying synaptic plasticity is not preceded or followed by D_1 receptor activation in the same neural population. Dopamine, therefore, can be viewed as the third factor in memory formation, controlling, in extension of the Hebbian model, persistence of hippocampal plasticity and memory. The critical role of dopamine in this gating mechanism implies that cellular consolidation in the hippocampus is controlled at the systems level by dopamine-releasing cells in the midbrain and/or brainstem that, in turn, are activated by a novelty (or salience) signal likely driven by complex computations involving multiple brain areas.

The hippocampal-VTA loop

Lisman and Grace suggested a hypothetical neural circuit that, they hypothesise, controls the entry of information into long-term memory (Lisman et al 2011, Lisman & Grace 2005, Otmakhova et al 2013). In the model, named the 'hippocampal-VTA loop' (figure 2.7), hippocampal formation and associated regions act as novelty detectors, comparing the incoming information with stored representations in real time. This contextual novelty signal reaches dopaminergic neurons in the midbrain through a polysynaptic pathway (as no direct projection from the hippocampus to VTA or SNc has yet been described) passing through the basal ganglia or the lateral septum. The loop is completed by the ascending projections from VTA/SNc to the hippocampal formation. The model has recently been updated to include LC neurons as another possible source of dopamine in the hippocampus (Otmakhova et al 2013). Dopamine released from VTA, SNc or LC terminals could theoretically induce cellular consolidation of information encoded in the hippocampus through an STC-like mechanism via activation of D1-like receptors. An important feature of the STC mechanism, illustrated by the behavioural tagging experiments (Ballarini et al 2013, Moncada & Viola 2007, Wang et al 2010), is that the dopamine-releasing stimulus does not need to occur at the same time as encoding of consolidated information, but can happen within a few hour window of opportunity determined by availability of PRPs and longevity of synaptic tags.

Descending pathways – controlling the dopaminergic learning signal

As described in chapter 2.3.2, VTA/SNc neurons respond to a variety of different signals, including unexpected rewards, reward predicting cues, aversive stimuli and perhaps even novelty. Similarly, LC neurons (which might under certain circumstances release dopamine in the hippocampus) respond to stimuli that are novel or significant to the animal. What is common among these types of stimuli is that they often occur in contexts or situations that are valuable for animal's survival, so it is logical that the resulting learning signal promotes long-term memory of

spatial and contextual representations in the hippocampus. However, such variety of different responses points to an array of different brain areas in control of the dopamine signal.

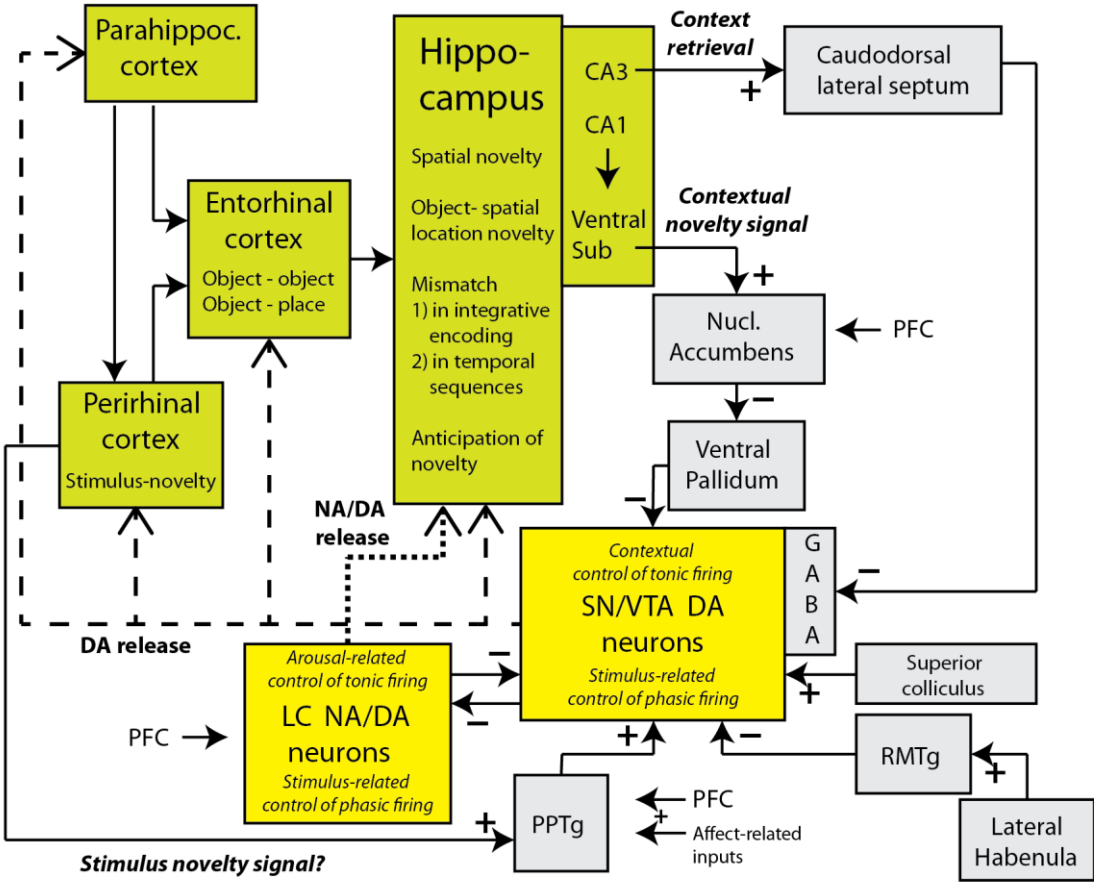
The reward signal in dopamine neurons likely comes from the laterodorsal tegmentum (LDT), which synapses onto VTA dopaminergic neurons preferentially connected with nucleus accumbens lateral shell. On the other hand, dopaminergic cells in VTA that receive input from the lateral habenula (LHb) and project to medial prefrontal cortex (mPFC) signal aversion. Consistent with this, optogenetic activation of LDT or LHb input to VTA causes place preference and place aversion, respectively (Lammel et al 2012). The spatial or contextual novelty signal is considerably more difficult to pinpoint, as there are no direct connections to midbrain dopamine cells from any structure in the hippocampal formation or associated structures in the medial temporal lobe (Watabe-Uchida et al 2012). Lisman and Grace (Lisman & Grace 2005) suggest that the novelty signal reaches VTA via the nucleus accumbens, which receives a direct input from the subiculum (Brog et al 1993) and sends dense projections to the VTA (Watabe-Uchida et al 2012). Although there is no direct evidence for this direct novelty signal, nucleus accumbens neurons have been shown to respond to novelty (Wood & Rebec 2004) and to mediate activation of VTA dopamine cells induced by stimulation of the subiculum (Floresco et al 2001). This pathway originates from the main hippocampal output and thus is likely to represent higher level contextual novelty (e.g. object – place – context). Additionally, another polysynaptic pathway linking hippocampus with VTA has been suggested by Luo and colleagues (Luo et al 2011), who found that stimulation of CA3 results in activation of dopaminergic neurons in VTA through a relay in the lateral septum. Notably, this input is necessary for linking contextual representations with reward (Luo et al 2011). The pathway linking non-contextual object novelty information with midbrain dopamine neurons is even more elusive, though anatomical studies have revealed a projection from perirhinal cortex to pedunculopontine tegmental nucleus, which is a major source of input to VTA dopaminergic cells (Otmakhova et al 2013). Overall, it is plausible that there are several different polysynaptic inputs from the medial temporal lobe to midbrain dopaminergic neurons, each conveying information about a different type of novelty.

Figure 2.7: The hippocampal-VTA loop.

The neural systems that control the entry of information to hippocampal long-term memory. Recent extension of the original Lisman and Grace model includes the locus coeruleus as a possible source of dopamine in the hippocampus.

Green: medial temporal lobe structures; yellow: catecholaminergic neurons. RMTg, rostromedial tegmental nucleus; PPTg, pedunculopontine tegmental nucleus.

Adapted from Otmakhova et al 2013.


Parahippoc. cortex
Entorhinal cortex
Object - object
Object - place
Perirhinal cortex
Stimulus-novelty
Hippo-campus
Spatial novelty
Object- spatial location novelty
Mismatch
1) in integrative encoding
2) in temporal sequences
Anticipation of novelty
CA3
CA1
Ventral Sub
Context retrieval
Caudodorsal lateral septum
Contextual novelty signal
Nucl. Accumbens
PFC
Ventral Pallidum
NA/DA release
DA release
Contextual control of tonic firing
SN/VTA DA neurons
Stimulus-related control of phasic firing
GABA
Superior colliculus
Arousal-related control of tonic firing
LC NA/DA neurons
Stimulus-related control of phasic firing
PFC
RMTg
PPTg
PFC
Affect-related inputs
Lateral Habenula
Stimulus novelty signal?

In contrast to VTA, functional investigation of inputs to LC neurons has been limited. As discussed earlier, most direct inputs to LC come from the cerebellum as well as from motor and arousal-related nuclei in the midbrain, pons and medulla, but a considerable fraction of inputs reaches LC from the neocortex (Schwarz et al 2015). Interestingly, input to LC from the prefrontal cortex (PFC) exerts a strong excitatory influence on LC neurons that spreads to the LC cells that do not directly project to the PFC, possibly through gap junctions (Jodo et al 1998). Computations in PFC have been implicated in task rule and contingency shifting (Birrell & Brown 2000, Dalley et al 2004, Morgan et al 2003), so it is conceivable that the learning-related contingency-change patterns of activity in LC neurons are driven by the PFC input. Similarly, not much is known about the stimulus novelty signal that modulates LC activity. There are no direct projections to LC from the medial temporal lobe structures hypothesised to detect novelty described in literature, which implies that such information needs to be relayed through other brain regions. The non-dopaminergic (putative GABAergic) projection from the VTA is a possible candidate as it could induce LC neuron spiking through transient disinhibition of LC neurons. Unfortunately, activity patterns of VTA GABA neurons during novelty exploration have to my knowledge not been reported, which makes this possibility purely speculative.

Dopaminergic consolidation signal in the hippocampus

The original concept of the hippocampal-VTA loop identified dopaminergic neurons in the VTA as the recipients of the novelty signal, and their hippocampal projections as the source of hippocampal dopamine that drives memory consolidation (Lisman & Grace 2005). However, the authors acknowledged that VTA efferents in the hippocampus are rather sparse, especially so in the stratum radiatum of CA1 where dopaminergic modulation of plasticity is the most thoroughly documented. In a recent evaluation of the 'hippocampal-VTA loop' hypothesis (Otmakhova et al 2013), three potential explanations that could account for this apparent mismatch were proposed:

1) **There are dopaminergic axons in CA1 stratum radiatum but they are not detectable by current methods.**
2) **Dopamine diffuses to radiatum from adjacent hippocampal regions (e.g. the alveus or CA1 stratum oriens) via volume transmission.**
3) **Dopamine released in radiatum does not come from VTA and instead is co-released from noradrenergic terminals of LC neurons.**

One might add that these possibilities need not be mutually exclusive. Catecholaminergic neurons in VTA and LC show partially overlapping but also distinct patterns of activation in response to

various environmental stimuli, and it could be that these two systems act in a complementary manner to drive hippocampal consolidation in different situations and with different effects. Sparse dopaminergic hippocampal innervation from VTA, as well as low degree of axon co-lateralisation imply localised dopamine release. In contrast, the 'broadcasting' nature of LC axonal arbours and their dense innervation of all hippocampal fields suggest a broad consolidation signal affecting all of the hippocampus and as well as many other brain areas.

Several previously discussed lines of evidence point to the VTA dopaminergic neurons as a likely ascending arm of the hippocampal consolidation loop. Firstly, pharmacological blockade of VTA impairs memory persistence while pharmacological enhancement of VTA neuron burst activity promotes it (Rossato et al 2009). Secondly, optogenetic stimulation of ChR2-expressing axons of VTA dopaminergic neurons in CA1 during learning promotes short-term retention of spatial reference memory in a D_1/D_5-dependent manner (McNamara et al 2014). However, it is not currently known whether activation of VTA dopaminergic terminals has to occur during encoding or, alternatively, within a certain time window (as postulated by the STC theory). What is more, the role of dopamine released from hippocampal LC terminals is still unclear. Therefore, in order to further expand our understanding of the ascending hippocampal consolidation mechanism it is imperative to assess whether activation of LC or VTA *separately* from memory encoding (but within the critical window) promotes memory consolidation in hippocampal D_1/D_5 dependent manner. Such experimental manipulation would require a method for temporally controlled activation of a small genetically-defined neural population, and the advent of novel tools such as the optogenetic toolbox (Fois et al 2014, Yizhar et al 2011) has made it possible to finally address these systems-level questions.

2.4 Optogenetic dissection of dopaminergic circuits

In 20th century tools for selective activation of spatially and genetically defined neural populations with considerable temporal precision were a dream of visionary scientists such as Sir Francis Crick. In one of his neuroscience-themed articles, Crick famously envisioned the next big step in systems neuroscience:

> "A major first step, then, is to identify the many different types of neuron existing in the cerebral cortex and other parts of the brain. One of the next requirements (…) is to be able to turn the firing of one or more types of neuron on and off in the alert animal in a rapid manner. **The ideal signal would be light** (…)." (Crick 1999)

Methods available at the time offered either high temporal resolution or high neuron-type specificity, but not both. Electrical pulses stimulate neural tissue with millisecond precision, but indiscriminately activate all cells within the stimulation area, as well as fibres of passage. On the other hand, molecular genetic techniques allow manipulation of strictly defined neural populations at the expense of temporal resolution. At the beginning of 21st century, introduction of optogenetic tools into neuroscience paved the way for investigation of the functional relationship between specific neural circuits and animal cognition in an unprecedented detail (Fois et al 2014, Yizhar et al 2011). This chapter will provide an overview of recent studies that used optogenetics to investigate dopaminergic circuits, and which served as an inspiration for the design of studies described in the experimental chapters of this thesis.

2.4.1 Overview of the optogenetic toolbox

Optogenetics is based on ectopic expression of light-sensitive ion channels (opsins) and pumps in various animal tissues and their subsequent activation via targeted light delivery. Channelrhodopsin-2 (ChR2), a cation channel from green alga *Chlamydomonas reinhardtii* (Nagel et al 2003), is a canonical example of such opsin. ChR2 can be expressed in neurons either via viral transfection or gene knock-in, and neurons expressing ChR2 depolarise when illuminated with blue light (Arenkiel et al 2007). Thus, ChR2-expressing neurons can be driven to fire action potentials at short latency (< 3 ms) in response to light pulses. On the other hand, hyperpolarising opsins such as the chloride pump halorhodopsin (NpHR) or the proton pump archaerhodopsin-3 (Arch) are efficient neural silencing tools, preventing almost all action potentials in a neuron when activated with light of appropriate wavelength. Spiking in specific neural populations can then be

controlled with millisecond precision in behaving animals via light delivered locally through implantable optic fibres.

Opsins can be efficiently delivered to target brain areas via injectable viral vectors (Davidson & Breakefield 2003), and can be expressed in genetically-defined populations of neurons using the Cre-lox system of conditional gene expression (Tsien et al 1996a). Recombination of target sites on the viral vector by Cre recombinase is extremely efficient and thus combination of these two techniques decouples the degree of opsin expression from the strength of the gene-specific promoter. This results in intracellular opsin levels that are high enough to efficiently influence neuronal activity irrespective of the targeted cell type. On top of that, precisely directed light delivery into target brain areas can further increase the precision of optogenetic manipulations.

2.4.2 Functional relationships between dopamine neurons and cognition

Cell-type specificity of optogenetics makes this technique ideally suited for interrogation of neuromodulatory systems and their role in animal cognition. In this section I will discuss several studies that used optogenetic tools to establish causal links between the activity of dopaminergic systems and cognitive processes.

Demonstration of causality between dopaminergic activation and behaviour

Tsai and colleagues (Tsai et al 2009) were the first to show that phasic activation of VTA dopaminergic neurons can serve as a positive reinforcer in behavioural conditioning. Mice expressing Cre recombinase in TH-positive neurons were injected into VTA with an adeno-associated virus (AAV) carrying Cre-dependent ChR2 coding sequence. The authors could then drive activity of VTA dopaminergic cells through implanted optic fibres, and established that phasic (50 Hz) but not tonic (1 Hz) stimulation of these neurons induces transient dopamine release in the striatum. Mice were then placed in a two-compartment conditioning apparatus, and received different pattern of stimulation (tonic or phasic) when they entered either of the two compartments. When mice returned to the apparatus after a delay, they spent considerably more time in the phasic chamber. Thus, authors were able to show the first direct link between phasic activation of VTA dopamine cells and positive reinforcement. Interestingly though, in contrast to electrical self-stimulation experiments (Cheer et al 2007), optogenetic activation of VTA dopaminergic neurons does not on its own drive lever pressing in an operant conditioning chamber (Adamantidis et al 2011), although it positively enhances lever pressing for food reward. This highlights the usefulness of genetically-targeted optogenetic tools, as discrepancies between

this study and earlier electrical self-stimulation studies might be due to activation of other cell populations and/or fibres of passage by electrical stimulation.

Activation of VTA dopaminergic axons in the hippocampus facilitates memory encoding

Dopamine signals are postulated to have a cognitive role beyond that in associative conditioning and reinforcement learning. Electrophysiological and behavioural experiments based on pharmacological manipulation or genetic ablation of hippocampal D_1/D_5 receptors pointed to the pivotal role of dopaminergic transmission in hippocampal memory persistence and perhaps also memory encoding (see section 2.3 for review). While the causal relationship between dopaminergic neuron activation and long-term memory is still unclear, a recent study established that optogenetic activation of dopaminergic VTA projection in the hippocampus promotes spatial learning (McNamara et al 2014).

Mice expressing ChR2 in DAT-positive VTA neurons were trained on a crossword-like maze made of a series of intersecting corridors with movable barriers blocking off certain routes. The task was to reach the new reward location and avoid dead-ends, and two alternating starting points were used in order to encourage allocentric navigation. Mice were given 20 consecutive trials to learn the location of food reward and typically achieved asymptotic performance (measured as path length to reward) after 15 trials. The new spatial memory decayed rapidly, with mice showing considerable forgetting even after 1 hour. Optical stimulation of ChR2-expressing axons of VTA neurons in dorsal CA1 *during* training trials had no effect on the learning curve but completely counteracted the rapid forgetting of the spatial memory after 1 hour.

A crucial point to consider is the nature of cellular and/or synaptic processes that might have been influenced by stimulation of dopaminergic axons in this experimental paradigm. While the results of this study have been interpreted by the authors as resulting from enhanced consolidation through D_1/D_5-mediated facilitation of LTP persistence (Atherton et al 2015), the 1 hour interval between encoding and retrieval corresponds to the duration of early LTP, and several studies have shown that spatial memories can be retained for several hours despite D_1/D_5 receptor blockade (Bethus et al 2010, O'Carroll et al 2006) (but see (Pezze & Bast 2012)). Therefore, a more plausible interpretation is that optogenetic facilitation of endogenous dopamine release in the hippocampus boosted memory encoding through facilitation of LTP induction (Granado et al 2008, Ortiz et al 2010, Otmakhova & Lisman 1996, Stramiello & Wagner 2008). While one could suggest that boost in capacity for LTP induction during training should also influence performance in later training trials, the paradigm involved short intertrial intervals (< 1 min)

(Colin McNamara, personal communication) and retention of spatial information for such short periods of time does not require NMDA receptor-dependent hippocampal plasticity (Steele & Morris 1999).

Enhancement of hippocampal short-term memory through optogenetic activation of dopaminergic axons in the hippocampus during learning by McNamara et al constitutes the first direct evidence linking the VTA dopaminergic system with memory (McNamara et al 2014). However, several outstanding questions need further clarification. Firstly, the role of dopaminergic innervation in the hippocampus in memory *consolidation* has not yet been determined. In particular, the STC theory postulates that dopaminergic activation can influence persistence of memories even if it occurs separately from memory encoding. Additionally, while the study by McNamara and colleagues (McNamara et al 2014) points to the functional role of hippocampal projections from VTA dopaminergic neurons in memory processes, further studies are needed to establish whether activation hippocampal LC terminals can serve a similar function.

2.4.3 Optogenetic identification of dopaminergic neurons *in-vivo*

Studies discussed in the previous section used optogenetics to determine the functional relationships between dopaminergic neurons and learning. More specifically, ChR2-mediated activation of dopaminergic neurons drove a cognitive change in animals performing a particular task. However, optogenetics can also be used in a way that does not influence behaviour *per se*, but allows for robust identification of specific neural populations in experiments where direct visualisation of neural tissue is not possible. In particular, single unit recording in whole animals often relies on identification of particular cell types through their firing patterns and electrophysiological properties. Such criteria are often very robust, as it is in case of hippocampal pyramidal cells and interneurons, but more often than not the relationship between physiological categories and genetic identify of neural populations is quite ambiguous. On the other hand, conditional opsin expression in genetically defined neural populations is characterised by high specificity, and neurons expressing light-sensitive opsins can be identified electrophysiologically by analysing their responses to optical stimulation. Therefore, optogenetic stimulation can be used as a tool to classify individually recorded neurons as belonging to a certain genetically-defined class.

The issue of neuron type identification is particularly important when recording single unit activity in VTA, which contains populations of dopaminergic (~70%), GABAergic (~25%) as well as glutamatergic (~2-3%) neurons. Pioneering recordings from rat SNc neurons *in vitro*

performed by Grace and colleagues (Grace & Bunney 1980, Grace & Bunney 1983, Grace & Bunney 1984, Grace & Onn 1989) established several physiological and pharmacological criteria that, according to the authors, reliably distinguished dopaminergic and non-dopaminergic neurons. According to these criteria, dopaminergic neurons exhibit large waveforms (duration of more than 2 ms) and fire at slow (2 – 10 Hz) firing rates with high incidence of burst firing (2 – 10 spikes with 80 – 160 ms interspike interval). Additionally, they are selectively inhibited by bath application of dopamine or D_2 receptor agonists. In contrast, non-dopaminergic (putative GABAergic) neurons tend to have short waveforms and fire tonically at high frequencies (> 10 ms).

Although these criteria were initially only confirmed in rat SNc slices, they were employed in virtually all electrophysiological studies of VTA and SNc neurons in rodents and primates for more than two decades (Ungless & Grace 2012). However, since then, several thorough studies called the reliability of these established criteria into question. Ungless and colleagues (Ungless et al 2004) recorded activity of VTA neurons of anaesthetised rats and used juxtacellular labelling and subsequent TH immunohistochemistry to categorise recorded neurons as dopaminergic or non-dopaminergic *ex vivo*. This challenging labelling technique only allowed for successful identification of 12 TH-positive and 8 TH-negative neurons, and even though a trend for shorter action potential half-widths (peak to trough) in TH-negative cells was observed, there was a considerable overlap between the two populations. The half-width cut-off point of more than 1.1 ms excluded all identified non-dopaminergic cells, but also a considerable proportion of identified TH-positive neurons. At the same time, the remaining identification criteria of dopamine neurons were questioned in a study of VTA neurons *in vitro* (Margolis et al 2006). Specifically, Margolis et al showed that at least in VTA around 50% of TH-negative neurons could also be inhibited by a D_2 receptor agonist. Additionally, *in vitro* spontaneous firing rates did not differ between the two groups.

A major breakthrough came from the Uchida laboratory, who used optogenetics to reliably identify dopaminergic and GABAergic neurons in VTA of behaving mice (Cohen et al 2012). Their method involved injecting a Cre-dependent viral vector carrying ChR2 coding sequence into VTA of two strains of mice, one expressing Cre in DAT-positive neurons (DAT-Cre) and one with Cre expression in neurons expressing GABA transporter (Vgat-Cre). Subsequently, a hybrid electrode array with an optic fibre surrounded by several tetrodes (an ‘optetrode’) was implanted at the same co-ordinates. Single units coming from ChR2-expressing dopaminergic or GABAergic neurons reliably fired action potentials in response to low-frequency light pulses

delivered through the optic fibre, and were classified as belonging to a particular neuronal type based on their light-responsiveness. Activity of these identified neurons could then be monitored in a behavioural paradigm (discussed in chapter 2.3.2). Surprisingly, dopaminergic and GABAergic neurons optogenetically identified by Cohen et al did not differ in mean waveform duration, which questions the validity of this criterion in mouse VTA recordings.

Overall, optogenetic identification of neuronal types in behaving animals is an very powerful method that, when coupled with single unit recording, is much more reliable for neuron type classification than electrophysiological or pharmacological criteria. This technique is therefore invaluable in experiments where recordings are made in heterogeneous brain areas (for example, in VTA) or in small brain nuclei that are genetically distinct from surrounding neural tissue (for example, in LC).

2.5 Aims of this thesis

The experiments presented in this thesis address the long-standing question in the field of memory consolidation research: what is the origin of the hippocampal dopamine signal that promotes consolidation of transient episodic-like memories? The choice of candidate dopamine-releasing nuclei was guided by the theory of hippocampal-VTA loop (Lisman & Grace 2005), which was recently extended to include LC as a possible source of hippocampal dopamine (Otmakhova et al 2013).

The conceptual divide between the processes of memory encoding and memory consolidation necessitates the choice of a behavioural paradigm where these two stages of memory formation can be robustly dissociated. Enhancement of memory persistence by novelty (i.e. "behavioural tagging") is a phenomenon that accurately illustrates this dissociation, with persistence of encoded memory being modulated by a novel event experienced *separately* from the weakly encoded event in question. If the novel event is experienced *after* weak memory encoding, it cannot influence the encoding process *per se*, and instead selectively affects the consolidation of the weakly encoded memory trace. Novelty induces the aura of enhanced memory consolidation through activation of hippocampal D_1/D_5 receptors (Moncada & Viola 2007, Wang et al 2010), presumably by dopamine, through a mechanism that parallels that of strong encoding (O'Carroll et al 2006, Wang et al 2010), and is thus used to investigate dopaminergic memory consolidation in this thesis. The everyday appetitive spatial memory paradigm on the event arena, which provides the background of daily transient memory traces that may be strengthened by unrelated novel experiences (Bast et al 2005, Wang et al 2010), was used as a platform for the optogenetic interrogation of the dopaminergic systems that mediate the novelty effect.

In order to conclude that area X mediates the dopaminergic novelty effect on memory persistence, and its activation is necessary *and* sufficient for this effect, three criteria must be fulfilled:

- **Catecholaminergic neurons in area X are activated by novelty**

- **Optogenetic stimulation of area X, substituted for novelty, mimics the novelty effect**

- **Optogenetic inhibition of area X during novelty exploration abolishes the novelty effect**

Experiments included in this thesis zero in on the first two criteria, using optogenetics to a) identify catecholaminergic neurons recorded during novelty exploration, and b) selectively activate catecholaminergic cells in VTA or LC as a substitute for novelty. The third, loss-of-function experiment is planned as a follow-up to the studies included in this document. Selective manipulation of neural activity in catecholaminergic neurons is possible due the use of a Cre-dependent viral vector carrying ChR2-coding sequence in mice expressing Cre recombinase in TH-positive cells (Th-Cre mice) (Lindeberg et al 2004, Tsai et al 2009). Figure 2.8a illustrates specificity of viral expression in VTA and LC of Th-Cre mice (Takeuchi, unpublished).

The structure of the experimental part of the thesis follows a logical progression:

1. Due to the use of Cre-mediated conditional opsin expression, critical tests outlined above have to be carried out in the mouse. Therefore, first and foremost, it is imperative to adapt the behavioural paradigm, originally developed for rats (Bast et al 2005, Wang et al 2010), for use with mice. This constitutes a replication of the previous results obtained in rats, and is described in detail in chapter 3. Importantly, this study involves analysis of the interplay between transient spatial memory traces and unrelated novelty experienced shortly (30 min) after weak encoding, as well as hippocampal D_1/D_5-dependence of the observed novelty effect.

2. Once the platform for investigation of the novelty effect is established, an electrophysiological survey of dopamine-releasing cells in two main catecholaminergic nuclei (VTA and LC), aimed to determine their responses to novelty, is conducted and described in chapter 4. ChR2 is conditionally expressed in TH-positive neurons of Th-Cre mice. Animals are also implanted with a hybrid 'optetrode' microdrive, consisting of an optic fibre surrounded by an electrode array, directed at VTA or LC. ChR2-expressing neurons are then identified *in vivo* by administration of blue light. Activity of catecholaminergic neurons in VTA and LC is then monitored during exploration of novel and familiar environments similar to the ones used in the event arena experiment described in chapter 3.

3. This is followed, in chapter 5, by the second behavioural study on the event arena, which aims to test the 'mimicry' criterion. Specifically, it aims to establish whether optogenetic activation of catecholaminergic cells in VTA or LC, separately from encoding, promotes persistence of transient memory traces in a way that is qualitatively and quantitatively

similar to the novelty effect. Pharmacologically, mimicry is assessed by investigating sensitivity of any observed effects to prior hippocampal D_1/D_5 receptor blockade.

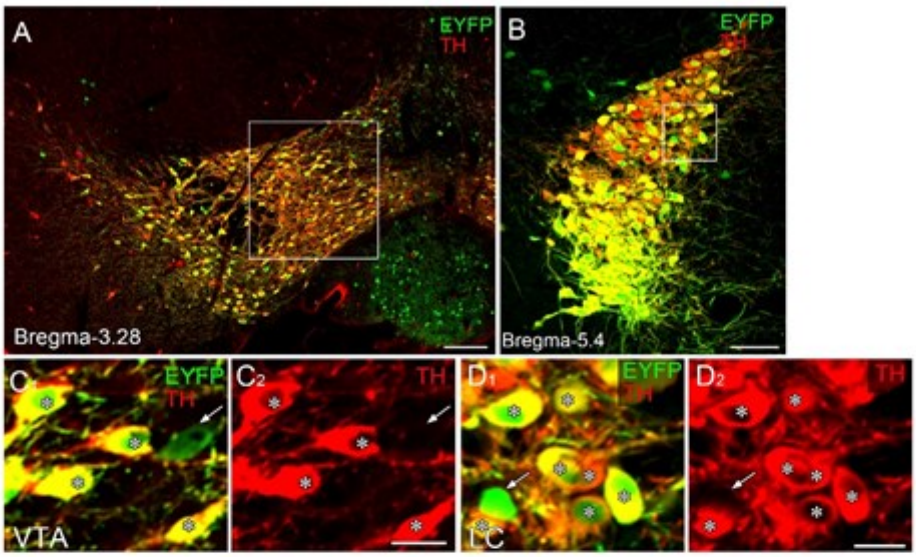
Methods used to address these aims are summarised in figure 2.8b.

Figure 2.8: Summary of methods used in the experiments included in this thesis.

(a) Cell-type specificity of optogenetic interventions was achieved by Cre-dependent expression of an adeno-associated virus (AAV) carrying ChR2-eYFP coding sequence. Expression of the viral vector in VTA and LC is highly specific to catecholaminergic cells. Quantification of viral expression was done with a eYFP-AAV control vector due to strong cytoplasmic signal. Mice used for quantification of viral expression were obtained from the same colony as mice used in the behavioural experiments. Green: eYFP, red: TH. Miwako Yamasaki and Tomonori Takeuchi, unpublished.

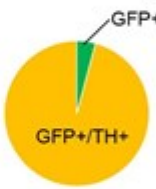
(b) Summary of methods used in each of the three studies described in this thesis.

a) Expression of Cre-dependent AAV in TH-Cre mice

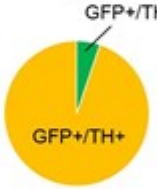


A, B, 200 um; C, D, 20 um

EFYP(+)/TH(+) / EYFP(+) 95.8 ± 2.9 % (VTA, 973 cells)



94.8 ± 1.3 % (LC, 906 cells)



b) Summary of methods

Step 1: Novelty effect on memory persistence in mice

Methods: Everyday apetitive DMP paradigm for mice
Novelty exploration paradigm for mice
Pharmacological blockade of hippocampal D1-like receptors

Step 2: Responses of VTA-DA and LC-NA neurons to novelty

Methods: Novelty exploration paradigm for mice
Single unit recording in behaving mice
Optogenetic identification of VTA-DA and LC-NA neurons *in vivo*

Step 3: Mimicry of the novelty effect by VTA-DA or LC-NA activation

Methods: Everyday apetitive DMP paradigm for mice
Optogenetic activation of VTA-DA or LC-NA neurons
Pharmacological blockade of hippocampal D1-like receptors

Chapter 3

Novelty Enhances Persistence of Everyday Memory in Mice

3.1 Introduction

Novel experiences have been shown to boost retention of transient memories encoded within a critical window of time (1-3 h) in a manner consistent with predictions of the Synaptic Tagging and Capture (STC) theory (Ballarini et al 2009, Moncada & Viola 2007, Redondo & Morris 2011, Wang et al 2010). This behavioural manifestation of the synaptic tagging and capture mechanism has been referred to as 'behavioural tagging'. Pharmacological studies of memory persistence in a variety of behavioural paradigms for rats have identified hippocampal D_1/D_5 dopamine receptors (Wang et al 2010) and β-adrenergic receptors (Moncada et al 2011) as critical mediators of the effect of novelty on persistence of unrelated memories. These findings, backed up by *in vitro* LTP experiments (Navakkode 2015), influenced formation of the neoHebbian framework for dopaminergic gating of memory persistence (Lisman et al 2011). This framework identifies ventral tegmental area (VTA) and locus coeruleus (LC) neurons as possible mediators of the novelty effect on retention of hippocampal memories.

A definite test of this hypothesis would involve a set of complementary 'behavioural tagging' experiments:

1) 'Mimicry' experiment where activation of either of these nuclei is substituted for novelty exploration.

2) 'Blocking' experiment, where activity in either of these brain areas is inhibited for the duration of novelty exploration.

The overarching aim of work described in this thesis is to use the optogenetic toolbox to characterise the neuromodulatory circuits that activate hippocampal dopamine and/or noradrenaline receptors during novelty exploration and thus promote initial memory consolidation in the hippocampus, with a particular focus on the 'mimicry' criterion.

The availability of numerous well characterised recombinase-driver mouse lines enables investigation of genetically and spatially-defined populations of neurons (such as neuromodulatory systems) on a scale that is unprecedented among mammalian organisms (Huang & Zeng 2013). Unfortunately, behavioural demonstrations of the tagging phenomenon have so far been limited to studies in rats and, to some degree, in humans (Ballarini et al 2013, Dunsmoor et al 2015). Recombinase-driver lines for the rat are becoming increasingly more available (Witten et al 2011), but the mouse is still at the apex of genetic flexibility in the laboratory setting. Designing a robust platform for investigation of behavioural tagging in mice would create new opportunities for the use of molecular genetic tools that in turn could shed light on the neuromodulatory circuitry involved in the initial consolidation in the hippocampus (Takeuchi et al 2014). A large body of evidence points to the role of dopamine (and, to a lesser degree, noradrenaline) in behavioural tagging. Accordingly, the group decided to use a Th-Cre mouse line, which expresses Cre recombinase in all populations of tyrosine hydroxylase-positive cells (including dopaminergic and noradrenergic neurons) (Lindeberg et al 2004), as a valuable tool for uncovering the potentially dissociable roles that these neuromodulatory systems play in novelty-associated enhancement of memory persistence.

The first step was to design a behavioural paradigm that demonstrates the tagging phenomenon in mice.

Investigation of everyday episodic-like memory in animal models poses a considerable challenge. Most widely used behavioural paradigms probe memory of events experienced by largely naïve animals, trained for no more than a couple of weeks. In contrast, episodic memories in real life situations are constantly encoded against the background of extensive past experience, which may influence the relative importance of newly encoded information. This background will likely influence the persistence of engrams in the brain. Behavioural paradigms that provide the animal with a similar kind of everyday experience but nonetheless enforce constant memory updating are particularly useful for examination of the cross-talk between 'strong' and 'weak' memories and thus for testing the behavioural predictions of the STC theory. Additional benefits of such longitudal studies lie in the opportunity to track each individual animal's performance over a

spectrum of experimental conditions (behavioural, pharmacological, optogenetic, etc.), and compare the effects of these manipulations with baseline performance. Wang and colleagues (2010) demonstrated behavioural relevance of the STC theory using a variant of a one-trial delayed matching to place (DMP) paradigm, previously developed by Bast et al. (2009), which involved rats searching for a food reward placed in one of 47 possible food locations in an event arena. Critical to the design of the study was the fact that food location changed every day, enforcing the requirement to encode the new location but eliminating the need to retain it in memory for more than a few hours. Using this protocol, it was possible to track individual animals over six months of daily training. Unexpected novelty exploration on occasional test days reliably enhanced persistence of these transient spatial memories, constituting a demonstration of one of the behavioural predictions of the STC theory.

The aim of this chapter is to establish an everyday spatial memory paradigm for mice, based on the study of Wang et al. (2010) and so set the stage for optogenetic interrogation of catecholaminergic circuits in relation to persistence of everyday memory. The critical hallmarks of a suitable experimental paradigm are:

1) Stable performance over many weeks of training

2) A 'weak' memory condition that can be rescued by unexpected novelty

3) Hippocampal D_1/D_5 and/or β-adrenergic receptor-dependence of the novelty effect

3.2 Methods

3.2.1 Subjects

The subjects were 13 tyrosine hydroxylase Th-IRES-Cre heterozygous male transgenic mice, backcrossed more than 20 times to the C57BL/6 strain (EM:00254) (Lindeberg et al 2004). The mice were at least 8 weeks old at the start of the behavioural experiment. The Th-IRES-Cre allele was identified by polymerase chain reaction using TH-P1 (5'-CCAAAGTTCCCAGCCCCTTCTCCAAC-3'), TH-P2 (5'-AACTGGTCGAGCGATGGATTTCCGT-3') and TH-P3 (5'-GCCAGGAACACTCCTGGAGACCTTTC-3'). Mice were food-restricted (85% of free-feeding weight monitored daily throughout the study, after behavioural training), given water *ad libidum* and kept under a 12 hour light/dark cycle. Behavioural testing was carried out during the light phase of the cycle. Mice were initially group-housed (2 mice per cage), and then transferred to individual cages after surgery. All procedures were compliant with the UK Animals (Scientific Procedures) Act 1986 and with the European Communities Council Directive of 24 November 1986 (86/609/EEC) legislation governing the maintenance of laboratory animals and their use in scientific experiments.

3.2.2 Behavioural apparatus

The event arena for mice

All behavioural experiments were conducted in an event arena – a square open field (120 x 120 cm) with walls made out of transparent Plexiglas (figure 3.1a). The name 'event arena' derives from it being an arena in which 'events' happen (e.g. finding food). Food was potentially available in sandwells which could occupy one or more of 25 possible locations in the floor of the arena, arranged in a 5 x 5 grid. The arena was covered with ~2 cm of sawdust and had two intramaze landmarks (a white metal cube located at row 3, column 2, and a black upright flash light at r3, c4). The Plexiglas sandwells could be fitted into any of the 23 remaining sandwell positions (positions occupied by internal cues were excluded); these were normally covered with plastic lids (level with the floor). Cheerios® cereals were used as food reward – half a Cheerio constituted one food pellet. Each sandwell had additional pellets placed under a metal mesh floor. These pellets were inaccessible to the animals and were placed there in order to minimise differences in olfactory cues between baited and non-baited sandwells. For bated sandwells, 4 pellets were placed on the metal mesh floor and sandwell was subsequently filled with bird sand (level with

the arena floor). Unbaited ('empty') sandwells were filled with bird sand but did not contain any accessible pellets.

The mice had access to the arena and sandwells from one of four start boxes on the perimeter of the event arena, one on each side. Start box walls and ceiling were made using black Plexiglas in order to limit the light levels inside (as if the start box was a kind of 'burrow'). The door, however, was made of transparent Plexiglas and could be opened and closed remotely by the experimenter. Mouse activity in start boxes was monitored by webcams (CCIR) installed in the start box ceiling and additional illumination for the camera was provided by infrared diode arrays. Additionally, each start box contained a pot with water and a small platform for cue pellets. The interior of the experimental room was designed to act as a polarising external cue, with walls painted white and a large, black window blind positioned north of the arena.

In addition, three 3-dimensional external cues (paper lampshades of different colours) were hanging from the ceiling at the level of the apparatus and adjacent to event arena walls (2 cues north-west and one cue south-west of the arena). Light level in the room was checked every day and was kept at 25-35 lux.

Activity in the arena was recorded by an overhead camera (CCIR) leading to a separate control room where computers and control equipment were located, together with the temporary holding space for the animals and sandwell preparation area. Data was recorded using custom-made Labview software (P. Spooner), using the image from camera placed above the arena.

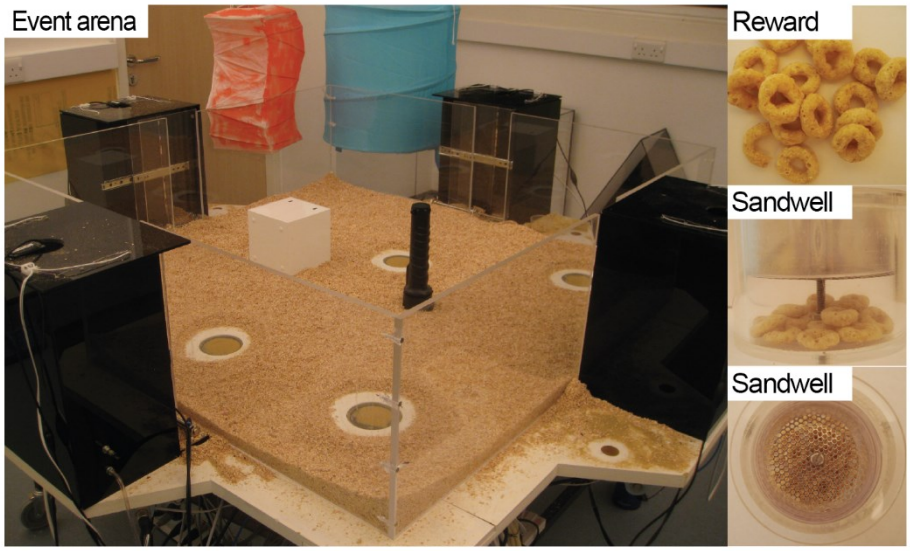
For novelty exploration, a square Plexiglas open field (80 x 80 cm) with transparent walls was placed in the middle of the event arena (figure 3.1b)). In order to maintain the novelty of the environment, a wide range of floor substrates that covered the floor of the box were used. This procedure had worked well with rats (Wang et al, 2010) and it was expected to also work for mice. Twenty different floor substrates were used during the experiment (with each substrate being used only once): rope pieces, shredded paper, shredded straws, lolly sticks, Lego blocks, pipe cleaners, cork pieces, ceramic baking beans, dried leaves, bamboo skewers, raffia, sea shells, feathers, bamboo leaves, cinnamon sticks, paper clips, packing foam, acrylic pom-poms, glass pebbles, and pine cones.

Figure 3.1: The event arena for mice.

