The Effects of Neuronal Calcium Sensor-1 Deletion on Mouse Behaviour and Neurophysiology

by

Enoch Ng

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Institute of Medical Science
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Abstract

While neuronal calcium sensor-1 (NCS-1) has been implicated in neurotransmitter release, synaptic plasticity, spatial cognition and a number of diseases, its role in mammalian behaviour, learning and motivation have not been thoroughly explored. Here we investigated the effects of *Ncs-1* deletion on mouse behaviour and explored potential underlying physiological mechanisms. Since NCS-1 is a dopamine-receptor interacting protein and has known roles in hippocampal synaptic plasticity, we focused on striatal-dependent motivated behaviours as well as hippocampal-dependent learning and memory. We hypothesized *Ncs-1* deletion would (1) impair motivated behaviour and decrease striatal dopamine signalling, (2) disrupt spatial learning and memory as well as hippocampal synaptic plasticity, and (3) produce endophenotypes of human neuropsychiatric disease. We found *Ncs-1* knockout (*Ncs-1*$^{-/-}$) mice were less willing to work for food than wildtype mice in operant conditioning tasks with high work requirements or when a less-preferred food was freely available. However, *Ncs-1*$^{-/-}$ mice preferred sweet foods as much as wildtype mice and showed intact Pavlovian incentive learning, operant acquisition and habit formation. At the level of striatal physiology, *Ncs-1*$^{-/-}$ mice displayed a 50% decrease in electrically evoked dopamine release from the nucleus accumbens core in acute slices. Dopamine half-life, dopamine receptor D2 (DRD2) levels, and dopamine transporter (DAT) levels were

relatively unaffected. Learning and memory was also relatively intact. *Ncs-1*$^{-/-}$ mice showed normal spatial reference memory, fear memory, and spontaneous object recognition, but were impaired in displaced object recognition. Subtle changes were found in hippocampal electrophysiology, with a trend for less metabotropic glutamate receptor (mGluR)-dependent LTD early on and reduced maintenance of LTP in the dentate gyrus. *Ncs-1*$^{-/-}$ mice had wildtype levels of locomotion, amphetamine-induced hyperlocomotion, sensorimotor gating, and anxiety-like behaviour but showed decreased social approach without impairments to actual social interaction behaviour. Our results suggest NCS-1 has a role in modulating effort to approach and work toward goals and rewards, via effects on presynaptic dopamine release.

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Contributions

The author performed all experiments described in this thesis, except as noted below:

Hosuk Mun – conducted the novel object recognition and displaced object recognition tests (Section 3.3.1).

Panayiotis (Peter) Servinis – assisted in certain behavioural experiments related to endophenotypes of human neuropsychiatric disease in Section 3.4, specifically open field, light/dark box, elevated zero maze, 3-chamber social approach, and reciprocal social interaction.

Dr. Ping Su – conducted subcellular fractionation of striatal tissue and immunoblotting for DRD2 and DAT (Section 3.5.1).

Dr. Rafael Koerich Varaschin – conducted fast-scan cyclic voltammetry to measure evoked dopamine release in acute slices (Section 3.5.2).

Dr. John Georgiou – conducted hippocampal slice electrophysiology experiments (Section 3.6). The author performed all subsequent analyses.

Dr. Qiuhong Jiang – prepared rat primary cortical neurons that I subsequently used for pilot testing a lentivirus to overexpress *Ncs-1* (Section 5.5).

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List of Abbreviations

6-OHDA	6-hydroxydopamine
A-O	action-outcome
A_{2A}	adenosine 2A
ACSF	artificial cerebrospinal fluid
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA	analysis of variance
AP2	adaptor protein 2
AP5	amino-5-phosphonopentanoic acid
ASD	autism spectrum disorders
BCA	bicinchoninic acid
BSA	bovine serum albumin
C-terminal	carboxy terminal
CA1	*Cornu Ammonis* area 1
CA3	*Cornu Ammonis* area 3
CaBP	calcium binding protein
CaM	calmodulin
CaMKII	Ca^{2+}/calmodulin-dependent kinase II
cAMP	cyclic adenosine monophosphate
CAPS	Ca^{2+}-dependent activator protein for secretion
CREB	cAMP response element-binding protein
CRF	continuous schedule of reinforcement
CS	conditioned stimulus
DA	dopamine
DHPG	(*S*)-3,5-dihydroxyphenylglycine
DLS	dorsolateral striatum
DMS	dorsomedial striatum
DNA	deoxyribonucleic acid
DNIP	DRD2 / NCS-1 interfering peptide
DRD2	dopamine receptor D2
DTT	dithiothreitol
ECL	electrochemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
fEPSP	field excitatory postsynaptic potential
FR	fixed ratio
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GCAP	guanylyl cyclase activating protein
GRK2	G-protein coupled receptor kinase 2
Het	heterozygote
IL1RAPL	interleukin 1 receptor accessory protein-like
KChIP	potassium channel interacting protein

KO	knockout
L	left
LTD	long-term depression
LTP	long-term potentiation
MAPK	mitogen activated kinase-like protein kinase
mGluR	metabotropic glutamate receptor
mRNA	messenger ribonucleic acid
N-terminal	amino terminal
NCS	neuronal calcium sensor
NCS-1	neuronal calcium sensor-1
NMDAR	N-methyl-D-aspartate receptor
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBS-BT	phosphate buffered saline with BSA and Triton X-100
PDB	protein databank
PICK1	protein interacting with PRKCA 1
PIT	Pavlovian-to-instrumental transfer
PP1	protein phosphatase 1
PP2B	protein phosphatase 2B
PPI	pre-pulse inhibition
PPi	pre-pulse + startling pulse trial
PR	progressive ratio
PRKCA	protein receptor kinase C alpha
PTM	post-translational modification
R	right
R102Q	missense mutation of amino acid 102 from arginine to glutamine
RI	random interval
RIPA	radioimmunoprecipitation assay
RMTg	rostromedial tegmental nucleus
RR	random ratio
S-	negative visual stimulus
S-R	stimulus-response
s.c.	subcutaneous
S+	positive visual stimulus
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
siRNA	small interfering ribonucleic acid
SNc	substantia nigra pars compacta
US	unconditioned stimulus
VGCC	voltage-gated calcium channel
VILIP	visinin-like protein
VTA	ventral tegmental area
WT	wildtype

1 Introduction

1.1 Order of presentation in literature review

What are the neural substrates and molecules involved in cognition? What processes in the brain support learning and memory, motivated, goal-directed behaviours, and the ability to flexibly adapt to a changing environment? These are some of the big questions in behavioural and cognitive neuroscience. There are many molecules important for normal neuronal function and behaviour; two of these are calcium and dopamine. Calcium-related processes, particularly as studied in the hippocampus, have been linked to long-term synaptic plasticity and spatial learning and memory. Dopamine signalling in the striatum has been found to be important for motivated behaviours and is disrupted in various neuropsychiatric diseases. This thesis is focused on Neuronal Calcium Sensor-1 (NCS-1), a highly sensitive calcium-binding protein that also modulates dopamine signalling. Given its ability to modulate both calcium- and dopamine-related processes, I studied the effects of knocking out *Ncs-1* on mouse behaviour and neurophysiology. In this chapter, I review the literature on four topics. First, I outline the historical and conceptual background for the study of memory in behavioural neuroscience (1.2). Second, I describe what is known about calcium and calcium binding proteins in neurophysiology and cognition, with a focus on hippocampus-related processes (1.3). There I also introduce NCS-1 in the context of its family members and discuss its roles in brain and behaviour. Third I review what is known about the cognitive functions mediated by the striatal circuits, particularly those dependent on dopamine (1.4).

1.2 Different types of cognitive processes depend on different brain networks

1.2.1 A brief history of the study of memory

Serious attempts to localize cognitive functions in the brain began in the 19th century (see reviews by (Koob et al., 2010; Milner et al., 1998)). Franz Joseph Gall studied the cerebral cortex and

localized mental functions to different regions by examining the skulls of people who were particularly strong in certain faculties. Pierre Flourens tested Gall's ideas through lesion studies and concluded that any part of the cerebral hemisphere can fulfill all the functions of the hemisphere. However, Gall's ideas of functional localization seemed to be vindicated by Broca's and Wernicke's discovery of areas critical for language as well as the motor area by Fritsch and Hitzig. Nevertheless, the debate about localization continued in the 20th century to Karl Lashley, arguably the most prominent figure in American neuropsychology at the time. Lashley systematically lesioned cortical areas in rats and tested them on mazes in an attempt to determine where memories are stored (the engram). He concluded that the site of the cortical lesion is unimportant, suggesting that all areas of the cerebral cortex are equally important functionally (equipotentiality) (Lashley, 1950). Instead of a discrete location for memory, impairments correlated with the size of the cortical lesion (mass action).

Taking into account Lashley's findings as well as others, Donald Hebb proposed that memories are stored in a distributed manner in assemblies of interconnected cells (Hebb, 1949). In particular, he also theorized that repeated neuronal activity acts to strengthen its connections to other neurons and its ability to cause them to fire (the Hebbian synapse). One of Hebb's students, Brenda Milner, made pivotal discoveries in her studies with the famous patient H.M., who had a bilateral temporal lobectomy to relieve seizures (Scoville and Milner, 1957). As a result of the lesion, H.M. could not form new memories (of a specific type, discussed later) though his other cognitive faculties were remarkably intact.

Studies of H.M. highlighted the role of the hippocampus in memory formation. Since then, much research has focused on the role of the hippocampus with the support of two other landmark discoveries. Bliss and Lomo found that repeated stimulation of inputs to the hippocampus can lead to profoundly enhanced synaptic responses that last for hours (long-term potentiation, LTP) (Bliss and Lomo, 1973). Today such long-term plastic changes in synaptic connections are hypothesized to be the basic mechanism for memory formation. At the same time, John O'Keefe also discovered the existence of cells in the hippocampus that fire reliably to only particular areas of an environment (place cells), revealing a mechanism by which the hippocampus could

help mediate spatial navigation (O'Keefe and Dostrovsky, 1971).

Studies into the induction of hippocampal LTP highlighted the role of calcium signalling particularly through NMDA (N-methyl-D-aspartate) receptors (NMDAR) as well as calcium sensors like calmodulin and its binding partner calcium/camodulin dependent kinase II (CaMKII). Morris and his colleagues began to make correlations between pharmacological blockade of NMDA receptors, LTP and spatial memory formation. In the early 1990s, advances in mouse genetics allowed more direct manipulation of molecular biology and observation of correlations with physiology and behaviour. This began with Alcino Silva's knockout of αCaMKII, leading to deficits in spatial learning and LTP (Silva et al., 1992a; Silva et al., 1992b).

Since then many studies with specific and conditional knockouts and transgenics have confirmed a link between LTP and memory. In the era of molecular and cellular cognition, four general strategies have been used to link molecules with behaviour: negative and positive alterations, nonintervention, and integration (Lee and Silva, 2010). Negative alterations involve deleting or mutating the gene and seeing its effects on behaviour. Positive alterations involve overexpression or upregulation of a gene's functions, with the expectation that it will produce phenotypes opposite to negative alterations. Non-interventions ask whether the molecules' activity precedes the behaviour being studied. And finally integration involves seeing how the gene or molecule fits into known mechanistic pathways for the behaviour of interest, so that predictions can be made about its potential role. My thesis involves the behavioural analysis of mice null for *Ncs-1*, a calcium sensor implicated in learning, memory and synaptic plasticity from previous negative, positive and nonintervention experiments (discussed in 1.3.4).

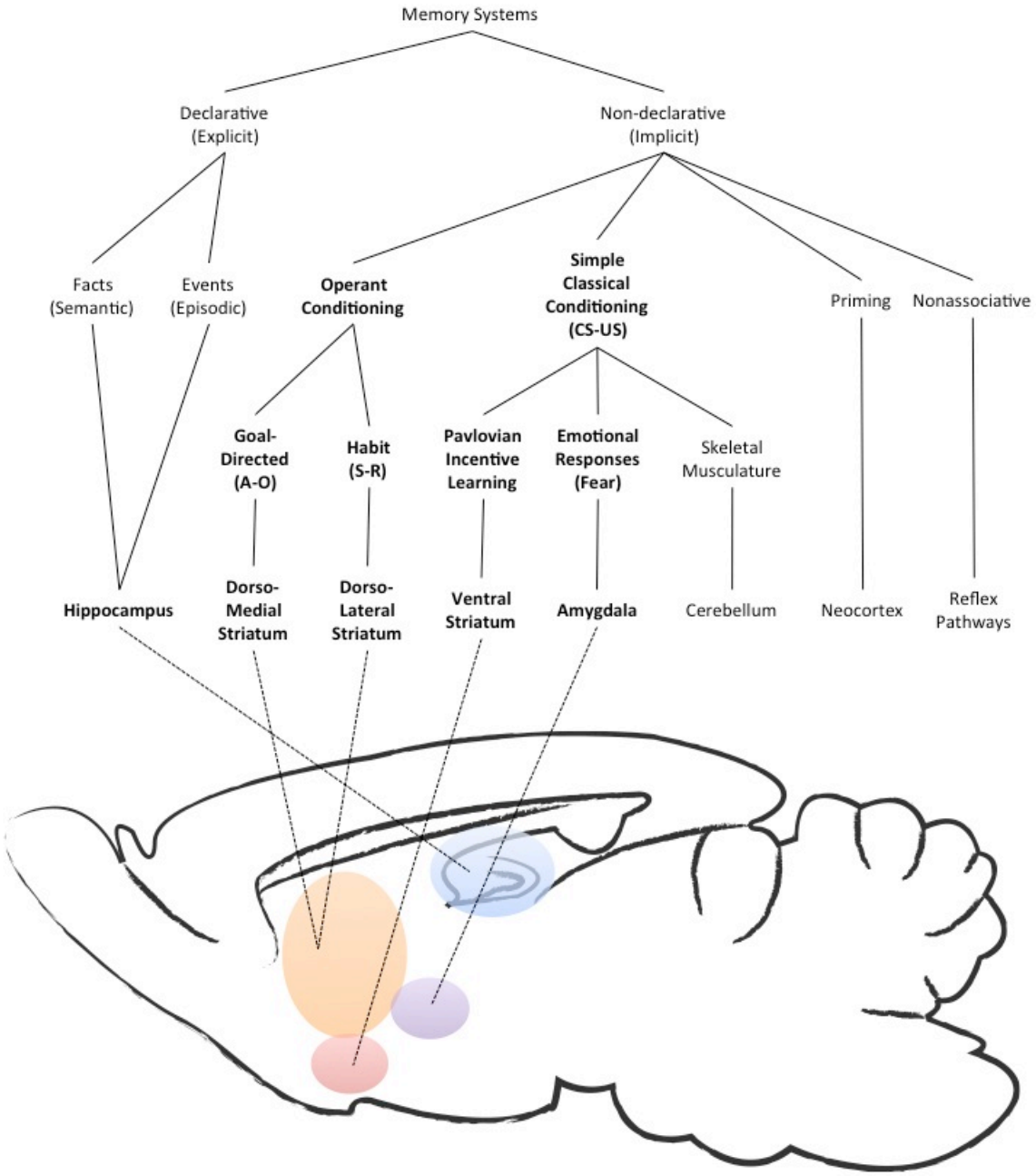

Figure 1-1. Multiple memory systems in the brain.
Adapted from (Squire and Zola, 1996). Different kinds of learning and memory involve different types of information processing and depend critically on different nodes in distributed networks of brain structures. A-O, action-outcome association. S-R, stimulus-response association. CS-US, conditioned stimulus – unconditioned stimulus association.

1.2.2 Different memory systems

While distinctions between different types of memory were not new, the impetus to find a biological (rather than only a philosophical or psychological) distinction between them began in earnest with the study of H.M (see review by (Squire, 2004)). While H.M. was impaired in remembering what he had just seen or heard just 30-40 s previously, he was able to learn a new motor skill requiring hand-eye coordination (mirror drawing) (Milner et al., 1968). Subsequent work from animal studies and other amnesic patients showing intact learning in a variety of domains led to the taxonomy of multiple memory systems. These memory systems operate in parallel and are distinguished by processing different types of information and having different brain regions which are thought to be particularly crucial for their formation (Figure 1-1) (McDonald and White, 1993; Squire and Zola, 1996).

The broadest distinction is between declarative and non-declarative memory. The declarative system is responsible for the conscious recollection of facts or events and involves learning associations between multiple stimuli or events. Declarative memories involve representations of the world, including spatial maps for navigation. The hippocampus and the temporal lobe is a critical region in the brain network underlying declarative memory formation. In contrast, non-declarative memories do not require conscious thought, are difficult to verbalize and are revealed by performance in the right situation. There are multiple types of non-declarative learning including procedural learning (such as the learning of movements in sport) and simple classical conditioning.

Procedural memory is expressed as skills and habits that allow one to automatically respond in a certain way when presented with a stimulus. Habits are formed by making stimulus-response (S-R) associations and depend on the dorsal striatum. The striatum is also critical for action-outcome (A-O) learning, where an animal learns about the consequences of its actions, for example, pressing a lever will result in food delivery. Both S-R and A-O learning are types of operant conditioning, in which associations are being made with an animal's actions (Yin et al.,

2008).

Finally, simple classical conditioning (Pavlovian learning) particularly of emotional memories involving threat depends critically on the amygdala. Here the association formed is between a conditioned stimulus (a tone or context) and an unconditioned stimulus (such as a footshock) (CS-US). In this way, the conditioned stimulus (CS), which was previously neutral will now evoke a response. For example, a previously neutral tone provokes freezing behaviour after it has been paired with a shock.

It is worth noting that the critical brain regions identified are not the only structures involved in these memory systems but rather are important hubs in their respective networks. For example, memory for threatening contexts engages not just the amygdala but the hippocampus as well. And certain Pavlovian processes such as learning to approach a stimulus associated with a food reward, or the ability for conditioned stimuli to impact operant responses engages the ventral striatum in addition to the amygdala. Moreover, brain structures such as the hippocampus, amygdala, and striatum are not just involved in learning and memory processes but mediate other behaviours as well. For example, while much research has focused on a role for the hippocampal formation in learning and memory since the studies on H.M., the hippocampus (particularly the ventral or anterior portion, in rodents and primates, respectively) is important in emotion and anxiety-related processes (Bannerman et al., 2014; Fanselow and Dong, 2010; Moser and Moser, 1998; Strange et al., 2014). Lesion studies suggest a functional dissociation between dorsal (or posterior) and ventral (or anterior) hippocampus, with the former mediating spatial cognition (Bannerman et al., 2003; Bannerman et al., 1999; Hock and Bunsey, 1998; Moser et al., 1993; Moser et al., 1995; Pothuizen et al., 2004) and the latter being involved in stress and emotional processes (Bannerman et al., 2002; Bannerman et al., 2003; Chudasama et al., 2008; Kjelstrup et al., 2002; Maren, 1999; McHugh et al., 2004; Richmond et al., 1999). The effects of chronic antidepressants may also depend on adult neurogenesis in the ventral hippocampus (O'Leary and Cryan, 2014; Perera et al., 2011; Sahay and Hen, 2007; Santarelli et al., 2003). For the purposes of my thesis, I focus on learning particularly dependent on the dorsal hippocampus and motivated behaviours thought to depend on the striatum, due to the ability for NCS-1 to interact

with calcium and dopamine signalling processes.

1.3 Calcium sensors, hippocampal plasticity, spatial learning

Changes in intracellular calcium concentration ([Ca^{2+}]) are a ubiquitous form of signalling used by cells. Calcium changes can be highly restricted in space and change dynamically over time and are categorized into waves, spikes, transients and puffs depending on their spatio-temporal features (Berridge et al., 2000). Calcium signalling is involved in nearly all aspects of cellular function including differentiation, proliferation, apoptosis and transcription (reviewed by (Berridge et al., 2000; Clapham, 2007)). Calcium signalling is similarly critical in neurons and neurophysiology, mediating functions across various time scales such as neurotransmission (<1 ms), short-term (>100 ms), long-term plasticity (10s of seconds to mins) and neurite and spine growth (hours to days) (reviewed by (Burgoyne, 2007)). The ability for changes in [Ca^{2+}] to mediate such a variety of functions in space and time depends in part on the many different proteins that detect different types of [Ca^{2+}] changes and interact with different downstream targets (Berridge et al., 2000; Burgoyne and Haynes, 2014). Increases in intracellular [Ca^{2+}] can come from: a) extracellular influx of Ca^{2+} for example through voltage-gated ion channels at the plasma membrane or b) the release of Ca^{2+} from intracellular stores, for example through inositol triphosphate receptors (IP_3Rs) at the endoplasmic reticulum. Below, I review the roles that calcium signals play in various neuronal functions, with a focus on those that affect the changes in brain connectivity thought to underlie learning and memory (1.3.1-2). I focus on extracellular influx of Ca^{2+} through ligand-gated ion channels, but release from intracellular Ca^{2+} stores play important roles as well, especially in synaptic plasticity (for reviews see (Baker et al., 2013; Maggio and Vlachos, 2014)). Next, I describe the various calcium binding proteins that transduce calcium signals in the brain, including neuronal calcium sensor-1 (NCS-1), which is the focus of my thesis (1.3.3). Finally, I describe the roles of NCS-1 in cognition, synaptic plasticity, the regulation of dopamine D2 receptors, and disease (1.3.4).

1.3.1 Calcium plays many roles in the nervous system

Fundamental aspects of neurophysiology include action-potential triggered neurotransmitter release across the synapse, short-term presynaptic plasticity, as well as long-term plasticity. All of these functions are regulated by calcium signalling and proteins that sense changes in [Ca^{2+}].

1.3.1.1 Neurotransmission

Action-potential evoked release of neurotransmitters is a primary means of quick information transfer between neurons, which depends critically on calcium. When the action potential travels across the axon to the presynaptic terminal, it causes membrane depolarization, causing voltage-gated calcium channels to open and allows transient Ca^{2+} influx. High local levels of Ca^{2+} are sensed by synaptotagmins that trigger fusion of the readily releasable pool of synaptic vesicles to the membrane and the release of neurotransmitters (Oheim et al., 2006; Südhof, 2004).

1.3.1.2 Short-term plasticity

Bursts of action potentials can lead to various changes in neurotransmission lasting tens of milliseconds to minutes; calcium and calcium sensors are again critical (reviewed in (de Jong and Fioravante, 2014; Regehr, 2012)). A common way to assess short-term plasticity is by giving a pair of electrical pulses that are closely spaced in time (paired-pulse stimulation) and assessing the ratio of response magnitude evoked by the second compared to the first pulse (paired-pulse ratio). In some conditions, the response evoked by the second pulse is smaller than that evoked by the first (paired-pulse depression; ratio < 1). In other cases, the second pulse will produce a larger response than that evoked by the first (paired-pulse facilitation; ratio > 1). The potential mechanisms for short-term plasticity are primarily presynaptic.

Short-term facilitation tends to occur at synapses with a low initial neurotransmitter probability when there is still some residual Ca^{2+} left in the presynaptic bouton as the second action potential arrives (Zucker and Regehr, 2002) (Figure 1-2 a). There is then a greater release probability with the second pulse. However, this does not explain the magnitude of facilitation seen in all synapses. Therefore other mechanisms have been proposed involving a high affinity, slow calcium sensor (a calcium sensor distinct from synaptotagmin-1) or a calcium sensor that enhances influx through voltage-gated calcium channels.

Various calcium-dependent mechanisms exist for short-term depression also. After bursts of activity, the readily releasable pool of synaptic vesicles can become depleted (Figure 1-2 b). Recovery from depression then, would depend on these vesicles being replenished, which takes several seconds and is dependent on calcium and certain calcium sensors. Another way depression could occur is via by calcium sensors down-regulating voltage-gated calcium channel function.

1.3.1.3 Long-term plasticity

Calcium and calcium sensors also mediate long-term changes in synaptic strength; here I briefly outline the major postsynaptic mechanisms for their induction. Long-term potentiation (LTP) and long-term depression (LTD) have been best characterized in their NMDAR-dependent forms in the Schaffer collateral pathway between CA3 and CA1 of the hippocampus (see Figure 1-3 for hippocampal anatomy) (LTP and LTD reviewed in (Lüscher and Malenka, 2015)).

Different patterns of activity induce NMDAR-dependent LTP and LTD. LTP requires pre- and postsynaptic neurons to be active together. This way, the depolarized state of the postsynaptic neuron removes the Mg^{2+} block of NMDAR, allowing presynaptic glutamate release to fully

activate NMDAR and allow large influxes of Ca^{2+}. In contrast, LTD involves repeated low frequency stimulation of the presynaptic neuron, leading to a smaller influx of Ca^{2+} through NMDAR, which allow a small amount of Ca^{2+} upon glutamate binding even under Mg^{2+} block. Therefore both LTP and LTD involve Ca^{2+} influx though at different levels (Figure 1-2 c-d).

The varying increases in Ca^{2+} levels are then transduced into different events by various effector molecules. In LTP, the large Ca^{2+} elevation[1] is detected by the calcium sensor calmodulin, leading to the activation of calcium/calmodulin-dependent kinase II (CaMKII). CaMKII then phosphorylates α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAR), increasing their ion channel conductance, and also facilitates the insertion of AMPAR into the postsynaptic membrane. In LTD, the modest Ca^{2+} increase is bound by calmodulin, leading to the activation of protein phosphatases such as calcineurin (protein phosphatase 2B, PP2B) and protein phosphatase 1 (PP1). These phosphatases dephosphorylate AMPAR and help trigger the internalization of AMPAR.

[1] Other factors in addition to differences in the amplitude of Ca^{2+} elevation likely contribute to the differential activation of kinases and phosphatases in LTP and LTD. This could include differences in the temporal dynamics of Ca^{2+} fluctuations resulting from different patterns of electrical stimulation.

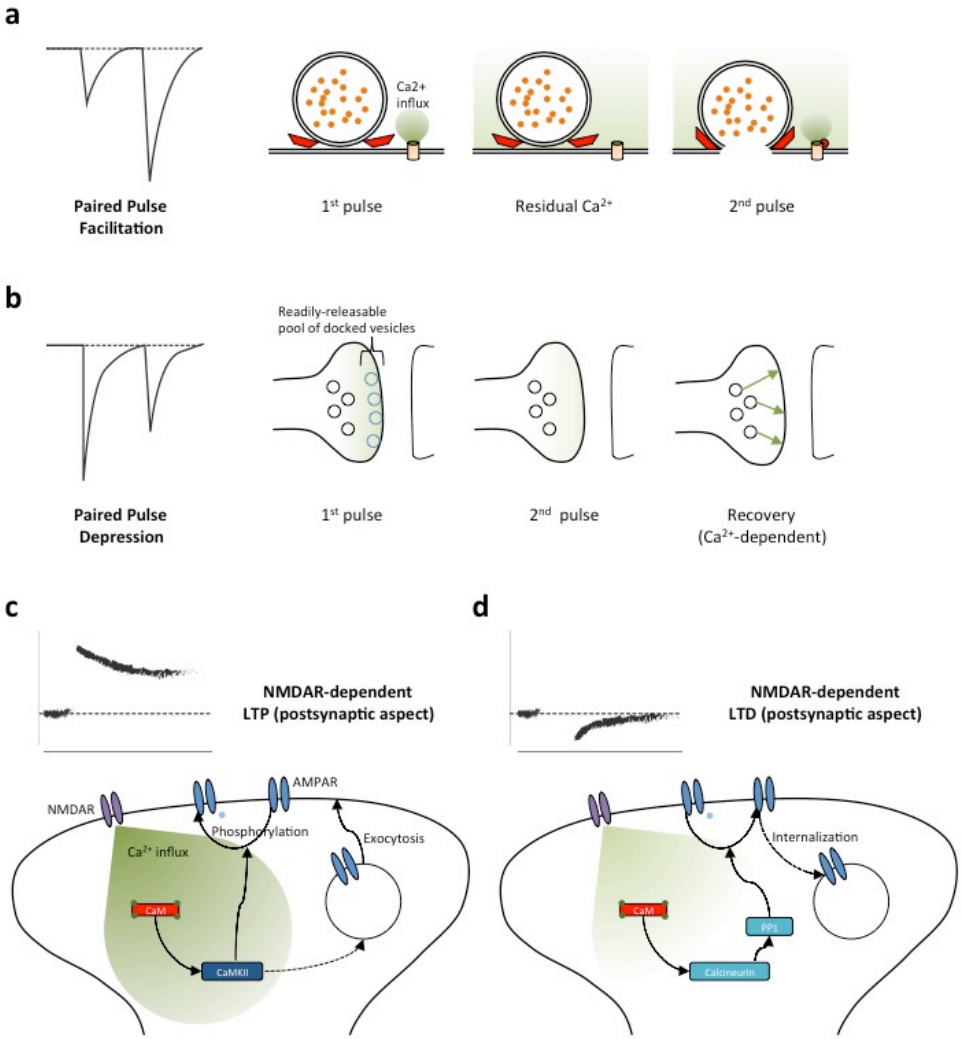

Figure 1-2. Calcium is involved in short-term and long-term plasticity.
(a) In paired-pulse facilitation, the second of a pair of pulses produces a larger response than the first. Transmitter release is calcium-dependent. Ca^{2+} influx occurs as membrane depolarization opens voltage-gated calcium channels. One mechanism for paired-pulse facilitation involves residual Ca^{2+} at the presynaptic terminal after the first pulse. The residual Ca^{2+} gets added to the Ca^{2+} influx triggered by the second pulse, leading to more transmitter release than the first pulse. **(b)** In paired-pulse depression, the second of a pair of pulses produces a smaller response than the first. One mechanism involves the depletion of a readily releasable pool of docked vesicles after the first pulse. Recovery from depression involves replenishing the readily releasable pool of vesicles with vesicles from other pools; this is a calcium-dependent process. **(c)** NMDAR-dependent LTP involves a large Ca^{2+} influx through NMDAR, which is detected by calmodulin (CaM) and leads to the activation of calcium/calmodulin dependent kinase II (CaMKII), which phosphorylates AMPAR and promotes the insertion of AMPAR. **(d)** NMDAR-dependent LTD involves a smaller Ca^{2+} influx through NMDAR, which is detected by calmodulin (CaM) and leads to the activation of phosphatases such as calcineurin (or protein-phosphatase 2B, PP2B) and protein-phosphatase 1 (PP1), which de-phosphorylate AMPAR and promote their internalization. **(a-b)** were adapted from (Xu-Friedman and Regehr, 2004).

There are many other molecules involved and other potential mechanisms underlying LTP and LTD; only the most widely accepted mechanisms were briefly outlined above. For example, a second major form of LTD is induced via metabotropic glutamate receptors (mGluR) using different signalling transduction pathways, although intracellular Ca^{2+} increases and calcium sensors are still involved (Collingridge et al., 2010). Stimulation of group I mGluRs leads to activation of phospholipase C and the formation of inositol triphosphate (IP_3), which then activate IP_3Rs at intracellular Ca^{2+} stores (which are physically linked to mGluRs by Homer), leading to the release of Ca^{2+} into the cytosol (Baker et al., 2013; Collingridge et al., 2010). Moreover, presynaptic changes can contribute to certain forms of long-term plasticity (Bliss and Collingridge, 2013). Therefore it is standard practice when testing for long-term plasticity to also make some rough measurements of presynaptic physiology such as baseline evoked neurotransmission (input/output curves) and short-term plasticity (paired-pulse stimulation).

Finally, synaptic plasticity also involves structural changes at the pre- and post-synaptic sites. Gene expression changes and local synthesis of new protein allows for the remodeling, addition and elimination of synapses (Bailey et al., 2015). For example, one type of ultrastructural remodeling that has been associated LTP induction is the formation of perforated synapses (Toni et al., 1999). Axospinous perforated synapses are characterized by aligned discontinuities or gaps in the presynaptic and postsynaptic densities (Nicholson and Geinisman, 2009). Often there is a small outgrowth from the spine head at the site of a postsynaptic density (PSD) discontinuity; this spinule grows into the presynaptic axon terminal (Nicholson and Geinisman, 2009). In addition to being larger, perforated synapses have many more AMPAR and NMDAR than nonperforated synapses (even after controlling for size) (Ganeshina et al., 2004a, b). The small spinules extending into the presynaptic side coupled with more AMPAR and NMDAR may strengthen the synapse by allowing more rapid elevations in intracellular Ca^{2+} in response to presynaptic firing than at non-perforated synapses.

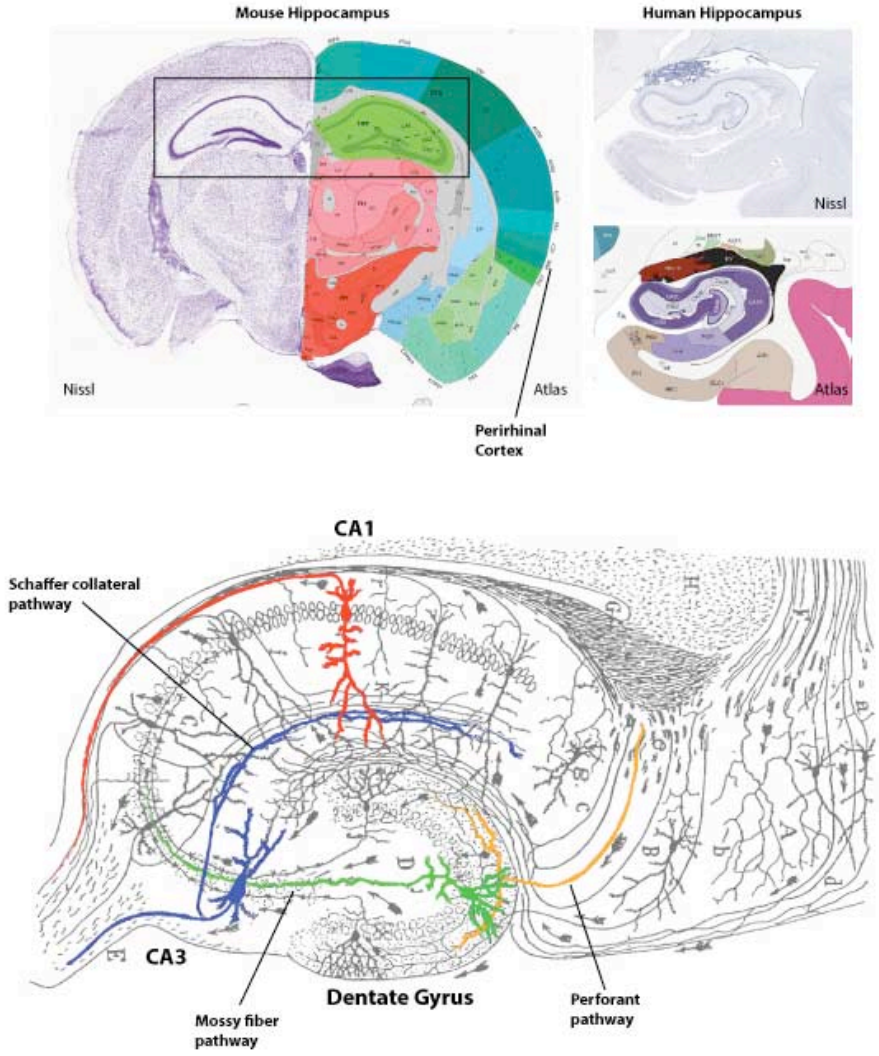

Figure 1-3. Anatomy of the hippocampus.
Top left image is a Nissl-stained coronal section from the Mouse Reference Atlas of the Allen Institute for Brain Science (Lein et al., 2007). Top right images are taken from the Human Brain Atlas of the Allen Institute for Brain Science, showing the hippocampus in a Nissl-stained section (Hawrylycz et al., 2012). Bottom panel shows the tri-synaptic hippocampal pathway as depicted by Cajal with cells in different hippocampal subregions highlighted (adapted from (Lüscher and Malenka, 2015)).

1.3.2 Calcium signalling in hippocampal long-term synaptic plasticity and memory

LTP and LTD have been heavily studied partly because they are thought to be the mechanisms that mediate information storage in learning and memory (Martin et al., 2000; Takeuchi et al., 2014). In particular, much work has focused on the link between hippocampal long-term plasticity and types of cognition mediated by the hippocampus and surrounding regions. Two tests commonly used to test declarative memory in rodents are the Morris water maze for spatial reference memory (1.3.2.1) and the spontaneous/displaced object recognition tests (1.3.2.2) (Clark and Martin, 2005). LTP has been associated with the former, whereas the latter has been associated with LTD though not necessarily in the hippocampus. LTD has also been associated with behavioural flexibility particularly in the form of spatial reversal (1.3.2.3). It is worth noting that while individual sets of experiments have seemed to associate different long-term synaptic changes with distinct types of learning and memory (as briefly described below), reality is likely more complex and the functions of such synaptic phenomena in behaviour may not be easily dissociable. Enhanced LTP does not necessarily result in improved memory (Jun et al., 1998; Uetani et al., 2000), nor does LTD necessarily improve forgetting. For example, LTD in the hippocampal CA1 region may be required for long-term spatial memory consolidation (Ge et al., 2010). Both LTP and LTD are likely mechanisms by which experiences can strengthen relevant synaptic connections while weakening or even eventually removing less relevant connections when forming memories. Moreover, LTP and LTD are important mechanisms for processes apart from spatial learning and memory, including experience-dependent plasticity during development and the formation of addictions to drugs of abuse (Malenka and Bear, 2004).

1.3.2.1 Spatial reference memory and LTP

The water maze is a standard test for allocentric spatial memory (memory based on distal cues)

and performance on this test can be modulated by manipulating molecules involved in LTP. In the water maze (Morris, 1984; Vorhees and Williams, 2006), a rodent is placed into a circular pool of water and needs to locate a platform hidden just below the water surface in order to escape (Figure 1-4 c-d). In the typical test for long-term spatial reference memory, the hidden platform is kept in the same location but the animal is released from a different starting point around the pool across daily trials. Use of different starting points encourages animals to use distal room cues to navigate rather than relying on egocentric strategies (when I am in this location then turn right or left etc.). Spatial learning is measured by the decline in latency or path length to find the hidden platform across trials and across days. Long-term spatial reference memory is typically measured by a probe test (typically 24 h) after the last training session. During the probe trial, the hidden platform is removed, and the memory is measured by the percentage time animals spend in the region of the pool (often the pool is divided into four quadrants) where the platform was located throughout training.

Pharmacological or genetic manipulations that prevent LTP induction also tend to impair water maze performance. Intracerebroventricular injections of AP5 (amino-5-phosphonopentanoic acid, an NMDAR antagonist) impair water maze learning at concentrations that also block LTP (Davis et al., 1992; Morris, 1989; Morris et al., 1986; Morris et al., 2013). However, such pharmacological findings are difficult to interpret as certain types of pre-training can rescue the ability of AP5 to impair water maze performance (Bannerman et al., 1995; Inglis et al., 2013). Subsequent experiments with genetic knockout mice have strengthened the link between LTP and spatial reference memory. Landmark studies by showed that knockout of αCaMKII impaired hippocampal LTP and spatial learning (Silva et al., 1992a; Silva et al., 1992b). Since then different mutations of αCaMKII and knockout mice of the different NMDAR subunits in specific brain regions have further solidified the link between LTP and spatial cognition (Lee and Silva, 2010; Silva, 2003).

1.3.2.2 Spontaneous/displaced object recognition and LTD

Novel (or spontaneous) object recognition and displaced object recognition (Figure 1-4 a-b) are commonly tested and have been associated with LTD in different brain regions. Both tests take advantage of the fact that rodents will prefer to investigate and explore novel stimuli (Aggleton, 1985). The tests are comprised of three phases: a sample (or familiarization), delay, and a test phase (see review by (Dere et al., 2007)). Rodents are first habituated to the empty arena, and then allowed to explore and familiarize themselves with some objects placed into the arena during the sample phase. They are then returned to their home cages for periods of 30 s to 24 h. In novel object recognition, one of the familiar objects from the sample phase is replaced with a novel object before the subject is returned to the arena for the test phase. Preferential exploration (nose within 2 cm) of the novel object over the familiar object is taken as an index of object recognition memory. In displaced object recognition, no new objects are introduced in the test phase but the spatial configuration of familiar objects is altered such that one object is in the same position but the other is moved to a new location. Spending a greater proportion of time exploring the displaced object over the stationary object is an index of object-location memory.

Lesion studies show that novel object recognition is primarily dependent on the perirhinal cortex and not on the hippocampus (Winters et al., 2004; Winters et al., 2008) especially for short delays, though there is still some debate over longer delays (Cohen and Stackman Jr., 2014). Object familiarity discrimination has been suggested to depend on perirhinal LTD particularly for long delays (24 h) (Brown and Bashir, 2002; Griffiths et al., 2008) but memory over short delays may depend on a different mechanism involving kainate receptors (Banks et al., 2012; Barker et al., 2006a; Barker et al., 2006b).

In contrast, lesion studies suggest displaced object recognition depends on the hippocampus particularly if encoded in an allocentric manner (Bussey et al., 2000; Good et al., 2007; Langston and Wood, 2010; Mumby et al., 2002; Save et al., 1992). Data from *in vivo* field recordings of freely moving rats as well as mice suggest a role for hippocampal LTD in displaced object

recognition; exploring shifts in the spatial configuration of objects facilitates endogenous LTD induction but attenuates LTP (Goh and Manahan-Vaughan, 2013a, b; Kemp and Manahan-Vaughan, 2008).

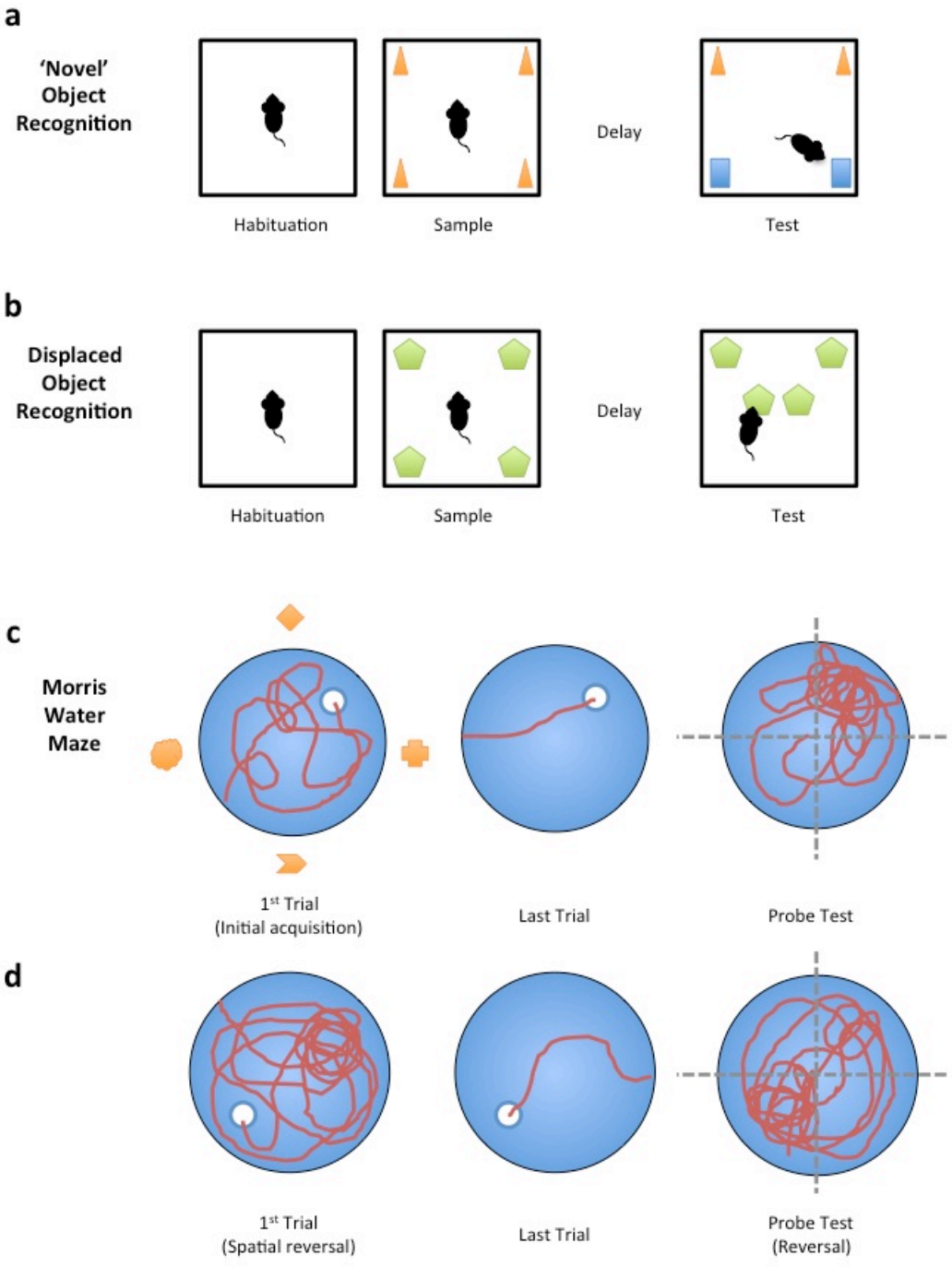

Figure 1-4. Common tests for declarative memory in the rodent.
(a) 'Novel' (or spontaneous) object recognition. Animals are habituated to an open field. They are then allowed to familiarize themselves with some objects in the sample phase. After a delay of seconds to hours, they are put back in the same environment to freely explore, but with some objects from the sample phase replaced by novel objects. Time spent exploring the novel objects relative to familiar objects is measured. **(b)** Displaced object recognition. The steps are similar to 'novel' object recognition, except in the test phase, no new objects are introduced. Rather, some of the familiar objects are displaced. Time spent exploring displaced objects relative to immobile objects is measured. **(c)** Initial acquisition in the Morris water maze. Path length to the hidden platform decreases with training. Spatial reference memory can be measured by path length within the target quadrant on a probe test 24 h after the last training trial. **(d)** Spatial reversal learning after initial acquisition. (Orange shapes represent extra-maze cues used by the animal to navigate in an allocentric manner; they are present throughout testing but I have only shown them in one of the images for simplicity's sake. Red tracings represent sample swim paths taken by the animal at different stages of training and testing. The white circle represents a platform hidden beneath the water surface.)

1.3.2.3 Behavioural flexibility and LTD

Recently, hippocampal LTD has also been linked to spatial reversal learning (Duffy et al., 2008; Kim et al., 2011; Mills et al., 2014; Morice et al., 2007; Nicholls et al., 2008). Spatial reversal learning can be tested in the water maze after an initial round of acquisition and probe testing shows animals have learned the location of the hidden platform. During reversal training, the location of the hidden platform is moved to the opposite quadrant (from where it was initially located). Changes in latency or path length during training and a final probe test are used as measures of flexibility. Animals with impaired spatial reversal learning would tend to perseverate and spend more time in the pool area where the platform was located during initial acquisition. It is worth noting though that spatial reversal is only one form of behavioural flexibility and dopamine signalling in cortico-striato-thalamic circuits is strongly implicated in flexibility (Floresco et al., 2009).

1.3.3 Calcium sensors

We have seen how calcium signalling is critical to fundamental aspects of neurophysiology including forms of long-term plasticity thought to underlie various forms of declarative memory. An obvious question arises as to how changes in [Ca^{2+}] can mediate so many different functions. Part of the answer lies in the existence of a large variety of calcium sensors that detect changes in calcium and initiate different biochemical cascades. Calcium sensors contribute to the specificity of calcium signals via their different tissue expression patterns, sub-cellular localizations, Ca^{2+}-binding affinities, and interactions with downstream proteins (Burgoyne and Haynes, 2012; Raghuram et al., 2012). Here I review a number of calcium sensors and the roles they play in neurophysiology and cognition. I focus on Neuronal Calcium Sensor-1 (NCS-1) and its family of proteins, as the role of NCS-1 in behaviour and physiology is the topic of my thesis.[2]

1.3.3.1 EF-hand proteins

The EF-hand is a common Ca^{2+}-binding motif which can bind a single calcium ion. Different EF-hands (even within the same protein) can vary considerably in their affinity for Ca^{2+} as well as the rate in which Ca^{2+} comes on and off (Burgoyne, 2007; Raghuram et al., 2012). The EF-hand super family of proteins include calcium buffers and calcium sensors. Calcium buffers have high affinity for Ca^{2+} but tend to display little change in protein conformation upon Ca^{2+} binding,

[2] It is important to note that while I focus on the role of Ca^{2+} and calcium binding proteins in neurons, calcium binding proteins expressed in astrocytes or glia can have important impacts on brain and behaviour. For example, the calcium binding protein S100 Beta, while only expressed in astrocytes, mediates LTP/LTD-related processes and affects hippocampal-dependent behavioural tasks. Gerlai, R., Wojtowicz, J.M., Marks, a., and Roder, J. (1995). Overexpression of a calcium-binding protein, S100 beta, in astrocytes alters synaptic plasticity and impairs spatial learning in transgenic mice. Learning & memory (Cold Spring Harbor, NY) 2, 26-39.

and are thought to primarily chelate Ca^{2+} though they may have other functions (e.g. calbindin, parvalbumin). Calcium sensors are characterized by the ability to bind and release Ca^{2+} over a physiological range of [Ca^{2+}] and have a Ca^{2+}-dependent change in protein conformation, which modulates their ability to bind target proteins (Burgoyne, 2007). The best studied EF-hand calcium sensor is calmodulin, which is ubiquitously expressed. Other EF-hand calcium sensors have more restricted expressions and have been categorized into two major families: the neuronal calcium sensors (NCS) and calcium-binding proteins/calneurons (CaBP) (Burgoyne, 2007; Haeseleer et al., 2002).

