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The role of neuroligin-1 in regulating presynaptic function and decision-making

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Abstract

Synapses are the basic computational units in the brain which higher level information processing depends on. Neuroligins are a family of postsynaptic cell adhesion molecules which bind to presynaptic neurexins to form a trans-synaptic complex. Neuroligin-1 (Nlgn1) is a member of the neuroligin family and has been extensively studied for its role in regulating postsynaptic function. However, whether Nlgn1 also regulates presynaptic function via the neuroligin-neurexin complex and how does its function at the synapse impact behaviour remain underexplored. This thesis aims to shed more light on these two outstanding questions. In Chapter 1, we assessed the impact of constitutive Nlgn1 deletion (*Nlgn1*$^{-/-}$) on the size of different populations of synaptic vesicles (synaptic vesicle pools) in the presynaptic terminal and the rate at which synaptic vesicles are mobilised to release neurotransmitters, a process called presynaptic exocytosis. We found that the loss of Nlgn1 moderately reduced the size of the total recycling pool of synaptic vesicles only when two sub-populations called the readily releasable pool and reserve pool were sequentially released. However, the rate at which the total recycling pool undergoes exocytosis was identical in *Nlgn1*$^{-/-}$ and WT synaptic terminals. In Chapter 3, we performed detailed dissections of the behavioural phenotype of *Nlgn1*$^{-/-}$ mice. Despite having robust and well-characterised impairment in postsynaptic mechanisms thought to be required for learning, *Nlgn1*$^{-/-}$ mice showed normal ability to learn complex associations and flexibility when the learnt associations changed in several instrumental conditioning tasks. However, we found that *Nlgn1*$^{-/-}$ mice were consistently less willing to overcome effort cost to earn rewards but were more willing to exert effort to escape from an aversive situation. To infer the underlying cognitive processes that generate these behavioural observations, we demonstrated in the form of a simple computational model and behavioural simulations that an increased subjective weighting on negative utilities could, at least in principle, capture the *Nlgn1*$^{-/-}$ behavioural phenotype across different tasks. Our findings provide new insights into the trans-synaptic function of Nlgn1 and identify a novel role of Nlgn1 in regulating utility trade-off in decision-making. Taken together, this thesis contributes towards a greater goal of neuroscience: understanding the generation of behavioural phenomena across different levels of the brain.

Declaration

This is to certify that:

I. The thesis comprises only my original work towards the PhD.
II. Due acknowledgement has been made in the text to all other material used.
III. This thesis is fewer than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

Signed

Jiaqi (Keith) Luo

September 2019

Preface

I. *Nlgn1*$^{-/-}$ mice used for all experiments in this thesis were generated from a colony established from mice provided by Professor Nils Brose at the Max Planck Institute for Experimental Medicine, Göttingen, Germany.

II. These studies were funded by an Australian Government Research Training Program (RTP) Scholarship awarded to me by the University of Melbourne, and a National Health and Medical Research Council Project Grant 1083334 awarded to Dr. Nithianantharajah.

III. No chapters are currently submitted for publication.

IV. All supplemental figures in this thesis address results in Chapter 3 therefore were attached to the end of Chapter 3.

Publications and conference abstracts

Publications

Luo J, Norris RH, Gordon SL, Nithianantharajah J (2018) Neurodevelopmental synaptopathies: Insights from behaviour in rodent models of synapse gene mutations. Prog Neuro-Psychoph 84:424-439.

Conference abstracts

Luo J, Brose N, Nithianantharajah J (2017) Neuroligin-1 regulate motivation and goal-directed behaviour. In: Australasian Neuroscience Society Annual Meeting 2017. Sydney, Australia.

Luo J, Nithianantharajah J (2017) Synaptic genes in cognition: investigating the role of neuroligin-1 In: Synaptic Dysfunction Symposium. Melbourne, Australia.

Luo J, Brose N, Nithianantharajah J (2018) Neuroligin-1 in complex cognition. In: Society for Neuroscience Annual Meeting 2018. San Diego, USA.

Luo J, Brose N, Nithianantharajah J (2018) Loss of neuroligin-1 at synapse leads to reduced motivation. In: Australian Appetitive Motivation Symposium 2018. Melbourne, Australia.

Luo J, Nithianantharajah J (2019) Neuroligin-1 deletion leads to a dissociable impact on motivation but not learning. In: International Behavioral Neuroscience Society Annual Meeting. Cairns.

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and heartfelt conversations. You are one of the kindest, most optimistic and resilient people I've met, and you always have so much faith in me. Your pint-half-full attitude towards life has made me a less gloomy person.

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Glossary of abbreviations

AZ	Active zone
PSD	Postsynaptic density
hPSD	human postsynaptic density
SNARE	Soluble NSF attachment proteins (SNAP) Receptor
GABA	γ-aminobutyric acid (GABA)
SynCAM	Synaptic cell adhesion molecule, an uncreatively named family of synaptic cell adhesion molecules
LRRT	Leucine rich transmembrane proteins
MGDA	Membrane-associated mucin domain-containing glycosylphosphatidylinositol anchor proteins
LNS domain	Laminin A, neurexin, and sex hormone-binding protein domain
PDZ domain	PSD95, Dlg1, ZO-1 domain
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
NMDAR	N-methyl-D-aspartate receptors
EPSC	Excitatory postsynaptic currents
mEPSC	Miniature excitatory postsynaptic current
LTP	Long-term potentiation
MWM	Morris water maze
PD	Pairwise discrimination learning
PAL	Paired associates object-location learning
FR	Fixed ratio
FST	Forced swim test

RL	Reinforcement learning
VTA	Ventral tegmental area

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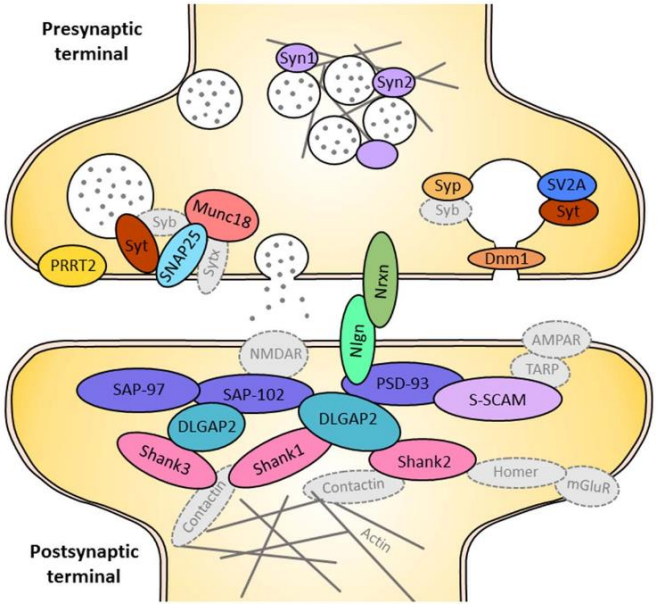
Chapter 1: Introduction

1.1 Synapses

1.1.1 Synaptopathy: diseases involving the synapse

The chemical synapse (synapse hereafter) is the site of inter-neuronal communication and forms the building blocks of higher-level computation in the brain. A synapse consists of a presynaptic and a postsynaptic terminal separated by a synaptic cleft (**Figure 1.1**). It is increasingly recognised that disease-causing synaptic dysfunction, or synaptopathy, plays a significant role in the pathophysiology of a wide range of neurological and neurodevelopmental disorders (Grant, 2012). Lending support to this notion, Bayes et al. (2011) isolated the proteome of the postsynaptic protein complex called postsynaptic density (PSD) from the human neocortex and systematically annotated the effects of mutations in human PSD proteins. These Mutations and their mouse orthologues are enriched in cognitive, affective and motor phenotypes, and they primarily cause neurological and psychiatric disorders. A similarly systematic analysis has not been performed for the presynaptic proteome therefore less is known about its disease relevance. However, we recently reviewed mutations in key synaptic proteins reported in neurodevelopmental disorders and found a partial dissociation in the disease relevance of pre- and postsynaptic mutations, which mirrors the structural and functional dissociations of pre- and postsynaptic proteins (Luo et al., 2018) (**Figure 1.1**). Mutations in presynaptic proteins often cause epilepsy, whereas mutations in postsynaptic proteins are more frequently identified in autism spectrum disorder and schizophrenia. Furthermore, we surveyed the behavioural phenotype of mouse models carrying mutations in these synaptic genes. We found a reasonably consistent mapping between human diseases and model phenotypes. Similar to the impacts of mutations in humans, mouse models of presynaptic mutations often develop seizures. Models of postsynaptic genes display a wider but less consistent range of behavioural phenotypes related to anxiety, locomotor function, social interaction, learning and memory mirroring the complex and heterogenous symptoms of psychiatric disorders. Together, genetic studies and animal models of disease-relevant synaptic genes offer empirical evidence for synaptic dysfunction being a major common cause of neurological and neurodevelopmental disorders, the nature and pathological mechanisms of which may be investigated by studying how synaptic proteins regulate synaptic function and impact behaviour.



Developmental disorder	Presynaptic genes (proteins)	Postsynaptic genes (proteins)
Epilepsy	STXBP1 (Munc18) DNM1 (Dynamin 1) SNAP-25 PRRT2 SYN1 (Synapsin 1) SV2A SYP (Synaptophysin)	DLG1 (SAP-97) MAGI2 (S-SCAM) SHANK3
Intellectual disability	SNAP-25 SYT1 (Synaptotagmin 1) SV2A SYP (Synaptophysin) DMN1 (Dynamin 1)	NLGN4 (Neuroligin 4) DLG2 (PSD-93) DLG3 (SAP-102) SHANK3
Autism Spectrum Disorder	SYN1 (Synapsin 1) SYN2 (Synapsin 2) NRXN1 (Neurexin 1)	NLGN1 (Neuroligin 1) NLGN3 (Neuroligin 3) NLGN4 (Neuroligin 4) DLG1 (SAP-97) DLG2 (PSD-93) DLGAP2 SHANK1 SHANK2 SHANK3
Schizophrenia	NRXN1 (Neurexin 1)	NLGN2 (Neuroligin 2) DLG2 (PSD-93) MAGI2 (S-SCAM)

Figure 1.1 Disease relevance of selected pre- and postsynaptic genes and the encoded proteins. Location of proteins at the synapse (left). Table (right) lists the disorders and genes (protein name in brackets). Figure modified from Luo et al., 2018.

1.1.2 Active zone and postsynaptic density

Presynaptic terminals are located on the axon and postsynaptic terminals along the dendrites of a neuron. In the presynaptic terminal, proteins cluster within areas called active zones (AZ) where neurotransmitters are released (**Figure 1.2A**). Similarly, postsynaptic proteins cluster to form large protein complexes called postsynaptic densities (PSD) in the postsynaptic terminal (**Figure 1.2B-C**). At the AZ, synaptic vesicles dock and fuse with the plasma membrane to release neurotransmitters. Briefly, this relies on 1) active zone proteins which define the docking and fusion sites for synaptic vesicles; 2) the SNARE complex formed between synaptic vesicles and the plasma membrane providing the mechanical force for membrane fusion; 3) Ca^{2+} channels and 4) gating proteins which ensure the precise timing of neurotransmitter release (Sudhof, 2012). Opposite to the AZ, the PSD complex typically consists of neurotransmitter receptors, postsynaptic scaffolding proteins and other downstream signalling (Sheng and Kim, 2011). Across the synaptic cleft, pre- and postsynaptic cell adhesion molecules are thought to physically and functionally couple the AZ and PSD to form a trans-synaptic column (Tang et al., 2016; Biederer et al., 2017; Haas et al., 2018).

1.1.3 The excitatory and inhibitory synapse

Broadly, synapses are either excitatory or inhibitory. Excitatory synaptic transmission uses glutamate as a major neurotransmitter and causes depolarisation in the postsynaptic terminal, whereas inhibitory synaptic transmission mainly relies on γ-aminobutyric acid (GABA) or glycine to cause polarisation. The excitatory and inhibitory synapse share a core set of proteins to regulate neurotransmitter release. In fact, SNARE-mediated fusion is thought to be a general scheme conserved across different types of membrane fusion (Sollner et al., 1993; McNew et al., 2000; Parlati et al., 2000). By contrast, the compositions of the excitatory and inhibitory PSD differ drastically (**Figure 1.2B-C**) (Sheng and Kim, 2011). In addition to receptors specialised for excitatory and inhibitory transmission, excitatory and inhibitory PSD often utilise different synaptic cell adhesion molecules and scaffolding proteins.

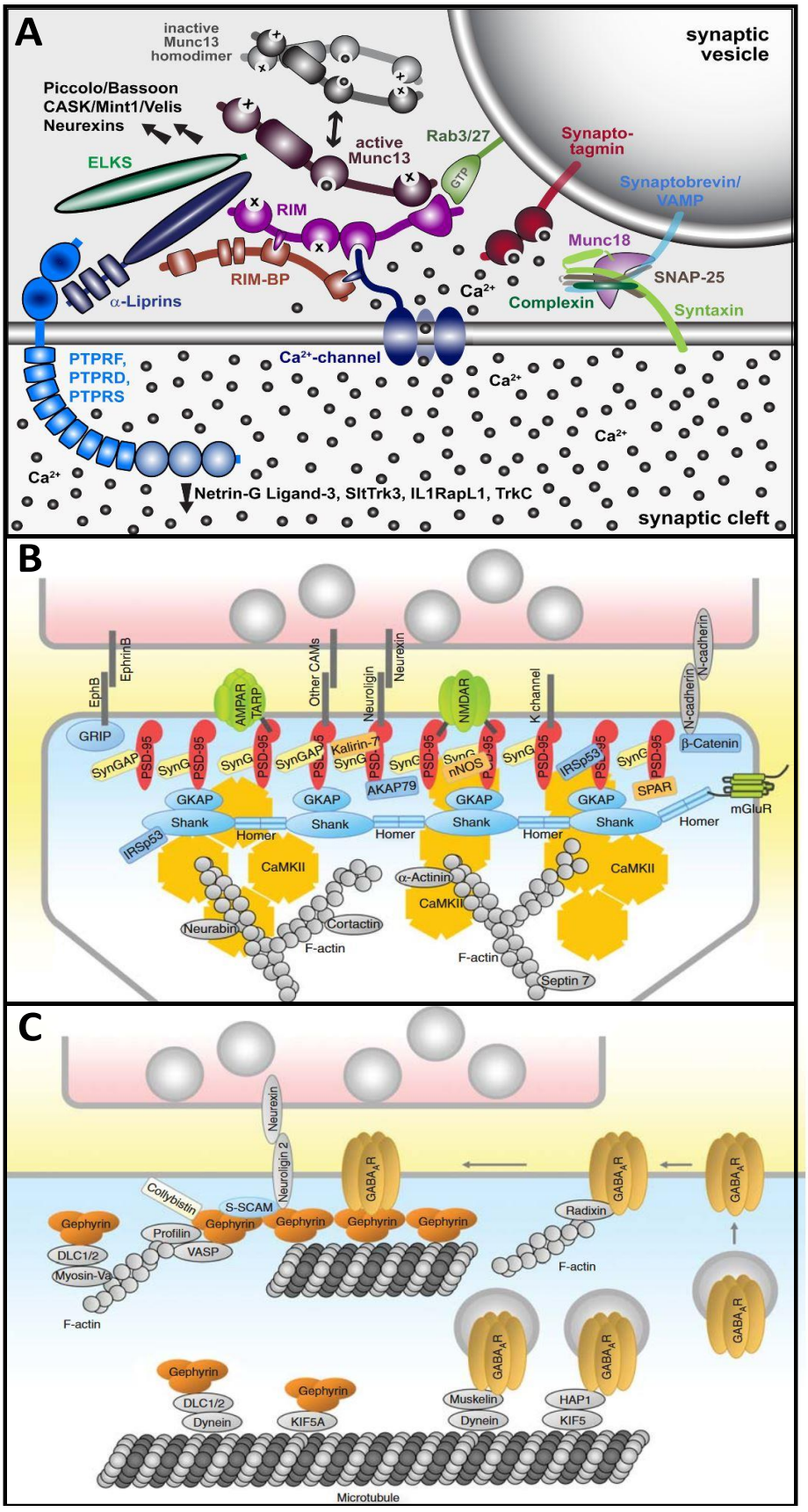


Figure 1.2 The presynaptic active zone and postsynaptic terminal. A) Protein machinery at the active zone. Modified from Sudhof 2010. B) The excitatory and C) inhibitory postsynaptic density. Modified from Sheng & Kim 2011.

1.2 The neuroligin-neurexin trans-synaptic complex

The pre- and postsynaptic terminal are connected by a diverse class of proteins collectively known as synaptic cell adhesion molecules, which play critical roles in establishing synaptic contact, assembly of synaptic machineries, excitatory/inhibitory synaptic specification, synaptic transmission and plasticity. Synaptic cell adhesion molecules can be homophilic where the same molecules provide binding from the pre- and postsynaptic terminal such as N-cadherins (Uchida et al., 1996), heterophilic where different pre- and postsynaptic cell adhesion molecules interact across the synaptic cleft such as neurexins and neuroligins (Ichtchenko et al., 1995; Nguyen and Sudhof, 1997) or both such as SynCAMs (synaptic cell adhesion molecules) (Biederer et al., 2002; Thomas et al., 2008). Despite some functional redundancy in synaptogenesis, different synaptic cell adhesion molecules are differentially required for the on-going function of a synapse. One such example is that constitutively deleting three of the four neuroligin isoforms caused severely impaired synaptic transmission and perinatal lethality without reducing the density and ultrastructure of synapses (Varoqueaux et al., 2006). The specific functional requirements of different synaptic cell adhesion protein families and members of the same family vary greatly due to different binding partners, synaptic localization, patterns of developmental expression and alternative splicing. Much of this complex molecular code of the synapse has yet to be deciphered (Missler et al., 2012; Sudhof, 2017).

On the presynaptic membrane, neurexins are thought to act as a presynaptic cell adhesion platform that provides binding to myriad postsynaptic cell adhesion molecules including neuroligins (Ichtchenko et al., 1995), leucine rich transmembrane proteins (LRRTMs) (de Wit et al., 2009; Ko et al., 2009a), cerebellins (Uemura et al., 2010; Matsuda and Yuzaki, 2011), latrophilins (Boucard et al., 2012), neurexophilins (Missler et al., 1998), dystroglycan (Sugita et al., 2001) and even the GABA-A receptor (Zhang et al., 2010) (**Figure 1.3**). Among them, the neurexin-neuroligin trans-synaptic complex is the most extensively studied synaptic cell adhesion complex (Sudhof, 2008). Two neurexin monomers extending from the presynaptic membrane bind to postsynaptic neuroligin (Nlgn) homo- or heterodimers in a calcium-dependent manner to mediate trans-synaptic cell adhesion (Nguyen and Sudhof, 1997; Arac et al., 2007). Neuroligins only bind to neurexins trans-synaptically but can form *cis* complexes on the postsynaptic membrane with the membrane-associated mucin domain-

containing glycosylphosphatidylinositol anchor proteins (MDGAs). While MDGA1 was initially shown to only bind to rat neuroligin-2 but not neuroligin-1 or 3, a later study using human neuroligins showed that both MDGA1 and 2 bound to human neuroligin-1 and 2 with similarly high affinity. MDGAs compete for the neuroligin binding interface with neurexins and are thought to suppress the formation of the neurexin-neuroligin complex (Lee et al., 2013; Pettem et al., 2013; Elegheert et al., 2017). Interestingly, there may also exist a population of postsynaptic neurexins which similarly serve as negative regulators for the trans-synaptic cell adhesion via a *cis* interaction (Taniguchi et al., 2007).

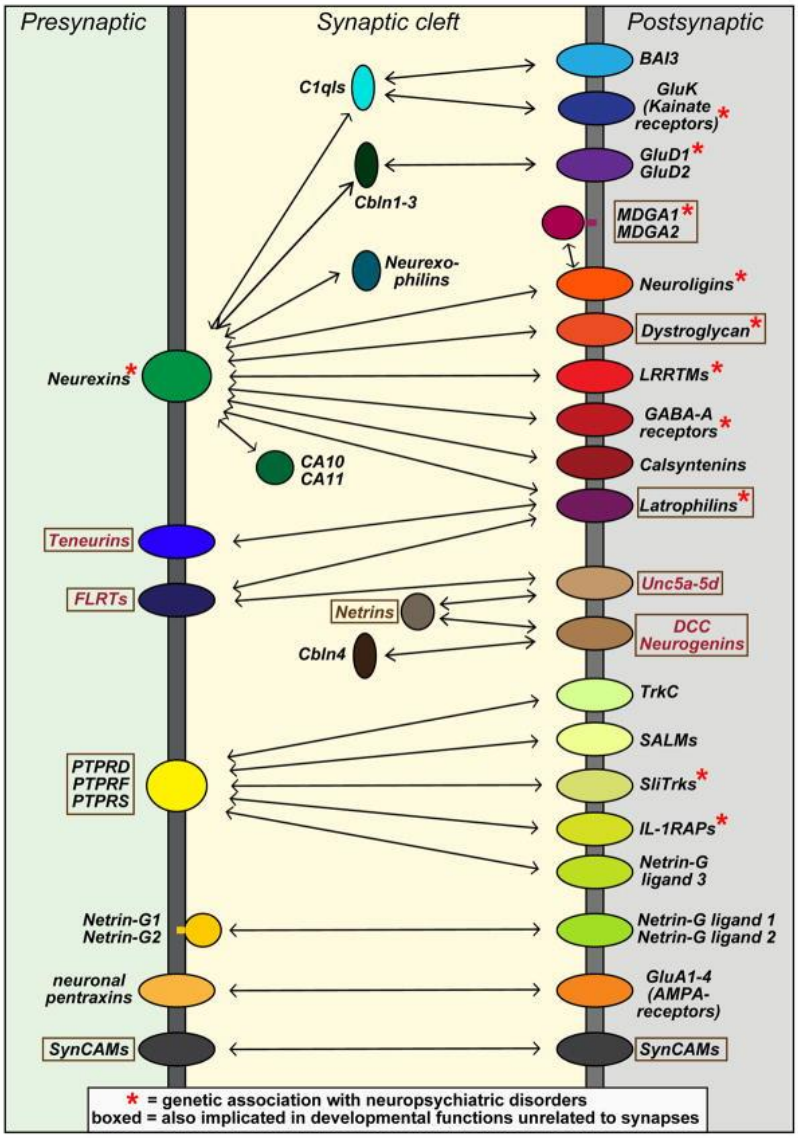


Figure 1.3 Interactions between pre- and postsynaptic cell adhesion molecules. Neurexins bind to a wide range of postsynaptic cell adhesion molecules whereas neuroligins only bind to neurexins trans-synaptically and MDGAs postsynaptically. Figure modified from Sudhof 2017.

1.2.1 Neurexins

Neurexin 1, 2 and 3 are presynaptic cell adhesion molecules encoded by three different genes (*Nrxn1-3* in mice, *NRXN1-3* in humans), each under the control of two independent promoters to produce α- and β-neurexins. α-neurexins possess six extracellular LNS (laminin A, neurexin, and sex hormone-binding protein) domains, while β-neurexins retain only the sixth, which mediates neuroligin binding (**Figure 1.4A**) (Ushkaryov et al., 1992; Ushkaryov and Sudhof, 1993; Ushkaryov et al., 1994). The extracellular domain of α-neurexins undergoes extensive alternative splicing at five canonical sites and β-neurexins at two (Ushkaryov et al., 1994). Together, two independent promoters and alternative splicing of the extracellular domains generate potentially over 1000 neurexin isoforms from just three genes, which is thought to constitute a molecular code specifying synaptic contacts between different subsets of neurons (Missler and Sudhof, 1998).

The intracellular C-termini of neurexins contain a PDZ (PSD95, Dlg1, ZO-1) domain through which neurexins bind to two important presynaptic adaptors: CASK and MINTs (**Figure 1.4B**) (Hata et al., 1996; Biederer and Sudhof, 2000). Cask, MINTs and VELIs form a tripartite complex thought to couple synaptic vesicle exocytosis machinery with synaptic cell adhesion complexes by its interaction with neurexins (Butz et al., 1998). A number of findings provide evidence for this model: first, the CASK/MINT/VELI tripartite complex binds to α-liprins and thereby indirectly to RIM, both of which are core AZ proteins (**Figure 1.2A**) (Schoch et al., 2002; Olsen et al., 2005; Sudhof, 2012). Second, deletion of CASK in mice reduced the expression of not only MINTs and VELIs but also neurexins suggesting neurexins function cooperatively with the tripartite complex (Atasoy et al., 2007). Third, MINTs recruit Munc18, essential for synaptic vesicle exocytosis, to neurexins by binding to both CASK and neurexin (Biederer and Sudhof, 2000; Verhage et al., 2000). Lastly and perhaps most convincingly, both CASK and MINTs bind directly to the N-type Ca^{2+} channels, which is uncoupled from neurotransmitter release by neurexin deletions (Maximov et al., 1999; Missler et al., 2003). Although this is an appealing model for synaptic exocytosis-adhesion coupling, it does not fully explain the function of neurexins at the synapse. Firstly, core AZ proteins such as RIM and RIM-BP (RIM binding protein) also bind to the N-type Ca^{2+} channels therefore it is unclear how neurexin deletions were enough to uncouple them from neurotransmitter release (Hibino et al., 2002; Kaeser et al., 2011). Moreover, neurexin 1/2/3 triple knockout causes

severer impairments than deletion of CASK or MINTs therefore neurexins likely have CASK/MINT/VELI-independent functions (Atasoy et al., 2007; Ho et al., 2008). Finally, the impairments caused by α-neurexin triple knockout can only be rescued by re-expressing neurexin-1α but not neurexin-1β suggesting that the extracellular domain of neurexins also perform diverse functions (Zhang et al., 2005).

Both the extracellular and intracellular domain of neurexins undergo activity-dependent proteolytic cleavage. Upon activation of glutamate receptors or depolarisation by potassium chloride (KCl), the extracellular domain of neurexins is cleaved by the metalloproteases ADAM10 and ADAM17 (Borcel et al., 2016). The membrane-bound C-terminus is subsequently cleared by presenilins, the catalytic subunits of the γ-secretase complex (Bot et al., 2011; Saura et al., 2011; Servian-Morilla et al., 2018). Importantly, intracellular cleavage by presenilins is downstream to the shedding of the extracellular domain suggesting the proteolytic processing of the extra- and intracellular domain of neurexins is coordinated presumably as a negative feedback mechanism. However, little is known about its physiological relevance and the signal transduction pathways involved.

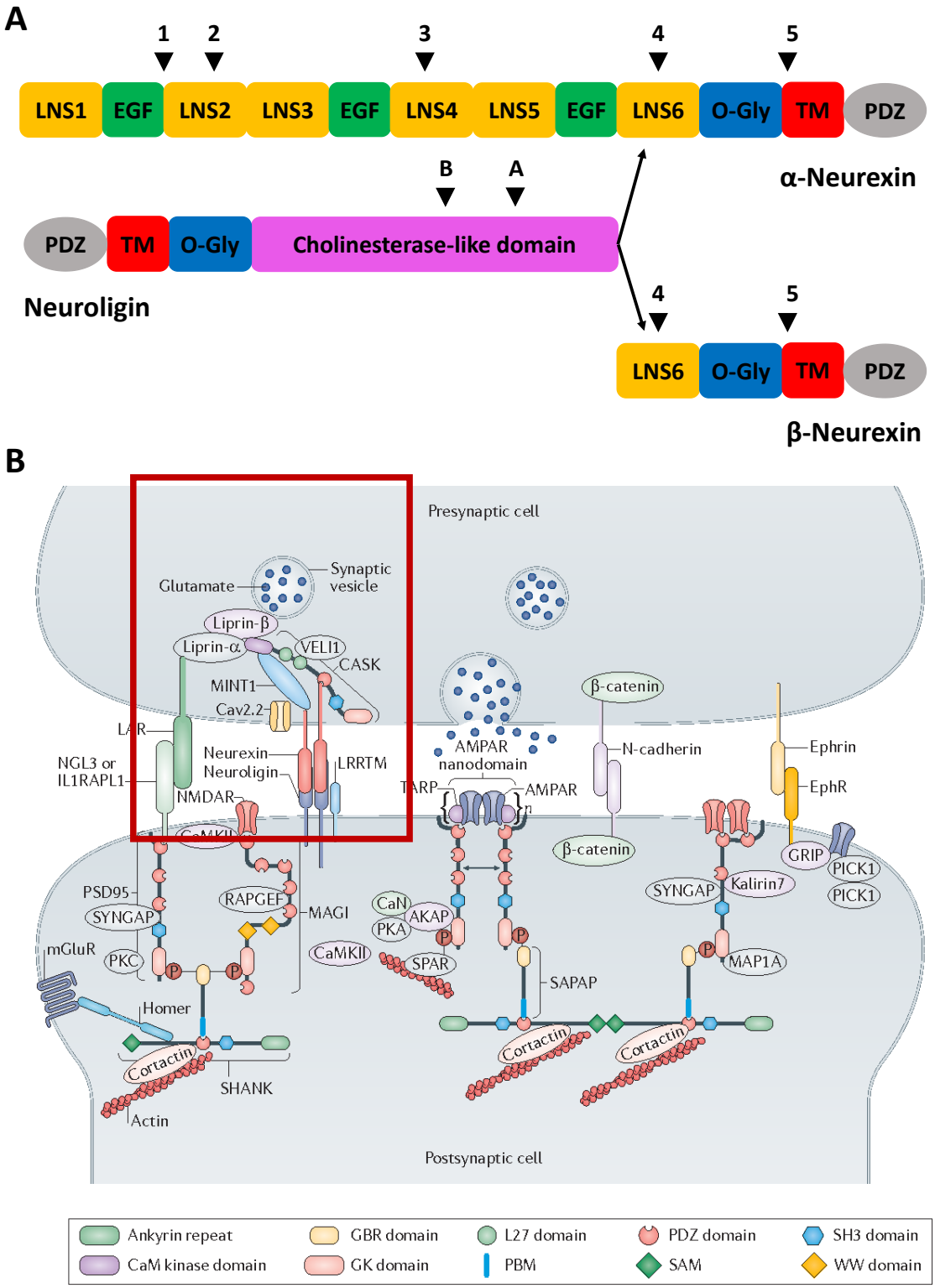


Figure 1.4 The neurexin-neuroligin trans-synaptic complex. **A**) Structural domains of neurexin and neuroligin. LNS: laminin A, neurexin, and sex hormone-binding protein domain, EGF: EGF (epidermal growth factor) -like domain, O-Gly: O-linked glycosylation region, TM: transmembrane domain, PDZ: PDZ (PSD95, Dlg1, ZO-1) domain. Arrowheads indicates alternative splicing sites. Splice site B is unique to neuroligin-1. The cholinesterase-like domain of neuroligins bind to the sixth LNS domain of neurexins. **B**) Neurexins and CASK/MINT/VELI tripartite complex links exocytic and cell adhesion machineries. Red box: synaptic vesicle, the Cask/MINTs/VELIs complex and the neurexin-neuroligin complex. Figure modified from Zhu et al., 2017.