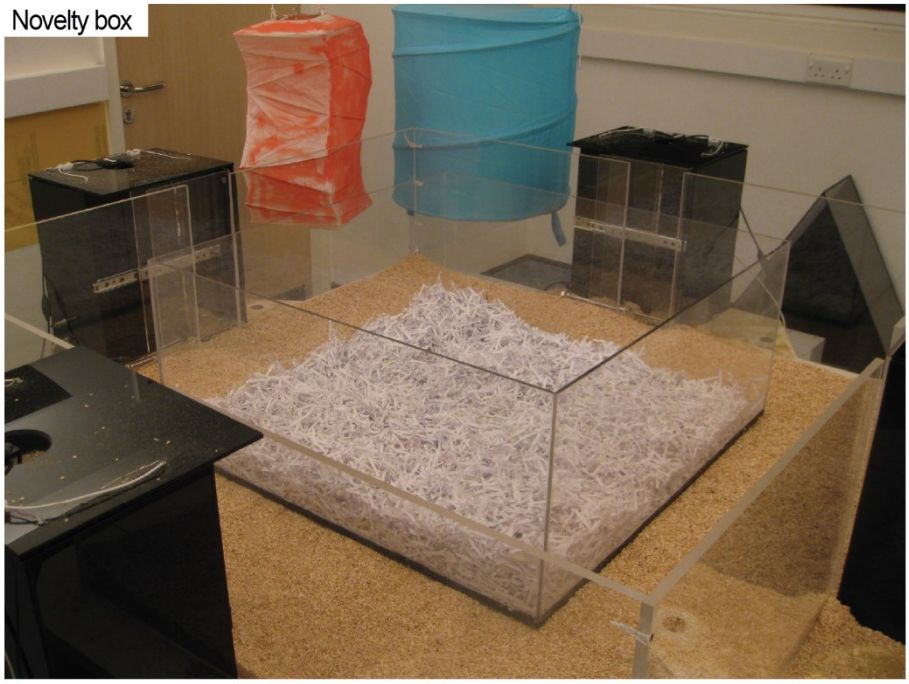
(a) The event arena with open sandwell positions. Four start boxes are located around the perimeter. Three-dimensional intra-, and extramaze cues were used to aid navigation around the arena. Twenty reward pellets were placed underneath a metal grid in each sandwell in order to mask olfactory cues.

(b) Novel open field used within the event arena, with the novel aspect of the exploration maintained by changing the floor substrate.

(a)



(b)



3.2.3 Training protocol

The behavioural protocol was adapted from (Wang et al 2010). The attempt to modify the protocol for use with mice while at the same time maintaining the one-trial nature of learning resulted in unstable performance (Tomonori Takeuchi, unpublished). Therefore, several additional modifications were made, including increased number of sample trials per day and increased number of food reward pellets available per sample trial.

Shaping and habituation

Mice were handled for 3 days before the start of food restriction and behavioural procedure. Habituation focused on teaching mice how to dig in the sand to retrieve food and how to carry it back to the start box. From day 1 to day 4, a sandwell-like dish with 5 pellets buried in sand was placed in the home cage overnight. Additionally, from day 4 onwards, 4 pellets were placed in a small dish inside the start box. On day 1 of habituation, mice were allowed to explore the entire open field (but not start boxes) for 2 x 60 minutes in groups of 8. On day 2, mice explored the arena for 2 x 10 min and explored start boxes (with closed door) for 3 x 10 min in groups of 2 mice. On day 3, mice explored the arena for 2 x 10 min with start box door open. They were encouraged to go back to start box after 10 min. On day 4, a sandwell with 4 pellets on top was placed at position (c3, r3) and mice explored the arena for 2 x 10 min with start box door open. This central sandwell position was not used during the subsequent training phases. On day 5, the same procedure as on day 4 was used with individual animals. Additionally, when they started eating a pellet they were encouraged to carry it back to the start box by gently tapping their tail. On days 6-8, the day 5 procedure was repeated but with some of the pellets buried deeper in the sand. By the end of day 8, most mice could retrieve 3 pellets from the bottom of the sandwell and carry them to the start box.

Everyday spatial memory protocol

The goal in each training session was to encode the position of a baited sandwell encountered during two consecutive sample trials, and then return to the same location during the choice trial. The choice trial was a retrieval test that involved a baited sandwell in a location that matched the sample location (the "correct" location; win-stay rule) in addition to 4 empty sandwells placed in other locations around the arena (the "wrong" locations) (figure 3.2a). Training sessions consisted of 2 sample trials followed by a choice trial and were conducted once daily, with 5-7 sessions per week.

Daily training protocol consisted of 2 sample trials followed by choice trial (intertrial interval ~ 10 min). Mice retrieved 2 food pellets in each trial (4 pellets per encoding session). In sample phase, the mouse was placed in the start box and allowed to explore the arena after ~1 min. Start of the trial was signalled by remote opening of the start box door by the experimenter. Once the mouse had retrieved one of the pellets from the sandwell and returned to the start box (or was encouraged to do so), the start box door was closed. The procedure was then repeated with the second pellet. After finishing the two-pellet sample trial, the animal was returned to its home cage. In the choice phase, a cue pellet was placed on the cue platform of the start box to help reactivate the memory trace. The start box door was opened after the mouse finished eating the cue pellet. During standard training sessions, food reward was buried in the same location as in the sample phase. When the mouse retrieved the pellet from the correct sandwell, it returned to the start box (or was encouraged to do so) and after it finished eating the procedure was repeated for the second pellet. If mouse could not retrieve the pellet after 10 min, a pellet was placed on top of the sandwell in order to make it easier for the mouse to locate it.

Before the start of the regular training, three days of easier 'pre-training' sessions were conducted in order to teach mice to follow the win-stay rule of the task. Similarly to normal training sessions, pre-training sessions consisted of two sample trials followed by a choice trial after 10 min delay. The difference was in the number of empty sandwells in the choice trial: only two sandwells were placed in wrong locations. The remainder of the procedure was the same as on regular training days.

Memory probe tests

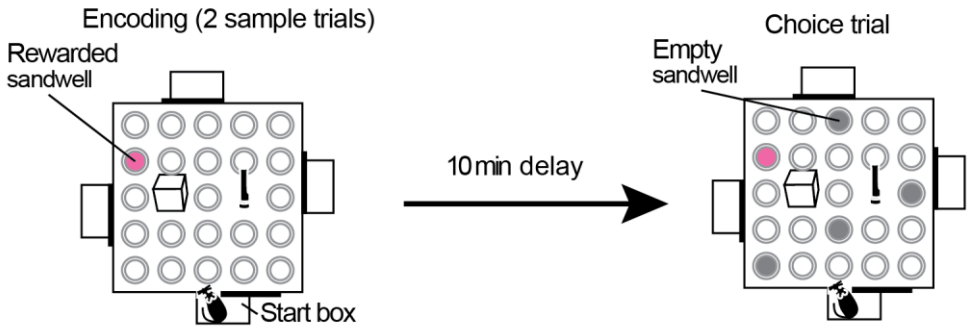
Critical memory tests were performed during 'probe' tests (that probed memory) – a session in which none of the sandwells contained any accessible food pellets. This was done to secure a highly sensitive "dig time" measure of memory (see below). The animals were allowed to search for the correct sandwell for 60 s, counted from the time it completely left the start box. After 60 s, the experimenter quietly entered the room and buried three pellets in the correct sandwell, allowing the mouse to retrieve them (one by one as in training). If the mouse did not dig in any of the sandwells during the first 60 s, the trial was extended by additional 60 s (this occurred rarely). As probe tests could be thought of as extinction trials, they were always separated by at least 2 days of regular training.

Figure 3.2: Daily training protocol on the event arena.

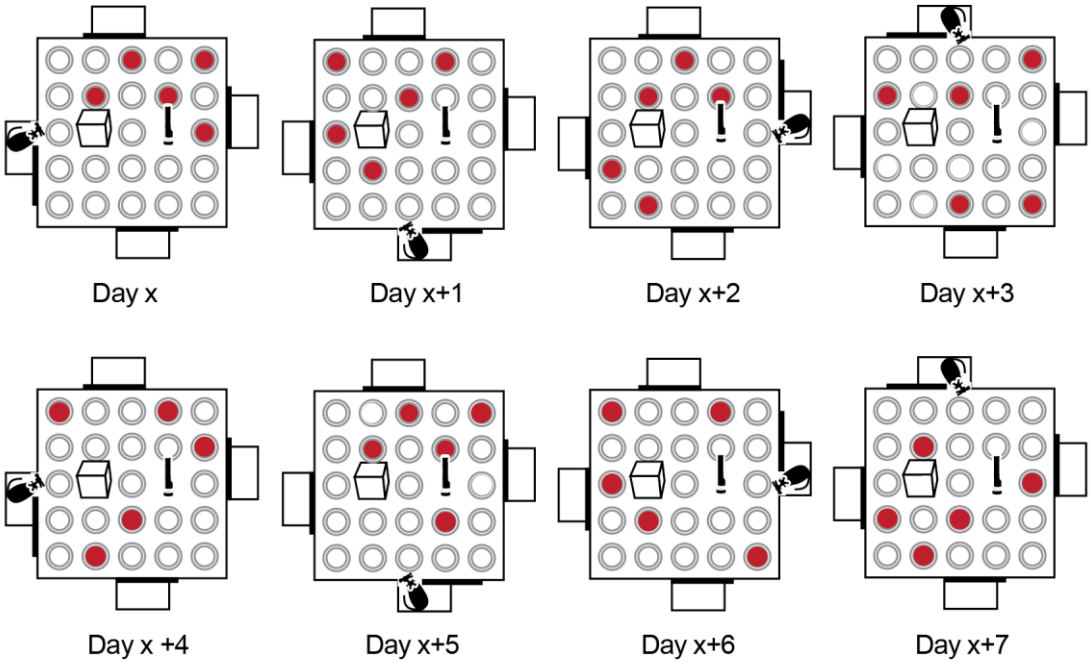
(a) Encoding (2 sample trials) and retrieval (choice trial) during training. Mice were trained to search for an open sandwell position during a sample trial and then go back to the same location during choice trial, ignoring the four empty sandwells. Each sample trial involved retrieval of two food pellets from the sandwell. Mice were encouraged to go back to the start box after they retrieved each pellet.

(b) Daily training schedule showing eight out of sixteen sandwell maps used on rotation throughout the main training phase. Starting position changed every day in a counter-clockwise direction.

(a)


Encoding (2 sample trials)
Rewarded sandwell
10 min delay
Choice trial
Empty sandwell
Start box

(b)


Day x
Day x+1
Day x+2
Day x+3
Day x +4
Day x+5
Day x+6
Day x+7

Throughout everyday memory training and testing, the starting location was the same throughout the daily session but changed every day in a clockwise pattern. On each training day one of 16 different possible sandwell configurations (maps) was used (figure 3.2b). Certain sandwell positions were excluded while designing the sequence of choice maps: the central sandwell position, three positions closest to the start box used on a given day, and all 5 sandwell positions used on the previous day. Additionally, sandwell locations in each choice map were never directly adjacent to each other. A single choice map was used for all mice on a given day, but the correct sandwell location was counterbalanced between subjects. Pilot tests (T. Takeuchi, unpublished observations) indicated that with a limited number of possible sandwell locations mice become very good at the task after extensive training, which results in 24 h memory even after 1 pellet encoding. Establishment of a stable weak memory condition is critical for this study, and therefore additional measures were taken in order to make sure that mice rarely experience the same sandwell location. Every time a map was used again, the correct sandwell location for each mouse was different from the previous time. Additionally, for every block of 16 training days each mouse experienced a particular sandwell location only once.

Behavioural training was performed jointly with Dr Tomonori Takeuchi.

3.2.4 Data collection and analysis

We used two measures of place memory during *regular training days*:

- **Latency to the start of digging in the correct sandwell, measured from the moment the mouse completely left the start box**
- **Number of errors, i.e. number of sandwells in wrong locations which the mouse dug before digging in the sandwell in correct location (measure taken only during choice trial)**

During *probe tests,* we recorded:

- **The time the mouse spent digging in each of the 5 sandwell locations.**

We defined "digging" as mouse putting both paws, as well as its snout into the sand, as seen on the image from the overhead camera. As an additional confirmation, we checked all sandwells for traces of digging after the choice trial.

For clarity, we computed a 'performance index' (PI) for each mouse on each training day.

- **Performance index was defined as the number of errors made during choice trial divided by the maximum number of errors and expressed as a percentage:** $\frac{number\ of\ errors}{max\ number\ of\ errors} \times 100\%$**.**

The maximum number of errors was **two** in pre-training sessions (three available sandwells) and **four** in training sessions (five available sandwells). Additionally, we normalized the digging time measure for each animal by expressing it as a percentage of total digging time during the 60 s retrieval phase of the probe test. The value expected for chance performance was 50% (in the PI) during training days, and 20% dig time in the correct sandwell location during probe tests.

It should be noted that a key design feature of the everyday memory test is that each animal serves as its own control with diverse test conditions repeated across days using a counterbalanced design. This within-subject design minimises animal use and enables the use of within-subject estimates of variability. Accordingly, variables in behavioural measures were analysed using repeated measures analysis of variance (ANOVA) or paired samples Student's t-tests. Assessment of performance on individual training days, probe tests or in single conditions was also done by comparison against the chance value of 20% using one-sample Student's t-tests.

3.2.5 Surgery

Delivery of drugs involved bilateral infusions into the dorsal hippocampus through previously implanted 26-gauge steel guide cannulae (2.5 mm length, 3.0 mm distance between cannulae; Plastics One) with stylets (33 gauge, Plastics One) that protruded 0.5 mm below the end of the cannulae.

Anaesthesia was induced using 5% isoflurane and mice were placed in the stereotactic frame. From this point onwards, anesthesia was maintained with 1-2% isoflurane. Air-flow was kept at 1000 ml/min throughout the surgical procedure. Dorsal surface of the skull was levelled to make sure that dorso-ventral (DV) co-ordinates of bregma and lambda points are less than 30 μm apart along the DV axis. Two holes (0.5 mm diameter) were drilled in the skull bilaterally above the dorsal hippocampus and underlying dura was removed. Three jeweller screws were placed in the skull in order to secure the head cap in place. The cannula implantation co-ordinates were (from bregma): anterior-posterior (AP), -2.10 mm; mediolateral (ML), ±1.50 mm; and dorsal-ventral (DV), -2.0 mm). Skull was covered with a layer of Metabond dental cement (C&B) followed by

extra layers of standard acrylic dental cement. Animals were given a subcutaneous injection of carprofen 1 hour before the end of surgery. The whole procedure took 2 hours to complete. Implantation surgery was performed after the first block of novelty probe tests (see: experimental design). Mouse weight was monitored daily until they regained their pre-operative weight.

Implantation of hippocampal cannulae was performed by Dr Tomonori Takeuchi.

3.2.6 Microinfusions and drugs

Bilateral infusion of drugs was done in home cages placed in the animal holding area (room adjacent to the testing room). The stylets in the guide cannulae were replaced by a double infusion cannula (33 gauge, Plastics One) connected to two 5 µl microsyringes (World Precision Instruments) in a microinfusion pump (Native Instruments) via flexible plastic tubing (C232CS, Plastics One) filled with Fluorinert. The tips of infusion cannulae projected 0.5 mm below the tip of the guide cannulae. Each microinjection involved 0.5 µl of drug injected per cannula, infused at a rate of 0.20 µl/min (2.5 min total infusion time). Infusion cannulas were left in place for additional 2.5 min after the end of infusion before being replaced with stylets in order to aid drug absorption. The whole procedure took 7 minutes to complete. Mice were habituated to the apparatus for six days and were given two test saline infusions before the drug infusion probe tests started. Habituation procedure was identical to the drug microinfusion procedure during subsequent probe tests, except that no drug was infused into the hippocampus.

Drug concentrations for infusions were 21.1 mM for propranolol ((S)-(-)-Propranolol hydrochloride, Tocris) (corresponding to 6.25 µg/µl) and two concentrations of SCH23390 ((R)-(+)-SCH 23390 hydrochloride, Tocris, Bristol, UK): 1.5 mM (corresponding to 0.5 µg/µl) and 3 mM (corresponding to 1 µg/µl). We used 0.9% NaCl (saline) in H_2O as a vehicle and for control infusions. Both vehicle and drug solutions were stored in 100 µl aliquots at -20°C until use.

3.2.7 Experimental design

The longitudal or repeated-measures design of the study made it possible to examine the effects of different manipulations in a within-subject fashion. The experiment was divided into blocks of probe tests in which mice experienced different experimental conditions in counterbalanced order, and these conducted against a backdrop of interspersed training days in which the stability of performance could be monitored.

After shaping, habituation and pre-training (12 days), mice were subjected to a series of 150 training sessions (1 session per day) conducted over 7 months. The experiment was divided into 4 blocks:

- **initial training (session 1 – 24),**
- **strong and weak encoding (session 25 – 45),**
- **weak encoding + novelty (session 46 – 65), and**
- **weak encoding + drug microinjection + novelty (session 66 – 150).**

Sessions 1-24: This block included three probe test sessions with the aim of characterising the acquisition phase of the task (probe test 1 – session 2, probe test – session 13, probe test 3 – session 24). Each probe test in this block mimicked a standard training day and consisted of 2 sample sessions (2 pellets each, 5 min intertrial interval) and a retrieval session after 10 min delay.

Sessions 25-45: The impact of reward magnitude on persistence of spatial memory was assessed. Mice were given 1, 2 or 4 sample trials (2 pellets each, 5 min intertrial interval) to encode position of the correct sandwell and were tested after a 1h or 24 h delay.

Sessions 46-65: These sessions examined the effect of novelty exploration after encoding on strength of 1 sample trial memory at two delay time points: 1 h and 24 h. Novelty exploration started 30 min after the start of the encoding trial and lasted for 5 min.

Sessions 65-150: Finally, we looked at the effect of intrahippocampal infusion of propranolol and SCH23990 20 min before novelty exploration (10 min after the start of encoding) on memory strength after a 24 h delay. Each probe test in this block was performed by two experimenters – the person who was handling animals during the task and scoring their performance was blind to the kind of drug the animal was microinjected with. We saw a significant reduction in mouse motor activity during novelty exploration after infusion of 3.0 mM SCH23390, and therefore decided to replicate the drug infusion block using a lower concentration of SCH23390 (1.5 mM). Drug infusion probe tests included 3 more drug conditions (for a total of 6 conditions per block): KN-93 - an inhibitor of CaM kinase II activation, KN-92 – an inactive derivative of KN-93, and dimethyl sulfoxide (DMSO) in saline (vehicle and control for KN-92 and KN-93). KN-93 was predicted to block tag setting during encoding and all three drugs were initially a part of a 'double dissociation' design with the same drugs infused either before novelty exploration or before encoding. Because of the need to repeat the first block of drug infusions, this design was abandoned half-way through this very long phase of the experiment. None of those infusions had

a detectable effect on mouse performance and, since they are not directly relevant to the catecholaminergic control of initial memory consolidationn discussed in this chapter, they are only used in the meta-analysis of mouse performance.

3.2.8 Perfusion and histology

After the experiment, animals were injected with euthatal and perfused transcardially with 4% paraformaldehyde (PFA) dissolved in PBS. Brains were removed and post-fixed in 4% PFA for a minimum of 24 h. Coronal sections (30 μm) were cut using a cryostat with one in every 5 sections recovered for histological analysis. Sections were then mounted on slides, stained with cresyl violet and coverslipped using DPX. The sections were examined with a light microscope under 20-fold magnification to verify cannula placements. Appropriate locations were then marked on the appropriate coronal sections from the Franklin and Paxinos mouse brain atlas (Franklin & Paxinos 1997).

3.3 Results

Mice were trained on a delayed match to place task in the event arena, where the goal was to search for food reward placed in one of twenty three possible locations in the open field. Each training day consisted of two encoding trials followed by a retrieval trial. During each encoding trial mice had the opportunity to encode the position of a baited sandwell containing two food pellets. Retrieval trial was conducted 10 minutes after the end of the second encoding trial (a total of 4 retrieved pellets), and included 4 non-rewarded sandwells scattered around the arena in addition to a rewarded sandwell placed in the location that matched that on the encoding trial. When mice achieved a stable performance level (less than 0.5 errors per day, after ~30 daily training sessions) several blocks of counterbalanced probe tests with different "conditions" were conducted over a period of six months.

Due to the longitudinal nature of the study there was an increased risk of losing animals due to illness, scarring of the implant, etc., and 1 animal had to be eliminated due to excessive scarring after training session 125. This change in number of subjects is reflected in changing number of reported degrees of freedom.

3.3.1 Task acquisition

Mice successfully learned the win-stay rule of the delayed matching to place task over several training days, as evidenced by the above chance performance during retrieval as well decreasing latency to the correct sandwell in later training sessions. Additionally, probe tests were conducted at three different time points during the acquisition phase, and established that (i) mice did not show any significant preference for the correct sandwell at the beginning of training, and (ii) by day 24, the animals had acquired the task, demonstrating a strong preference for the correct sandwell. Performance was stable for the duration of the experiment once the task rules were acquired.

Mice did not show preference for the correct sandwell at the beginning of training. Their performance on the three pre-training days was at chance level for both the first (one sample t-test, $t_{(12)} = 1.10$, $p > 0.05$) and the second pellet (one sample t-test, $t_{(12)} = 2.14$, $p > 0.05$) (Figure 3.3a). In contrast, their latency for the first pellet significantly decreased form pre-training day 1 to pre-training day 2 (repeated measures ANOVA, $F_{(2,24)} = 5.70$, $p < 0.01$; Turkey's multiple comparison test, day 2 < day 1, $p < 0.01$) (Figure 3.3b), possibly reflecting adaptation to changing reward location. There was no such effect on latency to retrieve the second pellet (repeated

measures ANOVA, $F_{(2,24)} = 0.43$, $p < 0.05$), indicating that mice quickly learned to update their memory of reward location despite several days of habituation with the central sandwell (figure 3.3c). Mice improved their performance during the acquisition phase of training (training days 1-27). There was a significant effect of day on performance index for both the first (repeated measures ANOVA, $F_{(23,276)} = 1.63$, $p < 0.05$) (figure 3.3d) and the second pellet (repeated measures ANOVA, $F_{(23,276)} = 2.26$, $p < 0.01$) (figure 3.3e). First pellet performance comparison against the chance level for the first 27 training days (excluding probe test days) shows that after chance performance on day 1 and 3, mice reliably scored above chance (with the exception of day 19). From day 20 onwards both first and second pellet scores reached a stable level above 70%, indicating that by then mice acquired the win-stay rule of the task. Similarly, there was a decrease in latency to dig in the correct sandwell as the training progressed (repeated measures ANOVA; first pellet: $F_{(23,276)} = 3.60$, $p < 0.0001$; second pellet: $F_{(23,276)} = 2.70$, $p < 0.0001$) (figure 3.4a and 3.4b).

As an additional quantification of the learning curve, we conducted a series of three unrewarded probe tests at different stages of the acquisition phase (day 2, day 13 and day 24, figure 3.4d and 3.4e). The encoding phase of the probe tests, as well as the delay between encoding and retrieval, matched the structure of training days (2 x 2 pellets, 10 min delay). Though mice did not show any sign of memory for reward location at the very early stage of acquisition (probe test 1), they progressively developed preference for the correct sandwell location during probe tests 2 and 3.

Overall, the mice progressively developed preference for the correct sandwell location over the course of three probe tests (repeated measures ANOVA, $F_{(2,24)} = 9.35$, $p < 0.001$, linear component, $F_{(1,12)} = 21.83$, $p < 0.001$) As expected, they did not preferentially dig in the correct location during the first probe test, conducted on day 2 of training ($t_{(12)} = 1.61$, $p > 0.05$; 1 out of 13 mice with preference $> 40\%$). There was a non-significant trend for preferential digging in probe test 2 ($t_{(12)} = 2.13$, $p = 0.054$, 7 out of 13 mice scoring $> 40\%$), which likely reflects variability in individual learning curves. By day 24, performance during probe test stabilised and became much higher than chance ($t_{(12)} = 4.76$, $p < 0.001$; 9 out of 13 mice scoring $> 40\%$), reflecting successful acquisition of all the task rules.

Figure 3.3: Acquisition of everyday spatial memory task – pre-training and performance index.

(a) Average performance index during three days of pre-training did not differ from chance.

(b) and (c) Latency to retrieve the (b) first and (c) second pellet during retrieval trial on 3 pre-training days

(d) and (e) Performance index during retrieval of the first (d) and second (e) pellet during the first 27 training days. Mice exhibited above chance performance on most training days, stabilizing by the fourth week.

* $p < 0.05$. ** $p < 0.01$, *** $p < 0.001$. Error bars represent ±1 S.E.M. Dashed lines indicate chance levels.

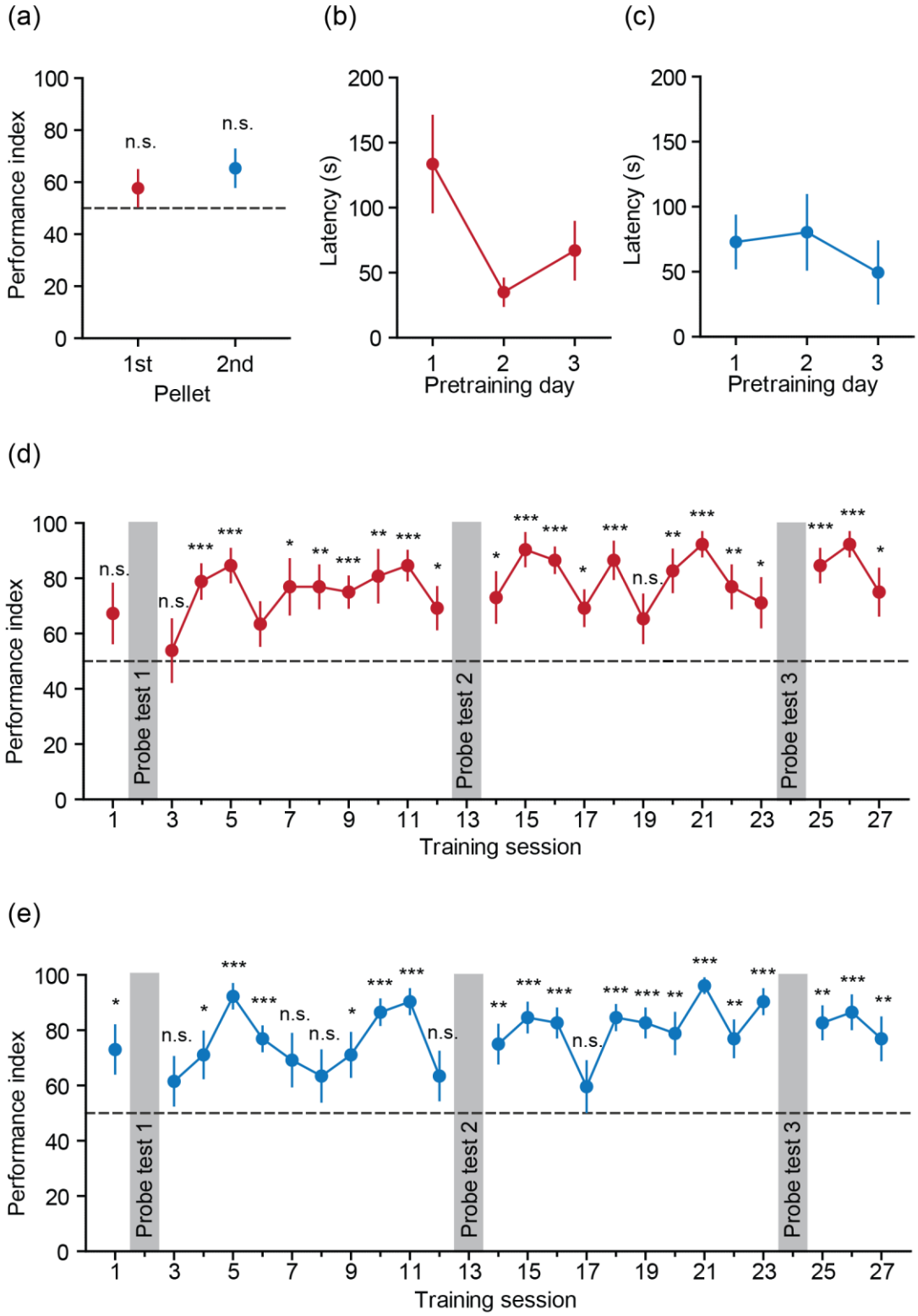

(a)
(b)
(c)
Performance index
Latency (s)
n.s.
1st
2nd
Pellet
Pretraining day
(d)
(e)
Probe test 1
Probe test 2
Probe test 3
Training session

Figure 3.4: Acquisition of everyday spatial memory task – latency and probe tests.

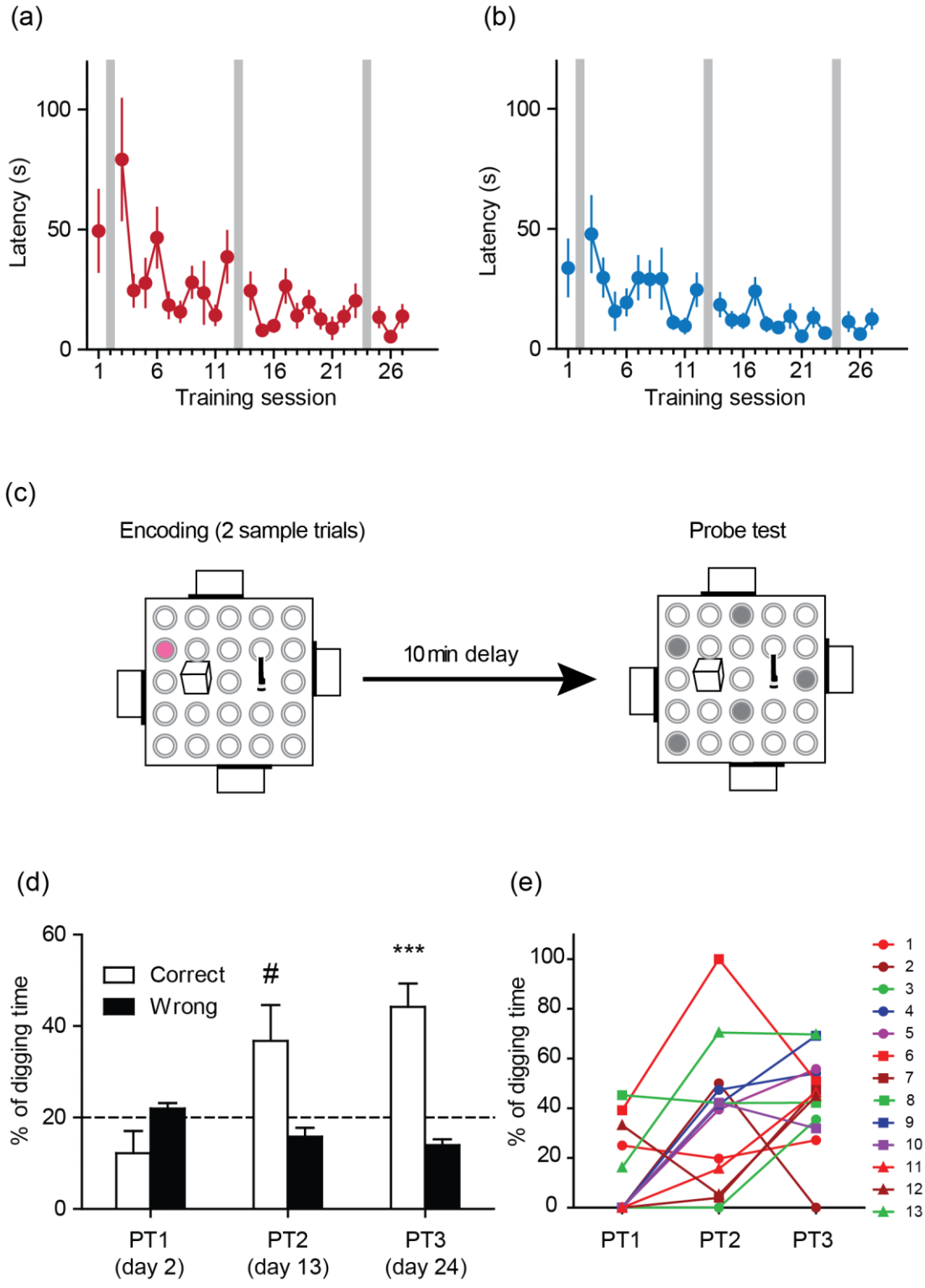
(a) and (b) Latency to retrieve (a) first and (b) second pellet during retrieval decreases in the first 3 weeks before reaching a steady state. Grey vertical bars represent probe test days.

(c) Protocol during probe test (PT) days 1-3. The encoding phase was conducted in the same way as during regular training days. During retrieval phase all 5 sandwells were empty so that a more sensitive measure of memory could be used.

(d) Animals developed preference for digging in the correct location during the acquisition phase of the experiment

(e) By PT3 12 out of 13 animals showed above chance preference for the correct location.

$p = 0.054$, *** $p < 0.001$. Error bars represent ±1 S.E.M. Dashed lines indicate chance levels.


(a)
Latency (s)
100
50
0
1
6
11
16
21
26
Training session
(b)
Latency (s)
100
50
0
1
6
11
16
21
26
Training session
(c)
Encoding (2 sample trials)
10 min delay
Probe test
(d)
% of digging time
60
40
20
0
Correct
Wrong
#
***
PT1
(day 2)
PT2
(day 13)
PT3
(day 24)
(e)
% of digging time
100
80
60
40
20
0
PT1
PT2
PT3
1
2
3
4
5
6
7
8
9
10
11
12
13

3.3.2 Stable performance over months of training

The qualitative picture, after the initial stages of training, was as follows. The mice would be active in the start box, as if waiting for the trial to start. Once the door was opened, they would pause at the entrance way to the arena and then run out, sometimes beside but often away from the side-walls, and typically find the single 'sample' sandwell in 10-15 sec. They would dig energetically in the sand, leaving a significant fraction of the sand on the floor surface adjacent, until they found the half-cheerio reward. Immediately, they would turn around and return to the start-box to eat it. Performance on the choice trial was consistently above 80% correct, for day after day. This means that the animals rarely dug at other sandwells than the correct one – though they did sometimes visit other wells. Again, once the food-pellet was found after digging, they would scurry back to the start-box to eat it.

The longitudinal, within-subject design of the study makes it possible to draw comparisons between probe tests conducted at different time points, provided mouse performance stays constant throughout the whole experiment. Indeed, after the initial rise in performance index from session 1 to 27, mice reached asymptotic performance on both the first and the second pellet, and performed at a stable level until the end of behavioural training (figure 3.5a and 3.5b), averaging 89.1% for the first pellet and, coincidentally, 89.1% for the second pellet (sessions 27 - 150).

3.3.3 Chance performance in non-encoding trials

In order to check whether high performance levels observed during training reflect genuine memory of the food location and are not due to cryptic olfactory artefacts, it was essential to conduct relevant control tests predicted to result in chance performance. These were rewarded choice tests that had not been preceded by an encoding trial. The first non-encoding test was performed as a single training day and was not counterbalanced. The second non-encoding test was performed at the end of the experiment and consisted of 2 counterbalanced training sessions with two conditions: 2 sample trials followed by retrieval after 10 min delay or retrieval trial not preceded by sample trials. Animals performed at chance level in both non-encoding tests, indicating that they cannot locate the reward sandwell using olfactory cues.

Figure 5c shows average performance index for sessions 61 – 65, with session 63 being the non-encoding session. Repeated measures ANOVA showed a significant effect of training day between sessions 61 and 65 ($F_{(4,48)} = 3.63$, $p < 0.05$). When compared against chance level, animals performed at chance level on the non-encoding day (one sample t-test, $t_{(12)} = 0.96$, $p > 0.05$),

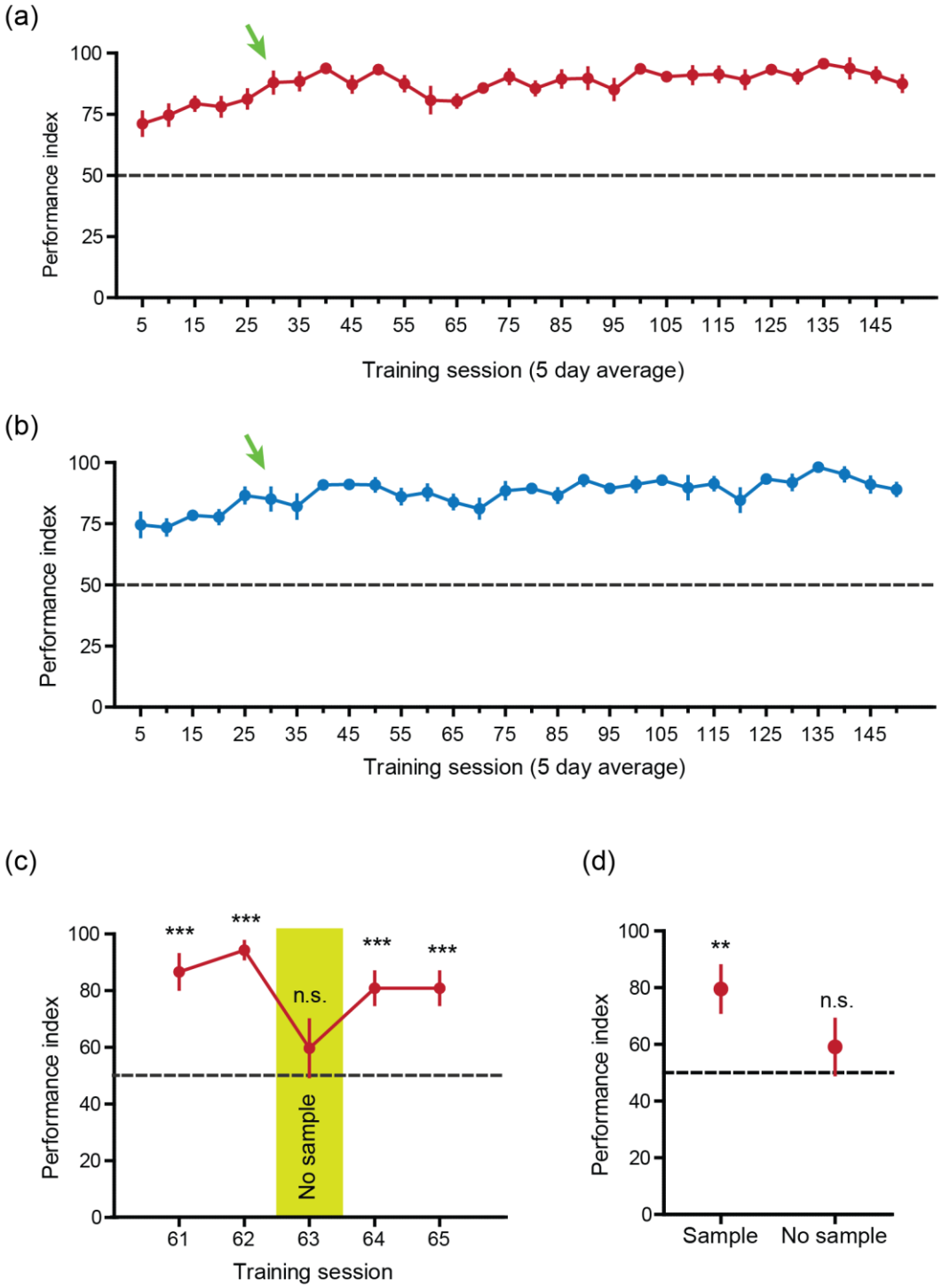
Figure 3.5: Performance throughout training and on non-rewarded training days.

(a) and (b) 5-day average of (a) first and (b) second pellet performance index during retrieval sessions on normal training days. Green arrows mark the point in time when critical probe tests began. Animals maintained stable performance levels for the duration of the main experiment phase.

(c) A no-sample training day conducted in order to establish whether animal performance is based on memory or cues (e.g. olfactory) coming from the rewarded sandwell.

(d) Second no-sample test, conducted at the end of the experiment, with both conditions counterbalanced across two training days. This was done in control for possible confounding effects of sandwell map difficulty on a given training day.

** $p < 0.01$, *** $p < 0.001$. Error bars represent ±1 S.E.M. Dashed lines indicate chance levels.


(a)
Performance index
100
75
50
25
0
5 15 25 35 45 55 65 75 85 95 105 115 125 135 145
Training session (5 day average)
(b)
Performance index
100
75
50
25
0
5 15 25 35 45 55 65 75 85 95 105 115 125 135 145
Training session (5 day average)
(c)
Performance index
100
80
60
40
20
0
***
***
n.s.
***
***
No sample
61 62 63 64 65
Training session
(d)
Performance index
100
80
60
40
20
0
**
n.s.
Sample
No sample

while on days 61-62 and 64-65 performance was significantly above chance (one sample t-test, all $p < 0.001$). In order to exclude the possibility that chance performance was a result of a particularly difficult map/sandwell position combination, another non-encoding test was performed at the end of the study, this time counterbalancing encoding and non-encoding conditions between two days (figure 3.5d). The result closely mimicked the earlier non-encoding test, with animals scoring above chance on retrieval trials preceded by encoding (one sample t-test, $t_{(10)}=3.63$, $p<0.01$), but not on retrieval trials without encoding (one sample t-test, $t_{(10)}=0.94$, $p>0.05$). Chance performance during the two non-sample tests indicates that mice did not use olfactory cues to find the reward during retrieval trials.

Overall, mice learned to perform the task during the first 27 training sessions, consistently showing above chance performance as measured by the number of errors made during standard training days and percentage of time spent digging in the correct sandwell during PT3. Furthermore, once mice achieved high and stable performance level, they maintained it for the duration of the arena experiment. This high average performance level of less than 1 error every 2 training days was not due to olfactory cues from the rewarded sandwell, as illustrated by the two non-sample tests performed at different stages of training.

3.3.4 Persistence of memory depends on the number of encoding trials

A critical step in investigation of the behavioural tagging phenomenon is establishment of a 'weak memory' condition which produces a memory that can be detected shortly after encoding but decays to baseline during the course of several hours, mimicking the temporal dynamics of early LTP. Once mouse performance during training was deemed stable, a series of counterbalanced probe tests was conducted in order to investigate the effect of the number of encoding trials on memory persistence. Mice were given 1, 2 or 4 sample trials during encoding, and were tested at two intervals (1 h and 24 h), resulting in a total number of six counterbalanced experimental conditions. While 2 and 4 sample trials resulted in significant preference for the correct sandwell position after 1 h as well as 24 h, 1 sample trial during encoding produced a robust weak memory condition, with strong preference for the correct sandwell after 1 h chance performance after 24 h.

