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The Developmental Trajectory of Synaptic Vesicle Recycling in Wild Type and Neurodevelopmental Disorder Models

Nawon Kim



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To mum, Nahee and Jonathon.

Abstract

The presynapse performs an essential role in brain communication via the activity-dependent release of neurotransmitters. This is made possible by exocytosis of synaptic vesicles (SV) that package the neurotransmitters, and these SVs are recycled via endocytosis. However, the sequence of events through which a presynapse acquires functionality is relatively poorly understood, which is surprising since mutations in genes essential for its operation are heavily implicated in neurodevelopmental disorders (NDDs). We addressed this gap in knowledge by determining the developmental trajectory of SV recycling pathways in wild type (WT) murine hippocampal neurons from primary cultures.

Using the genetically-encoded calcium indicator (GECI) GCaMP6f, we revealed that the majority of nerve terminals displayed activity-dependent calcium influx from 3 days *in vitro* (DIV). Synaptophysin-pHluorin (sypHy) imaging indicated that SVs are recycled via evoked exocytosis and endocytosis from the first week *in vitro* as well, although the number of responsive nerve terminals continued to increase until the second week *in vitro*. However, the most intriguing discovery was that activity-dependent bulk endocytosis (ADBE) was only observed from DIV 14 onwards. The delayed acquisition of ADBE was reported through a tetramethylrhodamine (TMR)-dextran uptake assay and was corroborated by examination of horse radish peroxidase (HRP) uptake via electron microscopy. This series of optical assays suggests that ADBE is acquired as an extra step of synaptic development.

After determining the developmental trajectory of SV recycling in WT neurons, I then investigated whether this was altered in NDD models. First, I tested SV recycling in *Fmr1* knockout (KO) neurons which model Fragile X Syndrome, one of the most common monogenic NDDs that shows high comorbidity to autism spectrum disorder (ASD). *Fmr1* KO neurons displayed reduced ADBE at DIV 14 compared to neurons from their littermate controls, as reported previously. Intriguingly, however, this deficit recovered at a later timepoint (DIV 21), indicating that the phenotype was not because of a mechanistic failure of ADBE due to the absence of FMRP. The mechanism behind this delay and the compensation will be the next key question to address. Through GST pull-down assay and mass spectrometry, I have identified Dynamin-1, a GTPase that constricts and fissions SVs during endocytosis, as a potential novel interacting partner of FMRP, opening up a new avenue for studying SV recycling in *Fmr1* KO neurons.

SV recycling was also investigated in *Dual Specificity Tyrosine Phosphorylation-Regulated Kinase 1A* (*Dyrk1A*) heterozygous (HET) neurons, which model DYRK1A syndrome, another monogenic NDD that is highly associated with ASD, intellectual disability and epilepsy. DYRK1A is located in the Down syndrome critical region on human chromosome (HSA) 21, meaning that it impacts neurodevelopment in a dosage-dependent manner. Similar to *Fmr1* KO neur-

ons, *Dyrk1a* HET neurons displayed reduced ADBE in comparison to WT neurons in both TMR-dextran and HRP uptake assay. However, in addition to depressed ADBE, they also showed reduced evoked exocytosis as well as SV pool size. This reduction in SV exocytosis was not due to altered activity-dependent calcium influx, since *Dyrk1a* HET neurons did not show any significant difference with WT neurons in calcium imaging using GCaMP6f. This finding highlights the presynaptic downscaling in *Dyrk1a* HET neurons downstream of activity-dependent calcium influx. The mechanism behind such phenotype could be further investigated based on the proteomics and phospho-proteomics data which highlighted the disrupted phosphorylation of SV recycling proteins. To summarise, it appears that *Dyrk1a* HET and *Fmr1* KO neurons may share depression of ADBE as a potential convergent phenotype but through separate pathology.

Lay Summary

Brain cells (neurons) communicate by releasing neurotransmitters from one neuron to the next. A neuron has a polarised structure of: a cell body (soma), dendrites, where neurons receive information from previous neurons in the circuit, and an axon, a major process deriving from the cell body that sends information to the next neuron in the circuit. The neurotransmitters are packed in small organelles called synaptic vesicles (SV) before they are released from nerve terminals, the tips of an axon that project to the next neuron. The SVs release the neurotransmitters by fusing to the plasma membrane (the external boundary of a neuron), and this process is named SV exocytosis. There are hundreds of SVs in individual nerve terminals and the number varies across different brain cell types. These SVs are recycled by retrieving their core components from the plasma membrane, a process called SV endocytosis. There are three modes of SV endocytosis – clathrin-mediated endocytosis (CME), ultrafast endocytosis (UFE) and activity-dependent bulk endocytosis (ADBE). The exocytosis and endocytosis of SVs are critical in maintaining brain communication and disruption in these processes is associated with neurodevelopmental disorders (NDDs), such as autism spectrum disorder (ASD), intellectual disability and epilepsy. This thesis aims to understand how neurons acquire SV recycling in brain cells grown on glass coverslips (*in vitro*), and to understand the altered developmental trajectory of SV recycling in neurons that bear the genetic mutations that mimic those found in NDDs.

First, I studied SV recycling in neurons harvested from rats that do not have any pathogenic mutation (wild type (WT)). Because SV exocytosis is triggered by the influx of calcium ions at the nerve terminals upon stimulation, I monitored the level of calcium influx in different timepoints as a first step. As expected, activity-dependent calcium influx increased over time and plateaued from day 14 *in vitro*. Next, to assess the level of SV exo- and endocytosis in individual nerve terminals, I performed a synaptophysin-pHluorin (sypHy) imaging. SypHy is a fluorescent protein (pHluorin) conjugated with a small SV cargo protein (synaptophysin). pHluorin, as the name suggests, is sensitive to pH, and fluoresces when it is exposed to a pH-neutral environment but does not fluoresce in an acidic environment. SypHy capitalises on the pH difference between the inside of a SV (acidic) and the outside of a neuron (neutral). When a SV is exocytosed, sypHy will be exposed to the extracellular space, where is pH-neutral, making it fluoresce, whereas when it is endocytosed, the pHluorin will not fluoresce because the inside of the SV is acidic. Surprisingly, the sypHy response was not significantly different across timepoints, indicating that the SV recycling process is activated early in neuronal development. However, when we looked into ADBE, it only started from day 14 *in vitro*, which means ADBE is acquired later than other modes of SV recycling. ADBE is a two-step process which consists of invagination of the bulk endosome from the plasma membrane, and then

budding of individual SVs from the bulk endosome. We used a chemical compound called tetramethylrhodamine (TMR) -dextran to report ADBE, as it is too big to be endocytosed by other modes of endocytosis but small enough to be endocytosed via ADBE when applied on neurons. This delayed recruitment of ADBE was further corroborated by electron microscopy, which is a high-resolution technique that visualises bulk endosomes inside nerve terminals.

In an effort to understand NDDs in the perspective of SV recycling, I investigated neurons from two rat models. Fragile X Syndrome is one of the most common monogenic NDDs linked to ASD, and it is caused by deficiency of the protein called Fragile X messenger ribonucleoprotein (FMRP). FMRP is encoded by the gene *Fmr1*, which means knocking out *Fmr1* models Fragile X Syndrome. In *Fmr1* knockout neurons, the level of ADBE was decreased compared to neurons from their WT littermate controls. A time-course analysis revealed that this was due to a delay in development of these neurons, as the level of ADBE recovered to WT levels a week later. Using different biochemical techniques, I tried to narrow down the binding partners of FMRP that could explain such delay. It would be of great importance to study why the *Fmr1* KO neurons show such delay and how it impacts development of the neural circuit in the future.

Next, dual-specificity tyrosine phosphorylation-regulated kinase 1A (DYRK1A) is an important enzyme in our body that affects development in a dosage-dependent manner. Located on human chromosome 21, too much of the protein is related to Down syndrome, whereas too little causes Dyrk1 syndrome, another NDD that shows high comorbidity with ASD. Neurons with half the usual amount of *Dyrk1a* (haploinsufficiency) showed reduced level of ADBE similar to *Fmr1* knockout neurons. However, these neurons also showed reduced exocytic capacity, which was not observed in *Fmr1* knockout neurons. In conclusion, the nerve terminals of *Dyrk1a* show weaker presynaptic strength, which could be an important question in understanding other NDDs too. Knowing that DYRK1A is a kinase that modifies other proteins via phosphorylation, we sought proteomics and phospho-proteomics to understand what proteins are affected by the haploinsufficiency of DYRK1A. With the potential downstream proteins shortlisted, it would be interesting to find out how the presynaptic strength is weakened and how it impacts circuit development in this model.

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Declaration

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

Nawon Kim

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Abbreviations

- ADBE** activity-dependent bulk endocytosis.
- AIS** axon initial segment.
- AMPA** α - amino-3- hydroxy-5- methyl-4- isoxazolepropionic acid.
- AP** action potential.
- AP-2** adaptor protein complex 2.
- APP** amyloid precursor protein.
- ASD** autism spectrum disorder.
-
- BAR** Bin/ Amphiphysin/ Rvs.
- BK** large-conductance calcium-activated potassium.
-
- CAM** cell adhesion molecule.
- CaMKII** Ca^{2+} /calmodulin-dependent protein kinase II.
- CAST** active zone-associated structural protein.
- CCK** cholecystoskinin.
- CDC** Centers for Disease Control and Prevention.
- CDK5** cyclin-dependent kinase 5.
- CHC** clathrin heavy chain.
- CK1** Casein kinase 1.
- CLTC** clathrin heavy chain.
- CME** clathrin-mediated endocytosis.
- CNS** central nervous system.
- CREB** cAMP response element-binding protein.
-
- DAB** diaminobenzidine.
- DIV** days in vitro.
- DRG** dorsal root ganglion.
- DTT** dithiothreitol.
- Dyn-1** Dynamin-1.
- DYRK1A** Dual-specificity tyrosine-regulated kinase 1 A.
-
- EGTA** ethylene glycol tetraacetic acid.
- ELKS** protein rich in the amino acids E, L, K, and S.
- EPSC** excitatory postsynaptic current.
-
- F-actin** filamentous actin.
- Fmr1** Fragile X Messenger Ribonucleoprotein 1.

FMRP Fragile X Messenger Ribonucleoprotein.

FXS Fragile X Syndrome.

G3BP1 Ras-GTPase-activating protein (GAP)- binding protein 1.

GABA gamma-aminobutyric acid.

GSK3 glycogen synthase kinase 3.

GST glutathione-S-transferase.

hiPSC human induced pluripotent stem cell.

HRP horse radish peroxidase.

HSA21 *Homo sapiens* chromosome 21.

IAA iodoacetamide.

ID intellectual disability.

iGluSnFR glutamate-sensitive fluorescent reporters.

IPSC inhibitory postsynaptic current.

IPTG Isopropyl β - d- 1- thiogalactopyranoside.

KCC2 K-Cl cotransporter 2.

KH K homolog.

KIF kinesin superfamily.

KIF5C Kinesin family member 5C.

KO knock out.

KRAB Krüppel-associated box.

LAR leukocyte antigen-related.

LTD long-term depression.

LTP long-term potentiation.

MeCP2 methyl-CpG-binding protein 2.

mGluR metabotropic glutamate receptor.

mnb minibrain.

MNTB medial nucleus of the trapezoid body.

mPFC medial prefrontal cortex.

NDD neurodevelopmental disorder.

NES nuclear export signal.

NKCC1 Na–K–Cl cotransporter 1.

NLS nuclear localisation signal.

NMDA N-methyl-D-aspartate.

PBS phosphate buffered saline.

PCA principal component analysis.

- PFA** paraformaldehyde.
- PI4KII α** phosphatidylinositol 4-kinase II α .
- PIP₂** phosphatidylinositol 4,5-bisphosphate.
- PKA** protein kinase A.
- PMCA** plasma membrane calcium ATPase.
- PMSF** phenylmethylsulfonyl fluoride.
- PS** phosphatidyl serine.
- PSD** postsynaptic density.
- PTV** Piccolo-Bassoon transport vesicle.
- RGG** arginine-glycine rich.
- RIM** regulating synaptic membrane exocytosis protein.
- RNA** ribonucleic acid.
- ROI** region of interest.
- RPTP** receptor-protein tyrosine phosphatase.
- RRP** readily releasable pool.
- SDS** sodium dodecyl-sulfate.
- SERCA** sarcoplasmic/endoplasmic reticulum calcium ATPase.
- SGIP1** SH3-containing GRB2-like protein 3-interacting protein 1.
- SK** small-conductance calcium-activated potassium.
- SNAP-25** Synaptosomal-associated protein 25.
- SNARE** soluble N-ethylmaleimide-sensitive fusion protein (NSF) attachment protein receptor.
- STV** synaptic vesicle transport vesicle.
- STXBP5** Syntaxin binding protein 5.
- SUMO** small ubiquitin-like modification.
- SV** synaptic vesicle.
- SV2A** Synaptic vesicle glycoprotein 2A.
- Syb-2** Synaptobrevin-2.
- syp-mOr2** synaptophysin-mOrange2.
- sypHy** synaptophysin-pHluorin.
- Syt-1** Synaptotagmin-1.
- Syt-7** Synaptotagmin-7.
- TCEP** tris (2-carboxyethyl) phosphine.
- TD** Tudor domain.
- TFA** trifluoroacetic acid.
- TMR** tetramethylrhodamine.
- TOM70** translocase of the outer mitochondrial membrane 70.
- UFE** ultrafast endocytosis.

UTR untranslated region.

VAMP-4 Vesicle-associated membrane protein.

vATPase vacuolar-type H⁺ ATPase.

vGaT vesicular GABA transporter.

VGCC voltage-gated calcium channel.

vGluT vesicular glutamate transporter.

WT wild type.

Chapter 1

Introduction

1.1 Synaptic Vesicle Recycling

Brain communication is made possible by transferring information between neurons, from a presynaptic neuron to a postsynaptic neuron. The chemical neurotransmission involves releasing neurotransmitters from the presynapse into the synaptic cleft, which bind to receptors on the postsynaptic membrane. At the presynapse, neurotransmitters are packed in small clear lipid bilayer organelles called synaptic vesicles (SVs). SVs were first identified in the amphibian neuromuscular junction in the 1950s, whose size ranged from 30 nm to 40 nm (De Robertis & Bennett, 1955; Palade, 1954). The number of SVs in a synapse varies from hundreds to over a thousand per bouton in central nervous system (Harris & Sultan, 1995; Qu, Akbergenova, Hu, & Schikorski, 2009). The SVs are enriched in an electron-dense area at the presynapse called the active zone, along with scaffolding proteins, ion channels and cytoskeleton. The active zone lies apposed to the postsynaptic density (PSD) at the postsynapse, another electron-dense area specialised in receiving chemical signals transmitted from the presynapse.

The influx of free Ca^{2+} ions through the voltage-gated calcium channels (VGCCs), either initiated by an action potential (AP) or spontaneously, trigger the fusion of the SVs to the plasma membrane, which results in the release of neurotransmitters into the synaptic cleft. Fused SVs are recycled via endocytosis, a process that selectively retrieves cargo proteins and lipids to replenish the SV pool. The regenerated SVs are reacidified by vacuolar-type H^+ ATPase (vATPase) and are refilled by neurotransmitter transporters using the H^+ gradient.

This recycling process, as demonstrated in the schematic figure 1.1, enables neurotransmission with remarkable speed and efficiency. Guided by the knowledge in exo- and endocytosis mechanism in neuroendocrine cells as well as the new molecular and optic tools, the life cycle and the molecular composition of SVs has been actively revealed (Jang, Kim, & Lee, 2021; Nosov, Kahms, & Klingauf, 2020). A growing number of studies on presynaptic neurotransmission now provide a detailed picture of the SV recycling process (Chanaday, Cousin, Milosevic, Watanabe, & Morgan, 2019).

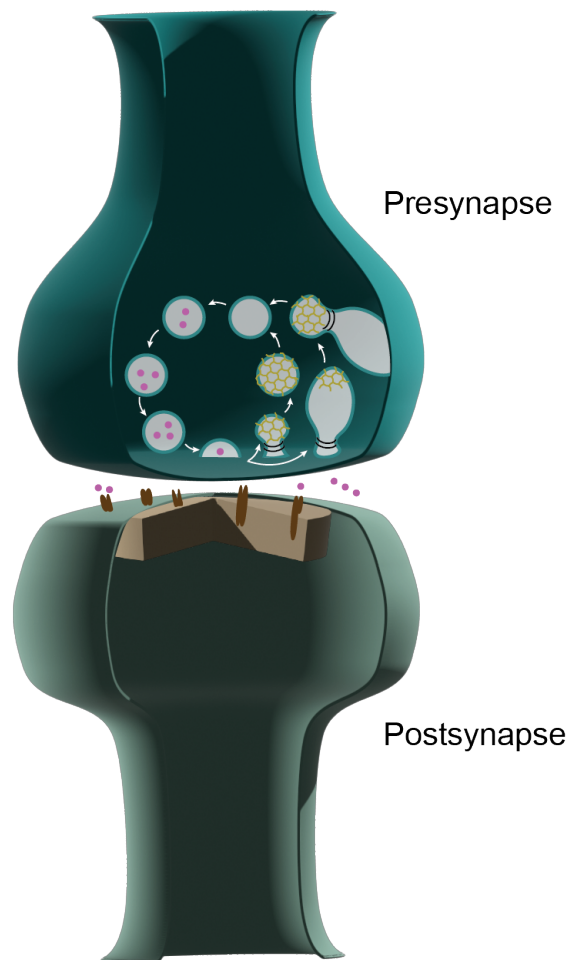


Figure 1.1: A summary of SV life cycle

The synaptic vesicle (SV)s are fused to the plasma membrane to release neurotransmitters (pink) into the synaptic cleft, after docking and priming at the presynapse. The SV proteins are retrieved and the SVs are recycled via different modes of endocytosis: clathrin-mediated endocytosis (CME), activity-dependent bulk endocytosis (ADBE) or ultrafast endocytosis (UFE). Light green indicates clathrin and black lines indicates Dynamin ring. The brown ovals at the postsynapse indicate receptors at the postsynaptic density (PSD).

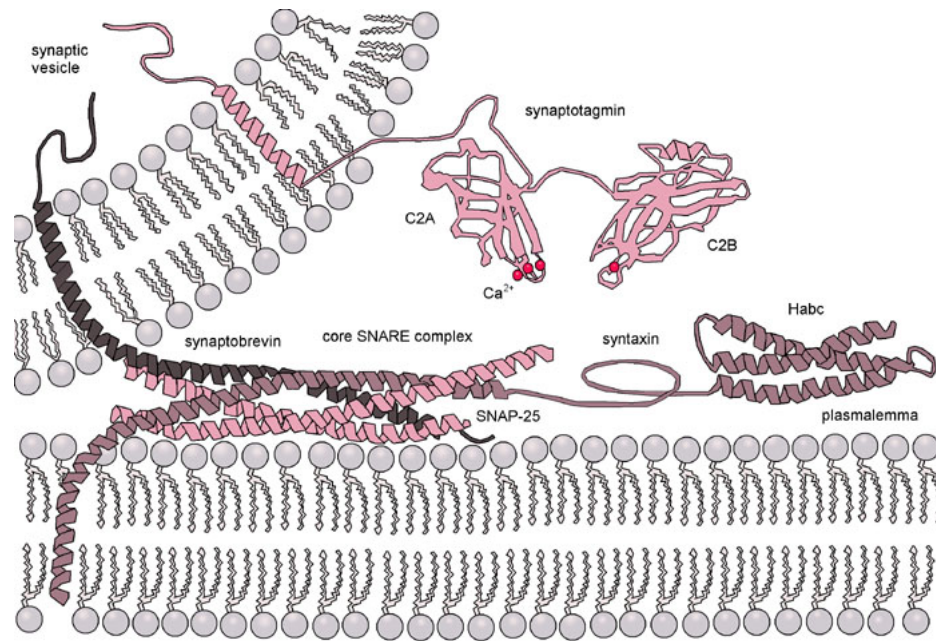


Figure 1.2: Simplified schematic of the SNARE complex

Image sourced online from "<http://en.wikipedia.org/wiki/Image:Exocytosis-machinery.jpg>" by Danko Dimchev Georgiev, M.D. (uploaded on 18 May 2006, accessed on 16 August 2024).

1.1.1 Synaptic vesicle exocytosis

SV exocytosis starts with docking and priming, the process in which soluble N-ethylmaleimide-sensitive fusion protein (NSF) attachment protein receptor (SNARE) proteins on the SV membrane, plasma membrane and in cytoplasm form a SNARE complex (docking). Then, the SNARE complex goes through conformational changes to become fusion-competent (priming). SNARE proteins refer to the proteins that harbor at least one SNARE motif, a 30-40 amino acid-long sequence that involves heptad repeats of hydrophobic residues. The SNARE proteins build a coiled-coil structure via this motif with other SNARE proteins (Wesolowski & Paumet, 2010) (schematic in figure 1.2). Based on the localisation, SNARE proteins are classified as either v-SNARE when on vesicular membrane (e.g., Synaptobrevin-2 (Syb-2)), or t-SNAREs when on the fusion target (e.g., Synaptosomal-associated protein 25 (SNAP-25) and Syntaxin 1).

Once a SNARE complex is formed, it would have a coiled-coil structure of four α -helices: two from SNAP-25 and one each from Syb-2 and Syntaxin-1 (Lou & Shin, 2016). Although not a SNARE protein itself, the assembly of the complex requires bridging of the vesicular and plasma membranes by Munc13, a large C2-domain protein (N. Brose, Hofmann, Hata, & Südhof, 1995; Imig et al., 2014; Papantoniou et al., 2023; Quade et al., 2019). Furthermore, not only does Munc13 physically bring a SV close to the plasma membrane, it primes a SV by opening Syntaxin-1 – Munc18 complex, so the linker region of Syntaxin-1 is exposed to form a SNARE complex (C. Ma, Li, Xu, & Rizo, 2011; S. Wang et al., 2017). Munc18 is a protein

that binds with Syntaxin-1 in advance to its opening by Munc13 (Burkhardt, Hattendorf, Weis, & Fasshauer, 2008; C. Ma et al., 2011). Munc18 might seem like a safety lock that inhibits formation of the SNARE complex, but it also functions as a guide for Syntaxin-1 towards the SNARE complex assembly, potentially by interacting with Syb-2 (Y. Lai et al., 2017; S. Wang et al., 2019). The participation of Munc18 in SNARE assembly explains the docking deficit in *unc-18* mutants in *C.elegans*, a *Munc18* homolog (Weimer et al., 2003). The role of Munc13 and Munc18 in docking and priming is further supported by the *in vitro* reconstitution assays, and by their murine knockout models which showed impaired SV fusion even though synaptogenesis was intact (Augustin, Rosenmund, Südhof, & Brose, 1999; Kalyana Sundaram et al., 2023; Leitz et al., 2024; Varoqueaux et al., 2002; Verhage et al., 2000). The clinical reports of genetic mutations in *MUNC13* or *MUNC18* (*STXBP1*) also highlight the importance of these two genes in neurodevelopment (Engel, Selcen, Shen, Milone, & Harper, 2016; Stamberger et al., 2016). Last but not least, Complexin also binds to SNARE complex and Syt-1 (Chen-Hwang, Chen, Elzinga, & Hwang, 2002). However, whether it plays an promoting or inhibitory role in exocytosis has been controversial, as different models showed contradicting results (U. Brose, 2010). The overview of the research on Complexins will be addressed more in detail in a later paragraph.

Next, docked and primed vesicles are fused to the plasma membrane upon Ca^{2+} influx through VGCCs. VGCCs are classified to five different groups: L- type ($\text{Ca}_v1.2$ and $\text{Ca}_v1.3$), P/Q- type ($\text{Ca}_v2.1$), N- type ($\text{Ca}_v2.2$), R- type ($\text{Ca}_v2.3$) and T- type ($\text{Ca}_v3.1$, $\text{Ca}_v3.2$, and $\text{Ca}_v3.3$). N-type and P/Q- type VGCCs have been identified as principal calcium channels regulating pre-synaptic exocytosis in hippocampal neurons (Koyanagi, Torturo, Cook, Zhou, & Hemmings Jr, 2019). These channels are activated by depolarisation, which leads to influx of free calcium ions from extracellular space into the cytoplasm.

After the influx, free intracellular calcium ions bind to Ca^{2+} sensors, one of which is the Synaptotagmin family. Both the mouse and human Synaptotagmin family have 17 isoforms of varying localisation, calcium affinity and kinetics, as reviewed in Wolfes and Dean (2020). Among them, Synaptotagmin-1, -2 and -7 are the mostly widely studied, and are known to cue different modes of exocytosis (C. Chen & Jonas, 2017). In evoked exocytosis, embedded on the vesicular membrane, Syt-1 regulates docking and priming by interacting with SNARE proteins, as well as anionic lipids such as phosphatidylinositol 4,5-bisphosphate (PIP_2), or phosphatidyl serine (PS) (Gruget et al., 2020; Ramakrishnan, Bera, Coleman, Rothman, & Krishnakumar, 2020). The mechanism by which Syt-1 coordinates with SNARE complexes, Munc13, Munc18 and complexins to release the primed SVs is discussed more in detail in "evoked exocytosis" section.

When SNARE complex zippers and fuses to the plasma membrane upon Ca^{2+} influx, the SV forms a fusion pore with the plasma membrane. The process of fusion pore expansion is still largely a mystery, especially in neurons due to the spatio-temporal limitations of the current technology. However, using nanodiscs and chromaffin cells, the transmembrane domain of Syb-2 and the balancing act between Complexin-2 and Syt-1 have been recognised as the determinants of the pore dynamics (Dhara et al., 2016, 2014; Z. Wu et al., 2021). Although it has been shown *in silico* that the majority of the neurotransmitters will be propelled to the synaptic cleft within 2 ms by simple diffusion, it is also possible that the SNARE complex or its accessory proteins pull the plasma membrane proximal to the fusion pore, or push the fused SV for a faster collapse (Rothman, Grushin, Bera, & Pincet, 2023). The biophysical mechanism behind the SV fusion requires further testing in neurons.

Synaptic vesicle pools

Before discussing the life cycle of a SV, it is worth noting that the SVs within a presynapse bear heterogeneous release probabilities, and hence compose different functional pools as confirmed *in vitro*, *ex vivo* and *in vivo* (Z. Li et al., 2005; Marra et al., 2012; Scimemi & Diamond, 2012). The nomenclature and the definition of each pool vary amongst studies, but in this thesis the SVs are first divided into two groups: recycling pool and resting pool. The recycling pool refers to the SVs that undergo exo- and endocytosis in response to neuronal depolarisation, whereas in the resting pool the SVs are unlikely to be mobilised by evoked neuronal activity, although they can be mobilised for spontaneous activity (Fredj & Burrone, 2009; Z. Li et al., 2005). The recycling pool can be further divided into two pools: readily releasable pool (RRP) and reserve pool. The RRP refers to the SVs that are docked and primed on the plasma membrane so that they can be immediately fused to the plasma membrane upon Ca^{2+} influx, guided by Munc13 and Munc18 (Augustin et al., 1999; Y. Lai et al., 2017; Toonen et al., 2006). The reserve pool is not docked onto the plasma membrane, but is fusion-competent enough to be mobilised under intense stimulation via the same molecular process when the RRP is depleted (Kaesler & Regehr, 2017). The classification is visualised in the schematic figure 1.3.

In a hippocampal synapse, it has been observed that about 10 - 20 % of total SVs account for the RRP and 50 % of the total SVs fall into the recycling pool (Denker & Rizzoli, 2010), but the partitioning is subject to different stages of development, presynaptic plasticity and models (Cazares et al., 2016; Kahms & Klingauf, 2018; Ratnayaka et al., 2012; Rey, Marra, Smith, & Staras, 2020; Rose, Schoenenberger, Jezek, & Oertner, 2013).

Previously, SVs crosslinking via Synapsin tetramers was suggested as the mechanism behind SV pool regulation (A. Ferreira, Kao, Feng, Rapoport, & Greengard, 2000; M. Zhang & Augustine, 2021). On the other hand, recent publications highlight liquid-liquid phase separation, as Synapsin and SVs form liquid condensates *in vitro* (Hoffmann et al., 2023; Pechstein et al., 2020; M. Zhang & Augustine, 2021). S.-H. Song and Augustine (2023) proposed different Synapsin-induced clustering depending on the type of the synapse.

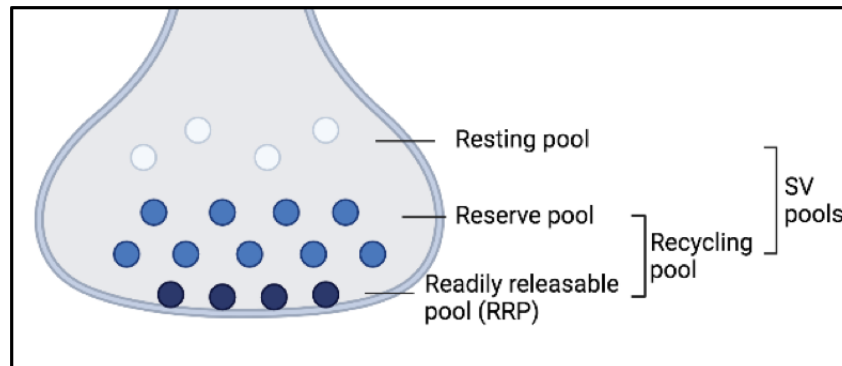


Figure 1.3: Different SV pools at the presynapse

Evoked exocytosis

Connectivity is an integral quality of the neural network and it derives from the ability to evoke activities from one neuron to another. Hence, evoked exocytosis, a type of exocytosis that is triggered by external signalling, plays an essential and a main role in brain communication. Evoked exocytosis can be further categorised into two subtypes based on the timescale: synchronous and asynchronous release.

Synchronous release refers to exocytosis of SVs within milliseconds after stimulation, by the fusion of RRP onto the plasma membrane. Synchronous release accounts for the majority of the release events in neuronal activity (> 90 %) (Kaesler & Regehr, 2014), to the point that the term synchronous release is only used in apposition to asynchronous release, whereas evoked exocytosis usually refers to synchronous release unless specified. Syt-1, a small calcium-binding protein that consists of a transmembrane domain, a linker sequence, and two tandem C-terminal domains (C2A and C2B), has been identified as the major calcium sensor in synchronous release (Fernandez et al., 2001; Geppert, Goda, et al., 1994; Nishiki & Augustine, 2004). So far, it has been established that Syt-1 docks a SV and then clamps it from fusing onto the plasma membrane spontaneously, while inducing zippering upon Ca^{2+} influx (H. Bai et al., 2016; Chicka, Hui, Liu, & Chapman, 2008; Littleton, Stern, Perin, & Bellen, 1994; Stepien & Rizo, 2021). This clamping is disinhibited by free Ca^{2+} ions binding to one of the two tandem cytosolic domains C2B, but the specific mechanisms behind this disinhibiting process is still under debate (H. Bai et al., 2016; Desai et al., 2000).

The firstly identified interaction partners to Syt-1 were SNARE complex proteins. SNAP-25 and Syntaxin have been recognised as calcium-dependent binding partners for Syt-1 (Chapman, Hanson, An, & Jahn, 1995; C. Li et al., 1995; X. Zhang, Kim-Miller, Fukuda, Kowalchuk, & Martin, 2002; Q. Zhou et al., 2015), but the functional role of the binding is not clear (Y. Park et al., 2015; Stein, Radhakrishnan, Riedel, Fasshauer, & Jahn, 2007). Recently, N. A. Courtney, Bao, Briguglio, and Chapman (2019) identified C2B domain of Syt-1 as the clamp of synchronous release, potentially via interaction with SNARE proteins.

Anionic phospholipids are another well-known binding partner of the C2B region of Syt-1 (Araç et al., 2006; Chapman & Davis, 1998; Gruget et al., 2020; Kiessling et al., 2018; van den Bogaart, Meyenberg, Diederichsen, & Jahn, 2012). Binding with PS bridges the two membranes in proximity, accelerating the SV fusion (Araç et al., 2006; Stein et al., 2007). It has also been suggested that the acceleration is achieved by bending the target membrane (Hui, Johnson, Yao, Dunning, & Chapman, 2009). PIP₂ on the plasma membrane, on the other hand, weakly binds to Syt-1 and enhances Ca²⁺ sensitivity, although the mechanism has not been clarified (J. Bai, Tucker, & Chapman, 2004; Gruget et al., 2020; L. Li et al., 2006; Y. Park et al., 2015). The role of the binding between Syt-1 and PIP₂ could be related to docking and priming, because increasing the concentration of PIP₂ in the plasma membrane positively correlated with the size of RRP in chromaffin cells (Milosevic et al., 2005). To incorporate the two protein-protein and protein-phospholipid binding perspectives, Voleti, Jaczynska, and Rizo (2020) suggests a scenario in which Syt-1 clamps SNARE complex at rest and it releases the SV by binding to PIP₂ instead of the SNARE complex upon Ca²⁺ influx, although this is not unanimously agreed upon (Y. Park et al., 2015).

Importantly, C2B regions of Syt-1 oligomerises to a ring structure on the vesicular membrane, forming a "halo" around the vesicular membrane (J. Wang et al., 2014). The oligomerisation has been explicated to reduce the release probability in resting state, whereas Ca²⁺ influx releases the oligomers, unclamping the SV to fuse with the plasma membrane, potentially by binding with anionic lipids (Bello et al., 2018; K. C. Courtney et al., 2021; Norman, Krishnakumar, Timofeeva, & Volynski, 2023; Tagliatti et al., 2020).

Despite various molecules being identified as binding partners of Syt-1, its disinhibiting process is still elusive, but there is growing evidence that Complexins play a modulatory role in this process. Complexins are small molecules ranging about 14 - 20 kDa and are expressed exclusively in the nervous system. They have been identified to interact with SNARE complex in the 1990s and have been revealed to stabilise the complex alongside other accessory proteins (X. Chen et al., 2002; McMahon, Missler, Li, & Südhof, 1995; Weninger, Bowen, Choi, Chu, & Brunger, 2008). There has been a heated debate regarding whether Complexins are the clamps that counteract Syt-1 or are facilitators of the synchronous release. Within experiments using mammalian Complexins, knockdown or knockout of Complexins resulted in reduced evoked release and increased spontaneous release in neurons (Maximov,

Tang, Yang, Pang, & Südhof, 2009; X. Yang, Cao, & Südhof, 2013). Hippocampal slices from Complexin 1 and 2 double knockout (DKO) mice had a reduced amplitude in evoked excitatory postsynaptic current (EPSC) as well as reduced release probability, which was confirmed later in culture (Reim et al., 2001; Xue et al., 2008). The reduced release probability but no change in the size of RRP pointed towards the facilitatory role in priming, as a downstream process to docking (Imig et al., 2014; López-Murcia, Reim, Jahn, Taschenberger, & Brose, 2019; Reim et al., 2001; X. Yang et al., 2013; Q. Zhou et al., 2017). On the contrary, Giraudo, Eng, Melia, and Rothman (2006) used SNARE proteins flipped inside-out in liposome fusion assays to prove that Complexins arrest the zippering of the SNARE complex. The arrest was released by Syt-1 and Ca^{2+} . Later, the inhibitory and facilitatory roles of Complexins were explained by the domain-specific functions of N-terminus and the accessory α -helix (Xue et al., 2009). Hence, Complexins are currently proposed as a double-faced regulator, or as a "checkpoint" protein downstream of docking but prior to fusion (Imig et al., 2014; F. Li et al., 2011; López-Murcia et al., 2024; Yoon et al., 2008). However, the discussion has not yet reached a consensus as to whether their relationship with Syt-1 is competitive or cooperative (N. A. Courtney et al., 2019; Jorquera, Huntwork-Rodriguez, Akbergenova, Cho, & Littleton, 2012; Liang et al., 2021; Ramakrishnan et al., 2020).

Asynchronous release, or delayed exocytosis, refers to the presynaptic activity that takes place tens to hundreds of milliseconds post-stimulation. Although synchronous release accounts for the majority of neuronal activity, asynchronous release is prominent in special types of synapses, such as inhibitory synapses from deep cerebellar nuclei to inferior olive (Best & Regehr, 2009; Turecek & Regehr, 2020), or excitatory synapses from cortico-striatal pyramidal cells to Martinotti cells in cerebral cortex (S. Deng et al., 2020). In hippocampal slices, it was found that cholecystoskinin (CCK)-positive basket cells use asynchronous release of gamma-aminobutyric acid (GABA) to inhibit network activity (Daw, Tricoire, Erdelyi, Szabo, & McBain, 2009; Hefft & Jonas, 2005). Recently, owing to the enhanced temporal resolution in microscopy, asynchronous release has been observed in cultured cortical and hippocampal neurons as well (S. Li et al., 2021; Malagon, Myeong, & Klyachko, 2023; Mendonça et al., 2022). Although the delay and the long-lasting nature of asynchronous release indicates that it has minimal role in relaying information, it is speculated that asynchronous release takes an important role in regulating plasticity of the postsynapse (S. Deng et al., 2020; Hefft & Jonas, 2005; Luo & Südhof, 2017).

It has been suggested that asynchronous release uses a higher-affinity calcium sensor than synchronous release based on its calcium-dependence but slower kinetics (Chamberland et al., 2020; Maximov & Südhof, 2005). The prime candidate so far is Synaptotagmin-7 (Syt-7) (Bacaj et al., 2013; Jackman, Turecek, Belinsky, & Regehr, 2016; Luo & Südhof, 2017). Syt-7 is an isoform of Syt-1, but unlike Syt-1, its expression is not limited to neural tissue (C. Li et al., 1995). Another distinction is that Syt-7 is enriched in the plasma membrane

in synapses, but not in the vesicular membrane, unlike Syt-1 (Marqu  ze, Berton, & Seagar, 2000; Sugita et al., 2001). In a membrane fusion assay using reconstituted vesicles, Syt-7 showed 400-fold lower $[Ca]_{1/2}$ (Ca^{2+} concentration needed to reach 50 % fusion response) than Syt-1, signifying substantially higher affinity to Ca^{2+} (Bhalla, Tucker, & Chapman, 2005). The disassembly kinetics of Syt-7 was distinctively slower than other isoforms, aligning with the long-lasting characteristic of the asynchronous release (Hui et al., 2009; Sugita, Shin, Han, Lao, & S  dhof, 2002). The publications on Syt-7 are not unanimous, however, as there are mixed results reported from murine Syt-7 knockout models (Bacaj et al., 2013; Maximov et al., 2008).

Meanwhile, another candidate Doc2 has been suggested as a distinctive calcium sensor for asynchronous release (J. Yao, Gaffaney, Kwon, & Chapman, 2011). However, Doc2 knock-down and knockout experiments have shown its role in spontaneous, but not in asynchronous release (Groffen et al., 2010; Pang et al., 2011; Ramirez et al., 2017). Even its participation in spontaneous release has been questioned recently (D  ez-Arazola et al., 2020).

The release site of asynchronous exocytosis is another point of controversy. While S. Li et al. (2021) has shown the alignment between asynchronous release sites and N-methyl-D-aspartate (NMDA) receptors at the postsynapse using zap-and-freeze fixed imaging technique, Mendon  a et al. (2022) showed stochastic distribution of the asynchronous release sites based on glutamate-sensitive fluorescent reporters (iGluSnFR) signalling in live imaging. These contrasting views on the nano-structure of the asynchronous release sites have been integrated in Malagon et al. (2023), where the authors classified asynchronous events into two groups based on their spatial profile.

Spontaneous exocytosis

Despite communal effort in its research, spontaneous exocytosis, or "miniature discharges" as denoted first in the 1950s, still remains contentious in multiple levels (Fatt & Katz, 1952). One of the questions was whether it is calcium-dependent. Whereas earlier papers define spontaneous exocytosis as a Ca^{2+} -independent activity (Knight, Von Grafenstein, & Athayde, 1989; Vyleta & Smith, 2011), later papers have defined spontaneous exocytosis as a Ca^{2+} -dependent, but AP-independent activity (Babiec & O'Dell, 2018; Glitsch, 2008; Truckenbrodt & Rizzoli, 2014; Tsintsadze, Williams, Weingarten, von Gersdorff, & Smith, 2017). The latter definition has been widely used in electrophysiology, and it can be conveniently measured with the application of tetrodotoxin to block AP firing. The Ca^{2+} dependence of spontaneous release spawns further questions, such as whether the source of the free Ca^{2+} is extracellular environment, or intracellular Ca^{2+} storage (Chanaday & Kavalali, 2022; Chanaday et al., 2021; Feldthouse, Vyleta, & Smith, 2023; Williams & Smith, 2018). Interestingly, there was a recent report on Ca^{2+} -independent, but VGCC-dependent exocytosis in mouse chromaffin cells (Moya-D  az et al., 2020). Ca^{2+} set aside, increasing evidence leads to an agreement that

the majority of spontaneous release activity is Ca^{2+} -dependent and the following discussions in this thesis would apply this definition, although there might be difference between excitatory and inhibitory synapses (Babiec & O'Dell, 2018; C. S. Wang, Monteggia, & Kavalali, 2023; Williams & Smith, 2018).

A number of studies have shown correlation between Syt-1 and spontaneous exocytosis, which led to the development of "release-of-inhibition" model where the clamping of SVs by Syt-1 tips the balance between spontaneous and evoked exocytosis (Bello et al., 2018; K. C. Courtney et al., 2021; N. A. Courtney et al., 2019; Norman et al., 2023; Xu, Pang, Shin, & Südhof, 2009). Doc2b has also been suggested as a Ca^{2+} sensor for spontaneous exocytosis (Groffen et al., 2010), but manipulating Doc2b in Syt-1-deficient neurons did not affect spontaneous exocytosis significantly in the following publications, contradicting the previous results (Díez-Arazola et al., 2020; Pang et al., 2011).

The next controversy lies in the SV pool implicated in spontaneous exocytosis. One view argues that the spontaneous exocytosis comes from a separate pool (resting pool) to evoked SV recycling (Fredj & Burrone, 2009; Ramirez, Khvotchev, Trauterman, & Kavalali, 2012; Sara, Virmani, Deák, Liu, & Kavalali, 2005; C. S. Wang et al., 2023), whereas another argues that spontaneous exocytosis share the same SV pool as evoked exocytosis (Duan, Kahms, Steinhoff, & Klingauf, 2024; Egashira, Kumade, Ojida, & Ono, 2022; Groemer & Klingauf, 2007; Ralowicz, Hokeness, & Hoppa, 2024; Wilhelm, Groemer, & Rizzoli, 2010). The hypothesis that Syt-1 and Complexin clamps SVs from fusing to the plasma membrane spontaneously support this view, as it means they share exocytosis machinery (Malsam et al., 2020; Xu et al., 2009). Some recent publications support a middle ground, demonstrating that spontaneous exocytosis takes place stochastically in terms of spatial distribution, compared to evoked exocytosis which is concentrated at the active zone, meaning the two modes are segregated but not exclusively (Astacio, Vasin, & Bykhovskaia, 2022; Farsi, Walde, Klementowicz, Paraskevopoulou, & Woehler, 2021; C. S. Wang et al., 2023).

Despite the challenges when studying spontaneous exocytosis, it is important that we advance our knowledge in its mechanism. To our understanding so far, not only does spontaneous exocytosis affect plasticity both presynaptically and postsynaptically, but also it seems to have a pivotal role in neural development (Andreae & Burrone, 2015; Andreae, Fredj, & Burrone, 2012; Garcia-Bereguain, Gonzalez-Islas, Lindsly, & Wenner, 2016; Grasskamp et al., 2023; Martini, Guillamón-Vivancos, Moreno-Juan, Valdeolmillos, & López-Bendito, 2021; Sutton, Wall, Aakalu, & Schuman, 2004). The developmental aspect will be discussed more in detail in the next section "Presynaptic development".

1.1.2 Synaptic vesicle endocytosis

SV endocytosis, the process of retrieving SV cargoes and regenerating SVs, plays an essential role in neurotransmission which can require up to hundreds of SVs at a time and can occur at frequencies over 100 Hz (Hennig & Lømo, 1985; Katz & Miledi, 1979). For the past few decades, it has been studied profoundly *in vitro* and *in vivo* (Denker et al., 2011; Gan & Watanabe, 2018). Based on the agreement that SV endocytosis efficiently caters to the high demand of SVs during neuronal activity, we have begun to understand its molecular mechanism in detail, despite some existing controversies.

The first of such controversies is "what initiates SV endocytosis?". The Ca^{2+} influx-activated phosphatase calcineurin dephosphorylates endocytic proteins downstream of calcium signalling (Cousin & Robinson, 2001). The role of calcineurin in SV endocytosis was disputed since, as it showed mixed results depending on the animal model (e.g., *Drosophila* (Kuromi, Yoshihara, & Kidokoro, 1997) versus rodents (Marks & McMahon, 1998)), types of the synapse (e.g., calyx of Held (Sun et al., 2010) versus cerebellar granule neurons (Clayton et al., 2009)) and different stages of development (Smillie, Evans, & Cousin, 2005; Yamashita, Eguchi, Saitoh, von Gersdorff, & Takahashi, 2010). To tackle this problem, X.-S. Wu et al. (2014) has recorded calyces, cerebellar neurons and chromaffin cells in a Calcineurin A subunit knockout model to test its involvement in both mature and immature synapses. On the other hand, Orlando, Schmitz, Rosenmund, and Herman (2019) and Ogunmowo et al. (2023) showed that the SV endocytosis can be induced by exocytosis without Ca^{2+} influx using sucrose-triggered fusion in cultured neurons.

Once initiated, SV cargoes are selectively recognised by adaptor proteins (Mayor, Parton, & Donaldson, 2014; Moreno-Layseca et al., 2021; Traub & Bonifacino, 2013). The adaptor proteins interact with Bin/ Amphiphysin/ Rvs (BAR) proteins and actin to invaginate the plasma membrane (McMahon & Gallop, 2005; Simunovic, Voth, Callan-Jones, & Bassereau, 2015). The invaginated SVs or endosomes are fissioned from the plasma membrane by the GTPase Dynamin-1 (Dyn-1). The recycled SVs are refilled with neurotransmitters using the $[\text{H}]^+$ gradient generated by vATPase to be rendered fusion-competent again (Cousin & Nicholls, 1997; Pulido & Ryan, 2021), or sorted following the endosomal pathways (Ivanova & Cousin, 2022).

The traditional view on SV endocytosis distinguishes different pathways: clathrin-mediated endocytosis (CME), ultrafast endocytosis (UFE) and activity-dependent bulk endocytosis (ADBE), depending on the kinetics and the invaginated product. There is an emerging view, however, that converges different modes of endocytosis into a single endosomal pathway (Arpino et al., 2022). Nonetheless, for the convenience of reviewing on previous publications, each mode of endocytosis is addressed separately in the following paragraphs.

Clathrin-mediated endocytosis

CME is the most well-known among the three different modes of presynaptic endocytosis. Clathrin polymerises and form a triskelion basket that coats and recycles SVs (Kaksonen & Roux, 2018). Clathrin-coated vesicles were first visualised in 1969 and its assembly has been elucidated down to atomic level later (Kanaseki & Kadota, 1969; Kovtun, Dickson, Kelly, Owen, & Briggs, 2020). CME starts with binding of the adaptor proteins, such as adaptor protein complex 2 (AP-2), to the synaptic cargo or lipids on the plasma membrane, which recruit clathrin to make a clathrin-coated pit (Kelly et al., 2014). AP-2 recognises Yxx Φ (Φ being either F, I, L, M or V) or acidic-dileucine motif ([DE]xxxL[L/I]), although not all the SV cargoes are recognised by AP-2 (Kelly et al., 2014; Traub & Bonifacino, 2013).

