NCK1 FUNCTIONS IN THE BRAIN TO REGULATE DENDRITIC SPINE FORMATION, COGNITION, AND BEHAVIOUR

by

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To Jiansong (Maggie) Qi, thank you

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Abstract

NCK1, encodes for the non-catalytic region of tyrosine kinase adaptor protein (NCK1). NCK1 has previously been shown to modulate cellular actin, although its role in CNS development and function remain unknown. Here I examined neurodevelopmental and behavioural defects using a murine model in which *NCK1* was inactivated. I show that NCK1 is ubiquitously expressed in neurons throughout the brain but does not grossly affect neuronal development. Nonetheless, loss of NCK1 leads to defects in memory, learning, and in anxiety-like behaviours. Examination of the hippocampal region revealed that NCK1 is necessary for normal synaptic density, and morphology of the postsynaptic density. Mechanistically, NCK1 affects synaptic structures by regulating the rate of actin turnover and polymerization, suggesting that NCK1 functions intrinsically in neurons to stabilize actin dynamics and promote synaptic integrity. In addition to memory defects, mice lacking NCK1 show context dependent defects in anxiety-like and stress hormone responses. These defects are ameliorated with treatment with Diazepam. The anxiety-like defects is linked with a loss of synaptic density in the basal lateral amygdala (BLA) and decreased neuronal activation in the prefrontal cortex and in inhibitory interneurons of the BLA, implicating NCK1 in inhibitory control of circuits important for regulating anxiety-like behaviours. Combined, my work suggests that NCK1 functions in the CNS to stabilize synaptic actin dynamics necessary for the development of neuronal circuits important for learning, memory and anxiety-like behaviours.

List of Abbreviations Used

$^{+ve}$ – positive

a.a. r – amino acid repeat

Abi-1 – Abl interactor 1

Abl1 – Abelson murine leukemia viral oncogene homolog 1

Abl2- Abelson murine leukemia viral oncogene homolog 2

ACK1 – activated Cdc42-associated kinase 1

ADF – actin-depolymerizing factor

ADP – adenosine diphosphate

AMCA – aminomethylcoumarin

AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

Ank – ankyrin

ANOVA – analysis of variance

AP-1 – activating protein 1

AP-2 – adaptor protein complex 2

Arp2/3 – actin related proteins 2/3

$ARPC_3$ – actin related protein subunit 2/3 complex C3

ATP – adenosine triphosphate

BA – basal amygdala

BDNF – brain-derived neurotrophic factor

BLA – basolateral amygdala

BM – medial amygdala

BMP – bone morphogenetic protein

CA1 – cornu ammonis 1

CA2 – cornu ammonis 2

CA3 – cornu ammonis 3

CA4 – cornu ammonis 4

cAMP – cyclic adenosine monophosphate

CAMs – cell adhesion molecules

CAP – Cbl-associated protein

Cap2 – F-actin capping protein subunit beta

CB1 – cannabinoid receptor type 1

CBL – Casitas B-lineage lymphoma

CC – coiled coil domain

CD69 – cluster of differentiation 69

CD79A – cluster of differentiation 79A

Cdc42 – cell division control protein 42 homolog

CeA – central nucleus of the amygdala

CEACAM3 – carcinoembryonic antigen-related cell adhesion molecule 3

CeL – lateral division of the central nucleus of the amygdala

CeM- medial division of the central nucleus of the amygdala

cGMP – cyclic guanosine monophosphate

CNS – central nervous system

COS1 – cells being CV-1 (simian) in origin and carrying the SV40 genetic material 1

Cox2 – cyclooxygenase 2

CP – cortical plate

CRIB – Cdc42- and Rac-interactive binding

Dab-1 – disabled-1

DCC – deleted in colorectal carcinoma

DIV – days *in vitro*

DLC – dynein light chain domain

DLG – Disc large

DLG4 – Disc large homolog 4

DLGAP – Discs large associated protein

DNA – deoxyribonucleic acid

DOCK180 – dedicator of cytokinesis 180kDa

E – embryonic day

E coli – *Escherichia coli*

E/I – excitatory/inhibitory

EC – entorhial cortex

EdU – ethynyl deoxyuridine

EGFR – epidermal growth factor receptor

EIF2AK3 – eukaryotic translation initiation factor 2-alpha kinase 3

EIF2B2 – eukaryotic translation initiation factor 2B, subunit 2 beta

ELKS – protein rich in amino acids E, L, K and S

ELMO1 – engulfment and cell motility 1

EphA3 – ephrin type A receptor 3

EphA4 – ephrin type A receptor 4

EphB1 – ephrin type B receptor 1

EphB2 – ephrin type B receptor 2

EPM – elevated plus maze

Eps8 – epidermal growth factor receptor pathway substrate 8

EPSC – excitatory postsynaptic current

ER – endoplasmic reticulum

ErbB2- erythroblastic oncogene (Erb) receptor tyrosine kinase -B2

ErbB3- erythroblastic oncogene (Erb) receptor tyrosine kinase -B3

ERK1 – extracellular signal-regulated kinase 1

ES – embryonic stem

EVH1 – Ena/VASP homology 1 domain

F-actin – filamentous actin

FAK – focal adhesion kinase

FASLG – Fas ligand

FH2 – formin homology-2

FRAP – fluorescence recovery after photobleaching

FXS – fragile X syndrome

G-actin – globular actin

GABA – gamma-aminobutyric acid

GAP – GTPase accelerating protein

GBD- GTPase binding domain

GDP –guanosine diphosphate

GEF – guanine nucleotide exchange factor

GFP – green fluorescent protein

GH1 – GKAP homology domain 1

GK - guanylate kinase-like

GKAP – guanylate kinase associated protein

GPI – glycosylphosphatidylinositol

GTP – guanosine-5'-triphoshate

GWAS – genome wide association studies

hnPNPK – heterogeneous nuclear ribonucleoprotein K

HPA – hypothalamic-pituitary-adrenal

i.p. – intraperitoneal injection

Iba1 - ionized calcium binding adaptor molecule 1

IL-2 – interleukin-2

ILK – integrin-linked kinase

IRE1α - inositol-requiring enzyme 1 alpha

IRE1β - inositol-requiring enzyme 1 beta

IRES – internal ribosome entry site

IRS1 – insulin receptor substrate 1

IZ – intermediate zone

KHDRBS1 – KH domain-containing, RNA-binding, signal transduction-associated protein 1