1.2.2 Neuroligins

Neuroligins are postsynaptic cell adhesion molecules encoded by four genes in mice (*Nlgn1-4*) and a fifth gene NLGN4Y on the human Y chromosome. While neurexins are pan-neuronally expressed, different members of neuroligins have distinct excitatory/inhibitory synaptic localisation. Nlgn1 is exclusively expressed at excitatory synapses; Nlgn2 is localised to inhibitory synapses; and Nlgn3 is expressed at both excitatory and inhibitory synapses (Song et al., 1999; Varoqueaux et al., 2004; Budreck and Scheiffele, 2007). Nlgn4 is the least evolutionarily conserved member of the neuroligin family and was initially found at glycinergic synapses in the mouse retina but recently reported to localise to glutamatergic synapses in humans (Bolliger et al., 2008; Hoon et al., 2011; Marro et al., 2019). Dimerisation of Nlgn is essential for their synaptic function (Dean et al., 2003; Ko et al., 2009b; Poulopoulos et al., 2012; Shipman and Nicoll, 2012a). Nlgn1 forms homodimers and heterodimers with Nlgn3 at excitatory synapses, and Nlgn2 form homodimers at inhibitory synapses whereas Nlgn1/2 or Nlgn3/2 heterodimers are rare (Poulopoulos et al., 2012).

Neuroligins bind to presynaptic neurexins via its extracellular catalytically inactive acetylcholine esterase domain. The interaction between neuroligin and neurexins is tightly regulated by alternative splicing of the extracellular domains of both molecules. Nlgn1, 2 and 3 share one splicing site called the A site although the amino acid sequences at the A site are not homologous between Nlgn1 and 2 and both partially overlap with Nlgn3 (Ichtchenko et al., 1995; Ichtchenko et al., 1996). Nlgn1 also possesses an additional splicing site called the B site, at which the presence of an insert biases Nlgn1 to selectively bind to β-neurexins but not α-neurexins (Boucard et al., 2005). The binding affinity between neuroligins and neurexins is further modulated by different alternatively spliced isoforms of neurexins (Koehnke et al., 2010). The C-termini of neuroligins directly interact with postsynaptic scaffolding proteins such as PSD-95 at excitatory synapses and gephyrin at inhibitory synapses, which are key organisers of the PSD protein complex (Meyer et al., 2004; Poulopoulos et al., 2009; Shipman and Nicoll, 2012a). Most of the neuroligin-binding scaffolding proteins belong to the membrane-associated guanylate kinase (MAGUK) superfamily. Most notably, PSD-95 and 93 of the Dlg family interact with Nlgn1 and 3 at excitatory synapses and S-SCAM of the MAGI family interact with Nlgn1 at excitatory and Nlgn2 at inhibitory synapses (Irie et al., 1997; Iida

et al., 2004; Sumita et al., 2007). It may be tempting to suggest that the synaptic localisation of neuroligins are determined by interaction with excitatory or inhibitory scaffolding proteins. However, the PDZ-binding motif for PSD-95/93 and gephyrin-binding motif are conserved across Nlgn1-3 suggesting that localising Nlgn1 and 2 involves more complex mechanisms than direct binding to excitatory or inhibitory postsynaptic scaffolds (Meyer et al., 2004; Poulopoulos et al., 2009). At the inhibitory synapse, Nlgn2 but not Nlgn1 additionally binds to collybistin, a GDP-GTP exchange factor required for membrane clustering of gephyrin, providing a possible mechanism for the localization of Nlgn2 to inhibitory synapses (Kins et al., 2000; Harvey et al., 2004; Poulopoulos et al., 2009). At the excitatory synapse, phosphorylation at Y782 on Nlgn1 prevents it from binding to gephyrin to allow preferential binding to PSD-95, a mechanism which we will discuss further in section 1.4 (Giannone et al., 2013).

Similar to neurexins, the extracellular domain of neuroligins is cleaved by the metalloprotease ADAM10 and additionally by MMP9 upon glutamate receptor activation or depolarisation by KCl. The resulting membrane-bound C-terminus is also subsequently cleared by presenilins (Peixoto et al., 2012; Suzuki et al., 2012; Bemben et al., 2019). By expressing a Nlgn1 construct the cleavage of which can be experimentally controlled, Peixoto et al. (2012) showed that shedding of the extracellular domain of Nlgn1 caused a reduction of neurexin-1β at the same synapse and reduced neurotransmitter release probability measured by miniature excitatory postsynaptic frequency (mEPSC) and paired pulse ratio. Using these same measures, Gjorlund and colleagues (2017) suggested that the cleaved extracellular domain of Nlgn1 suppressed presynaptic release by activating presynaptic metabotropic glutamate receptor 2 (mGluR2). It is worth noting that the studies discussed above inferred the functional consequences of neurexin and neuroligin proteolytic processing by either inducing proteolytic cleavage at synapses overexpression neuroligins/neurexins or observing the effect of directly administrating soluble extracellular domains. It is unclear whether proteolytic processing of endogenous neurexins and neuroligins under physiological conditions serves the same function.

Traditionally, neuroligins are thought of as postsynaptic cell adhesions molecules that mediate exclusively inter-neuronal synaptic cell adhesion. This was challenged by the recent evidence that Nlgn1-3 are all highly expressed in oligodendrocytes and astrocytes in both

humans and mice (Zhang et al., 2014; Zhang et al., 2016). In rat cerebellar neuron-glia co-cultures, knockdown of Nlgn3 by small interfering RNA (siRNA) reduced branching of oligodendrocytes. Reciprocally, saturating neurexin binding sites by soluble Nlgn1-fragments reduced myelination in neuron-glia co-cultures as well as organotypic slices (Proctor et al., 2015). An equally important role of neuroligin-neurexin interaction has been demonstrated in astrocytes. Astrocyte-specific knockdown of Nlgn1, 2 or 3 reduced astrocyte complexity measured by the number of intersections between astrocytic processes and the volume of neuropil infiltration by astrocyte (Stogsdill et al., 2017). A perhaps even more surprising finding reported in this study was that conditional knockout of Nlgn2 in astrocytes reduced the number of excitatory synaptic puncta labelled by colocalised VGLUT and PSD-95 but did not affect the number of inhibitory synapses labelled by VGAT and gephyrin. This resulted in a reduced frequency and amplitude of mEPSC and an increased frequency of miniature inhibitory postsynaptic currents (mIPSCs). Nlgn2 is classically considered an inhibitory postsynaptic cell adhesion molecule localised exclusively to inhibitory synapses and promoting inhibitory synaptic transmission. These data directly challenge the canonical neuron-centric view of excitatory/inhibitory specialisation of synaptic proteins.

1.3 Neuroligin-1

1.3.1 Neuroligin-1 in the formation and specification of excitatory synapses

Nlgn1 is arguably the most extensively studied member of the neuroligin family. Given that the neurexin-neuroligin complex forms a physical link between the pre-and postsynaptic terminal, much of the early and seminal studies on Nlgn1 focused on its role in inducing synapse formation. Initially, it was shown by co-culturing non-neuronal cells with pontine explants or hippocampal neurons that expressing Nlgn1 in non-neuronal cells could induce synapse formation on contacting axons via neurexin binding (Scheiffele et al., 2000; Chubykin et al., 2005). Reciprocal experiments were conducted by expressing neurexins in non-neuronal cells to induce postsynaptic terminals on contacting dendrites, which could be supressed by soluble Nlgn1 fragments (Nam and Chen, 2005). Similarly, overexpressing Nlgn1 in neuronal cultures or *in vivo* robustly increased the size and density of synapses (Prange et al., 2004; Chih et al., 2005; Chubykin et al., 2007; Dahlhaus et al., 2010; Budreck et al., 2013;

Hoy et al., 2013). However, Nlgn1 expression in non-neuronal cells but not Nlgn1 overexpression in neurons requires neurexin binding to induce synapse formation, suggesting that neurexin-independent mechanisms also contribute to the synaptogenic effect of Nlgn1 overexpression (Ko et al., 2009b). This is perhaps due to the presence of other synaptic cell adhesion molecules in the PSD and the fact that neurexins bind to a multitude of postsynaptic molecules (Sudhof, 2017). Nlgn1 may induce synapse formation by clustering other synaptic cell adhesion molecules via scaffolding proteins such as PSD-95 even without the ability to bind to neurexins itself. Overexpressing exogenous Nlgn1 is useful for understanding the *sufficient* conditions for synapse formation but may not generalise to what is *necessary* in the endogenous environment. Indeed, Nlgn1 constitutive knockout did not alter the density or size of excitatory or inhibitory presynaptic puncta in the hippocampus. Even more surprisingly, Nlgn1/2/3 triple knockout did not reduce the density of pre- (labelled by synapsin) or excitatory postsynaptic (labelled by PSD-95) terminals (Varoqueaux et al., 2006). However, postnatal knockdown of Nlgn1 in rats has been shown to reduce spine density in the dentate gyrus suggesting that there may be developmental compensation in constitutive knockout models (Shipman and Nicoll, 2012b). It also appears that the excitatory synapse has evolved a higher structural redundancy than the inhibitory synapse. While neurexin 1/2/3 triple knockout or 1/2 double knockout did not reduce the density of glutamatergic synapses, they did reduce the density of GABAergic and glycinergic synapses (Missler et al., 2003). Moreover, deletion of Nlgn2, the neuroligin isoform localised to inhibitory synapses, was sufficient to reduce the density and size of gephyrin puncta in the basal amygdala (Babaev et al., 2016).

A related idea suggests that Nlgn1 may not be essential for the formation of the synapse but plays an important role in driving the specialisation of excitatory synapses, or synaptic specification. Structurally, there is surprisingly little unequivocal evidence to suggest that Nlgn1 alone is the primary force driving excitatory specialisation. Expressing Nlgn1 in neurons robustly increases the density and size of both excitatory and inhibitory synapses (Prange et al., 2004; Chih et al., 2005; Levinson et al., 2005). Subsequently, it was shown that not all Nlgn1 splice variants specifically drive excitatory synaptic development. For example, an insert only at the A site of Nlgn1 completely abolishes the differential effect of Nlgn1 overexpression to promote excitatory specialisation (Chih et al., 2006). These data suggest

that Nlgn1 is likely to specify excitatory synapses by cooperating with other synaptic organisers. The most obvious candidates are the Dlg postsynaptic scaffolds such as PSD-95 and PSD-93. Nlgn1 colocalises with and directly binds to PSD95 and 93 at the excitatory PSD (Irie et al., 1997; Iida et al., 2004), which in turn bind to a wide range of postsynaptic proteins and most importantly to both of the major glutamate ionotropic receptors: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) and N-methyl-D-aspartate receptors (NMDAR) (Niethammer et al., 1996; Schnell et al., 2002; Dakoji et al., 2003). While expressing Nlgn1 alone promotes both excitatory and inhibitory synapse formation, co-expressing Nlgn1 with PSD-95 constrains the effect to excitatory synapses (Prange et al., 2004). Multiple studies have collectively pointed to a potential mechanism through which neurexins, Nlgn1 and PSD95 specify excitatory synapses synergistically. As mentioned above, the extracellular domain of Nlgn1 contains a splice site (B site) unique from other members of neuroligins that do not exclusively localise to excitatory synapses (Ichtchenko et al., 1996). An insert at the B site restricts Nlgn1 to β-neurexins binding, which triggers the phosphorylation of Y782 within the conserved gephyrin binding site by an unknown kinase(s) (Boucard et al., 2005; Giannone et al., 2013). Phosphorylation at Y782 then prevents Nlgn1 from binding to gephyrin and biases it towards interacting with PSD-95/93 and the associated excitatory postsynaptic machinery. Consistent with this view, Nlgn1 with a B site insert have the strongest preference for inducing excitatory synapses. Remarkably, artificially introducing the B site insert into Nlgn2 also causes it to preferentially induce excitatory synapses (Chih et al., 2006). The studies discussed so far concerns the effect of introducing exogenous Nlgn1 into neuronal cultures but do not address the necessity of endogenous Nlgn1 for synapse specification. The data collected from Nlgn1 constitutive knockout (*Nlgn1*$^{-/-}$) mice suggest that the loss of Nlgn1 does not significantly alter the balance between excitatory and inhibitory synapses at least in the CA1 and CA3 regions of the hippocampus, possibly due the compensation from an increased expression of Nlgn3 (Blundell et al., 2010).

1.3.2 Neuroligin-1 regulates synaptic alignment, transmission and long-term plasticity

1.3.2.1 Trans-synaptic alignment by Nlgn1

Since the advent of super-resolution microscopy, neuroscientists have been able to study the distribution of synaptic proteins at the sub-synaptic and even single molecule level (Dani and Huang, 2010; Huang et al., 2010). Instead of a homogenous distribution, AZ and PSD proteins form sub-synaptic nanoclusters which are aligned with each other across the synapse to form what has been termed trans-synaptic nanocolumns (MacGillavry et al., 2013; Nair et al., 2013; Broadhead et al., 2016; Tang et al., 2016; Haas et al., 2018). The obvious missing piece in the formation of a nanocolumn between AZ and PSD proteins is what physically connects them, namely synaptic cell adhesion molecules. So far, one study has shown that disrupting the nanocolumn by expressing in cultured neurons either a C-terminally truncated Nlgn1 to compete with endogenous Nlgn1 for neurexin binding or a Nlgn1 C-terminal peptide to compete for Dlg scaffold binding reduced the alignment between AMPAR and AZ protein RIMs. Functionally, this led to a reduced amplitude of miniature excitatory postsynaptic currents (mEPSC) (Haas et al., 2018). Although this study does not conclusively prove that endogenous Nlgn1 is required for trans-synaptic alignment because both the C-terminally truncated Nlgn1 and Nlgn1 C-terminal peptide may compete with other synaptic cell adhesion molecules, future studies can certainly investigate the role of Nlgn1 and other synaptic cell adhesion molecules in ensuring trans-synaptic alignment using knockout, knockdown and rescue approach.

1.3.2.2 NMDAR-mediated postsynaptic currents

How may the interaction between Nlgn1 and its synaptic binding partners regulate the efficacy of synaptic transmission? Nlgn1 overexpression in neuronal cultures robustly increases the amplitude of evoked excitatory postsynaptic currents (EPSCs) (Prange et al., 2004; Chubykin et al., 2007). This is not surprising given that Nlgn1 overexpression also increases the size and density of synapses. What was less expected is that Nlgn1 overexpression increases the amplitude of both AMPAR- and NMDAR-mediated EPSCs but its effect on NMDAR-mediated EPSCs is consistently greater. This is often represented as a relative increase in the NMDA/AMPA current ratio (Chubykin et al., 2007; Budreck et al., 2013;

Hoy et al., 2013). Conversely, in both *in vitro* and *in vivo* systems where the level of endogenous Nlgn1 was reduced by genetic knockdown or knockout, NMDAR-mediated EPSC amplitude and NMDA/AMPA ratio were robustly reduced to roughly 50% of control (Chubykin et al., 2007; Kim et al., 2008; Blundell et al., 2010; Jung et al., 2010; Budreck et al., 2013; Espinosa et al., 2015; Jiang et al., 2017). The regulation of NMDAR function appears unique to Nlgn1 as deletion of Nlgn3 which heterodimerises with Nlgn1 at excitatory synapses did not affect NMDA/AMPA ratio (Jiang et al., 2017), and expression of Nlgn1 alone was sufficient to rescue NMDA/AMPA ratio in Nlgn1-4 conditional knockout mice (Wu et al., 2019a). Furthermore, while most electrophysiological experiments use the hippocampus or hippocampal cultures as model systems, the requirement of Nlgn1 for normal NMDAR/AMPAR ratio has been demonstrated across multiple regions of the brain including the hippocampus (Chubykin et al., 2007; Budreck et al., 2013; Jiang et al., 2017), amygdala (Kim et al., 2008; Jung et al., 2010) and striatum (Blundell et al., 2010; Espinosa et al., 2015). How Nlgn1 exerts selective control over NMDAR but not AMPAR is still unclear. Budreck et al. (2013) showed that the extracellular domain of Nlgn1 was required for normal NMDA/AMPA ratio by directly binding to the NR1 subunit of NMDAR and retaining NMDAR in the PSD. However, a recent study showed that the intracellular domain of Nlgn1 but not neurexin binding site was required for rescuing NMDA/AMPA ratio in Nlgn1-4 conditional knockouts (Wu et al., 2019a). These two findings are not necessary contradictory. Budreck et al. (2013) demonstrated the requirement for the Nlgn1 extracellular domain by showing that a chimeric protein comprised of the extracellular domain of Nlgn1 and intracellular domain of Nlgn2 rescued NMDA/AMPA ratio. On the other hand, Wu et al. (2019a) showed that the Nlgn1 extracellular domain anchored to the membrane by glycosylphosphatidylinositol lipids could not rescue NMDA/AMPA ratio. A potential mechanism to reconcile these findings could be that both NR1 binding via the Nlgn1 extracellular domain and the PDZ binding motifs shared by the Nlgn1 and 2 intracellular domains are required for the synaptic retention of NMDAR by Nlgn1.

1.3.2.3 Long-term potentiation

The Hebbian theory broadly postulates that learning can be implemented by strengthening the connections between subsets of neurons whose firings are temporally and causally related (Hebb, 1949). Later, the discovery of the activity-dependent strengthening of synaptic

transmission, termed long-term potentiation (LTP), provided a plausible cellular mechanism for this theory and has since become one of the most studied neurophysiological phenomena (Bliss and Lomo, 1973; Dan and Poo, 2004; Nicoll, 2017). Depletion of Nlgn1 by genetic knockout or knockdown has been shown to completely or partially block the induction of LTP in the CA1 and dentate gyrus of the hippocampus and thalamic inputs to the lateral nucleus of the amygdala (Kim et al., 2008; Blundell et al., 2010; Jung et al., 2010; Shipman and Nicoll, 2012b; Budreck et al., 2013; Jedlicka et al., 2015; Jiang et al., 2017). LTP induced by either theta-burst stimulation and paired pre- and postsynaptic stimulation in these studies are NMDAR-dependent. Given that depletion of Nlgn1 by similar methods also robustly reduces NMDAR-mediated EPSCs, one obvious possibility is that impaired NMDAR-mediated transmission has a knock-on effect on the induction of LTP. However, recent findings suggest that Nlgn1 likely regulate NMDAR-mediated transmission and LTP by independent mechanisms. First, to minimise compensation by other synaptic cell adhesion molecules during synaptogenesis, Jiang et al. (2017) deleted Nlgn1 on postnatal day 21 using cre-recombinase and showed that Nlgn1 knockout abolished not only NMDAR-dependent LTP but also NMDAR-independent LTP. Subsequently, it was demonstrated that while normal NMDAR/AMPA ratio required the intracellular domain of Nlgn1 but not the extracellular neurexin binding site, the opposite is true for LTP (Wu et al., 2019a). It is unclear exactly how Nlgn1-neurexin binding contributes to LTP. One clue offered in the study was that dendritic spine expansion concomitant with LTP, presumably to accommodate the recruitment of additional PSD proteins, also required Nlgn1-neurexin binding but not the Nlgn1 intracellular domain. Nonetheless, this further begs the question how Nlgn1-neurexin binding contributes to dendritic spine expansion. Finally, the requirement of Nlgn1 for LTP does not dissociate whether Nlgn1 is responsible for the initial triggering of LTP via a trans-synaptic signalling cascade or the subsequent insertion of protein machinery into the synapse.

1.3.3 Trans-synaptic control of presynaptic structure and function by Nlgn1: an exciting area underexplored

Given the functional importance of Nlgn1-neurexin interaction and trans-synaptic signalling in general, it is surprising that few studies have investigated the potential role of Nlgn1 in regulating the structure and function of the AZ. Several studies have used mEPSC frequency or paired-pulse ratio as proxies to measure the impacts of Nlgn1 expression level on

presynaptic release probability. Overexpression of Nlgn1 typically increases release probability using these measures whereas Nlgn1 depletion has varying effects (Prange et al., 2004; Futai et al., 2007; Kim et al., 2008; Stan et al., 2010; Espinosa et al., 2015). Nlgn1 knockout in mice reduced mEPSC frequency in dorsal striatal D2-, but not D1-dopamine-receptor-expressing medium spiny neurons and Nlgn1 knockdown in the rat amygdala led to a moderately reduced mEPSC frequency which did not reach statistical significance (Kim et al., 2008; Espinosa et al., 2015). However, there are obvious drawbacks in using postsynaptic readouts to measure presynaptic function. Recordings of postsynaptic currents are, by definition, subject to changes in postsynaptic factors such as the alignment between PSD and AZ, abundance and intrinsic function of receptors. Given that Nlgn1 is a postsynaptic cell adhesion localised to the PSD, it seems hardly appropriate to use postsynaptic recording to assess presynaptic release probability. Moreover, neither mEPSCs nor paired-pulse ratio allows detailed examination of fundamental presynaptic functions such as the partitioning of synaptic vesicles into populations with different release probability (synaptic vesicle pools) and the rate at which synaptic vesicles are mobilised (synaptic exocytosis) and retrieved (synaptic endocytosis) upon stimulation.

So far, only one study has employed primarily presynaptic measures to investigate the role of Nlgn1 in AZ structure and function (Wittenmayer et al., 2009). The authors showed that the AZ complex and synaptic vesicles in immature neurons (5 days *in vitro* or DIV5) became destabilised and dissipated when challenged with actin depolymerisation but developed actin-independency in mature neurons (DIV13-15). Overexpression of Nlgn1 in immature neurons rendered AZ markers such as Bassoon, Piccolo and RIM1 resistant to actin depolymerisation whereas AZ complexes in mature *Nlgn1*$^{-/-}$ neurons were unstable. The effect was specific to the scaffolding proteins, as Nlgn1 overexpression did not stabilise synaptic vesicle markers. Nlgn1 overexpression might drive AZ actin-independence by binding to S-SCAM rather than the Dlg family postsynaptic scaffolds, as truncation of the S-SCAM-specific binding site on Nlgn1 but not the PDZ ligand alone abolished the effect. Notably, S-SCAM is a MAGUK postsynaptic scaffold that binds to both Nlgn1 and β-catenin, providing a potential link between the neuroligin-neurexin and the cadherin trans-synaptic complex (Nishimura et al., 2002; Iida et al., 2004). In the same study, the uptake of the fluorescent styryl dye FM4-64 via endocytosis was used to quantify the number of synaptic vesicles that

participate in exocytosis (the recycling pool) at a given synaptic terminal, and the kinetics of dye release was used to quantify the rate of exocytosis. Using these techniques, the authors reported that Nlgn1 overexpression increased the size of the recycling pool of synaptic vesicles and accelerated exocytosis whereas Nlgn1 knockout reduced recycling pool size but not exocytic rate. However, these data warrant cautious interpretation for three important reasons: first, FM dye is internalised by synaptic terminals via endocytosis therefore the amount of dye uptake depends not only on the size of the recycling pool but also the efficiency of endocytosis. Second, the raw fluorescence signal of FM4-64 in the uptake experiments was directly compared across samples therefore is susceptible to varying experimental conditions. Third, the *Nlgn1*$^{-/-}$ neurons in fact showed a notably slower release rate of FM4-64 especially in the early phase before the release reached plateau. However, details of statistical analysis were lacking in the article. In light of the current lack of research on the possibility that Nlgn1 trans-synaptically regulate presynaptic function, Chapter 2 of this thesis will explore the topic further.

1.3.4 The behavioural relevance of Nlgn1 function

Although far from solving the complete puzzle of the role of Nlgn1 at the synapse, we have learnt a great deal about its structure, binding partners, regulation of synaptic alignment, transmission and plasticity in the past two decades. By contrast, we know far less about the behavioural consequences of the known cellular functions of Nlgn1. The data on this topic gathered so far have been surprisingly scarce compared to the interest Nlgn1 receives at the cellular level. Blundell et al. (2010) performed the most comprehensive behavioural characterisation of the Nlgn1 constitutive knockout mouse model (*Nlgn1*$^{-/-}$) to date, where *Nlgn1*$^{-/-}$ mice were compared to wildtype (WT) controls in behavioural assays aimed at assessing pain sensitivity, locomotor activity, anxiety, repetitive behaviour such as grooming and marble burying, spatial learning, fear conditioning and social behaviour. Overall, the authors reported that *Nlgn1*$^{-/-}$ and WT mice were indistinguishable in most behavioural measures with the exception that *Nlgn1*$^{-/-}$ mice displayed increased grooming, which was interpreted as a stereotyped behaviour, and travelled a longer distance to locate the hidden platform in the Morris water maze test (MWM) suggesting an impairment in spatial learning (Blundell et al., 2010). In the MWM test, however, the learning trajectories of *Nlgn1*$^{-/-}$ and WT mice were perfectly parallel across sessions with no sign of different rates of learning. The

fact that *Nlgn1*$^{-/-}$ mice swam longer distance to locate the platform from the first session and throughout the test suggests that impaired spatial learning may be a questionable interpretation.

Other Nlgn1 studies involving behavioural assessment typically consist of extensive molecular and electrophysiological characterisation with targeted behavioural experiments to demonstrate that the physiological changes indeed have behavioural consequences. Knockdown of Nlgn1 in the rat amygdala reduces the ability to retain fear memory while overexpression of Nlgn1 impairs learning the location of the hidden platform in the MWM (Kim et al., 2008; Dahlhaus et al., 2010; Hoy et al., 2013). These data are robust suggesting that Nlgn1 may indeed play a role in learning and memory. Nonetheless, these in total amount to four studies, two of which examined gain-of-function overexpression models of Nlgn1 and all relied heavily on the MWM as the major or only paradigm for assessing learning. Collectively, the best we can say about the behavioural relevance of Nlgn1 as a synaptic cell adhesion molecule that regulates crucial aspects of synaptic function is that constitutive deletion of Nlgn1 does not cause global behavioural impairment and it may be required for some aspects of learning and memory. This means the extensive literature on the synaptic function of Nlgn1 has barely been exploited for investigating its behavioural consequences. Considering this, a major theme of this thesis is to conduct a thorough experimental and theoretical dissection of the behavioural role of Nlgn1 in the framework of decision-making.

1.4 Assessing learning and utility integration in mice

Behaviour encompasses an endless set of phenomena therefore all behavioural studies face the trade-off between breadth and depth. This thesis will focus on the behavioural processes we will refer to as “learning” and “utility integration”. However, their definitions are ambiguous and subjective therefore they would be poor behavioural descriptors without clarification. The following sections will clarify the functional definitions of learning and utility integration and the approaches employed to assess them in Chapter 3.

1.4.1 Learning: optimising behaviour

We consider learning as the process and ability to optimise one's behaviour towards a certain goal. In rodent experiments (mice in the current work), we typically measure, as a proxy, how optimally they act in a behavioural task. Note that, this critically depends on the existence of a well-defined and quantifiable optimal action within a behavioural task in order to minimize confounds. A good example for this is a choice task in which subjects choose from options that differ only in the resulting rewards and the optimal action is to choose the most highly rewarded option to the best of the subjects' knowledge. Therefore, in this case, the accuracy of choosing the best option can serve as a reliable measure for task knowledge or learning. We employed similar paradigms, in which there exists a "correct" response, to assess in mice the ability to learn complex associative learning tasks.

1.4.2 Utility integration: weighing the reward and the cost

Consider now that our options not only differ in rewards but also involve different costs (e.g. money, physical effort, pain etc.), such a decision problem does not contain an objectively optimal solution. A subject's choice depends on the relative and subjective weighting on rewards and cost, or positive and negative utility. Although choosing between options that differ only in their positive or negative utilities allows us to define an optimal action under the assumption that animals aim to maximise positive and minimise negative utility, most natural decisions involve weighing and combining both positive and negative utilities associated with the options. We refer to this process descriptively as utility integration. However, it is similar to the concept of motivation especially in rodent experiments where it is often assessed by measuring the willingness to exert physical effort for rewards (Pessiglione et al., 2017).

1.4.3 The case for in-depth behavioural analyses

Krakauer et al. (2017). argued in the thought-provoking and perhaps provocative perspective article that the complexity of behaviour had been too frequently neglected in neuroscience and the necessity or sufficiency of neural correlates for a behaviour alone does not qualify as mechanisms. Invoking the example used in the article, this often leads to findings in the form of "a bird's flight depends on its feathers", which is obviously incomplete as an explanation for flight because it tells us very little about *how* birds fly using feathers and it does not

generalise to flight in general (bats can fly whereas emu cannot). Instead, the authors suggested a framework of "pluralistic explanation" for behaviour, within which behavioural phenomena are analysed and explained across multiple levels. This highlights the need for a thorough understanding of behaviour in addition to searching for the underlying neural correlates. We argue that careful behavioural analyses should aim to articulate 1) the animals' goal in a task and the behaviours they exhibit to achieve it; 2) a precise definition of the underlying behavioural processes one intends to measure and their generalisability across different tasks or contexts; and 3) a mechanistic model that could, at least in principle, generate the observed behaviour through interactions among its components, and generate falsifiable predictions. The following sections will discuss the main approaches we employed to address these issues.

1.4.3.1 Descriptive and quantitative analysis of behaviour

The quantification of complex behaviour in a task is often reduced to a single canonical measure: spatial navigation in the MWM to the latency to platform, fear learning to the percentage of freezing, instrumental learning to the number of lever-presses. Although this approach allows high throughput behavioural assessment, it forgoes a detailed understanding of complex animal behaviour by reducing it to a single dimension. We argue that one should rather "decompress" behavioural analyses and aim to quantify *how* the animals are solving a given task. Dahlhaus et al. (2010) provided an encouraging example when assessing Nlgn1-overexpressing mice in the MWM. In addition to latency to platform, the authors also quantified different navigational strategies, which provided important details about what impaired spatial learning entailed in the study.

A related problem that needs to be addressed is the abuse of the *p* value in hypothesis testing. Perhaps due to our innate attraction to dichotomies (Melnikoff and Bargh, 2018), the $p = 0.05$ threshold renders statistical analyses maximally qualitative and minimally quantitative, conveniently dividing our scientific worldview into true or false hypotheses. Despite the over-reliance on the *p* value coming under heavy criticism in recent years (Nuzzo, 2014; Leek and Peng, 2015; Baker, 2016; Singh Chawla, 2017; Benjamin et al., 2018; Amrhein et al., 2019), changes have been slow to come. Note that there is nothing intrinsically erroneous about the *p* value and it should still be used it as *one* of the metrics to report and interpret data. However, in addition to concluding whether an effect is statistically significant,

the estimated effect size and its estimated precision should also be reported. To this end, we employ various statistical models in this thesis to estimate the effects of independent variables and their 95% confidence intervals.

