THE UNIVERSITY *of* EDINBURGH

Neuroprotection from the huntingtin-repressed transcriptional coactivator PGC-1α

by

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Abstract

The transcriptional coactivator PPARgamma coactivator 1alpha (PGC-1α) is a regulator of mitochondrial biogenesis and function and is decreased in the striatum of patients with Huntington's Disease (HD). HD is an autosomal dominant neurological disorder caused by a polyglutamine repeat in the huntingtin protein which leads to degeneration of striatal and cortical tissues. PGC-1α undergoes targeted downregulation by mutant huntingtin protein (mtHtt) and PGC-1α knockout mice have striatal lesions similar to HD transgenic mice. Exogenous PGC-1α partially reverses the toxic effects of mutant huntingtin in cultured striatal neurons while *in vivo* administration of PGC-1α to the striatum in a mouse model of HD reduces neuronal volume loss. Synaptic *N*-methyl-D-aspartate receptor (NMDAR)-activity can drive the expression of PGC-1α which is neuroprotective against oxidative and excitotoxic stress *in vitro* whereas extrasynaptic NMDAR expression is increased in HD. Excessive NMDAR activity, specifically through extrasynaptic rather than synaptic NMDARs, leads to excitotoxic death in neurons and its regulation has been targeted in the search for therapeutic interventions for multiple neurological disorders.

The data presented in this thesis show that the repression of PGC-1α by mtHtt may be significant in the dysregulation of NMDARs in HD. Both PGC-1α knockdown and mutant huntingtin are found to increase extrasynaptic NMDAR activity and excitotoxicity in a non-additive way, suggesting common regulatory mechanisms. Furthermore exogenous PGC-1α expression is sufficient to reverse this increase in extrasynaptic NMDAR currents and excitotoxicity by mtHtt. This thesis adds mechanistic insight into previous understanding of the synergistic roles of mtHtt, NMDAR activity and PGC-1α in HD.

Finally, we show that chronic knockout of PGC-1α in the PGC-1α$^{(-/-)}$ mouse causes distinct alterations in glutamatergic signaling that do not mimic the observation of acute knockdown of PGC-1α. We propose that the loss of PGC-1α in a number of neurological disorders contributes to concurrent increases in aberrant glutamate signaling and excitotoxicity in these diseases.

Declaration

This work was carried out in the School of Biomedical Sciences at the University of Edinburgh. I have composed the work and analysis presented in this thesis. The work complied in this thesis has not been submitted for any other degree or professional qualification.

Clare Puddifoot

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Chapter 1:

Introduction

1.1 Neurodegeneration

The prevalence of neurodegenerative disease (NDG) is rapidly escalating (Cowan and Kandel, 2001). Since advancing age is major factor in these diseases, as the aging population is expected to grow (Fig 1.1), neurodegenerative disease will continue to be a major public health issue. In the absence of effective treatments, NDGs will put an increasing burden on future economics. Uncovering the underlying molecular and cellular mechanisms responsible for the patho-progression of neurodegenerative disorders is key in the development of medical interventions.

Neurodegeneration refers to the progressive loss of neuronal function and viability associated with neurodegenerative diseases including, but not limited to, Alzheimer's disease (AD), Huntington disease (HD), Parkinson's disease (PD), and Amyotrophic lateral sclerosis (ALS). Despite the distinct profiles of neuronal loss associated with each disease (Table 1.1), advances in the last few decades have identified disruptions in subcellular processes common to all or some of these diseases (Table 1.1) including protein aggregation, mitochondrial dysfunction, defects in axonal transport, excitotoxicity and aberrant initiation of programmed cell death (Ikonomidou and Turski, 2002; Vila and Przedborski, 2003; Lashuel and Lansbury, 2006; De Vos et al., 2008)

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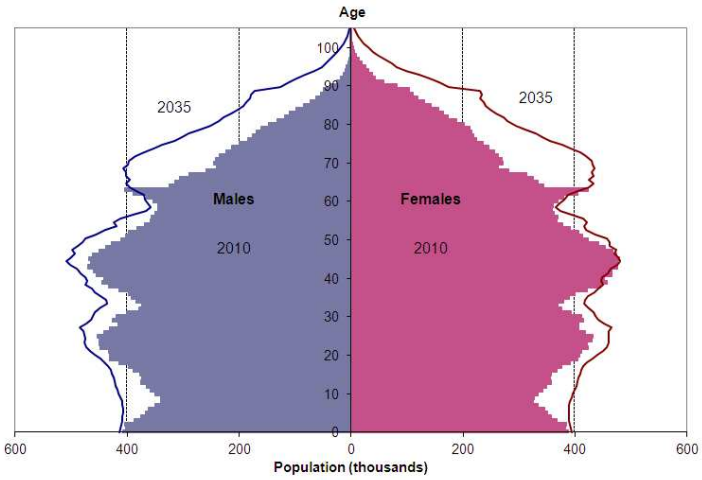

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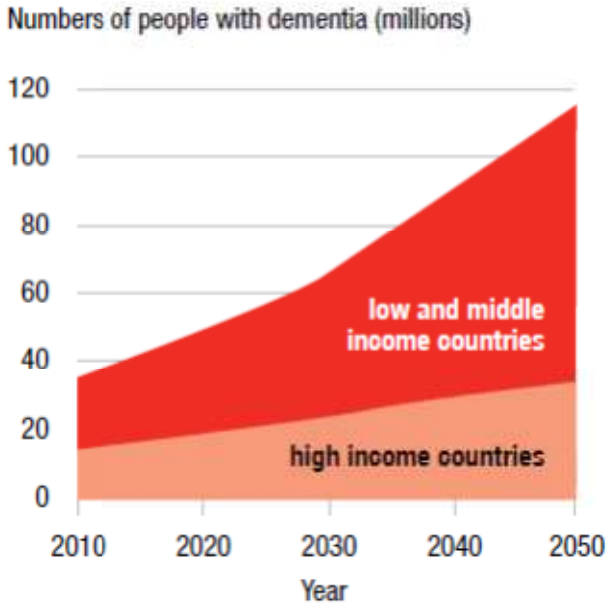

Fig 1.1 The projected increase in neurodegeneration highlights the significance of NDG research (A) Estimated and projected age structure of the United Kingdom population mid 2010- mid 2035 (UK Government Statistics; cited March 2012) (B) Projected world prevalence of dementia between 2010-2050. (World Alzheimer report 2010; cited March 2012).

Disease	Prevalence (per 100,000) (www.Neural.org.uk (cited April 2012)	Mode age of onset	Affected areas	Genes	Protein Accumulation	Mitochondria defects	Excitotoxicity/ NMDARs
Huntington's Disease	13.5	35-40y	Striatum, Frontal Cortex, Temporal Cortex. Specific loss of medium spiny neurons (The Huntington's Disease Collaborative Research Group, 1993)	Autosomal dominant inheritance of CAG repeat in Huntingtin gene (The Huntington's Disease Collaborative Research Group, 1993)	Huntingtin protein with large polyglutamine repeats forms cytoplasmic aggregates (Arrasate et al., 2004)	↓Complexes I-IV of respiratory chain (Turner & Schapira, 2010) ↑ Oxidative stress and free radical (Browne et al., 1999;Butterworth, 1998)	↑ Excitotoxicity (Okamoto et al., 2009) ↑ Extrasynaptic NMDARs (Milnerwood et al., 2010)
Alzheimer's Disease	1,000	>60y	Entorrhinal cortex, Hippocampus, Parahippocampal gyrus, Amygdala, Frontal, Temporal, Parietal and Occipital association cortices (reviewed Selkoe, 2001)	0.1% of AD Familial: Amyloid precursor Protein, presenilins1/2. Non familial: ε4 allele of the apolipoprotein E increases risk (Goate et al., 1991; Roses et al., 1996))	Cytoplasmic Tau aggregates & extracellular Aβ plaques (reviewed by Selkoe, 2001)	Mitochondiral fragmentation, altered metabolism, Excess ROS production (Reviewed by Santos et al., 2010)	Aβ regulates NMDAR trafficking (Snyder et al. 2005)
Parkinson's Disease	200	>60y	Substantia nigra. Specific loss of dopaminergic neurons (HenchCliffe and Beal 2008)	~5% known mutations : alpha-synuclein, parkin, leucine-rich repeat kinase 2, PTEN-induced putative kinase 1 (Kitada et al., 1998; Chen and Feany 2005;	α-synuclein aggregates form cytoplasmic Lewy body inclusions (Baba et al., 1998)	Complex I deficiency (Schapira et al., 2000) Excess anaerobic metabolism, Oxidative stress (reviewed Henchcliffe and Beal 2008)	
Amyotrophic Lateral Sclerosis	13.5	40-60y	Upper and lower motorneurons: bulbar, upper limb, thoracic, lower limb, motor cortex (reviewed Chio et al., 2009)	~5% hereditary, of which 20% carry mutation in SOD1. Mutation in *fused in sarcoma/translated in liposarcoma and TAR DNA-binding protein 43* in some sporadic cases (Watanabe et al., 2002; Kwiatkowski et al., 2008; Kabashi et al., 2008)	Cytoplasmic inclusion of SOD1, FUS or TDP-43 reported (Watanabe et al., 2002; Kabashi et al., 2011)	Vacuolation and degeneration of mitochondria, SOD1 accumulation in mitochondria, deficits in respiration. (reviewed by Dawson , 2004)	Aberrant glutamate reuptake causes excitotoxicity. Increase in Ca^{2+}-permeable AMPA-type Glutamate receptor (reviewed by Rao and Weiss, 2004)

Table 1.1 In this table I have collected examples of some of the common pathologies in cellular processes described in these Neurodegenerative diseases

1.1.1 Studying molecular cascades in NDGs

Identifying common phenotypes between NDGs highlights cellular cascades that may account for preferential vulnerability of neuronal tissue in these diseases, albeit offering no explanation for the regional-specificity of neuronal loss. However, with the end goal being the establishment of disease interventions, it is essential that we are able to distinguish between the causes and symptoms of pathology. This requires the analysis of relationships between these disease-associated components and assessment of their relative contribution to cellular health and function which may not be immediately evident for a number of reasons.

Firstly, disease-specific phenotypes may indeed be pathological but alternatively they could be protective compensatory mechanisms. One such example is the formation of mutant huntingtin inclusions in HD, long considered a hallmark of the disease (Davie et al., 1997), has in recent years been recognised as a compensatory protective mechanism and in such, restricting this process exacerbates cells death (Okamoto et al., 2009).

Secondly, although the apparent timecourse of cellular changes may correlate with the onset of the disease, this is not proof that such changes are pathological. For example Palop et al. (2006), argue that although neuronal death is what ultimately determines the onset of clinical phenotype in Alzheimer's diseases, the timecourse of neuropathological symptoms correlates more closely with alterations in synaptic function that occur when the remaining tissue is no longer able to compensate for lost synaptic inputs. Within this model, despite matching the timecourse of disease progression, disease-specific alterations in synaptic profiles may be considered as mechanisms that temporarily protect physiological function.

Finally, the pitfalls in antagonising known pathological cascades without fully understanding the physiological roles of the targeted protein

was highlighted by the failure of *N*-methyl-D-aspartate receptors (NMDAR)-antagonists in preclinical trials for the treatment of stroke (Ikonomidou and turski, 2002). Although it was known that NMDAR-activity contributed to pathology of stroke, it has since been shown that specific populations of NMDARs can couple to either pro-death or pro-survival cascades (Hardingham and Bading, 2010) and further understanding of the full molecular cascades are required in order to identify a safe and effective target for medical interventions. Together these models propose that studying the molecular basis of NDG is essential for both expanding our knowledge of the diseases as well as identifying potential therapeutic targets.

This thesis addresses the role of NMDAR signalling and excitotoxic cell death in neurodegenerative disease; the potential therapeutic benefits of enhancing an endogenous neuroprotective cascade, regulated by the transcriptional coactivator PGC-1α, that may be diminished in NDGs, is emphasised with specific interest in neuronal viability and function in HD. The work presented in this thesis is now published (Puddifoot et al., 2012; PGC-1α Negatively Regulates Extrasynaptic NMDAR Activity and Excitotoxicity; Journal of Neuroscience 35: 20; 6995-7000).

1.2 The excitotoxic theory of neurodegenerative disease

The ability of glutamatergic activity to induce neuronal cell death has been known for over 50 years (Lucas & Newhouse 1957; Curtis et al. 1959). On observation that injections of monosodium glutamate could induce neuronal necrosis in several regions of the brain, Olney termed this phenomena 'excitotoxicity' (Olney 1969). Glutamate is the main excitatory neurotransmitter in the mammalian brain and can activate a number of glutamate receptors both metabotropic and ionotropic. Ionotropic glutamate receptors (summarised in Fig 2.) contain a pore which opens upon receptor activation allowing the selective passage of ions into the cell across the plasma membrane. Studies by Choi (1988; 1987a; 1987b) and Tymianski et al. (1993) showed that excitotoxicity has an explicit requirement for Ca^{2+} influx into the neuron and the source of Ca^{2+} is specific to Ca^{2+} entry through the *N*-methyl-D-aspartate (NMDA) subclass of ionotropic glutamate receptors. For the decade that followed, many groups gathered evidence for the role of excessive NMDAR Ca^{2+} influx in neurodegenerative disease and stroke (Rothman & Olney 1986; Choi 1988; Lipton & Rosenberg 1994; Arundine & Tymianski 2004; Fan & Raymond 2007).

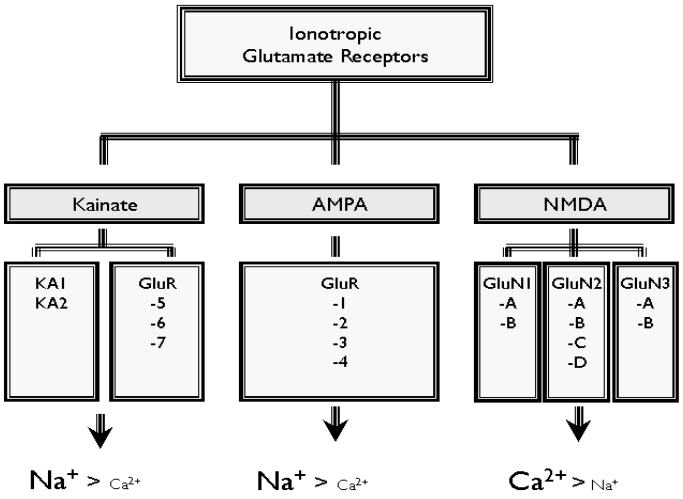

Figure 1.2 Ionotropic glutamate receptors There are three major classes of ionotropic glutamate receptors. All are heteromeric, composed of four subunits from the subsets outlined above. The receptors differ in their permeability to select ions; of interest is the high permeability of NMDARs to Ca^{2+} relative to other glutamate receptors.

1.2.1 NMDARs

NMDARs are a subfamily of glutamatergic receptors expressed almost ubiquitously throughout the CNS during pre and post natal development and thereafter. NMDARs are named after the selective agonist N-Methyl-D-Aspartate which distinguishes them from the two other ionotropic glutamate receptors: the α-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid (AMPA) receptor and the 2-carboxy-3-carboxymethyl-4-isopropenylpyrrolidine (kainate) receptor. Two major attributes of NMDARs that differentiates them from the other ionotropic glutamate receptors include (i) the necessary relief of a voltage-dependent inhibition by extracellular magnesium for ion permeation through the channel pore (Kutsuwada et al., 1992) and (ii) the receptor's high permeability to calcium ions upon activation. Combined these properties have implicated the NMDAR in a number of inter- and intra-cellular processes. Indeed, calcium influx into neurons has been shown to trigger diverse molecular cascades which lead to cellular and synaptic growth and development as well as neuroprotection and conversely pro-death signalling (Bliss & Collingridge 1993; Choi 1992; Hardingham et al. 2002; Papadia et al. 2008).

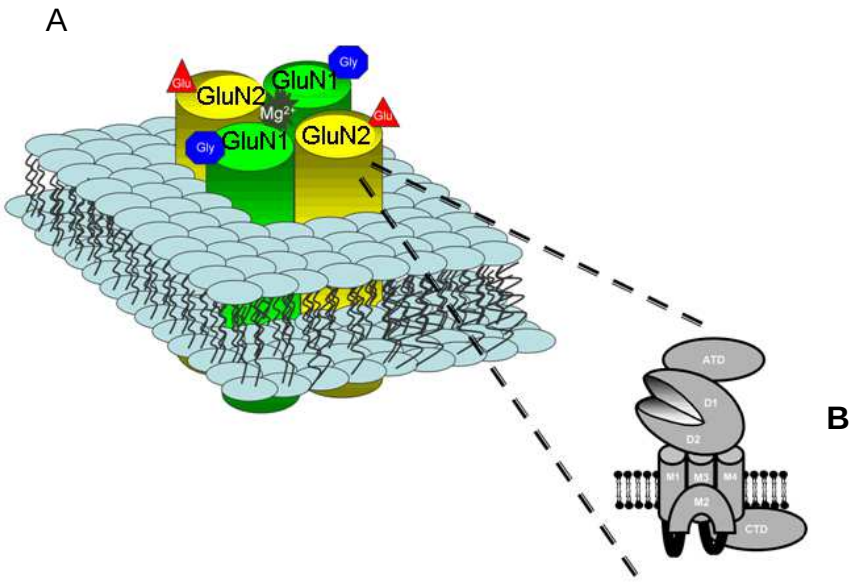

Figure 1.3 Cartoon illustration of the structure of NMDARs (A) NMDARs are found embedded in the post-synaptic membrane of neurons throughout the CNS. The NMDAR is composed of four subunits; the majority of receptors contain two glycine (Gly)-binding GluN1 and two glutamate (Glu)-binding GluN2 subunits as shown. At resting membrane potential (~ -70 mV) the ionic pore of the NMDAR is blocked by the presence of Mg^{2+} ions. The GluN2 subunit expressed (A-D) within any given recombinant is thought to define the majority of the functional properties of the receptor.(B) Each subunit is composed of four funtional domains, the amino terminal domain (ATD), the ligand binding domain (LB), the membrane spanning domain which includes the re-entrant loop (M1-4), and an intracellular C-terminal domain (CTD).Image in B previously published in Puddifoot et al., (2009).

1.2.2 NMDAR assembly, trafficking and distribution

There are seven possible NMDAR subunits: GluN1, GluN2A-D and GluN3A-B (Monyer et al., 1992; Das et al., 1998). Although some receptors do exist as triheteromers formed as GluN1/GluN2/GluN3 assemblies, the majority of NMDARs exist as an assembly of two GluN1 and two GluN2 subunits (Dingledine et al. 1999; Stephenson et al. 2008; Fig 1.3). It is the GluN2 subtype contained within any given recombinant that is thought to define the majority of the biophysical and pharmacological properties of the receptor; this includes the glutamate efficacy, calcium conductance level and potency of block by magnesium (Monyer et al., 1994; Cull-Candy et al., 2001).

The inclusion of specific NMDAR subunits within receptors is both temporally and spatially regulated throughout development (Monyer et al., 1994). GluN1 expression is high in most brain areas throughout development (Monyer et al., 1994), whereas the expression profile of GluN2 subunits varies during maturation (Cull-Candy & Leszkiewicz 2004). GluN2 subunit expression is dominated by GluN2B at early developmental stages and although GluN2B expression levels remain high through adulthood, as the cells mature the relative levels of GluN2A subunits expression increases becoming the most abundant subunit (Cull-Candy et al. 2001).

Once assembled in the endoplasmic reticulum, cell surface expression of the receptors requires the coordinated interactions of the NMDARs, membrane-associated guanylate kinases (MAGUKs) and kinesin KIF17–LIN10 motor complex to carry out the cytoplasmic and membrane interactions required for NMDAR trafficking (Gladding and Raymond, 2011). Receptors destined for the synapse are supported by a large protein complex termed the post-synaptic density (PSD). The PSD contains a network of scaffolding, adaptor and effector proteins which regulate

receptor trafficking, membrane stability as well as the activation of downstream signalling cascades (Sheng & Lee 2000).

NMDARs are also expressed at peri-synaptic sites (within 200–300 nm of the PSD) or extra-synaptic sites (throughout the dendritic membrane and soma), together termed extrasynaptic from herein (Gladding and Raymond, 2011). Studies in hippocampal cultures show a developmental change in the proportion of receptors at extrasynaptic sites from 75% at DIV5-7 (Tovar & Westbrook 1999) to 20-50% >DIV9 (Rosenmund et al., 1995). It has been shown that once at the cell surface, the location of receptor is not rigid, that is NMDARs can undergo lateral diffusion within the cell membrane (Tovar & Westbrook 2002; Groc et al. 2009). However this is contested by Harris and Petit (2007) who show that in acute slices, NMDAR expression is stable and once expressed at the cell surface there is no NMDAR mobility.

1.2.3 Excitotoxic cell death

Excessive calcium entry after prolonged NMDAR activity was found to induce both necrotic and apoptotic cell death depending of the stimulus intensity/duration (Bonfoco et al., 1995). Necrotic and apoptotic cell death are both morphologically and mechanistically distinct. Necrotic cell death is classically thought to result from ionic deregulation and rapid ATP depletion which leads to swelling of both the cell and cellular organelles with eventual breakdown of the plasma membrane and release of cellular content including proinflammatory molecules. In necrotic cell death, excessive Ca^{2+} influx is exacerbated by aberrant calpain cleavage of the Na^{+}/Ca^{2+} exchanger (NCX) which under normal conditions is responsible for Ca^{2+} extrusion (Bano et al., 2005).

In contrast apoptotic cell death, often termed 'programmed cell death', is characterised by cellular and nuclear shrinkage, chromatin condensation and genomic fragmentation without plasma membrane breakdown. Apoptosis requires the activation of cysteine proteases (caspases), it is ATP-dependent and can be activated either by ligation of the death receptor or by release of proapoptotic factors from the mitochondria (Zou et al., 1997; Ashkenazi and Dixit, 1998).

Although it was previously believed that there exists a threshold of activity above which NMDAR activity becomes excitotoxic (Lipton and Nakanishi, 1999; Hardingham and Bading, 2003), the current understanding has been shifted by the finding that NMDAR toxicity is conferred not simply by excessive amounts of activity, but rather by the activation of NMDARs located at extrasynaptic sites (Hardingham et al. 2002). For example, Hardingham et al. (2002) show that similar calcium loads through synaptic versus extrasynaptic NMDARs differentially

couple to CREB-dependent transcription; whereas synaptic activity induces CREB expression and activity and promotes cell survival, extrasynaptic NMDAR activation causes a dominant CREB shut-off and triggers cell death. Therefore, NMDAR activity provides an interesting paradox in which the activation of this protein complex can initiate the expression of either neuroprotective or neurodestructive transcriptional profile depending on the location of the receptor (Hardingham & Bading 2010; Fig 1.4).

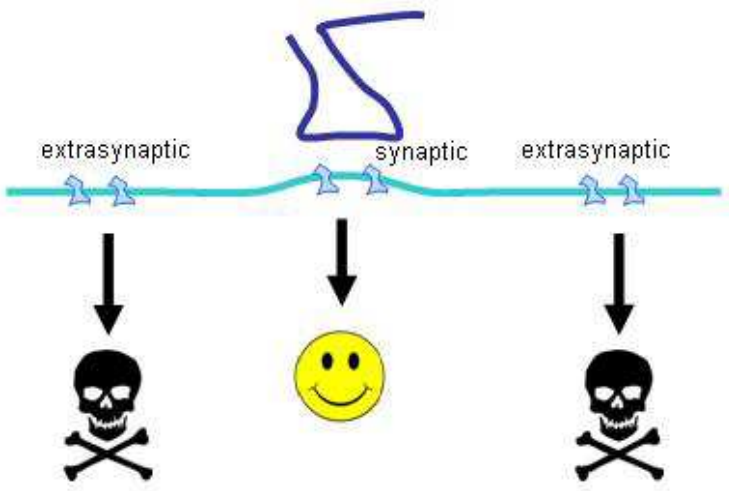

Fig 1.4 *Synaptic* NMDARs activation is pro-survival, *extrasynaptic* NMDARs activation is pro-death NMDARs transmit information from outside the cell to inside the cell. When NMDARs are activated at synaptic sites they couple to pro-survival pathways. When NMDARs at extrasynaptic sites are activated they couple to pro-death signaling cascades. In this figure the dendrite is depicted without spines representing the lack of spines in our cultured neurons at the time of experimentation.

1.2.4 What distinguishes synaptic and extrasynaptic NMDAR activity?

Despite a great deal of progression in identifying pro-survival and pro-death pathways coupled to synaptic and extrasynaptic activity (discussed in detail below), the basis for the differences in synaptic versus extrasynaptic NMDAR signalling is unknown. However, three independent theories have been proposed to explain these observations. The first is the potential differences in the subunit composition of the NMDAR complex at synaptic versus extrasynaptic sites leads to differential activation of signalling cascades. As well as determining the ligand efficacy, channel conductance and magnesium block of the NMDAR, the GluN2 subunits of NMDARs contain distinct cytoplasmic tails that can interact with intracellular proteins (Sheng et al., 1994; Zhong et al., 1994; Cull-Candy et al., 2001). Early in development the GluN2B subunit is most abundant and after the first post natal week, there is a switch at which point GluN2A expression is enriched (Stiegerwald et al., 2000). It has been suggested that extrasynaptic receptors retain a higher proportion of GluN2B subunits (Groc et al., 2006). However, Petralia et al. (2010) and Harris and Pettit (2007) report an even distribution of GluN2A and GluN2B receptors at synaptic and extrasynaptic sites. In addition, although Liu et al., (2007) found differential coupling of GluN2A and GluN2B to pro-survival and pro-death cascades respectively, the selectivity of the GluN2A agonist used has been disputed (Frizelle et al., 2006). Furthermore, GluN2B and GluN2A receptors have both displayed the ability to promote cell survival or cell death (von Engelhardt et al., 2007; Martel et al., 2009). Such investigations have been confounded by the absence of a selective GluN2A antagonist. However, an elegant study recently published by Martel et al., (2012) used chimeric NMDAR subunits with the C terminal domains (CTDs) of GluN2A and GluN2B

switched to demonstrate that the CTD of GluN2B promotes excitotoxicity better than that of GluN2A and this was independent of the identity of the other domains of the subunit.

A second theory is that there are differences in the components of the NMDAR signalling complexes at synaptic and extrasynaptic sites (Collins and Grant, 2007). However, a number of key members of the post-synaptic density membrane associated guanylate kinases (MAGUKs) are found at both synaptic and extrasynaptic sites. In particular the post-synaptic density protein 95 (PSD95) and disks large homologue 3 (SAP102) were found associated with extrasynaptic NMDARs in hippocampal cultures (Petralia et al., 2010).

The third model proposes that differences in the dynamics of calcium transients through extrasynaptic versus synaptic NMDARs affects the downstream signalling complexes. In contrast to synaptic NMDARs which are activated transiently after synaptic glutamate release, the timecourse of extrasynaptic NMDAR activation is chronic, resulting from increased background calcium concentrations. Despite evidence that the equivalent changes in calcium concentration from synaptic activity versus extrasynaptic NMDAR activity (Hardingham et al., 2002), this model suggest that it is the prolonged increase in calcium concentrations that may be responsible for the distinct coupling to pro-death versus pro-survival cascade.

1.3 Pro-survival and pro-death signalling from NMDARs

Studies in rodents both *in vivo* and *in vitro* have greatly contributed to our understanding of the distinct pathways activated/repressed by synaptic and extrasynaptic NMDAR activity. In cortical neuronal cultures, synchronous bursts of NMDAR activity can be stimulated by using the GABAR antagonist bicuculline to disinhibit circuit activity alongside the K^+ channel blocker 4-AP which depolarises cells increasing the probability that they will fire action potentials. Extrasynaptic NMDARs are pharmacologically isolated by first blocking synaptic NMDARs with the open channel blocker MK-801 and subsequently applying the agonist NMDA to the culture. *in vivo*, pathways downstream of synaptic NMDAR activity have been confirmed by analysis of cortical tissue from mice injected with MK-801.

Strikingly such analysis has shown that not only do NMDARs couple to distinct pathways depending on whether they are located at the synapse or at extrasynaptic sites, but in some cases, extrasynaptic NMDAR signalling opposes synaptic NMDAR signalling in a dominant manner (Hardingham & Bading 2010).

1.3.1 Synaptic NMDAR activity promotes cell survival

Abolishing synaptic NMDAR activity is highly detrimental to neuronal health (Ikonomidou et al., 2001). Antagonism of NMDAR activity *in vivo* has been shown to decrease the number of healthy cells and induce large-scale apoptosis (Gould et al., 1994; Ikonomidou et al., 1999; Pohl et al., 1999; Adams et al., 2002; Papadia et al., 2008) and amplifies neuronal loss in both acute traumatic brain injury and ongoing neurodegeneration (Ikonomidou et al., 2000). A multitude of studies have shown a powerful coupling of synaptic NMDAR activity to pro-survival signalling through both the induction/repression of gene expression and posttranslational modifications of existing proteins.

For over two decades molecular signalling cascades downstream of neuronal activity have been studied as an example of how genetic profiles can be regulated by physiological stimuli (Morgan et al., 1987). Indeed, synaptic NMDAR activity has been shown to bestow neuroprotective properties on the postsynaptic cell that enhance neuronal survival in the face of noxious stimuli (Papadia et al., 2008; Leveille et al., 2008; Lau et al., 2009). In addition, the acquired neuroprotection can outlast the timecourse of the activity itself (Papadia et al., 2005). This physiologically-driven neuroprotection was shown to have both transcriptional-dependent and independent factors and provides a robust protection by altering a number of fate-determining cascades. This includes; the repression of apoptotic genes and upregulation of anti-apoptotic genes, increase in pro-survival genes such as those involved in mitochondrial biogenesis and function, and the enhancement of antioxidative cascades (Hardingham and Bading 2010; Fig.1.4).

1.3.2 Synaptic NMDAR activity is anti-apoptotic

The phenomena known as ‘apoptosis’ was defined by Kerr et al., (1972) describing common morphological features observed during controlled cell death. Apoptosis, often referred to as ‘programmed cell death’ is thought to be a means of regulating cell populations during development and in the event of cellular stress such as DNA damage (Kerr et al., 1972; Renehan et al., 2001). Changes in cell morphology include pyknosis, chromatin condensation, nuclear fragmentation, plasma membrane blebbing, finally resulting in engulfment by resident phagocytes (Kerr et al., 1972). Despite common morphological traits, Kroemer et al., (2009) argue that heterogeneity of biochemical mechanisms which can induce programmed cell death indicate distinct subtypes of apoptosis. For example, there exist two known sub categories into which apoptotic events fall: intrinsic or extrinsic apoptosis. Extrinsic apoptosis is initiated by ligand activation of dedicated transmembrane death receptors from the tumour necrosis factor superfamily (Ashkenazi and Dixit, 1998) which can activate caspase -8 and caspase-9 (Riedl and Salvesen, 2007; Fig 1.5).

1.3.2a The intrinsic apoptosis cascade

In the intrinsic apoptotic pathway, Cytochrome C released from the mitochondria binds APAF1, initiating the formation of the apoptosome (Riedl and Salvesen, 2007). In turn, the apoptosome activates the initiator enzyme Caspase-9 which leads to the cleavage of and subsequently the irreversible activation of caspase-3 and caspase-7 (Riedl et al., 2001). Caspase-3 and Caspase-7 are responsible for executing the morphological changes of apoptosis.

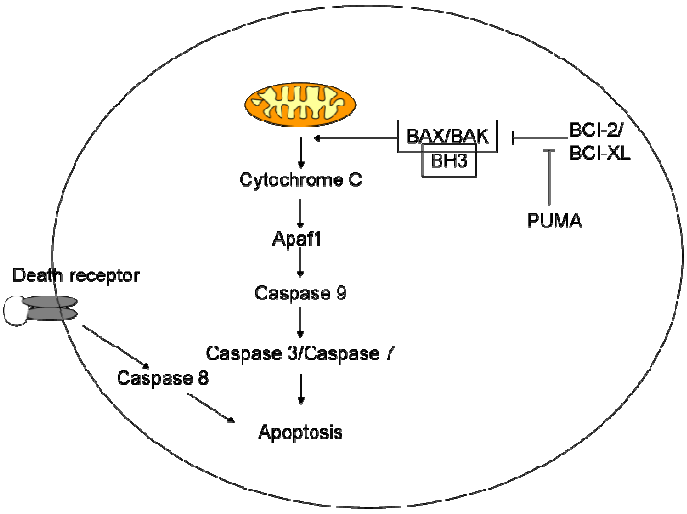

Figure 1.5 Intrinsic and extrinsic apoptosis: Two distinct routes to apoptosis, through mitochondrial (orange) cytochrome C release or activation of the death receptor. BAK, Bcl2-antagonist/killer; BAX, Bcl2-associated X protein; BH3 interacting domain death agonist; PUMA, pro-apoptotic Bcl2 homology domain 3 (BH3)-only member gene BCL-2, B-cell lymphoma 2; BCL-XL, B-cell lymphoma-extra large.

In addition, apoptosis is regulated by the Bcl-2 family of proteins, which can fall into three categories: pro-apoptotic multimeric domain members, pro-apoptotic BH3 only domain members or anti-apoptotic members. Examples of multidomain members include Bcl2-associated X protein (BAX) or BAK which form oligometric structures that permeabilise the outer mitochondrial membrane, allowing release of cytochrome-C. This requires the binding of BH3 domains. The anti-apoptotic members Bcl-2 and Bcl-XL antagonise the formation of BAX/BAK mediated pores, through binding to BH3 domains. However, the pro-apoptotic BH3 only Bcl-2 members such as BAD, BID, BIM and PUMA can inhibit this interaction by binding to antiapoptotic Bcl-2 proteins. This leads to the disinhibition of BAX and BAK (Youle and Strasser, 2008) and increased apoptosis (Fig 1.5).

1.3.2b Repression of pro-apoptotic genes

One mechanism of neuroprotection from ongoing synaptic NMDAR activity is the repression of endogenous apoptotic genes (Papadia et al., 2005; Lau and Bading, 2009; Léveillé et al., 2010; Soriano et al., 2011). The phosphoinositol-3-kinase (PI3K)/Akt pathway is a potent repressor of apoptotic genes and is activated by synaptic NMDAR activity (Papadia et al., 2005). Synaptic NMDA Ca^{2+} transients activate the calcium–binding protein calmodulin. Calmodulin activates phosphoinositol-3-kinase causing the synthesis of phosphatidylinositol trisphosphate which recruits Akt to the plasma membrane where is it phosphorylated by phosphoinositide dependent protein kinase (Brazil et al., 2004).

Akt phosphorylates and represses the pro-apoptotic genes; glycogen synthase kinase-3 (GSK-3), the BH3-only Bcl2 family member BAD, the BH3-only member gene *Puma* and the stress-activated protein kinase (SAPK) subtype c-Jun N-terminal kinase (JNK) (Hardingham & Bading 2010).

Synaptically activated Akt also represses the proapoptotic transcription factors forkhead box protein O (FOXO) and P53. FOXO1 and FOXO3 are the major FOXOs in neurons. FOXOs mediate the transcription of the pro-death genes including Bcl2-interacting mediator of cell death (Bim), Puma, Fas ligand (Fas1) and thioredoxin-interacting protein (Txnip) (Hardingham and Bading 2010). Akt phospohorylates FOXOs causing their nuclear exportation, and since Foxo1 is itself a FOXO target gene, this leads to prolonged repression of the FOXO1 pathway (Dick and Bading, 2010; Al-Mubarak et al., 2010). The synaptically repressed transcription factor P53 promotes the expression of the pro-death genes Bcl2-associated X protein (BAX) and apoptotic protease activating factor 1 (APAF1) (Lau and

Bading 2009). This potent activation of the PI3 Kinase/Akt pathway is specific to synaptic NMDARs (Soriano et al., 2006; Papadia et al., 2008).

1.3.2c Induction of pro-survival genes

Synaptic NMDAR Ca^{2+} influx, reinforced by the subsequent release of Ca^{2+} from internal stores, leads to an increase in both somal- and nuclear-calcium concentrations. One major pathway to neuroprotection from nuclear- Ca^{2+} is via the activation of nuclear Ca^{2+}/calmodulin-dependent protein (CaM) kinase IV and subsequent phosphorylation of the transcription factor cyclic-AMP response element binding protein (CREB) (G E Hardingham et al. 2001; Papadia et al. 2005; Lee et al. 2005). Outside the nucleus, these calcium transients also increase ERK1/2 phosphorylation of the CREB binding protein (CBP) and dephosphorylation of transducer of regulated CREB activity (TORC) leading to the nuclear translocation of these two transcriptional coactivators necessary for the activation of CREB (Screaton et al., 2004). Whereas continuous synaptic NMDAR activity can protect neurons independent of CREB activity, long lasting protection from NMDAR Ca^{2+} is blocked by the CREB isoform ICER (Papadia et al., 2005).