Once the clathrins are recruited to make a flat lattice on the plasma membrane (Avinoam, Schorb, Beese, Briggs, & Kaksonen, 2015; Nawara et al., 2022), the clathrin-coated pit is invaginated by nucleated actin, BAR proteins and by the intrinsic force driven from clathrin polymerisation (Obashi, Sochacki, Strub, & Taraska, 2023; Sochacki et al., 2021; Wei et al., 2024; C. Yang et al., 2022). Invagination results in Ω structure, whose neck is scaffolded by BAR proteins to be constricted and fissioned by oligomerised Dyn-1 (Daumke, Roux, & Haucke, 2014; Kishimoto et al., 2011; Kukulski, Schorb, Kaksonen, & Briggs, 2012; Simunovic et al., 2015; Takeda et al., 2018). After scission, a clathrin-coated vesicle is uncoated by recruitment auxilins, which sequentially recruits Hsc70, a chaperone protein that binds to auxilin and disrupts clathrin triskelia (Lemmon, 2001; Sousa & Lafer, 2015).

The ubiquitous presence of CME across kingdoms, species and cell types and the distinctiveness of its intermediate Ω structure hugely accelerated our understanding of its mechanism. In neurons, live-cell imaging techniques promoted CME as the main mode of SV endocytosis (Dickman, Horne, Meinertzhagen, & Schwarz, 2005; Granseth, Odermatt, Royle, & Lagnado, 2006; B. Zhang et al., 1998). However, experiments at physiological temperatures showed that clathrin-independent mode of endocytosis prevails in murine synapses, in contrast to the results in room temperature (Delvendahl, Vyleta, von Gersdorff, & Hallermann, 2016; Soykan et al., 2017). Furthermore, AP-2, which has been regarded as an exclusive adaptor protein to recognise cargoes in CME, was found to participate in clathrin-independent endocytosis as well (Kononenko et al., 2014; López-Hernández et al., 2022; Soykan et al., 2017). The temperature-dependence and non-specificity of the participating proteins cast doubt on the prevalence of CME.

Ultrafast endocytosis

UFE is a mode of SV endocytosis that takes place within 50 - 100 ms after stimulation at physiological temperatures. UFE became accessible for research owing to the development of flash-and-freeze electron microscopy as it allowed access to higher temporal resolution, down to 10 milliseconds post-stimulation. UFE has been reported in mouse hippocampal synapses *in vitro* and in *C. elegans* neuromuscular junction *in vivo* with similar kinetics (Watanabe, Liu, et al., 2013; Watanabe, Rost, et al., 2013). This was an important finding because previously CME was measured to retrieve synaptic cargoes within in the order of seconds (Balaji & Ryan, 2007), but UFE has shown that SV recycling activates within milliseconds. Watanabe and his colleagues have further shown that UFE takes place in the peri-active zone, forming either individual vesicles or endosomal intermediates, followed by budding of individual SVs in a clathrin, actin and Dynamin-dependent manner (Imoto et al., 2022; Watanabe et al., 2014). In following works, Synaptojanin-1 and Endophilin-A were identified to regulate neck formation in UFE, in coordination with BAR proteins to relieve the compressive force generated by exocytosis (Ogunmowo et al., 2023; Watanabe et al., 2018).

Activity-dependent bulk endocytosis

Activity-dependent bulk endocytosis (ADBE) is a two-step process that consists of initial invagination of the bulk endosome and the budding of the individual SVs from the bulk endosome (Arpino et al., 2022; Clayton & Cousin, 2009; Cousin, 2009). It was first reported 40 years ago but only has been studied in depth in the past two decades, catalysed by the reasoning that generating individual SVs from the plasma membrane via CME is not fast enough to sustain the neuronal activity (Miller & Heuser, 1984; Sankaranarayanan & Ryan, 2000). Indeed, a growing number of studies on ADBE showed its functional significance. It was soon established that ADBE generates fusion-competent SVs, sustains membrane tension after SV fusion, and provides a sorting platform for the presynaptic proteins especially during intense stimulation (Clayton, Evans, & Cousin, 2008; Hoopmann et al., 2010; Ivanova et al., 2021; Tran, Silva, & Marty, 2023).

It is true that the majority of ADBE molecules overlap with other modes of endocytosis (A. Kokotos, Peltier, Davenport, Trost, & Cousin, 2018). For example, Dyn-1 plays an important role in ADBE (Bonnycastle et al., 2023; Clayton & Cousin, 2009). However, some enzymes and cargo proteins have been recognised to be more specific for ADBE. First, Calcineurin has been reported as a key driver of the ADBE (Cheung & Cousin, 2013; A. C. Kokotos & Cousin, 2015; Sun et al., 2010). Vesicle-associated membrane protein (VAMP-4) has been indicated as an ADBE cargo that is selectively retrieved via bulk organelle (Ivanova et al., 2021; A. Kokotos et al., 2018; Nicholson-Fish, Kokotos, Gillingwater, Smillie, & Cousin, 2015). Recently, glycogen synthase kinase 3 (GSK3) has been highlighted as a regulator of ADBE through different pathways by phosphorylating Dyn-1 and phosphatidylinositol 4-kinase II α (PI4KII α) (Blumrich

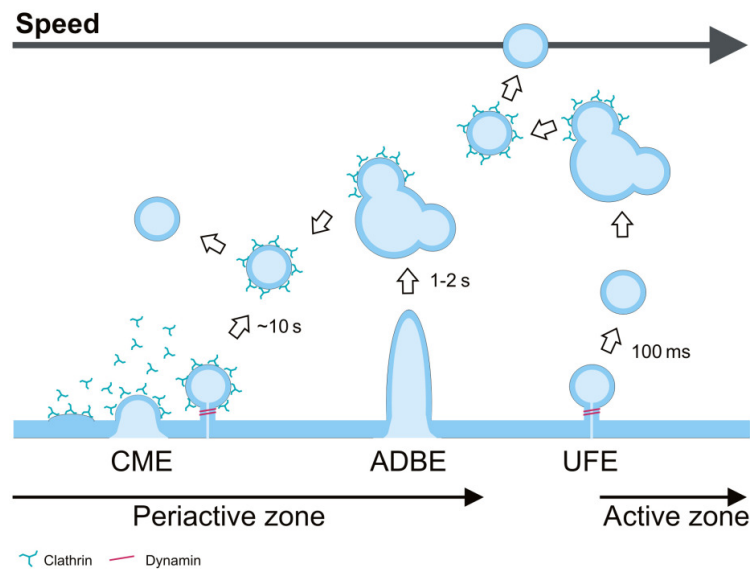


Figure 1.4: Simplified schematic of the SV endocytosis pathways

Image sourced from Soukup, Verstreken, and Vilain (2017).

et al., 2023; Clayton et al., 2010). The phosphorylation of Dyn-1 (S774) activates ADBE by modulating its affinity to Syndapin, whereas the phosphorylation of phosphatidylinositol 4-kinase II α (PI4KII α) (S47/51) leads to the binding of ADBE-regulating enzymes to PI4KII α , retaining the key ADBE molecules and inhibiting ADBE (Blumrich et al., 2023; Clayton et al., 2010; Imoto et al., 2022).

The majority of experiments on ADBE capitalises on the internalisation of fluid phase markers in *in vitro* systems, under artificial stimulation. Hence, there was skepticism of ADBE being an artefact rather than a physiological phenomenon. However, the frequency that was optimised for maximal ADBE (40 Hz) in primary culture of cerebellum granular neurons in Clayton et al. (2008) is within the range of the spontaneous firing rate in Purkinje cells and interneurons (Häusser & Clark, 1997). Furthermore, there is sufficient evidence of ADBE *in vivo*. In invertebrates, synaptic remodeling in *C. elegans* has been shown to utilise ADBE during development (Cuentas-Condori et al., 2023). In mammals, Körber, Horstmann, Sätzler, and Künér (2012) fixed and imaged calyx of Held after horse radish peroxidase (HRP) injection at MNTB in anaesthetised rats, and observed multiple HRP-labelled bulk endosomes at individual nerve terminals.

In summary, CME, UFE and ADBE can be summarised as the schematic figure 1.4.

1.2 Presynaptic Development

1.2.1 Morphological Development

Neuronal polarisation

The complex function of the presynapse described above is equipped and activated in parallel to the dynamic morphological development of a neuron. The morphological development from a progenitor cell to a polar neuron is a key step in acquiring neuronal identity. The morphological sequence of neuronal development has been established in the 1980s through observing primary neurons from embryonic hippocampi (Dotti, Sullivan, & Banker, 1988). This sequence starts with generation of processes, followed by axon specification and dendritogenesis as described in the schematic figure 1.5 (Fukata, Kimura, & Kaibuchi, 2002; Scott & Luo, 2001; Yoshimura, Arimura, & Kaibuchi, 2006).

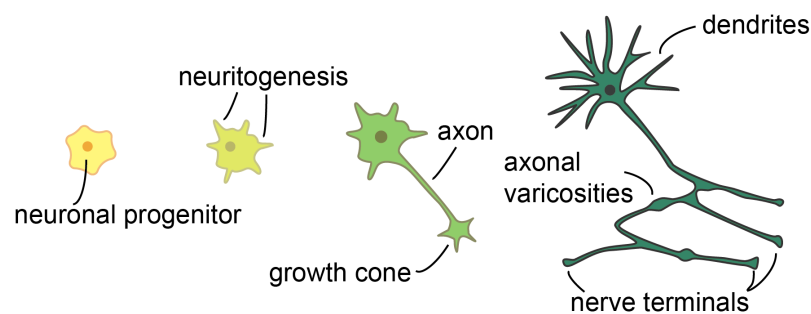


Figure 1.5: The developmental trajectory of neuronal morphology.

In a hippocampal primary culture, a neuronal progenitor starts to show protrusions that becomes processes within a day after plating. The major process specifies into an axon, and the minor processes develop into dendrites. A neuron reaches its morphological maturity within the first week *in vitro* (Dotti et al., 1988).

Axon specification and elongation is the first event in achieving the polarity of a neuronal progenitor, and it takes place as a result of a combination of intrinsic programming and extracellular cues. Axon guidance requires polymerisation and transport of microtubules, and extensive fusion and retrieval of the plasma membrane in order to expand or reduce the surface area in response to external cues. Aside from branches and nerve terminals, an axon would have two distinctive compartments: a growth cone and an axon initial segment (AIS) as shown in the schematic figure 1.6.

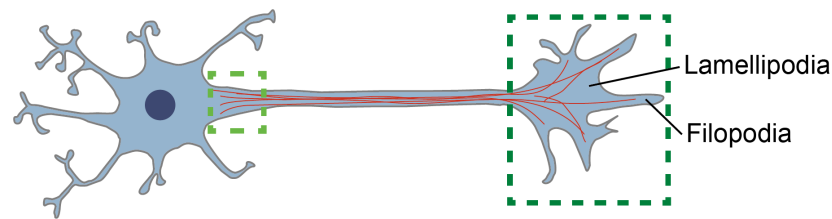


Figure 1.6: Compartments of a developing neuron.

The axon initial segment (AIS) (light green box) and it is an important compartment that maintains the axonal identity throughout the neuronal life by regulating action potential firing, intracellular transport and membrane diffusion. Growth cone (dark green box) is the driving vehicle of axon guidance. The growth cone shows amoeba-like motility by projecting and retracting dynamic protrusions (filopodia). Actin filaments (red) functions as the backbone of the axon and the growth cone.

Growth cone

The growth cone is a highly dynamic structure with an amoeba-like motility that functions as a guidance vehicle at the distal end of a growing axon. A growth cone is featured by filopodia, the protruding processes, and lamellipodia, the "webbing" between filopodia. The extension and retraction of these structures are dependent on the assembly and disassembly of cytoskeleton, such as microtubules and filamentous actin (F-actin), in response to substrate-bound cues (i.e., cell adhesion molecules (CAMs), laminin and fibronectin) and chemotropic cues (i.e., netrin, semaphorin), as reviewed in Lowery and Vactor (2009).

The motility of the growth cone requires quick adjustment of the surface area of the plasma membrane, for which SV exo- and endocytosis have been proposed as the mechanism. Indeed, clear vesicles have been first observed in the growth cone and this finding has been confirmed later (Nozumi, Nakatsu, Katoh, & Igarashi, 2017; Rees, Bunge, & Bunge, 1976; Sabo & McAllister, 2003). Asymmetric exo- and endocytosis induced by calcium signalling have been identified as the driving force for controlling the surface area (Hines, Abu-Rub, & Henley, 2010; Tojima et al., 2007; Tojima, Itofusa, & Kamiguchi, 2014). It is very likely that ADBE or UFE is utilised in this process. The endosomal structures in a growth cone was first identified by Dailey and Bridgman (1993). Other studies have shown clathrin-independence of SV recycling in growth cones in avian and mammalian neuronal cultures by using fluid phase markers such as FM dyes and dextran (Bonanomi et al., 2008; Kabayama et al., 2011). Super-resolution microscopy has demonstrated spatial segregation between CME and ADBE within the growth cone, as the retrieval of Synaptophysin proximal to filopodia in the growth cone did not colocalise with clathrin (Nozumi et al., 2017).

Axon initial segment (AIS)

Another core structure of the axon is the AIS, where the information transmitted from the dendrites are converged, and where the AP begins and propagates towards distal axons. Located at or next to the axon hillock, the AIS is featured with voltage-gated sodium (Na_v) and potassium (K_v) ion channels, a dense accumulation of cytoskeleton, CAMs, and the scaffolding protein Ankyrin G (Leterrier, 2016; Rasband, 2010). Assembling and maintaining the AIS is a keystone in presynaptic development, because it plays an important role in regulating AP firing and keeping axonal identity. Therefore, it is unsurprising that the AIS disruption is related to numerous neurological disorders such as stroke, schizophrenia, bipolar disorder and epilepsy (M. A. Ferreira et al., 2008; Lewis, Hashimoto, & Volk, 2005; Ogiwara et al., 2007; Oh et al., 2023; Schafer et al., 2009).

The AIS regulates AP based on the enrichment of Na_v and K_v channels (Clark, Goldberg, & Rudy, 2009; Kole & Stuart, 2012). The density of Na_v channels at the AIS is 40-50 times higher than in soma or dendrites (Kole & Stuart, 2012). The high local density of $\text{Na}_v1.6$, the most common subtype in mature pyramidal neurons, for example, causes a hyperpolarising shift of the activation threshold, facilitating the initiation of APs in pyramidal neurons (Hu et al., 2009; Kole et al., 2008). The distribution of the Na_v channels is not constant, however, as it was shown to redistribute in sensory input-dependent manner in avian magnocellularis neurons (Kuba, Ishii, & Ohmori, 2006; Kuba, Oichi, & Ohmori, 2010). K_v channels are also enriched in the AIS, and the reported subtypes enriched at the AIS are K_v1 , K_v3 , and K_v7 , which affects AP with when disrupted (Goldberg et al., 2008; Kole, Letzkus, & Stuart, 2007; Pan et al., 2006; Rowan, Tranquil, & Christie, 2014; Sabater, Rigby, & Burrone, 2021; Shah, Migliore, Valencia, Cooper, & Brown, 2008).

Aside from the electrical modulation, another important role of the AIS is to maintain neuronal polarity, or to maintain axonal identity (Rasband, 2010). The AIS has been suggested as a filter for intracellular transport, creating a physical barrier between the somato-dendritic compartment and axon based on the orientation of microtubules and on the efficacy of motor proteins Kinesin and Dynein (A.-H. Song et al., 2009). However, it is worth noting that there are publications that identify the pre-axonal zone, rather than the AIS as the sorting gate for intracellular transport (Fariás, Guardia, Britt, Guo, & Bonifacino, 2015; Petersen, Kaech, & Banker, 2014). Secondly, the AIS shows limited lipid mobility, which has been confirmed in a number of studies albeit the disagreement on the molecules that compose the diffusion barrier (Albrecht et al., 2016; Brachet et al., 2010; Nakada et al., 2003; Winckler, Forscher, & Mellman, 1999). Recently, Eichel et al. (2022) reported AP-2-dependent endocytic clearing at the AIS in *C. elegans*, mouse hippocampal neurons and human induced pluripotent stem cell (hiPSC), suggesting SV recycling as a mechanism for maintaining axonal identity. Cisternal organelles have been reported previously, but as a Ca^{2+} storage than as an intermediate for SV recycling (Sánchez-Ponce, DeFelipe, Garrido, & Muñoz, 2011).

1.2.2 Presynaptic differentiation

Active zone assembly

Active zone refers to the electron-dense area at the presynapse where SVs are docked and fused to release neurotransmitters into the synaptic cleft, in apposition to PSD. It is enriched with VGCCs, cytoskeleton and SV exo- and endocytic machineries, held together by scaffolding proteins such as Piccolo, Bassoon, regulating synaptic membrane exocytosis protein (RIM) and active zone-associated structural protein (CAST)/protein rich in the amino acids E, L, K, and S (ELKS). The macromolecular complex enables time-sensitive, fail-safe and plastic presynaptic activity which is essential for brain communication (reviewed in Südhof (2012) and Dresbach, Qualmann, Kessels, Garner, and Gundelfinger (2001). For plasticity see Glebov et al. (2017)).

Active zone assembly has been a popular topic since the early 2000s (Rizalar, Roosen, & Haucke, 2021). It was revealed that the presynaptic proteins are synthesised in soma and delivered to the nerve terminals in unitary, preassembled packets generated from *trans*-Golgi network, which explains the fast establishment of SV recycling within 40 mins after axo-dendritic contact (Ahmari, Buchanan, & Smith, 2000; Dresbach et al., 2006; Friedman, Bresler, Garner, & Ziv, 2000; Sabo, Gomes, & McAllister, 2006; Shapira et al., 2003; Zhai et al., 2001). There has been an eclectic array of reports about the composition of this precursor packet. Some called it Piccolo-Bassoon transport vesicle (PTV) or synaptic vesicle transport vesicle (STV) based on the protein cargoes, and they were reported as dense core vesicles (Dresbach et al., 2006; Sabo et al., 2006; Shapira et al., 2003; Zhai et al., 2001). Some called it "transport aggregates", which was reported as a combination of both dense-core vesicles and clear vesicles (Tao-Cheng, 2007). The latter idea aligns with the *in vivo* studies that show differential recruitment between some proteins and SVs at the nerve terminals (Easley-Neal, Fierro, Buchanan, & Washbourne, 2013; Lipton, Maeder, & Shen, 2018; Maeder, San-Miguel, Wu, Lu, & Shen, 2014). Recently, there have been more studies focusing on the transport mechanism of the active zone precursors. Rizalar et al. (2023) has identified ARL8A-KIF1A-PI_(3,5)P₂ pathway as a mechanism for the anterograde transport of the precursor vesicle. VGCCs are also transported and localised with active zone proteins although their individual presence is not indispensable for active zone assembly (Graf et al., 2012; Held et al., 2020; Radulovic et al., 2020). Another perspective suggests liquid-liquid phase separation by scaffolding proteins as a mechanism for active zone recruitment, but whether this is the case in physiological neurons remains to be answered (Liang et al., 2021; McDonald, Fetter, & Shen, 2020).

Trans-synaptic signalling

A mature synapse typically consists of a presynapse and a corresponding postsynapse, and these two components are physically linked by CAMs (Südhof, 2018). It was postulated that presynaptic and postsynaptic specialisation is instigated by axo-dendritic contact (Friedman et al., 2000). Artificial synapse formation assays have shown that the expression of postsynaptic CAMs in non-neuronal cells led to synaptic connection with the co-cultured neurons, and vice versa, the expression of presynaptic CAMs in non-neuronal cells recruited postsynaptic structures in the contacting neurons (Biederer et al., 2002; Graf, Zhang, Jin, Linhoff, & Craig, 2004; Nam & Chen, 2005; Scheiffele, Fan, Choih, Fetter, & Serafini, 2000). These studies made it evident that the trans-synaptic signalling induces presynaptic and postsynaptic differentiation, and the clinical cases of mutations in trans-synaptic molecules have stressed the significance of CAMs in neural development (de Agustín-Durán, Mateos-White, Fabra-Beser, & Gil-Sanz, 2021). However, there have been mixed views on the role of trans-synaptic signalling in synaptogenesis, as multiple works have shown that the SV clusters are fusion-competent before axo-dendritic contact (Kraszewski et al., 1995; Matteoli, Takei, Perin, Südhof, & De Camilli, 1992; Zakharenko, Chang, O'Donoghue, & Popov, 1999). Not only presynapses without a contacting postsynapse are functional, they mature regardless of axo-dendritic contacts as well (Andreae et al., 2012). How much does trans-synaptic signalling affect the presynaptic function still remains a conundrum. In the next two paragraphs, we will briefly discuss the most widely studied CAMs, neurexin and leukocyte antigen-related (LAR)-receptor-protein tyrosine phosphatase (RPTP)s.

Neurexin is a presynaptic CAM that binds to postsynaptic CAMs (e.g., neuroligins) trans-synaptically, and interacts with active zone proteins intra-synaptically (Luo, Sclip, Jiang, & Südhof, 2020; Südhof, 2017). Although a paper has suggested that neurexin organises active zone assembly by clustering SVs (Dean et al., 2003), triple knockout neurons from rodents did not show defects in the formation of synapses (L. Y. Chen, Jiang, Zhang, Gokce, & Südhof, 2017; Luo et al., 2020; Missler et al., 2003). Bury and Sabo (2014) has disproved the recruiting effect of neuroligin-mediated trans-synaptic signalling, because neither speed nor direction of the active zone proteins were affected by the trans-synaptic signalling. Instead, it was observed that the axo-dendritic contact site stabilises, or traps, the moving precursor packets. Interestingly, whereas neurexins might play a less active role in presynaptic differentiation, there are reports on trans-synaptic signalling in reverse direction, showing that presynaptic structures induce postsynaptic differentiation (Biederer et al., 2002). For example, Aoto, Martinelli, Malenka, Tabuchi, and Südhof (2013) has reported the regulatory role of neurexins on postsynaptic AMPA receptor trafficking. Sabo et al. (2006) has also observed that the dendritic filopodia "scan" an axon before stabilising at a contact site, and the preferred sites are where STVs pause during their transportation.

LAR-RPTP also has been highlighted as an essential presynapse organiser (Takahashi & Craig, 2013), but the mammalian neurons from triple-knockout model did not show a major synaptic phenotype (Emperador-Melero, de Nola, & Kaeser, 2021). On the contrary, aberration of liprin- α s, a downstream ligand of LAR-RPTP, resulted in "empty boutons" that made apposing contacts with the postsynapse but were lack of SVs (Marcó de la Cruz et al., 2024).

The inconsistent reports on the role of CAMs in presynaptic differentiation are quite puzzling. Hence, there is a new emerging view that argues there is no "master organiser" of the presynapse (Rizalar et al., 2021). This view agrees with the findings that the precursor components for the active zone can be transported without a postsynaptic cue, and the SVs can be recycled before the axo-dendritic contact (Kraszewski et al., 1995; Matteoli et al., 1992; Sabo et al., 2006; Zakharenko et al., 1999). This could be an example of degeneracy in nature, which refers to many-to-many relationship between proteins and functions in this case, pressured by the evolutionary selection process (Bernard, 2023; Edelman & Gally, 2001). The development of SV recycling, on the other hand, has a more distinctive requirement for individual proteins.

Synaptic vesicle recycling during development

So far the discussion has focused on the molecular recruitment and organisation at the presynapse, but the functional aspect of presynaptic development is critical in understanding brain development. When, where and how a nerve terminal acquires neurotransmission throughout the development determines the synaptic connection as well as the connectivity of the nervous system. Multiple neurodevelopmental disorder (NDD) reports on the genetic mutations in genes that participate in exo- and endocytosis at the nerve terminals again confirm its importance (Bonnycastle, Davenport, & Cousin, 2021; Guarnieri et al., 2017).

The first step of the presynaptic activity, Ca^{2+} influx via VGCC, and its relation with neurotransmission has been studied expansively in calyx of Held, a giant synapse in the auditory system with hundreds of active zones in medial nucleus of the trapezoid body (MNTB) (Baydyuk, Xu, & Wu, 2016). Simultaneously with hearing onset, the contribution of N- type ($\text{Ca}_v2.2$) VGCC decreases while P/Q- type ($\text{Ca}_v2.1$) increases (Iwasaki, Momiyama, Uchitel, & Takahashi, 2000; L.-G. Wu, Westenbroek, Borst, Catterall, & Sakmann, 1999). The coupling between Ca^{2+} influx and neurotransmitter release becomes tighter after hearing onset as well, meaning the Ca^{2+} influx triggers SV fusion more efficiently with fewer VGCCs (Fedchyshyn & Wang, 2005; Kochubey, Han, & Schneggenburger, 2009; L.-Y. Wang, Neher, & Taschenberger, 2008; Y.-M. Yang et al., 2010). This finding has led to a suggestion of the perimeter release model, where the SVs are released within a distance of (< 30 nm) a VGCC cluster and the perimeter coupling distance is shortened upon maturation (Nakamura et al., 2015). However, it is questionable whether the findings in calyx of Held can be generalised to typical central synapses because of its giant size and multiple active zones projecting towards a single principal

neuron (Baydyuk et al., 2016; Sierksma, Slotman, Houtsmuller, & Borst, 2020). So far, no major developmental transition in the mode of calcium coupling has been reported in small synapses, except the expression of N- type ($\text{Ca}_v2.2$) VGCC decreases on the axon throughout development, and P/Q- type ($\text{Ca}_v2.1$) VGCC regulates the majority of the presynaptic Ca^{2+} influx (Bornschein, Eilers, & Schmidt, 2019; Chaudhuri, Alseikhan, Chang, Soong, & Yue, 2005; Pravettoni et al., 2000; Qian & Noebels, 2001).

The second aspect that has caught the attention in the field is spontaneous exocytosis in developing neurons. In primary neuronal culture, spontaneous exocytosis has been recognised as the dominant mode of neuronal activity in the first week *in vitro* regardless of axo-dendritic contact (Andreae et al., 2012). In slices, Banerjee et al. (2022) has shown the decreasing spontaneous calcium activity in trigeminal ganglion in rodents from P0-16 to P60. Motor neurons and sensory neurons also exhibit spontaneous activity without external stimuli during development, implying spontaneous exocytosis could be an underpinning process for building neural circuits (Martini et al., 2021). Several theories have been suggested as the function of spontaneous exocytosis in developing neurons. It is possible that the spontaneous activity functions as a guide for postsynaptic differentiation both morphologically and functionally (Andreae & Burrone, 2015; Choi et al., 2014; Garcia-Bereguain et al., 2016; Sutton et al., 2004). Also, in retinogeniculate system, spontaneous exocytosis strengthens specific synaptic connections and it spares the synapse from pruning, resulting in eye-specific segregation of the circuit (Faust, Gunner, & Schafer, 2021). There have been many attempts to identify spontaneous exocytosis-specific molecules, such as Complexin or VAMP-4 but the suggested molecules are not exclusive to spontaneous exocytosis, nor do they show a decrease in the expression level later in the development (Lin et al., 2020; Malsam et al., 2020; Urbina & Gupton, 2020). It will be informative to identify the molecules in spontaneous exocytosis, as this will also help us solve the problem whether evoked and spontaneous exocytosis derive from the same pools.

The fact that there is a change in the dominant mode of exocytosis poses a question about the composition of SV pools. The hint of SV pool reorganisation come from electrophysiological data in the 1990s, which reported a decrease in the release probability in slices as the brain develops (Bolshakov & Siegelbaum, 1995; Reyes & Sakmann, 1999). Soon after, using live cell imaging and fixed electron microscopy, Mozhayeva, Sara, Liu, and Kavalali (2002) demonstrated three steps of SV pool development: from the absence of RRP to the predominance of RRP (~100 %), followed by balancing between RRP and reserve pool. The size of the reserve pool was previously associated to the expression level of Synapsins (A. Ferreira et al., 2000). Although Mozhayeva et al. (2002) did not delve into different modes of exocytosis, it suggested a possible explanation for the decreasing release probability of the SVs in the previous studies. It is possible that a change in spatial relationship between SVs and the calcium channels result in changes in release probability, hence changing the SV pool composition, as it was shown in

P14-21 hippocampal slices (Scimemi & Diamond, 2012). Interestingly, Rose et al. (2013) have shown that almost all of the SVs in hippocampal organotypic slice culture can be mobilised, indicating near absence of resting pool. Although the time frames are not exactly the same, the reduction in spontaneous calcium activity and the disappearance of resting pool might be correlated (Banerjee et al., 2022; Rose et al., 2013).

On the other hand, SV endocytosis in developing neurons largely remains unexplored, despite the fact that it was spotted early on in the 1990s (Coco, Verderio, De Camilli, & Matteoli, 1998; Matteoli et al., 1992). This is very surprising because endocytosis, like exocytosis, is a highly time-sensitive process that enables fidelity of neurotransmission and maintains membrane homeostasis. Therefore, understanding its acquisition is essential from painting a full picture of the presynaptic development, which is a central aim of this thesis.

1.3 Neurodevelopmental Disorders

NDDs are gaining more social awareness and scientific recognition in the modern society. However, still millions of individuals are speculated to be affected, which also heavily burdens their families and communities financially and (Fleming et al., 2020; Francés et al., 2022; Y. Yang et al., 2022; Zuvekas et al., 2021). National Health Statistics Reports from Centers for Disease Control and Prevention (CDC) in the United States reported that 17.8 % of the children and adolescents are diagnosed with at least one NDD between 2015 and 2018 (Zablotsky & Black, 2020). As we are beginning to understand the genetics of NDDs, a number of mutations in genes related to presynaptic SV recycling have been reported in clinical cases (Bonnycastle et al., 2021; Gidziela et al., 2023). This is not surprising considering that SV recycling is a critical part of neurotransmission, and its disruption will inevitably affect brain development. This thesis focuses on two of such NDDs: Fragile X Syndrome (FXS) and Dual-specificity tyrosine-regulated kinase 1 A (DYRK1A) syndrome. The overarching aim is to understand each condition more in depth in a novel perspective, and to contribute to searching for a convergent pathology which will provide a better approach for therapeutic intervention.

1.3.1 Fragile X Syndrome (FXS)

Clinical aspects of FXS

FXS is one of the most common X-linked monogenic NDDs that affects 1 in ~7,000 males and 1 in ~11,000 females (Hunter et al., 2014). Individuals affected by FXS show more than 50 % comorbidity with autism spectrum disorder (ASD) and intellectual disability (ID) in males and 20-30 % in females, with a smaller but significant association with epileptic seizure as well (Hagerman et al., 2017).

FXS is caused by the downregulation of the gene *Fragile X Messenger Ribonucleoprotein 1 (Fmr1)*, which encodes Fragile X Messenger Ribonucleoprotein (FMRP). The expression level of FMRP is downregulated as a result of the expansion of CGG repeats (> 200) at the 5'-untranslated region (UTR) that results in hypermethylation of the gene and its CpG island in the promoter region (Pieretti et al., 1991). In rare occasions, FMRP function is downregulated not because of epigenetic repression, but because of point mutations (e.g., I304N), but not all reported point mutations result in downregulation of FMRP (e.g., R138Q) (Quartier et al., 2017; J. B. Zang et al., 2009).

Physical characteristics of FXS include flat feet, large and prominent ears, large testicles and an elongated face with broad forehead (Hagerman & Hagerman, 2002; Lubala et al., 2018). Regional analysis in brains showed enlarged caudate nuclei in children and young adults (Eliez, Blasey, Freund, Hastie, & Reiss, 2001; Reiss, Abrams, Greenlaw, Freund, & Denckla, 1995), whereas reports on other brain regions are mixed. On a cellular level, post-mortem analysis shows immature postsynaptic structures (long, filopodial dendritic spines and high number of spines) in cortices in humans affected by FXS (Irwin et al., 2001). This is recapitulated in rodent models (Galvez & Greenough, 2005; Grossman, Elisseou, McKinney, & Greenough, 2006).

Fragile X Ribonucleoprotein (FMRP)

FMRP is a 632-amino acid-long ribonucleoprotein that regulates the translation of messenger-ribonucleic acid (RNA) at various subcellular locations. It is highly conserved across species and most abundantly expressed in the brain throughout development (den Broeder et al., 2009; Gholizadeh, Halder, & Hampson, 2015; Specchia et al., 2019; Tamanini et al., 1997). The protein is localised in cytoplasm, nucleus, pre- and postsynapse (Antar, Dichtenberg, Plociniak, Afroz, & Bassell, 2005; Dury et al., 2013; Monday, Kharod, Yoon, Singer, & Castillo, 2022). There are 11 isoforms in human FMRP depending on alternative splicing, and it is also post-translationally modified, either by phosphorylation, methylation or SUMOylation (Prieto, Folci, & Martin, 2020).

At the N-terminus, Tudor domain (TD) 1 and 2, as depicted in the schematic 1.7, have been suggested to provide platforms for RNA binding or protein-protein binding (Adinolfi et al., 2003; P.-Y. Deng, Kumar, Cavalli, & Klyachko, 2022; Myrick, Hashimoto, Cheng, & Warren, 2015; Ramos et al., 2006; Taha et al., 2021). In the central domain, there are nucleus localisation and export sequences (Eberhart, Malter, Feng, & Warren, 1996), followed by two K homolog (KH) motifs (KH1 and KH2), which also interact with mRNAs (reviewed in D'Annessa, Cicconardi, and Di Marino (2019)). The arginine-glycine rich (RGG) box at the C-terminus is another mRNA-binding domain that has been widely studied (D'Annessa et al., 2019). The structure of FMRP is reviewed more in detail in Fernández, Rajan, and Bagni (2013).



Figure 1.7: Structure of FMRP

From N-terminus, FMRP consists of Tudor domain (TD) 1, 2. There is nuclear localisation signal (NLS) and nuclear export signal (NES) in the central domain sandwiching K homolog (KH) domains. The arginine-glycine rich (RGG) box in the C-terminus interacts with mRNAs and regulates local translation.

Canonical roles of FMRP

FMRP is an RNA-binding protein, hence the early studies of FMRP focused on what it binds to, and the function of such binding. The most well-established sequence that binds to the RGG box is the guanine-quadruplex motif, although this motif does not account for all of the mRNA targets (Darnell et al., 2001; Maurin et al., 2018; Subramanian et al., 2011; Suhl, Chopra, Anderson, Bassell, & Warren, 2014). The binding of FMRP with mRNA has been reported to regulate transportation, stabilisation and translation of the target mRNAs (Richter & Zhao, 2021). It has been demonstrated that FMRP can inhibit translation by tackling either initiation or elongation steps (Darnell et al., 2011; Napoli et al., 2008), or interacting with a microRNAs in *Drosophila Fmr1* KO model (Jin et al., 2004; Muddashetty et al., 2011). At the subcellular level, it is established that FMRP regulates local protein synthesis in the postsynapse (Hagerman et al., 2017; Hale et al., 2021; Ifrim, Williams, & Bassell, 2015). One of the well-known circuitry defects stemming from the postsynaptic protein synthesis is the increased metabotropic glutamate receptor (mGluR) I- dependent long-term depression (LTD) in hippocampus (Huber, Gallagher, Warren, & Bear, 2002). According to this mGluR theory, FMRP deficiency results in increased expression of LTD-related proteins, such as Arc or MAP1b (Bear, Huber, & Warren, 2004; Hou et al., 2006; Lüscher & Huber, 2010; Pfeiffer & Huber, 2009). However, disinhibited protein synthesis is yet insufficient to account for a lot of the circuit phenotypes, such as downregulated expression of GABA receptors (Hong, Zhang, Ke, El Idrissi, & Shen, 2012; Sabanov et al., 2017) or a delay in the developmental GABA switch in *Fmr1* KO mouse models (He, Nomura, Xu, & Contractor, 2014). At the presynapse, on the other hand, research on local protein synthesis regulated by FMRP remains at its infancy, although the presynaptic mRNAs have been previously identified as targets of FMRP and translational machineries were localised at the presynapse (Darnell et al., 2011; Hafner, Donlin-Asp, Leitch, Herzog, & Schuman, 2019; Monday et al., 2022).

Noncanonical roles of FMRP at the presynapse

Research on FMRP primarily concentrated on the postsynapse, but for the past 15 years, growing evidence has shown its role in the function at the presynapse. The Klyachko group has played a major role in exploring AP broadening in *Fmr1* KO young adult mice, which was ascribed to the interaction between the N-terminus of FMRP and the $\beta 4$ region of the large-conductance calcium-activated potassium (BK) channel in hippocampus and cortical slices (P.-Y. Deng et al., 2013). The gating of the BK channel by FMRP has been confirmed again in *Drosophila* (Myrick, Deng, et al., 2015), and in rodent models it was further corroborated by genetic rescue experiments (P.-Y. Deng & Klyachko, 2016). Other potassium channels, such as sodium-activated potassium channel Slack-B or small-conductance calcium-activated potassium (SK) channel, have also been reported as interacting partners of FMRP via electrophysiological assays and co-immunoprecipitation (Brown et al., 2010; P.-Y. Deng et al., 2019). Y.-M. Yang et al. (2020) have shown that FMRP inhibits GABA release from basket cells by gating $K_v1.2$, at the presynapse and the deficiency of FMRP results in increased GABA release, leading to the reduced firing rate in Purkinje neurons in cerebellum. The Dolphin group, on the other hand, has shown that FMRP regulates the expression of N-type VGCC ($Ca_v2.2$), but similar experiments in different groups showed mixed results (Bonnycastle, Kind, & Cousin, 2022; P.-Y. Deng, Sojka, & Klyachko, 2011; Ferron, Nieto-Rostro, Cassidy, & Dolphin, 2014). Further regulation of ion channels by FMRP is summarised in P.-Y. Deng and Klyachko (2021).

Although it seems logical to speculate that the dysregulation of ion channels at the presynapse will result in changes in SV recycling, there have been few studies on this subject. P.-Y. Deng et al. (2011) has shown accelerated SV turnover and bigger SV pool size in *Fmr1* KO mice. The faster SV turnover is in line with the increased spontaneous SV recycling at the presynapse in *Fmr1* KO neurons. Bonnycastle et al. (2022) has shown an increase in the recycling pool, but did not show any difference in SV recycling kinetics. More specifically, Bonnycastle and her colleagues reported a decrease in ADBE at DIV 14 in primary neuronal cultures (Bonnycastle et al., 2022). This is potentially due to the lost binding between FMRP and the BK channel, but not due to the disinhibition of mRNA translation based on rescue experiments with FMRP with point mutations on the binding sites. The relationship between ADBE and the binding between FMRP and the BK channel has not been studied further.

1.3.2 Dual specificity tyrosine phosphorylation regulated kinase 1A (DYRK1A) syndrome

Clinical aspects of DYRK1A syndrome

DYRK1A syndrome is a recently-identified NDD that entails ID and speech impairment (van Bon, Coe, de Vries, & Eichler, 2021). It also shows high comorbidity with ASD, microcephaly and epilepsy (Courraud et al., 2021; Ji et al., 2015). It is estimated to affect 0.5 - 1 % of the population affected by developmental disorders (The Deciphering Developmental Disorders Study, 2015). DYRK1A syndrome is caused by *de novo* loss of function of the gene *DYRK1A*, which is located in human chromosome 21 in the Down syndrome critical region (21q22.13) (Courraud et al., 2021). As the name of the genetic loci suggests, its redundancy is related to Down syndrome, implicating that it is a dosage-sensitive gene playing a major role in neurodevelopment. Other features of the condition includes facial gestalt, feeding problems, hypertonia and abnormal gait (van Bon et al., 2021).

The gene *Dyrk1a* is homologous to *minibrain (mnb)* in *Drosophila*, the model where the gene was initially studied in the aspect of embryogenesis in the 1990s. *Mnb* was discovered and named as such because mutations in *mnb* resulted in malformation of neuroblastoma (Tejedor et al., 1995). The Chang group has played a leading role in exploring the role of *mnb* in the synapses, whose work will be discussed more in detail later in this section (C.-K. Chen et al., 2014; Peng et al., 2021).

In rodents, a *Dyrk1a* line was first generated and studied in Fotaki et al. (2002), where it was reported that the homozygous mutants (*Dyrk1a*^{-/-}) are not viable and the heterozygous mutants (*Dyrk1a*^{+/-}) showed developmental delay as well as microcephaly, phenocopying human symptoms. Later, more behavioral experiments revealed that the rodent models of *Dyrk1a*^{+/-} successfully phenocopy humans, as the animals show motor delay (Fotaki, Martinez De Lagran, Estivill, Arbones, & Dierssen, 2004), pronounced learning disabilities (Arque et al., 2008; Souchet et al., 2014) and seizures (Arranz et al., 2019; Raveau, Shimohata, Amano, Miyamoto, & Yamakawa, 2018).

On a cellular level, cortical pyramidal neurons from 7-month-old heterozygous mice showed shorter neurite length and fewer dendritic spines but higher neuronal density (Benavides-Piccione et al., 2005). Later studies confirmed the neurites to be shorter and less complex in hippocampal and cortical neurons (Dang et al., 2018; Levy, LaFlamme, Tsapralis, Crynen, & Page, 2021). Studies at the circuit level is still rudimentary, but some groups have shown circuit defects in heterozygous mice (Shih, Alipio, & Sahay, 2023; Souchet et al., 2014).

Although there is no study on human post-mortem tissue in DYRK1A syndrome, there are hiPSC lines developed in different groups, some of which will also be discussed later in this section in the context of presynaptic function (Ge et al., 2024; L. Ma et al., 2021).

DYRK1A

The DYRK family is a serine/threonine kinase family that plays an important role in regulating cell cycle (Aranda, Laguna, & Luna, 2011; Yoshida & Yoshida, 2023). It is a highly preserved kinase that was initially identified in yeast (Yak), and the term DYRK family was established with the discovery of its mammalian groups, DYRK1 and DYRK2 (Aranda et al., 2011). DYRK1A is one of the two members in DYRK1 subfamily. The 763 amino acid-long kinase is expressed ubiquitously regardless of tissue or cell type, in both nucleus and cytoplasm (Wegiel et al., 2004). In neurons, DYRK1A is expressed both in nucleus and cytoplasm, and its expression peaks peri-natally between E16 and P7 (Dang et al., 2018; Martí et al., 2003).

DYRK1A consists of a NLS sequence, followed by DYRK1A homology box, the catalytic domain and poly-histidine repeats as in the schematic figure 1.8 (Aranda et al., 2011). DYRK1A gains catalytic activity via auto-phosphorylation of a tyrosine residue in its catalytic domain (Y321), although this auto-phosphorylation seems dispensable after activation (Adayev et al., 2007; Lochhead, Sibbet, Morrice, & Cleghon, 2005). Once activated, DYRK1A specifically recognises and phosphorylates RPXS/TP site (Himpel et al., 2000).

The primary role of DYRK1A is to induce cell cycle exit (Aranda et al., 2011). There are numerous substrates identified in this regard, such as cyclins and P53, which point to the conclusion that DYRK1A inhibits proliferation and induces differentiation in neuronal progenitors through different pathways (J. Park et al., 2010; Soppa et al., 2014; E. J. Yang, Ahn, & Chung, 2001). However, the expression level of DYRK1A persists in post-mitotic neurons too, although the expression level decreases after P7 (Dang et al., 2018; Martí et al., 2003). This suggests some post-developmental functions of DYRK1A, which might be related to neurodegenerative disorders. Identified substrates of DYRK1A include amyloid precursor protein (APP), Tau protein and Parkin, the proteins associated with Alzheimer's disease and Parkinson's disease (Deboever, Fistrovich, Hulme, & Dunckley, 2022). Indeed, two longitudinal studies show significantly higher correlation between Down syndrome and neurodegenerative disorders. McCarron et al. (2017) showed 97.4 % of the Down syndrome subjects (> 35 y.o.) were

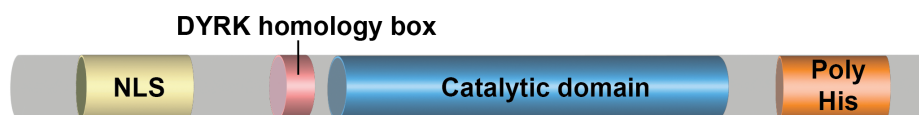


Figure 1.8: Structure of DYRK1A

The catalytic domain of DYRK1A is flanked by nuclear localisation signal (NLS) and DYRK homology box at the N-terminus, and poly-histidine repeats at the C-terminus.

diagnosed with dementia with the mean diagnosis age of 55. Hithersay et al. (2019) showed 5 times higher mortality rate of Alzheimer's disease in Down syndrome subjects. Hence, DYRK1A is highlighted as a potential therapeutic target for numerous disorders, although its non-specificity and dosage-sensitiveness make it a challenging target (Deboever et al., 2022).

Potential presynaptic functions of DYRK1A

There is compelling evidence that DYRK1A regulates dendritic development, based on the neuronal morphology and the down- or up-regulated genes in heterozygous mouse models (Arranz et al., 2019; Dang et al., 2018). It has also been suggested that DYRK1A plays a presynaptic role, because of its presynaptic colocalisation and its interaction with presynaptic proteins (Arque, Casanovas, & Dierssen, 2013). Endocytic proteins identified as substrates of DYRK1A are: Dynamin-1 (Chen-Hwang et al., 2002; Huang et al., 2004), Amphiphysin-1 (Murakami et al., 2006), Synaptojanin-1 (Adayev, Chen-Hwang, Murakami, Wang, & Hwang, 2006), α -Synuclein (E. J. Kim et al., 2006), and AP180 (Murakami, Bolton, Kida, Xie, & Hwang, 2012). Munc18 has also been recognised as a substrate of DYRK1A, although the electrophysiological data from a mouse line with phospho-mimetic Munc18 showed only minor phenotype in facilitation (Classen, Saarloos, Meijer, Sullivan, & Verhage, 2020).

Unfortunately, there is a very limited number of studies on the functional aspect of the pre-synapse in *Dyrk1a*^{+/-} mammalian neurons. The Chang group has explored the role of mnb in *Drosophila* and has found that its phosphorylation of Synaptojanin inhibits ADBE, whereas exocytosis was intact regardless of the catalytic activity of mnb (C.-K. Chen et al., 2014; Peng et al., 2021). On the other hand, C.-I. Wu et al. (2022) have generated neurons from trisomic hiPSC, which showed increased exocytosis in pHluorin imaging. This increased exocytosis was rescued by dosage-correction of DYRK1A. However, cerebellar granular neurons from trisomic mouse model Ts65Dn, which models Down syndrome, did not show any defect in SV exocytosis or endocytosis (Marland, Smillie, & Cousin, 2016). These mixed results could come from differences between species or difference in the stimulation paradigm. It is also possible that the various functions of DYRK1A are affected differentially depending on the dosage. Interestingly, Bessone et al. (2022) reported overexpression of DYRK1A accelerated antero-grade and retrograde vesicle movement in hiPSC, implicating its role in axonal transport. Although axonal transport may not directly relate to SV recycling, it could influence active zone assembly or synaptic maintenance. Investigating the presynaptic role of DYRK1A requires more expansive and thorough experiments, in order to understand the potential convergent pathology in NDDs.

1.4 Hypotheses

The purpose of this thesis is to contribute to the understanding of the presynaptic pathology of NDDs, with respect to SV recycling in rodent models of two example NDDs: FXS and DYRK1A syndrome. This requires understanding of the development of presynaptic function in WT neurons first. Based on literature, the three hypotheses are put forward:

1. Similar to the mode switch in SV exocytosis, immature WT neurons show different SV endocytosis to mature WT neurons.
2. Disrupted SV recycling underlies the neurotransmission defects in FXS and DYRK1A syndrome models.
3. SV recycling in NDD models is disrupted early in development.

1.5 Research Questions

Three research questions have been raised to test the hypotheses above:

1. How does a nerve terminal acquire optimal SV recycling in WT neurons?
2. Based on our knowledge that *Fmr1*^{-/-} neurons shows reduced ADBE on DIV 14 (Bonnycastle et al., 2022), is this because of a delay in the presynaptic development, or is this a mechanistic failure due to the absence of FMRP?
3. Do *Dyrk1a*^{+/-} neurons also show suboptimal SV recycling, like *Fmr1*^{-/-} neurons?

1.6 Experimental design

To answer to the research questions listed above, we took advantage of various assays in live-cell imaging as well as fixed-cell imaging to monitor different aspects of SV recycling. The assays include (1) calcium imaging to monitor activity-dependent Ca²⁺ influx using genetically-encoded calcium indicator GCaMP6f, (2) synaptophysin-pHluorin (sypHy) imaging to monitor the fusion and retrieval of overexpressed synaptophysin, (3) tetramethylrhodamine (TMR)-dextran (40kDa) uptake assay to report ADBE specifically, and (4) HRP uptake assay with subsequent electron microscopy to measure the extent of ADBE within individual nerve terminals.