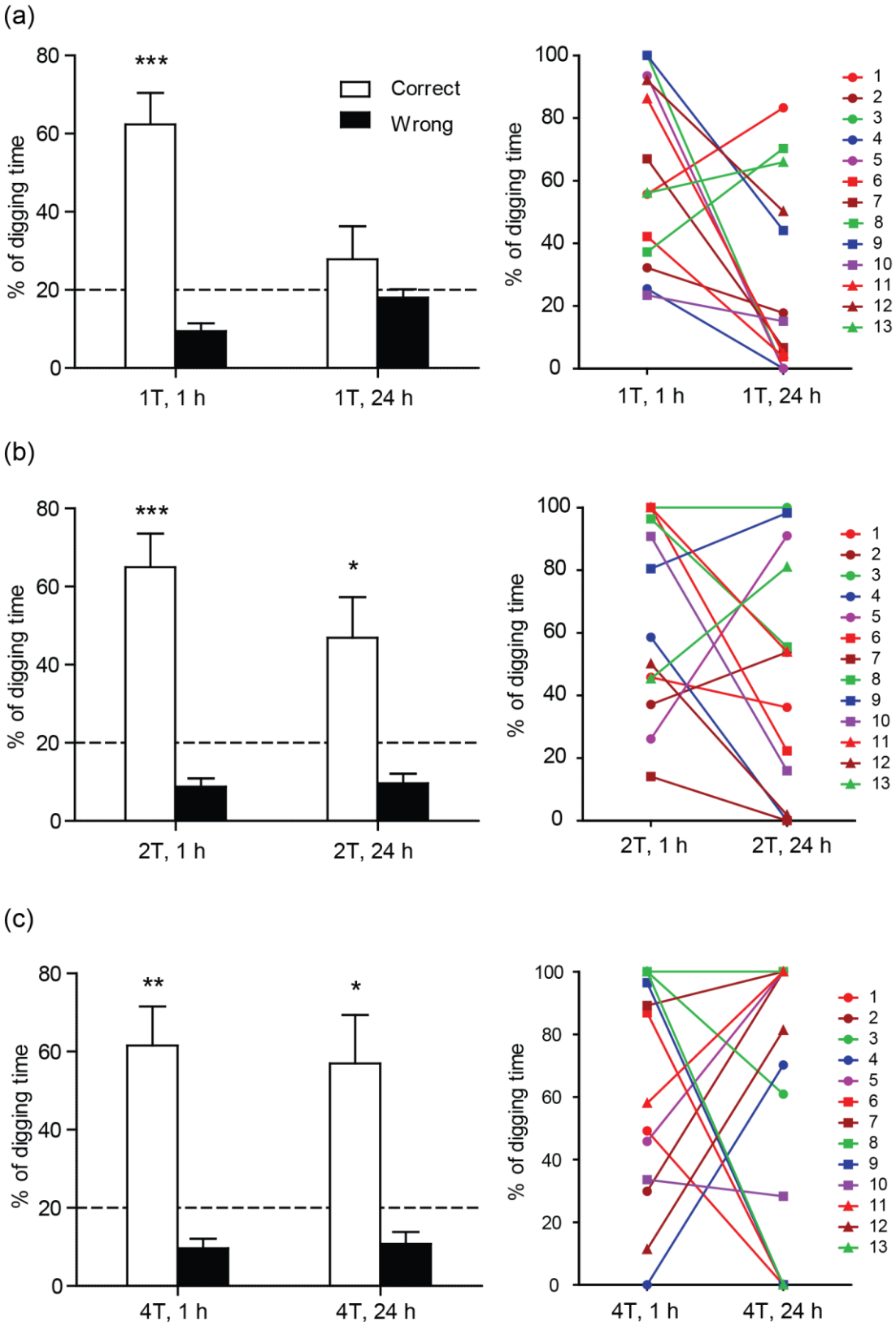
In order to quantify the temporal dynamics of memory traces formed as a result of different number of encoding trials, we compared animals' scores at two time intervals for each number of

Figure 3.6: Relationship between number of sample trials and memory retention.

(a) One sample trial encoding results in a sharp memory gradient. Memory for the correct location is detectable after 1 h but decays to baseline after 24 h.

(b) and (c) Increasing the number of samples to (b) two or (c) four results in persistent memory. In both cases mice show high preference for the correct sandwell after 24 h.

1T, 2T, 4T – number of encoding trials. * $p < 0.05$. ** $p < 0.01$, *** $p < 0.001$. Error bars represent ±1 S.E.M. Dashed lines indicate chance levels.


(a)
% of digging time
80
60
40
20
0
***
Correct
Wrong
1T, 1 h
1T, 24 h
100
80
60
40
20
0
1T, 1 h
1T, 24 h
1
2
3
4
5
6
7
8
9
10
11
12
13
(b)
% of digging time
***
*
2T, 1 h
2T, 24 h
2T, 1 h
2T, 24 h
(c)
% of digging time
**
*
4T, 1 h
4T, 24 h
4T, 1 h
4T, 24 h

sample trials. One sample trial resulted in lower preference for the correct sandwell location after 24 h than after 1h (paired t-test, $t_{(12)} = 2.86$, $p < 0.05$, 10 out of 13 animals showing decrease of preference) (figure 3.6a). Comparison of animal scores against chance level indicated significant preference for the correct location after 1 h (one sample t-test, $t_{(12)} = 5.25$, $p < 0.001$) that decayed to baseline after 24 h (one sample t-test, $t_{(12)} = 0.92$, $p > 0.05$). Two sample trials resulted in similar preference scores at both intervals (paired t-test, $t_{(12)} = 1.48$, $p > 0.05$, 8 out of 13 animals showing decrease of preference) (figure 3.6b), and comparison against chance level indicated significant preference after 1h (one sample t-test, $t_{(12)} = 5.25$, $p < 0.001$) as well as after 24h (one sample t-test, $t_{(12)} = 2.59$, $p < 0.05$). Similarly, with four sample trials, preference levels at two time intervals did not differ (paired t-test, $t_{(12)} = 0.26$, $p > 0.05$, 7 out of 13 animals showing decrease of preference)(figure 3.6c) and were both significantly higher than chance (one sample t-test; 1 h: $t_{(12)} = 4.16$, $p < 0.01$; 24 h: $t_{(12)} = 2.99$, $p < 0.05$). Taken together, the above comparisons indicate that one sample trial encoding results in a digging preference gradient that corresponds to a weak memory. Therefore, 1 sample encoding was used in all subsequent tests of memory persistence in this experiment.

3.3.5 Novelty exploration enhances memory persistence

Next step was to determine whether novel experiences can boost retention of unrelated spatial memories encoded close in time. In another set of counterbalanced probe tests mice were allowed to explore a novel open field 30 min after weak encoding (figure 3.7a) and retrieval session was conducted either 1 h or 24 h after encoding. As a control condition, mice were left in their home cages. We maintained the novel nature of exploration by changing floor substrates in the open field. While novelty exploration had no effect on preference for the correct sandwell location after 1h, after 24 h delay the mice in the novelty condition still showed high preference for the correct location while mice in the control condition (without novelty) performed at chance level.

There was no significant effect of novelty after 1 h post-encoding (paired t-test; $t_{(12)} = 0.91$, $p > 0.05$), with animals scoring above chance in both conditions (one-sample t-test; novelty: $t_{(12)} = 2.48$, $p < 0.05$; no novelty: $t_{(12)} = 4.04$, $p < 0.01$) (figure 3.7b). In contract, when tested after 24 hours, mice showed higher preference for correct digging if encoding was followed by novelty (paired t-test; $t_{(12)} = 2.24$, $p < 0.05$). When compared against chance, mouse performance was at baseline level without novelty (one-sample t-test; $t_{(12)} = 0.72$, $p > 0.05$) but differed from chance when encoding was followed by novelty exploration (one-sample t-test; $t_{(12)} = 3.17$, $p < 0.01$) (figure 3.7c).

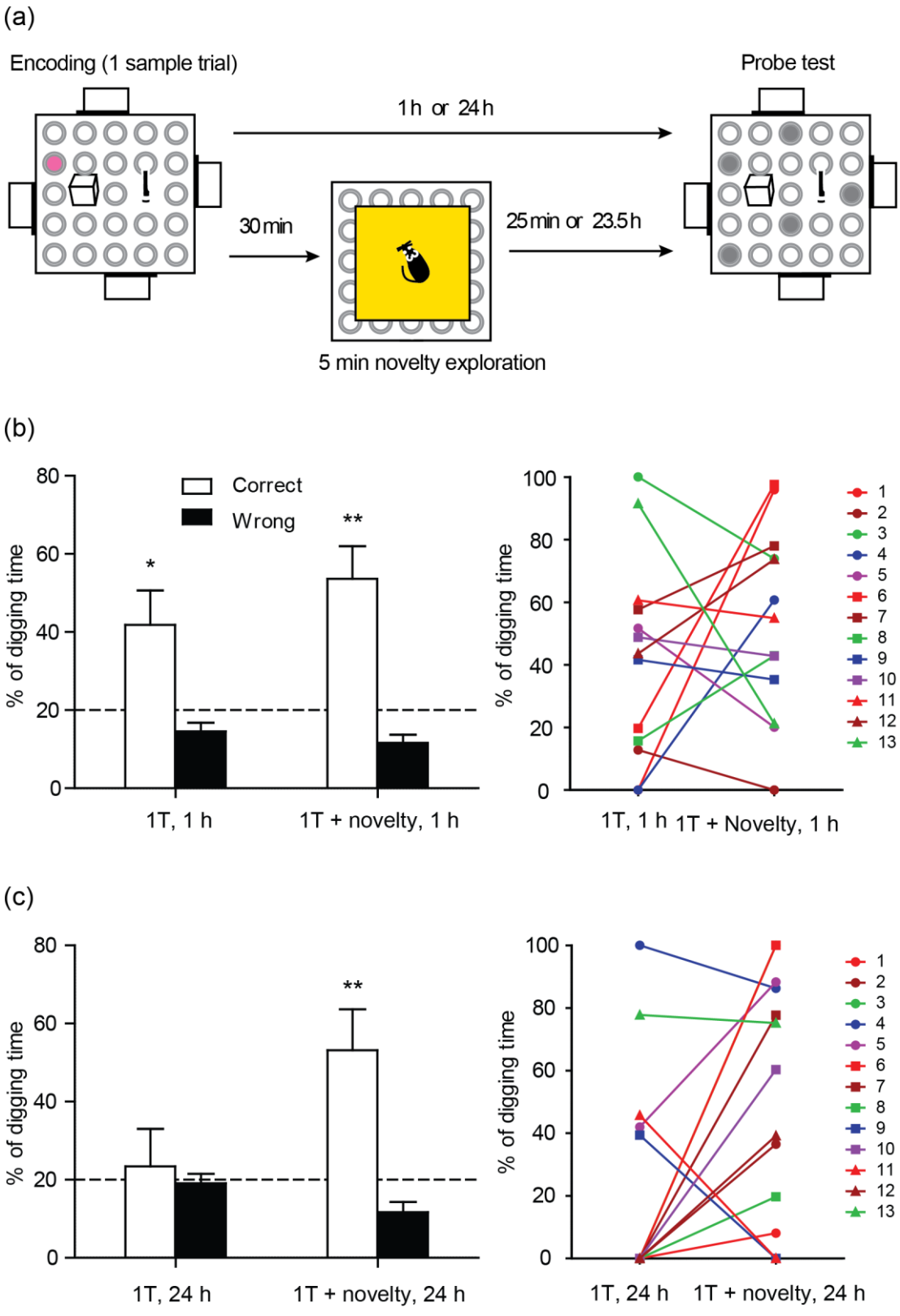
Figure 3.7: Persistence of spatial memory is enhanced by subsequent exploration of a novel open field.

(a) Protocol used in novelty probe tests. After one sample encoding mice either explored the novel open field or stayed in their home cages. Probe tests were conducted either 1h or 24 h after encoding.

(b) Novelty has no effect on 1 h memory, mice performed significantly higher than chance in both conditions.

(c) Novelty exploration boosts memory retention. Mice that explored the novel open field performed significantly higher than chance 24 h after encoding. Nine out of thirteen mice showed higher preference for correct sandwell position when encoding was followed by novelty exploration.

* $p < 0.05$. ** $p < 0.01$. Error bars represent ±1 S.E.M. Dashed lines indicate chance levels


(a)
Encoding (1 sample trial)
Probe test
1 h or 24 h
30 min
25 min or 23.5 h
5 min novelty exploration
(b)
Correct
Wrong
% of digging time
1T, 1 h
1T + novelty, 1 h
1T + Novelty, 1 h
(c)
1T, 24 h
1T + novelty, 24 h

3.3.6 The novelty effect requires activation of hippocampal D_1/D_5 receptors

We then asked whether the effect of novelty on memory persistence could be blocked by pharmacological blockade of hippocampal D1/D5 and/or β-adrenergic receptors. Mice were implanted with bilateral cannulae directed at the dorsal hippocampus (see figures 3.8a and 3.8b for histological identification of cannula positions). After mice recovered from surgery, a set of counterbalanced probe tests with one sample encoding followed by novelty exploration was conducted (figure 3.9a). Novelty exploration was preceded by intrahippocampal infusion of SCH23390 (selective D1/D5 receptor blocker), propranolol (selective β-adrenergic receptor blocker) or vehicle control (saline). Neither vehicle nor propranolol had any effect on correct digging after 24 h retention interval. In contrast, SCH23390 successfully blocked the effect of novelty on memory persistence, resulting in chance performance 24 h after encoding (figure 3.9b, left).

A repeated measures ANOVA detected a significant effect of drug treatment on preference for the correct sandwell after 24 h interval ($F_{(2,24)} = 3.83$, $p < 0.05$). Post-hoc pairwise comparisons using Dunnett's test against vehicle control unveiled a significant reduction in correct digging after SCH23390 infusion as compared with vehicle control (Dunnett's test, $p < 0.05$), but no effect of propranolol in comparison with vehicle infusion (Dunnett's test, $p > 0.05$). Additionally, vehicle and propranolol infusions did not bring mouse performance down to the chance level (one sample t-test, vehicle: $t_{(12)} = 4.38$, $p < 0.001$; prop: $t_{(12)} = 3.34$, $p < 0.01$).

As the concentration of SCH23390 (1 μg/μl) was relatively high (although used in other published experiments, e.g. Wang et al., (2010)), the whole block of probe tests was repeated using a lower SCH23390 concentration (0.5 μg/μl) (figure 3.9b, right).

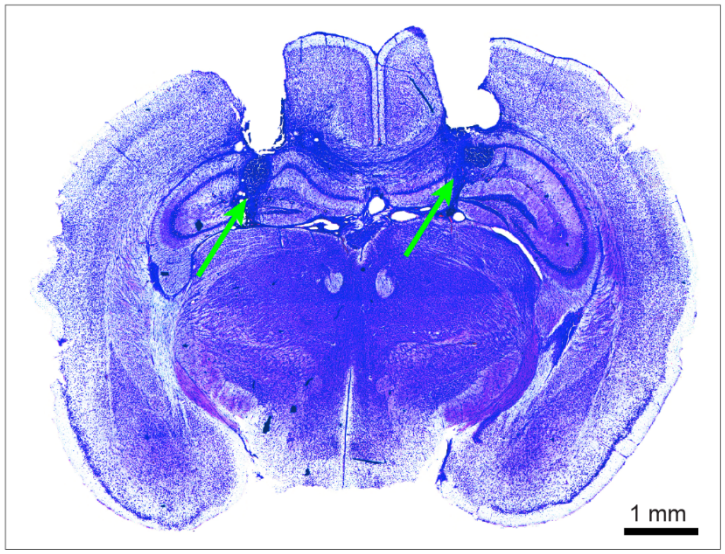
Surprisingly, this time repeated measures ANOVA did not reveal significant differences between groups ($F_{(2,22)} = 1.74$, $p > 0.05$). More detailed inspection of the data from the second round of probe tests revealed that while mice in propranolol condition exhibited correct digging significantly higher than chance (one-sample t-test; $t_{(11)} = 2.90$, $p > 0.05$), mice in saline condition failed to reach significance (one-sample t-test; $t_{(11)} = 2.11$, $p = 0.058$). We hypothesised it is due to a combination of three factors: repeated exposure to box exploration, lower animal number (one animal had to be perfused before the second block of pharmacology probe tests) and random variation. In order to address this, scores in propranolol and vehicle conditions were averaged between the two rounds in order to obtain a better estimate of mouse performance in these conditions.

Figure 3.8: Cannula placement in the dorsal hippocampus.

(a) Photograph of the cannula tracts in dorsal hippocampus. Green arrows indicate locations of cannula tips.

(b) Schematic representations of cannula locations in all 13 animals used in this study (black dots). Numbers on the side indicate distance from bregma.

(a)



(b)

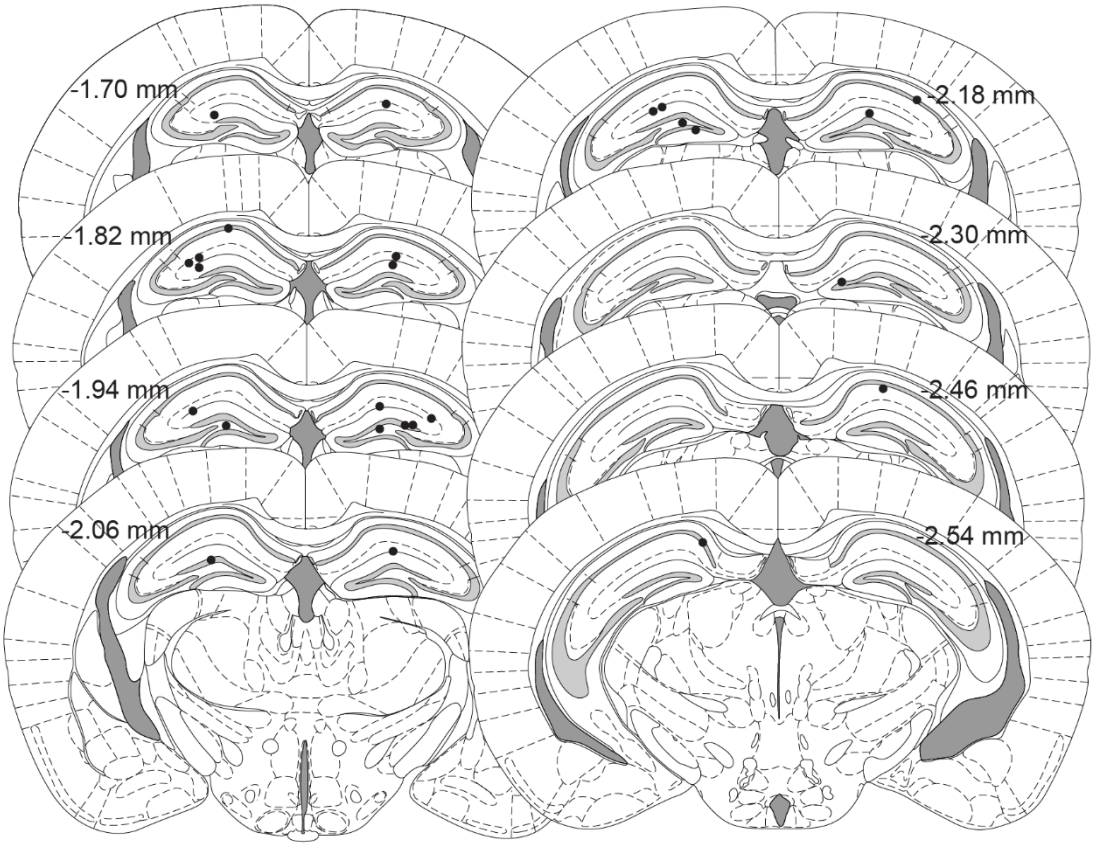
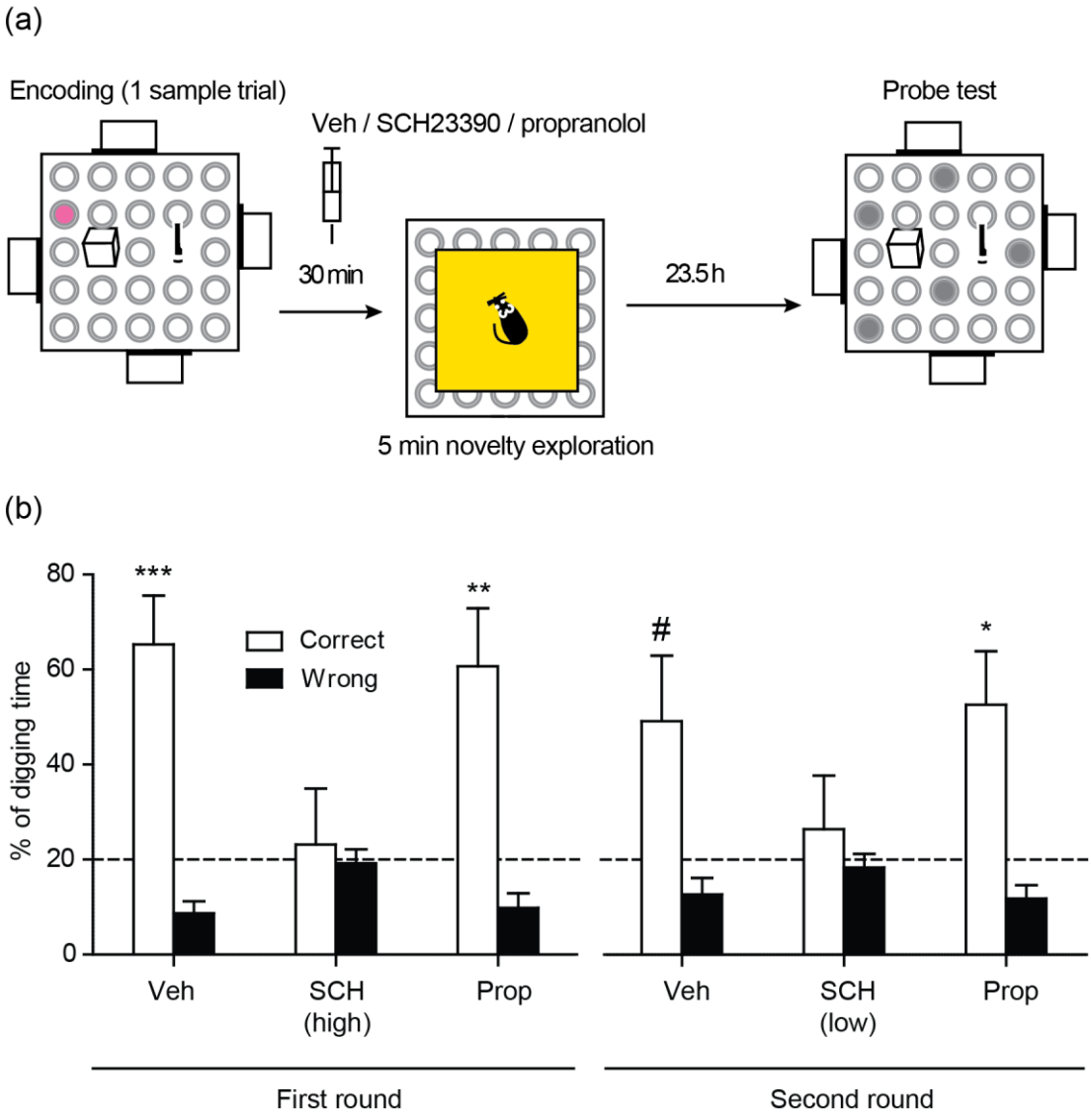


Figure 3.9: SCH23390 but not propranolol blocks the novelty effect on memory persistence.

(a) Protocol used in novelty probe tests with pharmacological intervention. Drug microinfusion was performed 10 minutes after memory encoding.

(b) Two rounds of pharmacological tests. (b, left) Infusion of high dose of SCH23390 (SCH high) but not propranolol (Prop) into dorsal hippocampus blocks the promoting effect of novelty exploration on memory persistence. (b, right) Intrahippocampal infusion of a lower dose of SCH23390 (SCH low) also leads to baseline performance. The results of the second round of PTs are hard to interpret because of lower performance of the vehicle control group in comparison with the first round.

p = 0.058 * $p < 0.05$. ** $p < 0.01$, *** $p < 0.001$. Error bars represent ±1 S.E.M. Dashed lines indicate chance levels.


(a)
Encoding (1 sample trial)
Veh / SCH23390 / propranolol
Probe test
30 min
23.5 h
5 min novelty exploration
(b)
% of digging time
80
60
40
20
0
***
**
#
*
Correct
Wrong
Veh
SCH (high)
Prop
Veh
SCH (low)
Prop
First round
Second round

Repeated measures ANOVA was then performed on three conditions (average saline, average propranolol, low SCH23390), and yielded a significant result ($F_{(2,22)} = 3.68$, $p < 0.05$). Additionally, it now transpired that in contrast to propranolol (Dunnett's test, $p > 0.05$), low SCH23390 blocks the effect of novelty (Dunnett's test, $p < 0.05$).

Separately, an intriguing observation was made during the habituation process for the hippocampal microinjection procedure. Mice were habituated to the injection procedure for 4 days, followed by a 24 hour probe test with weak (1 sample) encoding and saline injection, conducted in order to establish whether the microinjection procedure itself constitutes a novelty stimulus. Surprisingly, mice showed highly significant preference for the correct sandwell this probe test (49% average correct digging, one-sample t-test, $t_{(12)} = 1.93$, $p < 0.05$). After four more habituation sessions another 24 hour probe test was conducted, and this time animal performance was at chance (22% average correct digging; one-sample t-test, $t_{(12)} = 0.25$, $p > 0.05$). While these two probe tests were not counterbalanced and thus are not directly comparable, it is nonetheless an indication that memory persistence in this paradigm can be influenced by experimental procedures conducted without prior extensive habituation.

3.3.7 Meta-analysis of the novelty effect on memory persistence

An important caveat that became apparent during the course of the behavioural experiment was the problem of high variability in mouse performance which precludes more detailed analysis of individual probe tests. The variability was largely due to animals with low total digging times. Therefore, a meta-analysis was conducted that grouped all conducted probe tests into three categories: (i) good performance (e.g. 4 sample trial encoding, 24 h delay), (ii) poor performance (e.g. 1 sample encoding, 24h delay), and (iii) rescue by novelty. Absolute digging times of individual animals in correct and wrong sandwells were then averaged across categories.

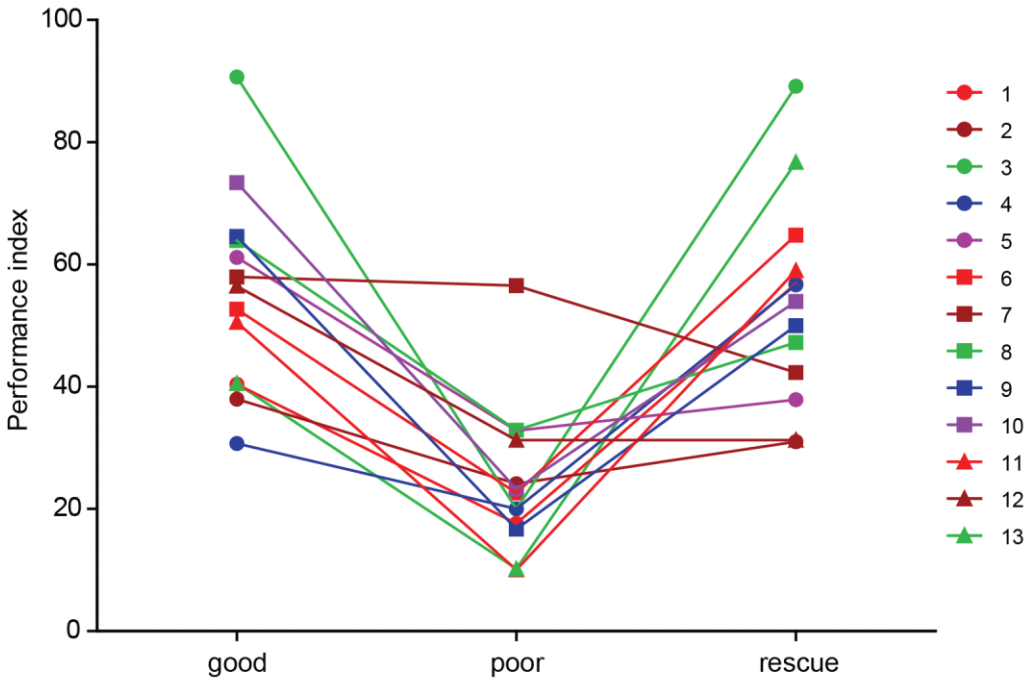
Meta-analysis of all experimental conditions shows that eleven out of thirteen mice clearly followed a V-shaped pattern of performance across the 'good', 'poor' and 'rescued' categories (repeated measures ANOVA of all animals across 3 categories, $F_{(2,24)} = 17.12$, $p < 0.0001$; quadratic component, reflecting the V-shaped distribution, $F_{(1,12)} = 30.09$, $p < 0.001$) (figure 3.10a). Interestingly, the difference between performance in 'good' and 'poor' conditions was highly correlated with the degree of enhancement of memory persistence by novelty (figure 3.10b), indicating that degree of memory enhancement by novelty could be explained by the degree of forgetting.

Figure 3.10: Meta-analysis of animal performance in probe tests.

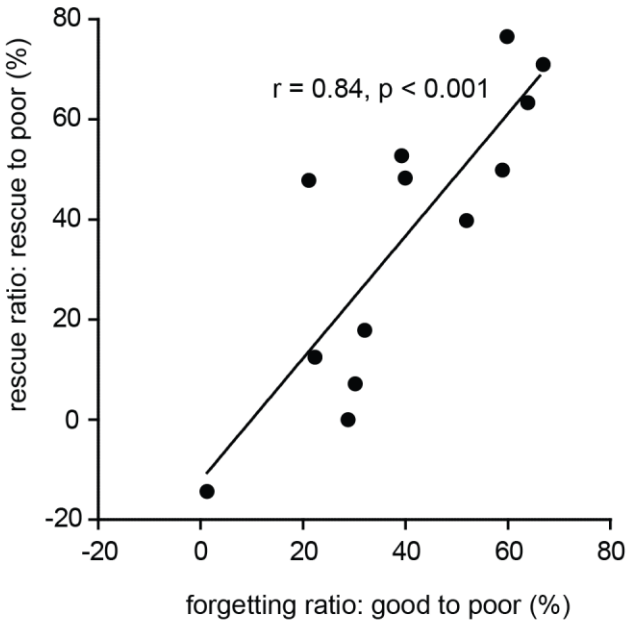
(a) Average performance of all experimental animals in probe test conditions classified into three categories: (i) good performance without novelty, (ii) poor performance, and (iii) rescue by novelty. Eleven out of thirteen animals showed a V-shaped pattern of performance across three categories.

(b) Degree of forgetting (forgetting ratio) was highly correlated with the degree of rescue of transient memories by novelty (rescue ratio).

(a)


Performance index
100
80
60
40
20
0
good
poor
rescue
1
2
3
4
5
6
7
8
9
10
11
12
13

(b)


r = 0.84, p < 0.001
rescue ratio: rescue to poor (%)
forgetting ratio: good to poor (%)
80
60
40
20
0
-20
-20
0
20
40
60
80

3.4 Discussion

We developed an 'everyday memory' test for mice – defined as a task in which a memory of the daily location of food reward is rapidly encoded, but it needs to be updated on a day-by-day basis. When animals reached asymptotic performance levels experimental protocol could be run for several months with no change to animal performance, giving the opportunity to conduct multiple tests on the same cohort of animals. The main findings were:

- **Persistence of spatial reference memory was critically dependent on the number of rewarded trials the animals were given, with 1 trial (2 pellets) producing a 'weak' memory that decayed to baseline after 24 hours.**
- **Unexpected novelty exploration 30 min after weak encoding rescued the decaying memory trace, resulting in significant preference for the correct location after 24 hours.**
- **the impact of novelty was blocked by infusion of a dopamine D_1/D_5 receptor antagonist into dorsal hippocampus, reflecting the results obtained in rats (Wang et al 2010)**.

The sensitivity of memory persistence to reward magnitude and to post-encoding novelty, and its sensitivity to SCH23390, both point to a critical role for dopamine in spatial memory consolidation in the mouse.

3.4.1 Everyday appetitive spatial memory protocol for mice

During daily training, mice performed above chance levels from training session 4 onwards, though their average performance did not reach asymptotic levels until much later (around session 27). Surprisingly, even though mice performed well on training days at the beginning of the experiment, their preference for the correct sandwell during PT2 (session 13) failed to reach statistical significance. There are several potential explanations for this discrepancy. First of all, different scoring methods were used during the probe tests (% digging time) and during standard training days (number of errors), and these two methods of scoring likely differ in their sensitivity, which makes direct comparisons between probe tests and regular training sessions difficult. Above chance performance early in training may also reflect the presence of residual olfactory cues that guided animals into the correct sandwell, though chance-level performance in non-encoding training sessions indicates that this is likely not the case. Another possible explanation for the discrepancy between probe test and training session scores early in the experiment but not

after extensive training may lie in the observed change in mouse behaviour on the arena as the rules of the task were being acquired. In general, mice learned to remember the route to the rewarded sandwell quite early in the training, but it was often evident that they dug in every sandwell the encountered on their trajectory to the correct location. This behaviour is reflected in high latency scores in the first two weeks of training which are partially due to mice spending a lot of time digging in empty sandwells. Failure to ignore incorrect sandwell locations arguably had a considerable impact on probe test scores – mice often reached the correct sandwell right before the 1 minute cut-off point, resulting in low digging time in that location. As the training progressed, mice learned the cost of digging in empty sandwells (energy expenditure and delay in finding reward) and as a result became more efficient at reaching the reward location. This is illustrated by very low latencies from session 15 onwards, as well as by significant preference for correct sandwell locations in probe tests conducted during the plateau phase of the experiment.

The premise of delayed match-to-place (DMP) protocols is that animals are given incentive to encode a specific location in the behavioural apparatus, and then act on it next time they return to the maze (win-stay rule). The crucial difference between rodent DMP protocols and spatial reference memory tasks is that in a DMP experiment the relevance (valence) of a particular location on the maze to the animal is not fixed across days. In the watermaze, it means that the platform location is different on each training day, enforcing the need for day-to-day updating of the spatial location of the goal (Steele & Morris 1999). Similarly, in the dry-land DMP task described in this chapter, the reward-location association is updated every day. In addition to the win-stay rule, the event arena DMP task involves another rule that complicates the behavioural readout – animals need to learn to ignore the empty sandwells during the retrieval phase. Successful learning of this rule is corroborated by the fact that the only cost of digging in the wrong sandwell is a short delay to retrieve the reward (i.e. the time spent digging). This rule was gradually acquired through extensive training, and from session 27 onwards mouse performance and behaviour in the maze was remarkably stable, allowing for comparison of mouse performance in probe tests conducted at different time points during the plateau phase.

Importantly, and in contrast to previous versions of the protocol (T. Takeuchi, unpublished) the ‘weak memory’ condition was stable for the duration of the critical phase of the experiment. After weak encoding animals performed at chance levels on 24 h probe tests that were conducted without novelty or when the effect of novelty was blocked pharmacologically. In the past version of the protocol a single encoding trial with variable number of pellets was used during encoding sessions (as opposed to variable number of two-pellet encoding trials in this protocol), mirroring

the protocol for rats described by Wang and colleagues (2010). Unfortunately, in the previous version of the protocol mice got better at remembering the food location as after several weeks of training, so that on probe tests conducted at later stages they were able to recall the correct reward location encoded with only 1 food pellet even after a 24 hour delay. A 'weak memory' condition (i.e. encoding protocol that results in 1h but not 24h memory) is critical for the design of the experiment, and we have found that a 'spaced' design with multiple encoding trials separated by 10 min intertrial intervals was associated with stable performance during training (with 2 two-pellet trials), but also, importantly, stable weak memory condition during probe tests (with 1 two-pellet trial).

3.4.2 Novelty promotes memory consolidation in the hippocampus

Environmental novelty has a profound effect on the circuit dynamics and plasticity in the hippocampus. It has been shown to promote depotentiation of recently potentiated synapses (Xu et al 1998a), as well as to lower the threshold for long term potentiation (LTP) (Li et al 2003) and long term depression (LTD) (Lemon & Manahan-Vaughan 2006, Manahan-Vaughan & Braunewell 1999), pushing the hippocampus into the state of increased plasticity that aids encoding of new information as well as promotes the establishment of a new cognitive map through global remapping of place cells (Colgin et al 2008, Dragoi et al 2003, Kitanishi et al 2015, Leutgeb et al 2005). Given these possibilities, scheduling novelty *prior* to encoding trials could give rise to a variety of effects on the subsequent encoding of memory.

However, environmental novelty also leads to upregulation of long term plasticity-related genes and second messengers - rats exposed to a novel open field show increased phosphorylation of hippocampal CREB (Winograd & Viola 2004) as well as increased expression of immediate early genes c-fos, arg3.1, zif268 and c-jun in hippocampal area CA1 (Guzowski et al 1999, Hall et al 2000, Pace et al 2005, Papa et al 1993). The STC framework postulates that some of these plasticity-related proteins (PRPs) could stabilise synaptic changes induced prior to their upregulation, with longevity of the synaptic tag induced at encoding and availability of newly synthesised PRPs acting together as determinants of the window for opportunistic cellular consolidation. The framework also allows for the possibility of PRP upregulation prior to encoding having an impact on memory persistence. However, scheduling novelty prior to encoding could, as just described, have a sensitising effect on encoding itself.

Accordingly, using post-encoding novelty, the findings of the experiment described in this chapter, together with those of Wang et al. (2010), are in line with the notion that novel,

unexpected experiences are PRP-promoting events that affect persistence of transient memories encoded within the window of opportunity.

Behavioural tests in the study described in this chapter were designed to mimic a 'weak before strong' experiment in a hippocampal slice, where E-LTP induced by weak tetanus at one set of synapses onto a population of CA1 neurons is transformed into L-LTP by subsequent delivery of a strong tetanus through an independent input onto the same population of cells. In slices, pharmacological blockade of dopamine D_1/D_5 receptors or blockade of *de novo* protein synthesis during the delivery of strong tetanus blocks L-LTP at both sets of inputs (Wang et al 2010), indicating that dopamine releasing afferents are activated by strong tetanic stimulation. In our behavioural protocol, encoding of the location of food reward can be conceptualised as being analogous to a weak tetanus. Similarly, unexpected novel experiences could be thought of as similar to a strong tetanus which, in addition to induction of Hebbian synaptic plasticity, causes dopamine release from neuromodulatory afferents to the hippocampal formation. One of the main consequences of D_1/D_5 receptor activation is upregulation of the cAMP-PKA-CREB signalling pathway – a cascade that is critical for maintenance of synaptic potentiation (Kandel 2012). Results of this study suggest that activation of the PRP-promoting pathway(s) does not need to occur at the same time as encoding of the memory trace to be maintained – these two events can in fact be separated in time, as postulated by the STC theory.

3.4.3 Relevance of the novelty effect to the STC theory

The main goal of this experiment was to establish whether the novelty effect on memory persistence can be observed in mice. Therefore, thorough testing of behavioural predictions of the STC theory is beyond the scope of this study. There exists, however, a large body of evidence from rat studies suggesting that novelty enhances memory persistence through a synaptic tagging and capture-like mechanism. Wang and colleagues (2010) used a similar everyday appetitive paradigm in rats to test the main predictions of the STC framework. They established that (1) effect of novelty is protein synthesis dependent – it can be blocked by protein synthesis inhibitors; (2) the effect of novelty is symmetrical, i.e. novel experience promotes consolidation of both future and past memory traces; (3) there is a critical window of opportunity for the novelty effect – novelty has no effect if it is experienced 6 hours after weak encoding; (4) memory of the novel experience itself is consolidated – if allowed to explore the same 'novel' box again 24 hours later, rats show marked decrease in exploratory activity.

3.4.4 Alternative explanations of the novelty effect

Results of this study (as well as the STC theory itself) are compatible with the neoHebbian model of episodic memory (Lisman et al 2011, Lisman & Grace 2005), which postulates that dopamine-dependence of late LTP at hippocampal synapses is the main gating mechanism for entry of information into long term memory. Nevertheless, it is important to point out that dopamine has profound effects on dynamics of the hippocampal circuit that might stem from, but in many cases reach beyond Hebbian (or neoHebbian) plasticity. Dopamine has been shown to dampen oscillatory activity in the gamma band (Weiss et al 2003) as well as facilitate occurrence of sharp wave-ripple complexes (SWRs) in hippocampal slices (Miyawaki et al 2014). Hippocampal gamma rhythms are widely believed to play a critical role in encoding and retrieval of episodic memories by synchronisation of activity in engram cell-assemblies (Bieri et al 2014). In contrast, SWR events occur in absence of theta and gamma rhythm and reflect offline reactivation (replay) of hippocampal engrams/cognitive maps (Csicsvari & Dupret 2014), which in turn promotes memory consolidation (Genzel et al 2014, Girardeau et al 2009, Girardeau & Zugaro 2011). SWR events are thought to drive a much slower process of systems consolidation in the cortex, but the rapid sequential firing of cells that forms the 'ripple', with interspike intervals corresponding to the window for spike timing-dependent plasticity (Dan & Poo 2004), creates an opportunity for additional strengthening of connections within the hippocampal cell-assemblies reactivated during SWRs. Thus, the intriguing switch in the state of the hippocampal network mediated by dopamine may reflect a shift away from encoding/retrieval and towards consolidation mode. It is possible that enhancement of memory consolidation by novelty illustrated in our experimental paradigm could be mediated by dopamine-dependent increase in reactivation of the hippocampal engrams encoded before the novel experience. Novel experiences were shown to promote reactivation of hippocampal cell assemblies in a radial maze task where some of the arms were initially inaccessible (Cheng & Frank 2008), though in this particular study increase in reactivation was limited to cell-assemblies representing novel parts of the maze and did not include representations of other, familiar compartments that the rats have explored. However, it remains to be established whether novelty promotes reactivation of unrelated experiences that involve a substantial learning component.

The effect of novelty on hippocampal reactivation was replicated in a recent landmark study by McNamara and colleagues (2014). Additionally, and in accordance with earlier pharmacological *in vitro* data (Miyawaki et al 2014), optogenetic activation of dopaminergic neurons in the ventral tegmental area (VTA) during novelty exploration caused further increase in SWR-associated

reactivation of hippocampal assemblies during subsequent rest period. Authors then went on to show that the same pattern of optogenetic activation of VTA neurons during learning of a hippocampus-dependent route-learning task ('crossword maze') resulted in enhanced memory of the route to reward measured 1 hour after encoding. Importantly, dopaminergic neurons were optogenetically activated during the encoding rather than consolidation phase of the task, which may reflect dopaminergic influence over LTP magnitude and induction threshold in CA1 (Li et al 2003, Otmakhova & Lisman 1996), rather than LTP persistence (Frey et al 1990, Wang et al 2010).