Importantly, although neuronal protection can last long after the stimulation has ceased, the associated CREB-phosphorylation is transient. CREB-activity is now widely accepted to promote neuronal survival; targets of activity-driven CREB include the pro-survival growth factor brain-derived neurotrophic factor (BDNF) and the immediate early gene; c-fos (Dragunow, 2004; Lee et al., 2005; Papadia et al., 2005; Greer and Greenberg, 2008) and understanding the full spectrum of gene changes

downstream of synaptic activity has been an ongoing goal of our lab and others. A recent study by (Zhang et al., 2009) identified a number of pro-survival genes regulated by nuclear Ca^{2+} signalling; this cohort of genes was termed Activity-regulated Inhibitor of Death (AID) genes. The AID genes were highlighted from a large number of genes alterations downstream of nuclear calcium signalling, due to their previous implications in apoptosis (Zhang et al., 2009). The AID genes include activating transcription factor 3 (*Atf3),* B-cell translocation gene 2 *(Btg2),* B-cell lymphoma 6 *(Bcl6),* growth arrest and DNA-damage-inducible 45 beta (*Gadd45b*), *Gadd45g,* inhibin beta A *(Inhba),* interferon activated gene 202B *(Ifi202B),* neuronal PAS domain protein 4*(Npas4),* nerve growth factor-induced gene B *and serine* protease inhibitor B2 (Serpinb). AID genes promote neuronal viability both *in vivo* and *in vitro* and the reduction of NMDA-induced break—down of mitochondrial membrane potential by Npas4, Bcl6, Inhibin b-A, Ifi202b, and Nr4a, suggest a common protective mechanism that builds defences against mitochondrial stress. In addition, we have recently shown that synaptic NMDAR activity induces the expression of the CREB-target gene peroxisome proliferator activated receptor gamma coactivator 1 alpha (PGC-1α); PGC-1α a transcriptional coactivator known to enhance the transcription of genes involved in mitochondrial biogenesis and function (Soriano et al., 2011).

1.3.3 Synaptic NMDAR activity reduces oxidative stress

Oxidative stress occurs when the production of reactive oxygen species outweighs the cell's ability to neutralise them via the intrinsic antioxidant pathways. The accumulation of oxidative damage into nucleic acids, lipids,

proteins or carbohydrates is thought to contribute to the patho-progression of many neurodegenerative diseases (Mariani et al., 2005). Synaptic NMDAR activity promotes the expression of antioxidant genes and protects against oxidative stress both *in vivo* and *in vitro* (Papadia et al., 2008). Again, this neuroprotective mechanism is distinct to synaptic NMDARs, *in vitro* bath application of NMDA which activates both synaptic and extrasynaptic NMDARs does not protect against antioxidant insults (Papadia et al., 2008). A key antioxidative pathway is the thiol-reducing thiorexodin-peroxiredoxin system. The thioredoxin system consists of thioredoxin reductase, thioredoxin and peroxiredoxins which detoxify peroxides by transferring reducing equivalents from NADH to peroxide. Synaptic activity enhances thioredoxin by repressing the FOXO target gene the thioredoxin inhibitor Txnip (Papadia et al., 2008). In conditions of increases oxidative stress the antioxdidant peroxiredoxin can become hyperoxidized, synaptic activity enhances the reduction of hyperoxidized peroxiredoxins by increasing the expression of sulfiredoxin and sestrin 2 (Papadia et al., 2008).

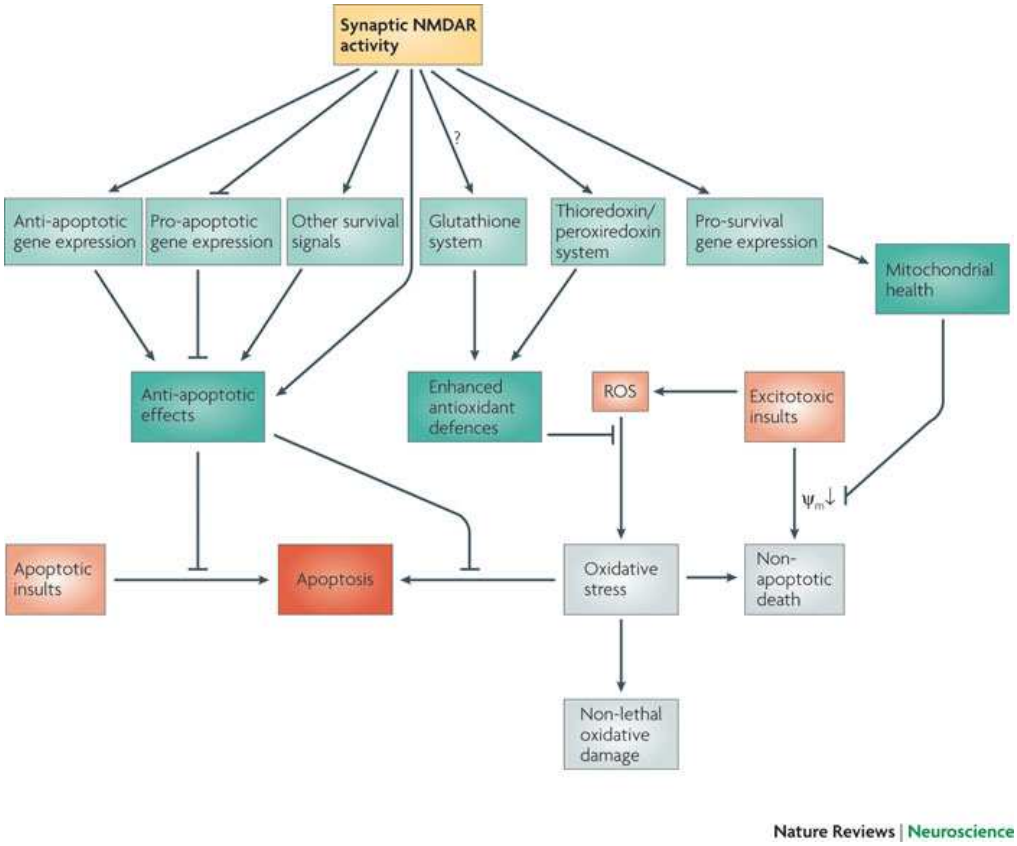

Figure 1.6 Synaptic NMDAR activity induces neuroprotective cascades Image from Hardingham and Bading (2010); Synaptic activity can prevent both apoptotic and non-apoptotic cell death by reducing endogenous pro-apoptotic cascades, enhancing antioxidant defences and promoting mitochondrial health.

1.4 Extrasynaptic NMDARs promote cell death

1.4.1 Extrasynaptic NMDAR activity and disease

The activation of extrasynaptic NMDARs has been mechanistically linked to both acute and chronic neuronal injury in ischemic stroke and HD. Extrasynaptic NMDAR activation requires the presence of glutamate outside of the synaptic cleft. This occurs in cerebral ischemia, where glutamate uptake by glia cells is impaired. Under these conditions the glial glutamate transporters no longer buffer extracellular glutamate, but rather release glutamate themselves. This leads to a build up of glutamate causing chronic depolarisation of the cell. The proximity of glia to extrasynaptic sites suggests that this glutamate release may activate extrasynaptic receptors (Petralia et al., 2010). Indeed mimicking this process *in vivo* with the glutamate transport inhibitor v L-trans-pyrrolidine-2,4-dicarboxylate (PDC), which causes glutamate transporters to pump glutamate out of the cell, causes excitotoxicity due to extrasynaptic NMDAR activation (Gouix et al., 2009). In addition, ischemia has recently been shown to increase the activity of extrasynaptic receptors via death-associated protein kinase DAPK 1 phosphorylation of the GluN2B NMDAR subunit which causes increased channel conductance (Tu et al., 2010).

A recent study by Milnerwood et al. (2010) identified a specific enhancement of both the expression and activity of extrasynaptic NMDARs in HD. This activity enhances the patho-progression of the disease (Okamoto et al. 2009) and as such is part of a positive feedback loop. Extrasynaptic NMDAR activity in HD is a major topic of this study and is introduced below.

1.4.2 Extrasynaptic NMDARs are pro-death

Site-specificity of NMDARs upstream of pro-death cascades is supported by the finding that extrasynaptic NMDARs alone is sufficient to induce equivalent levels of excitotoxic cell death as those seen after prolonged activation of all (synaptic and extrasynaptic) NMDARs together (Stanika 2009). Activation of extrasynaptic NMDARs leads to the activation or repression of a number of cellular cascades that ultimately leave the cell vulnerable to oxidative stress and apoptotic cell death (Hardingham and Bading, 2010). These include; mitochondrial calcium uptake and subsequent mitochondrial membrane depolarisation, the repression of anti-apoptotic genes downstream of synaptic NMDARs, the promotion of pro-apoptotic genes transcription, and aberrant calpain cleavage. (Fig 1.7)

1.4.2a Loss of mitochondrial membrane potential

Mitochondrial function is closely linked to excitotoxic events (Nicholls and Budd, 2000). Inhibiting mitochondrial calcium uptake strongly attenuates glutamate induced cell death (Stout el al., 1998). In fact, since calcium entering the cell via NMDARs is absorbed faster by mitochondria compared to calcium entry through voltage-dependent calcium channels or kainate receptors (Peng and Greenamyre, 1998), mitochondrial absorption dynamics is thought to play a role in the source specificity of NMDAR-toxicity. Calcium enters the mitochondria through the calcium uniporter which relies on the mitochondrial membrane potential.

The collapse of the mitochondrial membrane potential and the subsequent shift in the mitochondrial membrane permeability called the mitochondrial permeability transition (MPT) is a significant event that occurs early in excitotoxicity. A number of catastrophic events follow including the

depletion of cellular ATP, ionic imbalance, production of reactive oxygen species, and the release of cytochrome C which is known to induce the apoptotic cascade. Importantly, activation of extrasynaptic NMDARs, but not synaptic NMDARs, causes a rapid breakdown of the mitochondrial membrane potential (Hardingham et al., 2002).

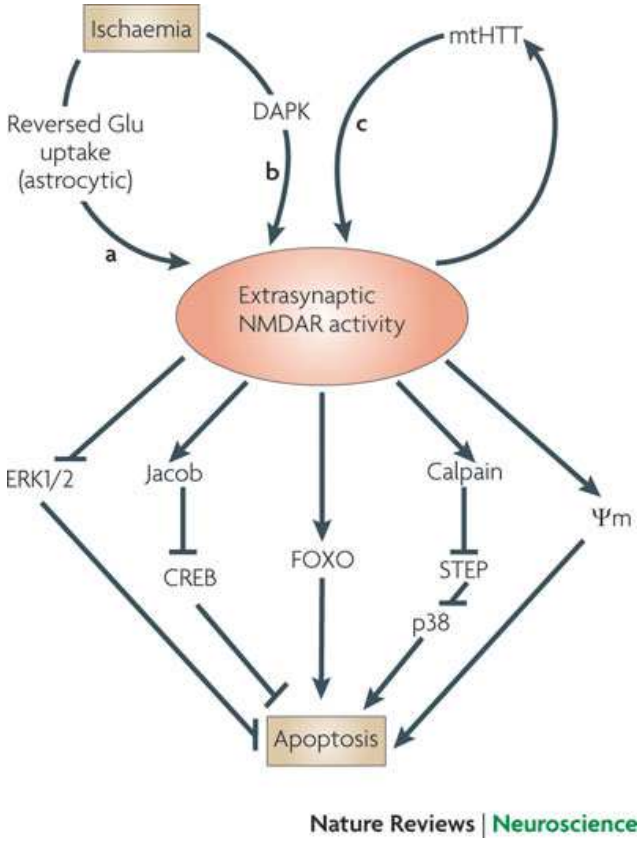

Figure. 1.7 Extrasynaptic NMDAR activity enhances pro-death signalling cascades Image from Hardingham and Bading (2010); Extrasynaptic NMDARs are activated during acute trauma; during ischemic insult, glutamate outside the synaptic cleft activates extrasynaptic NMDARs and extrasynaptic NMDAR conductance is enhanced by DAPK-phosphorylation of the NMDAR GluN2B subunit. Extrasynaptic NMDARs contribute to pathology of HD (HD); in HD the presence of mutant huntingtin protein enhances extrasynaptic NMDAR expression and activity. Extrasynaptic NMDAR activity causes a dominant 'shut-off' of neuroprotective cascades including ERK1/2 and CREB regulated transcription, blocking PI3K via FOXO translocation, Calpain mediated cleavage of p38 in addition to causing mitochondrial membrane depolarisation.

1.4.3b Expression of proapoptotic genes

Synaptic NMDAR activity promotes the nuclear export of the proapoptotic transcription factors FOXOs, whereas, extrasynaptic NMDARs have the opposite effect, causing the translocation and of FOXOs into the nucleus (Dick and Bading, 2010). As discussed above FOXOs regulates the transcription of pro-apoptotic genes and are known to contribute to oxidative and excitotoxic stress (Lehtinen et al., 2006; Lau and Bading, 2010).

1.4.2c Inactivation of CREB

As discussed above, CREB-dependent transcription is responsible for the long-lasting neuroprotection afforded by synaptic NMDAR activity (Papadia et al., 2005). In contrast to this, the bath application of NMDA is a very poor activator of CREB (Bading et al., 1993). An explanation for this was first shown in 2002; Hardingham et al., (2002) found that whereas synaptic NMDAR activity induces the phosphorylation of the CREB residue Ser-133, extrasynaptic NMDAR activity causes dephosphorylation of the same site, resulting in a dominant CREB-inactivation signal. This CREB dephosphorylation has since been shown to be regulated by the juxtasynaptic attractor of caldendrin on dendritic boutons proteins (JACOB). JACOB is a binding partner of the calcium binding protein caldendrin (Dieterich et al., 2008). Caldendrin binds JACOB in a calcium-dependent manner, preventing its nuclear import (Dieterich et al., 2008). Whereas synaptic NMDAR activity enhances caldendrin-retention of JACOB outside the nucleus, extrasynaptic NMDARs promotes nuclear import of (JACOB) which lead to CREB-dephosphorylation (Hardingham

et al., 2002). These CREB-shut off pathways are dominant over synaptic NMDAR-CREB activation.

1.4.2d Repression of Ras-ERK cascade

Extrasynaptic NMDARs also antagonise the Ras-ERK signalling pathway (Ivanov et al., 2006; Léveillé et al., 2008a). The extracellular-signal-regulated kinases 1/2 **(**ERK1/2) pathway enhances neuronal survival by promoting CREB dependent gene expression (Mayr and Montminy 2001). In resemblance to the bidirectional control of CREB-activation, the regulation of the ERK1/2 pathway by NMDAR activity is also strongly dependent on the population of receptors activated. In contrast to synaptic NMDAR activity which leads to ERK activation, extrasynaptic activity couples to ERK dephosphorylation and inactivation (Ivanov et al., 2005). In addition, extrasynaptic NMDAR activity represses the small GTPase Ras (Kim et al., 2005); Ras is upstream of ERK and is required for activation of the neuroprotective Ras-ERK pathway (Avruch et al., 2001).

1.4.2e Calpain activation

In 2009 Xu et al., (2009) demonstrated that extrasynaptic but not synaptic NMDAR activation can induce the calcium-activated cysteine proteases calpains, causing aberrant protein cleavage that contributes to excitotoxicity. Prior studies by Bano et al., (2005; 2007) have shown that calpain cleavage of the Na^{+}/Ca^{2+} exchanger 3 (NCX3) contributes to excitotoxic and oxidative stress. NCX3 ordinarily regulates calcium expulsion and thus its inactivation is thought to contribute to delayed calcium deregulation associated with excitotoxicity (Bano et al., 2005).

Activation of calpains by extrasynaptic NMDARs also leads to cleavage of STEP (Xu et al., 2009). STEP is a negative regulator of P38MAPK and activating STEP cleavage via extrasynaptic NMDARS induces disinhibition of P38MAPK which is neurotoxic in cerebellar granule cells and cortical neurons (Xu et al. 2009).

1.5 Huntington's Disease

Huntington disease (HD) is the most prevalent inherited neurodegenerative disease with an incidence of around 1 in 10,000 individuals. Characteristic symptoms include movement disorder, psychiatric and cognitive impairment followed by premature death normally within 10-15 years of disease diagnosis.

The neurodegeneration of HD leads to widespread loss/dysfunction in the cortex in early stages of the disease (Rosas et al., 2002), followed by significant cell loss in the caudate and putamen (Subramaniam and Snyder, 2011) which is thought to account for the motor, psychiatric and cognitive features of disease. Speculation that initial stages of HD are driven by cortical dysfunction with consequential malfunction in cortico–striatal pathways has been raised (Imarisio et al., 2008).

HD is inherited in an autosomal dominant manner and is caused by a CAG trinucleotide repeat in the HTT gene on chromosome 4, 17 codons downstream of the initiator ATG codon in exon 1. This mutation causes a polyglutamine expansion in the N terminal of the protein huntingtin (The HD Collaborative Research Group, 1993). It is currently believed that this polyglutamine insert causes a toxic gain of function on the mutant protein, which after sequential cleavage gives N-terminal fragments which contain the expanded polyglutamine region (Imarisio et al., 2008). A hallmark of the disease is the accumulation of insoluble mutant Huntington protein into cellular aggregates; this has recently been shown to be a protective mechanism which protects the cell from the toxicity of the protein (Arrasate et al., 2004). The presence of mutant huntingtin protein is known to disrupt a number of cellular processes including mitochondrial function, proteosome activity, synaptic and extrasynaptic NMDAR function and

excitotoxicity; as well as perturbing transcriptional pathways (Zeron et al., 2002a; Donaldson et al., 2003; Jenkins et al., 2005; Cui et al., 2006). However, the full interplay between these mechanisms and their contribution to disease pathology is not fully understood.

1.5.1 Increased excitotoxicity in HD

Initial evidence for a potential role of NMDAR activity in the neurodegeneration of HD came from studies demonstrating the ability to imitate the pathology of HD *in vivo* using NMDAR agonists. Injection of NMDAR agonists into the rodent striatum was seen to selectively destroy the medium spiny neurons sparing interneurons, thus mimicking the pattern of neuronal loss observed in HD (R J Ferrante et al. 1987; Beal et al. 1986). In addition, Hantraye et al. (1990) were able to replicate both the behavioural changes and neuropathology of HD in non-human primates by intrastriatal injection of the NMDAR agonist quinolinic acid. Subsequent investigations in YAC transgenic mice expressing the full length huntingtin with a polyQ expansion of 72 (YAC72) confirmed that excessive neuronal loss in response to quinolic acid was also observed *in vivo* in this HD mouse model (Zeron et al. 2002). Cultured MSN from YAC72 HD mouse model exhibited enhanced apoptotic cell death in response to NMDA stimulation (Zeron et al., 2002). In addition, cultured cortical neurons acutely expressing either full length or truncated exon 1 mtHtt, containing the polyglutamine repeat, also display increased excitotoxicity in response to glutamate stimulation (Okamoto et al. 2009). This toxicity was reversed by NMDA antagonists APV, Ifenprodil and interestingly low dose memantine, which selectively blocks extrasynaptic NMDARs (Okamoto et al. 2009).

In 2009, Okamoto et al. showed that while synaptic NMDAR activity reduces the toxicity of mutant huntingtin, extrasynaptic NMDAR activity has the opposite effect, increasing the patho-progression of the disease. This study provided mechanistic insight in to the opposing roles of NMDARs located at synaptic versus extrasynaptic sites, which have opposite effects on the formation of insoluble marcoinclusions.

1.5.2 NMDARs and mutant huntingtin inclusions

Aggregation of the mutant huntingtin protein into insoluble macroinclusions is a hallmark of the disease (Davies et al. 1997) ans is accompanied by striatal and cortical atrophy. The concurrence of aggregate formation and neuropathology of the disease lead to the prediction that inclusion formation was neurotoxic and disrupted cellular processes by sequestering proteins such as transcription factors (McCampbell, 2000) and components of the ubiquitin–proteasome system (UPS) (Donaldson et al., 2003). In contrast, multiple factors now suggest that rather than being toxic, the formation of these large insoluble inclusions are an example of one protective mechanism neurons use to counter the mutant protein. Like other neurodegenerative diseases, HD typically has a late onset, with symptoms presenting between 30-40 years of age. However, the mutant gene is present throughout life suggesting early mechanisms are in place to deal with mutant huntingtin. The argument that inclusion formation is an example of this is strongly supported by evidence that inclusion formation in certain cell types and brain regions does not correlate with their temporal vulnerability. For example, Gutekunst et al. (1999) found that mutant huntingtin aggregates were much more common in the cerebral

cortex than the striatum at a time when striatal loss was high and cortical atrophy was low. In addition Kuemmerle et al. (1999) reported disproportionate aggregation in striatal interneurons which are spared in HD compared to the more vulnerable medium spiny neurons.

Consistent with this theory, a recent study by Okamoto et al. (2009) has shown that synaptic and extrasynaptic NMDARs have opposing influence on the toxicity of mutant huntingtin by altering mutant huntingtin inclusion formation. Synaptic NMDAR activity was found to promote neuronal viability by enhancing the formation of non-toxic inclusions of the mutant huntingtin protein. Synaptic NMDAR activity increases the expression of the chaperonin T complex 1 ring complex (TRiC) subunit TCP1. TCP1 is a key mediator of inclusion formation and TCP1 knockdown significantly decreases protein aggregation, concurrently increasing neurotoxicity (Okamoto et al., 2009). In striking contrast, extrasynaptic NMDAR activity was found to activate a small GTPase called Rhes which causes sumoylation of mutant huntingtin, preventing inclusion formation and increasing toxicity (Okamoto et al. 2009; Subramaniam et al. 2011).

1.5.3 NMDARs in Huntington's disease

A large body of work from Lynn Raymond's lab has documented a striking increase in whole-cell NMDAR currents, synaptic NMDARs currents, NMDA-induced Ca^{2+} influx and increased NMDAR membrane insertion in MSN from the YAC72 HD mouse which expresses a mutant huntingtin that contains a 72 polyglutamine expansion (Zeron et al., 2002; Li, et al., 2004; Zeron et al. 2004; Fan et al. 2007). This agrees with early work showing mtHtt to selectively increase currents when coexpressed with recombinant GluN1/GluN2B NMDARs in non-neuronal cells but not

when coexpressed with GluN1/GluN2A. Interestingly, relative expression GluN2B NMDAR subunit compared to other GluN2 subunits is enriched in striatal MSNs (Christie et al. 2000; Li et al. 2004).

Until recently, contrasting evidence existed for the role of NMDAR activity in the YAC 128 HD mouse which expresses a mutant huntingtin that contains a 128 polyglutamine expansion. Although both groups agreed that there is increased NMDAR vulnerability in the YAC 128 mouse, while Zhang et al. (2008) found an increase in GluN2B NMDAR current density in the YAC128 mouse, Fernandes et al. (2007) failed to observe a change in the NMDAR current density in this model.

These conflicting results were resolved by a recent study demonstrating that mutant huntingtin, in the YAC128 mouse model of HD, selectively enhances the expression and function of NMDARs at extrasynaptic sites (Milnerwood et al., 2010). Changes in NMDAR expression in the YAC128 mouse were likely masked in the previous study by the combined study of synaptic and extrasynaptic whole cell currents (Fernandes el al., 2007). Milnerwood et al., (2010) found a specific enhancement of GluN2B-containing extrasynaptic NMDAR activity in the YAC128 mouse that persists into adulthood. This was accompanied by reduced CREB-phosphorylation consistent with a dominant extrasynaptic NMDAR signal (Milnerwood et al. 2010; Hardingham et al. 2002). The imbalance of CREB-signalling towards the CREB-shut off pathway further exacerbates neuronal health by disrupting pro-survival signalling. Of particular interest to this study is the ability of both extrasynaptic NMDAR activity and mutant huntingtin to disrupt PGC-1α coactivation of pro-survival pathways discussed below. In addition, the NMDAR antagonist memantine which is known to selectively block extrasynaptic NMDARs at low concentrations is neuroprotective in a mouse model of HD (Okamoto et al., 2009). *In vivo* administration of low doses memantine in YAC128 HD mice increased

aggregate formation, reduced striatal volume loss and improved motor function (Okamoto et al., 2009).

1.6 PGC-1α

The regulation and adaptation of biological processes in response to environmental cues requires coordinated changes in gene expression. Classically, this gene-regulation was thought to be predominantly regulated by DNA-binding transcription factors. More recently the biological control through the regulation of highly versatile transcriptional coactivators and corepressors has been appreciated (Lonard & O'Malley 2007; Spiegelman & Heinrich 2004). Transcriptional coactivators/corepressors alter transcriptional output without directly binding to DNA. Rather, they are proteins that bind activators or repressor proteins i.e transcription factors/repressors which contain a DNA-binding motif, and are subsequently able to mediate their navigation through the chromatin structure and facilitate interactions with transcriptional machinery.

The inducible family of proteins: the peroxisome proliferator activated receptor gamma coactivator 1 (PGC-1) family, coactivate the transcription of genes involved in mitochondrial biogenesis and oxidative metabolism and antioxidant defences (Lin et al., 2005). The PGC-1 family consists of three proteins PGC-1α, PGC-1β and PGC-related coactivator (PRC). Whereas PRC appears to be ubiquitously expressed, PGC-1α and PGC-1β are expressed in highly metabolic tissues including brown adipose, brain, heart and kidney tissue (Handschin, 2009). Of the three proteins, PGC-1α has been most widely studied and is of particular interest to us due to its emerging role in neuronal health. Downregulation of PGC-1α has been causally linked to the pathoprogression of HD and Parkinson's disease (Cui et al., 2006; Qin et al., 2009;) and exogenous expression of PGC-1α

has proved neuroprotective in models of these disorders as well as Alzhiemer's dieseaes, amyotrophic lateral scelrosis, ischemia and excitotoxicity (Chen et al., 2010; Cui et al., 2006; Luo et al., 2009; Qin et al., 2009; Shin et al., 2011a; Zhao et al., 2011). In this study, we investigate a novel mechanism of neuroprotection downstream of PGC-1α activity and describe how the loss of PGC-1α can increase the vulnerability of neurons to excitotoxic insult.

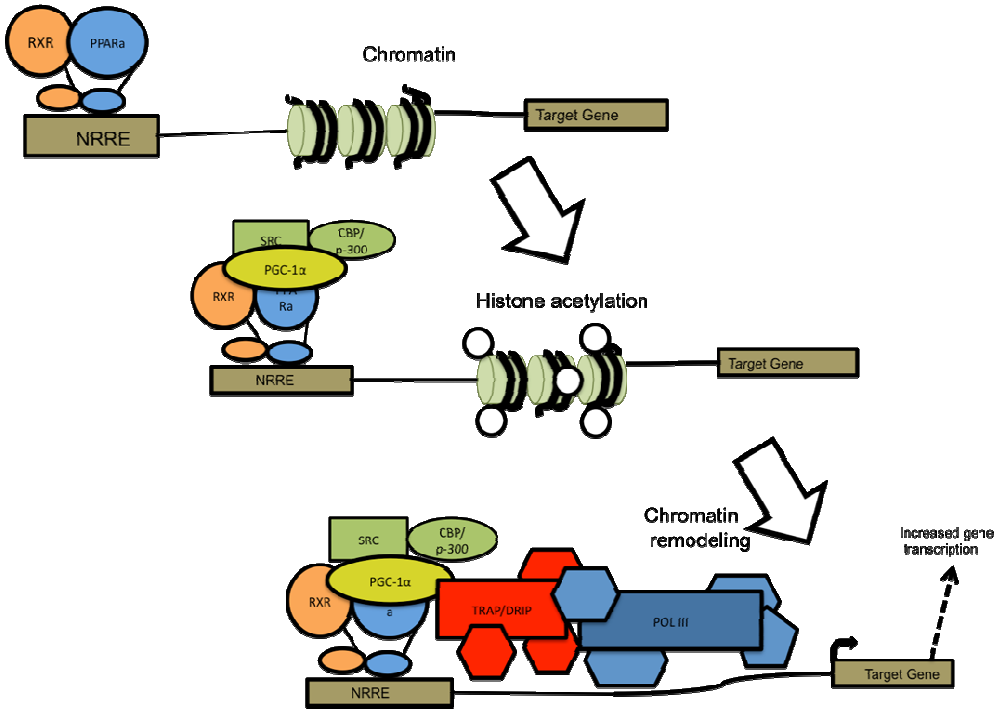

Figure 1.8 Function of PGC-1α PGC-1α acts as a platform for chromatin remodelling and histone modification. PGC-1α binds to its transcription factor targets (for example the RXR/PPARs complex), recruits histone acetylation transferase complexes including CBP/p300 and SRC, causing histone acetylation and chromatin remodelling. Histone acetylation by these proteins makes the chromatin more permissive for transcription. PGC-1α also recruites TRAP/DRIP facilitating interactions with transcriptional machinery. CBP/p300: cyclic adenosine monophosphate (cAMP) response element-binding protein p300 (CBP/p300), SRC: steroid receptor coactivator-1, TRAP/DRIP: the thyroid receptor-associated protein/vitamin D receptor interacting protein/mediator complex (TRAP/DRIP), NRRE: nuclear receptor response element**,** reviewed by Liu and Lin (2011).

1.6.1 PGC-1α: structure and function

Upon binding to its target, PGC-1α selectively activates the transcription of a subset of genes downstream of the target transcription factor (Schmidt & Mandrup 2011). PGC-1α increases transcriptional output by the recruitment of chromatin remodelling and histone modifying enzymes (Fig 1.8). At its N-terminus, PGC-1α has a strong transcriptional activation domain which recruits histone acetylation transferase complexes including 3'-5'-cyclic adenosine monophosphate (cAMP) response element-binding protein p300 (CBP/p300) and steroid receptor coactivator-1 (SRC). Histone acetylation by these proteins makes the chromatin more permissive for transcription. At its C-terminus, PGC-1α recruits the thyroid receptor-associated protein/vitamin D receptor interacting protein/mediator complex (TRAP/DRIP). TRAP/DRIP assists interactions with the transcriptional machinery (Liu & Lin 2011).

The ability of PGC-1α to coactivate an array of both nuclear receptor and non-nuclear receptor transcription factors enables the regulation of a number of distinct tissues-specific processes. For example, in brown adipose tissue (BAT), PGC-1α promotes adaptive thermogenesis after cold exposure by coactivating the nuclear receptors PPARγ and the thyroid hormone receptor TRβ on the promoter of uncoupling protein-1 (UCP-1). PGC-1α coactivation of hepatic nuclear factor-4alpha (HNF-4alpha) is necessary for fasting-induced gluconeogenesis in the liver (Yoon et al., 2001). In skeletal muscle, PGC-1α -coactivation of myocyte enhancer factor 2 proteins drive slow fibre gene expression (Lin et al. 2002).

Despite its functional versatility, loss of PGC-1α is generally associated with detrimental consequences in tissue function or viability (Handschin, 2009). Substantial evidence suggests that PGC-1α has a common role as a master regulator of mitochondrial biogenesis, fatty acid oxidation, electron

transport, and oxidative phosphorylation in an array of tissues including the heart, liver, skeletal muscle and the brain (Handschin, 2009). Exogenous expression of PGC-1α induces mitochondrial biogenesis by the coactivation of NRF-1, NRF-2, and the orphan nuclear receptor estrogen-related receptor α (Patti et al., 2003; Mootha et al., 2004).

1.6.2 PGC-1α in the brain

PGC-1α expression peaks in the rat brain between post-natal day 3-15 in the cortex, striatum, and hippocampus (Cowell et al., 2007). Neurological impairment is evident in the two current PGC-1α knockout (KO) mice. One PGC-1α KO displays movement disturbances including myoclonus, exaggerated startle response, dystonic postering, frequent limb clasping and has brain lesions in the striatum and in cortical layers V/VI, the nucleus accumbens, substantia nigra, hippocampus and mammalliary body (Lin et al. 2004). The other PGC-1α KO displays increased anxiety accompanied by microvacuolation in the pyramidal neurons of the cerebral cortex (Leone et al., 2005). PGC-1α null mice are also more sensitive to the neurodegenerative effects of the oxidative stressors MPTP and kainic acid affecting the substantia nigra and hippocampus, respectively (Leone et al., 2005).

Neurons are highly susceptible to oxidative stress by reactive oxygen species as in the absence of high levels of synaptic activity, neurons produce very little of the reactive-oxygen-species detoxifying enzymes (Mariani et al., 2005; Papadia et al., 2008). In addition, neurons are especially sensitive to defects in mitochondrial function for two reasons: (I) by converting the mitochondrial membrane potential to ATP via the ATP-synthase, mitochondria are the major source of cellular ATP. Neurons are subject to extreme fluctuations in ion concentrations due to action potential propagation and synaptic activity. The activity of ATP-driven membrane bound ionic pumps are required to maintain ionic homeostasis, if cellular ATP is depleted, the neurons can become depolarised and vulnerable to disruptions in calcium homeostasis. (II) Since the mitochondria are themselves required to buffer fluctuations in cytosolic Ca^{2+} through a calcium uniporter, Ca^{2+} overload may lead to

mitochondrial permeabilization through the activation of the permeability transition pore, causing further release of calcium and loss of mitochondrial membrane potential. In the brain PGC-1α promotes neuronal viability and increases oxidative capacity by promoting mitochondrial biogenesis (Wareski et al., 2009) and upregulating the reactive oxygen species (ROS)-detoxifying enzymes GPx1 and SOD2 (St-Pierre et al., 2006).

Altered metabolic function and mitochondrial health is a common feature of neurodegenerative disorders including HD, Alzhiemer's disease, Parksinson's disease, Amyotrophic lateral sclerosis, Ischemia and excitotoxicty (Henchcliffe and Beal, 2008; Knott et al., 2008; Sheng et al., 2011). Accordingly, models of the aforementioned neurological disorders have all displayed marked neuroprotection in response to exogenous PGC-1α expression (Cui et al., 2006; Luo et al., 2009; Okamoto et al., 2009; Qin et al., 2009; Chen et al., 2010; Mudò et al., 2011; Soriano et al., 2011; Zhao et al., 2011; Shin et al., 2011b).

1.6.3 Regulation of PGC-1α in neurons

Classically, synaptically-driven gene regulation is thought to occur by the activation of Ca^{2+} -responsive transcription factors (West et al., 2001). However, we have recently shown that the transcriptional co-activator PGC-1α is upregulated by synaptic NMDAR activity *in vitro* and is underexpressed after antagonism of synaptic NMDARs *in vivo* (Soriano et al., 2011). Synaptic activity can drive the upregulation of the major transcription factor CREB. CREB binds to and activates the PGC-1α

promoter increasing mRNA expression. Upregulation of PGC-1α outlasts the time window of activity-induced CREB-elevation in accord with the long-lasting neuroprotection afforded by synaptic activity (Papadia et al., 2005; Soriano et al., 2011).

PGC-1α can be both positively and negatively regulated by post-translational modifications. These include sumoylation and phosphorylation which regulate overall PGC-1α activity in response to environmental cues (reviewed by Liu & Lin 2011). In addition, PGC-1α pathway specificity can be governed by stimulus-induced phosphorylation preventing the interaction of PGC-1α with specific transcription factors (Lustig et al., 2011). In neurons, post-translational modifications of PGC-1α are linked to the calcium activated kinases ERK and P38 MAPK (Luo et al. 2009). Studies in myoblasts and more recently in neurons have shown that PGC-1α contains a negative regulatory domain and can recruit p160 myb binding protein (p160MBP) which reduces its transactivation ability (Puigserver et al. 2001; Wareski et al. 2009) . However, P160MBP –PGC-1α interaction is blocked by the phosphorylation of PGC-1α by p38 MAPK. Indeed, in a recent study we have shown that synaptic activity can increase the transactivation potential of PGC-1α and this relies of p38 MAPK phosphorylation of PGC-1α (Soriano et al., 2011).

1.7 PGC-1α and Huntington's disease

1.7.1 Mitochondrial defects in HD

Defects in energy metabolism and mitochondrial function are thought to be involved in the progressive pathology of HD. Not only do HD patients suffer weight loss despite sufficient calorific intake (Myers et al. 1991), early Positron Emission Topography (PET) and Nuclear Magnetic Resonance Spectroscopy (NMR) studies showed HD patients to have altered energy homeostasis in the striatum, occipital cortex and frontal cortex (Kuwert et al. 1990; 1993; Jenkins et al. 2005; 1998; Harms et al. 1997). Support for changes in mitochondrial function came from observations that administration of the complex II inhibitor 3-NP mimics HD striatal and cortical lesions as well as disease-associated behaviours in rats, primates and humans (Beal et al. 1993; Chyi & C. Chang 1999; Ludolph et al. 1991). Much evidence has since emerged for defects in respiratory chain function and expression of complexes I-IV in HD (Turner and Schapira, 2010). In addition, evidence for an oxidative stress component in HD comes from an increase of *in vivo* and *in vitro* free radical damage to DNA, lipids and proteins in HD (Butterworth, 1998; Browne et al., 1999; Greco et al., 2000).