For the first research question, the assays were conducted at five different time points in WT neurons from murine primary hippocampal cultures: DIV 3, 7, 10, 14 and 21 to cover all stages of presynaptic differentiation. To answer to the second research question, a TMR-dextran uptake assay was performed in *Fmr1*^{-/-} neurons at the same timepoints. Similar live-imaging techniques were used to investigate SV recycling in *Dyrk1a*^{+/-} neurons, but the experiments were only performed at DIV 14. Materials and methods for individual assays are addressed in the next chapter.

Materials and Methods

2.1 Animals

Adult animals over 2 months old were killed by rising exposure to CO₂ and death was confirmed by cervical dislocation. The embryos were subsequently killed by decapitation with death confirmed by destruction of the brain. All Schedule 1 procedures were performed under guidelines from the Animal Welfare and Ethical Review Body at the University of Edinburgh. All procedures involving animals were carried out in accordance with the Animals (Scientific Procedures) Act (ASPA) 1986 under the project license of M. Cousin (PP5745138). The colonies were maintained under 12:12 hour light: dark cycle and were fed with standard chow and water *ad libitum*.

2.1.1 Wild Type Rat

Sprague Dawley wild type (WT) rats (Crl:SD) were purchased from Charles River labs. A pair of a male and a female were set up overnight for weekly timed matings and were checked for plugs daily. The female was kept individually once a plug was found. Otherwise, females were not set up for two consecutive weeks in order to confirm there was no overlooked plugs. The animals used for timed mating were at least 8 weeks old and the embryos of either sex were taken at the age of embryonic day 18.5 (E18.5).

2.1.2 *Fmr1*^{-y} Rat

Long-Evans Hooded (LEH) *Fmr1*^{em1/PWC} female rats were taken from the in-house colony maintained by Lynsey Dunsmore, an animal lab manager at the University of Edinburgh. This line was initially designed by the lab of Dr Peter Kind in Centre for Discovery Brain Sciences (University of Edinburgh) and engineered by Envigo, previously Horizon Discovery (SAGE labs) (Asiminas et al., 2019). The exon 1 coding region of FMRP was replaced with eGFPnlsSV40pA cassette in this model. A heterozygous female was set up with a WT male rat from the same background for timed mating, in the same manner as for the Sprague Dawley WT rats. Because FXS is an X-linked disorder, only the male embryos were used to prepare *Fmr1*^{-y} (KO) and WT neurons. The sex of the embryos were identified by locating

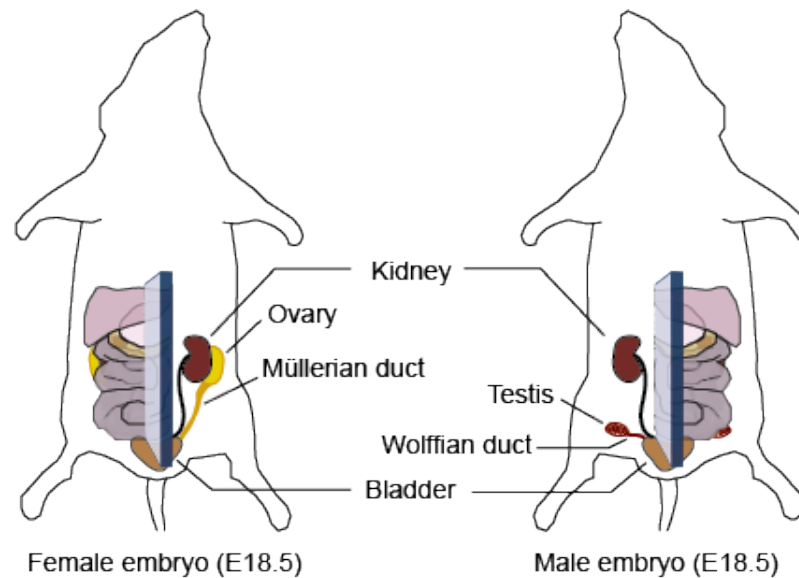


Figure 2.1: Anatomical positions of reproductive organs in male and female rat embryos at E18.5

The male embryos were selected for *Fmr1*^{-y} primary neuronal culture preparation by locating testes in the posterior end of the abdomen. Ovaries, on the other hand, are located dorsal to the kidneys.

the reproductive organs after abdominal dissection. This was possible because by E18.5, the testes are found in the posterior end of the abdomen next to the bladder in males under the digestive organs, whereas in females, the ovaries are found dorsal to the kidneys under the digestive organs as depicted in figure 2.1.

2.1.3 *Dyrk1a*^{+/-} Rat

LEH *Dyrk1a*^{em3Mcwi} male rats were also provided from the in-house colony at the University of Edinburgh. The model was originally generated by Medical College Wisconsin under support from Simons Foundation Autism Research Initiative (SFARI). In this model, five base pairs in exon 3 in *Dyrk1a* coding region (chr11:34,922,239-34,922,243) was deleted by injecting CRISPR and a gRNA (target site: GCGACGGTCA CTGTACTG **AT GTGAATGGGG**, deleted base pairs in bold). The *Dyrk1a*^{+/-} (HET) males were mated with WT females from the same background, in the same manner as previous animal lines stated above, to produce *Dyrk1a* HET and WT neurons.

2.2 Genotyping

After decapitation, the tails of the embryos were dissected and sent to Transnetyx (Tennessee, USA) for genotyping both *Fmr1* and *Dyrk1a* embryos. The primer sequences are listed below in table 2.1.

Fmr1	forward	TGGCATAGACCTTCAGTAGCC
	reverse	TATTTGCTTCTCTGAGGGGG
Dyrk1a	forward	CTTGGCTTGTGTGTTCCCAGG
	reverse	CAAGGGCTGGGGAGAATCAG

Table 2.1: Primer sequences for genotyping

2.3 Cloning

GST-tagged FMRP N-terminus WT was cloned by inserting 1-564 base pair of hEGFP-FMRP isoform 1 (obtained from Dr. B. Bardoni (Institut National de la Santé et de la Recherche Médicale, Institute Pharmacology Moléculaire Et Cellulaire Centre National de la Recherche Scientifique)) in a pGEX-KG vector (gifted from Dr C. Rickman, Herriot-Watt University). GST-tagged FMRP N-terminus bearing a point mutation R138Q was generated by cloning the insert from a plasmid previously generated in the lab to the same pGEX-KG vector (Bonnycastle et al., 2022). The sequences of the primers are in table 2.2.

After PCR amplification of the templates (denaturation: 95°C, 1 min; annealing: 52 °C, 45 sec; extension: 72 °C, 1 min), the insert and the vector were digested using enzymes Xho I (Thermo Fisher Scientific; FD0694) and Hind III (Thermo Fisher Scientific; CAT: FD0504) for 1 hour at 37 °C. The digested products were separated via 1 % agarose gel electrophoresis, from which the DNA was purified and used for ligation. Ligation was performed at RT overnight, and the ratio between vector to insert was optimised to 10:6. The ligation products were transformed into XL10-Gold onto agar LB plates added with ampicillin after 45 seconds of heat shock at 42 °C. The colonies were picked and grown for mini prep on the following day for sequencing. Reagents are listed in table 2.3.

FMRP N-terminus	forward	CTCGAGATGGAGGAGCTGGTGGTGAAGTGC
	reverse	AAGCTTCTATCTCGAGGCAAGCTGCCTTGAAGTC

Table 2.2: Primer sequences for cloning FMRP N-terminus in pGEX-KG vector

Product	Manufacturer	Cat #
pfu polymerase	Promega	M774A
pfu buffer	Promega	M776A
dNTP mix	Invitrogen	18427-013
Xho I	Thermo Fisher Scientific	FD0694
Hind III	Thermo Fisher Scientific	FD0504
10x FastDigest buffer	Thermo Fisher Scientific	B64
T4 ligase	Promega	M180A
Rapid ligation buffer	Promega	C671B
Zymoclean™ Gel DNA recovery kit	Zymo research	D4001
UltraPure™ Agarose	Invitrogen	16500
SYBR™ Safe DNA Gel stain	Invitrogen	S331002
LB Broth with agar (Lennox)	Sigma-Aldrich	L7025
LB Broth (Lennox)	Sigma-Aldrich	L7275
Mini prep kit	Zymo research	D4208S

Table 2.3: Cloning reagents

2.4 Primary neuronal culture

Once the cerebrum was removed from the decapitated embryonic heads, the hippocampi were revealed by a midsagittal cut of the cerebrum followed by removal of the midbrain. Primary hippocampi were acquired from both hemispheres and were dissociated in 10 U/mL papain (Worthington Biochemical Corporation, USA; CAT: LK003189) solution in 37 °C for 20 minutes. The cells were resuspended in DMEM/F12 (Gibco, Paisley, UK; CAT: 21331-020) supplemented with 10 % fetal bovine serum (FBS) (BioSera, France; CAT:S1810-500) and 1 % penicillin/streptomycin (Gibco, Paisley, UK; CAT: 15140-122). The cells were resuspended after centrifugation (260 g, room temperature (RT), 5 min) in Neurobasal (Gibco, Paisley, UK; CAT: 21103-049) supplemented with 2 % B-27 (Gibco, Paisley, UK; CAT: 17504-044), 1 % Glutamax (Gibco, Paisley, UK; CAT: 35050-038) and 1 % penicillin/streptomycin. Around 50,000 cells were plated on each 25 mm glass coverslip (VWR International Ltd, Lutterworth, UK) coated in 10 µg/mL laminin (Sigma Aldrich, Gillingham, UK; CAT: L2020) and 0.1 mg/mL poly-D-lysine (Sigma Aldrich; CAT: C7886). Two to three days after plating, 1 µM of cytosine β-D-arabinofuranoside (Sigma Aldrich, Gillingham, UK, CAT: C1768) was added in order to inhibit glial division.

2.5 Fluorescence imaging

The cells were imaged using a Zeiss Observer D1 epifluorescence microscope (Zeiss, Jena, Germany), Zeiss EC Plan-Neofluar 40X oil objective (numerical aperture: 1.30) and Zeiss AxioCam MR R3. Description of filters are addressed in each experiment. Images were acquired and saved using Zen Pro 2012 (blue edition) software. The exposure was set to 500 ms in all channels.

2.5.1 Calcium imaging

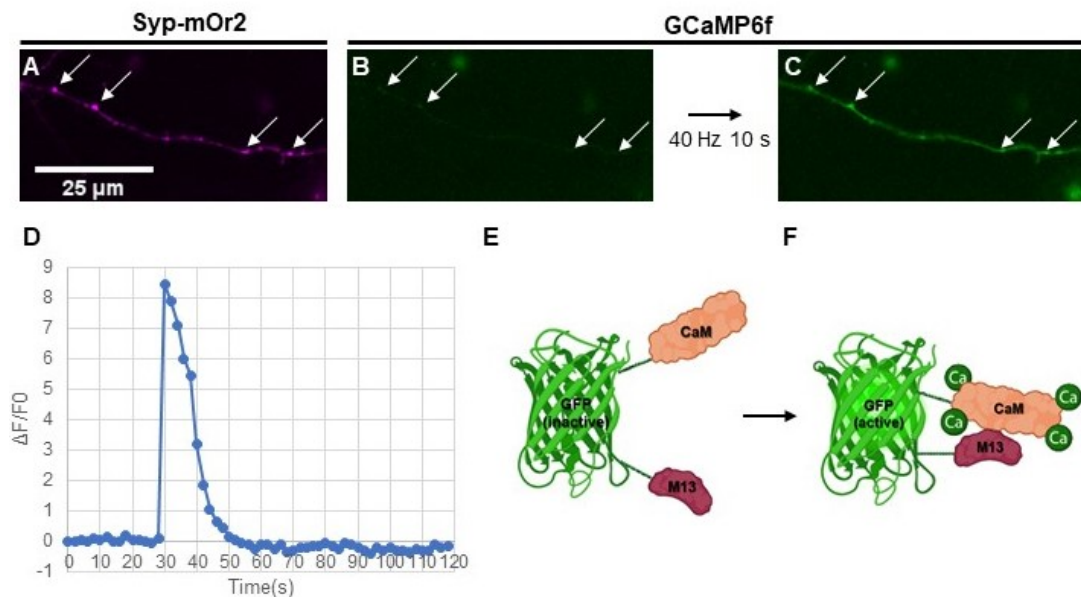


Figure 2.2: Calcium imaging protocol

The transfection with synaptophysin-mOrange2 (syp-mOr2) enables marking of the nerve terminals which are selected as region of interest (ROI)s (A), and transfection with GCaMP6f enables recording of Ca^{2+} events in the drawn ROIs (B, C). The cells were challenged with a train of 400 APs (40 Hz) and the timetraces were normalised to F_0 to measure the peak height and the decay following the end of the stimulation (D). This is because the binding between Calmodulin and calcium ions lead to the conformational change of EGFP (E, F) that makes it fluoresce. Panel E and F were created using BioRender.

For calcium imaging, pGP-CMV-GCaMP6f (GCaMP6f) was used to report the intracellular free calcium events. The plasmid was given by Douglas Kim from GENIE team (Addgene, USA; CAT: 40755) (T.-W. Chen et al., 2013). Synaptophysin-mOrange2 (syp-mOr2) was used to mark the nerve terminals, which was a gift from Shigeo Takamori, Doshisha University (Egashira, Takase, & Takamori, 2015). Primary cultures of hippocampal neurons were co-transfected with 0.8 µg of GCaMP6f and 0.8 µg of syp-mOr2 with 1 µL of Lipofectamine 2000 (Thermo Fisher Scientific, UK; CAT: 11668027) in 200 µL of unsupplemented Neurobasal media per coverslip 2-3 days prior to imaging. The coverslips with transfected cells were mounted

on a Warner Instruments imaging chamber embedded with parallel platinum wires (Warner instruments; CAT: RC-21BRFS) connected to a Digitimer LTD MultiStim system-D330 stimulator (current output: 100 mA, pulse width: 1 ms). Each coverslip was stimulated and filmed in imaging buffer (119 mM NaCl, 2.5 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 25 mM HEPES, 30 mM glucose at pH 7.4) supplemented with 50 μ M DL-2-Amino-5-phosphonopentanoic acid (AP-5) and 10 μ M 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (Armbruster & Ryan, 2011). Syp-mOr2 was visualised using ZEISS filter set 20 (excitation: BP 540-552 nm, emission: BP 575-640 nm, beamsplitter: 515 nm) and GCaMP6f was visualised using ZEISS filter set 46 (excitation: BP 490-510 nm, emission: 520-550 nm, beamsplitter: 515 nm).

After taking an image of syp-mOr2, GCaMP6f was imaged for 2 min at an interval of 2 seconds. The basal level of GCaMP6f fluorescence was recorded for 30 seconds prior to stimulation. The neurons were then subjected to stimulation for 10 seconds at 40 Hz. Representative images and a timetrace is shown in figure 2.2. Each coverslip was imaged for 80 seconds after stimulation to monitor fluorescence recovery.

The calcium imaging in *Dyrk1a* HET neurons used the same strategy except images were taken every 1 second for 2 minutes. The neurons were challenged with a train of 40 APs (20 Hz) after 30 seconds of baseline recording.

The acquired images were analysed using FIJI distribution of Image J (National Institutes of Health). After aligning images using Rigid body mode of StackReg plugin (<https://imagej.net/plugins/stackreg>), GCaMP6f images were collapsed in "maximum intensity" mode (Image > Stacks > Z project) and superimposed to syp-mOr2 image using "Merge Channels" (Image > Color > Merge Channels) (<https://imagej.net/imaging/z-functions>). Puncta that are both mOrange2 and EGFP positive were manually selected as regions of interest (ROIs) (5x5 pixels, 1.13 μ m in diameter). For time-course calcium imaging in chapter 3, F_0 is the average value of the fluorescence level in the four frames prior to the initiation of stimulation at each ROI, and the background fluorescence was measured by selecting 10 random ROIs. The time-traces of background was subtracted from time-traces of selected ROIs.

2.5.2 SypHy imaging

Primary neurons from hippocampal cultures were transfected with 1 μ g of synaptophysin-pHluorin (sypHy) with 1 μ L of Lipofectamine 2000 in 200 μ L of unsupplemented Neurobasal media per coverslip 2-3 days prior to imaging (Granseth et al., 2006). The plasmid was a gift from Leon Lagnado, previously at Laboratory of Molecular Biology, Cambridge University and now at Sussex University. Capitalising on the localisation of synaptophysin on SV membrane and the pH sensitivity of pHluorin, sypHy reports SV exo- and endocytosis at individual nerve terminals as explained in figure 2.3.

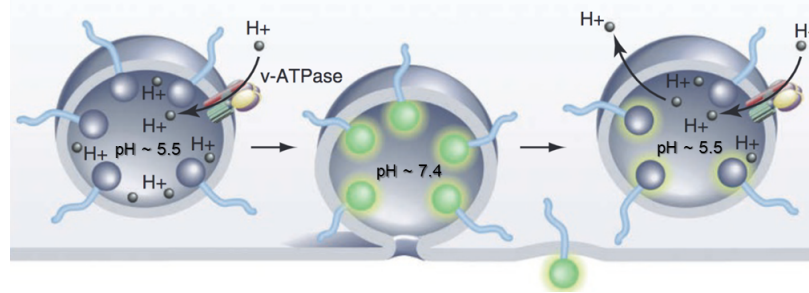


Figure 2.3: The principle of synaptophysin-pHluorin (sypHy) imaging

SypHy reports the exocytosis and endocytosis at individual nerve terminals based on the pH difference between reacidified SV lumen and extracellular space. When a sypHy-embedded SV fuses to the plasma membrane, pHluorin is exposed to the pH-neutral extracellular space, which unquenches the pHluorin and makes it fluoresce. The pHluorin is quenched again once sypHy is retrieved from the plasma membrane for SV recycling and it is reacidified as vacuolar-type H^+ ATPase (vATPase) transports H^+ ions into the SV. The quenching of the pHluorin leads to decrease in the fluorescence level (Granseth et al., 2006; Royle, Granseth, Odermatt, Drevier, & Lagnado, 2008). This figure was adapted from Kavalali and Jorgensen (2014).

For imaging, each coverslip was mounted on the imaging chamber and was imaged for one minute to record the basal level of sypHy fluorescence before stimulating at 40 Hz for 10 seconds, except for SV pool experiment. The coverslip was recorded for another three minutes after the stimulation to monitor the recovery of the fluorescence level. While imaging, the coverslip was perfused with imaging buffer supplemented with 50 μ M AP-5 and 10 μ M CNQX. After recovery, the coverslip was imaged continuously for the next two minutes under the perfusion of basic imaging buffer (69 mM NaCl, 50 mM NH_4Cl , 2.5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 25 mM HEPES, 30 mM glucose) in order to measure the total amount of pHluorin fluorescence which indicates the size of the total SV pool as indicated in the figure 2.4. The images were acquired every four seconds (excitation: 500 nm, dichroic filter beamsplitter: 515 nm, long-pass emission filter > 500 nm).

SypHy images were also analyzed using Image J. After aligning the images with Rigid body mode of StackReg plugin, sypHy positive puncta were manually selected as ROIs (5 x 5 pixels, 1.13 μ m in diameter). The ROIs were once again screened based on their timetraces, so that only the puncta that responded to both the stimulation and the basic imaging buffer were pooled together to calculate average timetraces using Time Series Analyser plugin ([https:// imagej.nih.gov/ij/plugins/time-series.html](https://imagej.nih.gov/ij/plugins/time-series.html)). The basal fluorescence level, F_0 , refers to the average of the four images prior to the stimulation. $\Delta F/F_0$ was normalised to the total amount of SV indicated from the fluorescence level in response to the basic imaging buffer, or to the maximum value (peak) during electrical stimulation. No background subtraction was performed in sypHy imaging.

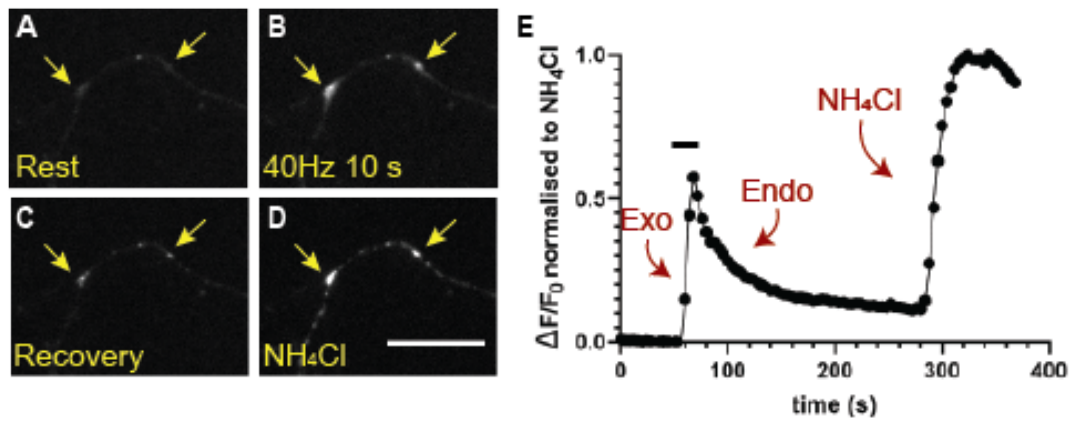


Figure 2.4: SypHy imaging protocol

A synaptophysin-pHluorin (sypHy)-transfected primary hippocampal neuron is monitored at rest (**A**), at stimulation of 400 APs (40 Hz) (**B**), and recovery (**C**) followed by perfusion with basic imaging buffer (**D**). An example of average timetraces of the ROIs is shown in panel **E**, which is normalised to the response to basic imaging buffer. An increase of the fluorescence indicates SV exocytosis and a decrease indicates SV endocytosis at nerve terminals. Yellow arrows indicate responsive nerve terminals. Scale bar: 15 μ m. The bar in panel **E** indicates period of stimulation (40 Hz 10 s).

The clathrin heavy chain (CHC) knockdown experiment used co-transfection of sypHy with shRNAs targeting CHC (shCHC) or its scrambled control (shScr) ligated into pSuper vector co-expressing mCerulean, as generated in (Nicholson-Fish et al., 2015). The shCHC targets AATCGCCCTTCTGAAGTCCT in *Rattus norvegicus*. The hippocampal neurons expressing shCHC or shScr were visualised (excitation: 430 nm, dichroic beamsplitter: 515 nm, long-pass emission filter > 500 nm) before sypHy response recording, which was conducted in exactly the same way as in the previous paragraph.

In the case of SV pool size experiments, the neurons were transfected with sypHy plasmid 2-3 days prior to imaging and were imaged under the same microscope with the same setting as the two previous paragraphs, except for the buffer and the stimulation setting. Here, the imaging buffer was supplemented with 1 μ M Bafilomycin 1A (Cayman chemical, Michigan, USA; CAT: 11038) in order to block the vATPase that reacidify recycled SVs, therefore the sypHy reports SV fusion exclusively. After recording baseline for the first minute, the neurons were challenged at 20 Hz for 2 seconds to mobilise the readily releasable pool (RRP), followed by 10 seconds of rest. The second stimulation was set at the same frequency (20 Hz) for 80 seconds to deplete the recycling pool at the nerve terminals. The neurons were perfused with basic buffer as stated previously to reveal the total SV pool size. The timetraces resemble the following figure 2.5.

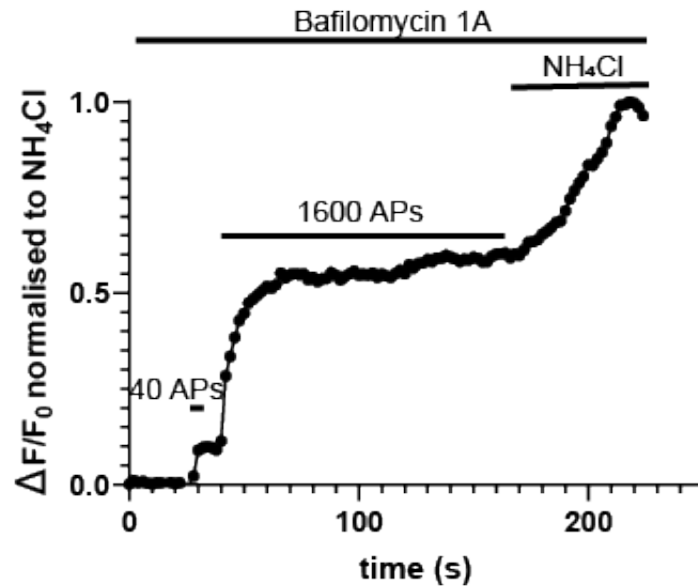


Figure 2.5: Protocol for SV pool size measurement

After baseline recording for 1 minute, the transfected hippocampal neurons were challenged with 40 APs (20 Hz) first to reveal the RRP. Then, the neurons were challenged with 1600 APs (20 Hz) to deplete the recycling pool. The neurons were imaged in the presence of Bafilomycin 1A to block SV reacidification. Perfusion with basic buffer at the end of imaging indicates total SV pool, to which the timetraces are normalised. Bars indicate the duration of stimulation (40 APs and 1600 APs), application of basic imaging buffer (NH_4Cl), or application of Bafilomycin 1A.

2.5.3 TMR-dextran uptake assay

TMR-dextran (40 kDa) (Thermo Fisher Scientific, Loughborough, UK; CAT: 1842) is a fluid phase marker that is less likely to be internalised at nerve terminals via CME but more likely to be internalised via ADBE due to size exclusion as the schematic shows in figure 2.6. Therefore, TMR-dextran puncta report the number of nerve terminals that performed ADBE in a field of view, the average of which indicates the prevalence of ADBE in a population of nerve terminals (Clayton et al., 2008).

Coverslips from primary neuronal cultures were mounted on the imaging chamber, and 200 μL of 50 μM TMR-dextran (40 kDa) dissolved in imaging buffer was applied on each coverslip. After stimulation for 10 seconds at 40 Hz using a Digitimer LTD MultiStim system-D330 stimulator in the presence of TMR-dextran, the coverslip was perfused with imaging buffer in order to remove uninternalised TMR-dextran particles. TMR-dextran was visualised using ZEISS filter set 20 (excitation: BP 540-552 nm, emission: BP 575-640 nm, beamsplitter: 515 nm) and each field was imaged in five Z-stacks manually. From each coverslip, 6-8 areas were imaged and brightfield images were taken in parallel as a reference for consistency in

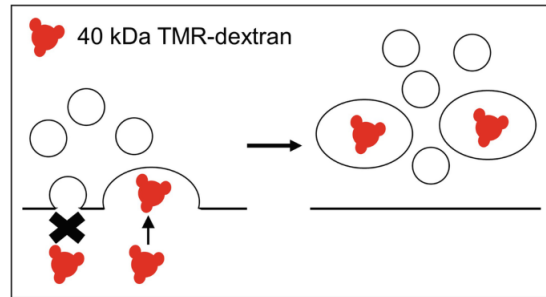


Figure 2.6: The principle of TMR-dextran uptake assay

A TMR-dextran uptake assay reports ADBE based on size exclusion. This figure was taken from Cousin and Smillie (2021).

neurite density. At least 7 coverslips from 3 independent preparations were imaged for these experiments, in both WT and *Dyrk1a* HET experiments. For each condition, one coverslip was exposed to TMR-dextran but was not challenged with stimulation as a control in order to measure spontaneous TMR-dextran uptake.

The number of fluorescent puncta was quantified using maximum entropy auto threshold plugin and analyze particles plugin on Image J (size = 0.23-0.91 μm^2 , circularity = 0.00-1.00) on the collapsed image in maximum intensity mode (<https://imagej.net/imaging/particle-analysis>). The protocol is summarised in figure 2.7. The number of TMR-dextran puncta from non-stimulated cells is subtracted from their corresponding groups (stimulated cells) in order to minimise the contribution from non-specific labelling.

2.6 Immunocytochemistry

The hippocampal neurons prepared on the coverslips were removed from the media and fixed in 4 % paraformaldehyde (PFA) (Sigma-Aldrich; CAT: 47608) in phosphate buffered saline (PBS) (10 mM phosphate buffer, 2.7 mM KCl and 137 mM NaCl, pH 7.4) for 15-20 min at RT. PFA was neutralised with 50 mM NH_4Cl in PBS for 10 min. After 3 washes in PBS, cells were permeabilised in 1 % Bovine serum albumin (BSA) and 0.1 % Triton X in PBS for 5 minutes at RT. Following a PBS wash, the cells were blocked with 1 % BSA in PBS for 30 min to prevent non-specific binding. Cells were stained with rabbit Synaptic vesicle glycoprotein 2A (SV2A) primary antibody (Abcam, Cambridge, UK, CAT: Ab32942) for 1-2 hour in 1:200 dilution at RT. After washing in PBS, cells were incubated in donkey-anti-rabbit Alexa Fluor 488 secondary antibody (Invitrogen, Paisley, UK, CAT: A21069) for 1 hour in the dark in 1:1000 dilution at RT. The coverslips were then mounted on slides using FluorSave (Millipore, Darmstadt, Germany; CAT: 345789) after washing in PBS and Milli Q water. Dried slides were imaged using ZEISS filter set 46 (excitation: BP 490-510 nm, emission: 520-550 nm, beamsplitter: 515 nm). Six to eight images were taken per coverslip in single Z-stack, with a brightfield image as a reference

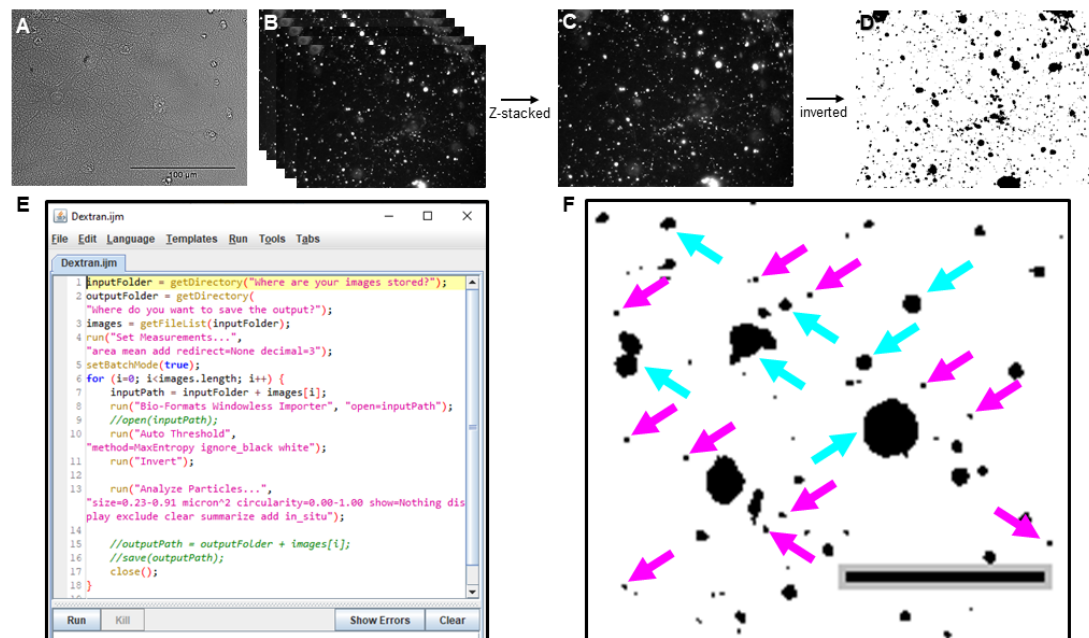


Figure 2.7: TMR-dextran uptake assay protocol

A snapshot of brightfield (A) is taken as a reference for neurite density at a field of view, alongside five fluorescence (TMR-dextran) images in a Z-stack (B). The stacks are collapsed (C) to capture maximal number of puncta in the field of view. The collapsed images are inverted (D) and the number of puncta is quantified using a macro script (E). The magenta arrows indicate representative puncta included in the analysis, whereas the cyan arrows indicate representative puncta excluded from the analysis (F). Scale bar: 25 μm .

for neurite density. α -SV2A puncta were quantified using maximum entropy auto thresholding plugin (method = "MaxEntropy", "ignore black white") and analyze particles plugin on Image J (size = 0.05-0.78 μm^2 , circularity = 0.00-1.00). Representative images and the macro script is shown in figure 2.8.

2.7 HRP uptake assay and electron microscopy

Each coverslip was mounted on the imaging chamber, where the cells were stimulated at 40 Hz for 10 seconds using Digitimer LTD MultiStim system-D330 stimulator, in the presence of 10 mg/ml horse radish peroxidase (HRP) (Sigma Aldrich, Gillingham, UK; CAT: P8250-50KU) in imaging buffer. After washing, the neurons were fixed with 2 % glutaraldehyde (Electron Microscopy Sciences, Hatfield, USA; CAT: 16019) and 2 % paraformaldehyde (Thermo Fisher Scientific, Ward Hill, USA; CAT: 43368) in 0.1 M Tris buffer for 30 minutes at 37 °C. HRP was then developed with SIGMAFAST 3,3'- diaminobenzidine (DAB) tablets (Sigma Aldrich, Gillingham, UK; CAT: D4293) dissolved in 0.1 M PB buffer for 10 minutes at RT. The tablets were dissolved as advised in the protocol provided from Sigma Aldrich. After washing in 0.1 M PB, the neurons were stained with 1 % osmium tetroxide (TAAB Laboratories Equipment Ltd,

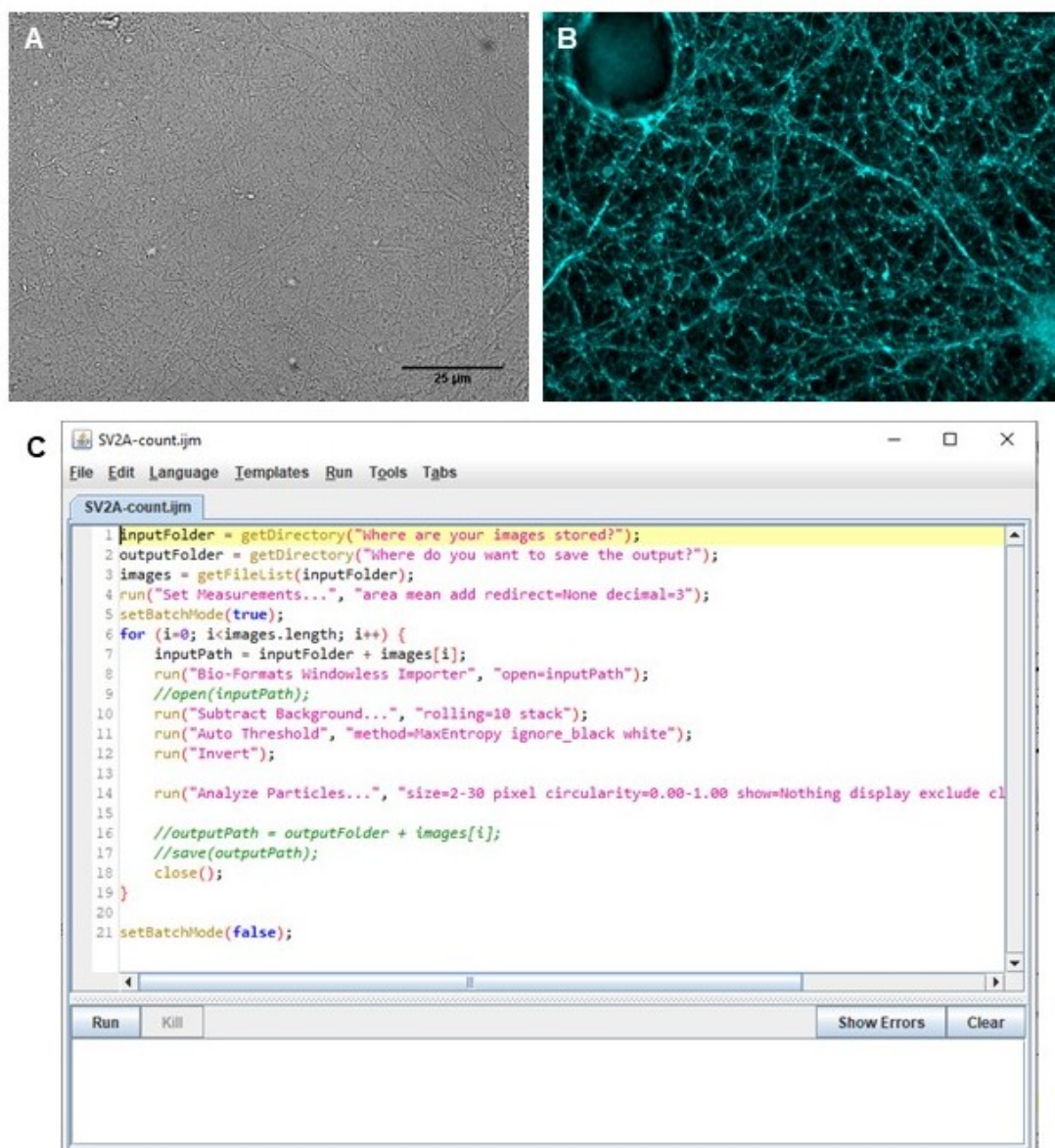


Figure 2.8: Immunocytochemistry imaging and analysis protocol

Representative images of brightfield (A) and fluorescence (B) imaging of a hippocampal neurons (DIV 14) from primary cultures labelled with α -SV2A. Panel C is a screenshot of the macro script for automated analysis.

UK; CAT: O015) for 30 minutes at RT. Followed by washes in 0.1 M Tris buffer and distilled water, the neurons were dehydrated sequentially with increasing concentration of ethanol (50 %, 70 %, 90 % and 100 %), and by propylene oxide (Electron Microscopy Sciences, Hatfield, USA; CAT: 20401) for 10 minutes each. The neurons were embedded in Durcupan epoxy resin (Sigma Aldrich, Gillingham, UK; CAT: 44610), after which the coverslips were removed by dipping the resin blocks in liquid nitrogen.

The samples were sectioned and mounted on grids using FEI Tecnai 12 transmission electron microscope by Steve Mitchell, the previous manager of Edinburgh Electron Microscopy lab at Kings Buildings, University of Edinburgh. The intracellular structures with HRP-labelled vesicles or endosomes were designated as nerve terminals and structures with diameter over 100 nm were quantified as bulk endosomes. Per coverslip, 10 - 30 fields of view were taken to calculate the average number of bulk endosomes.

2.8 Synaptosome preparation

Crude synaptosomes were prepared from over 2 months old female Sprague Dawley rats based on the lab protocol. The cerebrum was harvested following a Schedule 1 procedure as stated above, and was washed and homogenised in sucrose/EDTA buffer (sucrose 0.32 M, 1 mM EDTA, 5 mM Tris, pH 7.4). The brain lysate was centrifuged in 1,075 *g* for 10 minutes at 4 °C to remove the pellet (P1), then S1 was centrifuged in 20,200 *g* for 15 minutes at 4 °C to acquire P2 which contained the synaptosomes.

2.9 GST pull-down assay

Before the pull-down, P2 from synaptosomal pellet was resuspended and incubated in lysis buffer (150 mM NaCl, 25 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, pH 7.4) supplemented with 1% protease inhibitor cocktail (Sigma-Aldrich; CAT: P8849) on ice for 15 minutes with repeated vortexing. After spinning down at maximum speed for 5 minutes at 4 °C in a microfuge, the supernatant was collected for the glutathione-S-transferase (GST) pull-down assay.

The GST-tagged plasmids were transformed into BL21(DE3)pLysS Competent Cells (Promega; CAT: L1195) using heat shock at 42 °C for 45 seconds and were grown overnight. Once the bacteria culture reached its stationary phase on the following day, it was induced for protein expression with isopropyl β -D-1-thiogalactopyranoside (IPTG) (Cambridge Bioscience, CAT: 4026913) for 4 hours at 37 °C in a shaking incubator (RPM: 220). After washing in STE buffer (150 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0), the bacteria was treated with lysis buffer supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), protease inhibitor

cocktail as above, and 5 μ M lysozyme (Sigma-Aldrich; CAT: L6876). Following 30 minutes of incubation on ice, dithiothreitol (DTT) (Molekula group; CAT: M10166694) and 1% Triton X-100 (Sigma; CAT: T8787) were added before sonication. Sonication was performed at 10 kHz for 6 minutes with intermittent cooling on ice using a MSE Soniprep 150 in 50 mL plastic falcon tubes. The sonicated lysates were cleared via centrifugation at 18,000 *g* and were loaded onto pre-washed glutathione sepharose beads (Cytvia; CAT: 17075601) for 2 hours at 4 °C on a roller. The beads were washed extensively afterwards with PBS and 1.2 M NaCl/PBS to remove non-specific binding.

The beads linked with GST-tagged proteins are loaded into spin columns (MicroSpin™ G-50 Columns; Amersham Biosciences; CAT: 275330) and centrifuged at 780 *g* for 15 seconds at 4 °C. In each column, 250 μ l of lysis buffer supplemented with PMSF and protease inhibitor cocktail as stated in the previous paragraph were added with synaptosome in excess. The columns were rolled for 2 hours in 4 °C, followed by repetitive washing in ice-cold lysis buffer, 1.2 M NaCl/lysis buffer, and 20 mM Tris buffer. For elution, sodium dodecyl-sulfate (SDS) sample buffer (67 mM SDS, 2 mM EGTA, 9.3% glycerol, 12% β -mercaptoethanol, bromophenol blue, 67 mM Tris-HCl, pH 7.4) was added onto each column and the columns were spun at maximum speed after 2 minutes of incubation at RT. The eluted sample was boiled in 95 °C for 10 minutes to ensure denaturation.

2.10 Western blot

Samples were loaded into wells of SDS-PAGE gels (running gel: 0.1 % SDS, 10 % acrylamide (30 % Acrylamide/Bis Solution 37.5:1; Bio-rad; CAT: 1610158), 1 % APS and 0.1 % TEMED in 24 % of 1.5 M Tris-HCl (pH 8.8); stacking gel: 0.1 % SDS, 30 % acrylamide, 1 % APS and 0.1 % TEMED in 24 % of 1.5 M Tris-HCl (pH 6.8)) cast using Mini-PROTEAN Tetra Handcast Systems (Bio-rad; CAT: 1658001FC) and run at 120 V for 1.5 hours for separation in 25 mM Tris, 200 mM glycine and 0.1 % SDS in dH₂O. The bands were transferred to nitrocellulose membrane (Amersham; CAT: 10600002) at 100 V for 2 hours in transfer buffer (25 mM Tris, 200 mM glycine, 0.1 % SDS and 20% ethanol) in Mini Trans-Blot Electrophoretic Transfer Cell kit (Bio-Rad; CAT: 1703930). The membrane was first stained with 0.1 % Ponceau S (Sigma; P3504) and 5 % acetic acid to compare the total amount of proteins, and then blocked with blocking buffer (Li-Cor; CAT: 927-70001) diluted 1:1 with PBS.

After blocking, the membrane was incubated with primary antibodies as stated in table 2.4, overnight in 4 °C in 0.1 % Tween-20 (Sigma Aldrich; CAT: P1375) in PBS. The primary antibodies were removed on the next day, followed by washing with 0.1 % Tween-20 in PBS. After taking the primary antibodies off, the membrane was washed and incubated with

secondary antibodies for two hours at RT, followed by washing in 0.1 % Tween-20 in PBS and PBS without Tween-20 (table 2.4). When probing for phosphorylated proteins, TBS-blocking buffer (Li-Cor; CAT: 927-60001) was used instead of PBS-blocking buffer, and TBS was used instead of PBS for diluting antibodies and washing.

The membrane was dried and visualised via a Li-cor Odyssey M imager operated by Li-cor Acquisition 2.2 application. The acquired images were visualised and analysed for signal intensity using software Image Studio (ver. 5.5) ("Analysis" tab > "Add rectangle"). The background was subtracted using "local background" mode and "median" method. The rectangle was fit to the largest band on the blot and rectangles of these dimensions were consistently used for each condition. In the case of the Western blot experiment comparing the protein expression level between *Dyrk1a* HET and WT neurons (figure 5.5A), Synapsin signals were normalised to β -actin of the same lane. For the blot in figure 5.5D, Synapsin and p-Synapsin signals were normalised to β -actin, which were again normalised to WT.

Primary antibodies				
Target	Species	Manufacturer	Cat #	Dilution
α - GST	goat	Amersham Biosciences	Cytiva 27-4577-01	1: 2,000
α - Dynamin 1	rabbit	Proteintech	18205-1-AP	1: 1,000
α - p-Dynamin (s774)	sheep	Bio-rad	AHP899	1: 2,000
α - BK channel	mouse	NeuroMab	L6/60	1: 1,000
α - Synapsin 1/2	rat	Sysy	106 002	1:1,000
α - p-Synapsin 1 (s553)	rabbit	Abcam	32532	1: 500
α - β -actin	mouse	Sigma	A3854	1: 30,000
Secondary antibodies				
Target	Species	Manufacturer	Cat #	Dilution
α - goat	donkey	Li-cor	925-32214	1: 10,000
α - rabbit	donkey	Li-cor	925-68021	1: 10,000
α - mouse	donkey	Li-cor	926-32212	1: 10,000

Table 2.4: List of antibodies used in Western blot

2.11 Mass spectrometry (MS)

2.11.1 MS analysis of FMRP N-terminus GST pull-down assay samples

After running the pulled-down samples in a 50 mL 10 % polyacrylamide SDS gel, the gel was Coomassie-stained and the bands were cut out for destaining. The destaining step was performed with 50 % acetonitrile/ 50 mM ammonium bicarbonate solution. The bands were then reduced with dithreitol, alkylated with iodoacetamide following standard protocols from (Shevchenko, Tomas, Havli, Olsen, & Mann, 2006). The samples were then digested using

sequencing grade modified trypsin (Promega; CAT: V5111) at 37 °C overnight. The extraction was performed in two steps, the first in 1 % formic acid and 2 % acetonitrile, the second in 50 % acetonitrile. The peptides were cleaned up using Stagetips as stated in Rappsilber, Mann, and Ishihama (2007).

Purified peptides were separated over a 50 minutes gradient on an Aurora-25 cm column (IonOpticks Australia) using an UltiMate RSLCnano LC System (Dionex) coupled to a timsTOF FleX mass spectrometer (Bruker) via a CaptiveSpray ionization source. The gradient was delivered at a flow rate of 200 nL/min and washout was performed at 500 nL/min. The column temperature was set at 50 °C. For DDA-PASEF acquisition, the full scans were recorded from 100 to 1700 m/z spanning from 1.45 to 0.65 Vs/cm² in the mobility (1/K₀) dimension. Up to 10 PASEF MS/MS frames were performed on ion-mobility separated precursors, excluding singly charged ions which are fully segregated in the mobility dimension. Threshold and target intensity was set at 1750 and 14,500 counts, respectively.

Raw mass spectral data was processed using PEAKS Studio X-Pro Software (Bioinformatics LTD). Search was performed against Uniprot rat sequence database containing 20,865 entries with MS1 precursor tolerance of 20 ppm and MS2 tolerance of 0.06 Da. Fully-tryptic digestion allowing one missed cleavages, fixed modification of cystine [+57.02] and oxidation of methionine and deamination of asparagine and glutamine were also specified for database search. All the process starting from destaining was performed by Dr Dominic Kurian in Proteomics and Metabolomics Facility, Roslin Institute, University of Edinburgh.