1.3.3.2 Calmodulin

Calmodulin has 4-EF hand motifs (Chattopadhyaya et al., 1992), two in a C-terminal domain and two in an N-terminal domain with a highly flexible linker region between the two domains, forming a dumb-bell shape (Figure 1-7). Calmodulin is highly expressed in the brain (estimates of up to ~100 uM) and its flexible dumb-bell shape allows it to interact with many target proteins with more than 300 interactions described (Junker and Rief, 2009). Some of its functions include the regulation of glutamate receptors (O'Connor et al., 1999), ion channels (Saimi and Kung, 2002), and being involved in the induction and maintenance of long-term synaptic plasticity through its target proteins (Xia and Storm, 2005). For example, the calmodulin binding protein, CaMKII is involved in the phosphorylation of AMPAR in LTP (Barria et al., 1997) and CREB, a key nuclear transcription factor in memory (Deisseroth et al., 1998).

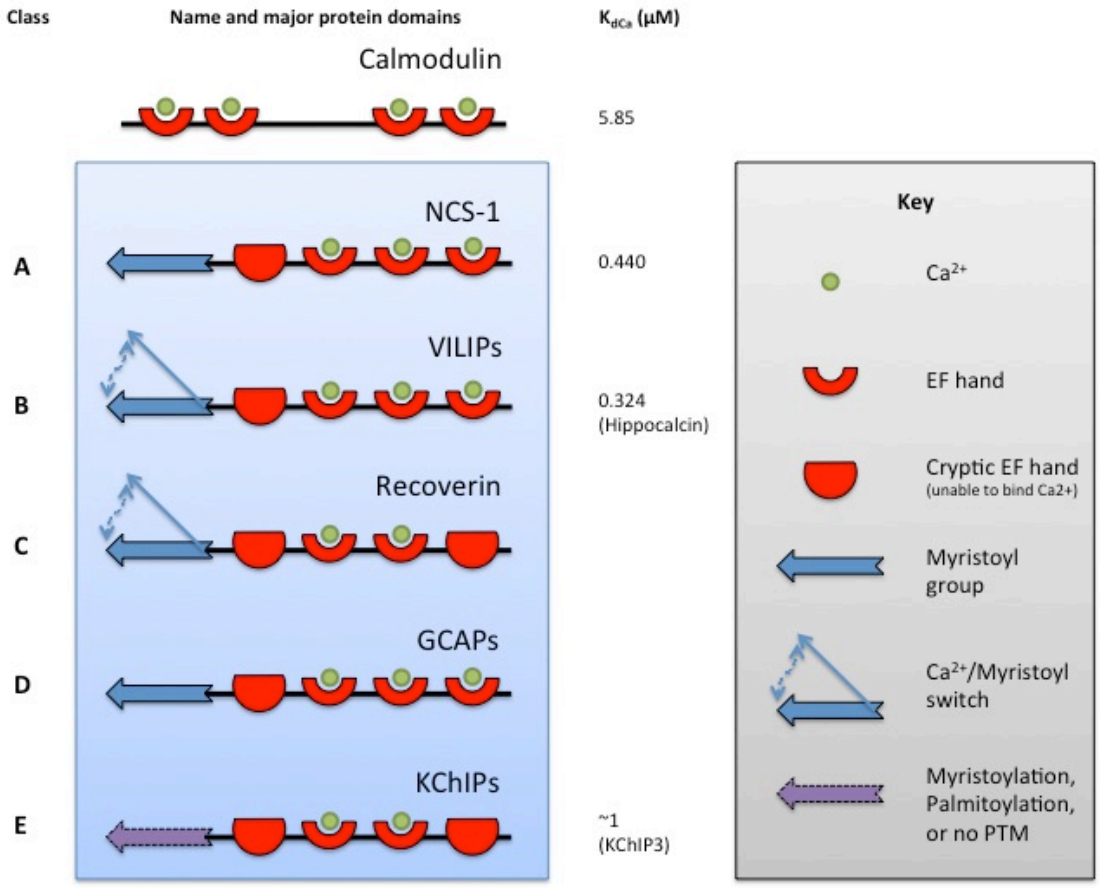

Figure 1-5. Structural motifs in the neuronal calcium sensor family of EF-hand proteins. Adapted from (Raghuram et al., 2012). Numbers for the average Ca^{2+} affinity (dissociation constants) for the EF-hands of selected proteins also from (Raghuram et al., 2012). PTM, post-translational modification.

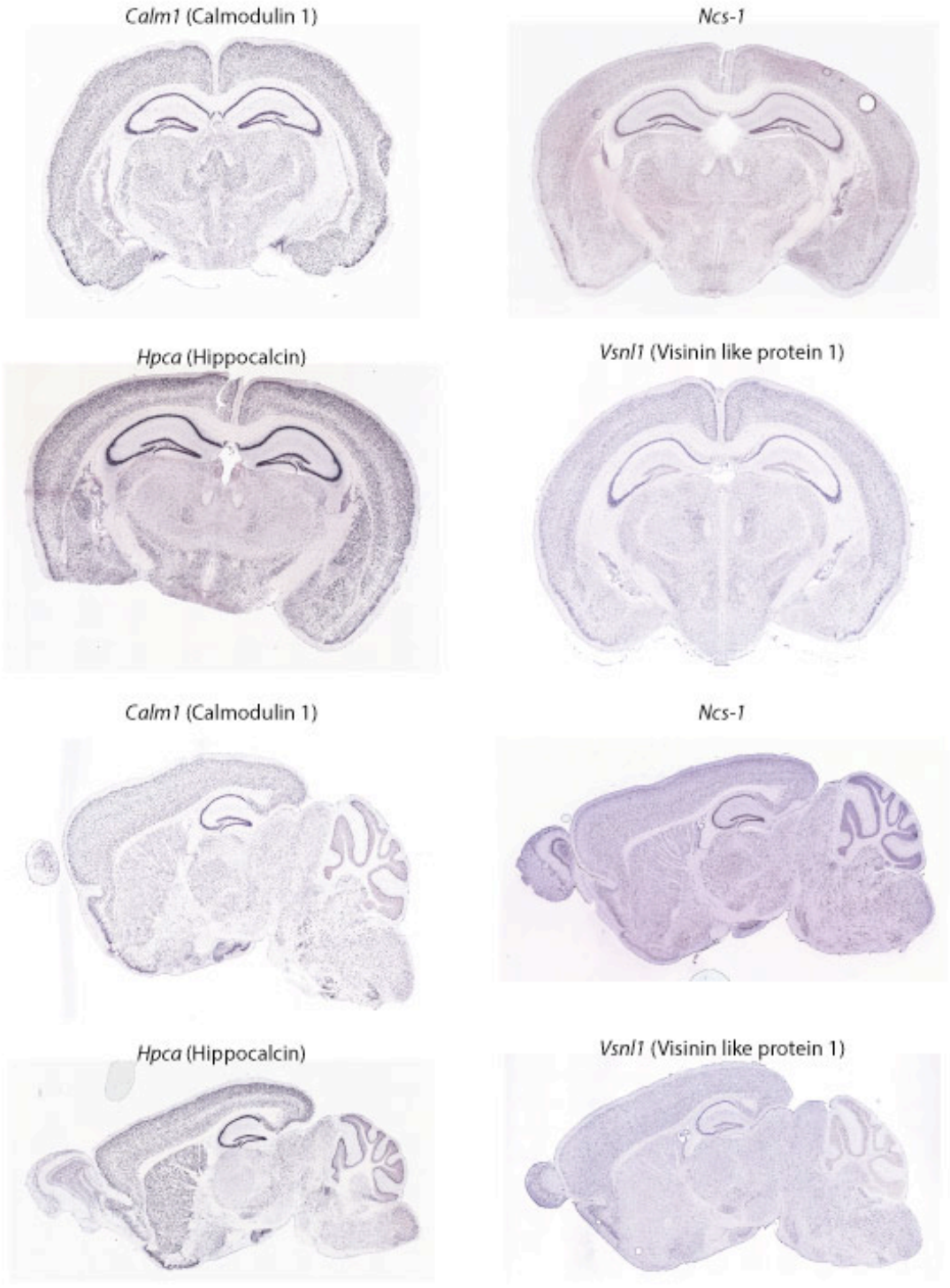

Figure 1-6. mRNA expression of various EF-hand proteins in mouse brain.
Images courtesy of the Allen Mouse Brain Atlas (Lein et al., 2007).

1.3.3.3 Neuronal calcium sensor proteins

The NCS family members all have 4-EF hands and have <20% sequence similarity to calmodulin (Burgoyne et al., 2004). They are expressed at lower levels than calmodulin (estimates of ~1-10 uM with hippocalcin slightly higher; (Raghuram et al., 2012)) and have more restricted expression profiles (Paterlini et al., 2000). They are structurally distinct from calmodulin in at least three ways. First, only two or three of their EF-hands are able to bind Ca^{2+} (Figure 1-5). Second, they have a globular protein structure that changes conformation less than calmodulin (Ames et al., 2006; Ames et al., 2012) and the NCS family tends to have a more restricted and specific set of target proteins. Third, NCS family members tend to have membrane-association motifs: either N-terminal myristoylation or palmitoylation. In some cases, there is a Ca^{2+}/myristoyl switch, where the myristoyl group becomes exposed only upon Ca^{2+} binding, such that the protein is cytosolic at low [Ca^{2+}] and translocates to membranes under higher [Ca^{2+}] (Ames et al., 1997; O'Callaghan et al., 2002). The NCS family of proteins have been divided into five classes (A-E) based on their amino acid sequence and their conservation across species (Burgoyne, 2007). Here I give a brief description of each class before returning to describe in more detail the ways Neuronal Calcium Sensor-1 (NCS-1) has been implicated in cognition, synaptic plasticity, and disease.

1.3.3.3.1 Class A: Neuronal calcium sensor-1 (NCS-1)

NCS-1 and homologous proteins have been found in yeast, worms, flies, rodents, and humans. Its protein sequence is fully conserved among mammals and there is 60% conservation between yeast and humans (Bourne et al., 2001). NCS-1 is widely expressed in the nervous system (Figure 1-6) (Chen et al., 2002; Jinno et al., 2002; Martone et al., 1999; Pongs et al., 1993) but is also found in tissues like the heart (Nakamura et al., 2011; Nakamura and Wakabayashi, 2012). Three of its EF-hands are functional and its N-terminal myristoyl domain is exposed allowing it to associate with membranes independently of Ca^{2+} (O'Callaghan et al., 2002) though it can

shuttle back and forth between the plasma membrane and cytosol (Handley et al., 2010). It has both Ca^{2+}-dependent and independent interactions with numerous target proteins (Burgoyne and Haynes, 2014; Haynes et al., 2006; Petko et al., 2009) and some of these targets are shared with calmodulin and other calcium sensors (McCue et al., 2010; Schaad et al., 1996) (Figure 1-8). Its most well studied interactions with established physiological significance are with Ca^{2+} channels and the dopamine D2 receptor. NCS-1 has a relatively high Ca^{2+} affinity (Aravind et al., 2008) (greater than calmodulin) so it may interact with certain binding partners when Ca^{2+} is lower than the threshold of activation for other calcium sensors.

NCS-1 was first identified as *Frequenin* in a Drosophila mutant where its up-regulation led to frequency-dependent facilitation of neurotransmitter release (Pongs et al., 1993). Since then NCS-1 has been implicated in many presynaptic and postsynaptic functions (see (Dason et al., 2012) for review). Presynaptically, there is evidence that NCS-1 enhances basal neurotransmitter release (Dason et al., 2009; Olafsson et al., 1997; Romero-Pozuelo et al., 2007; Saab, 2010; Wang et al., 2001) and that it modulates short-term plasticity with reports of it enhancing (Mallart et al., 1991; Pongs et al., 1993; Rivosecchi et al., 1994; Sippy et al., 2003) paired-pulse ratios in some studies and reductions in others (Dason et al., 2009; Romero-Pozuelo et al., 2007; Wang et al., 2001). Its presynaptic effects are mostly attributed to its ability to bind and regulate various Ca^{2+} channels (Dason et al., 2009; Hui et al., 2007; Hui and Feng, 2008; Romero-Pozuelo et al., 2007; Rousset et al., 2003; Tsujimoto et al., 2002; Wang et al., 2001; Weiss et al., 2000; Weiss and Burgoyne, 2001). Postsynaptically, it has been shown to be involved in long-term plasticity (Saab et al., 2009) and the regulation of G-protein coupled receptors (GPCRs) such as dopamine D2 receptors (DRD2)[3] (Kabbani et al., 2002), adenosine A_{2A} receptors (Navarro et al., 2012), and DRD2-A_{2A} heteromers (Navarro et al., 2014). Other functions that have been attributed to NCS-1 include neurite outgrowth regulation (see review by (Weiss et al., 2010)), neuroprotection, neuronal regeneration (Nakamura et al., 2006; Yip et al., 2010) and cardiac development (Guo et al., 2002; Nakamura et al., 2003).

[3] NCS-1 can, however, also interact with presynaptic D2 autoreceptors (DRD2 short isoform).

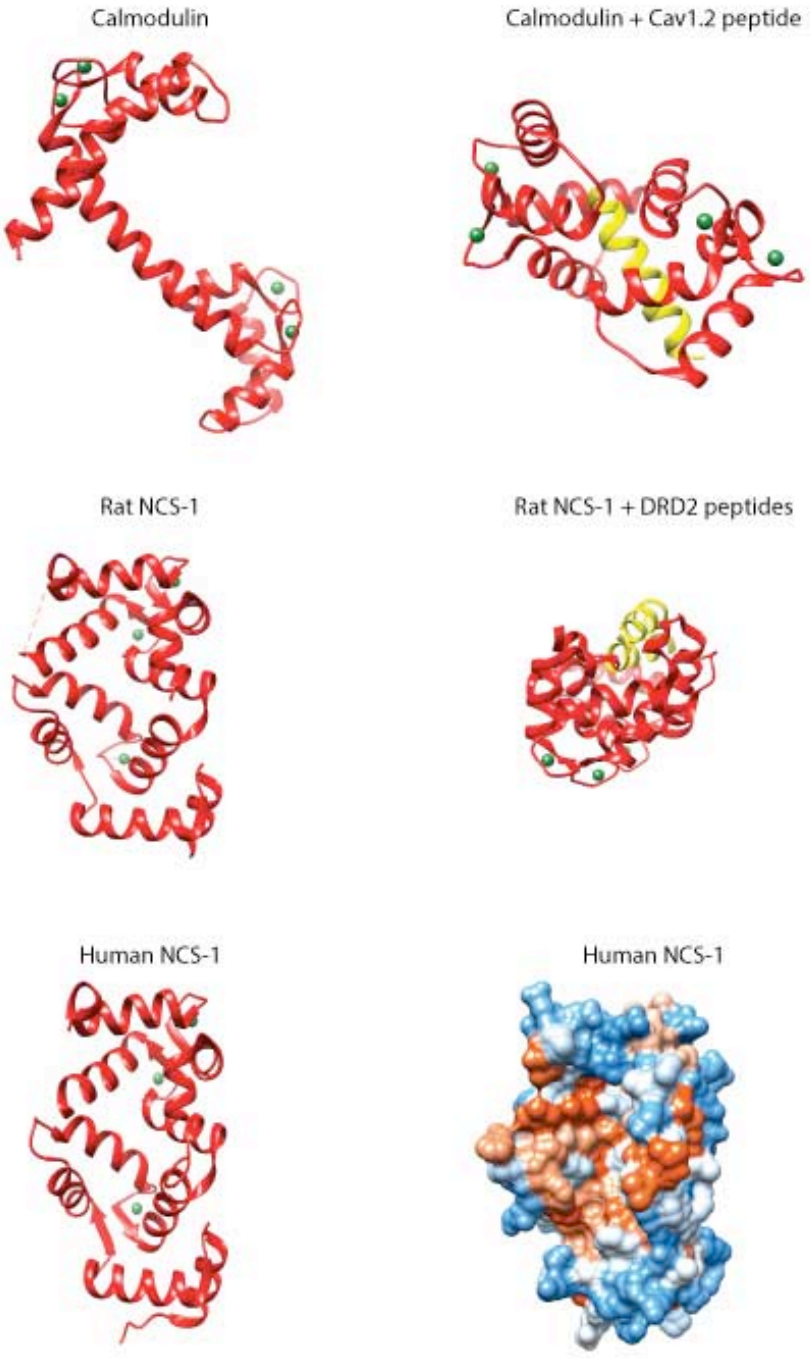

Figure 1-7. Structures of calmodulin and NCS-1.
Top row: Ca^{2+}-bound calmodulin (green spheres represent Ca^{2+}); note it has an N- and C-terminal EF-hand Ca^{2+} binding domains separated by a long flexible linker region (PDB 1CLL). The flexible linker region allows calmodulin to undergo large changes in conformation upon binding to its targets such as the IQ-like domain of the $Ca_v1.2$ calcium channel (target peptide is in yellow) (PDB 2F3Z).
Middle row: Ca^{2+}-bound rat NCS-1; note it only has 3 functional EF-hands (PDB 5AEQ). Ca^{2+}-

bound rat NCS-1 complexed with a human dopamine D2 receptor peptide fragment (PDB 5AER).
Bottom row: Ca^{2+}-bound human NCS-1 (PDB 1G8I). Surface hydrophobicity of human NCS-1. Images were obtained by loading PDB files into UCSF Chimera (Pettersen et al., 2004).

1.3.3.3.2 Class B: Visinin-like proteins (VILIPs)

The most studied member of the VILIPs is hippocalcin, which is most highly expressed in hippocampal neurons, though it is also expressed in other brain regions (Figure 1-6) (Paterlini et al., 2000). Hippocalcin has a Ca^{2+}/myristoyl switch, which allows it to translocate to the membrane with increases in [Ca^{2+}] (O'Callaghan and Burgoyne, 2003; O'Callaghan et al., 2002) from NMDAR (Dovgan et al., 2010). Indeed, hippocalcin has been implicated in NMDAR-LTD (Jo et al., 2008; Palmer et al., 2005) as well as LTD mediated by muscarinic receptor activation (Jo et al., 2010) by regulating AMPAR endocytosis through an interaction with AP2 (adaptor protein 2). Hippocalcin knockout mice show deficits in the probe test of the Morris water maze and decreased phospho-CREB responses to NMDA stimulation (Kobayashi et al., 2005). These mice were also impaired in an operant visual discrimination task. The other VILIPs have been less studied but have been implicated in regulating various calcium channels (Lautermilch et al., 2005), acetylcholine (Lin et al., 2002; Zhao et al., 2009) and purinergic receptors (Chaumont et al., 2008).

1.3.3.3.3 Classes C and D: Recoverins and Guanylyl-cyclase activating proteins (GCAPs)

Recoverins and GCAPs are both primarily expressed in the retina. Recoverin regulates light sensitivity by inhibiting retinal rhodopsin kinase in a Ca^{2+}-dependent manner (Chen et al., 2010; Klenchin et al., 1995; Komolov et al., 2009; Makino et al., 2004). The phosphorylation state of rhodopsin controls the light response in photoreceptors (Mendez et al., 2000). GCAPs are involved in light adaptation. They activate retinal guanylyl cyclases under low Ca^{2+} and inhibit them under high Ca^{2+} concentrations (Dizhoor and Hurley, 1996; Dizhoor et al., 1995; Gorczyca

et al., 1995). Knockout mice of GCAP1 and 2 show they are both required for full function, probably because they have differing affinities for Ca^{2+} (Koch, 2006; Mendez et al., 2001). This provides a good example of how a variety of calcium sensors may be needed to respond to the wide range of physiological Ca^{2+} concentrations to mediate physiological functions.

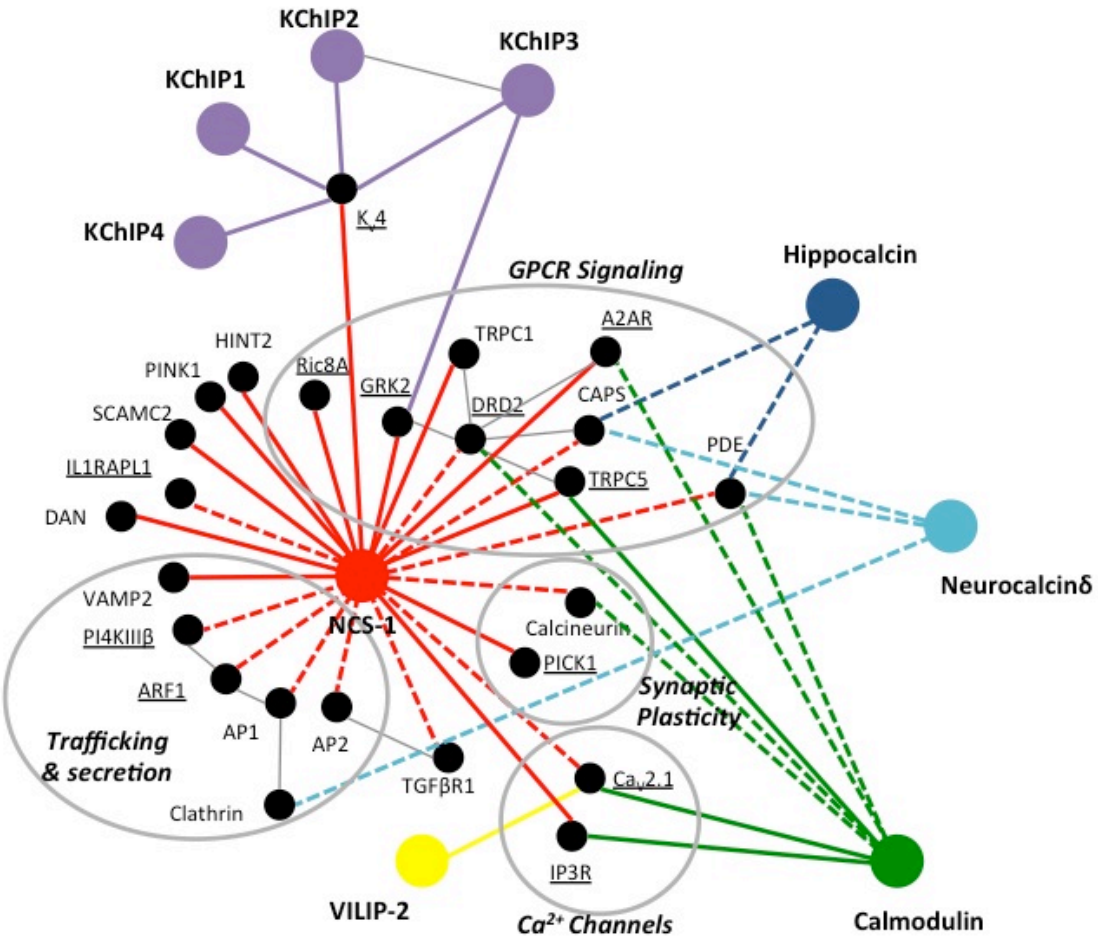

Figure 1-8. NCS-1 interactome.
NCS-1 interacts with many target proteins, some of which are shared with other calcium sensors, in both Ca^{2+}-dependent (dotted lines) and Ca^{2+}-independent manners (solid lines). Figure adapted from (McCue et al., 2010) and (Petko et al., 2009).

1.3.3.3.4 Class E: K+ channel interacting proteins (KChIPs)

KChIPs were named for their ability to interact with K_v4 potassium channels (An et al., 2000). The best studied is KChIP3 which is also known by the names calsenilin for its ability to interact

with presenilins (Buxbaum et al., 1998; Zaidi et al., 2002) and DREAM (downstream regulatory element (DRE) antagonist modulator) because it modulates gene transcription through DRE (Carrion et al., 1999; Cheng et al., 2002; Osawa et al., 2001). KChIP3 knockout mice show hypoalgesia (Cheng et al., 2002; Lilliehook et al., 2003), enhanced learning and memory including better contextual fear memory (Alexander et al., 2009), object recognition, classical conditioning and operant conditioning (Fontán-Lozano et al., 2009), as well as enhanced LTP (Fontán-Lozano et al., 2009; Lilliehook et al., 2003). Transgenic overexpression of a Ca^{2+}-insensitive KChIP3 mutant impaired NMDAR-mediated synaptic transmission, reduced LTD and impaired contextual fear memory (Wu et al., 2010). What use is there for KChIP3 then if eliminating it seems to enhance cognition? While decreased KChIP3 enhances NMDAR-mediated currents important to plasticity, it can also increase NMDAR-mediated excitotoxicity. Indeed knockdown of KChIP3 worsens NMDA-induced cell death in hippocampal cultures, whereas overexpression of KChIP3 into cell lines decreases measures of cell death in response to NMDA or oxygen-glucose deprivation (Zhang et al., 2010). Therefore calcium-dependent KChIP3 suppression of NMDAR function may have an important function in negative feedback regulation of NMDAR-mediated Ca^{2+} influx, thus preventing excitotoxicity (Wang and Wang, 2012).

1.3.3.4 Calcium affinity, kinetics and localization

Overexpression and knockout studies in various organisms show members of the NCS family have multiple non-redundant roles in the nervous system (Burgoyne and Haynes, 2012; McCue et al., 2010). While they are all calcium sensors, they have specific functional niches based on their affinity for Ca^{2+}, the kinetics of Ca^{2+} association and dissociation, their tissue expression and sub-cellular localization, and their effect on target proteins (see review by (Raghuram et al., 2012)). For example, the N-terminal EF-hands of calmodulin have a very fast binding capacity, but relatively low affinity for Ca^{2+} (Faas et al., 2007). This makes calmodulin well-suited to respond to fast and large changes in Ca^{2+}, such as near the openings of voltage-gated calcium channels (Raghuram et al., 2012). In contrast, NCS proteins like NCS-1 and hippocalcin have about 10-fold higher affinity for Ca^{2+} than calmodulin (Aravind et al., 2008; O'Callaghan et al.,

2003) which makes them sensitive to more gradual and longer-lasting changes in Ca^{2+} such as in NMDAR- or mGluR-LTD induction. Furthermore, even though many target proteins are shared by different calcium sensors, they can have different complex modulatory effects (Burgoyne and Haynes, 2014). The $Ca_v2.1$ (P/Q type) calcium channel, which conducts Ca^{2+} currents for the initiation of synaptic transmission is itself regulated by Ca^{2+}, via calcium sensor proteins. Calmodulin mediates calcium-dependent facilitation of the channel when small Ca^{2+} influxes through individual channels selectively activate its C-terminal lobe, whereas larger influx through multiple channels activates its N-terminal lobe, leading to calcium-dependent inactivation of the channel (Ben-Johny and Yue, 2014; DeMaria et al., 2001; Lee et al., 2003). CaBP1, VILIP-2 and NCS-1 also regulate this channel. For example, VILIP-2 inhibits calmodulin-dependent inactivation and enhances facilitation (Lautermilch et al., 2005; Nanou et al., 2012) and NCS-1 can enhance facilitation (Tsujimoto et al., 2002; Yan et al., 2014). However, CaBP1 tends to inactivate and decrease facilitation (Lee et al., 2002).

1.3.3.5 NCS-1 expression

NCS-1 is widely expressed in the nervous system of flies (Pongs et al., 1993), rats, (Martone et al., 1999; Paterlini et al., 2000), mice (Jinno et al., 2002; Olafsson et al., 1997) as well as humans (Chen et al., 2002). NCS-1 has also been found in non-neural tissues such as the heart, kidneys, testes and lungs (Gierke et al., 2004; Nakamura et al., 2011; Nakamura et al., 2003). NCS-1 has been found in neurons and astrocytes, but not oligodendrocytes or Schwann cells (Olafsson et al., 1997). NCS-1 expression can differ between cell types and regions. For example, NCS-1 is expressed in the Purkinje cells of the cerebellum but less so in the granule cell layer (Olafsson et al., 1997). In crayfish and frog presynaptic motor neuron terminals, NCS-1 homologues are found in much higher levels in phasic neurons than tonic neurons (Belair et al., 2005; Jeromin et al., 1999). Below I review studies of NCS-1 expression in rodents with particular focus on the hippocampus and striatum.

Three studies have studied the localization of NCS-1 in rat brain, identifying strong labeling in

dendrites and dendritic spines. Using immunolabeling to identify cells co-expressing NCS-1, nitric oxide synthase and calcineurin, Schaad et al, found NCS-1 in various cell types including hippocampal CA1 pyramidal neurons, striatal medium spiny neurons, and the axons of cerebellar basket cells (Schaad et al., 1996). Using immunohistochemistry, Martone et al found NCS-1 throughout the brain including the olfactory bulb and cortex, cerebral cortex, striatum, hippocampus, midbrain, cerebellar cortex, brainstem (Martone et al., 1999). The most intensely labeled regions were myelinated axons, the axons of cerebellar basket cells, large neurons in the brainstem. NCS-1 was strongly labeled in structures full of neurofilament protein, suggesting potential interaction with the cytoskeleton. Strong NCS-1 immunoreactivity was also found in the trans- Golgi network, suggesting a role in trafficking protein in the Golgi apparatus. Paterlini et al, used in situ hybridization to compare the expression of *Ncs-1* mRNA with the mRNA for several other neuronal calcium sensor proteins (hippocalcin, VILIP-1, 2, 3) (Paterlini et al., 2000). *Ncs-1* mRNA was found throughout the brain, including caudate putamen, nucleus accumbens, and the dendrites of hippocampal pyramidal and granule cells. In both the striatum and hippocampus, *Hpca* mRNA was more highly expressed than *Ncs-1*. However, *Ncs-1* expression is unique in hippocampal pyramidal cell in extending all the way out to the dendritic ends (whereas *Hpca* is found only at the proximal dendrite). This expression pattern suggests potential involvement in postsynaptic plastic changes in dendritic spines and locally regulated protein synthesis (Paterlini et al., 2000).

Two studies have investigated NCS-1 expression in the mouse brain. Olafsson et al, 1996 using immunoblotting detected NCS-1 as early as embryonic day 12 with gradual increases in protein levels during development. In the adult brain, the hippocampus showed the highest levels of NCS-1, with levels being similar in most other brain regions. In situ hybridization identified NCS-1 in most regions of the hippocampus including CA3 and CA1 pyramidal cells, dentate granule cells, and astrocytes (Olafsson et al., 1997). Immunohistochemistry showed staining primarily in the stratum radiatum and moleculare but not so much in pyramidal layer, suggesting that subcellularly NCS-1 is preferentially localized to neurites (Olafsson et al., 1997). Double-labeling experiments in primary hippocampal cell cultures revealed NCS-1 co-localization with MAP-2 (microtubule-associated protein 2, a marker for dendrites) and SV2 (synaptic vesicle protein 2, a marker for synapses) (Olafsson et al., 1997). Using a different antibody, Jinno et al

further characterized NCS-1 expression in the hippocampus (Jinno et al., 2002). Similar to previous studies, NCS-1 immunoreactivity was mostly in hippocampal dendritic layers and less so in principal cell layers. The most intensely labeled regions were the hilus of the dentate gyrus and the stratum lucidum of the CA3. Intense staining of hippocampal mossy fiber bundles and terminals (originating from dentate granule cells) suggest NCS-1 may play a role in regulating glutamate transmission from mossy fiber terminals and information transfer from the dentate gyrus to the CA3 (Jinno et al., 2002). Co-staining with synaptophysin (a marker for synapses) also revealed a complementary distribution pattern between synaptophysin-positive presynaptic terminals and NCS-1 immunoreactive dendrites in the stratum lacunosum-moleculare of CA3 and CA1 and the dentate molecular layer. This complementary distribution pattern suggests, NCS-1 may play a role in the transfer of information between the entorhinal cortex and hippocampus (Jinno et al., 2002).

1.3.4 NCS-1, cognition and plasticity

1.3.4.1 NCS-1 in learning and memory

Genetic manipulations in *C. elegans* and mice have demonstrated a role for NCS-1 in learning and memory. Knockout of *Ncs-1* in *C. elegans* impairs associative learning and memory while re-introducing NCS-1 to a specific interneuron was sufficient to rescue the effect (Gomez et al., 2001). Calcium signalling appears critical to this process, as re-introducing a mutant form of NCS-1 that cannot bind Ca^{2+} could not rescue the knockout animals (Gomez et al., 2001). On the other hand, over-expression of *Ncs-1* in *C. elegans* accelerated learning and enhanced memory (Gomez et al., 2001).

Similarly, in mice, over-expression of *Ncs-1* is associated with cognitive enhancement whereas knockout leads to impairments. A 40% increase of NCS-1 in the dentate gyrus led to increased spatial exploratory behaviour, accelerated spatial learning, and enhanced memory that was

dependent on the interaction between NCS-1 and the dopamine receptor D2 (DRD2) (Saab et al., 2009). Knockout of *Ncs-1* in mice led to decreased exploratory behaviour and impaired object recognition (de Rezende et al., 2014).

1.3.4.2 NCS-1 in hippocampal long-term plasticity

Long-term synaptic plasticity within the hippocampus is thought to be the mechanism for spatial learning and memory. NCS-1 has been shown to be involved in both long-term potentiation (LTP) and long-term depression (LTD) in the hippocampus (Figure 1-9). *In vivo* induction of LTP by electrical stimulation (Génin et al., 2001) or by chemical means (Brackmann et al., 2004) lead to increases in NCS-1 expression. Increasing *Ncs-1* expression in murine dentate gyrus was sufficient to both decrease the threshold for LTP induction and enhance the level of potentiation in the medial perforant path (Saab et al., 2009). Inhibiting the NCS-1 interaction with DRD2 abolished this enhancement and could also impair LTP in wild-type mice (Saab et al., 2009). Both NCS-1 and its interaction with PICK1 (protein interacting with protein kinase C, alpha 1) are necessary for metabotropic glutamate receptor (mGluR)-mediated LTD in rat perirhinal cortex but not for NMDAR-mediated LTD (Jo et al., 2008).

1.3.4.3 NCS-1 and the D2 Dopamine Receptor

While NCS-1 interacts with many proteins, its interaction with the dopamine receptor D2 (DRD2) has received much attention, as dopamine signalling is important in learning and in neuropathological processes. NCS-1 and DRD2 have been co-localized both presynaptically and postsynaptically in rat and primate brain (Kabbani et al., 2002; Negyessy and Goldman-Rakic, 2005). Initial deletion mapping suggests that the C-terminus of DRD2 (residues 428-436) interacts with the N-terminus of NCS-1 (Kabbani et al., 2002). Recent studies suggest DRD2 binds at the large solvent-exposed hydrophobic groove of NCS-1 (Lian et al., 2011; Pandalaneni et al., 2015) (Figure 1-7). Since NCS-1 interacts with the C-terminus of DRD2, it can interact

with both the long (postsynaptic) (Kabbani et al., 2002) and short (presynaptic) isoforms of DRD2 (Dragicevic et al., 2014), which differ in the presence or absence of 29 residues in the third intracellular loop (Giros et al., 1989; Monsma et al., 1989). NCS-1 modulates DRD2 (long isoform) function by attenuating G-protein-coupled receptor kinase 2 (GRK2)-mediated internalization and decreasing its phosphorylation, thus enhancing its downstream signalling effects (Kabbani et al., 2002) (Figure 1-9). These modulatory effects are Ca^{2+}-dependent; indeed NCS-1 and DRD2 often co-localize next to intracellular Ca^{2+} stores (Kabbani et al., 2002).

The DRD2/NCS-1 interaction has since been found to be important in hippocampal plasticity and spatial memory as well as modulating the activity of dopaminergic cells. Disrupting the DRD2/NCS-1 interaction with a cell-permeant peptide (DRD2/NCS-1 interfering peptide, DNIP) could reverse enhancements in LTP and spatial memory found in mice over-expressing *Ncs-1* in the dentate gyrus (Saab et al., 2009). Moreover, DNIP injections into the dentate gyrus of wild-type mice were sufficient to impair exploratory behaviour, LTP and spatial memory (Saab et al., 2009). More recently, NCS-1 has been found to play a role in modulating the activity of dopaminergic neurons in the substantia nigra via its interactions with DRD2 autoreceptors (short isoform) and the L-type calcium channel $Ca_v1.3$ (Dragicevic et al., 2014).

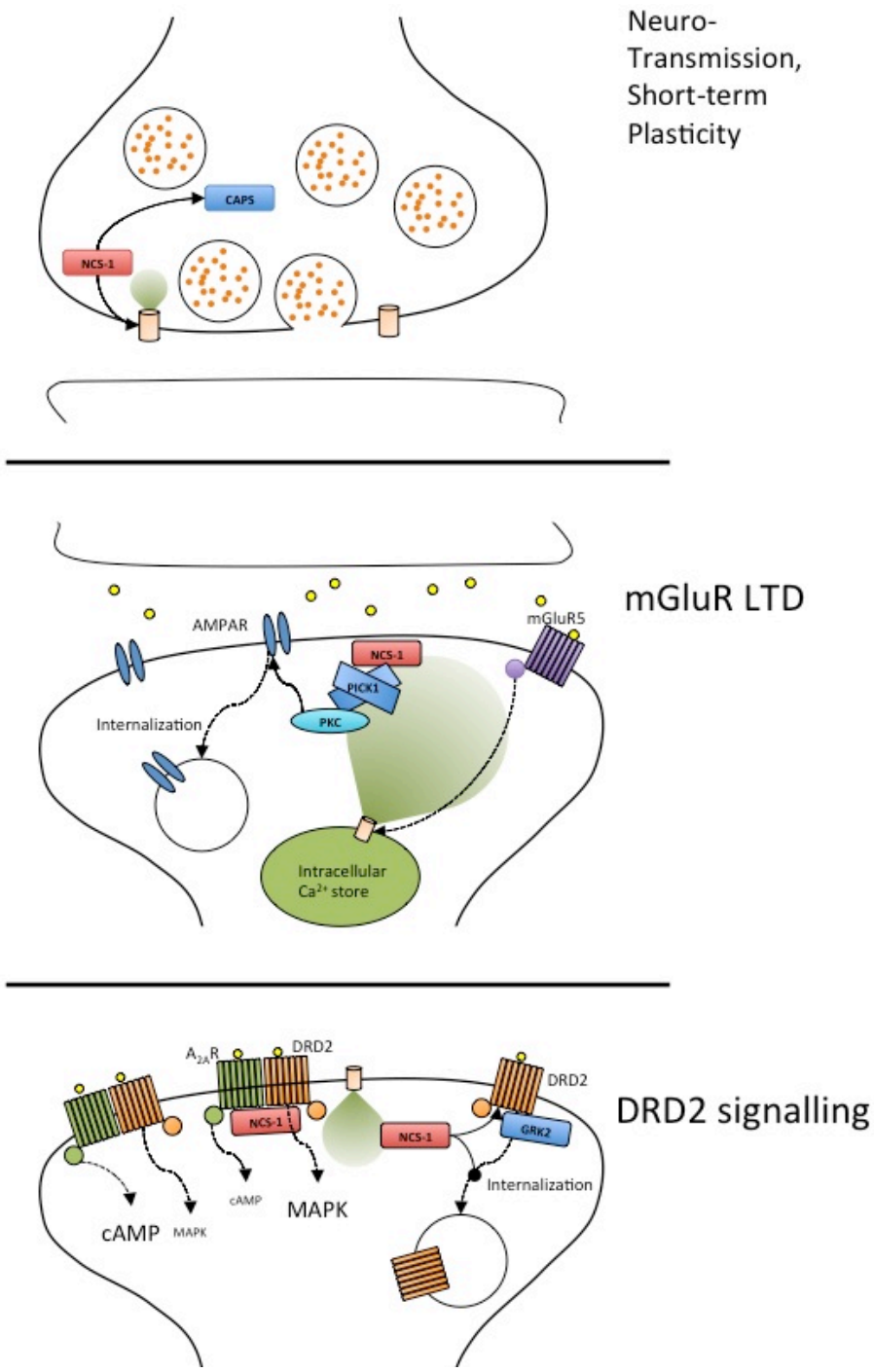

Figure 1-9. NCS-1 has roles presynaptically and postsynaptically.
Top panel: NCS-1 can modulate neurotransmission by interacting with voltage-gated calcium channels and CAPS (Calcium Activated Protein for Secretion). Figure adapted from (Dason et al., 2012).
Middle panel: NCS-1 is needed for mGluR-dependent LTD in the perirhinal cortex. Ca^{2+} influx leads to NCS-1/PICK1 interaction, which together help to promote AMPAR endocytosis (Jo et al., 2008).
Bottom panel: NCS-1 attenuates dopamine-induced DRD2 desensitization and internalization

(Kabbani et al., 2002). NCS-1 also modulates signaling of A_{2A}R-DRD2 heteromers, promoting MAPK signaling and decreasing downstream cAMP signaling (Navarro et al., 2014).

1.3.4.4 NCS-1 proteins and neuropsychiatric disease

1.3.4.4.1 Schizophrenia and bipolar disorder

A postmortem study found significantly higher NCS-1 protein levels in the dorsolateral prefrontal cortex of patients who had schizophrenia or bipolar disorder but not patients with major depression (Bai et al., 2004; Koh et al., 2003). These differences did not appear to be attributable to psychiatric medication as no differences in NCS-1 were found between patients who were drug-free or drug-treated at time of death (Bai et al., 2004; Koh et al., 2003). Moreover, chronic antipsychotic treatment of non-human primates did not lead to changes in NCS-1 protein or mRNA (Bai et al., 2004). Patients with schizophrenia or bipolar disorder show reductions in gamma band activity, a measure of neuronal synchrony important for cognitive processes (Özerdem et al., 2011; Uhlhaas and Singer, 2010). NCS-1 is a modulator of gamma band activity in the pedunculopontine nucleus, such that elevating NCS-1 decreases gamma band coherence (D'Onofrio et al., 2014).

NCS-1 has been implicated in schizophrenia and bipolar disorder due to its interactions with molecules affected by medications used to treat these diseases. NCS-1 interacts with the D2 dopamine receptor (DRD2), which is antagonized by antipsychotic drugs. Bipolar disorder is characterized by Ca^{2+} dysregulation and NCS-1 can increase the activity of the inositol 1,4,5-triphosphate receptor (InsP3R), a major regulator of intracellular Ca^{2+} stores (Schlecker et al., 2006). Therapeutic levels of the mood stabilizer lithium can inhibit the effects of NCS-1 on the InsP3R (Schlecker et al., 2006).

1.3.4.4.2 Autism spectrum disorder

NCS-1 has been implicated in autism spectrum disorder (ASD) due to its interaction with the IL1 receptor accessory protein like (IL1RAPL) (Bahi et al., 2003), which is involved in non-specific forms of X-linked mental retardation and also linked with ASD (Piton et al., 2008). One individual with ASD was also identified with a R120Q missense mutation in NCS-1 (Piton et al., 2008). Subsequent structural analyses suggest the R120Q mutation changes the Ca^{2+}-dependent component of NCS-1 cycling between the cytosol and plasma membrane (Handley et al., 2010) and reduces NCS-1 structural flexibility (Zhu et al., 2014).

1.3.4.4.3 Substance use

Two studies have found associations between variations in *NCS-1* and substance use. Dahl et al found an interaction between variations in *NCS-1* and *DRD2* in predicting the response of smokers to nicotine replacement therapy (Dahl et al., 2006). *NCS-1* polymorphisms are also associated with cocaine addiction in an African-American population (Multani et al., 2012).

1.3.4.4.4 Parkinson's disease

The death of dopaminergic neurons in the substantia nigra is the major pathophysiological event in Parkinson's disease and much research has focused on the particular vulnerability of these neurons to degeneration. NCS-1 modulates the activities of these dopaminergic neurons and *Ncs-1* mRNA is significantly elevated in the substantia nigra of patients who had Parkinson's disease (Dragicevic et al., 2014).

1.4 Cognitive processes dependent on striatal dopamine

The striatum is critically involved in instrumental conditioning where an animal learns to perform certain actions in order to obtain an outcome. Work over the past three decades has dissociated the roles that different striatal subregions play in the learning and performance of instrumental actions. After a brief overview of striatal anatomy (1.4.1), I review the evidence that the dorsal striatum is critical for instrumental acquisition and performance (1.4.2-1.4.4) whereas ventral striatal dopamine is involved in the motivational control of instrumental performance (1.4.5-1.4.6). I finish with a brief discussion of diseases involving impairments in striatal dopamine (1.4.7). While many other molecules and transmitter systems are involved in striatal function and neuropsychiatric disease, I will focus particularly on functions sensitive to manipulations of dopamine. Moreover, the majority of this section (1.4) is limited to the role of dopamine in motivated and approach behaviours associated with reward (particularly food), because that is the focus of my experimental work. Midbrain dopamine neurons also change firing in response to nonrewarding as well as aversive stimuli. Section 1.4.9 briefly describes the diversity of midbrain dopamine cells, their role in motivation and recent work on brain circuits that modulate striatal dopamine levels in response to aversive stimuli.

1.4.1 Anatomy of the striatum and the brain dopamine system

The rodent striatum can be subdivided into a dorsal and ventral portion (Figure 1-10). The ventral portion consists of the nucleus accumbens shell and core. The dorsal portion can be further functionally subdivided into a dorsomedial portion and dorsolateral portion, with the former mostly receiving projections from associative regions of the cortex and the latter receiving sensorimotor cortex projections (Alexander et al., 1986). The striatum is heavily innervated by dopaminergic projections from the midbrain. The nucleus accumbens mostly receives dopamine projections from the ventral tegmental area (VTA, A10) forming the mesolimbic pathway, whereas the dorsal striatum primarily receives projections from the substantia nigra pars compact (SNc, A9) forming the nigrostriatal pathway (German and

Manaye, 1993) (Figure 1-10). Both the dopamine D1 and D2 receptors are highly expressed in the striatum, though in distinct cell populations except in the nucleus accumbens (Gangarossa et al., 2013a; Gangarossa et al., 2013b; Gerfen and Surmeier, 2011).