The transcriptional coactivator PGC-1α regulates several genes involved in mitochondrial biogenesis, respiration and detoxification of reactive species, therefore the discovery that mutant Huntingtin negatively regulates PGC-1α expression and function revealed one mechanism by which mitochondrial function is disrupted in the disease (Cui et al., 2006; Weydt et al., 2006).

1.7.2 Repression of PGC-1α by mutant huntingtin

Striking similarities in the behavioural and neuropathological phenotype of the PGC-1α KO mouse and HD first suggested a possible role for PGC-1α in neurodegenerative disease (Lin et al. 2004). The PGC-1α knockout mouse displays a number of movement disturbances including stimulus induced myoclonus, exaggerated startle response, dystonic postering, frequent limb clasping and has brain lesions predominantly in the striatum, but also in cortical layers V/VI, the nucleus accumbens, substantia nigra, hippocampus and mammalliary body (Lin et al. 2004).

In 2006 two independent groups identified a relationship between disrupted PGC-1α activity and disease aetiology in HD (Cui et al., 2006; Weydt et al., 2006). A common loss of PGC-1α and PGC-1α-target genes encoding mitochondrial complexes was identified in all three of (i) human HD post mortem striatal tissue, (ii) striatal neurons of two HD mouse models, the HD KI mouse and HD N171-82Q mouse, and (iii) the HD striatal cell line STHdh^{Q111} (Cui et al., 2006; Weydt et al., 2006). PGC-1α rescued mitochondrial respiration in STHdh^{Q111} cells (Cui et al., 2006; Weydt et al., 2006) as well as toxicity in HD75 primary striatal cells (Cui et al., 2006) and lentiviral delivery of PGC-1α reduced striatal volume loss in the R6/2 HD mouse (Cui et al., 2006).

Finally, mtHtt is proposed to directly interact with the PGC-1α promoter and repress CREB-dependent transcription of PGC-1α mRNA (Cui et al., 2006). Cui et al. (2006) showed that mutant huntingtin occupies the PGC-1α promoter and disrupts promoter activity; furthermore reduced transcription of PGC-1α could be reversed overexpressing the transcription factor CREB alongside Transcription initiation factor TFIID subunit 4 (TAF4) a component of the RNA polymerase machinery. Together this

evidence suggests that the loss of PGC-1α in HD may results from direct disruption of PGC-1α expression by mtHtt.

Given the aforementioned role of PGC-1α in promoting neuronal health this describes one mechanism by which the cells become more vulnerable in HD and presents a potential therapeutic target. In addition, the interplay between PGC-1α, synaptic activity and mutant huntingtin expression increases in complexity and is summarised in Fig 1.9.

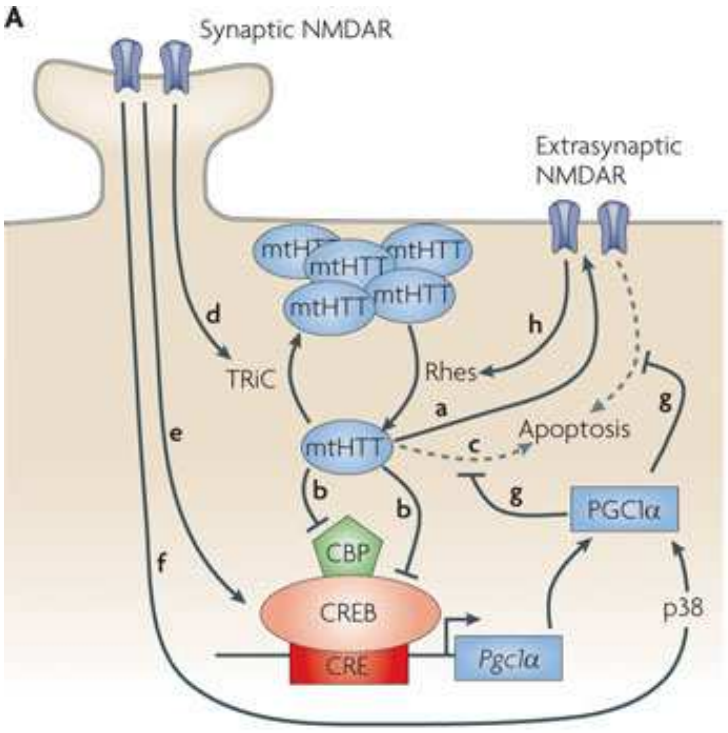

Figure 1.9 Interplay between PGC-1*α*, mtHtt and NMDARs in HD Image from Hardingham and Bading (2010). **Destructive cascade:** Although mutant huntingtin protein (mtHTT) can form insoluble aggregates which prevents its toxicity, mtHTT is toxic in its soluble form (c). MtHTT can repress the CREB-dependent transcription of PGC-1α (b) and can increase extrasynaptic NMDAR activity (a), exacerbating apoptotic cell death. In return, extrasynaptic NMDARs couple to the small GTPase Rhes (h) which reduces inclusion formation and increases the toxicity of mtHtt protein. **Protective cascade:** Synaptic NMDAR activity increases expression of the chaperonin T complex 1 ring complex (TRiC) which protects against mtHtt-apoptosis by inducing inclusion formation (d). Synaptic NMDARs also upregulate PGC-1α activity by promoting CREB-dependent transcription of PGC-1α gene (e) and by p38 phosporylation of PGC-1α protein (f). PGC-1α protects against mtHtt-induces cell death (g).

1.7.3 PGC-1α and other neurodegenerative diseases

PGC-1α: Parkinson's Disease

Parkinson's disease (PD) is a progressive neurodegenerative disease with the preferential loss of dopaminergic neurons of the substantia nigra. mRNA analysis shows a decrease in PGC-1α and the PGC-1α -target NRF-1 in PD striatal samples (Shin et al., 2001). This study found PGC-1α to be repressed by the Parkin-interacting substrate (PARIS) which is aberrantly overexpressed in one mouse model of autosomal recessive PD, the Parkin knock out mouse. Consistent with a role of PGC-1α in PD neuropathology, both transgenic expression of PGC-1α and pharmacological activation of PGC-1α caused an increase in the mitochondrial antioxidant expression and protect against cell degeneration in the MPTP model of PD (Mudo et al., 2011).

PGC-1α: Amyotrophic lateral sclerosis

Amytrophic lateral sclerosis (ALS) is a neurogenerative disease of the spinal cord and motor cortex attacking both the upper and motor neurons, leading to paralysis and death. ALS is thought to be mostly sporadic, however about 10% of cases are inherited, of which 20% are associated with mutations in the gene SOD1 (Zhao et al., 2011). Zhao et al., (2011) demonstrated that in crossing the PGC-1α transgenic mouse expressing human PGC-1α with the SOD1-G93A mouse model of ALS, they were able to rescue mitochondrial defects, motor neuron loss, motor performance and increase the life span in the double transgenic compared to the SOD1-G93A single transgenic mouse.

PGC-1α: Ischemia

in vivo and *in vitro* models of ischemia have implicated PGC-1α as part of an inducible neuroprotective cascade against the neuropathology which results from cardiovascular disorders. Transient global ischemia (TGI) is associated with delayed neuronal loss in the CA1 subfield of the hippocampus and increased abundance of toxic reactive oxygen species (Piantadosi et al., 1996). PGC-1α expression is upregulated in the rat model of TGI along with the ROS-detoxifying enzymes UCP2 and SOD2 (Chen et al., 2009). This is thought to be a protective mechanism against neuronal damage, and indeed, disrupting PGC-1α in this model prevents UCP2 and SOD2 production, increases oxidative stress and exacerbates neuronal loss (Chen et al., 2009). In agreement, cultured cortical neurons stimulated with the *in vitro* model of ischemia, oxygen-glucose-deprivation (OGD), have elevated PGC-1α expression, whereas virally-expressed PGC-1α suppresses apoptosis in OGD neurons (Luo et al., 2009).

PGC-1α: Alzheimer's disease

Alzhiemer's diease (AD) is an age-related progressive disorder characterised by the formation of two classical lesions (i) senile plaques composed of amyloid-β peptides and (ii) neurofibrillary tangles (Selkoe, 2001). A recent study by Qin et al. (2009) reports the decline of PGC-1α mRNA and protein concurrent with increased dementia rating, Aβ-plaque formation and Aβ peptide in AD human tissue. In addition, exogenous PGC-1α expression was able to reverse hyperglycemia induced

amyloidogeneis. This study revealed a potential role of PGC-1α as a protective agent against AD pathology.

1.8 Experimental Summary

PGC-1α is widely studied for its role in mitochondrial biogenesis and function. However, given the concomitant loss of PGC-1α and the increase in excitotoxicity and altered glutamatergic activity in a number of NDGs (table 1.1), we propose that PGC-1α expression and NMDAR excitotoxicity may be causally linked. In this thesis we aim to investigate the role of PGC-1α in excitotoxic cell death. We report the surprising finding that loss of PGC-1α increases neuronal vulnerability by altering NMDAR currents.

In **chapter three** we investigate the role of PGC-1α in excitotoxic cell death and NMDAR current amplitude. We present the surprising finding that siRNA knockdown of PGC-1α in mouse cortical cell cultures leads to increased extrasynaptic NMDAR currents and increased excitotoxic cell death. Finally overexpression of PGC-1α reduces extrasynaptic NMDAR currents and protects against NMDA insult.

In **chapter four** we explore the relationship between mutant huntingtin expression and PGC-1α on NMDAR currents and excitotoxicity. By expressing exon 1 of mutant huntingtin, containing a 148 CAG repeat, in cortical and striatal neuronal culture, we confirm that mutant huntingtin represses PGC-1α expression (Cui et al., 2006). We show that mutant huntingtin increases extrasynaptic NMDAR currents and excitotoxicity and this is non-additive to the effect of PGC-1α knockdown in these neurons. We also demonstrate that overexpression of PGC-1α can reverse

the increase in extrasynaptic NMDAR currents and cell vulnerability in both cortical and striatal cultures.

In **chapter five** we use cortical tissue from a PGC-1α knock out (KO) mice to investigate the influence of chronic loss of PGC-1α throughout embryonic development on AMPA and NMDA –type glutamate receptors. We show that cultured PGC-1α KO neurons do not have altered whole-cell NMDAR currents or vulnerability to NMDA. However, KO neurons display reduced whole cell AMPAR currents, AMPAR mEPSC frequency and mEPSC amplitude with concurrent changes in AMPAR subunit mRNA expression.

Chapter 2:

Methods

2.1 Primary rat and mouse culture

2.1.1 Animals used for this study

For acute study of the effect of plasmids transfection, E21 Sprague Dawley rats were used.

PGC-1α KO and WT pups were obtained from Alberto Camacho Morales (Cambridge). PGC-1α transgenic mice had been generated by Astrazeneca Transgenic and Comparative Genomics, Sweden and details of the creation of the knockout have been published in D'Errico et al., (2011). Briefly, a targeting construct containing a floxed neomycin phosphotransferase in place of exons 3-5 of PGC-1α was created from a C57 mouse BAC template and was electroporated into AZX1, a C57BL/6JOlaHsd-derived ES cell line. One of the clones that underwent desired recombination was expanded and injected into Balb/cOlaHsd blastocysts to generate chimeric mice. Chimeric males were bred to C57BL/6JOlaHsd females. Heterozygous PGC1α Δex3–5 floxed Neo mice were bred to a constitutive Cre-deleter strain of mice to generate the heterozygous PGC1α Δex3–5+/− mice lacking the Neo resistance cassette and leaving a single LoxP site at the deletion junction of intron 2 and 5. These mice were genotyped by using a forward primer (F2) in intron 2 and a reverse primer (R2) in intron 5. Heterozygous PGC1α+/− littermate mice were then intercrossed to generate homozygous PGC1α−/− mice. The absence or presence of PGC-1a RNA in wildtype, heterozygous or knockout mice was confirmed by

real time PCR analysis of RNA extracted from liver tissue(D'Errico et al., 2011). For routine genotyping, DNA was extracted from cerebellar tissue using the phenolchloroform-isoamyl alcohol method. Genotype was determined by the presence of either a KO- or WT-specific DNA fragment using traditional PCR analyses. PGC-1α -null mice have previously been shown to express defects in adaptive energy homeostasis and CNS hyperactivity. (Lin et al., 2004).

2.1.2 Primary cell culture

Cortical neurons from E21 Sprague Dawley rats and mice were cultured using the same technique with a couple of exceptions stated below. Neurons were cultured as described Bading et al., (2001) except that the growth medium was comprised of Neurobasal A medium + B27 (invitrogen), 1% rat serum and 1 mM glutamine. Mothers were killed by cervical dislocation and pups removed in sterile conditions. Rat embryos were culled by intraperitoneal injection of sodium pentobarbital (dose). Mouse pups were culled by decapitation.

The brain was removed and placed in dissociation medium (DM) (81.8 mM Na_2SO_4, 30 mM K_2SO_4, 5.84 mM $MgCl_2$, 252 μM $CaCl_2$, 1 mM HEPES, 0. 1% Phenol Red, 20 mM Glucose, 1mM Kyurenic. Cortical/striatal tissue was incubated in DM with 10 units/ml of papain (Worthington Biochemical Corporation) for forty minutes at 37°C. The brain was cut along the midline separated left and right hemispheres. In order to isolate the cerebral cortex, we removed the olfactory bulb,

cerebellum, medulla, pons, midbrain, thalamus, hypothalamus and basal ganglia, followed by extraction of the hippocampus (in rats only) all remaining structures were classified as cortical for this study. Cortices were then washed with DM and subsequently by growth medium containing NeuroBasal-A Medium, B-27 Supplement, Anti-Anti Supplement (anti-bacterial/anti-mycotic) (Invitrogen), 1mM glutamine and 1% Rat Serum (Harlan SeraLab). Mouse cortices were incubated in growth medium for 5-6 hours at RT to enable transit from a laboratory in Cambridge to Edinburgh. Corticies were triturated in 37°C growth medium using a 2 ml disposable plastic pipette.

The resulting cell suspension was then diluted using Opti-MEM (Invitrogen) supplemented with Glucose (20 mM), to obtain a concentration of one cortical hemisphere per 14 ml cell suspension. This solution was then plated on precoated sterile coverslip, with 0.5 ml of cell suspension used per well of a 24- well plate. Culture plates were incubated at 37 °C in a humified 5% CO2 atmosphere for two and a half hours, after which the cell suspension was removed and replaced with 1 ml of growth medium. At DiV 4, 1 ml of growth media supplemented with 9.6 μM cytosine-D-arabinofuranoside hydrochloride (AraC) (Sigma) was added to prevent proliferation of glial cells.

Experiments were carried out at 8-10 days *in vitro.* At this stage, cortical neurons have developed a network of processes, express functional N-methyl D-aspartate (NMDA)-type and AMPA/Kainate-type glutamate receptors and have formed synaptic contacts. For a lot of this study we are interested in the effect of plasmids/gene deletion on pyramidal neurons. For cell death and electrophysiological studies, pyramidal neurons were selected by morphology based on the presence of a large apical dendrite and pyramidal-shaped cell body.

2.2 Plasmid preparation

pcDNA-PGC-1α was a gift from P. Puigserver (Dana Farber Cancer Institute). PGC-1α was subcloned in the pEF/V5-His A vector by excising the cDNA from the pcDNA vector with EcoRI and AgeI digestion and blunting it to clone it in the EvorV site of pEF/V5-His A vector. Myc-wtHtt-N63-18Q (wtHtt(18Q)) and Myc-mtHtt-N63-148Q (mtHtt-(148Q)) were a gift from Stuart Lipton [Sanford-Burnham Medical Research Institute (Okamoto et al., 2009). For cell death and electrophysiologyical recordings pEGFP-N1 driven by the CMV promoter was coexpressed with the plasmid of interest to label transfected neurons (see below). For nucleofection, GFP was subcloned from the pEGFP-N1 into the pEF/V5-His A vector, this enabled high efficiency expression representative of other plasmids driven by EF-promoter. For plasmid generation, the GFP fragment was excised from pEGFP-N1 using Not1/EcoR1 and inserted into Not1/EcoR1 sites of pEF/V5-His A vector (Invitrogen)

2.3 Transfection of plasmids

Before transfection, neurons were transferred into trophic transfection medium ("TMits") supplemented with insulin-transferin-sertinin TMits was comprised of 10% MEM(Invitrogen) and 90% salt-glucose-glycine (SGG) medium (Bading et al., 1993) SGG:114 mM NaCl, 0.219% $NaHCO_3$, 5.292 mM KCl, 1mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Hepes, 1 mM glycine, 30 mM glucose, 0.5 mM sodium pruvate, 0.1% Phenol Red; osomlarity 325 mosm/l. Lipofectamine 2000 (Invitrogen) was used for all transfections as recommended concentration in TMits. Cells were placed in

transfection media for a minimum of four hours, before being tranfered into fresh TMits.

2.4 Nucleofection of plasmids

Nucleofection was carried out using Lonza Nucleofector. Cortical neurons were cultured in Neurobasal A medium + B27 (invitrogen), 1% rat serum and 1 mM glutamine (as above). Procedure followed user manual; 5ug DNA was used per 5 million cells. Effeciency of nucleofection was assessed by tracking expression of EF-GFP in control neurons. After 72 h, RNA was isolated from neurons for qPCR analysis.

2.5 RNA isolation, reverse transcriptase-polymerase chain reaction, and quantitative polymerase chain reaction.

RNA was isolated using the Qiagen RNeasy isolation reagents including the optional DNAse treatment, following passage of the cells through a QiaShredder column. cDNA was synthesized from 1-3 µg RNA using the Stratascript QPCR cDNA Synthesis kit (Stratagene). Briefly, the required amount of RNA (up to 3 µg) was diluted in RNase-free water (up to 7 µl final volume) and mixed on ice with 1x cDNA Synthesis master mix (10µl), random primers: oligo-dT primers 3:1 (total 2 µl- 200 ng) and either 1 µl RT/RNase block enzyme mixture (for RT reactions) or 1 µl water (for No RT control reactions). Reaction mixtures were mixed and spun down and incubated for 2 min. at 25°C, 40 min. at 42 °C and 5 min. at 95°C. cDNA was stored at -20°C.

Dilutions of this cDNA were subsequently used for real-time PCR (cDNA equivalent to 6 ng of initial RNA per 15 µl qPCR reaction) qPCR was performed in an Mx3000P QPCR System (Stratagene) using Brilliant SYBR Green QPCR Master Mix (Stratagene) according to the manufacturer's instructions. Briefly, the required amount of template was mixed on ice with 1x Brilliant SYBR Green Master Mix, the required concentration of forward and reverse primers, 30 nM ROX passive reference dye and water to the required reaction volume. Technical replicates as well as no template and no RT negative controls were included and at least 3 biological replicates were studied in each case. The sequence of the primers used are as follows:

PGC-1α –F: 5'-GAATGCAGCGCTCTTAGC-3',

PGC-1-R: 5'-GCT TTT GCT GTT GAC AAA TG-3'

GluN1 –F: 5'-CTGCGACCCCAAGATTGTCAA-3'

GluN1-R: 5'-TATTGGCCTGGTTTACTGCCT -3'

RPl13a-F 5'-GAGGTCGGGTGGAAGTACCA-3'

Rpl13A-R 5'-TGCATCTTGGCCTTTTCCTT-3'

complexin I, F 5′-CCACTGCAGGACATGTTCAA-3′

complexin I R 5′-TAAGATTGGTAGGGAGGGGG-3′

GluA1-F 5'- CAACAATCACAGGAACATGCG-3'

GluA1 -R 5'-GAGAACTGGGAACAGAAACGGT-3'

GluA2 -F 5'- GGAGCAAATGTCTCTGGATTTC-3',

GluA2 -R 5’-ATCACTTGGACAGCATCATACG-3’

GLUA3-F -5’ –TTCGGAAGTCCAAGGGAGAGT-3’

GluA -R; 5’CACGGCTTTCTCTGCTCAATG-3’

GluA4-F 5’-GGCTCGTGTCCGCAAGTC-3’

GluA4-R 5’-TTCGCTGCTCAATGTATTCATTC 3’.

The cycling program was 10 min. at 95 °C; 40 cycles of 30 sec. at 95 °C, 40 sec. at 60°C with detection of fluorescence and 30 sec. at 72 °C; 1 cycle (for dissociation curve) of 1 min. at 95 °C and 30 sec. at 55 °C with a ramp up to 30 sec. at 95 °C (ramp rate: 0.2°C/sec) with continuous detection of fluorescence on the 55-95 °C ramp. The data were analysed using the MxPro QPCR analysis software (Stratagene). Expression of the gene of interest was normalized to rpl13a, a commonly used control.

2.6 Electrophysiological recording and analysis

Recordings of agonist-evoked whole-cell currents were made 48-72 h after transfection. All recording were performed within a Faraday cage (Technical manufacturing Corporation (TMC)) mounted on a pressurised air table (TMC Micro-g Vibration isolation system), to prevent noise interference. Coverslips containing cortical neurons were transferred into a recording chamber containing artificial cerebrospinal fluid (aCSF) described below. Cells were visualised using a 40x water immersion lens (Carl Zeiss Germany). In all cases data was acquired using an Axopatch-1C amplifier (Molecular Devices, Union City, CA) with cells in whole-cell configuration held at -60 or -70 mV.

2.6.1 External recording solution: artificial cerebrospinal fluid (aCSF).

External recording solution was made of artificial cerebrospinal fluid (aCSF) containing (in mM): 152 NaCl, 2.8 KCl, 10 HEPES, 2 $CaCl_2$, 10 glucose, 20 µM strychnine, 50 glycine and 300nM tetrodotoxin (TTX), pH 7.3 (320-330 mOsm). The external solution was applied at a constant flow rate of 4mls per minute at room temperature (22-25°C)

2.6.2 Recording electrodes

Patch pipettes were made from thick-walled borosilicate glass with dimensions 1.5mm O.D. x 0.86 I.D containing a filament (Harvard Apparatus, Kent, UK). Glass was mounted onto a Flaming Brown Micorpipette Puller (Model 97; Sutter instruments Co. USA) and pulled into a patch electrode to have a final resistance between 5-10 MΩ. Electrodes were mounted on a headstage (Axon instrument company, MP-285) to enable fine manipulation.

2.6.3 Internal Recording solution

Pipettes were filled with an internal solution containing (in mM): 155 K-gluconate, 2 $MgCl_2$, 10 Na-HEPES, 10 Na-PiCreatine, 2 Mg_2-ATP and 0.3 Na_3-GTP, pH 7.3 (300 mOsm).

2.6.4 Whole-cell agonist-evoked currents

To record whole cell agonist evoked currents coverslips containing cortical neurons were transferred into a recording chamber containing artificial cerebrospinal fluid (aCSF). Cells were voltage clamped at -60mV and the agonist in question was applied for an average of 30 seconds and steady-state current recordings were made. Agonists concentrations were; 100 μM NMDA, 50 μM AMPA or 100 μM GABA. Agonist application and current recording was repeated twice for each cell for all agonist responses. Data were filtered at 1 kHz and digitized at 5 kHz for subsequent off-line analysis.

2.6.5 Recording extrasynaptic NMDAR currents

Recording of extrasynaptic NMDAR currents were performed 48-72h post transfection. Neurons were placed in Mg^{2+}-free aCSF composed of (in mM): 152 NaCl, 2.8 KCl, 10 HEPES, 2 $CaCl_2$, 10 glucose, 20 μM strychnine and 50 glycine, pH 7.3 (320-330 mOsm) supplemented with PTX (50 μM), TTX (300 nM) and MK-801 (10 μM) at room temperature. After a 10 minute incubation period, neurons were washed with MK-801-free aCSF and placed in a recording chamber. Patch pipettes and internal solutions were the same as described above. NMDA (100 μM) was applied for an average of 30 seconds and steady-state current recordings were made. Agonist application and current recording was repeated twice for each cell. Data were filtered at 1 kHz and digitized at 5 kHz for subsequent off-line analysis. Extrasynaptic NMDAR-current density was calculated as the steady-state current amplitude normalised to the cell capacitance. For each MK-801 stimulation 3 cells were recorded per coverslip and the mean current density per stimulation was calculated as n=1.

2.6.6 Miniature excitatory postsynaptic currents

For miniature excitatory postsynaptic currents (mEPSCs) recordings, coverslips containing cortical neurons were transferred into a recording chamber containing artificial cerebrospinal fluid (aCSF) containing (in mM) 150 NaCl, 2.8 KCl, 10 HEPES, 2 $CaCl_{2,}$ 10 glucose, 20 µM strychnine, 50 glycine and 1.3 Mg^{2+}, pH 7.3 (320-330 mOsm) and supplemented with 20 µM strychnine, 300 nM tetrodotoxin (TTX) and 50 µM picrotoxin (PTX). Patch pipettes and internal solutions were the same as described above. MEPSCs were recorded (Baxter and Wyllie, 2006) using an Axopatch-1C amplifier (Molecular Devices, Union City, CA). Events were recorded for 5-10 minutes (minimum of 300 events) from neurons clamped at −70 mV. Recordings were rejected if the cell holding current was higher than −100 pA. For data analysis, mEPSCs were filtered at 2 kHz and digitized at 10 kHz using WinEDR v6.1 software (John Dempster, University of Strathclyde, UK) and analyzed using MiniAnalysis software (Synaptosoft, Fort Lee, NJ). mEPSCs were manually selected with a minimum amplitude threshold of 10 pA (approximately 3 times the baseline noise level).

2.7 Induction of excitotoxicity and following the fate of transfected neurons

Following the fate of transfected neurons after induction of excitotoxicity was performed as described (Soriano et al., 2011). 24 hours after transfection neurons were transferred to a non-trophic growth medium ("TMo") containing 10% MEM(Invitrogen), 90% salt-glucose-glycine (SGG) medium (Bading et al., 1993) SGG:114 mM NaCl, 0.219% $NaHCO_3$, 5.292 mM KCl, 1mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Hepes, 1 mM glycine, 30 mM glucose, 0.5 mM sodium pruvate, 0.1% Phenol Red; osomlarity 325 mosm/l. After 24 hours in trophically-deprived medium, pictures of GFP-expressing neurons were taken using a Leica AF6000 LX imaging system, with a DFC350 FX digital camera. Neurons were exposed to 10µM-20µM NMDA in trophically deprived media for 1 h, after which 10 µM MK-801 (Tocris) was added to the neurons to block ongoing NMDAR activity. Using the automated cell-finder function within the Leica AF6000 LX software, images of the same neurons were taken 24 hours after insult.

Acute studies: Cell death was determined by counting the number of surviving GFP-expressing cells pre- and post-insult. Cell death was indentified by the absence of healthy GFP-expressing neurons. Analysis of cell death was performed blind to the plasmid transfection/geneotype in each case. In >90% of cases evidence of death was observed as fluorescent cell debris and fragmented nuclei, confirming death due to excitotoxicity rather then quenching of eGFP- fluorescent signal. This is also underlined by the fact that death measured by this technique is blocked by caspase inhibitors (Papadia et al., 2008). For each condition the fate of ~100-150 neurons was monitored over 3-6 independent experiments.

PGC-1α KO studies: (Performed by Karen Bell, unpublished) Neurons were transferred to a non-trophic growth medium ("TMo") containing 10% MEM(Invitrogen), 90% salt-glucose-glycine (SGG) medium (Bading et al., 1993) SGG:114 mM NaCl, 0.219% $NaHCO_3$, 5.292 mM KCl, 1mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Hepes, 1 mM glycine, 30 mM glucose, 0.5 mM sodium pruvate, 0.1% Phenol Red; osomlarity 325 mosm/l. After 24 hours, neurons were exposed to 10µM-20µM NMDA in trophically deprived media for 1 h, after which 10 µM MK-801 (Tocris) was added to the neurons to block ongoing NMDAR activity. 24 hours after insult neurons were fixed and subjected to 4′,6-diamidino-2-phenylindole (DAPI) staining, and cell death was quantified by the counting (blind) of the number of apoptotic nuclei as a percentage of the total. Neurons that die in response to exposure to excitotoxic levels of NMDAR agonists exhibit swollen cell bodies and pyknotic nuclei with small irregular chromatin clumps, a characteristic of necrotic cell death as opposed to apoptotic-like death (Fujikawa et al., 2000).

2.8 Stimulation of synaptic activity in cell culture

Bursts of action potential firing were induced by treatment of neurons with 50 µM bicuculline, and burst frequency was enhanced by addition of 250 µM 4-amino pyridine (Hardingham et al., 2001)

2.9 Luciferase reporter assays

PGC-1α-luc was a gift from A. Fukamizu [University of Tsukuba (Daitoku et al., 2003)]. pTK-RL was from Promega. GluN1-Luc was a gift from G. Bai (University of Maryland (Liu et al., 2003)).

Firefly luciferase-based reporter gene constructs [GluN1-Luc, PGC-1α-Luc] were transfected along with renilla expression vector (pTK-RL) and plasmid of interest (globin, 18Q, 148Q, efPGC-1α) at a ratio of 0.2:0.1:0.2. Neurons were stimulated where appropriate 24 h after transfection. Luciferase assays were performed using the dual glo assay kit (Promega) with Firefly luciferase-based reporter gene activity. In the case of NR-1-Luc reporter activity was normalised to the renilla control (pTK-RL plasmid).

2.10 Microarray analysis

RNA 6000 Nanochips in the Agilent 2100 Bioanalyzer were used to assess the quality of RNA. Following this, Affymetrix One-cycle cDNA Synthesis Kit and GeneChip Sample Cleanup Module were used to synthesize and purify double stranded cDNA Double-stranded. The double-stranded DNA was used as template for the *in vitro* transcription using GeneChip IVT Labelling Kit (Affymetrix) yielding biotin-labelled cRNA. Purified biotinylated target cRNA was then fragmented into short sequences. The hybridisation cocktail consisted of 15 μg fragmented biotin-labelled cRNA spiked with eukaryotic hybridisation control. Eighty microliters of the hybridisation cocktail was first hybridized to the test-chips to check the cRNA integrity and assess the system veracity. After that, the Mouse Genome 430A plus 2.0 microarrays (Affymetrix) were directly loaded with 130 μl of hybridisation cocktail solution and then placed in Genechip Hybridisation Oven 640 (Affymetrix) rotating at

60 rpm at 45 °C for 16 h. After hybridisation, the arrays were washed on Genechip Fluidics Station 450 (Affymetrix) and scanned using Genechip Scanner 3000 (Affymetrix) according to the manufacturer's procedure. Expression was calculated using the robust multiarray average algorithm implemented in the Bioconductor (http://www.bioconductor.org) extensions to the R statistical programming environment[24]. Robust multiarray average generates a background-corrected and quantile-normalized measure of expression on the log 2 scale of measurement.

2.11 Statistics

For all experiments a minimum of 3 independent cultures were used and the n number of cells analysed is stated throughout. For experiments that rely on spontaneous synaptic properties of the neuronal culture (mEPSCs, experiments, quantal block protocol) and for cell death experiments, the statistical n is equal to the number of independent cultures from single pups. In other cases, where we analyse the effect of the plasmid transfection on properties intrinsic to each cell (i.e., whole cell current), statistical n is equal to the number of cells analysed across a minimum of 3 independent cultures. In each figure the statistical n is underlined for clarity. For the majority of cases a one-way ANOVA or *t*-test is used. For cases in which we analyse the interaction of two independent variables (i.e. coexpression of PGC-1α or globin with mtHtt of wtHtt) two-way ANOVA is used followed by post hoc Dunnet's or as stated. Analysis of cell death was performed blind to the plasmid transfection/geneotype in each case. Analysis of electrophysiology, luciferase and RNA data was not blind.

Chapter 3:

Neuroprotection from PGC-1α-mediated repression of extrasynaptic NMDARs

3.1 **Chapter summary**

In this chapter we investigate the neuroprotective mechanisms downstream of PGC-1α signalling. We confirm that exogenous PGC-1α is neuroprotective in our *in vitro* model of excitotoxicity in cultured rat cortical neurons. We also find that knockdown the endogenous PGC-1α increases the vulnerability of neurons to excitotoxic cell death. These results are consistent with neurodegeneration previously reported in PGC-1α knockout mice as well as the neuroprotective capacity of PGC-1α in HD models. Having validated that PGC-1α is neuroprotective our *in vitro* model, we present the novel finding that PGC-1α expression mediates NMDAR current density in these neurons. Finally we show that whereas siRNA knockdown of PGC-1α leads to increased extrasynaptic NMDAR currents, overexpressing PGC-1α specifically represses extrasynaptic NMDAR currents. This repression of extrasynaptic NMDAR currents downstream of PGC-1α is surprising given its known role as a transcriptional regulator of mitochondrial biogenesis and function; however this result is consistent with the neuroprotective capacity of PGC-1α. Data from this chapter in now published in Puddifoot et al., (2012).

3.2 Results

3.2.1 PGC-1α regulates excitotoxicity in rat cortical cultures

In order to investigate the role of PGC-1α in excitotoxic cell death we cultured cortical neurons from embryonic day 21 Sprague-Dawley rats. We then used an *in vitro* overexpression model to assess whether enhancing PGC-1α levels alters the vulnerability of neurons to subsequent toxic insults. Neurons were transfected at DIV8 with PGC-1α or control (Globin) plasmids along with an eGFP-marker to label the neurons that were successfully transfected. In order to track the fate of transfected cells, 48h post transfection, GFP-expressing cells were imaged. By marking the positions of the images taken using LEICA AF6000 LX software, we were able to reimage the same cells for comparison after NMDA-insult enabling us to track the fate of individual cells.

We have previously shown stimulation with NMDA 20 μM (for 1 hour) triggers excitotoxicity in trophically-deprived neurons (Soriano et al., 2006). We induced excitotoxicity in the neuronal cultures by 1 hour stimulation with NMDA (20 μM), after which the stimulation was stopped by applying the NMDAR antagonist MK-801 (10 μM). 24h post stimulation images of the same cells were retaken to track cell fate. Excitotoxic cell death was quantified by counting the loss of GFP-positive cells as a percentage of the total GFP-positive cells before treatment. We found that overexpressing PGC-1α protects neurons against NMDA-induced cell death (Fig 3.1 $p<0.05$).

We next investigated whether the loss of endogenous PGC-1α had an effect on excitotoxic cell death, by using two independent on-target siRNAs against PGC-1α. To validate the siRNAs we overexpressed Flag-

tagged PGC-1α and confirmed the effectiveness of the siRNA in preventing PGC-1α expression (Fig. 3.2C). Knockdown of endogenous PGC-1α exacerbated neuronal death in response to a modest (10 μM) dose of NMDA (Fig. 3.2A,B) agreeing with studies by other groups (Luo et al., 2009; Chen et al., 2010).

This result is in agreement with the ability of PGC-1α to build antioxdative defenses and induce mitochondrial biogenesis, which should protect against the oxidative compenent of excitoxic cell death. However in Soriano et al. (2011) we found that, while exogenous PGC-1α protection against oxidative stress was occluded by co-transfection with the corepressor SMRT (Soriano et al., 2011), protection against exitotoxicity was uneffected by SMRT co-expression, suggesting these processes of neuroprotection are, at least in part, mechanistically distinct.