2.11.2 MS analysis of *Dyrk1a* HET synaptosome samples

Sample preparation

Synaptosomes (P2) were resuspended in lysis buffer (5 % w/v SDS, 50 mM TEAB pH 8.5) supplemented with cOmplete protease and phosphatase inhibitors (Roche). Proteins were quantified using a Pierce bicinchoninic acid (BCA) assay (ThermoFisher Scientific) according to manufacturer instructions, allowing standardisation of protein to 3 mg prior to digest. Next, proteins were denatured with 5 mM tris (2-carboxyethyl) phosphine (TCEP) at 60 °C for 15 min, alkylated with 30 mM iodoacetamide (IAA) at RT for 30 min in the dark, and acidified to a final concentration of 1.2 % phosphoric acid. Samples were then diluted 8-fold with 10 % TEAB (pH 7.2) in 90 % MeOH and added to S-trap midi (Protifi) columns. The columns were washed according to manufacturer's protocol, with a total of five washes with 90 % MeOH 10 % TEAB (pH 7.2), and trypsin (Worthington) added at a ratio of 1:10 enzyme:protein (300 µg: 3000 µg). A following digestion was performed for 20 h at 37 °C. A small amount (volume corresponding to 10 µg) of eluted peptides were dried and stored at -80 °C prior to LC-MS, with the remainder subjected to phosphopeptide enrichment. Peptides were resuspended in 80 µl of 3 % trifluoroacetic acid (TFA), 70 % acetonitrile, 250 mM lactic acid. After a centrifugation

for 1 min at 17000 *g*, 36 μ l (equivalent to ~1.5 mg peptides) of peptides was loaded onto self-packed stage-tips containing C8 (3M Empore) and 1.25 mg of 5 μ m TiO₂ beads (Titansphere, GL Life Science). Stage-tips were washed with the loading buffer, 3 % TFA, 70 % acetonitrile, and 0.1 % TFA. Phosphopeptides were eluted with 1 % NH₄OH (pH 10.5), and immediately subjected to Liquid Chromatography and Trapped Ion Mobility Mass Spectrometry (LC-TIMS-MS), which contains an intrinsic C18 cleanup.

Liquid Chromatography and Trapped Ion Mobility Mass Spectrometry (LC-TIMS-MS)

All samples were subjected to LC-MS/MS using an Evosep One LC-system with a 15 cm Aurora Elite C18 column with integrated captive spray emitter (IonOpticks), at 50 °C, in line with a timsToF-HT (Bruker Daltonics). For proteomics, peptides were resuspended in 0.1 % formic acid in HPLC grade H₂O (buffer A). A volume equivalent to 500 ng was loaded onto the LC-required C18-evotips according to the manufacturer's protocol. Next, the tims-ToF HT was operated in DIA-PASEF mode. Mass and IM ranges were 300-1200 *m/z* and 0.6-1.45 1/K₀, DIA-PASEF was performed as previously using variable width IM-*m/z* windows without overlap (Hermosilla-Trespaderne et al., 2024; Skowronek et al., 2022). TIMS ramp and accumulation times were 100 ms, total cycle time was ~1.8 seconds. Collision energy was applied in a linear fashion, where ion mobility = 0.6-1.6 1/K₀, and collision energy = 20 - 59 eV.

For phospho-proteomics, the peptides were acidified to a final concentration of 3 % TFA (pH <3) and 5 % of the eluate was loaded onto C18-evotips. The predefined Whisper-Zoom 20 SPD protocol was used, in which the gradient is 0-35 % buffer A-buffer B (0.1 % formic acid in acetonitrile), 200 nl/min, for 58 minutes, 20 samples per day were permitted. The timsToF-HT was operated in DDA-PASEF mode. Mass and IM ranges were 100-1700 *m/z* and 0.6-1.6 1/K₀. TIMS ramp and accumulation times were 100 ms, with a total of 10 PASEF ramps, with a target intensity of 20000 and threshold of 2500. Total cycle time was ~1.17 seconds. Collision energy was applied in a stepped-linear fashion based on ion mobility, where ion mobility = 0.6-0.85-1.17 1/K₀, and collision energy = 20-59-54 eV.

Data analysis

For proteomics, raw DIA-PASEF data files were searched using DIA-NN 1.9, using its *in silico* generated spectral library function, based on proteome FASTA files for *Rattus norvegicus* (downloaded from Uniprot on the 25th of January 2024, containing 8201 entries) proteomes and a common contaminants list (Demichev, Messner, Vernardis, Lilley, & Ralser, 2020; Frankenfield, Ni, Ahmed, & Hao, 2022). Trypsin specificity with a maximum of 2 variable modifications and 1 missed cleavage were permitted per peptide. Cysteine carbamidomethylation was set as fixed, oxidation of methionine and protein N-terminal acetylation were set as variable modifications. Peptide length and *m/z* was 7-30 and 300-1200, with charge states 2-4 included. Mass accuracy was fixed to 15 ppm for MS1 and MS2. Protein and peptide FDR

were both set to 1 %. Match between runs was used. All other settings were left as defaults. The protein group matrix output from DIA-NN underwent filtering to exclude contaminants and to include protein groups quantified by at least two peptides. Protein groups were log₂ transformed, median normalised, and filtered to include only those present in at least three of five replicates in any one condition. The log₂ intensity values were subjected to processing with limma, with significance inferred by T-test ($P < 0.05$) and fold change of < 0.75 or > 1.5 (Ritchie et al., 2015).

For phospho-proteomics, raw DDA-PASEF data files were searched using FragPipe v22.0, with the built in “LFQ-phospho” pipeline and the proteome FASTA files as above (Yu et al., 2020). This includes precursor and fragment mass tolerance of ± 20 . Cysteine carbamidomethylation was set as fixed. Oxidation of methionine, protein N-terminal acetylation and phosphorylation of STY were opted as variable modifications. Trypsin specificity with 2 missed cleavages were permitted. Peptide length was 7-50, and mass 500-5000 Da. Percolator was used for PSM validation, with peptide probability set to 0.5, and PTMprophet for PTM localisation (minimum probability 0.5). Quantification was performed with IonQuant (Käll, Canterbury, Weston, Noble, & MacCoss, 2007; Shteynberg et al., 2019; Yu et al., 2020). Post processing and analysis was performed using R with Rstudio. The combined site STY file from FragPipe underwent filtering to exclude contaminants and phosphosites with localization probability < 0.75 , and were required to be present in at least three of five replicates in any one condition. The filtered maxLFQ intensity values were log₂ transformed, subjected to random forest imputation using missForest, and then median normalised (Stekhoven & Bühlmann, 2012). Data were then processed using limma, with significance inferred by T-test ($P < 0.05$) and fold change of < 0.5 or > 2 .

2.12 Statistical Analysis

Data was analyzed using Microsoft Excel and Prism software version 10.1.2 (Graphpad Inc.). The error bars in the figures represent $\pm$ SEM and the n numbers indicate the number of coverslips. The number of coverslips and neuronal preparations are specified in Appendix A. The timetraces show average values.

The normality of the data distribution was assessed by Shapiro-Wilk normality test with significance level set at $\alpha = 0.05$. Comparison between three or more groups used one-way ANOVA followed by Dunnett's post hoc test for comparison against DIV 3. Kruskal-Wallis test was used in the case when any of the group failed the normality test, followed by Dunn's multiple comparison test. For comparison between two groups, unpaired Student's *t* test was used when both of the groups passed the normality test. Otherwise, Mann-Whitney test was used.

The Developmental Trajectory of SV Recycling in WT Neurons

3.1 Introduction

SV recycling is the foundation of the execution and maintenance of neurotransmission. Thus, it is not surprising that the dysfunction of SV recycling is associated with numerous NDDs (Bonnycastle et al., 2021). How neurons equip such functionality is naturally an important question in neurobiological as well as in clinical perspectives.

As discussed in the first chapter, the mechanism of synaptogenesis has been of interest as soon as the dissociated culture technique became available. Numerous studies have identified unitary delivery of presynaptic machinery, such as scaffolding proteins and synaptic vesicular proteins to the potential presynaptic sites (Ahmari et al., 2000; Easley-Neal et al., 2013; Rizalar et al., 2023; Sabo et al., 2006; Tao-Cheng, 2007). This was a major breakthrough that explained the fast assembly of the active zone after axo-dendritic contact, but a gap of knowledge remains regarding the functional development of the presynapse following its assembly.

The development of Ca^{2+} coupling to exocytosis was also widely studied, both in slices and in cultures. In calyx of Held, it has been established that the Ca^{2+} coupling tightens over development, and simultaneously the P/Q- type VGCC ($\text{Ca}_v2.1$) becomes the main contributor for the Ca^{2+} influx after hearing onset around P14 (Fedchyshyn & Wang, 2005; Iwasaki et al., 2000; Kochubey et al., 2009; L.-G. Wu et al., 1999; Y.-M. Yang et al., 2010). In a small typical synapse, however, there are limited reports on the tightening of Ca^{2+} coupling, although the composition of the VGCC subtypes seems to be regulated similarly to that in calyx of Held (Bornschein et al., 2019; Chaudhuri et al., 2005; Pravettoni et al., 2000). Whereas the distinction of the subtypes of the VGCCs is based on the pore-forming α_1 subunit, it is currently being revealed that the auxiliary subunits, β subunit and $\alpha_2\delta$ subunit play additional roles in VGCC signalling pathway and neurotransmitter release, opening a new avenue for research in the developmental aspect of Ca^{2+} coupling (Campiglio & Flucher, 2015; Cunningham, Sauvola, Tavana, & Littleton, 2022; Dolphin, 2018).

In response to the increase in the intracellular Ca^{2+} concentration, SVs fuse to the plasma membrane. Spontaneous SV exocytosis has been identified as the major mode of exocytosis in developing neurons, while evoked exocytosis becomes dominant later in development (Andreae et al., 2012; Banerjee et al., 2022). Spontaneous exocytosis is speculated to play a critical role in circuit development: postsynaptic guidance and synapse maintenance (Andreae & Burrone, 2015; Choi et al., 2014; Faust et al., 2021; Garcia-Bereguian et al., 2016; Sutton et al., 2004). Recently, Ralowicz et al. (2024) reported a positive correlation between the frequency of spontaneous exocytosis and the amplitude of evoked exocytosis, suggesting that spontaneous exocytosis could reinforce synaptic strength, although the causality was not proven.

SV endocytosis in the context of neuronal development, on the other hand, remains largely unexplored, except for the reports from the 1990s that showed SV cargoes are recycled in advance to axo-dendritic contact (Coco et al., 1998; Matteoli et al., 1992). This is surprising considering how important SV recycling is to maintain presynaptic activity. Furthermore, now that we are reshaping our understanding of SV endocytosis, from conventional CME-centered paradigm to endosomal recycling processes (ADBE and UFE), which is found to be the dominant mode of endocytosis, it is necessary that we understand the developmental trajectory in this new angle as well (Ivanova & Cousin, 2022).

In order to gain a more integrative perspective on the developmental trajectory of SV recycling in WT neurons, we used a palette of live and fixed imaging assays in hippocampal neurons from primary cultures at different ages from DIV 3 to 21. Here, we show that a neuron undergoes an increase in evoked Ca^{2+} influx and the recruitment of ADBE as a subsequent step to the activation of general SV recycling.

3.2 Results

3.2.1 Activity-dependent calcium influx increases as nerve terminals mature until second week *in vitro*

Neurotransmitter release is triggered by influx of free Ca^{2+} into the cytoplasm via VGCCs either from the extracellular space or from endoplasmic reticulum (Chanaday & Kavalali, 2022). To determine when activity-dependent Ca^{2+} influx starts in development and how it matures, we exploited the genetically encoded calcium indicator GCaMP6f with a nerve terminal marker syp-mOr2. The two plasmids were co-transfected 2-3 days prior to imaging for each timepoint (DIV 3, 7, 10, 14 and 21). After a snapshot of syp-mOr2 to mark the nerve terminals, the GCaMP6f fluorescence was continuously recorded before, during and after the neurons were challenged at 40 Hz for 10 seconds (figure 3.1A-C). This stimulation setting aims to maximise ADBE, which is a prime interest of this project (Clayton & Cousin, 2009).

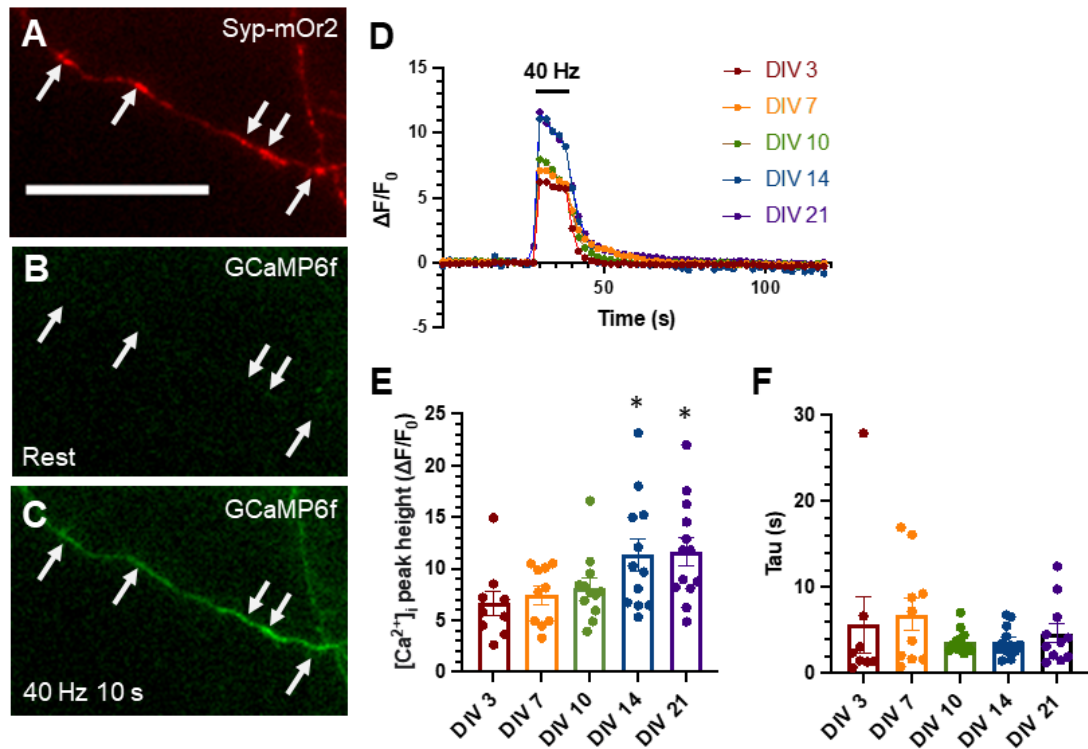


Figure 3.1: Activity-dependent calcium influx increases as nerve terminals mature until second week *in vitro*.

Primary cultures of hippocampal neurons derived from wild-type (WT) embryos were co-transfected with GCaMP6f and synaptophysin-mOrange2 (Syp-mOr2) 2-3 days prior to imaging and were imaged on days *in vitro* (DIV) 3, 7, 10, 14 and 21. Transfected neurons were challenged with a train of 400 APs (40 Hz).

A-C, Representative images of a syp-mOr2 transfected neuron (**A**, Syp-mOr2) and its GCaMP6f response before (**B**, Rest) and during (**C**, 40 Hz) stimulation. Scale bar: 25 μ m.

D, Mean traces displaying the average GCaMP6f fluorescent response at Syp-mOr2 positive puncta in response to stimulation ($\Delta F/F_0$). Bar indicates the period of stimulation (DIV 3 $n = 9$, DIV 7 $n = 10$, DIV 10 $n = 12$, DIV 14 $n = 12$, DIV 21 $n = 13$).

E, Mean evoked GCaMP6f peak height $\pm$ SEM (DIV 3 $n = 9$, DIV 7 $n = 10$, DIV 10 $n = 12$, DIV 14 $n = 12$, DIV 21 $n = 13$). One-way ANOVA ($p = 0.0167$) followed by Dunnett's multiple comparison test against DIV3 (DIV 14 $p = 0.0459$, DIV21 $p = 0.0268$).

F, Mean GCaMP6f decay time constants (tau τ) $\pm$ SEM (DIV 3 $n = 8$, DIV 7 $n = 10$, DIV 10 $n = 12$, DIV 14 $n = 12$, DIV 21 $n = 12$). One-way Kruskal-Wallis test non-significant. One coverslip from DIV 3 and DIV 21 each were excluded for not fitting in one-phase non-linear regression model. In all cases n = independent coverslips.

When challenged with 400 APs, nerve terminals from all five timepoints responded with an instant increase of fluorescence that signifies an increasing concentration of intracellular free calcium ions (figure 3.1A-C). Therefore, it is likely that the expression and the localisation of VGCCs are achieved from early in the development. There were some nerve terminals whose fluorescence level decreased sharply before the stimulation has ended, but the majority of the nerve terminals maintained the fluorescence level at least partially during the stimulation, producing trapezoid-like timetraces (figure 3.1D).

When normalised to the baseline fluorescence level ($\Delta F/F_0$), the peak height extracted from the timetraces increased approximately 1.7-fold from DIV 3 (6.632 ± 1.209) to DIV 14 (11.37 ± 1.589). This was followed by a plateau at DIV 21 (figure 3.1D and E). The substantial increase in the peak height indicates that there was a bigger Ca^{2+} influx upon stimulation from DIV 14 onwards. The increase of Ca^{2+} influx on the second week *in vitro* could be due to an increase in the number of VGCCs, or change in the composition of VGCC subtypes or subunits, which will be addressed in the discussion. Nevertheless, it is likely that the GCaMP reporter was saturated during stimulation, limiting the interpretation from the average peak heights in figure 3.1E. This also means the plateau of the peak height after DIV 14 requires further investigation.

On the other hand, the tau value, which was calculated to assess the kinetics of calcium clearance after the end of the stimulation, did not differ significantly across timepoints (figure 3.1F). This was expected, because in younger neurons (DIV 3 and DIV 7) some nerve terminals showed slower Ca^{2+} clearance generating shoulder-like timetraces, which were eliminated in the analysis to keep the data unimodal. The nerve terminals presenting shoulder-like timetraces accounted for about 20 % of the nerve terminal population, and were hardly seen from DIV 10 and onwards (figure 3.2). This indicates an immature calcium clearance system, potentially due to suboptimal expression, localisation or modification of plasma membrane calcium ATPase (PMCA) or sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) in developing neurons. The majority of the nerve terminals, however, showed similar speed in terms of calcium clearance after influx as represented in tau values in figure 3.1F.

3.2.2 Neurons acquire optimal SV recycling within the first week *in vitro*

The finding that the activity-dependent calcium influx increases until DIV 14 led to the question whether SV exo- and endocytosis also mature following the same trajectory. For this end, syHy was used to monitor SV recycling in individual nerve terminals under the same stimulation setting as in calcium imaging (400 AP, 40 Hz). As explained in figure 2.3, syHy fluoresces as it is exposed to the extracellular space as a result of exocytosis, and it becomes quenched when the endocytosed SV is reacidified by the vATPase. The neurons were filmed continuously before, during and after the stimulation, and throughout the final perfusion with basic buffer (NH_4Cl) (figure 3.3A-D).

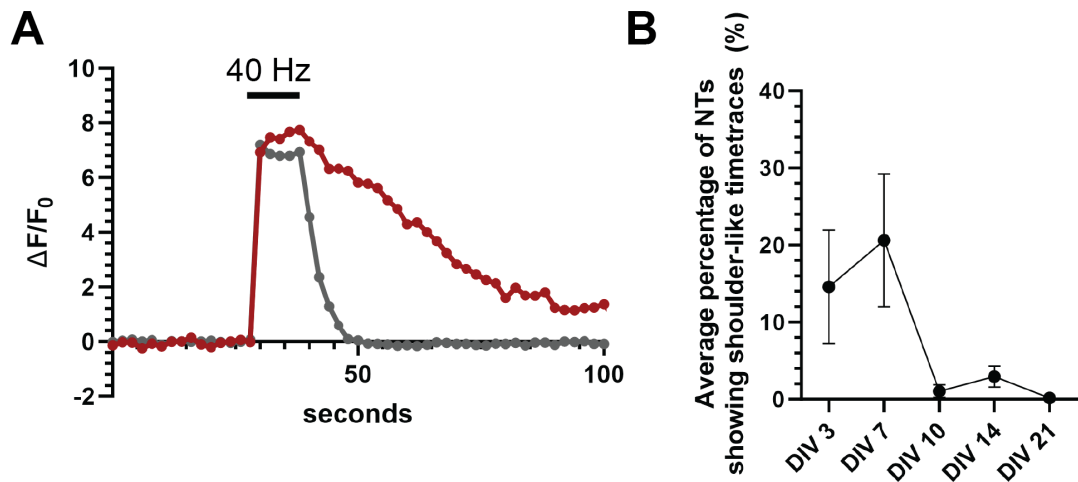


Figure 3.2: Some nerve terminals in developing neurons show slow Ca^{2+} clearance.

- A**, Example timetraces displaying a common trapezoid-like (grey) and a shoulder-trailing (red) GCaMP6f fluorescent response at syp-mOr2 positive puncta in response to stimulation (400 APs, 40 Hz). Both of the timetraces are from DIV 3, implying the bimodal maturity of the nerve terminals at this age. Bar indicates period of stimulation.
- B**, The percentage of nerve terminals showing shoulder-like timetraces per coverslip at each timepoint. The shoulder-like timetraces are defined as the ones that failed to recover halfway to the baseline within 5 s after the end of the stimulation. The dataset includes neuronal preparations that were previously excluded (figure 3.1) due to the presence of shoulder-like timetraces at DIV 3 or DIV 7 (n = 11, 14, 15, 14 and 15 for DIV 3, 7, 10, 14 and 21, respectively).

First of all, at all timepoints, we could find nerve terminals that showed a sypHy response upon stimulation. These nerve terminals displayed an instant increase of fluorescence level upon stimulation, which indicates fusion of SVs onto the plasma membrane (figure 3.3A and B). When the stimulation has finished, the fluorescence waned immediately and recovered to the baseline, indicating that the sypHy molecules were retrieved via SV endocytosis and were subsequently quenched via reacidification (figure 3.3C). The perfusion with imaging buffer containing NH_4Cl at the end of recording induces unquenching of all the sypHy molecules (figure 3.3D). Thus, NH_4Cl response indicates the total SV pool and its typically stronger than the response under the electrical stimulation.

When normalised to the NH_4Cl response, there was a trend of increasing sypHy response from DIV 3 to 14, but without any statistical significance (figure 3.3E and F). In other words, a similar portion of SVs within each nerve terminal were fused to the plasma membrane under the same stimulation, although it is possible that there was a change in the total size of the SV pool during development. This was unexpected because it was speculated that the bigger activity-dependent calcium influx from DIV 14 would correlate with the level of SV exocytosis (figure 3.1E and figure 3.3F).

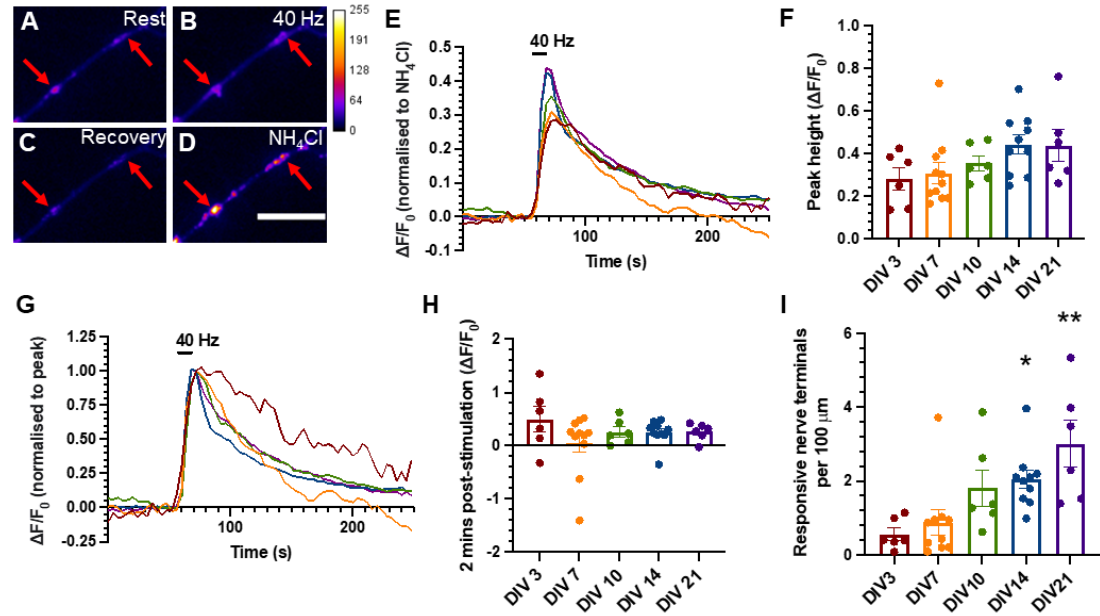


Figure 3.3: Neurons acquire optimal SV recycling within the first week *in vitro*.

Primary cultures of hippocampal neurons derived from wild-type (WT) embryos were transfected with synaptophysin-pHluorin (sypHy) 2-3 days prior to imaging and were imaged on days *in vitro* (DIV) 3, 7, 10, 14 and 21. Transfected neurons were challenged with a train of 400 APs (40 Hz) before exposure to a basic buffer (NH₄Cl) after 3 min.

- A-D**, Representative images of the sypHy response at DIV 14 at rest (**A**, Rest), during stimulation (**B**, 40 Hz), 2 minutes after stimulation (**C**, Recovery) and during NH₄Cl application (**D**, NH₄Cl). Arrows indicate examples of responsive nerve terminals. Scale bar: 10 μ m. A representation of the range of the Fire pseudocolour scale is displayed.
- E**, Mean traces displaying the average sypHy fluorescent response to stimulation at different timepoints ($\Delta F/F_0$). Traces are presented as a fraction of the total SV pool revealed by NH₄Cl application. Bar indicates the period of stimulation (DIV 3 n = 6, DIV 7 n = 11, DIV 10 n = 6, DIV 14 n = 10, DIV 21 n = 6).
- F**, Mean evoked sypHy peak height $\pm$ SEM (DIV 3 n = 6, DIV 7 n = 11, DIV 10 n = 6, DIV 14 n = 10, DIV 21 n = 6). Kruskal-Wallis test non-significant.
- G**, Mean traces displaying the average sypHy fluorescent response normalised to the stimulation peak ($\Delta F/F_0$). Bar indicates the period of stimulation (DIV 3 n = 6, DIV 7 n = 11, DIV 10 n = 6, DIV 14 n = 10, DIV 21 n = 6).
- H**, Mean evoked sypHy response measured 2 minutes after stimulation $\pm$ SEM (DIV 3 n = 6, DIV 7 n = 11, DIV 10 n = 6, DIV 14 n = 10, DIV 21 n = 6). Kruskal-Wallis test non-significant.
- I**, Mean number of responsive nerve terminals per 100 μ m of axon $\pm$ SEM (DIV 3 n = 6, DIV 7 n = 11, DIV 10 n = 6, DIV 14 n = 10, DIV 21 n = 6). Kruskal-Wallis test (p = 0.0003), followed by Dunn's multiple comparison test against DIV 3 (DIV 14 p = 0.012, DIV 21 p = 0.0039). In all cases n = independent coverslips.

Next, the level of SV endocytosis was gauged from the fluorescence level at 2 mins post-stimulation in the timetraces normalised to the peak, as it is indicated by how much the timetraces have recovered to the baseline (figure 3.3G). The data from DIV 3 and DIV 7 were more heterogeneous than those from the older timepoints, but the difference was not statistically significant (figure 3.3H). This means that the sybHy cargoes are retrieved efficiently, and thus SV recycling is functional at least from DIV 3.

Although the individual nerve terminals seem to have acquired functional SV recycling from the first week *in vitro*, only the puncta that were responsive to electric field stimulation were included in the analysis. However, there were notably fewer puncta that responded to the stimulation in younger neurons. In order to include this perspective, we calculated the average number of responsive nerve terminals per neurite length, which increased 3.6-fold from DIV 3 (0.565 ± 0.168) to DIV 14 (2.049 ± 0.250) (figure 3.3I). This data does not purport that the nerve terminals turned stimulation-responsive, as it is a measure of the number of responsive nerve terminals per distance. However, combined with the previous sybHy data, we could conclude that the number of responsive nerve terminals continue to increase until second week *in vitro*, and the responsive nerve terminals show optimal exo- and endocytosis regardless of age (figure 3.3).

3.2.3 Neurons recruit ADBE at a later developmental timepoint compared to other modes of SV endocytosis

SybHy enables monitoring of the SV cargo retrieval in individual nerve terminals, but it does not distinguish different modes of endocytosis. For this reason, TMR-dextran uptake assay was used to monitor ADBE specifically, as the TMR-dextran (40 kDa), a large fluid phase marker, is more likely to be internalised via ADBE than CME due to size exclusion. Thus, the neurons of the same age as calcium imaging or sybHy imaging were stimulated under the same setting (400 APs at 40 Hz), but in the presence of TMR-dextran to monitor the development of ADBE.

When challenged with 400 APs, the average number of the TMR-dextran puncta in a field of view were below 100 at DIV 3, 7, and 10. However, the number of TMR-dextran puncta increased 5-fold from DIV 10 (51.28 ± 17.18) to DIV 14 (271.50 ± 43.14), and plateaued at DIV 21. The TMR-dextran puncta of the non-stimulated coverslips were non-significant (DIV 3: 25.40 ± 2.32 , DIV 7: 24.68 ± 1.15 , DIV 10: 22.66 ± 0.99 , DIV 14: 51.03 ± 7.07 , DIV 21: 38.56 ± 1.14). This shows a big contrast to the sybHy results, which did not show any significant difference in neither SV exocytosis nor endocytosis across all timepoints (figure 3.3H, figure 3.4A-C). More specifically, the peak height of sybHy response at DIV 10 were comparable to DIV 14 (DIV 10: 0.3548 ± 0.0351 , DIV 14: 0.4425 ± 0.0448) (figure 3.3F). Endocytosis was

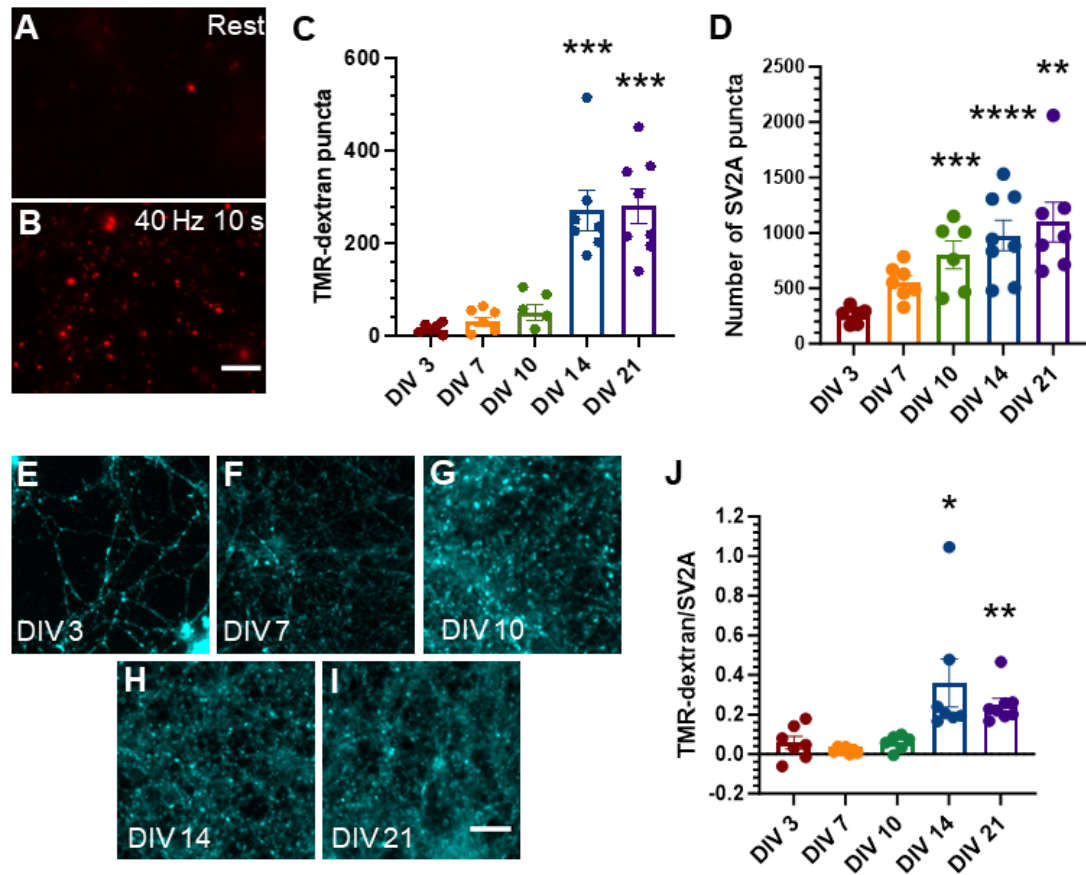


Figure 3.4: Neurons recruit ADBE at a later developmental timepoint.

Primary cultures of wild-type (WT) hippocampal neurons were challenged with an action potential train of 400 APs (40 Hz) in the presence of 50 μM tetramethyl-rhodamine (TMR)-dextran, which was immediately washed away on termination of stimulation.

A-B, Representative images of TMR-dextran-loaded nerve terminals either with (**B**, 40 Hz) or without (**A**, Rest) stimulation. Scale bar: 10 μm .

C, Mean number of TMR-dextran puncta $\pm$ SEM (days *in vitro* (DIV) 3 $n = 7$, DIV 7 $n = 7$, DIV 10 $n = 6$, DIV 14 $n = 7$, DIV 21 $n = 8$). Kruskal-Wallis test ($p < 0.0001$) followed by Dunn's multiple comparison test against DIV 3 (DIV 14 $p = 0.0009$, DIV 21 $p = 0.0004$).

D, Mean number of α -SV2A-positive puncta $\pm$ SEM (DIV 3 $n = 6$, DIV 7 $n = 7$, DIV 10 $n = 6$, DIV 14 $n = 8$, DIV 21 $n = 7$). One-way ANOVA ($p = 0.0004$) followed by Dunnett's multiple comparison test against DIV 3 (DIV10 $p = 0.0005$, DIV 14 $p < 0.0001$, DIV 21 $p = 0.0086$). Scale bar: 10 μm .

E-I, Primary cultures of WT hippocampal neurons from the same preparation were immunolabelled with α -SV2A antibodies. Representative images are displayed at DIV 3 (**E**), DIV 7 (**F**), DIV 10 (**G**), DIV 14 (**H**) and DIV 21 (**I**). Scale bar: 10 μm .

J, Mean number of TMR-dextran puncta normalised to α -SV2A-positive puncta $\pm$ SEM (DIV 3 $n = 6$, DIV 7 $n = 7$, DIV 10 $n = 6$, DIV 14 $n = 7$, DIV 21 $n = 8$). Kruskal-Wallis test ($p < 0.0001$) followed by Dunn's multiple comparison test against DIV 3 (DIV 14 $p = 0.0139$, DIV 21 $p = 0.0085$). In all cases $n =$ independent coverslips.

also similar between DIV 10 and DIV 14 (DIV 10: 0.2587 ± 0.0961 , DIV 14: 0.2475 ± 0.0739) (figure 3.3H). From tens of internalised TMR-dextran puncta in the first week to hundreds of them in the second week *in vitro*, on the contrary, looks as if a binary switch for ADBE has been activated at DIV 14.

Still, it is possible that this increase in the TMR-dextran uptake is because there were more nerve terminals at DIV 14 than DIV 10. Therefore, we needed to test whether these results from TMR-dextran uptake assay accurately represent the capacity of the nerve terminals to perform ADBE. Hence, the neurons from the same preparations as TMR-dextran uptake assay were fixed and immuno-stained for SV2A, a synaptic vesicular protein (figure 3.4D-I). The average number of SV2A puncta per field of view continued to increase from DIV 3 to 10. There was a trend of increase from DIV 10 to 14 but without statistical significance (figure 3.4D). In summary, the number of SV2A puncta in time-course drew a hyperbolic curve that leads us to believe that the increase of TMR-dextran puncta from DIV 10 to 14 was due to the recruitment of ADBE rather than synaptogenesis.

When the number of TMR-dextran puncta was divided by the number of SV2A puncta averaged from the same preparation to normalise the TMR-dextran uptake assay results, the values followed the same trend as the TMR-dextran puncta data from figure 3.4C, hovering around 0 and then increasing drastically at DIV 14 (figure 5.1J). It confirms once again that the neurons experience an extra maturation process that activates ADBE in the second week *in vitro*, simultaneously to the increase of the activity-dependent Ca^{2+} influx.

Whereas TMR-dextran uptake assay provides a binary readout of based on whether a nerve terminal has internalised TMR-dextran particles and thus performed ADBE or not, it misses the quantitative data within each nerve terminal. To address this limitation, the neurons were stimulated under the same setting but in the presence of HRP, another fluid phase marker, followed by processing and sectioning for electron microscopy (figure 3.5). After imaging, the number of HRP-labelled bulk endosomes (> 100 nm in diameter) were quantified per nerve terminal. A nerve terminal was defined as an intracellular structure with HRP-labelled SVs or bulk endosomes (figure 3.5A-E).

The quantification results show that the number of HRP-labelled bulk endosomes increased significantly from DIV 3 (1.053 ± 0.397) to DIV 14 (3.101 ± 0.290), which corroborates the results from TMR-dextran uptake assay (figure 3.4). However, it is worth noting that there was no dramatic increase in the number of HRP-labelled bulk endosomes compared to TMR-dextran uptake assay, for which the possible explanations will be discussed later.

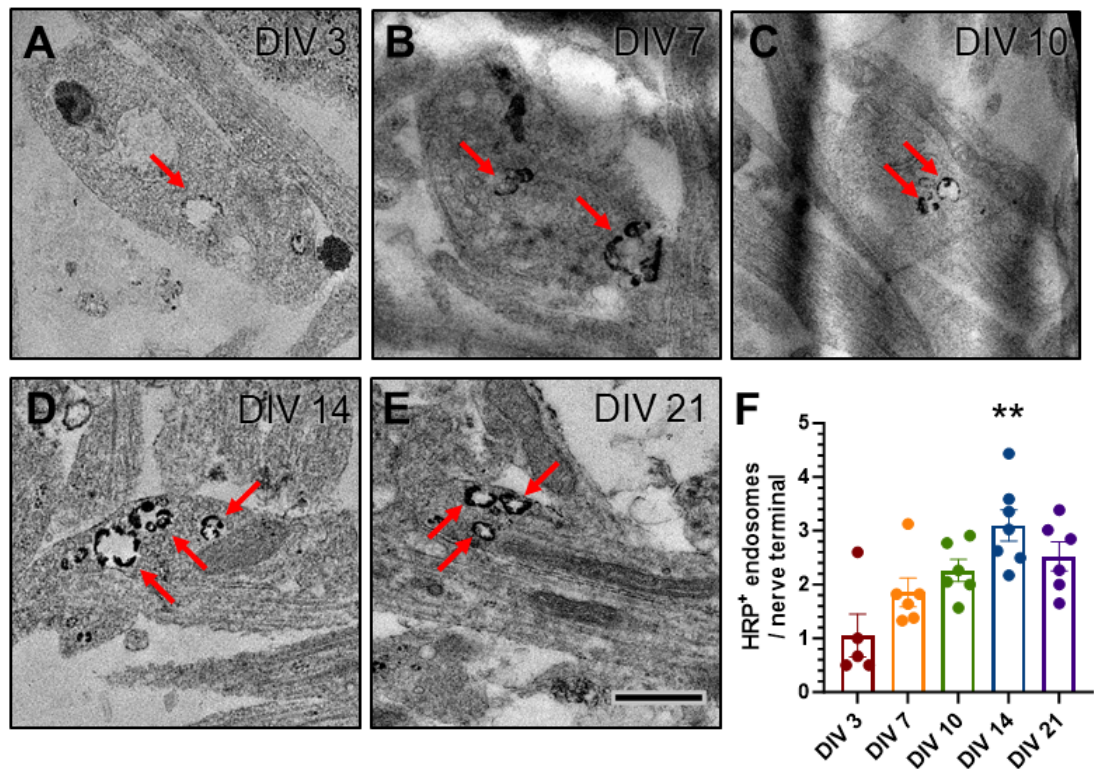


Figure 3.5: The number of endosomes generated via ADBE increases until the second week *in vitro*.

A-E, Primary cultures of wild-type (WT) hippocampal neurons were challenged with an action potential train of 400 AP (40 Hz) in the presence of 10 mg/ml horseradish peroxidase (HRP). Representative images displayed at DIV 3 (**A**), DIV 7 (**B**), DIV 10 (**C**), DIV 14 (**D**) and DIV 21 (**E**). Arrows indicate HRP-labelled endosomes. Scale bar: 500 nm.

F, Mean number of HRP-labelled endosomes per HRP-labelled nerve terminal ± SEM (DIV 3 $n = 5$, DIV 7 $n = 6$, DIV 10 $n = 6$, DIV 14 $n = 7$, DIV 21 $n = 6$). Kruskal-Wallis test ($p = 0.0069$) followed by Dunn's multiple comparison test against DIV 3 (DIV 14 $p = 0.0025$). In all cases $n =$ independent coverslips.

3.2.4 Identifying the mode of SV endocytosis in younger neurons

The data from calcium imaging, syHy imaging, TMR-dextran uptake assay and HRP uptake assay with electron microscopy point to the conclusion that the SV recycling is functional from the first timepoint of observation (DIV 3), but ADBE only begins from DIV 14. This raises another question, about the identity of the SV recycling in the first week *in vitro*, when ADBE hardly occurs.

One potential explanation is that CME accounts for the main mode of SV endocytosis in developing nerve terminals. To test this idea, syHy imaging was performed again at DIV 9-10, after 6 days of transfection with shRNAs targeting clathrin heavy chain (CHC) (shCHC) or a scrambled sequence (shScr) as a control. The period of transfection was extended from 2-3 days to 6 days to ensure the target was sufficiently knocked down (Wei et al., 2024).

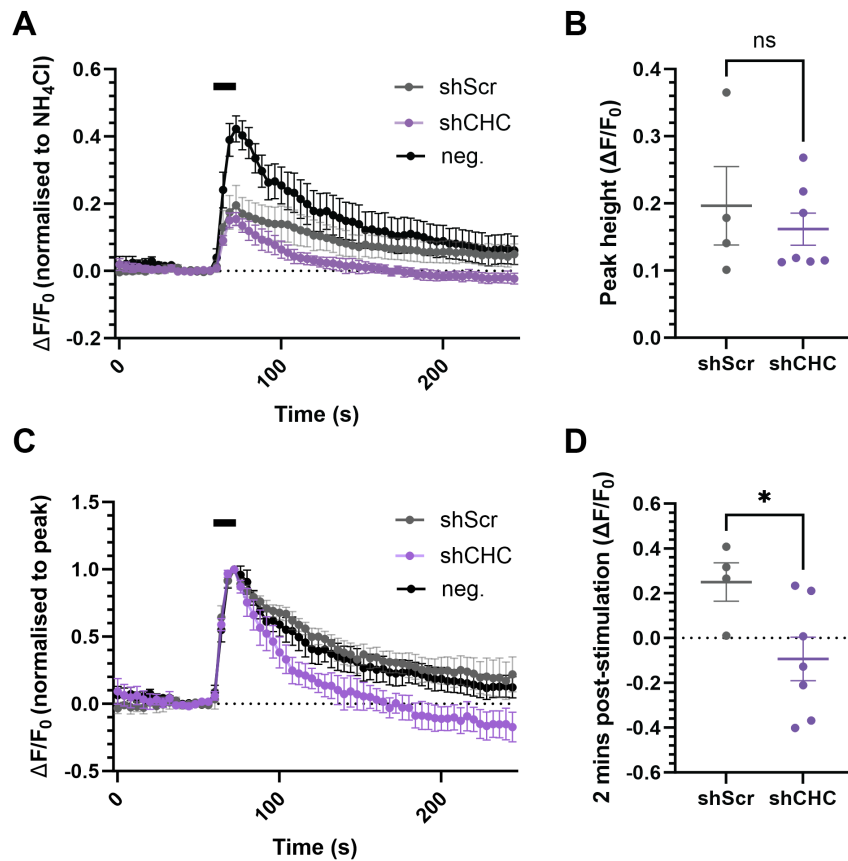


Figure 3.6: Clathrin heavy chain (CHC) knockdown reveals acceleration of SV endocytosis in developing neurons.

Primary neurons from hippocampal cultures were transfected with shRNAs targeting CHC (shCHC) or scrambled sequence (shScr) conjugated with mCerulean tag, alongside synaptophysin-pHluorin (syHy) plasmids. The neurons were transfected for 6 days prior to imaging, which was performed at DIV 9-10. After baseline recording, the transfected neurons were challenged with a train of 400 APs (40 Hz) before exposure to an basic buffer (NH_4Cl) after 3 mins.

- A,** Mean traces displaying the average syHy fluorescent response to stimulation in neurons transfected with shScr or shCHC $\pm$ SEM ($\Delta F/F_0$) (shScr $n = 4$, shCHC $n = 7$). The negative control was taken from the corresponding DIV 10 data in figure 3.3 for reference (mean $\pm$ SEM, $n = 6$). Traces are presented as a fraction of the total SV pool revealed by NH_4Cl application. Bar indicates the period of stimulation.
- B,** Mean evoked syHy peak height $\pm$ SEM (shScr $n = 4$, shCHC $n = 7$). Mann-Whitney test ($p = 0.9273$).
- C,** Mean traces displaying the average syHy fluorescent response normalised to the stimulation in neurons transfected with shScr or shCHC $\pm$ SEM ($\Delta F/F_0$) (shScr $n = 4$, shCHC $n = 7$). The negative control was pooled from the corresponding DIV 10 data in figure 3.3 for reference (mean $\pm$ SEM, $n = 6$). Bar indicates the period of stimulation.
- D,** Mean evoked syHy response measured 2 mins after stimulation $\pm$ SEM (shScr $n = 4$, shCHC $n = 7$). Unpaired Student's t test ($p = 0.0420$).

Both shScr- and shCHC- transfected neurons showed a smaller sypHy response when challenged with a train of 400 APs compared to the timetraces reported at DIV 10 in the time-course experiment from figure 3.3E (figure 3.6A). The peak height of both shScr- and shCHC- transfected neurons was about half of that at DIV 10 in WT neurons when normalised to NH_4Cl response (figure 3.6B). This could be a byproduct of plasmid toxicity, as there was no significant difference in the peak height between shScr and shCHC.

When normalised to the peak, however, shCHC-transfected neurons showed accelerated recovery to the baseline after stimulation compared to shScr-transfected neurons (figure 3.6C and D). Supported by the fact that the normalised timetraces of shScr-transfected neurons were comparable to that of the negative control (data from DIV 10 group in figure 3.3G), it is apparent that knocking down CHC results in faster endocytosis upon stimulation. Knocking down CHC was expected to either have no effect on sypHy response or slow down the SV endocytosis based on previous literature (López-Hernández et al., 2022; Nicholson-Fish et al., 2015). It is possible that in younger neurons, the knockdown has made ADBE more prominent, although the phenotypes in ADBE are not always represented in sypHy imaging according to previous literature (Bonnycastle et al., 2022; Ivanova et al., 2021).

3.3 Discussion

Using a variety of fixed and live cell imaging techniques, this chapter provides a well-rounded view on the development of SV recycling at the nerve terminals in culture. Calcium imaging utilising GCaMP6f and syp-mOr2 co-transfection showed that the amplitude of activity-dependent Ca^{2+} influx increased until DIV 14, although it is likely that the GCaMP6f signal is saturated. The experimental design chose 400 APs stimulation in order to keep the stimulation consistent throughout the study. The saturation problem could be circumvented by using a lower affinity genetically-encoded calcium indicator or a calcium dye such as Fluo5, or differential use of calcium chelators in future experiments. The decay after the calcium response, however, did not show a significant difference timepoints except for the shoulder-like traces observed at DIV 3 and 7. SypHy imaging uncovered the functional SV recycling at all timepoints, from DIV 3 to 21. TMR-dextran uptake assay and HRP uptake assay, on the other hand, revealed that ADBE is acquired and activated from DIV 14. In all experiments, the same setting of stimulation (40 Hz 10 s) was used consistently for maximal ADBE (Clayton et al., 2008). Except for sypHy imaging, all experiments indicated a divide between the neurons younger than 14 days and the neurons older than 14 days in primary cultures, signifying DIV 14 as a landmark in presynaptic maturation.