LA – lateral amygdala

LC-MS/MS – liquid chromatography with tandem mass spectrometry

LIMK – LIM domain kinase

LTD – long-term depression

LTP – long-term potentiation

MAGUK – membrane associated guanylate kinases

MAP2 – microtubule associated protein 2

MAP4K4 - mitogen-activated protein kinase kinase kinase kinase 4

mDia2 – mammalian Diaphanous-related formin

MEFs – mouse embryonic fibroblasts

MEK – mitogen activated protein kinase

mEPSC – miniature excitatory postsynaptic current

M_f – mobile fraction

Min6 – Mouse insulinoma 6

MINK1 – misshapen-like kinase 1

miRNA – micro ribonucleic acid

mPFC – medial prefrontal cortex

MRI – magnetic resonance imaging

MRM – multiple reaction monitoring

mRNA – messenger ribonucleic acid

MTA1 – metastasis-associated 1

MTA3 – metastasis-associated 3

Munc13 – mammalian homolog of Caenorhabditis elegans uncoordinated protein 13

MWM – Morris water maze

N-WASP – neural Wiskott-Aldrich syndrome protein

NCK – non-catalytic region of tyrosine kinase

NCK1-GFP – green fluorescent protein tagged NCK1

NCKIPSD – non-catalytic region of tyrosine kinase (NCK)-interacting protein with SH3 domain

NeuN – neuronal nuclei

NFAT – nuclear factor of activated T-cells

NGF – nerve growth factor

NGS – normal goat serum

NIK – NCK-interacting kinase

NMDA – N-methyl-D-aspartate

NMDA-R - N-methyl-D-aspartate receptor

NPFs – nucleation-promoting factors

NSF – N-ethylmaleimide sensitive factor

NW – northwest

OF – open field

p120-RasGAP – p120-Ras GTPase activating protein

p130CAS – p130 Crk-associated substrate

p62DOK1 – p62 docking protein 1

PAK – p21-activated kinase

PAK1 – p21-activated kinase 1

PAK2 – p21-activated kinase 2

PAK3 – p21-activated kinase 3

PAK4 – p21-activated kinase 4

PAK5 – p21-activated kinase 5

PAK6 – p21-activated kinase 6

PBS – phosphate buffer saline

PDGF-B – platelet-derived growth factor subunit B

PDGFR – platelet-derived growth factor receptor

PDZ – postsynaptic density protein 95, Drosophila disc large tumor suppressor 1, zonula occludens-1 protein

PERK – PRKR-like endoplasmic reticulum kinase

PFA – paraformaldehyde

PFC – prefrontal cortex

PFN1 – profilin 1

PFN2α - profilin 2α

PINCH – particularly interesting new cysteine-histidine-rich

PRK2 – protein kinase C-related kinase 2

ProSAP – proline-rich synapse-associated protein

PRR – proline rich region

PSD – postsynaptic density

PSD-95 – postsynaptic density protein 95

PV – parvalbumin

PV+ – parvalbumin-positive

Rab3 – Ras-related in brain 3

Rac1 – Ras-related C3 botulinum toxin substrate 1

Rho – Ras homologous

RhoA – Ras homologue family member

RIM – Ras-related in brain 3-interacting molecule

RIM-BP – Ras-related in brain 3-interacting molecule-binding proteins

RNA – ribonucleic acid

ROBO1 – roundabout homolog 1

ROBO2 – roundabout homolog 2

RRAS – Ras-related protein

SAM – sterile alpha motif domain

SAM68 – Src-associated substrate in mitosis of 68kDa

SE – southeast

SEM – standard error of the mean

SH2 – Src homology 3

SH3 – Src homology 3

Shank – SH3 and multiple ankyrin repeat domains protein

SNAP - soluble N-ethylmaleimide sensitive factor attachment protein

SNARE – soluble N-ethylmaleimide sensitive factor attachment protein receptor

SOCS7 – suppressor of cytokine signaling 7

Sox2 - sex determining region Y-box 2

SPIN90 – src homology 3 (SH3) protein interacting with non-catalytic region of tyrosine kinase (NCK), 90kDa

SPN – Shank/ProSAP N-terminus domain

SRM – selected reaction monitoring

SynCAM – synaptic cell adhesion molecule

t-PBS – triton X100 in phosphate buffer saline

Thy1 – thymocyte differentiation antigen 1

Tir – translocated intimin receptor

TNF – tumor necrosis factor

TNIK – tumor necrosis factor (TNF) receptor associated factor 2 (TRAF2) and non-catalytic region of tyrosine kinase (NCK)-interacting protein kinase

TNK2 – tyrosine kinase non-receptor 2

TRAF2 – tumor necrosis factor (TNF) receptor associated factor 2

UNC-5 – un-coordinated 5

VASP – vasodilator-stimulated phosphoprotein

VCA – verprolin cofilin acidic

VE – vascular endothelial

VEGFR1 – vascular endothelial growth factor receptor 1

VEGFR2 – vascular endothelial growth factor receptor 2

vs – versus

VZ – ventricular zone

WASH – Wiskott Aldrich syndrome protein and scar homologue

WAVE1 – Wiskott-Aldrich syndrome protein family member 1

WAVE2 – Wiskott-Aldrich syndrome protein family member 2

WIP – Wiskott-Aldrich syndrome/*WASL*-interacting protein family member 1

Wnt – wingless-related integration site

YFP – yellow fluorescent protein

β-gal – β-galactosidase

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Chapter 1: Introduction

This thesis reveals that the actin regulating adaptor protein, non-catalytic region of receptor tyrosine kinase adaptor protein 1 (NCK1), is necessary for regulating central nervous system (CNS) synapse number and morphology by modulating the stability of filamentous actin in dendritic spine. This change in synapse number reveals the importance of NCK1 in the formation of circuits necessary for normal cognition and behaviours including learning, memory, and anxiety-like behaviours.

To provide the rationale for these studies, my introduction will be divided into seven main parts. An introduction to 1) the neuron and the synapse; 2) actin and its regulators; 3) the NCK family of proteins, including their known signaling pathways; 4) mouse models, as they relate to behavioural studies that provide insight into how the hippocampus and amygdala function in learning, memory, and anxiety-like behaviours; 5) the role postsynaptic scaffoldings/adaptor proteins in synapse function, memory, and behaviour; and 6) the role of actin regulators in synapse function, memory and behaviour. Together these will provide background to provide the rationale for the work I undertook in my PhD studies.