It is conceivable that hippocampal D_1/D_5 receptor activation serves dual mnemonic function: (1) it promotes emergence and of new hippocampal cell-assemblies by lowering the threshold for LTP induction, which is reflected in increased pyramidal cell co-activation immediately after the novel experience; and (2) it enhances persistence of plastic changes that bind cell-assemblies together, resulting in a more long-lasting memory trace and, hypothetically, detectable reactivation over a longer time period. Indeed, the study by McNamara et al. (2014) involved a 1 hour delay between encoding and retrieval, which is usually associated with protein synthesis-independent phase of hippocampal LTP and thus did not specifically probe expression of consolidated engrams. A potential alternative explanation of their result is thus that enhanced memory at 1 h delay is due to the promoting effect of dopamine on encoding, rather than persistence, of the hippocampal memory trace. This is in contrast to the experiment described in this chapter, where encoding is separated in time from the subsequent dopamine pulse induced by novelty. It is currently unknown whether unexpected novel experiences can promote reactivation of other, separate memory traces encoded close in time, and it would be of interest to see whether novelty exploration after encoding could mimic the effects of VTA stimulation on both probe test performance and reactivation of the crossword maze-associated cognitive map in the McNamara et al. (2010) experiment. We did not observe a significant effect of novelty on memory after 1 hour delay, though the non-significant trend evident in our data could mean that baseline memory retained after 1h was too high to detect the novelty effect. Nevertheless, it is conceivable that cellular effects of dopamine on LTP threshold and/or availability of PRPs are a prerequisite for the enhanced re-activation of engram-bearing cell-assemblies during hippocampal SWRs.

3.4.5 Noradrenaline and memory persistence

The role of noradrenergic system in emotional memory is well documented (Joels et al 2011, LaBar & Cabeza 2006, van Stegeren 2008). Though emotional memory processing takes place

primarily in the amygdala, there is considerable evidence that noradrenergic innervation in the hippocampus is actively engaged during learning with a significant spatial or contextual component (Decker et al 1990, Murchison et al 2004). Learning in an inhibitory avoidance task is known to depend on hippocampal β-adrenergic receptors (Decker et al 1990), but Moncada and colleagues (2011) showed that this is also true for the effect of novelty on persistence of inhibitory avoidance memory in a 'weak-before-strong' behavioural tagging protocol. By infusing a β-adrenergic receptor blocker propranolol into dorsal dentate gyrus before rats explored a novel environment, Moncada et al managed to block the promoting effect of novelty on retention of the memory trace. Interestingly, as described earlier in this chapter, infusion of D_1/D_5 dopamine receptor antagonist SCH23390 into dorsal CA1 had the same effect, indicating that synergistic hippocampal release of both neuromodulators is necessary for the novelty effect. These findings can be interpreted in two ways: activation of β-adrenergic receptors may be selectively involved in the novelty-associated enhancement of emotional (or 'fear') memories, or, alternatively, they may be necessary for enhancement of memories regardless of their emotional content.

We did not observe any effect of hippocampal β-adrenergic receptor blockade on the novelty effect in our everyday spatial memory paradigm using the same propranolol concentration as was used by Moncada et al. (2011). This creates an intriguing possibility that the noradrenergic effects observed by Moncada and colleagues are task-dependent. While it is important to emphasise that our study was done in mice and the noradrenaline effect may be species-specific, it is conceivable that aversive place memories but not appetitive place memories engage β-adrenergic receptor-expressing neurons in the dentate gyrus. Consistent with this idea, L-LTP in the dentate gyrus but not in CA1 is sensitive to β-adrenergic antagonists (Swanson-Park et al 1999) and promoted by novelty (Davis et al 2004, Straube et al 2003a, Straube et al 2003b), rising a possibility that long term memory in our paradigm is not dependent on long-term plasticity in the dentate gyrus.

3.4.6 Possible sources of dopamine released during novelty exploration

As reviewed in the previous chapter, dopamine is released in the hippocampus during novel experiences (Ihalainen et al 1999) but the origin of this transient dopamine pulse is not entirely clear. The neoHebbian model of hippocampal consolidation postulates that dopaminergic projection from the ventral tegmental area (VTA) in the midbrain is the main source of hippocampal dopamine (Lisman et al 2011, Lisman & Grace 2005). Indeed, a sparse dopaminergic projection from VTA to the hippocampus has been identified in rats (Gasbarri et al 1994a, Gasbarri et al 1994b) as well as in mice (McNamara et al 2014, Stamatakis et al 2013).

Interestingly, recent evidence suggests that the bulk of hippocampal dopamine is released from the axons of locus coeruleus (LC) neurons (Smith & Greene 2012). LC is a small brainstem nucleus densely populated with noradrenergic neurons (Samuels & Szabadi 2008a, Samuels & Szabadi 2008b) that send substantial projections to a multitude of brain areas including the neocortex, basal forebrain and the limbic system (Jones et al 1977, Jones & Moore 1977, Maeda & Shimizu 1972). There is evidence that both VTA and LC neurons are activated by novel stimuli (Ljungberg et al 1992, McNamara et al 2014, Vankov et al 1995), which indicates that both of these nuclei are possible mediators of the novelty effect on memory persistence.

Chapter 4

Catecholaminergic Neurons in Mouse Ventral Tegmental Area and Locus Coeruleus are Activated by Novelty

4.1 Introduction

The experiments described in the previous chapter demonstrated in the mouse that a separate novel event experienced around the time of encoding of a transient spatial memory promotes consolidation of the transient memory trace in the hippocampus through activation of hippocampal D_1/D_5 dopamine receptors. Dopaminergic neurons in the VTA (VTA-DA neurons) have been hypothesised to provide this novelty-induced dopamine signal in the hippocampus (Lisman & Grace 2005). Interestingly, a recent study indicated that noradrenergic neurons of the locus coeruleus (LC-NA neurons) may under some circumstances co-release dopamine from their hippocampal terminals (Smith & Greene 2012). In order to provide more evidence for the three-way connection between dopamine-releasing neurons in VTA and/or LC, novelty and memory consolidation, it is imperative to characterise responses of these neurons to novel stimuli. Hence, the aim of the present study is to characterise the responses of mouse VTA-DA and LC-NA neurons to novelty.

An important point to consider is the choice of the novel stimulus. As described in the previous chapter, exploration of an open field with a novel floor substrate enhances memory persistence in mice. However, the exact features of a stimulus capable of inducing this effect are unclear. The

defining feature of the 'behavioural tagging' paradigm on the event arena is the interplay between daily repetitive training and rare, unexpected novel experiences. Hence, this study utilises simple novelty exploration paradigm that provides the same kind of mundane, everyday experience interspersed with unexpected, novel events. The 'novelty' stimulus used in this study is the same as the one used in the event arena experiment – an open field with a novel floor substrate. The 'familiar' open field is experienced daily but the 'novel' open field is experienced rarely and unexpectedly.

Most *in vivo* electrophysiological studies of dopaminergic and noradrenergic neurons use a mixture of electrophysiological and pharmacological criteria to distinguish these neurons from other neuron types. In case of dopaminergic neurons, the criteria are based on a set of pioneering studies of Grace and Bunney (Grace & Bunney 1983). Unfortunately, reliability of these criteria has recently been called into question (for review, see (Ungless & Grace 2012)). A recent landmark paper from the Uchida group in Harvard introduced a robust way of identifying VTA-DA neurons *in vivo* through simultaneous single unit recordings and optogenetic stimulation (Cohen et al 2012). By conditionally expressing the excitatory opsin ChR2 in DAT-positive neurons in VTA, they were able to reliably categorise neurons as dopaminergic or non-dopaminergic based on whether or not they fired short latency spikes in response to blue light pulses, which were delivered through an optic fibre coupled to the implanted microdrive. Activity of these optogenetically identified VTA-DA neurons could then be analysed while animals were performing a behavioural task.

Influenced by this approach, the present study of mouse VTA-DA and LC-NA neurons using Th-Cre mice and Cre-dependent ChR2-AAV viral vectors to conditionally express ChR2 in these cells. It is expected that conditional ChR2 expression in tyrosine hydroxylase (TH)-positive neurons will not only allow for accurate discrimination between VTA-DA neurons and other VTA neuron types, but also aid localisation of the LC, which due to its small size (~ 1500 neurons) is a very challenging target of electrophysiological recordings in the mouse.

4.2 Methods

4.2.1 Subjects

The subjects were tyrosine hydroxylase Th-IRES-Cre heterozygous male transgenic mice, backcrossed more than 20 times to the C57BL/6 strain (EM:00254; (Lindeberg et al 2004)). The Th-IRES-Cre allele was identified by polymerase chain reaction using TH-P1 (5'-CCAAAGTTCCCAGCCCCTTCTCCAAC-3'), TH-P2 (5'-AACTGGTCGAGCGATGGATTT CCGT-3') and TH-P3 (5'-GCCAGGAACACTCCTGGAGACCTTTC-3'). The mice were at least 8 weeks old at the time of surgery, were given food and water *ad libidum* throughout the experiment, and were kept under a 12 hour light/dark cycle. Behavioural testing was carried out during light phase of the cycle. A total of 8 mice were used for acute electrophysiological recordings (VTA = 4, LC = 4) and a total of 17 mice were used in the behavioural experiment (VTA = 10, LC = 7). All procedures were compliant with the UK Animals (Scientific Procedures) Act 1986 and with the European Communities Council Directive of 24 November 1986 (86/609/EEC) legislation governing the maintenance of laboratory animals and their use in scientific experiments.

4.2.2 Viral vectors

Cre-inducible AAV vectors carried a strong constitutive EF-1α promoter followed by a double-floxed (loxP and lox2722) hChR(H134R)-eYFP cassette (or eYFP cassette in case of the control vector). The coding sequence is in reverse orientation, but in presence of Cre it recombines so that transcription can be initiated. The sequence of both vectors also contains the Woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) that enhances expression of the protein. Viral vectors were serotyped with AAV5 and obtained from the University of North Carolina. The viral concentration was 8 x 10^{12} particles per ml for hChR2(H134R)-eYFP. A single batch of virus was used throughout the study.

4.2.3 Optetrode microdrive

An optetrode microdrive was used for chronic VTA and LC recordings. An optetrode, as the name implies, is a device that enables simultaneous cerebral co-location of an optical fibre for stimulation with tetrodes for multiple single-unit recording. Implants were made in-house based on a protocol from Ankeeva et al. (2010).

The design is based around a single screw threaded through a thumb nut. The screw has a vent through which the optic fiber/tetrode bundle is threaded. The sides of the screw are polished and held tightly inside the plastic casing so when the thumb nut is turned the screw moves vertically. Plastic casings were machined out of black Delrin by Carville Ltd according to the design obtained from the Deisseroth laboratory (Stanford, CA). A horizontal ridge was made around the plastic housing 2 mm from the bottom using a dental drill in order to provide a tighter grip for dental cement during implantation. A vented screw (LewVac , stainless steel, 2-56 thread, 1/2" length, 0.28" vent diameter) was threaded through a thumb screw (stainless steel, 2-56 thread size, 1/4" head diameter, 1/4" overall height), placed inside the plastic housing and secured with plastic interference pins (McMaster-Carr , 1/16" diameter, 1/4" length). The bottom 5 mm of the vented screw was polished on both sides using a rotary tool (Dremel), and its head was scored with a dental drill to provide a better grip for epoxy. It was necessary to place 1-3 small steel washers (0.1mm thickness) between the housing and the thumb screw to prevent unwanted vertical movement. Protective plastic tubing (Microlumen, 16 mm length, 0.02" inner diameter, 0.002" wall thickness) was attached to the corner hole 16 channel electrode interface board (EIB, Neuralynx Ltd.) using epoxy (RS Components) so that 0.5 mm of tubing protruded above the surface of the board. Isolation (5 mm) was stripped of both ends of the ground wire (50 mm). One end of the ground wire was flattened with smooth pliers in order to fit through the EIB hole and the other end was bent in a loop big enough to fit around a skull screw. The flat end of the ground wire was attached to the EIB with a gold pin (Neuralynx Ltd.).

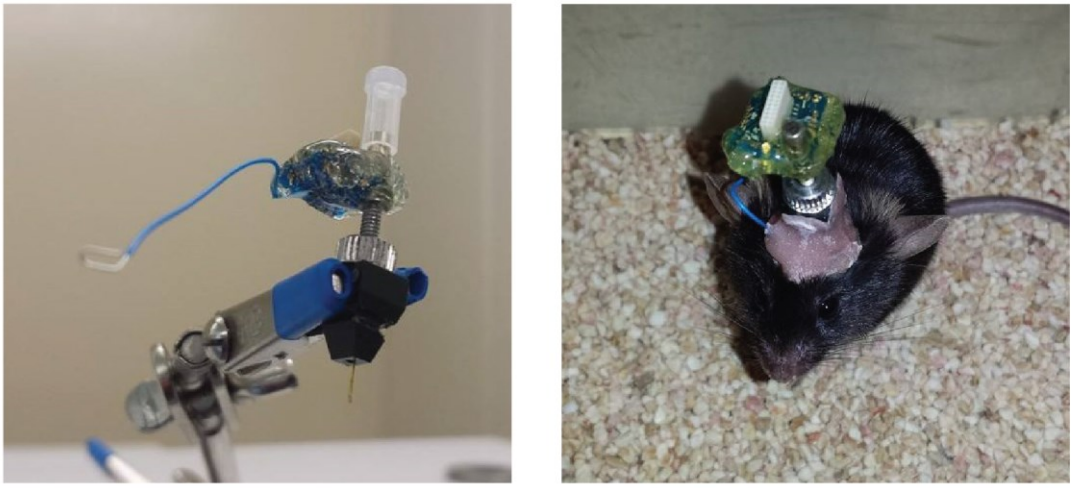
Tetrodes were made using 17 µm Pt/Ir wire (California Fine Wire). Tetrodes were twisted and then annealed together by exposing them to hot air for 10 seconds. Tetrodes were threaded through the protective tubing and connected to the EIB with gold pins. The tetrode-EIB assembly was then suspended above the plastic housing and threaded through the lumen of the vented screw. Implantable fiber-ferrules (Doric Lenses, ferrule: stainless steel, 2.5 mm diameter, 5 mm height; fiber: 200 µm core diameter, 0.22 NA) were cut to the right length (VTA: 24 mm; LC: 23 mm) with a diamond knife and the metal ferrule was scored with dental drill. The fiber-ferrule was inserted through the protective tubing and secured in place with super glue (RS Components). Exposed tetrode wires were covered with several layers of epoxy and extra care was taken to make sure that the fiber-ferrule and the vented screw are firmly attached to the EIB. Tetrodes were spaced evenly around the tip of the fiber and attached with super glue. Tetrode tips were then cut using sharp ceramic scissors at 0.5-1 mm from the fiber tip. Electrode tips were

Figure 4.1. The optetrode microdrive.

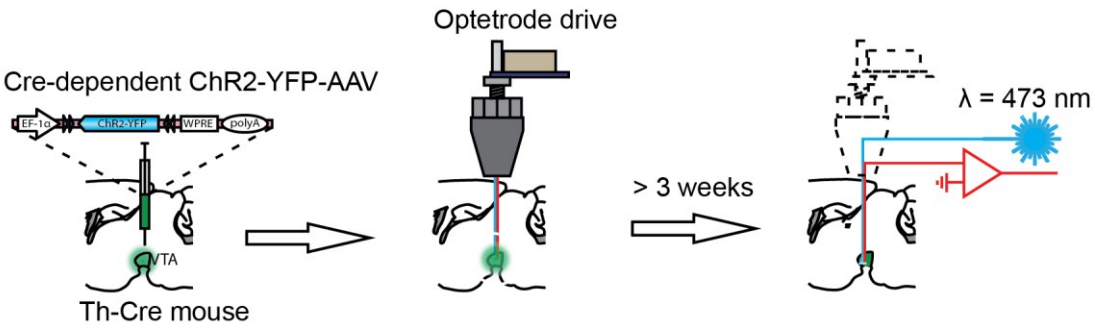
(a) Photograph of the optetrode microdrive before (left) and after implantation (right).

(b) and (c) Microinjection of the viral vector and implantation of the optetrode microdrive into (a) VTA and (b) LC.

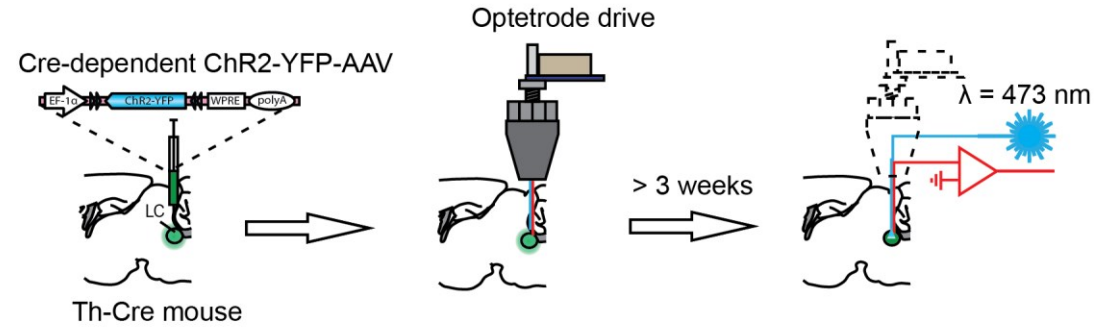
(a)



(b)


Optetrode drive
Cre-dependent ChR2-YFP-AAV
EF-1α
ChR2-YFP
WPRE
polyA
VTA
Th-Cre mouse
> 3 weeks
λ = 473 nm

(c)


Optetrode drive
Cre-dependent ChR2-YFP-AAV
EF-1α
ChR2-YFP
WPRE
polyA
LC
Th-Cre mouse
> 3 weeks
λ = 473 nm

then plated using colloidal gold (Neuralynx Ltd.) in order to reduce their impedance. Pulses of current (1 s duration, 0.2 μA intensity) were delivered until impedance of each wire was between 200-300 kΩ in saline solution. Figure 4.1 (a) shows the optetrode microdrive before and after implantation.

4.2.4 Surgery

Viral injection for acute optrode recordings

Anaesthesia was induced using 5% isoflurane and mice were placed in the stereotactic frame. From this point onwards, anaesthesia was maintained with 1-2% isoflurane. Air flow was kept at 1000 ml/min throughout the surgical procedure. Small hole (0.2 mm diameter) was drilled above left VTA or LC. The viral vector (1 μl) was injected at 0.1 μl/min into VTA (from bregma: anterior-posterior (AP), -3.52 mm; mediolateral (ML), 0.48 mm; and dorsal-ventral (DV), 4.4 mm) or LC (from bregma: AP, -5.45 mm; ML, 1.2 mm; and DV, 3.65 mm) using a high precision Nanofil syringe (World Precision Instruments) and UMP3 pump (World Precision Instruments) mounted directly on the stereotactic frame. The pump was started right before lowering the needle into the brain in order to ensure that the syringe did not get blocked during insertion. Target co-ordinates were reached after ~1 min, which indicates that around 0.9 μl of the virus reached the target injection area. Needle was left in the brain for an additional 10 min after the end of injection in order to prevent backflow. After the procedure, the skin was sutured or glued with Vet Bond. Vet Bond turned out to leave residues on the skull surface that made it more difficult to find stereotactic landmarks. Therefore suturing was the preferred method of scalp closure in experiments that involve subsequent acute electrophysiological recordings. Mice were given a subcutaneous injection of rhymadyl after surgery and their daily weight was monitored for 7 days post-surgery, and thereafter.

Viral injection and implantation of the optetrode microdrive

Anaesthesia was induced and maintained as described in previous section. Due to the fact that targeted structures were either very small (LC) or located on the ventral side of the brain (VTA), great care was taken to make sure that the surface of the skull did not deviate from horizontal plane by more than 30 μm along AP and ML axes. In order to further increase precision all stereotactic measurements were made from lambda. Zero co-ordinates for AP and ML direction were obtained at 3-5 points along the axes and the values were averaged. A hole (0.5 mm diameter) was drilled in the skull above left VTA or LC and underlying dura was removed. ChR2-AAV vector was injected into left VTA or left LC using the method described in previous

paragraph. Four jeweller's screws were placed in the skull in order to secure the head cap in place. Because of the posterior location of LC one of the screws had to be placed horizontally in the occipital bone after carefully detaching part of the occipitalis muscle with a scalpel blade. Microdrive was suspended from the sterotactic arm and the ground wire was attached to one of the skull screws. The implantation co-ordinates were (from bregma: AP, -3.52 mm; ML, 0.48 mm; and DV, 4.0 mm) for VTA and (from bregma: AP, -5.45 mm; ML, 1.0 mm; and DV, 2.8 mm) for LC. Optetrode was slowly lowered into the brain and the tetrode-optic fiber bundle was covered with several layers of petroleum jelly. Skull was covered with a layer of Metabond dental cement (C&B) followed by extra layers of standard acrylic dental cement. Animals were given a subcutaneous injection of carprofen 1 hour before the end of surgery. Diagrams in figure 4.1b and 4.1c illustrate viral vector microinjection and opetrode implantation into VTA and LC.

4.2.5 Acute *in vivo* optrode recording

All electrophysiological recordings were conducted at least 3 weeks after surgery in order to allow for sufficient expression of ChR2.

Apparatus

Recordings were made using a 125 μm 1 MΩ tungsten electrode (A-M systems) coupled to an optic fiber (200 μm core diameter, 0.22 NA). This "optrode" was prepared by stripping ~ 3 cm of cladding off the tip of the fiber, cutting the tip with a diamond knife in order to ensure that the tip is clean and flat, and attaching the fiber to the tungsten electrode so that the tip of the electrode protruded ~ 1 mm below the fiber tip. Electrode was connected to a differential AC amplifier (A-M Systems), the signal was band-pass filtered at 300 Hz – 5 kHz and amplified 10K times. Signal was then digitized at 20 kHz using a Lab View data capture card. Laser control and data collection was done using custom built Lab View software (P. Spooner). Laser stimulation was performed using a blue solid-state diode pumped laser (473 nm; Laser 2000) connected to a fibre optic patch cord (200 μm diameter, 0.22 NA).

Procedure

Anaesthesia was induced and maintained as described in the previous section. A hole (1 mm diameter) was drilled above left VTA (from bregma: AP, -3.52 mm; ML, 0.48 mm) or LC (from bregma: AP, -5.45 mm; ML, 1.0 mm). A skull screw was placed anterior to bregma and used as a reference for electrophysiological recordings. The optrode was slowly lowered towards the brain area of interest (3.0 mm for VTA, 2.0 mm for LC). Screening for light-evoked activity was done

by administering a series of 10 light pulses at 1 Hz (20-30 mW intensity, 5 ms duration), and optrode was lowered in 50 μm increments.

4.2.6 Behavioural apparatus

The apparatus was designed to mimic but simplify the event arena experience. The behavioural apparatus consisted of a rectangular wooden box with three compartments: screening compartment with space for the home cage, as well as 'familiar' and 'novel' open fields (figure 4.2a). the apparatus was surrounded by black curtains in order to minimise external cues. Light level in the apparatus was kept at 25-35 lux. There were no light sources inside the apparatus, the ambient light was coming in through the roof of the speed frame covered with semi-transparent white cloth. The floor of the familiar environment was covered in sawdust (the floor substrate on the event arena), and the floor of the novelty compartment was covered in one of eight novel substrates (figure 4.2b). Only the floor substrates that significantly enhanced memory persistence in the event arena experiment described in the previous chapter were used. The optical patch cord and the recording cable were fed through the side of the apparatus and suspended above the box using a pulley system. The pulley system and the overhead camera (CCIR) were installed on a rail above the apparatus so they could be moved directly above each compartment. Infra-red diode attached to the headstage amplifier was used for motion tracking. Diode light was tracked using the Axona data collection software.

4.2.7 *In vivo* single unit recording and optogenetic stimulation in behaving animals

Single unit recording

Unit activity was recorded using a 32 channel recording system and recording software (Axona). Signal was fed through a 16 channel unity gain headstage amplifier (Axona), band-pass filtered at 300-5000 Hz, amplified 1k-40k times and digitized at 50 kHz. Spike capturing was done on-line using amplitude threshold, and 2 ms spike windows were collected for offline data analysis. Recording sessions for novel and familiar environments were started immediately (~1 s) after the mouse was placed in the relevant compartment.

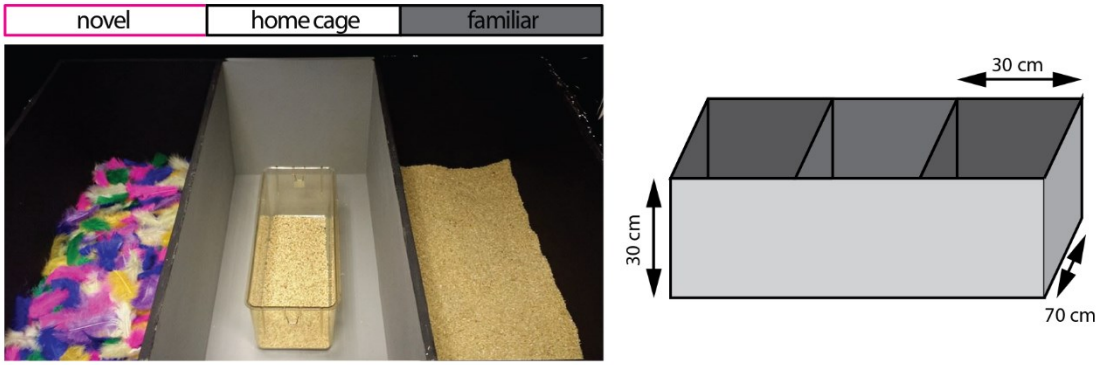
Figure 4.2. Experimental apparatus and novelty exploration protocol.

(a) Photograph and dimensions of the three-compartment experimental box.

(b) Novel and familiar substrates used in the study.

(c) Three versions of the daily screening and exploration protocol.

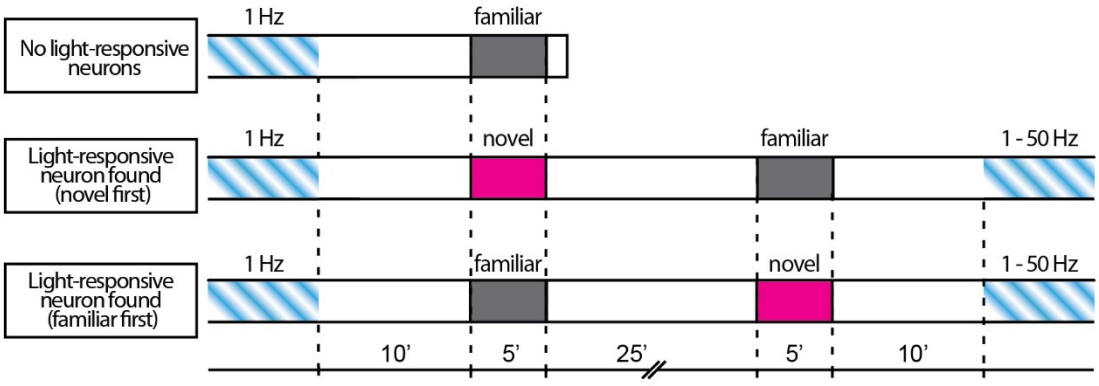
(a)


novel
home cage
familiar
30 cm
30 cm
70 cm

(b)


familiar
novel
sawdust
feathers
corks
straws
rope
pompoms
sticks
paper
Lego

(c)


No light-responsive neurons
1 Hz
familiar
Light-responsive neuron found (novel first)
1 Hz
novel
familiar
1 - 50 Hz
Light-responsive neuron found (familiar first)
1 Hz
familiar
novel
1 - 50 Hz
10'
5'
25'
5'
10'

Optogenetic stimulation. Laser stimulation was performed using a blue solid-state diode pumped laser (473 nm; Laser 2000) connected to a fibre optic patch cord (200 μm diameter, 0.22 NA). Epochs of 60 light pulses (1 Hz, 5 ms pulse duration) at different light intensities (0.1 – 20 mW) were then administered and each tetrode was screened for light-evoked spikes. Spikes were classified as 'light-evoked' if their latency from the onset of the light pulse was between 0 and 15 ms. All other spikes in the recording epoch were classified as 'spontaneous'. Units were classified as light-sensitive if: a) a cell fired a light-evoked spike in response to more than one third of light pulses, b) the shape of the mean light-evoked waveform of a unit was identical to the spontaneous waveform of the same unit. Additionally, units with basal firing rates above 20 Hz were excluded from this analysis because of intrinsically high probability of spiking within the 15 ms window after the light pulse.

4.2.8 Novelty exploration procedure

Ten days after surgery, the mice underwent 3 days of handling, followed by 5 days of habituation to the experimental apparatus. Each habituation day consisted of 30 min in the screening chamber (in their home cages), followed by 30 min of exploration of the familiar environment. Mice were not connected to any equipment for the first 2 days. For the remaining 3 days, mice were connected to the optical patch cord and the recording cable before being placed in the screening compartment. Extra days of habituation were given if on the fifth day mice still had difficulties pulling the recording cable and exploring the familiar compartment.

Following habituation, mice underwent daily screening sessions for light-responsive neurons. Each daily experimental session began with connecting the mouse microdrive to the optical patch cord and the recording cable, placing the mouse back in its home cage and moving the cage into the screening compartment. The animal was then screened for light-sensitive units. If no light-responsive cells were found, mouse was allowed to explore the familiar environment for 5 minutes and was then unplugged. If one or more recording channels showed a light-responsive unit, the mouse was subjected to the novelty exploration protocol after a 10 min delay (figure 4.2c). Mouse was first placed in the novel environment for 5 min (t = 0 - 5 min), followed by 25 min in the screening compartment (t = 5 - 30 min) and 5 min in the familiar environment (t = 30 - 35 min). The order of novel and familiar exploration was counterbalanced across all the trials. Baseline recording was performed half way between two exploration sessions (t = 20 - 25 min). These particular time delays were chosen in order to mimic the timing of the even arena experiment, where there was a 30 min delay between encoding and novelty exploration. In VTA-implanted

animals, a fraction of trials (n = 9) involved an additional 5 min exploration of the novel environment at t = 60 - 65 min. This was done in order to look for a possible habituation of DA neuron response on re-exposure to the 'novel' compartment.

Baseline activity (5 min) was recorded at half-time (t = 15 min) between the 'novel' and 'familiar' exploration sessions. This particular time point was done because at that point mice were sufficiently aroused to stay awake.

After the last exploration session mouse was transferred back to the screening compartment and presence of the light-sensitive unit was again confirmed with 1 Hz light stimulation. Subsequently, 50 light pulses at 1, 5, 10, 25 and 50 Hz were administered in order to quantify VTA-DA and LC-NA neuron responses to photostimulation at different frequencies. Stimulation trains were separated by at least 60 seconds. Mice were tethered to the recording cable for the duration of the protocol. At the end of each screening session microdrive was advanced by ~40 um (1/12 turn) to ensure that recordings are made from a different population of neurons. Novelty sessions were separated by at least one day and at least one exploration of the familiar environment only. This was done in order to make sure that novel stimuli are indeed unexpected.

4.2.9 Data analysis

Recorded spikes were clustered using Klusterkwik 1.5 unsupervised clustering algorithm (Ken Harris) based on their energy and first principal component of the waveform. Clusters were then corrected manually using Klusters spike sorting software (Lynn Hazan and Muchaël Zugaro), based on several additional parameters (width of waveform, amplitude, time at peak), as well as on auto- and crosscorrelograms. Data were analysed using Matlab R2012a (MathWorks). Firing patterns were characterised in terms of firing rate, rate of burst events, burst size, percentage of spikes fired in bursts and firing rate of spikes within bursts. Bursts were defined using classic criteria developed by Grace and Bunney (Grace & Bunney 1983) According to these criteria a burst event is a set of 2 or more spikes with interspike interval of less than 80 ms, followed by an interspike interval of more than 160 ms. These criteria were developed specifically for DA neurons, but are applicable for any neuron with low basal firing rate. Firing rates of LC-NA neurons do not generally exceed 5 Hz (Vankov et al 1995) so Grace and Bunney's burst identification criteria will be applied to these neurons as well.

4.2.10 Perfusion and histology

After the experiment, animals were injected with euthatal and perfused transcardially with 4% paraformaldehyde (PFA) dissolved in PBS. Brains were removed and post-fixed in 4% PFA overnight at 4^{o}C, transferred to 30% sucrose in PBS for 2 days and then kept at -80^{o}C.

For VTA-implanted animals, coronal sections were cut at 40 µm with a freezing microtome. Sections were washed in PBS, treated with 0.5% Triton X-100 in PBS and washed in blocking solution – 3% normal donkey serum. They were then incubated overnight at room temperature with a monoclonal mouse anti-tyrosine hydroxylase antibody (1:4000, ImmunoStar) and a polyclonal rabbit anti-GFP antibody (Invitrogen). Sections were then incubated with secondary antibodies (donkey anti-rabbit IgG Alexa Fluor 288 and donkey anti-mouse IgG Cy3, both 1:200). The most ventral position of the optic fibre tip was then localised on stained sections under a fluorescent microscope with 20-fold magnification. In sections from animals implanted with optetrode microdrives positions of electrode tips were calculated using the distance between the electrode tips and the tip of the fibre measured before implantation. Measurements were done in Image Pro.

Full histological analysis of electrode placement in LC-implanted animals has not yet been completed at the time of submission of this thesis.

4.3 Results

4.3.1 Acute optrode recordings in VTA and LC

Large, triphasic optically-evoked field potentials were observed in response to 1 Hz optical stimulation in both VTA and LC (figure 4.3a and 4.3b). No optically-evoked field responses were observed more than 0.5 mm above the target stereotactic co-ordinates, which indicates that the observed responses were specific to brain areas that express ChR2 and were not artefacts due to the Bacquerel effect. These light-evoked spikes are likely population responses rather than single neurons, as their amplitude scaled up with increasing light intensity and they were always time-locked to the light pulse.

4.3.2 Responses of VTA-DA neurons to novelty

Nine out of 10 implanted mice showed some degree of light-evoked activity in their local field potential (LFP). A total number of 15 light-responsive units (henceforth referred to as VTA-DA neurons) were identified in 5 VTA-implanted mice (see example on figure 4.5a and 4.5c). In the remaining mice (4 out of 9) light-evoked potentials did not pass the criteria for identified VTA-VTA-DA neurons, mainly because light-evoked potentials observed in those animals did not match spontaneous waveforms. Histological analysis revealed that in every mouse with identified VTA-DA neurons, the optetrode tract passed through the VTA (figure 4.4a and 4.4b). Each VTA-DA neuron identified in a given animal was recorded on a separate day, and a total number of 15 novelty sessions were conducted with VTA-DA neurons. In addition to 15 identified VTA-DA neurons, 48 neurons that did not show light-evoked spikes were recorded during novelty sessions. Fifteen of these neurons were recorded on the same tetrodes as optogenetically identified VTA-DA neurons.

Figure 4.3. Acute optrode recordings of light-evoked potentials in VTA and LC induced by photoactivation of ChR2-expressing neurons.

(a) and (b) Average field recording from (a) VTA and (b) LC in response to 10 pulses of blue light at 1 Hz and a close-up of one of the multiunit spikes in the train. Blue light pulse trains are represented by the blue lines below each trace. Each trace is an average of 10 sweeps.

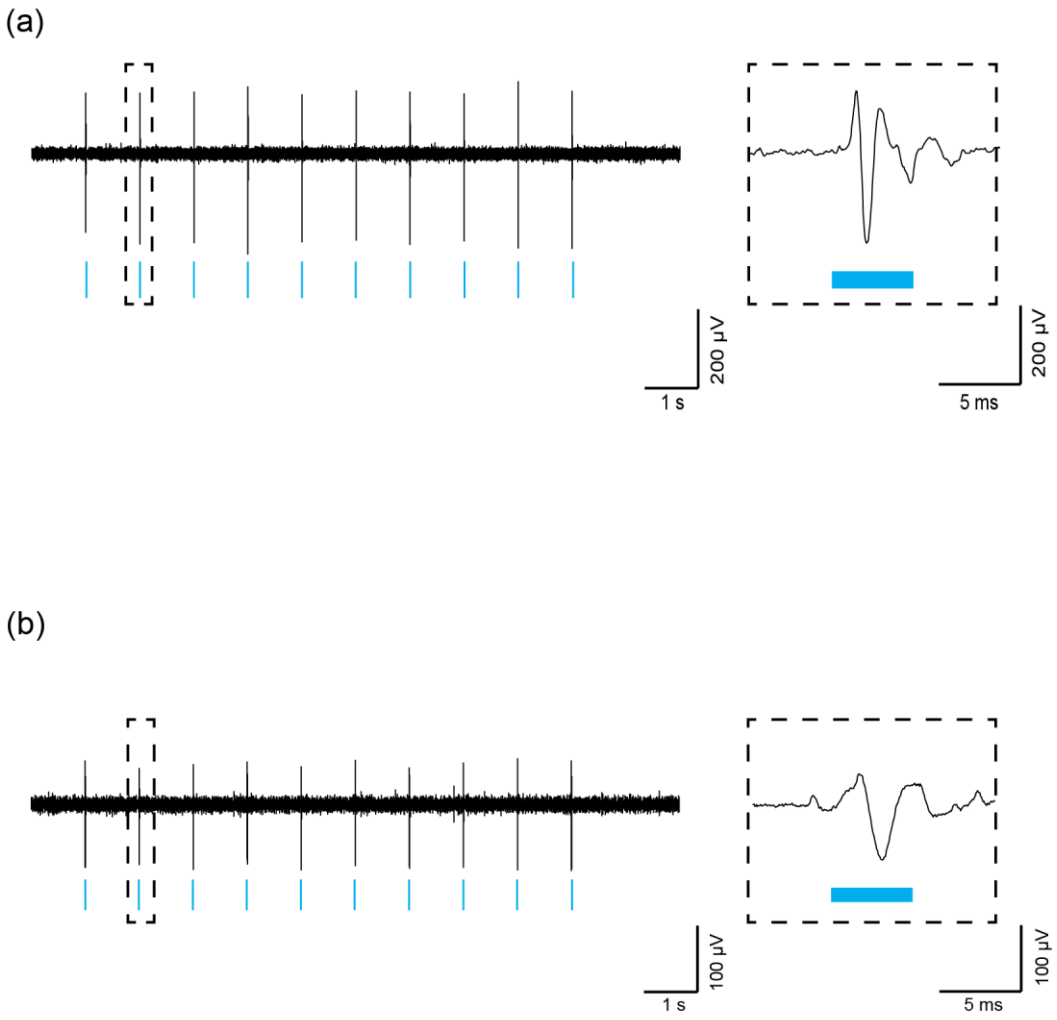
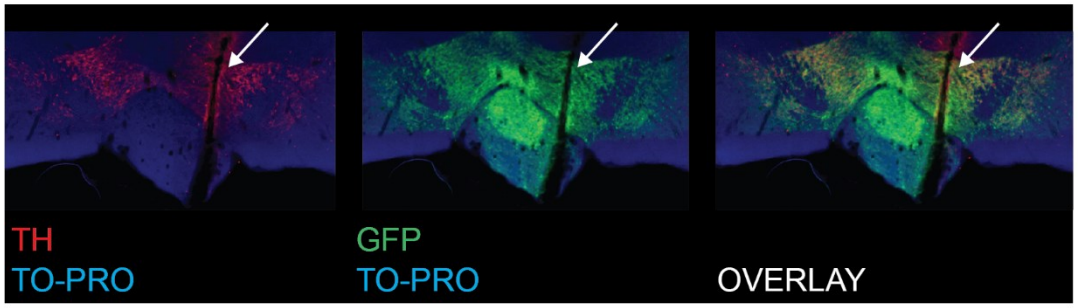

(a)
200 µV
1 s
200 µV
5 ms
(b)
100 µV
1 s
100 µV
5 ms

Figure 4.4. Location of electrode tips in VTA-implanted animals in which light-responsive neurons were identified.

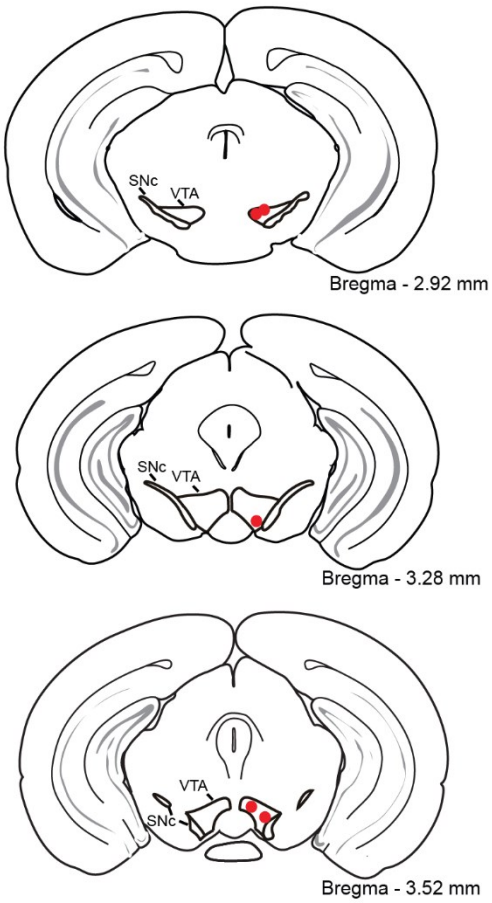
(a) Coronal section showing an optic fibre tract above the VTA and a single tetrode tract passing through the VTA. In this particular animal the microdrive was advanced all the way down.

(b) Predicted locations of the electrode tips at the time of termination in all 5 VTA-implanted animals in which light-responsive neurons were identified. In 4 animals in which electrode tracts were not clearly visible position of the electrode tips was extrapolated from the fibre tip.

(a)


TH
TO-PRO
GFP
TO-PRO
OVERLAY

(b)


SNc
VTA
Bregma - 2.92 mm
SNc
VTA
Bregma - 3.28 mm
VTA
SNc
Bregma - 3.52 mm

Optogenetically identified VTA-DA neurons

Identified VTA-DA neurons showed low to moderate basal firing rates that were largely consistent with published observations. Overall, basal firing frequency of VTA-DA neurons rarely exceeded 4 Hz and did not change substantially throughout the baseline recording period. Analysis of the percentage of spikes firing in bursts showed a broad range of different firing modes: from tonic (4.8% spikes in bursts (SiB) with 4.13 Hz firing rate) to highly phasic (84.3% SiB, 2.93 Hz firing rate). Baseline properties of all identified VTA-DA neurons are summarized in table 4.1.

Property	Mean	Range
Firing rate	3.12 Hz	0.87 – 5.52 Hz
Burst rate	0.48 Hz	0.07 – 0.80 Hz
Burst size	3.01 spikes	2.00 – 4.01 spikes
% spikes in bursts	47.5 %	4.80% - 84.3%
Intra-burst firing rate	25.8 Hz	14.6 Hz – 55.7 Hz

Table 4.1. Baseline firing properties of VTA-DA neurons.