The concept of presynaptic maturation has been around for some time, as it has been addressed in the first chapter. For example, it is now well-known that the coupling between calcium influx and SV exocytosis becomes tighter, the main mode of exocytosis switches from spontaneous to evoked, and the SV pools are reorganised (Andreae et al., 2012; Bornschein et al., 2019; Fedchyshyn & Wang, 2005; Mozhayeva et al., 2002). However, this project is valuable in that it brings an integrated assessment of the functional aspect of the presynaptic development under the same stimulating condition and at the same developmental timepoints. The observations reported in this project adds to the jigsaw of knowledge in presynaptic development, albeit some still existing gaps.

3.3.1 Evoked Ca^{2+} influx and efflux at nerve terminals in developing neurons

Through calcium imaging, we showed that the activity-dependent Ca^{2+} influx increased until the second week *in vitro*, although there is a possibility of saturation of the GCaMP reporter. There are multiple scenarios that could explain the enhanced amplitude of activity-dependent Ca^{2+} influx. Some of them are altered expression of VGCCs, change in the subcellular localisation of the VGCC subtypes, and change in the basal intracellular Ca^{2+} level.

A VGCC is composed of a pore-forming α_1 subunit, and auxiliary β and $\alpha_2\delta$ subunits. The isoforms of α_1 subunits determine the subtype of the VGCCs, whereas auxiliary subunits are reported to be promiscuous, binding to different α_1 subunits isoforms (Cunningham et al., 2022). Schlick, Flucher, and Obermair (2010) compared the mRNA level of VGCC subunits in hippocampal neurons in culture between DIV 5 and 24. Whereas most of the subunits did not show any obvious change between the two timepoints, there was a significant increase in the transcription level between the two timepoints in $\text{Ca}_v2.1$ (P/Q- type) and $\alpha_2\delta-2$. $\text{Ca}_v2.1$ is the major presynaptic VGCC, and it is natural to assume that the expression level of $\text{Ca}_v2.1$ increases as a neuronal progenitor acquires its neuronal identity. Indeed, an increase in the contribution of P/Q- type VGCC to the presynaptic activity has been recorded in culture (Scholz & Miller, 1995). An increase in the transcription of $\text{Ca}_v2.1$ *in vitro* aligns with the findings *ex vivo*, which showed that $\text{Ca}_v2.1$ accounts for higher proportion of the calcium current in calyx of Held, as well as cortical synapses later in development (Bornschein et al., 2019; Iwasaki et al., 2000; L.-G. Wu et al., 1999). The second subunit that showed an increase in its mRNA level, $\alpha_2\delta-2$, would also be an interesting topic for future studies, as it regulates localisation of the $\text{Ca}_v2.1$ α subunits (Hoppa, Lana, Margas, Dolphin, & Ryan, 2012). In addition, primary neurons deficient of $\alpha_2\delta$ subunits in hippocampal cultures showed reduced Ca^{2+} influx, making it another strong candidate that could be behind the increased activity-dependent Ca^{2+} influx at DIV 14 (Schöpf et al., 2021).

Although the mRNA level did not show any significant change in Schlick et al. (2010), both $\text{Ca}_v2.2$ (N-type) and $\text{Ca}_v1.1-1.4$ (L-type) VGCCs have been suggested that they are differentially regulated throughout development. N- type VGCC is another high-voltage activated calcium channel like P/Q- type VGCC. There are contradicting reports on whether the contribution of N- type VGCC increases or decreases as a neuron develops (Basarsky, Parpura, & Haydon, 1994; Pravettoni et al., 2000). In a different point of view, Bahls et al. (1998) demonstrated that the N- type VGCC shows more punctate expression in the second week *in vitro* rather than the linear expression along the axon in immature neurons, proposing a spatial reorganisation of the VGCCs. Nevertheless, $\text{Ca}_v2.2$ (N-type) VGCC was not linked to the amplitude of activity-dependent Ca^{2+} influx in these studies.

L-type calcium channels are differentially expressed in developing neurons. Obermair, Szabo, Bourinet, and Flucher (2004) have observed transient expression of $\text{Ca}_v1.2$ (L-type) in axon and the growth cone in immature neurons in culture. Kamijo et al. (2018) has also shown that the spontaneous activity in immature cortical neurons in culture is due to the activity of L-type VGCC. Similarly, in hippocampal cultures at DIV 8-11, it has been recognised as a major source of subthreshold Ca^{2+} activity (Lee et al., 2023). On the other hand, in mature cerebellar granule neurons, Cousin, Held, and Nicholls (1995) showed that L-type VGCC do not contribute to activity-dependent Ca^{2+} influx via blocking the L-type VGCC with nifedipine. These reports support the idea that the role of L-type VGCC is limited to development rather than maintaining neuronal activity. However, recent reports in cardiovascular studies have shown the effect of splice variants on voltage-dependence of L-type VGCCs, and these splice variants are developmentally regulated, although it was not investigated in central nervous system (CNS) neurons (Hofer et al., 2021; Panagiotakos et al., 2019).

It would be informative to establish a detailed profile of VGCC isoforms and subtypes to learn the developmental regulation of activity-dependent calcium influx. This can be achieved by using different subtype-specific VGCC blockers such as nifedipine against L- type, ω -agatoxin-IVA for P/Q-type and ω -conotoxin-GVIA for N-type (Cousin et al., 1995). Acute application of the blockers whilst calcium imaging would tell us if any specific isoform or subtype is responsible for the increase in the Ca^{2+} response at DIV 14.

What the calcium imaging approach in this project does not consider, is that the analysed timetraces are normalised to the baseline fluorescence level (F_0). Younger neurons are commonly observed to be more depolarised, hence it is possible that the younger neurons have higher intracellular Ca^{2+} concentration (Kirmse et al., 2015; Tyzio et al., 2003). It could be due to the spontaneous activity in developing neurons, or inward-rectifying potassium channels K_{ir} , or due to excitatory GABA signalling (Andreae et al., 2012; Ganguly, Schinder, Wong, & Poo, 2001; Mongiat, Espósito, Lombardi, & Schinder, 2009). This theory could not be tested in the current system as it would be fallacious to compare F_0 across coverslips as the total expression level of GCaMP6f within each neuron could be heterogeneous. To address this,

one could use an ionophore such as ionomycin, and calcium chelators such as ethylene glycol tetraacetic acid (EGTA) in order to measure the minimum and maximum GCaMP signals. This allows comparison of the response to the electrical field stimulation in a calibrated manner (McCombs & Palmer, 2008). A ratiometric calcium dye in a lower-density culture would also be an appropriate tool to measure basal intracellular Ca^{2+} level.

Last but not least, the qualitative observation of the nerve terminals that showed shoulder-like timetraces in developing neurons suggests immature calcium clearance system. This could be due to insufficient PMCA or SERCA. Both types of Ca^{2+} pumps have multiple variants that are expressed differentially in embryonic and adult brains, suggesting these "shoulder" timetraces also could be due to subtype differences (Britzolaki, Saurine, Klocke, & Pitychoutis, 2020; Mata & Sepulveda, 2010).

3.3.2 SV recycling in developing neurons

Via sybHy imaging, TMR-dextran uptake assay and HRP uptake assay with electron microscopy, we have monitored SV recycling in developing neurons (DIV 3, 7, and 10). It was previously known that the SVs are recycled in immature neurons in a Ca^{2+} -dependent manner in culture before axo-dendritic contact (Coco et al., 1998; Matteoli et al., 1992). Therefore, it was not surprising to see sybHy response from DIV 3. What was novel, however, was that there was no significance compared to mature neurons in sybHy response, despite the later recruitment of ADBE.

To begin with, the sybHy peak height (figure 3.3E and F) shows a trend of increase but without any statistical significance. This indicates that a comparable portion of SVs were fused to the plasma membrane upon stimulation. This contrasts to the increasing evoked calcium influx shown in figure 3.1E. It is possible that there was a difference in the number of SVs fused to the plasma membrane as the timetraces report in proportion to NH_4Cl response rather than the absolute numbers. Although there were studies on the reorganisation of the SV pool in culture, and decreasing release probability in slices, the total number of SVs in development has not been reported, probably due to the complications deriving from the heterogeneity across synapses (Bolshakov & Siegelbaum, 1995; Bouazza-Arostegui, Camacho, Brockmann, Zobel, & Rosenmund, 2022; Dobrunz & Stevens, 1997; Mozhayeva et al., 2002). Another blind spot of this project is spontaneous exocytosis. Spontaneous exocytosis is the main mode of exocytosis in immature neurons (Andreae et al., 2012), but here the nerve terminals with spontaneous activity were excluded from the analysis to look at the response to the given stimulation (400 APs) independently and exclusively. However, considering the importance of spontaneous exocytosis in development, it would be useful to expand the scope of our

understanding to spontaneous exocytosis. The key question would be how the transition from the spontaneous to evoked exocytosis takes place, and if there is a developmental change in the relationship between spontaneous and evoked exocytosis (Andreae et al., 2012; Ralowicz et al., 2024).

In terms of endocytosis, there was no significant difference across timepoints when comparing 2 mins post-stimulation in sypHy imaging (figure 3.3G and 3.3H). TMR-dextran uptake assay, on the other hand, suggested near-absence of ADBE in developing neurons (figure 3.4). This absence could be due to the smaller calcium influx (figure 3.1E), as ADBE requires dephosphorylation of the endocytic machinery triggered by calcium influx (Morton, Marland, & Cousin, 2015). The disparity between the deficient ADBE (TMR-dextran uptake assay) and functional SV recycling (sypHy imaging) is not uncommon, as reported in previous papers in our group (Bonnycastle et al., 2022; Ivanova et al., 2021; Nicholson-Fish et al., 2015). It is possible that the overexpression of exogenous reporters does not accurately represent the endocytotic pathway of the endogenous cargoes. For example, knocking down Syndapin-1, an endocytic protein that activates ADBE by interacting with Dynamin-1, did not alter sypHy traces (Cheung & Cousin, 2019; Clayton et al., 2009; Nicholson-Fish et al., 2015). Another pH-sensitive SV recycling reporter, cypHer, did not reflect the rapid decay observed in recordings of membrane capacitance, which indicates ADBE (Okamoto et al., 2016). Knocking in SV recycling probes into the endogenous genes and producing primary cultures from these genetically modified animals could circumvent this problem. Transgenic mouse lines with Syb-2 coupled with pHluorin have been produced and used to study SV recycling previously, but no comparison has been made between the endogenously expressed and exogenously expressed reporters (Miesenböck, De Angelis, & Rothman, 1998).

There is one more possible explanation for both exo- and endocytosis in sypHy results, the screening process during the analysis. The nerve terminals were screened for two criteria: responsive to both the electrical stimulation and the NH₄Cl perfusion. Thus, the nerve terminals included in the analysis could have been biased for the premature nerve terminals in culture, rather than representing an average nerve terminal at the given timepoint. This argument is further supported by Bouazza-Arostegui et al. (2022), where the authors showed an increase in the EPSC amplitude and release probability from DIV 11-12 to DIV 15-16 in autaptic hippocampal cultures.

The CHC knockdown data once again leads us astray, because knocking down CHC in developing neurons (DIV 9-10) resulted in acceleration of endocytosis after 6 days of transfection (figure 3.6C and 3.6D). This disagrees with the previous literature that decelerated or intact endocytosis when CHC was knocked down (López-Hernández et al., 2022; Nicholson-Fish et al., 2015; Wei et al., 2024). López-Hernández et al. (2022) showed that the retrieval of vGluT1 and SV2 were unaffected by knocking down CHC in physiological temperature. Nicholson-Fish et al. (2015), on the contrary, showed slower sypHy retrieval when CHC was knocked down

regardless of temperature. Wei et al. (2024) also supported the importance of CHC in SV endocytosis using siRNA and shRNA on bovine chromaffin cells, plus conditional knockout in neurons ($CHC^{LoxP/LoxP}$). In the conditional knockout experiments, the knockout of CHC showed severe deceleration of SV endocytosis from six days after transfection.

However, the major difference between the CHC knockdown experiment here and the ones in literature is the age of the neurons. Nicholson-Fish et al. (2015), López-Hernández et al. (2022) and Wei et al. (2024) performed shRNA transfection or conditional knockout at an age around one week *in vitro* and the cells were imaged around DIV 14. On the other hand, here the neurons were transfected with shRNA at DIV 3-4 and imaged at DIV 9-10. It is possible that either shCHC transfection at an earlier age made ADBE more prominent in syHy imaging by excluding the CME factor, although previous publications from our lab failed to report accelerated syHy recovery when ADBE was impaired. Alternatively, the developing neurons could have compensated for the reduced CHC level with exaggerated ADBE, which were not the case when transfected after DIV 7. The latter theory could be tested via single-neurite TMR-dextran uptake assay, which assays the TMR-dextran uptake of a transfected neuron.

3.3.3 SV recycling in mature neurons

The data from calcium imaging, syHy imaging and TMR-dextran uptake assay at DIV 14 and 21 recapitulated the SV recycling previously seen in the lab (e.g., WT data in Bonnycastle et al. (2022)). To summarise, the mature neurons have bigger activity-dependent Ca^{2+} influx, and performs SV endocytosis by recruiting ADBE.

However, there was one notable change in syHy imaging in mature neurons, the number of stimulation-responsive puncta (figure 3.3I). It would have been ideal if it was possible to quantify the total number of nerve terminals within a field of view, as what is shown in figure 3.3I could be due to either the increasing number of synapses or a nerve terminal turning from non-responsive to responsive. That would allow us to test whether the proportion of responsive nerve terminals increased significantly at DIV 14, which would confirm the simultaneous switch from spontaneous to evoked exocytosis, and from the lack of ADBE to predominant ADBE. Although not tested, this is a plausible scenario because evoked exocytosis entails fusion of more SVs than in spontaneous exocytosis, meaning evoked exocytosis would require a more effective cargo retrieval (Ogunmowo et al., 2023). Unfortunately, because of the high mobility of the syHy puncta especially in younger neurons, the whole neurites were saturated when perfused with the basic buffer, making it difficult to quantify the total number of nerve terminals. This could be addressed by live-labelling the nerve terminals with an antibody (e.g., α -SV2A) and selecting syHy puncta based on the live-labelling.

The recruitment of ADBE is a new finding as it has not been studied in younger neurons before. As mentioned earlier, it is possible that this recruitment is due to a bigger activity-dependent Ca^{2+} influx in the second week *in vitro* (figure 3.1E). More specifically, ADBE needs dephosphorylation of the endocytic proteins by Calcineurin, and the Calcineurin-dependence in SV endocytosis increases along the development of the primary culture of cerebellar granule neurons (Smillie et al., 2005). However, this contradicts with the findings in calyx of Held, whose SV endocytosis was Calcineurin-dependent in P7-10 but independent in P13-14 *ex vivo* (X.-S. Wu et al., 2014). Alternatively, vesicular calcium channel Flower was also shown to positively regulate ADBE, so its expression in younger neurons would be worth exploring (C.-K. Yao, Liu, Lee, Wang, & Wu, 2017).

In order to complement the binary readout from TMR-dextran uptake assay, we subjected the neurons to the same stimulation but in the presence of HRP, followed by the visualisation of subcellular HRP-uptake via electron microscopy. It should be noted that the average number of HRP-labelled bulk endosomes did not show as prominent a jump from DIV 10 to DIV 14 as in TMR-dextran uptake assay. However, this is not contradictory, as it is likely due to the screening for HRP-positive nerve terminals, which could obscure the absence of ADBE. Just like when we tried to quantify the total number of nerve terminals in sypHy imaging, it would have been ideal if we could quantify the total number of nerve terminals. However, due to technical limitations, it was challenging to distinguish a nerve terminal without any HRP labelling. Therefore, we have compromised to screen for any subcellular structure with at least one HRP-labelled SV or a bulk endosome. It would be helpful to use a genetic marker or an antibody as a reference.

What this series of live and fixed imaging assays did not look into is the SV pool size. In primary cultures, Mozhayeva et al. (2002) has shown a more heterogeneous distribution of the SV pools but in general higher proportion of the RRP at DIV 5 compared to mature neurons older than DIV 20, via hypertonic stimulation and electrical stimulation. The authors have identified the expansion of the reserve pool as the mechanism behind it, rather than the shrinkage of the RRP itself. On the other hand, Bouazza-Arostegui et al. (2022) has shown an increase in RRP size from DIV 11-12 to DIV 15-16, although there was no significant change in the release probability. There has not been a firm consensus on the developmental partitioning of the SV pools, probably because of the high heterogeneity across nerve terminals (Dobrunz & Stevens, 1997). This raises a need for studies on individual nerve terminals, as the average from a population may not be an accurate representation. Interestingly, a SV pool study raised a suspicion on the presence of the resting pool, the SV pool that is unlikely to be mobilised by electric stimulation (Rose et al., 2013). Using current injection, Rose et al. (2013) showed that all SVs were able to be mobilised in slice cultures, and the authors cast doubt on the maturity of primary cultures. However, whether almost all of the SVs are actually involved in synaptic activity *in vivo* is still questionable (Denker et al., 2011).

3.3.4 Understanding the divide between the developing and mature neurons

The results in this chapter expand our understanding of presynaptic development, focusing on the SV endocytosis (summarised in figure 3.1). One notable feature of the previous studies and the current results is that these changes were usually observed between neurons younger than 2 weeks and those older than 2 weeks (Andreae et al., 2012; Bouazza-Arostegui et al., 2022; Ganguly et al., 2001; Kato-Negishi, Muramoto, Kawahara, Kuroda, & Ichikawa, 2004; Schlick et al., 2010; Smillie et al., 2005). The synchronicity of the changes implicate that these different developmental changes might be a chain-reaction. However, neurons mature at different speeds depending on numerous factors such as neuronal subtypes, cell density, presence of glia, et cetera (Biffi, Regalia, Menegon, Ferrigno, & Pedrocchi, 2013; Blondel et al., 2000; Ruffolo Jr, Eisenbarth, Thompson, & Nirenberg, 1978). Synapses within a culture also show varying turnover rates depending on the age of the source of the primary culture and cell density (Heo et al., 2018; Jähne et al., 2021; Puro, De Mello, & Nirenberg, 1977). It would be even more challenging to apply this perspective *ex vivo* or *in vivo* as neurons are born, differentiate and migrate at different timepoints and at different speeds. Therefore, it is necessary to develop a tool to monitor maturation in a synapse-specific manner rather than looking at a population of synapses, in order to determine the mechanism behind the acquisition of bigger calcium influx, synchronised evoked exocytosis and ADBE.

	Developing NTs	Mature NTs	References
Evoked Ca^{2+} influx	Small	Large	Figure 3.1, Bouazza-Arostegui et al. (2022)
VGCCs at nerve terminals	P/Q-, N-, L- type	Increased contribution of P/Q- type	Bornschein et al. (2019); Scholz and Miller (1995)
Dominant mode of SV exocytosis	Spontaneous	Evoked	Andreae et al. (2012)
Dominant mode of SV endocytosis	Clathrin-mediated endocytosis *	Activity dependent bulk endocytosis	Figure 3.4 and figure 3.6

Table 3.1: A summary of the characteristics of developing and mature nerve terminals (NTs).

The table above summarises the key findings from this chapter. This was done by segregating NTs into developing (DIV 3-10) or mature (> 14 DIV) and reporting the dominant contribution in four different parameters. The previous findings referred to in this table exclude those in calyx of Held due to its peculiarity. Red text indicates findings from this chapter. VGCC: voltage-gated calcium channel; SV: synaptic vesicle

The Developmental Trajectory of SV recycling in *Fmr1* KO neurons

4.1 Introduction

Fragile X Syndrome (FXS), which affects 1 in 7,000 males and 1 in 11,000 females, is one of the most common monogenic neurodevelopmental disorders that shows high comorbidity with ASD and ID (Hagerman et al., 2017; Hunter et al., 2014). It is caused by the repression of the gene *FMR1*, which encodes FMRP (Pieretti et al., 1991). FMRP is expressed in neurons and is localised at both the presynapse and the postsynapse (Antar et al., 2005; Feng et al., 1997; Monday et al., 2022). FMRP negatively controls mRNA translation by stalling the elongation step, and it targets over a thousand mRNAs, the majority of which are synaptic, thus making it a major regulator of synaptic function (Darnell et al., 2001, 2011; Glock et al., 2021). Local mRNA translation at the postsynapse by FMRP has been studied extensively, and its regulation of local mRNA translation at the presynapse is also currently coming to light (Hagerman et al., 2017; Monday et al., 2022).

Despite the established role of FMRP as a regulator of local mRNA translation, the mechanism behind the AP broadening phenotype in hippocampal *Fmr1* KO neurons was revealed to derive from the non-canonical roles of FMRP at the presynapse (P.-Y. Deng et al., 2013). The subsequent studies confirmed the interaction between FMRP and the BK channel, the gating of which was controlled via the binding between FMRP and the pore-forming α and auxiliary β_4 subunits (P.-Y. Deng & Klyachko, 2016; P.-Y. Deng et al., 2013, 2011; Kshatri et al., 2020; Myrick, Hashimoto, et al., 2015). Other potassium and calcium channels have been reported to interact with FMRP as well, such as the SK channel, $K_v1.2$, and N-type VGCC (Brown et al., 2010; Ferron et al., 2014; Y.-M. Yang et al., 2020). Further channelopathies driven by the absence of FMRP is reviewed in P.-Y. Deng and Klyachko (2021).

Research on the non-canonical roles of FMRP was boosted by clinical cases which reported translation-independent but pathological point mutations, such as R138Q (Diaz, Scheiner, & Leon, 2018; Quartier et al., 2017). Prieto et al. (2020) showed that the expression level of FMRP in R138Q knock-in mouse model is comparable to that of WT but still phenocopies behavioral deficits in FXS. These case reports emphasise the importance of non-canonical protein-protein interactions of FMRP, which is speculated to be concentrated on the N-terminus of FMRP (Ramos et al., 2006; Taha et al., 2021).

Although it is generally agreed that FMRP regulates ion channels at the presynapse and its deficiency results in AP broadening, how FMRP controls presynaptic function, or SV recycling, is less clear. First, the SV pool is reported to be enlarged in *Fmr1* KO neurons (Bonnycastle et al., 2022; P.-Y. Deng et al., 2011). The activity-dependent Ca^{2+} influx in *Fmr1* KO neurons, however, is more controversial, as some groups observed exaggerated calcium influx in *Fmr1* KO neurons but others did not see a significant difference in the amplitude of evoked Ca^{2+} influx (Bonnycastle et al., 2022; P.-Y. Deng et al., 2011; Ferron et al., 2014). Evoked exocytosis is also contentious. Amplified exocytosis was reported in dorsal root ganglion (DRG), hippocampal and cerebellar neurons under electrical stimulation and hypertonic stimulation previously, but more recent publications did not see any significant difference in the sypHy response during 10 Hz or 40 Hz stimulation (Bonnycastle et al., 2022; P.-Y. Deng et al., 2011; Ferron et al., 2014; Subrahmanyam et al., 2021). Interestingly, Subrahmanyam et al. (2021) presented higher frequency and amplitude in spontaneous EPSC in *Fmr1* KO neurons, although whether this phenotype is developmental was not tested. In terms of endocytosis, Bonnycastle et al. (2022) unveiled reduced ADBE via live and fixed TMR-dextran uptake assays and HRP uptake assay followed by electron microscopy in hippocampal *Fmr1* KO neurons at DIV 13-15. The fact that the overexpression of FMRP with aforementioned missense mutation R138Q did not rescue the ADBE phenotype supports the idea that the presynaptic role of FMRP is more likely to be from non-canonical than canonical mechanisms. Another missense mutation I304N, which shows disrupted polyribosomal binding, rescued the reduced ADBE in *Fmr1* KO neurons, reinforcing this argument (Bonnycastle et al., 2022; Feng et al., 1997).

Based on the developmental trajectory of SV recycling in WT neurons delineated in the previous chapter, it was revealed that the reduced ADBE is a phenotype that *Fmr1* KO neurons share with developing WT neurons. This chapter asks whether the reduced ADBE in *Fmr1* KO neurons is a transient phenotype due to a developmental delay or is a permanent phenotype coming from the mechanistic failure due to the absence of FMRP. This was achieved with time-course analysis using a TMR-dextran uptake assay. Next, I performed a GST pull-down assay with the N-terminus of FMRP to identify potential binding partners that could explain such phenotype.

4.2 Results

4.2.1 *Fmr1* KO neurons display delayed acquisition of ADBE

To test if the reduced ADBE is a transient phenotype in *Fmr1* KO neurons, we performed a time-course TMR-dextran uptake assay at DIV 7, 10, 14 and 21 in KO and wild-type littermate controls (figure 4.1A and 4.1B). The number of TMR-dextran puncta in WT neurons recapitulated the trajectory from the previous chapter, starting from around 50 puncta at DIV 7 and 10, followed by a sharp increase at the second week *in vitro* that plateaus afterwards (figure 4.1B, figure 3.4). Secondly, *Fmr1* KO neurons showed a similar pattern to that of WT, the number of puncta remaining double-digits in the first week, but increasing substantially in the second week *in vitro* and plateauing at DIV 21 (figure 4.1B). This is comparable to the WT in that the time-course data show a step-like graph, but the extent of the increase was smaller in *Fmr1* KO.

When looking at the individual timepoints, there was a significant difference in the number of TMR-dextran puncta between WT and *Fmr1* KO neurons at DIV 14 (figure 4.1C, left). This reproduced the previous finding in Bonnycastle et al. (2022), which reported about a 25 % decrease in the number of internalised TMR-dextran puncta. The number of nerve terminals, which is represented by the number of α -SV2A-positive puncta (figure 4.1C, right), was comparable as it was previously reported when detected for SV2A, vesicular glutamate transporter (vGluT) and vesicular GABA transporter (vGAT) (Bonnycastle et al., 2022).

However, there was no significant difference a week later in culture, at DIV 21 (figure 4.1D, left). When stained for SV2A, the number of puncta were higher in general compared to DIV 14, but the values between the two genotypes were not significantly different (figure 4.1D, right). In conclusion, it is probable that the *Fmr1* KO neurons are capable of ADBE, but it develops slower than the WT, despite similar number of nerve terminals.

Meanwhile, the individual data points in figures 4.1C and 4.1D seem to be clustered in two groups. Suspecting there might be a bimodality in TMR-dextran uptake, a Warren Sarle's bimodality coefficient test was run on Matlab, and it signified that the data was unimodal (DIV 14 —WT: 0.89, KO: 0.9; DIV 21 —WT: 0.89, KO: 0.83) (> 0.55 is regarded as unimodal). The data was re-analysed per animals rather than per coverslips to see if the difference in the timepoint of ADBE recruitment was animal-dependent, but it did not show any animal-dependency. In short, *Fmr1* KO neurons acquire ADBE eventually, but in a delayed manner through an unknown mechanism. An age-dependent phenotype is not an unseen phenomenon, however, but rather a recurrent theme in recent studies in NDD research, which will be discussed more in-depth later.

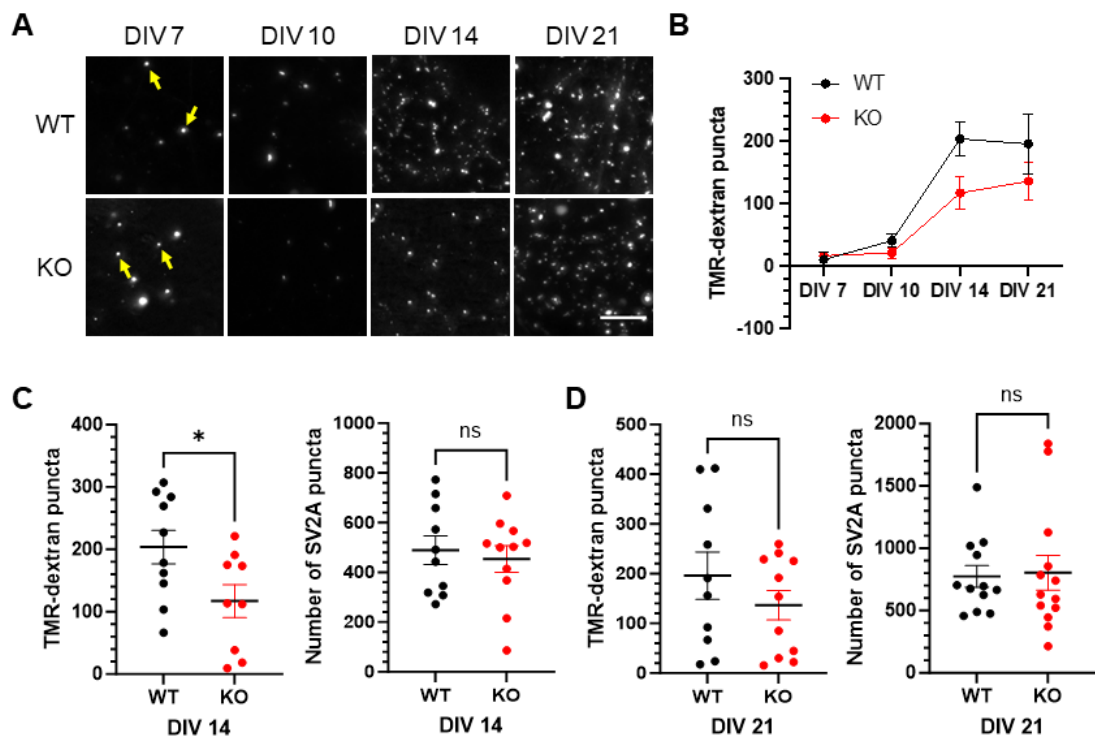


Figure 4.1: Developmental trajectory of *Fmr1* KO neurons compared to WT.

- A**, Representative images of primary cultures of *Fmr1* KO hippocampal neurons and their wild-type (WT) littermate controls challenged with an action potential train of 400 APs (40 Hz) in the presence of 50 μ M tetramethyl-rhodamine (TMR)-dextran at days *in vitro* (DIV) 7, 10, 14 and DIV 21. Scale bar: 10 μ m. Arrows indicate example puncta included in the analysis.
- B**, Mean number of TMR-dextran puncta of WT and *Fmr1* KO hippocampal neurons $\pm$ SEM (WT: n = 11, 11, 16 and 15 for DIV 7, 10, 14 and 21 respectively. KO: 12, 14, 16 and 15 for DIV 7, 10, 14 and 21, respectively).
- C, Left**, Mean number of TMR-dextran puncta at DIV 14 $\pm$ SEM (WT n = 10, KO n = 9). Unpaired Student's *t* test ($p = 0.0348$). **Right**, Mean number of α -SV2A-positive puncta at DIV 14 $\pm$ SEM (WT n = 10, KO n = 11). Unpaired Student's *t* test ($p = 0.6536$). The coverslips used for the TMR-dextran uptake assay and immunocytochemistry are from the same neuronal preparations.
- D, Left**, Mean number of TMR-dextran puncta at DIV 21 $\pm$ SEM (WT n = 10, KO n = 11). Mann-Whitney test ($p = 0.2920$). **Right**, Mean number of α -SV2A-positive puncta on DIV 21 $\pm$ SEM. (WT n = 12, KO n = 13). Mann Whitney's test ($p = 0.7689$). The coverslips used for TMR-dextran uptake assay and immunocytochemistry are from the same neuronal preparations. In all cases n = independent coverslips.

4.2.2 Potential binding partners of FMRP N-terminus at the presynapse

Although it appears that the depression of ADBE was developmental, it is also possible that there is a mechanistic requirement for FMRP in ADBE and its deficiency is compensated later in development. Because a few studies have shown that the presynaptic phenotypes are translation-independent by using truncated FMRP, FMRP with missense mutations that disrupt polyribosomal binding, or drugs that block mRNA translation, it was hypothesised that FMRP controls SV recycling via protein-protein interaction rather than translational regulation (Bonnycastle et al., 2022; P.-Y. Deng et al., 2019, 2013). To test this hypothesis, I decided to investigate the potential interaction partners of FMRP.

The current understanding of FMRP focuses on the C-terminus, where it interacts with mRNAs. However, there is little evidence that indicates the participation of its C-terminus in presynaptic activity. One study showed the activity-dependent local translation of actin at the presynapse, but it is not sufficient to explain the functional phenotypes in *Fmr1* KO neurons (Monday et al., 2022). This turns our eyes to the N-terminus of FMRP, which contains two Tudor domains (D'Annessa et al., 2019; Eberhart et al., 1996; Ramos et al., 2006). Previous studies identified that the BK channel is regulated by the N-terminus of FMRP, which is responsible for AP broadening in *Fmr1* KO neurons (Bonnycastle et al., 2022; P.-Y. Deng & Klyachko, 2016; P.-Y. Deng et al., 2013; Kshatri et al., 2020; Myrick, Hashimoto, et al., 2015).

Recently, a couple of studies performed proteomics analysis with the N-terminus of FMRP, and revealed its vast interactome including transcription and translation machinery as well as stress response proteins (He & Ge, 2017; Kieffer et al., 2022; Taha et al., 2021). However, these proteomics studies pulled proteins either from non-neuronal cell lysates (i.e., HEK293) or the nuclear fraction of the postnatal rat forebrains, limiting its interpretation in presynaptic phenotype observed in functional studies. Therefore, we decided to perform a GST pull-down assay with mass spectrometry from synaptosomes prepared from 2 month-old rat cerebra, to explore the potential binding partners of FMRP N-terminus (1-297 a.a) in a synaptic context.

For this end, GST-tagged FMRP N-terminus (WT or R138Q) was used as a bait to pull proteins down from synaptosomes to identify potential interacting partners. The GST empty vector was used as a negative control. R138Q was included in order to test if the identified candidates share the interaction site with the BK channel. During the experiment, eluted samples containing the extracted proteins were separated on a SDS-PAGE gel. There was no visible difference in terms of bands in the Coomassie-stained SDS-PAGE gel, therefore a few bands were randomly selected and excised for mass spectrometry as shown in the picture figure 4.2. Liquid chromatography - mass spectrometry was performed by Dr Dominic Kurian from Roslin Institute, University of Edinburgh.

After screening the mass spectrometry results based on the molecular weight, the most enriched proteins are shortlisted as the tables 4.1 and 4.2, with their known presynaptic function and their known interaction with FMRP. Mitochondrial proteins were excluded from the lists.

First of all, there were very few proteins that were previously known to bind to FMRP (tables 4.1 and 4.2). Among the proteins pulled down with WT FMRP N-terminus, Ras-GTPase-activating protein (GAP)- binding protein 1 (G3BP1) is a binding partner to FMRP reported previously (Taha et al., 2021). ARF-GTPase activating protein (GIT1), a protein that mediates intracellular trafficking and regulates SV exocytosis, was another hit that is indirectly linked to FMRP via GIT1-Arf6 actin polymerisation pathway (Briševac et al., 2021; Hoefen & Berk, 2006; Vitale et al., 2002). Other SV recycling- related proteins that have been identified in mass spectrometry but were not previously associated with FMRP are Rab3A and Dyn-1. Each protein will be addressed in detail in the discussion.

One interesting find from this experiment was Dyn-1, a GTPase that constricts and fissions individual SVs off the plasma membrane or bulk endosomes during SV endocytosis (Cheung & Cousin, 2019). Disruption in its activity results in epileptic encephalopathy, and the primary neurons expressing pathogenic Dyn-1 mutations show slower endocytosis (Bonnycastle et al., 2023). There has not been any report showing protein-protein interaction between FMRP and Dyn-1 so far, and there is a chance that R138Q has altered binding affinity to Dyn-1. However, because the bands from the SDS-PAGE gel were not excised uniformly, it was not possible to make quantitative comparison between the two genotypes. Therefore, we have proceeded with Western blot to confirm the binding between Dyn-1 and WT or R138Q FMRP N-terminus.

4.2.3 Dynamin-1 binds to FMRP N-terminus

To test the binding between FMRP N-terminus and Dyn-1, we again pulled down synaptosomal proteins using FMRP N-terminus WT and R138Q as baits, in the same way as we prepared samples for mass spectrometry.

As a preliminary experiment, we probed the pull-down samples for the BK channel (figure 4.3B). This is because the BK channel is already known to bind with FMRP, therefore making it a good substrate to validate the pull down experiment (P.-Y. Deng et al., 2013). We could identify signals in WT FMRP N-terminus. However, although the missense mutation R138Q is known to disrupt the binding between FMRP and the BK channel, there was no clear difference in the signal intensity between the two FMRP genotypes. More repeats followed by a quantitative analysis would be necessary to tell if the binding affinity between BK channel and R138Q FMRP N-terminus is altered. If not, the next question would be whether R138Q FMRP N-terminus has altered functionality in controlling the gating properties of the BK channel.

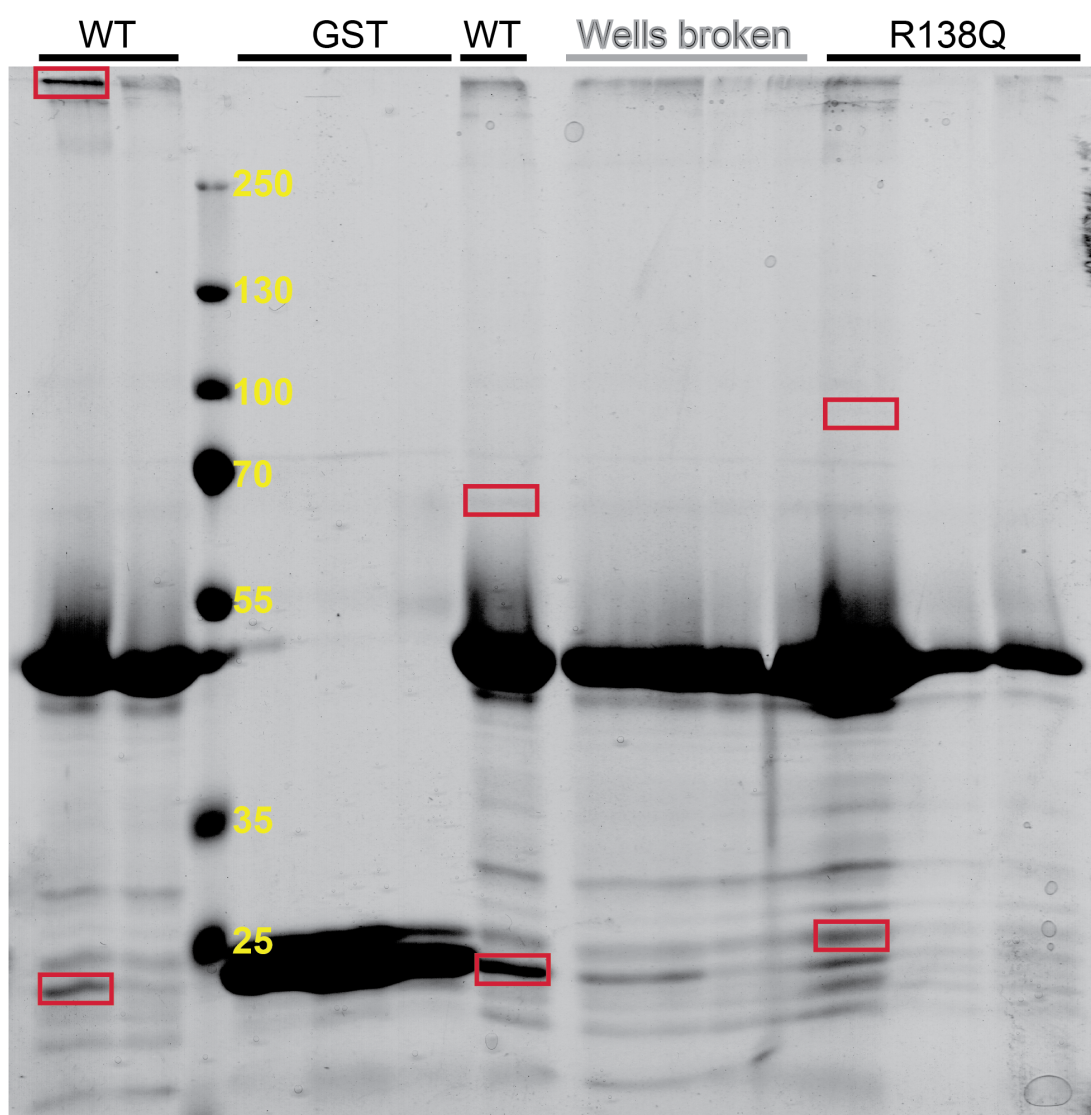


Figure 4.2: A picture of the SDS-PAGE gel separating pull-down eluates for mass spectrometry.

Proteins from synaptosomes were pulled using FMRP N-terminus, and the eluates were separated on a SDS-PAGE gel. The control group (GST) used an empty GST-tag, and the experiment groups used FMRP N-terminus WT (WT) or FMRP N-terminus mutant (R138Q).

Red boxes indicate the bands that were excised and processed for liquid chromatography-mass spectrometry. Unit for the ladder is kDa.

Protein	Presynaptic function	Interaction with FMRP
Na ⁺ /K ⁺ transporting ATPase subunits (α_2 , α_3)	Maintains resting membrane potential on the plasma membrane of a neuron, including axons (Young et al., 2008)	Unknown
G3BP stress granule assembly factor 1 (G3BP1)	Orchestrates stress granule assembly, which are also observed in axon (Sahoo et al., 2018)	Binds to N-terminus of FMRP (Taha et al., 2021)
Dihydropyrimidinase-related protein 2 (DRP2/CRMP2)	Mediates axon guidance via cytoskeletal reorganisation (Arimura & Kaibuchi, 2007)	Unknown
Synaptosomal-associated protein 25 (SNAP-25)	Comprises SNARE complex and regulates SV exocytosis (Lou & Shin, 2016)	FMRP is reported to regulate the translation of SNAP-25 but the protein-protein interaction is not known (Darnell et al., 2011)
Cysteine-rich protein 2 (CRIP2)	A transcription factor that regulates cell cycle, but its function at the presynapse is unknown	Unknown
Triosephosphate isomerase (TPI)	Participates in glycolysis, unknown at the presynapse	Unknown
Myeline basic protein	Promotes myelin membrane stacking (Raasakka et al., 2017), but no known function specifically at the presynapse	Unknown
CCHC- type zinc finger nucleic acid binding protein (CNBP)	Regulates transcription and translation but no known function at the presynapse	Unknown

Table 4.1: List of enriched proteins pulled with N-terminus of FMRP WT according to MS analysis, with their known presynaptic function and their interaction with FMRP.

When the pull-down samples were stained for GST-tag and Dyn-1, it showed a clear binding between Dyn-1 and both WT and R138Q FMRP N-terminus, confirming the mass spectrometry results (figure 4.3C). There could be a quantitative difference between WT and R138Q, but it would require more repeats for a valid quantitative analysis. How the binding between FMRP and Dyn-1 could affect presynaptic functions will be discussed later.

Meanwhile, it is known that the function of Dyn-1 in SV endocytosis is regulated by phosphorylation and dephosphorylation (Armbruster, Messa, Ferguson, De Camilli, & Ryan, 2013; Clayton et al., 2008; Imoto et al., 2022). Specifically, its phosphorylation by GSK3 on the serine residue 774 (S774) regulates ADBE (Clayton et al., 2010). Therefore, it was of interest to know

Protein	Presynaptic function	Interaction with FMRP
ARF-GTPase activating protein (GIT1)	Controls Arf6, which regulates SV recycling pathway between endosomes and individual SVs (Hoefen & Berk, 2006; Tagliatti, Fadda, Falace, Benfenati, & Fassio, 2016)	No report on the interaction between GIT1 and FMRP, but Arf6 has been shown to be dysregulated in <i>Fmr1</i> KO neurons (Briševac et al., 2021)
Dynamin-1	Fissures invaginated endosomes or SVs from the plasma membrane (Cheung & Cousin, 2019)	The mRNA that encodes Dyn-1 has been identified as target mRNA of FMRP, but no protein-protein interaction identified (Darnell et al., 2011)
14-3-3 protein ϵ	Regulates trafficking between endosomal reticulum and plasma membrane no direct evidence at the presynapse (Broadie, Rushton, Skoulakis, & Davis, 1997; Shikano, Coblitz, Wu, & Li, 2006)	Unknown
Rab3A	SV exocytosis (Geppert, Bolshakov, et al., 1994; Schlüter, Schmitz, Jahn, Rosenmund, & Südhof, 2004), and lipid raft endocytosis (Diaz-Rohrer et al., 2023)	Unknown

Table 4.2: List of enriched proteins pulled with N-terminus of FMRP R138Q according to MS analysis, with their known presynaptic function and their interaction with FMRP.

whether this binding with FMRP N-terminus was S774-phosphorylation-dependent. Despite the abundance of S774-phosphorylated Dyn-1 in the synaptosomal lysate (figure 4.3A), there was no phospho- S774- Dynamin-1 detected in the pull-down samples (figure 4.3D). This suggest that FMRP is binding exclusively to Dyn-1 dephosphorylated at S774.

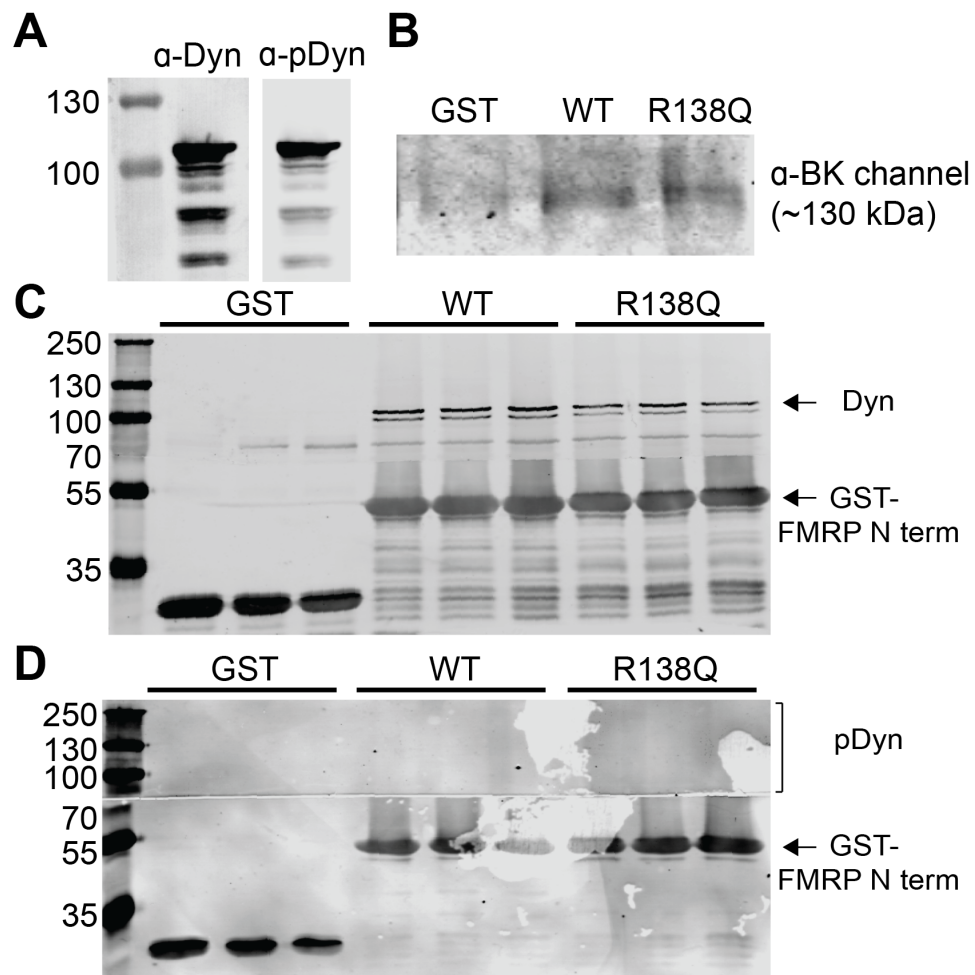


Figure 4.3: GST-pull down assay and Western blot confirms binding between FMRP N-terminus and Dynamin-1, but not phospho- S774- Dynamin-1.

A, Western blot of synaptosomal lysates immunostained for Dynamin-1 (Dyn-1) and phospho- S774- Dyn-1 (pDyn).

B, Proteins pulled-down with an empty GST-tag (GST), FMRP N-terminus WT and R138Q were immunostained for the BK channel.