1.4.3.2 Identifying a robust and generalisable behavioural phenotype

In most behavioural experiments, what we intend to measure is not a behaviour unique to a particular set of experimental conditions but a generalisable latent behavioural process. For instance, in fear conditioning, what one intends to measure is the ability to associate external cues with an aversive outcome and retain that association across time rather than freezing in the fear of an electrical footshock. Just like the impact of a manipulation on a synaptic mechanism is often assessed in multiple ways to demonstrate the underlying cause, one should also ask whether a behavioural phenotype seen in one task is generalisable. For instance, if a manipulation increases freezing towards footshocks, does it also increase freezing towards predator odours? In this case, the interpretation of which behavioural process is altered clearly depends on whether increased freezing is generalisable across the two conditions.

1.4.3.3 Explaining and interpreting behaviour with theoretical models

A theory articulates how the functional components of a system interact to produce the observed data. In behavioural neuroscience/experimental psychology, the Rescorla-Wagner model is one such example. In their seminal 1972 paper, Rescorla and Wagner proposed a precise theory describing how animals learn associations and the parameters affecting learning (Rescorla and Wagner, 1972) extending similar learning theories previously proposed by Bush and Mosteller (1951b, 1951a). The benefit of employing theoretical models to interpret behavioural data is that a model provides an explanation for *how* the behavioural data may be generated by the underlying cognitive processes and generates falsifiable behavioural predictions. In this thesis, the behavioural data are interpreted based on behavioural theories within the reinforcement learning framework (Sutton and Barto, 1998; Maia, 2009). We will demonstrate that the behavioural phenotype of Nlgn1 knockout (*Nlgn1*$^{-/-}$) mice across multiple tasks could be captured, in principle, by a single theoretical model.

1.5 Thesis overview and aims

Synapses are the fundamental computational units underlying higher-level information processing in the brain. Intuitively, understanding how the function of a synapse is regulated by its protein machinery lays the foundation for understanding how information is processed at the neuron, circuit and network level. Nlgn1 is a postsynaptic cell adhesion molecule, which binds to the presynaptic cell adhesion molecules neurexins to mediate synaptic alignment, specification, transmission and plasticity. Although the importance of trans-synaptic coupling has long been recognised, most of the studies conducted so far on the synaptic function of Nlgn1 has focused on postsynaptic mechanisms.

In Chapter 2, we aim to build a more comprehensive understanding of Nlgn1 function at the synapse and explore its potential role in regulating presynaptic function.

In contrast to the focus on synaptic function in Chapter 2, Chapter 3 will provide an in-depth dissection of Nlgn1's role in decision-making, through which we will interrogate the claim made above: understanding synaptic function in terms of its protein machinery lays the foundation for understanding higher-level information processing. Given the literature on the synaptic function of Nlgn1, what can we predict about its regulation of behaviour? Can we explain Nlgn1's role in behaviour in terms of its role at the synapse? Chapter 3 contains three interconnected sub-chapters. First, given the requirement of Nlgn1 for NMDAR function and LTP, we will investigate whether the loss of Nlgn1 is also required for mice to learn complex associative structures in several learning tasks which share overlapping and distinct features. Second, we will extend the behavioural assessment beyond learning the optimal action to the trade-off between positive and negative utilities in situations where there is no objectively optimal action. Third and finally, we will attempt to explain, in the form of a simple computational model, the altered cognitive processes underlying the behavioural phenotype of Nlgn1 knockout (*Nlgn1*$^{-/-}$) mice across a wide range of behavioural tasks.

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Chapter 2: Regulation of presynaptic function by Nlgn1

2.1 Introduction

The release of neurotransmitter is precisely controlled and tightly regulated in the presynaptic terminal. Neurotransmitters are stored in synaptic vesicles and released via fusion of synaptic vesicles with the plasma membrane (exocytosis). Fused synaptic vesicles are then retrieved from the plasma membrane (endocytosis) for the next round of exocytosis. Exocytosis and endocytosis of synaptic vesicles must be tightly coupled to avoid depletion of available synaptic vesicles (Wu et al., 2014). Furthermore, synaptic vesicles are typically divided into discrete populations or synaptic vesicle pools based on their propensity to undergo exocytosis. However, it is important to know that the categorisations of synaptic vesicle pools are not universally agreed upon and the exocytic tendency of a synaptic vesicle likely falls on a continuum depending on factors such as its molecular composition and distance to the release site (Rizzoli and Betz, 2005; Denker and Rizzoli, 2010; Alabi and Tsien, 2012; Crawford and Kavalali, 2015).

Historically, studies on pre- and postsynaptic function have focused on the *cis* regulation by pre- and postsynaptic proteins respectively. However, it is becoming increasingly clear that the pre- and postsynaptic terminal function as a coordinated trans-synaptic unit (Tang et al., 2016; Biederer et al., 2017; Haas et al., 2018). Synaptic cell adhesion molecules such as Nlgn1 are promising candidates for trans-synaptic regulation as they physically couple the AZ and PSD. It has been long recognised that the synaptic function of Nlgn1 depends partially on the trans-synaptic interaction with neurexins. Notably, proteolytic cleavage of the extracellular domain of Nlgn1 suppresses synaptic strength and Nlgn1 controls the induction of LTP by binding to neurexins (Peixoto et al., 2012; Suzuki et al., 2012; Wu et al., 2019). Furthermore, neurexins are required for coupling N-type Ca^{2+} channel to neurotransmitter release. It is plausible that the loss of Nlgn1 may also disrupt this coupling.

As discussed in chapter 1, previous studies on the trans-synaptic regulation by Nlgn1 have either relied heavily on postsynaptic measures or generated suggestive but inclusive findings (Prange et al., 2004; Futai et al., 2007; Kim et al., 2008; Wittenmayer et al., 2009; Stan et al., 2010; Espinosa et al., 2015). In light of this, we sought to investigate the effect of Nlgn1 deletion on the size of different synaptic vesicle pools and the rate of exocytosis using direct presynaptic measures. To directly probe the localisation and trafficking of synaptic vesicles,

we transfected cultured hippocampal neurons with pHluorin-tagged synaptophysin (SypHy) as a marker for synaptic vesicles (**Figure 2.1A**). Briefly, pHluorin is a pH-sensitive fluorescence reporter typically fused to the lumenal domain of vesicular proteins such as synaptophysin (Miesenbock et al., 1998; Granseth et al., 2006). Its fluorescence is quenched in the acidic interior of synaptic vesicles and increases upon synaptic exocytosis when vesicular and plasma membrane fuse and the pHluorin tag is exposed to the pH-neutral extracellular environment. Normally, pHluorin fluorescence is quenched again after synaptic vesicles are retrieved from the plasma membrane and undergo re-acidification. However, in our experiments, we blocked re-acidification with bafilomycin to prevent endocytosis from interfering with the quantification of the size of synaptic vesicle pool and exocytic rate.

To quantify the size of synaptic vesicle pools, we divide synaptic vesicles into three different pools based on the strength of stimulation required to mobilise them: 1) the readily releasable pool, which undergoes exocytosis upon a brief burst of electrical stimuli (30Hz for 2s); 2) the reserve pool, which requires prolonged stimulation to be mobilised (10Hz for 1200s) and 3) the resting pool which cannot be mobilised (**Figure 2.1B**). The readily releasable and reserve pool combine to form the total recycling pool which contain all the synaptic vesicles releasable by stimulation. We found that the loss of Nlgn1 moderately reduced the size of the total recycling pool of synaptic vesicles when the readily releasable and reserve pool were sequentially released but not in experiments where the total recycling pool was released with one single train of stimuli. Furthermore, we quantified the rate at which the total recycling pool of synaptic vesicles underwent exocytosis and found that it was not impacted by Nlgn1 deletion. In summary, our findings suggest that the loss of Nlgn1 from the postsynaptic terminal does not substantially affect the size of synaptic vesicle pools and exocytosis.

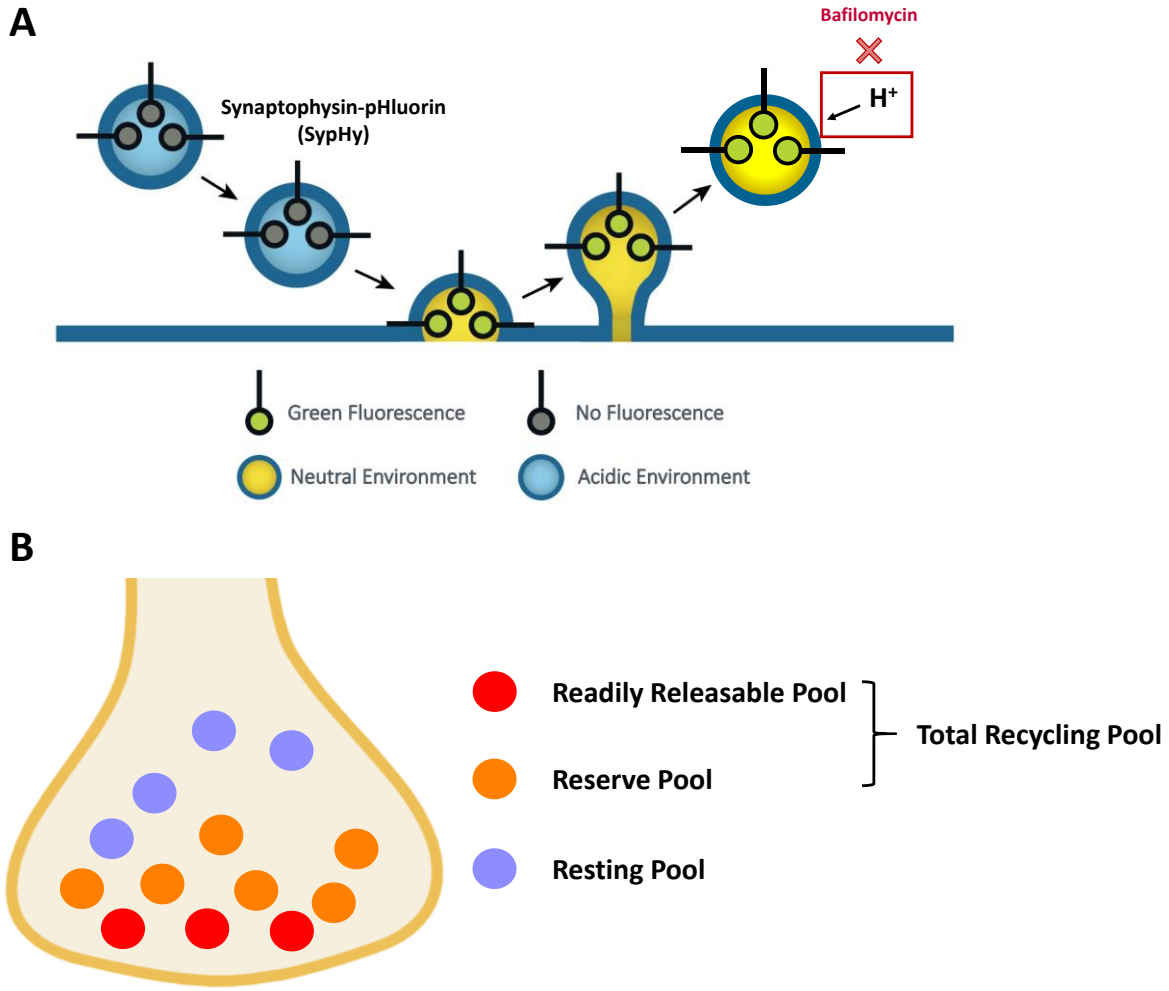

Figure 2.1 Using SypHy to report the localisation and trafficking of synaptic vesicles. A) Exocytosis causes an increase in SypHy fluorescence. Re-acidification (H^+) of synaptic vesicles upon endocytosis is blocked by bafilomycin in all our experiments. B) We divide synaptic vesicles into three categories based on the strength of electrical stimulation required to trigger their exocytosis. The readily releasable pool (red) are synaptic vesicles that undergo exocytosis in response to a short burst of electrical stimuli (30Hz for 2s). The reserve pool of synaptic vesicles (orange) require prolonged stimulation to be mobilised (10Hz for 120s) and resting pool (purple) cannot be mobilized. Although the readily releasable, reserve and resting pool are depicted with increasing distance from the plasma membrane, distance from the release site is not the only factor that influences the propensity to undergo exocytosis.

2.2 Methods

2.2.1 Materials

Synaptophysin-pHluorin (SypHy) was provided by Prof Leon Lagnado (University of Sussex, UK). Neurobasal media, B-27 supplement, L-glutamine, penicillin/streptomycin, Minimal Essential Medium (MEM), Dulbecco's Modified Eagle Medium (DMEM), Lipofectamine 2000 were obtained from Thermo Fisher Scientific. Bafilomycin A1, 6-cyano-7-nitroquinoxaline-2,3-

dione and DL-2-Amino-5-phosphonopentanoic acid were obtained from Sapphire Bioscience. All other reagents were obtained from Sigma-Aldrich.

2.2.2 Hippocampal neuronal cultures

Dissociated primary hippocampal enriched neuronal cultures were prepared from E16.5-18.5 WT and *Nlgn1*$^{-/-}$ C56BL/6J mouse embryos of both sexes (obtained from an in-house breeding colony) by trituration of isolated hippocampi to obtain a single cell suspension, which was plated at a density of 5 x10^5 cells/coverslip on poly-D-lysine and laminin-coated 25 mm coverslips. Cultures were maintained in neurobasal media supplemented with B-27, 0.5 mM L-glutamine and 1% v/v penicillin/streptomycin. After 72 hours cultures were further supplemented with 1 µM cytosine β-d-arabinofuranoside to inhibit glial proliferation. Cells were transfected after 7 days in culture with Lipofectamine 2000 as described (Gordon et al., 2011). In all experiments synaptophysin-pHluorin (SypHy) was co-transfected with mCherry empty vector (as a transfection marker). Cells were imaged after 13-15 days in culture.

2.2.3 Fluorescence imaging protocol

Hippocampal cultures were mounted in a Warner imaging chamber with embedded parallel platinum wires (RC-21BRFS) and placed on the stage of Zeiss Axio Observer 7 epifluorescence microscope for experiment quantifying exocytic rate and Leica SP8 confocal microscope for determining synaptic vesicle pool size and surface fraction of SypHy. Neurons expressing SypHy were visualized with a x40 oil immersion objective (NA 1.3) at 475nm (Zeiss) or 488nm (Leica) excitation wavelength and with a GFP filter. Neurons were subjected to continuous perfusion with imaging buffer (in mM: 136 NaCl, 2.5 KCl, 2 CaCl2, 1.3 MgCl2, 10 glucose, 10 HEPES, pH 7.4) throughout image acquisition at 37°C.

Surface fraction of SypHy was revealed by bathing neurons in imaging buffer, and then perfusing with acidic imaging buffer (20 mM 2-(N-morpholino) ethanesulfonic acid substituted for 10 mM HEPES, pH 5.5) to quench surface SypHy fluorescence and retain background autofluorescence. Neurons were washed in imaging buffer, and then exposed to alkaline imaging buffer to reveal total SypHy fluorescence. Z-stacks were captured at 30 seconds per frame for 27 frames. The surface fraction of SypHy as a percentage of total was calculated as [(basal fluorescence - acidic fluorescence) / (alkaline fluorescence - acidic fluorescence)] x 100.

To quantify the size of synaptic vesicle pools, the readily releasable and reserve pool were released sequentially by two separate trains of stimuli in the presence of 10 µM 6-cyano-7-nitroquinoxaline-2,3-dione and 50 µM DL-2-Amino-5-phosphonopentanoic to inhibit network activity and 1µM bafilomycin A1 to inhibit vesicle acidification. To release the readily releasable pool, neurons were stimulated with a train of 60 action potentials delivered at 30 Hz (100 mA, 1 ms pulse width). Subsequently, the reserve pool was released by a train of 1200 action potentials at 10Hz. Z-stacks were captured at 1 minute per frame for 13 frames. Neurons were subjected to continuous perfusion with imaging buffer throughout image acquisition and alkaline imaging buffer (50 mM NH_4Cl in imaging buffer recipe substituted for 50 mM NaCl) to reveal total SypHy fluorescence at the final stage of image acquisition.

To quantify the rate of exocytosis, neurons were stimulated with a train of 1200 action potentials at 10Hz in the presence of 10 µM 6-cyano-7-nitroquinoxaline-2,3-dione and 50 µM DL-2-Amino-5-phosphonopentanoic to inhibit network activity and 1µM bafilomycin A1 to inhibit vesicle acidification. Images were captured at 1 seconds per frame at baseline for 20 frames and 2 seconds per frame during and after stimulation for 80 frames. Neurons were subjected to continuous perfusion with imaging buffer throughout image acquisition and alkaline imaging buffer (50 mM NH_4Cl in imaging buffer recipe substituted for 50 mM NaCl) to reveal total SypHy fluorescence at the final stage of image acquisition. Change in fluorescence ΔF was quantified as $(F_t\text{-}F_0)/F_0$ where F_t is the raw fluorescence signal at time t and F_0 is the baseline fluorescence prior to stimulation. To quantify the size of synaptic vesicle pools, ΔF was normalised to the maximum fluorescence increase revealed by alkaline buffer (F_{max}) and expressed as a percentage. For quantification of exocytic rate, ΔF was normalised to the peak fluorescence increase during stimulation (F_{peak}).

2.2.4 Statistical analysis

Surface fraction of SypHy, size of synaptic vesicle pools and exocytic rates were quantified for individual synapses. Overall exocytic rate was quantified by fluorescence change to peak fluorescence change during stimulation and fitted with the function:

$$F_t = 1 - e^{-kt} + F_0$$

Early exocytic rate was fitted with the function:

$$F_t = kt + F_0$$

Here, F_t is the normalised fluorescence at time t, k the rate of exocytosis and F_0 is the intercept. Curve-fitting was perform using the MATLAB *fit* function.

To estimate the effect of Nlgn1 deletion while accounting for the potential intra-cluster correlations between synapses from the same neuron, and neurons prepared from the same culture, we employed a three-level (culture, neuron, synapse) mixed-effect general or generalised linear model with neurons and cultures treated as random intercepts. A gamma conditional response distribution and a log link function were specified for the mixed-effect generalised linear model to estimate the effect of Nlgn1 deletion on exocytic rates. Statistical analyses were performed in STATA v14.0 (Statacorp, College Station, Texas, USA).

2.3 Results

2.3.1 Similar surface/vesicle distribution of SypHy in *Nlgn1*$^{-/-}$ and WT synapses

The baseline fluorescence of SypHy-transfected synapses is emitted by plasma-membrane-localised SypHy (surface SypHy) whose lumenal domain is exposed to the pH-neutral extracellular space (**Figure 2.2A**). Given that we use fluorescence increase from baseline to monitor exocytosis, it is important to ensure the increase from baseline fluorescence is not confounded by differences in surface/vesicle SypHy localisation. Therefore, we compared the baseline distribution of SypHy between the plasma membrane and synaptic vesicles in *Nlgn1*$^{-/-}$ and WT synapses. To do this, we sequentially perfused neurons with acidic (to quench pHluorin fluorescence to residual autofluorescence and reveal surface level) and alkaline buffers (to reveal total pHluorin fluorescence) (**Figure 2.2B-C**). We quantified the presynaptic functions examined in this chapter for individual synapses and compared them between the genotypes because surface SypHy level, synaptic vesicle pool size and exocytic rates are properties of the synaptic terminal. To avoid treating synapses as independent samples, we employed three-level mixed-effect linear/generalised linear model to account for the potential correlations between synapses from the same neuron and neurons prepared from the same culture (**Methods**). We found that the loss of Nlgn1 had very little impact on the fraction of SypHy on the plasma membrane at baseline (**Figure 2.1D-E**). It is worth noting that WT synapses in these data showed both strong clustering within cultures and higher variance between cultures (different cultures indicated by different shades of blue and orange in

Figure 2.1D and E). We therefore interpret the data with caution and aim to confirm this finding in subsequent experiments.

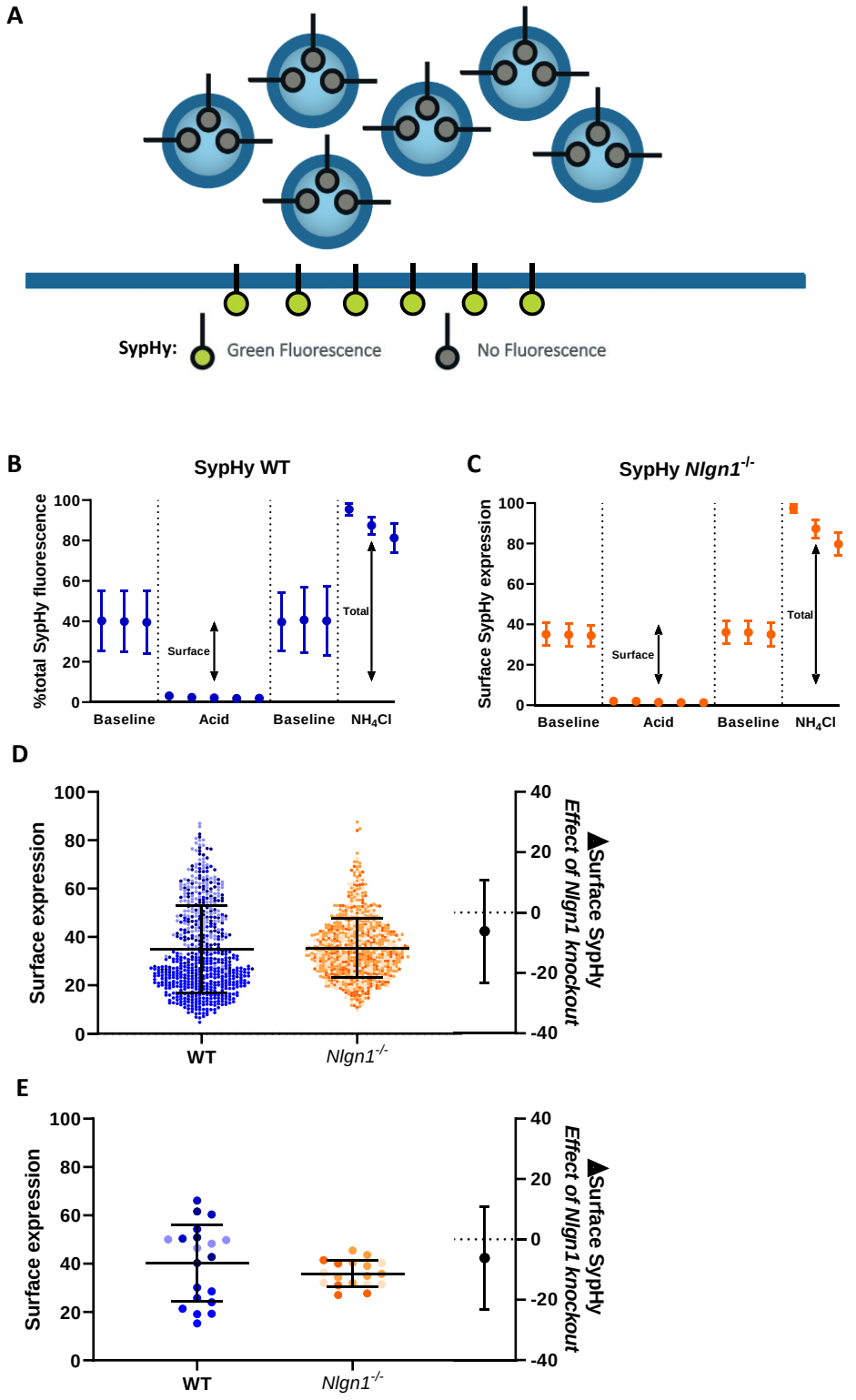

Figure 2.2 SypHy has dimilar distribution between synaptic vesicles and the plasma membrane in *Nlgn1*$^{-/-}$ and WT synapses. A) SypHy localised to the plasma membrane at baseline (surface SypHy) is the source of baseline fluorescence signal. B-C)

Experimental design for quantifying the surface level of SypHy in WT (B, blue circles) and *Nlgn1*$^{-/-}$ (C, orange circles). Values represent mean ± SD D) Baseline surface SypHy level of individual synapses and E) averages of synapses from the same neurons. Data points are colour-coded according to culture level clustering, values represent mean ± SD. The effect size of Nlgn1 deletion on surface level SypHy is displayed as forest plots on the right with 95% CI, Multi-level mixed effects general linear model.

2.3.2 Loss of Nlgn1 moderately reduces the total recycling pool upon sequential mobilisation of the readily releasable and reserve pool

We broadly divide synaptic vesicles into the readily releasable pool, the reserve pool and the resting pool based on the strength of stimulation required to trigger their exocytosis. We employed a two-step stimulation protocol to first release the readily releasable pool with a short burst of electrical stimuli (60 stimuli, 3Hz for 20s), and then the reserve pool with a prolonged train of stimuli (1200 stimuli, 10Hz for 120s). The fluorescence increase from baseline in response to the stimulations indicates exocytosis of synaptic vesicles (**Figure 2.3A)**. In addition, we perfused the neurons with alkaline buffer after stimulation to reveal the fluorescence of the total population of synaptic vesicles including the resting pool. The size of synaptic vesicle pools was measured by the increase in SypHy fluorescence from baseline normalised to the total SypHy fluorescence revealed by alkaline buffer. We observed a fractional reduction in both the readily releasable and the reserve pool in *Nlgn1*$^{-/-}$ compared to WT synapses, which did not reach statistical significance (**Figure 2.3B-C**). However, we found that Nlgn1 deletion caused a small (~6%) but significant reduction in the total recycling pool comprised of both the readily releasable and reserve pool (**Figure 2.1B**, **2.3B-C**). To examine whether the loss of Nlgn1 alters the contribution of the readily releasable to the total recycling pool, we compared their ratios and found that *Nlgn1*$^{-/-}$ and WT synapses were indistinguishable suggesting that the overall reduction in the total recycling pool affects both the readily releasable and reserve pool equally in *Nlgn1*$^{-/-}$ synapses. We also expressed the baseline SypHy fluorescence as a percentage of total SypHy fluorescence revealed by alkaline buffer to estimate the surface fraction of SypHy at baseline (similar to **Figure 2.3**). Consistent with the results obtained above, around 40% of SypHy is localised to the plasma membrane and 60% on synaptic vesicles at baseline for both *Nlgn1*$^{-/-}$ and WT synapses.

In summary, we observed that at baseline 60% of SypHy was localised to synaptic vesicles and 40% on the plasma membrane in both *Nlgn1*$^{-/-}$ and WT synapses (**Figure 2.4**). Of the vesicular SypHy, 23% was readily releasable and 37% was in the reserve pool in WT

synapses, leaving roughly 40% in the resting pool which could not be mobilised by stimulation. We found a 3% decrease in the size of the readily releasable and reserve pools in *Nlgn1*$^{-/-}$ synapses, which collectively constitutes a 6% reduction in the total recycling pool.

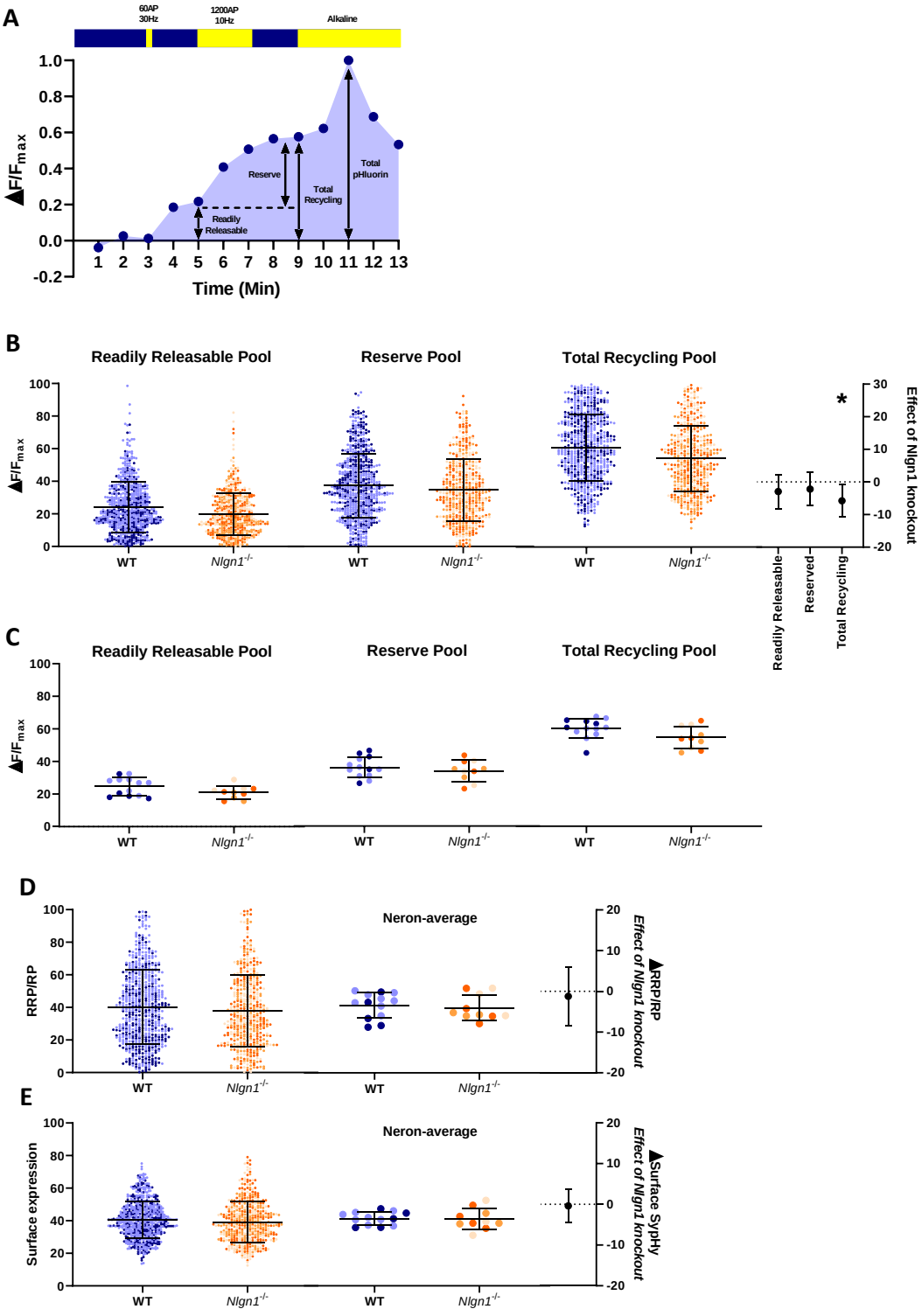

Figure 2.3 Impact of Nlgn1 deletion on the size of synaptic vesicle pools. A) Schematic of the two-step stimulation protocol and representative trace SypHy fluorescence increase from baseline normalised to the maximum change revealed by

perfusing neurons win alkaline buffer (F_{max}). B) Size of the readily releasable, reserve and total recycling pool of individual synapses, and C) averages of synapses from the same neurons. D) The ratio between the readily releasable and total recycling from individual synapses and averages of synapses from the same neurons. E) Baseline surface SypHy level of individual synapses and averages of synapses from the same neurons. Data points are colour-coded according to culture level clustering, values represent mean ± SD. The effect size of Nlgn1 deletion is displayed as forest plots on the right with 95% CI, Multi-level mixed effects general linear model.