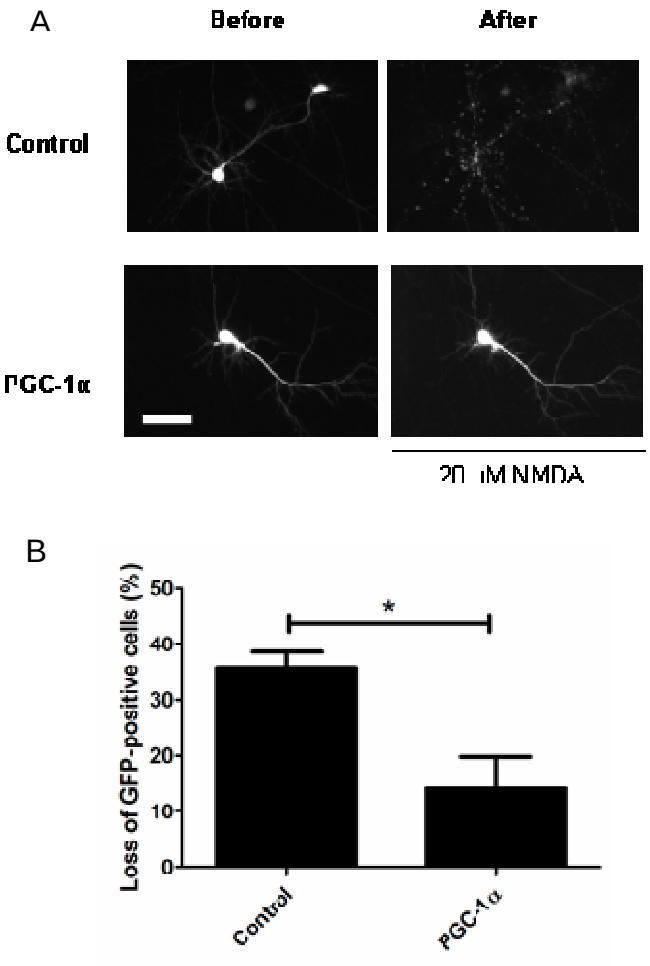

Figure 3.1 PGC-1α is neuroprotective against excitotoxic insult

(A) Images of neurons expressing GFP plus either a control (globin) plasmid or PGC-1α before and 24h after stimulation with NMDA (20 μM). Cells transfected (DIV7) were imaged 48h post transfection and excitotoxicity was induced by NMDA (20 μM) stimulation for 1h. 24h post stimulation images of the same cells were retaken to track cell fate. (B) Cell death was quantified by counting the loss of GFP-positive cells as a percentage of the total GFP-positive cells before treatment. Overexpression of PGC-1α strongly protects neurons against NMDA-induced cell death (150-300 cells from n=3 cultures were analysed per group). *t test $p<0.05$

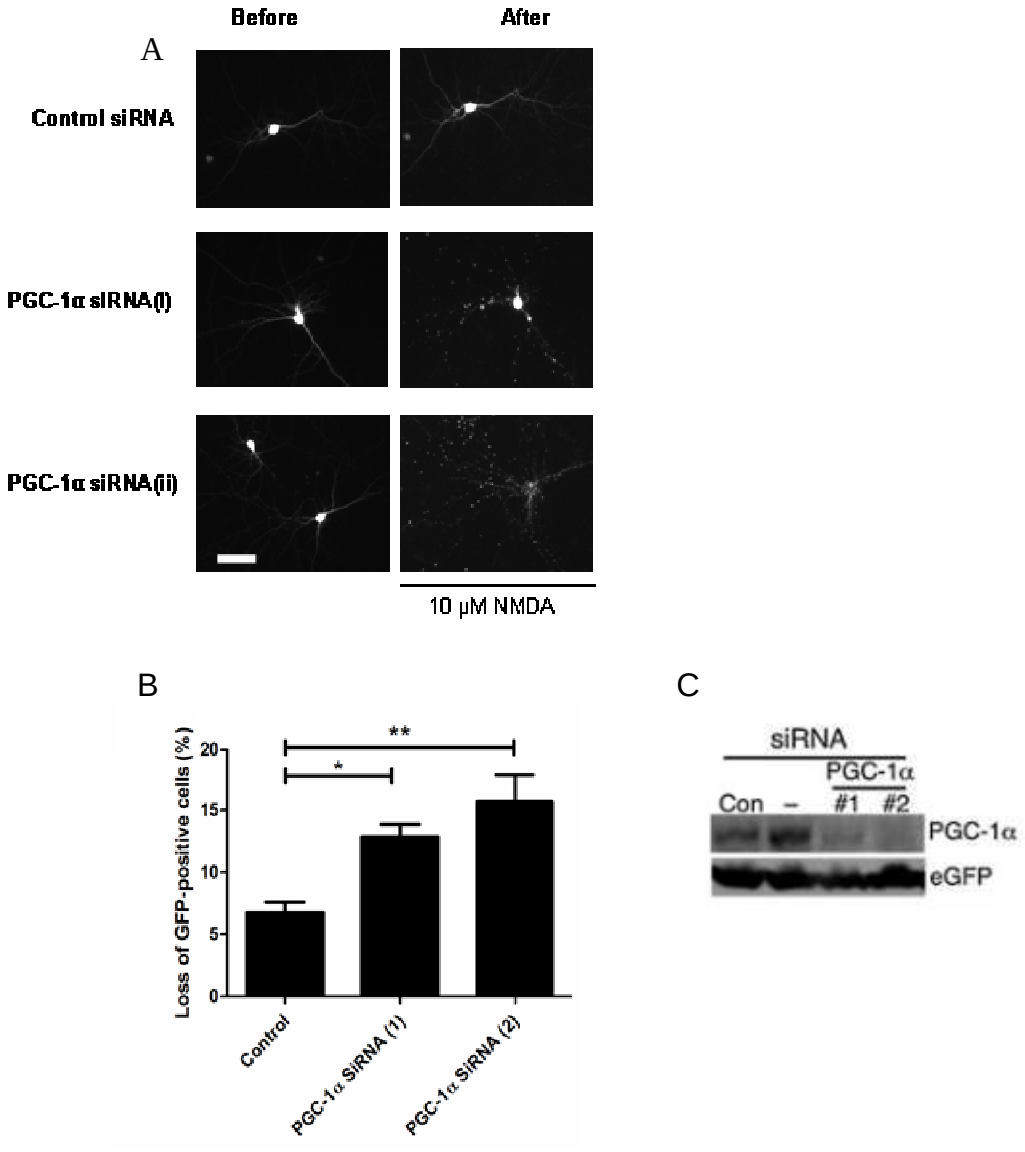

Figure 3.2 Loss of endogenous PGC-1α increases NMDA-induced excitotoxicity (A) Images of cortical neurons (DIV7) transfected with GFP plus either non-targeting control siRNA or one of two PGC-1α on target siRNA(i) or siRNA(ii) before and 24h after NMDA stimulation. **(B)** Cell fate was assessed by the loss of GFP-positive neurons and expressed as a percentage of GFP-positive cells before treatment. Knockdown of PGC-1α increased excitotoxicity in response to 10μM NMDA (150-300 cells from n=3 cultures were analysed per group). *$p<0.05$, **$p<0.01$ (C) To validate the siRNAs we coexpressed siRNAs (i) and (ii) with Flag-tagged PGC-1α and confirmed that both siRNAs repressed subsequent anti-flag signal compared to control siRNAs.

3.2.2 PGC-1α expression regulates whole-cell NMDAR currents

It has long been accepted that aberrant calcium signalling due to excess NMDAR activity can initiate the excitotoxic pathway (Olney, 1969; Choi, 1987; Tymianski et al., 1993) having a causal role in neuropathological disorders (Rothman and Olney, 1986; Choi et al., 1988; Lipton and Rosenberg, 1994; Arundine and Tymianski, 2004). Excitotoxic death in response to bath application of NMDA in neuronal cell culture is dose dependent (Soriano et al., 2006) and is proportional to the number of receptors activated and the corresponding Ca^{2+} current. We therefore investigated whether increased NMDA vulnerability in the absence of PGC-1α and conversely the neuroprotection afforded by exogenous PGC-1α may be due to direct/indirect regulation of cellular calcium influx through the NMDAR. We used whole-cell patch-clamp to examine the influence of exogenous PGC-1α overexpression or siRNA knockdown of PGC-1α on NMDAR current density in cultured cortical neurones.

Neurons were transfected at DIV8 with either PGC-1α, PGC-1α siRNA(i) or PGC-1α siRNA(ii) plus eGFP to mark transfected neurons. 48h post transfection, coverslips were transferred to Mg^{2+}-free aCSF and GFP-positive cells were voltage clamped at -60mV. Bath application of NMDA (100 µM) triggers an influx of positive ions into the cell as represented by the downward deflection in the current traces in Fig 3.3. The size of the deflection in the current traces is equal to the amplitude of the evoked current response. The total membrane capacitance is directly proportional to the membrane surface area (Eq 1.). Therefore, to account for differences in the size of neurons, the current amplitude is normalised to the membrane capacitance of the cell giving us the current density.

Eq.1 Total cell capacitance =specific membrane capacitance * total membrane area

Surprisingly, modifying the expression of PGC-1α led to changes in the NMDAR current density in these neurons. PGC-1α overexpression caused a 32% decrease in total whole-cell NMDAR currents (NMDA 100 μM, Fig.3.3) and correspondingly PGC-1α siRNAs caused a significant increase (siRNA(i) 38% siRNA(ii) 80%) in whole cell NMDAR responses (NMDA 100μM, Fig.3.4). This result presents the unexpected finding that the transcriptional coactivator PGC-1α is able to regulate NMDAR current density in cortical neurons. Furthermore this suggests that neuroprotection from PGC-1α may be due to the repression of NMDAR currents and subsequently reduced activation of excitotoxic cascades. This is consistent with the concurrent loss of PGC-1α and increased excitotoxicity in a number of neurodegenerative diseases as described in chapter 1.

NMDAR signalling to cell death is known to be regulated by the calcium influx through the extrasynaptic pool of NMDARs (Hardingham and Bading 2005). In addition, our lab has recently shown that the C-terminus of the GluN2B and GluN2A have differing affinities for the activation of cell-death cascades (Martel et al., 2012). We investigated whether PGC-1α expression protects against excitotoxicity by selectively altering the subunit composition of the NMDARs and secondly whether PGC-1α predominantly represses extrasynaptic activity.

3.2.3 PGC-1α overexpression does not alter the GluN2B subunit composition of NMDARs

One potential method of altering current density is by changing the efficacy of the NMDARs expressed in the neurons. The majority of the biophysical properties of the NMDARs are determined by the GluN2 subunit composition including the affinity of magnesium block of the

channel, single-channel conductance, and glutamate affinity (Monyer et al. 1994; Sucher et al. 1996; Meguro et al. 1993; Wyllie et al. 1996). In addition the C-terminal domain of the GluN2A and GluN2B subunits differentially couple to cell death (Martel et al., 2012). At the age of which these experiments are carried out (DIV 10-11), the GluN2B subunit is dominantly expressed in control neurons (Liu et al., 2004 and Fig 3.5). We investigated whether the increase in excitotoxicity and NMDAR currents in cells overexpressing PGC-1α could be due to altered ratio of GluN2 subunits expressed. We used the GluN2B selective antagonist Ifenprodil to test the proportion of the total NMDAR current that is mediated by GluN2B subunits. Ifenprodil had equal effect on agonist-evoked NMDAR currents in control and PGC-1α expressing neurons at DIV 10 and DIV 16 (Fig 3.5). This suggests that a switch in the dominant GluN2 subunits is not responsible for the reduction in NMDAR currents.

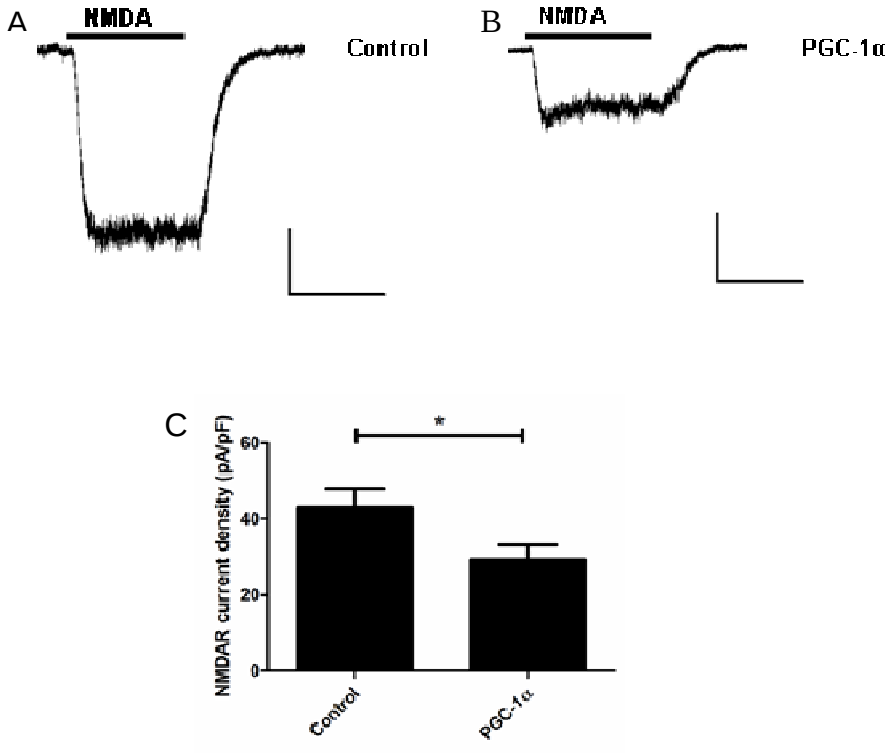

Figure 3.3 PGC-1α overexpression reduces agonist-evoked NMDAR currents Agonist evoked whole-cell NMDAR currents from cortical neurons transfected with GFP plus (A) globin (control) or (B) PGC-1α. Neurons were placed in Mg^{2+}-free external recording solution and voltage-clamped at -60 mV using whole-cell patch-clamp technique. NMDAR currents were evoked by applying NMDA (100 µM) until the cell reached a steady-state. (C) NMDAR- current density was calculated as the steady-state current amplitude normalised to the cell capacitance. Overexpression of PGC-1α caused a decrease in NMDAR current density. *t test $p<0.05$ (n=17 cells from N=4 cultures). Scale bar 5s by 300 pA

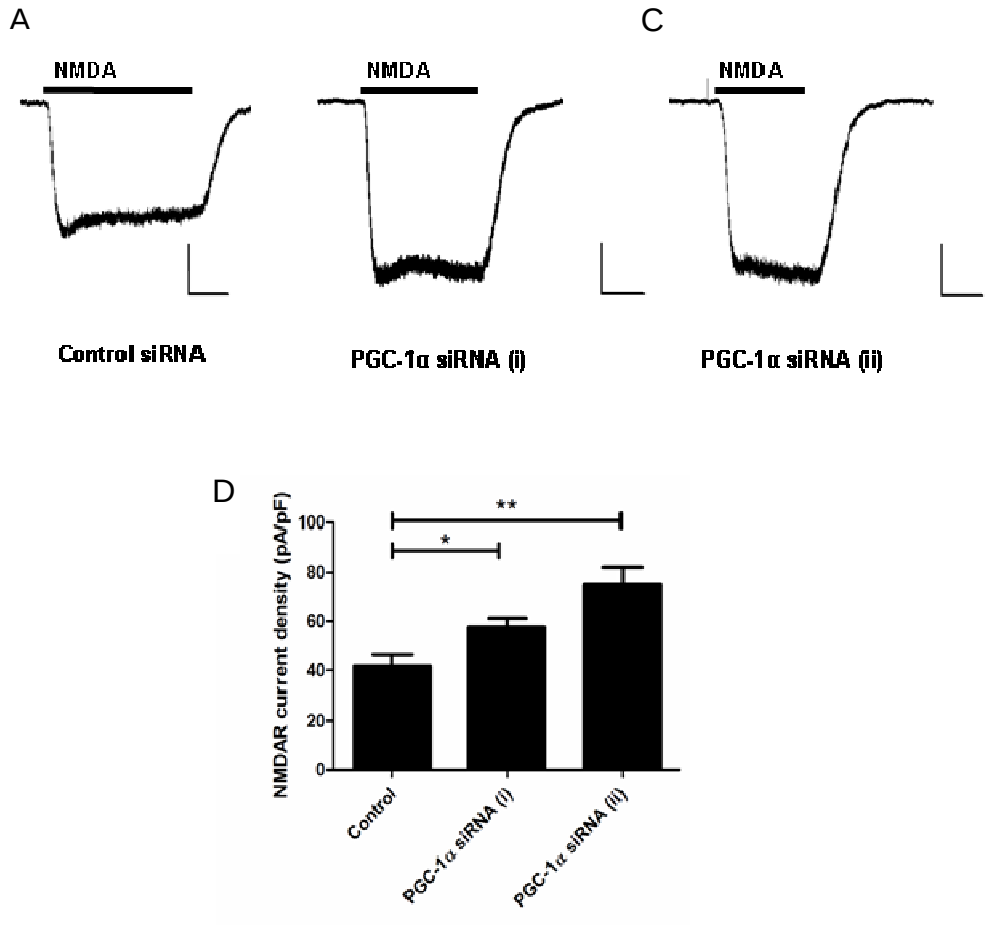

Figure 3.4 Knockdown of endogenous PGC-1α increases agonist-evoked NMDAR currents Agonist evoked whole-cell NMDAR currents from cortical neurons expressing GFP plus (A) control non-targeting siRNA, (B) PGC-1α siRNA(i) or (C) PGC-1α siRNA(ii). Neurons were placed in Mg^{2+}-free external recording solution and held at -60 mV using whole cell patch-clamp technique. NMDA (100 µM) was bath-applied to cells inducing an inward current. (D) NMDAR current density was quantified from the whole-cell current amplitude at steady-state normalised to cell capacitance. Neurons expressing either one of PGC-1α siRNA(i) or PGC-1α siRNA(ii) displayed greater than 30% increase in NMDAR current density compared to the control. One way ANOVA with post hoc Dunnett's test *$p<0.05$, **$p<0.01$ (n=18,14,5 from N=4 cultures). Scale bar 5 s by 400 pA

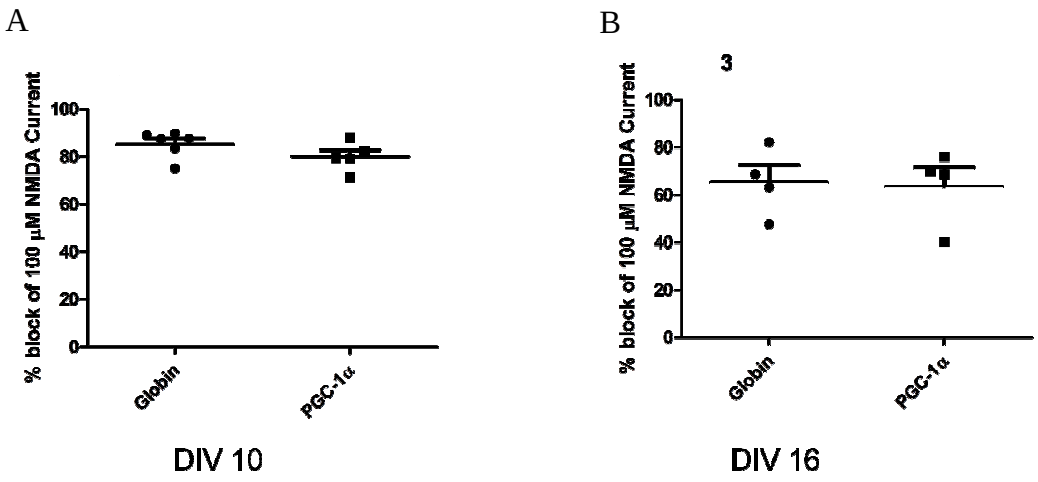

Fig 3.5 PGC-1α overexpression does not alter the subunit composition of NMDARs (A) The sensitivity of whole-cell NMDAR currents to block by the GluN2B antagonist Ifenprodil is comparable between control and PGC-1α expressing cells at (A) DIV10 and (B) DIV16 (n= 6,5 and 4,4 from 3 independent cultures each.

3.2.4 Excitotoxicity and *extrasynaptic* NMDAR activity

In the Central nervous system NMDARs reside both at synaptic sites where they partake in excitatory neurotransmission as well as at peri-synaptic and extra-synaptic sites throughout the dendritic membrane (grouped as 'extrasynaptic' from herein). In contrast to synaptic NMDAR activity, which is known to be essential for neuronal survival (Ikonomidou et al., 1999) extrasynaptic NMDAR activity is detrimental to neuronal health (Hardingham et al., 2002; Léveillé et al., 2008a; Gouix et al., 2009). Because excess activation of *extrasynaptic* NMDARs is an important mediator of excitotoxicity, we next investigated the influence of PGC-1α overexpression and knock-down on extrasynaptic NMDAR currents.

Pharmacological isolation of extrasynaptic NMDARs

To measure extrasynaptic NMDAR currents we pharmacologically isolated extrasynaptic receptors by first blocking synaptic NMDARs (Image 3.1). 48h post transfection, neurons were placed in Mg^{2+}-free recording solution supplemented with PTX (50 µM), TTX (300 nM) and MK-801 (10 µM). Under these conditions miniature excitatory synaptic potentials (mEPSPs) are generated by spontaneous presynaptic release of single quanta of glutamate which activates synaptic NMDARs. These receptors are then immediately and irreversibly blocked by the open-channel blocker MK-801. Extrasynaptic NMDARs, which remained closed during this 'quantal block' protocol, remain susceptible to activation by NMDA. Subsequent NMDA-evoked currents are recorded under voltage-clamp, which are now only mediated by extrasynaptic NMDARs. This protocol was previously used to study extrasynaptic signalling Papadia et al., (2008).

To ensure full blockade of synaptic NMDARs within the 10 minutes of MK-801/TTX/zero Mg^{2+} treatment, we conducted a timecourse experiment. Neurons were incubated for 0-30 minutes at room temperature after which NMDAR-currents were evoked. This revealed that by 10 minutes incubation in ACSF containing MK-801 blockade of synaptic NMDARs plateaus, and longer treatments have no further effect (Fig. 3.6).

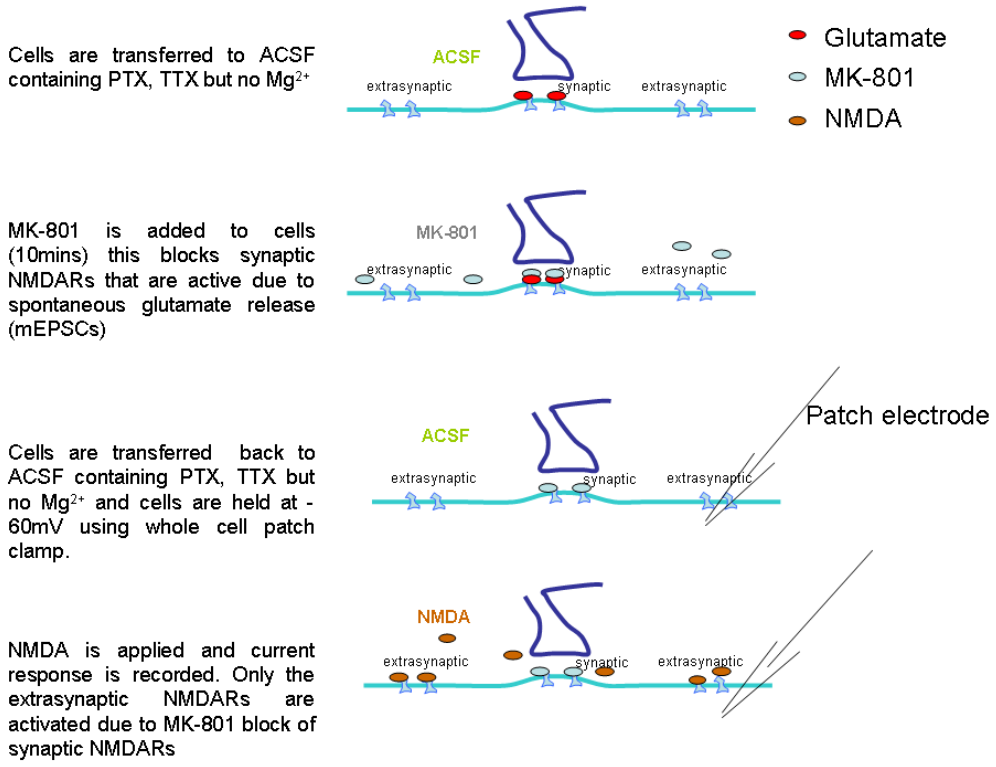

Image 3.1 Pharmacological isolation of extrasynaptic NMDARs Irreversible blockade of synaptic NMDARs by MK-801 during spontaneous mEPSPs enable us to isolate the extrasynaptic pool of NMDARs for further physiological analysis**.**

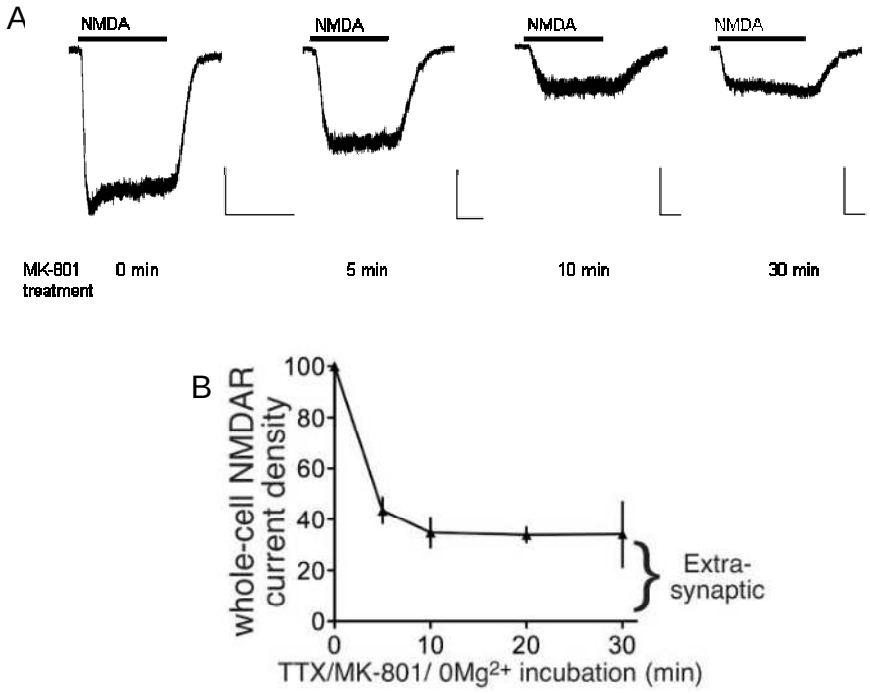

Figure 3.6 MK-801-blockade of synaptic NMDARs saturates by 10 min incubation 48h post transfection, neurons were placed in Mg^{2+}-free recording solution supplemented with PTX (50 µM), TTX (300 nM) and MK-801 (10 µM). Neurons were incubated for 0-30 minutes at room temperature. After incubation, neurons were washed with MK-801-free recording solution. Neurons were held under voltage-clamp (-60 mV) using whole-cell patch-clamp and NMDA (100 µM) -evoked currents were recorded. Extrasynaptic NMDAR-current density was calculated as the steady-state current amplitude normalised to the cell capacitance. (A) Example NMDA-responses from neurons expressing GFP plus after 0-30 minutes incubation with MK-801. (B) MK-801-reduction of whole-cell currents is saturated by 10 minutes stimulation implying maximal blockade of synaptic NMDARs (total of 17 cells analysed normalised to mean untreated).

3.2.5 PGC-1α does not affect miniature synaptic activity

The isolation of extrasynaptic NMDARs using the MK-801/TTX/zero Mg^{2+}-protocol relies on mEPSCs activity to open synaptic NMDARs which are subsequently blocked by MK-801. To ensure PGC-1α expression does not alter mEPSC properties we tested the frequency and amplitude of mEPSCs in neurons expressing PGC-1α siRNA. This was an important control specifically for the PGC-1α siRNA knockdown because in Fig 3.9 we show that increased currents remain post MK-801 block in siRNA-expressing cells. Had the mEPSCs been altered in siRNA-expressing cells, this could have been due to insufficient blockade of synaptic NMDARs.

Neurons were transfected (DIV7) with GFP plus control siRNA, PGC-1α siRNA(i) or PGC-1α siRNA(ii). 48h post transfection, neurons were placed in recording solution supplemented with PTX (50 μM), TTX (300 nM) and Mg^{2+} (1.3mM). mEPSCs were recorded in voltage clamp (-70 mV) for 5-10mins using whole-cell patch clamp. PGC-1α knockdown did not affect the frequency or amplitude of mEPSCs (Fig 3.7, p=0.76, 0.60 respectively, 1-way ANOVA (n=5-6)). We next confirmed that 10 minutes of MK-801/TTX/zero Mg^{2+} treatment was sufficient for saturation of the blockade of NMDA-induced currents to in siRNA-expressing cells (Fig 3.8), reassuring us that the remaining currents were mediated by extrasynaptic NMDARs (Fig 3.8). The experiments in Fig 3.7 and 3.8 indicate that the changes observed in subsequent experiments were due to alteration in the extrasynaptic pool of receptors, rather than the mEPSC properties of the neurons. In fig 3.8 current density after quantal block was normalised to the mean current in transfected (expressing the same construct) untreated (no quantal block) cells. This controls for difference in

the baseline current. This experiment also reveals that the proportion of the whole cell current that is blocked by the quantal block protocol differs between control and siRNA expressing cells. In PGC-1α siRNA cells Mk-801 blocks 63.4% of the mean current pre-quantal block compared to 78% in control cells. The effect of PGC-1α siRNA on the balance between synaptic and extrasynaptic NMDARs is the focus of the next set of experiments.

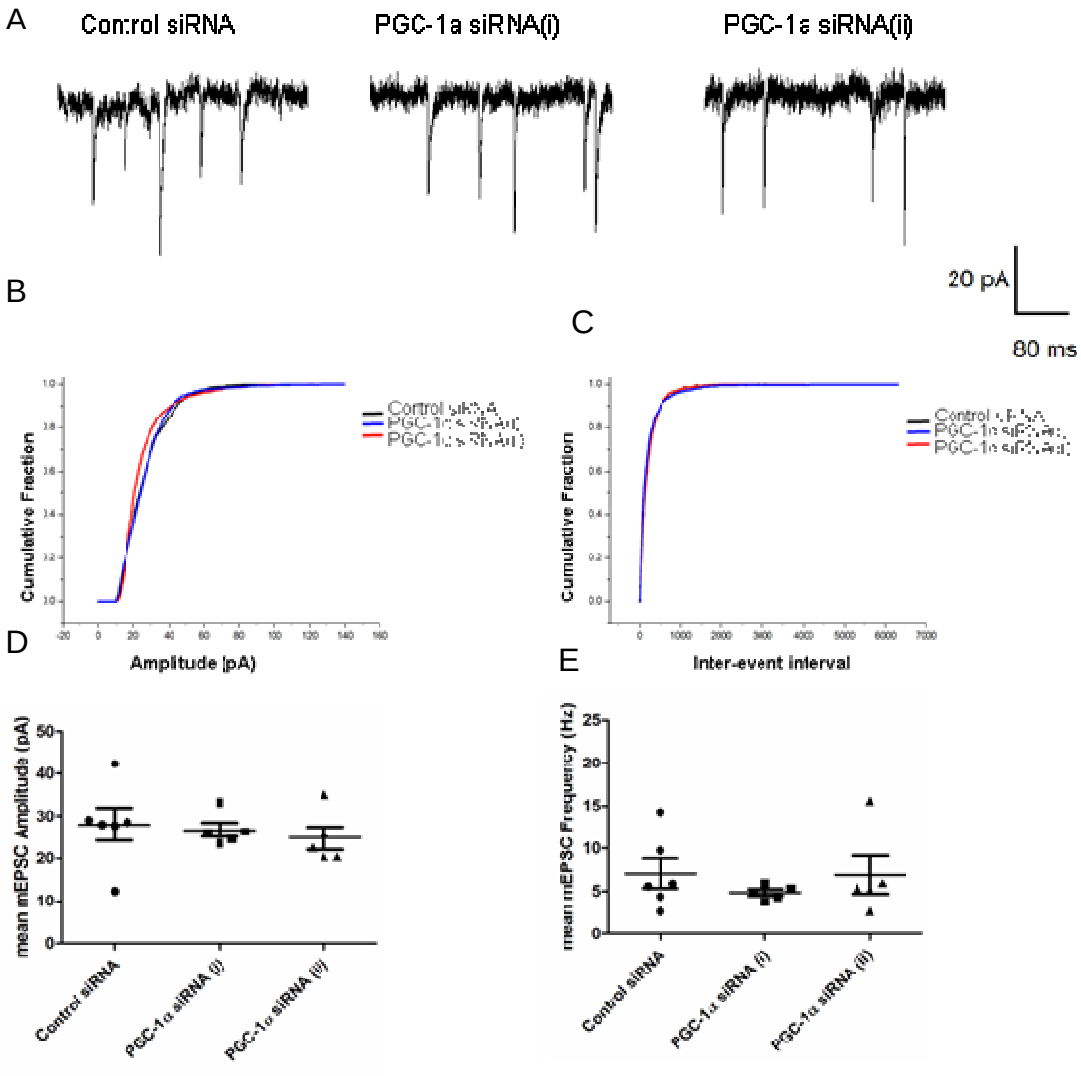

Figure 3.7 siRNA knockdown of PGC-1α does not alter mEPSC frequency or amplitude in cortical neuronal culture (A) Example mEPSC traces from cortical neurons transfected (DIV7) with GFP plus control siRNA, PGC-1**α** siRNA(i) or PGC-1**α** siRNA(ii). 48h post transfection, neurons were placed in recording solution supplemented with PTX (50 µM), TTX (300 nM) and Mg^{2+} (1.3mM). mEPSCs were recorded in voltage clamp (-70 mV) for 5-10mins using whole-cell patch clamp. (B) The amplitude distribution was similar for all three transfection groups. (C) Likewise, no change was observed in the inter-event interval distribution between groups (300 events per cell from n= 6, 5, 5 cells). No change was observed in either the (D) mean mEPSC amplitude or (E) mean frequency between groups (n= 6,5,5 cells) from 3 independent cultures.

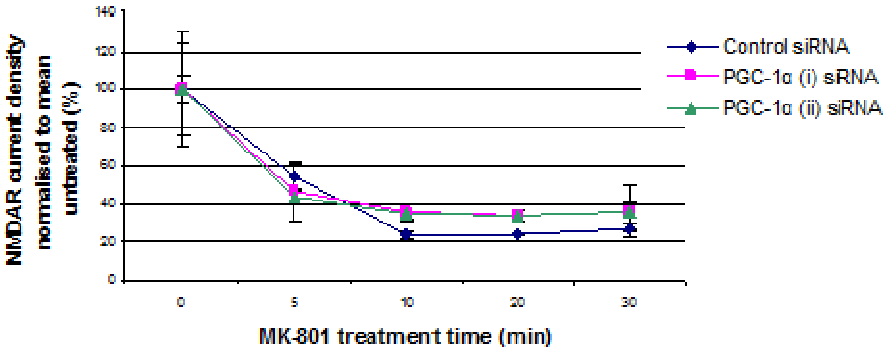

Figure 3.8 MK-801-blockade of synaptic NMDARs saturates by 10 min incubation in siRNA-expressing cells MK-801-reduction of whole-cell currents is saturated by 10 minutes MK-801-Mg2+/TTX incubation indicating maximal blockade of synaptic NMDARs. NMDAR current density was analysed following t=0,5,10,20,30 incubation with MK-801. Current response was normalised to the mean response pre-incubation to control for differences in baseline current between wtHtt and mtHtt. (total 17 cells expressing control siRNA; total 18 cells expressing PGC-1α siRNA(i); total 20 cells expressing PGC-1α siRNA(ii).

3.2.6 PGC-1α preferentially represses extrasynaptic NMDAR activity

To record extrasynaptic NMDAR currents, 48h post transfection, neurons were placed in Mg^{2+}-free recording solution supplemented with PTX (50 µM), TTX (300 nM) and MK-801 (10 µM) for 10 minutes at room temperature. After a 10 minute incubation, neurons were washed with MK-801-free recording solution. Neurons were held under voltage-clamp (-60 mV) using whole-cell patch-clamp and NMDA (100 µM) -evoked currents were recorded. Extrasynaptic NMDAR-current density was calculated as the steady-state current amplitude normalised to the cell capacitance. PGC-1α knockdown resulted in a striking increase in extrasynaptic NMDAR currents. Conversely, overexpression of PGC-1α greatly reduced extrasynaptic NMDAR currents (Fig 3.9)

Both the positive and negative manipulation of PGC-1α expression lead to a far greater change in extrasynaptic compared to synaptic NMDAR (Fig. 3.10). In agreement with studies on hippocampal neurons at >DIV9 (Rosenmund et al., 1995) assessment of NMDAR currents pre and post synaptic NMDAR blockade revealed that extrasynaptic NMDAR currents represent 32.4% of whole cell currents (Fig 3.11A, n=11). Thus we predict that alterations in whole-cell currents resulting from PGC-1α knockdown/overexpression are largely attributable to the changes in extrasynaptic NMDAR currents.

In order to directly confirm that PGC-1α adjusts the balance between synaptic and extrasynaptic NMDAR activity, we performed paired recordings of NMDA-evoked currents pre and post quantal block protocol in the same cell. From which we can extract the size of the synaptic and extrasynaptic pools of NMDARs in control cells versus cells expressing

PGC-1α. First, the total-whole cell current response to NMDA (100 µM) is recorded, followed by 10 minutes MK-801 quantal block protocol and finally the subsequent response to NMDA (100 µM), which is now mediated only by the extrasynaptic pool or receptors is recorded. Subtracting the NMDA response post quantal block from the response pre quantal block gives us the amplitude of the synaptic NMDAR current. PGC-1α does not alter the synaptic NMDAR current density (Fig 3.11B); however, PGC-1α overexpression caused a significant decrease in the current density of extrasynaptic NMDARs (Fig 3.11C).

Together this data shows that the concurrent neuroprotection and changes in NMDAR current density downstream of PGC-1α reflect alterations in the balance between synaptic and extrasynaptic NMDARs and that higher levels of PGC-1α pushes this balance in favour of the neuroprotective synaptic NMDARs.