C, Western blot of synaptosomes pulled down with GST conjugated with FMRP N-terminus WT and R138Q. The top half of the membrane was immunostained for Dyn-1, and the bottom half of the membrane was immunostained for GST-tag. *The GST tag (25 kDa) conjugated with FMRP N-terminus weighs approximately 50 kDa.

D, Same samples as panel C immunostained for pDyn (top) and GST-tag (bottom). In all cases ladder unit is kDa.

4.3 Discussion

In this chapter, we broadened our understanding of SV recycling in *Fmr1* KO neurons, and sought potential explanations for our findings. In the first section, via a time-course TMR-dextran uptake assay, we uncovered the delay in the presynaptic development in *Fmr1* KO neurons. In the second and the third section, via a mass spectrometry and Western blot, we identified potential binding partners of FMRP N-terminus, focusing on Dyn-1. These biochemistry experiments would be useful in designing future experiments to further dissect the mechanism behind the developmental delay.

4.3.1 The delayed recruitment of ADBE in *Fmr1* KO neurons

The TMR-dextran uptake assay in the first section indicated that the recruitment of ADBE is delayed in *Fmr1* KO neurons, rather than as a permanent defect (figure 4.1B). Even though neurons of both genotypes displayed a step-like trend in the number of internalised TMR-dextran puncta upon stimulation, *Fmr1* KO neurons showed significantly reduced TMR-dextran uptake at DIV 14. Strikingly, the difference between the two genotypes were not significant at DIV 21. This was independent of the number of nerve terminals (figure 4.1C and 4.1D, figure 4.4). However, the number of SV2A puncta at DIV 21 in both genotypes was overall larger than DIV 14, leaving the possibility of *Fmr1* KO neurons compensating for the deficient ADBE with synaptogenesis. It should be noted that the selection of field of view was not based on an objective criteria. Therefore, for a more thorough quantification of the number of nerve terminals at DIV 21, it would be useful to measure ADBE and the number of nerve terminals simultaneously via fixable fluorescein- dextran.

The results in this chapter should be interpreted in relation to Bonnycastle et al. (2022). This publication showed a successful rescue of reduced ADBE with FMRP I304N, the mutation that disrupts polyribosomal binding, but not with R138Q. This indicates that the interaction between FMRP and the BK channel is required for ADBE. However, experiments using a BK channel agonist and antagonist gave ambiguous results, because the BK channel agonist corrected the phenotype in *Fmr1* KO neurons, but when WT neurons were treated with BK channel antagonists, it did not phenocopy that of *Fmr1* KO (Bonnycastle et al., 2022). To investigate if the delay is due to the interaction between FMRP and the BK channel, it would be of prime interest to test the activity of the BK channel also at DIV 21. One indirect indicator of this would be the AP width, as reported in P.-Y. Deng et al. (2013). Electrophysiological assays in cultured neurons at the same timepoint DIV 14 and 21 would tell us if the AP broadening phenotype disappears after 3 weeks *in vitro*. Depending on whether the AP is broadened at DIV 21, the next step would be to study the other regulators of the BK channel that might have compensated for the loss of FMRP, such as Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) or VGCCs (Z.-W. Wang, 2008).

It is also possible that this delay is due to the RNA-binding properties of FMRP. Not only does FMRP inhibit translation at the presynapse, it also guides the localisation of mRNAs to pre- and postsynapse, although its selectivity and directionality is not yet fully understood (Antar et al., 2005; Jung et al., 2023; Monday et al., 2022; P. Zhang et al., 2015). There is a possibility that the rescue of the depressed ADBE by mutated FMRP (I304N) in *Fmr1* KO neurons is because of its binding and transportation of mRNA rather than the translation itself. Indeed, FMRP with the mutation I304N displays comparable binding to poly(A)-RNA as well as normal localisation, despite some contradicting observations in the I304N mouse line (Castrén, Haapasalo, Oostra, & Castrén, 2001; Feng et al., 1997; Schrier et al., 2004; J. B. Zang et al., 2009). If we suppose that FMRP drives faster delivery of ADBE machinery, the delay in ADBE recruitment can be explained by the delay of delivery. Unfortunately, there is very scarce literature on the axonal FMRP and its mRNA transportation for now.

Last but not least, Pfeiffer and Huber (2007) has suggested that FMRP promotes synaptic pruning in culture, and it is possible that ADBE is defective in a population of nerve terminals because immature synapses were not eliminated yet. This theory aligns with results in the previous chapter where we showed that younger WT neurons (DIV 3-10) do not, or at least minimally, perform ADBE (figure 3.4). However, this contradicts with the SV2A immunocytochemistry data, which did not show significant difference between genotypes at DIV 14 and 21. This requires a more thorough investigation when quantifying the number of nerve terminals.

4.3.2 The interaction partners of FMRP

In the second and third section of this chapter, we have identified the potential binding partners of FMRP N-terminus using GST pull-down assay. We took advantage of mass spectrometry to make a list of candidate substrates and performed Western blot which confirmed the binding between FMRP N-terminus and Dyn-1. The mass spectrometry also confirmed the binding between FMRP and G3BP1, which was reported previously and whose function was associated with FXS (Aulas et al., 2015; A. Lai, Valdez-Sinon, & Bassell, 2020; Taha et al., 2021; P. Yang et al., 2020). In addition, the mass-spectrometry proposed GIT1 and Rab3a. There is sufficient evidence to hypothesise that the interaction between FMRP and GIT1 affect presynaptic function, because GIT1 is localised at the presynapse, and the effector of GIT1, Arf6 is dysregulated in *Fmr1* KO neurons (Briševac et al., 2021; S. Kim et al., 2003; Meyer et al., 2006; H. Zhang, Webb, Asmussen, & Horwitz, 2003). Arf6 is a small GTPase that induces actin assembly and it was shown hyperactive in *Fmr1* KO neurons (Briševac et al., 2021). Most importantly, Meyer et al. (2006) showed an inverse correlation between exocytosis and the GIT1 expression in chromaffin cells, hinting that GIT1 negatively regulates exocytosis. Rab3a is a small GTPase localised on the SV membrane, whose knockout presented mixed phenotypes at the presynapse (Geppert, Bolshakov, et al., 1994; Geppert, Goda, Stevens,

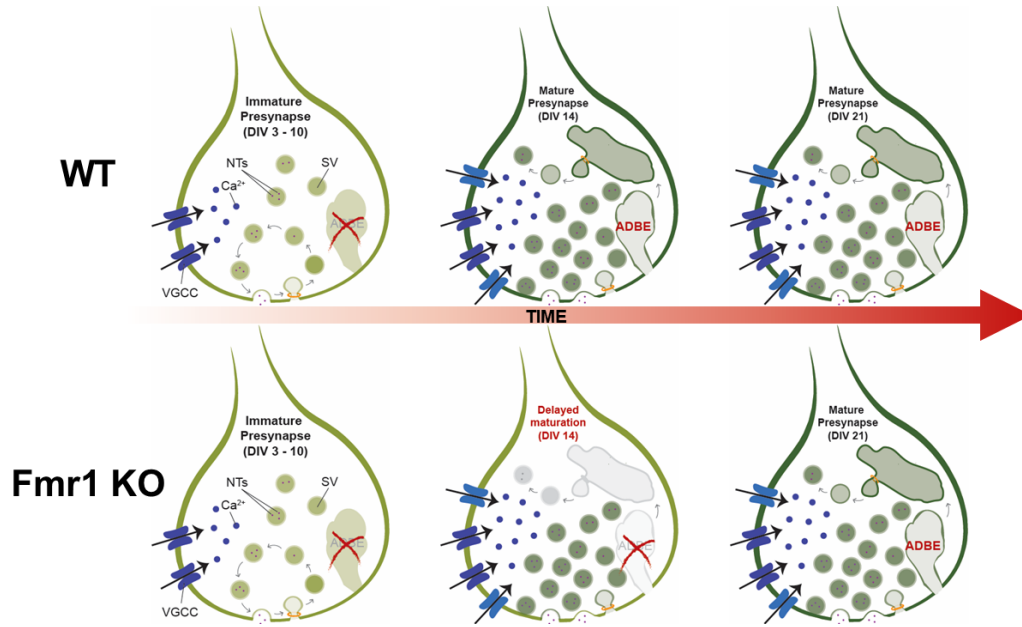


Figure 4.4: *Fmr1* KO neurons show delayed recruitment of ADBE.

The schematic figure summarises the developmental delay of ADBE recruitment in *Fmr1* KO neurons, which is deficient at DIV 14 but comparable to WT at DIV 21 in hippocampal neurons from primary cultures. The figure also collates the findings from the previous chapter, which demonstrated the developmental trajectory of SV recycling in WT regarding the activity-dependent Ca^{2+} influx and SV recycling. Bonnycastle et al. (2022) showed that *Fmr1* KO neurons do not exhibit reduced or exaggerated activity-dependent Ca^{2+} influx at DIV 13-15.

& Südhof, 1997; Johnston et al., 1991; Schlüter et al., 2004). In a different angle, Rab3 is recognised as an important sorting molecule for lipid rafts (sterols and sphingolipids) from endosomes to plasma membrane (Diaz-Rohrer et al., 2023). Thus, it is possible that Rab3 takes part in the SV recycling process indirectly, rather than directly regulating the SVs (Diaz-Rohrer et al., 2023). Nevertheless, the major limitation of this mass spectrometry approach is that it is not quantitative. A more thorough comparison would be required to discover the differential binding between FMRP N-terminus of WT and R138Q.

Western blot, on the other hand, confirmed the binding between Dyn-1 and GST-tagged FMRP N-terminus of both WT and R138Q. This is the first report on the protein-protein binding between Dyn-1 and FMRP N-terminus, except for the FMRP's targeting of mRNA that encodes Dyn-1 (Darnell et al., 2011). Additional blots showed that Dyn-1 does not bind to FMRP N-terminus when its S774 is phosphorylated. The binding between FMRP N-terminus and Dyn-1 is an interesting finding because both *Fmr1* KO neurons and *Dnm1*^{+/237W} neurons show reduced ADBE and broadened AP (Bonnycastle et al., 2023; P.-Y. Deng et al., 2013).

Furthermore, the BK channel has also been reported to bind with Dyn-1, potentially to control the channel density through endocytosis (Gorini et al., 2010). Therefore, it is possible that the three proteins, FMRP, the BK channel and Dyn-1 form a tripartite complex that controls SV recycling.

The first step to go down this avenue would be to repeat GST pull-down assay and Western blots with isolated N-terminus domains or with different point mutations in order to narrow down the binding site. A TMR-dextran uptake assay of *Fmr1* KO neurons transfected with various mutated FMRP N-termini would tell us which domain mediates ADBE, and if its binding with Dyn-1 affects ADBE. Last but not least, colocalisation of Dyn-1 and BK channel in *Fmr1* KO and WT neurons using super-resolution microscopy could be a pilot study to test whether these three proteins localise with each other and function as a tripartite.

4.3.3 Transient phenotypes in FXS

A NDD can have a varying degree of symptoms throughout one's lifetime. Likewise, there are multiple reports that demonstrate more severe symptoms in older patients with FXS than the younger group, and so is the case in animal FXS models (Martin, Lassalle, Brown, & Manzoni, 2016; Schneider, Ligsay, & Hagerman, 2013). There is also a growing list of literature on the transient phenotypes, the ones that are more pronounced in the younger age and are fully or partially recovered in the older age. These phenotypes are especially interesting, not only because they present us the plasticity of a developing brain, but also hint at a window of opportunity for therapeutical intervention.

On the molecular level, Tang et al. (2015) has shown that the presynaptic proteins such as Synapsin, SNAP-25, CaMKII α and postsynaptic scaffolding protein Shank are upregulated in whole brain synaptosomes at P17, but the expression level becomes comparable to the WT level at P45. However, this proteomics study is not brain-region specific, and did not associate their findings with any other circuitry or behavioral development as an attempt to explain this phenomenon. In a similar time frame, S. Yang, Yang, Park, Kirkwood, and Bao (2014) showed a decreased LTP at P16-20 in *Fmr1* KO neurons in auditory cortex, which is rescued to the WT level at P27-31. However, the hearing onset in rodents usually is at the end of their second postnatal week, in parallel to the onset of whisking (a high-frequency and repeated movement of whiskers) and eye opening (Fagiolini, Pizzorusso, Berardi, Domenici, & Maffei, 1994; Grant, Mitchinson, & Prescott, 2012; Kral & Pallas, 2010). The suggested time window in S. Yang et al. (2014) between the third and the fourth postnatal week does not coincide with the initial development of sensory onset.

Other publications on transient phenotypes employ a more earlier time frame. Harlow et al. (2010) presented an upregulated expression of NMDA receptor at P4-7 in *Fmr1* KO neurons in the barrel cortex, which was not significantly different from P14. The increased expression of NMDA receptor was associated with the increased LTP at the same age. In WT neurons, LTP

wanes after birth, but LTP starts decreasing only after the first postnatal week, suggesting a developmental delay in circuit development. The barrel cortex is a somatosensory cortex that receives sensory information from whiskers, and the results in Harlow et al. (2010) implicates that the molecular and circuit phenotypes that existed before the onset of whisking disappeared in parallel to when animals begin whisking (Grant et al., 2012; Staiger & Petersen, 2021). However, in the hippocampus, the opposite results were reported in a slightly later time frame. The AMPA receptor (AMPA) / NMDA receptor (NMDAR) ratio was higher in *Fmr1* KO animals younger than 2 weeks (Banke & Barria, 2020). In addition, the younger animals in this study showed lack of LTP and had bigger amplitude of mEPSC, which were all non-significant when older. The authors suggested an AMPA receptor subunit, GluA2 as the explanation for their findings, but there was no biochemical assay performed to support the argument. Live imaging of dendrites in the barrel cortex *in vivo* of *Fmr1* KO mice showed postsynapses with faster turnover rates and shorter lifetime at P10-12, whose difference was not significant at an older age, confirming the immature postsynaptic structure reported previously and adding a time dimension to our understanding of it (Cruz-Martín, Crespo, & Portera-Cailliau, 2010; Grossman et al., 2006). At the circuit level, another study has revealed that the firing of neurons is desynchronised with age in WT animals, but neurons show more synchronised activity in *Fmr1* KO animals in the barrel cortex (Gonçalves, Anstey, Golshani, & Portera-Cailliau, 2013). This phenotype was the most pronounced at P14-16 and was mitigated afterwards.

Not all transient phenotypes come from a developmental delay, however. Y. J. Song, Xing, Barbour, Zhou, and Jensen (2022) showed accelerated development of inhibitory circuit in auditory cortex at P12-13 in *Fmr1* KO mice. The electrophysiological studies at a later age demonstrated that the circuit composition in WT and KO animals become comparable in a month's time. The authors have attributed this transient circuit defect to the altered expression of GABA receptor subunits α_3 , although how it affects electrophysiological properties were unclear.

The majority of the reported phenotypes in FXS models are permanent, or reported from adult animals and are considered to be hard-wired. However, the intermittently reported transient phenotypes encourage us to look at the developmental aspect of FXS. The next big quest for the field would be to find out how the delay or acceleration in synaptic and circuit development affect cognitive and behavioral function, as FXS is a life-long condition. In parallel, understanding the molecular mechanism behind the shift would be imperative in tackling the disorder.

Of course, the translatability from culture to slices is limited. The sensory input, especially, is not reproduced in culture environment. However, increased spontaneous activity at the presynapse in *Fmr1* KO neurons have been reported both at P12-14 in slices and DIV 13-15 in culture (Y. J. Song et al., 2022; Subrahmanyam et al., 2021). Plus, the fact that the delay can

also be observed *in vitro* opens up more possibility for more detailed research in a time-course that is limited *ex vivo* or *in vivo* (figure 4.1B). For example, whether the delayed maturation of the presynapse (i.e., lack of ADBE and enhanced spontaneous release at DIV 14) and the delayed maturation of the postsynapse (i.e., filopodial dendritic spines and faster turnover rate) are related would be an interesting question in an attempt to explain the developmental phenotypes in the circuit level previously reported.

4.3.4 Critical window in neuronal development

Transient phenotypes seen in FXS is not a novel phenomenon. It resonates in other NDD models such as 22q11.2 deletion syndrome, SYNGAP1-related disorders, and FOXP1 syndrome (Amin, Marinaro, De Pietri Tonelli, & Berdondini, 2017; Clement, Ozkan, Aceti, Miller, & Rumbaugh, 2013; Miyoshi et al., 2021). In Amin et al. (2017), the GABA switch is delayed in culture from DIV 8 in WT to the second week *in vitro* in hippocampal neurons with micro-deletions that model 22q11.2 syndrome. The GABA switch refers to a developmental process when GABA signalling turns from excitatory to inhibitory, due to the change in the expression of chloride co-transporters, from Na–K–Cl cotransporter 1 (NKCC1), which exports chloride to K–Cl cotransporter 2 (KCC2), which imports chloride (Peerboom & Wierenga, 2021). Such a delay was also reported in a FXS model (He et al., 2014). In the medial prefrontal cortex (mPFC) of *Syngap1* heterozygous mice, the postsynapses display accelerated incorporation of a NMDA receptor GluN2A, hence the postsynapses show premature properties at P4-5 (Clement et al., 2013). Miyoshi et al. (2021) has identified the first postnatal week as the critical window, as manipulating *FoxG1* (another 1S SFARI gene) expression using doxycycline at a later timepoint showed lesser impact on behavioral and electrophysiological phenotypes compared to the manipulation in the first postnatal week (Miyoshi et al., 2021).

The age-dependent phenotypes in NDD models highlight the physiological importance of a critical window. The term critical window, or sensitive period, first appeared in developmental psychology to signify a period when an extrinsic or intrinsic manipulation could alter the course of development in an organism (Michel & Tyler, 2005). The term was initially used in cognitive and behavioral perspectives, and soon it was attributed to circuit and synaptic level as our understanding of developmental neuroscience advanced. In NDD studies, the concept of a critical window is being emphasised as we understand that such conditions are more than mere failures to reach certain developmental stages, but a misalignment of developmental sequences (Meredith, Dawitz, & Kramvis, 2012).

We still lack integrative understanding of the brain development. Nonetheless, from aforementioned studies, it is possible to delineate that the neural network, in general, undergoes dynamic changes and starts to stabilise past the second week after birth in rodents (Cruz-Martín et al., 2010; Gonçalves et al., 2013; Harlow et al., 2010; He et al., 2014). This is coincidental to the onset of sensory inputs, such as hearing, visual and tactile, but the causal link is insufficient between the sensory inputs and the circuit development (Fagiolini et al., 1994; Grant et al., 2012; Kral & Pallas, 2010).

It is possible that the critical window at the circuit level is reduced to the critical window in neuronal, even to molecular, development. For example, FMRP has been reported to have different roles in synaptogenesis depending on when it is expressed in slice cultures (T. Zang et al., 2013). Metabotropic GluR-dependent LTD, which is dysregulated in *Fmr1* KO neurons, was revealed to be independent of translation at P8-15, but not at P21-35, accentuating a translation-independent role of FMRP at a younger age (Nosyreva & Huber, 2005). The fact that SV recycling is indispensable in establishing a neural network, and the fact that the disruption of SV recycling results in NDDs that entail circuit deficits, make SV recycling a potential link between a critical window in the neuronal level and a critical window in the circuit level (Bonnycastle et al., 2021). How ADBE builds, strengthens or weakens synaptic connectivity, and hence the circuit, would be the next step to test this theory.

Disrupted SV fusion and ADBE in *Dyrk1a* HET neurons

5.1 Introduction

DYRK1A is a serine/threonine kinase that is encoded by the gene *DYRK1A* located within the Down syndrome critical region in *Homo sapiens* chromosome 21 (HSA21) (Courraud et al., 2021). Its haploinsufficiency due to loss of function leads to DYRK1A syndrome, which shows high comorbidity with ASD, ID and epilepsy (Courraud et al., 2021; Ji et al., 2015; The Deciphering Developmental Disorders Study, 2015; van Bon et al., 2021). Its overexpression due to full or partial trisomy is linked to Down syndrome, as the genetic locus suggests, implicating the dosage-dependence of DYRK1A in neurodevelopment (Altafaj et al., 2001; Martinez de Lagran et al., 2012; Murphy, Wilton, Aung-Htut, & McIntosh, 2024; Souchet et al., 2014). In line with its importance in neurodevelopment, DYRK1A expression level peaks between E16 and P7 in the neocortex in rodents, when neuronal proliferation and differentiation takes place at a maximum speed (Bockhorst et al., 2008; Dang et al., 2018; Mody et al., 2001).

The majority of the DYRK1A substrates are cell cycle regulators, such as cyclins, which encouraged the exploration of DYRK1A in the context of cancer (J. Park et al., 2010; Soppa et al., 2014; E. J. Yang et al., 2001). However, DYRK1A also colocalises with Synaptophysin, a presynaptic marker (Adayev et al., 2006). Furthermore, numerous SV recycling proteins have also been identified to be subjects of DYRK1A phosphorylation, including Dyn-1 and Synaptojanin-1 (Adayev et al., 2006; Chen-Hwang et al., 2002; Huang et al., 2004; E. J. Kim et al., 2006; Murakami et al., 2012, 2006). Although its phosphorylation is not confirmed in neurons, these findings lead us to speculate that DYRK1A regulates SV recycling at the presynapse.

On the functional level, the literature draws a perplexing image of what DYRK1A does at the presynapse. Trisomic hiPSC-derived neurons showed increased fusion of SVs when depolarised using KCl perfusion, and primary hippocampal neurons from trisomic mice (TgDyrk1a) showed slower endocytosis (Y. Kim, Park, Song, & Chang, 2011; C.-I. Wu et al., 2022). These

findings are refuted by other reports, however, where primary neurons from trisomic rodents (Ts65Dn) did not show any defect in SV recycling (Marland et al., 2016; C.-I. Wu et al., 2022). A conditional knockout (cKO) of *Dyrk1a* in mice showed downregulation of exocytic components according to RNA-seq and gene ontology analysis, but whether the cKO affected presynaptic neurotransmission was not tested (Brault et al., 2021). In *Drosophila*, there is a study which tested the relationship between Minibrain, a homologue of DYRK1A, and SV recycling. Peng et al. (2021) demonstrated that the deficiency of *minibrain* does not impact exocytosis but does abate ADBE. Manipulating the Ca^{2+} concentration and using a clathrin inhibitor chlorpromazine, the authors showed that Minibrain phosphorylates Synaptojanin on S1029 residue, which inhibits ADBE and promotes CME (Peng et al., 2021). However, the approach the authors used to distinguish different processes of SV recycling is ambiguous, and whether this finding translates to mammalian neurons is unknown.

In order to study whether SV recycling is affected in DYRK1A syndrome, we characterised SV recycling in primary neurons of hippocampal cultures from *Dyrk1a*^{+/-} (HET) rats and their littermate controls (WT). Using similar techniques used for WT neurons in chapter 3, we revealed that *Dyrk1a* haploinsufficiency leads to downregulation of ADBE and reduced evoked exocytosis, due to less mobile or less fusion-competent SV pools.

5.2 Results

5.2.1 *Dyrk1a* HET shows reduced ADBE

As a starting point to characterise SV recycling in *Dyrk1a* HET neurons, a TMR-dextran uptake assay was performed based on the contradicting reports that when *Minibrain* is mutated to lose its catalytic activity, ADBE is exaggerated in *Drosophila* neuromuscular junction (Peng et al., 2021). In addition, different NDD models show reduced ADBE, including *Fmr1* KO neurons as shown in the previous chapter, making it a potential convergent phenotype (Bonnycastle, Kind, & Cousin, 2024). The neurons were stimulated by a train of 400 APs at 40 Hz in the same manner as the previous TMR-dextran uptake experiments (figure 5.1A).

On the contrary to Peng et al. (2021), *Dyrk1a* HET neurons showed reduced number of TMR-dextran puncta compared to WT at DIV 14, as was the case in neurons from other NDD models (figure 5.1B) (Bonnycastle et al., 2024). The number of internalised TMR-dextran puncta averaged at 369.3 ± 60.40 in WT, whereas in HET the number of puncta averaged at 209.2 ± 17.85 , which translates to approximately 43 % decrease in HET compared to WT. There was no significant difference in the number of α -SV2A puncta ($p = 0.9654$), indicating that the reduced TMR-dextran uptake in HET is not due to a decrease in the number of nerve terminals, but due to partial loss of the capability to perform ADBE (figure 5.1C and D).

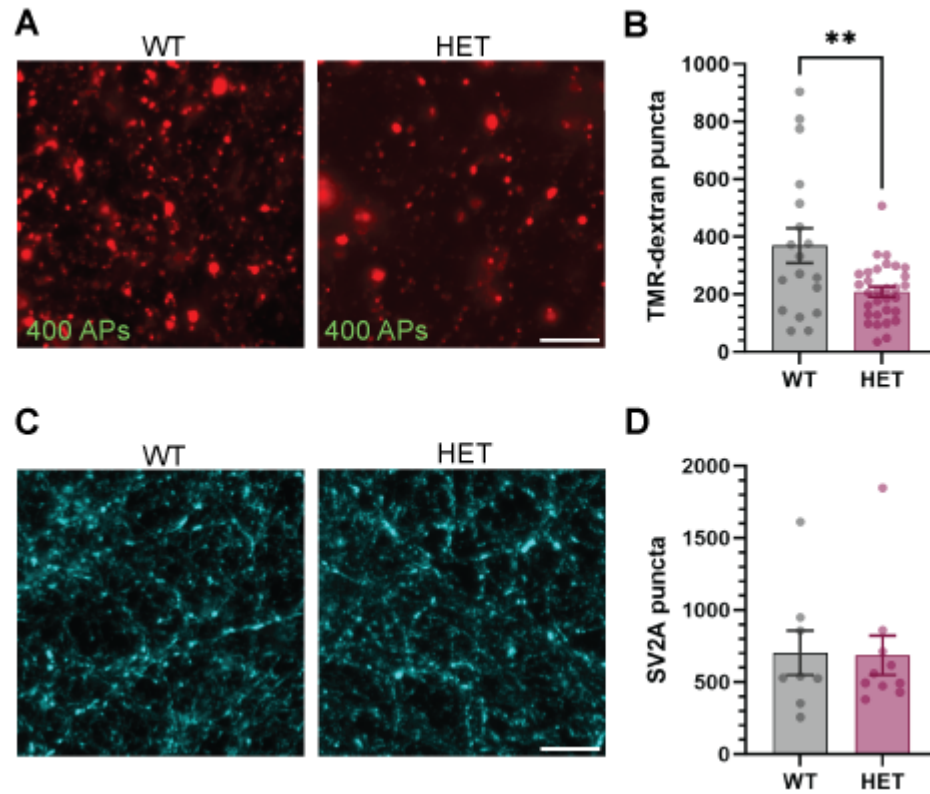


Figure 5.1: *Dyrk1a* HET neurons show reduced ADBE.

Hippocampal neurons of primary *Dyrk1a* HET and WT cultures (days *in vitro* (DIV) 14) were challenged with an action potential train of 400 APs (40 Hz) in the presence of 50 μ M tetramethylrhodamine (TMR)-dextran (40 kDa), with TMR-dextran immediately washed away on termination of stimulation.

- A,** Representative images of *Dyrk1a* WT and HET neurons loaded with TMR-dextran at DIV 14. Scale bar: 10 μ m
- B,** Mean number of TMR-dextran puncta $\pm$ SEM (WT n = 18, HET n = 31). Unpaired Student's *t* test (p = 0.0031).
- C,** Primary cultures of *Dyrk1a* WT and HET hippocampal neurons of the same age (DIV 14) were immuno-labelled with α -SV2A at DIV 14. Representative images of immuno-labelled neurons. Scale bar: 10 μ m
- D,** Mean number of α -SV2A positive puncta $\pm$ SEM (WT n = 8, HET n = 10). Mann-Whitney test non-significant. In all cases n = independent coverslips.

Because the TMR-dextran uptake assay only provides a binary readout of ADBE in a population of nerve terminals, a HRP uptake assay and electron microscopy was used to investigate ADBE in individual nerve terminals. The results showed a significantly reduced number of HRP-labelled bulk endosomes in *Dyrk1a* HET neurons when challenged with the same stimulation as the TMR-dextran uptake assay (400 APs) (figure 5.2). The average number of HRP-labelled bulk endosomes in a nerve terminal was 3.305 ± 0.1986 for WT and 2.693 ± 0.1531 in HET, which is a decrease for about 20 % in HET compared to WT.

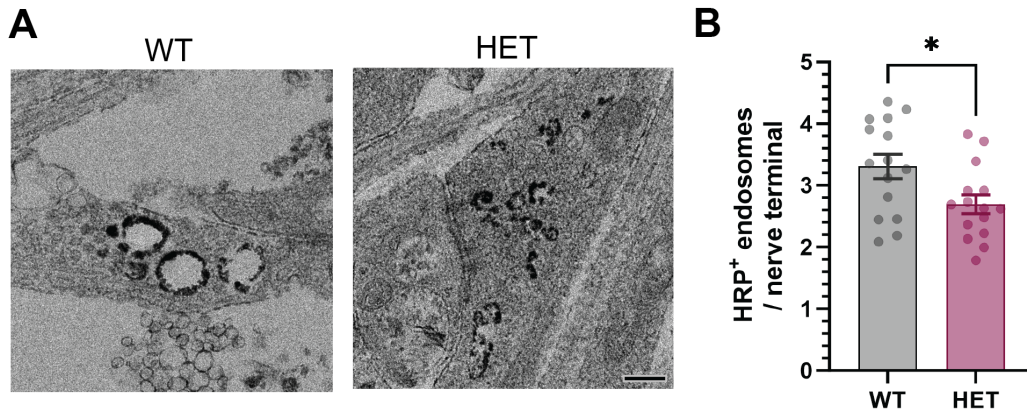


Figure 5.2: *Dyrk1a* HET neurons show reduced ADBE within a nerve terminal.

Hippocampal neurons of *Dyrk1a* WT (left) and HET (right) primary cultures were challenged with an action potential train of 400 APs (40 Hz) in the presence of 10 mg/ml horse radish peroxidase (HRP) and were fixed immediately afterwards for processing and sectioning for electron microscopy.

A, Representative images of HRP-labelled bulk endosomes at the nerve terminals of *Dyrk1a* WT and HET neurons at 14 days *in vitro*. Scale bar: 200 nm

B, Mean number of HRP-labelled endosomes per HRP-labelled nerve terminal $\pm$ SEM (WT $n = 15$, HET $n = 15$). Unpaired Student's *t* test ($p = 0.0265$). n = independent coverslips.

The reduced TMR-dextran uptake and HRP uptake in *Dyrk1a* HET is consistent with the phenotypes in *Fmr1* KO neurons. This underpins the idea that ADBE could be a convergent phenotype for NDD disorders (Bonnycastle et al., 2022).

5.2.2 *Dyrk1a* HET neurons show reduced evoked SV exocytosis

The reduced ADBE in *Dyrk1a* HET neurons shown in a TMR-dextran uptake assay and HRP-uptake assay raises more questions, as ADBE depends on numerous factors, such as evoked Ca^{2+} influx, SV exocytosis and phosphorylation or dephosphorylation of endocytic proteins (Blumrich et al., 2023; Cheung & Cousin, 2013; Cousin & Robinson, 2001; Morton et al., 2015). Therefore, we hypothesised that the downregulation of ADBE is not a stand-alone phenotype, and that the overall SV recycling is disrupted in *Dyrk1a* HET neurons. To understand the decrease in the level of ADBE in the context of SV recycling, syHy imaging was performed to assess the overall SV recycling in individual nerve terminals (figure 3.3A). This was the first attempt to monitor SV recycling in mammalian *Dyrk1a* HET neurons, although there are previous studies showing eclectic results in trisomic models that have an extra copy of *Dyrk1a* gene. The phenotypes reported in the mammalian neurons overexpressing DYRK1A are exaggerated SV exocytosis and decelerated SV endocytosis (Y. Kim et al., 2011; C.-I. Wu et al., 2022), although these are questioned in Marland et al. (2016).

Same as the syHy experiments with WT neurons in chapter 3, WT and HET neurons were challenged with a train of 400 APs (40 Hz) after baseline recording, followed by NH_4Cl perfusion after recovery in order to reveal the total SV pool at DIV 14 (figure 5.3A). When normalised to the total SV pool (NH_4Cl response), HET neurons showed a reduced peak height of approximately 31 % (WT: 0.5023 ± 0.072 , HET: 0.3455 ± 0.024) (figure 5.3B and 5.3C). This suggests a reduced net fusion of SVs. This could be because of the fewer number of SVs fusing onto the plasma membrane, or it could be because of the faster implementation of SV endocytosis, which will reduce the peak height in timetraces. Interestingly, a decrease in the peak height in syHy imaging was not observed in *Fmr1* KO neurons, implying a different pathological mechanism, although they shared the same phenotype of reduced ADBE (Bonnycastle et al., 2022).

On the other hand, when normalised to the peak, the timetraces were comparable between the two genotypes (figure 5.3D). When represented by the fluorescence level at 2 mins post-stimulation from the timetraces normalised to the peak, there was no significant difference between genotypes in SV endocytosis (WT: 0.054 ± 0.115 , HET: 0.097 ± 0.073) (figure 5.3E). In total, syHy imaging shows that *Dyrk1a* HET neurons do not show deficits in cargo retrieval, but has a potential deficit in SV exocytosis, which will be further explored in the next section.

5.2.3 *Dyrk1a* HET neurons show smaller SV pool size

To confirm the reduced evoked SV exocytosis in *Dyrk1a* HET neurons shown in figure 5.3B and 5.3C, we decided to perform another syHy imaging experiment but with Bafilomycin 1A, a drug that inhibits the vATPase on the SV membrane. Bafilomycin 1A blocks the reacidification of SVs, and hence allows the visualisation of SV exocytosis exclusively. What we used for the TMR-dextran uptake assay and syHy imaging, a train of 400 APs (40 Hz), is an intense stimulation that is expected to mobilise both the RRP and the reserve pool. In this experiment, however, we wanted a more delicate approach to gauge the individual SV pools. Applying the setting used in Bonnycastle et al. (2022), the syHy response was recorded in parallel to a sequence of stimulation first at 40 APs (20 Hz) and then followed by 1600 APs (20 Hz) in the presence of Bafilomycin 1A (figure 5.4A). The first short stimulation (40 APs) is designed to deplete the RRP, and the second stimulation (1600 APs) should mobilise the reserve pool (Bonnycastle et al., 2022; Murthy & Stevens, 1999). The interpretation of the syHy response reports the size of the individual SV pools in relation to the total SV pool, indicated by NH_4Cl application at the end of the recording.

As expected, the average timetraces showed a small increase upon the first stimulation (40 APs), followed by a bigger increase in the second stimulation (1600 APs), with minimal decrease in fluorescence after stimulations due to Bafilomycin 1A (figure 5.4). Under the first train of stimulation, the RRP was significantly reduced in HET than in WT for approximately

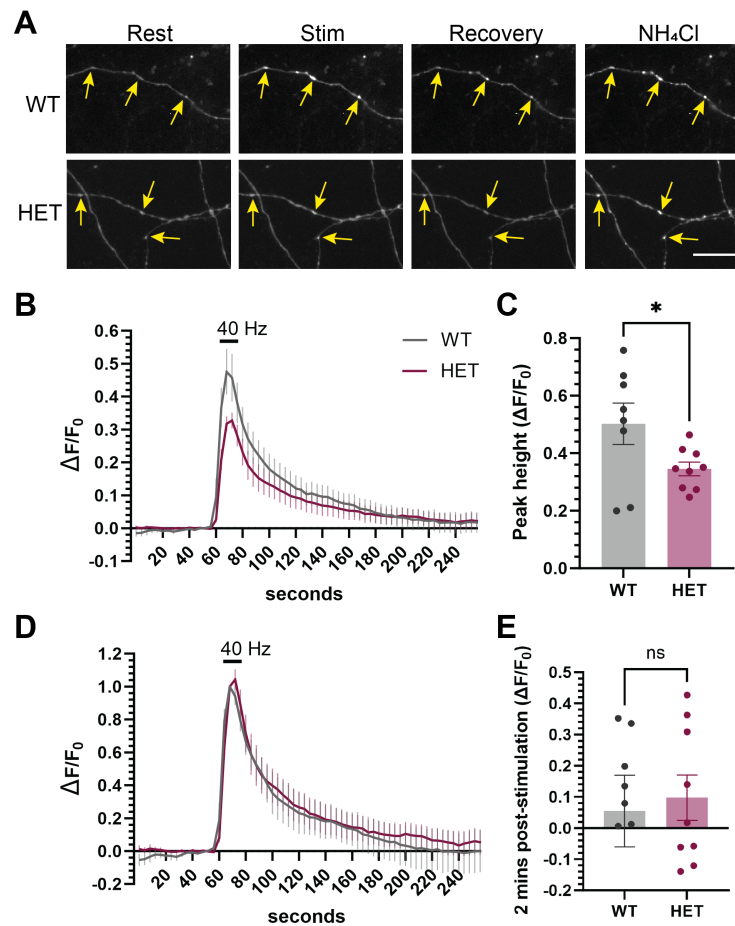


Figure 5.3: *Dyrk1a* HET neurons shows a potential decrease in SV exocytosis.

Hippocampal neurons from primary *Dyrk1a* HET and WT cultures were transfected with synaptophysin-pHluorin (syHy) 3 days prior to imaging and were imaged at 14 days *in vitro* (DIV). Transfected neurons were challenged with 400 APs (40 Hz) and were exposed to basic buffer (NH_4Cl) after 3 mins.

- A**, Representative images of the syHy response of transfected WT and HET neurons stimulated with 400 APs (40 Hz) (**Stim**) after baseline recording (**Rest**). The neurons were exposed to a basic buffer (**NH_4Cl**) after 3 mins of recovery (**Recovery**). Scale bar: 20 μm .
- B**, Mean traces displaying the average syHy fluorescent response $\pm$ SEM ($\Delta F/F_0$). Traces are presented as a fraction of the total SV pool revealed by NH_4Cl application. Bar indicates the period of stimulation. (WT $n = 8$, HET $n = 9$)
- C**, Mean evoked syHy peak height $\pm$ SEM. (WT $n = 8$, HET $n = 9$). Unpaired Student's t test ($p = 0.0465$).
- D**, Mean traces displaying the average syHy fluorescent response normalised to the stimulation peak $\pm$ SEM ($\Delta F/F_0$). Bar indicates the period of stimulation. (WT $n = 8$, HET $n = 9$)
- E**, Mean evoked syHy response measured 2 mins after stimulation $\pm$ SEM (WT $n = 8$, HET $n = 9$). Mann-Whitney test non-significant. In all cases $n =$ independent coverslips.

43 % (WT: 7.599 ± 0.944 , HET: 4.341 ± 0.771 , in percentage to total SV pool) (figure 5.4B). The reserve pool, as indicated by the second train of stimulation, was similarly reduced in HET neurons, for about 35 % (WT: 40.33 ± 2.932 , HET: 26.29 ± 3.179 , in percentage to the total SV pool) (figure 5.4C).

The size of both the RRP and the reserve pool were comparable to those reported in other studies, although slightly smaller in RRP (usually 10-20 %) (Denker & Rizzoli, 2010). In general, this data implicates smaller recycling pool in *Dyrk1a* HET neurons, which is the sum of the RRP and the reserve pool (figure 1.3). In summary, under the same stimulation, there are fewer SVs fusing onto the plasma membrane in relation to the total amount of SVs. This conclusion supports the idea that the reduced peak height in the previous sypHy experiment (figure 5.3C) is not because of faster implementation of SV endocytosis, but because the SV exocytosis itself was downregulated. Indeed, the extent of decrease in the size of SV pools was similar to the extent of decrease in the peak height in the sypHy imaging in figure 5.3C (~32 %). Although the two sypHy imaging experiments used different stimulations, it is compelling to believe that in *Dyrk1a* HET neurons, fewer SVs are mobilised when stimulated.

One limitation of sypHy imaging is that the analyses show SV exocytosis in proportion to the response to the NH_4Cl perfusion, and it lacks insight in the total number of SVs. It is possible that the total number of SVs is unchanged and there are fewer fusion-competent SVs, resulting in an enlarged resting pool. There are other studies that have quantified the total number of SVs at nerve terminals, which will be discussed more in-depth in the discussion.

One key player of maintaining the SV pools is Synapsin (M. Zhang & Augustine, 2021). It has been revealed that the absence of Synapsins results in a more dispersed distribution of SVs at the nerve terminals (Orenbuch et al., 2012; Vasileva, Horstmann, Geumann, Gitler, & Kuner, 2012). The phosphorylation-dependent binding of Synapsins with the SVs and the activity-dependent phosphorylation of the Synapsins led to the crosslinking theory, which explains the mobility of the SVs with the crosslinking of SVs via Synapsin oligomers (Chi, Greengard, & Ryan, 2003; Hosaka, Hammer, & Südhof, 1999; Jovanovic et al., 2001). Recently, based on the finding that Synapsins can form a distinct aqueous phase, liquid-liquid phase separation arose as another theory for maintaining and mobilising the SV pools (Hoffmann et al., 2023; Milovanovic, Wu, Bian, & De Camilli, 2018; Pechstein et al., 2020). A hybrid model also exists, which was suggested by S.-H. Song and Augustine (2023). The authors demonstrated that the regulation of SV pools are synapse type-dependent, GABAergic synapses via liquid-liquid phase separation of Synapsin 2 and glutamatergic synapses via biochemical crosslinking of Synapsin 2 tetramers.

Although the exact mechanism is still under debate, we thought it would be useful to investigate the expression level and the phosphorylation level of Synapsin in neuronal lysates to test the possibility of Synapsin being the link between *Dyrk1a* haploinsufficiency and the smaller recycling SV pool (figure 5.5). We hypothesised that the decrease in the SV pool size could be

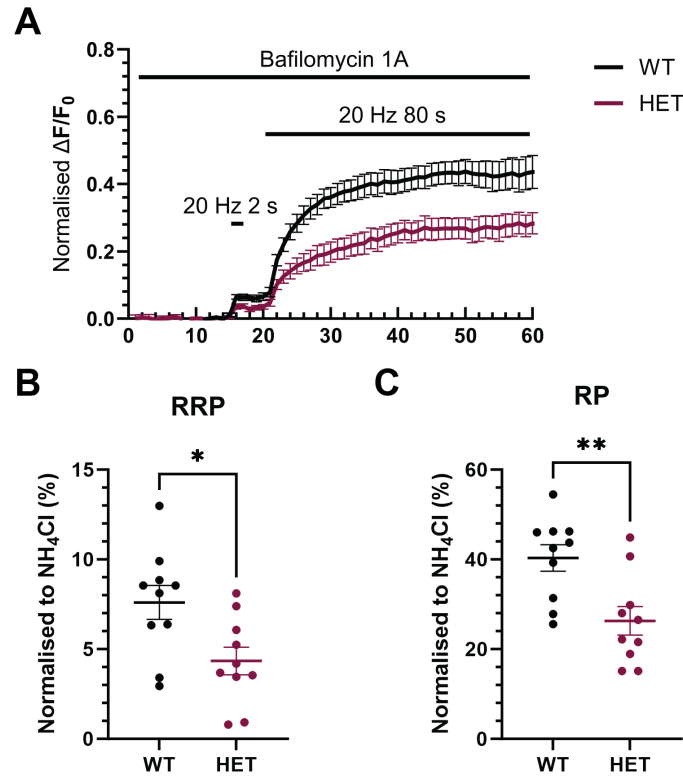


Figure 5.4: *Dyrk1a* HET neurons have smaller RRP and reserve pool.

Hippocampal neurons from primary *Dyrk1a* HET and WT cultures were transfected with syHy 3 days prior to imaging and imaged on 14 days in vitro (DIV) in the presence of 1 μM Bafilomycin 1A. Transfected neurons were challenged with two trains of APs (20 Hz, 2 s and 20 Hz 80 s) before exposure to basic buffer (NH_4Cl).

- A**, Mean traces displaying the average syHy fluorescent response of WT (grey) and HET (plum) hippocampal neurons is displayed $\pm$ SEM. Traces are $\Delta F/F_0$ and are normalized to the total SV pool, revealed by NH_4Cl application. Bars indicate periods of stimulation.
- B**, Mean syHy peak height $\pm$ SEM during 20 Hz 2 s stimulation (Readily releasable pool (RRP), % of total pool). WT $n = 10$ and HET $n = 10$. Unpaired Student's t test ($p = 0.0155$).
- C**, Mean syHy peak height $\pm$ SEM during 20 Hz 80 s stimulation (Reserve pool (RP), % of total pool). WT $n = 10$ and HET $n = 10$. Unpaired Student's t test ($p = 0.0045$).

explained by the disrupted regulation of the expression or the phosphorylation of Synapsin. Hence, a Western blot was performed to detect the expression levels of Synapsin 1/2 and phosphoserine Synapsin 1 in neuronal lysates harvested from primary cultures of *Dyrk1a* WT and HET at DIV 14. The reason why we chose to look at the phosphorylation of the serine residue 553 (S553) was because it is known to be phosphorylated by cyclin-dependent kinase 5 (CDK5) *in vivo*, whose inhibition using drugs or a phospho-null mutation resulted in reduced evoked exocytosis as well as dispersed organisation of the SV clusters, phenocopying the Synapsin knockout model (DeGiorgis et al., 2005; Matsubara et al., 1996; Verstegen et al., 2014).

However, there was no significant difference in the expression level of Synapsin 1/2 in hippocampal neuronal lysates between *Dyrk1a* WT and HET, when normalised to β -actin (figure 5.5A and 5.5B). The amount of S553 phospho-Synapsin 1 (p-Synapsin) between the two genotypes was also unaltered, when normalised to Synapsin 1/2 and then to WT (figure 5.5C). Next, based on the previous findings that Synapsin phosphorylation is activity-dependent, we stimulated the neurons by incubating in 50 mM KCl for 2 mins before lysing the neurons (figure 5.5D). The p-Synapsin level was still comparable regardless of the stimulation (figure 4.3D) (Chi et al., 2003). In summary, the expression of Synapsin or the phosphorylation of S553 is unaffected in *Dyrk1a* HET neurons.

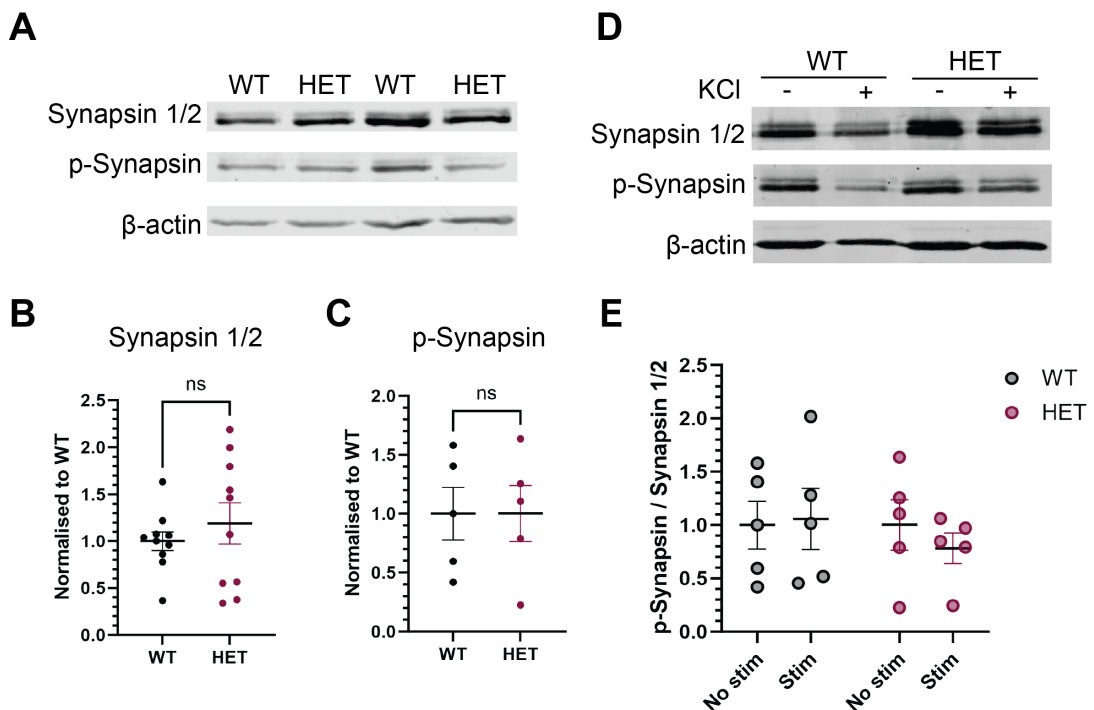


Figure 5.5: Western blot shows no significant difference in the expression level of Synapsin nor p-Synapsin between *Dyrk1a* WT and HET neurons.