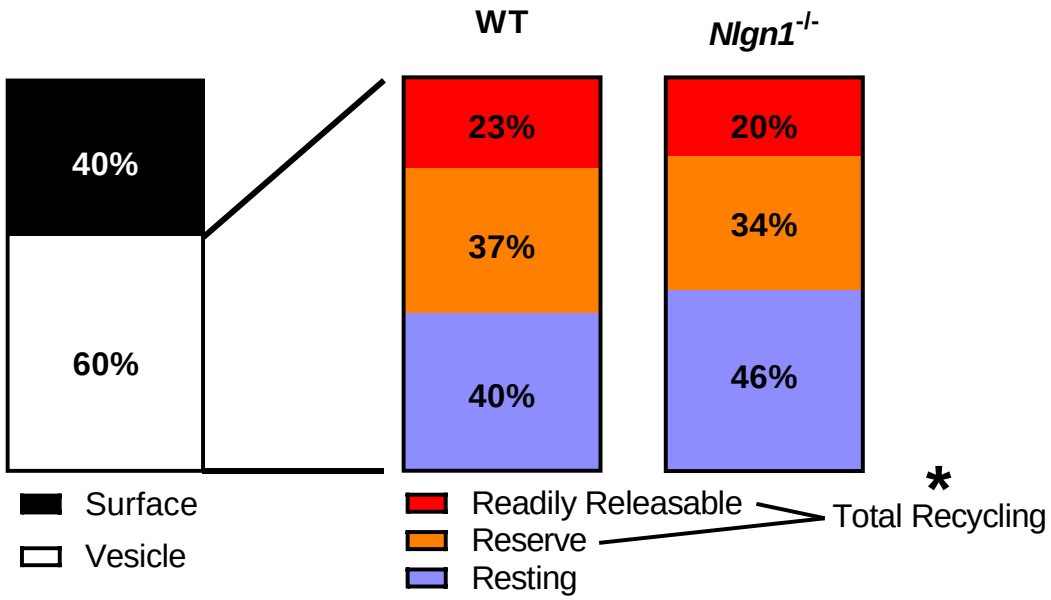

Figure 2.4 Distribution of SypHy on the plasma membrane (Surface), the readily releasable, reserve, total recycling and resting pool of synaptic vesicles in *Nlgn1*$^{-/-}$ and WT synapses. *$P < 0.05$.

2.3.3 Nlgn1 deletion does not impact exocytic kinetics of the total recycling pool of synaptic vesicles

Given that neurexins couple voltage-gated Ca^{2+} channels to the exocytic machinery, it is plausible that the loss of Nlgn1 will disrupt this coupling by disrupting the Nlgn1-neurexin trans-synaptic complex (Missler et al., 2003). Thus, we asked whether Nlgn1 deletion led to a slower rate of exocytosis. For this experiment, we released the entire total recycling pool with one single prolonged train of stimuli (1200APs, 10Hz for 120s) (**Figure 2.5A**). Exocytic rate was quantified for individual synapses by curve-fitting the fluorescence increase from baseline normalised to the peak increase during stimulation. Contrary to our expectation, synaptic vesicles at *Nlgn1*$^{-/-}$ and WT synapses underwent exocytosis at an indistinguishable rate (**Figure 2.4C**). Previous data suggest that Nlgn1 deletion may reduce release efficiency in the early phase of exocytosis (Wittenmayer et al., 2009), we therefore applied a linear fit to the early

phase of exocytosis (first ten seconds, **Figure 2.4B**) where fluorescence increase is largely linear. Similar to the overall exocytic rate, we found no difference in the early exocytic rate between the genotypes (**Figure 2.4D**). Together, these data suggest that Nlgn1 is not required for efficient synaptic exocytosis.

In this experiment, the asymptotic level of fluorescence increase during stimulation can be taken as an estimate for the size of the recycling pool once normalised to the total SypHy fluorescence revealed by alkaline buffer **(Figure 2.4E**). This can be compared with the estimates obtained from the previous experiments where the readily releasable pool and the reserve pool were mobilised sequentially and were combined to quantify the total recycling pool. Both sets of data give highly consistent estimates for the size of the recycling pool (~60%). However, unlike in the previous experiment where the readily releasable and reserve pool were sequentially released, we did not observe the same subtle reduction in the size of the recycling pool in *Nlgn1*$^{-/-}$ synapses when the total recycling pool was released with one single train of stimuli (**Figure 2.4F**).

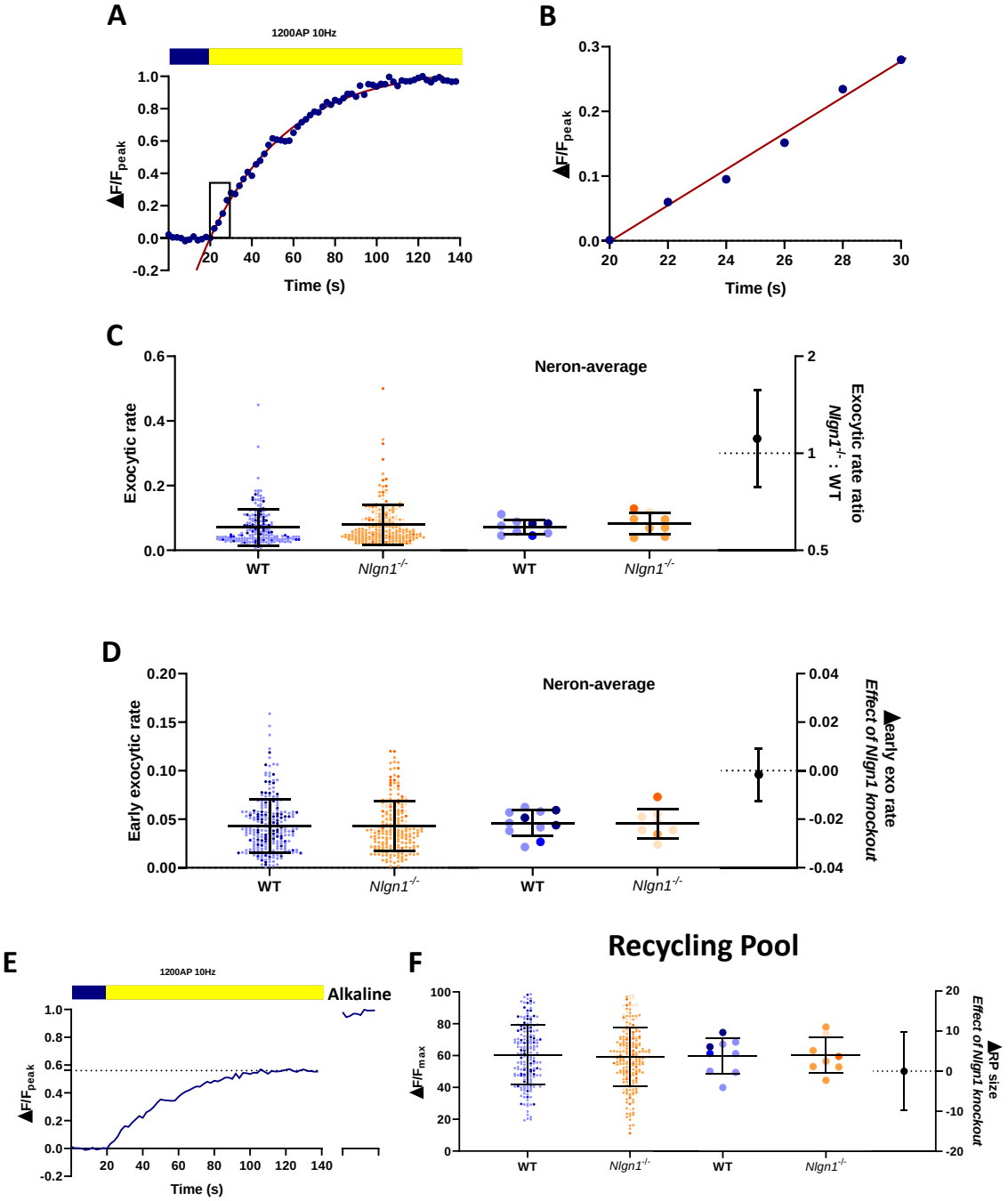

Figure 2.5 Similar rate of exocytosis in *Nlgn1*$^{-/-}$ and WT neurons. A) Representative trace of SypHy fluorescence increase from baseline normalised to the peak change during the simulation period (F_{peak}), curve-fitting was performed between the onset and termination of stimulation (red line). Square: early phase of exocytosis. B) Early phase of exocytosis, square in A. A straight line is fitted from the fist to the sixth frame after the onset of stimulation. C) Exocytic rate of individual synapses and averages of synapses from the same neurons. D) Early exocytic rate of individual synapses and averages of synapses from the same neurons. E) Representative trace of SypHy fluorescence increase from baseline normalised to the maximum change (F_{max}) revealed by alkaline buffer. F) Total recycling pool size of individual neurons and averages of synapses from the same neurons. Data points are colour-coded according to culture level clustering, values represent mean ± SD. The effect size of Nlgn1 deletion is displayed as forest plots on the right with 95% CI, Multi-level mixed effects generalised linear model for C, Multi-level mixed effects general linear model for D and E.

2.4 Discussion

In this chapter, we used SypHy to probe the impacts of Nlgn1 deletion on the size of the readily releasable pool, the reserve pool and collectively the total recycling pool of synaptic vesicles. We observed a 6% reduction in the total recycling pool in *Nlgn1*$^{-/-}$ synapses with roughly equal impacts on the readily releasable and reserve pool when the two pools were sequentially mobilised. However, we found no difference in the size of the total recycling pool between *Nlgn1*$^{-/-}$ and WT synapses in a different experiment where the total recycling pool was released by one single train of stimuli. Furthermore, we quantified the rate of exocytosis throughout or during the early phase of exocytosis of the total recycling pool and found that it was not altered by Nlgn1 deletion. Taken together, we found that the loss of Nlgn1 did not substantially impact the size of synaptic vesicle pools or the rate of exocytosis.

In contrast to a 60% reduction in the size of the total recycling pool previously reported in *Nlgn1*$^{-/-}$ hippocampal cultures (Wittenmayer et al., 2009), we found that the size of synaptic vesicle pools was not overtly affected by the deletion of Nlgn1. Several differences in experimental design and methodology may explain this discrepancy. We labelled synaptic vesicles by transfecting neurons with SypHy whereas Wittenmayer et al. (2009) stimulated neurons over 90s to induce fluorescent dye uptake. Importantly, synaptic vesicle labelling by fluorescent dye uptake is achieved by endocytosis during the stimulation period therefore the efficiency of labelling is heavily dependent on the rate of endocytosis and the pools of vesicles that undergo endocytosis. One possibility is that synapses lacking Nlgn1 undergo endocytosis at a slower rate therefore fewer vesicles were labelled by dye uptake. We have not assessed the impact of Nlgn1 deletion on the efficiency of endocytosis in this chapter. Future experiments investigating this will provide a more comprehensive understanding of the function of Nlgn1 in regulating the functional homeostasis of the presynaptic terminal. A second concern regarding the reported pool size difference in the previous study is that only 40% of the dye taken up during the uptake period could be subsequently released by depolarisation suggesting a mismatch between the identity of vesicles undergoing exocytosis during the labelling and release stage. Lastly, the authors used raw fluorescence signal as a direct readout of synaptic vesicle pool size and compared across cultures and experiments. This is prone to systemic errors because the raw fluorescence signal from FM dye uptake can vary greatly with experimental conditions such as the intensity of excitation laser/LED,

fluorescence decay and the effectiveness of washing. Using pHluorin fluorescence as a measure circumvents this issue because it allows us to normalise the fluorescence signal from synaptic vesicles undergoing exocytosis at a given synapse to the pHluorin fluorescence of the total population of synaptic vesicles (including the resting pool) at the same synapse.

A second surprising observation was the differential impact of Nlgn1 deletion on the size of the total recycling pool depending on whether it was sequentially released or by one single train of stimuli. Although we obtained highly consistent estimates for the size of the total recycling pool either using a two-step protocol to mobilise the readily releasable pool and reserve pool sequentially or one single prolonged stimulus train to mobilise the entire recycling pool, we only observed a reduction in *Nlgn1*$^{-/-}$ synapses when the readily releasable and reserve pool underwent exocytosis sequentially. We do not know the exact cause underlying the stimulation-dependent effect of Nlgn1 deletion on the size of the total recycling pool, but it may be attributed to the different stimulation patterns and timescales of image acquisition between the two experiments. In the first experiment, mobilising the readily releasable pool by a burst of high-frequency stimuli may have impacted the kinetics of the subsequent release of the reserve pool. An obvious way to address this possibility is to repeat the same experiment with a faster rate of image acquisition so that the exocytic rate of both the readily releasable pool and the reserve pool can be quantified and compared between *Nlgn1*$^{-/-}$ and WT synapses. As the sequential mobilisation of the readily releasable and reserve pool was conducted over 15 minutes, it is likely that spontaneously released synaptic vesicles had a non-negligible contribution to our measurements of the readily releasable pool and reserve pool, some of which may have come from the resting pool (Fredj and Burrone, 2009). Although changes in spontaneous release probability have not been reported in hippocampal neurons, future experiments may offer more evidence by directly monitoring spontaneous exocytosis using pHluorin reporters. In summary, the populations of synaptic vesicles undergoing exocytosis and their kinetics may be different in the two sets of experiments due to differences in stimulation patterns and experimental timescales. Further experiments are required to reveal the underlying causes.

Our findings so far suggest that disrupting the trans-synaptic interaction between Nlgn1 and neurexins does not substantially impact the size of synaptic vesicle pools and the efficiency of exocytosis. Importantly, our observations also highlight many exciting

outstanding questions that future experiments can be designed to address. Firstly, assessing the impact of Nlgn1 deletion on endocytosis will help resolve the disagreement between the lack of an effect of Nlgn1 deletion on the size of the total recycling pool in this study and the 60% reduction reported previously. Secondly, assessing spontaneous exocytosis in *Nlgn1*$^{-/-}$ and WT synapses will provide a better understanding on the stimulation-dependent effect of Nlgn1 deletion on the size of the total recycling pool. Furthermore, future experiments are needed to shed light on whether the requirement of Nlgn1 for LTP depends on a potentiation of exocytosis given that LTP induction requires the trans-synaptic interaction between Nlgn1 and neurexins (Wu et al., 2019). Finally, investigation of the synaptic function of Nlgn1 and other synaptic proteins in other cell types in addition to hippocampal and cortical neurons with greatly benefit our understanding of the interaction between the function of a synaptic protein and its cellular environment.

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Chapter 3: Nlgn1 deletion differentially impacts utility trade-off but not associative learning

3.1 Introduction

In a changing environment, the most fundamental challenge for the brain is to guide behaviour towards maximizing survival. Understanding how this is orchestrated by the physical components of the brain, from circuits to regions, to cells types and ultimately protein machineries, is arguably the principal goal of neuroscience. An important approach towards this goal is by investigating synaptic communication between neurons and how it is regulated by synaptic proteins, the clinical relevance of which is supported by the evidence that human mutations in synaptic genes disrupt cognitive phenotypes and are over-represented in neurodevelopmental disorders (Bayes et al., 2011; Fromer et al., 2014). Decades of research has generated an enormous amount of molecular and functional data on key synaptic proteins and significantly advanced our knowledge of the basic structure and operation of synapses.

Neuroligin-1 (Nlgn1) is a member of the extensively studied neuroligin family of postsynaptic cell adhesion molecules. It binds to neurexins on the presynaptic terminal to form trans-synaptic complexes at excitatory synapses (Ichtchenko et al., 1995; Song et al., 1999) and are critical for synapse specification, maintenance, function and plasticity. At the postsynaptic density (PSD), Nlgn1 directly binds to postsynaptic scaffolds including PSD-95 aligning PSD components with neurotransmitter release sites (Irie et al., 1997; Iida et al., 2004; Haas et al., 2018), promotes the retention of α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptors (AMPARs) (Heine et al., 2008; Mondin et al., 2011) and *N*-methyl-D-aspartate receptors (NMDARs) by indirect intracellular (Barrow et al., 2009) and direct extracellular interactions (Budreck et al., 2013) during early dendritic spine development and in mature synapses. Functionally, NMDAR-mediated postsynaptic currents have been consistently shown to be decreased by Nlgn1 knockout or knockdown (Nam and Chen, 2005; Chubykin et al., 2007; Kim et al., 2008; Blundell et al., 2010; Jung et al., 2010; Budreck et al., 2013; Espinosa et al., 2015; Jiang et al., 2017; Wu et al., 2019) and conversely, increased by Nlgn1 overexpression (Hoy et al., 2013). Further, Nlgn1 is essential for synaptic plasticity and the induction of both NMDA receptor-dependent and -independent long-term potentiation (LTP) (Chubykin et al., 2007; Kim et al., 2008; Blundell et al., 2010; Jung et al., 2010; Shipman and Nicoll, 2012; Budreck et al., 2013; Jedlicka et al., 2015; Jiang et al., 2017; Wu et al., 2019).

In contrast to the extensive molecular and physiological studies, how the documented synaptic signalling changes translate to changes in decision-making and complex behaviour has been less explored, with the exception of some evidence for altered learning and memory (Kim et al., 2008; Blundell et al., 2010; Hoy et al., 2013). While these studies collectively covered a wide array of behavioural assays, they relied on the primary measures of the behavioural assays as a direct readout for the underlying cognitive processes (e.g. increased swim distance in MWM equals impaired spatial memory) therefore did not dissect the complex behaviour within each assay in detail. Further, there was no experimental and theoretical decomposition of the behavioural phenotype to uncover common causes that can account for behavioural changes across assays.

In this chapter, we assessed male and female Nlgn1 knockout (*Nlgn1*$^{-/-}$) mice on two key decision problems: 1) the ability to learn and exploit the associative structure of the environment and 2) the trade-off between potential rewards and costs, or positive and negative utilities associated with potential actions. Despite consistent report of NMDAR hypofunction and reduced LTP in *Nlgn1*$^{-/-}$ mice, they show no overt impairment in learning reward contingencies based on complex visual and spatial information. However, we found that *Nlgn1*$^{-/-}$ mice were less motivated to exert effort for potential rewards across multiple tasks but were more willing to exert effort to escape an aversive situation. Under the assumption that different types of positive and negative utilities may be integrated into two opposing common neural currencies, we suggest that the seemingly divergent phenotype of *Nlgn1*$^{-/-}$ mice in reward- and punishment-driven tasks may be explained an increased weighting on negative utilities. Our data reveal a novel role of Nlgn1 in distinct components of decision-making, which highlights the importance of detailed investigation on the behavioural impact of synaptic proteins and the challenges in relating behaviour to cellular mechanisms.

3.2Methods

3.2.1 Animals and housing

Nlgn1$^{-/-}$ mice were obtained from Prof. Nils Brose, generated by homologous recombination of embryonic stem cells deleting exon sequences covering the translational start site and 546

bp of 5' coding sequence of the murine *Nlgn1* gene (Varoqueaux et al., 2006), and backcrossed more than 10 generations on a C57BL/6 background. *Nlgn1*$^{-/-}$ mice, and WT littermate matched controls were generated by mating heterozygous females and males. Mice were weaned at 3-4 weeks of age and housed in mixed genotype groups of 2-4 per individually ventilated cage (IVC) with food and water available *ad libitum*. Bedding consisted of sawdust chips 2cm deep and tissue paper for nesting material. At ~10 weeks of age, mice were moved from individually ventilated cages to open top cages in a humidity and temperature-controlled holding room maintained on a 12:12-hour reversed light/dark cycle (lights off at 07:00). All mice were housed under identical conditions. Mice were acclimatised to these conditions for a minimum of one week prior to handing. Pre-training began at ~12 weeks of age and all behavioural testing was conducted during the dark active phase of the cycle, with the experimenter blinded to genotype during behavioural testing. All procedures were approved by the Florey Institute of Neuroscience and Mental Health Animal Ethics Committee.

3.2.2 Rodent touchscreen operant tasks

3.2.2.1 Apparatus

Touchscreen testing was conducted in the Bussey-Saksida mouse touchscreen operant system (Campden Instruments Ltd, UK). Stimulus presentation, task parameters and data recording were controlled through Whisker Server and ABET II Touch software (Campden Instruments Ltd, UK).

3.2.2.2 Pre-training

Pretraining and food restriction were conducted as previously described (Horner et al., 2013; Mar et al., 2013; Nithianantharajah et al., 2013). Before testing, mice were first food restricted to 85 – 90% free-feeding body weight. Mice were then trained through five phases or instrumental conditioning to associate nose-poking visual stimuli on the touchscreen with the delivery of a liquid reward (strawberry flavoured milk, Devondale, Australia). Briefly, mice were habituated to the touchscreen chamber and to consuming liquid rewards from the magazine for two 20-minute sessions and required to consume two freely available rewards each session (phase 1, Habituation). For phases 2–5, a trial did not advance until the reward was consumed. In phase 2 (Initial Touch), a single visual stimulus was displayed on the screen

for 30 s, after which the disappearance of the stimulus coincided with delivery of a 20 µL reward, presentation of a tone and illumination of the reward magazine. A nose-poke response to the stimulus during the 30s window was rewarded with a 3x reward (criterion: 30 trials in 60 min). In phase 3 (Must Touch), mice were required to nose-poke visual stimuli that on the screen to obtain a reward. A trial did not advance otherwise (criterion = 30 trials in 60 min). Mice then learnt to initiate a new trial with a head entry into the reward magazine (phase 4, Must Initiate, criterion = 30 trials in 60 min). In phase 5, responses at a blank part of the screen during stimulus presentation produced a 5-second timeout (signalled by illumination of the house light) to discourage indiscriminate screen responding (criterion = 21/30 correct responses in 60 min on 2 consecutive days). Only phase 3 – 5 were not required for progressive ratio and fixed ratio testing.

3.2.2.3 Pairwise visual discrimination and reversal learning

The Pairwise discrimination (PD) and reversal learning task were conducted similarly to previously described (Horner et al., 2013; Mar et al., 2013). Briefly, mice were trained to discriminate between two novel, equiluminescent visual stimuli (left and right diagonal stripes) displayed pseudorandomly across two locations with equal number of appearances at each location. Response to one stimulus resulted in reward delivery (S+, correct response), followed by a pseudorandom trial (maximum 30 per session); response to the other stimulus resulted in a 5-second timeout, illumination of the house light followed by a correction trial. The same stimulus configuration was presented on correction trials until a correct response was made and a reward was delivered. Correction trials were not counted towards the trial limit or percentage of correct responses of a session. The designation of S+ and S- was counterbalanced within genotype and sex groups. Mice were trained to an acquisition criterion of 80% correct responses across two consecutive sessions. Following the acquisition of the visual discrimination task, mice were immediately moved on to the reversal leaning task, where the previously acquired reward contingencies were reversed. Reversal learning was assessed across 20 sessions.

3.2.2.4 Object location paired-associates learning

The paired associates object-location learning (PAL) task was conducted as previously described (Horner et al., 2013). Briefly, mice were trained to acquire reward associations jointly defined by visual stimuli (flower, plane and spider) and their assigned correct spatial

locations on the touchscreen (left, centre and right, respectively). For each trial, only two objects were presented: one object in its correct location (S+) and the other object in one of two incorrect locations (S−); therefore, there were six possible trial types. A nose-poke to the S+ resulted in delivery of a reward followed by a pseudorandom trial (maximum 36 per session), and incorrect responses resulted in a 5 s time-out followed by correction trial. Acquisition of the PAL task was assessed across 40 sessions.

3.2.2.5 Instrumental extinction learning

The instrumental extinction learning task was conducted similar as previously described (Mar et al., 2013; Nithianantharajah et al., 2013). Mice were first trained to associate response to a single white square displayed on the touchscreen with a reward (30 trials under 12.5 minutes across five consecutive sessions). Following acquisition, instrumental extinction was assessed where responses were no longer rewarded. During extinction, the visual stimulus was displayed for 10 seconds on each trial, which could result in either a response or omission. Mice were given 30 trials per session and assessed across six sessions.

3.2.2.6 Progressive ratio

The touchscreen progressive ratio testing has been described in detail previously (Heath et al., 2015). Briefly, a naïve cohort of mice first underwent phase 1 and 2 of pre-training, followed by one session of fixed ratio 1 (FR1), FR2, FR3 and three sessions of FR5 training where a fixed number of nose-pokes (1, 2, 3 and 5 respectively) resulted in a reward. Mice were required to complete 30 trials within 60 minutes in each of the FR sessions. Once training criterion was reached, mice advanced to the progressive ratio training where the number of nose-poke responses required to obtain a reward incremented by 4 after every trial (1, 5, 9, 13 etc.). If no response to the touchscreen or magazine entry was detected for five minutes, the session ended, and the animal was removed from the chamber.

3.2.2.7 Fixed ratios

A naïve cohort of mice first underwent phase 1 and 2 of pre-training followed by three sessions of FR1 and were required to complete 30 trials in each session before advancing to the test phase. During the test phase of serial FR testing, mice were given 60 minutes per session to make as many responses as they were willing to and sessions did not terminate

due to inactivity. Mice were trained through three sessions of FR1, FR5, FR20 and FR40 sequentially.

3.2.2.8 Fixed ratio 20 with water rewards

Following the serial FR testing, mice were water-restricted with access to water limited to one hour per day. Water-restricted body weight was maintained between 85 – 90% of free-feeding body weight. Mice were tested on FR20 for three sessions where 20 nose-poke responses resulted in a water reward. After each session, mice were returned to home cage and given one-hour free access to water. Water restricted body weights were recorded daily.

3.2.3 Other behavioural testing

3.2.3.1 Spontaneous locomotor activity

Following progressive ratio testing, mice were assessed for spontaneous locomotor activity in a novel environment in a 60-minute session under low stress conditions (20lux red light). The same test was repeated with a naïve cohort of mice that had not been exposed to environments outside their home cage.

3.2.3.2 Accelerating rotarod

Following the spontaneous locomotion testing, mice were assessed for motor coordination and learning on the accelerating rotarod. Daily training consisted of three 5-minute trials during which the speed of the rotarod accelerate from 4 to 40 rpm. Mice were trained for three consecutive days and nine trials in total. Latencies to fall off the rotarod were manually recorded. Testing was conducted under 20lux red light.

3.2.3.3 Forced swim test (FST)

Mice were individually placed into a beaker of water (23 – 25°C) for five minutes with experimenter blinded to the genotype of the mice. Each session was video-recorded, and mobility time was scored using the ForcedSwimScan software (CleverSys Inc, VA, USA).

3.2.4 Data analysis

The effect size of independent variables on dependent variables were estimated together with 95% confidence intervals and statistical significance using various 2-level mixed-effect general or generalised linear models (StataCorp, TX, USA). Mice was treated as level-2 clusters and random intercepts. Binary *dependent variables* (correct/incorrect response,

response/omission) were analysed trial-by-trial using the gllamm (generalized linear latent and mixed models) program (Rabe-Hesketh et al., 2005) with a logit link or the xtlogit (mixed-effects logit models) program, whereby the effects of *independent variables* were expressed as odds ratios with an odds ratio of 1 indicating no effect (e.g. an effect of session > 1 indicates response accuracy improves over sessions). Latency data were analysed using the qreg2 program (quantile regression with robust and clustered standard errors) (Machado et al., 2011) from the 0.05- to 0.95-quantile at 0.05 steps to allow distribution-wide comparisons, whereby the effects of *independent variables* were expressed as latency difference for the modelled quantile with 0 indicating no effect (e.g. an effect of genotype > 0 for a given quantile indicates the *Nlgn1*$^{-/-}$ mice have longer latencies for that quantile). Other dependent variables were analysed using the xtreg (mixed-effects linear models) program if the dependent variable was normally distributed or median regressions (qreg2) otherwise, whereby the effects of *independent variables* were expressed as differences between the analysed means or medians respectively with 0 indicating no effect. *Independent variables* included genotype (WT as 0 and *Nlgn1*$^{-/-}$ as 1), sex (female as 0 and male as 1), location of the correct stimulus (left as 0 and right as 1 for two potential locations, three separate binary variable for left, centre and right for three potential locations), session and trial number within session (treated as continuous variables). To analyse the effect of correction trials and reoccurring trials on accuracy, two additional binary variables were included in the models (1 if a trial is a correction trial or a reoccurring trial, 0 otherwise). Heteroskedasticity-robust standard errors adjusted for clustering within animals were used for all analyses.

3.2.5 Behaviour simulation

3.2.5.1 Agent

A simple reinforcement learning agent learnt the utility of an action following the classic Rescorla-Wagner rule (Rescorla and Wagner, 1972):

$$Q_{t+1}(A) = Q_t(A) + \alpha \cdot [r_t - Q_t(A)] \quad (1)$$

Here, $Q_t(A)$ is the learnt utility of a given action A on trial t, α is the learning rate, r_t is the reinforcement received on trial t. Actions can have both positive and negative utilities (e.g. responding may result in rewards but also incurs effort). The net utility of a given action is

given by the linear combination of its positive and negative utilities, the relative importance of which is controlled independently by β_P and β_N respectively:

$$U(A) = \sum_i [I \cdot \beta_P + (1 - I) \cdot \beta_N] \cdot Q_i(A) \quad (2)$$

Here, $U(A)$ is the net utility of action A, $Q_i(A)$ is the different positive or negative utilities of A, I is the indicator function:

$$I = \mathbb{1}_{Q(A_i) \geq 0}(Q(A_i)) \quad (3)$$

Such that,

$$I = \begin{cases} 1 \; if \; Q(A_i) \geq 0 \\ 0 \; if \; Q(A_i) < 0 \end{cases}$$

Action selection is given by a softmax function on the net utilities of potential actions

$$P(A) = \frac{e^{U(A)}}{\sum_i e^{U(A_i)}} \quad (4)$$

Here, $P(A)$ is the probability of choosing action A, which depends on the net utility of A compared to that of alternative actions. For all our simulations, a choice is made between only two actions.