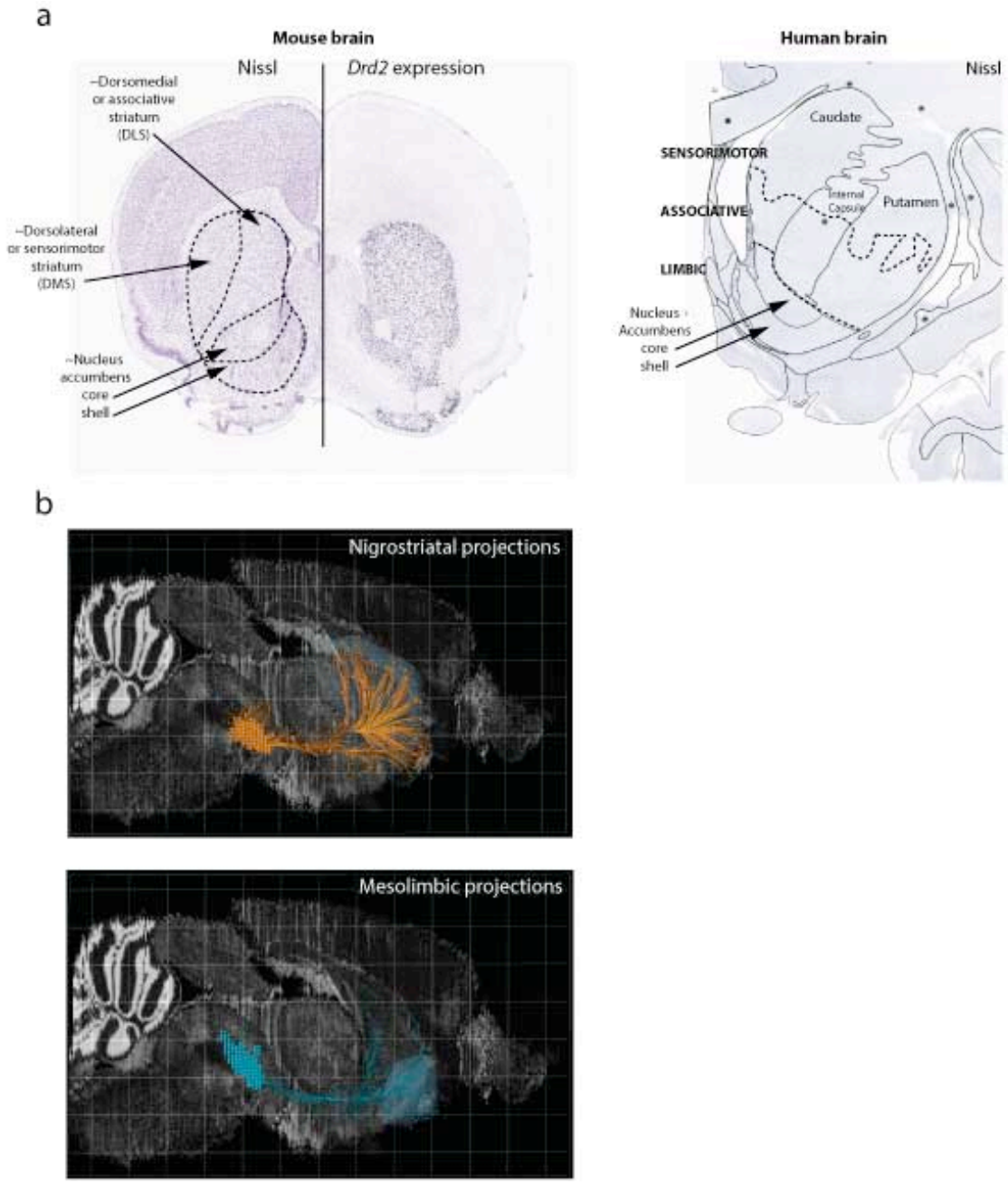

Figure 1-10. Striatal anatomy and dopamine projections.
(a) Left, approximate functional subdivisions of the striatum as adapted from (Yin et al., 2008). Dopamine D2 receptor mRNA expression by *in situ* hybridization. Images courtesy of the Allen Mouse Brain Atlas (Lein et al., 2007): experiment RP_Baylor_102735 image 16. Right, Nissl-stained section of the human brain showing the functional subdivisions of the striatum adapted

from (Feekes and Cassell, 2006). Image courtesy of the Allen Human Brain Atlas (Hawrylycz et al., 2012). **(b)** Approximate visualizations of nigrostriatal (top) and mesolimbic (bottom) dopamine projections. The blue translucent shapes mark the dorsal striatum (top) and the nucleus accumbens (bottom). Images obtained using the Brain Navigator with data from the Allen Mouse Brain Connectivity Atlas (Oh et al., 2014): experiments 160539283 and 156314762.

1.4.2 Dorsal striatum mediates instrumental learning

There are two types of instrumental behaviour that involve distinct learning processes: goal-directed action and habit (reviewed in (Yin and Knowlton, 2006)). If an animal is goal-directed, it has learned an association between an action and the particular outcomes of the action (action-outcome, A-O learning). In contrast, if an animal is behaving habitually, it has learned a stimulus-response (S-R) association, such that the stimulus elicits a reflexive response regardless of the outcomes of the response. For example, when rewards are withheld (extinction trials), animals behaving habitually will continue to responding for quite a while and display a significantly slower decay in responses than goal-directed animals, who will extinguish responding much sooner. Habits are likely to form after extensive training, particularly under random interval schedules[4]. Goal-directed actions allow animals to respond flexibly to changes in the environment and outcomes but require the continuous use of limited cognitive resources such as attention. Habitual actions allow for a more efficient use of resources when the behaviour has been repeated without much change in outcome, but this is associated with a loss of cognitive control.

1.4.3 Distinguishing between goal-directed actions and habits

Goal-directed actions and habits can be distinguished according to two criteria (Dickinson,

[4] In random interval (RI) schedules, the reinforcer is delivered at the first nosepoke after a random time interval. The time intervals in the long run average to a certain length of time, for example, 30 s or 60 s (RI 30 s, RI 60 s schedules). Since animals cannot predict when the next nosepoke will lead to reinforcement, the optimal strategy is to nosepoke continuously during the session, which is habit inducing.

1985). First, goal-directed actions are sensitive to changes in outcome value, whereas habits are not. Second, while goal-directed actions are sensitive to changes in the contingency relationship between response and outcome, habits are not. Accordingly, outcome devaluation and contingency degradation are the two most common behavioural tests for probing whether an action is goal-directed or habitual.

In outcome devaluation, the value of a food reward is decreased by either pre-feeding the animal to satiation just before testing or associating the food with gastric malaise by pairing it with lithium chloride injections. In the sensory-specific devaluation paradigm, animals are pre-fed or satiated on consecutive days with either reward pellets or lab chow just prior to a short extinction trial, in which responses are recorded but no rewards are delivered (Figure 1-11 b). If an animal is acting in a goal-directed manner, it will respond significantly less on the day it was satiated with reward pellets relative to the day it was satiated on lab chow. In contrast, an animal that has formed a habit will respond to a similar extent on both days.

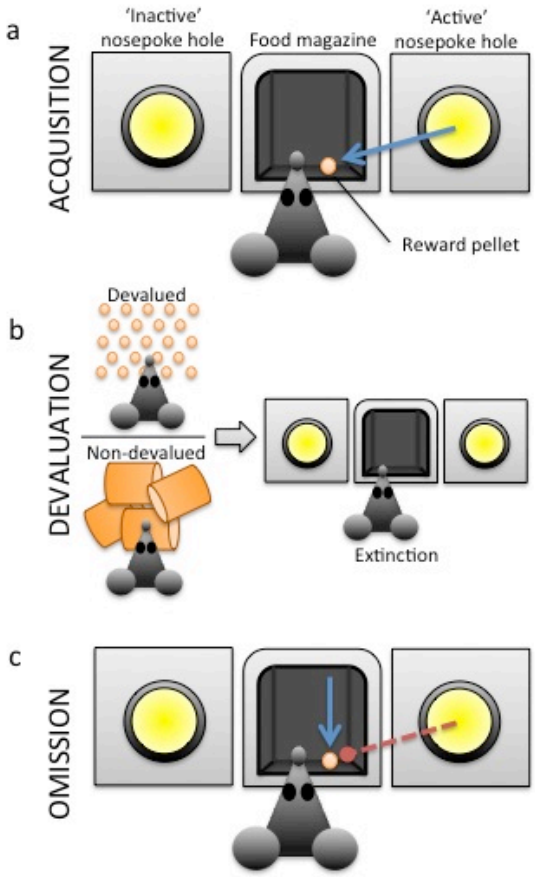

Figure 1-11. Sensory-specific devaluation and omission tests.
(a) Instrumental acquisition. Mice learn make nosepoke responses into an 'active' nosepoke hole to receive reward pellets from the food magazine. **(b)** Sensory-specific devaluation. Mice are pre-fed with either reward pellets (thus devaluing the reward) or home chow (leaving the value of the reward pellets intact) on different days and their operant responses recorded during a short extinction session when no rewards are delivered. Mice under goal-directed control will show significantly decreased nosepoking in the devalued compared to the valued condition. But mice that have formed a habit (due to extended training) would nosepoke similar amounts on devalued days than on non-devalued days. **(c)** Omission. The contingency between nosepoking and food pellet delivery is changed such that food pellets are delivered at random time intervals and nosepokes delay food delivery. Goal-directed behaviours will quickly decrease under omission contingencies whereas habitual responding will decrease at a slower rate.

Another way to test if a habit has formed is by testing whether animals are sensitive to a degradation of the contingency between response and outcome (Figure 1-11 c). For example, whereas nosepokes had previously led to food delivery, now food is delivered on a random interval and nosepokes delay or prevent food delivery. Subjects performing habitually will be

insensitive to this change and continue to nosepoke for a while whereas those under goal-direction will quickly cease their operant responses.

1.4.4 Dorsal striatal control over instrumental action

Goal-directed actions and habitual behaviour are dependent on different dorsal striatal subregions. Lesioning or inactivating the dorsolateral striatum can cause an animal that was performing habitually to become sensitized again to changes in reinforcer value or contingency (Quinn et al., 2013; Yin et al., 2004, 2006). In contrast, lesioning or inactivating the dorsomedial striatum abolishes goal-directed learning, such that animals become insensitive to devaluation and degradation (Quinn et al., 2013; Yin et al., 2005a; Yin et al., 2005b). Genetic depletion of dopamine in the projections that go to the dorsal striatum impairs the acquisition of goal-directed actions, whereas restoring dopamine can rescue these deficits (Darvas and Palmiter, 2009, 2010; Palmiter, 2008). Specific lesions of the nigrostriatal projections to the dorsolateral striatum impair habit formation without having much effect on lever pressing rate (Faure et al., 2005). Table 1-1 provides a summary of the functional domains of the dorsal striatum.

The ventral striatum, however, does not seem to be critical to goal-directed learning. After lesions to the nucleus accumbens, animals are still sensitive to outcome devaluation and degradations of the action-outcome contingency (Corbit et al., 2001; de Borchgrave et al., 2002) and can readily respond for food on fixed ratio 1 schedules (Cardinal and Cheung, 2005). Similarly, depletion of nucleus accumbens dopamine has little effect on fixed ratio 1 acquisition or performance (Aberman and Salamone, 1999; McCullough et al., 1993; Salamone et al., 1995). Restoration of dopamine to the dorsal striatum in dopamine deficient mice is sufficient to rescue effects on instrumental acquisition[5] (Darvas and Palmiter, 2009, 2010). What then is the role of

[5] In line with this, the firing of mesolimbic dopamine neurons thought to code for "reward prediction error" recorded by Schultz and colleagues is theorized to underlie stimulus-reward (CS-US) learning and in its current theoretical formulation does not address instrumental learning (see Yin, H.H., Ostlund, S.B., and Balleine, B.W. (2008).

mesolimbic dopamine? Is it important for reward? We discuss this in the next section.

Table 1-1. Instrumental actions depend on distinct learning processes and dorsal striatal substrates.

	Goal-directed action	Habitual action
Association learned	Action-outcome (A-O)	Stimulus-response (S-R)
Sensitive to changes in outcome value?	Yes	No
Sensitive to contingency degradation?	Yes	No
Neural substrate in striatum	Dorsomedial or associative striatum (DMS)	Dorsolateral or sensorimotor striatum (DLS)
Advantages	Flexibly adapt to changes	Automatic
Disadvantages	Uses lots of cognitive resources	Loss of impulse control

Reward-guided learning beyond dopamine in the nucleus accumbens: The integrative functions of cortico-basal ganglia networks. European Journal of Neuroscience 28, 1437-1448.).

1.4.5 A brief history of motivation and dopamine

Mesolimbic dopamine is thought to underlie motivation and specific aspects of reward. Here we outline a conceptual history of motivation and how mesolimbic dopamine came to be associated with it.

1.4.5.1 Theories of motivation

In addition to learning and memory, another concept in psychology used to understand behaviour is motivation. Motivation can be defined as "a state of desire or energy to carry out a certain action, triggered by intrinsic and extrinsic factors, which can be aversive or appetitive" (Pennartz et al., 2011). Here I give a brief overview of two influential theories on motivation (for reviews see (Berridge, 2004; Berridge, 2007)). Early theories on motivation such as by Hull were based around the idea of homeostasis and drive reduction. What 'drives' behaviours are biological needs or imbalances. For example, animals seek food and water because they are reducing a drive for hunger and a drive for thirst. Drive reduction was intuitive but was not supported by experimentation. For example, animals that were intravenously fed their needed amount of nutrients would still eat by mouth if given a chance. This suggests the very act of eating and tasting is in itself pleasurable and motivating. Moreover, brain electrical stimulation experiments gave results totally contrary to drive reduction theory. Brain sites that promoted eating behaviour upon electrode stimulation were the same sites that animals found rewarding in intra-cranial self-stimulation procedures.

Furthermore, drive reduction theory left many issues of motivation unexplained, leading to the development of incentive motivation theory by Bolles, Bindra and Toates (reviewed by (Berridge, 2004)). For example, individuals are also motivated by incentives, which are stimuli associated with and predictive of a hedonic reward (light cue predictive of food, a Pavlovian process). In this theory, the incentive (CS) is not only predictive of reward but becomes

perceived as a reward in its own right. In other words, obtaining the incentive itself becomes able to promote seeking behaviours. Finally, while physiological drives are not the same as motivation, they do still have an important modulatory effect on the values of incentives. For example, being in a state of hunger would enhance the incentive value of a light cue that had been associated with food. Incentive motivation theory continues to guide and has been refined and elaborated upon by modern studies of motivation.

1.4.5.2 Dopamine = reward? Not quite

For many years, people have studied midbrain dopamine projections to the striatum as a neural substrate for motivation. A hypothesis that lives on particularly in the general public is the idea that dopamine signals pleasure in the brain; in the scientific literature it was named the anhedonia hypothesis by Wise (Wise, 1982). The hypothesis was based on the effects of neuroleptic drugs (dopamine receptor antagonists). When it was discovered that rats would work for electrical self-stimulation of particular brain areas, the search was on for brain areas that mediate pleasure. Analyses revealed that these sites of stimulation were dopaminergic fibres. Neuroleptics decreased rat self-stimulation at these sites. Subsequently it was found that neuroleptics also disrupted operant responding for other positive reinforcers including food, water, psychostimulants and opiates. On the other hand, studies found that many things we find pleasant lead to dopamine release particularly in the ventral striatum (nucleus accumbens) from natural rewards like food, sex, social and cognitive rewards to various drugs of abuse.

However, studies by Berridge showed that dopamine is important for only certain aspects of reward and hedonia was not one of them (Berridge et al., 1989; Pecina et al., 1997; Smith et al., 2011). For example, lesions of mesolimbic dopamine projections do not change facial expressions of “liking” in response to sweet treats into expressions of disgust. This led him and Robinson to propose the incentive salience model, which elaborated on and divided incentive motivation into two components: ‘liking’ and ‘wanting.’ ‘Liking’ corresponds to the hedonic or

"pleasurable"[6] effects of an incentive, which they found to depend not on dopamine but on opioid systems (Berridge, 2007; Berridge and Robinson, 1998). In contrast, 'wanting' or the ability for the incentive to motivate behaviour (incentive salience) was hypothesized to depend on mesolimbic dopamine. Moreover, some midbrain dopamine neurons have also been found to fire phasically in a way that may underlie stimulus-reward (CS-US) learning (Bromberg-Martin et al., 2010; Schultz, 2007; Schultz et al., 1997). They fire strongly in response to unpredicted rewards, do not fire for fully predicted rewards, and depress when a predicted reward is omitted (reward-prediction error).

Elaborating on incentive salience, work by Salamone and others suggests that nucleus accumbens dopamine is particularly critical for the vigour or effort with which animals will pursue or work for reinforcers or incentives without affecting their primary motivational drive (Barbano and Cador, 2007; Phillips et al., 2007; Salamone and Correa, 2002; Salamone et al., 2007). In other words, dopamine is not critical for a desire or appetite to eat food, but that animals will not be willing to expend much effort for reward without dopamine input. There have been suggestions that a subset of dopamine neurons or overall tonic levels of dopamine may code for the effort dimension of motivation (Niv, 2007; Niv et al., 2007; Pasquereau and Turner, 2013).

In summary, while there is still debate on the roles of mesolimbic dopamine, studies have led to a more nuanced understanding of dopamine and how it does not mediate reward in the common sense of the word. Dopamine does not mediate the hedonic effects or 'liking' responses associated with receiving a reward. Rather, mesolimbic dopamine is important for the motivational aspects of reward, making an organism "want" the natural reward or incentive. Mesolimbic dopamine also promotes approach behaviours and efforts to obtain rewards. There

[6] Note it is not known if animals experience "pleasure" in the sense of experiencing the subjective feelings that we human beings associate with the term "pleasure." What is known, however, is that animals, including mice, show facial expressions, seeking behaviours and other physiological responses to "pleasurable" stimuli like sweet foods that are homologous to the types of responses we display toward things we feel to be "pleasurable."

may also be a role for mesolimbic dopamine in stimulus-reward (CS-US) learning of incentives. The following section further elaborates on how dopamine in the ventral striatum is important for motivation. Note, striatal dopamine also has a role in aversive motivation and avoidance behaviour; this is described briefly in section 1.4.9.

1.4.6 Ventral striatal dopamine is critical for the effort dimension of motivation

The ventral striatum appears to modulate the motivational control of instrumental actions. There are many dimensions to motivation, which can be captured by three critical distinctions made in the field: 1) seeking versus taking, 2) direction versus activation, and 3) "wanting" versus "liking" (reviewed in (Salamone and Correa, 2012)).

1.4.6.1 Seeking vs Taking

Motivated behaviour can be divided into two phases in time (Czachowski et al., 2002; Foltin, 2001). In the seeking phase, an animal acts to approach a stimulus in the face of work costs, delays, and changes in the environment. Afterward, in the taking phase, the animal has direct interaction with the goal stimuli for example, eating food or copulating with a sexual partner. The construct of seeking can be measured behaviourally through the ability to perform operant conditioned behaviours.

1.4.6.2 Direction vs Activation

Motivation also has directional and activational components (Cofer and Appley, 1964; Salamone, 1988). Direction refers to whether an animal's actions are toward or away from the

stimuli. There are often substantial obstacles between an animal and its goal stimuli, whether physical distances, obstructions or delays in time. Activation refers to the energy or effort, which an animal will put into initiating and sustaining actions to approach stimuli.

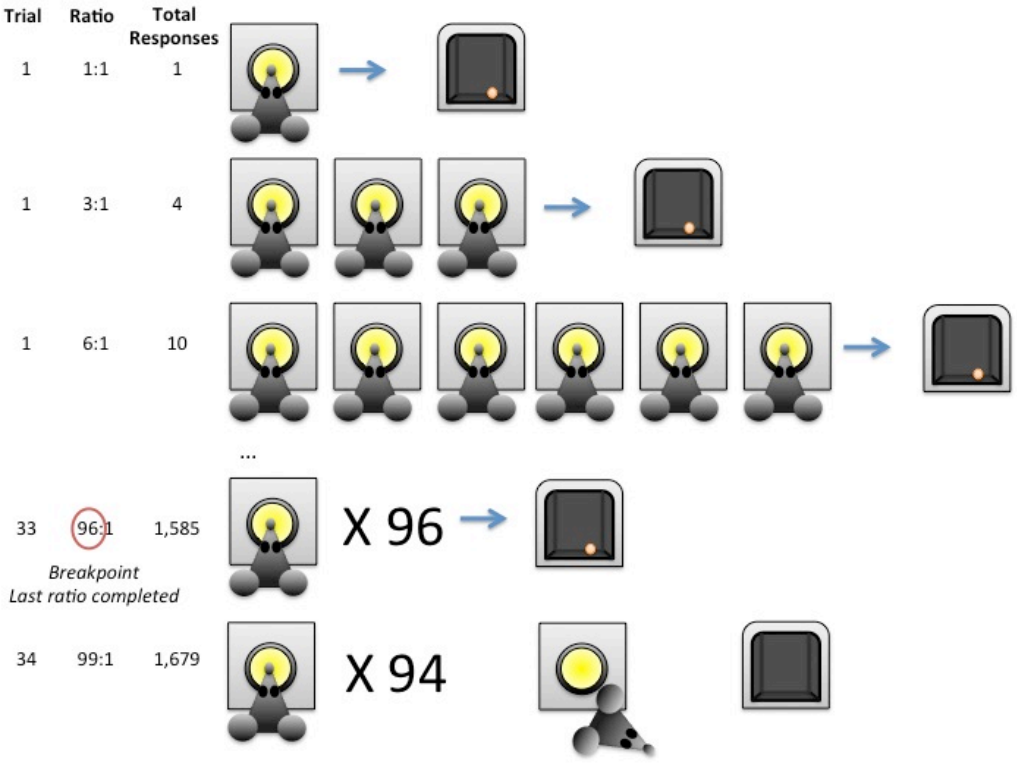

Figure 1-12. Progressive ratio task for assessing motivation to work for food rewards. Progressive ratio task for assessing motivation to work for food rewards. This diagram shows a progressive ratio 3 (PR3) task, where the ratio of nosepokes required for one food pellet delivery increases by 3 every trial in an arithmetic progression. The task continues until either the animal stops responding for a certain period of time or the session ends. In this example, the animal gives up at trial 34, having completed a total of 1,679 responses, receiving 33 rewards and having a last completed ratio of 96 which is counted as its breakpoint.

Behavioural activation is most commonly assessed in two ways: through the progressive ratio task (Hodos, 1961) and the concurrent choice task (effort-related choice task) (Salamone et al., 1991). Both tasks involve operant conditioning for a food reinforcer, for example, nosepoking or lever pressing for a chocolate-flavoured food pellet. In the progressive ratio task, the ratio of operant responses to reinforcer delivery increases with each trial until the point at which the

animal stops responding or the session finishes (Figure 1-12). The ratio at which the animal stops responding is called the breakpoint and is seen as a measure of willingness to work for the reinforcer.

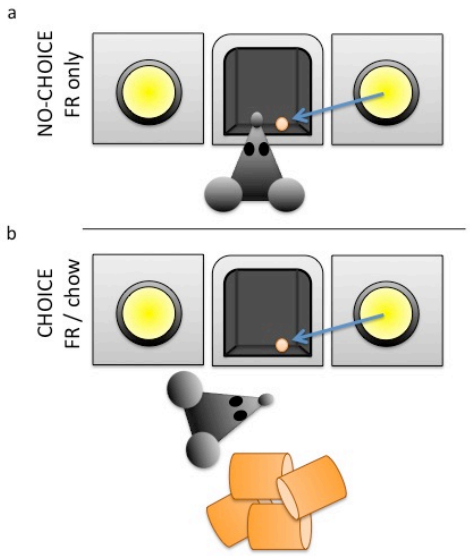

Figure 1-13. Concurrent choice task for effort-related choice behaviour.
Concurrent choice task for effort-related choice behaviour. On no-choice days, mice respond for food on a certain fixed ratio (most commonly FR5). On choice days, mice can either nosepoke for food rewards on the same fixed ratio or eat freely available food chow (which they do not prefer as much as reward pellets). Decreases in nucleus accumbens dopamine will tend to significantly decrease nosepoking for pellet rewards on choice days (relative to no-choice days) and also increase lab chow consumed relative to a control condition. Pre-feeding or appetite suppressants will tend to decrease responding on choice and no-choice days as well as lab chow consumption on choice days.

In the concurrent choice task, there are sessions where animals must choose between working for a relatively preferred food and spending their time eating freely available lab chow inside the operant box (Figure 1-13). A fixed ratio is chosen, such that control animals readily respond for the food reward on “no-choice” days (food available only via operant responding), and still get most of their food through operant responding on “choice” days when lab chow is present in the box. Manipulations that lower behavioural activation would substantially decrease operant responses on “choice” days and increase the amount of lab chow consumed.

1.4.6.3 Wanting versus Liking

Finally, another qualitative distinction in motivation is between “wanting” and “liking” (Berridge, 2007; Berridge and Robinson, 1998). “Liking” refers to an animal’s hedonic response to a stimulus, for example, of pleasure or disgust. “Wanting” or incentive salience describes the degree to which a stimulus or its associated cues draws attention or elicits approach and pursuit.

One way to measure “liking” with respect to food stimuli is observing and coding facial expressions in response to taste (taste reactivity paradigm) (Berridge et al., 1989). For example, “liking” reactions to pleasant tastes include rhythmic protrusions of the tongue and licking of the lips. This is seen in rodents, nonhuman primates and human infants.

Another way to measure hedonic “liking” is via preference tests. In the sucrose preference test, a bottle with sucrose-infused water and a bottle with plain water are made freely available in a home cage. Control animals will normally ingest significantly more of the sucrose water than the plain water. Disruption of this preference is interpreted as a form of anhedonia. A common test of social motivation is the 3-chamber social approach task, where time spent exploring a chamber with a novel non-social object is compared with time spent in a chamber with a novel conspecific (Kaidanovich-Beilin et al., 2011; Nadler et al., 2004). Control mice show a strong preference for exploring the novel mouse over the novel object. Failure to show a preference is seen to reflect social anhedonia.

“Wanting” can be measured by Pavlovian approach and conditioned reinforcement (Browne et al., 2014; O'Connor et al., 2010). In the Pavlovian approach, investigators measure the degree to

which a cue associated with a reward can itself elicit approach behaviours (Figure 1-14 a). For example, if illumination of a stimulus light predicts and overlaps with food delivery, the stimulus light coming on can elicit the animal to approach the food magazine or the stimulus light even prior to food delivery. Conditioned reinforcement tests the ability for a conditioned stimulus itself to be used as a reinforcer for operant responding (Figure 1-14 b). Continuing with the example above, conditioned reinforcement would involve animals being willing to lever press or nosepoke just to get stimulus light illumination. Greater ability to elicit approach or (thus acting as a reinforcer in itself) would be interpreted as greater "wanting."

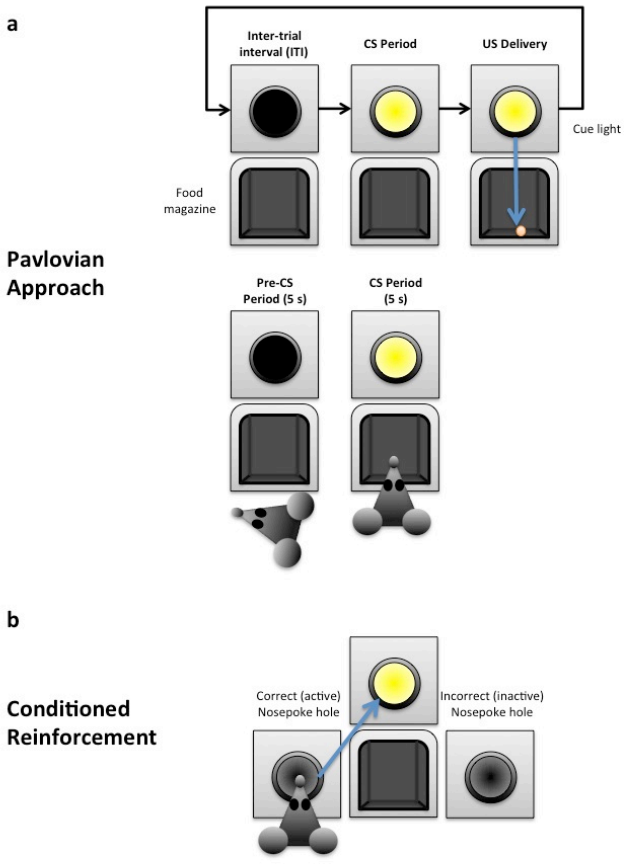

Figure 1-14. Pavlovian approach and conditioned reinforcement.
(a) In Pavlovian incentive learning, a light cue (CS) precedes and predicts food pellet (US) delivery. Learning is measured as the number of entries into the food magazine during the 5 s CS period (prior to US delivery) relative to a 5 s period just prior to the CS.
(b) In conditioned reinforcement, the animal learns to make an operant response (nosepoke) for the CS (cue light coming on) even in the absence of US, suggesting the CS itself has taken on 'rewarding' properties or incentive salience. Conditioned reinforcement can be measured as the number of nosepokes into an active nosepoke hole (that leads to CS delivery) relative to an inactive nosepoke hole (that has no consequence).

1.4.6.4 Nucleus accumbens dopamine is important for seeking, activation and "wanting"

These dimensions of motivation are differentially sensitive to dopamine signalling in the ventral striatum or nucleus accumbens (summarized in Table 1-2). Overall, antagonism of dopamine receptors or depleting dopamine in the accumbens has larger effects on seeking, activation and "wanting" and less effects on taking, direction, and "liking" (Salamone and Correa, 2012). Dopamine receptor blockade or depletion has little effect on total food intake (reviewed in (Baldo and Kelley, 2007)), but it does decrease conditioned locomotor activity to repeated food presentation (Barbano and Cador, 2006), thus showing the importance of dopamine in seeking but not taking.

Evidence from two types of behavioural experiments show that nucleus accumbens dopamine is critical for the exertion of effort to obtain food. The first line of evidence comes from fixed ratio and progressive ratio experiments. Nucleus accumbens dopamine depletion by local injections of 6-hydroxydopamine (6-OHDA) do not significantly affect fixed ratio 1 (FR1, 1:1 of lever press:reward) schedules, showing no effect on directionality (they still approach/seek food) nor the ability to acquire and perform the simplest operant conditioning task (1 lever press = 1 food reward) (Ishiwari et al., 2004; McCullough et al., 1993; Salamone et al., 1995). However, accumbens dopamine depletion does decrease operant responses when higher ratios of lever-pressing to reward were used such as in an FR50 schedule (50:1 of lever press:reward) (Aberman and Salamone, 1999; Salamone et al., 2001). Indeed progressive ratio performance is impaired by dopamine receptor antagonists or accumbens dopamine depletion (Aberman et al., 1998; Hamill et al., 1999).

Second, concurrent choice task performance is modulated by dopamine. Animals given

dopamine receptor blockers or receiving accumbens dopamine depletion decrease lever pressing for preferred food pellets and eat more of the freely available lab chow (Cousins and Salamone, 1994; Cousins et al., 1994; Nowend et al., 2001; Salamone et al., 1991). 6-OHDA injections into various striatal regions show that the nucleus accumbens core is the critical region for allocating effort in food-related processes (Cousins et al., 1993; Sokolowski and Salamone, 1998). The fact that animals lever pressed similarly on days when no lab chow was freely available highlights again that decreased dopamine is not leading to a general motivational deficit decreasing appetite for food. Recent developments of a hybrid progressive ratio / concurrent choice task show similar results (Randall et al., 2015; Randall et al., 2012).

Nucleus accumbens dopamine is not required for hedonic "liking" but is involved in Pavlovian approach processes. 6-OHDA lesions of dopamine projections do not affect facial responses of "liking" to sweet tastes (Berridge and Robinson, 1998; Berridge et al., 1989). Dopamine receptor antagonism or dopamine depletion does, however, impair Pavlovian approach processes (Di Ciano et al., 2001; Parkinson et al., 2002). While psychomotor stimulants enhance responding for conditioned reinforcers, dopamine receptor antagonists decrease responding and block the enhancing effects of stimulant drugs (Beninger et al., 1980; Fletcher and Higgins, 1997; Robbins, 1975; Taylor and Robbins, 1986; Wolterink et al., 1993). Dopamine signalling in the nucleus accumbens is also critical for Pavlovian-to-Instrumental Transfer (PIT), where a conditioned stimulus (CS) can enhance the rate of instrumental responding (Dickinson et al., 2000; Lex and Hauber, 2008; Murschall and Hauber, 2006).

Genetic manipulations in mice produced effects generally consistent with the pharmacological and lesion studies described above. Dopamine deficient mice that have dopamine selectively restored in the dorsal striatal subregions show impaired progressive ratio performance (Darvas and Palmiter, 2009, 2010) and Pavlovian conditioned approach (Darvas et al., 2014). In contrast, DAT knockdown mice that have chronically elevated extracellular dopamine show higher motivation to work for food reward in the progressive ratio task and the concurrent choice task without having a general effect on appetite for food (Cagniard et al., 2006). Specific deletion of dopamine D2 autoreceptors on dopaminergic neurons leads to elevated dopamine synthesis and

release, hyper-locomotion, increased sensitivity to cocaine and motivation to work for food (Bello et al., 2011b). Viral vector-mediated over-expression of postsynaptic DRD2 in the nucleus accumbens of adult mice selectively enhanced willingness to work for food reward; over-expression in the dorsal striatum however, had no such effect (Trifilieff et al., 2013).

Table 1-2. Different dimensions of motivation are differentially sensitive to nucleus accumbens dopamine manipulations.

	Tested by	Nucleus accumbens dopamine critical?
Seeking	Approach responses	Yes
Taking	Direct interaction with stimulus i.e. eating	No
Direction	Approach or avoid	No
Activation (effort)	Progressive ratio Concurrent choice	Yes
"Liking"	Preference tests Facial expressions of "like" or "dislike"	No
"Wanting"	Pavlovian approach Conditioned reinforcement Pavlovian-to-Instrumental Transfer	Yes

1.4.7 Disrupted striatal dopamine in neuropsychiatric disease

A number of neuropsychiatric diseases are associated with disruptions in striatal dopamine

including Parkinson's disease, schizophrenia, and addiction. A greater understanding of striatal function and the role of dopamine signalling in these regions may shed light on these disorders.

Parkinson's disease is classically characterized by the motor signs of tremor, rigidity and akinesia (Lang and Lozano, 1998a, b) but often also involves cognitive impairments (Koerts et al., 2009) as well as dementia (Svenningsson et al., 2012). The pathological hallmark of Parkinson's is the loss of nigrostriatal dopamine projections. The most susceptible neurons are those in the ventrolateral substantia nigra pars compacta that project to the posterior putamen (Fearnley and Lees, 1991; Kish et al., 1988). These regions are the parts of the dorsal striatum involved in habitual control of actions, therefore some have argued that the symptoms of Parkinson's disease can be seen as a relative loss of automatic habits and increased reliance on goal-directed actions (reviewed in (Redgrave et al., 2010)). However, the hypothesis of disrupted habit formation in Parkinson's is still relatively untested and has not found support in patients with mild Parkinson's disease (de Wit et al., 2011). In contrast to the loss of automaticity associated with the disease, the use of dopaminergic drugs to address the motor symptoms of Parkinson's disease sometimes leads to disruptions in impulse control (see review in (Okai et al., 2011)).

Schizophrenia is characterized by positive symptoms such as hallucinations and delusions, negative symptoms such as decreased motivation, and cognitive symptoms such as decreased working memory and behavioural flexibility (van Os and Kapur, 2009). A meta-analysis of imaging studies shows a consistent and significant elevation of presynaptic striatal dopamine release in schizophrenia (Howes et al., 2012). The enhanced striatal dopamine release has been associated with the positive symptoms of schizophrenia (Howes and Kapur, 2009; Laruelle et al., 1999; Woodward et al., 2011). Recent data using higher resolution positron emission tomography (PET) suggests the locus of increased synaptic dopamine in schizophrenia is the associative striatum (Kegeles et al., 2010). Interestingly goal-directed action appears to be disrupted in schizophrenics, specifically the ability to put together knowledge about action-outcome contingencies with changes in outcome value to guide choice (Morris et al., 2014). Though striatal dopamine depletions can impair effort, preclinical evidence suggests in some

cases that too much striatal dopamine signalling can be disruptive. Transient transgenic over-expression of *Drd2* in dorsal and ventral striatal medium spiny neurons of mice decreased motivation to work for food (Drew et al., 2007; Simpson et al., 2011; Ward et al., 2012).

Stimulant drugs and alcohol increase dopamine release in the human striatum, and addiction to these substances is associated with decreased dopamine receptor availability and blunted dopamine release (reviewed in (Nutt et al., 2015)). It has been hypothesized that drug addictions arise from drug-seeking behaviours which are initially goal-directed, but which become increasingly habitual (Everitt and Robbins, 2005). Exposing rats to amphetamines enhances habit formation (Nelson and Killcross, 2006). Recent work on cocaine-seeking behaviour in rats supports this hypothesis, showing that control over such behaviour shifts from the dorsomedial to the dorsolateral striatum (Murray et al., 2012; Zapata et al., 2010). Functional connections between the ventral and dorsal striatum through dopaminergic neurons in the midbrain ("spiralling connections") also appear to be critical to drug seeking behaviours (Belin and Everitt, 2008).

1.4.8 Summary

The striatum contains distinct functional domains (Yin and Knowlton, 2006; Yin et al., 2008) (Table 1-3). The dorsal striatum is critical for instrumental actions with the dorsomedial striatum mediating goal-directed actions under the control of action-outcome contingencies, and the dorsolateral striatum underlying habits under the control of stimulus-response contingencies. Nucleus accumbens dopamine signalling is critical for the exertion of effort and certain Pavlovian processes and is not needed for hedonic responses or the performance of low-effort instrumental actions. Greater understanding of how these circuits underlie motivated behaviour and how they are modulated by dopamine may lead to insights about neuropsychiatric diseases that include disruptions in motivation and cognition such as schizophrenia, addiction and Parkinson's disease.

Table 1-3. Functional domains in the striatum.

	Receives DA from	Behaviour mediated	Behaviour test	Implications in disease
Dorsolateral / sensorimotor striatum (DLS)	Dorsal substantia nigra pars compacta	Habitual (S-R) instrumental actions	Insensitive to changes in outcome and contingency	Controls established drug habits; impaired function Parkinson's disease
Dorsomedial / associative striatum (DMS)	Ventral substantia nigra pars compacta	Goal-directed (A-O) instrumental actions	Sensitive to changes in outcome and contingency	Elevated dopamine here and impaired goal-directed actions in schizophrenia
Nucleus accumbens core	Lateral ventral tegmental area	Effort	Progressive rato, concurrent choice	Elevated dopamine here in response to stimulant drugs and alcohol
		Pavlovian preparatory responses	Pavlovian approach, conditioned reinforcement	
Nucleus accumbens shell	Medial ventral tegmental area	Pavlovian consummatory responses		Controls actions during initial drug-taking

1.4.9 Addendum: the diversity of dopamine signals and dopamine in aversive motivation

While much work has focused on the role of dopamine in reward learning and positive motivation, midbrain dopamine neurons also fire in response to things that are not necessarily rewarding including novelty (Lisman and Grace, 2005; Redgrave and Gurney, 2006; Redgrave et al., 1999), sensory salience (Horvitz, 2000) and aversive stimuli (Abercrombie et al., 1989; Anstrom and Woodward, 2005; Matsumoto and Hikosaka, 2009b). Indeed, there is increasing recognition of the large diversity of dopaminergic neurons (Lammel et al., 2014) with recent studies in mice showing large populations of VTA neurons that change firing in response to aversive stimuli like a tail pinch (Cohen et al., 2012; Zweifel et al., 2011). In order to explain the available data, Bromberg-Martin et al hypothesize the existence of at least two populations of midbrain dopamine neurons and three types of dopamine signals (Bromberg-Martin et al., 2010). One population of motivational value-coding neurons is activated by rewards or omission of an aversive event and inhibited by aversive stimuli or reward omission. A second population is sensitive to motivational salience; they will be excited by anything that is motivationally relevant (whether positive/rewarding or negative/aversive) and have little response to motivationally neutral stimuli. Both classes of neurons are thought to contribute to an alerting signal. Value-coding neurons are thought to provide signals important for learning about rewards or punishments and the evaluation of outcomes. Dopamine signals for salience are thought to support the orienting of attention toward stimuli, cognitive processing, and provide the behavioural activation to animate action (sustained engagement, approach, avoidance etc.). And alerting signals help with the quick detection of important stimuli. Midbrain dopamine neurons are not homogenous and provide a variety of dopamine signals that project to various brain structures to support motivated and adaptive behaviour. This more nuanced perspective of dopamine is important to keep in mind given the strong focus on dopamine in reward in both the scientific and public arena.

Indeed, recent work has identified a brain circuit thought to be critical for the dopamine signal for motivational value, particularly for aversive stimuli and avoidance behaviours (reviewed in

(Barrot et al., 2012; Barrot and Thome, 2011; Proulx et al., 2014)). This circuit involves the lateral habenula, the rostromedial tegmental nucleus (RMTg, also known as the tail of the VTA) and the midbrain dopamine neurons of the VTA and substantia nigra. Electrophysiology recordings in non-human primates have revealed a role for this circuit in coding reward prediction errors and sending aversion-related signals. The lateral habenula appears to code a 'negative' reward prediction error in that it gets excited in response to aversive events or stresses and becomes inhibited by unexpected rewards (Matsumoto and Hikosaka, 2007, 2009a). A major output of the lateral habenula is the RMTg, which sends GABAergic (inhibitory) projections to dopamine cells in the VTA and substantia nigra (Balcita-Pedicino et al., 2011). In effect then, aversive events that activate the lateral habenula (via the RMTg) will tend to inhibit at least certain populations of midbrain dopamine neurons (e.g. medial SNc) (Matsumoto and Hikosaka, 2009b). Not all midbrain dopamine neurons are inhibited by punishment, however; in monkeys the lateral SNc is excited by punishment (Matsumoto and Hikosaka, 2009b). In rats, while the dorsal VTA is inhibited by footshocks, the ventral VTA is excited (Brischoux et al., 2009). In mice, the lateral habenula also has some direct excitatory projections to VTA dopamine neurons (Lammel et al., 2012). Perhaps these populations of cells, which get excited in response to both rewarding and aversive stimuli, energize actions for approaching rewards or avoiding punishments. Work in rodents implicates the lateral habenula-RMTg-VTA circuit in avoidance behaviours and response to aversive stimuli. Lesioning the RMTg decreases conditioned freezing, unconditioned freezing as well as open arm avoidance (Jhou et al., 2009). Optogenetic stimulation of lateral habenular terminals in the RMTg promotes passive, active and conditioned avoidance behaviours (Stamatakis and Stuber, 2012). Of relevance to human psychiatric disease, opiate and cannabinoid action at the RMTg may contribute to the disinhibition and hyperactivity of VTA dopamine neurons in addiction processes (Barrot et al., 2012). Furthermore, evidence is emerging that depression in humans and animal models of depression-like states is associated with higher lateral habenula activity (Morris et al., 1999; Shumake et al., 2003). And a case study has shown promising outcomes from deep brain stimulation (to decrease lateral habenular activity) in patients with treatment-resistant depression (Kiening and Sartorius, 2013; Sartorius et al., 2010).

1.5 Thesis Aims

While NCS-1 has been implicated in transmitter release, synaptic plasticity, spatial cognition, and a number of neuropsychiatric diseases, its role in mammalian behaviour, learning and memory have not been thoroughly explored. The central aim of this thesis was to investigate the effects of *Ncs-1* deletion on mouse behaviour and to explore potential physiological mechanisms that may underlie any behavioural changes. Based on NCS-1 being a dopamine-receptor interacting protein and its known roles in hippocampal synaptic plasticity, we focused our analyses on striatal-dependent operant conditioning and motivated behaviours, as well as hippocampal-dependent spatial learning and memory. In accordance with our behavioural analyses, we studied striatal dopamine processes and hippocampal electrophysiology. A secondary aim was to screen for any behavioural endophenotypes relevant to neuropsychiatric disease. Our hypothesis had three specific predictions, which we tested.

1.6 Hypothesis

1.6.1 *Ncs-1* deletion impairs motivated behaviour and decreases striatal dopamine signalling

NCS-1 interacts with the DRD2 and they are both expressed in the striatum. Indeed, the striatum is the brain region where DRD2 is most highly expressed. The striatum is critical for operant conditioning and ventral striatal dopamine signalling through DRD2 is important for motivation. We predicted that *Ncs-1* knockout mice would show impairments in striatal-dependent learning processes such as action-outcome learning, stimulus-response learning, or motivated behaviours. Since, NCS-1 can modulate neurotransmitter release as well as affect DRD2 internalization, we investigated the effects of *Ncs-1* deletion on nucleus accumbens dopamine release and striatal DRD2 expression.

1.6.2 *Ncs-1* deletion impairs spatial cognition, flexibility, and hippocampal synaptic plasticity

Overexpressing *Ncs-1* in mouse dentate gyrus enhances spatial learning as well as LTP. Therefore, we tested the hypothesis that deletion of *Ncs-1* would lead to deficits in spatial cognition and hippocampal LTP. Knockdown of *Ncs-1* in perirhinal cortex impairs metabotropic glutamate receptor (mGluR) LTD but it is not known if it is needed for LTD in other brain regions such as the hippocampus. We predicted that *Ncs-1* knockout mice would show impaired hippocampal LTD as well as behavioural flexibility, since LTD has been associated with flexibility.

1.6.3 *Ncs-1* deletion is associated with endophenotypes relevant to neuropsychiatric disease

Dysregulation of calcium signalling and dopamine signalling are associated with disease. Indeed, NCS-1 has been implicated in a range of diseases including schizophrenia, bipolar disorder, autism, Parkinson's and addiction. We used a broad behavioural screen to test the prediction that the absence of NCS-1 may contribute to endophenotypes relevant to human neuropsychiatric disease.

2 Methods

2.1 Mice

Ncs-1 knockout (*Ncs-1*$^{-/-}$) mice congenic with C57BL/6J (backcrossed to C57BL/6J >10 generations) were a kind gift from Olaf Pongs and colleagues at the University Medical Center Hamburg-Eppendorf (backcrossing information from personal communication with Joanna Hermainski). Mice were re-derived at the Toronto Centre for Phenogenomics (TCP) and bred for our studies. Mice were housed 1-5 mice per cage in a controlled environment (7AM lights on, 7PM lights off) on corncob bedding with home chow and water available *ad libitum*. In general mice used for behavioural testing were group housed (>2 mice per cage) unless otherwise specified e.g. separation for food restriction and operant conditioning. All experiments were approved by the local committee on animal care ethics at the Toronto Centre for Phenogenomics and conformed to the national guidelines.

For behavioural studies and fast-scan cyclic voltammetry, adult males (8-25 weeks old) were used from heterozygote-heterozygote crosses, unless otherwise indicated. For other physiology and biochemistry assays, male mice from homozygote-homozygote and wildtype-wildtype crosses were sometimes used as well. Some female mice were used in measurements of dentate gyrus LTP.

An outline of the gene targeting strategy for creating the mice is below; for details please refer to dissertation of Joanna Hermainski (2012). A targeting construct was used containing the last four translated exons of wildtype *Ncs-1*, with a modification of the last translated exon to code for enhanced green fluorescent protein (EGFP) in frame; these regions were flanked by *lox P* sites. Embryonic stem cells of 129 strain origin (R1 line, derived from a cross between 129X1/SvJ and 129S1/Sv-+^{p} +$^{Tyr\text{-}c}$ Kitl$^{Sl\text{-}J}$/+) (Nagy et al., 1993) with the targeted locus (after electroporation and homologous recombination) were injected into C57BL6/J blastocysts to produce chimeric mice. Chimeric mice were mated to C57BL/6J to produce heterozygous knockin mice that express NCS-1-EGFP fusion protein. NCS-1-EGFP knockin mice were crossed with CMV-Cre transgenic mice (B6.C-Tg(CMV-cre)1Cgn/J). This transgenic line has *cre* under control of a human cytomegalovirus (CMV) minimal promoter ($P_{Bi\text{-}2}$) that is expressed in all tissues, likely before implantation during early embryogenesis (Schwenk et al, 1995). The CMV-Cre mice were originally generated by introducing the CMV-Cre construct into a BALB/cJ derived BALB/c-I

embryonic stem cells and the resulting mice backcrossed to BALB/c for 8 generations then to C57BL/6J for 10 generations. The NCS-1-EGFP x CMV-Cre cross led to deletion of the last four exons coding for NCS-1-EGFP across all tissues including germ cells, producing the *Ncs-1*$^{-/-}$ mice. The *Ncs-1*$^{-/-}$ mice were subsequently backcrossed to C57BL/6J greater than 10 generations; this strain therefore has a background congenic with C57BL/6J with potential contributions from 129 strain alleles flanking the deletion site. After receiving and re-deriving the mice, we maintained the *Ncs-1*$^{-/-}$ strain via heterozygote-heterozygote crosses and further backcrosses to C57BL/6J.

Genotyping was done using by PCR amplification using 3 primers from DNA extracted from tail snips or ear hole punches (5'-GTCCACCCATACCAATCACT-3', 5'-ACAGAGAATCCAAAGCCAGC-3', 5'-TTGTGCTGGAGAAGGGAGAG-3'). Separation of amplification products by gel electrophoresis produces one 398 bp band for wildtype mice, one 514 bp band for homozygous knockout mice, and both product sizes are found from amplification of heterozygote tissue.

2.2 Weight and food restriction

Mice were food-restricted to 85% of baseline weight for operant conditioning tasks as well as Pavlovian approach and conditioned reinforcement (unless otherwise specified). Food restriction was used in order to help motivate mice to learn and perform these tasks. Mice were individually housed, weighed daily, and gradually brought down to 85% of baseline weight over 5 days. Mice were maintained at 85% baseline weight by daily weight monitoring and feeding them an appropriate amount of home chow daily after testing in the operant chambers. Mice were fed some reward pellets (20-mg chocolate-flavoured pellets, Bio-Serv) two days prior to the start of training in the operant chamber, in order to familiarize them with the food.