The three classical criteria for identification of VTA-DA neurons are length of the waveform (biphasic duration of ≥ 1.1 ms), sensitivity to D_2 agonists and firing rate below 10 Hz (Grace & Bunney 1983, Ungless & Grace 2012). None of VTA-DA neurons identified in this study had waveforms with biphasic duration greater than 0.6 ms (see figure 4.5e for waveforms of all identified VTA-DA neurons). Still, a comparison between biphasic duration of waveforms of identified VTA-DA neurons and light-unresponsive VTA neurons showed a significant difference between the two (independent samples t-test, $t_{(28)} = 2.82$, $p < 0.01$) (figure 4.5b). Only putative light-unresponsive neurons recorded on the same **day** and **tetrode** as VTA-DA neurons were included in the waveform width analysis in order to increase the likelihood that they come from VTA. Although these neurons may include some unidentified dopaminergic cells, their shorter waveforms indicate that they are likely nondopaminergic.

Figure 4.5. Mouse VTA-DA neurons identified using optogenetics.

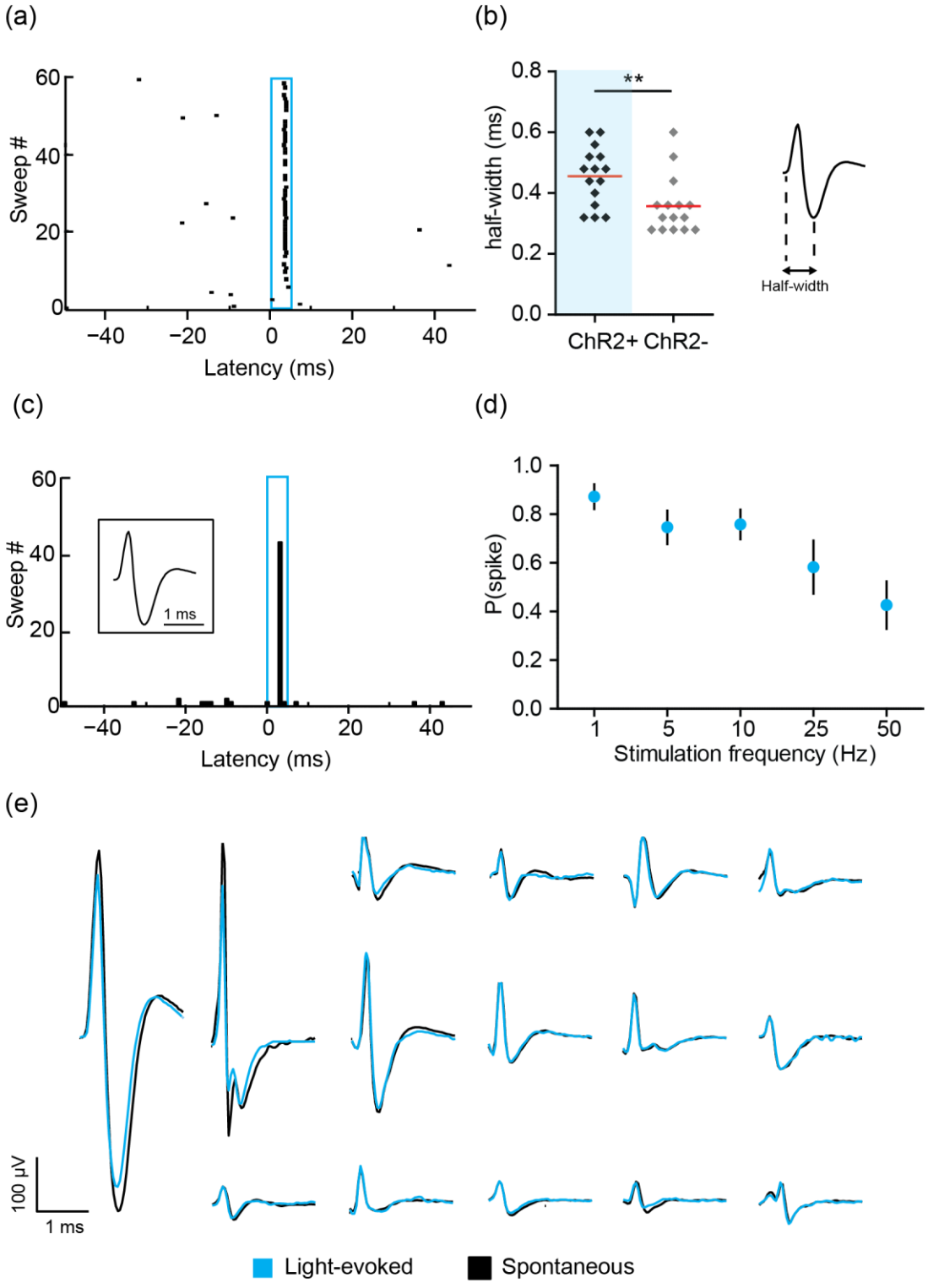
(a) Raster plot of spikes fired by an identified VTA-DA neuron during a single photostimulation epoch consisting of 60 pulses at 1 Hz. Only the spikes fired within 50 ms before and after stimulus onset are shown. Blue vertical stripe represent the light pulse.

(b) Half-width of ChR2-positive (putative DA) unit waveforms and waveforms of ChR2-negative units recorded on the same day and tetrode (putative non-DA). Putative VTA-DA units tend to have broader waveforms than other neurons coming from VTA (ChR2+, n = 15; ChR2-, n = 15).

(c) Histogram and waveform of the unit raster plot shown in (a).

(d) Firing probability of ChR2+ neurons in VTA in response to light trains at different frequency (n = 10).

(e) Average spontaneous and light evoked waveforms of all identified VTA-DA-neurons. In all cases there is a high degree of overlap between the two waveform shapes. ** $p < 0.01$. Error bars represent ±1 S.E.M.


(a)
Sweep #
Latency (ms)
(b)
half-width (ms)
**
ChR2+ ChR2-
Half-width
(c)
Sweep #
1 ms
Latency (ms)
(d)
P(spike)
Stimulation frequency (Hz)
(e)
100 µV
1 ms
Light-evoked
Spontaneous

The responsiveness of recorded neurons to laser stimulation was also tested across different frequencies, revealing that while ChR2-expressing VTA-DA neurons readily responded to 1 Hz stimulation (average spike probability: 86%), probability of spiking decreased with increasing stimulation frequency, with 50 Hz stimulation evoking a spike with only 43% chance (figure 4.5d).

Activity of VTA-DA neurons in familiar and novel environments

The next step is to compare the firing patterns of VTA-DA neurons in novel and familiar environments. Basal firing rates of VTA-DA neurons recorded in home cages were also included in the analysis in order to determine whether VTA-DA neuron firing rates during exploration differ from baseline. A key finding of this thesis is the observation that, on average, the firing rates of putative VTA-DA neurons was higher in a novel environment, although the magnitude of change is modest. While the mean rate of firing in the familiar environment was 3.50 Hz, that in the novel environment typically exceeded 4 Hz. Another important observation is that in novel environments VTA-DA cells also shifted their firing mode towards burst pattern, firing ~25% more bursts in novel than in familiar environment.

Analysis of average firing rates of identified VTA-DA neurons in all three conditions revealed stronger activation by novelty, as compared with both familiar environment and home cage baseline. The average values for each VTA-DA neuron in each condition (novel, familiar, home cage) were computed and a repeated measures analysis of variance (ANOVA) was performed on all three conditions. There was a highly significant effect of experimental condition on firing rate of VTA- DA neurons ($F_{(2,28)} = 11.92$, $p < 0.001$), illustrating that average firing rates of VTA-DA neurons were sensitive to the compartment of the experimental apparatus (either novel, familiar, or home cage). *Post hoc* tests using the Bonferroni correction revealed that VTA-DA neurons showed increased firing rate during exploration of the novel compartment, as compared with familiar compartment ($p < 0.01$) and with baseline firing rate in home cage ($p < 0.01$) (figure 4.6a). Twelve out of 15 cells showed higher firing rates in response to novelty compared to familiar exploration. There were no significant differences in mean firing rates between familiar compartment and home cage ($p = 0.37$).

Figure 4.6. Average firing patterns of VTA-DA neurons in novel and familiar environments.

(a) Mean firing rates of VTA-DA neurons are elevated in novel environments

(b) Average frequency of burst events observed in VTA-DA neurons are elevated in novel environments

(c) Average size of bursts fired by VTA-DA neurons

(d) Average frequency of spikes within bursts in VTA-DA neurons

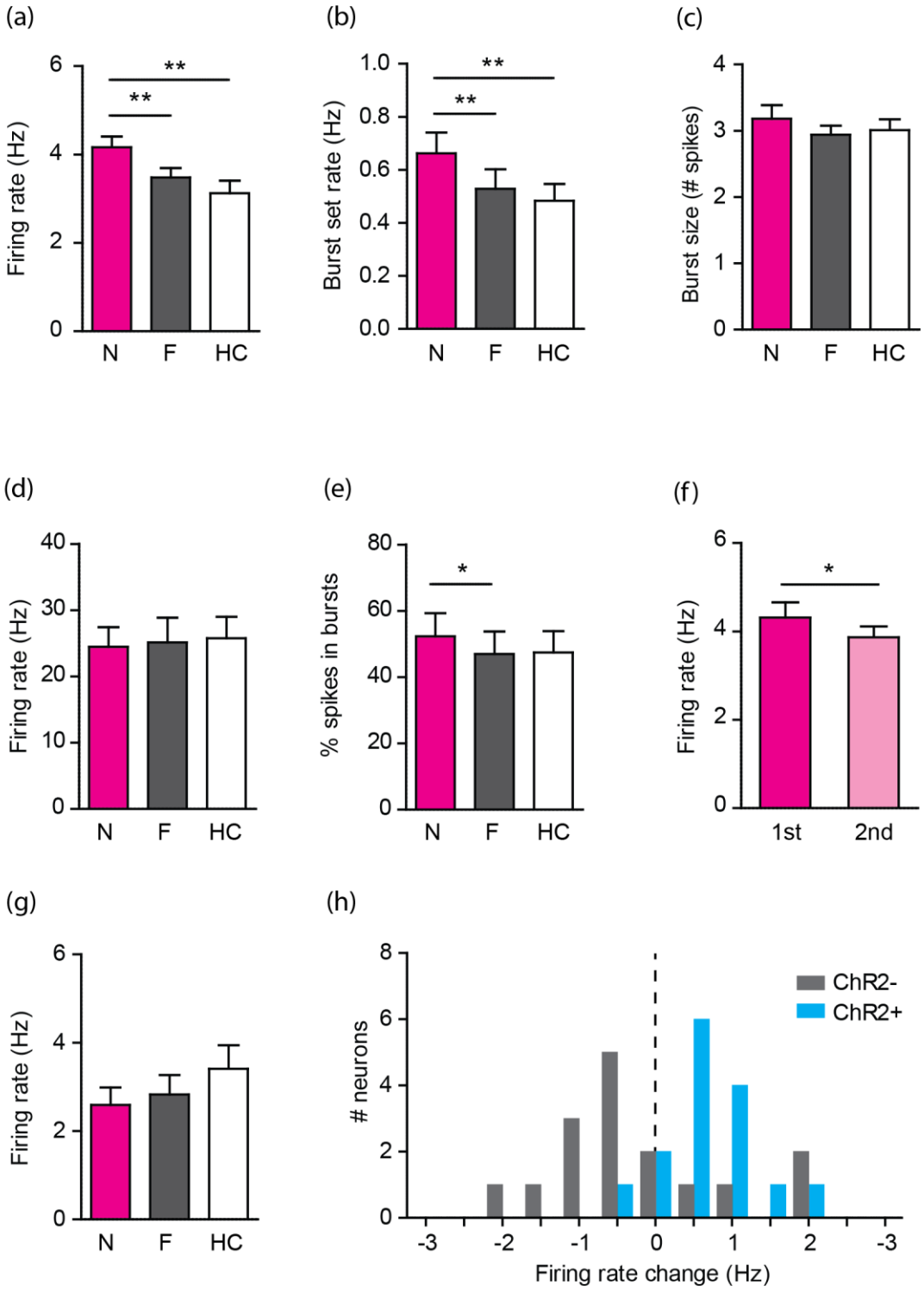
(e) Percentage of spikes fired in bursts in VTA-DA neurons

(f) Mean firing rates in VTA-DA neurons during the first and second exploration of the 'novel' compartment. VTA-DA neurons attenuate their firing rate after re-introduction to the novel box.

(g) Firing rate of all light-unresponsive neurons recorded during novelty sessions with firing rates not exceeding those of identified VTA-DA neurons (< 7 Hz).

(h) Histogram of the degree of differential firing (calculated as (firing rate in novel) – (firing rate in familiar) of light responsive (VTA-DA) and light unresponsive (putative non-DA) cells. Majority of ChR2+ neurons (putative VTA-DA) increase their firing rates in novel environment. This pattern is not observed in ChR- neurons (putative non-DA).

N, novel; F, familiar; HC, home cage * $p < 0.05$, ** $p < 0.01$. Error bars represent ±1 S.E.M.


(a)
Firing rate (Hz)
6
4
2
0
**
**
N
F
HC
(b)
Burst set rate (Hz)
1.0
0.8
0.6
0.4
0.2
0.0
**
**
N
F
HC
(c)
Burst size (# spikes)
4
3
2
1
0
N
F
HC
(d)
Firing rate (Hz)
40
30
20
10
0
N
F
HC
(e)
% spikes in bursts
80
60
40
20
0
*
N
F
HC
(f)
Firing rate (Hz)
6
4
2
0
*
1st
2nd
(g)
Firing rate (Hz)
6
4
2
0
N
F
HC
(h)
neurons
8
6
4
2
0
ChR2-
ChR2+
-3
-2
-1
0
1
2
-3
Firing rate change (Hz)

Similar differences were found in the rate of burst events (ANOVA, $F_{(2,28)} = 10.88$, $p < 0.001$; novel vs familiar, $p < 0.01$; novel vs baseline, $p < 0.01$) (figure 4.6b). ANOVA also detected differences in burst size (ANOVA, $F_{(2,26)} = 3.37$, $p < 0.05$), but pairwise comparisons failed to detect significant differences between groups (figure 4.6c). There were no significant differences in intra-burst firing rate ($F_{(2,28)}=0.63$, $p=0.54$), with neurons invariably firing bursts of spikes at ~ 25 Hz (figure 4.6e). Percentage of spikes that were part of a burst event was higher in novel than in familiar condition (ANOVA, F $F_{(2,26)} = 3.60$, $p < 0.05$; novel vs familiar, $p < 0.05$), but unfortunately pairwise comparisons did not detect significant differences between exploration and home cage baseline (figure 4.6d). These data collectively indicate that on a population level VTA-DA cells selectively increase their firing rates in novel environments, while at the same time their firing pattern becomes more 'bursty'. Comparisons of firing rates gave the most robust results and thus the remainder of data analysis will focus exclusively on differences in firing rates.

These results so far indicate that VTA-DA neuron firing frequency and the frequency of bursts selectively increases in response to novelty. In order to establish whether VTA-DA neurons react to the 'novelty' feature of the environmental stimulus or merely to environmental enrichment, a subset of trials (n = 9) included a second exposure to the novel floor substrate. Mean VTA-DA neuron firing rate decreased during second exposure to the same floor substrate compared to the first (paired t-test, $t_{(8)} = 2.33$, $p < 0.05$) (figure 4.6f). VTA-DA neurons are therefore likely modulated by the perceived novelty of the stimulus.

It was of interest to check whether the increase in firing rates of VTA-DA neurons reflects a more global change in excitability that is not specific to identified VTA-DA neurons. All of the VTA-DA neurons recorded in this study had firing rates below 7 Hz (maximum firing rate across all conditions: 6.42 Hz). Therefore, firing rates of slow-spiking (< 7 Hz) neurons recorded on **all** tetrodes during novelty sessions were analysed. This included 15 light-responsive (VTA-DA) neurons and 16 light-unresponsive neurons. These slow-spiking neurons could include ChR2-negative VTA-DA cells as well as other cell types. Nevertheless, these slow-spiking, light-unresponsive neurons were not significantly modulated by novelty (figure 4.6g). In order to compare light-responsive (VTA-DA) and light-unresponsive neurons, firing rate difference (novel - familiar) was computed for every neuron (figure 4.6h) and it was observed that VTA-DA neurons constitute a unique subpopulation of the recorded slow-spiking neurons. The activity of most slow-spiking (< 7 Hz) light-unresponsive neurons is negatively modulated by novelty. Direct comparison of firing rate difference scores between the two groups shows a significant difference between the two (independent t-test, $t_{(29)} = -2.98$, $p < 0.01$). These data collectively

show that activity of VTA-DA neurons is selectively increased in response to environmental novelty and this activation by novelty is specific to the identified VTA-DA neurons, indicating that the majority of slow-spiking light-unresponsive neurons was likely non-dopaminergic.

Averaging parameters over a whole session could occlude dynamic changes in firing patterns throughout 5 min of exploration. Therefore, the next step was to look at whether firing rates changed in time. Plotting firing rates in 1 min bins in both novel and familiar conditions revealed a different trend for each group. The paradoxical result observed was that the firing rate in the familiar environment gradually increased whereas that in the novel environment it was sustained over time (figure 4.7a). Interestingly, dynamics of fast-spiking (putative GABAergic) cells recorded on the same sessions exhibited an inverse pattern of activity (figure 4.7e).

Results of a 2x5 factorial ANOVA (condition vs 'time bin') showed, again, that that there is a significant effect of condition on firing rate ($F_{(2,14)} = 17.99$, $p = 0.001$), but notably showed both an effect of time on firing rate ($F_{(4,56)} = 3.41$, $p < 0.05$) and an interaction between time and condition ($F_{4,56)} = 4.68$, $p < 0.01$). VTA-DA neurons fire at a steady rate in the novel box throughout the session (one-way repeated measures ANOVA, $F_{(4,56)} = 0.36$, $p = 0.84$) but during exploration of the familiar compartment they increase their firing rate towards the end of the session (one-way repeated measures ANOVA, $F_{(4,56)} = 9.93$, $p < 0.001$). Direct comparison of firing rates in novel and familiar environments in each time bin indicates that neurons show differential firing only in the first 3 minutes (figure 4.7a).

Several representative examples of light-responsive neurons modulated by novelty are shown in figure 4.8. In all cases, both firing rate and burst rate in the **first minute** of exploration of the novel compartment is higher than in the corresponding time bin of familiar exploration. This was true for 14 out of 15 recorded VTA-DA neurons. Interestingly, recorded neurons exhibited diverse firing patterns. Cell #1 and cell #2 tend to fire tonically with occasional short bursts of 2-3 spikes with interspike interval of around 50 ms. In contrast, cell #3 and cell #4 are more 'bursty' in nature, firing longer and faster bursts (4-5 spikes, 25 ms average interspike interval).

One possible explanation for the slow increase in firing rate of VTA-DA neurons in familiar environment is that their activity is related to motor movement. However, to the contrary, movement speed declined in time during exploration of the familiar compartment (figure 4.7c). Additionally, comparison of average mouse speed in novel and familiar compartments did not reveal any significant differences (paired t-test, $t_{(14)} = -0.95$, $p > 0.05$)(figure 4.7b). Average speed

Figure 4.7. Firing rate changes in VTA neurons and mouse motor activity during novel and familiar exploration

(a) Firing rate of identified VTA-DA neurons in novel and familiar environments, averaged in 1 minute bins. VTA-DA neuron firing rate stays at a constant, elevated level throughout the recording session. In contrast, in familiar environments VTA-DA neurons initially fire at baseline levels, but later progressively increased their firing rates. Differential firing can only be observed in the first 3 minutes.

(b) Average mouse movement speed during exploration. There is no significant difference in average speed of the animal during familiar and novel exploration.

(c) Mouse movement speed during the trial. When averaged in 1 min bins, changes in animal speed in both conditions largely follow the same pattern.

(d) Comparison of VTA-DA neuron firing rates averaged for each mouse. All 5 mice show differential VTA-DA neuron firing in the first minute of exploration.

(e) Firing rates of fast-spiking neurons recorded during novelty sessions averaged in 1 minute bins. Fast-spiking non-DA neurons in the VTA (n = 7) show differential firing in the first two minutes of exploration.

(f) Average firing rates of fast-spiking neurons in all three conditions. Fast-spiking VTA neurons selectively increase their firing rate in familiar environments.

N, novel; F, familiar; HC, home cage. * $p < 0.05$, ** $p < 0.01$. Error bars represent ±1 S.E.M. Red bars in (a) and (c) mark average firing rate in home cage.

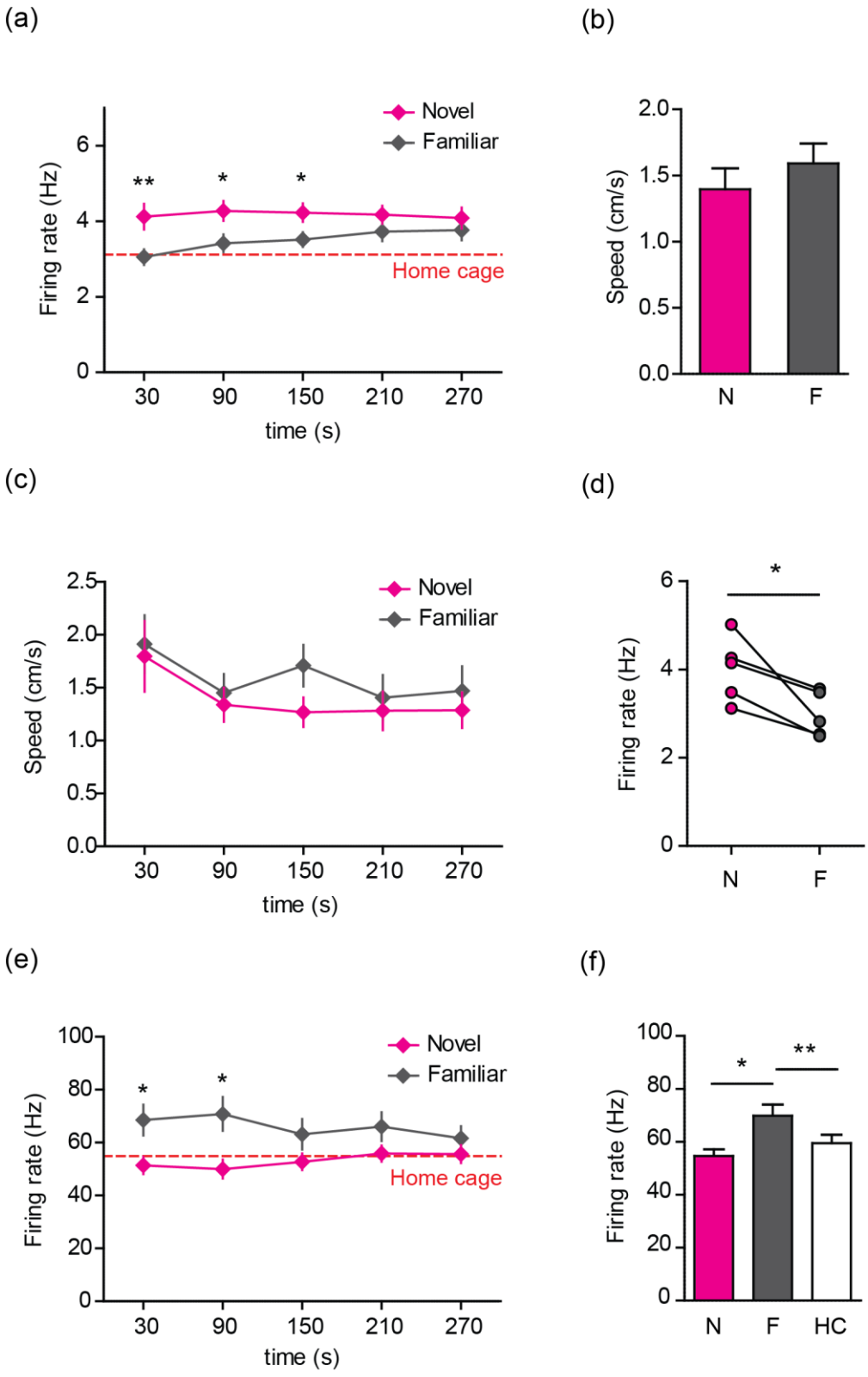
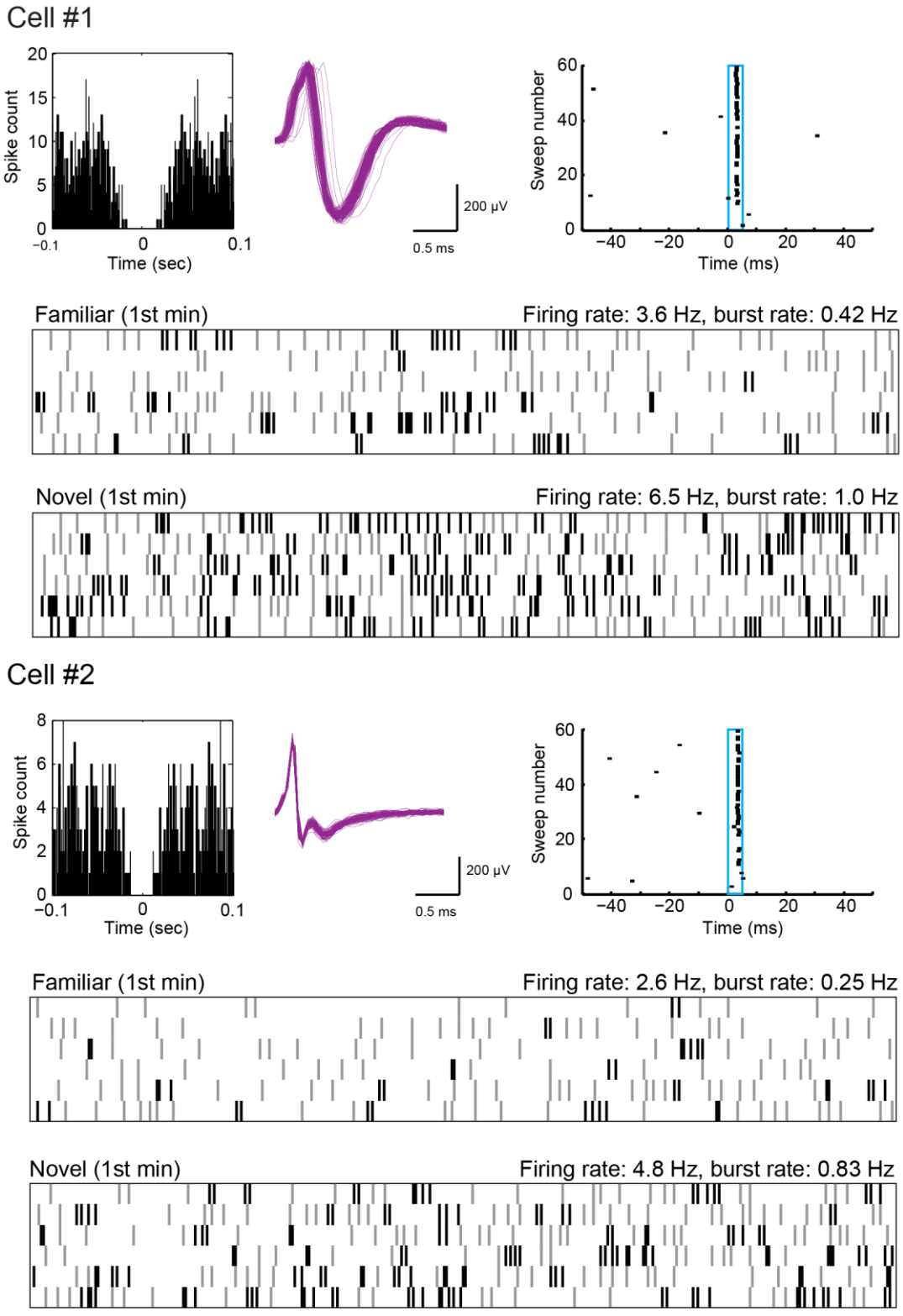
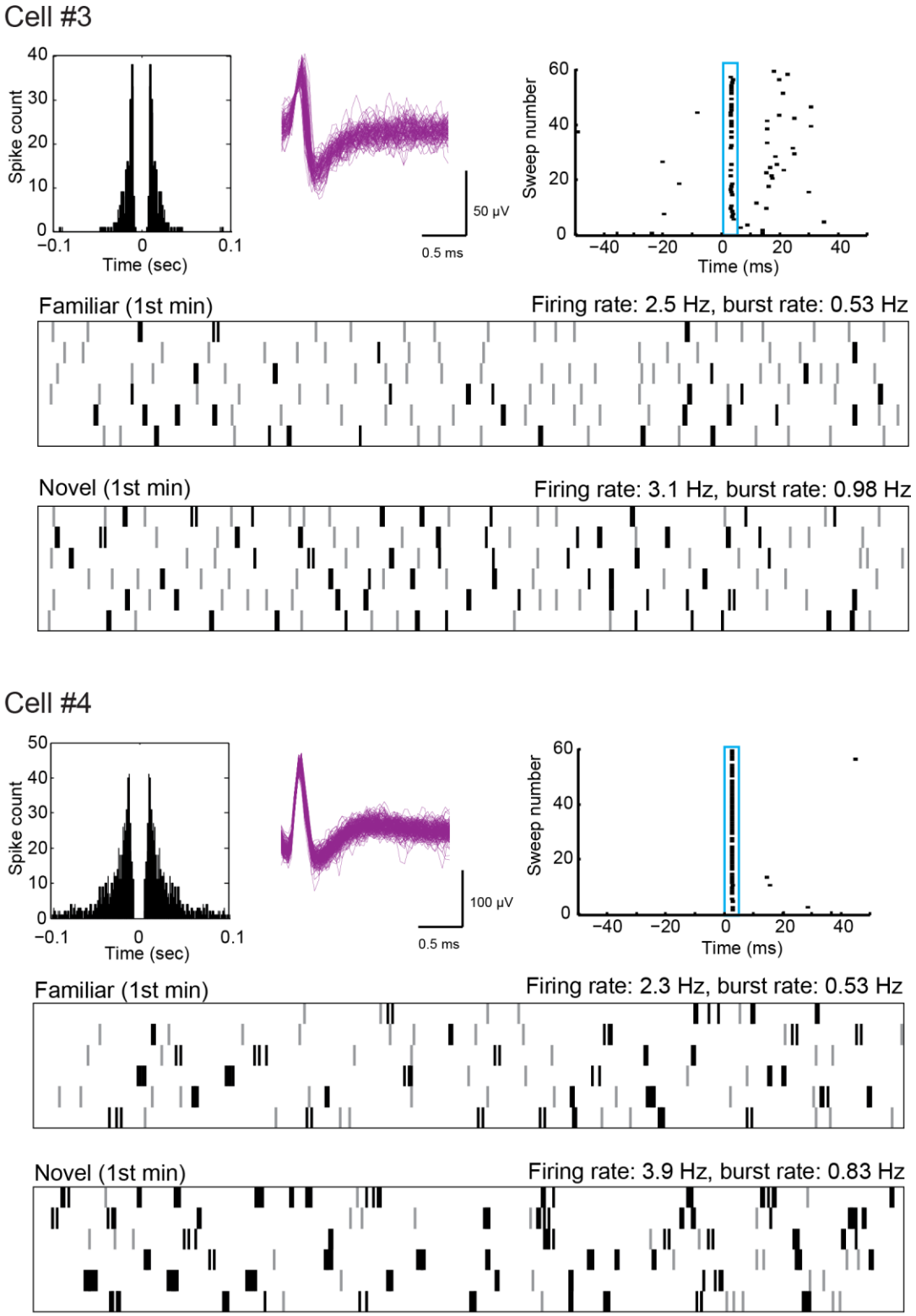

(a)
Novel
Familiar
Firing rate (Hz)
**
*
*
Home cage
time (s)
(b)
Speed (cm/s)
N
F
(c)
Novel
Familiar
Speed (cm/s)
time (s)
(d)
*
Firing rate (Hz)
N
F
(e)
Novel
Familiar
Firing rate (Hz)
*
*
Home cage
time (s)
(f)
*
**
Firing rate (Hz)
N
F
HC

Figure 4.8. Examples of optogenetically identified VTA-DA cells activated by novelty.

Each panel includes an interspike interval histogram and overlaid unit waveforms (top left), a raster plot with light activated spikes (top right) and raster plots from the first minute of exploration with burst events highlighted in black (bottom).

Cells #1 and #2 have more tonic firing patterns while cells #3 and #4 are more phasic.


Cell #1
Spike count
Time (sec)
200 µV
0.5 ms
Sweep number
Time (ms)
Familiar (1st min)
Firing rate: 3.6 Hz, burst rate: 0.42 Hz
Novel (1st min)
Firing rate: 6.5 Hz, burst rate: 1.0 Hz
Cell #2
Spike count
Time (sec)
200 µV
0.5 ms
Sweep number
Time (ms)
Familiar (1st min)
Firing rate: 2.6 Hz, burst rate: 0.25 Hz
Novel (1st min)
Firing rate: 4.8 Hz, burst rate: 0.83 Hz


Cell #3
Spike count
Time (sec)
50 µV
0.5 ms
Sweep number
Time (ms)
Familiar (1st min)
Firing rate: 2.5 Hz, burst rate: 0.53 Hz
Novel (1st min)
Firing rate: 3.1 Hz, burst rate: 0.98 Hz
Cell #4
Spike count
Time (sec)
100 µV
0.5 ms
Sweep number
Time (ms)
Familiar (1st min)
Firing rate: 2.3 Hz, burst rate: 0.53 Hz
Novel (1st min)
Firing rate: 3.9 Hz, burst rate: 0.83 Hz

values for each minute show a similar trend in both conditions. A 2x5 factorial ANOVA (condition x time bin) indicates a significant effect of time ($F_{4,56}$= 2.98, $p < 0.05$), effect of condition ($F_{(1,14)} = 0.90$, $p > 0.05$) and no interaction between the two ($F_{4,56}$) = 0.38, $p > 0.05$). It is therefore unlikely that VTA-DA neuron firing rate in VTA is related to motor activity.

The number of identified VTA-DA neurons in each mouse varied considerably (range: 1 to 6 neurons). It is therefore important to examine whether the observed effect is not biased by data obtained from a single animal. VTA-DA neuron firing rates in novel and familiar conditions were averaged for each animal. Animal averages followed the pattern observed in individual VTA-DA neurons, with all 5 animals showing higher VTA-DA neuron firing rates in the first minute of exploration, in novel environments compared with the familiar environment (1st minute average, paired t-test, $t_{(4)} = 2.56$, $p < 0.05$) (figure 4.7d).

Firing rates of fast-spiking neurons in VTA are modulated by novelty

A total number of 15 light-unresponsive neurons were recorded during novelty exploration sessions on the same tetrodes as identified VTA-DA neurons. Neurons with units detected on the same tetrode are likely in close proximity to each other, so it can be assumed that most of these neurons do come from VTA. A proportion of these neurons (n = 7) had very high firing rates (> 40 Hz) and short waveforms (biphasic duration ≤ 0.36 ms). One of the known and reliable properties of VTA-DA neurons is their low basal firing rate (< 10 Hz) and therefore these fast-spiking neurons were classified as putative non-dopaminergic (non-DA) neurons. Neurons included in this group showed firing rates above 40 Hz in all experimental conditions (mean baseline firing rate: 59 Hz, range 49 – 74 Hz). The remaining neurons (n = 7) had highly diverse firing properties (mean baseline firing rate 11 Hz, range 0.20 – 23 Hz). Curiously, fast-spiking VTA neurons showed a pattern of activity changes that is very different to that observed in identified VTA-DA neurons: they increased their firing rates selectively in familiar environments.

Average firing rates of fast-spiking VTA neurons were higher during familiar exploration as compared with both novelty exploration and home cage baseline (repeated measures ANOVA, $F_{(2,12)} = 13.06$, $p < 0.001$)(figure 4.7f). Post hoc pairwise comparisons with Bonferroni correction revealed increased mean firing rate in familiar environment in comparison with baseline ($p < 0.01$) as well as with the novel compartment ($p < 0.05$). This firing pattern contrasts with that of VTA-DA neurons, which tend to spike more in novel environments. It is conceivable that these fast-spiking VTA neurons act as a gating mechanism that controls VTA-DA neuron spiking. If this is the case, changes firing pattern in familiar environment should show a pattern opposite to

that of VTA-DA neurons. Analysis of their average firing rates in 1 minute bins with a 2-way ANOVA (time x condition) showed a significant effect of condition ($F_{(1,6)} = 17.88$, $p > 0.01$) but did not reveal any significant effects of time ($F_{(4,24)} = 0.29$, $p > 0.05$). Importantly, there was a significant interaction between time and condition ($F_{(4,24)} = 2.72$, $p < 0.05$) (figure 4.7e), indicating that the temporal dynamics of activity of these fast spiking neurons depend on whether the environment is novel or familiar.

4.3.3 Responses of LC-NA neurons to novelty

Another set of animals was implanted with optetrode microdrives directed at the locus coeruleus (LC). In reviewing these data, it should be born in mind that LC is extremely small in the mouse and thus the expectation was that, despite the care taken during surgery, accurate placement within LC may not be achieved in every animal. Ten light-responsive units (identified LC-NA neurons) were nonetheless observed, but in only 3 out of 7 LC-implanted mice. The number of neurons identified in each mouse ranged from 2 to 4 (see figure 4.9a and 4.9c for an example). There were a total of 8 novelty trials with identified LC-NA neurons.

In addition, 31 light-unresponsive neurons were recorded during novelty exploration sessions. Twelve of these light-unresponsive neurons were recorded during the same novelty sessions and on the same tetrodes as LC-NA neurons. These neurons likely come from outside LC, which is a highly homogenous structure in terms of neurotransmitter content, with an overwhelming majority of neurons staining positive for TH (Szabadi 2013).

Optogenetically identified LC-NA neurons

Identified LC-NA neurons had low firing rates during baseline recordings. Three out of nine LC-NA neurons were completely silent during baseline recording. Very few burst events (classified according to Grace and Bunney criteria) were observed during baseline recordings. Only 3 out of 7 spiking neurons fired more than a few bursts during the whole 5 min session, but the most frequently bursting neuron fired as many as 10.8 bursts per minute (see table 4.2 for summary of basal firing properties).

All identified LC-NA neurons had relatively broad waveforms lasting from 0.40 up to 0.72 ms (biphasic duration). Eight out of ten neurons had a stereotypical LC-NA neuron biphasic waveform with a pronounced afterhyperpolarisation tail (figure 4.9e). Mean waveform duration of identified LC-NA neurons was significantly higher than that of light-unresponsive units

Figure 4.9. LC-NA neurons in mouse LC identified using optogenetics.

(a) Raster plot of spikes fired by an identified LC-NA neuron during a single photostimulation epoch consisting of 60 pulses at 1 Hz. Only the spikes fired within 50 ms before and after stimulus onset are shown. Blue vertical stripe represent the light pulse.

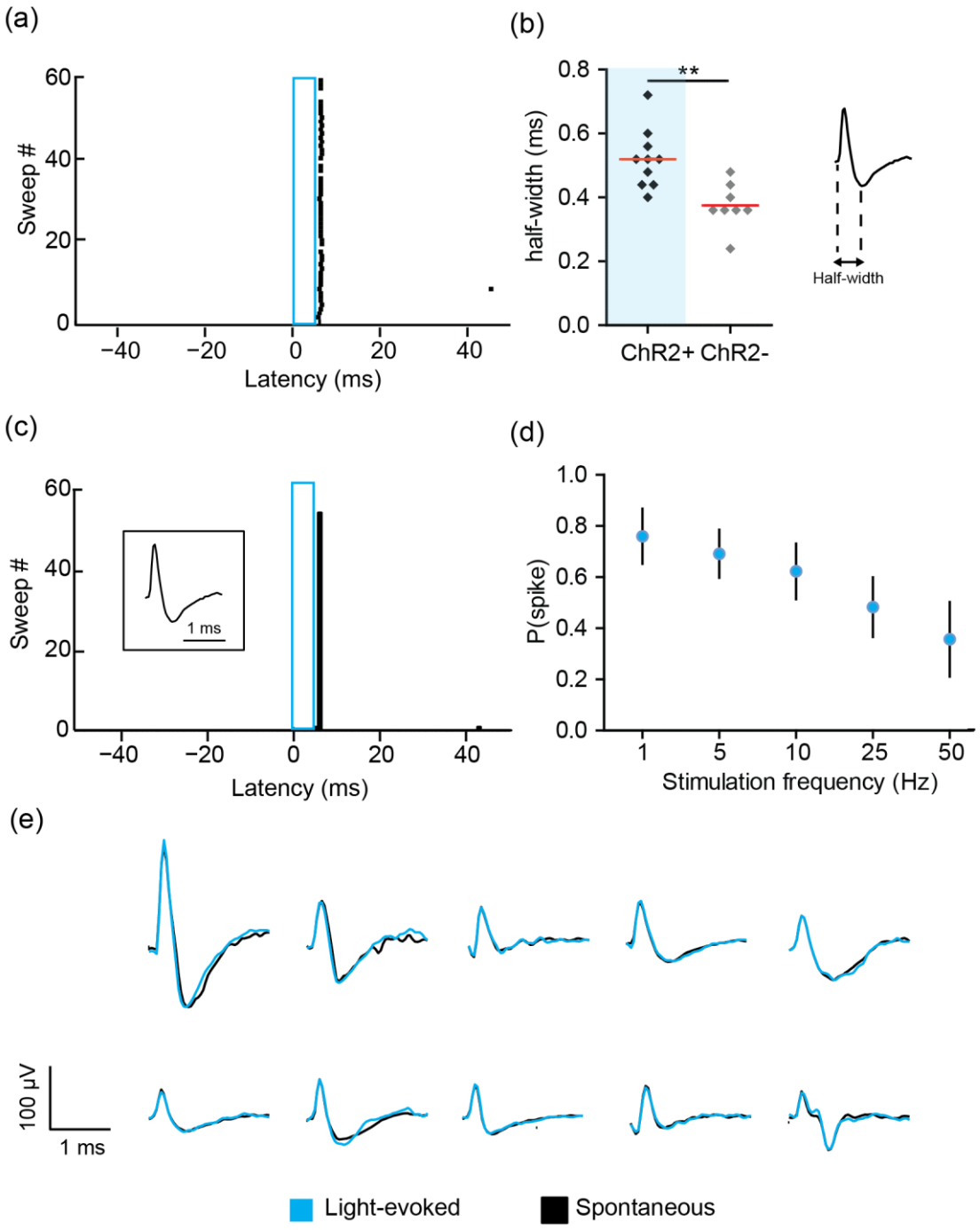
(b) Half-width of ChR2-positive (putative LC-NA) unit waveforms and waveforms of ChR2-negative units recorded on the same day and tetrode (putative non-NA neurons coming from outside LC). Putative NA units tend to have broader waveforms than other nearby neurons (ChR2+, n = 10; ChR2-, n = 8).