3.2.5.2 Simulations

Binary choice (two-armed bandit) task. The reinforcement learning agent learnt to choose between a correct and an incorrect response for 30 trials per session and 20 sessions. The correct response was always rewarded, and the incorrect response never rewarded. Both correct and incorrect responding incurred a negative utility of -1 representing the physical effort of responding.

Serial fixed ratio tasks. The agent was trained sequentially through FR1, 5, 20 and 40 for three session each ratio requirement where it chose between responding or resting. Responding resulted in a reward if the ratio requirement was met (positive utility) and incurred a negative utility of -1 representing the physical effort. Resting results in no reward but incurs a much smaller effort-related negative utility of -0.2. Note that the designation of the alternative action as resting is arbitrary. The general idea is that an animal chooses between responding and some other low-reward-low-effort actions. Time elapsed as the agent chose to either

respond or rest, the session ended when a cumulative time counter exceeded 2700 roughly corresponding to a 2700-second or 45-minute session.

Forced swim test. The forced swim test was simulated as a choice between swimming and resting. Swimming was initialised with a utility of 0 representing that the agent initially believed that swimming will lead to a neutral outcome, and a utility of -1 represent the effort of swimming. Resting had a large negative utility of -10 representing the possibility of drowning but incurred no effort. Every time the agent chose to swim, it received a reinforcement of -9 thereby gradually learnt by equation (1) that swimming did not drastically improve the situation therefore reduced mobility over time. For simplicity, the agent made 300 decisions over 300 timesteps roughly corresponding to a 5-minute session.

3.3 Results

3.3.1 Nlgn1 is not essential for acquiring complex associative structures

Both LTP and NMDAR function are synaptic mechanisms thought to be required for learning and memory (Morris et al., 1986; Rogan et al., 1997; Giese et al., 1998; Moser et al., 1998; Reynolds et al., 2001; Whitlock et al., 2006; Brigman et al., 2008; Nabavi et al., 2014; Nicoll, 2017). Based on the established consistent requirement of Nlgn1 for NMDAR function and multiple forms of LTP, we hypothesised that Nlgn1 may be important for acquiring associative structures of the environment and use them to optimise action selection. To address this, we assessed male and female mice lacking the gene for *Nlgn1* (*Nlgn1*$^{-/-}$) and control wildtype (WT) littermates in a series of rodent touchscreen cognitive tests, in which mice were required to respond via nose-pokes to different visual stimuli displayed on a touchscreen under different situations. Of note, we observed no interaction between genotype and sex for most our analyses except for Figure 3.6E (data separated by sex shown in Supplemental Figure 11), therefore we combine both sexes and present the data by genotype for clarity. Animals first underwent phases of instrumental pre-training to learn to nose-poke simple visual stimuli displayed on the touchscreen in order to obtain a strawberry milk reward (Horner et al., 2013; Nithianantharajah et al., 2013). We observed that *Nlgn1*$^{-/-}$ and WT mice required similar number of training sessions across all pre-training phases indicating loss of Nlgn1 does not impact the capacity to acquire simple instrumental conditioning

(**Supplemental Figure 1**). Following pre-training, we introduced the mice to the pairwise visual discrimination task (PD) where two visually similar stimuli were presented pseudorandomly between two locations (left or right side of the touchscreen), and responses to one stimulus were rewarded (S+) while responses to the other were unrewarded (S-) (**Figure 3.1A**). The PD task therefore requires the mice to perceptually discriminate the stimuli and selectively respond to the rewarded or correct stimulus regardless of stimulus location. The primary measure used to track learning across sessions is accuracy (percentage of correct responses) and the mice were trained to reach a learning criterion (80% accuracy over two sessions). We found that *Nlgn1*$^{-/-}$ mice required similar numbers of trials to reach the criterion as WT littermate controls (**Figure 3.1B**). To understand how key variables including genotype, sex and session affect accuracy, we estimated the effect of these variables on trial outcomes (correct/incorrect responses) using a mixed-effect generalised linear model (Rabe-Hesketh et al., 2005) (**Methods**). The effect of genotype is the estimated effect of the *Nlgn1*$^{-/-}$ genotype on correct responding relative to WT expressed as an odds ratio. Consistent with our analysis on the number of trials to learning criterion, there was no effect of genotype on trial outcomes (**Figure 3.1C**). However, note that the overall effect of genotype across sessions is not a sensitive measure for the PD task since all the mice were trained to the same learning criterion. The effect of session measures the improvement on response selection per session and therefore provides insights into the rate of learning across sessions (session n+1 compared to session n). As expected, the effect of session was greater than 1 and highly significant representing a robust upward learning curve. To examine whether the rate of improvement differ between the genotypes, we estimated the strength of genotype x session interaction and found that the interaction was small and did not reach significant indicating that both *Nlgn1*$^{-/-}$ and WT mice improved at a similar rate (**Figure 3.1C**).

To increase associative complexity of the task and incorporate spatial information into defining the reward contingencies, we next assessed the mice in the object-location paired associates learning task (PAL) which requires mice to not only learn the visual features of three visual stimuli (flower, plane, spider) but also their unique rewarded location on the touchscreen (left, center, right respectively). On each trial, only two of the three stimuli are presented; one displayed in its correct location (S+) and the other in an incorrect location (S-) (**Figure 3.2A**). The correct response on any given trial is therefore jointly defined by both

visual and spatial information of the stimuli. As expected, the effect size of session is smaller in PAL compared to PD and the average learning curve required 40 sessions to reach plateau reflecting the increase in task difficulty (**Figure 3.2B-C**). Despite this, we again observed no significant effect of genotype or interaction between session and genotype indicating that both *Nlgn1*$^{-/-}$ and WT mice were able to acquire the complex associations in PAL to similar degrees and at similar rates (**Figure 3.2C)**

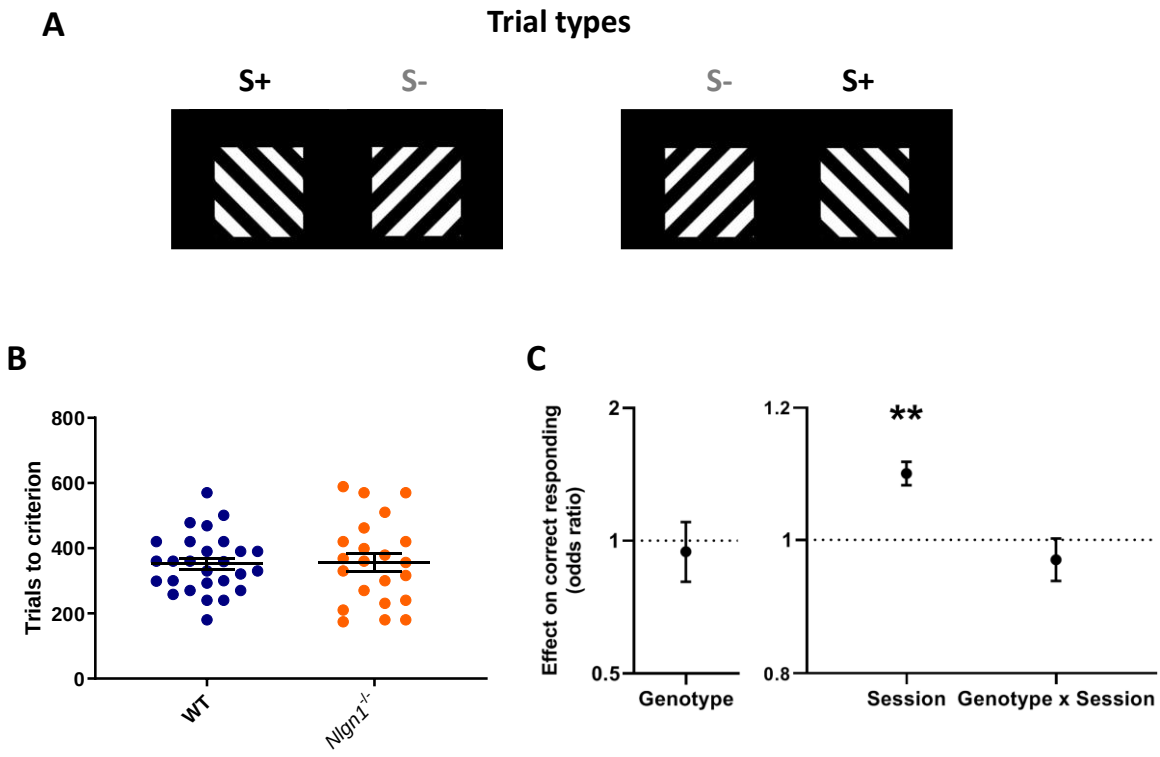

Figure 3.1 *Nlgn1*$^{-/-}$ mice displayed normal acquisition of the pairwise visual discrimination (PD) task. **A)** Visual stimuli used in the PD task. S+: rewarded stimulus, S-: unrewarded stimulus, S+ and S- were counterbalanced across groups. **B**) Number of trials *Nlgn1*$^{-/-}$ and WT mice required to reach training criterion (80% correct responses across two consecutive sessions), two-way mixed ANOVA, values represent means ± SEM. **C)** Effect of genotype, session and their interaction on correct responding, GLLAMM (logistic link function), $^{**}p < 0.005$ significantly different from 1, values represent the estimated effect of the variables on the odds of correct responding (odds ratio) ± 95% CI.

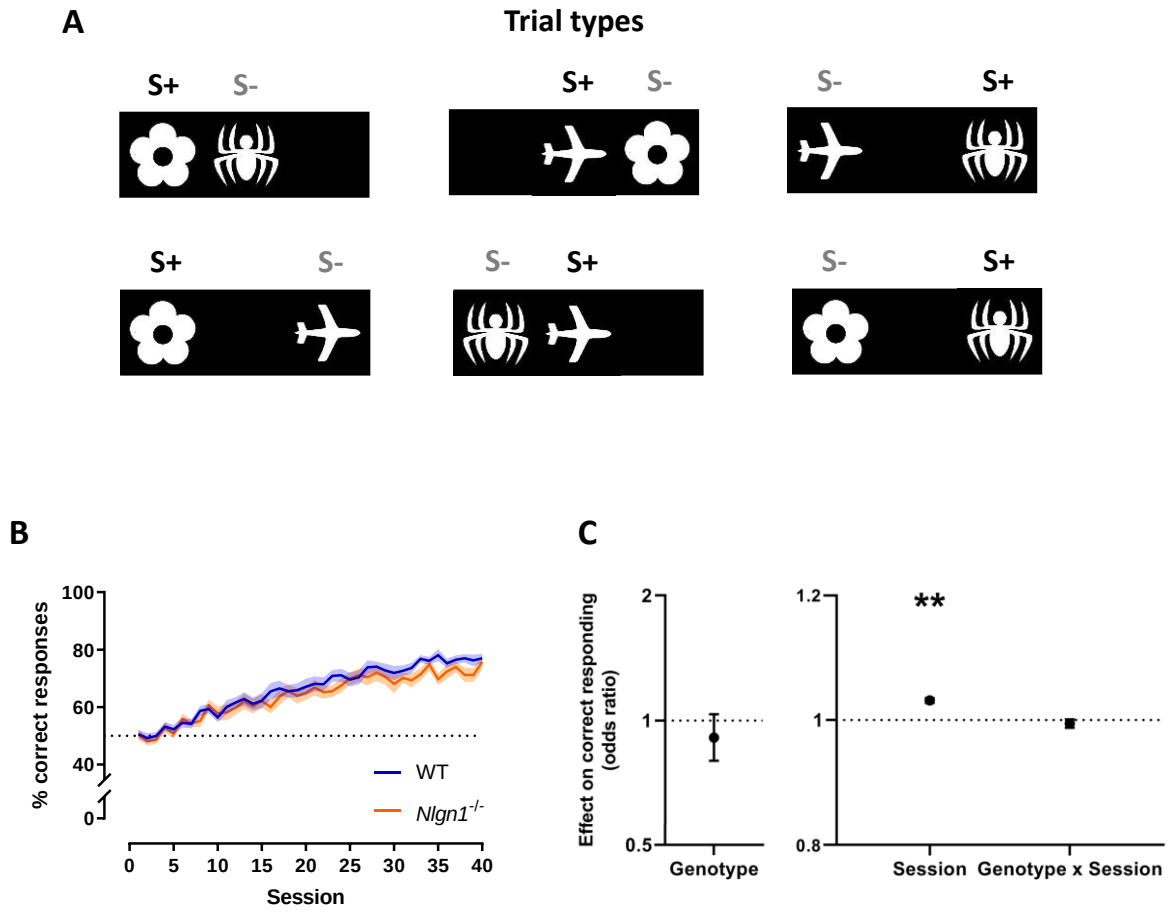

Figure 3.2 PAL *Nlgn1*$^{-/-}$ mice displayed normal acquisition of the object-location paired associates learning (PAL) task. **A)**: Visual stimuli and their correct location used in the PAL task. S+: rewarded stimulus, S-: unrewarded stimulus. **B**) Learning curve showing accuracy across sessions, dotted line indicates chance-level responding. Learning curve plotted for data visualization only, values represent means ± SEM. **C)** Effect of genotype, session and their interaction on correct responding, GLLAMM (logistic link function), $^{**}p < 0.005$ significantly different from 1, values represent the estimated effect of the variables on the odds of correct responding (odds ratio) ± 95% CI.

3.3.2 Nlgn1 deletion does not alter the reversal and extinction of learnt associations

Adapting to dynamic environments where the outcome of a response is not always stable requires the ability to inhibit learnt responses once they no longer yield positive outcomes and explore other alternatives. To assess the requirement of Nlgn1 for flexible adjustment of response selection, once the mice had reached the learning criterion on PD training (**Figure 3.1**), we reversed the reward contingencies so that the previously rewarded stimulus was now unrewarded and vice versa (**Figure 3.3A**). The mice displayed a strong tendency to respond to the previously rewarded stimulus at the beginning of reversal learning as expected, then shifted their responding gradually (**Figure 3.3B**). Similar to the initial acquisition of

associations in the PD task, we found no difference in response accuracy between the genotypes (effect of genotype) or the rates of reversal across sessions between *Nlgn1*$^{-/-}$ and WT mice (genotype x session interaction, **Figure 3.3C**).

We next investigated the rate at which the *Nlgn1*$^{-/-}$ and WT mice extinguish a learnt response without competing alternatives in an instrumental extinction learning task. The mice were first trained on an acquisition phase to robustly respond to a simple stimulus (white square) and once a stable performance criterion was reached (**Methods**), the mice were tested on the extinction phase in which responses were no longer rewarded (**Figure 3.3D**). On each trial, the mice can either respond or omit a response within a 10-second window. One challenge in quantifying the rate of instrumental extinction learning is that learning only takes place after a response. As a result, an animal which has extinguished less therefore responded more will receive more learning opportunities and vice versa. We minimised the difference in learning opportunities by limiting the number of trials per session to 30 and assessed learning across sessions (**Figure 3.3E, Supplemental Figure 2**). We observed no impact of genotype on whether the mice responded or omitted a trial (effect of genotype, **Figure 3.3F**). The rates of extinction within a session (effect of trial) and across sessions (effect of session) are both less than 1 indicating than the mice extinguish responding both within and across sessions (**Figure 3.3F**). However, neither the effect of trial or session differed significantly between *Nlgn1*$^{-/-}$ and WT mice suggesting that the rate of instrumental extinction was similar for both genotypes (genotype x trial, genotype x session interaction, **Figure 3.3F**). To confirm potential differences in extinction rate was not masked by difference in learning opportunities, we also analysed the rate of extinction as the effect of cumulative responses and again found no difference between *Nlgn1*$^{-/-}$ and WT mice (data not shown).

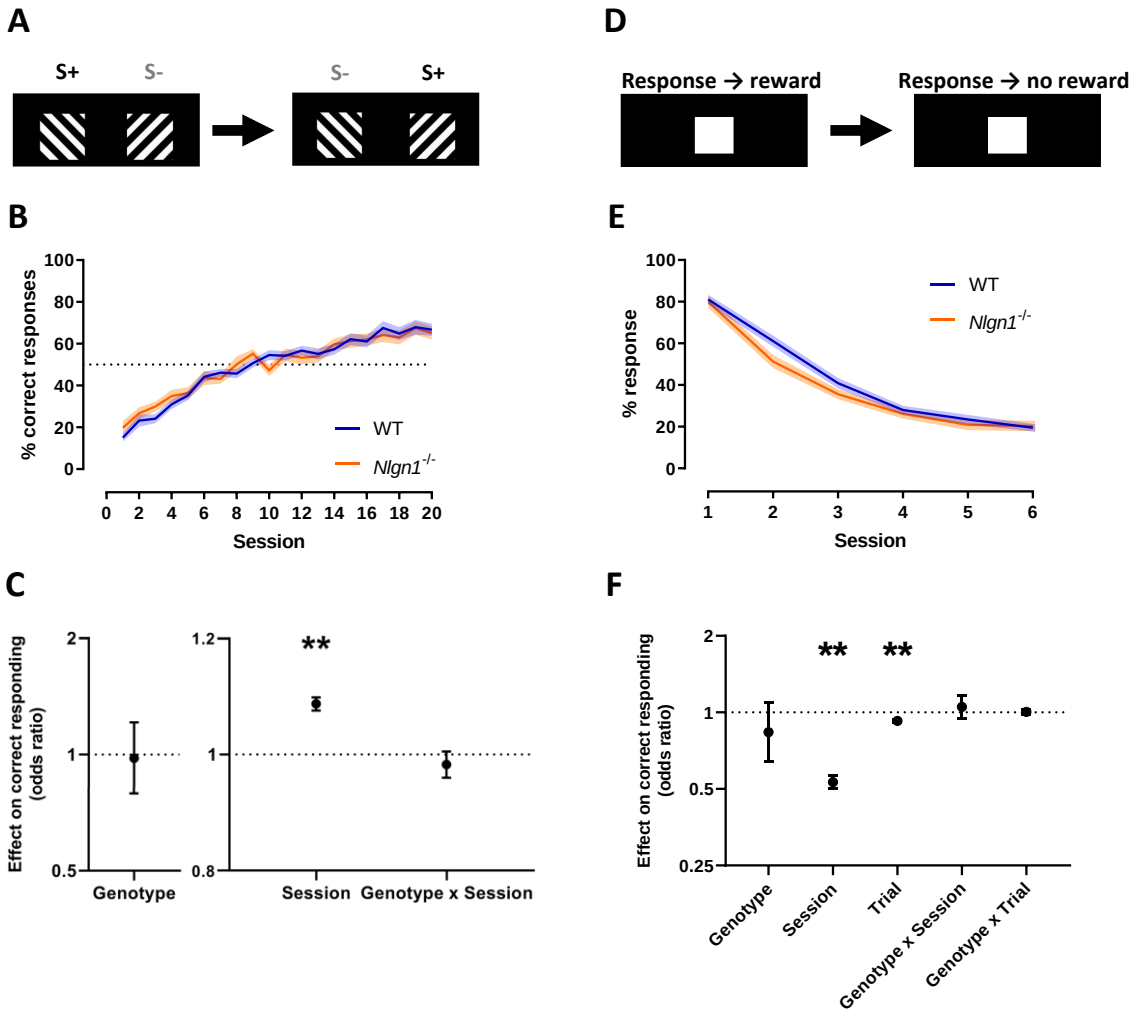

Figure 3.3 *Nlgn1*$^{-/-}$ mice displayed normal reversal and extinction of learnt associations. **A)** Visual stimuli and task design of the reversal learning task, S+: rewarded stimulus, S-: unrewarded stimulus, S+ and S- were counterbalanced across groups. **B**) Learning curve showing accuracy across sessions, dotted line indicates chance-level responding. Learning curve plotted for data visualization only, values represent means ± SEM. **C**) Effect of genotype, session and their interaction on correct responding, GLLAMM (logistic link function), $^{**}p < 0.005$ significantly different from 1, values represent odds ratio ± 95% CI. **D**) Task design of the extinction learning task. **E**) Learning curve showing percentages of responses across sessions. learning curve plotted for data visualization only, values represent means ± SEM. **F**) Effect of genotype, session, trial within a session and their interactions on responding, GLLAMM (logistic link function), $^{**}p < 0.005$ significantly different from 1, values represent the estimated effect of the variables on the odds of correct responding (odds ratio) ± 95% CI

3.3.3 Mice display perseverative responding regardless of genotypes

Previous work reported that *Nlgn1*$^{-/-}$ mice displayed increased self-grooming (Blundell et al., 2010), which was thought to represent repetitive and stereotypic behaviour common in autism spectrum disorder (Crawley, 2007; Kalueff et al., 2016). We were therefore interested in exploring indications of perseverative response selection in PD, PAL and reversal learning. In these tasks, a correct response was followed by a pseudorandom trial where stimuli were displayed in a pseudorandom and counterbalanced manner across locations, whereas an

incorrect response was followed by a "correction trial" where the same stimuli were displayed at the same locations repeatedly until the mice switched its response to make a correct response and earn a reward (**Supplemental Figure 3A**). The average number of correction trials committed per incorrect response termed perseveration index (PI) is thought to measure perseverative responding (Brigman et al., 2008; Horner et al., 2013; Mar et al., 2013; Piiponniemi et al., 2017; Zeleznikow-Johnston et al., 2018). However, to our knowledge, it has not been demonstrated quantitatively that mice indeed perseverate over the incorrect stimulus on correction trials (more likely to respond incorrectly). Correction trials are typically excluded from accuracy calculation because they are not pseudorandom. To ask whether the mice perseverated on correction trials, we included correction trials into the dataset and estimated the effect of correction trials on correct responding. We found that the mice were indeed drastically less accurate on correction trials compared to standard pseudorandom trials suggesting a tendency of reselecting the same incorrect response previously selected (**Figure 3.4A**). Similarly, when the same stimulus configuration happened to reoccur on consecutive pseudorandom trials (reoccurring trials), the mice were more likely to reselect the correct response previously selected (**Figure 3.4B**). Thus, we demonstrate that mice indeed tend to perseverate in the PD, PAL and reversal task. Having established this, we asked whether the degree of perseverative responding was affected by Nlgn1 deletion. We did not find a strong genotype x correction trial or genotype x reoccurring trial interaction indicating that mice of both genotypes displayed comparable degree of perseverative responding on both type types (**Figure 3.4C - D**).

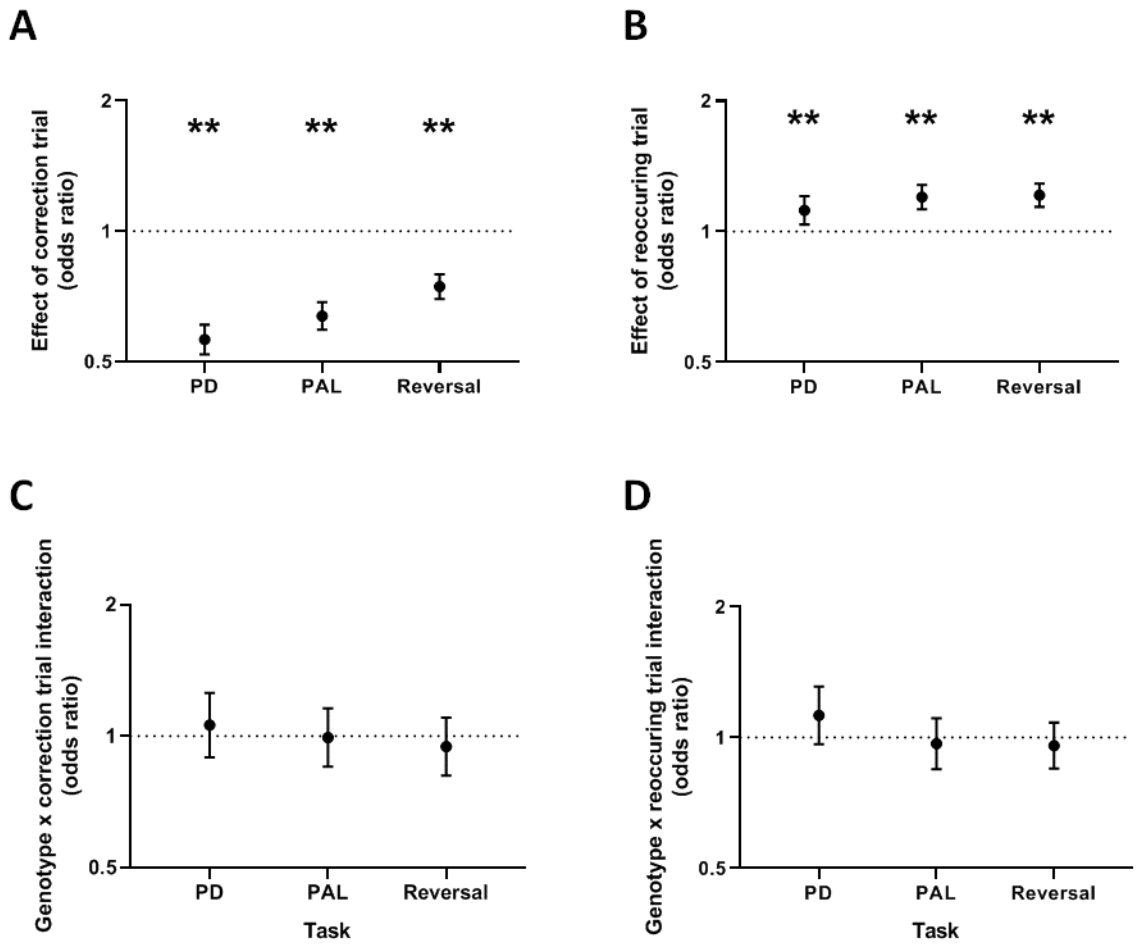

Figure 3.4 *Nlgn1*$^{-/-}$ and WT mice displayed similar degree of preservation in response selection. **A**) Effect of correction trial on correct responding, mice in general were less accurate on correction trials (effect sizes < 1). **B**) Effect of reoccurring pseudorandom trial on correct responding, mice in general were more accurate on pseudorandom trials with reoccurring stimulus configurations (effect size > 1). **C**) Interaction between the effect of correction trial and genotype. **D**) Interaction between the effect of reoccurring pseudorandom trial and genotype. GLLAMM (logistic link function), $^{**}p < 0.005$, values represent the estimated effect of the variables on the odds of correct responding (odds ratio) ± 95% CI.

3.3.4 *Nlgn1*$^{-/-}$ mice are slower to perform actions instrumental for rewards

So far, we have examined the requirement of Nlgn1 for action selection between two alternatives, which require the same amount of physical effort and differ only in expected rewards. However, like most natural decision problems, the touchscreen tasks are free-operant tasks where mice are free to choose a wide range of actions (e.g. sniffing, rearing, resting), instead of choosing actions instrumental for earning a reward (trial initiation, responding, reward collection). To ask whether *Nlgn1*$^{-/-}$ and WT mice differ in the level of engagement in performing the instrumental actions despite similar response accuracy, we

analysed latencies of trial initiation, approaching the touchscreen and reward collection (**Supplemental Figure 3B**). Response latency was separated into time taken to arrive in front of the touchscreen (approach latency) and from then to making a response (discrimination latency). This is because discrimination latencies positively predicted accuracy whereas approach latencies did not (**Supplemental Figure 4**), suggesting discrimination latency reflect the decision between correct and incorrect response whereas approach latency reflects the decision between to respond or not. We analysed the latency data with median regressions (**Methods**) and found that *Nlgn1*$^{-/-}$ mice were slower to initiate trials and collect rewards across all three tasks (**Figure 3.5A, B, see Supplemental Figure 5 for latency learning curves**). The differences in approach latency were less robust possibly because the mice were not forced to pause between trial initiation and approaching the touchscreen therefore learn to combine the two into one action sequence (**Figure 3.5C**). We performed quantile regressions from the 0.05-th to the 0.95-th quantile (median being the 0.5-th quantile) to analyse distribution-wide differences and obtained similar results throughout the distribution (**Supplemental Figure 6**). By contrast, discrimination latencies were nearly identical between the *Nlgn1*$^{-/-}$ and WT mice (**Figure 3.5D**).

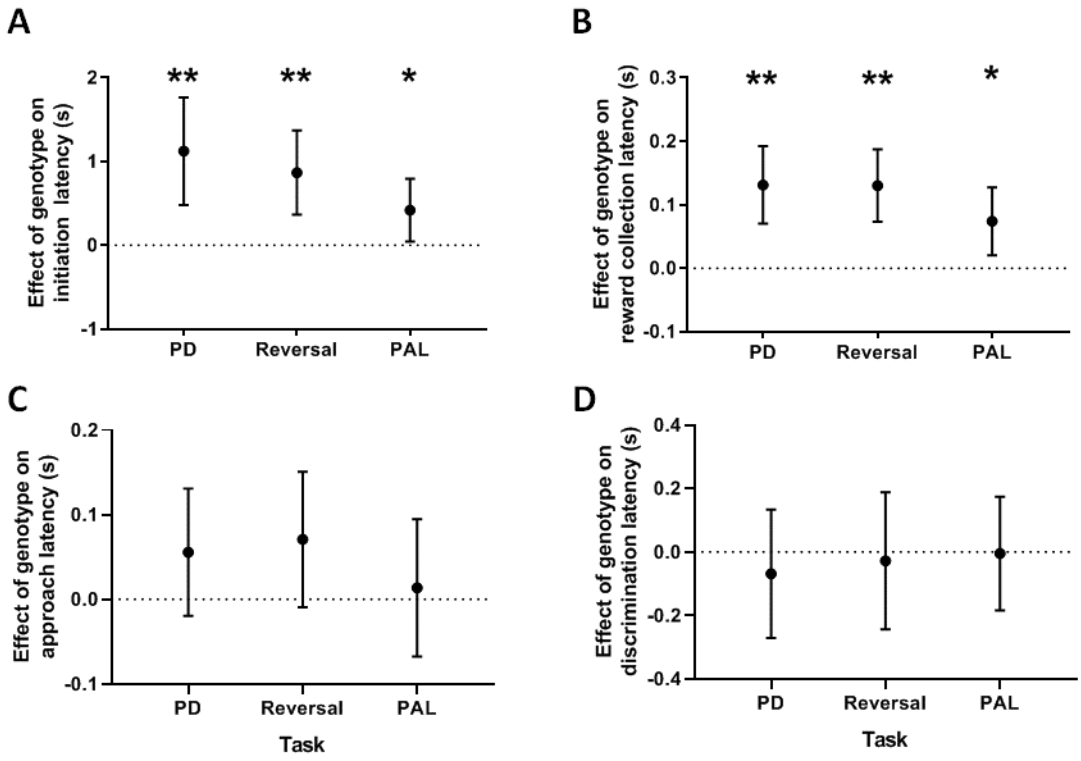

Figure 3.5 *Nlgn1*$^{-/-}$ showed lower task engagement in PD, reversal learning and PAL, task arranged in the order of training. *Nlgn1*$^{-/-}$ mice consistently took longer to **A**) initiate new trials and **B**) collect rewards after a correct response compared to WT controls (effect of genotype > 0). **C**) The difference between *Nlgn1*$^{-/-}$ and WT mice was less robust for approach latencies and did not reach statistical significance. **D**) *Nlgn1*$^{-/-}$ and WT mice showed very similar discrimination latencies. Quantile regression (median), *$p < 0.05$, **$p < 0.005$, values represent estimated latency difference between *Nlgn1*$^{-/-}$ and WT mice ± 95% CI.