2.3 Operant acquisition

The ability for mice to learn to make nosepoke responses in order to get food reinforcers was tested, because subsequent tests of food motivation build upon this simple operant response. Moreover, acquisition of simple operant responding may give a rough index of cortico-dorsomedial striatal function (Hart et al., 2014). Mice were tested in a mouse operant chamber (Med Associates Inc. ENV-307A) with a house light, pellet dispenser (ENV-203-20), and two nosepoke response holes (ENV-313M) at either side of pellet receptacle (ENV-303M). The chamber was housed within a sound-attenuating cubicle (ENV-022MD). Chambers were cleaned with 70% ethanol between subjects. In general, the house light was kept on for the duration of a session unless otherwise specified. Procedures were adapted from (Cagniard et al., 2006).

Mice (7 WT and 8 KO littermates from Het x Het crosses; 2 additional WT came from a cross between a Het x C57BL/6J cross) were trained under a fixed ratio 1 (FR1) schedule with only one active nosepoke hole; responses in the inactive nosepoke hole were recorded but had no consequence. The side of the active nosepoke hole was counterbalanced across subjects in each group. A session lasted 45 min or until a subject earned 50 pellets (20-mg chocolate flavoured pellets, Bio-Serv). During the first two days of training, a variable time 60 s (VT 60 s) schedule was in effect in addition to FR1, such that pellets were also delivered on time intervals averaging 60 s with a range of 0 to 120 s. On the fifth and sixth days of training, both nosepoke holes were primed with ground up reward pellet powder. All mice reached a criterion of 50 lever presses within one session by the eighth day of FR1 training.

2.4 Progressive ratio

Progressive ratio performance was used as a test of effort or willingness to work for a reinforcer (Markou et al., 2013; Stewart, 1974), since we predicted that mice lacking NCS-1 would show lower motivation. Subjects were moved to a progressive ratio 3 (PR3) schedule for 5 days. This task was adapted from (Cagniard et al., 2006). In PR3, the first nosepoke response is reinforced, then the number of responses in the active nosepoke hole required per pellet delivered increased by three after each successful trial. After PR3, mice were tested on a PR7 schedule for three weeks, five days per week. Three different food deprivation conditions were used: 85% baseline weight for week 1; 92% baseline weight for week 2; and no food deprivation for week 3. Each session lasted 45 min or until no responses were in the active nosepoke hole for 5 min, whichever came first. The last ratio (number of nosepokes required to get a pellet) completed was defined as the breakpoint. Number of nosepokes into the inactive nosepoke hole was also recorded as a measure of nonspecific activity.

2.5 Food preference test

Food preference was tested as an index of hedonic experience (‘liking’), since decreased preference for sweet reinforcers (often interpreted as anhedonia) can contribute to any decreases in food motivation we find. Subjects were again food deprived to 85% baseline weight. They were given 30 min free access to 5-6 g of home chow or chocolate-flavoured food pellets in a clean empty cage. The amounts of food left were weighed after the session and the amount of each food consumed calculated. Percent preference for chocolate-food pellets was calculated as (100% x (amount of chocolate-flavoured pellets eaten) / (total amount of food consumed)).

2.6 Concurrent choice

Subjects were tested on a concurrent choice task for four weeks, five days a week. The concurrent choice task was conducted to assess another dimension of food motivation, namely effort-based decision making (Markou et al., 2013; Salamone et al., 1991). While responding on progressive ratios of reinforcement tests willingness to work for a reinforcer, the concurrent choice task examines behaviours when animals are confronted with a choice between a high effort/preferred reward option and a low effort/less preferred option. We adapted the task from (Cagniard et al., 2006). Mice had the choice to nosepoke for preferred chocolate-flavoured food reward on a fixed ratio (FR) schedule or to eat less preferred but freely available home chow on the floor of the operant chamber. Days one, three and five of each week involved such a 'choice' or 'FR/chow' condition. Days two and four of each week consisted of a 'no-choice' or 'FR-only' condition, where no home chow as available and food could only be obtained by nosepoking for reward pellets on the fixed ratio schedule. A different FR schedule was used for each week in the following order for the 'choice' and 'no-choice' days: FR30 (week 1), FR20 (week 2), FR10 (week 3), FR5 (week 4). Sessions lasted 30 min. The number of nosepokes and quantity of food consumed (chocolate pellets and home chow) were recorded. The percentage of food obtained by nosepoking was calculated.

2.7 Sensory-specific satiety test

To test whether subjects' actions were under control of the action-outcome (A-O) goal-directed system that is sensitive to changes in outcome value, we performed a sensory-specific satiety test (Rossi and Yin, 2012). Response to sensory-specific satiety was used to help confirm subjects were not making nosepoke responses out of habit (during previous progressive ratio or concurrent choice trials), but were likely making choices based on outcome value and effort required. We adapted the procedure from (Trifilieff et al., 2013). Subjects were moved to a random ratio 10 (RR10) schedule for one day, and then an RR20 schedule for three days prior to the devaluation days (animals need to complete a random number of nosepokes prior to

reinforcement, with the number of nosepokes being 10 or 20 on average). On satiety testing day one, half of the subjects were given free access to home chow (non-devalued condition) and the other half were given free access to chocolate-flavoured reward pellets (devalued condition) for one hour. After the hour of free access, subjects were placed in operant chambers for a 5 min extinction session (with the houselight on and nosepokes being recorded but having no consequence). Subjects underwent two more days of RR20. On satiety testing day two, the subjects that were in the devalued condition (satiated on chocolate-flavoured pellets) were now in the non-devalued condition (satiated on home chow) and those that were previously in the non-devalued condition were now in the devalued condition. A five min extinction session again followed the one hour of free food access. The amount of food eaten during the free access period and the number of nosepokes during the extinction session were recorded. Subjects under action-outcome control would be expected to nosepoke much less on the day when pre-fed chocolate-flavoured pellets than on the day when pre-fed with home chow. Subjects behaving habitually would nosepoke similar amounts whether in the devalued or non-devalued conditions.

2.8 Sucrose preference test

The sucrose preference test is a measure of hedonic preference, with a preference for sucrose solution being typical (Nestler and Hyman, 2010). Mice (9 WT, 7 KO; these mice had undergone fear conditioning 3 weeks prior) were individually housed in home cages with access to sippers from two water bottles; one bottle contained 2% sucrose solution and the other distilled drinking water. Subjects were housed this way for seven days in total, with the first three days being used to acclimate the mice to the arrangement and measures from the last four days being used to measure sucrose preference. Water and sucrose solutions were weighed daily to calculate daily consumption by each subject. The side of the home cage with sucrose solution was counterbalanced across subjects. The placement of the bottles was also switched daily to reduce any confounds from side bias. Sucrose preference was calculated as (100% x (sucrose solution consumed) / (total water and sucrose solution consumed)) and averaged over the four days of testing.

2.9 Progressive delay

The progressive delay tests the ability for mice to continue responding despite lengthening delays between nosepoke response and reinforcer delivery. This test was used to help ensure that differences found in responding for high ratios of reinforcement were indeed due to differences in effort discounting rather than differences in delay discounting (Markou et al., 2013). High ratios of reinforcement alone cannot distinguish the two since it takes longer for mice to complete a high ratio than a lower ratio (thus not only effort is being manipulated but time to reinforcer delivery as well). Mice (10 WT, 12 KO) underwent FR1 training and were kept on FR5. They were then moved to a progressive delay 3 s schedule for five days; the task was adapted from the "progressive interval" task in (Simpson et al., 2011). Chocolate-flavoured pellets were delivered on a FR1 schedule but with a delay between nosepoke and pellet delivery that increased by 3 s every trial. Each session lasted 45 min. Number of rewards earned was recorded as a measure of performance. Mice were restricted to 85% baseline weight during this task.

2.10 Pavlovian approach and conditioned reinforcement

Pavlovian approach and conditioned reinforcement were used to assess incentive motivation or 'wanting.' Procedures were adapted from (Browne et al., 2014). During Pavlovian approach training, the nosepoke holes in the operant chamber were absent and a yellow stimulus light was placed above the food receptacle. During Pavlovian conditioning, a compound CS was presented prior to delivery of chocolate-flavoured pellets on a random time 60 s schedule. 30 CS-US pairings occurred per session, which lasted 40 min on average. Daily sessions were conducted for 14 days. The CS (conditioned stimulus) consisted of houselight off and a 10 s illumination of the yellow stimulus light; a pellet (US, unconditioned stimulus) was delivered 5 s into CS presentation. The main measures were the number of head entries into the food magazine during

the 5 s CS periods prior to US delivery and during the 5 s just before each CS period (pre-CS period). Pavlovian learning was demonstrated by a greater number of head entries during the CS period relative to the pre-CS period.

During conditioned reinforcement, nosepoke holes were introduced on either side of the food magazine. One nosepoke hole was active and the other inactive; the active side was counterbalanced across subjects in each group. The compound CS (houselight off + yellow stimulus light on), followed by the sound of the pellet dispenser (that was devoid of pellets) was delivered on a random ratio 2 (RR2, responses are reinforced randomly but on average every 2 times) schedule of reinforcement. Each session lasted 40 min. Daily sessions occurred for 10 days. The first session was a pre-test session to familiarize animals to nosepoke holes and lasted until 10 nosepokes were made into the active nosepoke hole or 40 min had elapsed. The number of nosepokes into the active and inactive holes were measured. Conditioned reinforcement was demonstrated by a greater number of nosepokes into the active compared to the inactive nosepoke hole.

2.11 Habit formation

Random interval schedules were used to promote the formation of habits (behaviour under stimulus-response control). Habits were distinguished from goal-directed (action-outcome) behaviour by being insensitive to changes in outcome devaluation through sensory-specific satiety. Habit formation was used as an index of dorsolateral striatum; procedures were adapted from (Rossi and Yin, 2012). Mice (7 WT, 8 KO) were trained for six days on a FR1 task. They were then moved to a random interval 30 s (RI 30 s) task for two days followed by six days of random interval 60 s (RI 60 s). Under such schedules of reinforcement, chocolate-flavoured pellets were delivered with the first nosepoke after a random interval that on average lasts 30 or 60 s. RI schedules are habit-inducing because animals are unable to predict when their next nosepoke will lead to reinforcement, so they will tend to continuously nosepoke at high rates

throughout the session. After the sixth day of RI60 training, animals were subjected to sensory-specific satiety as described previously in section 2.7 except that the two days of satiety testing were done on consecutive days.

2.12 Spontaneous and displaced object recognition

Object recognition tests were used to assess short-term memory for objects and for the placement of objects in space (Antunes and Biala, 2012; Clark and Martin, 2005). Experiments were conducted in clear plexiglass chambers (42 x 42 x 42 cm) and adapted from (Saab et al., 2009). The test consisted of three phases. In phase one, a subject was allowed to explore and familiarize itself with four identical objects placed at the corners of the open field for 15 min. The subject was returned to its home cage for a 3 min (delay interval). In the displaced object phase, two of the objects from the previous place were displaced towards the centre of the arena and the animal allowed to explore them for 5 min. Before the next phase, the subject was again returned to its home cage for 3 min. Finally, in the spontaneous object discrimination phase, two objects were present, one familiar (from the previous phases) and one novel. Again, the subject was allowed to explore for 5 min. Each phase was video-recorded. Videos were viewed and manually scored using EthoVision XT8 (Noldus) for time spent exploring the objects in the latter two phases. Active investigation by sniffing within 1 cm of an object was scored as object exploration; contact with the object was not required. Rearing or in rare cases standing on objects was not considered object exploration. Discrimination measures were calculated as percentage of total exploration time spent exploring the different kinds of objects (displaced and non-displaced or novel and familiar) in the latter two phases. Such discrimination measures show the relative interaction between the different objects, and are commonly used in the literature in place of absolute differences in time exploring the different objects because they correct for potential biases in exploratory activity between subjects or conditions (Akkerman et al., 2012a).

2.13 Water maze learning and spatial reversal

Testing in the water maze was used to test for spatial learning, long-term spatial reference memory, as well as spatial reversal learning; procedures were adapted from (Vorhees and Williams, 2006). The water maze consisted of a circular pool (110 cm in diameter) filled with water (24.5 – 26° C) made opaque with non-toxic white tempera. The pool was divided into four equal quadrants: northeast, northwest, southeast and southwest. The platform (12 cm diameter) was submerged 1 cm below the water surface. Distal cues were fixed onto each wall. Activity in the water maze was recorded using a CCD camera on the ceiling above the pool centre; automated tracking was performed using HVS Water 2020. Subjects were handled daily for 5 min for at least 4 days prior to training. During the visual cued trial and initial acquisition, the hidden platform was placed in the southeast quadrant. Six trials were performed per day in two sets of three trials (inter-trial interval within a set was ~30 s; inter-set interval was 1-2 h). On each trial, the subject was released from the edge of the pool (facing the pool wall) and allowed to swim to find the hidden platform. Each trial lasted until the subject found the platform or until 60 s had elapsed. If the subject did not find the platform after 60 s, it was placed on the platform by hand. Every time a subject was on the platform it was left there for 15 s before being returned to a cage where it was warmed under a heat lamp. Subjects were released from a different point at the edge of the pool every day (north, west, southwest, and northeast) in a pseudorandom order. Latency to the target (s), path length (m), and percent time in each quadrant were recorded, and cumulative distance to target (Gallagher's measure) calculated.

Initial spatial acquisition was performed with a hidden platform and the curtains pulled away to allow for allocentric spatial navigation using distal cues. A probe test day was performed in between the sixth and seventh days of spatial acquisition and also after the last day of initial acquisition. During probe test days, the platform was removed. Subjects were placed at the edge of the pool facing the wall opposite that of the target quadrant and allowed to swim for 60 s. Only one trial was performed on probe test days.

After initial acquisition and a probe test of memory for the initial hidden platform location, reversal learning was conducted for the next three days prior to a reversal probe test. During reversal learning trials, the hidden platform was moved to the opposite quadrant (northwest). Six trials were performed per day with subjects being released from a different starting point each day at the pool's edge (south, east, northeast, southwest).

2.14 Waterbox flexibility

The waterbox was used to test for measures of behavioural flexibility, specifically of strategy set shifting and reversal learning. Traditionally, behavioural flexibility in rodents have been tested using food reinforced tasks (in cross mazes or operant chambers etc.), but a water-based task was chosen due to potential differences in food motivation in mice lacking NCS-1.

Apparatus

A visual water box was fabricated based on the design of Prusky et al (2000) and Wong & Brown (2006) (Prusky et al., 2000; Wong and Brown, 2006). The trapezoidal pool had a release chute (35 x 7 x 20 cm) and a 41 cm long midline divider. White plexiglass was used for all the walls except for the wall opposite the release chute (or wide end of the water box) which was transparent. Two identical computer-controlled monitors were placed facing this wide end of the water box. A movable escape platform of transparent plexiglass (20 x 5 x 12 cm) was placed below the monitor displaying the positive visual stimulus (S+). The pool was filled with 14 cm of water (2 cm above escape platform). No additives to the water were needed as reflections of the lit monitors off the water surface made the platform invisible from the water level. The stimuli displayed on the monitors were created on and controlled using Microsoft Powerpoint. The positive visual stimulus (S+) had vertical black and white gratings. The negative visual stimulus (S-) was a uniform grey screen. Room lights were turned off during testing.

Procedure

Testing involved 4 phases: pre-training, visual-cue discrimination, strategy shift to egocentric spatial response, spatial reversal. The procedure was adapted from the visual water box procedure of (Wong and Brown, 2006) and the cross maze procedure from (Floresco et al., 2006). On each test day, mice were removed from their home cage and placed into cages with no bedding but with an absorbent cloth on the bottom together with their cage-mates. Mice were tested in groups of 5-7 for 10 trials per day (12 trials during pre-training) with an inter-trial interval of approximately 5-10 minutes. Between trials, mice were placed under a heat lamp. During testing, mice were transported between the resting cage and the water box with a plastic container. Pre-training took one day; for all subsequent phases, mice were tested for 10 trials a day until they reached a criterion of 8 or more correct trials over two consecutive days.

Pre-training

Over 12 trials, mice were gradually shaped to swim to a hidden platform that was placed below the monitor displaying the S+ (alternating black/white vertical stripes). The S+ and hidden platform were alternated between the two sides of the midline divider separating the two monitors according to the sequence LLLRRRLRLRLR. On trial one, mice were released directly onto the hidden platform and left there for _ min. Afterward, mice were released from gradually increasing distances from the platform (17.5, 35, 52.5, 70 cm) until the last two trials when they were released from the release chute. If a subject did not find the platform within 60 s, it was guided to the platform with a clear plastic paddle and allowed to stay on the platform for 10 s before removal. During each trial, latency to reach the platform was recorded.

Visual-cue discrimination

The S+ and the hidden platform were always placed together and mice were trained to find the hidden platform below the S+. The S+/platform was alternated between the left and right sides pseudo-randomly. The number of trials with the S+/platform on the left and right were balanced daily and the S+/platform was not placed on the same side more than three consecutive trials

within a day. A trial was considered correct if the mouse swam from the release chute to the platform without entering into the opposite arm (breaking the plane perpendicular to the end of the divider on the side of the tank without the platform). An error was scored if the mouse entered the wrong arm (arm without the platform) prior to finding the platform or took more than 60 s to find the platform. If a mouse took more than 60 s to find the platform, it was guided to the platform. In the case of an error, the mouse was immediately made to run another trial before returning to its resting cage. Every time the mouse found the platform, it was left for 10 s prior to removal.

Shift to spatial response

Once a mouse reached criterion on the visual-cue discrimination phase, it needed to shift to always locating the platform in the arm of one side regardless of where the S+ was displayed. The platform was always placed in the side arm opposite of each individual's side bias. Side bias was defined as the side the animal swam to first the most times on the first day of visual-cue discrimination. If they showed no bias on the first day, they were randomly assigned a side bias.

Reversal

After an individual learned to always find the platform on the side opposite its side bias, they needed to shift to find the platform on the side to which it was biased (spatial reversal).

Analysis

Learning and behavioural flexibility were measured on this task based on number of errors committed prior to reaching criterion for each of the phases after pre-training. Errors committed in the shift to spatial response phase were further sub-divided into three types: perseverative, regressive and never-reinforced errors. Perseverative errors were committed when a mouse made an error on a trial where the S+ and platform were located on different sides. Once a mouse made 6 or more correct trials in a day, all subsequent perseverative errors were considered regressive

errors. Never-reinforced errors occurred when a mouse made an error on a trial where the S+ and platform were located on the same side.

2.15 Fear conditioning

Pavlovian conditioning was conducted by associating a mild footshock to a tone and spatial context, and memory assessed by measuring freezing behaviours when re-exposed to the tone or spatial context (with footshock absence). Fear conditioning was used as an index of amygdala function (Maren, 2008). The Med Associates Inc. NIR video fear conditioning system was used. Two different types of context were used. Context A consisted of houselights being on, metal rod flooring, a rectangular enclosure and 70% ethanol olfactory cue. Context B consisted of houselights off, a smooth white plexi-glass floor, an A-frame and a 0.5% acetic acid olfactory cue. The testing chambers were cleaned with 1:5:1 Clidox solution between animals.

On the training day, mice (9 WT, 7 KO) were conditioned within context A. The training procedure involved 120 s of baseline period followed by pairing a tone CS (30 s, 2800 Hz, 85 dB) with a mild shock delivered through the metal rod flooring (2s, 0.75 mA) that co-terminates with the tone CS. The tone-shock pairing was followed by 150 s of no stimulus. 24 h after conditioning, mice were returned to context A for 300 s and their levels of activity and freezing behaviour measured. 48 h after conditioning, mice were placed in context B and their levels of freezing measured during 120 s of no stimulus followed by 180 s of tone presentation.

2.16 Open field locomotion and light/dark box

Subjects were placed into an open field and locomotor activity recorded as an index of general motor activity in a novel environment (Hall and Ballachey, 1932); this is an important measure

as almost all other behaviour tests depend on some measure of motor activity. Procedures were adapted from (Saab et al., 2009). Open field locomotion (8 WT, 12 Het, 9 KO) was conducted in clear plexiglass chambers (42 x 42 x 42 cm, AccuScan Instruments) and activity detected via infrared beam breaks and Versamax software. Animals were allowed 30 min of free activity and exploration in the open field arena.

The light/dark box was used as a measure of anxiety-like behaviour (Pellow et al., 1985). For the light/dark box (7 WT, 11 Het, 9 KO), the open field arena was divided into two compartments with an open doorway between them. The dark compartment was enclosed and made of black plexiglass (with holes to allow for infrared beams to shine through). The light compartment was open, with overhead lighting adjusted to about 200 lux. Sessions lasted five minutes. Percentage of time spent in the light compartment was calculated as an index of anxiety-like behaviour; more time in the light compartment is interpreted as less threat avoidance.

2.17 Accelerating rota-rod

Motor learning was tested using an accelerating rota-rod for mice (Jones and Roberts, 1968) (Panlab/Harvard Apparatus). Subjects (10 WT, 12 KO) were given three trials a day, with 15 min inter-trial intervals, for three consecutive days. Subjects were placed onto the rod while it was rotating slowly at the constant speed of 4 rpm. Rotation was then accelerated (4 to 40 rpm in 300s) and the latency for subjects to fall off or latency for one full passive rotation was recorded.

2.18 Amphetamine-induced locomotion

Amphetamine-induced locomotion and sensitization to amphetamine were used as indices of response to psychostimulant drugs. The open field apparatus described in 3.16 were used.

Subjects (9 WT, 8 KO) had previously gone through operant conditioning and open field locomotion. On day 0, mice were allowed 15 min to habituate to the open field arena, then given a saline injection (10 ml/kg subcutaneous) and returned to the open field arena for 90 min. For the next five days, subjects were allowed 15 min habituation in the open field, injected with 2.5 mg/kg d-amphetamine (Sigma) subcutaneously, then returned to the open field for 90 min. Subjects were then given a three day "withdrawal" period were no drug was administered. After the "withdrawal" period, subjects were given 15 min habituation to the open field, challenged with 2.5 mg/kg amphetamine and returned to the open field for 90 min. 2.5 mg/kg is one of the higher doses in the range that still primarily yields hyper-locomotion rather than stereotypy in C57BL/6 mice as found by (Yates et al., 2007). This dose was chosen because we were looking to detect a decrease in amphetamine-induced locomotion.

2.19 Elevated zero maze

Time spent in the closed quadrants of the elevated zero maze were used as an index of anxiety-like behaviour (Shepherd et al., 1994). Behaviour on the elevated zero maze (34" height x 24" outside diameter; Med Associates Inc.) was recorded using an overhead camera linked to a computer running EthoVision XT 8.0 software (Noldus). Mice (8 WT, 11 Het, 7 KO) were initially placed on an open area and their movements tracked. A session lasted 5 min. Percentage time spent in the open or closed areas of the circular track were calculated.

2.20 Tail suspension test

The tail suspension test was used to obtain an index of the latency for mice to stop struggling and became immobile when suspended by their tail (Steru et al., 1985). Subjects (5 WT (4 female, 1 male), 4 Het (3 female, 1 male), 6 KO (4 female, 1 male)) were suspended by their tails using

tape (3M Durapore) on an apparatus that automatically detects escape movements (Bioseb). The test lasted 6 min.

2.21 Pre-pulse inhibition

Pre-pulse inhibition of the acoustic startle reflex provides an index of sensorimotor gating; procedures were adapted from (Geyer and Dulawa, 2003). Subjects (10 WT, 13 HET, 12 KO) were placed in an animal holder placed on top of a load cell platform connected to a transducer and amplifier to record startle responses. All of this was contained within a sound attenuated box that contained a fan and speakers (San Diego Instruments). The session began with a 5 min acclimation period (background noise from fan only, 65 dB). The session continued by the presentation of different types of trials, each of which were presented 10 times in pseudorandom order with an inter-trial interval that varied randomly between 20-30 s. There were four categories of trials: pre-pulse only, pre-pulse + startling pulse (PPi), startle pulse only, no stimulus. In pre-pulse only trials, 20 ms of white noise stimuli were presented (at 5, 15, 20 or 25 dB above background). In pre-pulse + pulse trials, a 20 ms pre-pulse (5, 15, 20 or 25 dB above background) were delivered 80 ms prior to a 40 ms startling pulse of white noise (110 dB). (10 trials of each pre-pulse intensity were delivered pseudorandomly during the session for both the pre-pulse only and pre-pulse + pulse categories of trials). In startle only trials, the 40 ms 110 dB of white noise was presented alone. In no stimulus trials, only the background noise was present and baseline movements were recorded. Maximal peak amplitude detected was used to determine the acoustic startle response. Percentage pre-pulse inhibition (PPI) for each level of pre-pulse intensity was calculated as (100% x (startle only) – (PPi) / startle only).

2.22 3-chamber social approach

3-chamber social approach was used as a measure of sociability; procedures were adapted from (Kaidanovich-Beilin et al., 2011). The apparatus was made of clear Plexiglass (24 ¾" x 16 ¾" x 8 ¾") with three chambers (each 16 ¾" x 8") with guillotine doors in the walls separating chambers. The apparatus was cleaned with Clidox in-between subjects. An overhead CCD camera and EthoVision XT 8.0 software were used to track and record activity. The task was split into two phases. In the habituation phase subjects (10 WT, 13 HET, 11 KO) were placed in the middle chamber and allowed to freely explore all chambers. In the social preference phase, a novel inverted wire cup (Galaxy Pencil & Utility Cup, Spectrum Diversified Designs) was placed on one side chamber, while an age-matched novel male C57BL6/J mouse was placed under an identical inverted cup in the other side chamber. Which side chamber contained the stranger mouse or the empty inverted cup was counterbalanced across subjects. Time spent in each chamber was extracted from the behavioural tracking software. Time spent sniffing the new objects was tracked in EthoVision and operationalized as time when the nosepoint was within 2 cm of the object without the body or tail points being concurrently within that 2 cm circumference. Planned comparisons (two-tailed T tests) were made between time spent in the stranger room and time spent in the empty cup room (and between time sniffing the stranger and time sniffing the empty cup) based on the recommendations for statistical analysis found in the protocol by (Yang et al., 2011).

2.23 Social interaction

Subjects (4 WT, 6 KO) were tested for reciprocal social interactions with age-matched novel C57BL/6J mice in a clean cage with clean bedding for 15 min (adapted from (Lipina et al., 2013; McFarlane et al., 2008)). Behaviour was recorded using video cameras and EthoVision XT 8.0 software. The following social interaction behaviours with the novel C57BL/6J were manually coded for subject mice: freezing (immobile for > 5 s), digging, allogrooming (grooming the other mouse), self-grooming, push-crawl (pushing past the partner or crawling over/under the partner), face sniffing, anal sniffing and mounting.

2.24 Immunofluorescence

Mice were anaesthetized with isoflurane and transcardially perfused with 15 mL of phosphate buffered saline (PBS) followed by 15 mL of 4% paraformaldehyde in PBS. Brains were rapidly removed and post-fixed overnight in 4% paraformaldehyde. 40 μm coronal sections were sliced using a vibratome (Leica VT1000S) and stored at -4 degrees Celsius in PBS. Sections were blocked for 1 h at room temperature in PBS containing 5% bovine serum albumin (BSA) and 0.1% Triton X-100 (PBS-BT). Sections were incubated overnight with primary antibody in PBS-BT (1:200 Rockland chicken anti-NCS-1; 1:50 NeuroMab mouse anti-DRD2) with gentle rocking. The next day sections were washed thrice with PBS. They were then incubated at room temperature for 2 h in secondary antibody diluted in PBS-BT (1:100 Jackson ImmunoResearch Cy3-conjugated anti-chicken, 1:100 Jackson ImmunoResearch Cy5-conjugated anti-mouse). Slices were washed thrice in PBS, and then mounted onto glass slides with ProLong Gold Antifade (Life Technologies) and glass coverslips for subsequent imaging.

2.25 Immunoblots

Mice were sacrificed by cervical dislocation and brains quickly removed and frozen in liquid nitrogen. Whole brains were lysed using a motorized homogenizer (Fisher Powergen 125) in radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors (Roche) on ice. Samples were centrifuged, supernatant collected and protein concentrations were determined using the BCA assay (Pierce) and a spectrophotometre. 20 μg of whole brain lysate was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto nitrocellulose membranes (Bio-Rad). Membranes were blocked PBS with 5% BSA for 1 h, then incubated overnight at 4 degrees Celsius with primary antibodies (1:1000 BioMol rabbit anti-NCS-1; 1:1000 Millipore rabbit anti-DRD2; 1:5000 Abcam mouse anti-GAPDH). Membranes were washed thrice with PBS, then incubated for 1 h at room temperature with secondary antibodies (1:10,000 Licor IR800 anti-rabbit, IR680 anti-mouse). Membranes were

washed thrice with PBS again and then imaged using the Licor Odyssey Infrared Imaging System.

2.26 Slice biotinylation

Slice biotinylation was conducted to obtain a measure of membrane proteins at the cell surface.

Slices

The protocol was adapted from (Mahadevan et al., 2014). Age-matched littermate wildtype and knockout mice (30-40 days old) were used. Mice were anaesthetized with isoflurane, transcardially perfused with 15 mL of ice-cold sucrose ACSF. The brain was rapidly removed and placed into an icy slurry of sucrose ACSF. 300 µm coronal slices were cut using a vibratome in sucrose ACSF (26 mM $NaHCO_3$, 10 mM glucose, 180 mM sucrose, 1.25 mM NaH_2PO_4, 3 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$) saturated with carbogen (95% O_2 / 5% CO_2; all ACSF solutions were carbogenated). Slices were recovered in a 1:1 mix of sucrose ACSF and normal ACSF (26 mM $NaHCO_3$, 10 mM glucose, 124 mM NaCl, 1.25 mM NaH_2PO_4, 3 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$) at room temperature for 20 min, followed by a second 20 min recovery period in normal ACSF.

Biotinylation

Slices were then incubated in 1 mg/mL biotin (Pierce Sulfo-NHS-SS-Biotin) in carbogen-saturated ACSF for 1 h at 4 degrees Celsius with gentle rotation. Slices were then washed twice with 100 mM glycine in normal ACSF for 5 min at 4 degrees Celsius to quench excess biotin. Finally slices were washed twice for 5 min each in normal ACSF and once in sucrose ACSF before being frozen onto chilled glass slides on powdered dry ice.

Pulldown

Sections were thawed on ice at a later date and the striatum dissected out and placed in RIPA buffer with protease inhibitors (Roche). Samples were sonicated (Q-Sonica with microtip) on ice. Samples were centrifuged, supernatant collected and quantified using the bicinchoninic acid (BCA) assay (Pierce). 25 µg of striatal protein was set apart as a measure of input or total protein. 100 µg of striatal protein in a total volume of 200 µl (made up using RIPA with protease inhibitors) was mixed with 60 µl of 50% slurry of high capacity streptavidin agarose beads (Pierce) and rotated for 3 h at 4 degrees Celsius. Samples were centrifuged and the supernatant corresponding to the unbound fraction were frozen and stored. The beads were then washed three times in RIPA buffer. After the last wash, all solution was removed from beads and the biotin-bound fraction was eluted by adding 50 µl of 2X SDS sample buffer containing dithiothreitol (DTT) followed by a 1 h at room temperature incubation with mixing. The whole volume of this biotin-bound fraction was used in subsequent SDS-PAGE and immunoblotting.

Immunoblotting

SDS-PAGE and immunoblotting was performed as described in 1.25 except for the following changes. The primary antibodies used were: 1:200 Santa Cruz rabbit anti-DRD2 and 1:1000 Invitrogen mouse anti-Transferrin receptor. The secondary antibodies used were: GE Healthcare ECL Horseradish peroxidase conjugated donkey anti-rabbit and sheep anti-mouse. Bands were detected using chemiluminescence (Amersham ECL Prime, GE Healthcare or Western Lightning Plus-ECL, Perkin-Elemer) and film (Denville Scientific). Films were scanned and densitometric analysis performed using ImageStudioLite (Licor).

2.27 Subcellular fractionation

Subcellular fractionation was used to get measures of striatal proteins in total lysates as well as cytosolic and membrane fractions. Mice were sacrificed by cervical dislocation, brains collected and rapidly dissected on ice for striatum which were then frozen in liquid nitrogen. Striata were dissected according to the Richfield1 protocol on the Mouse Phenome Database at the Jackson Laboratory (http://phenome.jax.org/db/q?rtn=projects/docstatic&doc=Richfield1/Richfield1_Protocol). Tissues were homogenized in lysis buffer (50 mM Tris-HCl, pH = 7.4; 150 mM NaCl; 1% NP-40; 1 mM EDTA) with protease inhibitors (Sigma) on ice and shaken at 4° C for 1 h. Samples were then centrifuged and supernatant collected (total protein). The supernatant was then centrifuged at 14,000 g for 1 h at 4° C and the supernatant set apart into new tubes (cytoplasmic fraction). The pellet was dissolved in lysis buffer with 1% Triton-X and protease inhibitors (Sigma), and then shaken at 4° C for 1 h, followed by centrifugation at 500 g for 10 min, and supernatant collected (membrane fraction).

Immunoblotting was performed as described in 3.25 except for the following changes. Blocking was done in 5% non-fat milk. The following primary antibodies were used: Santa Cruz mouse anti-DRD2, Millipore rat anti-DAT, Sigma mouse anti-α-tubulin. Membranes were washed six times, 5 min each. Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature. Bands were detected using chemiluminescence (Clarity Western ECL, Bio-rad) and the ChemiDoc MP system (Bio-rad).

2.28 Fast-scan cyclic voltammetry in acute slices

Fast-scan cyclic voltammetry was used to measure electrically-evoked dopamine release from the nucleus accumbens core in acute coronal slices (Patel and Rice, 2013). Matched pairs of knockout and wildtype mice were used on each experiment day. Mice were anaesthetized with halothane, sacrifice and brains harvested. 300 μm coronal slices were made in ice-cold ACSF.

Slices were recovered in room temperature ACSF for at least 40 min then transferred to a 32° C recording chamber. The carbon fibre electrode (calibrated to 1 μM dopamine) was positioned in the nucleus accumbens core. The stimulating bipolar electrode was placed 200 μm away. After electrode positioning, slices were equilibrated for 20 min; during this time no stimuli were given. Afterward, different types of electrical stimulation were given for the next three phases. In the first stabilization phase, single pulses were given every 2 min 10 min. Second, single pulses were alternated with double pulses (100 Hz) and responses were recorded for 20 min. Third, trains of 20 pulses (10 Hz) were given for 10 min. After the fifth train, the media was changed to ACSF containing 5 μM sulpiride. Single/double pulse stimulation and train stimulations and recording were repeated as above.

Current peaks in nA, detected by oxidation of electrically evoked dopamine release were translated into μM by comparing these values to a 1 μM dopamine standard solution. For single pulses, slices showing dopamine peaks above or below two standard deviations of the mean were excluded (1 KO). This procedure was not repeated for other parameters. The paired pulse ratio was calculated as ((double pulse evoked response) – (immediately preceding single pulse evoked response) / (single pulse evoked response)). Half-lives of dopamine peaks were calculated by fitting a one-phase exponential decay starting at the maximum value of the peak. In some recordings it was not possible get a good fit (1 WT, 1 KO).

2.29 Hippocampal slice electrophysiology

Electrical stimulation and field recordings were performed on acute hippocampal slices to measure hippocampal function and long-term plasticity. Mice were anaesthetized with isoflurane, decapitated and brains quickly removed and placed in ice-cold ACSF. The hippocampi were dissected out and 400 μm slices made using a McIlwain chopper. Slices were allowed to recover in carbogenated ACSF (26 mM $NaHCO_3$, 10 mM glucose, 124 mM NaCl, 1.25 mM NaH_2PO_4, 3 mM KCl; with variable $MgCl_2$ and $CaCl_2$ concentrations depending on the

preparation as specified later) at room temperature for 1-2 h and then transferred to a chamber perfused with 30° C ACSF. Stimulating and recording electrodes were placed along the medial perforant path or the Schaffer collateral pathway. Recordings were obtained using 3 MΩ glass electrodes (containing ACSF without Ca^{2+}) and an Axopatch 1D amplifier (Axon instruments) set to 5 kHz low-pass filtering and digitized at 20 kHz using a Digidata 1200 and pCLAMP8 software. Responses were electrically evoked by pulses spaced 40 ms apart that produced field excitatory postsynaptic potentials (fEPSPs) 40% of the maximum spike-free fEPSP size. Responses were evoked and collected every 20 s throughout the experiment and the slope (10-50% of the fEPSP rising phase) was measured. Input/output data were obtained by recording responses with 0.5, 1, 2, 3, 5, and 7.5 mA stimulation. Short-term plasticity was assessed by recording responses to paired pulses delivered 10, 20, 40, 60, 160, 260, 400, and 600 ms apart.

Dentate gyrus medial perforant path LTP

The protocol from (Saab et al., 2009) was used, because they had found enhancements in LTP with transgenic overexpression of *Ncs-1* in the dentate gyrus, which led us to predict that *Ncs-1* deletion would produce the opposite effect (impaired LTP). Adult male and female mice were used (8-24 weeks old). ACSF contained 2.5 mM $CaCl_2$ and 1.3 mM $MgCl_2$. Only slices that showed stable baseline responses to electrical stimulation (paired pulses 40 ms apart at 40% maximum spike-free fEPSP size every 20 s) over at least 30 min were given tetanic stimulation for LTP induction. The stable baseline helped provide a rough measure of the health of the preparation. LTP induction and recording was done in the presence of 10 μM bicuculline (Tocris) to block $GABA_A$ receptor-mediated inhibition. LTP was induced with 100 Hz tetanus (0.15 ms pulses delivered in 4 trains of 0.5 s duration; trains spaced 20 s apart).

Dentate gyrus medial perforant path mGluR-LTD

LTD was induced chemically using S-DHPG (group I metabotropic glutamate receptor agonist) and in the presence of D-AP5 (NMDAR antagonist), because a previous study had identified that NCS-1 is necessary for mGluR-dependent LTD but not NMDAR-dependent LTD in rat perirhinal cortex (Jo et al., 2008). Mice (21-30 days old) were used. ACSF contained 2.0 mM $CaCl_2$ and 1.3 mM $MgCl_2$. Slices were perfused with D-AP5 (50 μM; Cayman Chemical) for 5

min, then perfused with S-DHPG (50 μM; Cayman Chemical) in the continued presence of D-AP5 for 10 min.

CA1 Schaffer collateral mGluR-LTD

Recordings were also made from the CA1 as a control since the phenomenon of S-DHPG-induced hippocampal LTD was first characterized in the rat CA1 region (Palmer et al., 1997). The same types of subjects, conditions and induction protocol were used as in the dentate gyrus, except that D-AP5 was not present in all cases.

2.30 List of primary antibodies

The table below (2-1) lists the vendor, catalogue number and conditions at which each primary antibody was used for the figures with immunofluorescence or immunoblotting.

Table 2-1. Primary antibodies used.

Figure	Protein	Vendor	Catalogue #	Condition
3-1 a-b 3-14 a	NCS-1 immunofluorescence	Rockland	600-901-216	1:200 in 5% BSA, 0.1% Triton X
3-1 c 3-14 b	GAPDH immunoblot	Abcam	AB9484	1:5000 in 5% BSA
3-1 c	NCS-1 immunoblot	Biomol/Enzo	BML-NL3750	1:1000 in 5% BSA
3-14 a	DRD2 immunofluorescence	UC Davis/NIH NeuroMab	N186/29	1:50 in 5% BSA, 0.1% Triton X
3-14 b	DRD2 immunoblot	Millipore	AB5084P	1:1000 in 5% BSA
3-14 c 3-14 d 3-14 e 3-14 f	DRD2 immunoblot	Santa Cruz	SC-5303	1:200 in 5% non-fat dry milk
3-14 c	TfR immunoblot	Invitrogen	13-6800	1:1000 in 5% non-fat dry milk
3-14 d 3-14 e 3-14 f	DAT immunoblot	Millipore	MAB369	Non-fat dry milk
3-14 d 3-14 e 3-14 f	α-tubulin immunoblot	Sigma Aldrich	T6074	Non-fat dry milk

2.31 Statistical analysis

Sample sizes were chosen based on those used in the literature. As done in the literature, electrophysiological and fast-scan cyclic voltammetry recordings from different acute slices from the same animal were counted as biological replicates. However, no more than 2-3 slices from the same animal were used.

Results are given as mean $\pm$ standard error of the mean. Statistical analyses were conducted on GraphPad Prism 6.0. Data were analyzed using the two-tailed Student's t-test, one-way ANOVA, two-way ANOVA, or two-way ANOVA with one repeated measure. Significant main effects or interactions in ANOVA were followed by post hoc tests with corrections for multiple comparisons. The table below (2-2) describes the type of statistical test used for the results in each figure. Parametric tests assume a near normal distributions and homogenous variances. These assumptions sometimes did not hold true (Table A-1 in the Appendix). Nevertheless, parametric tests were still used, as they tend to be relatively robust even when there are failures to meet these assumptions, especially if sample sizes are similar (Glass et al., 1972).

Table 2-2. Statistical tests used for each figure.

Figure	Data	Statistical test	Correction for multiple comparisons during post-hoc tests
3-2 a-b	Baseline weight, open field	Unpaired two-tailed t tests	
3-2 c-d	FR1 nosepokes, PR3 breakpoints	Two-way repeated measures ANOVA (factors = time, genotype)	
3-2 e-f	PR7	Two-way ANOVA (factors = genotype, target weight)	
3-2 g	Progressive delay 3s	Two-way repeated measures	

		ANOVA (factors = time, genotype)	
3-3 a		Two-way ANOVA (factors = schedule of reinforcement, genotype)	
3-3 a-e	Concurrent choice	Two-way ANOVA (factors = schedule of reinforcement, genotype)	
3-3 f	Sensory-specific satiety	Two-way ANOVA (factors = devalued/non-devalued, genotype)	
3-4 a	Taste preferences	Unpaired two-tailed t tests (note unequal variances between genotypes for sucrose preference)	
3-4 b-f	Pavlovian approach, conditioned reinforcement, habit formation	Two-way repeated measures ANOVA for each genotype (factors = time and (b) preCS/CS, (c) active/inactive nosepoke, or (d-f) genotype)	Bonferroni's for 3-4 d
3-4 g	Sensory-specific satiety (after habit formation)	Two-way ANOVA (factors = devalued/non-devalued, genotype)	
3-5 a-b	Novel/displaced object preference	Unpaired two-tailed t tests (planned comparisons between % preference for novel/displaced objects and 50% chance)	
3-6 a	Water maze acquisition	Two-way repeated measures ANOVA (factors = time, genotype)	
3-6 b-c	Probe tests	Two-way ANOVA (factors = genotype, quadrant)	
3-6 d	Visible platform	Unpaired two-tailed t test	
3-7 a-b, d	Cue-based learning, Shift to turn-based, Reversal	Unpaired two-tailed t tests	
3-7 c	Error analysis	Two-way ANOVA (factors = error type, genotype)	
3-8 a	Context fear	Two-way repeated measures ANOVA (factors = time, genotype)	
3-8 b	Tone fear	Two-way ANOVA (factors = presence/absence of tone, genotype)	
3-9 a-b	Open field locomotion, Accelerating rota-rod	Two-way repeated measures ANOVA (factors = time, genotype)	
3-10 b-f	Open field locomotion (amphetamine)	Two-way repeated measures ANOVA (factors = time, genotype)	
3-11 a-c	Light-dark box, elevated zero maze, tail suspension test	One-way ANOVA	
3-12 a-b	Pre-pulse inhibition,	Two-way ANOVA (factors = pre-	

	acoustic startle response	pulse intensity, genotype)	
3-12 c	Startle response to pulse	One-way ANOVA	
3-13 a	Social approach chamber time	Unpaired two-tailed t-tests (planned comparisons between % time spent in stranger and empty cup chambers for each genotype)[7]	
3-13 b	Social approach sniffing tie	One-way ANOVA	
3-13 c	Social interaction behaviours	Unpaired two-tailed t-tests	
3-14 b	Whole brain DRD2	One-way ANOVA	
3-14 c-f	Immunoblots	Unpaired two-tailed t-tests; paired t-test for striatal slice biotinylation	
3-15 b-c, e-f	Fast-scan cyclic voltammetry	Unpaired two-tailed t-tests	
3-16 a	fEPSP 70-80 min	Two-way ANOVA (factors = genotype, time)	Sidak's correction
3-16 c	Paired pulses	Two-way ANOVA (factors = inter-pulse interval, genotype)	
3-17 a	fEPSP 10-20 min	Two-way ANOVA (factors = genotype, time)	Sidak's correction
3-17 c	Paired pulses	Two-way ANOVA (factors = inter-pulse interval, genotype)	
3-18 a	fEPSP 10-20 min	Two-way ANOVA (factors = genotype, time)	
3-18 c	Paired pulses	Two-way ANOVA (factors = inter-pulse interval, genotype)	

[7] These comparions were planned based on recommendations for statistical analysis in the protocol by Yang et al, 2011.

Yang, M., Silverman, J. L., & Crawley, J. N. (2011). Automated three-chambered social approach task for mice. *Current Protocols in Neuroscience / Editorial Board, Jacqueline N. Crawley ... [et Al.]*, *Chapter 8*(July), Unit 8.26. doi:10.1002/0471142301.ns0826s56

3 Results

3.1 Order of presentation

I first present behavioural results followed by measures of physiological mechanisms that may underlie the behavioural phenotypes. The first section deals with striatal-dependent behaviour, followed by hippocampal-dependent spatial learning and memory (3.2), and a general behavioural screen for phenotypes relevant to neuropsychiatric disorders (3.3). Finally, I describe measures of striatal dopamine signalling (3.4) and hippocampal electrophysiology (3.5).

3.2 *Ncs-1* deletion specifically decreases effort to work for food

Since *Ncs-1* interacts with the dopamine D2 receptor, which is most highly expressed in the striatum and is implicated in diseases with aberrant motivation, we tested the hypothesis that deleting *Ncs-1* impairs motivated behaviours dependent on intact striatal function. After confirming the absence of NCS-1 in knockout mice, we employed a series of operant conditioning tasks to test learning as well as motivation for food rewards. We tested different dimensions of motivated behaviour including effort, hedonic preference ('liking'), and incentive motivation ('wanting').

3.2.1 *Ncs-1* deletion confirmed

Ncs-1 knockout (*Ncs-1*$^{-/-}$) mice were generated using gene targeting by Olaf Pong's group at the University Medical Center Hamburg-Eppendorf (Figure 3-1 a; see Section 2.1 for detailed description). *Ncs-1*$^{-/-}$ mice were fertile, could reach the same age as wildtype mice and do not shown any changes in gross brain morphology (Hermainski, 2012). After re-deriving the mice at

the Toronto Centre for Phenogenomics, we confirmed the absence of NCS-1 through immunoblots of whole brain lysates (Figure 3-1 b) and immunofluorescent staining of coronal slices showing the striatum and hippocampus (Figure 3-1 c-d).