1.1: The neuron

The neuron is the principal output cell of the nervous system and has evolved to be a specialized cell that can transmit electrical and chemical signals

to other neurons, muscle cells, and gland cells. The neuron can be divided into three main regions, the soma, the axon, and the dendritic arbour (also refered to simply as dendrites). The soma is the main cell body of the neuron and contains the nucleus. The axon contains the output machinery of the neuron and is the region responsible for transmitting electrical and chemical signals to other neurons, muscles, and glands. Dendrites including dendritic spines contain the input machinery of the neuron, and are important as they function to receive signals from other neurons. Dendrites are branched and diverse depending on the neuronal type and function.

Neurons maintain electrically polarized membranes; meaning that they actively pump positive-ions out of their cytosol and into the extracellular space to maintain a resting membrane potential that is relatively more negative intracellularly then it is extracellularly. Therefore, neurons can transmit electrical signals down their membranes and into distal regions by altering their membrane potentials (relative charge along the membrane) in the direction of dendrites to soma to axon. This forms the basis of neuronal communication within the nervous system. To control this electrical communication the nervous has evolved two distinct types of neurons, excitatory neurons and inhibitory neurons. Indeed, nervous system communication and function is dependent on the development and maintenance of proper excitatory/inhibitory balance.

1.1.1: Excitatory neurons

Excitatory neurons function to produce electrochemical excitatory potentials and promote depolarization in the receiving neuron. The excitatory neurons make up the primary projection neurons of the cortex, hippocampus, basolateral amygdala and spinal cord. Glutamate is the primary excitatory neurotransmitter in the CNS and acts on two classes of receptors, the ionotropic glutamate receptors and the metabotropic glutamate receptors. Ionotropic glutamate receptors function as ion channels that when activated by glutamate allow positively charged ions to enter the neuron and depolarize its membrane. There are three main types of ionotropic glutamate receptors, N-methyl-D-aspartate (NMDA) receptors, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, and kainate receptors. While metabotropic glutamate receptors, upon glutamate activation, activate intracellular signaling cascades that can also lead to changes in neuronal excitability by modulating ion channels and intracellular calcium dynamics (Platt, 2007).

1.1.2: Inhibitory neurons

Inhibitory neurons function to suppress excitatory potentials and therefore function to constraint neuronal outputs. The striatum and striatal-like brain regions, including the central nucleus of the amygdala, are made up of principal inhibitory neurons, while the cortex, spinal cord, and basolateral amygdala contain inhibitory interneurons that regulate the firing of the principal excitatory neurons. Different populations of inhibitory interneurons exist and differ based on

their soma-size, dendritic branching, their axon targets, and are classified according to their differential expression of calcium binding proteins and neuropeptides (Babaev et al., 2018; Marín, 2012). The largest and most-studied class of inhibitory interneuron in the CNS is the parvalbumin-positive (PV+) interneurons. The majority of PV+ interneurons directly target the soma of excitatory neurons and have a fast-spiking activity, therefore they exert strong inhibitory control over excitatory neuronal networks (Veres et al., 2017). Interestingly, PV+ interneurons form both chemical and electrically coupled networks and can therefore synchronize neuronal firing in large networks by generating oscillatory activity (Buzsáki & Wang, 2012; Muller et al., 2005).

There are two inhibitory neurotransmitters that are released from inhibitory neurons, gamma-aminobutyric acid (GABA) and glycine. GABA activates two different classes of receptors, GABA-A receptors and GABA-B receptors. GABA-A receptors are inotropic chloride channels, that when activated by GABA, open allowing the negatively charged chloride ions to enter the neuron and hyperpolarize the membrane. They are fast-acting channels that are responsible for fast inhibitory neurotransmission. Benzodiazepines, such as diazepam, act as positive allosteric modulator of GABA-A receptors (K. S. Smith & Rudolph, 2012). Therefore, diazepam acts as a nervous system depressant by inducing a conformational change to the GABA-A receptor that promotes the binding of GABA to the GABA-A receptor, results in more frequent and prolonged opening of the GABA-A chloride channel, and in increased hyperpolarization of neurons.

GABA-B receptors are metabotropic receptors that when activated by GABA activate signaling cascades that can directly inhibit neurotransmitter release and/or activate inwardly rectifying potassium channels resulting in membrane hyperpolarization (Gassmann & Bettler, 2012). Finally, glycine acts on glycine receptors, which similar to GABA-A receptors, are chloride ion channels (McCracken et al., 2017).

1.1.3: The synapse

Neuronal communication occurs at specialized signaling junctions known as synapses (Fig. 1.1). Synapses are formed between a presynaptic neuron and a postsynaptic cell (often another neuron). The synaptic structure allows for the rapid yet controlled exchange of electro-chemical signaling to enable the propagation of biochemical reactions between two neuronal cells. The synapse itself is composed of a presynaptic terminal, synaptic cleft (the extracellular gap between the presynaptic neuron and the postsynaptic cell), a postsynaptic membrane, and a postsynaptic membrane associated protein networks which includes postsynaptic receptors, intracellular scaffolding proteins, and signaling molecules. When the presynaptic terminal becomes sufficiently depolarized, it results in the release of neurotransmitters, stored in presynaptic vesicles, into the synaptic cleft space. Here the neurotransmitters migrate and engage receptors on the postsynaptic membrane resulting activation of neurotransmitter-specific postsynaptic receptors. Activation of postsynaptic receptors results in conformational changes that either opens ion-channels resulting in changes to

the membrane potential, or that trigger signaling cascades that result in changes to protein composition.

1.1.4: The presynaptic active zone and calcium dependent neurotransmitter release

Presynaptically, the synapse consists of a presynaptic active zone and a perisynaptic zone. Within the presynaptic active zone, neurotransmitters are stored in vesicles, known as synaptic vesicles, following their synthesis from various amino acid precursors. Synaptic vesicles in the presynapatic active zone can be docked and primed for rapid release following depolarization of the presynaptic zone (Südhof, 2013). Vesicular fusion with the presynaptic membrane is a calcium-dependent process. Therefore, the active zone is rich in voltage-gated calcium channels. When the membrane at the active zone is sufficiently depolarized it results in the opening of the voltage-gated calcium channels. Calcium ions then rush into the active zone where they promote the fusion of the vesicular membrane to the presynaptic membrane, and neurotransmitter release. The core of the active zone is made up of five evolutionary conserved protein families, Ras-related in brain 3 (Rab3)-interacting molecule (RIM), mammalian homolog of *Caenorhabditis elegans* uncoordinated protein 13 (Munc13), RIM-binding proteins (RIM-BP), α-liprin, and protein rich in amino acids E, L, K and S (ELKS), and one mammalian specific family Piccolo/Bassoon (Gundelfinger et al., 2016). These six active zone protein families' complex together and function to regulate neurotransmitter release by a