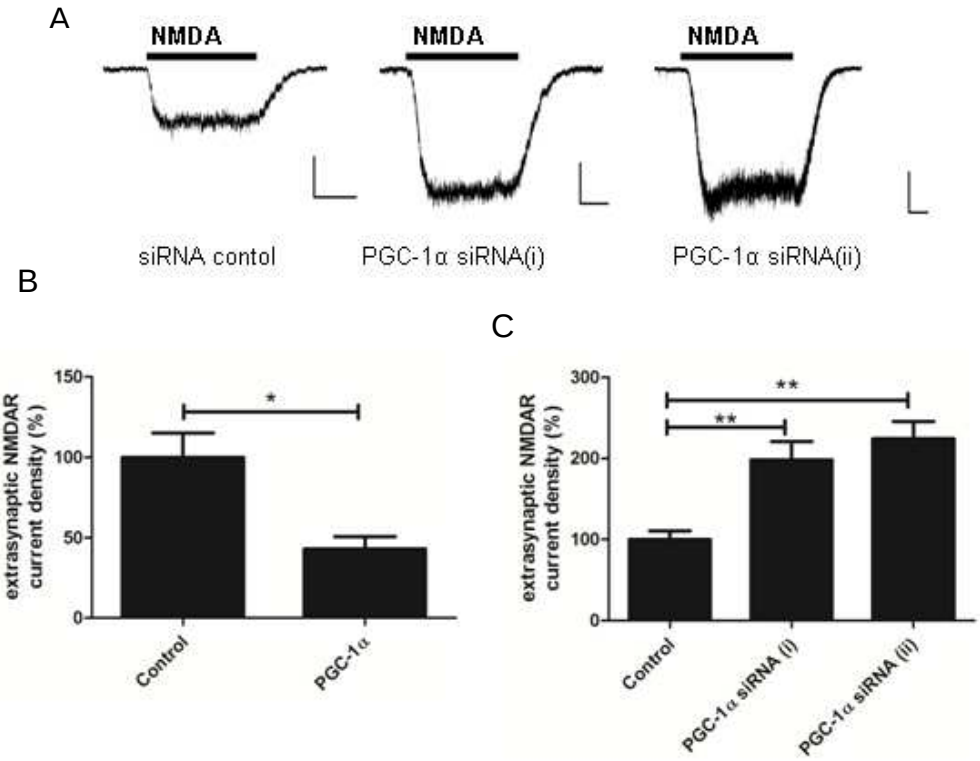

Figure 3.9 Extrasynaptic NMDAR current density is increased in cortical neurons expressing PGC-1α siRNA and decreased in neurons overexpressing PGC-1α 48h post transfection, neurons were placed in Mg^{2+}-free recording solution supplemented with PTX (50 µM), TTX (300 nM) and MK-801 (10 µM) for 10 minutes at room temperature. After a 10 minute incubation, neurons were washed with MK-801-free recording solution. Neurons were held under voltage-clamp (-60 mV) using whole-cell patch-clamp and NMDA (100 µM) -evoked currents were recorded. Extrasynaptic NMDAR-current density was calculated as the steady-state current amplitude normalised to the cell capacitance. (A) Example traces of extrasynaptic NMDAR-currents from neurons expressing control or PGC-1α siRNAs (B) Overexpression of PGC-1α lead to a reduction in the extrasynaptic NMDAR current density (n=4-5 stimulations from 4 cultures **t*-test $p<0.05$). (C) Conversely, extrasynaptic NMDAR current density is increased by the siRNA knockdown of PGC-1α (n=5-7 stimulations from 5 cultures, **$p<0.01$)

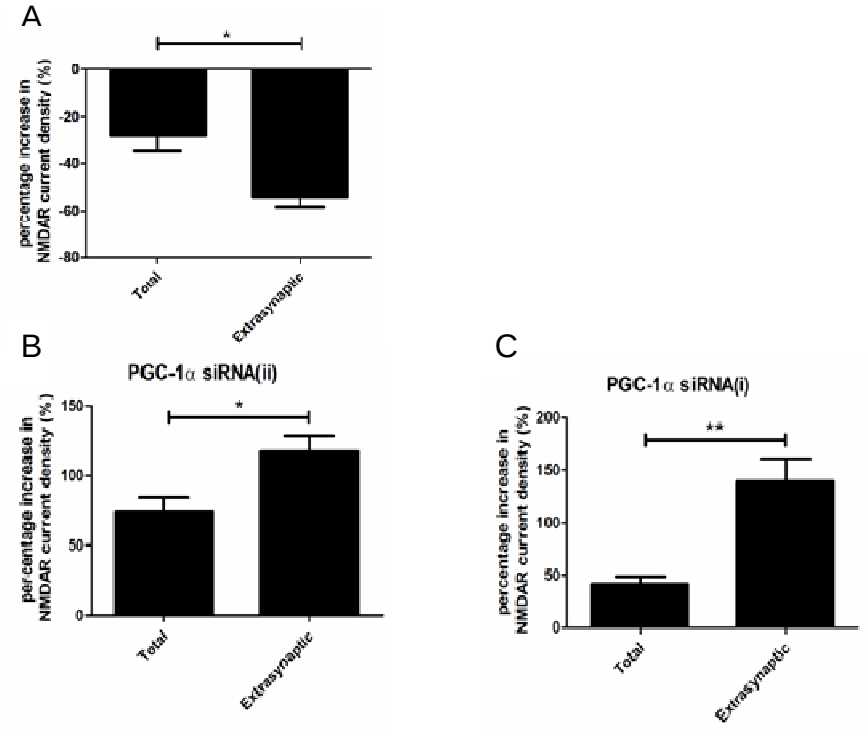

Figure 3.10 The effect of PGC-1α siRNA and PGC-1α overexpression is greater on extrasynaptic NMDAR-currents compared to the total whole-cell agonist-evoked currents (A) PGC-1α overexpression caused a greater reduction in whole cell NMDAR current density than total cell NMDAR current density (mean increase: -54.42% ± 3.966 extrasynaptic, -28.04 ± 6.225 total, N=4,4 **t*-test $p<0.05$). (B, C) PGC-1α siRNA(i) and (ii) caused a greater increase in extrasynaptic NMDAR currents compared to the total cell NMDAR current density (PGC-1α siRNA (i) mean increase: 139.0% ± 22.03 extrasynaptic, 41.02% ± 7.613 total n=5,4 respectively ***t*-test $p<0.01$. PGC-1α siRNA (ii) mean increase: 117.3% ± 11.29 extrasynaptic, 73.86% ± 10.59 total, n=5,4 respectively

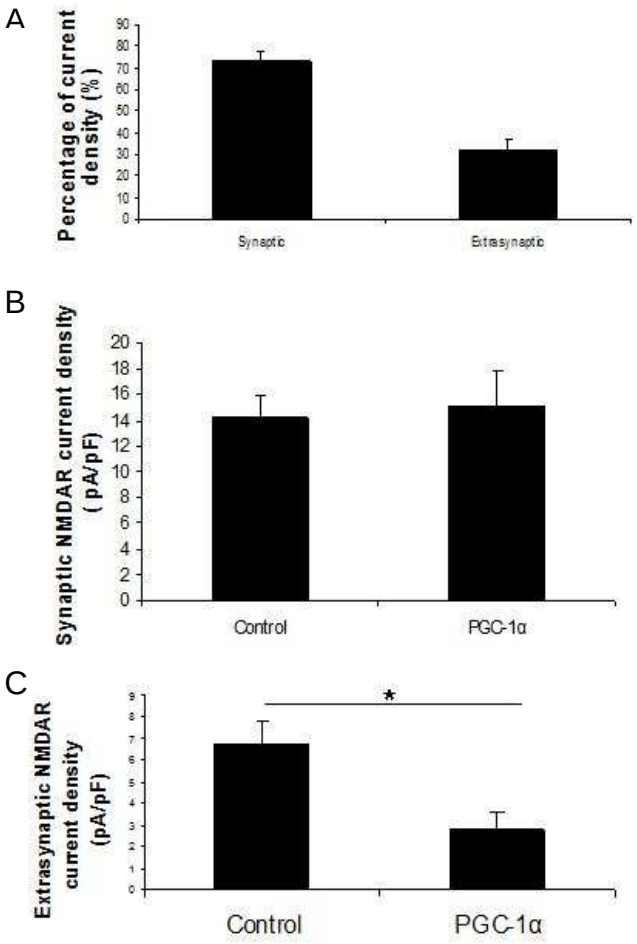

Fig 3.11 PGC-1α overexpression preferentially represses extrasynaptic NMDARS but not synaptic NMDARS To directly assess the distinct effects of PGC-1α overexpression on synaptic versus extrasynaptic pools of NMDAR receptors we performed paired recordings of total followed by extrasynaptic currents in the same cell. First the total-whole cell current response to NMDA (100 µM) is recorded, followed by 10 minutes MK-801 quantal block protocol and finally the subsequent response to NMDA (100 µM), which is now mediated only by the extrasynaptic pool or receptors. (A) At DIV10-11, the extrasynaptic NMDARs represent 32.4% of the total pool of NMDARs (n=11 from 3 independent cultures) (B) PGC-1α does not alter the synaptic NMDAR current density, measured by subtracting the current response after quantal block from that before quantal block, and normalising to cell capacitance. (C) PGC-1α overexpression caused a significant decrease in the current density of extrasynaptic NMDARs activated by NMDA (100 µM) post quantal block $p<0.05$; n =11, 5 cells from 3 independent cultures)

3.2.7 Exogenous PGC-1α expression leads to decreased GluN1 mRNA and GluN1 promoter activity.

In view of the fact that the known processes downstream of PGC-1α lead to the positive regulation of transcriptional activity, these results raise the surprising finding that PGC-1α activity can repress the activity of the NMDAR. Since NMDAR activity can be regulated at many stages from transcription, to trafficking, to post translational modifications of active receptors, we next asked whether PGC-1α - regulation of NMDARs involved changes in transcription of the ubiquitous NMDAR subunit GluN1. Nucleofection of PGC-1α in cortical cultures lead to a 21±6% decrease in GluN1 mRNA expression (Fig 2.10) with a parallel 102±2% increase in the PGC-1α target cytochrome C compared to control (GFP-expressing) cells.

We then tested whether exogenous PGC-1α expression interferes with the GluN1-promoter activity. To do this, we coexpressed PGC-1α and a 5.4kb GluN1-promoter (a gift from Guang Bai; (Bai et al., 2003)). PGC-1α overexpression reduced the activity of a luciferase reporter of the GluN1-promoter (Fig. 2.10, 43±9%, p=0.009). Together this data shows that mRNA of the NMDA GluN1 subunit is negatively regulated by PGC-1α.

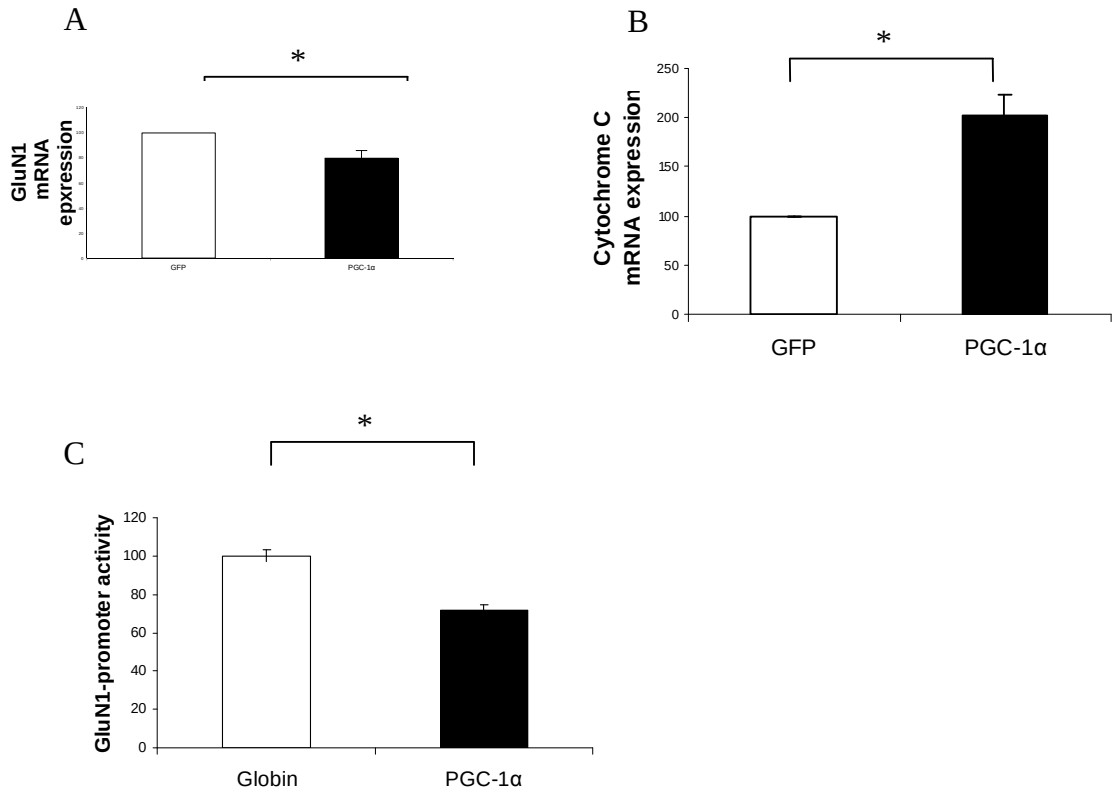

Figure 3.12 Exogenous PGC-1α increases GluN1 mRNA expression and promoter activity (A,B) Dissociated cortical rat neurons were nucleofected with either PGC-1α or GFP plasmids. Exogenous PGC-1α expression (A) decreased the mRNA expression of GluN1 (n= 5 nucleofections from 5 independent cultures, paired *t*-test $p< 0.05$) and (B) increased the mRNA expression of cytochrome C (n= 5 cultures, paired *t*-test, $p<0.01$). (C) GluN1-promoter activity was assayed by luciferase activity in cells expressing the GluN1-promoter-luciferase reporter with either Globin (control) or PGC-1α. PGC-1α significantly decreased GluN1 promoter activity (n= 3 cultures, *t*-test $p< 0.05$).

3.3 Discussion

3.3.1 Summary of experimental results

This study shows the potent neuroprotective capacity of PGC-1α expressed in cortical neurons by the regulation of NMDAR activity specifically at extrasynaptic sites. We have previously shown PGC-1α expression is part of an endogenous activity-dependent pro-survival signalling cascade and in reducing PGC-1α expression, neurons become more vulnerable to both oxidative and excitotoxic stress (Soriano et al., 2011). The known role of PGC-1α in upregulating the transcription of genes involved in mitochondrial biogenesis and function as well as ROS-detoxifying enzymes is thought to explain one mechanism in which it exerts its protection against these two insults (St-Pierre et al., 2006). Indeed, excitotoxic cell death also shares many phenotypic components with oxidative-stress including the loss of mitochondrial membrane potential and increased ROS-production (Nicholls et al. 2012; Hardingham & Bading 2010). However, the selective disruption of the anti-oxidative potential of PGC-1α by coexpression of the corepressor SMRT, which does not affect PGC-1α signalling to anti-excitotoxic neuroprotection (Soriano et al., 2011) suggested a second independent mechanism might exist.

Here we presents the novel finding that upregulation and conversely depression of PGC-1α expression in cortical neurons causes a bidirectional control of extrasynaptic NMDAR currents and vulnerability to NMDA-induced death. This suggests that the neuroprotection afforded by PGC-1α signalling is partially due to the repression of the neurotoxic extrasynaptic NMDAR-signalling. In addition, we found that the regulation of NMDARs may be in part due to the transcriptional repression of the ubiquitous GluN1 subunit of NMDARs. One would assume that the mechanisms

underlying this transcriptional suppression are indirect due to the coactivating nature of PGC-1α on its target transcription factors. Although our results are inconclusive as to how the loss of PGC-1α could directly/indirectly bring about an increase in NMDAR expression, we do know that a resulting excess of NMDARs are preferentially located at extrasynaptic sites. Whether NMDARs always selectively occupy extrasynaptic when overly abundant or whether this loss in extrasynaptic retention is downstream of PGC-1α-signalling is unstudied. However, recently published data from our lab shows that overexpression GluN1/GluN2A of GluN1/GluN2B cDNAs does not change the proportion of receptors at synaptic and extrasynaptic sites (Martel et al., 2012). Below, I will discuss known regulatory pathways involved in the expression, stability and function of NMDARs.

3.3.2 Potential routes to altered NMDAR-activity

There are two possible explanations for the increase in the total whole-cell current density in cells after PGC-1α knockdown (i) an increase in the function of pre-existing receptors, (ii) an increase in the NMDAR expression across the postsynaptic membrane. Evidence for increased surface expression and forward trafficking of GluN2B-containing receptors by the PGC-1α- repressor mtHtt in HD models (Milnerwood et al., 2010) combined with the repression of the GluN1-promoter downstream of exogenous PGC-1α (this study), supports the latter. These two pieces of evidence suggest the involvement of post-translation modifications and transcriptional regulation respectively. Given its known role as a transcriptional coactivator, how might PGC-1α-repress the expression of NMDARs?

3.3.3 Transcriptional regulation of NMDAR subunit expression

Transcription of genes encoding the NMDAR subunits is the first stage in the physiological regulation of NMDAR expression and appears to retain the susceptibility to environmental cues throughout development and maturation (Bai and Hoffman. 2009). With that said the majority of factors known to bind to the regulatory cis elements within the GluN1, GluN2 or GluN3 promoters act to enhance transcriptional output. These include the following transcription factors; Specific Protein family (SP-1), Early growth response family (Egr), T box (Tbr), Fos, Jun, cAMP response element-binding (CREB), and nuclear respiratory factor-1 (NRF-1) (Reviewed by Bai and Hoffman 2009, Dhar & Wong-Riley 2009).

Of interest, NRF-1 activity is positively regulated by the PGC-1α activity (Wu et al. 1999). This potential PGC-1α -NRF-1-GluN1 pathway would in theory oppose our findings the PGC-1α represses GluN1 transcription. However, by nature transcriptional coactivators upregulate only a selection of the genes transcribed by their target transcription factor and there is no evidence that PGC-1α activity is involved in NRF-1-dependent transcription of GluN1.

Although reduced NMDAR-expression is a phenotype of neurological disorders including schizophrenia (Olney & Farber, 1995), little is know about specific repressors counteracting the transcription of the NMDAR subunits. However, all three of GluN1, GluN2A, GluN2B promoters contain the repressor element RE-1/NRSF. The *trans* factor RE1 silencing transcription factor (REST) or neuron-restrictive silencer factor (NRSF) (REST/NRSF) binding to the RE1 element repressing GluN1 and GluN2B expression (Bai et al., 2003). We have yet to investigate whether REST/NRSF activity may be regulated by PGC-1α. Alternatively transcriptional repression could occur via epigenetic remodelling of the GluN promoters. Promoters often contain clusters off CpG dinucleotides termed CpG islands and methylation of these CpG islands negatively regulates transcription. CpG island have been found in the promoter or proximal regions of GluN1, GluN2A, GluN2B (Bai and Kusiak, 1993; Suchanek et al., 1995; Liu et al., 2003; Kim et al., 2006). This represents another potential target for the repression of NMDAR- expression. In addition, although there is no evidence that the GluN subunits are subject to chromatin remodelling themselves, such modifications are known to regulate the GluN-activating transcription factor CREB (reviewed Bai and Hoffman, 2009; Levenson & Sweatt 2005).

Of note, a number of independent groups have shown that mRNA-transcription, specifically of GluN1 is not rate-limiting in the expression of

functional NMDARs (Huh and Wenthold, 1999; Chazot and Stephenson, 2002; Prybylowski et al., 2002). Pryblowski et al., (2002) found that increasing GluN1-mRNA alone in granule or cortical cells is insufficient to increase NMDAR-currents and rather the co-expression of GluN2 is the rate-limiting step and is able to recruit more GluN1 to the surface. This is consistent with the theory that there is an excess pool of unassembled GluN1 subunits retained within the ER that are rapidly degraded if unrecruited (Huh and Wenthold, 1999; Chazot and Stephenson, 2002). Of course a significant upregulation of receptor expression may require de novo transcription; however increased transcription alone may be insufficient to push more active receptors to the plasma membrane. In agreement with this, we have found that overexpressing a GluN1 plasmid in neurons causes no further increase in whole-cell current amplitude (experiment performed by Marc-Andre Martel; unpublished data).

Vazhappilly & Sucher (2004) reported translational regulation of transcribed-mRNA as a second regulatory step; however, consistent with pool of unassembled subunit, much more focus has been paid to the regulation of assembly, trafficking and receptor stability of NMDARs.

3.3.4 Receptor stability at synaptic and extrasynaptic sites

Throughout maturation NMDARs are trafficked in and out of the plasma membrane between synaptic and extrasynaptic sites (Groc et al., 2006; Bard and Groc, 2011). Protein-protein interactions play an important role in regulating the stability of NMDARs. Even before leaving the endoplasmic reticulum, nascent NMDARs bind to MAGUKs such as PSD-95, PSD-93, SAP-102 which facilitate cytoskeletal interactions necessary for receptor trafficking to both synaptic and extrasynaptic sites (Groc et al.,

2006; Petralia et al., 2010). Once at the plasma membrane, NMDARs can directly interact with the cytoskeletal mesh, by binding to the actin binding protein, α-actinin (Wyszynski et al., 1997; Dunah et al., 2000) and the cytoskeletal protein, spectrin (Wechsler and Teichberg, 1998). However, NMDAR-MAGUK interactions continue to regulate NMDAR stability by promoting the protein-protein interactions required for post-translational modifications such as phosphorylation, ubiquitination, palmitoylation or calpain cleavage. Some specific modifications that retain receptors at synaptic sites over extrasynaptic sites have been identified.

For example, specific phosphorylation of tyrosine (tyrosine1472) by Src and serine (Serine1480) by casein kinase II on the GluN2B-subunit are known to promote and disrupt synaptic retention of the receptor respectively. In contrast Src- phosphorylation of GluN2B tyrosine Y1336 promotes extrasynaptic retention. NMDARs have two C-terminal cysteine clusters that once palmitoylated enhance synaptic trafficking and stability (Prybylowski et al., 2002; Hayashi et al., 2009). Similarly synaptic targeting of PSD-95 is thought to require its palmitoylation by the palmitoyl-acyl-transfrase (PAT), huntingtin interacting protein 14 (HIP14) (Huang et al., 2004; Huang and El-Husseini, 2005). Monoubquitination of GluN1 by F-box protein Fbx2 activity also regulates localisation (Hicke, 2001), whereas, polyubiquitination recruits the ubiquitin/proteasome system and targets receptors for degradation (Ehlers, 2003). The C-terminal of the NMDAR can be cleaved by calpain, and although this appears not to effect the biophysical function of the receptor (Guttmann et al. 2001; Puddifoot et al. 2009) it can disrupt interactions between the NMDAR and proteins of the post synaptic density. With such a complex mesh of NMDAR-regulating proteins there are many candidates for the indirect suppression of NMDAR activity by PGC-1α regulation of proteins involved in transcription right through to membrane stability. Given that

the mechanisms for targeting NMDARs to synaptic versus extrasynaptic sites are not well understood, this requires further investigation before we can derive a substantial hypothesis as to how PGC-1α might alter receptor distribution.

3.3.5 Mitochondrial function and NMDARs

One explanation for the increase in NMDAR currents downstream of PGC-1α is that deregulation of NMDAR activity is a possible by-product of disruptions in mitochondrial function. In such knocking down PGC-1α disrupts the transcription of mitochondrial genes including members of the electron transport chain such as complex II. Consistent with this theory, the complex II inhibitor 3-NP, which mimics both behavioural and pathological phenotypes of HD, can induce LTP by increasing synaptic NMDAR activity in the corticostriatal pathway (Calabresi et al., 2001; Gubellini et al., 2004). Furthermore, in addition to the essential role of mitochondrial-ATP production for ionic homeostasis in neurons, ATP has the ability to repress both GluN1/GluN2B-currents and NMDA-induced neurotoxicity in cultured hippocampal neurons (Ortinau et al., 2003). It is possible that the ability of PGC-1α to repress NMDAR-receptors is via regulatory mechanisms downstream of its known enhancement of mitochondrial biogenesis and function.

Additional clues may be sought from neurodegenerative diseases known to display increased excitotoxicity and NMDAR deregulation as well as PGC-1α repression. The role of PGC-1α in neurodegeneration has been most widely studied in HD (Cui et al., 2006; Weydt et al., 2006; Okamoto et al., 2009). An imbalance of synaptic and extrasynaptic NMDAR activity also contributes to the toxicity of HD. In the next chapter we investigate whether mutant huntingtin-induced changes in NMDAR expression and function are due to known repression of PGC-1α.

Chapter 4:

PGC-1α repression underlies the effect of mtHtt on extrasynaptic NMDAR currents

4.1 Chapter summary

In this chapter we investigate the relationship between the HD protein mutant huntingtin (mtHtt) and PGC-1α in excitotoxic cell death and the regulation of NMDAR currents. We find that overexpressing the N-terminal fragment of the mutant huntingtin protein containing a 148 polyglutamine repeat (mtHtt (148Q)) in both cortical and striatal neurons increases excitotoxicity. We also show that mtHtt (148Q) increases NMDAR currents in both striatal and cortical neuronal culture. In addition, mtHtt (148Q) causes a specific increase in the extrasynaptic NMDAR currents in cortical cultures. These results are consistent with studies in YAC128 HD mouse model (Milnerwood et al., 2010; Fan et al., 2007) and confirm our *in vitro* model of the actions of mutant huntingtin in neurons. Given the known repression of PGC-1α by mutant huntingtin and the increase in extrasynaptic NMDARs and excitotoxicity after knockdown of PGC-1α we show in chapter three, we hypothesized that mutant huntingtin-repression of PGC-1α may mediate its effects on NMDAR currents and excitotoxicity. In this chapter we report that in the absence of PGC-1α MtHtt has no further effect on NMDAR currents or excitotoxicity. We also show that restoring high levels of PGC-1α reverses the effect of mutant huntingtin on NMDAR currents and excitotoxicity. Together this data suggests that PGC-1α may be downstream of mtHtt in the regulation of NMDAR currents and that there may exist a common mechanism by which these two proteins mediate their effects on NMDAR currents. Data from this chapter in now published in Puddifoot et al., (2012).

4.2 Results

4.2.1 MtHtt increases excitotoxicity in primary cortical neurons

Previous studies have shown enhanced excitotoxicity in mouse models of HD (Zeron et al., 2002; Zhang et al., 2008). We therefore investigated whether expressing the N-terminal mtHtt exon 1 (mtHtt (148Q)) containing the polyglutamine repeat in cultured neurons enhanced excitotoxicity compared to N-terminal wtHtt (wtHtt (18Q)) control. Our studies focussed initially on cortical neurons, and subsequently striatal neurons, both of which are effected in HD. Cortical neurons were transfected with GFP plus either control (globin), wtHtt (Q18) or mtHtt (148Q). Neurons transfected at DIV7 were imaged 48h later and stimulated with a low dose of NMDA (10 μM) for 1h. Stimulation was stopped by the addition of MK-801(10 μM) and 24h after stimulation neurons are re-imaged to track cell fate. Cell death was quantified as the loss of GFP-positive cells as a percentage of GFP-positive cells before insult. Cell death in untreated neurons was quantified by the same means and subtracted from the total death in NMDA-treated cells. Consistent with aforementioned studies, we observed an increase in vulnerability to sub-toxic doses of NMDA (10 μM) in neurons expressing mtHtt (148Q) compared to wtHtt (18Q) or control (Globin)-expressing cells (fig 4.1). This indicates that our *in vitro* model of mutant huntingtin expression in neuronal cells mimics the neurotoxic phenotype of HD mouse models. Using this *in vitro* expression system in neuronal cultures, we can analyse the effect of acute manipulation of genes of interest on certain aspects of cell physiology.

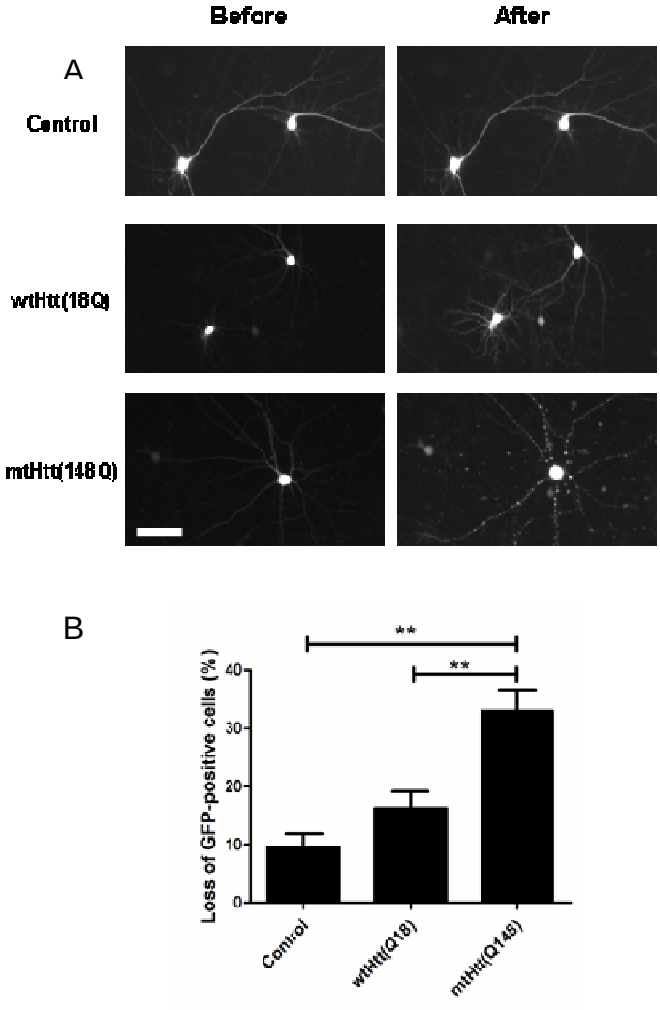

Figure 4.1 Mutant Huntingtin mtHtt (148Q) increases vulnerability to excitotoxicity *in vitro* (A) Cortical neurons expressing GFP plus either control (globin), wtHtt (Q18) or mtHtt (148Q) before and 24h after NMDA stimulation. Neurons transfected at DIV7 were imaged 48h later and stimulated with a low dose of NMDA (10 µM) for 1h. (B) Cell death was quantified as the loss of GFP-positive cells as a percentage of GFP-positive cells before insult. MtHtt (148Q) dramatically increased vulnerability of cortical neurons to a low dose of NMDA (150-300 cells from n=4 cultures **$p<0.01$)

4.2.2 MtHtt increases whole-cell NMDAR currents in cortical neurons

Previous studies have shown the YAC HD mouse models have increased NMDAR expression in the cortical and striatal neurons (Zeron et al., 2002; Zeron et al., 2004; Li et al., 2004; Fan et al., 2007) with a specific enhancement of extrasynaptic NMDARs (Milnerwood et al., 2010). These changes in NMDAR expression are thought to account for increased excitotoxicity in the YAC HD mouse models, and blocking the extrasynaptic NMDARs with the selective extrasynaptic NMDAR antagonist memantine rescues neuronal health (Milnerwood et al., 2010; Okamoto et al., 2009). We next investigated whether the increased vulnerability we observed after *in vitro* transfection of mtHtt(148Q) corresponds to changes in NMDAR currents in these cells.

Cultured cortical rat neurons were transfected with GFP plus control plasmid (Globin), wtHtt (18Q) or mtHtt (148Q) at DIV 8 and whole-cell patch-clamp analysis of NMDA-evoked currents was performed at DIV10. Neurons were transferred to Mg^{2+}-free external recording solution and GFP-expressing cells were voltage-clamped at -60 mV using whole-cell patch-clamp technique. NMDA (100 µM) was bath-applied to the cells evoking an inward current. NMDAR- current density was calculated as the steady-state current amplitude normalised to the cell capacitance. Expression of mtHtt (148Q) caused an increase in NMDAR current density compared to control or wtHtt (18Q) (fig 4.2; $p<0.05$). This result agrees with data from the YAC HD mouse model and previous *in vitro* studies that show mtHtt-driven increase in excitotoxicity results in part from increased NMDAR currents (Milnerwood et al., 2010; Fan et al., 2007).

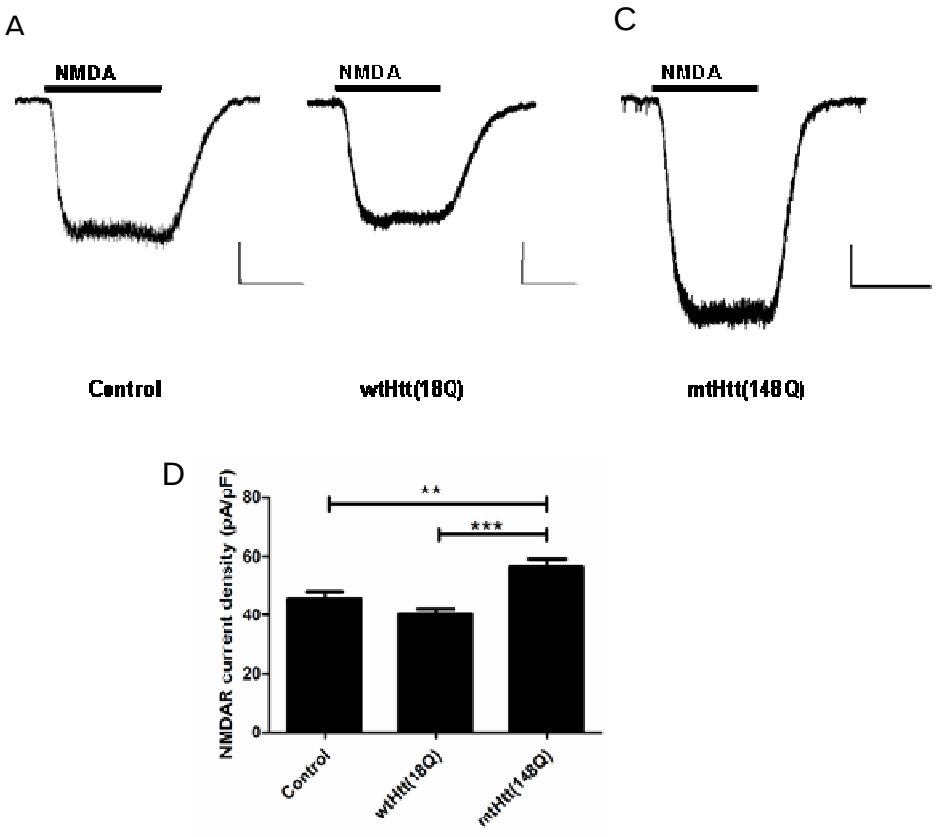

Figure 4.2 MtHtt (148Q) expression *in vitro* increases whole-cell NMDAR currents Agonist-evoked whole-cell currents from cortical neurons transfected with GFP plus (A) control plasmid (Globin) (B) wtHtt (18Q) (C) mtHtt (148Q). Neurons were transferred to Mg^{2+}-free external recording solution and voltage-clamped at -60 mV using whole-cell patch-clamp technique. NMDA (100 µM) was bath-applied to the cells evoking an inward current (D) NMDAR- current density was calculated as the steady-state current amplitude normalised to the cell capacitance. Expression of mtHtt (148Q) caused an increase in NMDAR current density compared to control or wtHtt (18Q) cells *one way ANOVA followed by post hoc Dunnett's test $p<0.01$, $p<0.001$ (n=17 cells from N=6 cultures). (Traces scale bar 5 s by 300 pA)

Our next aim was to test whether this increase is whole cell current and concurrent cell death was due to a preferential increase in extrasynaptic NMDAR activity as found in Milnerwood et al., (2010). To do this we used the 'quantal block' protocol described in chapter 2. To validate the use of this protocol, we first investigated whether mtHtt-expression altered mEPSCs amplitude or frequency, as well as confirming the saturation of the synaptic component of the whole cell current is reached by 10 minutes MK801/TTX/zero Mg^{2+}.

4.2.3 No change in mEPSCs in cells expressing mtHtt (148Q)

To study the effects of mtHtt expression on mEPSCs, neurons were transfected (DIV7) with GFP plus wtHtt (18Q) or mtHtt (148Q). 48h post transfection, neurons were placed in recording solution supplemented with PTX (50 µM), TTX (300 nM) and Mg^{2+} (1.3mM). MEPSCs were recorded in voltage clamp (-70 mV) for 5-10 min using whole-cell patch-clamp. Analysis of mEPSC recordings showed that the amplitude and inter-event-interval distributions were similar for both wtHtt (18Q) and mtHtt (148Q)-expressing cells. Furthermore, no change was observed in mean amplitude or frequency between groups (Fig 4.3, mtHtt vs. wtHtt; p=0.26, 0.31 respectively, *T*-test (n=6)). This confirmed that the synaptic mEPSC frequency and amplitude were unaffected by mtHtt (148Q)-expression in our cortical cultures.