3.3.5 Nlgn1 deletion reduces the motivation to overcome response effort for rewards

We hypothesised based on the increased latencies that the Nlgn1 deletion caused a reduction in the motivation to exert effort for potential rewards. To test this, *Nlgn1*$^{-/-}$ and WT mice were trained sequentially across fixed ratio 1, 5, 20, 40 tasks (FR1, 5, 20, 40). We expected *Nlgn1*$^{-/-}$ mice to show a greater reduction in the number of responses made at the higher ratio requirements where responding has a lower utility, resembling the well-characterised model of amotivation by accumbal dopamine depletion (Aberman and Salamone, 1999; Niv et al., 2007; Collins and Frank, 2014). Indeed, we found that the *Nlgn1*$^{-/-}$ mice made significantly fewer responses than WT controls on FR5, 20, 40 but not FR1, and the reduction was greater at the higher ratio requirements (**Figure 3.6A – B, Supplemental figure 7**). The increasing difference between *Nlgn1*$^{-/-}$ and WT response counts across ratio requirements was primarily

driven by the non-linear increase in the latency to re-engage in responding after consuming a reward (post-reinforcement pause, **Figure 3.6C, Supplemental Figure 8A),** and the average time spent per response subsequently (**Fig. 6D, Supplemental Figure 8B, Supplemental Figure 9**). Using a separate cohort of mice, we were able to reproduce the same motivational phenotype of the *Nlgn1*$^{-/-}$ mice in a progressive ratio task where ratio requirements progressively increased within a session until the mice stopped responding (**Supplemental Figure 10**).

One possible explanation for the reduced motivation in the *Nlgn1*$^{-/-}$ mice was taste insensitivity towards the caloric contents of strawberry milk rewards. To examine whether the motivational phenotype of the *Nlgn1*$^{-/-}$ mice was specific to high-sugar-high-fat rewards, we water-restricted the *Nlgn1*$^{-/-}$ and WT mice and tested them on FR20 for water rewards. Similar to what we observed with strawberry milk rewards, *Nlgn1*$^{-/-}$ mice made significantly fewer responses compared to WT with a stronger effect in females (**Fig. 6E, Supplemental figure 11A**). Importantly, there is a significant and positive correlation between the numbers of responses for strawberry milk and water rewards indicating that mice more motivated by strawberry milk also tended to respond more for water. Together, these data suggest that Nlgn1 deletion led to a reduction in instrumental responding motivated by both hunger and thirst (**Fig. 6F, Supplemental Figure 11B**).

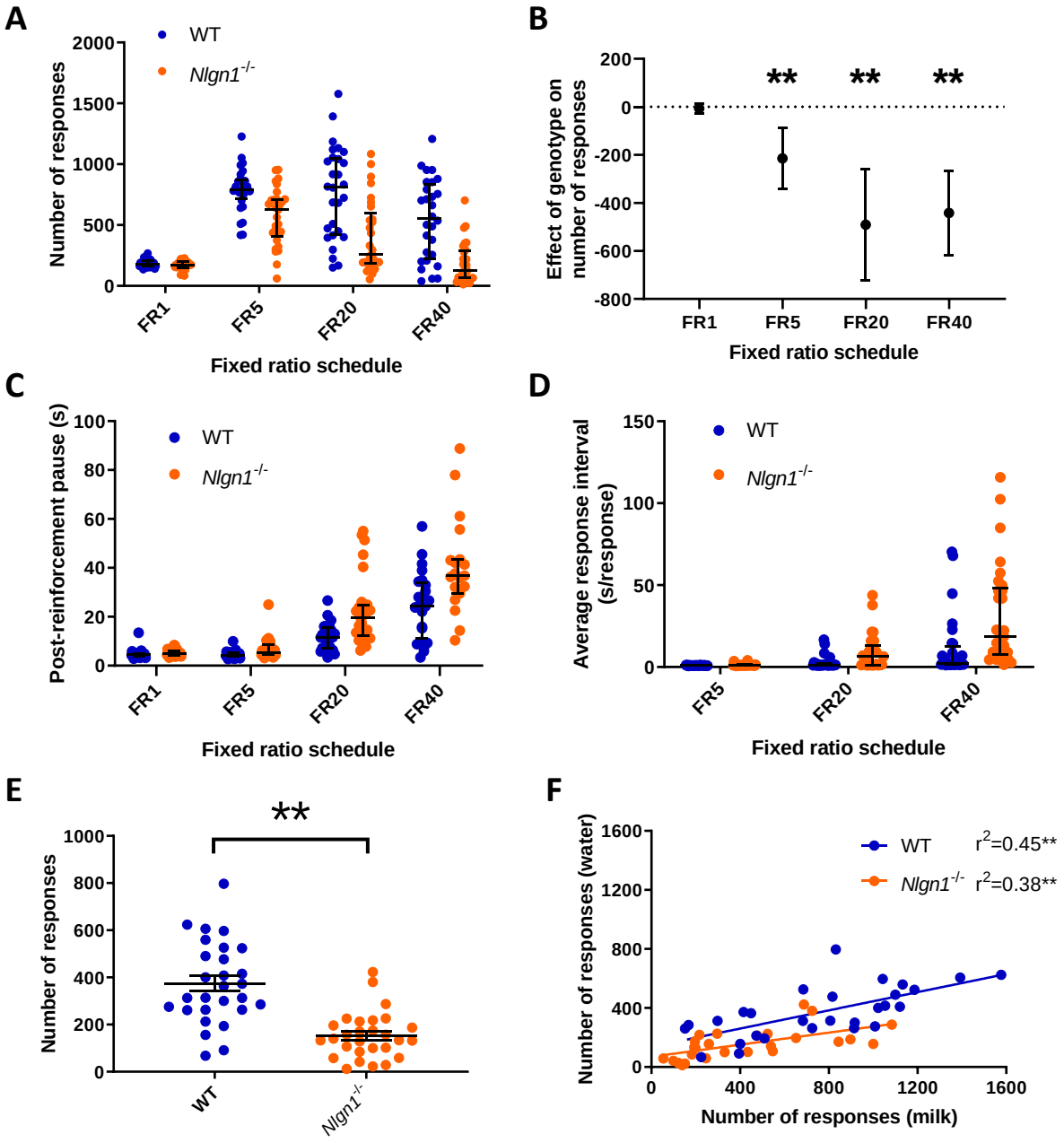

Figure 3.6 *Nlgn1*$^{-/-}$ mice were less motivated to respond for rewards. **A**) Total number of responses averaged over three sessions per ratio requirement. Solid line indicates median, upper and lower dotted line indicate the 3rd and 1st quartile respectively. **B**) *Nlgn1*$^{-/-}$ mice made fewer responses in the higher ratio requirements (effect of genotype < 0), 1uantile regression (median), $^{**}p < 0.005$, values represent estimated effect of genotype on response counts ± 95% CI. **C**) Post-reinforcement latency: time to the first response after consuming a reward, averaged over three sessions per ratio requirement. Note data could not be gathered from animals which did finish at least one trial. **D**) Average time spent per response after the animal has made the first response of a trial, averaged across three sessions per ratio requirement. See **Supplemental Figure 9** for a response-by-response breakdown of inter-response intervals. **E**) *Nlgn1*$^{-/-}$ mice made fewer responses for water rewards on FR20, linear regression, $^{**}p < 0.005$ values represent means ± SEM. **F**) Number of responses made for milk rewards positively correlated with number of responses made for water rewards, linear regression, $^{**}p < 0.005$

3.3.6 Beyond amotivation: *Nlgn1*$^{-/-}$ mice exert less effort to earn rewards but more to escape from aversion

To investigate the generalizability of the motivational phenotype of *Nlgn1*$^{-/-}$ mice, we asked whether they were also less willing to exert effort outside the operant environment. First, we allowed the mice to explore a novel environment under low stress conditions and compared *Nlgn1*$^{-/-}$ and WT mice on the decision between exploration and resting (**Methods**). Consistent with a reduced willingness to overcome the cost of physical effort, *Nlgn1*$^{-/-}$ mice covered a shorter distance compared to WT controls and spent more time resting (**Figure 3.7A-B**). Furthermore, when the mice engaged in exploration, *Nlgn1*$^{-/-}$ and WT mice did so with a similar velocity indicating similar intrinsic motor function between the genotypes, which was confirmed using the accelerating rotarod test (**Figure 3.7C, Supplemental Figure 12**). However, we note that while we were able to reproduce this phenotype across two touchscreen-trained cohorts of mice, the difference was much smaller between naïve *Nlgn1*$^{-/-}$ and WT mice, which had not been habituated to any novel environment outside their home cages.

Motivation often refers to the ability to overcome physical effort to achieve a desirable outcome (Salamone and Correa, 2012; Pessiglione, 2014; Salamone et al., 2016; Pessiglione et al., 2017). In the laboratory, this is typically assessed by training animals to perform instrumental actions to obtain rewards. However, if Nlgn1 deletion increases the weighting specifically on physical effort, it follows that *Nlgn1*$^{-/-}$ mice would be less willing to exert effort to earn rewards as well as to avoid punishment. To test this, we employed the forced-swim test (FST) where the mice initially struggled/swam in the hope of escaping the aversive situation and mobility declined over time as an adaptive response to fatigue and learning that swimming did not improve the aversive situation (learnt helplessness) (West, 1990; Molendijk and de Kloet, 2015; de Kloet and Molendijk, 2016). Surprisingly, contrary to an increased weighting on physical effort, the *Nlgn1*$^{-/-}$ mice spent significantly more time swimming than WT controls (**Figure 3.7D**). This surprising finding contradicts a specific role of Nlgn1 in estimating the cost of physical effort, rather Nlgn1 deletion may lead to an increased aversion towards punishment or negative utility in general. We suggest that in the case of the FST, both the fear of drowning and the effort of swimming incurred negative utilities, and the

effect Nlgn1 deletion magnified the aversion towards fear more than effort because the former was a more severe punishment.

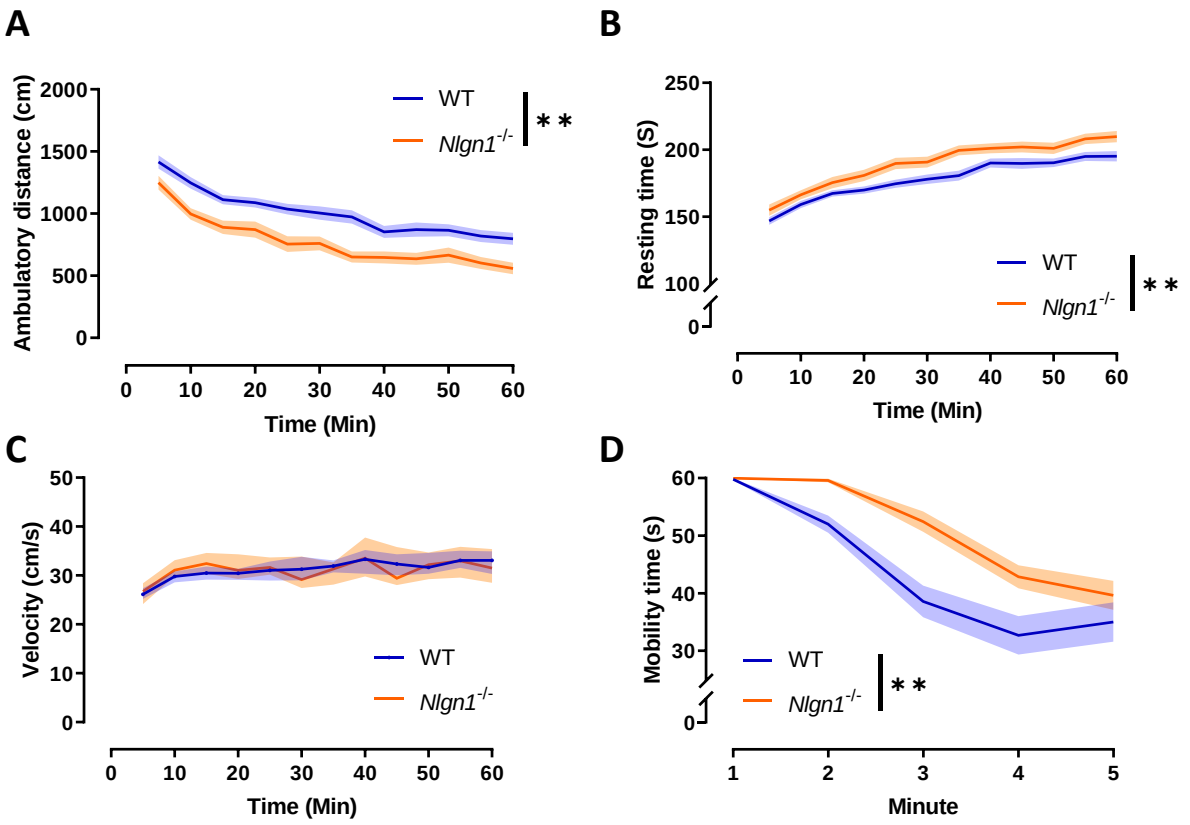

Figure 3.7 *Nlgn1*$^{-/-}$ mice show reduced exploration in a novel environment but increased mobility in the FST. (**A**) *Nlgn1*$^{-/-}$ mice covered a shorter ambulatory distance compared to WT controls in a 60-minute session of spontaneous locomotor activity test under dim red light. (**B**) *Nlgn1*$^{-/-}$ mice also showed an increase in resting time during the session. (**C**) Both *Nlgn1*$^{-/-}$ and WT mice moved at the same velocity during the locomotor activity test. Linear regression, $^{**}p < 0.005$, values represent means ± SEM. (**D**) *Nlgn1*$^{-/-}$ mice showed increased mobility time in the forced-swim test, main effect of genotype, time bins were collapsed for analysis, two-way ANOVA. $^{**}p < 0.005$, values represent means ± SEM.

3.3.7 Increased weighting on negative utilities captures the behavioural phenotype of *Nlgn1*$^{-/-}$ mice across tasks

In the case that *Nlgn1*$^{-/-}$ mice are generally less motivated to overcome the cost of physical effort but spent more time swimming in the FST driven by an independent mechanism such as an exaggerated fear response, others have provided detailed theoretical accounts for the potential cognitive mechanisms causing the decrease in motivation (McClure et al., 2003; Niv et al., 2007). Therefore, we considered the possibility that the seemingly opposite motivational phenotypes of *Nlgn1*$^{-/-}$ mice in reward- and punishment-driven tasks were

caused by the same mechanism. To this end, we sought a theoretical model that can capture the key behavioural observations of the *Nlgn1*$^{-/-}$ mice across tasks including: 1) normal binary choice between a correct and incorrect response, 2) fewer responses emitted on sequential FR tasks and 3) higher mobility in the FST. We visualised the behavioural predictions of the theory by simulating the behaviour of a simple reinforcement learning (RL) model in the simplified versions of the abovementioned tasks. This allows us to describe precisely and unambiguously the assumptions, architecture and predictions of the theory. We compared the simulated effect of changing the parameters in the theoretical model to the effect of Nlgn1 deletion in the experimental data. Importantly, we assumed parameters in the learning rule such as learning rate and perceived reward magnitude were not impacted by Nlgn1 deletion based on the normal learning curve of *Nlgn1*$^{-/-}$ mice in PD, PAL, reversal and extinction learning, therefore only altered the parameters controlling the weighting on learnt positive (rewards) and negative (punishment including physical effort) utilities in selecting an action (**Methods**).

The simulated agent selects actions by comparing their net utilities, calculated by summing the positive and negative utilities of a given action (**Figure 3.8A**). We first considered the effect of reducing the weighting on positive utilities (β_P) in the calculation of net utilities (**Supplemental Figure 13**). Reducing the weighting on positive utilities predicts the phenotype of the *Nlgn1*$^{-/-}$ mice in the FR tasks because it reduces the importance of rewards. On the contrary, reducing the weighting on positive utilities renders the choice between correct and incorrect response more random in the simulated binary choice task because accuracy depends on the difference between the positive utilities associated with the correct and incorrect response. This does not resemble our observations in the PD, PAL and reversal learning task therefore fails to capture the *Nlgn1*$^{-/-}$ phenotype. Next, we consider the effect of Nlgn1 deletion as increasing the weighting on negative utilities (β_N). We found increasing β_N 1) did not alter the learning curves because correct and incorrect responding required the same physical effort (**Figure 3.8B, E**); 2) reduced the number of responses in the simulated FR tasks because the potential rewards of responding were more heavily discounted by the physical effort incurred (**Figure 3.8C, F**), and 3) increased mobility in the FST because increasing β_N had a greater effect on greater punishment, under the assumption that the aversion of being in water has a greater negative utility than the physical effort of swimming

(**Figure 3.8D, G**). Therefore, an increased weighting on negative utilities can, at least in principle, capture the divergent phenotype of *Nlgn1*$^{-/-}$ mice across reward- and punishment-driven tasks.

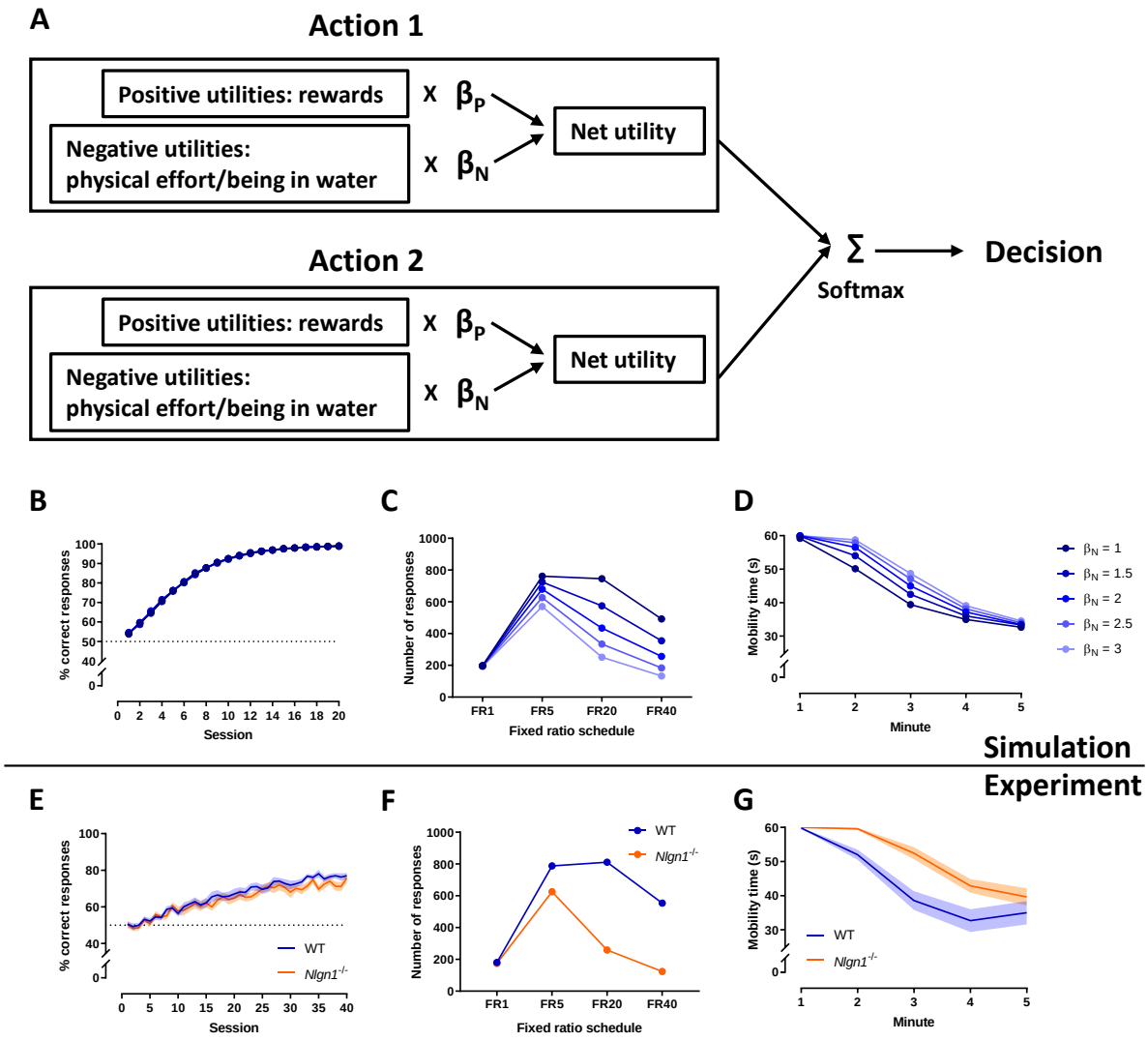

Figure 3.8 Simulated effect of increasing the weighting on negative utilities (β_N) in the calculation of net utilities captures the *Nlgn1*$^{-/-}$ behavioural phenotype across tasks. **A**) RL agents integrates positive and negative utilities into a net utility for each action. Two separate parameters: β_P and β_N control the weighting on positive and negative utilities in the calculation of a net utility for each action. The model assumes that different types of positive and negative utilities are under the general control of β_P and β_N, (e.g. β_N affect both the weighting on physical effort as well as the aversion of being in water where appropriate). Net utilities of potential actions are then fed into a softmax function for probabilistic action selection where actions with higher net utilities are selected with higher probabilities. **B-D**) Increasing β_N from 1 to 3 at steps of 0.5 has no impact on the choice between correct and incorrect responding (**B**), reduces the number of responses made in simulated FR tasks where the choice is between responding (high-effort action) and resting (low-effort action) (**C**) and increases mobility in the simulated FST where the choice is between swimming (high-effort action) and resting (low-effort action) (**D**). **E-G**) For comparison, experimental data from the object-location paired associates learning (PAL) task (**E**), the fixed ratio tasks (**F**) and the forced swim test (**G**).

3.4 Discussion

Building on the extensive molecular and cellular literature on the synaptic function on Nlgn1, we investigated how the loss of Nlgn1 might affect components of decision-making. Contrary to our initial hypothesis, we found that Nlgn1 was not required for learning complex associative structures, or the subsequent reversal and extinction of learnt associations despite the consistent findings that Nlgn1 is required for NMDAR function and LTP induction. While the *Nlgn1*$^{-/-}$ mice demonstrated intact associative learning, they were consistently less motivated to overcome the effort cost to earn rewards across multiple tasks. However, the *Nlgn1*$^{-/-}$ mice were more motivated to exert effort to escape from an aversive situation in the forced swim test. We suggest that the divergent phenotype of *Nlgn1*$^{-/-}$ mice can be explained by an increased weighting on negative utilities. Our findings suggest a novel, valence-dependent role of Nlgn1 in reward-punishment trade-off, which contributes to linking the synaptic function of Nlgn1 with its behavioural consequences.

Based on the findings that the loss of Nlgn1 causes NMDAR hypofunction and impaired LTP, we initially hypothesised that it would lead to an impaired ability to learn complex associations in the environment. It is widely held that LTP and NMDAR function are required for various forms of learning (Morris et al., 1986; Rogan et al., 1997; Giese et al., 1998; Moser et al., 1998; Reynolds et al., 2001; Whitlock et al., 2006; Brigman et al., 2008; Brigman et al., 2013; Nabavi et al., 2014; Nicoll, 2017). Indeed, deletions of NMDAR subunits have been shown to impair performance in both PD and reversal learning tasks highly similar to those reported in the current study. It is therefore surprising that *Nlgn1*$^{-/-}$ mice were indistinguishable from WT mice in learning these same tasks as well as the PAL task, which was demonstrably more complex. It is possible that the loss of Nlgn1 did have a minor impact on learning which we failed to detect. Indeed, *Nlgn1*$^{-/-}$ mice improved their response accuracy at a fractionally slower rate in the PD and PAL task compared to WT controls, which would reach statistical significance using more liberal error estimates for the effect sizes (standard errors assuming homoscedasticity). However, the assumption of homoscedasticity has not impact on the effect size itself therefore the subtle decrease in the rates of improvement nonetheless stands in contrast with the robust and consistent reduction in LTP and NMDAR function caused by loss Nlgn1. There are other possibilities that may account for the lack of overt learning impairment. For example, both LTP and NMDAR-mediated transmission are

reduced but not abolished in *Nlgn1*$^{-/-}$ mice therefore may not be sufficient to disrupt learning and cognitive flexibility.

However, instead of exhaustively listing to all the possible explanations for why animals with a reduction in LTP and NMDAR hypofunction do not display a deficit in learning and memory, it is worth highlighting that it is not clear why and how the ability to optimise one's behaviour adaptively should depend on NMDAR function and LTP based on the current knowledge (or lack thereof) on how complex behaviour may be implemented by well-studied synaptic mechanisms. Here, LTP and NMDAR function information processing at the synaptic level whereas adaptive behaviour emerges from the dynamics and cooperation of large populations of neurons across brain regions and circuits. There are many levels of analysis between complex behaviour and synaptic function: the characterisation of behaviour, neural computational algorithms that underlie behaviour, population- and circuit-level motifs that underlie neural computation, neuron-level information processing that underlies population- and circuit-level motifs and finally synaptic mechanisms that underlie neuron-level information processing. The gaps between these levels need to be bridged before complex behaviour can be reduced to synaptic mechanisms.

Action selection generally requires weighing both the expected positive and negative consequences of available actions. We showed that *Nlgn1*$^{-/-}$ mice were less willing to exert effort to earn rewards in the touchscreen tasks but were more willing to exert effort in the FST. One possibility is that Nlgn1 deletion decreases the willingness to overcome effort cost and the increased mobility in the FST was caused by an independent mechanism. In this case, there would be no need to postulate a single theory to capture both phenotypes. Theoretical models have been proposed to address the dissociation between learning and motivation, which could potentially explain why *Nlgn1*$^{-/-}$ mice were normal in associative learning but less willing to exert effort for rewards (Niv et al., 2007; Dayan, 2012). According to these theories, an animal could be less motivated to overcome the effort cost of responding yet maintain the normal ability to choose between a correct and an incorrect response due to either an underestimation of the average reward rate of the environment independent of any particular action, or an overestimation of the effort costs of potential actions. On the other hand, the cause for the *Nlgn1*$^{-/-}$ mice's increased mobility in the FST is less clear. One possibility is that *Nlgn1*$^{-/-}$ mice have an exaggerated fear response. We know very little about

how Ngln1 regulate fear response. *Nlgn1*$^{-/-}$ mice demonstrated intact fear memory while Nlgn1 knockdown rats showed an impairment in a Pavlovian fear memory task. However, it is unclear how much the mechanisms underlying freezing behaviour in Pavlovian fear conditioning overlap with swimming in the FST. Furthermore, *Nlgn1*$^{-/-}$ mice displayed normal behaviour in three different classical anxiety tests (Blundell et al., 2010) suggesting that the phenotype in the FST is unlikely to be due to differences in stress response.

We are also interested in the possibility that the divergent phenotypes of the *Nlgn1*$^{-/-}$ mice in the touchscreen tasks and FST are caused by alterations in a common underlying decision process. We showed that an increased weighting on negative utilities in the calculation of net utilities could, in principle, capture the seemingly opposite phenotypes. Two key questions arise from this interpretation. First, is it plausible to assume a common currency for effort cost and other forms of punishment? Conceptually, this seems reasonable since physical cost, like other forms of punishment, is a decision variable which animals should seek to minimise all else being equal (Skvortsova et al., 2014). Empirically, fMRI data suggest that hemodynamic response of the anterior insula cortex correlates with both physical cost and other forms of punishment (Seymour et al., 2005; Pessiglione et al., 2006; Samanez-Larkin et al., 2008; Prevost et al., 2010; Skvortsova et al., 2014). Second, is it plausible that the brain affords dissociable neural computation and hardware for reward and punishment? It is generally accepted that there is at least partial dissociation between the neural implementation of reward and punishment (Pessiglione and Delgado, 2015; Seymour et al., 2015; Tye, 2018). Notwithstanding the lack of a consensus, reward and punishment processing may be differentially implemented by 1) firing rates and pause duration of midbrain dopaminergic neurons respectively (Maia and Frank, 2011), distinct subsets of dopaminergic neurons (Matsumoto and Hikosaka, 2009; Brooks and Berns, 2013; Hauser et al., 2017) or opponent dopaminergic and serotoninergic signalling (Daw et al., 2002; Dayan, 2012).

One major limitation in our interpretation regarding Nlgn1's role in utility weighting is that while our model accommodates the current findings in both reward-driven operant tasks and the FST, we do not have evidence to support that *Nlgn1*$^{-/-}$ would exert more effort to avoid punishment in an operant paradigm comparable to the touchscreen tasks. To address this, we attempted to train mice to press a lever to either avoid an upcoming footshock or to

terminate it. Unfortunately, the mice were unable to acquire the action-avoidance contingency in both task variants. It appears that in the face of an aversive outcome, mice can escape but struggle to acquire trained actions to prevent it. This impairment was also recently reported in rats (Jean-Richard-dit-Bressel et al., 2019). The authors showed that while rats froze in response to footshock and the conditioned cue, they did not robustly learn to lever-press to prevent the punishment.

A second caveat in our approach is that we were only able to perform simulations of behavioural data by choosing model parameters *a priori* but not estimation of model parameters *a posteriori* from data. In our experiments, parameter estimation was prevented due to two reasons. First, we observed that mice were drastically less accurate on correction trials compared to pseudorandom trials in the early sessions of PD and PAL followed by a rapid improvement much faster than the learning rate on pseudorandom trials. Clearly, certain elements of the correction trial bias mice away from making correct responses in the early sessions which was overcome more rapidly than the learning of reward contingencies. However, we failed to identify a model structure or parameter that could adequately capture this behaviour, which prevents us from estimating model parameters from the 2-alternative forced choice tasks (PD, reversal learning, PAL). Second, while parameter estimation for 2-alternative forced choice tasks is straightforward since the behavioural outcome of interest (binary choice on a given trial) is simple and discretised neatly into trials, building a model of the task and the agent for fixed- and progressive ratio tasks is far more complicated. One cannot simply fit the number of responses produced by the animals but must construct a meaningful model representing how an animal decide whether to respond or perform a myriad of other actions at any given moment. This requires knowledge about not only how animals make a response to the touchscreen but also what other actions they perform in the touchscreen and the durations of these actions. Due to this complexity, parameter estimation from ratio tasks would be either highly incomplete or relies on too many arbitrary assumptions. As a result, they are almost never performed.