number of mechanisms. First, they function as scaffolds that recruit calcium channels and anchor them to the active zone (Han et al., 2011; Kaeser et al., 2011; Nishimune et al., 2012). Second, they scaffold neurotransmitter-containing vesicles at the active zone, a process referred to as docking (Gracheva et al., 2008; Han et al., 2011). Third, they are involved in priming synaptic vesicles for neurotransmitter release (Augustin et al., 1999; Deng et al., 2011; Koushika et al., 2001; Schoch et al., 2002). Priming involves the association of synaptic vesicles with the soluble N-ethylmaleimide sensitive factor (NSF) attachment protein (SNAP) receptor (SNARE) complexes, which are important for membrane fusion, and prepares the vesicle for rapid membrane fusion after calcium detection (Imig et al., 2014). Ultimately, the core active zone protein complex is critical for tethering vesicles and channels to a specified zone that restricts and controls neurotransmitter release. The collection of synaptic vesicles that are either docked or primed in the active zone is referred to as the readily releasable pool of synaptic vesicles, since they are the vesicles most rapidly fused upon stimulation.

1.1.5: The perisynaptic zone of the presynaptic neuron

The second major component of the presynapse is the perisynaptic zone, which is defined as the region surrounding the active zone. The perisynaptic zone contains the recycling and reserve pool of synaptic vesicles, as well as presynaptic receptors that modulate neurotransmitter release such as the endocannabinoid cannabinoid receptor type 1 (CB1) receptor, and transsynaptic

cell adhesion molecules (CAMs) (Südhof, 2012). The recycling pool of synaptic vesicles consists of 10-20% of synaptic vesicles in the presynaptic terminal (Denker & Rizzoli, 2010). They are the most mobile vesicles and get docked to the active zone when a docking site is available following calcium-induced fusion of the readily releasable pool of vesicles (Denker & Rizzoli, 2010). However, the majority (80-90%) of synaptic vesicles in the presynaptic neuron are found in the reserve pool. Reserve pool vesicles are much less mobile and require either intense stimulation, or the depletion of the recycling pool of vesicles, to be released (Denker & Rizzoli, 2010).

Transsynaptic cell adhesion molecules (CAMs) are another important player in the perisynaptic zone of the presynaptic terminal. CAMs are transmembrane proteins with large extracellular domains and form homo- or heterodimer interactions across the synaptic cleft (Gorlewicz & Kaczmarek, 2018; Missler et al., 2012). CAMs are critical for synapse integrity as they function to hold the synapse together, coordinate the precise alignment of the pre- and postsynaptic zones, and through their trans-synaptic signaling may directly play a role in synaptic plasticity by altering synapse size, number, or composition (Südhof, 2018). Synaptic CAMs include proteins from the Integrin, Cadherin, Neurexin, and synaptic cell adhesion molecule (SynCAM) families (Südhof, 2018).

1.1.6: The postsynaptic density

The post-synaptic side of the synapse has evolved to detect neurotransmitters and to respond by transducing the neurotransmitter signal into electrical or biochemical changes in the postsynaptic cell. In neurons, the protein dense region on the postsynaptic neuron, directly opposite the presynaptic active zone, is called the postsynaptic density (PSD). Unlike the conserved composition of the presynaptic active zone, the makeup of the PSD is diverse and depends on the neuronal -type, -environment, and -function. Indeed, over a thousand distinct proteins have been identified in the PSD (Grant, 2019). The majority of inhibitory synapses occur at symmetric synapses that have relatively thin PSDs, while the majority of excitatory synapses occur at asymmetric synapses that have thicker PSDs. Therefore, the excitatory PSD can be further divided into two distinct regions, the core and the pallium (Dosemeci et al., 2016), while the inhibitory PSD is lacking the pallium. The PSD core is made up of ionotropic and metabotropic receptors that are concentrated at the membrane, and are constrained by a dense protein network of scaffolding proteins (Kaizuka & Takumi, 2018; M. Sheng & Kim, 2011). Scaffolding proteins contain multiple protein binding domains that anchor specific proteins together into large complexes and this often restricts their mobility. The pallium is made up of a second layer of scaffolding proteins that do not directly bind to the neurotransmitter receptors, but instead bind to other scaffolding proteins, as well as to cytoskeletal components including actin-filaments, adaptor proteins, and signaling proteins (Dosemeci et al., 2016; M. Sheng & Kim, 2011). Interestingly,

after neuronal stimulation the pallium becomes thicker (when viewed under the electron microscope), suggesting that activity-dependent recruitment of proteins occurs in this region (Dosemeci et al., 2001). Postsynaptic CAMs are also present in the PSD and play a parallel role to the presynaptic CAMs, making them important for synapse alignment, formation and plasticity (Kaizuka & Takumi, 2018). Intriguingly, the PSD has been shown to be very dynamic, and continuously undergoes actin-dependent continuous remodeling in the form of receptor trafficking and changes to the composition of intracellular proteins (Blanpied et al., 2008; Minerbi et al., 2009). Importantly, signaling mechanisms that are dependent on enzyme activation and lead to post-translational modifications of target proteins, including phosphorylation, ubiquitination, and proteasome-mediated protein degradation, can also alter PSD composition (Coba et al., 2009; M. Sheng & Kim, 2011). Indeed, the dynamic nature of the PSD has been directly linked to proper brain functioning, especially in the context of learning and memory, and behavioural regulation of fear and anxiety (Fonseca et al., 2006; Karpova et al., 2006; Nagura et al., 2012). Therefore, it is not surprising that mutations in many different PSD proteins are associated with human neuropsychiatric disorders (Bayés et al., 2011; M. Sheng & Kim, 2011). Despite recent evidence outlining activity-induced regulation of the PSD, it is still unclear of which proteins and signaling pathways are involved, especially in the modification occurring in the pallium.