(c) Histogram and waveform of the unit shown in (a).

(d) Firing probability of ChR2+ neurons in LC in response to light trains at different frequency (n = 7).

(e) Average spontaneous and light evoked waveforms of all identified LC-NA neurons. In all cases there is a high degree of overlap between the two waveform shapes.

** $p < 0.01$. Error bars represent ±1 S.E.M.


(a)
Sweep #
Latency (ms)
(b)
half-width (ms)
**
ChR2+ ChR2-
Half-width
(c)
Sweep #
1 ms
Latency (ms)
(d)
P(spike)
Stimulation frequency (Hz)
(e)
100 µV
1 ms
Light-evoked
Spontaneous

recorded on the same day and tetrode (n = 9; independent t-test, $t_{(16)} = 3.66$, $p < 0.01$) (figure 4.9b). As in case of VTA-DA neurons, there was a significant overlap between two groups. However, the possibility that some LC-NA neurons did not respond to light pulses cannot be excluded.

We also investigated spiking probability of LC-NA neurons in response to light pulses at different frequencies, and found a similar pattern to the one observed in VTA-DA neurons: relatively high spiking probability in response to 1 Hz stimulation (76%) which then decreased with progressively higher stimulation frequency (figure 4.9d).

Property	Mean	Range
Firing rate	0.70 Hz	0 – 1.49 Hz
Burst rate	0.04 Hz	0– 0.02 Hz
Burst size*	2.08 spikes	2.00 – 2.19 spikes
% spikes in bursts	5.84 %	0 – 25.1%
Intra-burst firing rate*	22.4 Hz	17.2 Hz – 30.7 Hz

*Table 4.2. Baseline firing properties of LC-NA neurons. * LC-NA neurons silent during baseline recording were excluded.*

Firing patterns of LC-NA neurons in familiar and novel environments

LC-NA neurons reliably increased their firing rates in response to novelty. For example, one neuron with a mean baseline firing rate of 0.98 Hz increased its firing rate to 2.04 Hz (> 100% increase) during the animal's exposure to the novel floor substrate. Across the 10 neurons monitored, the mean doubling of firing rate was observed - from 0.70 Hz in the home-cage to 1.68 Hz in the novel environment.

Detailed analysis revealed a significant effect of experimental condition on firing rate (repeated measures ANOVA, $F_{(2,9)} = 8.48$, $p < 0.01$). *Post hoc* pairwise comparisons showed that mean firing rate of LC-NA neurons was selectively increased in novel environments ($p < 0.01$ versus familiar, $p < 0.05$ vs baseline, Bonferroni correction) (figure 4.10a). The pattern was consistent across identified LC-NA neurons - 9 out of 10 neurons had their peak firing rate in the novel

compartment, with the remaining one neuron being the most active during home cage recording. ANOVA also detected an effect of condition on burst frequency ($F_{(2,9)} = 4.15$, $p < 0.05$) (figure 4.10b) and percentage of spikes fired in bursts ($F_{(2,9)} = 4.57$, $p < 0.05$) (figure 4.10c) but pairwise comparisons with Bonferroni correction failed to detect differences between conditions.

As some neurons did not fire any bursts during exploration, burst size and intraburst firing rate were not compared between conditions. As in case of VTA-DA neurons, we asked whether increase of LC-NA neuron firing is a consequence of a general increase in excitability. First, firing rates of ChR2-negative neurons with matching firing rates (< 4 Hz; n = 11) were analysed in all three conditions (novel, familiar, home cage), and did not show any significant differences (one-way ANOVA, ($F_{(2,20)} = 0.83$, $p > 0.05$) (figure 4.10d). We then calculated the difference between average firing rates in novel and familiar environments for ChR2-positive neurons and the ChR2-negative neurons with matching firing rates (figure 4.10e). Identified LC-NA neurons showed significantly higher degree of differential firing than firing rate-matched putative non-NA neurons ($t_{(19)} = -2.84$, $p < 0.01$).

Temporal dynamics of putative LC-NA neurons were analysed in order to investigate the possible habituation of response. Interestingly, a quite different pattern was now observed to that described above for the VTA-DA neurons. Whereas VTA-DA neurons showed the paradoxical increase in firing rate in the familiar environment, putative LC-NA neurons showed a steady decline in the increased firing rate observed in responsive to novelty. That is, the novelty-associated enhancement appeared to habituate (Fig. 4.11a).

Minute-by-minute analysis of LC-NA neuron spiking during novel and familiar trials showed a different trend for each condition. Results of a 2x5 factorial ANOVA (condition x time bin) showed a significant effect of condition on firing rate ($F_{(1,9)} = 18.08$, $p < 0.01$) and importantly found an interaction between condition and time bin ($F_{(4,36)} = 4.35$, $p < 0.05$) (figure 4.11a). Analysis of each condition separately indicates that identified LC-NA neurons progressively decreased their firing rate during exploration of the novel compartment after initial peak (one-way ANOVA, $F_{(4,36)} = 2.69$, $p < 0.05$), but fired at a consistently low level in the familiar box (one-way ANOVA, $F_{(4,36)} = 0.45$, $p = 0.78$). Comparisons of firing rate between novel and familiar conditions in each 1-min bin indicate that LC-NA neurons exhibit differential firing during the first 3 minutes of exploration, after which the response habituates.

Transient increase of LC-NA neuron firing rate during exposure to environmental novelty could be attributed to changes in motor activity during the trial. There was a weak trend for animals to

explore the novel environment to a lesser degree (figure 4.11b). However, the overall difference was not statistically significant (paired samples t-test, $t_{(7)} = -1.30$, $p = 0.24$). There was an overall decrease in motor activity in time (2x5 ANOVA, condition x time bin, $F_{(4,28)} = 6.92$, $p < 0.01$) but no effect of condition ($F_{(1,7)} = 2.07$, $p = 0.19$) and no interaction between the two ($F_{(4,28)} = 1.27$, $p = 0.30$) (figure 4.11c). Additionally, all animals showed the same pattern of LC neuron activation by comparing firing rates of LC-NA neurons averaged for each animal (1st minute of exploration, paired t-test, $t_{(2)} = -18.00$, $p < 0.01$) (figure 4.11d). Together, these results indicate that LC-NA neurons are transiently activated by environmental novelty.

Figure 4.12 shows four representative examples of recorded LC-NA neurons. All neurons have very similar waveform shapes, although their firing patterns differ substantially. Cell #1 and cell #2 had relatively high firing rates for LC neurons in, with cell #2 firing at as much as 6 Hz in the first minute of novelty exploration, with quite high firing rate (3 Hz) in the familiar compartment as well. Cell #3 and cell #4 were almost completely silent during exploration of the familiar compartment. In the novel compartment, cell #4 showed a tonic firing pattern, while cell #3 occasionally fired long bursts of action potentials. Overall, despite apparent similarity in waveform shape and modulation of firing rate by novelty, recorded LC-NA neurons exhibit considerable variability in their firing patterns.

Figure 4.10. Average firing patterns of LC-NA neurons in novel and familiar environments.

(a) Average firing rates of LC-NA neurons in each condition. LC-NA neurons selectively increase their firing rate in novel environments.

(b) Average frequency of burst events in each condition.

(c) Average size of bursts in each condition

(d) Percentage of spikes fired in bursts in each condition

(e) Average firing rates of all ChR2- neurons recorded during novelty sessions with firing rates not exceeding those of identified LC-NA neurons (< 4 Hz).

(f) Histogram of the degree of differential firing (calculated as (novel firing rate) – (familiar firing rate)) of cells in (a) and (d). Majority of ChR2+ neurons (putative LC-NA) increase their firing rates in novel environment. This pattern is not observed in ChR- neurons (putative non-NA)

N, novel; F, familiar; HC, home cage. * $p < 0.05$, ** $p < 0.01$. Error bars represent ±1 S.E.M.

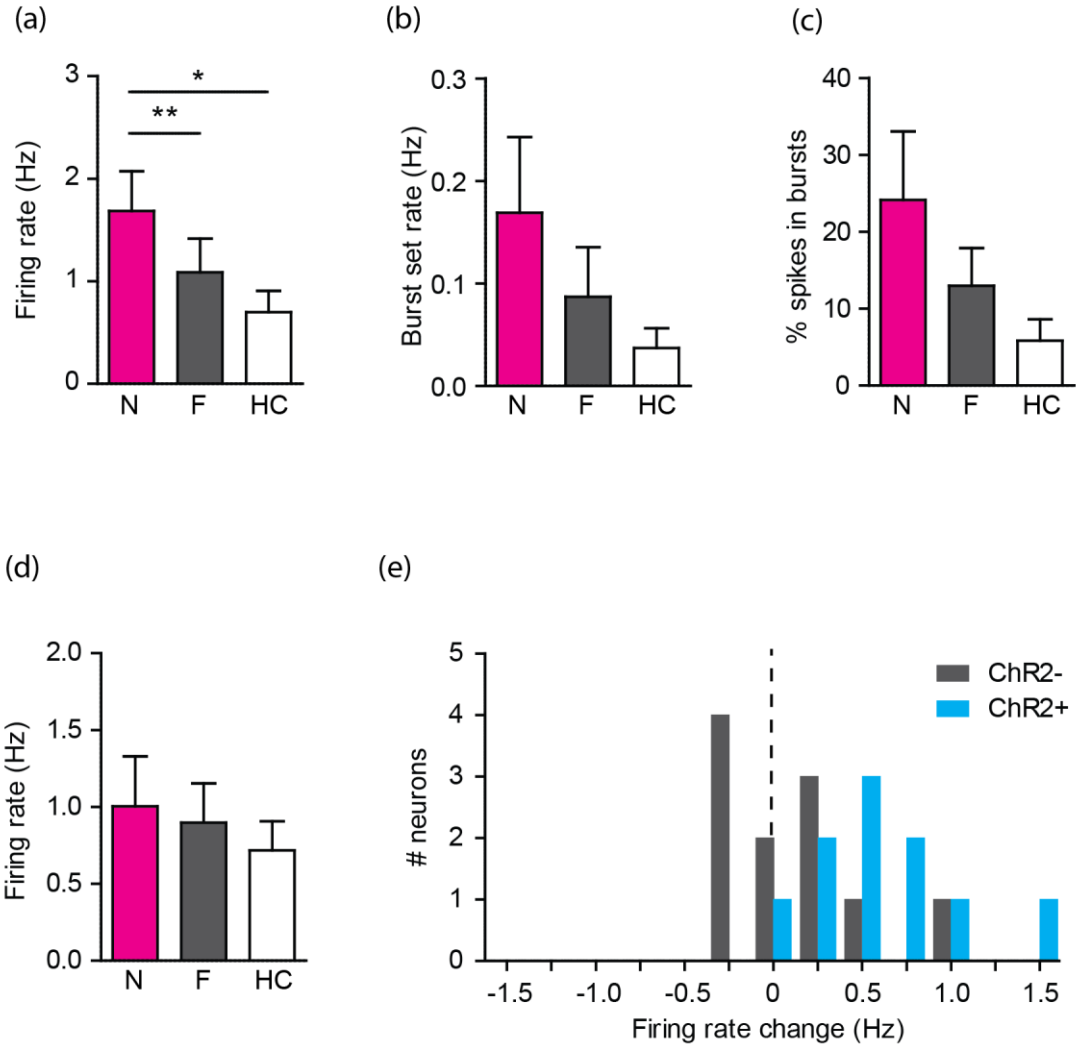

(a)
Firing rate (Hz)
3
2
1
0
*
**
N
F
HC
(b)
Burst set rate (Hz)
0.3
0.2
0.1
0.0
N
F
HC
(c)
% spikes in bursts
40
30
20
10
0
N
F
HC
(d)
Firing rate (Hz)
2.0
1.5
1.0
0.5
0.0
N
F
HC
(e)
neurons
5
4
3
2
1
0
ChR2-
ChR2+
-1.5
-1.0
-0.5
0
0.5
1.0
1.5
Firing rate change (Hz)

Figure 4.11. Firing rate changes in LC neurons and mouse motor activity during novel and familiar exploration

(a) Average firing rate of identified LC-NA neurons in novel and familiar environments, averaged in 1 minute bins. LC-NA neuron firing rate in the familiar environment stayed at a constant, low level throughout the recording session. In contrast, in novel environments LC-NA neurons initially increased their firing rates. Their activity then decreased as the trial progressed. Differential firing can only be observed in the first 3 minutes.

(b) Average mouse movement speed during exploration of novel and familiar environments.

(c) Mouse movement speed during the trial. When averaged in 1 min bins, changes in animal speed in both conditions largely follow the same pattern.

(d) All 3 mice show differential LC-NA neuron firing in the first minute of exploration. Two of the animals produced similar average values in both conditions.

N, novel; F, familiar; HC, home cage. * $p < 0.05$, ** $p < 0.01$. Error bars represent ±1 S.E.M. Red bar in (a) marks average LC-NA neuron firing rate in home cage.

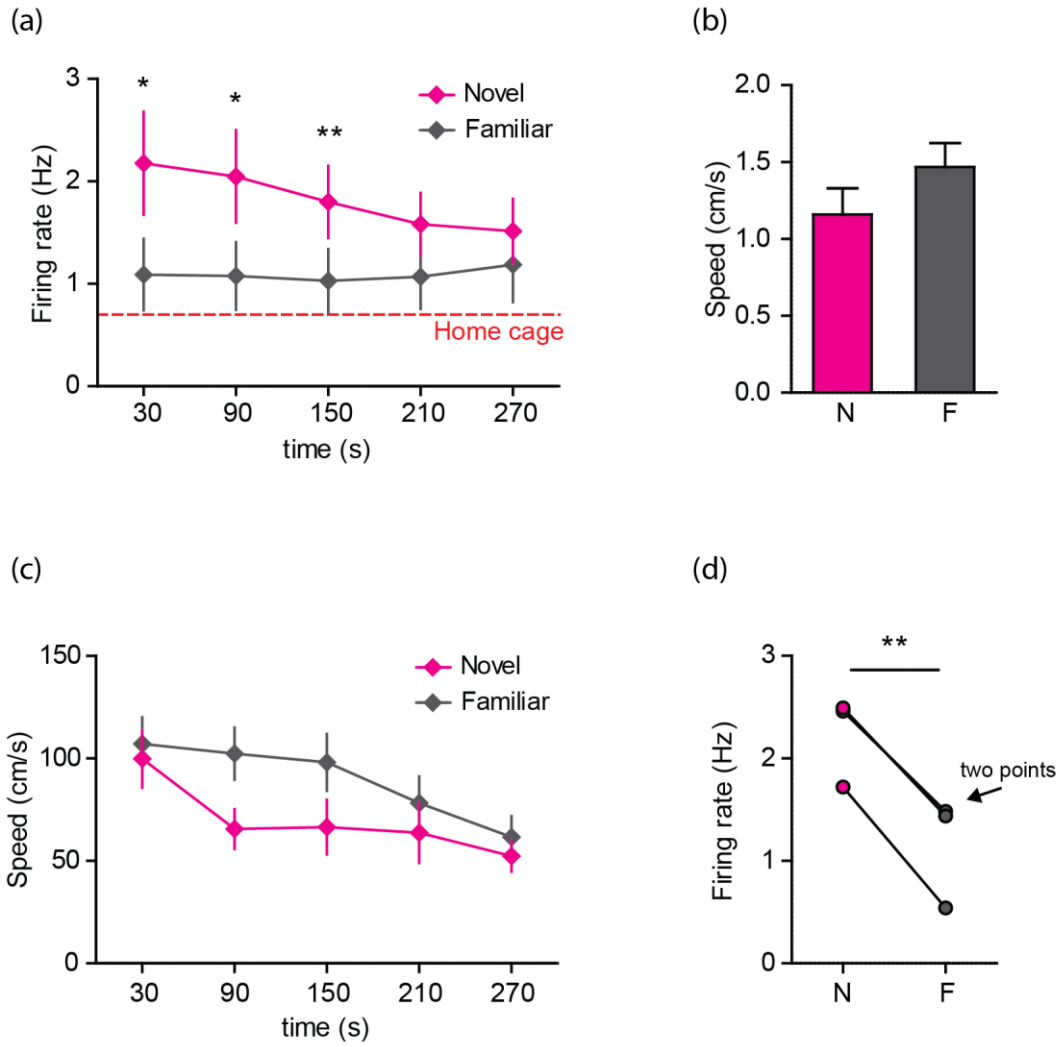
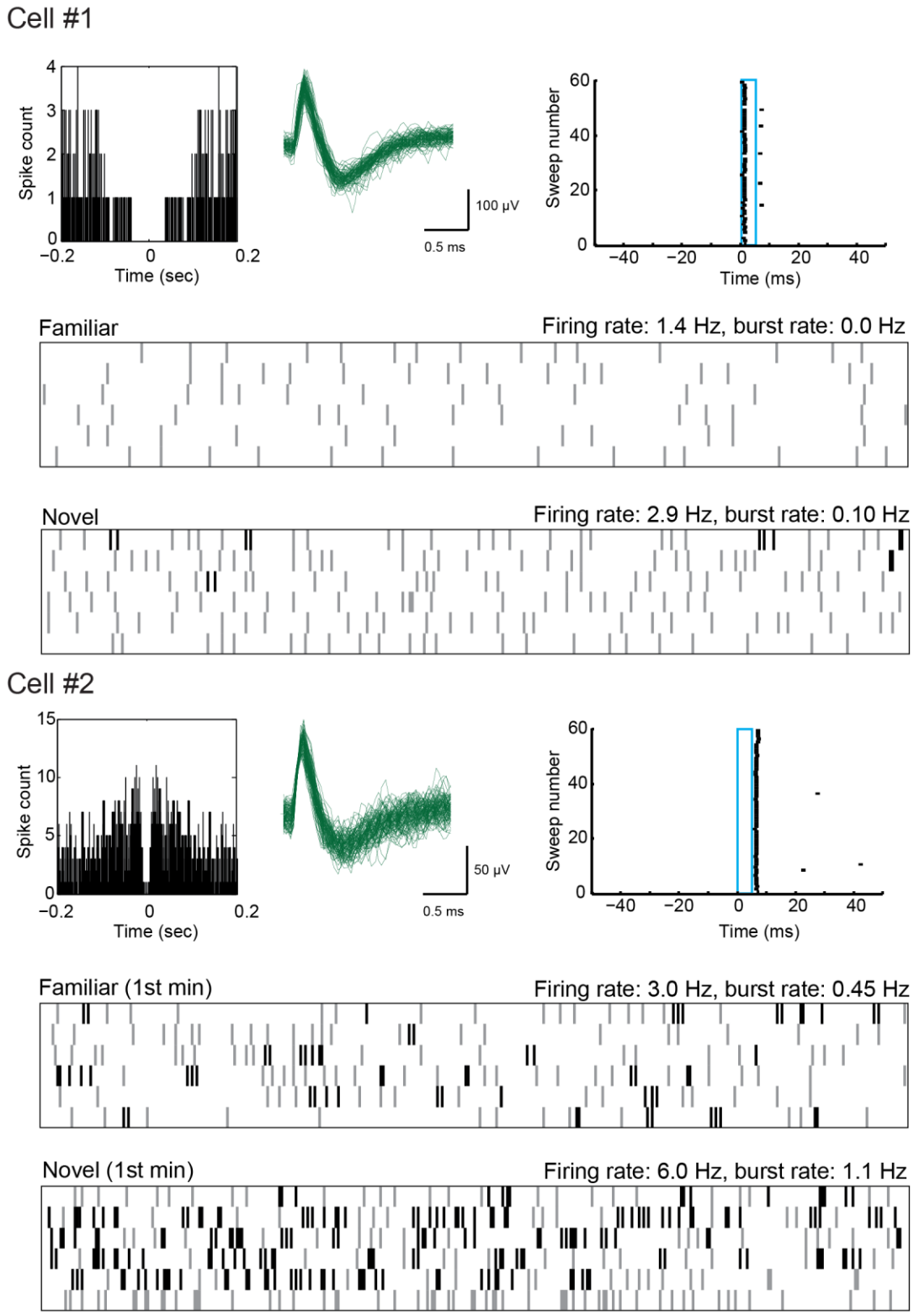
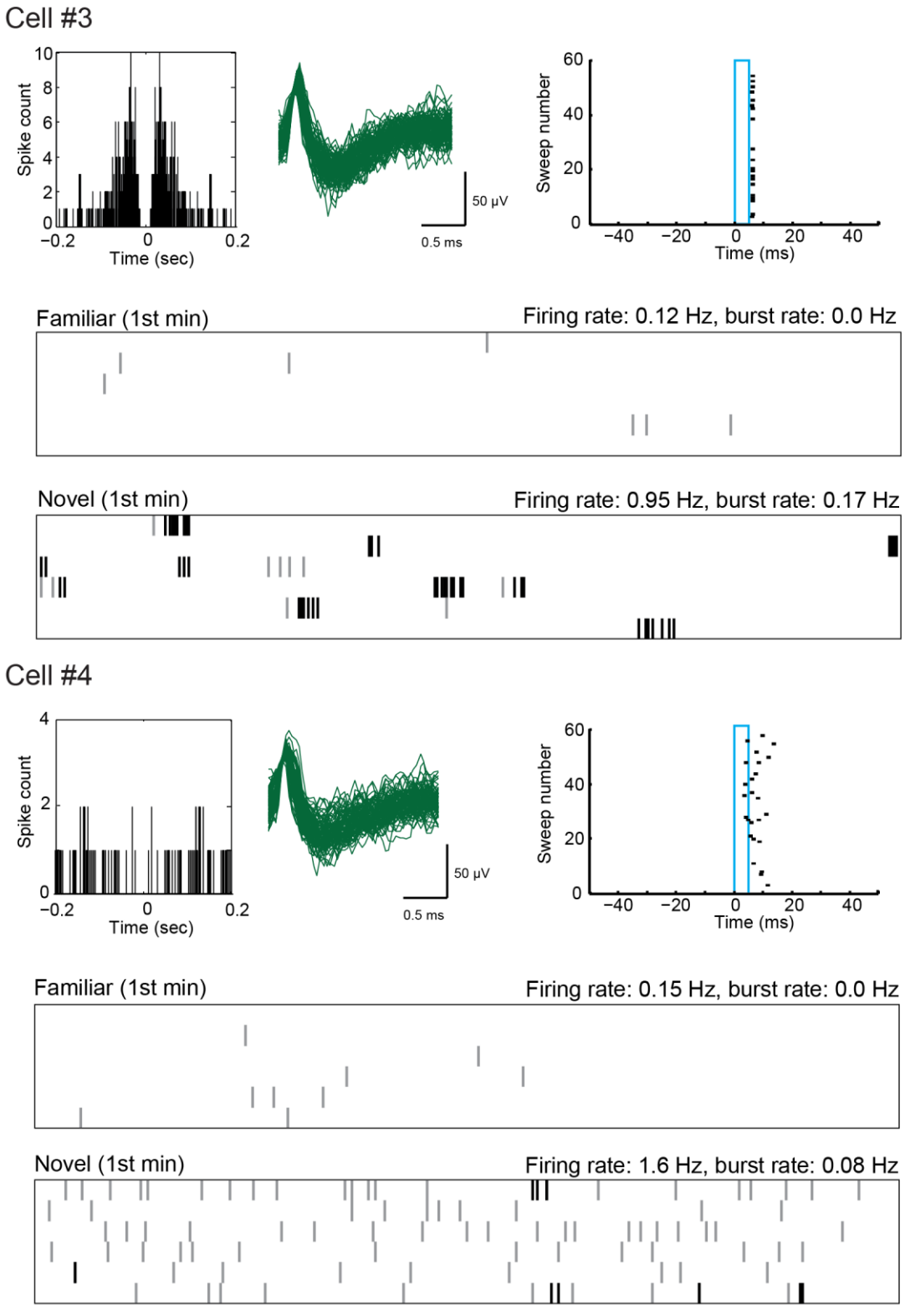

(a)
Firing rate (Hz)
3
2
1
0
*
*
**
Novel
Familiar
Home cage
30
90
150
210
270
time (s)
(b)
Speed (cm/s)
2.0
1.5
1.0
0.5
0.0
N
F
(c)
Speed (cm/s)
150
100
50
0
Novel
Familiar
30
90
150
210
270
time (s)
(d)
Firing rate (Hz)
3
2
1
0
**
two points
N
F

Figure 4.12. Examples of optogenetically identified NA cells activated by novelty.

Each panel includes an interspike interval histogram and unit waveform (top left), raster plot with light activated spikes (top right) and raster plots from the first minute of exploration with burst events highlighted in black (bottom). Cells #1 and #2 show relatively high firing rates in both novel and familiar environments, while cells #3 and #4 are almost quiet in the familiar environment.


Cell #1
Spike count
Time (sec)
100 µV
0.5 ms
Sweep number
Time (ms)
Familiar
Firing rate: 1.4 Hz, burst rate: 0.0 Hz
Novel
Firing rate: 2.9 Hz, burst rate: 0.10 Hz
Cell #2
Spike count
Time (sec)
50 µV
0.5 ms
Sweep number
Time (ms)
Familiar (1st min)
Firing rate: 3.0 Hz, burst rate: 0.45 Hz
Novel (1st min)
Firing rate: 6.0 Hz, burst rate: 1.1 Hz


Cell #3
Spike count
Time (sec)
50 µV
0.5 ms
Sweep number
Time (ms)
Familiar (1st min)
Firing rate: 0.12 Hz, burst rate: 0.0 Hz
Novel (1st min)
Firing rate: 0.95 Hz, burst rate: 0.17 Hz
Cell #4
Spike count
Time (sec)
50 µV
0.5 ms
Sweep number
Time (ms)
Familiar (1st min)
Firing rate: 0.15 Hz, burst rate: 0.0 Hz
Novel (1st min)
Firing rate: 1.6 Hz, burst rate: 0.08 Hz

4.3.4 Comparison of novelty response magnitude in VTA-DA and LC-NA neurons

As described in previous sections, temporal dynamics of activation in VTA-DA neurons during exploration of a novel (or familiar) environment are very different from dynamics exhibited by LC-NA neurons. In order to draw more direct comparisons between novelty-associated activation of those two areas, each neuron's firing rate was normalized to the mean baseline firing rate of all optogenetically identified neurons in the corresponding brain area. This way, the degree of firing rate modulation can be compared between the two populations of neurons. In doing so, an intriguing observation transpired – while on average VTA-DA neurons showed only modest difference in firing rate between novel and familiar environments (~33%), LC-NA neuron firing rate at the beginning of the novel exploration (i.e. when the floor substrate is the most 'novel') was twice as high as during familiar exploration.

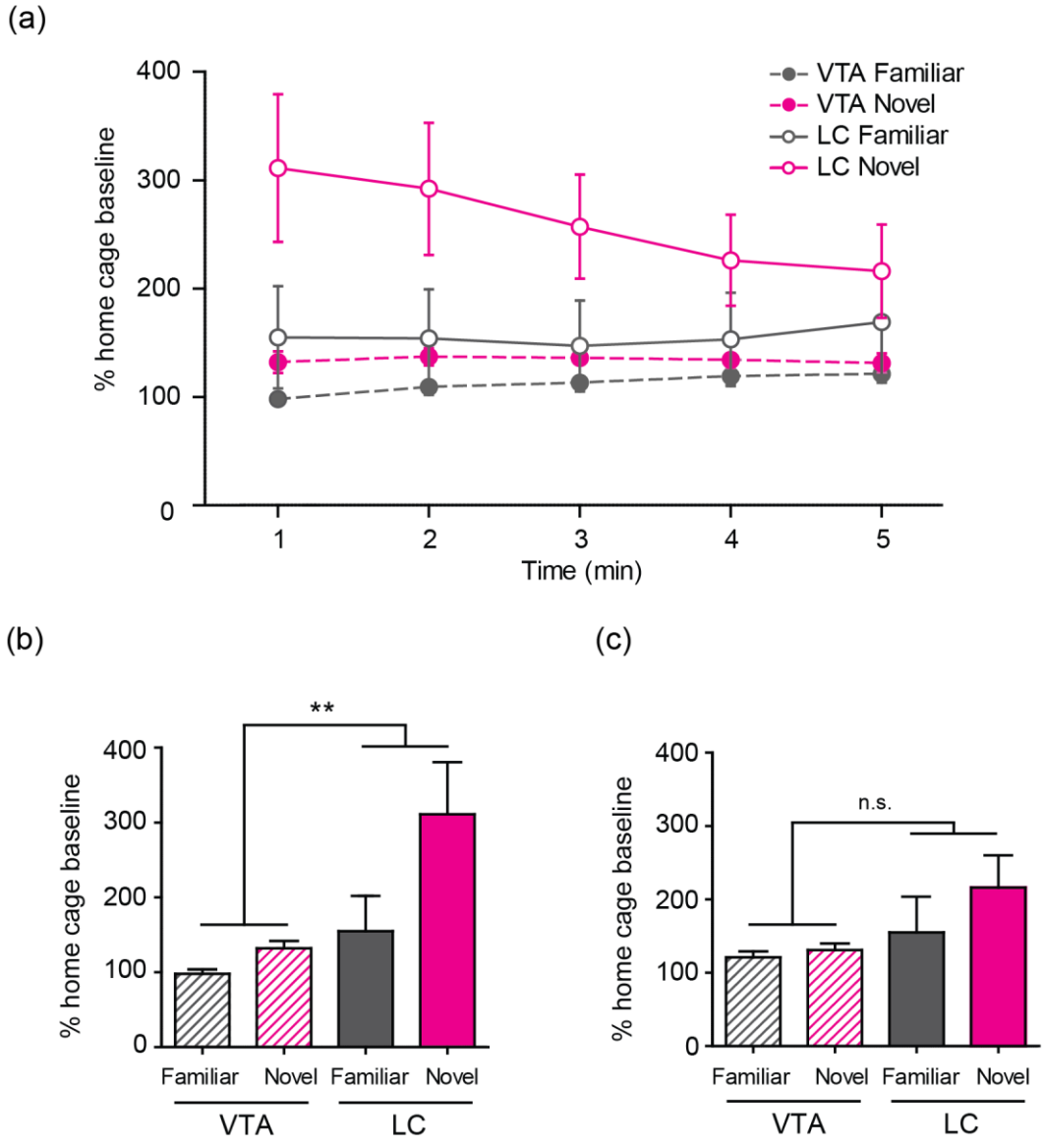
In order to compare the magnitude of response to novelty in optogenetically identified VTA and LC neurons, normalised firing rate of each neuron throughout each trial was split into 1 min bins. Binned and normalised firing rates were then analysed with a 3-way ANOVA (time x brain area x condition), resulting in a statistically significant three-way interaction ($F_{(2.03,,46.65)} = 3.75$, $p < 0.05$). This three-way interaction indicates that the difference in modulation by novelty between these two populations of neurons varies as a function of time. Figure 4.13a illustrates this result in a graphical form. LC-NA neurons increase their firing rates at the beginning of the trial to a much greater extent than VTA-DA neurons (significant brain area x condition interaction in the 1st min, $F_{(1,23)} = 11.99$, $p < 0.01$)(figure 4.13b). This difference in modulation by novelty disappears by the end of the trial (area x condition, 5th min, $F_{(1,23)} = 1.78$, $p < 0.05$) (figure 4.13c). This is arguably the most important finding reported in this chapter – it indicates that even though neurons in both areas show modulation by novelty, LC-NA neurons are more strongly activated by novelty than VTA-DA neurons.

Figure 4.13. Comparison of normalised firing rates in VTA-DA and LC-NA neurons in novel and familiar environments.

(a) LC-NA neurons are strongly modulated by novelty, whereas VTA-DA neurons show only modest modulation.

(b) and (c) The difference in degree of activation is the most pronounced in the first minute of exploration, disappearing completely by the end of the trial.

n.s., not significant; * $p < 0.05$, **$p < 0.01$. Error bars represent ±1 S.E.M


(a)
VTA Familiar
VTA Novel
LC Familiar
LC Novel
% home cage baseline
400
300
200
100
0
1
2
3
4
5
Time (min)
(b)
**
% home cage baseline
400
300
200
100
0
Familiar
Novel
Familiar
Novel
VTA
LC
(c)
n.s.
% home cage baseline
400
300
200
100
0
Familiar
Novel
Familiar
Novel
VTA
LC

4.4 Technical challenges

Conditional expression of ChR2 in genetically and spatially-defined populations of neurons allowed us to reliably classify single units recorded in brain areas that are either very small (LC) or highly heterogeneous (VTA). Doing this was one of the most exciting moments of the laboratory work – namely to turn on the blue light briefly and see an immediate response. Combining unit recording with optogenetics was a challenging task, and the following section will deal with practical aspects of this method as well as with technical limitations of our study.

Successful identification of a neuron based on its light-responsiveness is corroborated by the fact that it is often surrounded by other ChR2-expressing cell bodies and neuropil. Illumination of the brain area with high proportion of ChR2-expressing neurons will inevitably result in activation of a large neural population, giving rise to a substantial multiunit field potential registered on the electrode. In fact, the most reliable indicator of the electrode being close to the target area was presence of a time-locked multiunit spike in the field potential, similar to the one observed in optrode recordings in anaesthetised animals. Such spikes were observed on all electrodes with light-responsive units in LC and VTA.

The population spike was the most prominent in unreferenced recordings but could also be often detected in differential mode (with all wires of one tetrode referenced to a single wire of another tetrode). This posed a substantial challenge as light-evoked spikes fired by an isolated unit could be occluded by that high-amplitude light-evoked response. We found that differential recording within a tetrode (using one wire as a reference for the remaining three) often alleviated this problem. We hypothesise it is due to the fact that amplitude of the light-evoked potential is more similar on wires that are close together in space, and thus differential recording within a tetrode (i.e. referencing one wire of the tetrode to another one) causes them to cancel each other out. We performed all our recordings in this configuration, which in addition to minimising the light-evoked response substantially decreased the amount of background noise. However, in a small fraction of recordings, we still observed a residual light-evoked spike that could occlude light-evoked spikes. In order to overcome it, we made use of the fact that short light pulses at high light intensities can evoke multiple spikes in ChR2-expressing neurons. In all of these cases, a second light-evoked spike (latency of 10-15 ms from light onset) perfectly matched the spontaneous waveform.

Second, an important point to consider while performing unit recordings coupled with optogenetic stimulation is sufficient light delivery to the recording sites. Light emitted from the optic fibre

forms a cone of light in the brain tissue, with a radius that increases with the distance from the fibre tip and intensity that decreases exponentially with distance. The radius of the light cone at a given distance from the fibre is dependent on the fibre's numerical aperture (NA) and, to some degree, on the refractive index of the implanted brain area (Aravanis et al 2007, Yizhar 2012, Al-Juboori et al 2013). Tips of the electrodes have to be positioned sufficiently far from the tip of the fibre for the light beam to illuminate most of the area around the recording site. At the same time, enough light to activate ChR2-expressing neurons has to reach the recording area. We experimented with distances ranging from 200 to 1000 μm and archived best results with 1000 μm distance from the tip of the fibre to the tip of the tetrodes. This value is likely highly dependent on the NA of the fibres used, as higher NA translates to a larger angle of light scattering and would thus provide wider illumination at shorter distances from the fibre tip.

4.5 Discussion

A series of single unit recordings in mouse VTA and LC was performed during exploration of novel and familiar environments, using conditional expression of ChR2 in TH-positive neurons as an in vivo marker for catecholaminergic cells. This method made it possible to reliably identify these neurons based on their light-responsivity. We have shown that activity of both VTA-DA neurons and LC-NA neurons is positively modulated by novelty, which implicates catecholaminergic neurons in both of these structures as possible sources of hippocampal dopamine. Interestingly, when normalised to their baseline firing rates, LC-NA neurons showed much higher percentage modulation of their firing rates than VTA-DA neurons, especially so in the first minute of novelty exploration.

4.5.1 Mouse VTA-DA neurons are modestly activated by novelty

VTA-DA neurons showed a modest (~20%) increase in average firing rate and a similar (~25%) increase in burst firing in novel environments as compared with the familiar compartment.

Dopaminergic neurons have been previously shown to respond to novel events in primates (Ljungberg et al 1992). Authors of this study recorded unit activity of midbrain dopaminergic neurons in behaving macaques. Neurons were recorded during different stages of acquisition of a behavioural task, which involved retrieval of reward from a box in front of the animal. The box door was controlled remotely by the experimenter, and the first stage of training involved habituation to the opening of the door. Two electrophysiologically identified dopaminergic neurons recorded in the first stage of training showed short latency (<200 ms) bursts of activity in response to box opening. Both of these dopaminergic cells showed habituation of the burst response after repeated presentation of the stimulus, and none of the neurons recorded in subsequent stages of the task increased their firing rate in response to door opening. Rapid habituation of recorded dopaminergic neurons to the repeated event points to their role in novelty detection. However, due to very low sample size (n = 2 neurons recorded during habituation) one should be cautious when interpreting results of these recordings. In contrast, a study of VTA neuron responses to repeated visual and auditory stimuli in awake cats found no evidence of response habituation even after 50 or more stimulus presentations (Horvitz et al 1997). Though we did not observe any habituation of VTA-DA neuron response after several minutes of novelty exploration, during 2nd exploration of the 'novel' (once explored) environment on the same day VTA-DA neuron response was attenuated by ~10%. This reduction in firing rate suggests that the novel nature of the environment contributes the surge of VTA-DA neuron spiking.

In a recent landmark study, McNamara et al. (2014) used high density tetrode recording to monitor activity of VTA neurons in mice exploring new and familiar environments. Using a similar technique to the one described in this chapter, they identified VTA-DA neurons through conditional expression of ChR2 in dopamine transporter cre (DAT-Cre) mice. Though they only managed to successfully identify 5 VTA-DA neurons using optogenetics (C. McNamara, personal communication), they expanded their dataset by including all other recorded VTA cells with firing rates lower than the highest firing rate of an optogenetically identified VTA-DA neuron (11 Hz). Analysis of firing patterns of 157 VTA neurons showed an average firing rate increase of 0.56 ± 0.14 Hz in novel environments, as compared with familiar exploration. Criteria used in the study described in this chapter were much stricter and thus the number of neurons (15) included in the main analysis is substantially lower. Still, the average firing rate difference reported for VTA-DA in the present experiment is 0.68 ± 0.16 Hz, which agrees with the value obtained by McNamara and colleagues. Of interest is the fact that rate-matched light-unresponsive neurons recorded in the present study were not modulated by novelty, which may explain why the average change in firing rates reported by McNamara et al. (2014) is slightly lower than the one reported in this chapter.

A closer look at dynamics of VTA-DA neuron activity in the present study uncovers a rather peculiar phenomenon. While during exploration of the novel compartment VTA-DA neurons fired at a consistently elevated level, in the familiar environment they initially fired at baseline levels but by the end of the 5 min trial increased their firing rates to the level observed in the novel environment. Therefore, it seems that perceived familiar nature of an environment merely delayed VTA-DA neuron activation. Unfortunately, the short trial duration in our experiments did not allow us to determine whether this delayed increase in VTA-DA neuron spiking in familiar environments was of sustained or transient nature. This progressive increase in VTA-DA neuron spiking rate towards the end of the trial could not be explained in terms of locomotor activity alone. However, it is conceivable that behaviours other than active exploration could lead to activation of midbrain dopaminergic centres. Dopamine release has long been associated with grooming in rodents (Murray & Waddington 1989, Stivers et al 1988, Wiegant et al 1977), though mostly as a cause, rather than consequence of this type of behaviour. We often observed that after about 2-3 min in the familiar compartment, the mice often lost the incentive to actively explore the environment and were instead focusing on grooming behaviours. Due to low resolution of our video recordings, we were not able to quantify the occurrence of these behaviours throughout recording sessions. As an indirect measure, we looked at possible association between animal's

instantaneous speed and VTA-DA neuron firing rate, but did not find any significant correlations between the two (data not shown).

Interestingly, we found that a population of fast-spiking (>40 Hz) neurons in VTA increased their firing rate selectively in familiar environments. These neurons are likely GABAergic, as in contrast to dopaminergic neurons GABAergic neurons in VTA often show high basal firing rates *in vivo* (Cohen et al 2012, Tan et al 2012). These fast-spiking neurons attenuated their firing rate towards the end of the trial – an opposite pattern to the one observed in VTA-DA neurons. GABA neurons in VTA synapse directly onto and functionally inhibit VTA-DA neurons (Ciccarelli et al 2012, Tan et al 2012), so it is tempting to speculate that progressive increase of VTA-DA neuron firing rate observed while mice were exploring the familiar environment may be mediated by decrease in local inhibitory tone.

Baseline firing rates of VTA-DA neurons optogenetically identified in this experiment are within the range of mouse VTA-DA neuron firing rates reported in other studies that used either optogenetic (Cohen et al 2012) or classical Grace and Bunny methods (Robinson et al 2004, Zweifel et al 2009) (see table 4.3). Of note is the absence of VTA-DA neurons with firing rates higher than 5.5 Hz in the present study. This discrepancy could be attributed to larger sample sizes in these studies (17–32 DA neurons), as VTA-DA neurons with firing rates close to 10 Hz seem to be relatively rare. Another possibility could be a different approach to baseline recording – in present study we used animal's home cage as the baseline recording chamber, whereas all other studies reported in table 3. performed their recordings directly inside the experimental apparatus. Additionally, animals in Cohen et al (2012) were head-fixed, which could influence firing rates of VTA-DA neurons.

Firing rate (Hz)		Burst rate (Hz)		Burst size (spikes)		% Spikes in bursts		Ref.
Mean	Range	Mean	Range	Mean	Range	Mean	Range	
4.6	0.8 - 12.7	n/a	n/a	4.0	2.0 - 19.3	48.9	0.3 - 93.8	Robinson et al 2004*
3.62	n/a	~ 0.65	~ 0-1.7	n/a	n/a	~ 60	~ 0 - 85	Zweifel et al 2009*
~ 5	~ 0 - 10	n/a	n/a	n/a	n/a	n/a	n/a	Cohen et al 2012**
3.1	0.9 - 5.5	0.48	0.07 – 0.80	3.0	2.0 - 4.0	47.5	4.8 – 84.3	Present study**

*Table 4.3. Basal properties of VTA-DA neurons of awake mice reported in literature. *VTA-DA neurons identified using Grace and Bunney criteria; ** VTA-DA neurons identified using optogenetics.*

4.5.2 Mouse LC-NA neurons are strongly activated by novelty

Results of our LC recordings indicate that mouse LC-NA neurons show robust activation in response to environmental novelty, with an average 100% increase in firing rate in the first minute of novelty exploration as compared to the familiar environment. In contrast to VTA-DA neurons, a large proportion of LC-NA neurons were silent or nearly-silent during home cage recording - 7 out of 10 identified cells had baseline firing rates equal to or less than 0.01 Hz. Firing patterns of LC-NA neurons are highly modulated by the sleep/wake cycle (Aston-Jones & Bloom 1981a, Szabadi 2013) so their low firing rates may be indicative of the animal resting or being asleep. Indeed, often when a putative LC-NA neuron fell silent during screening, the mouse was observed to be resting quietly and was probably asleep. Waking up the mouse was enough for it to start spiking again. Identified LC-NA neurons showed huge variation in their firing rates during exploration. Some fired at frequencies of 3-4 Hz, while others generally did not exceed 1 Hz. Irrespective of their preferred firing rates, all of the recorded neurons spiked more readily in novel than in familiar environments. One of the neurons had its peak activity during the home cage recording – possibly an indication of a transient increase in animal's arousal level or a sleep to wake transition (Carter et al 2010, Sara & Bouret 2012).