4.2.4 'Quantal block' of synaptic NMDARs

As in the previous chapter, we applied the 'quantal block' protocol using TTX/MK-801/ zero Mg^{2+} to selectively block synaptic NMDARs. This protocol requires that all of the synaptic NMDARs are activated during the quantal block period, such that they can be blocked by the irreversible open-channel antagonist MK-801. To confirm that the expression of mtHtt(148Q) does not effect the rate of blockade of synaptic NMDARs we performed a time-course experiment in which the coverslips containing the neuronal cultures were bathed in TTX/MK-801/ zero Mg^{2+} for increasing amounts of time to establish the time point of saturation of MK-801 antagonism. 48h after transfection with globin (control), mtHtt(18Q) or mtHtt(148Q) plus an eGFP marker, neurons were placed in Mg^{2+}-free recording solution supplemented with PTX (50 µM), TTX (300 nM) and MK-801 (10 µM). Neurons were incubated for 0-30 minutes at room temperature, and then washed with aCSF to remove residual MK-801. Coverslips were transferred to patch-clamp recording chamber containing aCSF supplemented with PTX, TTX and Zero Mg^{2+}. GFP-expressing neurons were voltage-clamped at -60mV using whole cell patch clamp and NMDAR-currents were evoked. We confirmed that 10 minutes stimulation was sufficient to reach a saturated blockade of the synaptic proportion of the whole cell current and the time course of synaptic NMDAR blockade by MK-801 did not differ between wtHtt(18Q)-expressing and mtHtt(148Q)-expressing cells (Fig 4.4). In this experiment we can see that both mtHtt(148Q) and wtHtt(18Q) reach saturating MK-801 block by 10 minutes incubation; in addition, we observe reduction in the percentage of the mean whole cell current that is blocked by MK-801 in mtHtt(148Q) neurons. In mtHtt(148) cells only 54% of mean pre-treatment current is blocked by MK-801, compared to 64% in wtHtt-neurons. MtHtt-mediated changes specific to extrasynaptic NMDARs are analysed below.

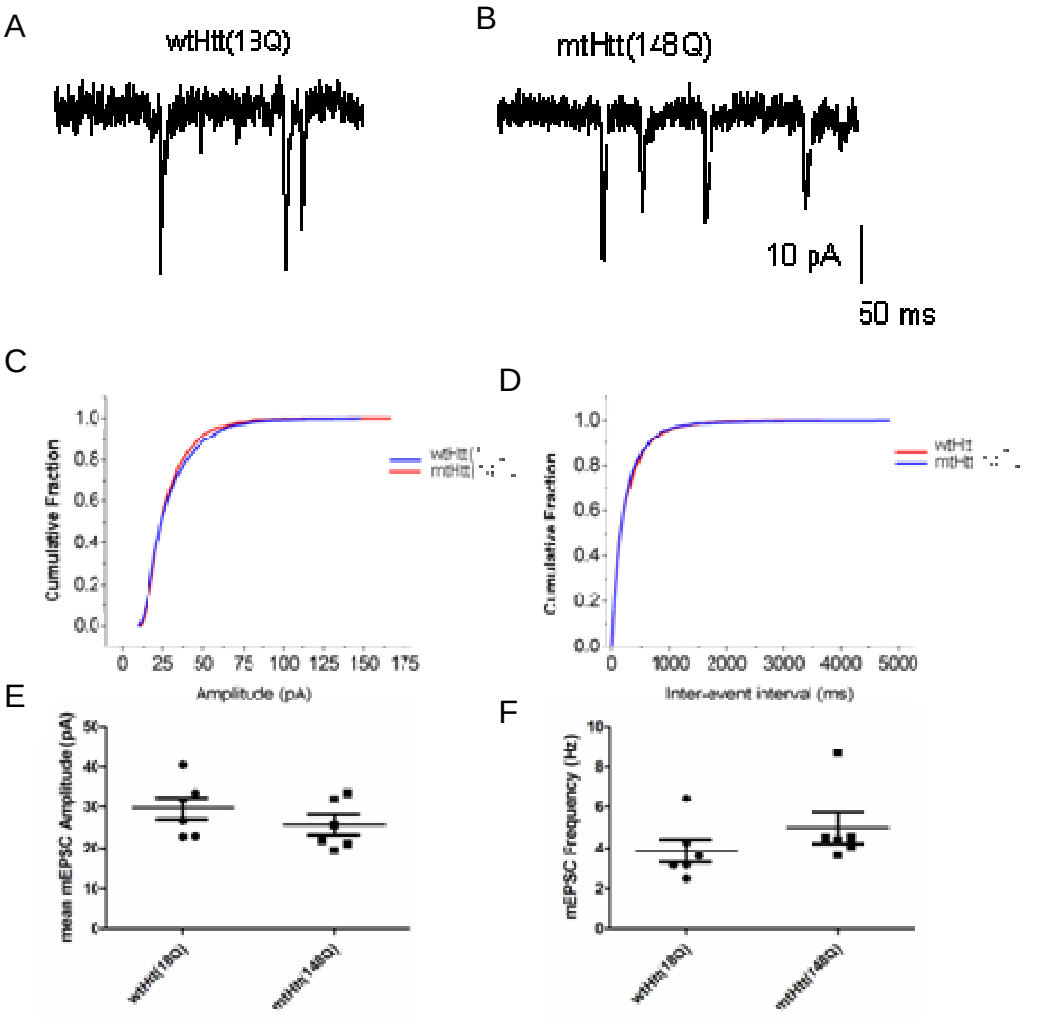

Figure 4.3 mtHtt(148Q) expression does not alter mEPSC frequency of amplitude in cortical neuronal culture (A) Example mEPSC traces from cortical neurons transfected (DIV7) with GFP plus wtHtt (18Q) or mtHtt (148Q). 48h post transfection, neurons were placed in recording solution supplemented with PTX (50 µM), TTX (300 nM) and Mg^{2+} (1.3mM). MEPSCs were recorded in voltage clamp (-70 mV) for 5-10 min using whole-cell patch-clamp. (B) The amplitude distribution was similar for both wtHtt (18Q) and mtHtt (148Q)-expressing cells. (C) Likewise, no change was observed in the inter-event-interval distribution between groups (300 events per cell from N=6 cells). No change was observed in either the (D) mean mEPSC amplitude or (E) mean frequency between groups (n= 6, 6 cells).

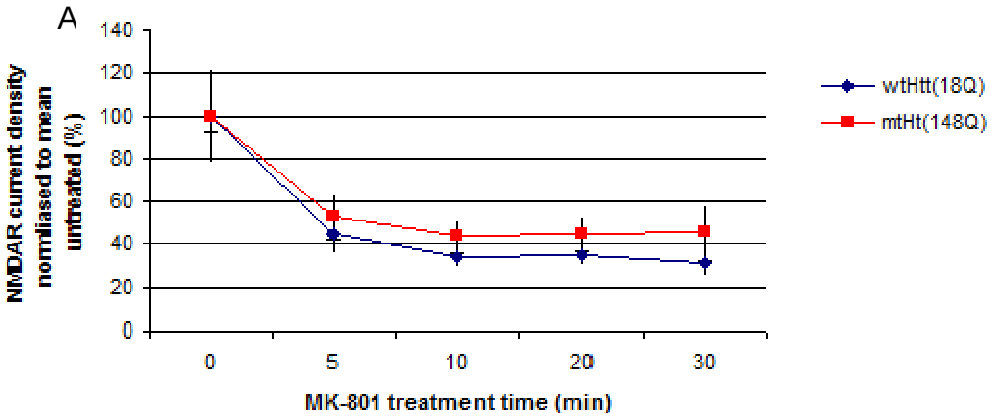

Figure 4.4 MK-801-blockade of synaptic NMDARs saturates by 10 min incubation Neurons were incubated for 0-30 minutes at room temperature in aCSF containing PTX (50 µM)/TTX (300 nM)/MK-801 (10 µM). After which NMDAR-currents were evoked. Current response was normalised to the mean response pre-incubation to control for differences in baseline current between wtHtt and mtHtt. The time course of synaptic NMDAR blockade by MK-801 did not differ between wtHtt (18Q)-expressing (n= 20 cells total) mtHtt (148Q) - (n=17 cells) saturating by 10 minutes incubation time in both cases.

4.2.5 MtHtt enhances extrasynaptic NMDAR currents

Milnerwood et al., (2010) presented evidence for enhanced extrasynaptic NMDAR activity in the YAC128 model of HD. In addition whereas increased extrasynaptic NMDAR signalling increases excitotoxic cell death, synaptic NMDAR activity is neuroprotective (reviewed Hardingham and Bading, 2010). We therefore applied the quantal block protocol described in chapter 3 to assess whether the extrasynaptic current density is altered after *in vitro* expression of mtHtt(148Q) compared to wtHtt(18Q) control. Neurons were stimulated for 10 minutes with aCSF containing MK-801/TTX/zero Mg^{2+} to block spontaneously active synaptic NMDARs. Agonist activation of the remaining extrasynaptic NMDARs, which were not active during the quantal block protocol, was measured by subsequent bath application of NMDA (100 µM). MtHtt (148Q)expression led to an increase in extrasynaptic NMDAR currents compared to wtHtt (Fig. 4.5). Furthermore, the effect of mtHtt (148Q) on extrasynaptic currents was far greater than its effect on total currents (mean increase: 139.0±22% extrasynaptic, 41.02±7.2% total current (synaptic+extrasynaptic) N=5, 4 $p<0.01$). At this stage of development *in vitro*, the extrasynaptic pool represents ~30% of the total population of receptors as described in chapter 3; this indicates a preferential effect of mtHtt(148Q) on expression levels of extrasynaptic NMDARs over synaptic NMDARs (Fig. 4.6). As discussed in Milnerwood et al., (2010), this increase in extrasynaptic NDMAR activity may account for the increased vulnerability of neurons expressing the mutant huntingtin protein.

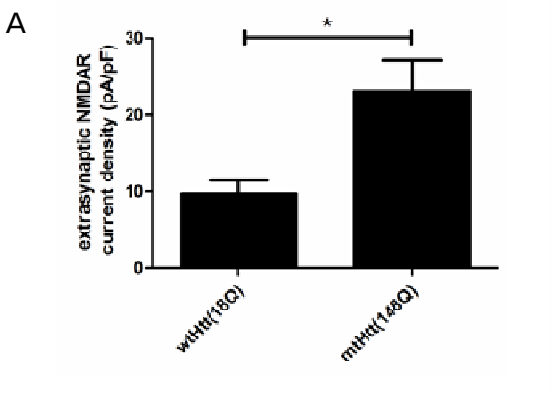

Figure 4.5 Extrasynaptic NMDAR current density is increased in cortical neurons expressing mtHtt(148Q) 48h post transfection, neurons were placed in Mg^{2+}-free recording solution supplemented with PTX (50 µM), TTX (300 nM) and MK-801 (10 µM) for 10 minutes at room temperature. After a 10 minute incubation, neurons were washed with MK-801-free recording solution. Neurons were held under voltage-clamp (-60 mV) using whole-cell patch-clamp and NMDA (100 µM) -evoked currents were recorded. Extrasynaptic NMDAR-current density was calculated as the steady-state current amplitude normalised to the cell capacitance. (A) Expression of mtHtt (148Q) significantly increased extrasynaptic NMDAR current density (n=4, 4 stimulations from 4 cultures, t test *$p<0.05$)**.**

A

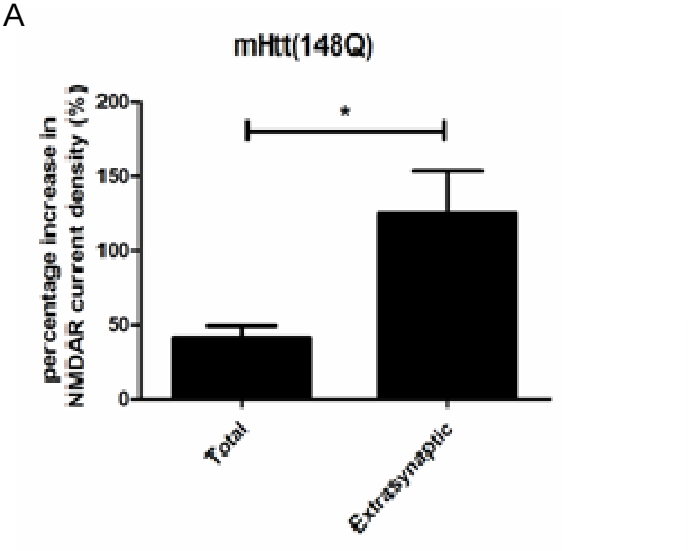

Figure 4.6 The effect of mtHtt-expression is greater on extrasynaptic NMDAR-currents compared to the total whole-cell agonist-evoked currents (A) mtHtt(148Q)-overexpression has a greater effect on the extrasynaptic NMDAR-currents compared to the effect on the total whole-cell current (mean increase 125.1% ± 28.86 extrasynaptic, 40.79% ± 8.486 total, n=4,6 respectively **t*-test $p<0.05$**)**

4.2.6 Does mtHtt alter NMDAR activity via the repression of PGC-1α?

In chapter 3 we described the bidirectional control of extrasynaptic NMDAR currents downstream of PGC-1α. Mutant huntingtin protein has previously been shown to suppress PGC-1α expression by directly interacting with the PGC-1α promoter (Weydt et al., 206). Therefore, we proposed that the effects of mtHtt on extrasynaptic NMDAR currents could be due to its known effects on suppressing PGC-1α expression. If this was the case we would expect the effects of PGC-1α knockdown and mtHtt (148Q) expression to be non-additive and occlude each other. Secondly, since overexpression of PGC-1α is known to counter both the toxicity of mutant huntingtin and suppress extrasynaptic NMDAR activity (this study) we hypothesised that exogenous PGC-1α expression should rescue the effect of mtHtt (148Q) on extrasynaptic NMDAR currents and toxicity.

We first confirmed the repression of PGC-1α by mtHtt (148Q) - expression in cultured cortical neurons compared to wtHtt (18Q) control. MtHtt(148Q) reduced PGC-1α-promoter activity assayed by the coexpression of mtHtt(148Q) or wtHtt(18Q) with a 5.4kb PGC-1α-promoter-luciferase reporter (Fig 4.7) (Bai et al., 2003).

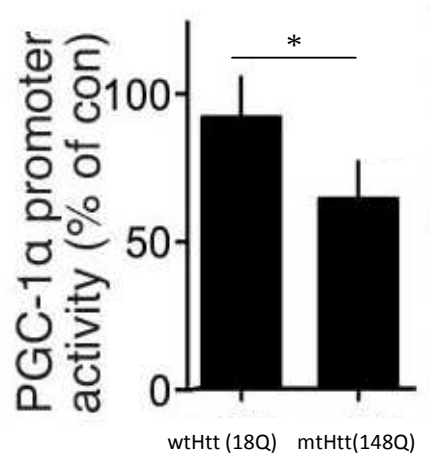

Figure 4.7 mtHtt expression significantly reduces PGC-1α promoter activity Cultured cortical neurons coexpressing a PGC-1α-promoter-luciferase reporter plus either wtHtt(18Q) of mtHtt(148) were assayed for luciferase activity 48h post transfection. 24h after transfection neurons were stimulated with Bic/4AP to induce higher levels promoter activity (Soriano et al., 2011). The expression of mtHtt (148Q) significantly reduced promoter activity, n= 3 cultures, ttest $p<0.05$.

4.2.7 MtHtt (148Q) and PGC-1α knockdown cause a non-additive increase in excitotoxicity

Since we have evidence that mtHtt represses PGC-1α transcription in this model we hypothesised that this repression of PGC-1α may contribute to the mtHtt (148Q) increase in excitotoxicity. To test this, we analysed whether mtHtt (148Q) expression increased excitotoxicity in neurons that no longer express PGC-1α. PGC-1α siRNA was coexpressed with mtHtt (148Q) or wtHtt (18Q) plus an eGFP marker. Images of the same neurons were taken pre and 24h post a 1h NMDA (10 µM) stimulation.

PGC-1α knockdown and mtHtt (148Q) both increased excitotoxicity compared to control (Fig 4.8). However, in neurons transfected with PGC-1α siRNA, mtHtt (148Q)-expression had no additional effect on NMDA-induced excitotoxicity (Fig 4.8, Two way, interaction factor $p=0.0379$). This suggests that the PGC-1α pathway is involved in the mtHtt excitotoxic pathway and that inhibiting PGC-1α expression is sufficient to mimic excitotoxicity of mtHtt. This agrees with data from the PGC-1α KO mice in which striatal lesions closely replicate those seen in HD mice (Cui et al., 2006).

4.2.8 MtHtt (148Q) and PGC-1α knockdown cause a non-additive increase in NMDAR current density

We next tested the combined effects of mtHtt (148Q) and PGC-1α knockdown on whole cells NMDAR currents. Neurons were transfected with wtHtt (18Q) or mtHtt (148Q) in the presence of either control non-targeting siRNA or PGC-1α-targeting siRNA. Whole-cell current density was increased by mtHtt (148Q)-expression in control siRNA-expressing

neurons (*t*-test **p<0.01). Concurrently, siRNA knockdown of PGC-1α caused an increase in NMDAR-current density in wtHtt (18Q)-expressing cells (*t*-test *P<0.05). However, no significant difference in NMDAR current density was observed in cells co-expressing wtHtt (18Q) versus mtHtt (148Q) in PGC-1α siRNA-expressing cells (Fig 4.9) this result suggests for the first time, that the repression of PGC-1α by mutant huntingtin may account in part for the observed alteration in NMDAR activity reported in HD.

4.2.9 MtHtt-increase in extrasynaptic NMDAR-currents is occluded by knockdown of PGC-1α

Finally, we investigated whether the expression of mtHtt in cells expressing PGC-1α siRNA had further increase in extrasynaptic NMDAR activity. Expression of mtHtt (148Q) significantly increased extrasynaptic NMDAR current density in control siRNA neurons but not in neurons expressing PGC-1α siRNA. Likewise, these observations support the hypothesis that mtHtt increases extrasynaptic NMDAR activity, at least in part, via repression of PGC-1α.

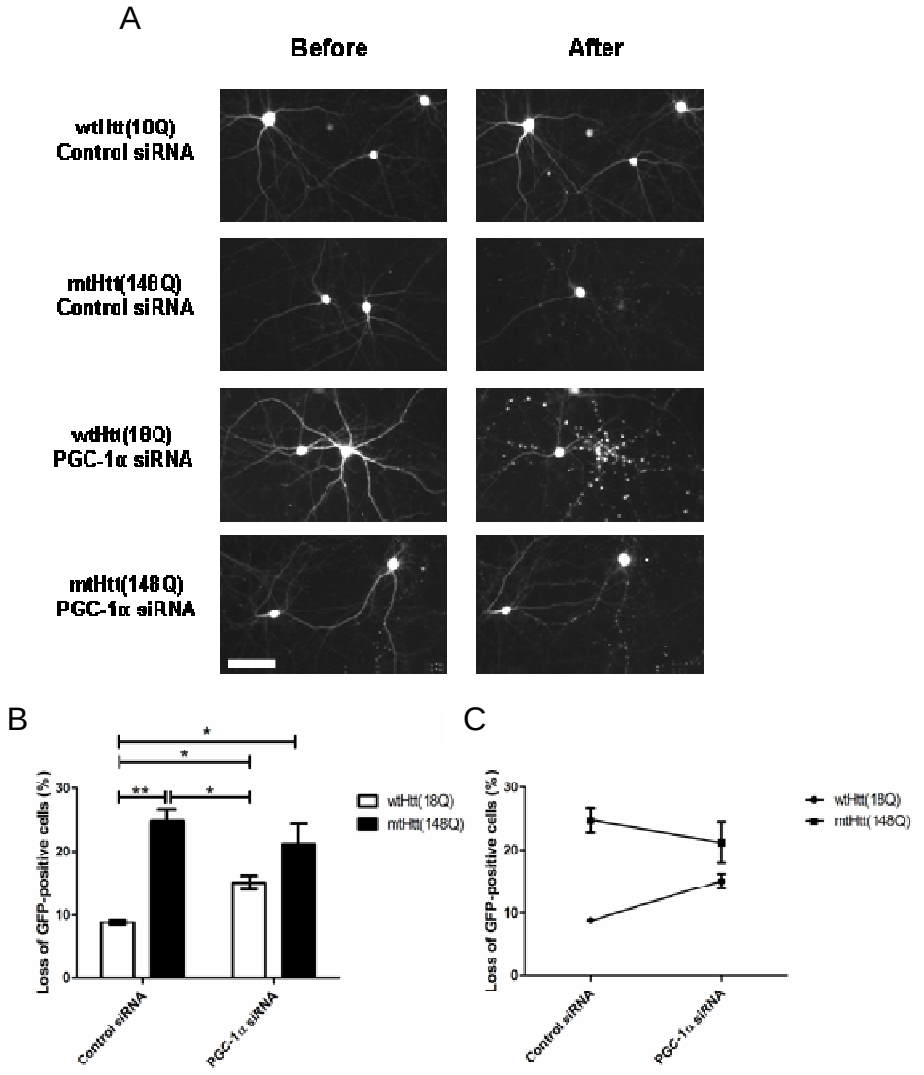

Figure 4.8 MtHtt(148Q) expression and siRNA knockdown of PGC-1α have a non-additive effect on excitotoxicity (A) Images pre and 24h post 1h NMDA (10 µM) stimulation. Cortical neurons were transfected with wtHtt (18Q) or mtHtt (148Q) in the presence of either control non targeting siRNA or PGC-1α targeting siRNA (PGC-1α siRNA (i)). (B) MtHtt (148Q) expression caused an increase in NMDA-induced cell death in control cells. No significant increase in cell death by mtHtt (148Q) compared to wtHtt (18Q)-expressing cells was observed in cells co-expressing PGC-1α siRNA. (n=3, for each culture, 50-100 cells were analysed per group for each stimulation) **t*-test $p<0.05$ **$p<0.01$. (C) Two-way ANOVA results show a significant interaction factor ($p=0.0379$) between the effects of PGC-1α siRNA-expression and mtHtt (148Q)-expression on the loss of GFP-positive cells.

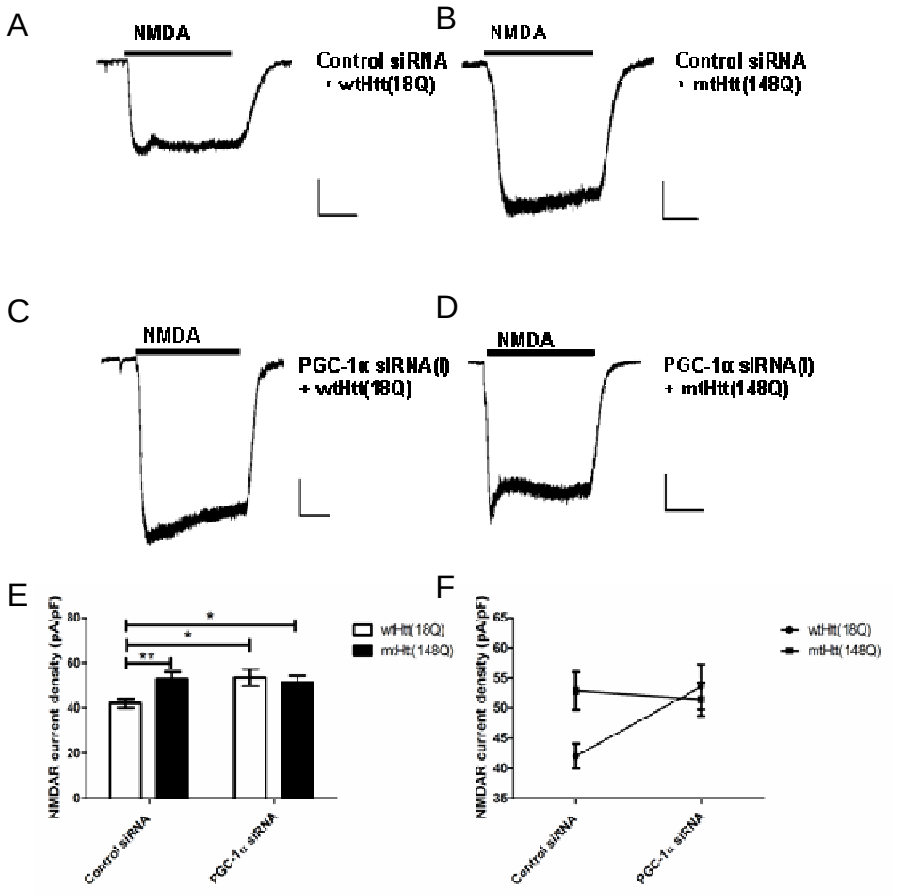

Figure 4.9 Co-expression of MtHtt (148Q) and PGC-1α-siRNA results in a non-additive increase in agonist-evoked NMDAR currents Neurons transfected with (A, C) wtHtt (18Q) or (B, D) mtHtt (148Q) in the presence of either (A, B) control non-targeting siRNA or (C, D) PGC-1α-targeting siRNA (PGC-1α siRNA (i)). (E) Whole-cell current density was increased by mtHtt (148Q)-expression in control siRNA-expressing neurons (*t*-test **p<0.01). Concurrently, siRNA knockdown of PGC-1α caused an increase in NMDAR-current density in wtHtt (18Q)-expressing cells (*t*-test *P<0.05). No significant difference in NMDAR current density was observed in cells co-expressing wtHtt (18Q) versus mtHtt (148Q) in PGC-1α siRNA-expressing cells. (F) Two-way ANOVA results indicate a significant interaction between the effect of PGC-1α siRNA–expression and mtHtt (148Q)-expression on whole-cell NMDAR current density. In the context of reduced PGC-1α, mtHtt (148Q) has a different effect on whole-cell NMDAR currents. (Traces scale bar 5 s by 400 pA; n= 21, 17, 38, 21 cells from 4 cultures)

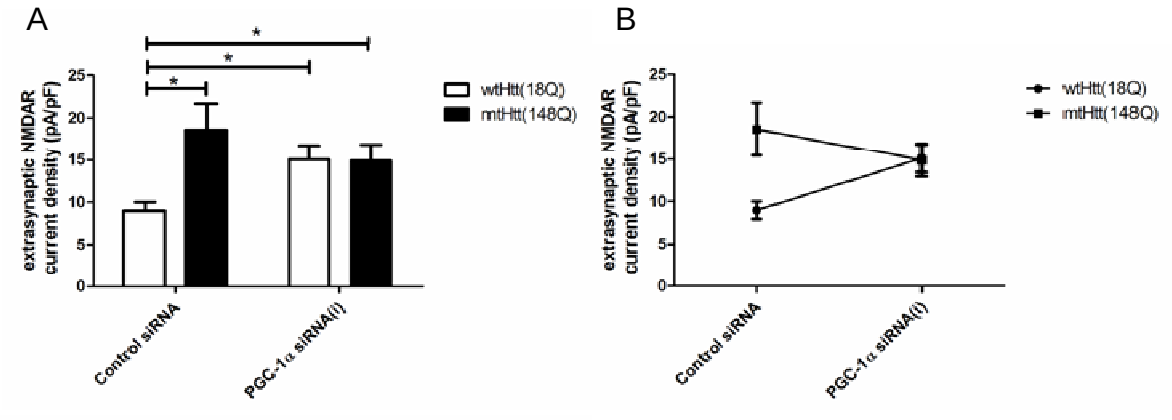

Figure 4.10 Increase of extrasynaptic NMDAR current density by mtHtt(148Q)-expression and siRNA knockdown of PGC-1α is non-additive (A) Expression of mtHtt(148Q) significantly increased extrasynaptic NMDAR current density in control neurons but not in neurons expressing PGC-1α siRNA (n=4,4,4,4 stimulations from 4 cultures, t test *p<0.05)**.** (B) Two way ANOVA results show a significant interaction between PGC-1α siRNA and mtHtt (148Q)-expression on extrasynaptic NMDAR currents (Interaction P=0.037).

4.2.10 PGC-1α rescues mtHtt (148Q)-mediated increase in excitotoxicity

Given that mtHtt and PGC-1α knockdown occlude each other's effect on excitotoxicity and NMDAR deregulation, we next tested the hypothesis that expression of exogenous PGC-1α can rescue the changes in extrasynaptic NMDAR activity and toxicity by mtHtt. Previous studies have shown that PGC-1α overexpression can protect against mtHtt toxicity in STHdh^{Q111} HD cell line, *in vivo* in the R6/2 cells the death as well as against cell death induced by TTX blockade of synaptic activity in primary striatal cells expressing mtHtt (Okamoto et al., 2009). Cortical neurons were transfected with GFP plus either control (globin) or PGC-1α alongside either wtHtt (Q18) or mtHtt (148Q). Coexpression of PGC-1α completely reversed the effect of mtHtt on excitotoxicity (Fig 4.10, mean cell death: 30.51 % ± 3.79 in mtHtt(148Q) + globin cells, 0.5404 % ± 2.64 in mtHtt(148Q) + PGC-1α cells, $p<0.001$). PGC-1α expression can counteract the toxicity of mtHtt.

4.2.11 PGC-1α rescues mtHtt (148Q)-induced increase in NMDAR current density

The ability of PGC-1α to rescue mtHtt-increases in excitotoxicity combined with the repression of NMDAR currents by PGC-1α expression, shown in chapter 1, suggests that exogenous PGC-1α may reverse the increase in NMDAR currents by mtHtt. To test this hypothesis, we again transfected cortical neurons with GFP plus either control (globin) or PGC-1α alongside either wtHtt (Q18) or mtHtt (148Q). MtHtt expression caused an increase in NMDAR currents in control cells; the increase in NMDAR

currents was significantly reduced in cells expressing exogenous PGC-1α expression (Fig 4.11).

4.2.12 PGC-1α overexpression rescues mtHtt (148Q) increase in extrasynaptic currents

We tested the effect of mtHtt on extrasynaptic NMDAR currents in cells co-expressing exogenous PGC-1α. The increase in extrasynaptic NMDAR current density in mtHtt-expressing cells was rescued by the coexpression of PGC-1α (Fig 4.12). Indeed, analysis by two-way ANOVA shows that in the context of exogenous PGC-1α expression, the effect of mtHtt on excitotoxicity and extrasynaptic NMDAR currents is significantly decreased (Fig 4.11, Fig 4.12).

Summary: Together this data shows that the repression of PGC-1α by mtHtt contributes to enhanced extrasynaptic NMDAR currents and excitotoxicity in HD. Given that mtHtt has no further effect on NMDAR currents or excitotoxicity in the absence of PGC-1α , this suggests that the effect of mutant huntingtin and loss of PGC-1α share common mechanisms in mediating these effects. Consistent with this theory, restoring PGC-1α expression reverses the changes in NMDAR currents and excitotoxicity induced by mutant huntingtin expression.

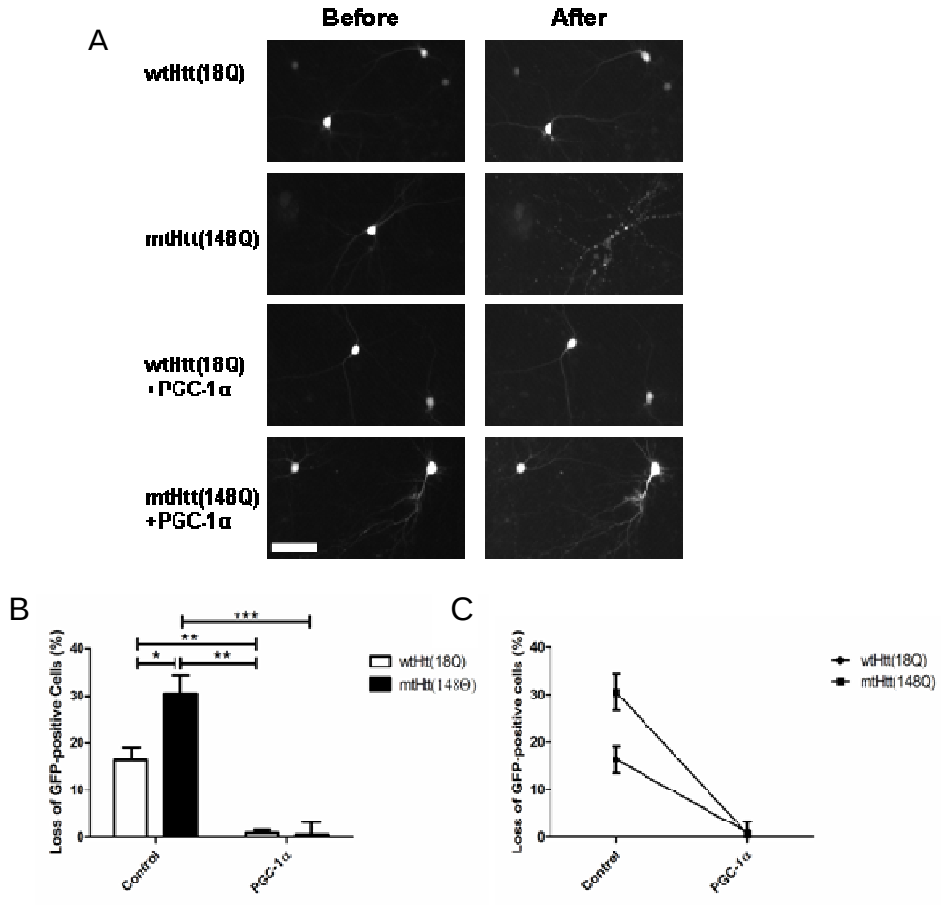

Figure 4.11 PGC-1α rescues mtHtt(148Q)-induced increase in excitotoxicity Cortical neurons were transfected with GFP plus either control (globin) or PGC-1**α** alongside either wtHtt(Q18) or mtHtt(148Q) (A) Images before and 24h after NMDA stimulation. Neurons transfected at DIV7 were imaged 48h later and stimulated with a low dose of NMDA (10 µM) for 1h. (B) Cell death was quantified as the loss of GFP-positive cells 24h post stimulation. In control cells, mtHtt (148Q) increased excitotoxic cell death (**t*-test $p<0.5$). Whereas co-transfection of PGC-1**α** dramatically rescued mtHtt(148Q)-induced NMDA vulnerability (mean cell death: 30.51 % ± 3.79, 0.5404 % ± 2.64 in control + mtHtt(148Q) and PGC-1**α** + mtHtt(148Q) respectively, ****t*-test $p<0.001$) (n=6,5,4,5 cultures for each culture, a total of 200-300 cells were analysed per group for each stimulation). (C) Two way ANOVA results show a significant interaction between PGC-1**α** -overexpression and mtHtt (148Q) -expression on loss of GFP-positive cells (Interaction p=0.024). This results indicates that in the context of PGC-1α overexpression the effect of mtHtt (148Q)

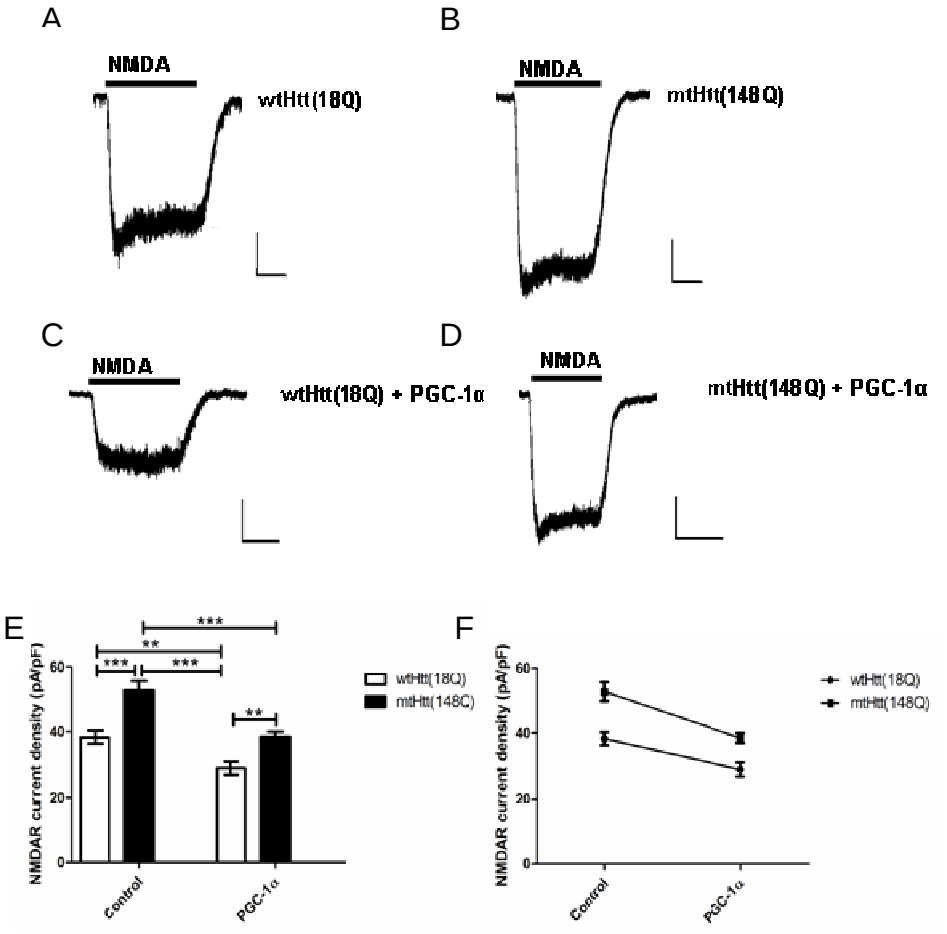

Figure 4.12 PGC-1α rescues mtHtt (148Q)-induced increase in NMDAR current density Agonist-evoked whole-cell currents from cortical neurons transfected with (A, C) wtHtt (18Q) or (B, D) mtHtt (148Q) in the presence of either (A, B) control (globin) plasmid or (C,D) PGC-1α. (E) PGC-1α overexpression caused a significant decrease in NMDAR-current density in both wtHtt(18Q) and mtHtt(148Q)-expressing neurons (** *t*-test $p<0.01$, ***t-test $p<0.001$). Therefore, even though mtHtt(148Q)-expression increased NMDAR-current density compared to wtHtt(18Q) in both control and PGC-1α-expressing neurons(*t*-test ***$p<0.001$, **$p<0.01$), in PGC-1α-expressing neurons this resulted in levels similar to control (mean: 38.29 pA/pF ± 1.98, 38.48 pA/pF ±1.5 respectively). (n= 30,19,43, 28 cells from 4 cultures (A-D respectively).
(F) Two-way ANOVA results indicate mtHtt(148Q) has a similar effect in control or PGC-1α -expressing neurons. Scale bar represents 5 s by 300 pA.