1.1.7: Dendritic spines

The majority of glutamatergic excitatory synapses occur on dendritic spines ((Tønnesen & Nägerl, 2016). Dendritic spines are actin-rich post-synaptic structures that function to compartmentalize electrical and biochemical signals. Typically, mature dendritic spines consist of a thin neck region and a thicker head region, giving them a characteristic mushroom shape. However, dendritic spines are dynamic and can change shape and size in response to repetitive stimulation (Sala & Segal, 2014). In fact, dendritic spines are diverse in terms of shape and size, even on spines found on the same dendrite. Spine necks can range from 50-500nm in diameter and up to 3μm in length. While spine head volume can range from 0.01-1.0μm^3 (Tønnesen & Nägerl, 2016). Although spine morphology exists on a continuum, many studies classify dendritic spines into four simplified categories, mushroom, thin, stubby, and filopodia-like. Mushroom-shaped spines have a stereotypical thin neck and large head and are thought to be fully mature with a functional PSD. Thin spines have thinner heads and stubby spines have no clear distinction between head and neck. Finally, filopodia-like spines lack any head, or PSD, and are thus thought to be immature. Indeed, the size of spine head is correlated to the size of the PSD and with the amplitude of the excitatory postsynaptic currents (Arellano et al., 2007; Harris & Stevens, 1989; M. Matsuzaki et al., 2001; Noguchi et al., 2011). Therefore, larger heads correlate with more proteins in the PSD and more ionotropic glutamate channels at the membrane. In fact, the induction of long-term potentiation (LTP), an experimentally induced model of cellular learning defined by in an increase in

synaptic strength and sensitivity at a specific synapse, results in spine head enlargement (Harvey & Svoboda, 2007; Lang et al., 2004; M. Matsuzaki et al., 2004). Interestingly though, while head enlargement occurs seconds after the initiation of a LTP protocol, the increase in PSD size occurs more slowly, sometimes over tens of minutes (Bosch et al., 2014). Therefore, there are separate but complimentary processes occurring in dendritic spines during synaptic remodeling. Conversely, long-term depression (LTD), an experimentally induced extinguishing of cellular memory that reduces synaptic strength and desensitizes synapses, has been shown to be associated with modifications of the dendritic spine including dendritic spine shrinkage (Nägerl et al., 2004; Zhou et al., 2004). Although there is considerable diversity in dendritic spine morphology, there are consistent differences in spine density when comparing neuron-type, brain region, developmental age, and disease state (C.-C. Chen et al., 2014).

In addition, the growth of de novo dendritic spines can be induced by a number of different experimental protocols both *in vitro* and *in vivo*. These include electrical stimulation, glutamate uncaging experiments (that directly activate glutamate receptors at specific spine heads), changes in sensory experiences (such as sensory deprivation), environmental enrichment, and behavioural learning paradigms (Fu et al., 2012; Holtmaat & Svoboda, 2009). In the cerebral cortex, for example, although dendritic branches are thought to remain stable after development, dendritic spines are constantly formed and eliminated in a process referred to as spine turnover. The major site of

neuroplasticity that occurs with experience and learning is reflected in spine turnover (C. H. Bailey et al., 2015; Bosch & Hayashi, 2012). However, the rate of spine turnover changes over development, with sensitive developmental periods such as infancy and adolescence showing the highest rates. Although dendritic spines remain dynamic in adulthood, the overall density is thought to remain constant until the onset of frailty (Mostany et al., 2013; Zuo et al., 2005). Nonetheless, it is well established that dendritic spines are highly dynamic during development as well as during learning and memory consolidation. This plasticity is of great interest and understanding the underlying mechanisms that lead to this change and/or contribute to this plasticity, is an important area of current neuroscience research.

1.1.8: Effectors that contribute to dendritic spine plasticity

A number of molecular effectors have been shown to influence dendritic spine formation, maintenance and elimination. Given that dendritic spines are actin rich structures, unsurprisingly, intracellular actin regulating proteins and signaling pathways have been shown to be key regulators of dendritic spine dynamics (S. Basu & Lamprecht, 2018). These include, actin binding and cytoskeletal proteins; small guanosine-5’-triphoshate (GTP)ases; cell surface receptors, extracellular matrix and adhesion molecules; receptor tyrosine kinases and soluble kinases; postsynaptic scaffolding proteins and adaptor proteins; micro ribonucleic acid (miRNA), messenger RNA (mRNA) binding proteins and transcription factors (that are mostly associated with altering protein expression

levels of previously identified dendritic spine regulators); and steroid hormones, namely estradiol and glucocorticoids (Sala & Segal, 2014). However, central to the mechanism of action of many of the molecular influencers in all the above classes is their ability to alter the actin cytoskeleton. In fact, over-expression of actin in neurons has been shown to increase dendritic spine density (O. L. Johnson & Ouimet, 2006). In addition, neurons undergoing spinogenesis upregulate actin, and both new and established spines incorporate exogenous actin (O. L. Johnson & Ouimet, 2006). It is therefore no surprise that a number proteins, mRNA, and miRNAs that regulate actin are involved in spine formation, maintenance and elimination (Sala & Segal, 2014). In this context, it is important to introduce actin and some basic cell biology surrounding how actin is modulated.