The present study focused on dissecting the complex behavioural phenotype of *Nlgn1*$^{-/-}$ mice but did not identify a cellular mechanism causally related to the behavioural changes. The selective impact of Nlgn1 deletion on behaviour may arise from differential expression and/or functional roles of Nlgn1 in different neuronal populations. Although Nlgn1 is

ubiquitously expressed across the rodent brain (Song et al., 1999; Varoqueaux et al., 2006), heterogenous expression pattern and functional roles of Nlgn1 have been reported between the CA1 and dentate gyrus of the hippocampus (Shipman and Nicoll, 2012), dorsal and ventral striatum, and different populations of medium spiny neurons within the striatal sub-regions (Rothwell et al., 2014; Espinosa et al., 2015). An obvious candidate pathway via which Nlgn1 may exert its impact on motivation is dopamine neurons in the ventral tegmental area (VTA) as well as their targets in the dorsal and ventral striatum. Future experiment may perform recording on the firing of dopamine neurons in the VTA while *Nlgn1*$^{-/-}$ and WT mice performing instrumental tasks. In the same tasks, fast-scan cyclic voltammetry and fluorescence imaging of dopamine sensors (e.g. dLight) can provide insight into whether dopamine concentration differs in the target regions between *Nlgn1*$^{-/-}$ and WT mice while performing instrumental actions. Another pathway of interest is serotoninergic signalling based on the reports that enhanced serotonergic transmission promotes overcoming effort cost and some serotonergic neurons in the dorsal raphe nucleus fire in response to punishment (Cohen et al., 2015; Meyniel et al., 2016; Bailey et al., 2018). The vast majority of the current data on the synaptic function of Nlgn1 have been collected from hippocampal pyramidal neurons. Our findings suggest that investigating the synaptic function of Nlgn1 in other regions and cell types may help inform the cellular mechanisms underlying the behavioural phenotype of *Nlgn1*$^{-/-}$ mice. Furthermore, it is almost certain that we have not fully resolved the complexity of the behavioural phenotype of the *Nlgn1*$^{-/-}$ mice. Importantly, we were not able to directly quantify the animals' behaviour when they were not engaged in trained instrumental actions nor did we record their movements in the tasks, both of which would have significantly improved our behavioural characterisation. Nonetheless, we have revealed a novel and robust role of Nlgn1 in behaviour, which generalises across reward-driven and potentially punishment-drive tasks. We have also offered a precise theoretical model for the cognitive process underlying the *Nlgn1*$^{-/-}$ behavioural phenotype, which makes unambiguous behavioural predictions These predictions will allow future experiments to test the validity of the model. For example, although training mice to lever-press to avoid footshock has proven difficult, there may be other ways to train animals to perform operant behaviour to avoid an milder aversive stimulus such as optogenetically induced thirst (Allen et al., 2017; Leib et al., 2017). Although it is tempting to isolate specific parts or circuits that cause the behavioural phenotype of *Nlgn1*$^{-/-}$ mice, it is important to note that identifying the

cognitive processes disrupted is a prerequisite for understanding the meaning behind neurophysiological changes caused by loss of Nlgn1.

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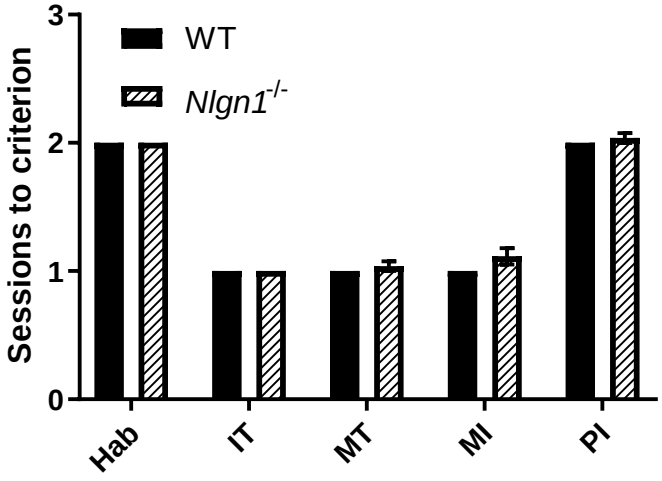
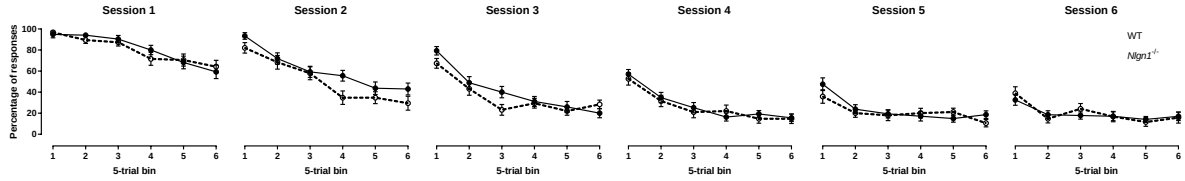
Supplemental material

Supplemental Figure 1



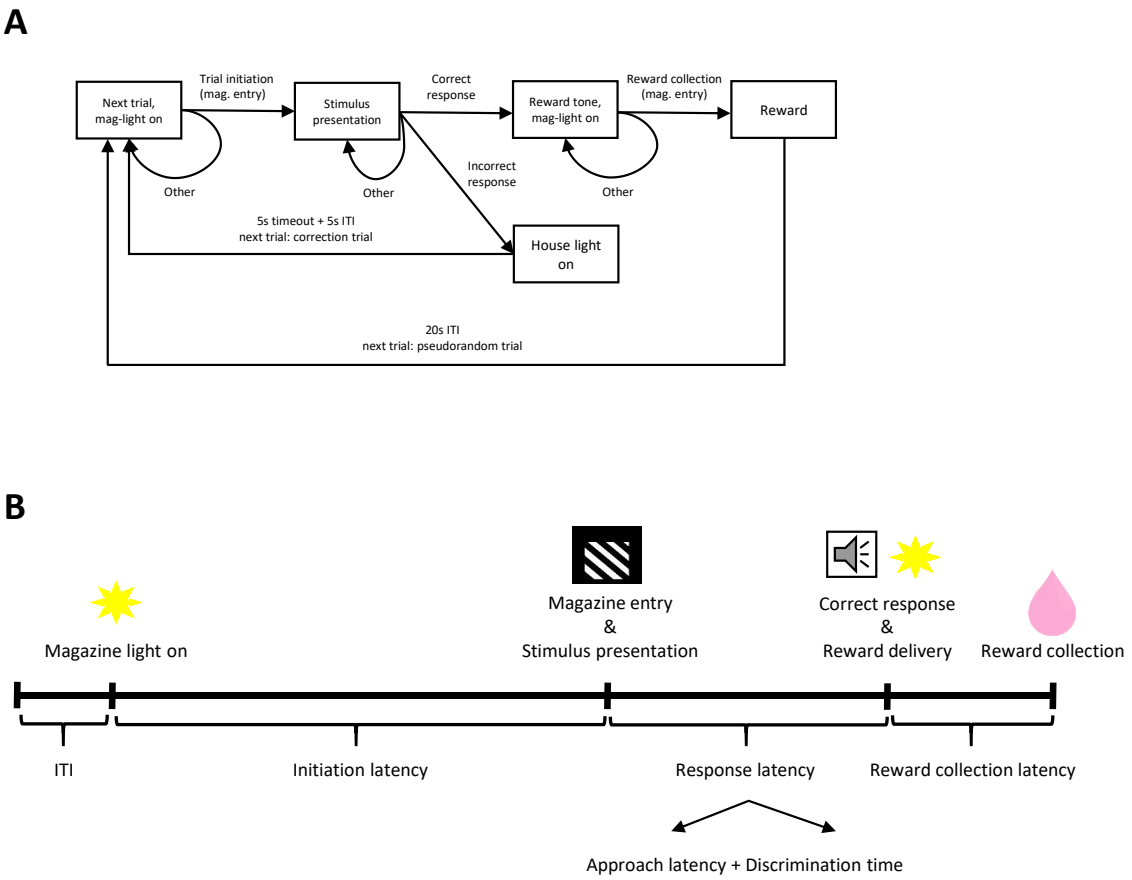
Touchscreen pre-training. Mice were trained through several phases to acquire nose-poking visual stimuli displayed on the touchscreen and were required to reach the training criterion of each phase before advancing to the next phase. Habituation (Hb): mice were habituated to touchscreen chamber for 20 minutes on 2 days and were required to consume strawberry milk rewards freely available from the magazine. Initial touch (IT): a single visual stimulus was displayed on the touchscreen for 30 seconds, the disappearance of which coincided with delivery of a strawberry milk reward, the reward tone and illumination of the magazine. If the animal touched the stimulus during stimulus presentation, a triple reward was delivered. Must touch (MT): Mice were required to nose-poke a visual stimulus displayed on the touchscreen to earn a reward on 30 trials. Must initiate (MI): mice were required to voluntarily initiate a new trial by a head entry into the reward magazine. Punish incorrect (PI): in addition to the requirements of the previous phases, responses to a blank location of the touchscreen during stimulus presentation resulted in a 5-second timeout, illumination of the house-light and were not rewarded. *Nlgn1*$^{-/-}$ and WT mice required similar number of sessions to reach pre-training criteria.

Supplemental Figure 2



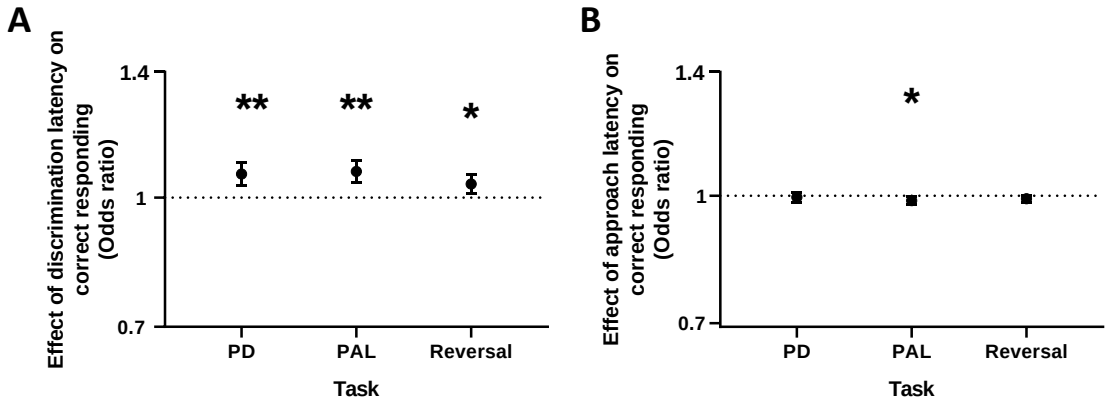
Instrumental extinction learning curves. Percentages of responses in the extinction phase of the instrumental extinction learning task across 5-trial bins within and across sessions. Learning curve plotted for data visualization only, values represent means ± SEM.

Supplemental Figure 3



Task dynamics and latency measures of the PD, reversal learning and PAL task **A**) Task-state transitions of the PD, reversal learning and PAL task. Rectangular boxes represent task states and associated cues. Arrows represent transitions to the next state or staying in the current state. Labels above arrows represent actions leading to transitions. ITI: inter-trial intervals, mag-light: magazine light. **B**) Latency measures of the PD, PAL and reversal learning task. Illumination of the magazine light signals the availability of the next trial after an ITI, initiation latency measures the time from the end of ITI to trial initiation by head entry into the magazine. Head entry triggers the presentation of stimuli, approach latency measures the time from exiting the magazine to arriving in front of the touchscreen. Discrimination latency measures the time from arriving in front of the touchscreen to nose-poking one of the stimuli. If the response is correct, a reward tone and the magazine light signal the delivery of a reward, reward collection latency measures the time from delivery of the reward tone to head entry into the magazine.

Supplemental Figure 4



Discrimination latency but not approach latency positively predicts response accuracy. **A**) Increase in discrimination latencies predicted increased response accuracy across PD, PAL and reversal learning. **B**) Conversely, increased approach latency predicted a slight decrease in response accuracy across PD, PAL and reversal learning (**Supplemental Table 1**) Logistic regression, *$p < 0.05$, **$p < 0.005$ significantly different from 1, values represent odds ratio ± 95% CI.

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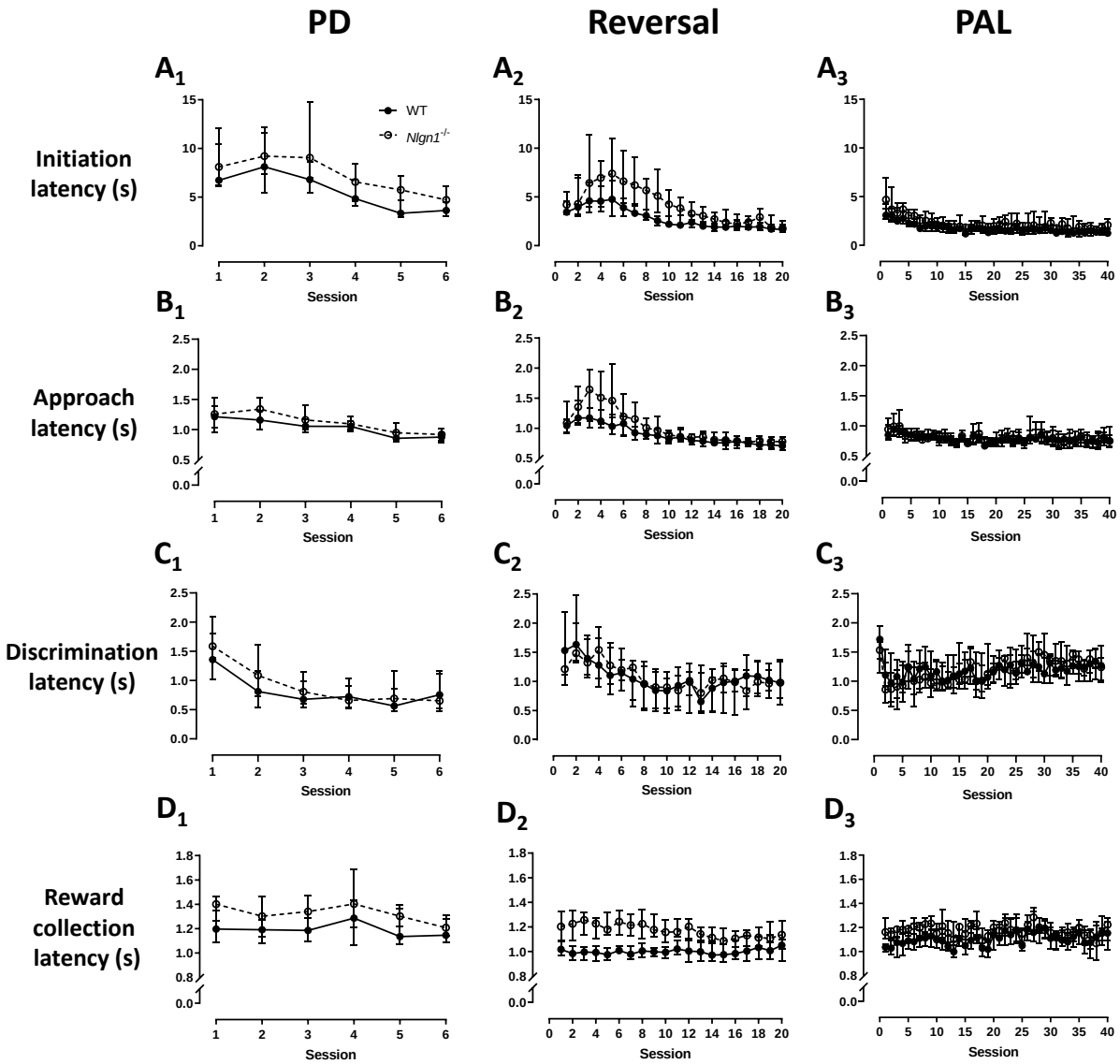
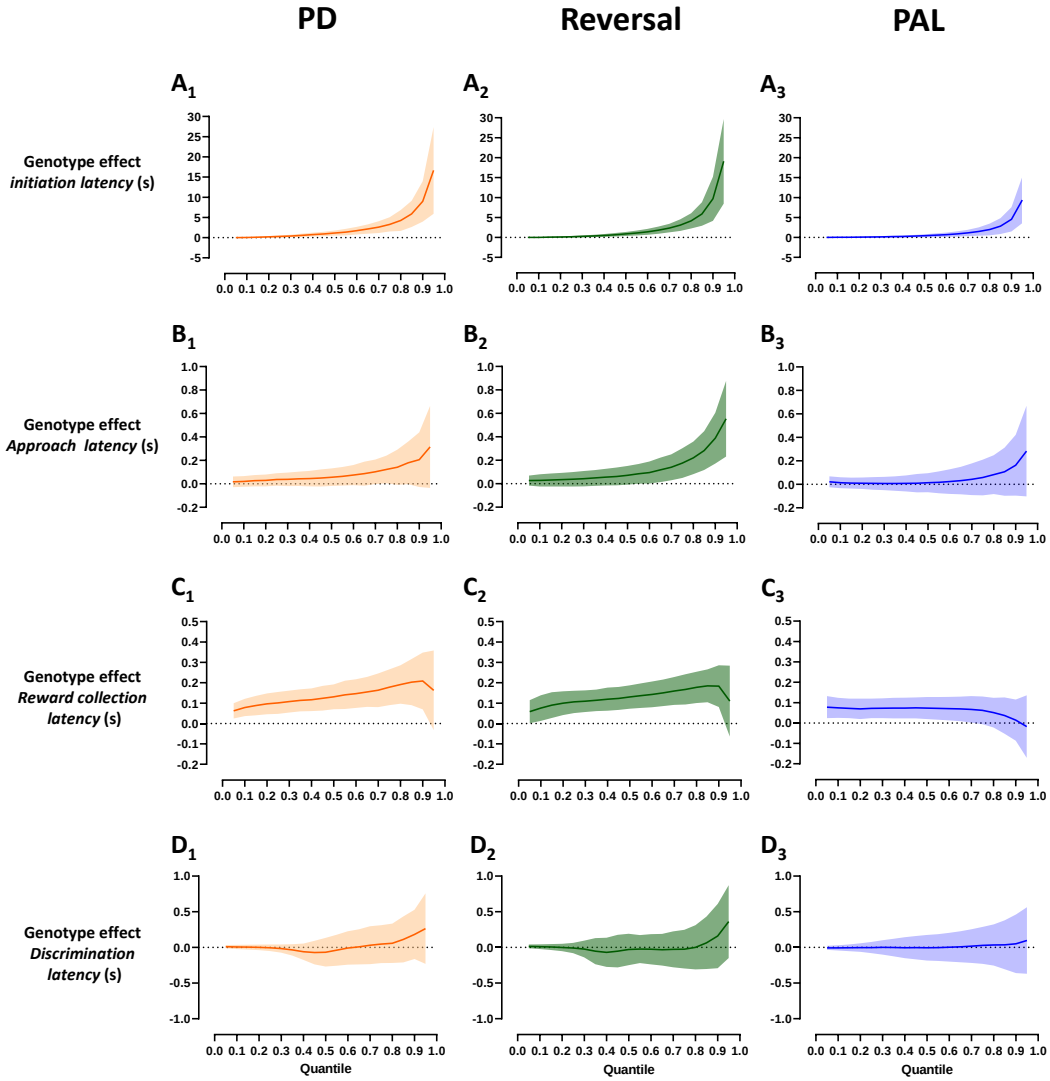
Supplemental Figure 5



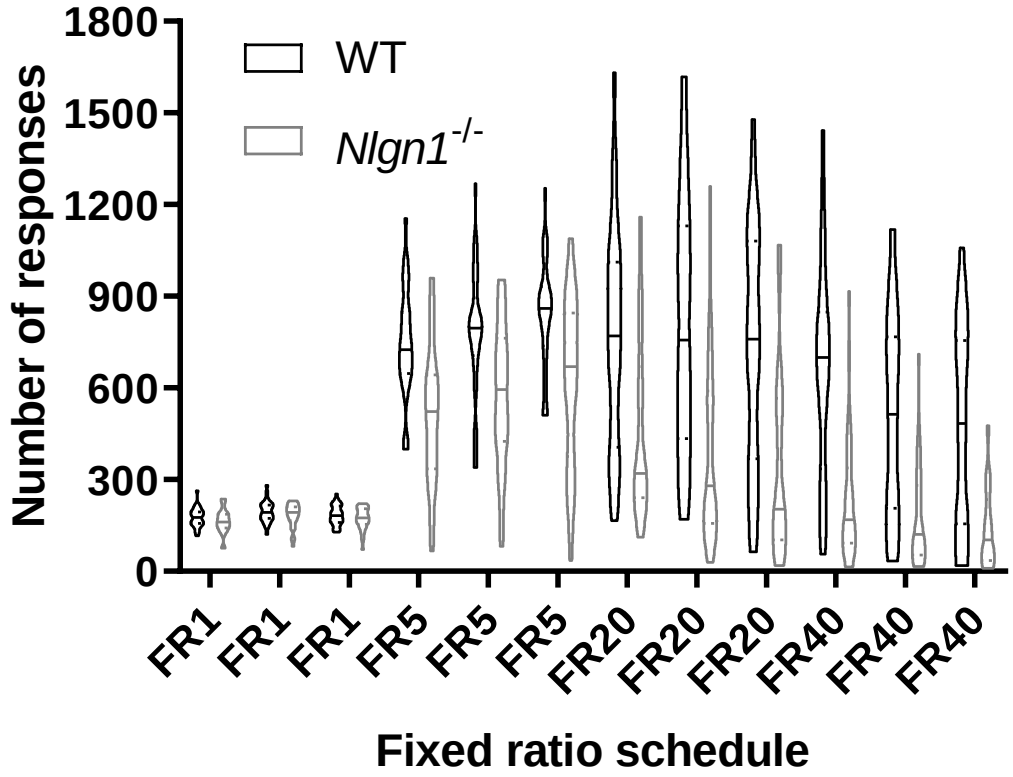
Latency learning curves. Learning curves showing the median A) initiation latency, B) approach latency, C) discrimination latency and D) reward collection latency across the PD, reversal learning and PAL task arranged in the order of training (column 1 – 3 respectively). Only the first six sessions of PD prior to the first mouse reaching criterion were visualised. Values represents median ± 95% CI.

Supplemental Figure 6



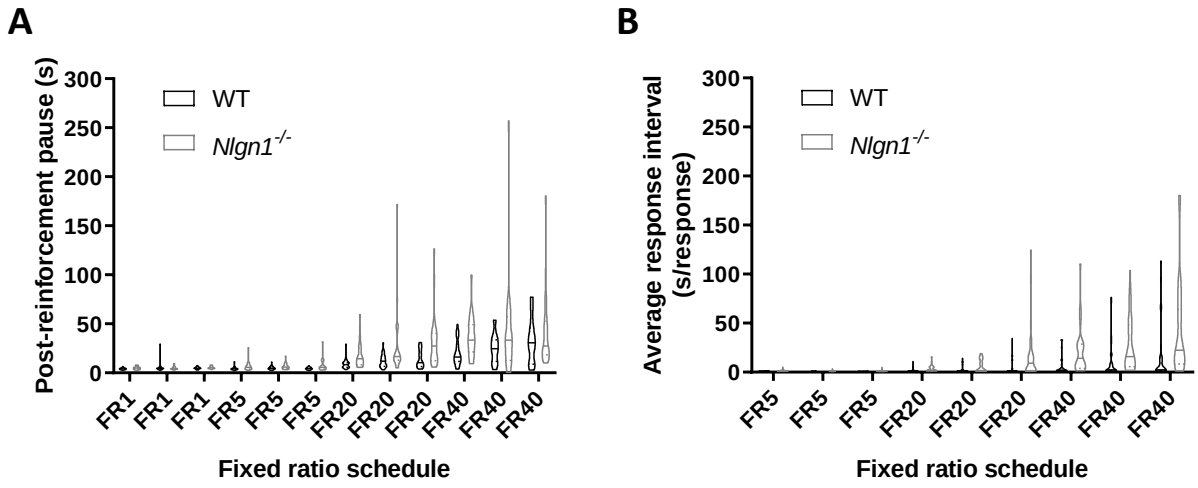
Effect of genotype on latency measures. Quantile regressions on latencies from the 0.05-th to the 0.95-th quantile at steps of 0.05. *Nlgn1*$^{-/-}$ mice showed a general increase in **A1-A3**) initiation latency and **B1-B3**) approach latency **D1-D3**) reward collection latency whereas **C1-C3**) Genotype has very little effect on discrimination latency. Quantile regression values represent estimated latency difference between *Nlgn1*$^{-/-}$ and WT mice ± 95% CI.

Supplemental Figure 7



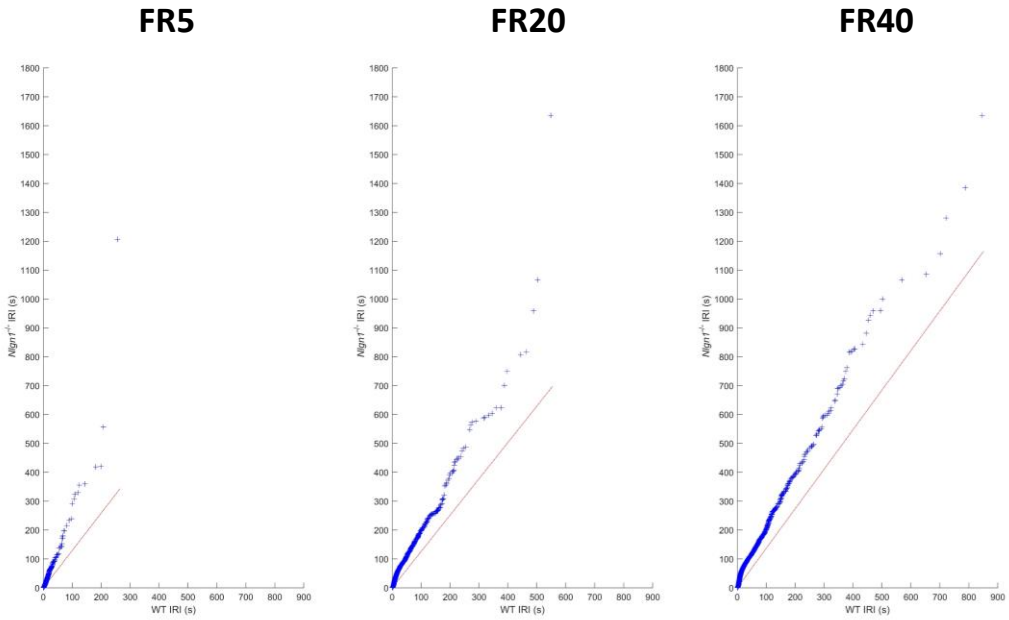
Number of responses by *Nlgn1*$^{-/-}$ and WT mice for three sessions per ratio requirement, Solid line indicates median, upper and lower dotted line indicate the 3rd and 1st quartile respectively.

Supplemental Figure 8



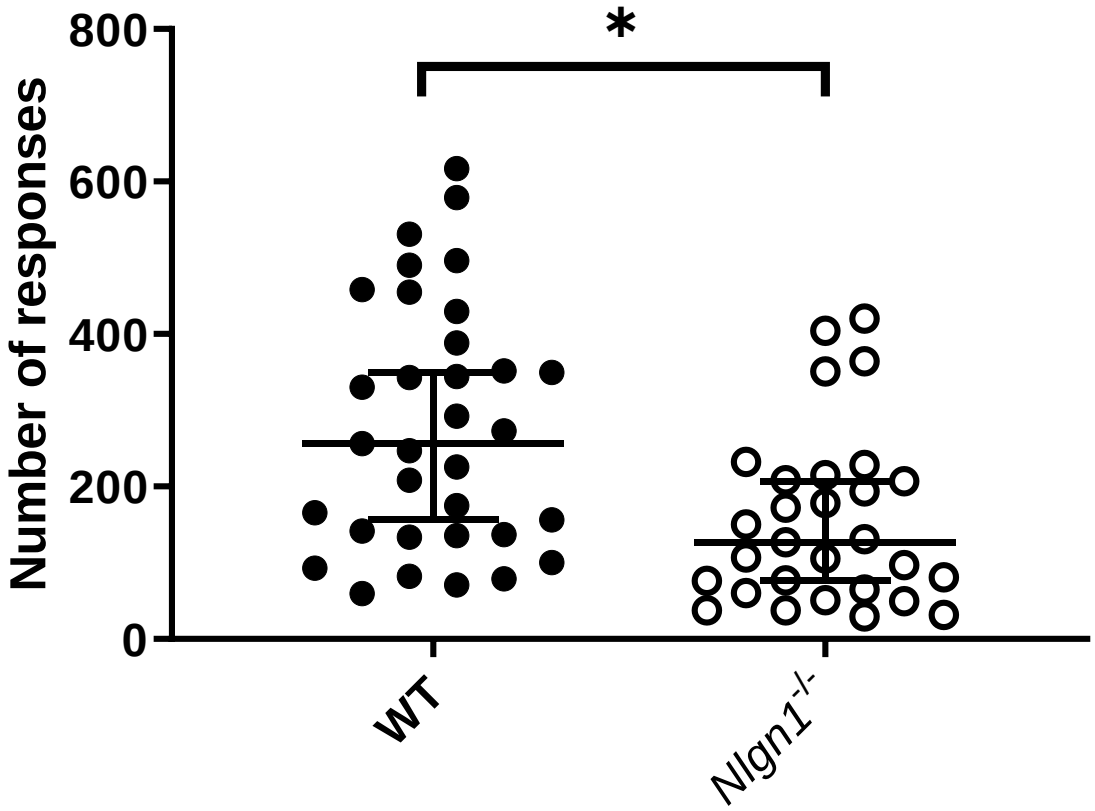
Post-reinforcement latency and average response interval across sessions of fixed ratio tasks. A) Post-reinforcement latency: time to the first response after consuming a reward. Note data could not be gathered from animals which did finish at least one trial. **B**) Average time spent per response after the animal has made the first response of a trial. Solid line indicates median, upper and lower dotted line indicate the 3rd and 1st quartile respectively.