- A**, Representative images of blots containing *Dyrk1a* HET and WT. The blot was probed for Synapsin 1/2, p553-Synapsin 1 (p-Synapsin) and β -actin. Synapsin 1/2 and p-Synapsin Molecular weights (MWs): ~75 kDa. β -actin MW: ~42 kDa.
- B**, Comparison of the relative expression level of Synapsin 1/2 (WT: n = 10, HET: n = 10). Student's *t* test non-significant.
- C**, Relative expression level of p-Synapsin normalised to Synapsin 1/2 expression level (WT: n = 5, HET: n = 5). Student's *t* test non-significant.
- D**, Representative images of blots containing non-stimulated and stimulated neuronal lysates of *Dyrk1a* WT and HET. The blot was probed for Synapsin, p-Synapsin and β -actin.
- E**, Stimulation (50 mM KCl) did not alter the expression level of p-Synapsin in both WT and HET neurons (In all conditions n = 5). 2-way ANOVA non-significant.

5.2.4 Evoked calcium influx is not altered in *Dyrk1a* HET neurons

Investigating from ADBE to SV recycling, we were curious if these phenotypes stemmed from a more upstream activity, the activity-dependent Ca^{2+} influx. Although 40 APs and 1600 APs are assumed to deplete the recycling pool, a possibility remains that the difference derives from the smaller Ca^{2+} influx in *Dyrk1a* HET neurons. To explore this avenue, calcium imaging was performed using the same approach as WT neurons, by co-transfecting the neurons with *syp-mOr2* to mark the nerve terminals and *GCaMP6f* to report the concentration of intracellular free Ca^{2+} ions (figure 5.6A). Here, the neurons were challenged with a train of 40 APs (20 Hz) to answer whether the difference in the size of the RRP can be attributed to the smaller activity-dependent Ca^{2+} influx (figure 5.4).

When the neurons were stimulated, *Dyrk1a* HET neurons did not show any significant difference compared to WT in timetraces nor the peak heights (WT: 0.359 ± 0.132 , HET: 0.493 ± 0.081) (figure 5.6B and C). This narrows down the presynaptic defect in *Dyrk1a* HET neurons to the SV recycling pathway, such as the mobility or the fusion-competence of the SVs rather than its upstream processes.

5.2.5 Potential substrates of DYRK1A at the presynapse

In literature, the identified substrates of DYRK1A include Dynamin-1, Synaptojanin-1, Amphiphysin -1, α -Synuclein, AP180 and Munc18-1, all of which are heavily associated with SV recycling (Adayev et al., 2006; Chen-Hwang et al., 2002; Classen et al., 2020; E. J. Kim et al., 2006; Murakami et al., 2012, 2006; Peng et al., 2021). It is likely that the reduced SV exocytosis and ADBE in *Dyrk1a* HET neurons are relevant to the dysregulation of its substrates. However, it should be noted that not all of the identified substrates are validated in neurons, which is crucial considering DYRK1A is expressed prevalently in all cell types and its role is not limited to the regulation of synaptic activity (Fagerberg et al., 2014; Rammohan et al., 2022). In order to identify DYRK1A substrates specifically at the synapses and to reveal potential mechanism behind the presynaptic downscaling, we collaborated with Dr Andrew Frey from Newcastle University who performed LC-MS proteomics and phospho-proteomics with synaptosomes prepared from 5 *Dyrk1a* HET cerebra and 5 littermate controls (2 months old, female).

Before comparing the phosphorylation level between the two genotypes, we proceeded with proteomics to test if there was any significant difference in the protein expression level. First of all, the proteomics data showed low variability across individuals, as presented in the principal component analysis (PCA) plot (Appendix B). This gives us confidence that the proteomics and phospho-proteomics results do not derive from the variability among individual animals.

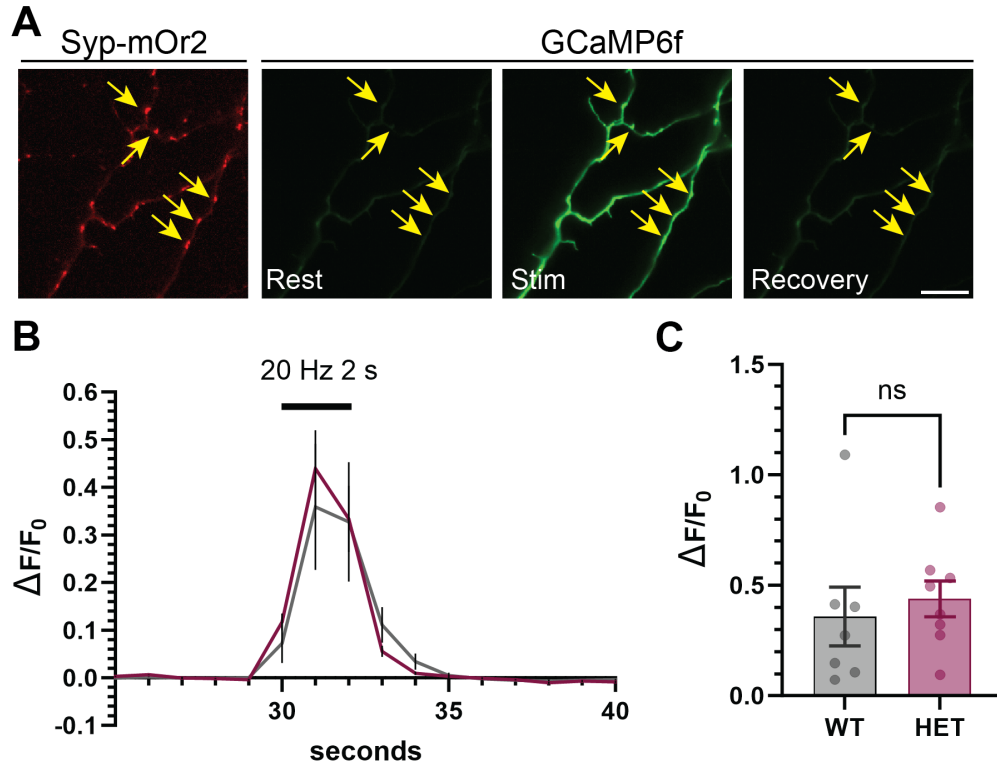


Figure 5.6: *Dyrk1a* HET neurons show comparable evoked Ca^{2+} influx to WT.

Hippocampal neurons from primary cultures of *Dyrk1a* HET and WT (DIV 14) were co-transfected with synaptophysin-mOrange2 (syp-mOr2) and GCaMP6f 3 days prior to imaging. The neurons were challenged with 40 APs (20 Hz) after baseline recording, and continued to be recorded for 1 min.

- A**, Representative image of a syp-mOr2 transfected neuron and its GCaMP6f response before (**Rest**), during (**Stim**) and after stimulation (**Recovery**). Scale bar: 10 μm .
- B**, Mean traces displaying the average GCaMP6f fluorescent response at syp-mOr2 positive puncta in response to stimulation in HET (plum) and WT (grey) neurons $\pm$ SEM ($\Delta F/F_0$). WT $n = 7$, HET $n = 8$. Bar indicates the period of stimulation.
- C**, Mean evoked GCaMP6f peak height $\pm$ SEM (WT $n = 7$ and HET $n = 8$). Mann Whitney's test non-significant.

Secondly, the proteomics analysis showed increased (> 1.5 fold) and decreased (< 0.75) expression level of some proteins in HET synaptosomes, which was not surprising because DYRK1A regulates protein expression by phosphorylating transcription factors (Aranda et al., 2011). There were six proteins over-expressed in the HET synaptosomes: Ciao1, Cdk18, Rab28, Arl2bp, Clic6 and Eif1ad. There were 57 under-expressed proteins in the HET synaptosomes, whose molecular function was focused to KRAB domain binding (Annotation version and release date: GO Ontology database, released on 2024-06-17; Annotation data

set: GO Molecular function). Neither the proteins over-expressed nor under-expressed in HET synaptosomes were directly linked to SV recycling pathway. Meanwhile, the expression level of DYRK1A was reduced to 78 % in *Dyrk1a* HET synaptosomes, which will be verified via Western blot in future experiments.

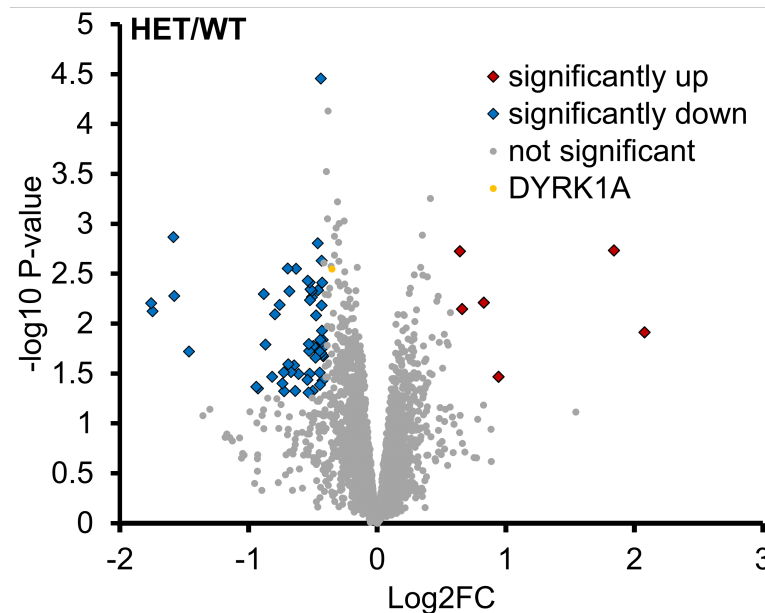


Figure 5.7: A volcano plot showing the over- and under-expressed proteins in *Dyrk1a* HET and WT synaptosomes.

For multiple comparison, t-test was performed without correction at the threshold of 1.5-fold for increase and 0.75-fold for decrease. Full list is available in Appendix B. The data was produced and visualised by Dr Andrew Frey from Newcastle University.

The phospho-proteomics was also first checked for its validity before comparisons between genotypes (B), and the following analysis implied that a number of proteins are over- or under-phosphorylated in *Dyrk1a* HET neurons (figure 5.8).

When the whole list of the over- or under- phosphorylated proteins were analysed using PANTHER overrepresentation test, the over-phosphorylated proteins were heavily involved in "presynaptic signal transduction" and "active zone assembly" (fold enrichment >100), meaning the downstream effects of DYRK1A haploinsufficiency results in over-phosphorylation of proteins involved in these two processes (figure 5.9). On the other hand, under-phosphorylated proteins were enriched in "regulation of SV docking" (fold enrichment >100), which aligns with the downscaled presynaptic strength reported earlier in this chapter.

Zooming in from the network level, the individual proteins were visualised via annotation from STRING database and classified using a GO enrichment analysis tool (biological process) (figure 5.10 and 5.11). The proteins relevant to SV recycling were circled red on the edge of the nodes, which formed a cluster in both over- and over- phosphorylated networks. The clustered proteins in over-phosphorylated group were: SNAP91 (AP180), Piccolo, Synapsin-1, SV2A

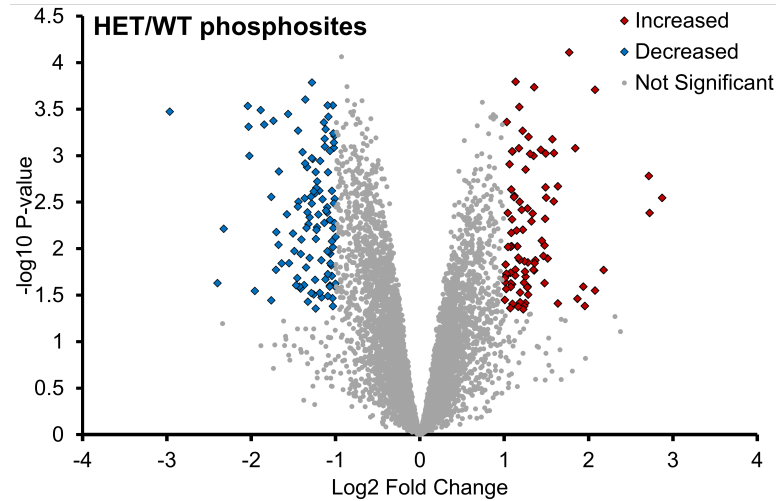


Figure 5.8: A volcano plot showing the over- and under- phosphorylated proteins in *Dyrk1a* HET and WT synaptosomes.

For multiple comparison, t-test was performed without correction at the threshold of 2-fold for increase and 0.5-fold for decrease. Full list is available in Appendix B. The data was produced and visualised by Dr Andrew Frey from Newcastle University.

and GIT1. SV2A, a glycoprotein on the SV membrane had the highest fold change ($\log_2\text{FC} = 2.72$). Synapsin-1, which was probed for in Western blot in the previous section, also showed more than 2-fold of increase in phosphorylation (S566). In the under-phosphorylated group, the proteins that are associated with SV recycling are: Kinesin family member 5C (KIF5C), SH3-containing GRB2-like protein 3-interacting protein 1 (SGIP1) (activator of AP2 (Hollinger et al., 2014)), Synaptojanin-1, SNAP91 (different peptide to the over-phosphorylated sequence), clathrin heavy chain (CLTC) and Dyn-1.

5.3 Discussion

In this chapter, we investigated the presynaptic function in *Dyrk1a* HET neurons from an rodent model of DYRK1A syndrome. Using various imaging techniques, we revealed that *Dyrk1a* HET neurons perform reduced ADBE, both in a population of nerve terminals and within individual nerve terminals at DIV 14 (figure 5.1 and figure 5.2). This is potentially associated with the smaller evoked SV exocytosis observed in syHy imaging, which would implicate less demand for SV endocytosis (figure 5.3). The dampened SV exocytosis does not stem from the smaller Ca^{2+} influx when stimulated, as calcium imaging using GCaMP6f showed no significant difference between HET and WT, although it is possible that the GCaMP reporter is saturated under 40 APs (figure 5.6). Together with the syHy imaging showing smaller SV recycling pool, our data suggest that *Dyrk1a* haploinsufficiency causes presynaptic downscaling, due to dysregulation of a process downstream to Ca^{2+} influx (figure 5.4). In order

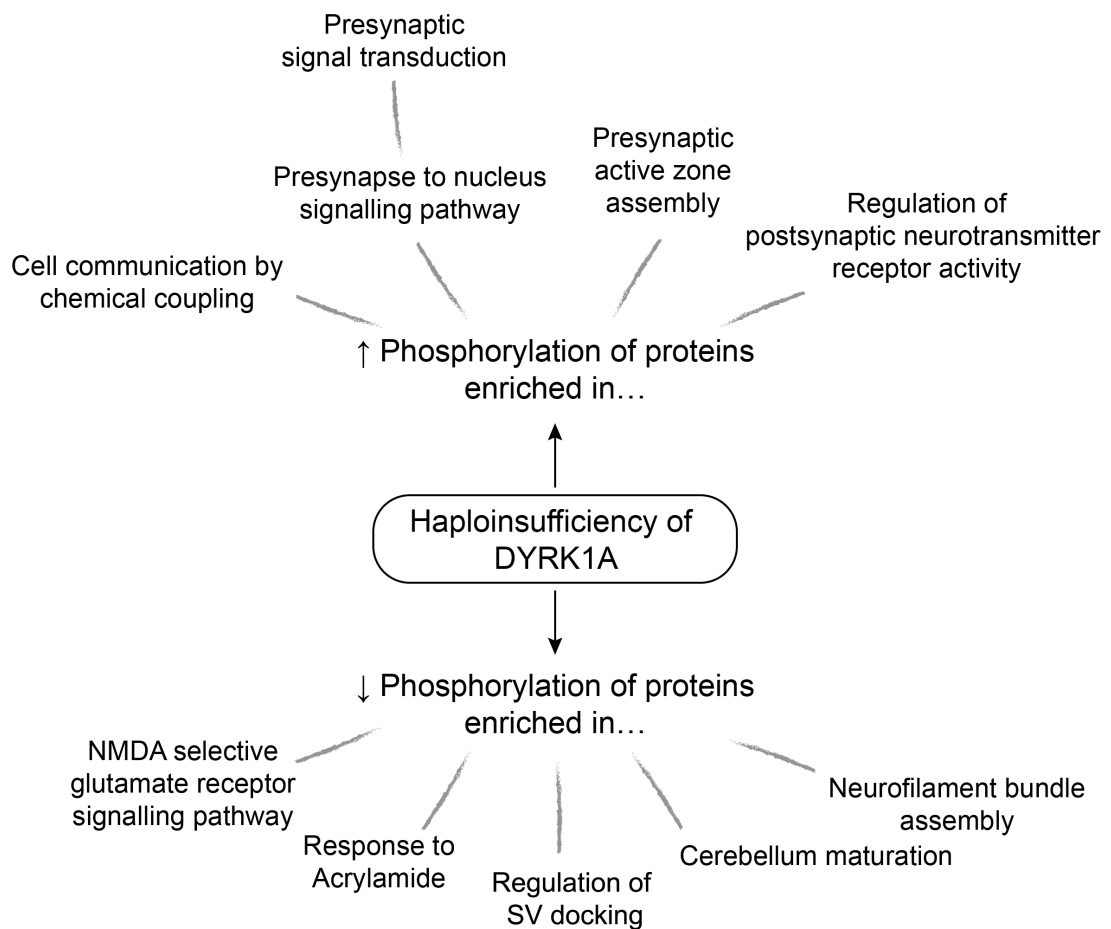


Figure 5.9: The biological processes that are enriched among over- and under- phosphorylated proteins in *Dyrk1a* HET neurons according to GO analysis.

Analysis type: PANTHER overrepresentation Test (Released 20240807); Annotation Version and Release Date: GO Ontology database DOI: 10.5281/zenodo.12173881 Released 2024-06-17; Reference list: *Rattus norvegicus* (all genes in database); Annotation data-set: GO biological process complete; Test type: Fisher's exact, Correction: Calculate false discovery rate.

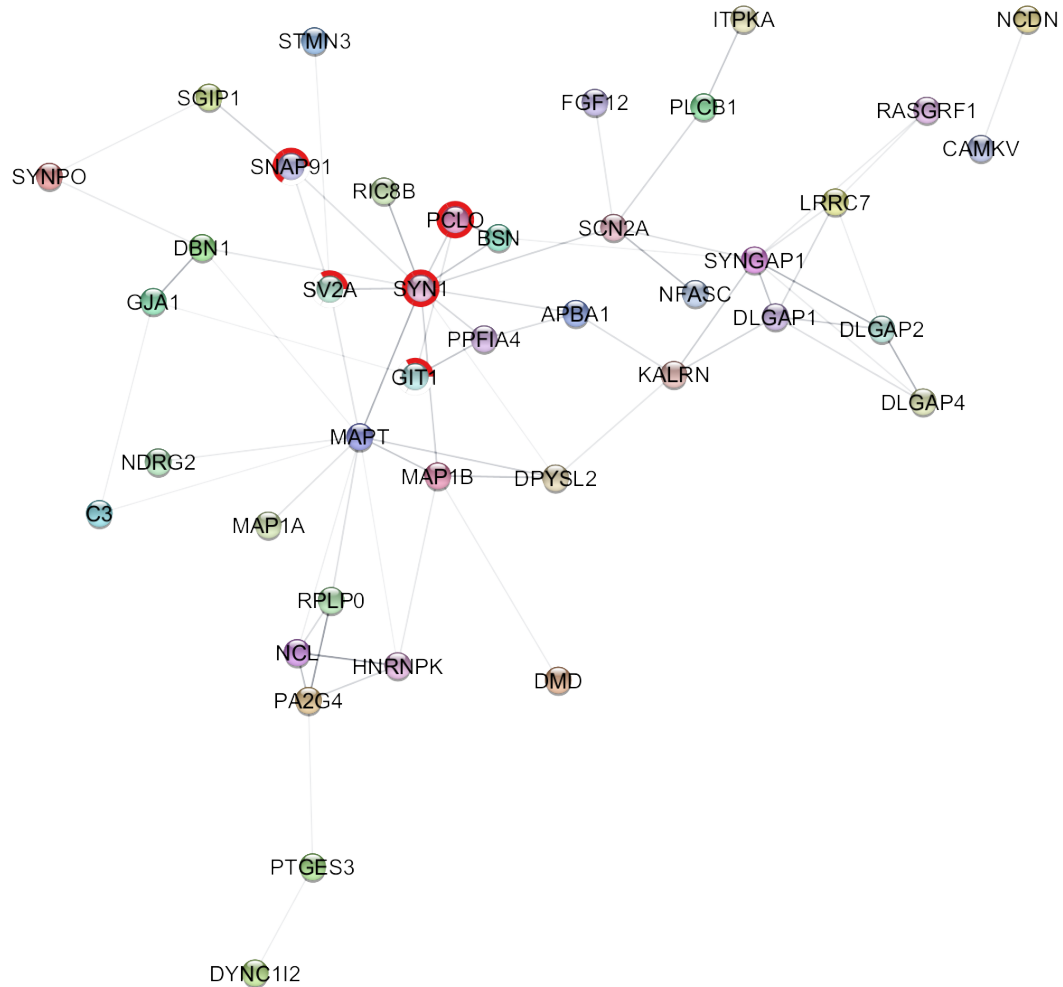
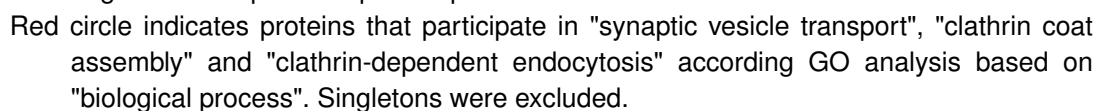


Figure 5.10: A network of over-phosphorylated proteins in *DYRK1A* HET synapses.

The proteins that show a significant increase in phosphorylation in *DYRK1A* HET compared to WT were visualised as a network using STRINGapp (STRING: protein query) on Cytoscape (3.10.2) software (Doncheva, Morris, Gorodkin, & Jensen, 2018). The nodes indicate proteins, and edges indicate potential protein-protein association.

Red circle indicates proteins that participate in "synaptic vesicle cycle", "synaptic vesicle clustering" and "synaptic vesicle localisation" according to GO analysis based on "biological process". Singletons were excluded.



to untangle the mechanism behind such phenotype, we performed Western blot to assess the expression level and the phosphorylation level of Synapsin, a protein known to organise SV pools (figure 5.5) (M. Zhang & Augustine, 2021). However, both the expression level and the phosphorylation level at S553 remained comparable with or without stimulation. Last but not least, we performed proteomics and phospho-proteomics studies with synaptosomes to shortlist more candidate proteins for future experiments. Although the samples were largely consistent, the validation of proteomics data revealed that the expression level of DYRK1A at the synaptosomes were reduced to 78 %. This was not surprising, because it has been widely reported that the copy number of a gene does not linearly correlate to the expression level of the protein, including in Down syndrome (Dowjat et al., 2007). Nonetheless, we are planning to perform Western blot in neuronal lysates and synaptosomal lysates in order to further corroborate the under-expression of DYRK1A. Proceeding with phospho-proteomics, the most over-phosphorylated presynaptic proteins in *Dyrk1a* HET synaptosomes was SV2A. Interestingly, the under-phosphorylated presynaptic proteins included Dyn-1.

It is worth noting, however, that there are caveats to these experimental designs. First of all, when analysing HRP-uptake and electron microscopy data, the nerve terminals were screened for HRP-positive nerve terminals as was the case in chapter 3, rather than including all the nerve terminals. This could have inflated the number of HRP-labelled bulk endosomes higher as the unlabelled nerve terminals are disregarded, which could explain the milder decrease compared to the TMR-dextran uptake assay or sypHy imaging data. Nevertheless, even if the HRP-uptake and electron microscopy understates the ADBE phenotype, it does not undermine the presynaptic downscaling narrative of this chapter. Secondly, sypHy imaging shows response in proportion to the total SV pool, which would disregard a change in the number of total SVs. Third, the synaptosomes used in phospho-proteomics are prepared from 2-month-old rats, different from DIV 14 neurons that were used to report phenotypes in imaging. Therefore, the candidate targets of DYRK1A would require verification in primary neurons. Lastly, in calcium imaging, the reporters could have been saturated. This could be addressed by using a different genetically encoded calcium indicator or calcium dye with lower affinity or using calcium chelators. Nevertheless, the fact that *Dyrk1a* neurons showed less sypHy response after 1600 APs, which should have depleted the reserve pool even if the Ca^{2+} influx was smaller, backs up the argument that the SV recycling pool is smaller in size (figure 5.3). How the presynaptic downscaling can be interpreted in relation to the phospho-proteomics data, and how our findings relate to the preexisting literature will be discussed next.

5.3.1 Potential mechanisms for the presynaptic downscaling in *Dyrk1a* HET neurons

Because DYRK1A is a protein kinase, it is likely that the deficient phosphorylation of exo-/endocytic proteins in *Dyrk1a* HET neurons result in the presynaptic downscaling in *Dyrk1a* HET neurons, which is why the phospho-proteomics is the cornerstone of this project. Pre-synaptic proteins that participate in SV recycling were picked up in both over- and under-phosphorylated groups, narrowing down the paths forward of this project.

Among the proteins that showed more than 2-fold increase in phosphorylation, SV2A had the highest fold change. The phosphorylation site recognised in the peptide sequence is threonine 84 (T84), whose phosphorylation is known to mediate binding with Syt-1 (N. Zhang et al., 2015). The phosphorylation of T84 of SV2A by Casein kinase 1 (CK1) enhances its binding with Syt-1, whose blockage accelerated the retrieval of Syt-1 from the plasma membrane (N. Zhang et al., 2015). Whether the over-phosphorylation is due to the competitive relationship between DYRK1A and CK1, or is due to a compensation by CK1 for the deficiency of DYRK1A is unknown. Synapsin-1 was also enlisted in the over-phosphorylated group, a protein we suspected to be behind the smaller SV pool size (figure 5.5). However, the phosphorylation site picked up in the peptide sequence (S566 in rat; S568 in human) is not the same site we used the phosphoserine antibody for (S551 in rat; S553 in human). The S566 is known to be phosphorylated by CaMKII, which contributes to dissociation of Synapsin-1 from SVs (Benfenati, Neyroz, Böhler, Masotti, & Greengard, 1990; Chi et al., 2003; Czernik, Pang, & Greengard, 1987; Z.-W. Wang, 2008). It would be informative to confirm the phospho-proteomics data with a phosphoserine 566- Synapsin-1 antibody.

Among the proteins that were under-phosphorylated in *Dyrk1a* HET neurons, on the other hand, were Synaptojanin-1, Syntaxin binding protein 5 (STXBP5) (Tomosyn), and Dyn-1. Synaptojanin is an established substrate of Mnb/DYRK1A, although the phosphorylation site reported in literature (S1029) does not match with the sequence produced by the phospho-proteomics data (T1217) (Adayev et al., 2006; Peng et al., 2021). Synaptojanin is a phosphatase that targets $PI_{4,5}P_2$, thereby regulating the uncoating of the clathrin coat around recycled SVs (Chang-Ileto et al., 2011). It also participates in the neck formation in UFE through interaction with Endophilin (Watanabe et al., 2018). Interestingly, nerve terminals of Synaptojanin knockout neurons were reported to have fewer SVs and slower SV endocytosis (Verstreken et al., 2003; Watanabe et al., 2018). In order to test if Synaptojanin-1 mediates the presynaptic downscaling in *Dyrk1a* HET neurons, a confirmation of the phospho-proteomics study with kinase assay should be preceded before functional assays. Tomosyn, on the other hand, also results in smaller SV pools when overexpressed, and bigger SV pools when knocked down (Cazares et al., 2016). Although Tomosyn is a novel target of DYRK1A, one can speculate that the haploinsufficiency of *Dyrk1a* results in hyper-activity of Tomosyn, which downregulates the SV mobility. In neurons, the phosphorylation of Tomosyn by protein kinase

A (PKA) at S724 has been related to SNARE complex formation, but the site does not match with the phospho-proteomics data (S760) (Baba, Sakisaka, Mochida, & Takai, 2005). Lastly, the recognised phosphorylation site of Dyn-1 (S817) is not a major phosphorylation site *in vivo* nor the phosphorylation site predicted (S857) based on the DYRK1A consensus sequence RPX(S/T)P (Graham et al., 2007; Himpel et al., 2000). The nearest phosphorylation site identified *in vivo* is S822, although the relationship between the two phosphorylation sites are unknown (Graham et al., 2007).

Alternatively, it is possible that the presynaptic phenotypes in *Dyrk1a* HET neurons is not directly associated with the phosphorylation of exo-/ endocytic machinery. This is because DYRK1A is expressed globally, in both nucleus and cytosol as well as the nerve terminals, and its expression is not limited to neurons (Aranda et al., 2011). One essential aspect of SV recycling is its energy metabolism. Pulido and Ryan (2021) showed that the vATPase on SV membrane consumes 44 % of the synaptic energy at a resting state in hippocampal neurons, meaning that maintaining SV pools is an energy-expensive process. In a similar context, Myeong, Stunault, Klyachko, and Ashrafi (2024) showed the correlation between the spatial localisation of the mitochondria at the nerve terminals and the SV release probability. DYRK1A regulates the import of the metabolite precursors from the cytoplasm into the mitochondria by phosphorylating translocase of the outer mitochondrial membrane 70 (TOM70), which indicates that it is responsible for generating energy that the presynapse requires for SV recycling (Kreimendahl & Rassow, 2020; Marada et al., 2024; Walter et al., 2021). It is reasonable to theorise that the deficiency of DYRK1A would result in insufficient delivery of metabolite precursors into the mitochondria, and therefore limits the presynaptic capacity. This idea could be tested with a pilot experiment that inhibits mitochondria translocase in WT neurons using drugs such as oligomycin, and monitor if it phenocopies *Dyrk1a* HET neurons (Myeong et al., 2024).

There is a possibility that the phenotypes reported in this chapter are transient, as was the case in *Fmr1* KO neurons. A major role of DYRK1A is cell cycle exit by phosphorylating cyclins (Rammohan et al., 2022). Overexpression of DYRK1A in neuronal cell lines induced cell cycle exit, whereas the inhibition of DYRK1A increased proliferation rate in a glioblastoma cell line (Recasens et al., 2021; Soppa et al., 2014; Yabut, Domogauer, & D'Arcangelo, 2010). It is possible that the downscaling of the nerve terminals in DIV 14 *Dyrk1a* HET neurons is due to a longer proliferation and shorter differentiation time period, making the nerve terminals of *Dyrk1a* HET neurons less mature at DIV 14. It would be interesting to assess evoked exocytosis or SV pool size at DIV 21 to test this theory.

5.3.2 Presynaptic defects in *Dyrk1a* HET neurons in literature

Although the mechanism is in veil, the reduced SV exocytosis, ADBE, and SV pool size align with some of the previous publications but not all of them. In trisomic hiPSC-driven neurons, C.-I. Wu et al. (2022) recorded higher sypHy response when stimulated with 50 mM KCl, which was rescued when the copy number of *DYRK1A* was corrected. Combined with the sypHy imaging data reported in this chapter, we can speculate that DYRK1A has a dosage-dependent function in evoked exocytosis. However, in murine trisomic model Ts65Dn, there was no phenotype in sypHy response in hippocampal neurons, when challenged with a train of 300 APs at 10 Hz (Marland et al., 2016). It is possible that the presynaptic downscaling is not as visible under a train of mild stimulations (300 APs @ 10 s) compared to an intense stimulation (50 mM KCl or 400 APs @ 40 Hz). In terms of SV endocytosis, Y. Kim et al. (2011) reported slower endocytosis when DYRK1A was overexpressed, which contradicts with the non-significant data in trisomic mice (Marland et al., 2016). These discrepancies could come from the difference in species, or from whether the model uniquely targets *Dyrk1a* or multiple genes that are triplicated in Down syndrome, or from different experimental designs. So far, the effect of DYRK1A overexpression is inconclusive.

Another relevant study to this project is Classen et al. (2020), which showed that Munc18-1 is phosphorylated by DYRK1A at T479 residue. The authors explored the function of this phosphorylation by overexpressing Munc18-1 with phospho-null and phospho-mimetic point mutations in Munc18-1 knockout neurons. Using electrophysiological parameters, the authors concluded that the neurons with the phospho-mimetic mutant have enhanced recovery of SV pool after its depletion (Classen et al., 2020). Although Munc18 was not enlisted in the over- or under-phosphorylated groups in phospho-proteomics results, it is possible that the deficient phosphorylation of Munc18-1 by *Dyrk1a* haploinsufficiency results in reduced ADBE, hence undermining the presynaptic strength. If overexpressing phosphomimetic Munc18-1 in *Dyrk1a* HET neurons rescue ADBE, it would be a good indication that the presynaptic downscaling is mediated by Munc18-1.

Meanwhile, in haploinsufficiency models, deficiency of DYRK1A leads to undergrowth of cortical layers, and shorter and simpler dendrites (Benavides-Piccione et al., 2005; Dang et al., 2018; Levy et al., 2021). In the functional aspect, Shih et al. (2023) has reported an increase in the amplitude of optically-evoked EPSCs and a decrease in optically-evoked IPSCs in pyramidal neurons in hippocampal slices from *Dyrk1a* HET mice when stimulated via channelrhodopsins, but the paired-pulse ratio was not significantly different between genotypes. It is likely that the *Dyrk1a* haploinsufficiency model also has postsynaptic deficits, which was not studied in this thesis. The contradiction between the strong presynaptic phenotypes in this thesis and the comparable paired-pulse ratio in Shih et al. (2023) could be due to the different maturity of the systems. The animals used in Shih et al. (2023) were 2-3 months old, leaving the possibilities open that there might be a developmental phenotype, as was the

case in FXS-modelling neurons in the previous chapter. Considering that DYRK1A is most abundantly expressed perinatally and its expression decreases from P14 (Dang et al., 2018), it is of great importance to explore younger animals, which is why we plan electrophysiological assays in younger animals in the near future.

Last but not least, the most relevant study in *Dyrk1a* haploinsufficiency is Peng et al. (2021). Using two different stimulating protocols, the authors monitored neuromuscular junctions in *Drosophila* and argued that Minibrain suppresses ADBE by phosphorylating Synaptojanin (Peng et al., 2021). However, a flaw of their experimental design is that the two protocols do not ensure the same amount of Ca^{2+} influx nor evoked exocytosis. The protocol designed to induce CME used perfusion in 90 mM KCl and 0.5 mM Ca^{2+} , whereas clathrin-independent protocol used 60 mM KCl with 2 mM Ca^{2+} , plus application of a clathrin inhibitor chlorpromazine. A validation step is required to ensure that the amplified FM dye loading, which does not distinguish CME and ADBE, is not due to an increase in Ca^{2+} influx or SV exocytosis, especially because ADBE requires these two upstream activities (Morton et al., 2015). On the other hand, the authors have convincingly shown that Mnb phosphorylates the S1029 in Synaptojanin using phospho-mimetic Synaptojanin (S1029E), which positively correlated to TMR-dextran uptake (Peng et al., 2021). It would be interesting to test if the phospho-mimetic Synaptojanin alters SV pool organisation, hence rescuing the presynaptic phenotypes in *Dyrk1a* HET neurons.

5.3.3 Dosage-dependence of *Dyrk1a*

Both DYRK1A haploinsufficiency syndrome and Down syndrome are NDDs that are comorbid with ID and epilepsy, as well as microcephaly. Down syndrome results from complete or partial trisomy of HSA21, which contains 233 genes, and 168 of them are conserved in mice scattered in *Mus musculus* chromosome (MMU) 10, 16 and 17 (Antonarakis et al., 2020). Based on the clinical reports of partial HSA21 trisomy, some genes were revealed to be more penetrant than the others, leading to the establishment of Down syndrome critical region at 21q22.13 (Korenberg et al., 1994; Murphy et al., 2024; Pelleri et al., 2019). The genes in the Down syndrome critical region include *amyloid precursor protein (APP)* and *DYRK1A* (Duchon & Herault, 2016; Murphy et al., 2024; Snyder et al., 2020). Triplicating one or more of these genes, there are a few trisomic rodent models available (e.g., Ts65Dn), which show a spectrum of behavioral and cognitive deficits (Aziz et al., 2018; Jiang et al., 2015). In murine trisomic models, genetically correcting the copy numbers of *Dyrk1a* or inhibiting DYRK1A activity rescued behavioral and electrophysiological phenotypes at least partially, emphasising the potential causative role of DYRK1A in Down syndrome (Jiang et al., 2015; Neumann et al., 2018; Nguyen et al., 2018). It is difficult to attribute all the findings in these trisomic models to *Dyrk1a*, as it is not the only gene triplicated in these models. Still, the studies that used transgenic mice with an extra copy of *Dyrk1a* or the studies that overexpressed DYRK1A in

vitro allow us some insights to the kinase (Jiang et al., 2015; Martinez de Lagran et al., 2012). Compiling the findings in neurons from DYRK1A haploinsufficiency (+/- or +/-loss-of-function mutation) and redundancy models (transgenic triplicates or overexpression), it is obvious that the two different pathology rarely results in phenotypes in the opposite ways (table 5.1). The morphological phenotypes and the behavioral phenotypes of haploinsufficiency and redundancy models are overlapping, implying that DYRK1A affect neurodevelopment in a dosage-dependent manner, but the dosage does not correlate to the phenotype linearly.

5.3.4 Presynaptic downscaling in other NDDs

Downscaled neurotransmission at the presynapse has been repeatedly reported in other NDD models, although it does not apply to all NDD models. For example, mutations in *STXBP1*, or *Munc18-1* result in encephalopathy heavily associated with epilepsy and ID. More than 60 % of the reported cases are truncated mutations, meaning its deficiency is detrimental for neurodevelopment (Stamberger et al., 2016). Primary hippocampal neurons from *Munc18-1*^{+/-} mice showed a smaller RRP, which was extrapolated from the hypertonic sucrose response in culture (Toonen et al., 2006). The authors confirmed this finding via electron microscopy, the analysis of which showed fewer docked SVs per nerve terminal. There was a trend of decrease in the total number of vesicles and the length of the active zone compared to WT, but it was not statistically significant. Surprisingly, when *Munc18-1* was overexpressed using viral transduction in WT, the number of total vesicles as well as the number of docked vesicles increased (Toonen et al., 2006). The theme of altered SV pool size is repeated in Classen et al. (2020), which showed enhanced replenishment of RRP after intense stimulation using primary neurons overexpressing phospho-mimetic Munc18-1. However, neurons with phospho-null Munc18-1 showed normal morphological features and electrophysiological properties (Toonen et al., 2006). In addition, there are more than one phosphorylation sites in Munc18-1 targeted by different kinases and it is not known how different phosphorylation sites affect one another (Barclay et al., 2003; Meijer et al., 2018). Thus, it is difficult to say if DYRK1A is the only kinase that regulates presynaptic scaling via Munc18-1, nor to say Munc18-1 is the only substrate of DYRK1A in regulating presynaptic activity.

Another NDD that shows dosage-dependence is Rett syndrome. Rett syndrome is an X-linked NDD caused by the loss of function of methyl-CpG-binding protein 2 (MeCP2), a transcription factor that represses transcription by binding to CpG dinucleotides (Amir et al., 1999). Chao, Zoghbi, and Rosenmund (2007) compared mouse lines with different MeCP2 dosage, -/y, +/y and *Tg1* that has double MeCP2 expression level. In culture, the authors have demonstrated that the dosage of *MeCP2* positively correlates to the amplitude and frequency of mEPSC, the size of RRP, and synaptic density. It would be interesting to measure the mEPSC of *Dyrk1a* HET neurons and the SV pool size in Rett syndrome in order to test if DYRK1A syndrome and Rett syndrome share same neuronal pathology.

Levels	Haploinsufficiency models	Redundancy models
SV recycling	↓ exocytosis ↓ ADBE ↓ recycling pool	↑ Exocytosis (C.-I. Wu et al., 2022) Slower endocytosis (Y. Kim et al., 2011) *
Molecular	↓ expression of GAD67 (Souchet et al., 2014) ↓ phosphorylation of S6 ribosomal protein (Levy et al., 2021)	↑ expression of GAD67 and vGAT1 (Souchet et al., 2014)
Cellular	Higher synaptic density (Arranz et al., 2019; Benavides-Piccione et al., 2005) Shorter neurites and simpler dendritic structure (Benavides-Piccione et al., 2005; Dang et al., 2018; Levy et al., 2021)	Shorter neurites (Dang et al., 2018) Simpler and immature dendritic structure (Martinez de Lagran et al., 2012) Delayed neuronal differentiation and migration (Dang et al., 2018; Kurabayashi & Sanada, 2013; Pons-Espinal, de Lagran, & Dierssen, 2013)
Tissue & Circuit	Decrease in brain mass (Fotaki et al., 2002; Levy et al., 2021) ↑ amplitude of oEPSC and ↓ amplitude IPSC of pyramidal neurons in hippocampus (Shih et al., 2023)	Increase in brain volume (Sebrié et al., 2008) ** ↓ gamma power in prefrontal cortex (Ruiz-Mejias et al., 2016)
Behavioral	Delayed motor development (Fotaki et al., 2002) Impaired learning and social deficits (Arranz et al., 2019; Levy et al., 2021; Raveau et al., 2018) More susceptible for seizures (Raveau et al., 2018; Souchet et al., 2014)	Motor deficits (de Lagrán et al., 2004; Souchet et al., 2014) Impaired learning (Altafaj et al., 2001)

Table 5.1: The phenotypes of *Dyrk1a* haploinsufficiency and redundancy models in literature.

Red text indicates findings reported in this thesis. * This contradicts to Marland et al. (2016), which did not see neither exocytic nor endocytic phenotype in transgenic mice (Ts65Dn).

**This is not consistent with the microcephaly symptom of Down syndrome.

Although not tested for dosage-dependence, a missense mutation in Neuroligin-3 (R451C) found in two ASD patients also share similar downscaling of the presynapse in slices (Etherton et al., 2011; Jamain et al., 2003). Etherton et al. (2011) showed that Neuroligin-3 R451C knock-in mouse have fewer SVs per boutons as well as smaller nerve terminals in adult hippocampus via electron microscopy. This aligns with the findings in STXBP1 encephalopathy model and Rett syndrome model mentioned above (Chao et al., 2007; Toonen et al., 2006). It would be very informative to quantify the number of SVs in *Dyrk1a* HET neurons, but it was not possible due to the quality of the electron microscopy images.

Although a few NDD models share the phenotypes of presynaptic downscaling, this does not apply to all the NDD models. For example, a point mutation in Synaptophysin (T198I) which leads to ID and epilepsy does not lead to defects in SV recycling when modelled in hippocampal neurons (Harper, Mancini, van Slegtenhorst, & Cousin, 2017). Another disclaimer is FXS, as *Fmr1* KO neurons showed bigger recycling pool compared to WT (Bonnycastle et al., 2022; P.-Y. Deng et al., 2011). However, the upscaling or downscaling of the presynapse could serve as a common frame for understanding different NDDs.

In summary, we showed the presynaptic downscaling *Dyrk1a* HET neurons, which is represented by a series of consistent phenotypes: dampened SV exocytosis, ADBE and smaller SV recycling pool. This is a first attempt in characterising SV recycling in a DYRK1A syndrome model, and it suggests presynaptic strength as a tool to understand the NDD pathology. I believe DYRK1A is a useful starting point in understanding NDDs because it has dosage-dependent effects. In the table 5.1, haploinsufficiency and redundancy often have inverse effects in molecular and subcellular level, but the phenotypes begin converging from the morphological level, and to behavioral level eventually. DYRK1A syndrome and Down syndrome encapsulate the mystery of the development of the nervous system, which has only just started to reveal its silhouette.

Chapter 6

Discussion

This thesis aims to contribute to our understanding of NDDs in the light of SV recycling. For this I have investigated SV recycling in three different animal lines: WT, *Fmr1* KO, which models FXS and *Dyrk1a* HET, which models DYRK1A syndrome. Using a palette of live and fixed imaging techniques, I have first characterised the developmental trajectory of SV recycling in WT primary neurons from murine hippocampal cultures. Most interestingly, the TMR-dextran uptake assay showed an activation of ADBE from the second week *in vitro*, which was simultaneous to the enhanced activity-dependent Ca^{2+} influx. Electron microscopy corroborated this finding with an increase in the number of HRP-labelled bulk endosomes internalised during the stimulation. These results highlighted ADBE as an extra landmark of presynaptic development, which comes later than the general SV recycling reported via syHy imaging.

Based on the developmental trajectory established in WT neurons, I discovered that the reduced ADBE in *Fmr1* KO neurons is due to a developmental delay, rather than a permanent defect caused by the absence of FMRP. This finding accentuates the importance of the time component when studying NDDs. Using a GST-pull down assay, I sought potential binding partners of FMRP that could be responsible for the delayed recruitment of ADBE. Dyn-1 was confirmed as a binding partner to FMRP via mass spectrometry and Western blot. Whether this is relevant to ADBE requires further experiments.

In the last results chapter, I characterised SV recycling in *Dyrk1a* HET neurons. Calcium imaging, syHy imaging, TMR-dextran uptake assay and electron microscopy showed a smaller SV recycling pool, dampened evoked exocytosis as well as reduced ADBE. The phenotypes point towards the reduced capacity of the presynapse, despite the normal activity-dependent Ca^{2+} influx. The downscaling of the presynaptic strength was surprising, as it was not seen in other NDD models with a similar ADBE phenotype. This suggests that although ADBE might be a convergent phenotype, each model could have a different pathological mechanism. Using phospho-proteomics of synaptosomal lysates, I tried to narrow down the candidate proteins that deliver the presynaptic roles of DYRK1A. The phospho-proteomics data revealed over-phosphorylation or under-phosphorylation of proteins that heavily participate in SV recycling in *Dyrk1a* HET synaptosomes.

Here, I would like to discuss how the developmental trajectory of SV recycling contributes to the understanding of neuronal development in general. Next, I would like to discuss how appreciating the time component in NDD research can contribute to therapeutic intervention. Last but not least, inspired by the presynaptic downscaling in the DYRK1A syndrome model, I would like to discuss how dissecting the mechanism behind synaptic strength can help us understand the connectivity in the brain network.

6.1 Putting ADBE in the context of neurodevelopment

Presynaptic development starts with delivery of the presynaptic proteins: active zone scaffolding proteins, ion channels and exo-/ endocytic proteins. These proteins are established to be transported in unitary packets along the microtubules in the axon via motor proteins and adaptor proteins (Dresbach et al., 2006; Rizalar et al., 2023; Sabo et al., 2006; Sabo & McAllister, 2003; Shapira et al., 2003; Tao-Cheng, 2007; Zhai et al., 2001). For example, it has been suggested recently that kinesin superfamily (KIF) 1A and its cargoes are unloaded from the microtubules when they have a higher concentration of GTP-bound tubulin, the building block of microtubules whose nucleotide can bind with either GTP or GDP (Guedes-Dias et al., 2019; J. Zhou et al., 2023). Guedes-Dias et al. (2019) implied that the GTP-tubulin functions as a stop signal during SV delivery. Nonetheless, how GTP-tubulin or GDP-tubulin are differentially distributed along the axon is still unknown. Aside from the cytoskeleton perspective, a number of trans-synaptic signalling proteins and glia-secreted proteins are reported to stabilise the presynaptic structure (Akins & Biederer, 2006; Christopherson et al., 2005; Siddiqui & Craig, 2011).