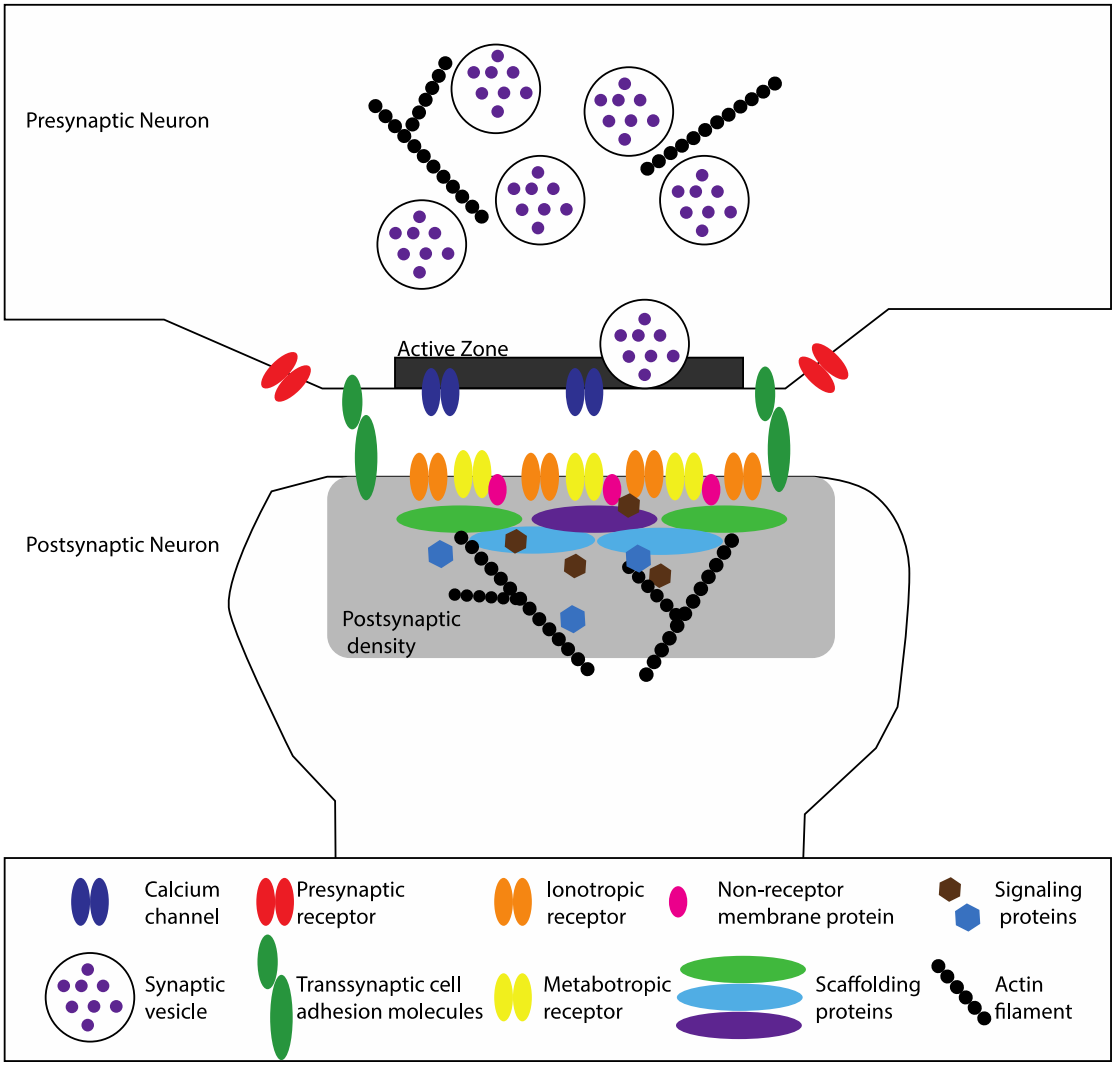

Figure 1.1: The synapse. A pictorial representation of a synapse onto a postsynaptic dendritic spine. Synaptic zone consists off the presynaptic neuron, the synapse, and the postsynaptic neuron. Presynaptic neuron contains neurotransmitter-filled synaptic vesicles that get shuttled, docked and primed in the voltage-gated calcium channel containing synaptic active zone. When the membrane is sufficiently depolarized the voltage-gated calcium channels open and calcium flows into the active zone and stimulates the fusion of the primed vesicle membranes with the presynaptic membrane resulting in neurotransmitter release from the presynaptic active zone. The presynaptic neuron also contains presynaptic receptor proteins that can influence neurotransmitter release and reuptake. The postsynaptic neuron consists of a protein dense region known as

the postsynaptic density (PSD). Ionotropic, metabotropic neurotransmitter receptors, and other non-receptor membrane proteins are found at the membrane across from the presynaptic active zone. The PSD contains various scaffolding, adaptor, and other signaling proteins important for converting the neurotransmitter-receptor signal into physiological and structural changes in the postsynaptic neuron. Both the presynaptic and postsynaptic zones contain a highly dynamic actin cytoskeleton, made up of actin filaments, important for shuttling vesicles and proteins, and for maintaining the shape of the zones and keeping the membranes in close proximity to each other. Finally, the perisynaptic space contains transsynaptic cell adhesion molecules that coordinate the precise alignment of the presynaptic active zone to the postsynaptic density.

1.2: Actin and its regulators

1.2.1: Actin

As in all cells, actin exists in two primary states in neurons. A 42kDa actin monomer, known as globular (G)-actin, or as a linear polymer known as filamentous (F)-actin. The process of F-actin assembly is dependent on energy being produces by adenosine triphosphate (ATP) hydrolysis. Indeed, G-actin is an ATPase. ATP-bound G-actin promotes the binding of G-actin to other actin monomers or actin filaments. As soon as actin trimers are formed, they elongate rapidly into F-actin depending on the abundance of ATP-bound G-actin present (Blanchoin et al., 2014). F-actin assembles asymmetrically, where G-actin is always preferentially incorporated into one end (known as the barbed-end) and removed from the opposite end (known as the pointed end). Therefore, actin filaments have directionality and grow at the barbed end and are disassembled at the pointed end (Pollard, 1986). Interestingly, F-actin is dynamic and constantly undergoes a process known as treadmilling, where actin monomers are continually incorporated into the barbed-end and removed from the pointed end while keeping the overall filament length constant. Indeed, 85% of actin filaments are constantly remodeling in the spine head with a 44-second turnover rate (Yan et al., 2016). Actin dynamics have been shown to play a crucial role in a number of cellular processes, including cell migration (Schaks et al., 2019), cytokinesis (Robinson & Spudich, 2000), endo/exocytosis (Mooren et al., 2012; Porat-Shliom et al., 2013), axon guidance (Dent et al., 2011), and morphogenesis during development (Munjal & Lecuit, 2014). Importantly, a number of molecular

regulators of actin assembly (1.2.2), disassembly (1.2.3), and stabilization (1.2.4) have been identified (Fig. 1.2).

1.2.2: Regulators of actin filament assembly

Factors that promote actin filament assembly include actin related proteins 2/3 (Arp2/3), the formin family, and profilin (Spence & Soderling, 2015). The architecture of the actin cytoskeleton within the postsynaptic dendritic spine is predominately branched actin filaments (Korobova & Svitkina, 2010). Actin filament branching requires the activation of the Arp2/3 complex (Korobova & Svitkina, 2010). The Arp2/3 complex associates with preexisting actin filaments and promotes the incorporation of free actin monomers to F-actin at a 70degree angle. This creates a newly branched barbed end to which G-actin can bind, polymerize, and elongate (B. A. Smith et al., 2013). However, in order for actin branching to occur Arp2/3 must be activated by nucleation-promoting factors (NPFs), such as neural Wiskott-Aldrich syndrome protein (N-WASP) (R. Rohatgi et al., 1999), Wiskott-Aldrich syndrome protein family member 1 (WAVE1) (Sweeney et al., 2015), or Wiskott Aldrich syndrome protein and scar homologue (WASH) (Linardopoulou et al., 2007). Each of these NPFs has their own spatial and temporal expression, as well as specific upstream regulators. For example, WAVE1 is enriched within the sub-membrane region, 20-100nm from the membrane, surrounding the spine head and is activated by the Ras homologous (Rho) GTPase Ras-related C3 botulinum toxin substrate 1 (Rac1) (Soderling et al., 2007; Yan et al., 2016).