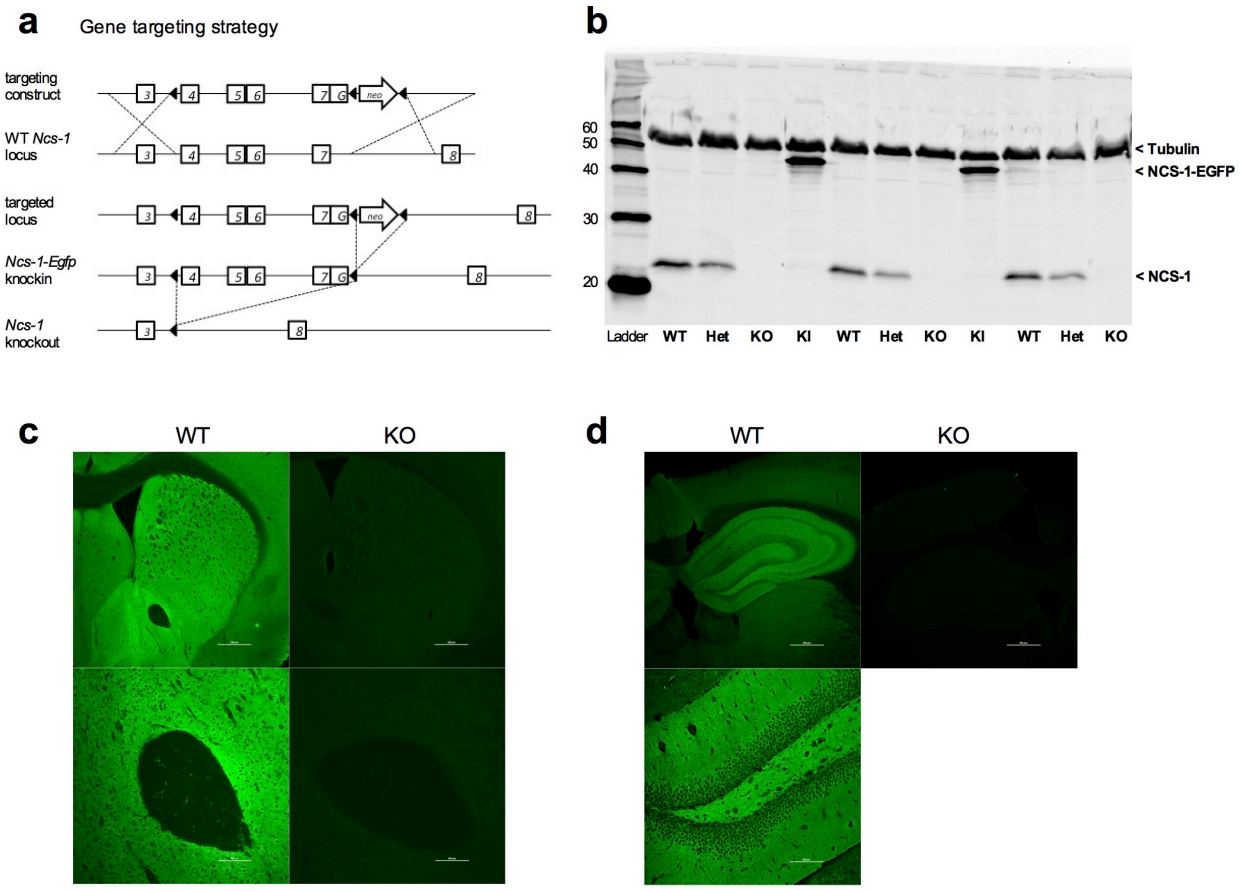

Figure 3-1. *Ncs-1*$^{-/-}$ mice have undetectable levels of NCS-1 protein.
(a) Gene targeting strategy for *Ncs-1*$^{-/-}$ generation. (b) Immunoblots for NCS-1 and β-tubulin III in whole brain lysates from mice wildtype (WT), heterozygous (Het), or null (KO) for *Ncs-1* or that express *Ncs-1-Egfp* (KI). NCS-1 protein could not be detected in *Ncs-1*$^{-/-}$ mice, despite it being detectable in WT and Het mice and NCS-1-EGFP fusion protein being detected in KI mice. (c-d) Immunostains for NCS-1 in coronal slices of wildtype and knockout mice. **(c)** Top panels show 4X images of whole striatum (scale bars = 500 μm); bottom panels show 20X images of nucleus accumbens (scale bars = 100 μm). **(d)** Top panels show 4X images of hippocampus (scale bars = 500 μm); bottom panel shows 20X image of dentate gyrus (scale bars = 100 μm).

3.2.2 *Ncs-1*$^{-/-}$ mice readily acquire operant conditioning

Ncs-1$^{-/-}$ mice show similar or slightly lower total locomotor activity levels (WT: 4470 $\pm$ 473.6 cm, n = 17; KO: 3773 $\pm$ 249.3 cm, n = 18; $t_{33} = 1.324$, $p = 0.1946$) (Figure 3-2 b) to wildtype littermates and weigh on average 3.8 g more (WT: 25.86 $\pm$ 1.095 g, n = 9; KO: 29.67 $\pm$ 0.8131 g, n = 9; $t_{16} = 2.796$, $p = 0.0130$) (Figure 3-2 a). Mice were food deprived to 85% baseline weight and trained on operant conditioning paradigms where they nosepoke for chocolate-flavoured pellets. Both genotypes readily acquired a fixed ratio 1 (FR1) task with a 1:1 ratio of nosepoke to food reward (time effect, $F(7,105) = 46.09$, $p < 0.0001$; non-significant (ns) genotype effect, $F(1,105) = 0.88$, $p = 0.3620$; ns interaction, $F(7,105) = 0.5245$, $p = 0.8143$) (Figure 3-2 c), demonstrating intact action-outcome learning.

3.2.3 *Ncs-1*$^{-/-}$ mice show decreased motivation to work for food

To test willingness to work for food reward, we employed progressive ratio schedules of reinforcement, where the ratio of nosepokes per reward increased by three or seven (PR3 and PR7) on succeeding trials within a session. The last ratio reached before mice stopped responding for five minutes or the 45-minute session ended was defined as the breakpoint. *Ncs-1*$^{-/-}$ mice had significantly lower breakpoints than wildtype mice on both a PR3 (genotype effect, $F(1,60) = 6.23$, $p = 0.0247$; time effect, $F(4,60) = 20.36$, $p < 0.0001$; ns interaction, $F(4,60) = 1.1789$, $p = 0.1428$) and PR7 schedule (genotype effect, $F(1,45) = 8.50$, $p = 0.0055$; food deprivation effect, $F(2,45) = 37.78$, $p < 0.0001$; ns interaction ($F(2,45) = 1.064$, $p = 0.35$) (Figure 3-2 d-e). On the PR3 schedule, *Ncs-1*$^{-/-}$ mice reached breakpoint at ratios requiring on average 14.5 nosepokes less than wildtype mice, meaning they stopped nosepoking or the session ended 3 trials earlier than wildtype mice. Similarly on the PR7 schedule, *Ncs-1*$^{-/-}$ mice reached breakpoint at ratios requiring on average 27 nosepokes less than wildtype mice, which translates to *Ncs-1*$^{-/-}$ mice giving up about 3-4 trials ahead of wildtype mice.

Breakpoint differences were not due to activity differences in the operant chamber as the number of responses in an inactive nosepoke hole was similar between genotypes (ns genotype effect, $F(1,45) = 0.58$, $p = 0.5658$; food deprivation effect, $F(2,45) = 7.835$, $p = 0.0012$; ns interaction, $F(2,45) = 0.5767$, $p = 0.5658$) (Figure 3-2 f). Progressive ratio performance can also be affected by the fact that the interval between the start of each trial and reinforcer delivery progressively lengthens since it takes more time to complete each ratio. To control for this, we compared the genotypes on a progressive delay schedule, in which a reward is given each trial after one nosepoke but the delay between the first nosepoke and food delivery increases by 3 s every trial. *Ncs-1*$^{-/-}$ mice earned a similar number of rewards as wildtype mice on a progressive delay schedule (ns genotype effect, $F(1,20) = 0.05562$, $p = 0.8159$; time effect, ($F(4,80) = 5.319$, $p = 0.0008$; ns interaction, $F(4,80) = 1.038$, $p = 0.3929$), suggesting breakpoint differences were not confounded by differences in response to delays in reward (Figure 3-2 g).

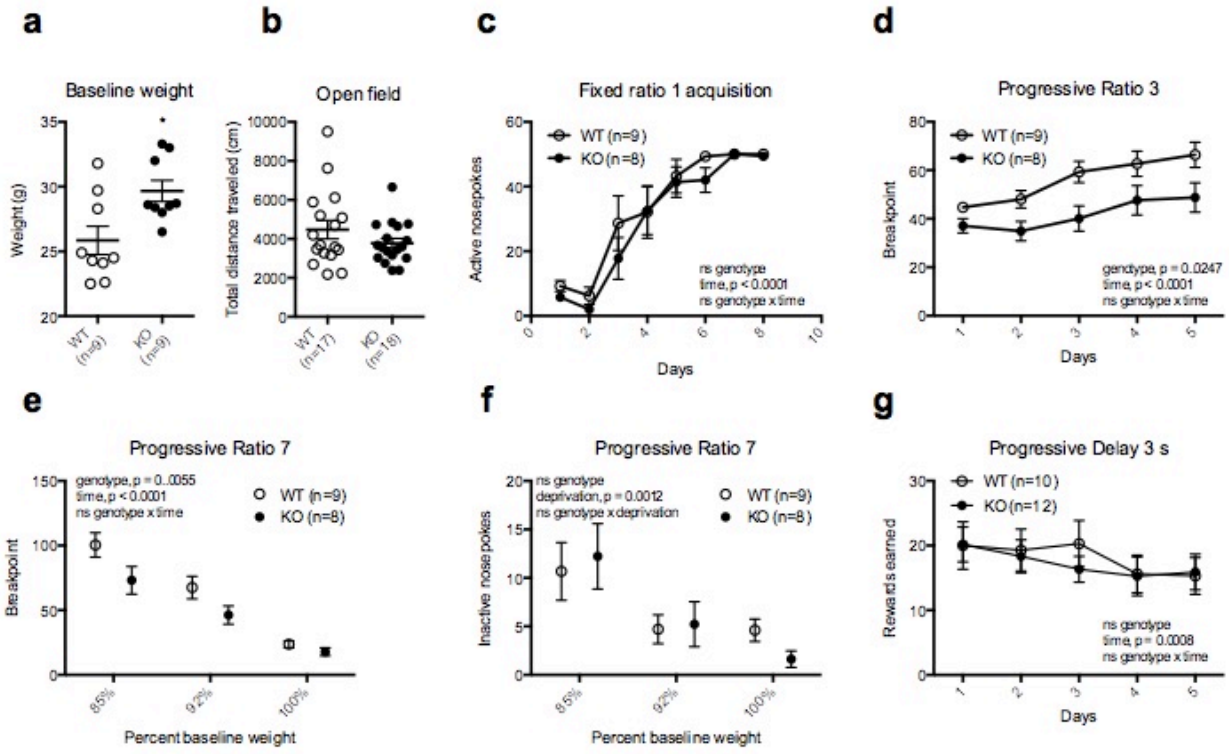

Figure 3-2. *Ncs-1*$^{-/-}$ mice show higher weight and a decreased motivation to work for food in progressive ratio tests.
(a) *Ncs-1*$^{-/-}$ mice have a higher baseline weight than wildtype mice. **(b)** *Ncs-1*$^{-/-}$ mice show similar levels of open field locomotion to wildtype mice. **(c)** *Ncs-1*$^{-/-}$ mice readily acquire operant conditioning on an FR1 schedule. *Ncs-1*$^{-/-}$ mice show lower breakpoints on a **(d)** progressive ratio 3 schedule and **(e)** progressive ratio 3 schedule when deprived to 85% baseline weight. **(f)**

The genotypes show similar levels of activity in the inactive nosepoke hole. **(g)** *Ncs-1*$^{-/-}$ mice obtain similar numbers of rewards as wildtype mice in a progressive delay task, suggesting breakpoint differences in previous panels are not due to differences in ability to tolerate delays in reward. * = $p < 0.05$. Error bars = SEM. ns = non-significant.

3.2.4 *Ncs-1*$^{-/-}$ mice choose the less effortful option

We next tested willingness to work for a preferred food reward (chocolate-flavoured pellets) when a less preferred food (home chow) was concurrently available. Over four weeks, mice were given a choice between rewards (home chow or pellets) for three days of the week and tested on regular fixed ratio (FR) schedules the other two days. *Ncs-1*$^{-/-}$ mice nosepoked significantly less than wildtype mice during FR-only days (genotype effect, $F(1,60) = 23.02$, $p < 0.0001$; schedule effect, $F(3,60) = 4.532$, $p = 0.0063$; ns interaction, $F(3,60) = 1.564$, $p = 0.2075$) from ~25% at FR5 to ~50% less at high ratios such as FR30 and FR20, suggesting decreased motivation particularly under high work requirements (Figure 3-3 a). On choice days, *Ncs-1*$^{-/-}$ mice nosepoked less for chocolate pellets than wildtype mice (genotype effect, $F(1,60) = 6.206$, $p = 0.0155$; ns schedule effect, $F(3,60) = 1.045$, $p = 0.3794$; ns interaction, $F(3,60) = 0.8559$, $p = 0.9677$), consumed more home chow (genotype effect, $F(1,60) = 6.78$, $p = 0.0116$; schedule effect, $F(3,60) = 8.195$, $p = 0.0001$; ns interaction, $F(3,60) = 1.205$, $p = 0.3156$), and obtained a lower percentage of their food from nosepoking (genotype effect, $F(1,60) = 8.09$, $p = 0.0061$; schedule effect, $F(3,60) = 19.42$, $p < 0.0001$; ns interaction, $F(3,60) = 0.6910$, $p = 0.5611$) (Figure 3-3 b-d). The difference on the choice days was most pronounced under the FR5 schedule, when *Ncs-1*$^{-/-}$ mice nosepoked ~40% less than wildtype mice and ate ~50% more chow. Nevertheless, overall on choice days, wildtype mice ate more total food (reward pellets and chow) than *Ncs-1*$^{-/-}$ mice (genotype effect, $F(1,60) = 4.937$, $p = 0.0301$; schedule effect, $F(3,60) = 26.25$, $p < 0.0001$; ns interaction, $F(3,60) = 1.385$, $p = 0.2560$). Together these data suggest *Ncs-1*$^{-/-}$ mice are more likely to choose a less effortful option.[8]

[8] This interpretation comes with the caveat that the greater weight of *Ncs-1*$^{-/-}$ mice could also contribute to this difference in effort-related decision making for food, potentially due to differences in metabolism relative to wildtype mice. Future studies with conditional, site-specific knockout mice that do not display differences in weight would be needed to rule out this alternate explanation.

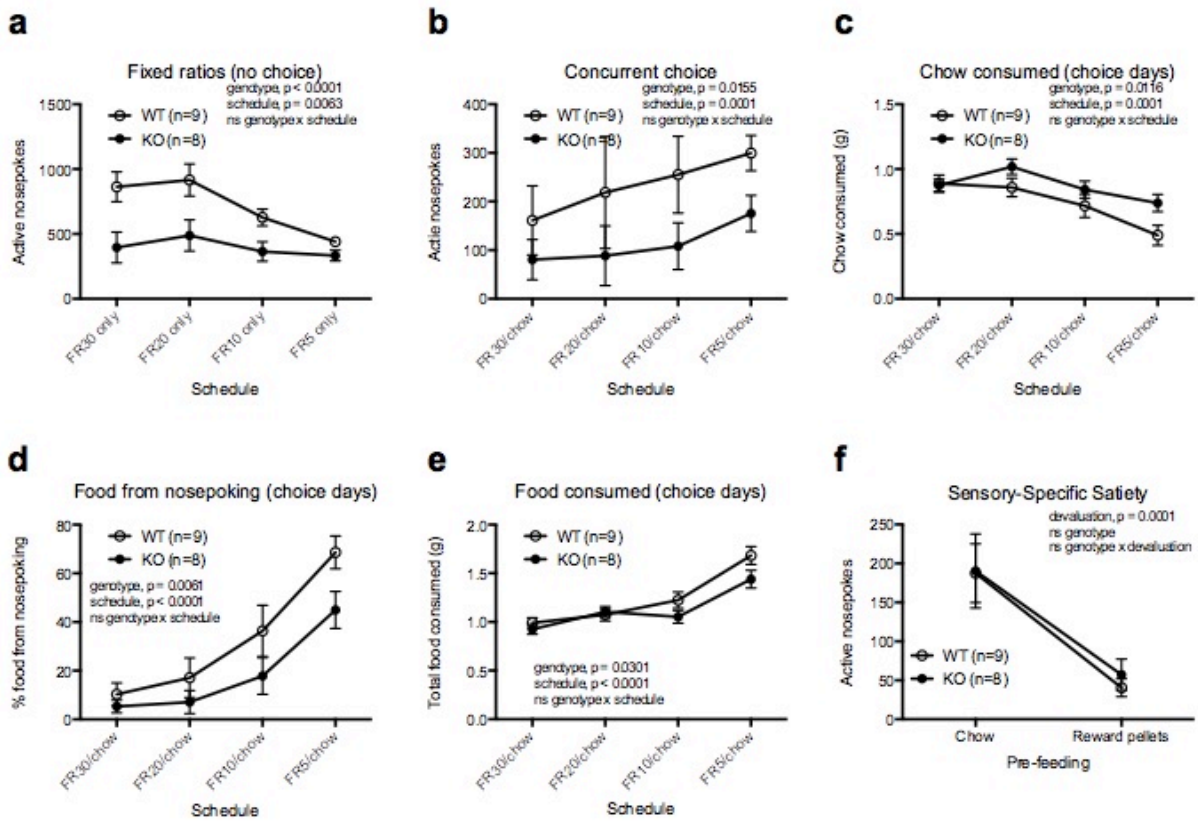

Figure 3-3. *Ncs-1*$^{-/-}$ mice choose the less effortful option more than wildtype mice in an effort-related task and can encode changes in outcome value.
(a) *Ncs-1*$^{-/-}$ mice nosepoke significantly less than wildtype mice under higher ratios (FR30, FR20) but nosepoke similarly at FR5. **(b)** *Ncs-1*$^{-/-}$ mice nosepoke less than wildtype mice on choice trials when free home chow is available, even on the FR5 schedule. **(c)** *Ncs-1*$^{-/-}$ mice tend to consume more chow than wildtype mice on choice days. **(d)** *Ncs-1*$^{-/-}$ mice obtain less of their food from nosepoking on choice days. **(e)** The genotypes eat similar amounts of food. **(f)** Both genotypes nosepoke significantly less when satiated on reward pellets compared to home chow, showing sensitivity to changes in outcome value. Error bars = SEM. ns = non-significant.

3.2.5 *Ncs-1*$^{-/-}$ mice can represent changes in reward value

The differences in the effort-based choice task were not due to habitual responding on the part of wildtype mice or an inability to represent changes in reward value in the *Ncs-1*$^{-/-}$ mice, as both *Ncs-1*$^{-/-}$ mice and wildtype mice reduced their responding to similar degrees after being satiated on reward pellets (devaluation effect, $F(1,30) = 19.05$, $p = 0.0001$; ns genotype effect, $F(1,30) =$

0.09, $p = 0.7606$; ns interaction, F(1,30) = 0.04673, $p = 0.8303$) (Figure 3-3 f), indicating that they were sensitive to a decrease in reward value.

3.2.6 *Ncs-1*$^{-/-}$ mice do not 'like' or 'want' foods less and readily form habits

While deletion of *Ncs-1* clearly impaired effort, we wondered if other dimensions of motivation were affected. To test if *Ncs-1*$^{-/-}$ mice were anhedonic and 'liked' sweet food rewards less than wildtype mice, we tested their preference for chocolate-flavoured pellets compared to home chow, and plain water compared to 2% sucrose solution. *Ncs-1*$^{-/-}$ mice, similarly to wildtype mice showed a preference for reward pellets over chow (food type effect, F(1,32) = 16.05, $p = 0.0003$; ns genotype effect, F(1,32) = 2.812, $p = 0.1033$, ns interaction, F(1,32) = 0.2421, $p = 0.6261$) and sucrose over water with no significant genotype effects (fluid type effect, F(1,28) = 71.10, $p < 0.0001$; ns genotype effect, F(1,28) = 0.1107, $p = 0.7419$; ns interaction, F(1,28) = 0.1448, $p = 0.7064$) (Figure 3-4 a).

We also tested for differences in incentive motivation, which can be measured by the ability of a conditioned stimulus (CS) that is paired with an unconditioned stimulus like a drug of abuse or food to itself become 'wanted', such that the CS alone can serve as a reinforcer for behaviour. Both genotypes readily learned to approach the food magazine when a cue light signaling food delivery came on (Figure 3-4 b). Significantly more magazine entries were made during the CS period than during an equivalent period of time just before the CS and the number of CS period magazine entries increased over time while preCS magazine entries were relatively stable (WT: CS-preCS effect, F(1,16) = 4.611, $p = 0.0474$; time effect, F(13,208) = 2.672, $p = 0.0017$; interaction, F(13,208) = 4.203, $p < 0.0001$) (KO: CS-preCS effect, F(1,26) = 78.70, $p < 0.0001$; time effect, F(13,338) = 14.73, $p < 0.0001$; interaction, F(13,338) = 18.00, $p < 0.0001$). Moreover, both genotypes nosepoked for the presentation of the stimulus light alone serving as a conditioned reinforcer (Figure 3-4 c). Subjects nosepoked significantly more in the active response hole than the inactive one (WT: active-inactive effect, F(1,16) = 7.938, $p = 0.0124$;

time effect, $F(9,144) = 3.699$, $p = 0.0003$; ns interaction, $F(9,144) = 1.299$, $p = 0.2421$) (KO: active-inactive effect, $F(1,26) = 15.95$, $p = 0.0005$; ns time effect, $F(9,234) = 0.0924$; ns interaction, $F(9,234) = 0.2149$, $p = 0.9922$). Comparing the genotypes on these two tasks, *Ncs-1*$^{-/-}$ mice actually learned Pavlovian approach quicker and showed a greater difference between preCS and CS magazine entries than wildtype mice (interaction, $F(13,273) = 1.944$, $p = 0.0256$; genotype effect, $F(1,21) = 4.760$, $p = 0.0406$; time effect, $F(13,273) = 21.96$, $p < 0.0001$), particularly on days 8 and 9 (multiple comparisons between genotypes with Bonferroni's correction show $t_{294} = 3.463$, $p < 0.01$ on day 8 and $t_{294} = 3.109$, $p < 0.05$ on day 9) (Figure 3-4 d). However, the genotypes did not differ in nosepoking for conditioned reinforcement (ns interaction, $F(9,189) = 1.196$, $p = 0.2998$; ns genotype effect, $F(1,21) = 1.415$, $p = 0.2474$; ns time effect, $F(9,189) = 0.9329$, $p = 0.4977$) (Figure 3-4 e).

Yet another dimension of motivation is the ability for behaviours to come under habitual control (stimulus-response learning), such that organisms respond in a way insensitive to changes in the value of an outcome. We induced habitual nosepoke responses using random interval (RI) schedules, which promote near constant nosepoking by mice over each session. Again *Ncs-1*$^{-/-}$ mice tended to nosepoke less in the schedules with higher work demands (interaction, $F(13,169) = 2.38$, $p = 0.0058$; time effect, $F(13,169) = 33.22$, $p < 0.0001$; ns genotype effect, $F(1,13) = 3.915$, $p = 0.0694$; post hoc tests with Bonferroni's correction $p < 0.05$ for genotype differences for the last two days of RI 60s: $t_{182} = 3.204$, $t_{182} = 3.004$, respectively) (Figure 3-4 f). Nevertheless, both genotypes readily formed habits as evidenced by an insensitivity to reinforcer devaluation. After one hour of pre-feeding with pellets, both wildtype mice and *Ncs-1*$^{-/-}$ mice continued to nosepoke. Indeed, they nosepoked at similar levels to days when they were pre-fed with home chow (ns prefeeding effect, $F(1,26) = 2.12$, $p = 0.1577$; ns genotype effect, $F(1,26) = 0.5719$, $p = 0.4563$; ns interaction, $F(1,26) = 0.01239$, $p = 0.9122$) (Figure 3-4 g).

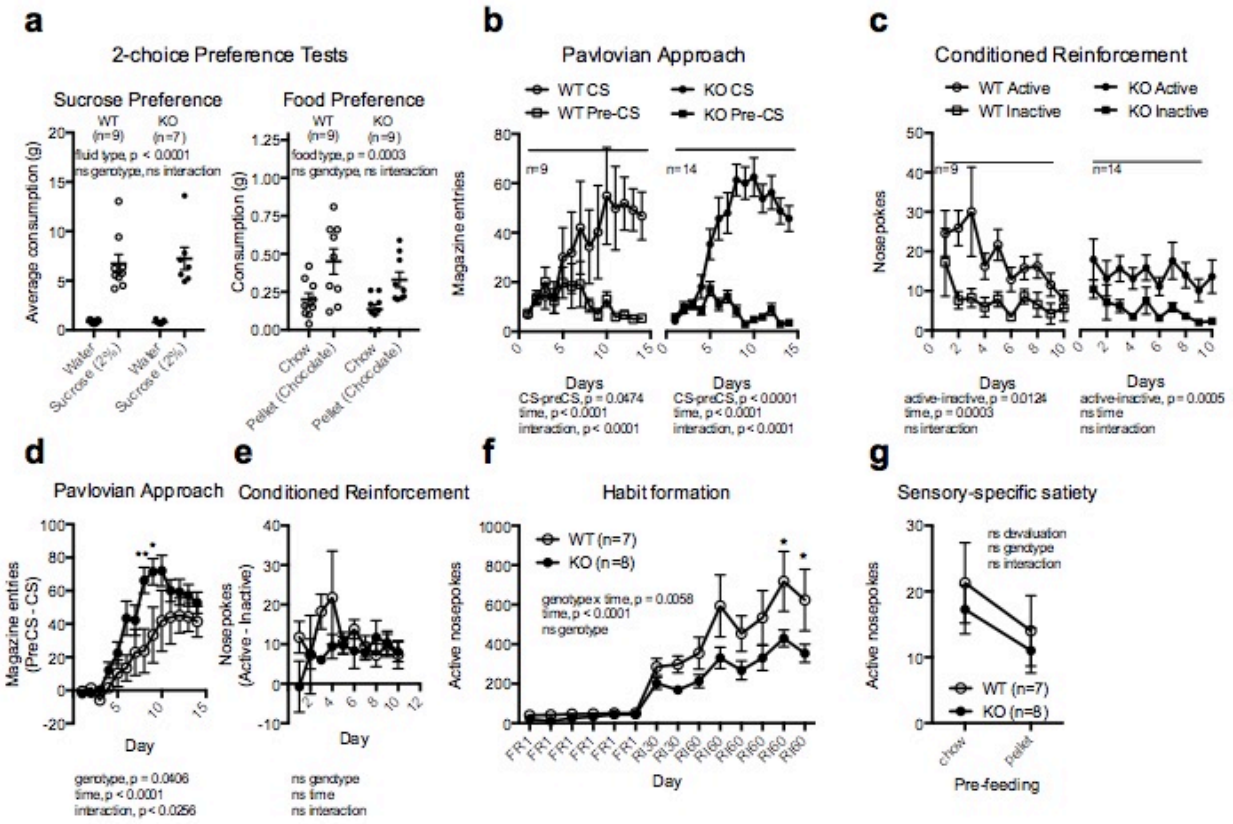

Figure 3-4. *Ncs-1*$^{-/-}$ mice do not 'like' or 'want' food rewards less and have intact habit formation.

(a) *Ncs-1*$^{-/-}$ mice show similar levels of preference as wildtype mice for chocolate reward pellets over home chow and for a sucrose solution over pure water. **(b, d)** Both genotypes readily learn to approach a food magazine upon the turning on of a light stimulus paired with reward delivery. **(c, e)** Both genotypes readily learn to nosepoke to turn on the cue light, suggesting intact incentive motivation or "wanting." **(f-g)** Both genotypes form habits after 6 days of RI60 training, and were insensitive to outcome devaluation as measured by sensory-specific satiety. * = $p < 0.05$. ** = $p < 0.01$. Error bars = SEM.

3.2.7 Summary

We found that *Ncs-1* deletion in mice specifically impairs behavioural activation or effort to work for food rewards, without affecting action-outcome learning, habit formation or other dimensions of motivation such as hedonic preference and incentive motivation. Moreover, it is unlikely that differences in operant responses under high work requirements seen across different paradigms are due to differences in motor function, ability to represent changes in reward value, or differences in ability to work for delayed words.

3.3 *Ncs-1* deletion may impair spatial pattern separation

Given previous work showing hippocampal NCS-1 can enhance spatial learning and that NCS-1 is required for mGluR LTD, we tested whether *Ncs-1* deletion impairs spatial learning and memory dependent on the hippocampus as well as behavioural flexibility. We employed a series of behavioural assays for different forms of spatial cognition from short-term object recognition memory to long-term spatial reference memory in the Morris water maze. Behavioural flexibility was tested using water-based tasks rather than food-motivated tasks given the differences we found in the *Ncs-1*$^{-/-}$ mice for motivated behaviour.

3.3.1 *Ncs-1*$^{-/-}$ mice show impaired displaced object preference

In order to test short-term spatial memory, we used the novel object preference and displaced object preference tests. Object preference was calculated as percent total exploration time spent interacting with various objects. Both genotypes preferred exploring the novel object over the familiar object, spending ~30% more of their time with the novel object (planned comparison of novel versus 50% chance preference; WT: $t_{12} = 3.780$, $p = 0.0026$; KO: $t_{12} = 2.741$, $p = 0.0179$) (Figure 3-5 a). These comparisons to a fictive 50% chance group were made as recommended in

(Akkerman et al., 2012b). The two genotypes showed similar levels of preference for the novel object (planned comparison, $t_{12} = 0.7463$, $p = 0.4698$). In contrast, in the displaced object test, *Ncs-1*$^{-/-}$ mice spent equal amounts of time exploring the displaced and stationary objects (planned comparison of displaced versus 50% chance preference; $t_{36} = 1.620$, $p = 0.1140$), while wildtype mice spent ~63% of their time exploring the displaced object (planned comparison of displaced versus 50% chance preference; $t_{34} = 4.670$, $p < 0.0001$) (Figure 3-5 b). There was a non-statistically significant trend for *Ncs-1*$^{-/-}$ mice to have lower displaced object preference than wildtype mice (planned comparison, $t_{35} = 1.949$, $p = 0.0593$). Velocities ($t_{35} = 1.170$, $p = 0.2498$) and distance traveled ($t_{35} = 1.076$, $p = 0.2892$) were similar between knockout and wildtype mice when testing for displaced object preference (Figure 3-5 b), suggesting differences in displaced object preference are unlikely to be due to activity differences. These data may suggest a selective impairment in spatial location memory.

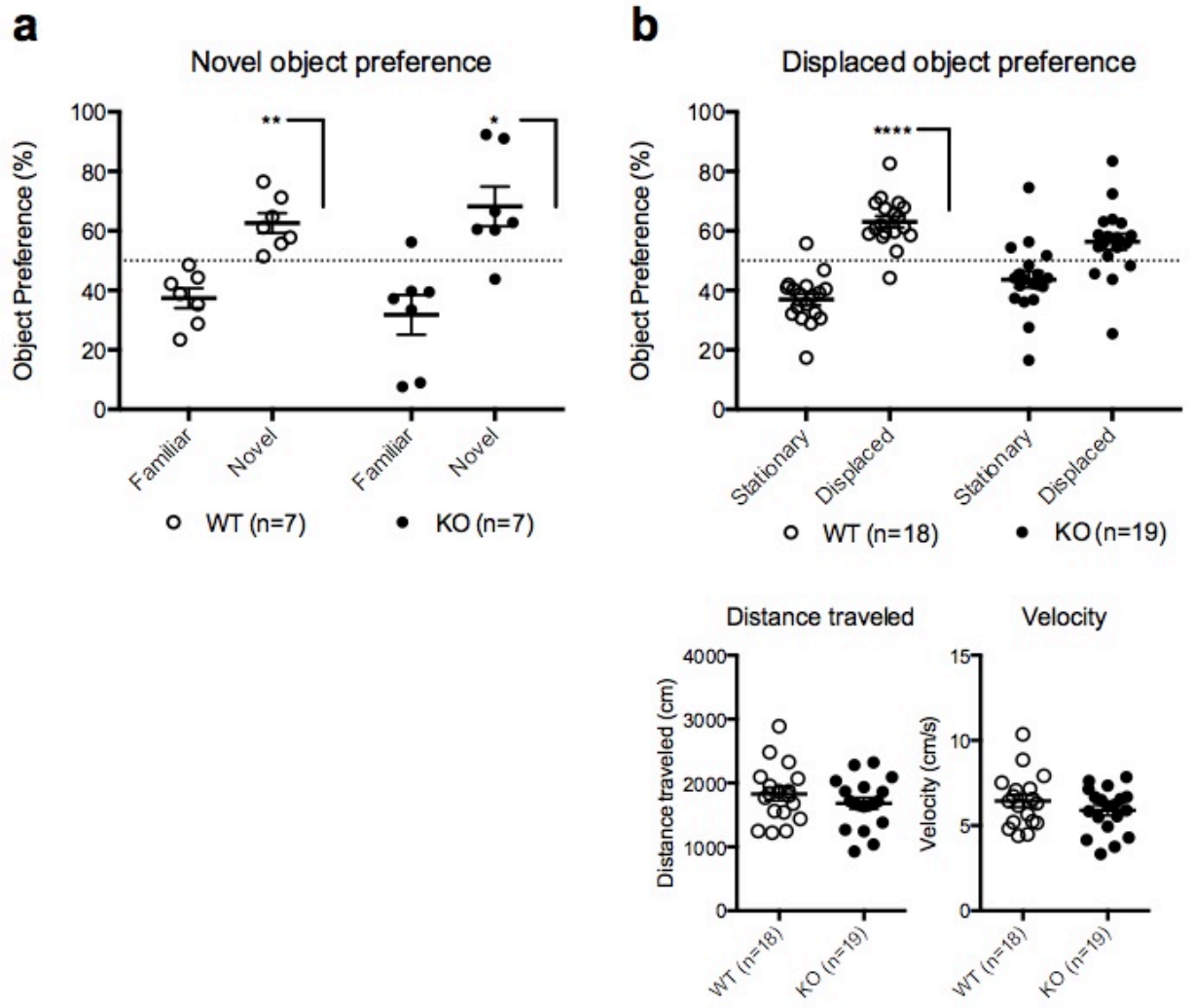

Figure 3-5. *Ncs-1*$^{-/-}$ mice show impaired displaced object recognition.
(a) Both genotypes show a preference for exploring a novel object. **(b)** *Ncs-1*$^{-/-}$ mice showed no preference for exploring a displaced object. The two genotypes showed similar amounts of distance traveled and similar velocities during the displaced object testing phase. **** = $p <$ 0.0001. ** = $p < 0.01$. * = $p < 0.05$. Error bars = SEM.

3.3.2 *Ncs-1*$^{-/-}$ mice show normal spatial reference memory

Spatial learning and long-term spatial reference memory were assessed in the Morris water maze. Both genotypes learned the location of the platform at the same rate (ns genotype effect, $F(1,18) = 0.1115$, $p = 0.7423$) with both genotypes finding the hidden platform more efficiently with

practice (time effect, F(14,252) = 22.45, $p < 0.0001$; ns interaction, F(14,252) = 0.3822, $p = 0.9791$) (Figure 3-6 a). A probe test 24 hours after the last acquisition trial on day 12 showed that both genotypes spent the highest percentage of their total swimming path in the target south-east quadrant (quadrant effect, F(3,72) = 19, $p < 0.0001$; ns genotype effect, F(1,72) = 1.105e-5, $p = 0.9974$; ns interaction, F(3,72) = 0.7415, $p = 0.5308$) (Figure 3-6 b). Both genotypes spend more of their swimming path in the target quadrant than the non-target quadrants. The two genotypes also had similar performance on the probe test when evaluated in terms of cumulative distance to target, which has been shown to be the most sensitive measure of water maze probe test performance (Maei et al., 2009) ($t_{18} = 0.5709$, $p = 0.5751$) (Figure 3-6 d).

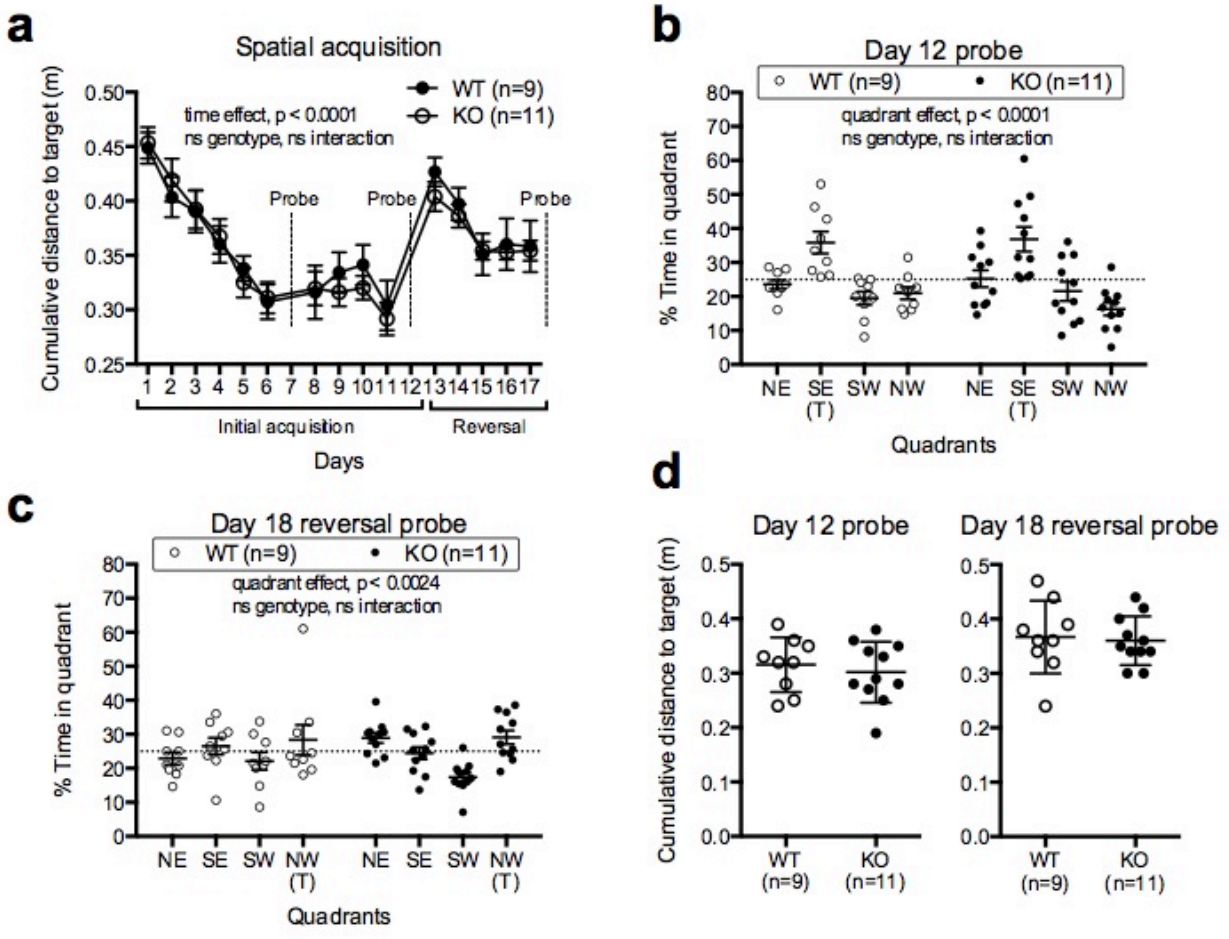

Figure 3-6. *Ncs-1*$^{-/-}$ mice show intact spatial learning and spatial reference memory in the Morris water maze.
(a) *Ncs-1*$^{-/-}$ mice learned the location of a hidden platform at a similar rate to wildtype mice. **(b)** Day 12 probe trial. **(c)** Neither genotype perseverated at the old target quadrant. **(d)** The genotypes had similar cumulative proximity measures to the target location on the probe and reversal probe days. (T) = target quadrant. Error bars = SEM.

After the day 12 probe test, the hidden platform was moved to the opposite quadrant to test for the spatial reversal dimension of behavioural flexibility. *Ncs-1*$^{-/-}$ mice and wildtype mice performed similarly. On a probe test 24 hours after the last reversal training trial, neither genotype perseverated in the old target quadrant, and both showed a very slight trend toward spending more time in the new target quadrant (quadrant effect, $F(3,72) = 5.290$, $p < 0.0024$; ns genotype effect, $F(1,72) = 8.596e\text{-}6$, $p = 0.9977$; ns interaction, $F(3,72) = 1.996$, $p = 0.1222$) (Figure 3-6 c) and they had similar cumulative distances to the new target quadrant ($t_{18} = 0.2659$, $p = 0.7933$) (Figure 3-6 d).

3.3.3 *Ncs-1*$^{-/-}$ mice show normal behavioural flexibility

To test the ability to shift strategy or behavioural flexibility and for an additional measure of spatial reversal, we used the visual water box. Both genotypes reached a criterion of >80% performance for two consecutive days in learning to associate a visual cue (vertical black and white stripes) with a hidden escape platform, but the *Ncs-1*$^{-/-}$ mice committed significantly more errors before reaching criterion ($t_{16} = 2.901$, $p = 0.0104$) (Figure 3-7 a). *Ncs-1*$^{-/-}$ mice and wildtype mice committed similar numbers of errors before shifting from the visual cue-based strategy to an egocentric turn-based strategy to find the hidden platform ($t_{16} = 0.5528$, $p = 0.5880$) (Figure 3-7 b). An analysis of the types of errors committed during the strategy shift did not show any significant differences between genotypes (perseverative: $t_{16} = 0.6702$, $p = 0.5123$; regressive: $t_{16} = 1.177$, $p = 0.2565$; never-reinforced: $t_{16} = 0.4128$, $p = 0.6852$) (Figure 3-7 c). Reversal was tested by moving the hidden platform to the opposite side of the water box after mice had successfully shifted from the cue- to the turn-based strategy. Again, the genotypes made a similar number of errors prior to reaching criterion ($t_{16} = 0.2397$, $p = 0.8136$) (Figure 3-7 d). Together with the Morris water maze spatial reversal data, these results from the visual water box suggest that *Ncs-1* deletion does not impair behavioural flexibility.

3.3.4 *Ncs-1*$^{-/-}$ mice show normal fear memory

After testing striatal-dependent behaviours (Section 3.1) and hippocampal-dependent spatial learning, we also tested integrity of fear memory formation, which is primarily amygdala-dependent. Moreover, a qualitatively different and perhaps stronger reinforcer than food or water (foot shock) is used in this paradigm. *Ncs-1*$^{-/-}$ mice showed similar amounts of freezing when exposed to the same context where it had received tone-shock pairings 24 hours before (ns genotype effect, $F(1,14) = 0.40$, $p = 0.5395$; time effect, $F(4,56) = 79.30$, $p < 0.0001$; ns interaction, $F(4,56) = 0.8138$, $p = 0.5217$) (Figure 3-8 a). Levels of freezing were also similar between genotypes when they were exposed to the shock-associated tone 48 hours post-conditioning ($t_{14} = 0.2352$, $p = 0.8174$) (Figure 3-8 b).

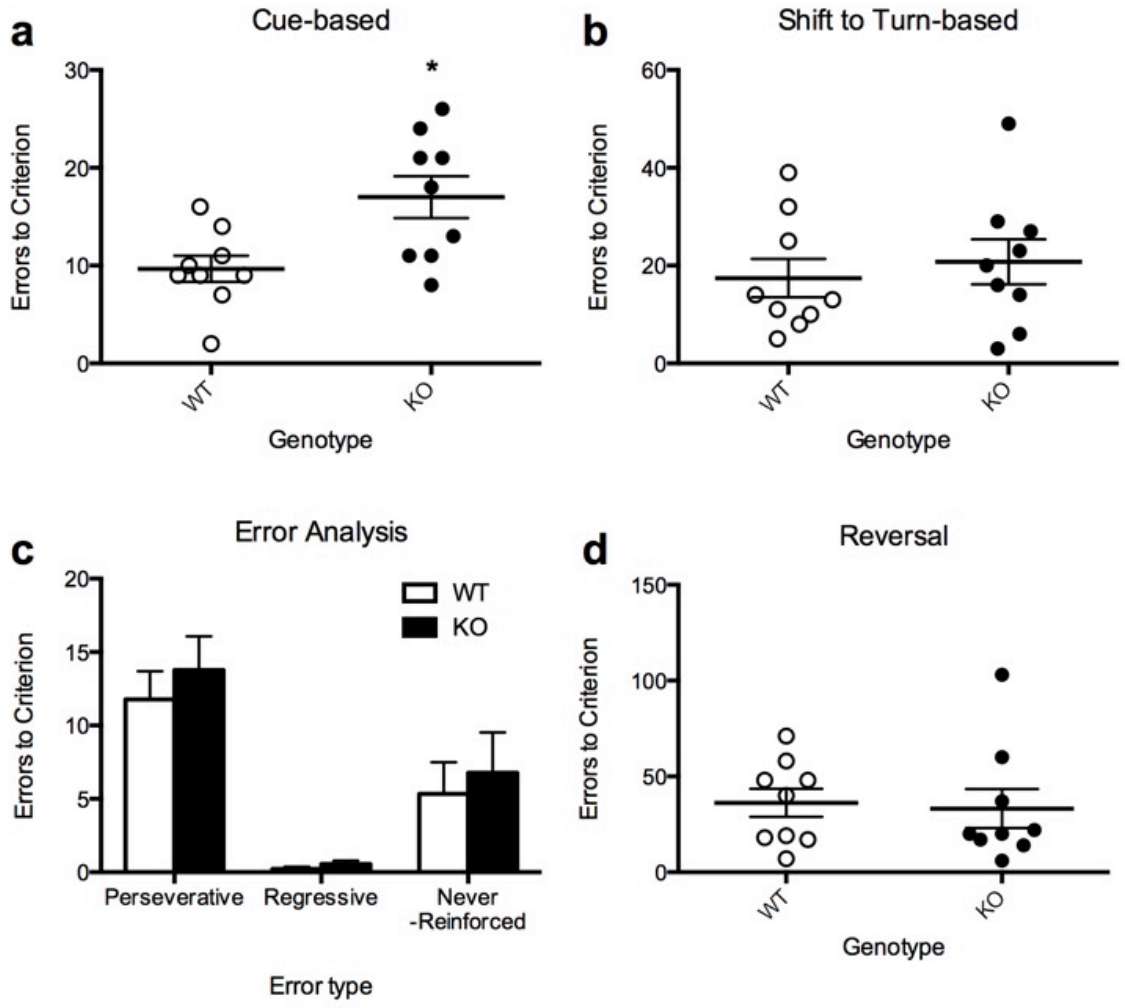

Figure 3-7. *Ncs-1*$^{-/-}$ mice show intact strategy shifting and spatial reversal in the visual water box but slower initial acquisition.
(a) *Ncs-1*$^{-/-}$ mice took longer to learn the initial cue-based strategy to finding the hidden platform. **(b)** The genotypes took similar amounts of time to shift to a turn-based strategy. **(c)** The genotypes committed similar numbers of the various types of errors during the shift to a turn-based strategy. **(d)** The genotypes took similar amounts of time to learn a reversal within a turn-based strategy. * = $p < 0.05$. Error bars = SEM.

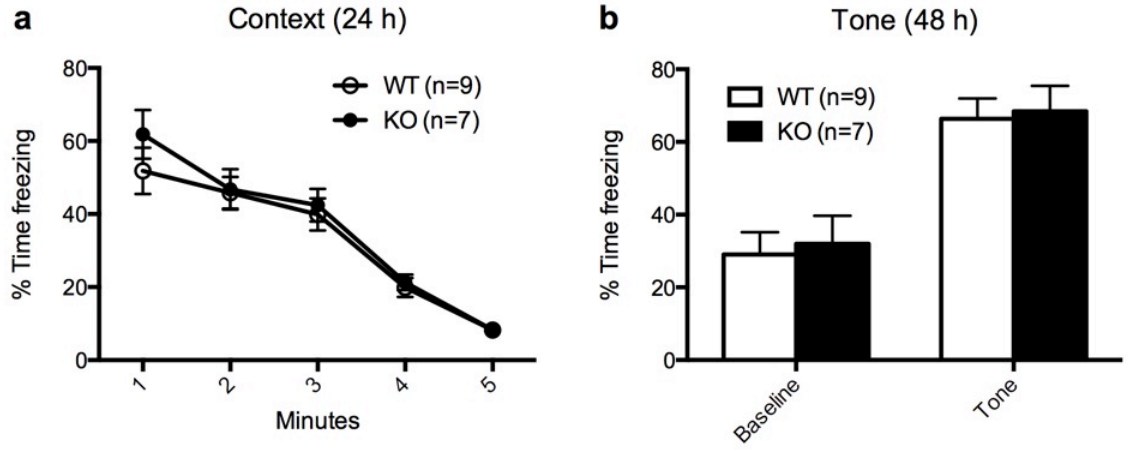

Figure 3-8. *Ncs-1*$^{-/-}$ mice show intact memory for a fearful context and a fearful tone. *Ncs-1*$^{-/-}$ mice and wildtype mice showed similar levels of freezing to **(a)** a fearful context 24 h post-conditioning and **(b)** to the tone CS in a novel context 48 h post-conditioning. Error bars = SEM.