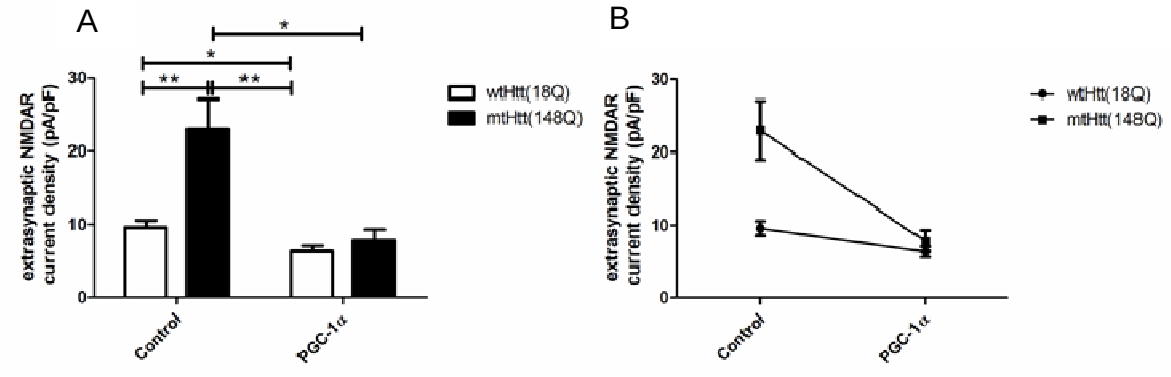

Figure 4.13 PGC-1α overexpression rescues mtHtt(148Q)-induced increase in extrasynaptic current density (A) Overexpression of PGC-1α occluded the effect of mtHtt(148Q)-expression of extrasynaptic NMDAR current density (n=7,5,4,4 stimulations from 4 cultures **t*-test $p<0.05$ $^{**}p<0.01$) (B) Two way ANOVA results show a significant interaction between PGC-1α overexpression and mtHtt(148Q)-expression on extrasynaptic NMDAR currents (Interaction P=0.006).

4.2.13 Striatal neurons

Many studies have reported that the medium spiny neurons of the striatum are particularly vulnerable to mtHtt toxicity (Ferrante et al. 1991; Graveland et al., 1985; Klapstein et al., 2001). In HD patients a specific loss of PGC-1α is observed in the caudate nucleus of the striatum (Cui et al., 2006). We therefore wanted to test the relative effects of mtHtt and PGC-1α on the vulnerability to excitotoxicity and NMDAR currents in striatal cultures.

One major caveat to the study of striatal NMDAR population is that glutamatergic inputs onto striatal neurons originate from outside the striatum. We therefore hypothesised that changing our cell culture protocol to enrich for striatal neurons restricts the glutamatergic input these cells receive in culture, and therefore we cannot guarantee the (stable) presence of spontaneous release of glutamate in such cultures and we can no longer use the 'quantal block' method to isolate extrasynaptic NMDAR currents.

However, we were able to test whether mtHtt expression and PGC-1α knockdown have an additive effect on total NMDAR currents and excitotoxicity in cultured striatal cells, enriched with DARPP-32 positive neurons. Secondly, we examined whether PGC-1α could rescue mtHtt-increase in toxicity and NMDAR currents in striatal cultures.

4.2.14 A non-additive increase in excitotoxicity by mtHtt expression and PGC-1α knockdown in striatal cultures

Striatal neurons were transfected with wtHtt(18Q) or mtHtt(148Q) in the presence of either control non targeting siRNA or PGC-1α targeting siRNA (PGC-1α siRNA(i)). Images were taken before and 24h after NMDA stimulation and cell death was quantified as the percentage loss of GFP-positive cells. Both mtHtt(148Q) and PGC-1α knockdown caused an increase in NMDA-induced cell death in control cells. No significant increase in cell death by mtHtt(148Q) compared to wtHtt(18Q)-expressing cells was observed in cells co-expressing PGC-1α siRNA. Thus, PGC-1α knockdown and mtHtt(148Q) expression increased NMDAR in a non-additive manner (Fig 4.12B).

4.2.15 PGC-1α rescues mtHtt-increase in excitotoxicity in striatal cells

We next assessed whether PGC-1α reverses mtHtt-induced increase in excitotoxic vulnerability in striatal cells as it does so in cortical cells. Striatal neurons were transfected with GFP plus either control (globin) or PGC-1**α** alongside either wtHtt (18Q) or mtHtt (148Q). Neuronal death was stimulated with a toxic dose of NMDA (20 µM) for 1h. In control cells, mtHtt (148Q) increased excitotoxic cell death (**t*-test $p<0.5$). Whereas co-transfection of PGC-1**α** dramatically rescued mtHtt (148Q)-induced NMDA vulnerability (Fig 4.12C).

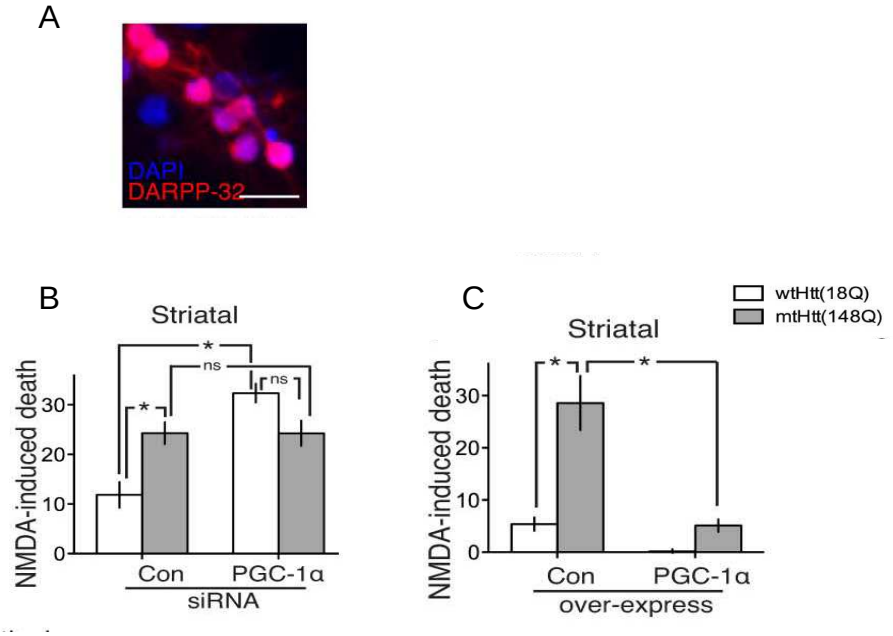

Figure 4.14 NMDA-induced death in mtHtt-expressing cells in striatal cultures in the context of PGC-1α knockdown or overexpression

(A) DARPP-32 -enriched striatal cultures. In our hands striatal cultures were approx. 50% DARPP-32^{+}.**(B)** Striatal neurons were transfected with wtHtt(18Q) or mtHtt(148Q) in the presence of either control non targeting siRNA or PGC-1α targeting siRNA (PGC-1α siRNA(i)). Both mtHtt(148Q) and PGC-1α knockdown caused an increase in NMDA (10μM)-induced cell death in control cells. No significant increase in cell death by mtHtt(148Q) compared to wtHtt(18Q)-expressing cells was observed in cells co-expressing PGC-1α siRNA. **(C)** Striatal neurons were transfected with GFP plus either control (globin) or PGC-1**α** alongside either wtHtt(Q18) or mtHtt(148Q). Neuronal death was stimulated with a toxic dose of NMDA (20 μM) for 1h. In control cells, mtHtt(148Q) increased excitotoxic cell death (*t-test $p<0.5$). Whereas co-transfection of PGC-1**α** dramatically rescued mtHtt(148Q)-induced NMDA vulnerability ($p<0.05$, n=3 cultures, 50-100 cells were analysed per group for each stimulation)

4.2.16 In striatal cultures, mtHtt and PGC-1α knockdown increase NMDAR currents non-additively

To test whether mtHtt and PGC-1α alter NMDAR currents in striatal neurons and whether they do so in an additive way, we carried out the following experiment. Striatal neurons were transfected with wtHtt(18Q) or mtHtt(148Q) in the presence of either control siRNA or PGC-1α siRNA. Reflecting results from studies in cortical neurons, whole-cell currents were increased by both mtHtt(148Q)-expression and by PGC-1α knockdown in striatal cultures. However, as we saw in the cortical neurons, in the context of PGC-1α knockdown, mtHtt(148Q) caused no further increase in NMDAR currents compared to wtHtt(18Q)-expressing cells (Fig 4.13A). This result suggests that in the absence of PGC-1α mtHtt can no longer exert its effects on NMDAR current density. From this we can extract that mtHtt and PGC-1α knockdown share a common mechanism in the regulation of NMDAR currents in striatal neurons and potentially, mtHtt requires the presence of PGC-1α to be able to drive NMDAR currents away from baseline levels. An alternative explanation is that intrinsic regulation of NMDAR expression prevents extrasynaptic NMDARs increasing above a certain threshold. Given that the excitotoxic cell death is also non-additive in mtHtt(148) and siRNA-expressing cells, we can assume that the failure to see an additive effect of mtHtt(148Q) and siRNA is not due to excitotoxicity of high-expressing cells.

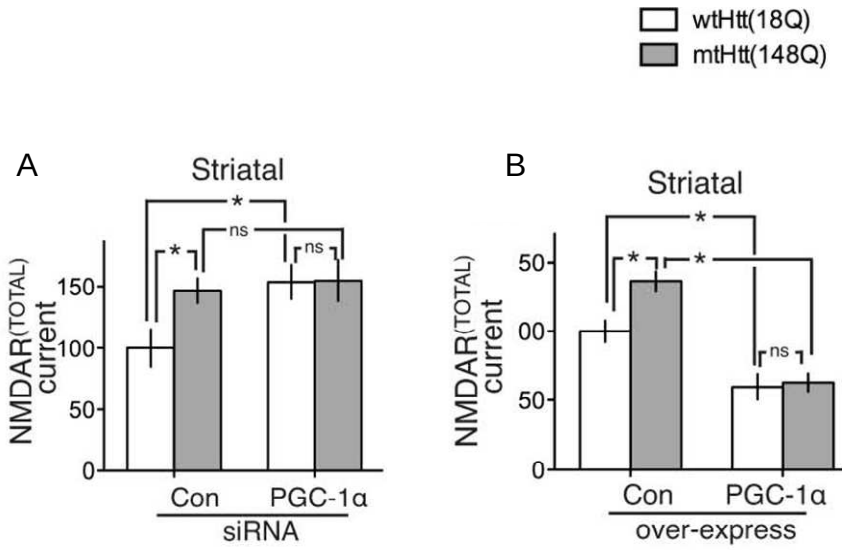

Figure 4.15 In striatal cultures, mtHtt and PGC-1α knockdown increase NMDAR currents non-additively, while PGC-1α overexpression rescues mtHtt-induced increase in currents (A) Striatal neurons transfected with wtHtt(18Q) or mtHtt(148Q) in the presence of either control siRNA or PGC-1α siRNA. Whole-cell current density was increased by mtHtt(148Q)-expression in control siRNA-expressing neurons. Concurrently, siRNA knockdown of PGC-1α caused an increase in NMDAR-current density in wtHtt(18Q)-expressing cells. However, no significant difference in NMDAR current density was observed in cells co-expressing wtHtt(18Q) versus mtHtt(148Q) in PGC-1α siRNA-expressing cells. (B) Striatal neurons transfected with wtHtt(18Q) or mtHtt(148Q) in the presence of either control (globin) plasmid or PGC-1α. PGC-1α overexpression caused a significant decrease in NMDAR current density. Moreover, in cells expressing exogenous PGC-1α, mtHtt no longer increased NMDAR currents (ttest *$p < 0.05$; $n = 5–7$ (A) and 6-8 (B)).

4.2.17 PGC-1α overexpression rescues mtHtt-induced increase in currents

In preceding experiments, we describe the ability of PGC-1α to protect against mtHtt-induced increase the total whole-cell and extrasynaptic NMDAR currents in cultured cortical neurons. We now show that PGC-1α can protect against excitotoxic cell death in striatal neurons and investigate whether this is concurrent with the repression of mtHtt-induced increase in NMDAR currents. To investigate whether PGC-1α could rescue augmented NMDAR currents in mtHtt-expressing striatal neurons, the cells were transfected with wtHtt(18Q) or mtHtt(148Q) in the presence of either control (globin) plasmid or PGC-1α. PGC-1α overexpression caused a significant decrease in NMDAR current density in cells expressing wtHtt(18Q). Furthermore, exogenous expression of PGC-1α prevented mtHtt-mediated increases in NMDAR currents (Fig. 4.13B).

This result is consistent with our results in cortical cells and agrees with the anti-excitotoxic capacity of PGC-1α in striatal neurons presented above (Fig 4.14). In addition this present a novel explanation for the neuroprotection of virally expressed PGC-1α in the striatum of HD mice reported by Cui et al., (2006)

Summary: These data support the notion that mtHtt-repression of PGC-1α contributes to the deregulation of NMDAR currents and excitotoxicity in striatal neurons as well as cortical neurons, the major sites of neurodegeneration in HD.

4.3 Discussion

This study confirmed our hypothesis, that pathological upregulation of extrasynaptic NMDAR activity by mutant huntingtin is occluded by PGC-1α knockdown and conversely rescued by PGC-1α overexpression in both cortical and striatal neurons. This finding suggests that the mutant huntingtin repression of PGC-1α is mechanistically linked to the pathological extrasynaptic activity in HD.

4.3.1 Consequences for neurological disease

HD: A reciprocal relationship between mtHtt-toxicity and the balance of synaptic and extrasynaptic NMDAR activity was identified by two independent studies in 2009-10. Okamato et al., (2009) found that synaptic NMDAR activity suppressed mtHtt-toxicity by inducing the expression of the chaperonin subunit TCP-1 which leads to formation of non-toxic inclusions of the mutant huntingtin protein. In contrast, extrasynaptic NMDARs activate the GTP-binding protein Rhes, which causes sumoylation and disaggregation of mutant huntingtin (Okamoto et al., 2009; Subramaniam et al., 2011b). In addition, Milnerwood et al., (2010) described an increase in extrasynaptic NMDAR-activity, CREB-dephosphorylation and increased toxicity in the YAC128 HD mouse. MtHtt is known to disrupt the CREB-target PGC-1α by both directly interacting with and suppressing the PGC-1α promoter and by the repression of CREB-activity downstream of exaggerated extrasynaptic NMDAR activity. Together these results present a complex three-tiered feedforward signaling cascade. The presence of mutant huntingtin in HD represses the CREB-driven PGC-1α expression. Loss of PGC-1α increases extrasynaptic NMDAR activity. Extrasynaptic NMDARs disrupt both inclusion formation and the CREB-signaling cascade causing further

repression of PGC-1α. The current study builds on our previous understanding that the mtHtt-repression of PGC-1α contributes to HD phenotype by its known regulation of mitochondrial function and biogenesis. The mechanisms behind the imbalance of extrasynaptic and synaptic NMDAR signaling downstream of PGC-1α remain unknown. Milnerwood et al., (2010) describe an increase in protein expression of both GluN1 and GluN2B in 'non-PSD' membrane fractions from striatal and cortical cells, consistent with the ifenprodil-sensitivity of augmented extrasynaptic NMDAR activity this is attributed to increased forward trafficking of GluN2B-containing receptors (Fan et al., 2007; Milnerwood et al., 2010).

The mechanisms behind increased NMDAR expression in HD remain relatively unexplored. However, many studies point to defects in protein-protein interactions and post-translational modifications of NMDARs in HD that may lead to defects in synaptic targeting. Endoplasmic retention and forward trafficking is mediated by PKA phosphorylation of GluN1 S896 and S897 (Tingley et al., 1997; Scott et al., 2003). There are reports of increased GluN1 S897 phosphorylation in the HD striatum, however this is still under dispute (Jarabek et al., 2004; Ariano et al., 2005). Retention of NMDARs at the plasma membrane is regulated by interactions with PSD-95, enhanced binding of GluN2B to PSD-95 may alter extrasynaptic stability of NMDARs (Fan et al., 2009; Milnerwood et al., 2010). In addition, elevated calpain levels are reported in the YAC HD mouse model (Cowan et al., 2008). As discussed in chapter 2. calpain cleavage of the GluN2B C-terminus disrupts interactions with proteins of the post synaptic density, and has been found to increase functional membrane bound receptors (Prybylowski et al., 2005). Our results indicate that mtHtt-repression of PGC-1α is part of the signaling cascade that leads

to increased extrasynaptic NMDAR activity, but the common mechanisms downstream await investigation.

Alzheimer's disease: Alzheimer's disease (AD) is an age-related progressive disorder characterised by the formation of two classical lesions: senile plaques composed of amyloid-β peptides and neurofibrillary tangles (Selkoe, 2001). A recent study by Qin et al. (2009) reports the decline of PGC-1α mRNA and protein concurrent with increased dementia rating, Aβ plaque formation and Aβ peptide in AD human tissue. In addition, exogenous PGC-1α expression was able to reverse hyperglycemia induced amyloidogeneis.

In striking resemblance to HD a reciprocal relationship has been discovered between the balance of synaptic and extrasynaptic NMDAR activity and the toxicity of Aβ peptides. Excess Aβ peptide in Alzhiemer's disease brain is thought to selectively reduce glutamatergic synaptic transmission by reducing synaptic retention of NMDARs (Snyder et al., 2005). Aβ is though to prevent the degradation of the tyrosine phosphatase STEP with leads to inactivation of the tyrosine kinase Fyn, as well as enhanced GluN2B Y1472 dephosphorylation, resulting in reduced exocytosis and increased endocytosis of synaptic NMDARs (Kurup et al., 2010). In return, chronic extrasynaptic NMDAR activity increases the production of Aβ peptides, exacerbating toxicity (Bordji et al., 2010). Combining this with our findings, PGC-1α is able to reduce the production of Aβ peptide by reducing amyloidogeneis (Qin et al., 2009) and indirectly by the repression of extrasynaptic NMDAR activity and subsequent Aβ production.

4.3.2 Current therapeutic targets: Extrasynaptic NMDAR activity

Given the necessity of synaptic NMDAR signaling for cell survival and function, pharmacological interventions for the prevention of excitotoxic cell death associated with neurodegenerative disease have been complicated. In accord, the use of NMDAR antagonists for the treatment of excitotoxicity associated with ischemic brain injury in stroke proved to be poorly tolerated and ineffective (Ikonomidou & Turski 2002; Muir 2006). Current understanding of the dichotomy of NMDAR signaling to pro-death and pro-survival cascades depending on their location means that selective targeting of the extrasynaptic NMDAR cascade is desired. The biophysical properties of the NMDAR antagonist memantine enable it to be both effective at reducing excitotoxic damage and well tolerated *in vitro* and *in vivo* (Chen & Lipton 2006; Chen et al. 1998; Léveillé et al. 2008). Firstly, memantine is a non-competitive, open channel blocker of NMDARs and in such is highly effective at blocking the prolonged activation of extrasynaptic NMDARs (Chen & Lipton 2006). In contrast, the voltage-dependence and fast-off rate of memantine means it does not accumulates in the synaptic cleft and is ineffective at blocking synaptic NMDARs at low doses (Chen & Lipton 2006; Xia et al. 2010). Results from two independent groups support the potential neuroprotection afforded by memantine antagonism of extrasynaptic NMDARs in HD. (Okamoto et al., 2009, Milnerwood et al., 2010). Low dose memantine was shown to restore phospho-CREB activity in the YAC128 striatum consistent with antagonism of the dominant extrasynaptic CREB-shut off pathway (Milnerwood et al., 2010). In addition injection of low dose memantine at either 2 months (Milnerwood et al., 2010) or 12 months (Okamoto et al. 2009) prevented motor learning deficits in a HD mouse model.

5.3 Future potential: Targeting PGC-1α

The observation that PGC-1α expression can repress neurotoxic extrasynaptic NMDAR currents and reverse the vulnerability of HD models, proposes an alternative target to intervene against excitotoxicity aside from pharmacological antagonism of the receptors themselves. Due the diverse functions of PGC-1α targets in an array of tissues, strategies for enhancing its function would ideally be tissue specific, if not target specific. Furthermore, transcriptional coactivators by definition do not possess DNA or ligand binding domains; as a result, pharmacological activation of PGC-1α is unfeasible. Regulation of PGC-1α activity is restricted to increasing its expression, stability or interaction with its targets.

Transglutaminase 2 is a selective corepressor of nuclear genes and is upregulated in HD (Karpuj et al., 2002). A recent study has shown that PGC-1α is a target of TG2 activity and inhibition of TG2 is sufficient to derepress both PGC-1α and cytochrome C in HD models (McConoughey et al., 2010). This was achieved not only by RNAi knockdown and genetic knockout of TG2, but also by the application of a novel peptide, the transglutaminase inhibitor ZDON. ZDON restored PGC-1α expression and protects YAC128 primary striatal neurons from NMDA-excitotoxicity (McConoughey et al., 2010). Interesting, the ameliorating effects of ZDON were not associated with alterations in mitochondrial biogenesis, consistent with the additional neuroprotective properties of PGC-1α expression outside the mitochondria as described in this study.

Chapter 5:

PGC-1α knockout mice have alterations in AMPA but not NMDA- type glutamate receptors

5.1 Introduction

PGC-1α is highly expressed in multiple brain regions in mice including cerebral cortex, striatum and substantia nigra (Tritos et al., 2003). Previous studies in mice have shown PGC-1α expression to protect against neuronal stress in a number of neurodegenerative diseases including HD, Alzhiemer's disease, Parkinson's disease, Amyotrophic lateral sclerosis and Ischemia (Chen et al., 2010; Cui et al., 2006; Luo et al., 2009; Qin et al., 2009; Shin et al., 2011; Zhao et al., 2011). In addition to our findings of the role of PGC-1α in excitotoxicity (Soriano et al., 2011; Puddifoot et al., 2012) this clearly demonstrates a functional role for PGC-1α signalling in the brain. In accord two independent groups have reported neurological impairment in two PGC-1α knockout (KO) mouse lines discussed below.

Lin et al., (2004) created PGC-1α null mice by LoxP deletion of exon 3-5; although these mice were viable through embryogenesis only 50% survived to adulthood. Lin et al., (2004) showed that in addition to brain lesions in the striatum and cortical layers V/VI, the nucleus accumbens, substantia nigra, hippocampus and mammalliary body, 3 month old PGC-1α knockout mice displayed behavioural abnormalities including exaggerated startle response, dystonic postering and frequent limb clasping. Finally, primary striatal neurons isolated from the PGC-1α knockout mice showed aberrant neurite outgrowth (Lin et al., 2004).

Another PGC-1α KO mouse was created by Leone et al., (2005) using neomycin based gene targeting of exon 4-5 of PGC-1α resulting in a recombination/insertion of a repeat in exon 3, ultimately causing premature termination at amino acid 255 which results in an unstable PGC-1α transcript (Leone et al., 2005). These KO mice displayed increased anxiety accompanied by microvacuolation in the pyramidal neurons of the basal ganglia, cerebral cortex, hippocampus and brainstem (Leone et al., 2005).

PGC-1α null mice were also more sensitive to the neurodegenerative effects of the oxidative stressors MPTP and kainic acid affecting the substantia nigra and hippocampus, respectively (Leone et al., 2005).

Given the changes in NMDA-type glutamate receptor expression and potent neuroprotection downstream of mutant huntingtin repressed-PGC-1α, described in chapters 3 and 4 (now published; Puddifoot et al., 2012), we proposed that alterations in glutamate signalling may contribute to the neurological phenotypes observed in the PGC-1α knockout mouse.

In order to test our hypothesis we used cortical neurons from the PGC-1α knockout mice created by AstraZeneca Transgenic and Comparative Genomics, Sweden (D'Errico et al., 2011). We obtained PGC-1α KO and WT pups from Alberto Camacho Morales (Cambridge); knockout pups contain LoxP delta deletion between exons 3-5 of PGC-1α gene. PGC-1α KO and WT mice were produced by crossing heterozygote mice. Subsequently, neuronal cultures were made from E.16.5 PGC-1α$^{(-/-)}$ and PGC-1α $^{(+/+)}$ pups.

The chronic loss of PGC-1α in the knockout mouse enables us to investigate whether losing PGC-1α throughout development may affect glutamatergic signalling both at post synaptic and presynaptic sites. Transient knockdown of PGC-1α described in chapters 3 and 4 is limited to studying acute changes in the post synaptic cell due to the specific knockdown in a small (<5%) number of cells making it highly unlikely that presynaptic cells incorporate the siRNA. Finally, whereas low/variable efficiency of transfection hinders mechanistic insight into functional changes after acute knockdown of PGC-1α, RNA analysis of the PGC-1α knockout is possible in this model.

5.2 Results

5.2.1 PGC-1α$^{(-/-)}$ display no change in whole-cell NMDAR currents

Cortical neurons cultured from embryonic day 16.5 PGC-1α$^{(-/-)}$ and PGC-1α $^{(+/+)}$ mice were used to study the expression of two major glutamate receptors; AMPAR and NMDAR. Knock out and wildtype pups were bred by crossing two PGC-1α heterozygotes; each pup was then cultured independently enabling us to compare neurons from PGC-1α $^{(+/+)}$ and PGC-1α$^{(-/-)}$ littermates. Both AMPA and NMDA-receptors are essential for excitatory neurotransmission in the central nervous system and the regulation of their expression both independently and with respect to each other has been the focus of many neurophysiological studies (reviewed by Rousseaux et al., 2008).

Our specific interest in the expression profile of NMDARs is due to their critical role in neuronal health (Hardingham and Bading, 2010). Activation of synaptic NMDARs bestows long-lasting neuroprotection, whereas activation of NMDARs located outside of the synapse triggers pro-death signalling cascades (Hardingham et al., 2002). In chapter three, we have shown that neurons lacking PGC-1α, due to targeted siRNA knockdown of PGC-1α in neuronal cultures, are more susceptible to NMDA-induced cell death and have greater amplitude of whole-cell NMDAR currents, resulting from the preferential enhancement of extrasynaptic NMDAR currents.

We undertook our studies in the PGC-1α knockout mice on observation of increased whole cell current amplitude and cell death after siRNA knockdown of PGC-1α *in vitro*, but prior to our understanding of the

distinct role played by extrasynaptic NMDARs in this cascade. We therefore hypothesised that the PGC-1α knockout mouse would display increased neuronal vulnerability and increased NMDAR currents. To investigate cell vulnerability, our colleague, Karen Bell, carried out cell death assays on neuronal cultures from the PGC-1α$^{(-/-)}$ and PGC-1α$^{(+/+)}$ mice. Low dose NMDA (10 µM) was used; a dose sufficient to induce death after PGC-1α knockdown with siRNA *in vitro* (Chapter 3). Cells were stimulated with NMDA (10 µM) for 1h, after which the stimulation was stopped by applying the NMDAR antagonist MK-801 (10 µM). 24h post stimulation, the cells were fixed with 4% PFA and DAPI was used to stain the cell nulcei. Images of the cells were taken to enable quantification of cell death based on the morphology of the nucleus. NMDA stimulation had comparable effect on PGC-1α$^{(+/+)}$ and PGC-1α$^{(-/-)}$ neurons (Data not shown), indicating no change in the vulnerability of these cells to this insult.

We next tested whether the change in whole-cell NMDAR currents after siRNA knockdown of PGC-1α was mimicked in the PGC-1α knockout neurons. To do this, we used whole-cell patch clamp to hold cultured cortical neurons from PGC-1α$^{(-/-)}$ and PGC-1α$^{(+/+)}$ mice at -60mV in Mg^{2+}-free external recording solution. We measured the current response to bath application of NMDA (100 µM) in cultured cortical neurones. No changes in whole-cell NMDAR current density was observed in PGC-1α$^{(-/-)}$ compared to PGC-1α$^{(+/+)}$ mice (Fig 5.1C). In order to calculate the current density, we measure the cell capacitance of the neurons. The capacitance is proportional to the membrane surface area of the cell, and thus represents the size of the neuron. We found no difference in the capacitance of neurons from PGC-1α$^{(-/-)}$ and PGC-1α$^{(+/+)}$ mice (Fig 5.1D).

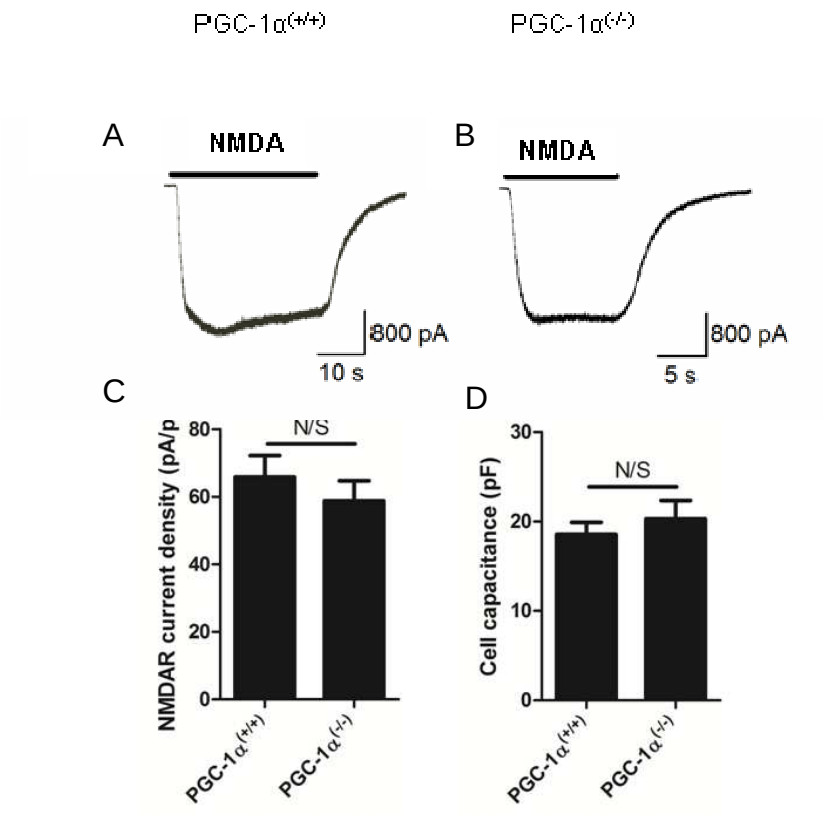

Figure 5.1 No change in NMDAR-current density was observed in neurons from PGC-1α$^{(-/-)}$ mice Agonist evoked whole-cell NMDAR currents were recorded from (A) PGC-1α(+/+) and (B) PGC-1α(-/-) mouse cortical neurons. Neurons were placed in Mg^{2+}-free external recording solution and voltage-clamped at -60 mV using whole-cell patch-clamp technique. NMDAR currents were evoked by applying NMDA (100 µM) until the cell reached a steady-state. (C) NMDAR current density was calculated as the steady-state current amplitude normalised to the cell capacitance. No difference was observed in NMDAR current density between PGC-1a+/+ and PGC-1a(-/-) neurons. (D) We observed no change in cell membrane capacitance; an estimate of membrane surface area . * paired t test p=0.31 (n=6 pups from independent litters compared to WT littermate control. The statistical n represents each pup and is the mean of 4-14 cells from each pup, ttest p>0.05).

5.2.2 PGC-1α$^{(-/-)}$ mice have reduced AMPAR current density

Despite observing no change in NMDAR whole-cell currents/NMDA-induced death, given the striking neurological phenotype previously reported in PGC-1α knockout lines, we were interested in investigating whether changes in another subtype of glutamate receptors, the AMPA receptors, may occur. AMPA receptors are responsible for fast excitatory neurotransmission in the CNS (Hollmann and Heinemann, 1994) and the dynamic regulation of AMPA receptor insertion and internalisation from the synaptic membranes plays an integral role in synaptic plasticity (reviewed by Malenka, 2003).

AMPA receptors are named after the artificial glutamate analog AMPA which selectively activates this subclass of receptors. AMPARs are tetramers composed of four types of subunits, designated as GluA1, GluA2, GluA3, and GluA4. Unlike NMDARs, most AMPARs have very low permeability to Ca^{2+} ions due to the incorporation of an edited GluA2 subunit which has a positive arginine residue which repels Ca^{2+} ions. This is thought to prevent AMPAR activity from contributing to excitotoxicity (Kim et al., 2001). However, AMPA receptor activity is necessary to depolarise the cell and relieve the Mg^{2+}-block from NMDAR pores, and therefore plays a role in their activation.

To assess whether neurons from PGC-1α$^{(-/-)}$ mice have changes in AMPAR expression, we used whole-cell patch clamp to record AMPA-induced currents in cultured cortical neurons from PGC-1α$^{(-/-)}$ and PGC-1α$^{(+/+)}$ mice. Neurons were voltage clamped at -60mV in external recording solution. We measured the current response to bath application of AMPA (50 μM). AMPAR steady state current response was normalised to cell

capacitance to calculate the AMPAR current density in these cells. We observed a significant reduction in AMPAR current density in PGC-1α$^{(-/-)}$ neurons compared to PGC-1α$^{(+/+)}$ (Fig 5.2C).

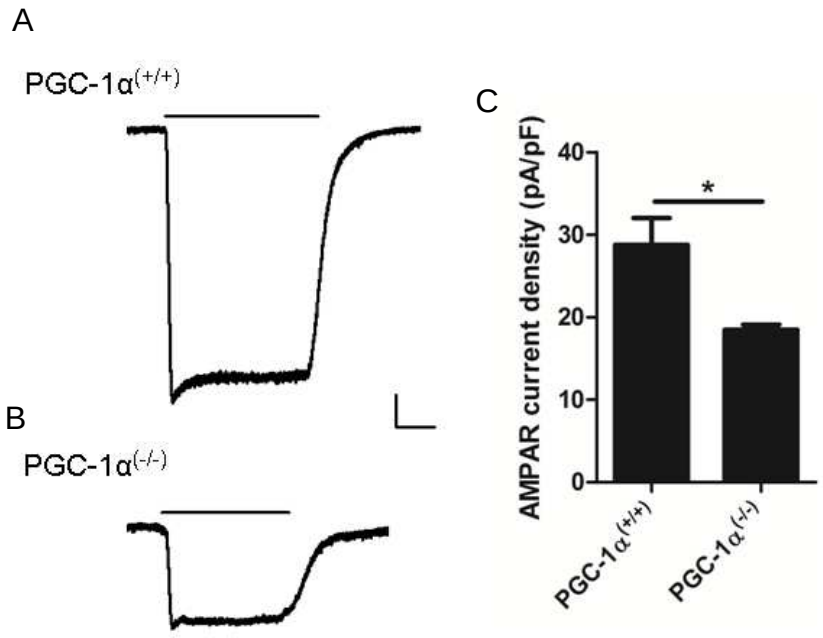

Figure 5.2 Cortical neurons from PGC-1α$^{(-/-)}$ mice had reduced agonist-evoked AMPAR current density compared to wild type controls Agonist evoked whole-cell AMPAR currents were recorded from (A) PGC-1α$^{(+/+)}$ and (B) PGC-1α$^{(-/-)}$ mouse cortical neurons. Neurons were placed in external recording solution and voltage-clamped at -60 mV using whole-cell patch-clamp technique. AMPAR currents were evoked by applying AMPA (50 µM) until the cell reached a steady-state. (C) AMPA-current density was calculated as the steady-state current amplitude normalised to the cell capacitance. We observed a significant reduction in AMPAR current density in PGC-1a$^{(-/-)}$ neurons compared to PGC-1α $^{(+/+)}$. *paired t test $p<0.05$ (n=4 pups from independent litters compared to WT littermate control. The statistical n represents each pup and is the mean on 6-12 cells from each pup).