While the Arp2/3 complex and its activators regulate actin branching, the formin family facilitate the linear polymerization of actin. Formins contain a formin homology-2 (FH2) domain that forms a homodimer and loops around the barbed end of actin filaments and promotes the addition of G-actin onto the preexisting F-actin (Courtemanche, 2018). Formins are especially important for driving the formation of cellular protrusions, such as the filapodia-like spine precursors (Vicente-Manzanares et al., 2009). Finally, profilin is a G-actin binding protein that facilitates the nucleotide exchange of adenosine diphosphate (ADP) to ATP that promotes the incorporation of G-actin into F-actin. Profilin gets rapidly recruited to spines following neuronal activation, binds to actin, promotes ATP loading that allows G-actin to interact with actin assembling factors such as WAVE1 (in branching) and the formins (in elongation), and which finally results in the elongation of the F-actin polymer (Spence & Soderling, 2015).

1.2.3: Regulators of actin filament disassembly

Actin filament disassembly is equally important to assembly to maintain proper actin dynamics since disassembly is the primary source of free G-actin (Spence & Soderling, 2015). Therefore, disassembly is required for new growth and further branching. F-actin disassembly is also important for actins role in cellular functions, including synaptic plasticity mechanisms in neurons (Cingolani & Goda, 2008; Spence & Soderling, 2015). Actin-dependent synaptic plasticity mechanisms include the recruitment and/or endocytosis of receptors, which result in electrophysiological changes at synapses, and structural changes in

spine size and number (S. Basu & Lamprecht, 2018). Cofilins are actin-depolymerizing factors (ADFs) and are the primary regulators of actin disassembly. Cofilin promotes the severing of F-actin at the pointed end and leads to the release of G-actin that can then be recycled by growing F-actin (Pavlov et al., 2007). Therefore, the regulation of cofilin is critical for actin treadmilling.

1.2.4: Regulators of actin filament stabilization

Although the dynamic nature of actin is critical for proper synaptic function, actin filament stabilization is also essential and highly regulated, especially in the context of synaptic adhesion (Cingolani & Goda, 2008). Capping proteins, such as F-actin capping protein subunit beta (Cap2) and epidermal growth factor receptor pathway substrate 8 (Eps8), bind to the barbed end of actin filaments to stabilize and restrict elongation by blocking the G-actin binding site (Spence & Soderling, 2015).

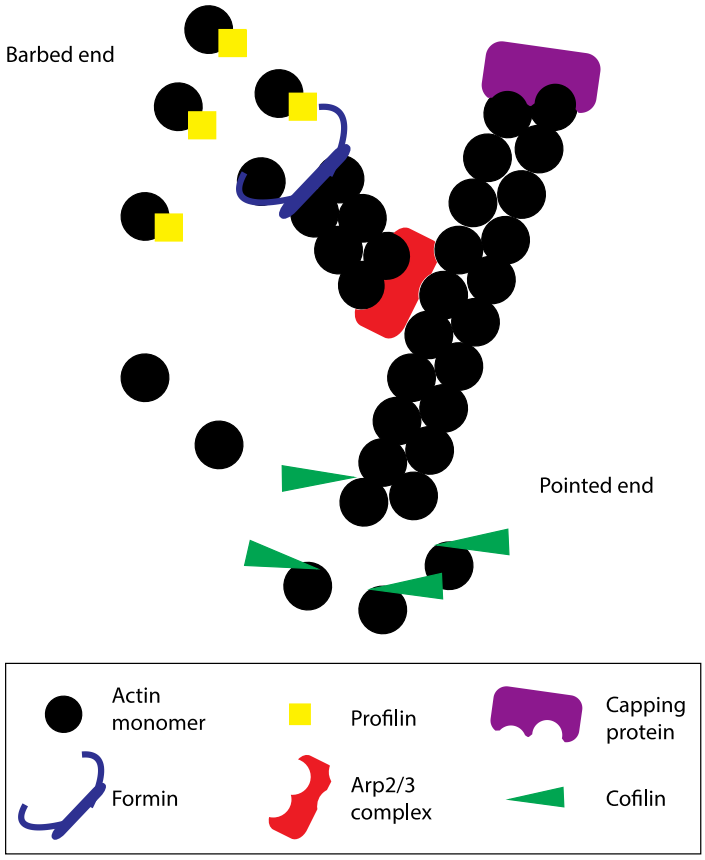

Figure 1.2: F-actin and its regulators. A pictorial representation of a branched actin filament. Actin monomers (G-actin) contain two binding sites that allows for the association of two other actin monomers, which enables their polymerization into actin filaments (F-actin). A number of regulatory proteins are involved in promoting the assembly, disassembly, and stabilization of actin filaments. The location of the actin binding sites on the actin monomers gives actin filaments directionality, where the actin filament is assembled at the barbed end and disassembled at the pointed end. The interaction between G-actin, profilin, and formin promotes F-actin assembly at the barded end. Cofilin severs F-actin at the pointed end leading to disassembly. The newly freed G-actin is now able to bind to profilin and get reincorporated into the barbed end in a process known as treadmilling. Importantly, F-actin can grow both linearly or it can branch. Branching requires the association of the Arp2/3 complex with F-actin and G-actin. Finally, capping proteins can bind to the ends of F-actin to stabilize and restrict elongation.

1.2.5: The Rho family of GTPases

Some of the best-studied regulators of actin cytoskeletal dynamics are members of the Rho family of GTPases. In humans, there are over 20 proteins that fall into the Rho family (Wennerberg & Der, 2004), but Rac1, cell division control protein 42 homolog (Cdc42), and Ras homologue family member A (RhoA) are currently the most studied. The Rho GTPases function upstream of many of the actin regulating and actin binding proteins previously introduced and are thus thought to play a role in actin assembly, disassembly, and stability (A. Hall, 1998; Spiering & Hodgson, 2011). Indeed, expression of dominant-negative Rho family of GTPases in cell culture severely disrupts actin dynamics resulting in cellular defects in a variety of actin regulated events including cell migration (Ridley, 2001), cytokinesis (Jordan & Canman, 2012), endo/exocytosis (Chi et al., 2013), axon guidance (A. Hall & Lalli, 2010), and developmental morphogenesis (Duquette & Lamarche-Vane, 2014). The Rho family of GTPases function as GTPases and are able to bind GTP and hydrolyze it to guanosine diphosphate (GDP). Consequently, the Rho-GTPases can exist in two separate confirmations that alter their binding affinities for other intracellular proteins. Hence why, Rho GTPases are classically thought of as molecular switches that are “on” (more likely to bind other proteins) in the GTP-bound form, and “off” (less likely to bind other proteins) in their GDP-bound form. GTPases are also further regulated by GTPase accelerating proteins (GAPs) that function to speed up the hydrolysis of GTPase bound-GTP to GDP, and thus function to turn “off” the GTPase. Guanine nucleotide exchange factors (GEFs) also regulate

GTPases by promoting the release of GDP from the GTPase, freeing the GTPase to bind GTP, and turning back “on”.