3.3.5 Summary

We found *Ncs-1*$^{-/-}$ mice show a specific impairment in recognizing a subtle shift in spatial configuration and distances (spatial pattern separation) without affecting object recognition or spatial reference memory acquisition in the Morris water maze. Behavioural flexibility whether in the form of simple spatial reversal or the more complex shift from a cue- to turn-based strategy was unaffected by *Ncs-1* deletion. Context and cue fear memory were also intact in *Ncs-1*$^{-/-}$ mice.

3.4 *Ncs-1* deletion impairs social approach

Since *Ncs-1* deletion had effects on striatal- and hippocampal-dependent cognition and NCS-1 has been implicated in various diseases including schizophrenia, bipolar disorder, autism spectrum disorders, substance use, and Parkinson's disease, we tested *Ncs-1*$^{-/-}$ mice for a variety

of behavioural endophenotypes. We included endophenotypes that cut across many types of disorders including measures of motor activity, anxiety, sensorimotor gating, and sociability.

3.4.1 *Ncs-1*$^{-/-}$ mice show normal locomotor activity and motor learning

General locomotor activity and motor learning are important measures because all behaviour tests must be interpreted in light of any differences in motor activity and many diseases involve some change in motor activity. For example, the manic episodes of bipolar disorder may involve hyperlocomotion and enhanced rota-rod motor learning has been used as a proxy for repetitive motor behaviours found in forms of autism.

Mice wildtype, heterozygous and null for *Ncs-1* traveled similar distances in a novel open field (ns genotype effect, $F(2,26) = 1.186$, $p = 0.3215$; time effect, $F(5,130) = 42.33$, $p < 0.0001$; ns interaction, $F(10,130) = 1.784$, $p = 0.0696$) (Figure 3-9 a), suggesting *Ncs-1* deletion does not have obvious effects on baseline locomotion. *Ncs-1*$^{-/-}$ mice and wildtype mice also demonstrated similar motor learning and performance on the accelerating rota-rod (ns genotype effect, $F(1,20) = 0.006316$, $p = 0.9374$), with latency to fall increasing over time for mice of both genotypes at similar rates (time effect, $F(8,160) = 3.339$, $p = 0.0015$; ns interaction, $F(8,160) = 0.8272$, $p = 0.5798$) (Figure 3-9 b).

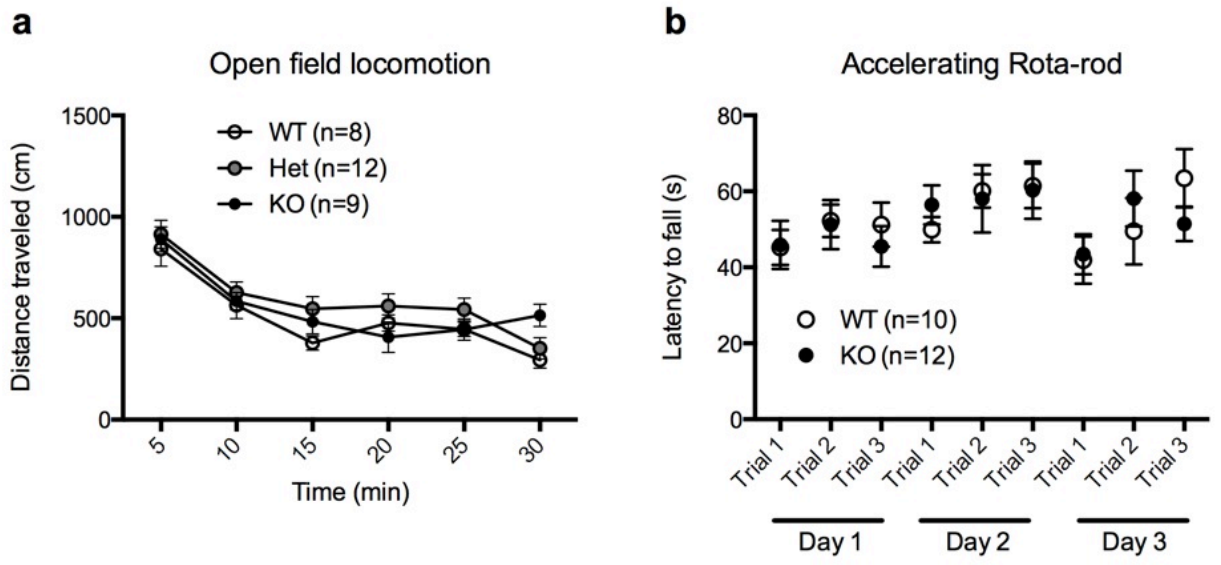

Figure 3-9. *Ncs-1*$^{-/-}$ mice show normal locomotor activity in an open field and normal motor learning.
(a) *Ncs-1*$^{-/-}$ mice travel similar distances in a novel open field. **(b)** *Ncs-1*$^{-/-}$ mice and wildtype mice show similar latencies to fall when learning to stay on an accelerating rotating rod. Error bars = SEM.

3.4.2 *Ncs-1*$^{-/-}$ mice show normal locomotor response to amphetamine

Locomotor response to amphetamine has been used as an endophenotype for schizophrenia and sensitization to repeated amphetamine (AMPH) administration is thought to underlie aspects of the addiction process. *Ncs-1*$^{-/-}$ mice show amphetamine-induced hyperlocomotion (paired t tests comparing SAL vs. d1 AMPH: WT, $t_7 = 8.408$, $p < 0.0001$; KO, $t_6 = 10.09$, $p < 0.0001$) as well as sensitization, similar to wildtype mice (two-way repeated measures ANOVA of AMPH-injected days: time effect $F(5,65) = 7.823$, $p < 0.0001$; ns genotype effect, $F(1,13) = 3.499$, $p = 0.0841$; ns interaction, $F(5,65) = 0.2507$, $p = 0.9380$) (Figure 3-10 a). There may be a non-significant trend for *Ncs-1*$^{-/-}$ mice to have slightly lower locomotor activity in response to amphetamine. This can be seen in response to acute amphetamine (ns genotype effect, $F(1,13) = 1.761$, $p = 0.2073$; time effect, $F(17,221) = 39.75$, $p < 0.0001$; ns interaction, $F(17,221) = 0.9861$, $p = 0.4749$), and in the response to repeated amphetamine (ns genotype effect, $F(1,13) = 3.251$, $p = 0.0946$; time effect, $F(17,221) = 44.25$, $p < 0.0001$; ns interaction, $F(17,221) = 0.7350$, $p =$

0.7650) and during a challenge after 3 days of withdrawal (ns genotype effect, F(1,13) = 3.109, p = 0.1013; time effect, F(17,221) = 78.09, $p < 0.0001$; ns interaction, F(17,221) = 1.458, p = 0.1122) (Figure 3-10 d-f). However, *Ncs-1*$^{-/-}$ mice also tended to have lower locomotion during the habituation phases (ns genotype effect, F(1,13) = 4.120, p = 0.0634; time effect, F(6,78) = 9.309, $p < 0.0001$; ns interaction, F(6,78) = 0.5634, p = 0.7581) (Figure 3-10 c).

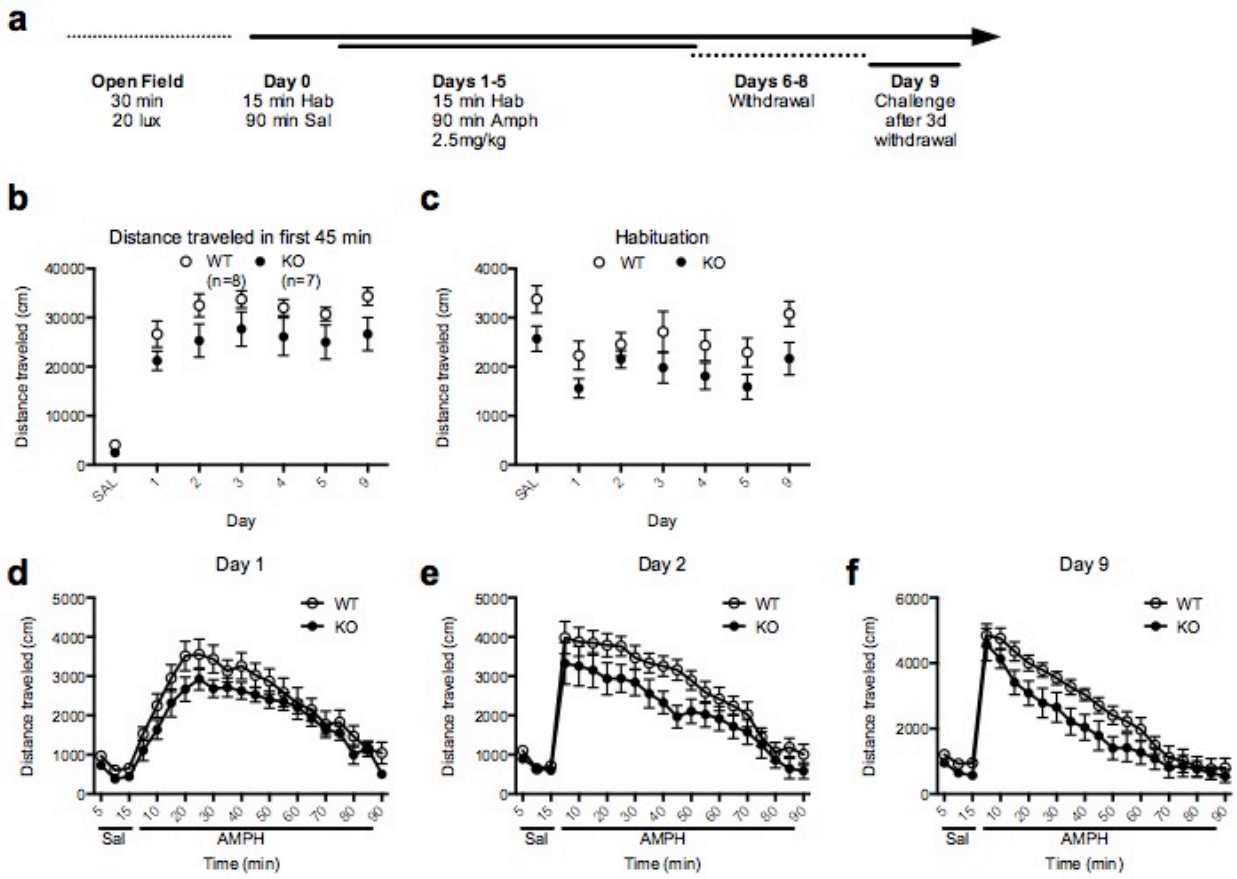

Figure 3-10. *Ncs-1*$^{-/-}$ mice show a trend toward lower open field locomotion in response to amphetamine, with intact sensitization.
(a) Experiment progression. **(b)** Total distance traveled over the first 45 minutes of each experiment day. **(c)** Distance traveled during the habituation period of each day. **(d-f)** Distance traveled in 5 min bins during an acute response to 2.5 mg/kg amphetamine s.c. (day 1), initial sensitization (day 2), and during a challenge after 3 days of withdrawal (day 9), respectively. Error bars = SEM.

3.4.3 *Ncs-1*$^{-/-}$ mice have normal levels of anxiety and behaviour on the tail suspension test

The light-dark box and elevated zero maze were used to assess anxiety-related behaviour. Wildtype, heterozygous and *Ncs-1* null mice spent similar proportions of time in the light compartment in the light-dark box ($F(2,24) = 0.1323$, $p = 0.8767$) (Figure 3-11 a) and in the open quadrants of the elevated zero maze ($F(2,24) = 1.944$, $p = 0.1669$) (Figure 3-11 b), suggesting decreased NCS-1 does not affect anxiety-like behaviour. The three genotypes also spent similar times immobile during the tail suspension test ($F(2,13) = 0.04914$, $p = 0.9522$) (Figure 3-11 c).

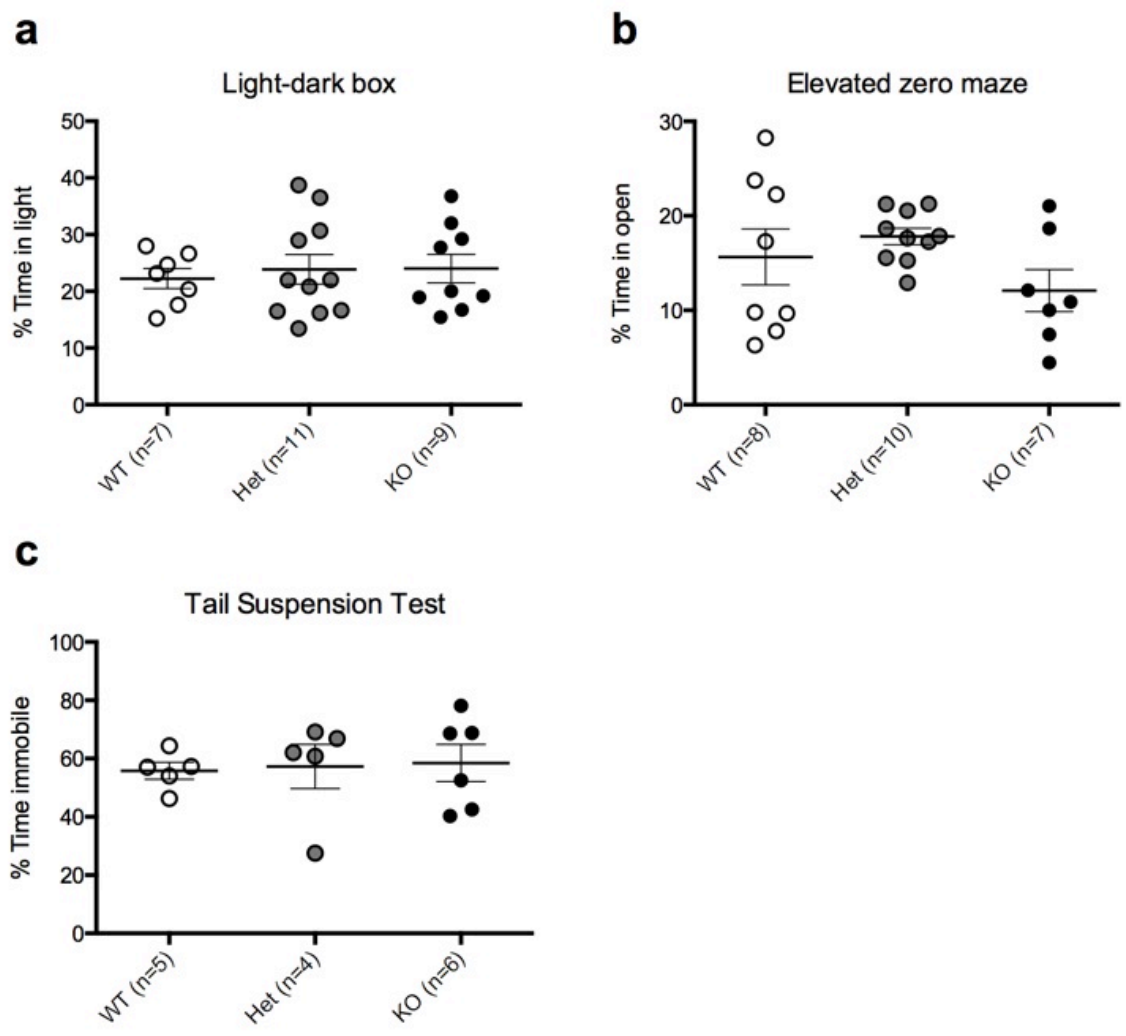

Figure 3-11. *Ncs-1*$^{-/-}$ mice show normal levels of anxiety and similar performance to wildtype mice in the tail suspension test.

The three genotypes spend similar proportions of time **(a)** in the lit portion of the light-dark box, **(b)** in the open quadrants of the elevated zero maze, and **(c)** staying immobile during tail suspension. Error bars = SEM.

3.4.4 *Ncs-1*$^{-/-}$ mice show normal pre-pulse inhibition

Pre-pulse inhibition of the startle response is a measure of sensorimotor gating and is disrupted in schizophrenia as well as other neuropsychiatric diseases. Similar levels of pre-pulse inhibition were found across genotypes (ns genotype effect, $F(2,128) = 1.17$, $p = 0.8268$; ns interaction, $F(6,128) = 0.1133$, $p = 0.9947$) and levels increased with pre-pulse intensity (pre-pulse intensity effect, $F(3,128) = 48.58$, $p < 0.0001$) (Figure 3-12 a). Levels of startle were also similar across genotypes during trials when only a pre-pulse (ns genotype effect, $F(2,128 = 1.17$, $p = 0.3126$; ns interaction, $F(6,128) = 0.5656$, $p = 0.7571$; pre-pulse intensity effect, $F(3,128) = 26.62$, $p < 0.0001$) (Figure 4-12 b) or a pulse ($F(2,31) = 0.1986$, $p = 0.8209$) (Figure 3-12 c) were delivered.

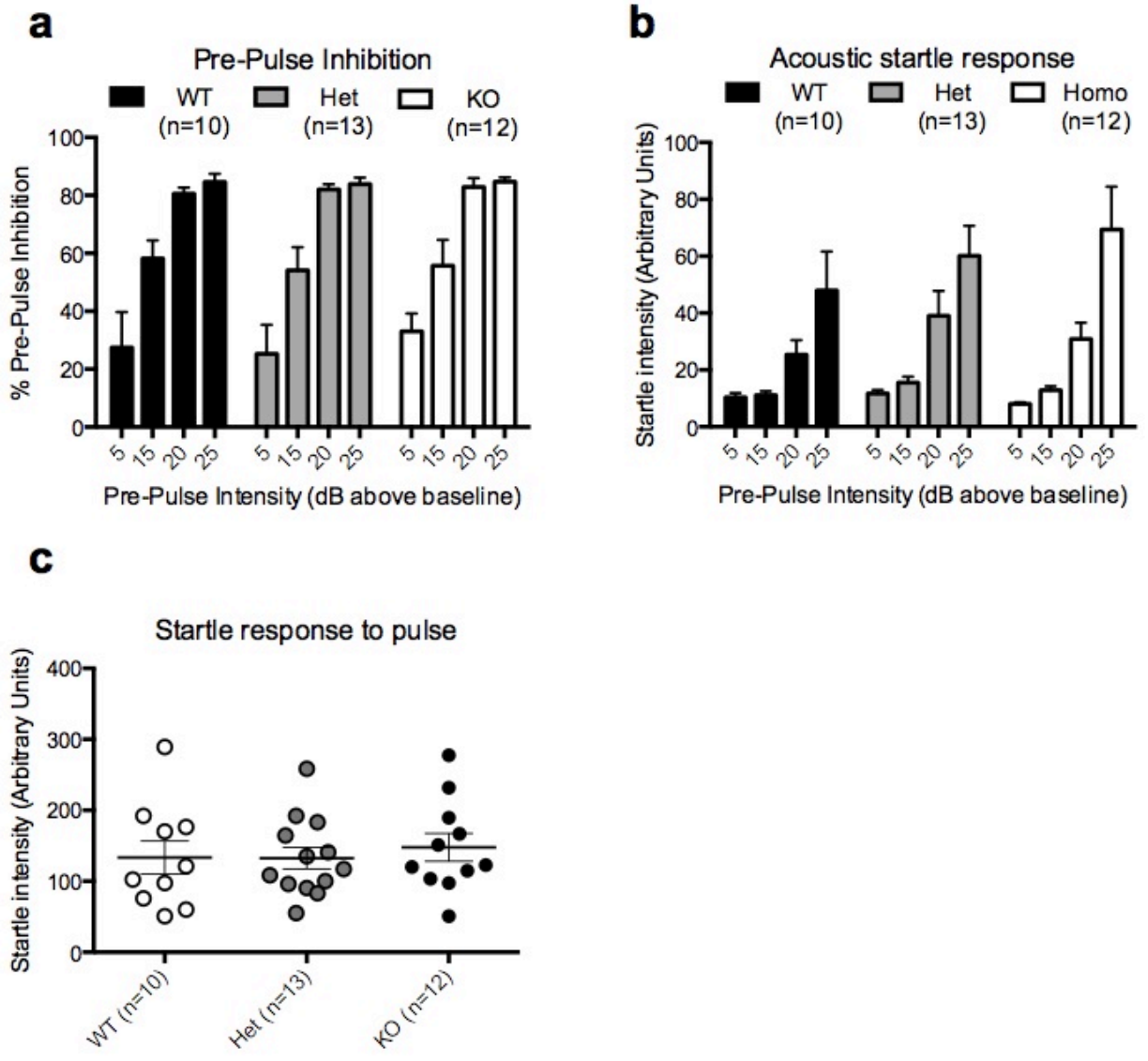

Figure 3-12. ***Ncs-1*$^{-/-}$ mice show intact sensorimotor gating of the acoustic startle response.** **(a)** The genotypes show similar levels of pre-pulse inhibition of the acoustic startle response. The genotypes do not differ in their startle responses to **(b)** pre-pulses alone or to the **(c)** startling pulse alone. Error bars = SEM.

3.4.5 *Ncs-1*$^{-/-}$ mice show decreased social approach

The 3-chambered social approach test and reciprocal social interaction tests were used as measures of sociability, which are decreased in disorders like autism and schizophrenia. While both wildtype (planned comparison, $t_{18} = 5.97$, $p < 0.0001$) and heterozygote mice (planned comparison, $t_{24} = 4.651$, $p = 0.0001$) spent significantly more time (> 50%) in a chamber with a stranger than in a chamber with an empty cup, homozygous *Ncs-1*$^{-/-}$ mice spent roughly equal proportions of time in those two chambers (planned comparison, $t_{20} = 1.681$, $p = 0.1083$) (Figure 3-13 a). Similar results were found when measuring time spent sniffing (nose-point within 2 cm of object) an empty cup only or a novel social partner under an empty cup (Stranger vs. Empty planned comparisons: WT, $t_{18} = 3.526$, $p = 0.0024$; Het, $t_{22} = 3.022$, $p = 0.0063$; KO, $t_{20} = 1.033$, $p = 0.3139$) (Figure 3-13 b). These planned comparisons were used as recommended by the protocol in Yang et al, 2011. When coding for various behaviours during a ten minute free reciprocal social interaction with a stranger C57BL6/J, *Ncs-1*$^{-/-}$ mice spent similar amounts of time and sometimes even more time than wildtype mice in social behaviours such as push-crawl, face sniffing and allogrooming (Figure 3-13 c).

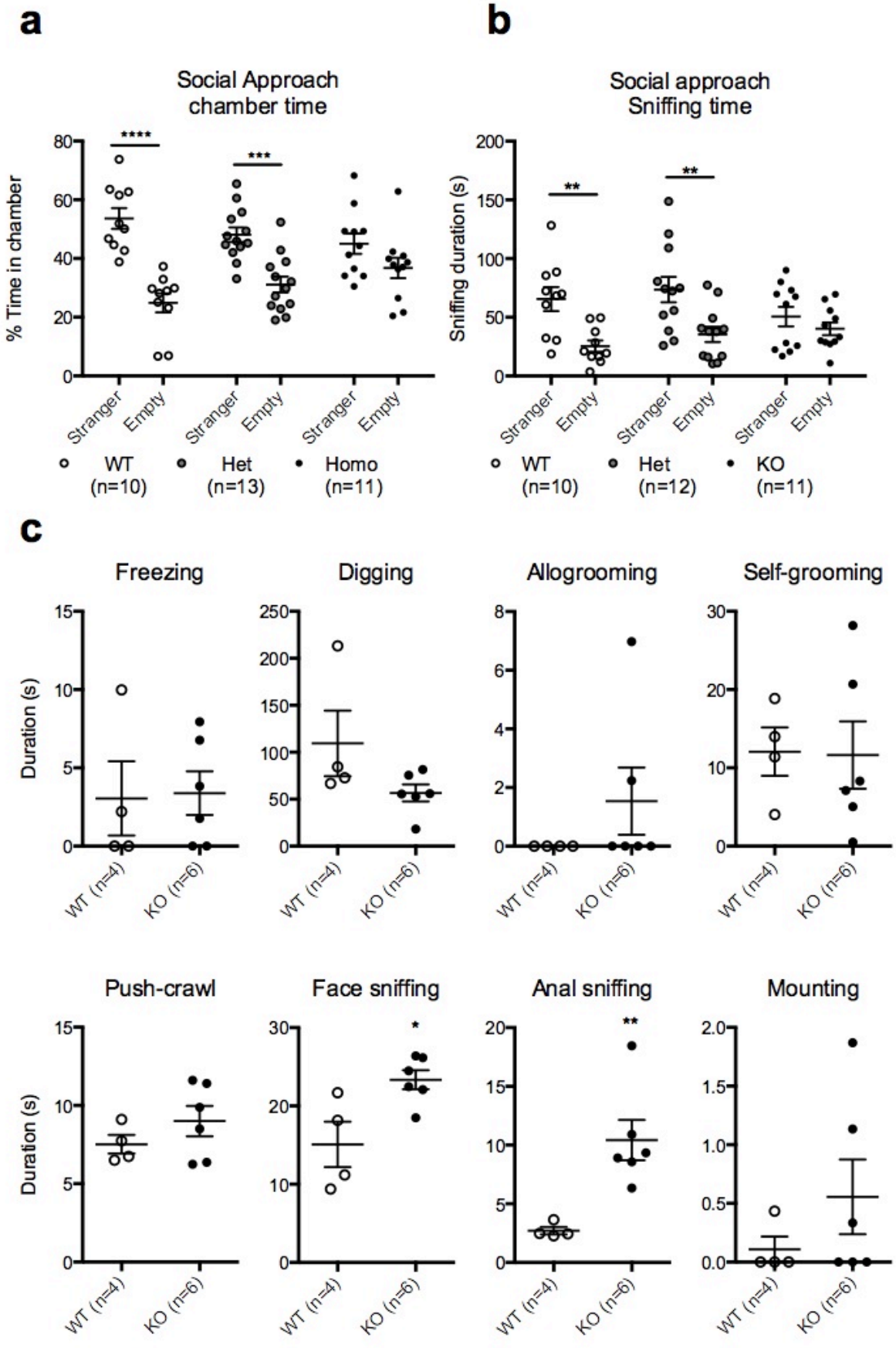
a
Social Approach
chamber time
% Time in chamber
****
***
Stranger
Empty
WT
(n=10)
Het
(n=13)
Homo
(n=11)
b
Social approach
Sniffing time
Sniffing duration (s)
**
**
WT
(n=10)
Het
(n=12)
KO
(n=11)
c
Freezing
Digging
Allogrooming
Self-grooming
Duration (s)
Push-crawl
Face sniffing
Anal sniffing
Mounting
*
**
WT (n=4)
KO (n=6)

Figure 3-13. *Ncs-1*$^{-/-}$ mice show decreased social approach but intact social interactions. **(a)** *Ncs-1*$^{-/-}$ mice show no preference for spending time in the chamber with a novel social partner, whereas wildtype and heterozygous mice prefer spending time in the social chamber to time in the chamber with an inverted empty cup. **(b)** Similar results were found when measuring time spent sniffing (nose-point within 2 cm of inverted wire cup) the novel social or novel non-social stimuli. **(c)** *Ncs-1*$^{-/-}$ mice spend similar times in various non-social behaviours (top panel) and similar if not more time in various social behaviours (bottom panel) in a reciprocal social interaction test (with stranger C57BL/6J). * = $p < 0.05$. ** = $p < 0.01$. *** = $p < 0.001$. Error bars = SEM.

3.4.6 Summary

Taken together, *Ncs-1*$^{-/-}$ mice are normal across a variety of behavioural endophenotypes including locomotor activity, motor learning, anxiety, behavioural despair, and pre-pulse inhibition. Deletion of *Ncs-1* however, seems to decrease social approach behaviours without impairing actual social interactions when individuals are in close contact.

3.5 *Ncs-1* deletion decreases striatal dopamine release

Striatal dopamine signalling is known to be important for the effort dimension of motivation, and *Ncs-1*$^{-/-}$ mice show a specific deficit in effort. Therefore, we hypothesized that the *Ncs-1*$^{-/-}$ mice have decreased striatal dopamine signalling. We tested our hypothesis by measuring dopamine release at the nucleus accumbens as well as DRD2 levels for two reasons. First, both measures have been linked to changes in effortful behaviour. Second, both measures could plausibly be changed in *Ncs-1*$^{-/-}$ mice given the role of NCS-1 in exocytosis and its ability to interact with and attenuate internalization of DRD2.

3.5.1 *Ncs-1*$^{-/-}$ mice have normal levels of striatal DRD2

We confirmed that both NCS-1 and DRD2 are expressed in the striatum using immunofluorescence (Figure 3-14 a). Whole brain DRD2 was measured by immunoblot and we found *Ncs-1*$^{-/-}$ mice had similar levels relative to wildtype mice ($F(2,6) = 1.520$, $p = 0.2923$) (Figure 3-14 b); the small sample size used limits strong conclusions but additional measurements in striatal tissue do show similar results (relatively similar levels across genotypes). In order to get a measure of surface DRD2, which are the population that has been associated with effort, we biotinylated acute coronal slices containing striatum, pulled down biotin-bound proteins, and immunoblotted for DRD2. Again, densitometry showed similar but slightly decreased (18% lower) levels of biotinylated cell surface DRD2 in *Ncs-1*$^{-/-}$ mice relative to wildtype mice ($t_4 = 1.391$, $p = 0.2367$) (Figure 3-14 c).

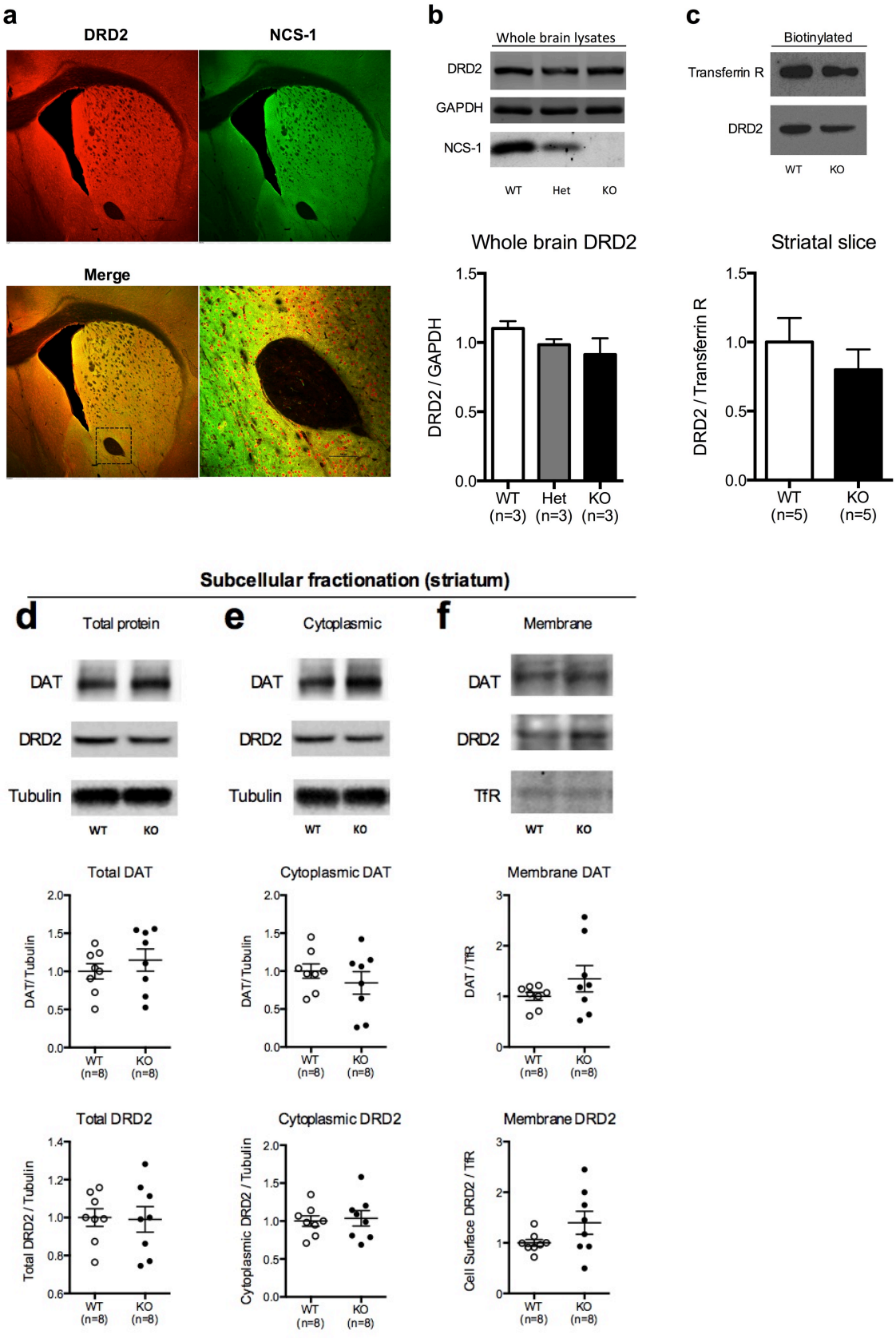
a
DRD2
NCS-1
Merge
b
Whole brain lysates
DRD2
GAPDH
NCS-1
WT
Het
KO
Whole brain DRD2
DRD2 / GAPDH
1.5
1.0
0.5
0.0
WT (n=3)
Het (n=3)
KO (n=3)
c
Biotinylated
Transferrin R
DRD2
WT
KO
Striatal slice
DRD2 / Transferrin R
1.5
1.0
0.5
0.0
WT (n=5)
KO (n=5)
Subcellular fractionation (striatum)
d
Total protein
e
Cytoplasmic
f
Membrane
DAT
DRD2
Tubulin
TfR
WT
KO
Total DAT
DAT/ Tubulin
Cytoplasmic DAT
DAT/ Tubulin
Membrane DAT
DAT / TfR
Total DRD2
Total DRD2 / Tubulin
Cytoplasmic DRD2
Cytoplasmic DRD2 / Tubulin
Membrane DRD2
Cell Surface DRD2 / TfR
WT (n=8)
KO (n=8)

Figure 3-14. *Ncs-1*$^{-/-}$ mice have similar levels of dopamine receptor D2 to wildtype mice. **(a)** NCS-1 and DRD2 are both found in the striatum and nucleus accumbens. All panels are 4X images except for the bottom-right, which is a 20X image taken of the region enclosed by the box in the bottom-left panel. Upper left quadrant scale bar = 500 μm. Lower right quadrant scale bar = 100 μm. **(b)** The genotypes show similar levels of whole brain DRD2 with a trend toward decreased DRD2 in *Ncs-1*$^{-/-}$ mice. **(c)** *Ncs-1*$^{-/-}$ mice show similar levels of striatal surface DRD2 in a coronal slice biotinylation experiment. **(d-f)** Subcellular fractionation revealed similar levels of total striatal DRD2 and DAT (d), cytoplasmic DRD2 and DAT (e), as well as membrane DRD2 and DAT (f). Error bars = SEM.

We used subcellular fractionation to get another measure of membrane DRD2 as well as DAT (dopamine transporter) (Figure 3-14 d-f). Similar levels of total striatal DAT ($t_{14} = 0.8374$, $p = 0.4164$) and DRD2 ($t_{14} = 0.1184$, $p = 0.9074$) were found across genotypes. *Ncs-1*$^{-/-}$ mice also had wildtype levels of DAT ($t_{14} = 0.88894$, $p = 0.3888$) and DRD2 ($t_{14} = 0.2917$, $p = 0.7748$) in striatal cytoplasmic fractions. Membrane fractions revealed equivalent levels of DAT ($t_{14} = 1.287$, $p = 0.2188$) between genotypes and a trend for more cell surface DRD2 though this was not statistically significant ($t_{14} = 1.675$, $p = 0.1162$).

3.5.2 *Ncs-1*$^{-/-}$ mice show decreased nucleus accumbens dopamine release

Given the similar levels of DRD2 between *Ncs-1*$^{-/-}$ and wildtype mice, we wondered if there may be a difference in presynaptic dopamine release. We focused on dopamine release from the nucleus accumbens core, as this is one of the striatal regions where dopamine levels have been most associated with effortful behaviour. Fast scan cyclic voltammetry was used to measure electrically-evoked dopamine release from the nucleus accumbens core in acute coronal slices. Release was measured under normal conditions in artificial cerebral spinal fluid (ACSF) only or in the presence of sulpiride (Figure 3-15 a, d). We used 5 μM sulpiride, which blocks dopamine D2 autoreceptors to help rule out any confounding influences it may have on dopamine release given the ability for NCS-1 to interact with DRD2.

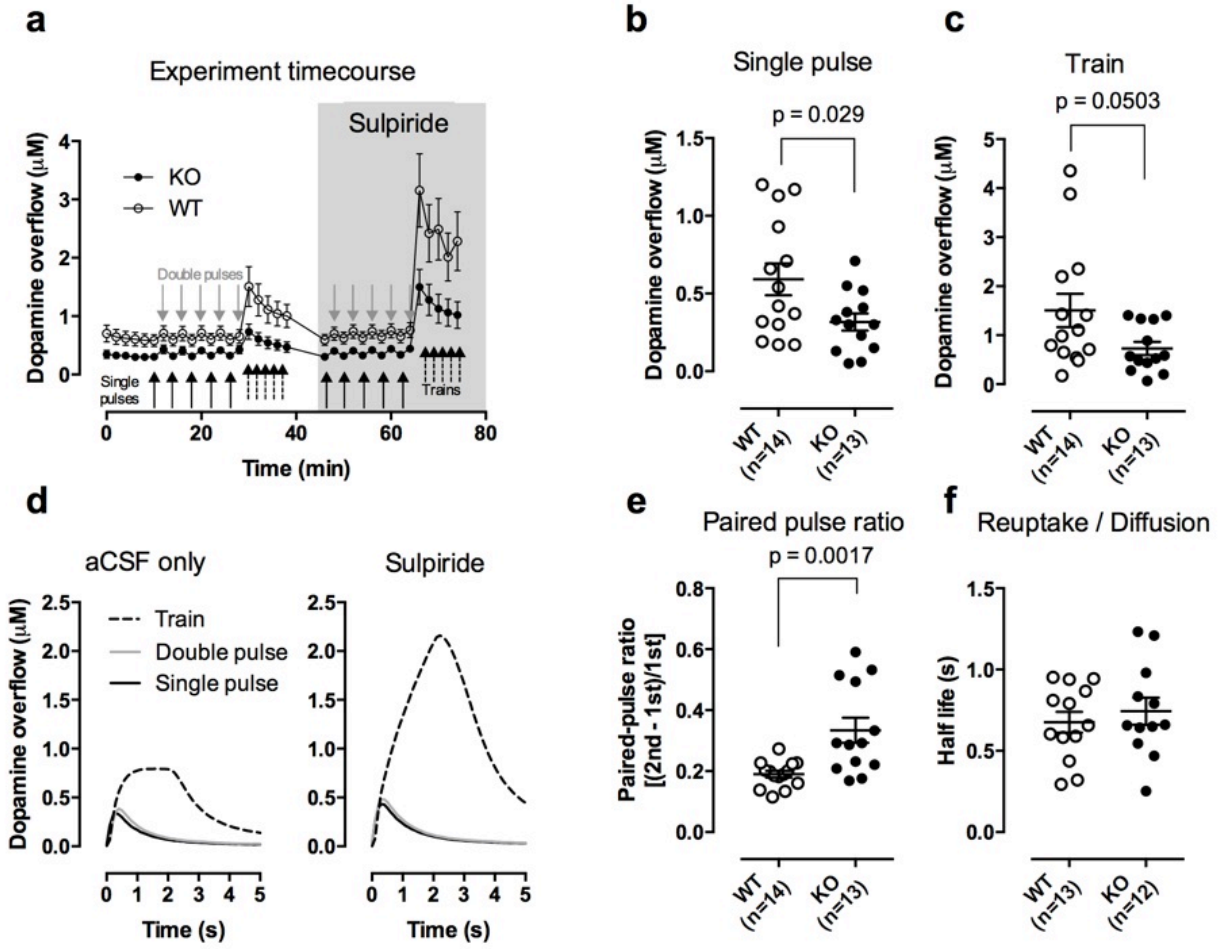

Figure 3-15. *Ncs-1*$^{-/-}$ mice show lower nucleus accumbens dopamine release in slice.
(a) Experiment time-course. *Ncs-1*$^{-/-}$ mice show lower dopamine release in response to **(b)** single pulse and **(c)** train stimulation. **(d)** Representative traces for electrically stimulated dopamine overflow as measured by cyclic voltammetry. **(e)** *Ncs-1*$^{-/-}$ mice have a lower probability of release. **(f)** Dopamine half-life is similar across genotypes. Error bars = SEM.

Ncs-1$^{-/-}$ mice showed ~50% lower dopamine release in response to single pulses ($t_{25} = 2.322$, $p = 0.0287$) and train stimulation ($t_{25} = 2.057$, $p = 0.0502$) (Figure 3-15 b-c). As expected, dopamine D2 autoreceptor blockade increased dopamine release. However, *Ncs-1*$^{-/-}$ mice still showed lower dopamine release in the presence of sulpiride, suggesting that *Ncs-1* deletion may be modulating dopamine release independently of DRD2 (Figure 3-15 a). Moreover, *Ncs-1*$^{-/-}$ mice had a paired-pulse ratio close to twice that of wildtype mice, suggesting a lower probability of dopamine release ($t_{25} = 3.511$, $p = 0.0017$) (Figure 3-15 e). Differences in dopamine release were not a

result of different rates of dopamine reuptake as the dopamine half-life following single pulse stimulation was similar between genotypes ($t_{23} = 0.6507$, $p = 0.5217$) (Figure 3-15 f).

3.5.3 Summary

In accordance with a behaviours suggesting decreased motivation, we found decreases in dopamine signalling in the striatum of *Ncs-1*$^{-/-}$ mice. Presynaptic dopamine release in the accumbens core and probability of release were significantly reduced in *Ncs-1*$^{-/-}$ mice without effects on dopamine reuptake or dopamine D2 autoreceptor-mediated feedback regulation. In contrast, DRD2 levels were similar between genotypes in whole brain lysates and at the striatal cell surface.

3.6 *Ncs-1* deletion decreases hippocampal long-term plasticity in a time-dependent manner

Given our previous work showing NCS-1 overexpression enhances dentate gyrus LTP (Saab et al., 2009), a study showing involvement of NCS-1 in perirhinal cortex mGluR LTD (Jo et al., 2008), and impaired displaced object preference in the *Ncs-1*$^{-/-}$ mice (Figure 3-5), we assessed hippocampal long-term plasticity in knockout mice. We predicted that *Ncs-1* deletion would decrease dentate gyrus LTP and as well as mGluR LTD. We focused on the dentate gyrus due to our previous work in that region and because spatial pattern separation is a function attributed to the dentate gyrus (Kesner, 2013a, b). We also measured mGluR LTD in the CA1 for comparison.

3.6.1 *Ncs-1*$^{-/-}$ mice show lower late-phase medial perforant path LTP

We assessed medial perforant path LTP in response to high frequency stimulation and found similar levels of LTP between adults of the two genotypes just after induction (~300%) (Figure 3-16 a). However, starting from about 50 min on, levels of LTP in *Ncs-1*$^{-/-}$ mice begin to drop reaching ~150% of baseline by 70 min, whereas wildtype mice hold steady at ~200% of baseline. Repeated measures ANOVA of a $\log_{10}$ transformation of datapoints following the tetanus (Figure 3-16 a, inset) revealed a significant genotype x time interaction (interaction, $F(44,396) = 2.043$, $p = 0.0002$; time effect, $F(44,396) = 26.25$, $p < 0.0001$; ns genotype effect, $F(1,9) = 1.485$, $p = 0.2540$; post-hoc comparisons with multiple corrections did not show statistical significant results). The significant genotype x time interaction indicates that LTP decreases sooner over time in *Ncs-1*$^{-/-}$ slices relative to wildtype slices, particularly in the late-phase (~50 min post-tetanus). Presynaptic function was similar between genotypes as revealed by similar input-output functions (Figure 3-16 b) as well as responses to paired pulses of different intervals (ns genotype effect, $F(1,96) = 2.333$, $p = 0.1300$; inter-pulse interval effect, $F(7,96) = 7.615$, $p < 0.0001$; ns interaction, $F(7,96) = 0.4752$, $p = 0.8504$) (Figure 3-16 c).

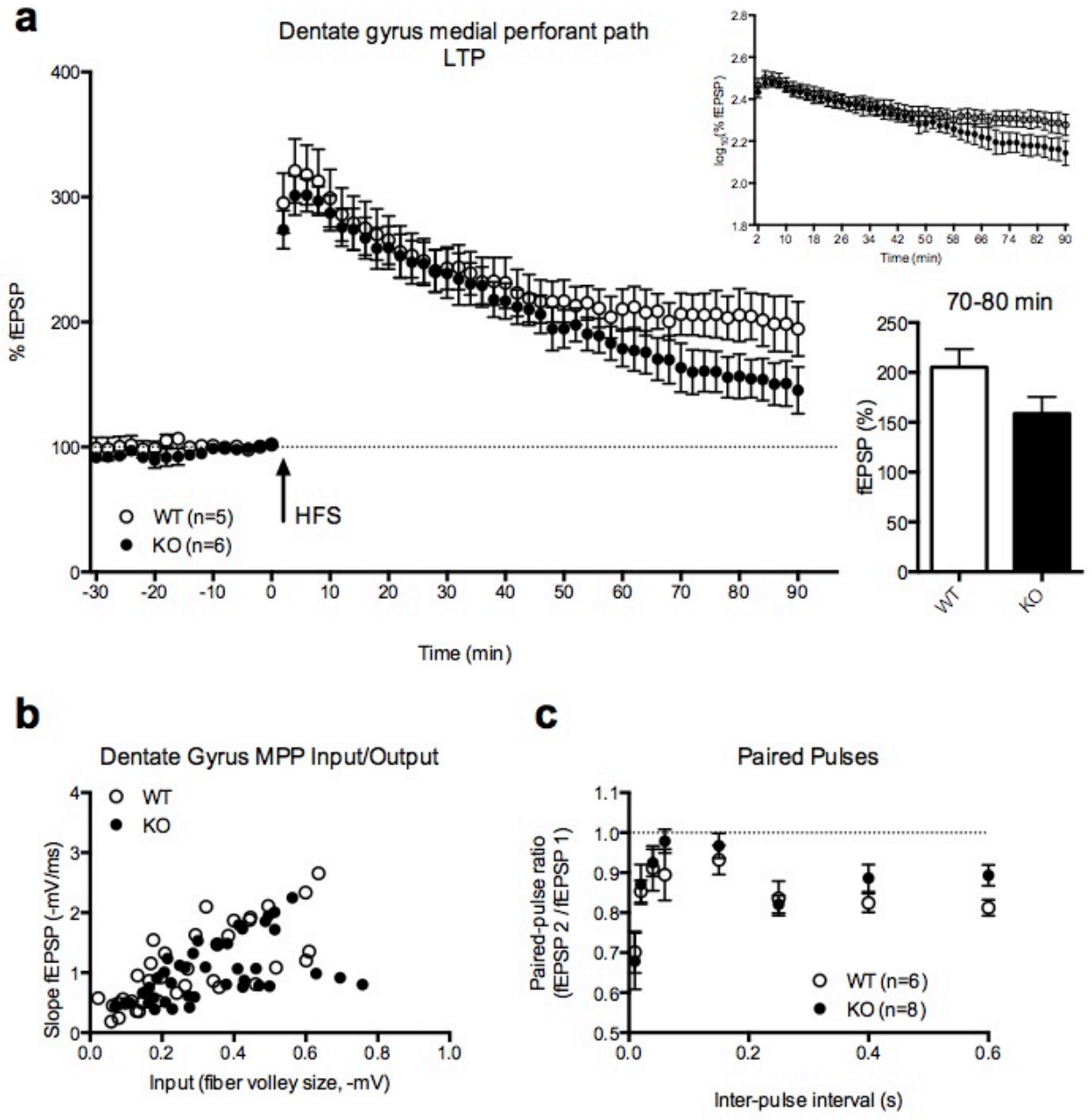

Figure 3-16. ***Ncs-1*$^{-/-}$ mice show normal levels of LTP in the medial perforant path but show a reduced late-phase LTP.**
(a) LTP was induced using four trains of 0.5 s 100 Hz tetanic stimulation delivered 20 s apart. The inset shows a $\log_{10}$ transformation of the data post-tetanus. Bar graph shows average values during the 70-80 min time interval. **(b)** Input/output functions are similar between genotypes. **(c)** Levels of paired pulse depression are similar between genotypes. Error bars = SEM.