To our knowledge, this is the first successful recording from identified LC-NA neurons in freely moving mice. However, a number of studies looked at activity of LC-NA neurons in different behavioural paradigms using other mammalian species. Evidence for a role of LC-NA neurons in novelty detection is largely based on work by the Sara laboratory, who recorded activity of LC-NA neurons in behaving rats exploring a hole board maze (Vankov et al 1995). LC-NA neurons discharged robust bursts the first time a rat approached an empty hole during habituation sessions, but were largely quiet on subsequent visits. On a fraction of trials, a small object was placed in one of the holes, and on trials with objects LC-NA neurons transiently increased their firing rate when rats first peeked into the hole but, again, did not respond on subsequent approaches. In our study, firing rate of identified LC-NA neurons in novel environments seems to peak in the first minute of exploration at almost three times the basal level and then decreases monotonically for the first couple of minutes until it reaches a steady state. In contrast, in familiar environments, LC-NA neurons fire with a constant, low rate. Habituation of response to novelty in our study closely reflects the findings reported by Vankov and colleagues.

4.5.3 Light-induced spikes and light stimulation frequency

Light-responsive cells readily responded to photostimulation at 1 Hz, firing a spike in response to 86% of pulses on average in case of VTA-DA neurons and in response to 76% of pulses in case of LC-NA cells. Increasing stimulation frequency led to higher rate of spike failure, with an average of only 43% of light pulses at 50 Hz evoking a VTA-DA neuron spike (36% for LC-NA neurons). This observation is in line with *in vitro* patch clamp recordings from VTA-DA neurons conducted by Karl Deisseroth's group using the same mouse line and AAV vector (Tsai et al 2009). In contrast, Cohen et al (2012) could optogenetically drive VTA-DA neurons in DAT-Cre *in vivo* at frequencies up to 50 Hz with spiking probability approaching 100%. In our study the pattern varied among the light-responsive neurons - some cells reliably followed photostimulation at up to 50 Hz with over 90% probability, while others completely failed to follow photostimulation at frequencies of 25 Hz and higher. Reasons for this discrepancy are unclear, but we hypothesise that it could be due to the level of illumination of ChR2-positive neurons used in our study. Though our laser had sufficient power output to be able to activate all illuminated neurons in the vicinity of the electrode tip, we exclusively used optic fibres with 0.22 NA, which produce a relatively narrow light cone. This was deliberate, as limited tissue illumination should activate fewer neurons and thus increase the chance of observing light-induced single unit spikes. However, it is conceivable that neurons that failed to follow high frequency photostimulation trains were positioned partially outside the illuminated area. This, however, does not explain the results of the slice experiment reported by Tsai and colleagues (2009), where a large area of the slice was illuminated.

4.5.4 LC-NA neurons are likely mediators of the novelty effect

A recent landmark study by Smith and Greene (2012) established that noradrenergic neurons are capable of co-releasing dopamine in the hippocampus, making it a viable candidate for mediating the novelty effect on memory persistence. An efficient machinery for gating of entry to long term memory through hippocampal dopamine release should be characterised by a relatively high signal-to-noise ratio, which means that the basal dopamine concentration in the hippocampus should be well below the critical concentration of dopamine required to activate hippocampal D_1 and/or D_5 receptors and initiate synthesis of plasticity-related products (PRPs). Characterisation of relationship between firing rate of VTA-DA or LC-NA neurons and hippocampal dopamine release is beyond the scope of this thesis. However, it is logical to assume that dopamine concentration at target sites is a (not necessarily linear) function of (1) density of projections in

target area and (2) firing rates of dopamine-releasing neurons projecting to the brain area in question. As mentioned in the previous chapter, projections of LC-NA neurons to the hippocampus vastly outnumber those of VTA-DA (see figure 2.4). Therefore, even though firing rates of LC-NA neurons in novel environments are on average 60% lower than VTA-DA neurons, the total amount of dopamine released from hippocampal LC-NA axon terminals might be larger than from VTA-DA terminals. Importantly, the 100% increase in LC-NA neuron firing rate in the first minute of novelty exploration may provide the all-or-none dopamine pulse and thus the high signal-to-noise ratio required for an efficient long-term memory buffer.

So far, the evidence for the role of LC-NA and/or VTA-DA neurons in mediating the novelty effect on memory persistence is largely indirect. In order to unequivocally establish whether either of these nuclei play a role in dopamine-mediated consolidation of transient memory traces it is imperative to conduct a mimicry experiment on the event arena, in which optogenetic activation of either of these nuclei is substituted for novelty exploration. Both VTA-DA and LC-NA neurons are activated by novelty to some degree and therefore are possible components of the behavioural tagging circuitry. Therefore, it is critical to investigate the effects of optogenetic activation of both of these nuclei on persistence of everyday spatial memories.

Chapter 5

Optogenetic Activation of Locus Coeruleus Enhances Persistence of Everyday Memory

5.1 Introduction

Electrophysiological and anatomical evidence identifies neurons in the ventral tegmental area (VTA) and locus coeruleus (LC) as potential sources of hippocampal dopamine (Lisman et al 2011, Lisman & Grace 2005, Smith & Greene 2012). We have previously shown that activity of both VTA-DA and LC-NA neurons is modulated by novelty, though LC-NA neurons show a much stronger relative modulation of response than VTA-DA neurons. Additionally, anterograde tracing experiments performed in our laboratory concluded that while both of these nuclei project to the hippocampus, axons from LC vastly outnumber those from VTA (T. Takeuchi, see figure 2.4). As all the evidence so far has been indirect, we designed a set of combined optogenetic and pharmacological experiments to establish whether activation of these brain areas is sufficient to enhance memory persistence in a manner that mimics the novelty effect.

One of the behavioural predictions of Synaptic Tagging and Capture (STC) theory is that activation of cellular pathways that lead to *de novo* synthesis of plasticity-related products (PRPs) is sufficient to boost persistence of transient synaptic modifications, and with it retention of transient memory traces. At a systems level, the neoHebbian framework postulates that dopamine release and subsequent activation of D_1/D_5 receptors accomplishes the same goal (Lisman et al 2011). However, a prediction that is unique to STC theory is that the dopamine pulse does not need to happen during encoding, and can influence persistence of memories encoded in the past,

provided the synaptic tags are still present at relevant synapses (Redondo & Morris 2011). Keeping that it mind, the experiment described in this chapter was designed to test this prediction. By optogenetically activating catecholaminergic neurons in these nuclei 30 min after encoding, substituting for novelty, it should be possible to rescue a weak memory trace that would normally be undetectable after 24 hours.

Optogenetics allows for precise bidirectional control of neural activity in genetically- and spatially-defined neural populations. As described in previous chapter, using Th-Cre mouse line it is possible to selectively express ChR2 in dopaminergic neurons of VTA or noradrenergic neurons of LC, depending on the site of injection. Inducing spiking activity in these populations of neurons through delivery of short blue light pulses should cause release of neuromodulators (dopamine in case of VTA, dopamine and noradrenaline in case of LC) throughout the brain. Such experiment on its own would be hard to interpret, given the global nature of neuromodulator release. However, if the boosting effect of optogenetic stimulation on memory is blocked by prior local infusion of a D_1/D_5 antagonist into the hippocampus (but not by a β-adrenergic receptor antagonist), it will be possible to conclude that (1) hippocampal dopamine is a critical mediator of the effect of optogenetic stimulation, and (2) optogenetic stimulation closely mimics the effect of novelty on memory persistence.

5.2 Methods

5.2.1 Subjects

The subjects were 14 thyrosine hydroxylase Th-IRES-Cre (henceforth referred to as Th-Cre) heterozygous male transgenic mice (see chapter 3 methods for details). Eight Th-Cre mice received ChR2-eYFP-AAV injection and six mice received injections of the control vector (eYFP-AAV).

5.2.2 Viral vectors

See chapter 4 methods for details of Cre-inducible AAV vectors used. The viral concentration was 8 x 10^{12} particles per ml for hChR2(H134R)-eYFP and 4 x 10^{12} particles per ml for the eYFP control vector. A single batch of virus was used throughout the study.

3.2.3 Behavioural apparatus

Behavioural apparatus used was the same as the one described in chapter 3.

3.2.4 Training protocol

Animal training was conducted as described in chapter 3.

5.2.5 Surgery

Due to very small size of targeted brain nuclei, surgical procedures were modified to allow for even greater precision. Additionally, long duration of the procedure (9-11 hours) called for introduction of additional measures aimed at increasing animal survival rate. Anaesthesia was induced using 5% isoflurane and mice were placed in the stereotactic frame. From this point onwards, anaesthesia was maintained with < 1% isoflurane, occasionally increased to 1.5% during skull drilling. Air flow was kept at 1000 ml/min throughout the surgical procedure. Breathing rate, heart rate and blood oxygenation level was monitored throughout the surgery using MouseOx Plus system (Starr Life Sciences Corp.) coupled to a thigh sensor. Temperature was measured using a rectal probe coupled to a closed-feedback heating pad and kept at 36.9-37.4°C, and mouse was additionally insulated with a cotton wool blanket to prevent heat loss. We found that very low level of isoflurane (~ 0.8%) was critical for ensuring quick recovery after

anaesthesia. On-line readout of breathing rate proved invaluable at assessing adequate levels of anaesthesia, and mice were generally kept at 90-130 breaths per second.

The critical step during surgery was to precisely measure the midline, bregma and lamba co-ordinates. Midline was measured directly on the brain surface through the large hole drilled above VTA on both sides of the brain. In order to increase the precision even further, portions of lambdoid and coronal sutures were cleaned of connective tissue with a sharp probe and 30% hydrogen peroxide, and all measurements were performed on anterior ridges of the sutures. Bregma was defined as the point of intersection between coronal and sagittal suture. If there was more than one point of intersection (one on each side), the co-ordinates were averaged. In contrast, anterior-posterior (AP) co-ordinate of lambda was defined as the line of best fit going through the lateral portions of lambdoid suture, and obtained by averaging the AP measurement at 3-5 points on each side. Pilot studies indicated that this is the most precise and reproducible measurement of lambda. Mouse skull was then tilted along the medial-lateral axis until it the difference in dorsal-ventral (DV) co-ordiantes of bregma and lambda was less than 30 μm. The same adjustment was performed along the sagittal axis (difference between midline ± 2.0 mm in ML less than 30 μm).

Subsequently, small holes (0.2 mm diameter) were drilled above LC and a larger hole (1.0 mm diameter) was drilled above VTA, across the midline. The viral vector (1 μl per side) was injected at 0.1 μl/min into VTA (from lambda: AP, +1.20 mm; ML, ± 0.5 mm; and DV, -4.4 mm) or LC (from lambda: AP, -1.30 mm; ML, 1.15 mm; and DV, 3.4 mm) using a high precision Nanofil syringe (World Precision Instruments) and UMP3 pump (World Precision Instruments) mounted directly on the stereotactic frame. The pump was started right before lowering the needle into the brain in order to ensure that the syringe did not get blocked during insertion. Target co-ordinates were reached after ~1 min, which indicates that around 0.9 μl of the virus reached the target injection area. Needle was left in the brain for an additional 10 min after the end of injection in order to prevent backflow.

Two kinds of bilateral fibre-ferrule implants (Doric Lenses) were used. A dual ferrule (1.0 mm fibre spacing, 200 μm core diameter) was implanted vertically into VTA (from lambda: AP, +1.15 mm; ML, ± 0.5 mm; and DV, -4.3 mm). Subsequently, a two-ferrules implant (1.8 mm fibre spacing, 200 μm core diameter) was implanted into LC at -30° angle to coronal plane (from lambda: AP, +3.63 mm; ML, ± 0.90 mm; and DV, -3.02 mm). This required carefully detaching

part of the occipitalis muscle with a scalpel blade, and drilling two small holes (0.2 mm diameter) in the occipital bone.

Additionally, small holes were drilled bilaterally anterior to dorsal hippocampus and bilateral 26-gauge steel microinjection guide cannulae (4.0 mm length, 3.0 mm distance between cannulae; Plastics One) with stylets (33 gauge, Plastics One) were inserted into the dorsal hippocampus at a 40° angle to coronal plane (from bregma: AP, +3.70 mm; ML, ± 0.15 mm; and DV, - 2.40 mm).

Due to long duration of the surgical procedure, mice were given several subcutaneous fluid injections at different stages: 1 ml of 0.9% saline at the start and ~3h into surgery, and 1 ml of glucose solution during recovery from anaesthesia. Additionally, they were given a subcutaneous injection of carprofen after surgery and their daily weight was monitored for 7 days post-surgery.

Surgery was performed jointly with Dr Tomonori Takeuchi.

5.2.6 In vivo optogenetic stimulation

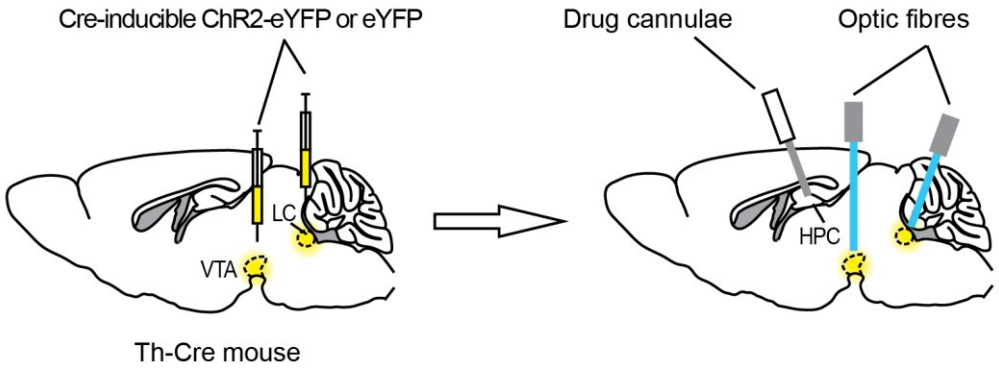
Laser stimulation was performed using two blue solid-state diode pumped lasers (473 nm; Laser 2000) connected to either a dual fibre optic patch cord (VTA) or two single fibre optic patch cords (LC; both patch cords: 200 μm core diameter, 0.22 NA). Both lasers were synchronously controlled using custom built Lab View software (P. Spooner) via TTL pulses. Laser power was measured using a photometer (Thor Labs) and calibrated to 18-19 mW at the beginning of each photostimulation day, and then checked again at the end of the day in order to make sure that patch cords were not damaged during the session. Photostimulation was performed in animal's home cage, in the control room adjacent to the event arena room. Mouse was plugged in 2 minutes before the start of stimulation and unplugged 2 minutes after the last light pulse was delivered. Burst photostimulation protocol was chosen in order to maximize neurotransmitter release from in regions efferent to activated brain areas (Tsai et al 2009). Mice were given a series of 20 blue light pulses (5 ms duration) at 25 Hz every 5 seconds for 5 minutes. The same stimulation protocol was used for VTA and LC stimulation. The chosen stimulation frequency is within the physiological range – catecholaminergic neurons in VTA and LC with intraburst firing rates of more than 25 Hz were observed during novelty exploration (see table 4.1 and 4.2).

Figure 5.1. Implantation of optic fibres and intrahippocampal cannulae.

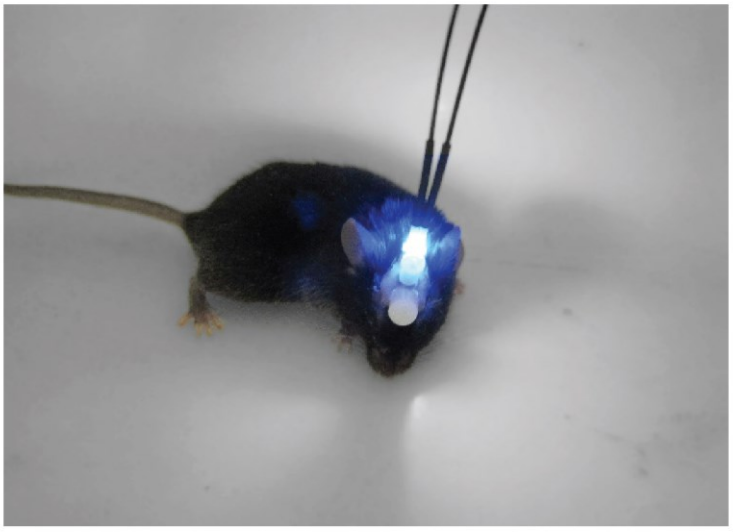
(a) Mice were injected bilaterally with either ChR2-eYFP or eYFP AAV into both VTA and LC, and then implanted with bilateral optic fibres above both of these nuclei. Additionally, a bilateral drug cannula implant was inserted into the dorsal hippocampus.

(b) Implanted mouse connected to an optic fibre patch cord.

(a)


Cre-inducible ChR2-eYFP or eYFP
Drug cannulae
Optic fibres
LC
VTA
HPC
Th-Cre mouse

(b)



After implantation and recovery from surgery animals were extensively habituated to the photostimualtion procedure in order to eliminate 'novelty' effect of the procedure on memory persistence. First, they underwent 10 days of connecting and disconnecting the patch cords (5 min per animal per day). Subsequently, animals were habituated to the whole procedure described in the previous paragraph (except for blue light delivery) for 6 days (3 days for LC, 3 days for VTA). Due to a substantial leak of light through the connection between the patch cord and the implanted ferrules, animals were habituated to the procedure for additional 3 days, this time using a connecting sleeve that blocks light delivery to the brain but still produces secondary light emission (a 'mock light on' condition). When the laser light was on, mouse was still able to see flashes of light, but the light did not reach the animal's brain.

5.2.7 Microinfusions and drugs

Intrahippocampal microinfusion was performed according to the procedure described in section 3.2.7. Animals were habituated to the injection procedure for 5 days before the pharmacology probe tests. Habituation procedure was identical to the drug microinfusion procedure during subsequent probe tests. Animals received saline infusion on 3 days and no infusion on the remaining 2 days.

Drug concentrations for infusions were 21.1 mM for propranolol ((S)-(-)-Propranolol hydrochloride, Tocris) (corresponding to 6.25 μg/μl) and 3 mM for SCH23390 ((R)-(+)-SCH 23390 hydrochloride, Tocris, Bristol, UK) (corresponding to 1 μg/μl). We used 0.9% NaCl (saline) in H_2O as a vehicle and for control infusions. Vehicle and drug solutions were stored in 100 μl aliquots at -20°C until use.

5.2.8 Experimental design

Due to the time constraints on probe test days (2 animals per hour) the number of animals in each group had to be substantially smaller than the number of animals used in the previous experiment. Therefore, in order to decrease the variance in animal scores, all critical probe tests involving photostimulation were conducted at least twice and the digging time scores for each animal were averaged.

After habituation, shaping and pre-training (12 days) mice underwent 95 daily training sessions. The experiment was divided into three blocks:

- **initial training (session 1 – 46)**
- **photostimulation, (session 47 – 83)**
- **photostimulation with pharmacology (session 84 – 125)**

In first block, after 15 days of standard training followed by a probe test with 2 sample sessions and 10 min delay between encoding and retrieval, animals were divided into two groups. One group (n = 8, henceforth referred to as ChR2 group) received intracerebral injections of ChR2-eYFP-AAV into LC and VTA. The other group (n = 6, henceforth referred to as control group) was injected with the eYFP-AAV control vector. Both groups were then implanted with bilateral optic fibre implants into VTA and LC, and, additionally, with bilateral microinfusion cannulae directed at dorsal hippocampus. Animals in both groups were performance-matched, based on performance on training days 1 – 15. After recovery from surgery animals were trained for additional 30 days before the probe tests started. In between training days three 24 hour probe tests were conducted: with 1, 2, and 4 sample trial encoding. These probe tests were conducted in order to confirm that 1 sample encoding produces weak memory and 4 sample encoding results in strong memory. Only the 1-sample encoding probe test was part of the initial design and therefore all three tests were not counterbalanced.

In the second block, two sets of 3 counterbalanced 24 hour photostimulation probe tests were conducted with 3 conditions. Animals received either VTA photostimulation (*VTA light ON*), LC photostimulation (*LC light ON*), or no photostimulation at all (*light OFF*). Due to unexpected results in block 2 (4 sample encoding not producing strong memory in ChR2 group, described in results section), 4 sample encoding was used as weak encoding in this block. In all cases, light was turned on 30 min after the start of encoding. In the *light OFF* condition, mice were subjected to the same photostimulation procedure, with the exception of activating the lasers. Animals in this condition had patch cords connected to either VTA or LC fibre ferrules in an alternating manner.

Block 3 involved two sets of counterbalanced 24h probe tests with 4 conditions.The first three conditions involved photoactivation of LC, preceded by microinfusion of one of three drugs: SCH23390 (*LC Light ON + SCH*), propranolol (*LC light ON + prop*) or vehicle (*LC light ON + veh*). The fourth condition (*LC light OFF + veh*) was equivalent to the 'light OFF' condition from

the previous block and served as a negative control for 24h memory persistence – animals received saline infusion and subsequently patch cords were plugged in to the LC fibre ferrules but lasers were not activated. In all cases, drug microinfusion was performed 10 min after the start of encoding and photostimulation was performed 30 minutes after the start of encoding. Addition of the microinjection procedure into the protocol forced the need to decrease the number of encoding sample trials - each sample trial takes about 4 minutes to complete. First round of probe tests in this block was completed with 2 sample trials and second round was completed with 1 sample trial.

3.2.9 Data collection and analysis

Data collection and analysis was performed as described in chapter 3.

Additionally, percentage digging scores from block 3 were analysed with a repeated measures ANOVA followed by a *post hoc* a set of orthogonal comparisons based on *a priori* predictions. The first orthogonal comparison is based on the hypothesis that the novelty effect and the LC photostimulation effect share a common neural mechanism:

- *LC light OFF + veh:* control condition without LC stimulation results in **baseline** performance
- *LC light ON + veh:* LC photostimulation enhances memory persistence, resulting in **high preference** for correct location
- *LC light ON + SCH: i*nfusion of SCH23390 prior to LC stimulation blocks the memory enhancing effect, resulting in **baseline** performance
- *LC light ON + prop:* infusion of propranolol prior to LC stimulation has no effect on digging preference, resulting in **high preference** for correct location

The first two of the predictions are derived from the results of block 2 probe tests. The remaining two predictions are derived from the experiment described in chapter 3, based on the hypothesis that the LC photostimulation effect mimics the novelty effect, and thus both phenomena have the same pharmacological profile.

In order to avoid a false positive result of the previous comparisons, another set of orthogonal comparisons was conducted, this time assuming that LC photostimulation **does not** pharmacologically mimic the effect of novelty. In this set of comparisons, SCH23390 is predicted to have no effect but propranolol blocks the effect of LC photostimulation:

- *LC light OFF + veh:* control condition without LC stimulation results in **baseline** performance
- *LC light ON + veh:* LC photostimulation enhances memory persistence, resulting in **high preference** for correct location
- *LC light ON + SCH: i*nfusion of SCH23390 prior to LC stimulation blocks the memory enhancing effect, resulting in **baseline** performance
- *LC light ON + prop:* infusion of propranolol prior to LC stimulation has no effect on digging preference, resulting in **high preference** for correct location

3.2.10 Perfusion and histology

After the experiment, animals were injected with euthatal and perfused transcardially with 4% paraformaldehyde (PFA) dissolved in PBS. Brains were removed and post-fixed in 4% PFA for a minimum of 24 h. Complete histological analysis was not yet performed at the time of submission of this thesis.

5.3 Results

Fourteen Th-Cre mice were trained on the everyday appetitive spatial memory task introduced in chapter 3. After initial training, mice were divided into two groups that underwent the same surgical procedure, the only difference being the type of Cre-dependent viral vector injected into VTA and LC: ChR2-eYFP vector (ChR2 group) or eYFP vector (control group). One of the strengths of the behavioural protocol used in this study is the ability to trace performance of individual animals through a battery of experimental conditions. Therefore, we decided to implant each animal with optic fibres into both VTA and LC and compare the effects of optogenetic activation of these nuclei in each animal. This experimental design provided us with two orthogonal experimental controls: a within-subject control (light on versus light off) and a between-subject control (ChR2 versus control), controlling for any non-optogenetic effects of light delivery.

5.3.1 Task acquisition

Animals showed successful acquisition of the behavioural paradigm in 15 training sessions before surgery, as evidenced by above chance performance on 12 out of 15 training days as well as significant preference for the correct sandwell location on a probe test conducted on day 16. Subsequently, animals were divided into two experimental groups based on their performance on training days 1-15.

As observed in the previous cohort of animals, mouse performance during retrieval of the first pellet (out of two) during the three pre-training days was at chance level ($t_{(13)} = 1.24$, $p > 0.05$)(figure 5.2a). In contrast, mice learned to successfully retrieve the second pellet during pre-training, with average performance index of 76% ($t_{(13)} = 4.21$, $p = 0.001$), indicating quick acquisition of the win-stay rule. Performance during training days was significantly above chance already on the first training day, and only occasionally dropped to chance during the first 15 days of training (figure 5.2c). Similarly, latency to retrieve the first pellet during choice trial was relatively low from the start of the training and decreased further (with the exception of day 13) as the training went on (figure 5.2b). In order to unequivocally establish that the mice have acquired the procedure, an unrewarded probe test (10 min delay) was conducted on training day 16. As expected, mice showed a highly significant preference for the correct sandwell location ($t_{(13)} = 3.17$, $p < 0.01$). Mice were then separated into two experimental groups based on their

Figure 5.2. Acquisition of everyday spatial memory task.

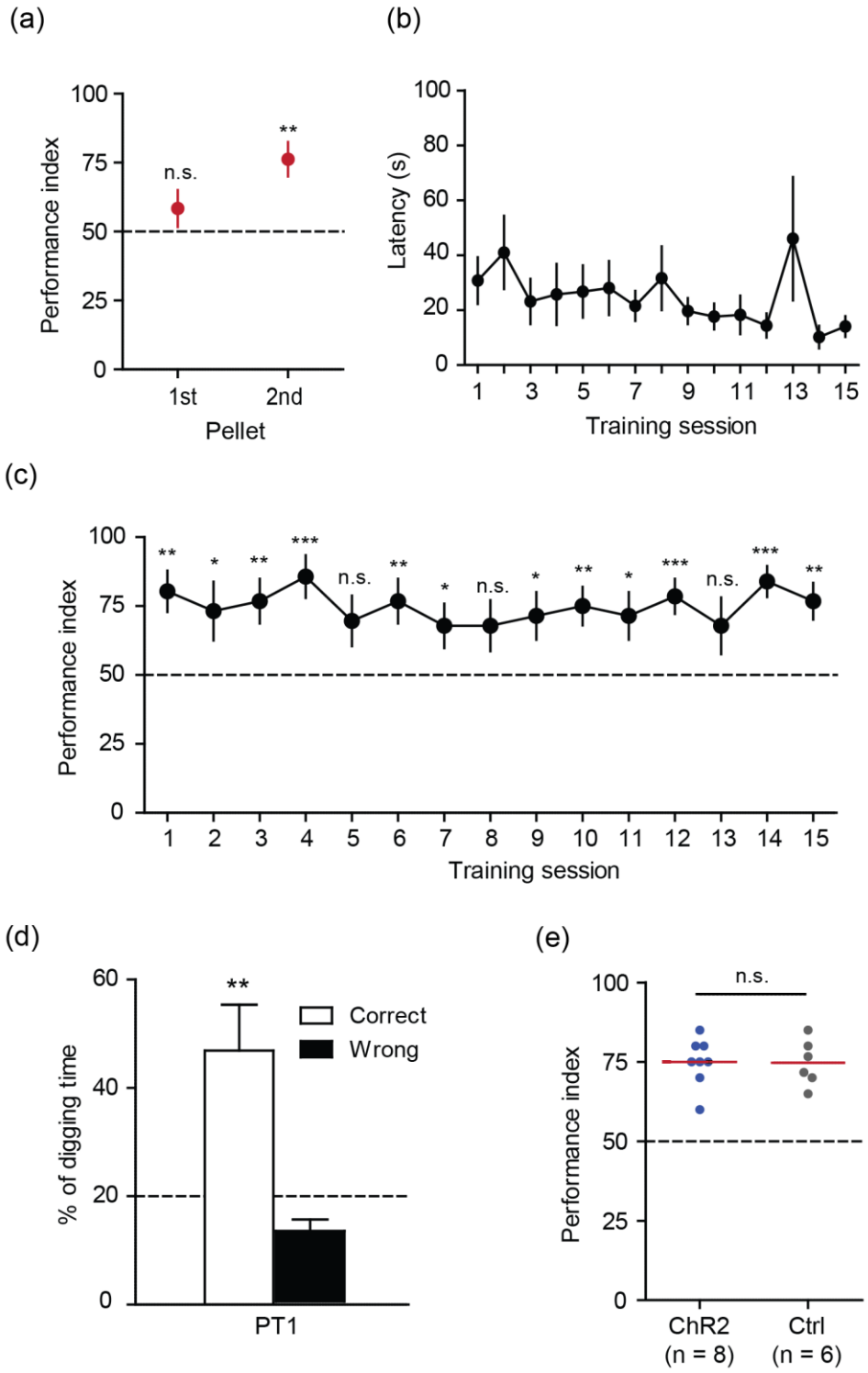
(a) Average scores from 3 pre-training sessions show chance performance for the first pellet and above-chance performance for the second pellet.

(b) Latency to retrieve the first pellet decreases as animals are learning the task. (c) In this cohort, animals performed above chance from the first day of training and maintain a stable performance level for 15 training sessions before surgery.

(c) 10 min probe test conducted on training day 16, above chance performance. n = 14.

(d) Before surgery animals were divided into two experimental groups based on their performance during training. Each point on the graph represents average 1st pellet performance index for a single animal. Red horizontal lines indicate average performance index for each group.

n.s., not significant; * $p < 0.05$, ** $p < 0.01$. Error bars represent ±1 S.E.M. Dashed lines indicate chance levels.


(a)
Performance index
100
75
50
25
0
n.s.
**
1st
2nd
Pellet
(b)
Latency (s)
100
80
60
40
20
0
1
3
5
7
9
11
13
15
Training session
(c)
Performance index
100
75
50
25
0
** * ** *** n.s. ** * n.s. * ** * *** n.s. *** **
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Training session
(d)
% of digging time
60
40
20
0
**
Correct
Wrong
PT1
(e)
Performance index
100
75
50
25
0
n.s.
ChR2
(n = 8)
Ctrl
(n = 6)

average performance scores during training days. Figure 5.2d shows distribution of average performance index scores for each animal (average performance index for each group in pre-surgery training: Control = 74.6%, ChR2 = 75.0%).

5.3.2 Stable performance over months of training

Due to complex nature of surgery and several obstacles that were encountered (and overcome) while performing the procedure, some animals had to wait for 2-3 months before their training continued. After all animals recovered from surgery their daily training sessions continued. Mice achieved asymptotic levels of performance after 15 training days post-surgery (figure 5.3a). Similarly, latency to retrieve the first pellet, though high in the first few training sessions, fell down to asymptotic levels during first 15 training days post-surgery (figure 5.3c). Both ChR2 and Control group reached similar performance levels (average performance index for each group during plateau phase (session 31 – 125): Control = 80.2%, ChR2 = 86.7%), with considerably more variability among animals in Control group (figure 5.3b).

Throughout the experiment, animals underwent 101 standard training sessions and 24 probe tests. Overall, performance on training days of both groups increased from session 1 to 125 ($F_{(100,1200)} = 2.02$, $p < 0.0001$), but there were no differences in performance levels between groups ($F_{(1,1200)} = 3.65$, $p > 0.05$) and no interaction between the two ($F_{(100,1200)} = 1.11$, $p > 0.05$). The longitudal design of the experiment forces the need to establish that there were no differences in animal performance during the period when critical optogenetic stimulation probe tests were performed (block 3 and block 4, sessions 48 - 125). There was no significant effect of time ($F_{(56,672)} = 1.34$, $p > 0.05$) or group ($F_{(1,672)} = 4.52$, $p > 0.05$) during training days in block 3 and 4.

5.3.3. The ‘weak memory’ condition

A series of three 24h probe tests (with 1,2 and 4 sample trials) was then conducted in order to establish the weak memory condition for this cohort of animals. Due to small number of animals in each group and lack of counterbalancing results of these probe tests were only treated as pilot data. To our surprise, there was a considerable difference in probe test performance between groups (figure 5.3d and 5.3e).

Figure 5.3. Performance throughout the experiment.

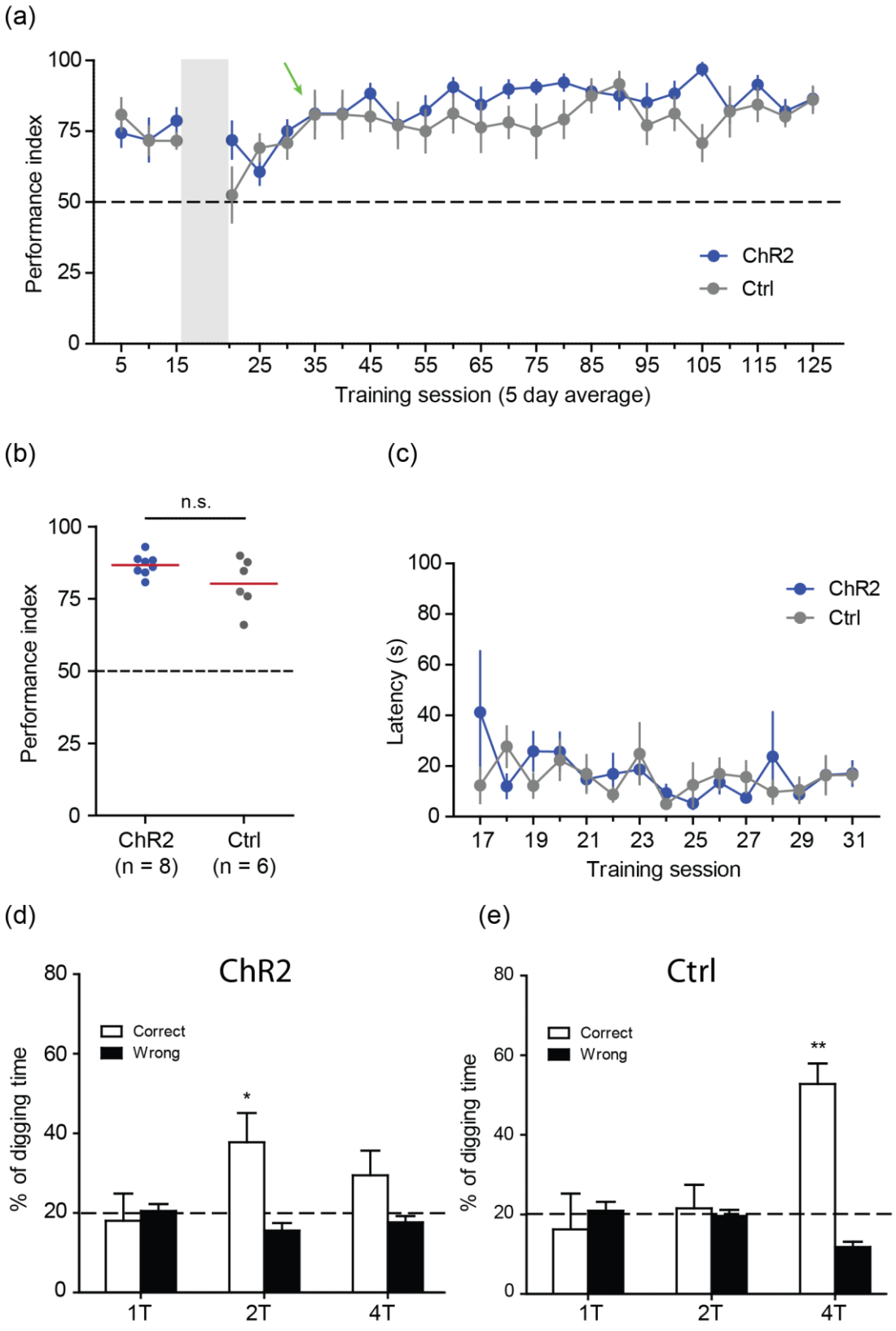
(a) Both ChR2 and control group reached asymptotic performance levels after 30 training days. Grey area indicates a ~ 3 month break in training due to surgery complications.

(b) Both groups show similar performance during the plateau phase of training (session 30-120).

(c) Both groups showed similar latencies to retrieve the first pellet during choice trials after recovery from surgery. surgery animals were divided into two experimental groups based on their performance during training. Each point on the graph represents average 1st pellet performance index in sessions 32-125 for a single animal. . Red horizontal lines indicate average performance index for each group.

(d) and (e) 24h probe test scores with 1, 2 and 4 sample trials for (d) ChR2 and (e) control groups.

* $p < 0.05$, ** $p < 0.01$. Error bars represent ± 1 S.E.M. Dashed lines indicate chance levels.


(a)
Performance index
100
75
50
25
0
5
15
25
35
45
55
65
75
85
95
105
115
125
Training session (5 day average)
ChR2
Ctrl
(b)
n.s.
Performance index
ChR2
(n = 8)
Ctrl
(n = 6)
(c)
Latency (s)
Training session
ChR2
Ctrl
(d)
ChR2
% of digging time
Correct
Wrong
1T
2T
4T
(e)
Ctrl
% of digging time
Correct
Wrong
1T
2T
4T

While Control animals scored highly above chance with 4 pellet encoding (52.8% average correct digging, $t_{(5)} = 6.32$, $p < 0.01$), ChR2 animals fell short of the 30% mark ($t_{(7)} = 1.52$, $p > 0.05$). In contrast, with 2 pellet encoding, the effect was reversed, with ChR2 group performing high (37.8%, $t_{(7)} = 2.41$, $p < 0.05$) and control animals performing at chance level (21.5%, $t_{(5)} = 0.26$, $p > 0.05$). With only 1 sample trial, both groups performed at chance level (ChR2, 37.8%, $t_{(7)} = 0.29$, $p > 0.05$; control, 37.8%, $t_{(5)} = 0.42$, $p > 0.05$). After careful deliberation, we decided to run photostimulation probe tests with 4 sample trials and then, if the baseline performance is too high, repeat the block using a smaller number of trials. The reasoning behind it is that LC or VTA photoactivation effect on memory is likely to be weaker than the effect of novelty (e.g. because of incomplete coverage of the relevant brain area by the light beam), and thus with a borderline memory condition the effect should be easier to detect. Nevertheless, if the difference between groups during probe tests becomes apparent in control conditions of counterbalanced photostimulation blocks, it will not be possible to directly compare light effects in two groups.

5.3.4 Optogenetic stimulation of LC-NA neurons enhances memory persistence

If increased activity of VTA-DA and/or LC-NA neurons is sufficient to produce enhancement of transient everyday memories in the same manner novelty does, then substituting optogenetic stimulation of these nuclei for novelty should produce the same effect. In the previous experiment on the event arena (see chapter 3), animals were allowed to explore a novel box 30 min after encoding. Similarly, in this block of counterbalanced probe tests animals were underwent bilateral optogenetic stimulation of VTA or LC in their home cages 30 minutes after encoding (figure 5.4a and 4.4b). Due to ambiguous results described in the previous section, an extra condition was added in which animals did not receive any light stimulation, and which served to monitor their baseline performance. This set of probe tests with three conditions (‘*VTA light ON’*, *‘LC light ON’* and ‘*light OFF’*) was repeated twice and digging times for each mouse were averaged between the two rounds.

Surprisingly, and to our relief, in absence of stimulation both ChR2 and control groups performed at chance level (19.5% and 20.6%, respectively) (figure 5.4c and 5.4d). In contrast, if their LC-NA neurons were stimulated after encoding, ChR2 mice spent over 52% of their time digging in the correct sandwell after 24 hours, while control mice still performed close to chance level. In case of VTA stimulation, neither group performed higher than chance, but ChR2 mice showed a considerable trend for digging in the correct location.

Figure 5.4. Photostimulation probe tests.

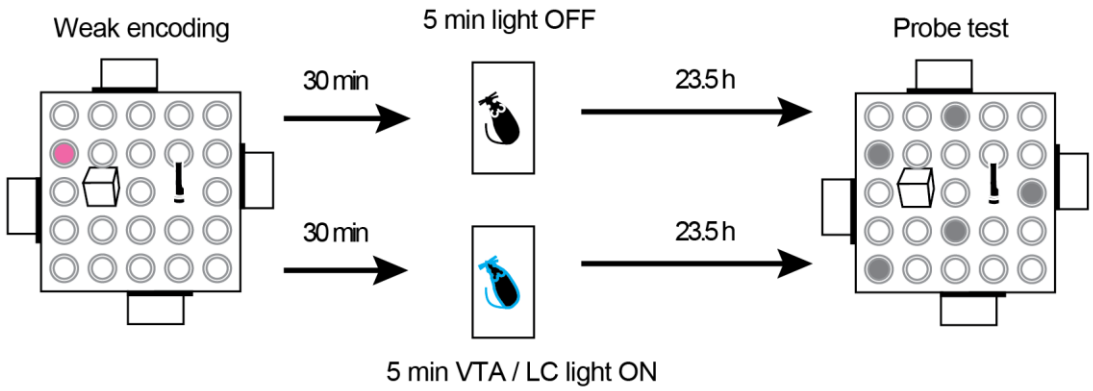
(a) Protocol used in this block of probe tests. Thirty minutes after four sample encoding mice were placed in their home cage and connected to the patch cords. Blue light pulses were then delivered to VTA or LC.

(b) Burst stimulation protocol used for optogenetic stimulation.

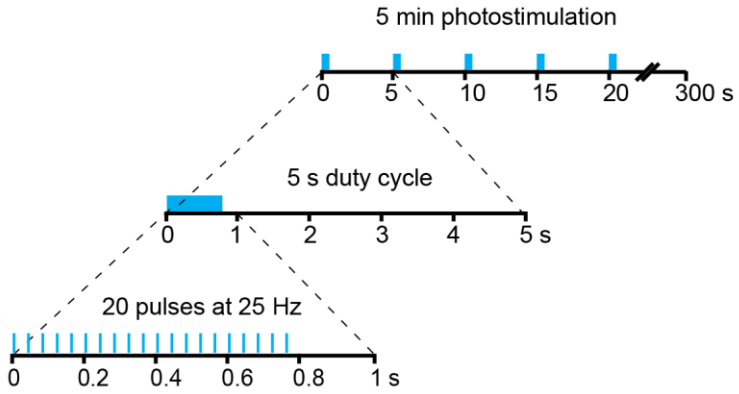
(c) and (d) probe test results for (c) ChR2-eYFP and (d) eYFP groups. Both groups show chance performance with no photostimulation. In contrast, optogenetic activation of LC neurons after weak encoding results in above chance performance in ChR2-eYFP but not in eYFP group. Of interest is the non-significant trend in the VTA photostimulation condition.

n.s., not significant; ** $p < 0.01$. Error bars represent ± 1 S.E.M. Dashed lines indicate chance levels.