Supplemental Figure 9



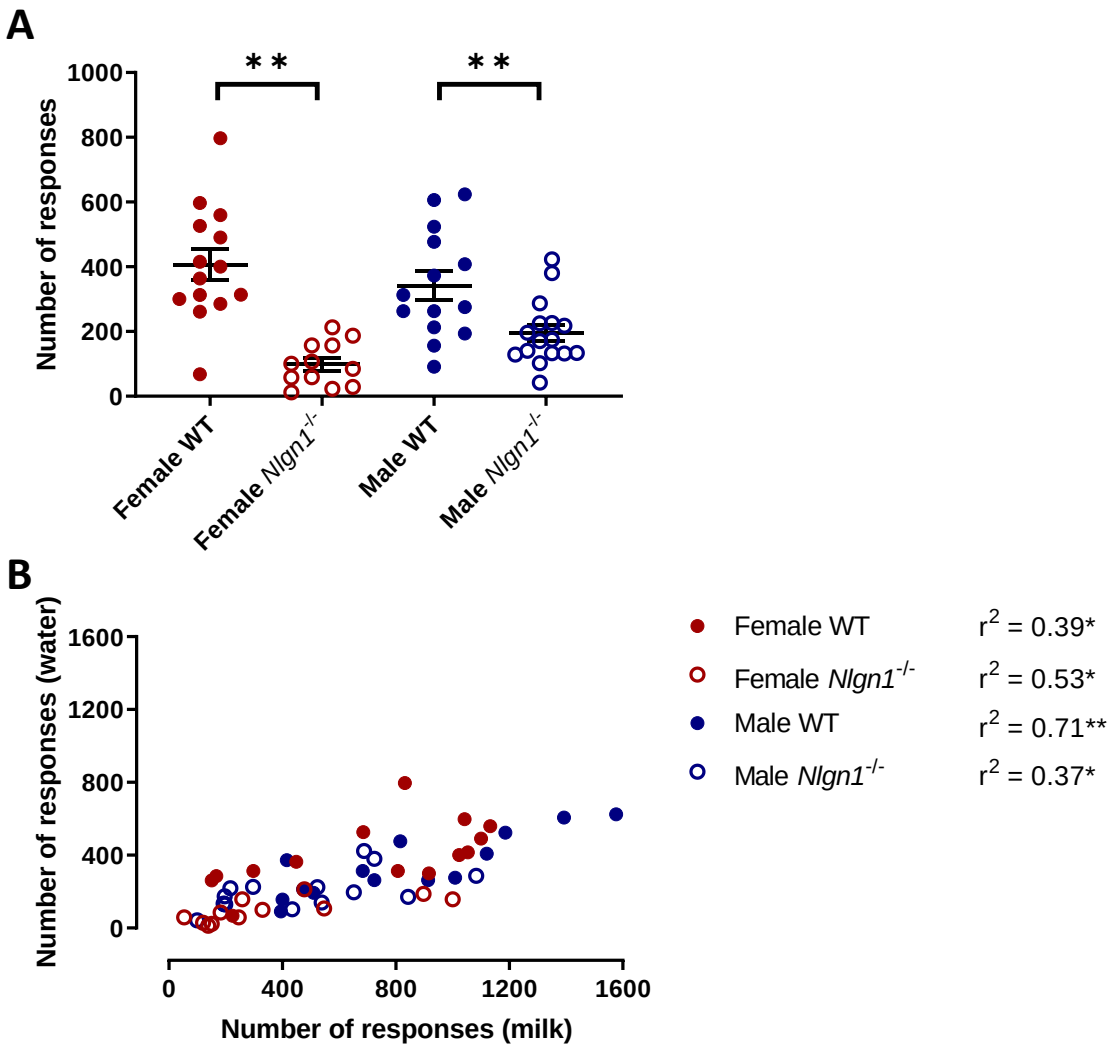
Response-by-response comparison of inter-response intervals between *Nlgn1*$^{-/-}$ and WT mice. Quantile-quantile plots comparing the distribution of inter-response intervals (IRI) by *Nlgn1*$^{-/-}$ and WT mice. The *Nlgn1*$^{-/-}$ distribution is shifted towards longer IRI. Red line indicates identical distribution (y=x).

Supplemental Figure 10



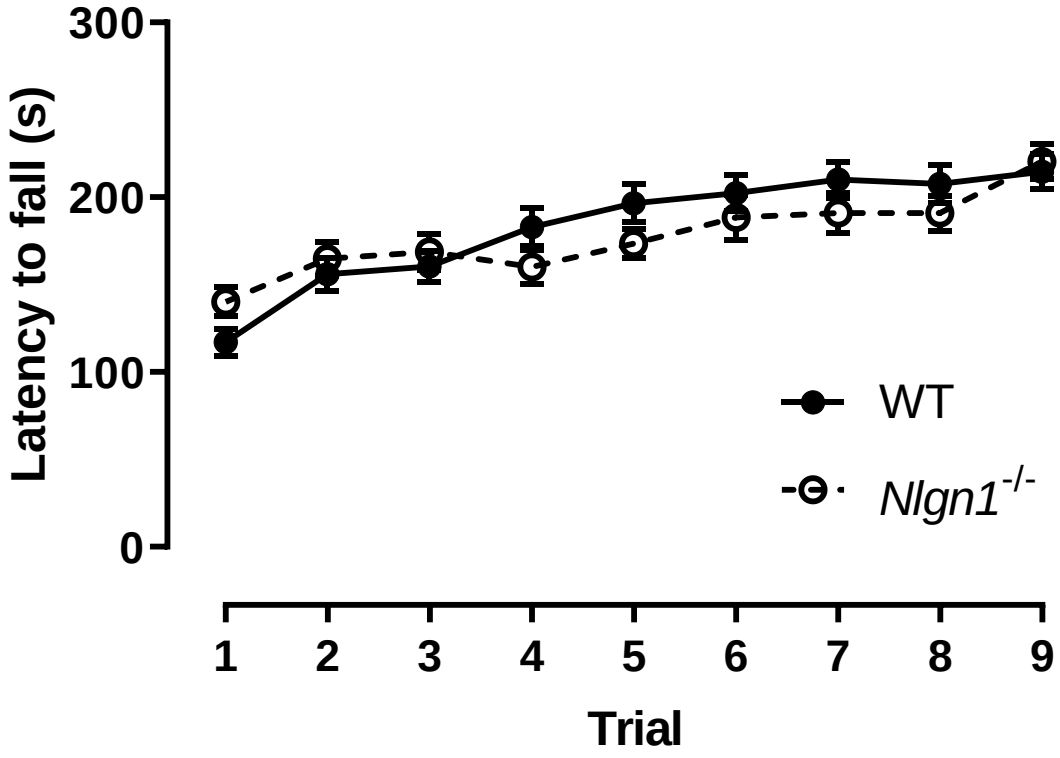
Total number of responses in the progressive ratio task. A separate cohort of *Nlgn1*$^{-/-}$ mice made fewer responses than WT controls in the progressive ratio task. Quantile regression (median), *P < 0.05, values represent median ± 95% CI.

Supplemental Figure 11



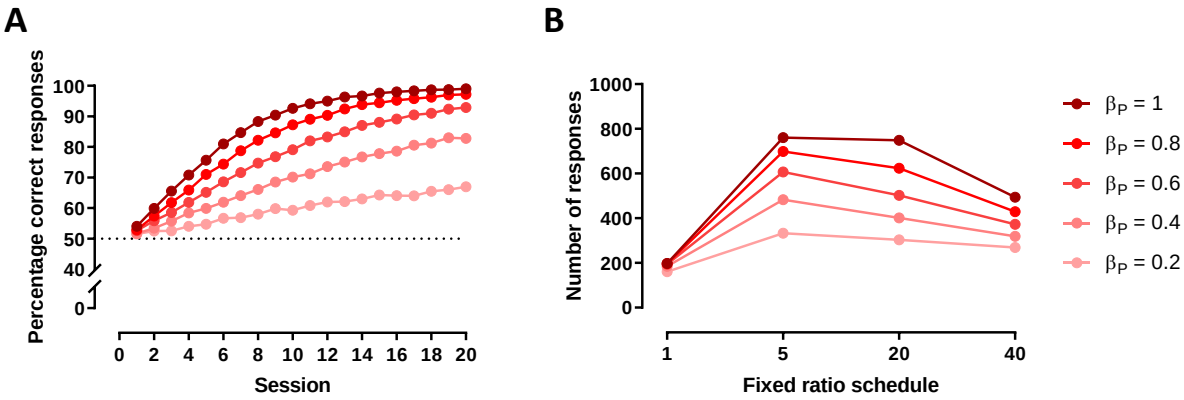
Female *Nlgn1*$^{-/-}$ mice showed a greater reduction in number of responses made for water rewards. **A**) There was a significant interaction between genotype and sex effect on the number of responses made for water rewards (**Supplemental Table 1**). nonetheless both male and female *Nlgn1*$^{-/-}$ made significantly fewer responses than WT controls. Linear regression, $^{**}p < 0.005$. **B**) The positive correlations between number of responses made for milk and water rewards were significant for each individual sex and genotype group.

Supplemental Figure 12



Nlgn1$^{-/-}$ mice displayed normal motor coordination and learning on the accelerating rotarod test. *Nlgn1*$^{-/-}$ mice showed similar latency to fall off the accelerating rotarod compared to WT controls across multiple 5-minute trials. Linear regression, values represent means ± SE

Supplemental Figure 13



Simulated effect of decreasing the weighting on positive utilities (βP) in the calculation of net utilities. **A**) Decreasing β_P from 1 to 0.2 at steps of 0.2 increases randomness in the choice between the correct and incorrect response leading to a flatter learning curve. **B**) Decreasing β_p reduces number of responses in the simulated FR tasks.

Chapter 4: General Discussion

4.1 Summary of findings

I embarked on this PhD project with the aim to further the current knowledge on the function of Nlgn1 both at the synapse and in behaviour and ultimately, how its synaptic function may impact behaviour. Importantly, the *Nlgn1*$^{-/-}$ mouse model serves as good tool for understanding the relationship between synaptic function and behaviour because of its robust and well-characterised physiological phenotype. At the synapse, we found that Nlgn1 does not play an essential role in regulating the size of synaptic vesicles pools or the rate of exocytosis therefore its primary synaptic function appears to be postsynaptic. We also investigated the behavioural consequences of Nlgn1 deletion. Despite the robust reduction in NMDAR function and impaired LTP, *Nlgn1*$^{-/-}$ mice were able to learn complex associative structures and adapt flexibly to changes in previously learnt associations. However, we found that the loss of Nlgn1 led to a decrease in the willingness to exert effort for rewards across all the reward-driven instrumental-conditioning tasks without affecting associative learning in even the most complex tasks. Surprisingly, *Nlgn1*$^{-/-}$ mice were more willing to exert effort to avoid punishment. Finally, to explain the nature of the altered cognitive mechanisms that generated the divergent *Nlgn1*$^{-/-}$ behavioural phenotype across tasks, we simulated behaviour using a simple reinforcement learning model and propose that the behavioural results could, at least in principle, be captured by an increased weighting on negative utilities in calculating the net utilities of potential actions.

Discussion on the direct implications and limitations of the current findings to the Nlgn1 literature has been included as part of Chapter 2 and 3 therefore will not be reiterated here. Rather this final chapter will discuss more broadly the limitations and strength of this PhD project and outstanding challenges for future studies. Finally, I reflect on the important conceptual and philosophical lessons learnt over the course of the past four years as a PhD student.

4.2 Limitation: a lack of neural correlates

The goal of neuroscience is arguably to explain the behavioural outputs of an organism in terms of the physical components of the brain and their interactions: from direct motor

control such as moving an arm, to overt symptoms of brain diseases such as neurodegeneration, to more abstract modalities such as mood changes. In order to explain how behaviour is generated by neural implementation, neuroscientists may carefully observe neural correlates during behaviour to generate hypotheses about the potential mechanisms; then inactivate the neural correlate to test its necessity for the target behaviour; and finally, artificially generate the neural correlate to prove that it is sufficient to generate the target behaviour.

In this project, the only coarse neural correlates for the *Nlgn1*$^{-/-}$ behavioural phenotype are the global deletion of Nlgn1 and its previously reported impacts on synaptic function. Our study only establishes that the behavioural phenotype of *Nlgn1*$^{-/-}$ mice depends on the expression of Nlgn1 but falls short of offering insights into *how* synaptic dysfunction caused by Nlgn1 deletion leads to altered behaviour. The explanatory power of future experiments could be significantly improved by assessing behaviour and recording neural activity in parallel, which would allow correlations to be drawn between behaviour and the underlying neural patterns. Importantly, recording neural activity in behaving animals provides an intermediate level of analysis. In doing so the problem of relating behaviour to synaptic function can be decomposed into 1) relating behaviour to neural activity, and 2) relating neural activity to synaptic function, which will help bridge the wide gap between behaviour and synapses.

4.3 Strength: careful behavioural characterisation

4.3.1 Embracing the complexity of behaviour

If our goal is to understand how behaviour is generated by the brain, it should be self-evident that understanding the nature of the behaviour is just as important as recording or manipulating the underlying neural correlates. This project focuses on characterising the *Nlgn1*$^{-/-}$ behavioural phenotype in detail using three main strategies. First, we quantified the animals' behaviour not with the question "are they impaired?" but "how do they behave?". The subtle difference between the two mindsets is that the former focuses on a binary categorisation whereas the latter tries to capture the complexity in behaviour. While treating behaviour as a dichotomy allows a succinct summary of behavioural phenotypes, it often

leads to oversimplification especially in studying complex behaviour. For example, using latency to platform alone to characterise spatial learning in the Morris water maze is an oversimplification of the complexity in spatial navigation and it seems unlikely that by doing so we will achieve an understanding of spatial learning. Instead of relying only the primary readout from each behavioural task to diagnose complex behaviour as "impaired" or "normal", we used multiple behavioural measures to reconstruct to the best of our ability an empirical description of the animals' behaviour. Furthermore, we estimated the effect size of a large set of variables on behavioural readouts, which provided a more comprehensive and quantitative understanding of the factors that influence behaviour than testing for statistical significance alone.

4.3.2 Testing the generalisability of behaviour

A second approach we employed to elucidate the *Nlgn1*$^{-/-}$ phenotype was to test its generalisability across different tasks. As behavioural neuroscientists, we are generally interested in the underlying cognitive processes that generate the observed behaviour rather than behaviour specific to a particular task. Therefore, we want to describe a behavioural phenotype generalisable across different task environments. Conversely, we may disprove a theory by discovering inconsistencies between tasks. In this project, we were able to demonstrate that *Nlgn1*$^{-/-}$ mice were consistently less willing to exert effort for rewards in different reward-driven tasks and for different reward types. However, we observed the seemingly opposite behaviour when the motivational drive for effort exertion was switched to escaping a punishing environment. Although much of the *Nlgn1*$^{-/-}$ phenotype can be observed in a single task (e.g. normal response accuracy and increased latencies in PD, reversal learning or PAL), examining behaviour across tasks greatly informed our interpretation. On the one hand, the consistent findings in reward-based paradigms demonstrate the robustness of the *Nlgn1*$^{-/-}$ phenotype under non-aversive conditions. On the other hand, the finding that *Nlgn1*$^{-/-}$ mice were more willing to exert effort in the forced swim test rejects reduced willingness to exert effort as an overall explanation for the *Nlgn1*$^{-/-}$ phenotype.

4.3.3 A theory-based, operationalised interpretation of behaviour

Lastly, we offered a hypothesis about how the *Nlgn1*$^{-/-}$ phenotype may be generated by the underlying cognitive processes in the form of a simple computational model and behavioural

simulations. We often label cognitive processes with concepts which we introspectively relate to (e.g. motivation, perseveration, flexibility) instead of functional descriptions of their effects on behaviour. However, the definitions of these intuitive concepts are often ambiguous and subjective especially for rodents. As a result, they often do not articulate how observable behaviour may be generated neither do they offer testable predictions. One way to circumvent this ambiguity is to represent cognitive processes in precise theoretical models, such that they are defined according to their modelled effects rather than subjective interpretation. This allows us to explicitly present 1) the relationships between the cognitive constructs included in the model (e.g. weights on positive and negative utility); 2) the assumptions of the model (e.g. a common neural currency for different types of negative utilities) and consequently, 3) the model's behavioural predictions (e.g. *Nlgn1*$^{-/-}$ mice will work harder to avoid punishment in general). This is not to claim that theoretical models always provide an accurate interpretation of the generative process of behaviour. In fact, all behavioural models are incomplete, rest on many assumptions and simplifications, and capture at best the essence but not the details of behaviour. However, combining behavioural data and theoretical models makes interpretations transparent with regards to their predictions, assumptions and incompleteness, thereby making improvement possible.

4.4 Unresolved behavioural complexity

4.4.1 Recording and quantifying spontaneous untrained behaviours

Through carefully analysing the behaviour of the *Nlgn1*$^{-/-}$ mice, we have also come to recognise some great challenges. In most instrumental conditioning experiments including ours, we only record and quantify a small subset of actions displayed by animals during a task focusing on the ones which animals are trained to perform such as initiating a trial, responding to the stimuli and collecting a reward. However, animals also exhibit many spontaneous untrained behaviours (e.g. resting, exploration, grooming), which are hidden from our quantitative analyses. As a result, the duration spent in performing untrained actions contributes to the latency to perform a recorded trained action. For example, response latency is composed of both the time taken to make a response and the time spent engaging in other unrelated actions before the animal commits to responding, and the two cannot be

separated in *post hoc* analyses. Quantifying untrained actions is difficult but neglecting them has important implication in our understanding of decision-making and is reflected in formal theories of action selection. Current models attempt to capture action selection in free operant tasks mostly by assuming that animals decide which action to perform and the optimal latency to its execution (McClure et al., 2003; Niv et al., 2007; Dayan, 2012; Collins and Frank, 2014). These accounts render the solution to the optimal action and the optimal latency to perform it tractable given parameters such as reward rates and effort cost. However, the many extreme values in our latency data (10s of seconds, up to minutes) can only be realistically explained by taking into account engagement in untrained actions and the duration over which these actions last. Most commonly, a mouse would enter a state of inactivity especially when probability of earning a reward is low. This cannot be accurately described as the animal choosing an extremely long latency to make the next response. Characterising and quantifying at least a subset of the untrained actions which mice typically exhibit will greatly enhance our understanding of action selection in free operant tasks.

4.4.2 Learning to avoid punishment

We made the generalisation between overt punishment (being in water) and physical effort in our interpretation of the behavioural phenotype of *Nlgn1*$^{-/-}$ mice yet we could not find strong empirical evidence to support or contradict this assumption. It seems conceptually obvious that animals make decisions by considering both the reward (positive utility) and punishment (negative utility) associated with their options (though this does not imply optimality). This implies that the neural representations of different types of reward and punishment need to be compressed onto a common scale and compete against each other. Historically, the neuroscience of decision-making has primarily focused on reward maximisation. This has led to reasonably robust evidence for the common neural representation of different types of rewards, the neural computation to optimise reward maximisation and the neural substrates implementing the computation (Schultz et al., 1997; O'Doherty et al., 2003; Balleine and O'Doherty, 2010; Haber and Knutson, 2010; Levy and Glimcher, 2012). On the contrary, much less is known about the neural representation and computation of punishment. It is not clear when a lack of reward becomes punishment, whether different types of punishment integrate into a common neural currency and the neural computational algorithms employed to optimise punishment minimisation, with some

evidence suggesting it may be optimised similarly to reward maximisation (Skvortsova et al., 2014; Pessiglione and Delgado, 2015; Palminteri and Pessiglione, 2017).

The disparity is partly due to the lack of rodent instrumental tasks for avoiding punishment (active avoidance tasks). The current available active avoidance tasks typically train animals to move to a safe location to avoid punishment (Diehl et al., 2019). The obvious drawback of these tasks is that moving to a safe location is more difficult to quantify and manipulated than lever-presses and nose-pokes, which are temporally discrete actions and the economics of which can be easily manipulated (e.g. number of lever-presses/nose-pokes required to avoid punishment). There is also evidence to suggest that rodents (rats and mice) do not robustly learn the contingency between an instrumental action and an aversive outcome. In previous studies, significant proportions of the experimental populations did not adjust their behaviour to avoid aversive outcomes (Jean-Richard-dit-Bressel et al., 2019; Stelly et al., 2019). Jean-Richard-dit-Bressel et al. (2019) showed that rats failed to change their behaviour despite demonstrating fear to the aversive outcome itself. We made similar observation in our experiments using mice (data not shown). We attempted multiple paradigms yet failed to train mice to lever-press to avoid footshocks. More sophisticated tasks using less behaviourally disruptive punishment are needed in order to assess neural correlates of punishment in rodents. One possibility is to induce mild but unpleasant bodily sensation such as training mice to lever-press to terminate thirst-inducing optogenitic stimulation (Allen et al., 2017; Leib et al., 2017).

4.5 From behaviour to synapses: a gap worth bridging?

Neuroscience spans across many levels, from behaviour to neural activity of large populations, to the wiring of neurons between and within populations, to a single neuron, to synapses on a neuron, to proteins at a synapse, and each of these levels can be further subdivided (**Figure 4.1**). Therefore, it is not trivial to ask at which level is the phenomenon one tries to understand, and at which level is one trying to explain it. I embarked on this PhD project because of a fascination with how complex behaviour is generated by physical components of the brain. Above all, the project rests on the premise that understanding the function of a protein at the synapse will improve our understanding of how the brain

generates behaviour. In this final section of the thesis, I will reflect on this premise and consider what may be done in the future to better bridge the gaps between behaviour and synapses.

4.5.1 The case against synapse-level complexity for understanding behaviour

We know that behaviour causally depends on proteins at the synapse. However, it does not follow that an explanatory model of behaviour must include features at the level of synapses. In building a theory to explain a phenomenon, we abstract away lower-level features that do not improve our description and prediction about the system. I do not need to know how the transistors in this computer work in order to write my thesis and knowing how the transistors work will not improve its quality. This is hardly a new argument but a powerful one that has been voiced by prominent neuroscientists (Marr, 1982/2010; Carandini, 2012; Krakauer et al., 2017). Theoretical models of behaviour and cognition typically go as far as treating neurons as the unit of analysis (e.g. neural network models) with the notable exception of dividing a neuron into three compartments (Larkum, 2013). This is echoed by influential findings on the physical substrates of neural computation, such as the cellular basis of memory engram (Liu et al., 2012), place-cells and grid cells in spatial navigation (O'Keefe and Dostrovsky, 1971; Hafting et al., 2005), which consider the firings of neurons as the “atoms” of information processing in the brain. Much of our current understanding of neural computation and biological/artificial intelligence does not seem to have benefited from synapse biology. So, why should neuroscientists interested in behaviour investigate at the synapse level?

4.5.2 The case for synapse-level complexity for understanding behaviour

Despite the wide gap between behaviour and synapses, there is strong practical incentive to study the roles of synaptic proteins in behaviour even without achieving full explanation. We know that mutations in genes encoding synaptic proteins are strongly associated with psychiatric disorders (International Schizophrenia, 2008; Kirov et al., 2008; Marshall et al., 2008; Walsh et al., 2008; Glessner et al., 2009; Neale et al., 2010; Williams et al., 2010). Identifying the genetic architecture and penetrant mutations of these disorders will improve disease prevention and management, even without fully understanding why a mutation causes a disorder. However, findings on disease-associated genes from genetic studies are not always robust and consistent. It is highly likely that each mutation only contributes to a subset of symptoms of a disorder but are involved in multiple disorders. Therefore, instead

of mapping disorders to genes, it may be more productive to study the genetics of dissociable cognitive processes impacted in psychiatric disorders. while the task of recapitulating complex human psychiatric disorders using rodent models seems daunting for various reasons beyond the scope of our discussion here, rodent models are powerful tools for mapping evolutionarily conserved cognitive modules to gene mutations (Nithianantharajah et al., 2013; Norris, 2019). Careful behavioural characterisation of rodent models carrying synaptic gene mutations may provide valuable and practical insights into the genetic underpinnings of behavioural disturbances in humans.

More importantly, we simply do not know the level of details required for a full understanding of the neural computation underlying intelligence and behaviour. Even though we do not yet know how synaptic complexity may improve our understanding of neural computation or intelligence in general, there are strong enough reasons to believe that it will. We do not know how animals achieve learning generalisable across domains with far less training data compared to artificial intelligence. It is likely that the advantage that biological intelligence has over its artificial counterparts is in part due to the brain's enormous anatomical and molecular complexity including complex signaling at the synapse, which has been constrained and optimised throughout evolution (Sinz et al., 2019; Zador, 2019). In light of this, rodent models carrying synaptic gene mutations serve as good tools for investigating the behavioural consequences of synaptic mechanisms, especially for well charaterised synaptic proteins causing discrete synaptic dysfunction. In the case of Nlgn1, the extensive literature on its synaptic function allows us to investigate the impact of NMDAR hypofunction and impaired LTP on behaviour. Although deleting a synaptic gene in a mouse model generally does not inform us about *how* the deletion causes the behavioural phenotype, it is useful for testing the behavioural necessity of the impacted synaptic mechanisms.

4.5.3 Bridging the gaps between behaviour and synapses: computation, algorithm and implementation

Regardless of whether it is necessary or possible to reduce neural computation and behaviour all the way down to the function of a synaptic protein (or for that matter any molecule in the brain), It appears that this is a quest which neuroscientists are innately invested in. Even if we have fully understood the brain's operations in terms of the dynamics of neuronal ensembles, most of us would not consider this to be the end of neuroscience. There is a strong sense that

we will not be satisfied until we have a molecular explanation for everything the brain does. As there are many levels of investigation between behaviour and the molecular machinery of the brain, the main challenge then is to bridge the gaps between these levels (**Figure 4.1**). David Marr's three levels of analysis provide a useful conceptual tool for the problem. Marr argued that to understand a hierarchical system means understanding the problem it tries to solve (the level of *computation*), the abstract rules employed to solve the problem (the level of *algorithm*) and the physical substrates implementing the algorithms (the level of *implementation*) (Marr, 1982/2010). This framework can be applied to each level between behaviour and synaptic proteins such that each level serves as the computation for the level below and implementation for the level above, connected by algorithms (not depicted for the two end levels, **Figure 4,1**). For example, the dynamics of large neuronal populations may serve as the implementation for a given behaviour, and the computation for neuronal circuitry. This is not just a truism but a view contrary to common practice. While we are relatively good at the reduction from computation to implementation (consider the circuits/cell types/molecules involved different behaviours), the constraints that computation places on implementation (e.g. the ethological relevance of the behaviour) and the algorithms connecting computation and implementation (e.g. the computational algorithms through which circuits implement a behaviour) are taken far less seriously. The combinatorial explosion of complexity inherent in scaling up from synaptic proteins to behaviour sometimes makes the task seem impossible. To overcome this, we divide the overall problem into more tractable, inter-dependent sub-problems in the hope that solving the sub-problems will lead to a solution to the overall problem. The tendency of unidirectional reduction hinders communication between neuroscience subfields across different levels and therefore must be overcome if we are truly invested in constructing a coherent account of brain function from behaviour to molecules.

4.5.4 Embracing the absurd

On a final note, one must also consider seriously the possibility that the task is in fact impossible and the implication of such a possibility. We may uncover some general principles on which the brain operates but we may never fully understand all of its complexities. Indeed, even artificial neural networks are quickly becoming too complex for human understanding. If we cannot even understand the intelligence we create, there is no reason to believe that

our brain is capable of understanding its own complexity. At a personal level, we hope and often assume that our research will somehow contribute to the overall goal of neuroscience. But for most of us, years of hard work will turn out to be utterly inconsequential because we base our work on the wrong premise. The discrepancy between our desire to understand the brain and the very real possibility that we lack the capacity for such a task or that we will spend years asking the wrong questions is a source of absurdity. If escaping the absurd by taking a leap of faith or ignoring it altogether seems unsatisfactory, how then can someone who feels the absurd vividly not be paralysed by an overwhelming sense of uncertainty? We may remind ourselves that the ability to recognise the absurd is a uniquely human characteristic and it need not be a source of despair or agony (Nagel, 1971). Perhaps one could take up Camus' advice by shaking their fists at the universe and proclaiming in defiance that the endeavour itself is enough to fill a person's heart and one must imagine the scientist happy (Camus, 1955). As Alyosha Karamazov puts it in *The Brothers Karamazov*, "Certainly, love it, love it regardless of logic, it must be regardless of logic, and it's only then one will understand the meaning of it." Above all, I consider this to be the most important lesson from my PhD.

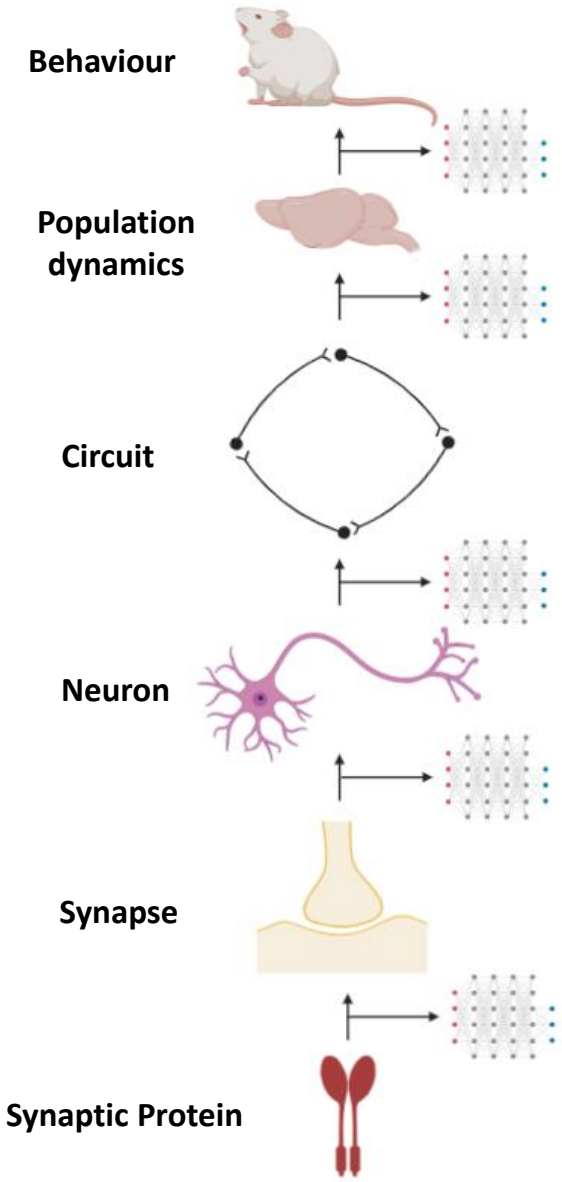


Figure 4.1 Understanding the relationship between behaviour and components of the brain across multiple levels. The levels between behaviour and synaptic proteins are coarsely divided into behaviour, the brain-wide activity, circuits of the brain, neurons in a circuit, synapses on a neuron and proteins at a synapse. Each level except the end levels serves as the computation for the level below and the implementation for the level above. Network icons represent the algorithm through with the implementation achieve the computation. Note that there more levels above behaviour such as ethology, and below synaptic proteins such as genetics, which are not depicted here.

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