3.6.2 *Ncs-1*$^{-/-}$ mice show decreased early-phase medial perforant path mGluR LTD and show lower paired-pulse ratios

There was a trend for juvenile *Ncs-1*$^{-/-}$ slices to show less depression soon after addition of *S*-DHPG (Figure 3-17 a). Repeated measures ANOVA of the data following drug application revealed a significant genotype x time interaction, indicating that early on, *Ncs-1*$^{-/-}$ slices do not depress as much over time than wildtype slices (interaction, $F(44,484) = 1.959$, $p = 0.0004$; time effect, $F(44,484) = 1.743$, $p = 0.0029$; ns genotype effect, $F(1,11) = 0.3380$, $p = 0.5725$). Input-output functions were similar between genotypes (Figure 3-17 b). However, in the conditions we used (2 μM : 2 μM, Ca^{2+} : Mg^{2+}) juvenile *Ncs-1*$^{-/-}$ slices showed lower responses to paired pulses than wildtype slices (genotype effect, $F(1,96) = 40.50$, $p < 0.0001$; inter-pulse interval effect, $F(7,96) = 4.105$, $p = 0.0006$; ns interaction, $F(7,96) = 0.3982$, $p = 0.9013$) (Figure 3-17 c), particularly at the 10 ms and 40 ms inter-pulse intervals. Indeed, while wildtype slices showed evidence for some paired-pulse facilitation (ratios > 1.0) at multiple points, *Ncs-1*$^{-/-}$ slices consistently showed paired-pulse depression throughout.

3.6.3 *Ncs-1*$^{-/-}$ mice show a non-significant trend toward decreased Schaffer collateral mGluR LTD

Both wildtype and *Ncs-1*$^{-/-}$ slices showed stronger depression in response to *S*-DHPG in the CA1 relative to the dentate gyrus (Figure 3-18 a). Repeated measures ANOVA of the data following drug application only revealed a main effect of time (time effect, $F(44,308) = 8.068$, $p < 0.0001$; ns genotype effect, $F(1,7) = 0.01706$, $p = 0.8997$; ns interaction, $F(44,308) = 0.3114$, $p > 0.9999$). Input-output functions (Figure 3-18 b) and paired-pulse ratios (ns genotype effect, $F(1,64) = 0.2718$, $p = 0.6039$; interval effect, $F(7,64) = 35.46$, $p < 0.0001$; ns interaction effect, $F(7,64) = 0.3495$, $p = 0.9276$) (Figure 3-18 c) were similar between genotypes, suggesting no differences in presynaptic function at the CA1.

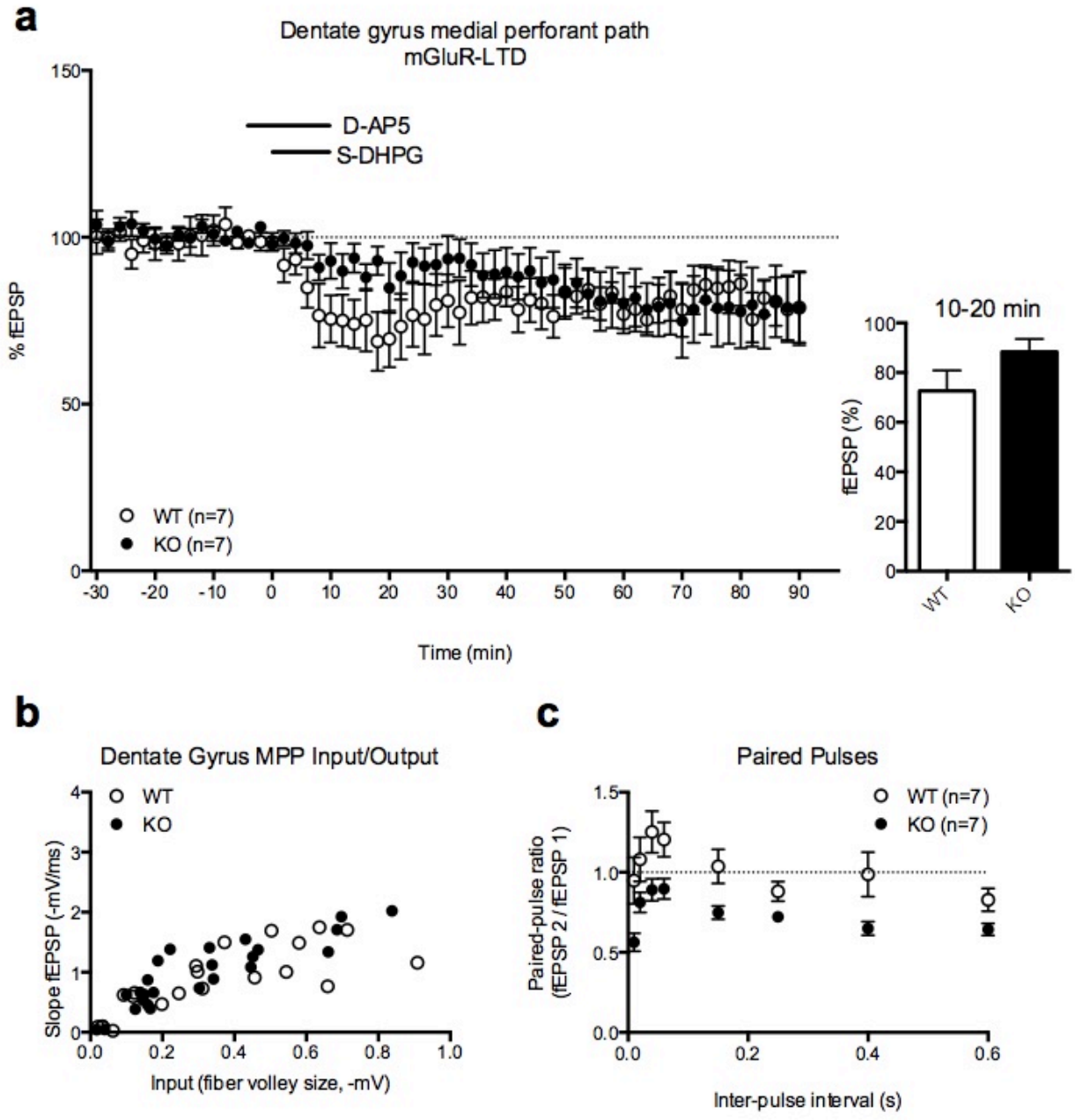

Figure 3-17. *Ncs-1*$^{-/-}$ mice show toward impaired early-phase mGluR-LTD in the medial perforant path.

(a) LTD was induced using 10 min of *S*-DHPG (50 μM) in the presence of D-AP5 (50 μM). The bar graph shows average values during the 10-20 min time period. **(b)** Input/output functions are similar between genotypes. **(c)** *Ncs-1*$^{-/-}$ mice show a trend for lower paired pulse ratios to wildtype mice. * = p <0.05. Error bars = SEM.

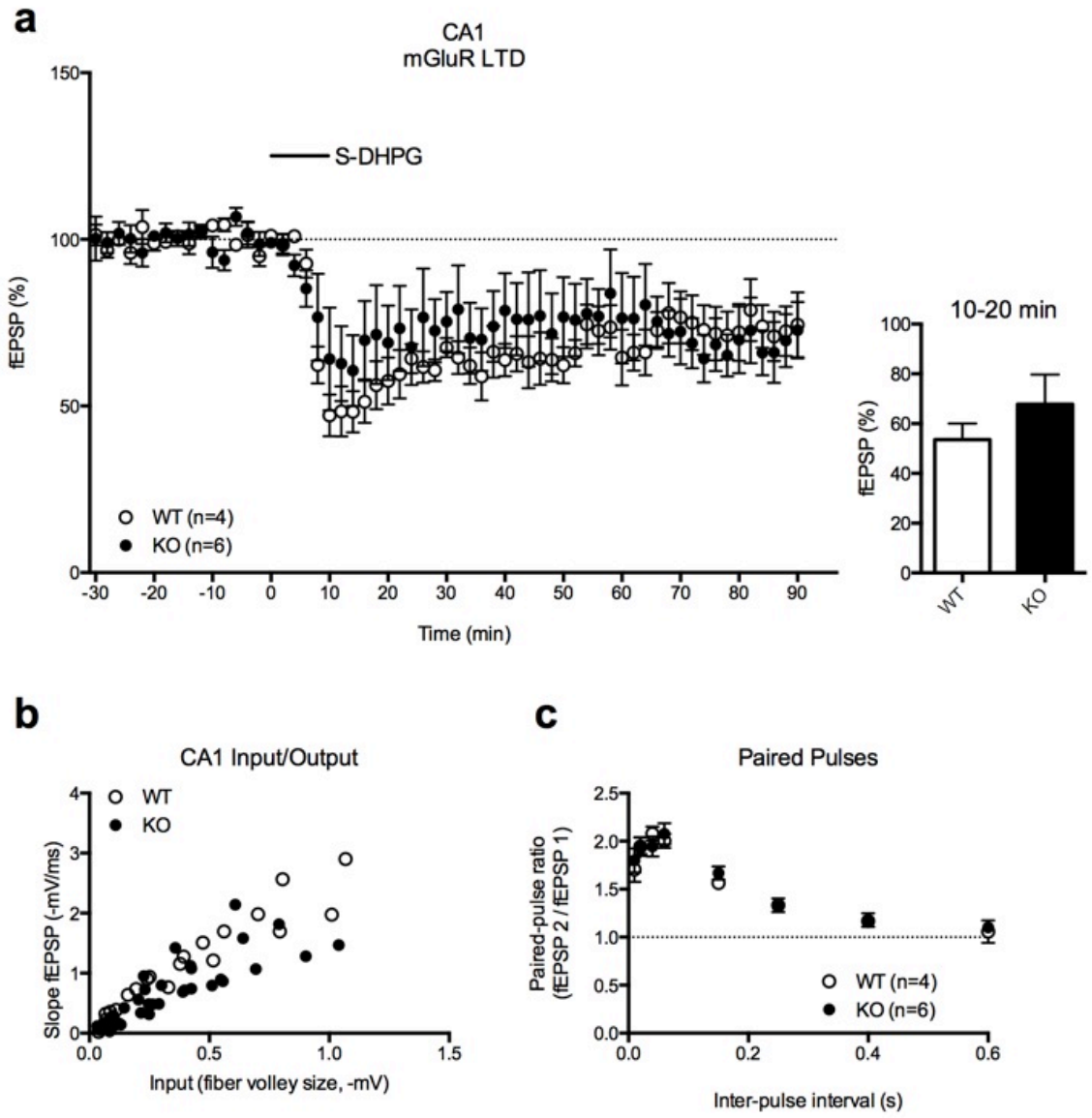

Figure 3-18. ***Ncs-1*** $^{-/-}$ **mice show normal hippocampal CA1 mGluR-LTD.**
(a) LTD was induced by 10 min of *S*-DHPG (50 μM) with or without D-AP5 (50 μM). The bar graph shows average values during the 10-20 min time period. **(b)** Input/output functions were similar between genotypes. **(c)** The genotypes showed similar levels of paired pulse facilitation. Error bars = SEM.

3.6.4 Summary

Ncs-1$^{-/-}$ mice showed decreased long-term plasticity in late-phase LTP in the dentate gyrus and decreased early-phase of LTD in response to *S*-DHPG at the dentate gyrus. Proxies for presynaptic function were similar between genotypes in juvenile CA1 and in the adult dentate gyrus. However, the use of juvenile mice together with 2 μM : 2 μM, Ca^{2+} : Mg^{2+} ACSF revealed a significant decrease in paired-pulse ratios in *Ncs-1*$^{-/-}$ mice relative to wildtype mice.

3.6.5 Repetitions

Since a large battery of behavioural tests was run, there is the possibility that some statistically significant differences were found by chance alone. Certain tests were repeated multiple times with similar results, suggesting that at least these differences can be reliably replicated. Moreover, certain datasets (e.g. total distance traveled in Figure 3-2 b, as well as social approach in Figure 3-13 a-b) came from combining multiple independent cohorts of mice, making it less likely the differences (or lack of differences) were due to chance alone.

The following results have been replicated:

- *Ncs-1*$^{-/-}$ mice are less willing to work for food under various progressive ratio schedules of reinforcement in two additional cohorts, despite similar performance on fixed ratio 1 schedules (Figures A-1, A-2).
- *Ncs-1*$^{-/-}$ mice tend to not work as much for preferred food but eat chow instead in the concurrent choice task (Figure A-1).
- *Ncs-1*$^{-/-}$ mice show amphetamine-induced hyperlocomotion and sensitization, but there is a trend for them to have slightly less hyperlocomotion under 2 mg/kg amphetamine (Figures A-1, A-2).

- *.Ncs-1*$^{-/-}$ mice perform at wildtype levels in the water maze spatial learning, memory and reversal (Figure A-3).

4 Discussion and Conclusions

The discussion of how our results accord with our predictions, fit with and contribute to the existing literature, as well as limitations to our approach is divided into four sections. Behavioural data are discussed together with relevant physiology where possible. Data on motivated behaviour and striatal dopamine signalling are discussed together (4.1), followed by results on learning, memory and hippocampal electrophysiology (4.2), and findings on endopohenotypes relevant to disease (4.3). Section 4.4 describes the limitations and complexities from our use of a mouse with constitutive deletion of *Ncs-1* obtained from gene targeting. The concluding section is an integrated discussion of the role of NCS-1 on motivation and learning (4.5).

4.1 The role of NCS-1 in motivation and dopamine release

Though a role for NCS-1 in dopamine-related cognitive processes had long been speculated (Bergson et al., 2003; Koh et al., 2003), the only evidence for this had come from previous work in our lab showing a role for DRD2 in NCS-1 mediated enhancements in hippocampal-dependent spatial learning and long-term potentiation (Saab et al., 2009). It was not known what role NCS-1 plays in striatal-dependent cognitive processes, which are modulated by dopamine.

Here, we predicted that *Ncs-1* deletion would impair motivated behaviour and decrease striatal dopamine signalling. We found that *Ncs-1*$^{-/-}$ mice showed impaired motivation, but specifically in the dimension of effort, without affecting hedonic preference, Pavlovian approach, conditioned reinforcement or operant learning (Table 4-1). In accordance with this deficit in willingness to work for food, we found decreased presynaptic dopamine release at the nucleus accumbens core in *Ncs-1*$^{-/-}$ mice, but only subtle changes in DRD2 levels. Thus, we demonstrate for the first time a role for NCS-1 in effort and accumbens core dopamine release.

Table 4-1. Motivated behaviour and accumbens dopamine in *Ncs-1*$^{-/-}$ mice.

Test / Procedure	Measure of	Relative to Wildtype
Fixed ratio 1 (FR1)	Instrumental learning	=
Progressive ratio	Effort	<
Concurrent choice	Effort-based choice	<
Sucrose, food preference	"Liking"	=
Conditioned reinforcement	"Wanting"	=
Sensory-specific satiety	Sensitivity to changes in reward value after - goal-directed learning - habit formation	= =
Immunoblotting	Whole brain DRD2	=
Slice biotinylation	Striatal surface DRD2	=
Subcellular fractionation	Striatal DRD2 (whole, cytosolic, membrane fractions) Striatal DAT (whole, cytosolic, membrane fractions)	= =
Fast scan cyclic voltammetry	DA release Probability of release Reuptake / diffusion Dopamine D2 autoreceptor function	< = =

4.1.1 Motivation for food

Overall, decreased effort coupled with lower accumbens core dopamine fits well with the literature on the role of dopamine in food-motivated behaviour. Just as accumbens dopamine depletion has little effect on FR1 but reduces responding on higher ratio schedules (Aberman et al., 1998; Ishiwari et al., 2004; McCullough et al., 1993; Mingote et al., 2005; Salamone et al., 1995; Salamone et al., 2001), *Ncs-1*$^{-/-}$ mice show normal FR1 performance but decreased nosepoking on higher ratio schedules such as FR30, FR20 and RI-60 s (Figure 3-3 a; Figure 3-4 d). Similarly, decreased motivation in progressive ratio schedules in *Ncs-1*$^{-/-}$ mice (Figure 3-2 d-e) is consistent with literature showing decreases in accumbens dopamine signalling and progressive ratio performance (Aberman et al., 1998; Darvas and Palmiter, 2009; Hamill et al., 1999). Conversely, elevations in signalling enhance motivation (Bello et al., 2011a; Cagniard et al., 2006; Trifilieff et al., 2013; Zhang et al., 2003). The trend for *Ncs-1*$^{-/-}$ mice to nosepoke less for chocolate-flavoured pellets and consume more chow in the concurrent choice task is consistent with the effects of decreased accumbens dopamine signalling on this task (Cousins and Salamone, 1994; Cousins et al., 1994; Salamone et al., 2002; Salamone et al., 1991; Sokolowski et al., 1998). The effect on concurrent choice behaviour found in the *Ncs-1*$^{-/-}$ mice may be smaller than those found in other studies because the decrease in striatal dopamine signalling in the *Ncs-1*$^{-/-}$ mice may be more subtle than those produced by 6-OHDA injections or dopamine receptor blockade.

The specific effect on effort found in *Ncs-1*$^{-/-}$ mice sparing other dimensions of motivated behaviour is in line with a large body of work that differentiates the aspects of food motivated behaviour that are sensitive to dopaminergic manipulations (reviewed in (Kelley et al., 2005; Salamone and Correa, 2012)). Hedonic responses to and preferences for food reward are intact in rodents with decreased dopamine signalling (Berridge et al., 1989; Cannon and Palmiter, 2003; Pecina et al., 1997). Similarly, *Ncs-1*$^{-/-}$ mice show intact preference for chocolate-flavoured pellets over lab chow and preference for sucrose solution over pure water (Figure 3-4 a). Ventral striatal dopamine even at 30% of normal levels is sufficient to support Pavlovian approach processes in mice (Darvas et al., 2014) and increases in accumbens dopamine signalling enhance

motivation without affecting Pavlovian approach (Cagniard et al., 2006) or incentive valuation (Trifilieff et al., 2013). *Ncs-1*$^{-/-}$ mice, which show an ~50% decrease in evoked dopamine release in slices (Figure 3-15 b) have normal Pavlovian approach and conditioned reinforcement behaviours (Figure 3-4 b-c). Furthermore, the lack of effect of *Ncs-1* deletion on conditioned reinforcement may be due to an insufficient reduction in extracellular striatal dopamine (Cador et al., 1991), or a compensation by other brain regions such as the amygdala (Cador et al., 1989).

Action-outcome learning and habit formation are highly dependent on processes in the dorsal striatum (Quinn et al., 2013; Yin et al., 2004, 2005a, 2006); these types of learning are intact in the *Ncs-1*$^{-/-}$ mice (Figures 3-2 c, 3-3 f, 3-4 d-e) suggesting relatively normal dorsal striatal function in the *Ncs-1*$^{-/-}$ mice. Taken together, the pattern of impaired and intact motivated behaviour in *Ncs-1*$^{-/-}$ mice matches well with a specific disruptions to ventral striatal dopamine signalling.

4.1.2 Striatal dopamine

There are at least four possible loci at which ventral striatal dopamine signalling could be altered to produce changes in motivated behaviour: at the level of presynaptic release (Zhang et al., 2003), feedback inhibition of release by dopamine D2 autoreceptors (Bello et al., 2011a), reuptake (Cagniard et al., 2006), and changes in surface receptor levels (Trifilieff et al., 2013). We found only small and statistically insignificant changes in whole brain DRD2 levels and striatal surface DRD2 levels (Figure 3-14 b-c). The small effect of *Ncs-1* knockout on baseline levels of DRD2 may not be too surprising, since no changes in baseline DRD2 were found even with a 10-fold overexpression of *Ncs-1*; differences in surface DRD2 and DRD2 internalization were found only upon dopamine stimulation (Kabbani et al., 2002). Future experiments should examine the effects of *Ncs-1* deletion on agonist-induced DRD2 internalization.

We found *Ncs-1* deletion decreases presynaptic dopamine release and increases the paired-pulse

ratio (Figure 3-15 b, c, e), but not rates of reuptake (Figure 3-15 f) or response to dopamine D2 autoreceptor blockade (Figure 3-15 a) were no different than wildtype mice. This finding argues for a role of NCS-1 in regulating frequency-dependent facilitation and contributes to the existing literature on the function of NCS-1 in neurotransmission, most of which has been based on work in *Drosophila* and *Xenopus* with only a few studies in mammalian cells or tissues (reviewed in (Dason et al., 2012)). Our findings are consistent with studies in *Drosophila* where deletion of *Frequenin* or application of an inhibitory C-terminal peptide reduced basal levels of evoked release and enhanced paired-pulse facilitation (Dason et al., 2009; Romero-Pozuelo et al., 2007) Unpublished observations in mouse hippocampal slices also show that an interfering C-terminal peptide decreases basal synaptic transmission and enhances paired-pulse facilitation (Saab, 2010). A large body of literature has highlighted the role of NCS-1 modulation of voltage-gated calcium channels in presynaptic phenotypes (Dason et al., 2012; Dragicevic et al., 2014; Lian et al., 2014; Yan et al., 2014). Future work on the mechanism by which NCS-1 modulates dopamine transmission can focus on such NCS-1/voltage-gated calcium channel interactions.

Our findings of decreased dopamine release at the nucleus accumbens core suggests that at least in this region, NCS-1 may play a unique role in modulating dopamine release that is non-redundant with other calcium sensor proteins. As we did not measure dopamine release from other sites within the ventral striatum (nucleus accumbens core shell) or in the dorsal striatum, we can only speculate as to the function of NCS-1 on dopamine in those areas. Indeed, there are differences in the regulation of dopamine release in the dorsal and ventral striatum, particularly by voltage-gated calcium channels (Brimblecombe et al., 2015; Rice et al., 2011).

The fact that *Ncs-1*$^{-/-}$ mice show amphetamine-induced hyperlocomotion slightly below or at wildtype levels as well as sensitization would suggest that nucleus accumbens dopamine synthesis, vesicular storage and the release machinery hijacked by amphetamine are functional. Amphetamine is thought to act as a dopamine "releaser" (Fleckenstein et al., 2007), enhances exocytotic dopamine release and decreases dopamine uptake (Daberkow et al., 2013). Viral restoration of dopamine to the nucleus accumbens alone (but not the caudate putamen alone) of dopamine deficient mice is sufficient to rescue amphetamine-induced hyperlocomotion (Heusner

et al., 2003). Taken together, our data suggest NCS-1 plays a role in the modulation of frequency-dependent dopamine release, but might not play a detectable role in the actions of amphetamine in the nucleus accumbens (at least as reflected in locomotor behaviour). Another interpretation is that amphetamine acts in such a way that it can overcome the effects the absence of NCS-1 modulation may have e.g. on voltage-gated calcium channel function. Indeed, recent studies show that amphetamine can activate various voltage-gated calcium channels via depolarization mediated by dopamine transporters (Cameron et al., 2015).

4.1.3 Limitations

There are at least three limitations to our experimental approach here. First, we are drawing interpretations based on the association between behavioural data and acute slice recordings of one striatal subregion. A stronger argument could be made using *in vivo* voltammetric or microdialysis measurements of dopamine from various striatal subregions concurrent with behaviour tasks. Nevertheless, we do have a crude and indirect index of *in vivo* dopamine release in from the effects of amphetamine on locomotor activity.

Second, our measurements of striatal surface DRD2 were obtained from biotinylation of thin acute coronal sections. Since acute slices are not one-cell layer thick and there may be some damage to cells in the process, it is unlikely that we obtained a very pure measure of surface DRD2 levels. Moreover, even with the use of subcellular fractionation, we cannot not distinguish between pre- and postsynaptic DRD2. Biotinylation in primary cell cultures would provide a more pure measure of surface DRD2, while immunogold labelling for DRD2 and electron microscopy would allow one to differentiate presynaptic, postsynaptic and extrasynaptic DRD2.

Third, our interpretations of *Ncs-1*$^{-/-}$ mice showing decreased motivation for food must be tempered by the fact that *Ncs-1*$^{-/-}$ mice weigh more than wildtype mice (Figure 3-2 a)

(Hermainski, 2012; Nakamura et al., 2011). While *Ncs-1*$^{-/-}$ mice show similar levels of food intake and activity level to wildtype mice, they show a metabolic phenotype with a predisposition toward altered glucose regulation (Hermainski, 2012). It is unlikely though that metabolic difference could fully account for the behavioural phenotypes we find for example, by changes in appetite. *Ncs-1*$^{-/-}$ mice show similar levels of food intake during food restriction (Figure 3-3 e) and tend to consume more lab chow than wildtype mice in the concurrent choice task (Figure 3-3 c). This latter effect is contrary to the effect of pre-feeding or appetite suppressants on the concurrent choice task, which not only decrease lever pressing for preferred food but also amount of chow consumed as well (Salamone et al., 2002). The tight fit between the pattern of behavioural and physiological phenotypes and the literature on dopamine-dependent food motivation is consistent with our current interpretation. Moreover, *Ncs-1*$^{-/-}$ mice appear to have impairments in non-food related motivation as found in decreased social approach (Figure 3-13 a). Additional experiments testing *Ncs-1*$^{-/-}$ mice motivation for social stimuli or drugs of abuse would help to clarify this issue. Nevertheless, it may be worth investigating if the metabolic phenotype may contribute to aspects of motivation given recent work showing insulin could induce LTD in the ventral tegmental (VTA) neurons, and local insulin injections into the VTA reduced food-related anticipatory behaviours and food conditioned place preference (Labouebe et al., 2013). Note however, that progressive ratio performance was unaffected in Laboube et al's study. Ultimately, the potential confound of metabolic and weight factors contributing to the difference in willingness to work for food rewards can likely only be determined using tissue or cell-type specific conditional knockout mice that do not possess a metabolic phenotype. Alternatively, *Ncs-1*$^{-/-}$ mice could be given drugs that do not cross the blood-brain barrier to rescue or correct the metabolic effects. If the difference in motivated behaviour remains despite the lack or correction of a metabolic phenotype, this would suggest the motivational phenotype is not a by-product of the metabolic phenotype.

4.1.4 Summary

Ncs-1 deletion appears to specifically impair willingness to work for food via a decrease in nucleus accumbens core dopamine release. The effort dimension of motivation may have been

more affected than Pavlovian incentive motivation because the former is more sensitive to changes in dopamine, while Pavlovian approach can occur even with 30% of baseline dopamine signalling. Intact instrumental conditioning, habit formation and rota-rod performance suggest that dorsal striatal function and dopamine signalling may be relatively unaffected by *Ncs-1* deletion. Future studies should assess the mechanisms by which NCS-1 modulates dopamine release such as through the regulation of voltage-gated calcium channels especially since there are differences in the roles of these ion channels in gating dopamine release from the dorsal compared to the ventral striatum (Brimblecombe et al., 2015). While how dopamine signalling codes for effort is not yet known (some theorize it comes from tonic dopamine (Niv et al., 2007) or firing from a subset of dopaminergic neurons (Pasquereau and Turner, 2013)), it would be interesting to investigate why this class of signalling may be particularly dependent on NCS-1 function.

Based on our results and what is known from the literature, the following speculative model can be used to describe the links between NCS-1, dopamine and motivated behaviour (Figure 4-1). Similar to other synaptic terminals, dopamine release in the nucleus accumbens is regulated by P/Q-type voltage-gated calcium channels ($Ca_v2.1$) (as well as N-type channels) (Brimblecombe et al., 2015). Calcium sensor proteins like calmodulin and NCS-1 can bind to the IQ-like motif in the C-terminal domain of the α_{1A} channel subunit to modulate its function in a Ca^{2+}-dependent manner (Burgoyne and Haynes, 2014; Tsujimoto et al., 2002; Yan et al., 2014). Since NCS-1 has a higher Ca^{2+} affinity than calmodulin (though the latter is more highly expressed), NCS-1 may regulate $Ca_v2.1$ function at lower ranges of Ca^{2+}, with calmodulin displacing NCS-1 as Ca^{2+} concentrations increase (Lian et al., 2014). Studies in *Drosophila*, rat hippocampal cell cultures, Calyx of Held and superior cervical ganglion cultures all suggest NCS-1 acts to increase $Ca_v2.1$ channel activity and thus facilitates synaptic transmission (Dason et al., 2009; Sippy et al., 2003; Tsujimoto et al., 2002; Yan et al., 2014). I speculate then, that *Ncs-1* deletion in the mouse, removes calcium-dependent facilitation of $Ca_v2.1$ channels in a manner that cannot be compensated for by other calcium sensor proteins (at least in the case of nucleus accumbens core dopaminergic terminals). As a result, while the $Ca_v2.1$ channels are functional, still allowing for dopamine transmission, they will tend to inactivate sooner in the absence of NCS-1, leading to decreased dopamine release and initial probability of release in response to electrical stimulation.

Reduced facilitation of P/Q type currents then, presumably interferes with the patterns of dopamine release that code for effort or behavioural activation, thus decreasing the willingness with which animals will work for reward.

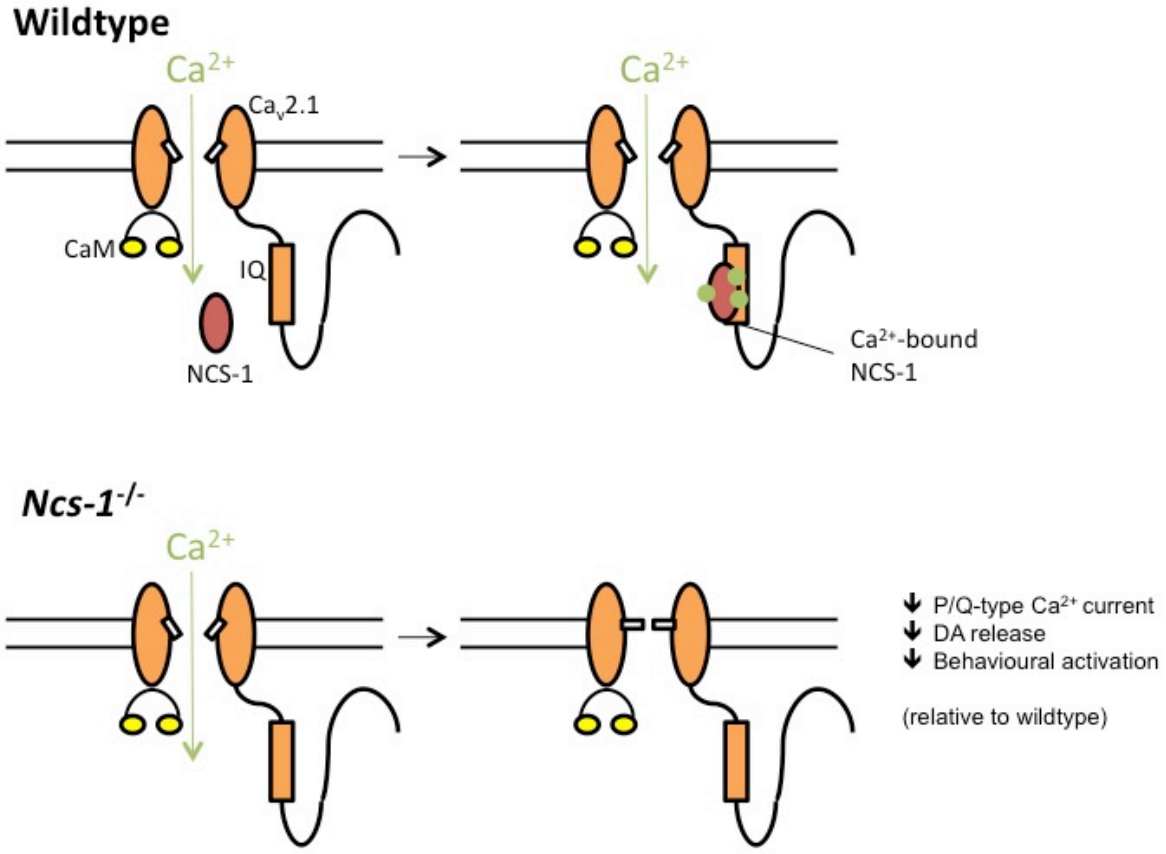

Figure 4-1. *Ncs-1* deletion might decrease $Ca_v2.1$ channel activity, thus decreasing dopamine neurotransmission in the nucleus accumbens and motivated behaviour. Note this is a speculative model. Schematics show P/Q-type voltage-gated calcium channels ($Ca_v2.1$) in nucleus accumbens core dopaminergic terminals. In wildtype neurons, membrane depolarization opens $Ca_v2.1$ channels, allowing the influx of Ca^{2+}, which is detected and bound by NCS-1. Ca^{2+}-bound NCS-1 then interacts with the IQ-like motif in the C-terminus of the α_{1A} channel subunit, acting to increase P/Q-type Ca^{2+} currents by delaying channel inactivation or perhaps facilitating opening of nearby channels. In *Ncs-1*$^{-/-}$ neurons, the absence of NCS-1 may lead to an earlier inactivation of $Ca_v2.1$ channels, decreasing presynaptic Ca^{2+}, leading to lower dopamine neurotransmission from the nucleus accumbens core, and interfering with the dopamine signals that code for effort. Calmodulin (CaM) is likely present as well, but for some reason is unable to compensate for the lack of NCS-1 at least in the nucleus accumbens core. The schematics were adapted from (Budde et al., 2002).

Motor and other functions mediated by the dorsal striatum may not be affected by *Ncs-1* deletion, potentially because dorsal striatal dopamine release is modulated by a greater variety of voltage-gated calcium channels including L-type, P/Q type, N type and T type channels (Brimblecombe et al., 2015). Amphetamine-induced hyperlocomotion may still be found in *Ncs-1*$^{-/-}$ mice, perhaps because its effects may not be primarily dependent on calcium-dependent modulation of voltage-gated calcium channels, but on DAT (DAT mediated dopamine release and DAT-mediated depolarization that activates $Ca_v2.1$ channels).

4.2 The role of NCS-1 in learning, memory and hippocampal electrophysiology

Overexpressing *Ncs-1* in murine dentate gyrus enhances spatial learning and LTP, but the effect of decreasing NCS-1 on spatial learning is unclear (Saab et al., 2009). Moreover, while NCS-1 is required for perirhinal mGluR LTD (Jo et al., 2008), whether this holds true for other brain regions is unknown. We hypothesized that knocking out *Ncs-1* would impair spatial learning and memory as well as hippocampal long-term plasticity. We also expected impaired behavioural flexibility since this has been associated with hippocampal LTD. Consistent with our hypothesis, we found that *Ncs-1* deletion led to a deficit in spatial cognition, but only in displaced object recognition (Table 4-2). Spontaneous object recognition, spatial reference memory acquisition, behavioural flexibility, as well as memory for a fearful context or cue were unaffected. Unexpectedly, *Ncs-1*$^{-/-}$ mice showed slower acquisition in a water-based visual discrimination task. Field recordings from hippocampal slices revealed decreased long-term plasticity in late-phase LTP in the dentate gyrus, and early-phase mGluR LTD in the dentate gyrus and CA1.

Table 4-2. Learning, memory and electrophysiology in *Ncs-1*$^{-/-}$ mice.

Test / Procedure	Measure of	Relative to Wildtype
Novel object recognition	Short-term object memory	=
Displaced object recognition	Short-term spatial memory for spatial configuration	<
Water maze initial acquisition	Spatial reference memory	=
Spatial reversal in the water maze	Behavioural flexibility	=
Waterbox visual discrimination learning	Operant learning	<
Waterbox strategy shifting and reversal learning	Behavioural flexibility	=
Fear conditioning	Simple classical conditioning	=
Dentate gyrus electrophysiology	Paired pulse ratio (2.5:1.3 Ca^{2+}:Mg^{2+})	=
	Paired pulse ratio (2:2 Ca^{2+}:Mg^{2+})	<
	LTP	$\leq$ later stages
	mGluR-LTD	$\leq$ early stage
CA1 electrophysiology	Paired pulse ratio (2:2)	=
	mGluR-LTD	=

4.2.1 Object recognition and LTD

Our finding of impaired displaced object recognition together with intact spontaneous object recognition with use of a short 3 min delay (between sample and test) is similar to Saab et al (2009). They found that dentate gyrus infusions of a DRD2/NCS-1 interfering peptide could reverse enhancements in *Ncs-1* over-expressing mice and impaired displaced object preference (with a 2 min delay) in control mice while sparing preference for a novel object. In contrast, de Rezende et al, found impaired spontaneous object memory in a distinct line of *Ncs-1*$^{-/-}$ mice, though with a 24 h delay; they did not test displaced object memory (de Rezende et al., 2014).

Spontaneous object recognition depends primarily on the perirhinal cortex and can occur without the hippocampus, particularly at short delay intervals (<10 min) between the sample and test phases (see reviews by (Cohen and Stackman Jr., 2014; Dere et al., 2007; Winters et al., 2008)). Evidence suggests visual familiarity discrimination in the perirhinal cortex depends on LTD (Brown and Bashir, 2002; Griffiths et al., 2008). The difference in findings between our study and de Rezende's may be attributable to the different sample-test delay interval used (3 min versus 24 h respectively). Considering both studies together, we can make two statements about the role of NCS-1 and mGluR-LTD in object recognition. First, impaired long-term spontaneous object recognition (24 h as in de Rezende) in *Ncs-1*$^{-/-}$ mice may be due to impaired mGluR-LTD, since NCS-1 is required for perirhinal mGluR-LTD and AMPAR internalization (Jo et al., 2008). Second, short-term spontaneous object recognition (3 min as in our study) may not require NCS-1 because it depends on mechanisms other than perirhinal NMDAR- or mGluR-LTD (Barker et al., 2006b; Brown and Banks, 2014). Consistent with these suggestions, local perirhinal infusions of mGluR antagonists or NMDAR antagonist impairs novel object preference with a long delay (24 h) but not with a short (15-20 min) delay (Barker et al., 2006a; Barker et al., 2006b). Rather, novel object preference with short delays (<20 min) seems to rely on kainate receptors (Banks et al., 2012; Barker et al., 2006a).

In contrast to object recognition, recognizing object displacement is hippocampal-dependent

(Bussey et al., 2000; Good et al., 2007; Langston and Wood, 2010; Mumby et al., 2002; Save et al., 1992). Consistent with our hypothesis that *Ncs-1*$^{-/-}$ mice would display deficits in spatial cognition dependent on the hippocampus, we found impaired displaced object preference as well as a trend toward reduced early phase LTD in the dentate gyrus and the CA1 (Figures 3-17, 3-18).

In vivo dentate gyrus LTD is facilitated by large directional spatial cues including changes in spatial configuration of cues similar to a displaced object task (Kemp and Manahan-Vaughan, 2008). Novel spatial object recognition also facilitates *in vivo* hippocampal LTD induction in the CA1 in a manner dependent on NMDAR and mGluR (Goh and Manahan-Vaughan, 2013a, b). We speculate that changes in dentate gyrus function may underlie displaced object preference as evidence from computational models and animal studies implicates the dentate gyrus in detecting changes in spatial configuration (reviewed in (Kesner, 2013a, b)). Mice with a GluN2A (NMDAR subunit) deletion show a selective deficit in a displaced object task and disrupted dentate gyrus LTP and LTD similar to *Ncs-1*$^{-/-}$ mice (Kannangara et al., 2014). This recent study taken together with our data may suggest that both NMDAR- and mGluR-LTD are needed for displaced object recognition even with short delays (we used a 2 min delay; Kannangara et al used a 5 min delay).

Our speculation that the dentate gyrus may be particularly affected in the *Ncs-1*$^{-/-}$ mice is also supported by decreased paired-pulse ratios under lower extracellular Ca^{2+} conditions found in the dentate gyrus but not the CA1. Lower extracellular calcium tends to attenuate depression and favour facilitation by decreasing initial probability of transmitter release. While *Ncs-1*$^{-/-}$ mice still showed a typical triphasic response curve (Blaise and Bronzino, 2000) with an early inhibitory phase, relative facilitation in the middle, followed by late-onset inhibition, their response curve was shifted down toward depression relative to wildtype mice. Indeed, the lower calcium conditions led us to see some facilitation (ratios > 1) in wildtype mice during the middle phase, but none was seen in *Ncs-1*$^{-/-}$ mice. Interestingly, the input/output curves were very similar between genotypes, suggesting the paired pulse difference may not be due to differences in initial probability of release.

4.2.2 Spatial reference memory and LTP

Our results here are consistent with findings in rat hippocampal cultures showing NCS-1 can enhance facilitation without affecting basal transmitter release (Sippy et al., 2003). Similar findings have been found in calyx of Held (Tsujimoto et al., 2002) and superior cervical ganglion neurons (Yan et al., 2014). Note however, this is different from nucleus accumbens core dopamine release, where *Ncs-1*$^{-/-}$ slices showed decreased evoked dopamine release together with an increase in paired-pulse ratios. This suggests that NCS-1 may have slightly different roles in different synapses; indeed there are many differences between dopamine and glutamate transmission (see (Rice et al., 2011).

We expected *Ncs-1* deletion to impair Morris water maze performance and dentate gyrus LTP, because overexpressing *Ncs-1* in the dentate gyrus was associated with improved one-trial water maze learning and enhanced LTP (Saab et al., 2009). Contrary to our predictions, *Ncs-1*$^{-/-}$ mice showed Morris water maze performance similar to wildtype mice and only showed a trend toward decreased late-phase LTP. Our results are similar to those of mice null for *Hpca* (Hippocalcin), a related calcium sensor with high expression in the hippocampus (Paterlini et al., 2000). *Hpca* knockout mice show intact CA1 LTP and normal Morris water maze learning, but in probe trials they show fewer crossings of the former platform location than wildtype mice (Kobayashi et al., 2005). *Ncs-1*$^{-/-}$ mice also show a trend toward spending less time in the target quadrant than wildtype mice on a probe trial (Figure 3-6 b). Therefore, while increasing hippocampal *Ncs-1* may enhance spatial learning and long-term plasticity, deletion of any one neuronal calcium sensor (at least NCS-1 or hippocalcin) is insufficient to drastically disrupt spatial learning perhaps due to compensation by many other calcium sensors.

The observation that dentate gyrus LTP drops sooner than in wildtype mice is consistent with at least two explanations. First, *in vivo* dentate gyrus LTP in rats is associated with rapid elevations

in *Ncs-1* mRNA within an hour that are sustained for at least 3 h, suggesting it may play a role in the transition to late-phase LTP (Génin et al., 2001). Second, dopamine is known to potentiate late-phase LTP (Frey et al., 1989; Huang and Kandel, 1995). While we only measured dopamine release in the nucleus accumbens core, it is possible that hippocampal dopamine may also be decreased in *Ncs-1*$^{-/-}$ mice.

4.2.3 Behavioural flexibility

While impairments in LTD have been associated with disrupted spatial reversal (Kim et al., 2011; Nicholls et al., 2008), *Ncs-1*$^{-/-}$ mice displayed intact spatial reversal in the Morris water maze. Lack of impairment in spatial reversal may not be too surprising since most studies associating LTD and spatial reversal learning have focused on NMDAR-mediated LTD, which is hippocalcin-dependent, and only one study has associated flexibility with mGluR-mediated LTD (Eales et al., 2014). Also, LTD is not absolutely necessary for flexibility as type I adenylyl cyclase knockout mice show enhanced flexibility despite lacking LTD (Zhang and Wang, 2013).

Behavioural flexibility (including strategy shifting and reversal) also involves dopamine signalling in cortico-striato-thalamic circuits (Darvas and Palmiter, 2011; Floresco et al., 2009; Haluk and Floresco, 2009). *Ncs-1*$^{-/-}$ mice did not show impairments in strategy shifting or reversal of an egocentric response. Presumably the decrease in striatal dopamine release found in *Ncs-1*$^{-/-}$ mice was not sufficient to lead to the type of deficits found in intra-accumbens dopamine antagonism (Haluk and Floresco, 2009) or in dopamine-deficient mice (Darvas and Palmiter, 2011).

Unexpectedly, *Ncs-1*$^{-/-}$ mice committed more errors before meeting criterion in a waterbox visual discrimination task (Figure 3-7). This task required discriminating between alternating black and white vertical stripes with a pure grey background and associating the former with an approach

response that would lead to a hidden escape platform. Forming this type of association is likely dependent on the striatum, especially since the images we used are quite dissimilar. It is unlikely that visual perceptual deficits account for this difference due to normal performance of *Ncs-1*$^{-/-}$ mice in the spatial version of the water maze (Figures 3-6 and A-6) as well as a visual cued version. Nevertheless, further testing on visual perception would be required to definitively rule out this possibility. Perhaps the stress associated with a water-based task unmasked a slight deficit in instrumental learning that we did not detect in food-based operant tasks. *Hpca* knockout mice demonstrated a similar but more severe visual discrimination deficit (in a food based operant task). While *Ncs-1*$^{-/-}$ mice are able to learn visual discrimination though at a slower rate, *Hpca* knockout mice performed at chance levels throughout initial acquisition into a reversal training phase (Kobayashi et al., 2005).

Ncs-1$^{-/-}$ mice also showed normal memory for a fearful context and a fearful cue, which in general depend on the amygdala and the former on the hippocampus as well (reviewed with cautions in (Maren, 2008)). This is consistent with intact aversive memory in de Rezende's study of *Ncs-1*$^{-/-}$ mice using an inhibitory avoidance task.

4.2.4 Limitations

There are three limiting factors to our approach and interpretation. First, our novel and displaced object preference data were only performed with a short delay of 3 min, precluding us from making conclusions regarding long-term recognition memory. Second, our use of a knockout animal does not allow us to resolve whether NCS-1 may play a role in the encoding, consolidation or retrieval phases of memory, for instance in displaced object recognition. To resolve the role of NCS-1 in the various stages of memory, acute and temporary manipulations to NCS-1 would be needed for example, using micro-infusions of cell-permeant inhibitory peptides or siRNA. Third, while we have made use of the literature to associate various measures of

hippocampal plasticity with behaviour, one must keep in mind that these are correlations between a freely behaving animal and physiological measures in an acute brain slice. Stronger conclusions would require *in vivo* electrophysiology in freely behaving knockout mice.

4.2.5 Summary

Overall our findings suggest that *Ncs-1* deletion has selective effects on displaced object memory and subtle effects on hippocampal plasticity. Since the dentate gyrus is thought to mediate detection of subtle shifts in spatial configuration and we found trends toward pre- and postsynaptic changes in that hippocampal subregion, we suspect that NCS-1 may have a special role in the dentate gyrus. *Ncs-1*$^{-/-}$ mice were deficient in short-term displaced object recognition, potentially because it requires LTD-like mechanisms and *Ncs-1*$^{-/-}$ mice showed a trend for attenuated early-phase LTD. In contrast, long-term spatial reference memory appeared relatively normal. This may be because *Ncs-1*$^{-/-}$ mice showed relatively normal levels of early LTP induction and only a trend toward decreased maintenance of LTP.

4.3 NCS-1 and endophenotypes of neuropsychiatric disease

We hypothesized that *Ncs-1*$^{-/-}$ mice would display behavioural endophenotypes relevant to neuropsychiatric disease such as disruptions in sensorimotor gating, greater anxiety or altered sociability. We found that *Ncs-1*$^{-/-}$ mice performed at wildtype levels in the assays we employed except for displaying a lack of social preference in the 3-chamber social approach task (Table 4-3).

Table 4-3. Behavioural endophenotypes of human neuropsychiatric disease in *Ncs-1*$^{-/-}$ mice.

Test / Procedure	Measure of	Relative to Wildtype
Open field	Locomotion	=
Accelerating rota-rod	Motor learning	=
Amphetamine induced locomotion	Acute hyperlocomotion to a psychostimulant	=
Sensitization to amphetamine		=
Light/Dark box	Anxiety-like behaviour	=
Elevated zero maze	Anxiety-like behaviour	=
Tail suspension	Immobility in response to tail suspension	=
Pre-Pulse Inhibition	Sensorimotor gating	=
3-chamber social approach	Social motivation / preference	<
Reciprocal social interaction		=

4.3.1 Motor activity

Ncs-1$^{-/-}$ mice showed normal open field locomotion and motor learning on the rota-rod, which suggests that differences found in other behaviours are unlikely to be by-products of motor deficits or decreased activity. *Ncs-1*$^{-/-}$ mice also demonstrated hyperlocomotion to amphetamine and behavioural sensitization with repeated amphetamine administration. It is unlikely that

ceiling effects could fully explain the lack of a genotype difference in hyperlocomotion in response to 2.5 mg/kg amphetamine, because similar results (no genotype differences) were found using a lower, 1 mg/kg dose (Figure A-4 e). Sensitization to drugs of abuse is used as a model of drug-induced neuroplasticity and sensitization to amphetamine involves nucleus accumbens LTD and AMPAR endocytosis (Brebner et al., 2005; Choi et al., 2013). Intact amphetamine sensitization in the *Ncs-1*$^{-/-}$ mice suggests NCS-1 may not be critical for this form type of LTD (that underlies sensitization) in the nucleus accumbens.