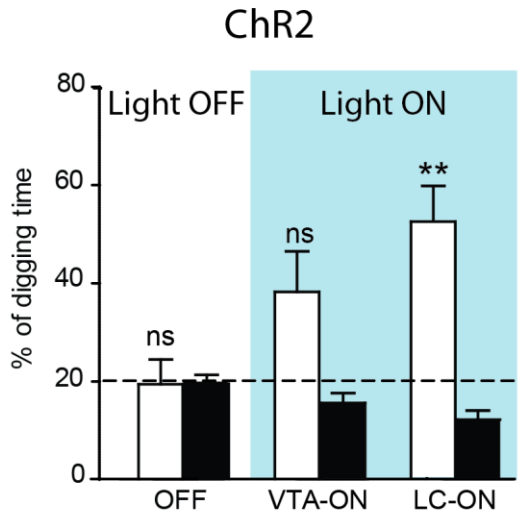
(a)


Weak encoding
5 min light OFF
Probe test
30 min
23.5 h
30 min
23.5 h
5 min VTA / LC light ON

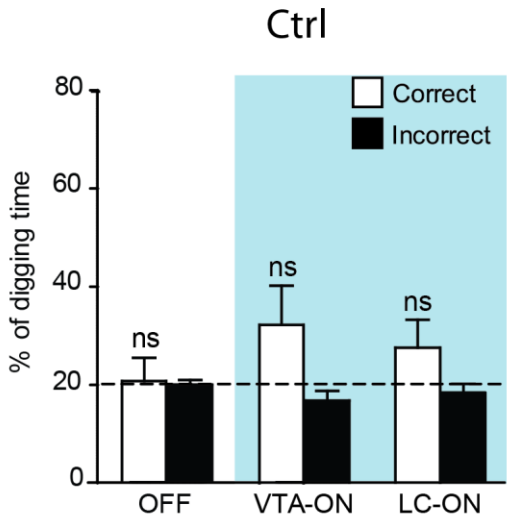
(b)


5 min photostimulation
0
5
10
15
20
300 s
5 s duty cycle
0
1
2
3
4
5 s
20 pulses at 25 Hz
0
0.2
0.4
0.6
0.8
1 s

(c)


ChR2
Light OFF
Light ON
% of digging time
80
60
40
20
0
ns
ns
**
OFF
VTA-ON
LC-ON

(d)


Ctrl
Correct
Incorrect
% of digging time
80
60
40
20
0
ns
ns
ns
OFF
VTA-ON
LC-ON

Overall, there was a significant of light stimulation in this series of probe tests (3x2 repeated measures ANOVA; $F_{(2,24)} = 6.33$, $p < 0.01$). By comparing the effects of LC photostimulation with the control 'light OFF' condition, it transpired that the effect of light is stronger in ChR2 group than in control mice (2x2 repeated measures ANOVA, interaction, $F_{(1,12)} = 5.66$, $p < 0.05$). Individual comparisons of light effects in both groups showed that while ChR2 animals spent significantly more time digging in the correct location if they had received LC photostimulation (paired samples t.test, *'LC light ON'* vs *'light OFF'*, $t_{(7)} = 3.70$, $p < 0.01$). In contrast, light directed at LC had no effect on digging preference of control mice (paired samples t.test, *'LC light ON'* vs *'light OFF'*, $t_{(5)} = 2.07$, $p > 0.05$). Comparison of effects of VTA illumination in both groups did not show an interaction between group and condition (2x2 repeated measures ANOVA, interaction, $F_{(1,12)} = 0.33$, $p > 0.05$), indicating that two experimental groups did not differ in their response to VTA illumination. Strangely, main effect of light was observed ($F_{(1,12)} = 6.50$, $p < 0.05$), which indicates that there may be a non-specific effect of laser illumination not related to optogenetic manipulation of VTA-DA neurons.

5.3.5 Effect of LC-NA stimulation depends on hippocampal D_1/D_5 receptors

Given the ambiguous results of VTA photostimulation in the experimental block, the rest of the experimental tests focused entirely on LC. The goal of the next block of probe tests was to look more closely at the mechanism by which LC activation boosts retention of everyday memories. As reported in chapter 3, the novelty effect on memory persistence can be blocked by prior infusion of dopamine D_1/D_5 receptor antagonist SCH23390 into dorsal hippocampus, which indicates that hippocampal dopamine is a critical mediator of this phenomenon. In contrast, β-adrenergic receptor antagonist propranolol infused into the same brain area had no effect on mouse performance. In order to fully demonstrate mimicry of the novelty effect by optogenetic LC stimulation, it is critical for both of these phenomena have the same pharmacological profile. Therefore, another set of counterbalanced probe tests was designed to test whether the effect of post-encoding stimulation of LC-NA neurons on 24 hour memory can be blocked by prior infusion of SCH23390 or propranolol through previously implanted hippocampal cannulae (figure 5.5a).

Our working hypothesis is that LC is the mediator of dopamine-dependent effect of novelty on memory persistence. Therefore, the three main predictions arising from previous data are (1) optogenetic stimulation of LC-NA neurons will, again, lead to significant preference for the correct sandwell after 24 hours, (2) prior hippocampal infusion of SCH23390 will block the effect

of LC photostimulation, and (3) prior hippocampal infusion of propranolol will fail to block the effect of LC photostimulation on memory. If, on the other hand, the effect of LC stimulation on memory is mediated by β-adrenergic receptors, outcomes of predictions (2) and (3) will be reversed: propranolol will bring mouse performance back to baseline while SCH23390 will have no effect.

Previous block of probe tests was conducted using 4 sample trial encoding, which, despite not producing long term memory on its own, does not parallel the 1 sample trial encoding protocol used in the novelty cohort. In order to demonstrate that LC activation can enhance persistence of memories encoded with weaker training protocol, we decided to lower the number of encoding trials. Bearing in mind that this cohort of animals may have a different sensitivity to reward magnitude than the cohort described in chapter 3, the first and second round of probe tests in this block were conducted with 1 and 2 encoding samples, respectively, and the digging times for each mouse were averaged. As in the previous block, an extra condition with no light stimulation (but with vehicle infusion) was included to monitor whether or not this encoding protocol on its own produces long term memory.

Unsurprisingly, light administration had no effect in the control animals as they performed at chance level in all conditions (figure 5.5c). In contrast, the effect of LC stimulation was evident in the ChR2 mice, as they exhibited very high preference for the correct sandwell (56.2%) in that condition, while scoring merely 23.8% in the control condition in which laser was not turned on (figure 5.5b). The difference between performance of ChR2 mice in these two conditions also demonstrates lack of influence of post-encoding intrahippocampal vehicle infusion on the memory-boosting effect of optogenetic LC activation. Importantly, and in line with our predictions, propranolol failed to block the optogenetic effect, while infusion of SCH23390 prior to light stimulation brought mouse performance back to chance level. The key conditions of a successful mimicry experiment have therefore been met: just like novelty, photoactivation of LC neurons 30 min after encoding reliably enhances persistence of everyday spatial memory in a hippocampal dopamine D_1/D_5 receptor, but not β-adrenergic receptor-mediated manner.

Overall, there was a significant group effect among experimental conditions (2x4 repeated measures ANOVA, group effect, $F_{(1,12)} = 5.01$, $p < 0.05$), which justified a separate analysis of light effect in each group. Unsurprisingly, there was no effect of condition in the control group (1x4 repeated measures ANOVA, main effect, $F_{(3,15)} = 0.84$, $p > 0.05$). In contrast, analysis of variance performed on ChR2 group showed a significant difference among conditions (1x4

repeated measures ANOVA, main effect, $F_{(3,21)} = 3.18$, $p < 0.05$). Further differences among experimental conditions (*LC light OFF + veh, LC light ON + veh, LC light ON + prop, LC light ON + SCH*) in the ChR2 group were explored using orthogonal comparisons based on *a priori* predictions. If the effect of LC photostimulation is mediated by β-adrenergic receptors and therefore doesn't mimic the novelty effect, propranolol but not SCH23390 should bring the performance down to baseline. This comparison, (*LC light ON + veh, LC light ON + SCH versus LC light OFF + veh, LC light ON + prop*) failed to reveal a significant difference ($F_{(1,21)} < 0.01$, $p > 0.05$). If, on the other hand, the effect of LC stimulation is mediated by dopamine D_1/D_5 receptors, the opposite should be true: SCH23390 should bring mouse performance back to baseline but propranolol should have no effect. This orthogonal comparison (*LC light ON + veh, LC light ON + prop versus LC light OFF + veh, LC light ON + SCH)* turned out to be significant ($F_{(1,21)} = 9.23$, $p < 0.01$), revealing a strong difference between conditions predicted to result in memory enhancement and conditions predicted to produce chance performance.

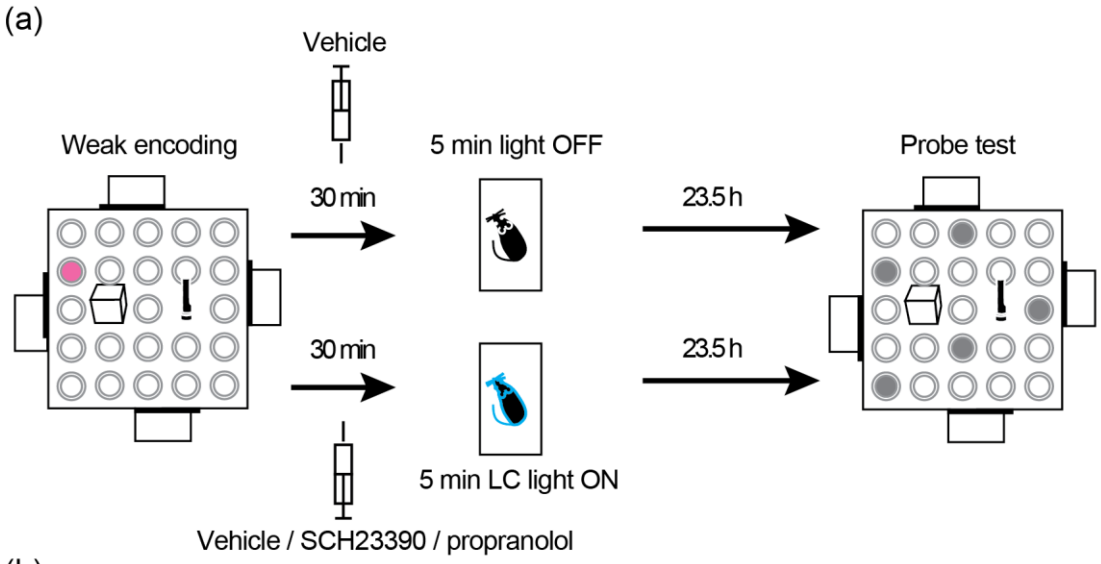
Additional comparisons within the ChR2 group did not reveal any differences in performance between *LC light ON + veh*, and *LC light ON + prop* conditions (paired t.test, $t_{(7)} = 0.34$, $p > 0.05$), or between *LC light OFF + veh*, and *LC light ON + SCH* conditions (paired t.test, $t_{(7)} = 0.42$, $p > 0.05$). Overall, results indicate that while in the control animals light and drug delivery had no significant effect on 24 hour memory, in the ChR2 group LC photostimulation 30 min after encoding resulted in memory enhancement 24 hours later. This boost in memory retention was not affected by post-encoding propranolol infusion prior to photostimulation, but was completely blocked by post-encoding infusion of SCH23390.

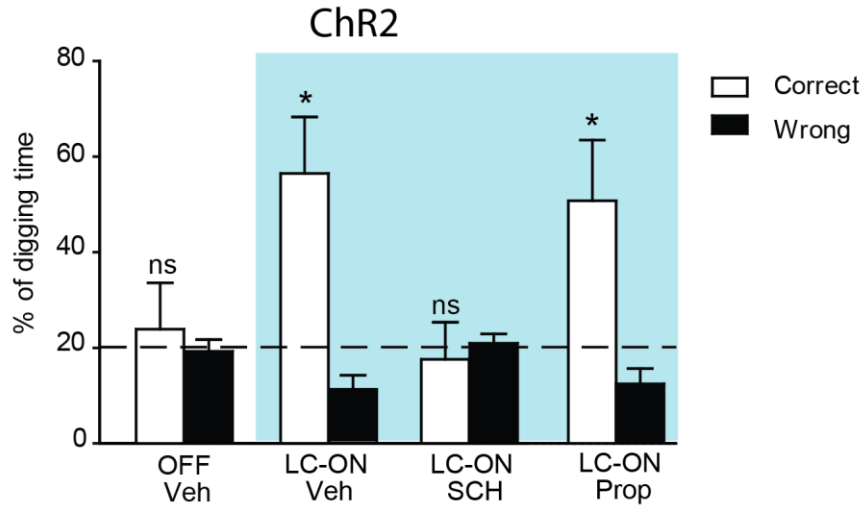
Figure 5.5. Photostimulation probe tests with pharoacological blockade of hippocampal D_1/D_5 or β-adrenergic receptors.

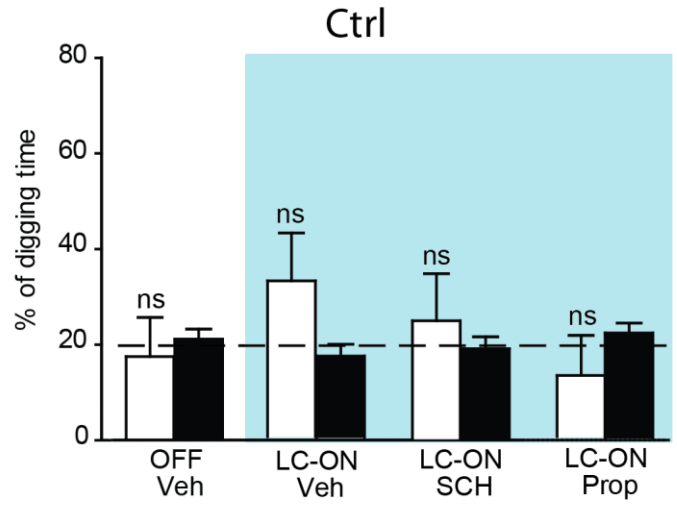
(a) Protocol used in this block of probe tests. Drug infusion was performed 10 min after the start of encoding. LC stimulation was performed 30 minutes after the start of encoding.

(b) and (c) probe test results for (b) ChR2-eYFP and (c) eYFP groups. Both groups show chance performance with no photostimulation. In contrast, optogenetic activation of LC neurons after weak encoding results in above chance performance in ChR2-eYFP but not in eYFP group. Blocking hippocampal D_1/D_5 but not β-adrenergic receptors before photostimulation blocks the effect of optogenetic LC activation on memory persistence.

n.s., not significant; ** $p < 0.01$. Error bars represent ± 1 S.E.M. Dashed lines indicate chance levels.


(a)
Vehicle
Weak encoding
5 min light OFF
Probe test
30 min
23.5 h
30 min
23.5 h
5 min LC light ON
Vehicle / SCH23390 / propranolol


(b)
ChR2
% of digging time
80
60
40
20
0
Correct
Wrong
*
*
ns
ns
OFF Veh
LC-ON Veh
LC-ON SCH
LC-ON Prop


(c)
Ctrl
% of digging time
80
60
40
20
0
ns
ns
ns
ns
OFF Veh
LC-ON Veh
LC-ON SCH
LC-ON Prop

5.3.6 Meta-analysis of the optogenetic LC-NA stimulation effect

Additional analysis was performed on probe tests from block 3 and 4 in order to look at effects of LC stimulation in more detail. All conditions in these blocks (except for *VTA light ON* condition in block 3) were divided into two categories: those where baseline performance was expected (i.e. *light OFF* from block 3, and *LC light OFF + vehicle* and *LC light ON + SCH23390* from block 4) and those where rescue of weak memory was predicted (*LC light OFF* from block 3, and *LC light ON + vehicle* and *LC light ON + propranolol* from block 4).

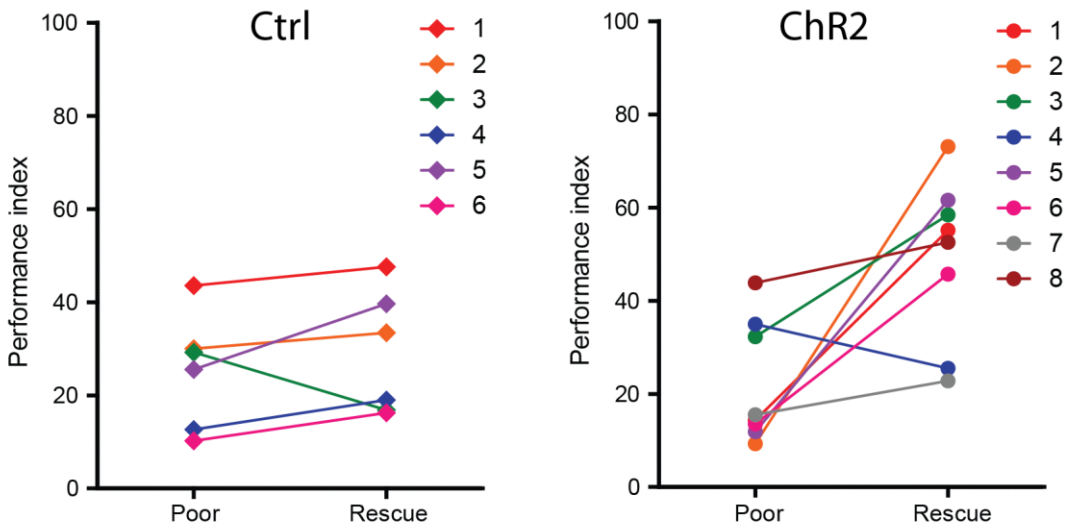
Across all conditions analysed in this section, 7 out of 8 animals from the ChR2 group showed increased preference in 'rescue', as compared to the 'poor memory' conditions (figure 5.6a, right), with the largest gains made by animals with low baseline performance levels. Unexpectedly, majority of animals in the control group (5 out of 6) also showed elevated performance in the 'rescue' conditions, although most gains were of no more than a few percent. This overall difference in memory enhancement is illustrated by a significant interaction between *Group* and *Condition* in this pooled set of data (2x2 repeated measures ANOVA, $F_{(1,12)} = 5.15$, $p < 0.05$). Additional comparisons reveal that while in ChR2 animals light has a significant rescue effect on memory (paired t.test, $t_{(7)} = 3.18$, $p < 0.05$), the modest trend for memory enhancement in control animals is not statistically significant (paired t.test, $t_{(5)} = 1.00$, $p > 0.05$).

Figure 5.6. Meta-analysis of memory persistence enhancement by optogenetic LC stimulation.

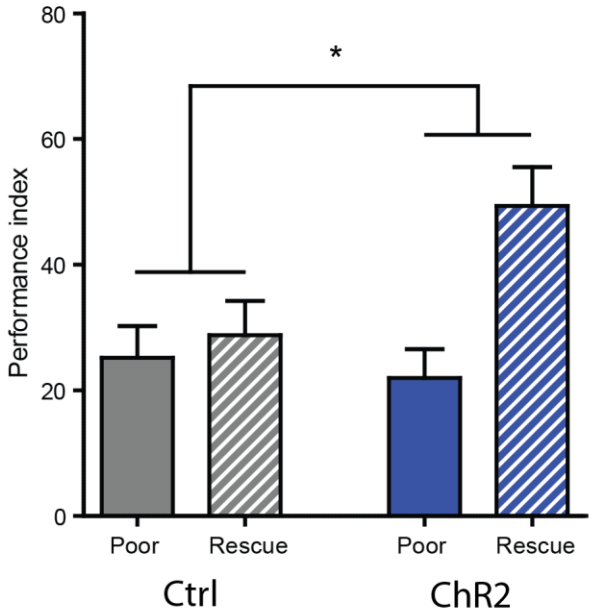
(a) Average digging time for each animal in ChR2 and Control groups in poor memory conditions and conditions where memory was rescued by LC activation. All scores from block 3 (photostimulation) and block 4 (photostimulation with pharmacology) except 'VTA light ON' scores were used in meta-analysis.

(b) Optogenetic LC stimulation had a large effect on memory for the correct sandwell location in ChR2 but not in Control group.

(a)


Ctrl
ChR2
Performance index
100
80
60
40
20
0
Poor
Rescue
1
2
3
4
5
6
7
8

(b)


Performance index
80
60
40
20
0
*
Poor
Rescue
Poor
Rescue
Ctrl
ChR2

5.4 Discussion

The everyday appetitive spatial memory paradigm introduced in chapter 3 was used to determine whether optogenetically-driven spiking activity of LC-NA or VTA-DA neurons is sufficient to mimic the effect of novelty on memory persistence. While light delivery into mouse VTA produced an effect that was not specific to animals that expressed ChR2 in VTA-DA neurons, optogenetic activation of LC-NA neurons 30 min after encoding reliably produced memory enhancement in animals expressing ChR2 but not in animals expressing the control vector in the same neural populations. Importantly, this LC-mediated memory enhancement was completely blocked by prior infusion of a D_1/D_5 dopamine receptor antagonist SCH23390 into dorsal hippocampus, mirroring the pharmacological dependence of the novelty effect on activation of hippocampal D_1/D_5 dopamine receptors reported in chapter 3. Overall, results of critical tests described in this chapter are in line with a model of behavioural tagging in which LC-NA neurons, activated by novelty, release dopamine in the hippocampus, which in turn initiates *de novo* synthesis of plasticity-related proteins (PRPs) through activation of D_1/D_5 receptors and downstream signalling cascades.

3.4.1 Animal performance

As in chapter 3, stability of animal performance throughout the critical phase of the experiment is crucial. Additionally, in order to be able to compare the effect of light administration in ChR2-expressing animals and animals expressing the control vector, animals expressing ChR2 should not differ in behaviour from control animals during standard training as well as in control conditions during probe tests. Overall, the two groups performed at similar, stable levels during later phases of training, although there was a trend for control group to perform slightly worse, on average. This minor (6% overall difference) trend for lower performance index in control mice was observed despite careful performance matching of the two groups prior to surgery, and was mostly due to a single animal performing worse than others, although still above chance.

Although uneven performance of the two groups during pilot probe tests with varying reward magnitude raised concerns, both groups performed on similar levels in control 'Light OFF' conditions in both blocks of optogenetic probe tests. Such longitudal experimental design, involving many months of training, may produce occasional drifts in animal performance and is therefore not ideal for between-subject comparisons. Therefore, incorporating a baseline performance condition in all critical probe tests was a necessary step in assuring that the two experimental groups are comparable. Unfortunately, due to the sheer number of experimental

conditions we were unable to incorporate a positive control for 24h memory in control mice (e.g. novelty exploration). Still, the highly significant preference for the correct sandwell in the pilot 24 h probe test with 4 sample trials indicates that control animals show 24 hour memory of the correct sandwell in certain experimental conditions.

A striking observation in this cohort of animals was that certain reward magnitudes did not produce stable memory readout in probe tests. For example, While during pilot reward magnitude probe tests 4-sample encoding produced highly significant preference for the correct sandwell in the control group, control animals performed at chance levels in the first block of optogenetic stimulation probe tests (which involved 4 pellet encoding). Additionally, ChR2 group failed to show 24 h memory after 4-sample encoding at any stage of the experiment, but LC stimulation reliably enhanced its memory persistence and resulted in good 24 hour performance on probe tests. In contrast, in the chapter 3 experiment, mice overall showed very good 24 hour memory after 4-sample encoding, although 4-sample memory was only looked at in block 2 (session 25 – 45), so relatively early in training. In contrast, the present cohort of animals underwent and extended training regime before the first block of optogenetic probe tests, where 4-sample encoding was used (session 47 – 83). This was mainly due to initial post-surgery complications and the need to delay further surgeries for a period 2 months, which in turn lead to considerable drop in animal performance once the training was resumed. It is possible that relationship between reward magnitude and 24 hour memory changes in later stages of training. Nevertheless, despite varying reward magnitude during critical probe tests in the present cohort, the two critical conditions have been met: (1) the encoding regime used in a particular block of probe tests did not, on its own, produce 24 h memory in either group, and (2) memory enhancement by LC stimulation was independent of reward magnitude (compare figures 5.3 and 5.4).

5.4.2 Optogenetic stimulation of LC-NA neurons promotes memory consolidation

Locus coeruleus (LC) is a small brainstem nucleus composed almost entirely of neurons expressing noradrenergic markers. It sends dense projections to a large number of cortical and subcortical structures, including all hippocampal subfields (see figure 2.4). It is the source of most of the noradrenaline released in the central nervous system, and thus it is not surprising that it is a critical modulator of many cognitive functions. The role of LC in promoting wakefulness and arousal is well established (Aston-Jones & Bloom 1981a, Carter et al 2010, Sara & Bouret 2012, Takahashi et al 2010), but LC neurons are also strongly modulated by salient stimuli of either positive or negative valence (Sara & Segal 1991). Notably, LC neuronal response to a conditional

stimulus in a conditioning paradigm is inversely proportional to rat performance during all conditioning stages (habituation, associative learning, reversal and extinction), indicating that LC neurons may convey learning signals about cognitively significant stimuli. Role of the noradrenergic system in consolidation of fear memory is also well documented and thought to be mediated by β-adrenergic receptor activation in the amygdala (McGaugh et al 2002). Results of the study outlined in this chapter point to LC as an important modulator of spatial memory of positive valence, which is in line with physiological evidence for LC activation by salient reward and novelty (Aston-Jones & Bloom 1981a, Vankov et al 1995). Importantly, in our paradigm LC stimulation was delivered at a considerable delay after the encoding trial was finished (10-15 min in block 3, 20-25 min in block 4), which excludes the possibility that LC activation enhanced animal's ability to encode new information, e.g. via enhanced attention or arousal. Instead, LC activation boosted retention of a memory trace that had already been encoded and it did so in absence of any salient stimuli (animals underwent stimulation in their home cages).

Meta-analysis of memory-enhancing effect of post-encoding LC stimulation indicated that the effect is noticeable in all but one ChR2-expressing animal. Histological analysis of fibre tracts and virus expression has not yet been performed at the time of submission of this thesis, and it is possible that the LC-targeted fibres missed their target co-ordinates in the poor-performing animal. Nevertheless, even in absence of histological data, it is evident through 'behavioural histology' that LC viral expression and fibre placement was successful in most animals. Curiously, animals expressing the control vector also showed a small (though non-significant) trend for memory enhancement after LC-targeted light delivery. Although extra care was taken to habituate mice to the faint flashes of light leaking through the patch cord – ferrule junction, the possibility that these sensory stimuli still managed to weakly activate neuromodulatory systems promoting memory consolidation cannot be excluded.

5.4.3 LC-NA neurons enhance memory persistence through D_1/D_5 receptors

Just like the effect of novelty, the effect of LC-NA neuron stimulation on memory persistence was completely abolished by hippocampal microinfusion of a D_1/D_5 dopamine receptor blocker, but was not affected by prior infusion of a β-adrenergic antagonist. This superficially paradoxical observation that activation of a classically noradrenergic brain area leads to a dopamine receptor-mediated memory enhancement is, to our knowledge, the first indication that LC modulates cognitive function through dopamine release.

The idea that LC neurons are capable of co-releasing dopamine has been pondered in the literature (Devoto & Flore 2006), and has recently gained momentum due to the landmark study by Smith and Greene (2012). Authors of this study used an *in vitro* hippocampal slice preparation to investigate the well-known phenomenon of enhancement of basal glutamatergic synaptic transmission by amphetamine, which depends on hippocampal D_1/D_5 dopamine receptors and was thought to occur through release of endogenous dopamine from VTA-DA afferents. Curiously, neither loss of tyrosine hydroxylase (TH) expression in VTA or pharmacological blockade of the dopamine transporter (DAT) had any effect on the amphetamine-induced enhancement of glutamatergic transmission. In contrast, loss of TH in LC was sufficient to completely block the enhancement in synaptic transmission, as was the pharmacological blockade of noradrenaline transporter (NET). These results strongly point to LC terminals as a major site of dopamine release in the hippocampus, but the ultimate proof of the critical role of LC-released dopamine in hippocampal physiology would be a demonstration that optogenetic stimulation of hippocampal LC terminals leads to enhanced persistence of long term potentiation (LTP) at CA3-CA1 synapses, which itself is a D_1/D_5 dopamine receptor-mediated phenomenon. Indeed, pilot LTP experiments conducted by the same group indicate that this may be the case (Robert Greene, personal communication).

Several other phenomena could theoretically account for dopamine receptor dependence of the boosting effect of LC stimulation on memory retention: (1) LC stimulation could activate VTA-DA neurons, which in turn would release dopamine in the hippocampus, (2) noradrenaline release in the hippocampus could stimulate dopamine release from dopaminergic fibres, and (3) noradrenaline could directly activate dopamine receptors.

Although indirect activation of VTA-DA neurons through stimulation of LC-NA neurons is a possible explanation, previous studies established that burst stimulation of LC, as well as local application of dopamine or noradrenaline, all have an inhibitory effect on VTA-DA cell firing (Grenhoff et al 1993, Guiard et al 2008a). Also, while in VTA-DA neuron photostimulation arguably resulted in modest memory enhancement (see next section for discussion), the effect was numerically smaller than that observed after LC-NA neuron activation, and it is unlikely that indirect stimulation of VTA would have a larger effect than its direct stimulation.

Indirect activation of dopaminergic fibres by noradrenaline is another possibility. Although not much is known about nonsynaptic interactions between noradrenergic and dopaminergic terminals, to author's knowledge there is no evidence of noradrenergic receptors on presynaptic

terminals of VTA-DA neurons, and β-adrenergic receptor blockade does not affect the LC photostimulation effect. Still, the possibility cannot be completely excluded, as the nonsynaptic interaction could be mediated by α-adrenergic receptors or perhaps by an intermediate messenger.

Finally, noradrenaline could directly bind to D_1 or D_5 receptors in the hippocampus. Noradrenaline and dopamine are structurally very similar, and there is indeed evidence of noradrenaline acting as agonist of the D_4 (but not D_2) dopamine receptor, although with 10-100 fold lower affinity than dopamine (Lanau et al 1997, Newman-Tancredi et al 1997). However, to author's knowledge, no evidence of considerable affinity of noradrenaline towards D_1-type receptors has been published so far.

5.4.4 Non-specific effect of light delivery into VTA

Although light delivery into VTA neurons resulted in moderate memory enhancement, it did so independently of whether or not ChR2 was expressed in VTA-DA neurons. There a few possible reasons for this non-specific effect: (1) light delivery into VTA produces a non-specific effect (e.g. change in brain temperature) that increases VTA-DA neuron firing rate and therefore produces memory enhancement, (2) secondary light emission during light-delivery into VTA produces visual stimuli that in turn act as 'novelty', and (3) above-chance trend for correct sandwell preference by control mice in *VTA light ON* condition is a result of chance fluctuations in animal performance, exacerbated by small number of animals in this group.

Although focused light is under certain circumstances capable of exciting neurons directly (Hirase et al 2002, Hirase et al 2012) appreciable non-specific effects of VTA-directed blue light have not been previously reported in literature despite widespread use of eYFP control vectors in optogenetic studies of VTA neurons involving ChR2 (Adamantidis et al 2011, McNamara et al 2014, Tsai et al 2009, Tye et al 2013, Witten et al 2011). It is therefore unlikely that direct illumination of VTA with short light pulses (amounting to 2% of overall stimulation time) would cause any major physiological changes in neurons in absence of opsin expression.

Closer examination of average performance levels with or without VTA stimulation reveals an interesting trend: even though animals in both groups perform better if light is delivered into VTA, ChR2 animals, on average, tend to score higher than control animals in *VTA light ON* condition. In addition, meta-analysis of poor-performance conditions (figure 5.5b) indicates average baseline performance of control animals was slightly higher than ChR2 animals (25.2% versus 22.0%). Therefore, it is not unlikely that the score of control animals in *VTA light ON*

condition (32.5%) is a result of fluctuations in baseline performance. Additionally, as mentioned before, meta-analysis of LC stimulation conditions exposed a small (~ 4%) trend for memory enhancement in control animals after light delivery into LC. Even though secondary light emissions from patch cord-VTA ferrule junctions were much weaker than from patch cord-LC ferrule junctions, VTA-directed fibre-ferrules were implanted anterior to LC-directed ferrules and therefore were close to the animal's field of view. Therefore, VTA-directed light pulses acting as visual 'novelty' stimuli could be among the causes for weak memory enhancement in both groups.

Even though, overall, there was a mild effect of VTA illumination on memory enhancement across all experimental animals, neither ChR2 nor control groups on their own showed above chance performance in *VTA light ON* condition. Most importantly, VTA-DA neuron stimulation in ChR2 animals failed to enhance memory persistence to a degree comparable with the effect of novelty reported in chapter 3. Still, caution has to be exerted while interpreting this result as fibre placement and virus expression have not yet been inspected in this cohort of animals. McNamara and colleagues (2014) recently reported that activation of hippocampal VTA-DA projection during encoding has a robust effect on memory retrieval after 1 h. Weak trend for enhanced memory in our experimental paradigm might be a manifestation of the same mechanism, and it would be of interest to investigate whether VTA-DA neuron stimulation has a more pronounced effect on 1 h memory in our experimental paradigm.

5.4.5 Technical considerations

A critical aspect of experimental design in this chapter is the ability to zero in on a specific neural projection as well as specific neuromodulator. Therefore, it was imperative to use a combination of optogenetic stimulation and pharmacological blockade. While designing the experiment, a choice had to be made between two experimental configurations, each with its own advantages and drawbacks. One possibility was to activate VTA-DA or LC-NA terminals via fibres implanted above the dorsal hippocampus and then administer receptor blockers systemically via an intraperitoneal (i.p.) injection. The obvious drawback of this method is that it would be impossible to look at effects of VTA and LC terminal stimulation in the same batch of animals. Moreover, due to the body-wide action of an i.p.-injected drug, one could expect a large variety of physiological side-effects that could affect animal performance or even constitute a visceral 'novelty' stimulus of their own. Finally, LC neurons are known for their extended axonal collaterals, with a large proportion of individual cells sending projections to many different brain areas (Loughlin et al 1986a, Loughlin et al 1986b). Therefore, antidromic activation of LC

neurons through stimulation of their hippocampal terminals could still lead to a brain-wide release of noradrenaline and dopamine.

An alternative experimental configuration would be so stimulate the cell bodies (causing brain-wide neuromodulator release) and inhibit dopamine receptors locally via intrahippocampal infusion of receptor blocker. This particular design was chosen partially due to the possibility of performing both manipulations (VTA-DA and LC-NA neuron stimulation) in the same batch of animals. Though a robust and complete block of LC-NA stimulation effect on memory by prior hippocampal infusion of SCH23390 was observed, it does not exclude the possibility that LC activation causes dopamine release indirectly, e.g. via activation of VTA-DA neurons. Indeed, LC sends projections to VTA, and some studies observed VTA-DA neuron activation after LC stimulation (Grenhoff et al 1993), though inhibition of VTA-DA neurons by noradrenergic receptor agonists (Paladini & Williams 2004) as well as by noradrenaline and dopamine (Guiard et al 2008a) has also been reported. Crucially, if the effect of LC-NA neuron stimulation was mediated by VTA-DA neurons, one would expect the effect of VTA-DA neuron stimulation to be at least as pronounced, while in the present study only a modest trend for enhanced memory persistence was observed.

Using the everyday appetitive spatial memory paradigm for mice, we were able to show that activation of LC-NA neurons is sufficient to promote consolidation of recently encoded spatial memories. Importantly, it does so through activation of hippocampal D_1/D_5 receptors, presumably via dopamine released from LC terminals in the hippocampus. In contrast, we failed to observe a robust and specific effect of VTA activation, partially due to apparent non-specific light effect. Overall, the outcome of this study is consistent with recent electrophysiological data (Smith & Greene 2012), and points to LC as the pivotal hub in the brain-wide network that controls entry to long-term memory.

However, this does not exclude the possibility of VTA and LC dopaminergic modules both influencing persistence of hippocampal memories in a complementary fashion. In fact, stimulation of VTA-DA axons in the hippocampus during encoding was recently shown to boost spatial memory at shorter timescales (McNamara et al 2014), which indicates that there are two parallel dopaminergic systems exercising control over long-term memory. Nevertheless, it is clear that the concept of 'hippocampal-VTA loop' (Lisman & Grace 2005), has to be updated to reflect the prominent role of LC in hippocampal memory consolidation.

Chapter 6

Conclusions and General Discussion

Dopamine release in the hippocampus is conceptualised as an important gating mechanism that controls the persistence of hippocampal memory traces (Lisman and Grace 2005), although the exact origin of hippocampal dopamine is not known. Possible sources include the sparse dopaminergic projections from the VTA and/or dense noradrenergic projections from the LC, which have recently been shown to co-release dopamine in the hippocampus (Smith & Greene, 2012).

This thesis uses the phenomenon of 'dopamine-dependent enhancement of memory persistence by novelty' as a behavioural tool to probe the hippocampal dopaminergic system. The experiments included here shed more light on the relative role of different dopamine-releasing nuclei in hippocampal memory consolidation. Overall, the evidence presented in this thesis supports the notion that dopamine released from hippocampal LC terminals promotes memory consolidation and mediates the effect of novelty of memory persistence.

In support of this, it is shown that:

- In a novel everyday appetitive spatial memory task for mice, persistence of normally transient spatial memories can be enhanced by a *separate* novel event experienced *after* the initial encoding. The novelty effect is dependent on hippocampal D_1/D_5 receptor activation at the time of novelty exploration, which indicates that it is mediated by hippocampal dopamine release.

- Analogous novel events are associated with increased firing frequency of both VTA-DA and LC-NA neurons, confirming that dopaminergic circuitry is actively engaged during

the novelty effect. Moreover, the relative modulation of LC-NA neuron firing rate by novelty is higher for than in VTA-DA neurons and their activity profile is more consistent with a novelty response, which makes them more likely to mediate the novelty effect on memory persistence.

- Optogenetic activation of LC-NA neurons, mimicking their activation by novelty, is sufficient to enhance persistence of transient spatial memories in a way that parallels the effect of novelty. Furthermore, the effect of LC-NA activation is dependent on hippocampal D_1/D_5 receptor activation, indicating that it is mediated through hippocampal dopamine release.

To the author's knowledge, these experiments constitute the first evidence that LC-NA neurons can promote entry of information to long-term memory through hippocampal dopamine release. Although this does not exclude the possibility that LC-NA neurons indirectly activate midbrain dopaminergic nuclei that in turn project to the hippocampus, two lines of evidence argue against it: (1) LC-NA neurons exert a largely inhibitory influence on VTA-DA neurons, especially after burst stimulation (Grenhoff et al 1993, Guiard et al 2008a), (2) in the present experiments, direct optogenetic stimulation of VTA-DA neurons produced ambiguous effects on memory persistence (although corroborated by the apparent non-specific light effect), consistent with modest memory enhancement.

Importantly, this does not exclude the possibility that VTA-DA and LC-NA dopamine-releasing systems both modulate the hippocampal memory system in a complementary fashion. In fact, stimulation of VTA-DA axons in the hippocampus during encoding was recently shown to boost spatial memory at shorter timescales (McNamara et al 2014), which indicates that there are two parallel dopaminergic systems exercising control over long-term memory. Indeed, there are fundamental differences between VTA-DA and LN-NA circuitry that may reflect their relative contributions to neural processing. The output structure of the midbrain dopaminergic cell populations seems to be modular and non-divergent, with single neurons projecting directly to specific brain areas through largely uncollateralised axons (Bentivoglio & Morelli 2005). In contrast, individual LC-NA neurons receive inputs from and send projections to many diverse brain areas, suggesting high input integration and output divergence (Schwarz 2015). What is more, LC-NA neurons are linked through gap junctions and show high degree of synchronous activity (Aston-Jones & Cohen 2005) which further reinforces their role as a unitary 'broadcasting' system. Therefore, while VTA-DA neurons may provide local dopamine release

in a particular brain region in response to a particular stimulus, while LC-NA neurons induce a brain-wide network reorganisation through global noradrenaline and possibly also dopamine release. It was proposed that LC-NA neurons co-release dopamine only as a consequence of high spiking activity (Otmakhova et al 2013), largely due to saturation of vesicular Dbh. Neurons in LC are strongly activated by novel, salient and alerting stimuli (Sara, 2009), and a mechanism based on Dbh saturation would enable them to supply a consolidation-promoting dopamine pulse during flashbulb memory-like experiences while at the same time maintain low basal levels of dopamine in other circumstances.

Future directions

The present experiments satisfy two out of three criteria, outlined in section 2.6, that would constitute a proof that LC-NA neurons mediate the dopamine-mediated effect of novelty on memory persistence: (1) LC-NA neurons are activated by novelty and (2) their activation is sufficient to mimic the novelty effect. However, the third 'necessity criterion' still remains unexplored. An ideal demonstration of the link between LC-NA activation and the novelty effect would involve a loss-of-function experiment on the event arena, whereby spiking activity in LC is inhibited during novelty exploration. Just as the LC-NA activation mimicked the novelty effect, LC inhibition (e.g. via an inhibitory opsin) is predicted to mimic the blocking effect of SCH23390 on the novelty enhancement of memory.

Such an experiment would demonstrate that LC is a vital component of the behavioural tagging mechanism. However, there are several caveats to the experimental design that should be discussed. First of all, inhibition of LC may entail behavioural and cognitive consequences that could make the interpretation of the behavioural result somewhat problematic. LC is the hub of the network that regulates animal's arousal as well as the sleep-wake cycle (Berridge et al 2012), and it has been demonstrated that LC activation promotes wakefulness, whereas inhibition of LC activity promotes sleep (Carter et al 2010). Therefore, the hypothetical blockade of the novelty caused by optogenetic LC inhibition could be explained by its indirect effects on animal's ability or incentive to explore the novel open field. While a similar critique can be directed at the 'mimicry' experiment described in this thesis, effects of LC-NA activation in that experiment were blocked by local infusion of SCH23390 into the hippocampus, indicating that the effect is (a) localised to the hippocampus and (b) dopaminergic. Such approach is not possible in a 'loss of function' experiment. However, potential brain-wide effects of LC inactivation may be

minimised by local optogenetic inhibition of LC-NA axons in the hippocampus instead of LC-NA cell bodies.

More generally, the present study does not provide a direct proof of dopamine release from LC, relying instead on the pharmacological profile of the observed behavioural effect. Thus, a direct observation of dopamine release from LC terminals is needed before one can conclude with certainty that it is indeed the unexpected major source of dopamine in the central nervous system.

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