Once the precursors are delivered to a potential presynapse, the nerve terminals release neurotransmitters spontaneously without action potentials. This spontaneous exocytosis switches to evoked exocytosis as the nerve terminals mature. The predominance of spontaneous exocytosis in immature neurons has been reported in culture, in slices and *in vivo* (Andreae et al., 2012; Banerjee et al., 2022; Martini et al., 2021). It has been elucidated that the spontaneous activity serves the function of guiding postsynaptic contacts and circuit refinement (Andreae & Burrone, 2015, 2018; Choi et al., 2014; Faust et al., 2021; Garcia-Bereguain et al., 2016; Martini et al., 2021; Sutton et al., 2004). The time window that shows the transition from spontaneous to evoked exocytosis in culture corresponds to the heightened activity-dependent Ca^{2+} influx and the recruitment of ADBE. It is known that the Ca^{2+} influx is required for ADBE, but whether the absence of ADBE in younger neurons is solely due to the insufficient Ca^{2+} remains to be confirmed (Morton et al., 2015). The change in the activity-dependent Ca^{2+} influx could be due to the compositional change of VGCC subtypes, a change in the distribution of the VGCCs, or spatial tightening of Ca^{2+} -sensing topography, which is reported around the second postnatal week (Bornschein et

al., 2019; Fedchyshyn & Wang, 2005; Schlick et al., 2010; Scholz & Miller, 1995). It is also possible that the small Ca^{2+} fluctuations at the presynapse cause spontaneous exocytosis in young neurons, whose amplitude is not large enough to require ADBE. In this scenario, the disappearance of spontaneous activity can be explained by the tighter Ca^{2+} coupling, which allows desensitisation towards small Ca^{2+} fluctuations. This scenario combines different aspects of presynaptic development, but the causality of the sequence needs to be tested.

Other landmark transitions that happen in parallel to the ones mentioned above are GABA shift and desynchronisation. Around P10 in rodents, GABA signalling changes from excitatory to inhibitory due to the expression of KCC2 (Rivera et al., 1999). There are many theories that could explain this shift, both extrinsic and intrinsic, but an agreement has not yet been made (Peerboom & Wierenga, 2021). In culture, there are contradicting reports on the existence of GABA shift (Ganguly et al., 2001; Kato-Negishi et al., 2004). On the other hand, Rochefort et al. (2009) and Golshani et al. (2009) showed desynchronisation of neurons in the visual and the barrel cortex *in vivo* around P14. This desynchronisation was independent of sensory input, as the sensory deprivation did not affect the desynchronisation. Some literature suggests that the inhibitory GABA signalling promotes network desynchronisation, but more evidence is needed to link the two (Burman, Brodersen, Raimondo, Sen, & Akerman, 2023; Manseau et al., 2010).

In summary, our next challenge is to connect our fragmented understanding of neuronal development and use it to enhance our understanding in circuit development. The new technology will accelerate this process, as the super-resolution fluorescence microscopy is coming close to providing a synapse-level resolution in tissues (Linsley et al., 2019; Michalska et al., 2024).

6.2 Through the hourglass: time-dependence in NDDs

The majority of the NDDs are lifelong conditions, although some symptoms improve with age (Antolini & Colizzi, 2023). However, a number of animal models that phenocopy clinical NDD cases showed transient phenotypes on the circuit, cellular and molecular level, which were apparent during the development but not in adulthood. Among NDDs one such case is FXS. The results in this thesis showed that the recruitment of ADBE is delayed in *Fmr1* KO neurons at DIV 14, but not at DIV 21. The Chattarji group showed that spontaneous exocytosis was exaggerated at DIV 13-15 in the same model (Subrahmanyam et al., 2021). These two findings *in vitro* point to the conclusion that the *Fmr1* KO neurons at DIV 14 are less mature than WT. In slices, the transient phenotypes were often reported within the first two postnatal weeks. Harlow et al. (2010), Banke and Barria (2020) and Cruz-Martín et al. (2010) showed upregulated receptor expression and higher protein turnover at the postsynapse in the first week, which was recovered in the second week. The GABA shift that usually takes place around P10 in WT mice was delayed to P15 in *Fmr1* KO, although this finding is still disputed

(He et al., 2014; Y. J. Song et al., 2022). Furthermore, defects in the plasticity of the auditory cortex disappeared in a similar timeframe (H. Kim, Gibboni, Kirkhart, & Bao, 2013). Gonçalves et al. (2013) has shown that the neurons are hyper-synchronised in the barrel cortex, although the phenotype was mitigated, not rescued at a later age.

Aside from FXS, data from other monogenic NDD models also highlight the importance of the protein of interest at the early ages of development. For example, Clement et al. (2013) deleted one of the *Syngap1* alleles using genetic engineering at two different timepoints, P1 and P60. The animals whose *Syngap1* haploinsufficiency was driven at P1 phenocopied *Syngap1*^{+/-}, whereas the other group, which experienced haploinsufficiency in adulthood, did not have any significant change in their AMPA/NMDA ratio. Similarly, in Angelman syndrome, deletion of *UBE3A* at the embryonic stages phenocopied animals harbouring *UBE3A* haploinsufficiency, whereas deletion at the third postnatal week or later did not have any significant effect. However, not every NDD gene shares the same trend of the heavier affliction the earlier the ablation. For example, the electrophysiological defects in Rett syndrome could be rescued even when MeCP2 expression level was corrected after appearance of symptoms (20-42 weeks) (Guy, Gan, Selfridge, Cobb, & Bird, 2007).

In a nutshell, these studies mentioned above emphasise the importance of a critical window, or sensitive period, when the nervous system is more susceptible to change (Dehorter & Del Pino, 2020; LeBlanc & Fagiolini, 2011; Meredith et al., 2012). Depending on the brain region and the circuit, the exact timeframe and the number of this period can be variable (Dehorter & Del Pino, 2020). The time-course studies provide us with an insight that the lifelong behavioural and cognitive deficits in NDDs might arise from transient cellular phenotypes, which reduces the concept of a sensitive period in psychology to a neuronal critical window in cell biology. How such transient changes in the pre- and post-synaptic level during the neuronal critical window make subtle but significant changes in the circuit that are powerful enough to lead to lifelong conditions, would be the next key question to answer.

The reason why understanding the sensitive period is so important is because it provides a guideline for clinical intervention (Hadders-Algra, 2021; Marín, 2016). Indeed, meta-analysis of reports on early intervention in children with ASD showed a decrease in the effectiveness in social communication past the age of 4 (Fuller & Kaiser, 2020). For more effective diagnosis and intervention, it is critical to determine why the sensitive period is so vital, based on the molecular and physiological mechanisms.

6.3 Presynaptic component in the synaptic strength

Synaptic strength is a deterministic factor in the connectivity between two neurons, and therefore between different brain regions. It is influenced by post- and presynaptic elements, which include the number of SVs fused to the plasma membrane, the number of postsynaptic receptors and their kinetics. Both the presynaptic and postsynaptic strength are subject to change, which is an essential mechanism behind brain plasticity (Sejnowski, 1999).

Postsynaptic plasticity in short-term can be achieved by the desensitisation or saturation of the receptors potentially based on the spatial distribution of the receptors (Blitz, Foster, & Regehr, 2004; Polenghi et al., 2020). Postsynaptic plasticity in long-term, on the other hand, first came to light with the discovery of LTP and LTD, the phenomenon that refers to activity-dependent change in the neuron's excitability (Kombian & Malenka, 1994). One of the key mechanisms was revealed to be the insertion and retrieval of AMPA receptors from the postsynaptic membrane (Kessels & Malinow, 2009). Transcription factors were also discovered as a major driver of postsynaptic plasticity, including cAMP response element-binding protein (CREB) (Alberini, 2009). LTD was further highlighted when hippocampal neurons from FXS showed exaggerated LTD, which developed into mGluR theory (Bear et al., 2004; Huber et al., 2002; Lüscher & Huber, 2010; Pfeiffer & Huber, 2009).

Presynaptic plasticity, however, is relatively less explored. There are reports showing short-term and long-term plasticity at the presynapse and that presynaptic plasticity is affected by the size of the RRP (Monday, Younts, & Castillo, 2018; Regehr, 2012; Stanton, Winterer, Zhang, & Müller, 2005). Recently, Monday et al. (2022) has revealed the local translation regulated by FMRP at the presynapse, implying that there could be translation-dependent long-term plasticity at the presynapse too. However, how much the local translation at the presynapse accounts for the plasticity is an open question, as presynaptic proteins are dominantly translated from the soma (Glock et al., 2021).

Although it is posited that the highly dynamic aspect of the synapse derives from the plasticity of the postsynapse, there is growing evidence that shows that the presynapse maintains homeostasis in compensation to perturbations in neurotransmission (i.e., blockade of postsynaptic receptors), which was labelled "presynaptic homeostatic plasticity" (Davis & Müller, 2015; Nair, Muttathukunnel, & Müller, 2021). For example, Goel et al. (2020) produced hyper-innervated NMJs in *Drosophila*, and reported that hyper-innervation results in a decrease of release probability in compensation to the hyper-connectivity. It is possible that the same is the case in *Dyrk1a* HET neurons, which raises a suspicion for postsynaptic upscaling. It would be of great importance to find out whether the total synaptic strength is disrupted, and whether there was compensation between the pre- and postsynaptic components in order to find out the source of functional disruption. Currently, there are three different perspectives explaining the presynaptic homeostatic plasticity. First, the presence of presynaptic neurotransmitter receptors indicate a local feedback system within the presynapse (Lovinger et al., 2022; Pinheiro

& Mülle, 2008). It was shown that GABA_BRs are recruited at the presynaptic terminals after NMDAR activation (Hannan, Gerrow, Triller, & Smart, 2016). The presence of a glutamate-gated chloride channel in axon in *Drosophila* also indicates an autocrine feedback system (X. Li et al., 2021). The second theory is via the trans-synaptic signalling pathway. Chipman et al. (2022) and Orr, Fetter, and Davis (2022) showed that the postsynaptic receptors or trans-synaptic CAMs are necessary for presynaptic potentiation. Lastly, the structural change of the active zone has been suggested as another mechanism behind presynaptic plasticity. Mrestani et al. (2021) has shown that the active zone proteins are distributed more densely after inducing presynaptic homeostatic plasticity, and Müller et al. (2022) has related this to the phosphorylation of RIM at the active zone. However, it is worth noting that presynaptic plasticity is not unanimously agreed upon. There is a possibility that the homeostatic response alters the number of connections, rather than the individual synaptic strength, which was shown by pharmacological blocking of circuit activity and via computational modelling (Bartley, Huang, Huber, & Gibson, 2008; J. Kim & Tsien, 2008; Sukenik et al., 2021).

In chapter 5, I showed that *Dyrk1a* HET neurons have weaker presynaptic strength, which was reported as reduced SV exocytosis and smaller SV pools. Whether this is a presynapse-autonomous deficit or compensation for a postsynaptic deficit is unknown. It is of prime interest to locate the presynaptic downscaling in the context of the whole synapse, and eventually in the context of connectivity in order to explain the pathological mechanisms of NDDs.

Appendix A

Statistical report

A.1 Statistical report for WT data

A.1.1 Calcium imaging

Table A.1: Statistics for figure 3.1E.

Group	Mean $\pm$ SEM	No. of coverslips/ No. of preps	Comparison	p value
DIV 3	6.632 $\pm$ 1.209	9/3	N/A	N/A
DIV 7	7.445 $\pm$ 0.888	10/3	DIV 7 vs. DIV 3	0.9781
DIV 10	8.179 $\pm$ 0.935	12/3	DIV 10 vs. DIV 3	0.8055
DIV 14	11.370 $\pm$ 1.589	12/3	DIV 14 vs. DIV 3	0.0459
DIV 21	11.690 $\pm$ 1.360	13/3	DIV 21 vs. DIV 3	0.0268

One-way ANOVA (DF = 4, F = 3.340, p = 0.2047), Dunnett's multiple comparisons test.

Table A.2: Statistics for figure 3.1F.

Group	Mean $\pm$ SEM	No. of coverslips/ No. of preps	Comparison	p value
DIV 3	5.608 $\pm$ 3.259	8/3	N/A	
DIV 7	6.837 $\pm$ 1.892	10/3	DIV 7 vs. DIV 3	>0.9999
DIV 10	3.711 $\pm$ 0.391	12/3	DIV 10 vs. DIV 3	>0.9999
DIV 14	3.683 $\pm$ 0.5109	12/3	DIV 14 vs. DIV 3	>0.9999
DIV 21	4.629 $\pm$ 1.084	12/3	DIV 21 vs. DIV 3	>0.9999

Kruskal-Wallis test (DF = 4, H = 2.938, p = 0.5683), Dunn's multiple comparison test.

Table A.3: Statistics for figure 3.2B.

Group	Mean $\pm$ SEM	No. of coverslips/ No. of preps
DIV 3	14.591 $\pm$ 26.408	12/3
DIV 7	20.622 $\pm$ 8.615	12/3
DIV 10	1.066 $\pm$ 0.843	15/3
DIV 14	2.942 $\pm$ 1.364	14/3
DIV 21	0.189 $\pm$ 0.130	15/3

A.1.2 SypHy imaging

Table A.4: Statistics for figure 3.3F.

Group	Mean $\pm$ SEM	No. of coverslips/ No. of preps	Comparison	p value
DIV 3	0.282 $\pm$ 0.051	6/4	N/A	N/A
DIV 7	0.308 $\pm$ 0.049	11/5	DIV 7 vs. DIV 3	>0.9999
DIV 10	0.355 $\pm$ 0.035	6/3	DIV 10 vs. DIV 3	>0.9999
DIV 14	0.443 $\pm$ 0.045	10/4	DIV 14 vs. DIV 3	0.1639
DIV 21	0.437 $\pm$ 0.074	6/4	DIV 21 vs. DIV 3	0.3789

Kruskal-Wallis test (DF = 4, H = 8.373, p = 0.0788), Dunn's multiple comparison test.

Table A.5: Statistics for figure 3.3H.

Group	Mean $\pm$ SEM	No. of coverslips/ No. of preps	Comparison	p value
DIV 3	0.495 $\pm$ 0.237	6/4	N/A	N/A
DIV 7	0.049 $\pm$ 0.172	11/5	DIV 7 vs. DIV 3	0.6901
DIV 10	0.259 $\pm$ 0.096	6/3	DIV 10 vs. DIV 3	>0.9999
DIV 14	0.248 $\pm$ 0.074	10/4	DIV 14 vs. DIV 3	>0.9999
DIV 21	0.260 $\pm$ 0.065	6/4	DIV 21 vs. DIV 3	>0.9999

Kruskal-Wallis test (DF = 4, H = 20.86, p = 0.7210), Dunn's multiple comparison test

Table A.6: Statistics for figure 3.3I.

Group	Mean $\pm$ SEM	No. of coverslips/ No. of preps	Comparison	p value
DIV 3	0.565 $\pm$ 0.168	6/4	N/A	N/A
DIV 7	0.880 $\pm$ 0.335	11/5	DIV 7 vs. DIV 3	>0.9999
DIV 10	1.815 $\pm$ 0.491	6/3	DIV 10 vs. DIV 3	0.171
DIV 14	2.049 $\pm$ 0.250	10/4	DIV 14 vs. DIV 3	0.012
DIV 21	3.001 $\pm$ 0.630	6/4	DIV 21 vs. DIV 3	0.004

Kruskal-Wallis test (DF = 4, H = 26.15, p = 0.0003), Dunn's multiple comparison test

A.1.3 TMR-dextran uptake assay

Table A.7: Statistics for figure 3.4C.

Group	Mean $\pm$ SEM	No. of coverslips/ No. of preps	Comparison	p value
DIV 3	11.88 $\pm$ 5.97	7/3	N/A	N/A
DIV 7	30.04 $\pm$ 10.33	7/3	DIV 7 vs. DIV 3	>0.9999
DIV 10	51.28 $\pm$ 17.18	6/3	DIV 10 vs. DIV 3	>0.9999
DIV 14	271.50 $\pm$ 43.14	7/3	DIV 14 vs. DIV 3	0.0009
DIV 21	281.50 $\pm$ 37.44	8/3	DIV 21 vs. DIV 3	0.0004

Kruskal-Wallis test (DF = 4, H = 26.15, p < 0.0001), Dunn's multiple comparison test

Table A.8: Statistics for figure 3.4D.

Group	Mean $\pm$ SEM	No. of coverslips/ No. of preps	Comparison	p value
DIV 3	258.7 $\pm$ 30.1 $\pm$	6/3	N/A	N/A
DIV 7	559.6 $\pm$ 56.9	7/3	DIV 7 vs. DIV 3	0.1849
DIV 10	803.0 $\pm$ 126.0	6/3	DIV 10 vs. DIV 3	0.0005
DIV 14	976.6 $\pm$ 136.0	8/3	DIV 14 vs. DIV 3	<0.0001
DIV 21	1099.0 $\pm$ 179.4	7/3	DIV 21 vs. DIV 3	0.0086

One-way ANOVA (DF = 4, F = 7.141, p = 0.0004), Dunnett's multiple comparisons test

Table A.9: Statistics for figure 3.4J

Group	Mean $\pm$ SEM	No. of coverslips/ No. of preps	Comparison	p value
DIV 3	0.057 $\pm$ 0.032	7/3	N/A	N/A
DIV 7	0.019 $\pm$ 0.005	7/3	DIV 7 vs. DIV 3	>0.9999
DIV 10	0.057 $\pm$ 0.016	6/3	DIV 10 vs. DIV 3	>0.9999
DIV 14	0.359 $\pm$ 0.121	7/3	DIV 14 vs. DIV 3	0.0139
DIV 21	0.248 $\pm$ 0.033	8/3	DIV 21 vs. DIV 3	0.0085

Kruskal-Wallis test (DF = 4, H = 25.32, p < 0.0001), Dunn's multiple comparison test

A.1.4 HRP-uptake assay and electron microscopy

Table A.10: Statistics for figure 3.5F

Group	Mean $\pm$ SEM	No. of coverslips/ No. of preps	Comparison	p value
DIV 3	1.053 $\pm$ 0.397	4/2	N/A	N/A
DIV 7	1.855 $\pm$ 0.268	6/2	DIV 7 vs. DIV 3	>0.9999
DIV 10	2.261 $\pm$ 0.206	6/2	DIV 10 vs. DIV 3	0.2656
DIV 14	3.101 $\pm$ 0.290	7/2	DIV 14 vs. DIV 3	0.0025
DIV 21	2.528 $\pm$ 0.270	6/2	DIV 21 vs. DIV 3	0.0604

Kruskal-Wallis test (DF = 4, H = 14.14, p = 0.0069), Dunn's multiple comparison test

A.1.5 CHC knockdown with syHy imaging

Table A.11: Statistics for figure 3.6B and D

Panel	Group	Mean $\pm$ SEM (CS/preps)	Comparison	p value
B	shScr	0.197 $\pm$ 0.058 (4/3)	Mann Whitney test	0.927
	shCHC	0.162 $\pm$ 0.024 (7/3)		
D	shScr	0.250 $\pm$ 0.085 (4/3)	Unpaired t test	0.042
	shCHC	-0.094 $\pm$ 0.097 (7/3)		

B: U = 13, D: t = 2.369, DF = 9. CS: coverslips.

A.2 Statistical report for *Fmr1* KO data

A.2.1 TMR-dextran uptake assay

Table A.12: Statistics for figure 4.1B

Genotype	Group	Mean $\pm$ SEM	No. of coverslips/ No. of preps
WT	DIV 7	10.54 $\pm$ 11.39	6/3
	DIV 10	40.94 $\pm$ 10.64	5/2
	DIV 14	203.67 $\pm$ 26.77	10/3
	DIV 21	195.93 $\pm$ 47.73	10/3
KO	DIV 7	16.86 $\pm$ 5.58	7/3
	DIV 10	21.28 $\pm$ 8.37	6/2
	DIV 14	117.01 $\pm$ 26.45	9/3
	DIV 21	136.32 $\pm$ 29.53	11/3

Table A.13: Statistics for figure 4.1C

Graph	Genotype	Mean $\pm$ SEM (CS/preps)	Comparison	p value
Left (Dextran)	WT	203.67 $\pm$ 26.77 (10/3)	Unpaired <i>t</i> test	0.0348
	KO	117.01 $\pm$ 26.45 (9/3)		
Right (Immuno)	WT	489.8 $\pm$ 57.6 (10/3)	Unpaired <i>t</i> test	0.6536
	KO	454.0 $\pm$ 53.2 (11/3)		

Left: $t = 2.294$, $DF = 17$, Right: $t = 0.4560$, $DF = 19$. CS: coverslips

A.3 Statistical report for *Dyrk1a* HET data

A.3.1 TMR-dextran uptake assay

Table A.14: Statistics for figure 5.1B and D.

Panel	Genotype	Mean $\pm$ SEM (CS/preps)	Comparison	p value
B	WT	369.3 $\pm$ 60.40 (18/6)	Unpaired <i>t</i> test	0.0031
	HET	209.2 $\pm$ 17.85 (31/6)		
D	WT	702.6 $\pm$ 153.6 (8/3)	Mann-Whitney test	0.9654
	HET	686.5 $\pm$ 136.6 (10/3)		

B: $t = 3.116$, $DF = 47$, D: $U = 39$. CS: coverslips

A.3.2 HRP-uptake assay and electron microscopy

Table A.15: Statistics for figure 5.2B.

Genotype	Mean $\pm$ SEM	Coverslips / Preps	Comparison	p value
WT	3.299 $\pm$ 0.184	15/4	Unpaired <i>t</i> test	0.0265
HET	2.740 $\pm$ 0.150	15/4		

$t = 2.329$, DF = 31.

A.3.3 SyPhy imaging

Table A.16: Statistics for figure 5.3C and E.

Panel	Genotype	Mean $\pm$ SEM (CS/preps)	Comparison	p value
C	WT	0.502 $\pm$ 0.072 (8/4)	Unpaired <i>t</i> test	0.0465
	HET	0.346 $\pm$ 0.024 (9/4)		
E	WT	0.054 $\pm$ 0.115 (8/4)	Unpaired <i>t</i> test	0.8884
	HET	0.097 $\pm$ 0.073 (9/4)		

C: $t = 2.170$, DF = 15, E: U = 34. CS: coverslips

Table A.17: Statistics for figure 5.4B and C.

Panel	Genotype	Mean $\pm$ SEM (CS/preps)	Comparison	p value
B	WT	7.599 $\pm$ 0.9437 (10/3)	Unpaired <i>t</i> test	0.0155
	HET	4.341 $\pm$ 0.7707 (10/3)		
C	WT	40.33 $\pm$ 2.932 (10/3)	Unpaired <i>t</i> test	0.0045
	HET	26.29 $\pm$ 3.179 (10/3)		

C: $t = 2.170$, DF = 15, E: U = 34. CS: coverslips

A.3.4 Western blot

Table A.18: Statistics for figure 5.5B and C.

Panel	Genotype	Mean $\pm$ SEM (CS/preps)	Comparison	p value
B	WT	1.000 $\pm$ 0.1018 (10/5)	Unpaired <i>t</i> test	0.4459
	HET	1.190 $\pm$ 0.2216 (10/5)		
C	WT	1.000 $\pm$ 0.2238 (5/3)	Unpaired <i>t</i> test	0.9950
	HET	1.002 $\pm$ 0.2373 (5/3)		

B: $t = 0.7793$, DF = 18, C: $t = 0.006519$, DF = 8. CS: coverslips

Table A.19: Statistics for figure 5.5E.

Group	Condition	Mean $\pm$ SEM	No. of coverslips/ No. of preps
WT	No stim	1.000 $\pm$ 0.224	5/3
	Stim	1.057 $\pm$ 0.285	5/3
HET	No stim	1.002 $\pm$ 0.237	5/3
	Stim	0.783 $\pm$ 0.143	5/3

2-way ANOVA, Genotype effect: $p = 0.6670$, Stimulation effect: $p = 0.4995$.

A.3.5 Calcium imaging

Table A.20: Statistics for figure 5.6C.

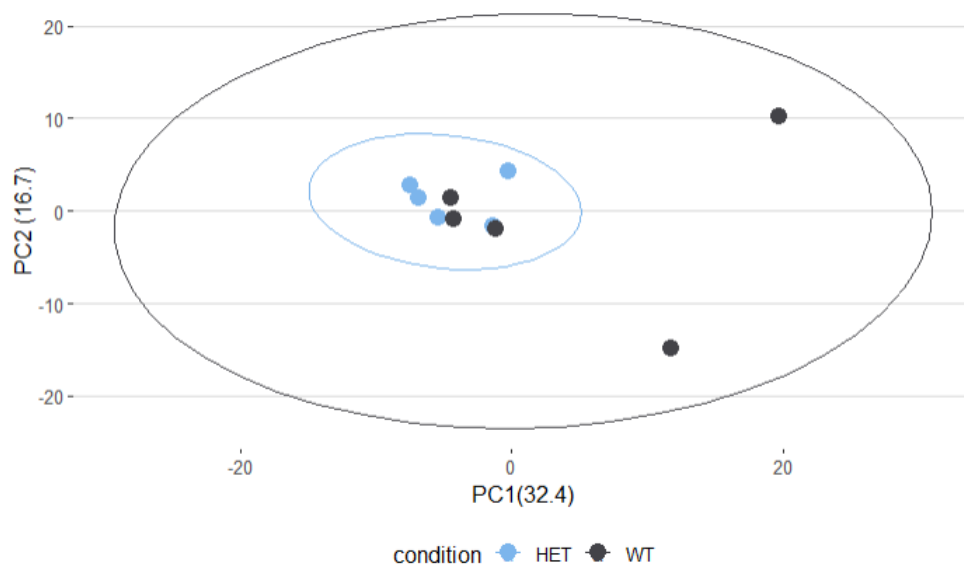
Genotype	Mean $\pm$ SEM	Coverslips/ preps	Comparison	p value
WT	0.359 $\pm$ 0.132	7/2	Mann-Whitney test	0.3357
HET	0.439 $\pm$ 0.081	8/2		

$U = 19$.

DYRK1A Proteomics and Phospho-proteomics data

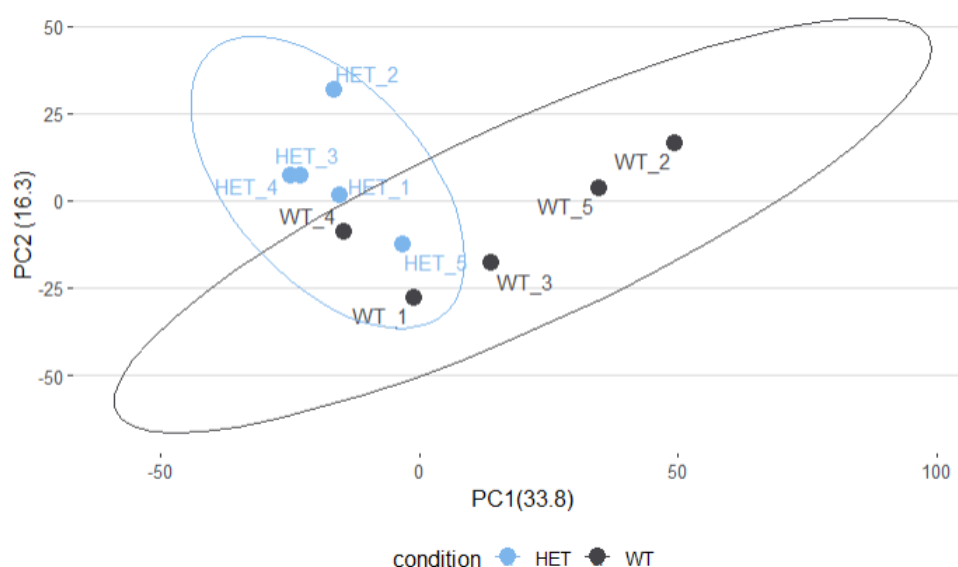
B.1 Validation of synaptosomal preparation

Figure B.1: Validation of *DYRK1A* HET and WT synaptosomes for proteomics analysis.



Five synaptosome samples were prepared from five *Dyrk1a* HET and WT animals each, for both proteomics and phospho-proteomics analysis. The PCA plot shows consistent properties across individuals, except for two WT samples. This graph was made by Dr Andrew Frey from Newcastle University.

Figure B.2: Validation of *DYRK1A* HET and WT synaptosomes for phospho-proteomics analysis



The PCA plot shows consistent properties across individuals in phospho-proteomics analysis. This graph was made by Dr Andrew Frey from Newcastle University.

B.2 Proteomics data

Table B.1: Over-expressed proteins in *Dyrk1a* HET synaptosomes

Genes	log ₂ FC	p value
Ciao1	2.078353232	0.012151229
Cdk18	1.840258867	0.001833831
Rab28	0.942606223	0.034217677
Arl2bp	0.828979724	0.006157711
Clic6	0.660986564	0.007139853
Eif1ad	0.643503511	0.001880308

Fold change threshold > 1.5 fold

Table B.2: Under-expressed proteins in *Dyrk1a* HET synaptosomes

Genes	log ₂ FC	p value
Gng7	-0.418845302	0.021195543
Acox3	-0.422335888	0.014531746
Pcsk1	-0.424786673	0.020681662
Cuta	-0.425788763	0.037353728
Slc5a11	-0.427131509	0.003880941
Slc44a1	-0.42728997	0.01771204

B2m	-0.429330552	0.01175379
Pcp4	-0.431533438	0.006522874
Timm10b	-0.432839522	0.002348219
Slco3a1	-0.433980799	0.015308313
Ggps1	-0.435481359	0.019709717
Grn	-0.437998144	3.50E-05
Plip	-0.441653819	0.014550263
Pex19	-0.445880366	0.041065244
Hmgcr	-0.446021307	0.031008328
Cyb5d2	-0.450439272	0.018973912
Hsd11b1	-0.45916769	0.004587467
Fbxo9	-0.462063748	0.001568573
Tcn2	-0.475041998	0.008268897
Cartpt	-0.480048264	0.022002505
Gtf2i	-0.49020207	0.017060069
Mapkapk3	-0.497315098	0.016735736
Ninj2	-0.501020565	0.046910419
Gjb1	-0.50672382	0.005429545
Pdyn	-0.507355748	0.004705837
Mtpn	-0.519283903	0.004531303
Timm8a	-0.523087692	0.005826429
Neu3	-0.524516733	0.003864232
Slc6a5	-0.525645657	0.031727212
Ikbkg	-0.530025575	0.018743731
Tor2a	-0.53075687	0.015999041
Ldlrap1	-0.534352366	0.049055203
Qprt	-0.539082241	0.003687391
Nup58	-0.542830308	0.036656338
Trim28	-0.612131946	0.031997487
Pkia	-0.631557644	0.002817109
Cdh20	-0.636814794	0.046984585
Map1lc3b	-0.645844907	0.026313731
Prph	-0.670384831	0.030422522
Rnf34	-0.68366893	0.004737532
Slc38a2	-0.691199706	0.025536209
Nqo2	-0.695758428	0.002795517
Mblac1	-0.723930998	0.047684169

Cul7	-0.725488412	0.030570837
Gramd2b	-0.736235964	0.039870305
Slc13a5	-0.758650352	0.006444192
Hdac1	-0.79497641	0.00801527
Snrnp200	-0.816951443	0.034077916
Krt18	-0.868549021	0.016149833
Myl3	-0.881721366	0.005069375
Hnrnpm	-0.92731345	0.04458578
Cyp4b1	-0.939794981	0.042994729
Ube4a	-1.464338251	0.019060125
Mybbp1a	-1.577248782	0.005239392
Sstr3	-1.585431177	0.001355909
Mx2	-1.746352615	0.007493546
Nono	-1.75780619	0.006250048

Fold change threshold < 0.75 fold

B.3 Phospho-proteomics data

Table B.3: Over-phosphorylated proteins in *Dyrk1a* HET synaptosomes

Genes	Peptide sequence	log ₂ FC	p value
Ndrp2	LSRsRtASLtSAASIDGSR	2.871448206	0.002836471
Sv2a	DEEEGGASSDAtEGHDEDEIYEGE	2.723796045	0.004131756
Dlgap1	AMGDEDSGSDSTsPKPSPK	2.715149529	0.001651178
Gja1	MGQAGStISNSHAQPFDFPDDNQNA	2.177395857	0.016967313
Taok1	STSVTSQIsNGSHMSYT	2.077206817	0.000195475
Ptges3	MGGDEDVDLPEVDGADDDSQDsDDEK	2.075542172	0.028216407
Dnb1	LsSPVLHR	1.956301773	0.041374564
Palm	EENQtGPTTtPsDTQDLDMK	1.867096122	0.034499807
Git1	DLDDQHDYDsVAsDEDTDQEPLPSAG	1.841555937	0.000830605
Unc5a	HTLHHsSPTSEAEDFVSR	1.769789763	7.74E-05
Prkar2b	sIEIPAGLTELQGFVEVLR	1.635667987	0.038886951
Dlgap2	TTSKPLISVTAQSSTESTQDAYQDSR	1.634947104	0.002135711
Stmn3	SPSDLSPESPVLSSPPK	1.589580687	0.000939734
Camkv	SATPATDGSVTPATDGSItPATDGS	1.586764679	0.003095281
Ablim2	TSSSIVSVPASsTSGSPSR	1.571368182	0.000665876

Epb41l1	GACsTPELPQFESVK	1.515380208	0.012752645
Map1b	IAELEEEERSQGsTSNsDWMK	1.495450618	0.002833462
Ppp1r2	EQEsSGEEDNDLSPEER	1.491590907	0.000946445
Mapt	SGERSGYsPGSPGtPGSR	1.488711749	0.002188412
Trim2	TGNAYLTAELSTPDGsVADGEILDNK	1.484953712	0.00477849
Ncdn	AEEASDISQQVANLAIstPTTPGPAWP	1.478325987	0.023523129
Fgf12	EPsLHEIGEK	1.477710211	0.009209694
Nfasc	EDPKEEDGSFDySDEDNKPLQGSQTS	1.464644722	0.011952679
Lrrc7	GDEDFQsDSDSFNPTLWEEQR	1.444395903	0.008222646
Pdap1	KSLDSDEsEDEDDDYQQK	1.43284863	0.000862234
Plcb1	NTYSDSSSVFEPSSPGAGEADtEsDDD	1.36875228	0.013293009
Ric8b	GDNWYSEDEDtDTEEYK	1.355785483	0.000183436
Adra2a	DGDALDLEESsssEHAERPQGPgKPER	1.35476773	0.017129178
Apba1	LHHYDERSDGEsDsPEKEAEFAPYPR	1.347055076	0.001005637
Epb41l1	DKSDsETEGLVFAR	1.346852071	0.016925123
Mapt	AKTDHGAEIVyKsPVVSGDTSPR	1.305821623	0.000955783
Dync1i2	SVSTPSEAGSQDsGDGAVGSR	1.287633852	0.000626395
Rplp0	VEAKEESEEsDEDMGFLFD	1.279794313	0.01408238
Bsn	HsDSGSDSKHEASASSSAAAAAAR	1.276400452	0.003693948
Pclo	SSCSEYSPSIEsDPEGFEISPEK; SSCSEYSPSIEsDPEGFEIsPEK	1.251136351	0.0384383
Dlgap2	TTSKPLISVTAQsStESTQDAYQDSR	1.245376544	0.01761725
Lrrc7	TPsPFEDR	1.239545706	0.013623447
Sgip1	WVHFSDASPEHVtPELTPR	1.236751037	0.041353906
Dmd	sVTELGDsLK	1.220354657	0.006246293
Ndr3	SRTHSTssSIGSGESPFSR	1.218282779	0.000540094
Syn1	QAsISGPAPPK	1.205655161	0.003811519
Map1a	TEATQGLDYVPSAGTISPTSsLEEDK	1.188063411	0.029579137
Ogfr1	CDSTWQtDsEPEPEQPGPAGGGEGQE	1.183771285	0.013254446
Myo18a	SRDESASETstPsEHSAAPSPQVEVR	1.183485764	0.003130929
Rasgrf1	KPTSDILKEESDDDQsDVDDTEVsPPtPK	1.178014266	0.000299052
Pclo	ASYDSVEDsSEsENsPVVR	1.174421203	0.000832594
Srcin1	GSEPETPSEKIEGSNGAAtPSAPVCGS	1.167560296	0.012534275
Itpka	RYsWVQLAGHTGSFK	1.165217743	0.042112132
Ncl	KEDsDEDEDEDEDDSDDEDEDEDE	1.155542317	0.009438307
Syngap1	SSPAYCTsSDITEPEQK	1.134214228	0.000160198
Bsn	TLPASTPSYTSgTSPTSLsSLEEDSDS	1.131966353	0.016839099

Snap91	sSPATTVTsPNSTPAK	1.120892917	0.002704753
Septin4	LDPYDSsEDDKEYVGFATLPNQVHR	1.115297773	0.00278874
Dclk1	VCSsMDENDGPGEESDEGFQIPATITE	1.097303388	0.03924726
Ppfia4	LLsPVSR	1.093601906	0.024158593
Scn2a	GSLFsPR	1.084745657	0.002314413
Pclo	SHSSQNSQQsPKPSVIK	1.082465175	0.006755451
Bsn	SHGPLLPTIEDsSEEEELREEEELLR	1.073854124	0.018195256
C3	AFYEHAPKQtFsAEFEVK	1.073492866	0.009516304
Pa2g4	sGEDEQQEQTIAEDLVVTK	1.071368167	0.043649261
Ablim2	RLDVEDSsFDQDSR	1.044603335	0.009567512
Dpysl2	TVTPASsAKTsPAK	1.041353646	0.004137624
Mtfr1l	NAsVPNLR	1.029840666	0.000435191
Map1a	DYVPSAGtISPTSSLEEDKGFKsPPCEDF	1.028031147	0.019200137
Hnrnpk	EGLQLPSPtATSQLPLESDAVECLN	1.026559817	0.018606277
Synpo	LYNAPVQDSLQPTAVsPTYSSDISPV	1.022995557	0.027198111
Syn1	DASPGRGsHSQTPSPGALPLGR	1.015337513	0.014804149
Dlgap4	AMGDEDSDEsGGGSPKPSPK	1.007392257	0.020677488
Kalrn	DGVEDGDSQGDGsSQPDTISIASR	1.00724053	0.035520816

Fold change threshold > 2 fold; Overlapping peptide sequences were excluded. Small letters indicate detected phosphorylation site.

Table B.4: Under-phosphorylated proteins in *Dyrk1a* HET synaptosomes

Genes	Peptide sequence	log ₂ FC	p value
Map2	PPAHEAGySTLAQSYTSDHPSEL	-2.965991045	0.000336575
Rmdn3	sQSLPNSLDYAQTSEIR	-2.400159706	0.023477658
Necap1	VTIPPPSSSVAISNHVtPPPIPK	-2.039950864	0.000291457
Caskin1	AHGtPPPVSPPKPPPTAPK	-2.031258142	0.000487404
Grin2b	QSVMNSTAtMNNTHSNILR	-2.02204159	0.001002379
Mapt	NVSSTGSIDMVDsPQLATLADEVSA	-1.958544907	0.028486184
Sptan1	KLDPAQsASRENLLLEEQGSIALR	-1.888079218	0.000323254
Ppp1cb	YQYGGLNSG RPVt PPR	-1.846949406	0.000463755
Map1a	GHWDDGtINDSDLEK	-1.76228153	0.035864956
Mfn2	QVStAMAEIR	-1.738203745	0.000422866
Cend1	NNHsNLKPAPTVPAAAPSSPDT	-1.703052668	0.00662417
Dlg4	RySPVAK	-1.67642462	0.00909257
Nefh	IGFGPsPFSLTEGLPK	-1.671494669	0.00148229

Plppr4	PGSSQLVHIPEETQENIS ^t SPK; VSIQSRPGSSQLVHIPEETQENIS ^t SPK	-1.640664818	0.014381625
Map1a	KPSPFLsPSGDHEANGPGETSLNPP	-1.577528558	0.004264874
Dlgap3	TVHTQGQWAYR	-1.562561262	0.000355677
Map1b	NYAYQKPES ^t TESPDEEDYDYESHEK	-1.506186922	0.006863777
Camk2d	ENIPTPALEPQ ^t TVIHNP ^t DGNKESTES	-1.467811695	0.024751878
Pde4d	LMHSSsLTNSCIPR	-1.4548122	0.020729745
Kif5c	AHSAQIAKPIRPGHYPA ^s SPTAVHAVR	-1.446772153	0.003537267
Gnas	AHLRPPsPEIQVADPPTPR	-1.445759441	0.000538661
Zdhhc5	LLPTGPPHREPsPVR ^y DNLSR	-1.44003721	0.00313963
Mbp	NIVTPR ^t PPPSQ GK	-1.428247982	0.025635722
Gap43	SATTDAA ^t ATSPKAE ^t EPsKAGDAPSEEK	-1.414684229	0.02682062
Nefh	RPADIRsPEQVK	-1.413314045	0.011434382
Chrna4	APPQHLPLsPALTR	-1.406193316	0.007963657
Nefm	EVAKES ^t PKEEK	-1.372876472	0.02464106
Gja1	KVAAGHELQPLAIVDQRPS ^s R	-1.365857194	0.002869034
Eepd1	REHGPF ^r sVEDLVR	-1.360849534	0.001214644
Sgip1	EKVViPPAASDIPADSPAGPPGPPGSA	-1.360022146	0.000248753
Mbp	AsQKRPSQR	-1.350613464	0.015910051
Lysmd1	ARsYGSLVQSSCSPVR	-1.337513596	0.001329424
Psmf1	LsSPPREFPPATAR	-1.333698142	0.004074943
Matr3	RDsFDDRGP ^s SLNPVLDYDHGSR	-1.327702106	0.037213776
Syngap1	GLNSSMDMARLPsPTK; LPsPTKEKPPPPPPGGGK	-1.321683506	0.005441203
Bcas1	RRQsLGGFLK	-1.307501198	0.012568364
Gja1	QAsEQNWANYSAEQNR	-1.291337701	0.002766731
Hcn2	ASRPLSAsQPSLPHGAPAPsPAASAR	-1.287458858	0.030018828
Ptbp2	TSLLAVPGALsPLAIPNAAAAAAAAAAGR	-1.28252871	0.003271597
Camsap1	VKAPVHFVEPLsPTGVPGHR	-1.282117503	0.001062575
Snap91	ESSLTADLLSVDFAAPsPASTASP	-1.279864591	0.000163659
Grm1	RGPPVAT ^t PPLPPHLTAEETPLFLADS	-1.270949943	0.001085358
Camk2a	LHDsISEEGHHYLIFDLVTGGELFE	-1.260127142	0.03112568
Sv2b	AKGtPEKVFTVSHIK	-1.25815459	0.002420935
Camsap2	RFsPSQVPIQTR	-1.242651331	0.021601949
Tmem175	FMGHREsPAHNVEPF ^s IDLHAPLSK	-1.241724227	0.002526897
Zdhhc5	LLRQsPPLAGREEEPGLGDSGIQSTPG	-1.236864194	0.001502156
Ank3	SGSLSSsPSNTPSASPLK	-1.235501468	0.044148284

Uba3	ENCPACSQLPQNIQF _s PSAK	-1.233146751	0.007873169
Inpp5j	RPIPPADGCLHTPVQAAGLAT _s PPR	-1.230020629	0.002216371
Scn2a	FSsPHQSLLSIR	-1.226448983	0.006298277
Mark1	RFsDHAGPSIPPAVS _Y TK	-1.219845307	0.005874561
Synj1	VSAGRLtPESQSKPLETSK	-1.218014878	0.001897069
Add1	AAVVtSPPPTTAPHKER	-1.202754406	0.004297696
Itm2c	AAASGPASASAPAAEILLtPAREERPPR	-1.191514481	0.002376937
Atp2b2	SSLYEGLEKPESR _t sIHNFMAHPEFR	-1.186770333	0.02985132
Hmgcs1	SLDEGVGLVHSNTATEHIP _s PAK	-1.183257986	0.001136395
Thrap3	RIDIsPSTFR	-1.167669486	0.01334846
Os9	EQAILALTStLDKLIK	-1.167012489	0.033482591
Marcks1	DAIEPAPPSQEAEAKGEVAPKEtPK	-1.137536278	0.000438947
Wipf3	GGsTPPALGDLFAGGFVLRPAGQR	-1.134416772	0.005273433
Cend1	SAsSPKPD _T KVPQATAEAK	-1.128728268	0.000658536
Atp2b3	EAGHGPGKDEM _t DEELAE _G EEEEIDH	-1.128072138	0.000800713
Nek6	AGQPSHMPHGGsPNHLCHVLGPAHPP	-1.119402532	0.021390592
Cacna1a	LATGEPA _s PHDSLGHSGLPSPAK	-1.1168288	0.000519773
Armc9	LGELSGEKLLTTEYLGIMTNtGKAR	-1.113878059	0.003921667
Nefh	SPAEAK _s PAEAKPPAEAK; SPVVAK- sPAEAK _s PAEAK	-1.099667975	0.003594484
Ppp1r12a	FPTSTTKIsPKEEER	-1.097685164	0.010652549
Slc15a2	NEsKETLFSPVSTEEMLP _{PPP} _s PPK	-1.095339574	0.000287993
Lzts1	SGAILHsSPESTNHQLHPMPDPKPK	-1.093020303	0.032254674
Sgip1	GKPGVG _D VS _{PP} SPPIH _s SPPIAP	-1.091179555	0.018755487
Bcas1	EPAPCVQQPTVEVNALQTGDKtPKR	-1.089829306	0.001508233
Pebp1	QVMNRPSSIsWDGLDPGKLYTLVLT	-1.086005393	0.000381311
Caskin1	QVLPSGVsHFTPPQTPTK	-1.083545791	0.010872992
Exoc2	PMLDLNDARPSVLGHLSQTAsLKR	-1.06839078	0.025550255
Tjp1	FNHNLLPSETVHKPELSSKPPPsPK	-1.067041728	0.004889521
Map4	AAVGLTGNDIAtPPNKELPPsPEKK	-1.066020227	0.000891055
Dnm1	GSALGGAPPVPsRPGASDPFPGP	-1.065716586	0.024921389
Kcnc1	HIPRPPQLGsPNYCK	-1.061938055	0.014410018
Mbp	RGsGKVPWLK	-1.061709788	0.011329415
Sipa1l1	FHGLSsPQSPFPSTPTSR	-1.050913622	0.015136882
Mink1	LVDFGVSAQLDRtVGRR	-1.044521053	0.008348662
Stxbp5	KLsLPTDLKPDLDVK	-1.044260132	0.002386693
Sgip1	LARPFPTGtPPPLPPK	-1.042540857	0.019630945

Hspa14	LVsPEDVAR	-1.037571865	0.034103121
Septin5	LA ^t PEDKQDIDK; LA ^t PEDKQ- DIDKQYVGFATLPNQVHR; SKLA ^t PEDKQDIDK	-1.033039148	0.000287315
Numbl	GPAQPGHVSPTPA ^t TSPGEKGEAGT	-1.032643909	0.041629147
Dync2h1	VYSAIsSSK	-1.032515549	0.009643022
SLC16A2	MLSHDPDPNGELLPGsPTPEEPI	-1.027915111	0.006026679
Impact	KKtEEEEVEGEDDPILEHPPENPVK	-1.027283754	0.000574098
Micall1	APSA ^s PLVLHASR	-1.021294574	0.003256457
Pafah1b2	ELFsPLHALNFGIGGD ^t TTR	-1.019643777	0.000822367
Cltc	GILR ^t PDTIR	-1.01383896	0.000614208
Clip2	KIsGTTALQEALKEK	-1.011165872	0.002919381
Caskin1	RPPEGHPTP ^{RPA^s} PDPGR	-1.011026094	0.005309599
Alpl	MILPFLVLAIG ^t CLTNSFVPEK	-1.000704548	0.023793388
Rasgrf2	KLsLTSSLNSR	-1.000485925	0.007479283
Map1a	PGGHFMTSDSSLTKSPEsLSSPAMEDLAV	-1.000148172	0.025819966

Fold change threshold < 0.5 fold; Overlapping peptide sequences were excluded. Small letters indicate detected phosphorylation site. Sequences matching the DYRK1A recognition sequence (RPXS/TP) were marked in red color (Himpel et al., 2000).

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