4.3.2 Anxiety-like behaviours

We found that *Ncs-1*$^{-/-}$ mice show normal levels of anxiety-like behaviour and wildtype levels of immobility during the tail suspension test, in contrast to a recent report in a distinct line of *Ncs-1*$^{-/-}$ mice. While de Rezende et al (2014) found *Ncs-1*$^{-/-}$ mice spent significantly less time in the open arms of an elevated plus maze and less time in the centre of an open field, we found *Ncs-1*$^{-/-}$ mice spent similar proportions of time in the open quadrant of an elevated zero maze and in the light compartment of the light/dark box. Also, while de Rezende et al identified increased immobility in homozygous and heterozygous knockout mice in the forced swim test and tail suspension test, we found similar levels of tail suspension immobility across genotypes. The difference in results regarding immobility may be a sex difference, as our results in the tail suspension test were primarily from female mice (all our other behaviour tests used males) while de Rezende used only male mice. Two other factors could contribute to these differences. First genetic background differences can remain despite repeated backcrossing due to the flanking allele effect (Crusio, 2004a; Gerlai, 1996). The founder mouse for the knockout line used by de Rezende et al has a mixed C57BL/6 and BALB/c background (C57BL/6 ES cell; BALB/c blastocyst) (Mühlemann, 2005). These mice were re-derived and backcrossed to C57BL/6 mice at least ten times prior to behavioural testing (de Rezende et al., 2014). In contrast, the gene targeting strategy used for the knockout line we tested involved the use of a 129/Sv-derived ES line (R1) and C57BL/6J blastocyst; the line has been back-crossed to C57BL/6J over ten generations and kept on a C57BL/6J background since (Hermainski, 2012). Second, differences in tests used, lab environments, and testing conditions could certainly contribute the different

findings (Crabbe et al., 1999). Differences in maternal behaviour due to background effects may also be a contributing factor.[9]

4.3.3 Sociability

While *Ncs-1*$^{-/-}$ mice were impaired in 3-chamber social approach, they showed wildtype levels of reciprocal social interaction. Similarly, de Rezende et al found normal social interactions in *Ncs-1*$^{-/-}$ mice but they did not test the 3-chamber social approach. This pattern of social behaviour may reflect impaired motivation to approach a social partner, without actual disruptions in the ability for social interaction. A deficit in social motivation would suggest that *Ncs-1*$^{-/-}$ mice show a more general deficit in motivation that extends beyond food-reinforced operant behaviours. The deficit in social motivation is unlikely to be due to differences in maternal care or prior social interactions as mice were reared by heterozygous parents and then weaned into group cages that included mice of other genotypes. Our interpretation is of course limited by the fact that we did not test the various dimensions of social motivation as we did for food. Effort tests for social reinforcers have been recently developed and can be used in future studies (Bai et al., 2014; Martin et al., 2014).

4.3.4 Summary

Our results suggest that deletion of *Ncs-1* does not appear to produce any obvious behavioural endophenotypes relevant to neuropsychiatric disease except for a selective deficit in social approach. Indeed, of the diseases where NCS-1 levels have been assessed (schizophrenia, bipolar

[9] Note however that in de Rezende and in our experiments, mice were raised by heterozygous parents prior to weaning and then were subsequently group-housed.

disorder, Parkinson's disease), elevations in NCS-1 have been found. *Ncs-1* deletion may have relatively subtle effects due to the large number of related calcium sensors that may be able to compensate for its effects. Elevations of NCS-1 however, may be more deleterious because NCS-1 has a higher calcium affinity than many calcium sensors such that it can outcompete other calcium sensors. For example, Yan et al, show that introduction of NCS-1 can functionally compete with calmodulin when introduced into superior cervical ganglion cells (Yan et al., 2014).

4.4 Limitations to use of a constitutional knockout obtained via gene-targeting

Our interpretation that the behavioural and physiological phenotypes differences we find may be directly attributed to *Ncs-1* deletion comes with three caveats. First is the so-called flanking allele problem caused by the gene-targeting manipulations in 129 strain-derived embryonic stem cells that are then transferred to C57BL/6J. Second, our use of a constitutional knockout suggests potential compensatory changes may contribute to the phenotypes we see. Third, epistatic interactions may occur between the general genetic background and the targeted locus.

4.4.1 Flanking allele problem

Most gene targeting manipulations, including the one used to generate the *Ncs-1*$^{-/-}$ mice we used, are generated in embryonic stem (ES) cells derived from the 129 family of mice (Section 2.1). These ES cells are then usually injected into a blastocyst (often C57BL/6 as in our case) to get chimera hopefully with germline derived from the ES cells. Since 129 strains of mice tend to be poor breeders, have corpus callosum dysgenesis and are poor learners, such chimeras are often mated to C57BL/6J. This common practice, however, introduces an important confound as first pointed out by Gerlai in 1996 (Gerlai, 1996; Gerlai, 2001). Due to the linkage of genes to the targeted locus, the alleles flanking the targeted locus will be of 129-type whereas the alleles in

the wildtype mice will be of C57BL/6J-type. Therefore phenotypic differences between wildtype and mutant mice could also be due to these flanking alleles that differ between genotypes, because they come from different background strains. This limitation applies to the *Ncs-1*$^{-/-}$ mice we used and would require future experiments to rule out such potential confounds.

Even with extensive backcrossing to C57BL/6J (in our case >10 generations), which can reduce the size of the flanking region, it is practically impossible to eliminate all 129-derived alleles surrounding and linked to the targeted locus. For example, even after 11 generations of backcrossing to C57BL/6J, a null mutation for *Kcc2* (a K-Cl cotransporter) co-segregated with agouti fur colour because the agouti locus is only 5 cM away from the *Kcc2* locus (Crusio, 2004a; Woo et al., 2002). The only way to obtain co-isogenic strains where the mutant and wildtype mice differ only at the one targeted locus would be to cross the chimera to the same strain as that used for ES cell derivation.

There are several strategies that could be used to address the flanking allele problem (Wolfer et al., 2002). First, we could generate inducible knockout mice, for example by crossing the NCS-1-EGFP knockin mouse to an inducible Cre strain. Then we could use as controls mice that have the targeted gene locus but have not been fed drug to induce Cre-mediated deletion of *Ncs-1*. Second, we could rule out flanking allele effects if the phenotypes in the constitutional knockout mice we used could be rescued by re-introducing the knocked out gene. Third, Wolfer et al has provided some breeding strategies that deal with the flanking allele problem by comparing littermates in which the alleles of flanking genes always derive from the ES-cell donor strain (Wolfer et al., 2002). For example, a backcross-outcross strategy can be used to distinguish between flanking allele effects and the effects of a targeted mutation that is recessive (Wolfer et al., 2002). Mutants that are congenic with C57BL/6J are outcrossed with inbred 129 mice to generate a reverse F1 (RF1) generation. RF1 heterozygotes are homozygous for 129-type alleles flanking the target region. Therefore, phenotypic differences between RF1 heterozygous mice and RF1 wildtype mice that resemble the ‘mutant’ phenotype suggest that recessive 129-type alleles may be contributing to the ‘mutant’ phenotype.

4.4.2 Compensatory effects

The constitutive deletion of *Ncs-1* could lead to a number of developmental, physiological or behavioural compensatory effects that could affect the phenotypes we observe in at least two possible ways. First, genetic redundancy, for example from genes that code for other calcium sensors could potentially mask phenotypes or make them relatively subtle. As mentioned previously, compensatory effects, for example upregulation of other calcium sensors in the hippocampus might play a role in the subtle effect *Ncs-1* deletion has on spatial learning. Assuming that the phenotypes we observe are truly due to the deletion of *Ncs-1*, our results suggest that there is not full redundancy between NCS-1 and other calcium sensor proteins particularly in the nucleus accumbens core. Nevertheless, our current experiments have not ruled out a second possibility, which is that compensatory changes in other genes may actually contribute to or be the cause of the phenotypes observed.

There are several ways future studies can address whether compensatory effects are affecting the phenotypes we found. First, one could use gene microarrays to see if there are any genes that are significantly upregulated or downregulated in *Ncs-1*$^{-/-}$ mice relative to wildtype littermates. Second, one could minimize the potential for developmental compensations by using inducible Cre lines to knockout *Ncs-1* in adulthood. Another way is to interfere with function or interactions at the level of the NCS-1 protein. For example, cell-permeant peptides have been used previously in rodent and *Drosophila* to disrupt the interaction between the C-terminus of NCS-1 and target proteins that bind there (Romero-Pozuelo et al., 2007; Saab, 2010; Tsujimoto et al., 2002). In future, such interfering peptides could be used to see whether acute disruptions of NCS-1 to its C-terminal binding partners produces phenotypes similar to or even more profound than those found in the constitutive knockout mouse.

4.4.3 Genetic background

In addition to the flanking allele problem and compensatory effects, polymorphisms in the general genetic background (not just at loci linked to the target mutation) could contribute to phenotypes due to epistatic interactions (Crusio, 2004b). For example, neuronal nitric oxide synthase knockout mice only show increased aggression on a hybrid 129Sr/SvJae-C57BL/6J background but not when backcrossed 5 generations to C57BL/6J (Crusio, 2004b; Le Roy et al., 2000). Thus, our results primarily pertain to the effects of constitutive deletion of *Ncs-1* on a primarily C57BL/6J background with some alleles flanking the deletion site that may come from 129 strains. To test the generalizability of the effects we found, future studies may want to examine the effects of *Ncs-1* deletion on the background of other inbred, hybrid or even outbred strains.

4.5 Conclusions

We explored the effects of *Ncs-1* deletion on various types of cognition dependent on different networks of brain regions (see Section 1.2.2 and Figure 1-1). *Ncs-1* deletion had only subtle effects on declarative memory processes, with a deficit in displaced object recognition and slight changes to hippocampal plasticity. Operant conditioning and classical fear conditioning were similarly intact in *Ncs-1*$^{-/-}$ mice. The largest effect of *Ncs-1* deletion was in decreasing motivation to work for food rewards and to approach novel social partners together with an approximately 50% decrease in evoked dopamine release from the nucleus accumbens core. The most significant changes in neurophysiology appear to be in neurotransmission and short-term plasticity as levels of striatal DRD2 and DAT were not significantly different from wildtype mice and a difference in short-term plasticity was also observed in the dentate gyrus under low Ca^{2+} conditions. Therefore, our studies highlight a role for NCS-1 in the modulation of presynaptic dopamine release, particularly in the nucleus accumbens core where it supports effortful action.

What is the role of NCS-1 in cognition in the context of other calcium sensors? Calmodulin is ubiquitous and has many roles, but is critical for NMDAR-dependent LTP and LTD thought to underlie many learning and memory processes. Hippocalcin has a role in NMDAR-dependent LTD, long-term spatial reference memory and operant acquisition. While decreasing KChIP3 appears to have cognitive enhancing effects due to an elevation of NMDAR function, this also has the downside of excitotoxicity, suggesting KChIP3 has an important role in the negative feedback regulation of NMDAR. All these calcium sensors are involved in learning, memory and long-term plasticity. While NCS-1 also has effects on mGluR-LTD and displaced object recognition, it seems to be unique from other calcium sensors in its ability to modulate motivation, particularly the dimension of effort. Effort and effort-related decision-making are critical aspects to cognition and survival. The ability to learn what actions lead to reward or are necessary for escape from danger is not enough for life. An organism needs to be able to sustain activity in the face of obstacles, challenges and delays. Moreover, an organism must continuously make decisions based on its current energy status and the value of a particular outcome relative to the work required to obtain it. NCS-1 modulation of presynaptic dopamine release as well as dopamine receptor signalling pathways may contribute to these types of neural calculations.

In summary NCS-1 may not be primarily involved in learning or memory *per se* but appears to play a role in modulating behavioural activation. Overexpression of *Ncs-1* promotes exploratory behaviour, which enhances spatial learning and long-term plasticity in a dopamine-dependent fashion (Saab et al., 2009). Conversely knockout of *Ncs-1* leads to decreased recognition of spatial novelty and willingness to work for food reward or to approach a novel social partner (with no deficits in actual feeding behaviour or social interaction). In other words, knockout of *Ncs-1* seems to decrease effort expenditure and promote energy conservation, which may help explain their higher weights. Indeed the effects of modulating NCS-1 levels on mouse behaviour are consistent with aspects of the energy expenditure hypothesis of dopamine (Beeler et al., 2012). I speculate then that, NCS-1 may be an important molecular player in translating neuronal

activity and subtle shifts in Ca^{2+} into the types of dopamine signals that mediate motivation and behavioural activation.

5 Future Directions

5.1 Can A_{2A} antagonists rescue the motivational deficit found in *Ncs-1*$^{-/-}$ mice?

Medium spiny neurons in the striatum that express DRD2 also express adenosine A_{2A} receptors ($A_{2A}R$) (Ferre et al., 1997; Svenningsson et al., 1997). Indeed DRD2 and A_{2A} receptors form functional heteromers (Ferre et al., 1991a; Ferre et al., 1991b; Ferre et al., 2011; Ferre et al., 1991c). Recent work has shown that NCS-1 interacts with A_{2A} receptors as well as $A_{2A}R$ -DRD2 heteromers, modulating their function (Navarro et al., 2014; Navarro et al., 2012) (Figure 5-1 shows coronal expression patterns of the genes for these proteins in mouse brain).

There is also mounting evidence that various $A_{2A}R$ antagonists can reverse motivational deficits caused by DRD2 antagonists or dopamine depletion. $A_{2A}R$ antagonists but not adenosine A_1 receptor antagonists could reverse the effects of DRD2 antagonists or dopamine projection disconnections on concurrent choice tasks, restoring the levels of operant responses and decreasing chow consumption (Farrar et al., 2007; Farrar et al., 2010; Mingote et al., 2008; Nunes et al., 2010; Salamone and Correa, 2009; Worden et al., 2009). Similar results have been found in mice using a T-maze version of the concurrent choice task. $A_{2A}R$ antagonists could reverse the effects DRD2 antagonism and *Adora2a* knockout mice were resistant to the effects of DRD2 antagonism on the effort-based choice in the T-maze task (Pardo et al., 2012).

Given these findings, I hypothesize that $A_{2A}R$ antagonism or crossing with *Adora2a* (adenosine A_{2A} receptor gene) knockout mice could reverse the motivational impairments found in *Ncs-1* knockout mice. It would also be interesting to explore the effects of *Ncs-1* knockout on the A_{2A} and $A_{2A}R$-DRD2 signalling pathways and how these processes correlate with behaviour.

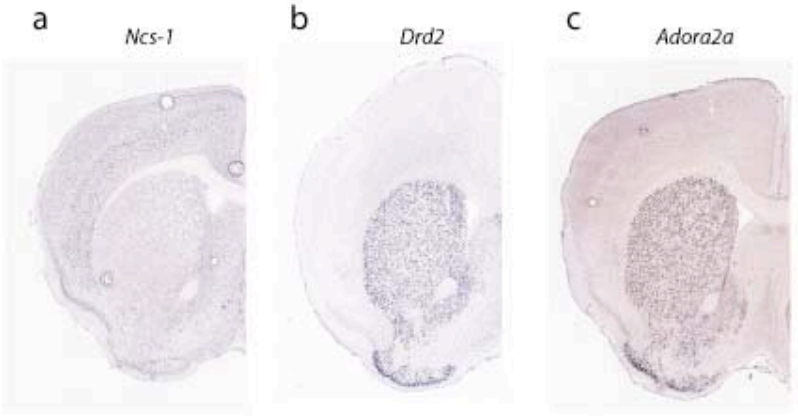

Figure 5-1. *Ncs-1, Drd2,* and *Adora2a* are expressed in the striatum.
Images courtesy of the Allen Mouse Brain Atlas, (Lein et al., 2007). *In situ* hybridization data for **(a)** *Ncs-1* mRNA, experiment RP_070116_02_A02, **(b)** *Drd2* (dopamine receptor D2) mRNA, experiment RP_Baylor_102735, and **(c)** *Adora2a* mRNA (codes for the A_{2A} receptor), experiment RP_050712_03_E04 in coronal slices of C57BL/6J mice.

5.2 What is the role of NCS-1 in behavioural responses to drugs of abuse and in ventral striatal long-term plasticity?

Our experiments focused on the role of NCS-1 in operant responding for food. A natural extension of this work would be whether knockout of *Ncs-1* would affect motivated behaviour for drugs of abuse. Would *Ncs-1*$^{-/-}$ mice be less willing to work for the self-administration of stimulants or opiate drugs? Would they be more or less vulnerable to the formation of drug-seeking habits that appear to depend on ascending spiralling dopamine projections connecting the ventral and dorsal striatal regions (Belin & Everrit, 2008)? Moreover, what is the effect of NCS-1 manipulation on addiction-related changes in synaptic plasticity such as LTP or LTD in the VTA and the nucleus accumbens (Kauer and Malenka, 2007). Though not focused on the study of addiction, Dragicevic et al show that giving cocaine to juvenile mice elevates substantia nigra *Ncs-1* mRNA leading to lasting changes in dopamine D2 autoreceptor responses (Dragicevic et al., 2014).

5.3 What is the mechanism by which NCS-1 modulates dopamine transmission in the nucleus accumbens core?

Our studies suggest that *Ncs-1* knockout mice have altered presynaptic release of dopamine in the nucleus accumbens core. NCS-1 is known to interact with a number of voltage gated calcium channels that could modulate presynaptic transmitter release (Dragicevic et al., 2014; Lian et al., 2014; Tsujimoto et al., 2002; Yan et al., 2014). Electrophysiology, calcium imaging (with calcium-sensitive reporter dyes), cell-permeant inhibitory peptides and specific calcium channel blockers could be used to explore how modulations in NCS-1 levels affects presynaptic calcium signals, channel function and transmitter release. It would also be interesting to see whether NCS-1 plays similar roles in other striatal dopamine projections or in other synapses.

5.4 Are there alterations to striatal NCS-1 in neuropsychiatric diseases?

Neuronal calcium sensor-1 mRNA and protein are increased in the dorsolateral prefrontal cortex of patients who had schizophrenia that do not appear to be attributable to the use of psychiatric medication (Bai et al., 2004; Koh et al., 2003). A recent study has found significantly elevated *Ncs-1* mRNA in the substantia nigra of patients who had Parkinson's disease (Dragicevic et al., 2014). Given our findings that NCS-1 can modulate dopamine transmission in the nucleus accumbens, it would be interesting to systematically test NCS-1 levels in the dopaminergic projections to various striatal subregions in diseases with known striatal dopamine disruptions such as schizophrenia, addiction, Parkinson's disease (Section 1.3.4.4).

5.5 What are the effects of overexpressing *Ncs-1* in the striatum or cortex?

Based on the findings from postmortem brain studies in section 5.4, the effects of overexpressing *Ncs-1* in specific cortical or striatal subregions could be explored using viral vectors in rodents. For example, would overexpression of *Ncs-1* in the prefrontal cortex of mice lead to deficits in executive function relevant to schizophrenia as well as changes in cortical morphology or neurophysiology (Figure 5-2)? Calcyon, which was identified together with NCS-1 as a molecule upregulated in schizophrenic dorsolateral prefrontal cortex, impairs working memory and response inhibition in adulthood when transgenically upregulated in mice (Vazdarjanova et al., 2011).

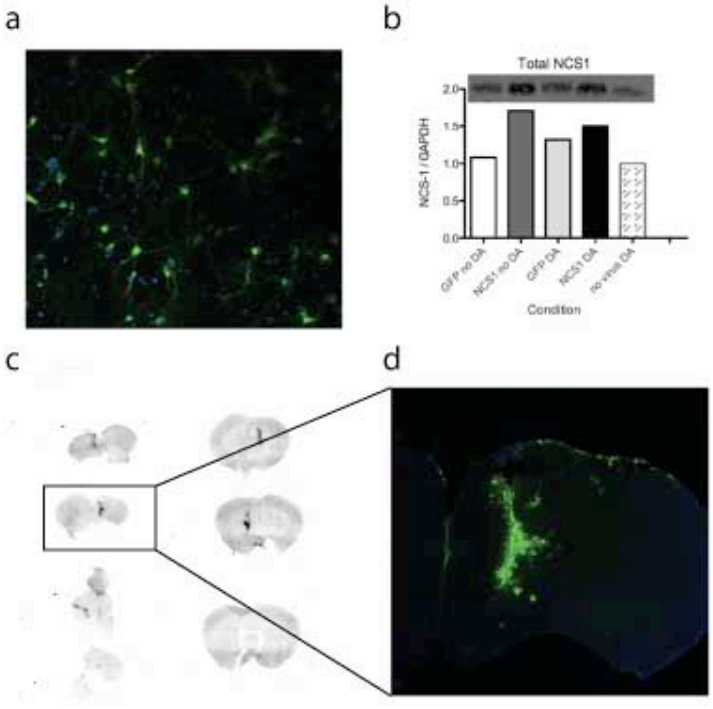

Figure 5-2. Pilot data showing *Ncs-1* overexpression by a lentivector that also expresses *Egfp*.
(a) Rat primary cortical neurons infected with the *Ncs-1* lentivector. **(b)** Immunoblots comparing NCS-1 protein levels in neurons infected with EGFP-only virus, *Ncs-1* containing virus or no virus, and then treated or not treated with 10 uM dopamine. **(c)** Coronal slices of mouse brain showing the site of stereotaxic injection targeting the prelimbic and infralimbic cortex. **(d)** GFP expression in a coronal brain section showing cells infected by the lentivector.

5.6 What compensatory changes, if any, are there to the deletion of *Ncs-1*?

Future work can explore if there are compensatory changes in gene expression to *Ncs-1* deletion particularly in genes that code for other calcium sensors and the Neuronal Calcium Sensor family (Section 1.3.3). Microarray analyses for differential gene expression could be done followed by validation by real-time PCR and western blots. Manipulations could then potentially be done to counteract compensatory changes and see if it worsens or improves the phenotypes found in *Ncs-1* knockout mice. For example, if levels of Hippocalcin are increased in the hippocampal formation as a result of *Ncs-1* deletion, it may be interesting to see the effects of knocking down *Hpca* expression or to explore the phenotypes of *Hpca/Ncs-1* double knockout mice.

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Appendix

Table A-1. Tests for assumptions of parametric tests.
The D'Agostino & Pearson omnibus normality test was used. To test homogeneity of variances, the F test was used to compare two distributions and the Brown-Forsythe test used to compare standard deviations of greater than two distributions.

Figure	Data	Statistical test	Distribution significantly non-normal?	Variances significantly different?
3-2 a	Baseline weight,	Unpaired two-tailed t tests	No	No
3-2 b	Open field	Unpaired two-tailed t test	Yes	Yes
3-2 c	FR1 acquisition	Two-way repeated measures ANOVA	Yes for some (WT: d3, 5, 6; KO: d2, d8)	Yes for some
3-2 d	Progressive ratio 3	Two-way repeated measures ANOVA	No	No
3-2 e	Progressive ratio 7 breakpoints	Two-way ANOVA	No	Yes, but not when comparing genotypes
3-2 f	Progressive ratio 7 inactive nosepokes	Two-way ANOVA	Yes for some (KO: 92%, 100%)	Yes, but not when comparing genotypes
3-2 g	Progressive delay 3 s	Two-way repeated measures ANOVA	No	No
3-3 a	Fixed ratios (no choice)	Two-way ANOVA	No	No
3-3 b	Concurrent choice (active nosepokes)	Two-way ANOVA	Yes for some (KO: FR30/chow, FR20/chow)	No
3-3 c	Concurrent choice (chow consumed)	Two-way ANOVA	No	No
3-3 d	Concurrent choice (% preferred)	Two-way ANOVA	Yes for some (KO: FR30/chow, FR20/chow)	No
3-3 e	Concurrent choice (total food consumed)	Two-way ANOVA	No	No
3-3 f	Sensory-specific	Two-way ANOVA	No	Yes, but not

	satiety			when comparing genotypes
3-4 a	Sucrose preference	Two-way ANOVA	Yes for some (WT: Sucrose); n's too small to test KOs	No
	Food preference	Two-way ANOVA	No	Yes
3-4 b	WT Pavlovian approach	Two-way repeated measures ANOVA	Yes	Yes
	KO Pavlovian approach	Two-way repeated measures ANOVA	Yes	Yes
3-4 c	WT Conditioned reinforcement	Two-way repeated measures ANOVA	Yes	No
	KO Conditioned reinforcement	Two-way repeated measures ANOVA	Yes	Yes
3-4 d	Pavlovian approach	Two-way repeated measures ANOVA	Yes for some (WT: d9, 10; KO: d3, d4)	Yes
3-4 e	Conditioned reinforcement	Two-way repeated measures ANOVA	Yes for some (WT: d2, 4; KO: d1, 2, 6, 8, 9, 10)	Yes
3-4 f	Habit formation	Two-way repeated measures ANOVA	Yes; n's too small to test WTs	Yes
3-4 g	Sensory-specific satiety	Two-way ANOVA	No; n's too small to test WTs	No
3-5 a	Novel object preference	Planned comparisons (Unpaired, two-tailed t-test)	n too small to test	No
3-5 b	Displaced object preference	Planned comparisons (Unpaired, two-tailed t-test)	No	No
	Distance traveled	Unpaired, two-tailed t-test	No	No
	Velocity	Unpaired, two-tailed t-test	No	No
3-6 a	Spatial acquisition	Two-way repeated measures ANOVA	Yes for some (WT: d13, 17)	No
3-6 b	Spatial probe quadrant time	Two-way ANOVA	No	No
3-6 c	Reversal probe quadrant time	Two-way ANOVA	Yes for WT:NW quadrant	No
3-6 d	Spatial probe proximity	Unpaired two-tailed t-test	No	No
	Reversal probe	Unpaired two-	No	No

	proximity	tailed t-test		
3-7 a	Cue-based water box	Unpaired two-tailed t-test	No	No
3-7 b	Shift to turn-based	Unpaired two-tailed t-test	No	No
3-7 c	Error analysis	Two-way ANOVA	Yes for WT: never-reinforced errors	Yes but not between genotypes
3-7 d	Reversal	Unpaired two-tailed t- test	Yes for KO	No
3-8 a	Context fear	Two-way repeated measures ANOVA	Yes for WT 1st min; n's too small to test KO	No
3-8 b	Tone fear	Unpaired two-tailed t-test	No; n's too small to test KO	No
3-9 a	Open field locomotion	Two-way repeated measures ANOVA	No	Yes
3-9 b	Accelerating rota-rod	Two-way repeated measures ANOVA	Yes for KO: d2 trial3, d3 trial2	No
3-10 b	Amphetamine-induced locomotion	Two-way repeated measures ANOVA	No; n's too small to test KO	No
3-10 c	Habituation	Two-way repeated measures ANOVA	Yes for WT: d3; n's too small to test KO	No
3-10 d	Day 1 AMPH	Two-way repeated measures ANOVA	Yes for WT: 80 and 90 min; n's too small to test KO	No
3-10 e	Day 2 AMPH	Two-way repeated measures ANOVA	No; n's too small to test KO	No
3-10 f	Day 9 AMPH	Two-way repeated measures ANOVA	Yes for WT: 85 and 90 min; n's too small to test KO	No
3-11 a	Light-dark box	One-way ANOVA	No; n's too small to test WT	No
3-11 b	Elevated zero maze	One-way ANOVA	No; n's too small to test KO	Yes
3-11 c	Tail suspension test	One-way ANOVA	n's too small to test	No
3-12 a	Pre-pulse inhibition	Two-way ANOVA	Yes for WT 5; Het 5; KO 15, 20	Yes
3-12 b	Acoustic startle response	Two-way ANOVA	Yes for WT 20,25; Het 20; KO 20	Yes
3-12 c	Startle response	One-way ANOVA	No	No

	to pulse			
3-13 a	Social approach chamber time	Planned comparisons (Unpaired two-tailed t-test)	No	No
3-13 b	Social approach sniffing time	Planned comparisons (Unpaired two-tailed t-test)	No	No
3-13 c	Freezing	Unpaired two-tailed t-test	n too small to test	No
	Digging	Unpaired two-tailed t-test	n too small to test	Yes
	Allogrooming	Unpaired two-tailed t-test	n too small to test	Cannot test
	Self-grooming	Unpaired two-tailed t-test	n too small to test	No
	Push-crawl	Unpaired two-tailed t-test	n too small to test	No
	Face sniffing	Unpaired two-tailed t-test	n too small to test	No
	Anal sniffing	Unpaired two-tailed t-test	n too small to test	Yes
	Mounting	Unpaired two-tailed t-test	n too small to test	No
3-14 b	Whole brain DRD2	One-way ANOVA	n too small to test	No
3-14 c	Biotinylation	Paired two-tailed t-test	n too small to test	No
3-14 d	Total DAT	Unpaired two-tailed t-test	No	No
	Total DRD2	Unpaired two-tailed t-test	No	No
3-14 e	Cytoplasmic DAT	Unpaired two-tailed t-test	No	No
	Cytoplasmic DRD2	Unpaired two-tailed t-test	No	No
3-14 f	Membrane DAT	Unpaired two-tailed t-test	No	Yes
	Membrane DRD2	Unpaired two-tailed t-test	No	Yes
3-15 b	Single pulse stimulated DA release	Unpaired two-tailed t-test	No	Yes
3-15 c	Train stimulated DA release	Unpaired two-tailed t-test	Yes for WT	Yes
3-15 e	Paired pulse ratio	Unpaired two-	No	Yes

		tailed t-test		
3-15 f	Half-life	Unpaired two-tailed t-test	No	No
3-16 a	Dentate LTP	Two-way repeated measures ANOVA	n too small to test	No (for log transformed data)
3-16 c	Dentate paired pulses	Two-way repeated measures	No; n too small to test WT	No
3-17 a	Dentate mGluR LTD	Two-way repeated measures ANOVA	n too small to test	No
3-17 c	Dentate paired pulses	Two-way repeated measures ANOVA	n too small to test	No
3-18 a	CA1 mGluR LTD	Two-way repeated measures ANOVA	n too small to test	No
3-18 c	CA1 paired pulses	Two-way repeated measures ANOVA	n too small to test	No

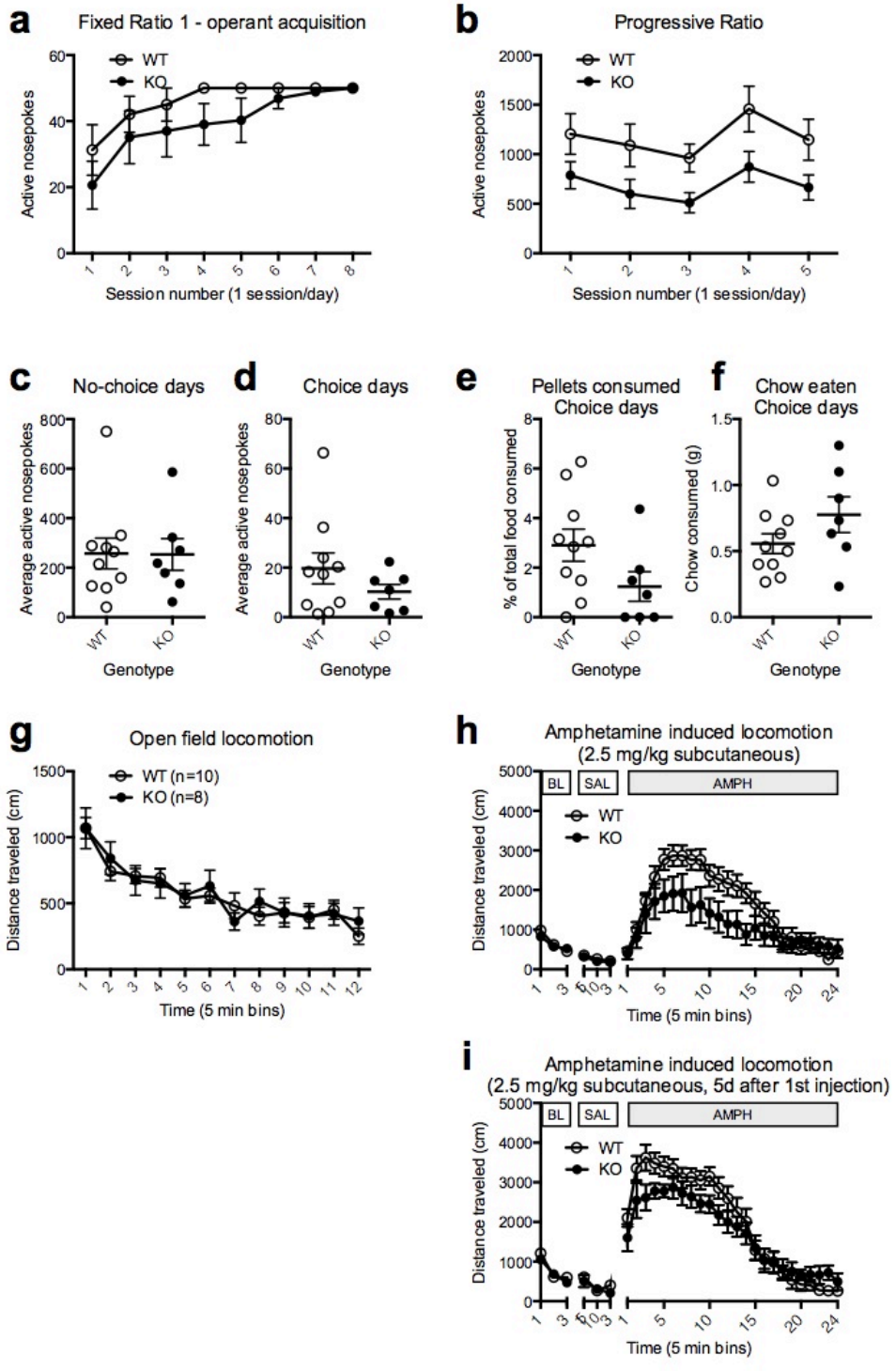

Figure A-3. *Ncs-1*$^{-/-}$ mice show lower motivation and a trend toward dampened response to amphetamine, despite normal operant learning and open field locomotion.
Note that male and female mice were grouped together in these results (WT: 3 female, 7 male; KO: 3 female; 5 male). **(a)** *Ncs-1*$^{-/-}$ mice learn to respond for food on a fixed ratio 1 schedule in at a similar rate to wildtype mice. Two-way repeated measures ANOVA only reveals a main

effect of time (time effect, $F(7,112) = 8.206$, $p < 0.0001$; ns genotype effect, $F(1,16) = 2.191$, $p = 0.1582$; ns interaction, $F(7,112) = 0.6199$, $p = 0.7385$). **(b)** *Ncs-1*$^{-/-}$ mice are less willing to work for food reinforcers in a progressive ratio schedule of reinforcement in which the ratio increases by 'x', where x starts at one and doubles after every eighth reinforcer delivery. Two-way repeated measures ANOVA shows main effects of genotype and time but no significant interaction (genotype effect, $F(1,16) = 4.698$, $p = 0.0456$; time effect, $F(4,64) = 5.139$, $p = 0.0012$; ns interaction, $F(4,64) = 0.1924$, $p = 0.9415$). **(c-f)** Concurrent choice testing. The two genotypes respond similarly on a FR5 schedule on no-choice days ($t_{15} = 0.04560$, $p = 0.9642$). There may be a slight trend for *Ncs-1*$^{-/-}$ mice to respond less than wildtype mice on choice days ($t_{15} = 1.186$, $p = 0.2540$), to obtain a lower percentage of their food from reward pellets ($t_{15} = 1.796$, $p = 0.0927$), and to consume more chow ($t_{15} = 1.524$, $p = 0.1483$). **(g)** Mice from the two genotypes traveled similar distances over 60 min in the open field (ns genotype effect, $F(1,16) = 0.03401$, $p = 0.8560$; time effect, $F(11,176) = 24.82$, $p < 0.0001$; ns interaction, $F(11,176) = 0.7154$, $p = 0.7228$). **(h-i)** There is a trend for *Ncs-1*$^{-/-}$ mice to show blunted responses to 2.5 mg/kg amphetamine. Significant genotype x time interactions were found for the first response to amphetamine (interaction, $F(23, 368) = 3.578$, $p < 0.0001$; time effect, $F(23,368) = 28.52$, $p < 0.0001$; ns genotype effect, $F(1,16) = 2.896$, $p = 0.1082$) and for response to a second injection 5 d after the first injection (interaction, $F(23,345) = 2.636$, $p < 0.0001$; time effect, $F(23,345) = 64.33$, $p < 0.0001$; ns genotype effect, $F(1,15) = 1.053$, $p = 0.3211$). Data shown as mean $\pm$ SEM.

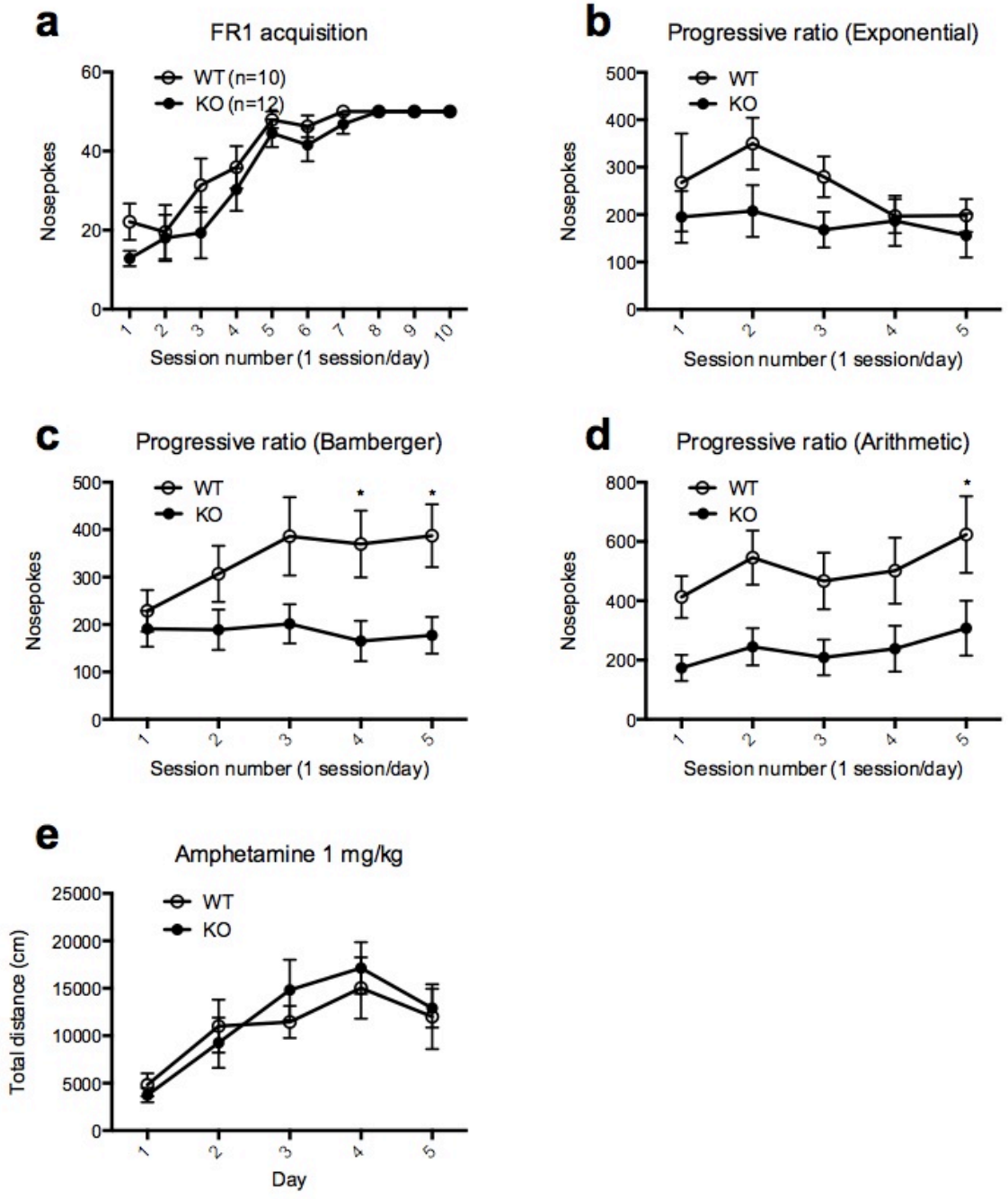

Figure A-4. *Ncs-1*$^{-/-}$ mice are less willing to work for food across different progressive ratio schedules and show similar hyperlocomotion to wildtype mice in response to 1 mg/kg amphetamine.

Male and female mice were grouped together in these results (WT: 6 female, 4 male; KO: 5 female, 7 male). **(a)** *Ncs-1*$^{-/-}$ mice learn to respond for food on a fixed ratio 1 schedule in at a similar rate to wildtype mice. Two-way repeated measures ANOVA only reveals a main effect of time (time effect, $F(9,180) = 34.04$, $p < 0.0001$; ns genotype effect, $F(1,20) = 1.387$, $p = 0.2527$; ns interaction, $F(9,180) = 0.7959$, $p = 0.6205$). **(b-d)** *Ncs-1*$^{-/-}$ mice are less willing to work for food reinforcers under various progressive ratio schedules of reinforcement: using an exponential

progression: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, …etc., derived from the formula [(5 x $e^{0.2n}$) – 5] rounded to the nearest integer (ns genotype effect, $F(1,20) = 1.628$, $p = 0.2166$; ns time effect, $F(4,80) = 2.042$, $p = 0.0963$; ns interaction, $F(4,80) = 0.9134$, $p = 0.4603$), the Bamberger progression in which the ratio doubles after every eighth reward delivery (genotype effect, $F(1,20) = 10.37$, $p = 0.0043$; ns time effect, $F(4,80) = 1.029$; ns interaction, $F(4,80) = 1.265$; post hoc comparisons between genotypes with Bonferroni's correction show significant differences on the last two days, $p < 0.05$), and an arithmetic progression in which the ratio increases by 3 after every reward delivery (genotype effect, $F(1,20) = 6.106$, $p = 0.0226$; time effect, $F(4,80) = 6.869$, $p < 0.0001$; ns interaction, $F(4,80) = 0.4177$, $p = 0.7954$). **(e)** *Ncs-1*$^{-/-}$ mice show a similar levels of hyperlocomotion and sensitization in response to repeated daily injections of 1 mg/kg amphetamine (ns genotype effect, $F(1,19) = 0.5269$, $p = 0.4768$; time effect, $F(4,76) = 27.12$, $p < 0.0001$; ns interaction, $F(4,76) = 0.4887$, $p = 0.7440$). Data shown as mean $\pm$ SEM. * = $p < 0.05$.

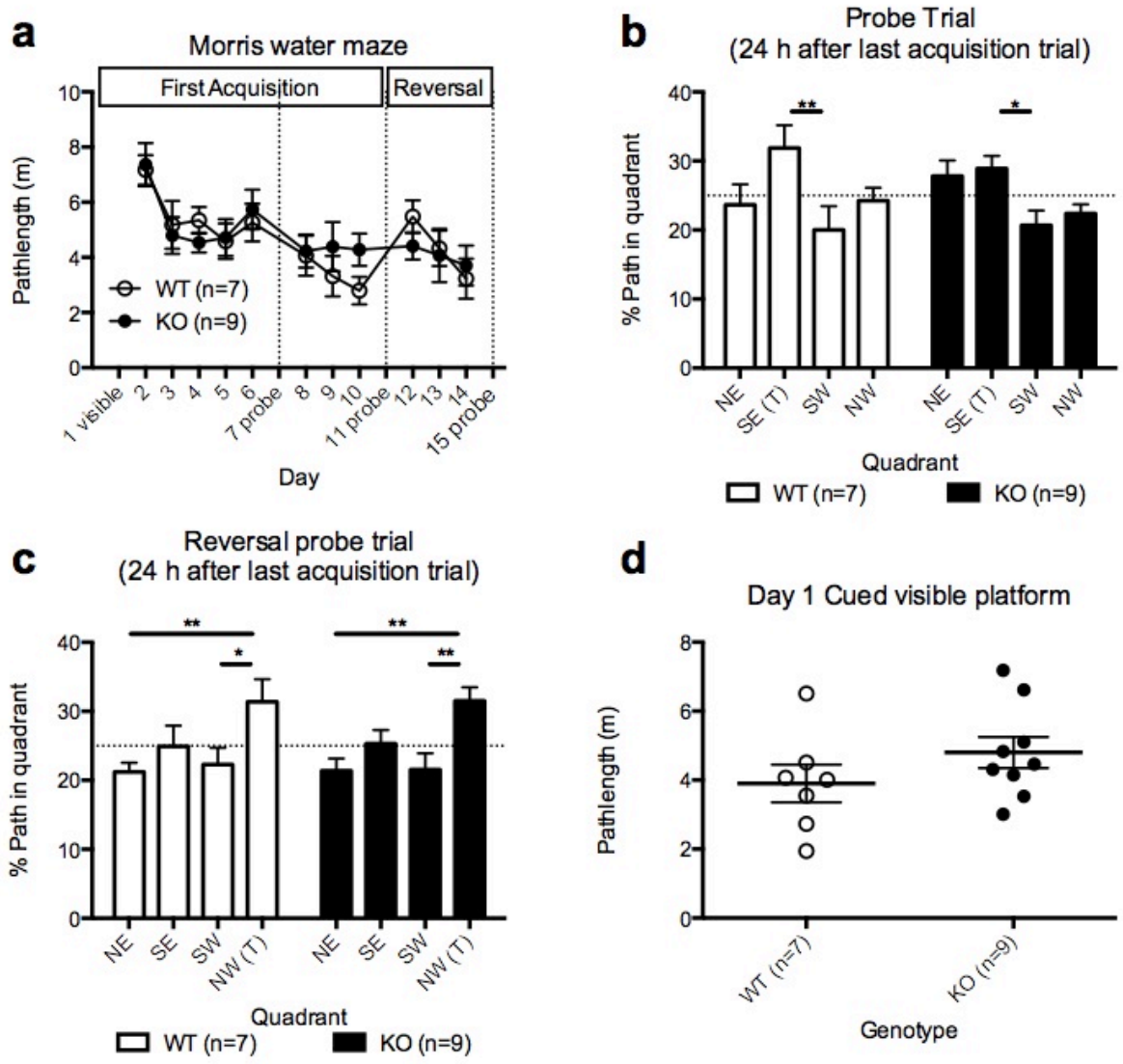

Figure A-5. ***Ncs-1*$^{-/-}$ mice show similar spatial learning and memory performance to wildtype mice in the water maze.**
(a) *Ncs-1*$^{-/-}$ mice learned the location of a hidden platform at a similar rate to wildtype mice (ns genotype effect, $F(1,14) = 0.1082$, $p = 0.7471$; time effect, $F(10,140) = 5.252$, $p < 0.0001$; ns interaction, $F(10,140) = 0.6452$, $p = 0.7729$). **(b)** Day 11 probe trial. Two-way ANOVA reveals main effect of quadrant (quadrant effect, $F(3,56) = 06.322$, $p = 0.0009$; ns genotype effect, $F(1,56) = 6.679e\text{-}6$, $p = 0.9979$; ns interaction, $F(3,56) = 0.8769$, $p = 0.4596$; post hoc comparisons of quadrants within each genotype with Bonferroni's correction yielded some significant differences as shown on the graph). **(c)** Both genotypes readily learned to switch looking for the hidden platform in the opposite quadrant to the initial acquisition (quadrant effect, $F(3,56) = 8.134$, $p = 0.0001$; ns genotype effect, $F(1,56) = -8.715e\text{-}14$, $p < 0.9999$; ns interaction, $F(3,56) = 0.02262$, $p = 0.9953$; post hoc tests with Bonferroni's correction yielded some significant differences as shown on the graph). **(d)** The genotypes traveled similar distances to find a visible platform on day 1 ($t_{14} = 1.278$, $p = 0.2221$). ** = $p < 0.01$. * = $p < 0.05$. (T) = target quadrant. Error bars = SEM.