5.2.3 PGC-1α$^{(-/-)}$ mice have reduced AMPAR GluA1-4 mRNA expression

The current response to bath application of the AMPA-receptor agonist AMPA (50 μM) depends on a number of variables including (i) the number of receptors expressed on the cell (ii) the conductance of each receptor channel (iii) the efficacy of agonist binding and channel activation. It is well accepted that both subunit composition and post-translation modifications contribute to the diversity of AMPAR responses and can play a key role in synaptic plasticity (reviewed by Lu and Roche, 2011). However, since in the absence of external signalling, steady state protein levels are highly dependent on mRNA expression (Lu et al., 2007), in order to determine whether any changes in expression of each AMPAR subunit contribute to decreased AMPAR responses, we analysed RNA extracted from neuronal cultures from PGC-1α$^{(-/-)}$ and PGC-1α$^{(+/+)}$ mice using qPCR. Interestingly, PGC-1α$^{(-/-)}$ neurons have lower mRNA expression of all four AMPA receptor subunits; GluA1, GluA2, GluA3, and GluA4 (Fig 5.3).

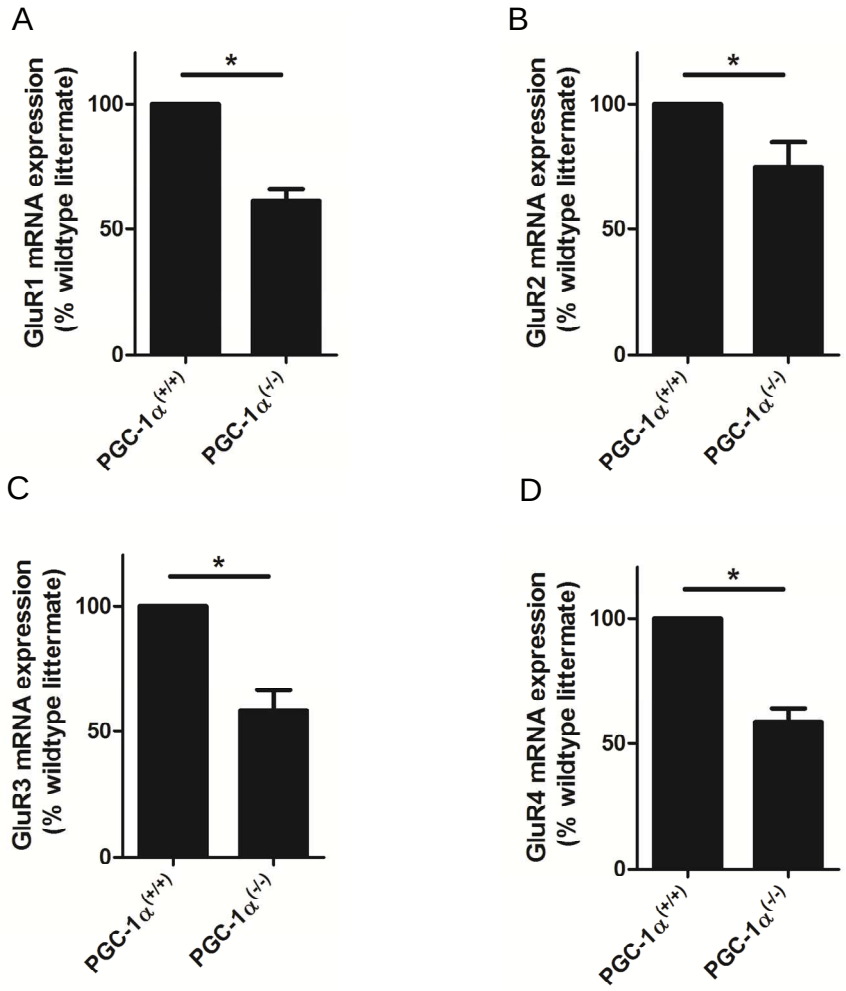

Fig 5.3 PGC-1α$^{(-/-)}$ neurons have reduced mRNA expression of AMPAR subunits GluA1-4 RNA was collected DIV9 from neuronal cultures from PGC-1α$^{(-/-)}$ and PGC-1α$^{(+/+)}$ mice. Quantification of mRNA expression was done by qPCR. (A) PGC-1α$^{(-/-)}$ neurons have lower expression of GluA1 (B) GluA2 (C) GluA3 (D) GluA4 mRNAs (n = 7 PGC-1α$^{(-/-)}$ and 7 PGC-1α$^{(+/+)}$ mice from 7 independent litters; paired *t test* $p<0.05$).

5.2.4 Cortical neurons from PGC-1α$^{(-/-)}$ mice have reduced mEPSC frequency and amplitude

Given the evidence for lowered AMPAR expression in the PGC-1α$^{(-/-)}$ neurons we hypothesised that knockout cells may have smaller AMPAR quantity at synapses. If this was the case, we would expect to see a drop in the amplitude of mEPSCs in the knockout neurons. To test our hypothesis we recorded miniature excitatory post synaptic currents (mEPSCs) from PGC-1α$^{(-/-)}$and PGC-1α$^{(+/+)}$ neurons and analysed the frequency and amplitude of AMPAR-mediated mEPSCs. Neurons were placed in recording solution supplemented with PTX (50 µM), TTX (300 nM) and Mg^{2+} (1.3mM; this blocks NMDAR activity). mEPSCs were recorded in voltage clamp (-70 mV) for 5-10mins using whole-cell patch clamp. We observed a reduction in both the mean amplitude (Fig.5.3C) and mean frequency (Fig.5.3D) of mEPSC events in the PGC-1α$^{(-/-)}$ cells. Further analysis showed that the amplitude distribution of PGC-1α$^{(-/-)}$ neurons was skewed to the left, indicating an absence of high amplitude events (Fig.5.4E). Likewise the distribution of inter-event-interval (i.e.i) is altered in the PGC-1α$^{(-/-)}$ neurons (Fig5.3F). The i.e.i is the time between mEPSC events, a trend towards higher i.e.i indicates the absence of high frequency events.

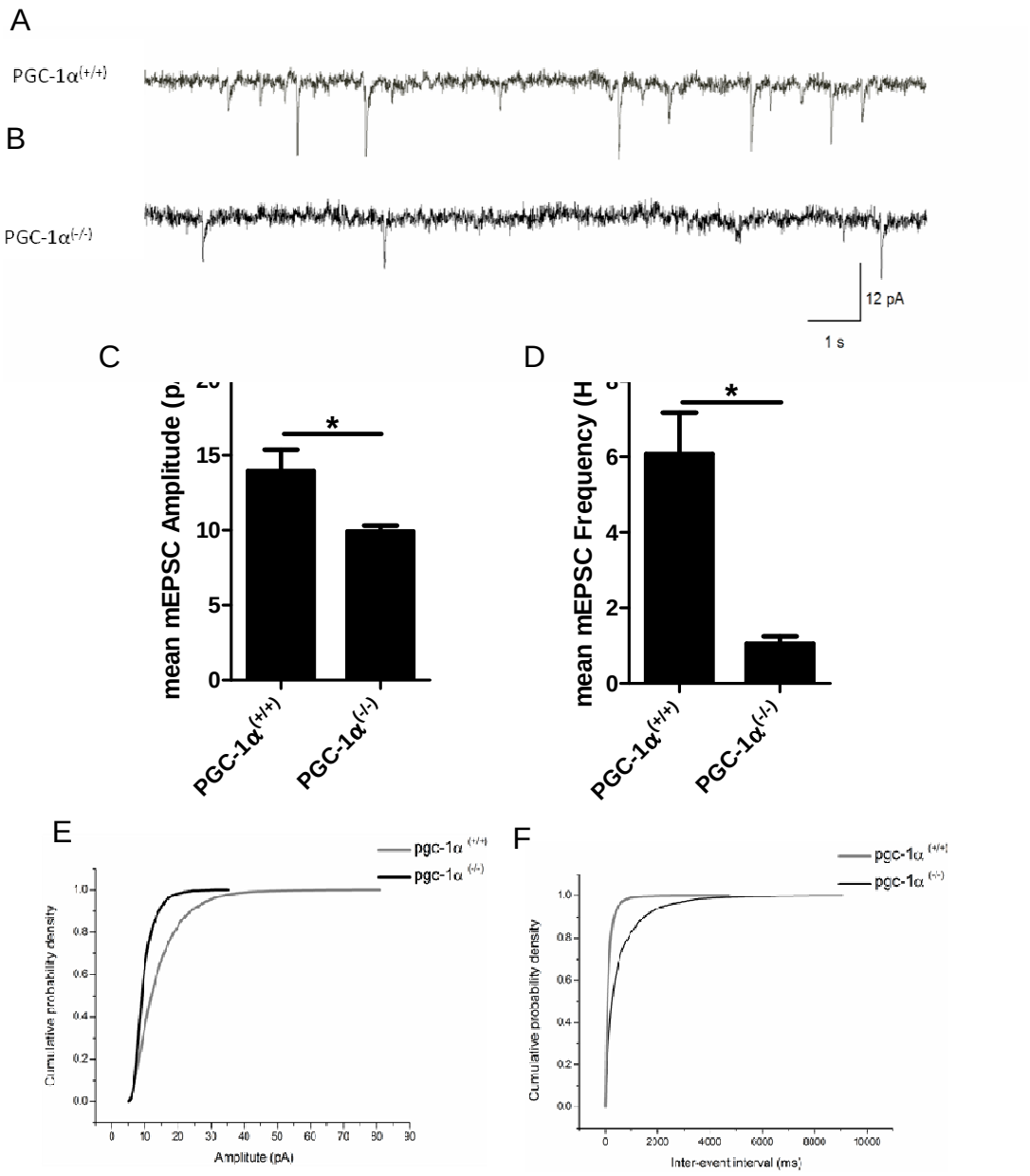

Figure 5.4 Cultured cortical neurons from PGC-1α$^{(-/-)}$ mice have reduced mEPSC frequency and amplitude (A) Example mEPSC traces from (A) PGC-1α$^{(+/+)}$ and (B) PGC-1α$^{(-/-)}$ mouse cortical neurons. Neurons were placed in recording solution supplemented with PTX (50 μM), TTX (300 nM) and Mg^{2+} (1.3mM). mEPSCs were recorded in voltage clamp (-70 mV) for 5-10 mins using whole-cell patch clamp. We observed a reduction in both the mean amplitude (C) and mean frequency (D) of mEPSC events in the PGC-1α$^{(-/-)}$ cells. (E) Furthermore, the amplitude distribution of PGC-1α$^{(-/-)}$ neurons was skewed to the left, indicating an absence of high amplitude events. (F) The distribution of inter-event-interval (i.e.i) is altered in the PGC-1α knockout mice. Mean amplitude and frequency *t test $p<0.05$; cumulative distributions KS test $p<0.05$ (from n= 6, 7, cells, each from 6 independent cultures, minimum 300 events were recorded per cell).

The 30% reduction in mEPSC amplitude corresponds to the (38%) reduction in whole cell current response to AMPAR application as well as the 25-40% reduction in AMPAR GluA1-4 subunit mRNA expression. Together this provides a strong argument for reduced AMPAR signalling in the PGC-1α knockout neurons.

Of interest is the change in mEPSC frequency in these cells. mEPSC frequency is determined by both the number of synapses on the post synaptic cell, and the frequency of release of glutamate from pre-synaptic cells. We have yet to investigate whether this is a presynaptic or postsynaptic mechanism. In order to do this, future experiments could include the overexpression of PGC-1α along with a fluorescent marker into knockout neurons, to test whether this recues the mEPSC profile and AMPAR expression in the post synaptic cell. Due to the mouse line no longer being available in the UK, these experiments were not carried out in this study. However the reduction in AMPAR mRNA and whole-cell currents suggest that control of AMPAR expression is disrupted in the PGC-1α$^{(-/-)}$ neurons. Our final analysis, outlined below, suggests that this may be exacerbated by reduced AMPAR exocytosis in the PGC-1α$^{(-/-)}$ neurons.

5.2.5 Decreased complexin I: a candidate for altered AMPAR exocytosis

Finally, with the aim of gaining mechanistic insight into changes in AMPAR profile in PGC-1α$^{(-/-)}$ we performed microarray analysis on PGC-1α$^{(-/-)}$ and PGC-1α$^{(+/+)}$ neurons. Two way ANOVA analysis of microarray data from PGC-1α$^{(-/-)}$ mice (n=3 PGC-1α$^{(-/-)}$ and 3 PGC-1α$^{(+/+)}$) from 3 independent litters indicates a significant effect of PGC-1α knockout on the regulator of exocytosis: complexin I (-1.8 fold change in complexin 1 expression in PGC-1α$^{(-/-)}$ compared to PGC-1α$^{(+/+)}$ $p<0.01$; Fig 5.5). We confirmed this reduction in complexin I in neuronal cultures from PGC-1α$^{(-/-)}$ compared to their littermate controls using qPCR (Fig 5.6). We also confirmed the reduction of the PGC-1α target cytochrome C (ref) as a positive control (Fig 5.6) (n = 7 PGC-1α$^{(-/-)}$ and 7 PGC-1α$^{(+/+)}$ mice from 7 independent litters). A recent study indicates a role for complexin I in AMPAR exocytosis (Ahmad et al., 2012), suggesting a possible mechanism of altered AMPAR expression in the PGC-1α$^{(-/-)}$ neurons, this is discussed below.

Transcript ID	gene_assignment	Gene Symbol	RefSeq	p-value(Knockout)	Fold-Change(K vs. W)
10429515	neurotoxin 1	Lynx1	NM_011838	0.0243824	-2.25167
10562637	cyclin B1	Ccnb1	NM_172301	0.0205658	-1.88321
10532180	complexin 1	Cplx1	NM_007756	0.00494886	-1.87722
10530029	leucine-rich repeat LGI family, member 2	Lgi2	NM_144945	0.0217192	-1.82116
10360053	Purkinje cell protein 4-like 1	Pcp4l1	NM_025557	0.0362984	-1.81874
10459138	solute carrier family 6 (neurotransmitter transporter, L-	Slc6a7	NM_201353	0.0349109	-1.80795
10364784	receptor accessory protein 6	Reep6	NM_139292	0.0191009	-1.77551
10411739	cyclin B1	Ccnb1	NM_172301	0.0227779	-1.75852
10570963	zinc finger	Zmat4	NM_177086	0.0358941	-1.67828
10455071	protocadherin beta 7	Pcdhb7	NM_053132	0.0173976	-1.67519
10579341	mitochondrial membrane protein-like 2	Mpv17l2	NM_183170	0.0258289	-1.63443
10583732	low density lipoprotein receptor	Ldlr	NM_010700	0.00846083	-1.63144
10364102	coiled-coil-helix-coiled-coil-helix domain containing 10	Chchd10	NM_175329	0.04714	-1.62595
10427162	major facilitator superfamily domain containing 5	Mfsd5	NM_134100	0.0370484	-1.62546
10594774	cyclin B2	Ccnb2	NM_007630	0.00837279	-1.62346
10566993	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylg	Galntl4	NM_173739	0.0185161	-1.58489
10514128	tetratricopeptide repeat domain 39B	Ttc39b	NM_027238	0.0466711	-1.58343
10526553	VGF nerve growth factor inducible	Vgf	NM_001039385	0.0369659	-1.57186
10456171	serine peptidase inhibitor, Kazal type 10	Spink10	NM_177829	0.0432687	-1.56523
10400095	interferon-related developmental regulator 1	Ifrd1	NM_013562	0.0462099	-1.56398
10390560	SH3 and cysteine rich domain 2	Stac2	NM_146028	0.0447392	-1.53977
10356345	natriuretic peptide precursor type C	Nppc	NM_010933	0.00753253	-1.53618
10594963	unc-13 homolog C (C. elegans)	Unc13c	NM_001081153	0.0282571	-1.52939
10476042	transglutaminase 3, E polypeptide	Tgm3	NM_009374	0.0313984	-1.52277
10478572	ubiquitin-conjugating enzyme E2C	Ube2c	NM_026785	0.00188072	-1.5221
10484307	frizzled-related protein	Frzb	NM_011356	0.0121291	-1.51989
10533401	cut-like homeobox 2	Cux2	ENSMUST00000111752	0.028687	-1.51033
10545835	RIKEN cDNA 1700040I03 gene	1700040I03Rik	BC115452	0.0393797	-1.50535
10518833	calmodulin binding transcription activator 1	Camta1	NM_001081557	0.00331519	-1.50483
10570634	RIKEN cDNA 4930467E23 gene	4930467E23Rik	ENSMUST00000098907	0.0110811	1.50702
10576328	melanocortin 1 receptor	Mc1r	NM_008559	0.0416114	1.51834
10408074	Hist1h4b // histone cluster 1	Hist1h4b	NM_178193	0.0468423	1.51913
10408225	histone cluster 1, H4c	Hist1h4c	NM_178208	0.0136299	1.52464
10583314	TATA box binding protein (Tbp)-associated factor, RNA polym	Taf1d	BC056964	0.0016867	1.52513
10362574	U3A small nuclear RNA	Rnu3a	NR_002842	0.0130753	1.59117
10570432	small nucleolar RNA, H/ACA box 3	Snora3	AF357390	0.0390803	1.60528
10556206	small nucleolar RNA, H/ACA box 3	Snora3	AF357390	0.0413357	1.60588
10598087	NADH dehydrogenase subunit 6	ND6	ENSMUST00000082419	0.00801001	1.71437
10465244	Malat1	Malat1	NR_002847	0.00167957	2.15965
10530406	gamma-aminobutyric acid (GABA) A receptor, subunit alpha	Gabra2	NM_008066	0.0253005	3.63057

Fig 5.5 Microarray analysis of cultured cortical neurons from PGC-1α$^{(-/-)}$ and PGC-1α$^{(+/+)}$ mice Microarray analysis identified genes with statistically different expression in the PGC-1α$^{(-/-)}$ and PGC-1α$^{(+/+)}$ neurons. This table presents all genes with greater than 1.5 fold decrease (grey) or increase (orange) in expression between wildtype and knockout neurons. (N= 3 KO and 3 WT littermates from 3 independent cultures). Complexin I (red) had one of the greatest decreases in expression in the PGC-1α$^{(-/-)}$. The biggest increase in the PGC-1α$^{(-/-)}$ compared to PGC-1α$^{(+/+)}$ was seen in the GABA(A) receptor subunit Gabr2a, this is not addressed in this study.

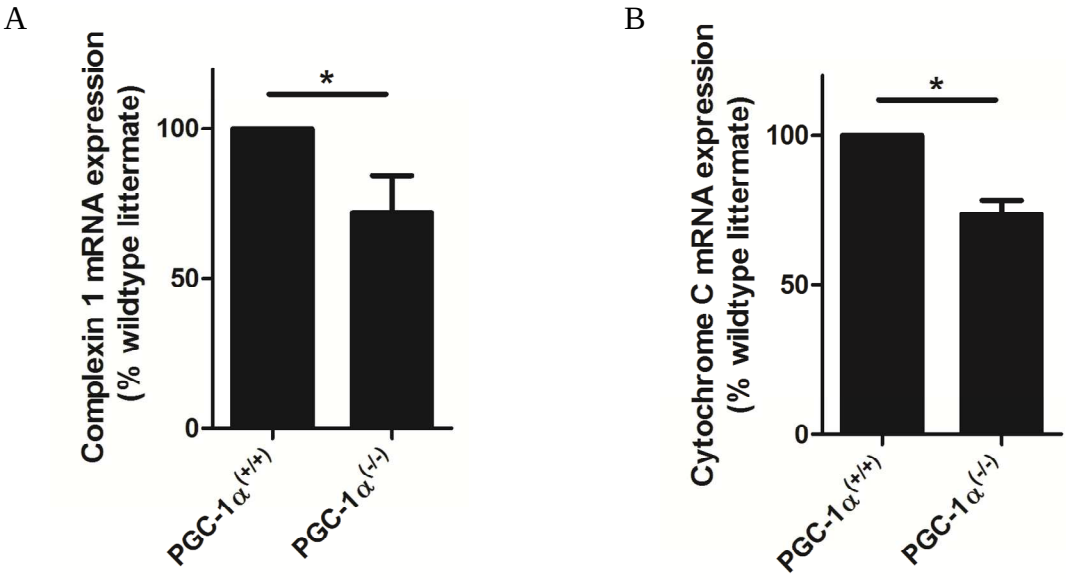

Fig 5.6 PGC-1α$^{(-/-)}$ neurons have reduced complexin I mRNA expression RNA was collected DIV 7-9 from neuronal cultures from PGC-1α$^{(-/-)}$ and PGC-1α$^{(+/+)}$ mice. Quantification of mRNA expression was performed by syber green qPCR. (A) PGC-1α$^{(-/-)}$ neurons have lower expression of complexin I. (B) The PGC-1α-target gene cytochrome C was also reduced in the PGC-1α$^{(-/-)}$ neurons, acting as a positive control. n = 7 PGC-1α$^{(-/-)}$ and 7 PGC-1α$^{(+/+)}$ mice from 7 independent litters; paired *t test* $p<0.05$).

5.3 Discussion

5.3.1 Disregulation of AMPARs in PGC-1α(-/-) neurons

This study clearly shows an alteration in AMPAR response to both bath application of the AMPAR agonist AMPA, and spontaneous excitatory activity. In addition we identify two potential mechanisms for the reduction in AMPAR responses.

Firstly, reduced steady state expression of AMPAR subunit (GluR1-4) mRNA could limit the rate of translation of the subunit proteins, leading to a reduction in the steady state function of the receptor complex. In order to directly test this, future studies may involve overexpressing GluR1-4 under the control of highly active promoters which, if the hypothesis is true, should counteract any affect of mRNA deficiencies and rescue the reduction in AMPAR currents. The mechanisms by which loss of PGC-1α may alter expression of GluR1-4 remains unclear, but could be potential way of linking energy homeostasis to highly energy demanding synaptic activity.

Secondly, the reduction in complexin I in PGC-1α$^{(-/-)}$ neurons may limit AMPAR exosytosis in these cells. At the presynaptic site, complexions play an essential role in calcium-dependent vesicle fusion as well as being necessary to restrain the exocytosis SNARE proteins to prevent inappropriate vesicle release (Yang et al., 2010). In such, presynaptic

knockdown of complexin I causes an increase in mEPSC frequency (Maximov et al., 2009) in striking contrast to the reduction in mEPSC frequency in the PGC-1α$^{(-/-)}$ neurons (Fig 5.4). However, a recent study by Ahmad et al. (2012) has found that on the postsynaptic side, viral knockdown of complexin I and complexin II in hippocampal neurons disrupts AMPA receptor exocytosis during LTP. This result is consistent with our observed reduction of AMPAR whole cell currents, mini amplitude, and mini frequency, and proposes a change in the development of AMPAR synapses due to disruption in AMPAR delivery to the synapse; although we cannot be sure that this mechanism would affect the steady state AMPAR expression measured here, it could potentially have a potent affect if altered during development, such as in our knockout system. In order to assess whether this hypothesis is true, it would be necessary to do a number of rescue experiments. For example, analysing AMPAR expression in PGC-1α$^{(-/-)}$ cells after transfection of complexins would enable us to study the contribution of these proteins, at postsynaptic site, to the observed phenotype, whereas the transfection of PGC-1α itself into PGC-1α$^{(-/-)}$ neurons should rescue the loss of complexin, GluR1-4 mRNA and may allow recovery of AMPAR profile. Together these findings strongly suggest alterations in AMPAR expression is the mechanism behind changes in AMPAR responses.

5.3.2 Discrepancies between acute and chronic PGC-1α knockdown on NMDARs.

In chapters three and four we demonstrate a functional role for PGC-1α signalling in the determining the amplitude of agonist-evoked NMDAR currents. Loss of PGC-1α was found to increase extrasynaptic NMDAR currents with concurrent increase in vulnerability to NMDA-induced cell death in rat cortical and striatal cultures (Chapter 3; Puddifoot et al., 2012). We therefore proposed that NMDAR signalling may be altered in the PGC-1α$^{(-/-)}$ mice. In this chapter we report no change in the whole-cell NMDAR current density in cortical neuronal cultures from PGC-1α$^{(-/-)}$) mice compared to PGC-1α$^{(+/+)}$. Furthermore we found no change in vulnerability to NMDA in these cultures (Karen Bell). There are a number of possible explanations for this discrepancy.

Firstly, it is possible that the differential effects of PGC-1α$^{(-/-)}$ loss in the acute versus the chronic scenario is determined by developmental aspects. Throughout maturation NMDAR expression is dynamically regulated at the level of transcription, translation and post translational modifications. Transcription factors known to enhance transcriptional output of NMDAR subunits GluN1-3 include Specific Protein family (SP-1), Early growth response family (Egr), T box (Tbr), Fos, Jun, cAMP response element-binding (CREB) (reviewed by Bai and Hoffman 2009). Vazhappilly & Sucher (2004) suggest that translational regulation of GluN subunit proteins can alter the rate of NMDAR expression. In addition, the regulation of receptor trafficking and stability at the plasma membrane can be altered by post-translation protein modifications (Hayashi & Huganir, 2009; Huang & El-Husseini, 2005). This suggests that many regulatory

elements coordinate to control the expression of functional NMDARs on the plasma membrane and a number of sequential steps exist that could potentially compensate for any disruption in this system to maintain synaptic homeostasis. The ability of the knockout neurons to compensate for the chronic loss of PGC-1α seems to be the most plausible explanation for the discrepancies observed in the chronic knockout versus acute knockdown. Indeed, compensation for the loss of synaptic proteins by enhancing proteins with homologous functions has previously been observed in studies of mice with specific knockout of MAGUK proteins (Elios et al., 2006). Whereas siRNA knockdown of endogenous PSD-95 or PSD-93 disrupts AMPA receptor expression at excitatory synapses, in adult PSD-95/ PSD-93 double knockout animals, SAP-102 is upregulated and compensates for the loss of synaptic AMPA-Rs (Elios et al., 2006). It is possible that compensation takes place at any stage in the unmapped pathway between PGC-1α signalling and the amplitude of NMDAR currents.

However, we cannot rule out alternative explanations for the differential effects of PGC-1α knockout and knockdown in these experiments. For example at the time in which these experiments were carried out, we did not have our current understanding of the specific changes in extrasynaptic NMDARs that occur after siRNA knockdown in rat neuronal cultures; we therefore omitted to test the proportion of NMDARs at synaptic and extrasynaptic sites in the PGC-1α$^{(-/-)}$. In the acute studies, we found siRNA knockdown of PGC-1α to have a *significantly higher effect on extrasynaptic currents compared to the whole* cell currents (Chapter 3; Fig 3.9), therefore it is possible that changes that occur at extrasynaptic NMDARs, which can make up between 25-50% of NMDARS at this age point (Rosemund et al., 1994), are masked in this study. The PGC-1α

mouse line used in this study was frozen before this result came to light; therefore we have yet to test this hypothesis. However, changes in the ratio of extrasynaptic and synaptic NMDARs that would normally alter neuronal vulnerability (Hardingham and Bading, 2010) are inconsistent with the absence of increased vulnerability in the PGC-1α$^{(-/-)}$ neurons undermining this explanation.

An alternative possibility is that PGC-1α$^{(-/-)}$ does cause disturbances in NMDAR activity levels but those cells with prolonged elevated NMDAR activity are not surviving during the culture process. Due to the housing location of the PGC-1α$^{(-/-)}$ mice, the PGC-1α$^{(-/-)}$ and PGC-1α$^{(+/+)}$ mouse brains were subject to an extended culture protocol which could have lead to increased cellular stress. This scenario was previously seen in early studies in post mortem tissue from HD patients in which radiolabeled ligand binding assays show a disproportionate loss of glutamate receptors in striatal tissue from HD patients (Young et al., 1988). In this case the expression of glutamate receptors are thought to be reduced, as the neurons with higher levels of NMDAR activity are the most vulnerable and have already died, however, we have no evidence to suggest cell death occurred in our cultures.

Finally, I must cknowledge that species differences could contribute to discrepancy in data since the acute studies reported in chapter 3 were performed in rat neurons rather than mice. However, this seems unlikely given that much this work performed in rat neurons agrees with data from HD mouse models including the upregulation of extrasynaptic NMDAR currents and excitotoxicity downstream of mutant huntingtin (Milnerwood et al., 2010).

Chapter 6:

Summary of findings presented in this thesis

In this thesis I studied the role of PGC-1α in excitotoxic cell death. I identified the unexpected neuroprotective mechanism of PGC-1α by the downregulation of extrasynaptic NMDARs. Furthermore, I show that the repression of PGC-1α by mutant huntingtin protein in Huntingtin's disease may contribute to excessive extrasynaptic NMDAR activity and excitotoxicity in this disease. By showing that mtHtt does not exacerbate toxicity or extrasynaptic currents in cells in which PGC-1α is knocked down, my data suggest that the action of mtHtt on PGC-1α may be significant in the deregulation of NMDARs by this protein (summarised in Fig 6.1).

This data adds mechanistic insight into previous understanding of the synergistic roles of mtHtt, NMDAR activity and PGC-1α in HD. During the course of this study two groups reported a reciprocal relationship between mtHtt toxicity and extrasynaptic/synaptic NMDAR signalling in HD. Synaptic activity protects against mtHtt toxicity by increasing mtHtt inclusion formation and upregulating pro-survival transcriptional activity, including the expression of PGC-1α (Okamoto et al., 2009; Soriano et al., 2009). In contrast, extrasynaptic activity represses inclusion formation, and CREB-dependent PGC-1α expression (Okamoto et al, 2009; Hardingham et al., 2002). PGC-1α transcription is further repressed by mtHtt antagonising the PGC-1α promoter (Cui et al 2006). Exacerbating this cascade is the upregulation of extrasynaptic NMDARs by mutant huntingtin (Milnerwood et al., 2010). This thesis addresses the increase in extrasynaptic NMDAR signalling in HD, showing that the repression of PGC-1α contributes to the increase in excitotoxicity by tipping the balance of NMDAR expression toward the neurotoxic extrasynaptic pools. Since extrasynaptic activity represses CREB-dependent PGC-1α expression this represents a neurotoxic positive feedback loop.

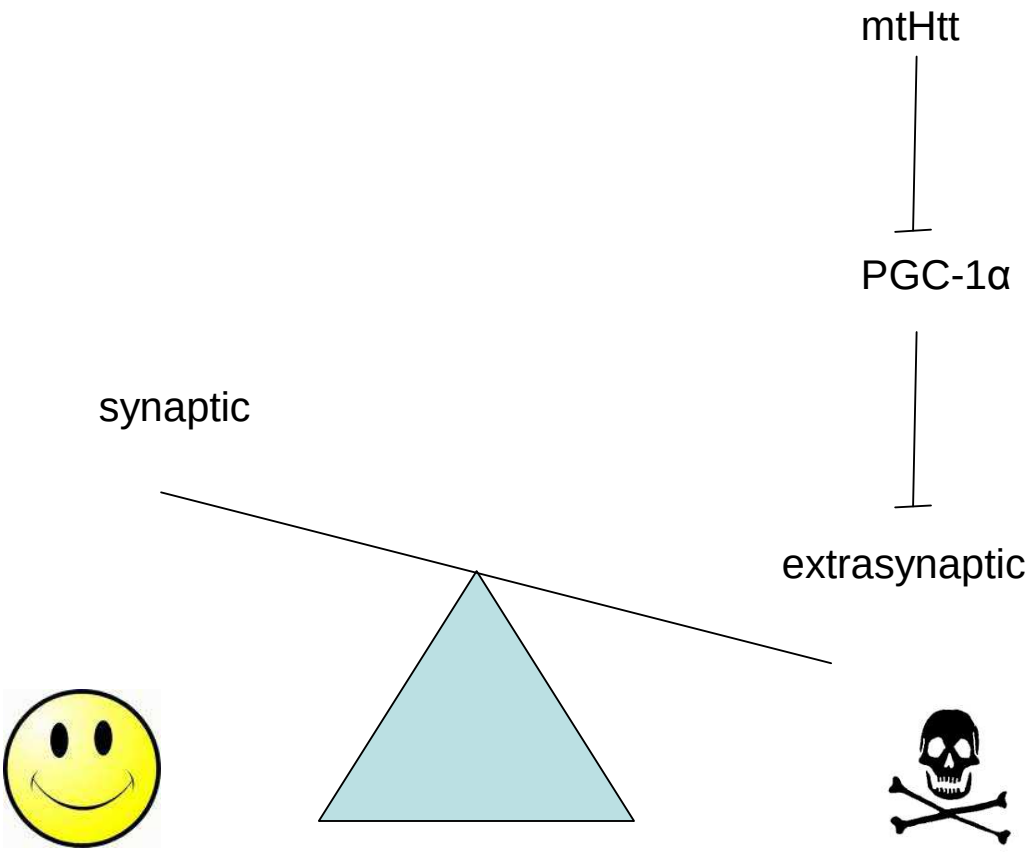

Fig 6.1 This study provides mechanistic insight in to the role of PGC-1α in NMDAR excitotoxicity in HD MtHtt has also been shown to preferentially increase extrasynaptic NMDAR currents. Tipping the balance of NMDAR activity in favour of excitotoxic extrasynaptic NMDAR pools increases excitotoxicity in HD. This thesis provides evidence that mtHtt regulates extrasynaptic NMDARs and excitotoxicity via the repression of PGC-1α which I show to be a potent repressor of extrasynaptic NMDAR currents and excitotoxic cell death.

I also show the ability of PGC-1α to rescue both the excitotoxicity and enhanced extrasynaptic NMDAR currents caused by mtHtt expression. This suggests that targeting PGC-1α may provide therapeutic benefits in HD. A recent study by McConoughey et al (2010) identify a novel compound ZDON, a transglutaminase 2 inhibitor capable of upregulating endogenous PGC-1α. ZDON was able to protect neurons against mtHtt-mediated increase in excitotoxicity. Future studies may address whether this compound can reverse the effects of mtHtt on extrasynaptic NMDAR currents. In addition to being a potential therapeutic, such experiments would confirm the ability of endogenous PGC-1α expression to counteract this imbalance in the extrasynaptic pool of NMDARs.

Prior to this thesis, PGC-1α was widely studied for its capacity to increase mitochondrial biogenesis and antioxidant defences (Handschin et al., 2009) and although it was known that PGC-1α expression is neuroprotective, my finding that this transcriptional coactivator can repress NMDAR activity was surprising. One theory derived from this study is that mitochondrial health and energy metabolism might be intrinsically linked to the regulation of NMDAR expression and localisation. This would imply that the effects of altering PGC-1α on NMDAR currents may be downstream of its ability to boost mitochondrial health and dampen the toxic effects of reactive oxidative species. In order to directly test the role of mitochondrial health on glutamatergic receptor expression, it would be necessary to study the effects of mitochondrial stressors on NMDAR currents. Alternatively it would be interesting to test whether altered NMDAR currents in response to loss of PGC-1α persist in conditions where the mitochondrial health is maintained. Opposing this theory is the ability of the PGC-1α-inducer ZDON to protect against NMDA- excitotoxicity in the absence of increased mitochondrial function (McConoughey et al., 2010). Much is left to be understood about the regulation of extrasynaptic and synaptic

NMDAR expression in both physiological and pathological conditions and whether this is directly related to the mitochondrial dysfunction apparent in many neurological disorders (Table 1.1) may prove interesting.

Finally, I present findings from my study on chronic loss of PGC-1α in the PGC-1α knockout mouse. Although the chronic knockout of PGC-1α did not mimic the effects observed in acute knockdown of PGC-1α *in vitro*, I found that loss of PGC-1α during embryogenesis altered both the whole cell expression and synaptic activity of AMPA receptors, microarray analysis indentified the regulator of exocytosis complexin I as a potential regulator of this process in addition to an observed reduction in AMPA subunit mRNA. As seen from the microarray data presented here, a number of changes occur in response to transgenic knockout of a gene and full analysis of glutamate receptor signalling throughout development and adulthood may need to be considered to elucidate the effects of chronic knockout of PGC-1α. This thesis provides some mechanistic insight into the role of PGC-1α in the regulation of glutamate receptor currents throughout embryonic development and in an *in vitro* model of HD.

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