Rho GTPases function to alter actin dynamics through two major mechanisms of action. The first is by activating actin regulators and actin binding proteins (Sit & Manser, 2011). Many actin-regulating proteins have auto-inhibitory domains, including formins (Otomo et al., 2005; Rose et al., 2005) and N-WASP (J. Lane et al., 2014), while others are found in protein complexes that block their actin regulating domains, like WAVE (J. Lane et al., 2014). However, actin-regulating proteins are modular (they contain multiple protein binding domains), and when GTP-bound Rho GTPases are present they bind the actin regulating proteins (often at a Cdc42- and Rac-interactive binding (CRIB) domain) and cause conformational changes that release the autoinhibited actin associating domains allowing them to modulate the actin cytoskeleton (J. Lane et al., 2014). The second mechanism by which Rho GTPases function to alter the actin network is by recruiting actin-regulating proteins to specific loci within the cell (Sit & Manser, 2011), again through their ability to bind actin regulating proteins, and therefore regulating their action in place and time in response to specific local signaling events. Thus, the spatial and temporal recruitment of the Rho GTPase proteins themselves and GEF and GAP proteins that modulate their ‘on/off’ state is critical to understand how localized regulation of actin dynamics occurs. Certainly, functional domains within the Rho-GEF and Rho-GAP proteins can regulate the recruitment of the actin modulating proteins; however, other

‘adaptor’ proteins including the non- catalytic region of tyrosine kinase (NCK) family of proteins have been shown to be important in this regard.

1.3: Non-catalytic region of tyrosine kinase (NCK) adaptor protein

1.3.1: Adaptor proteins

In the context of the nervous system, and in particular the synapse, there are over one thousand highly conserved proteins in the postsynaptic proteome of vertebrate excitatory synapses (Grant, 2019). For neurons to have specific and appropriate responses to external stimuli requires the recruitment and integration of numerous proteins that initiate and maintain signaling cascades in a time sensitive and order specific manner. Therefore, when a cell surface receptor at an excitatory synapse is stimulated it initiates cellular signals often in the form of posttranslational modifications, like phosphorylation, and must correctly select (from the over one thousand different types of proteins) specific binding partners at almost instantaneous time scales. In order to achieve what seems like an impossible task, cells, including neurons, evolved to rely on adaptor proteins (Emes et al., 2008; Pawson & Scott, 1997). Adaptor proteins contain multiple protein binding domains that link together various and specific proteins into complexes. These complexes then allow for signal transduction to be highly regulated, not only by the protein binding domains present in the specific adaptor protein, but also by the subcellular location of the adaptor protein and its proximity to other binding partners. Therefore, adaptor proteins organize

molecular signaling networks in both space and time and can be thought of as master regulators of cell signaling in both a spatial and temporal fashion.

1.3.2: NCK proteins consists of 3 SH3 domains and an SH2 domain

An important family of adaptor proteins studied in the context of actin dynamics in cell biology is the non-catalytic region of tyrosine kinase (NCK) adaptor protein family. The NCK family of adaptor proteins consists of two members, NCK1(NCKα) and NCK2(NCKβ/Grb4). NCK proteins have no intrinsic enzymatic activity, but instead are thought to function as adaptor proteins that bridge tyrosine phosphorylation with downstream effectors involved in modulating the actin cytoskeleton. These 47kDa proteins primarily consist of four modular protein-binding domains: three N-terminal Src homology 3 (SH3) domains and one C-terminal Src homology 2 (SH2) domain. The SH3 domains have been shown to bind proline-rich motifs. However, each SH3 domain has its own specificity in protein binding, where certain proteins only bind to one specific SH3 domain. For example, the second SH3 specifically binds a number of signaling proteins that possess a PxxPxRxxS motif (where P is proline, x is any amino acid, R is arginine, and S is serine), including p21-activated kinase (PAK), NCK-interacting kinase/mitogen-activated protein kinase kinase kinase kinase 4 (NIK/MAP4K4), synaptojanin, protein kinase C-related kinase 2 (PRK2), and Wiskott-Aldrich syndrome/*WASL*-interacting protein (WIP) (Table 1). At the other end, the SH2 domain specifically binds phosphorylated tyrosine residues at YDxV/P/D motifs (where Y is tyrosine, D is aspartic acid, x is any amino acid, and

P is proline) that include receptor tyrosine kinases, cell adhesion molecules, and tyrosine phosphorylated docking proteins, such as $p62^{DOK-1}$ and $p130^{CAS}$ (Table 1). Therefore, NCK1 and NCK2 connect receptor and non-receptor tyrosine kinases via their SH2 domains to larger protein complexes bound to their SH3 domains. Importantly, given NCK1 and NCK2's small size and their sensitivity in recognizing protein phosphorylation, they can functionally act as switches that when coupled to protein phosphorylation, or dephosphorylation, can turn on and off entire signaling cascades.

Critically, the *NCK1* and *NCK2* genes are highly conserved across species. The *Mus musculus* NCK proteins are 96% identical, in terms of amino acid identity, to their *Homo sapiens* counterparts (M. Chen et al., 1998), making the mouse an attractive model to study NCK function. Direct comparison of the murine NCK1 and NCK2 protein sequences to each other, reveals they are 68% identical (M. Chen et al., 1998). Nonetheless, the protein binding domains (SH3 and SH2) of NCK1 and NCK2 are between 77-82% similar (M. Chen et al., 1998). Thus, most of the differences between the NCK1 and NCK2 proteins are found in the linker regions between the protein binding domains. Whether these regions functionally contribute to any differences between NCK1 and NCK2 remains to be determined.

SH2 BINDING PARTNERS

PROTEIN	NCK SELECTIVITY	SYSTEM	REFERENCE
CD79A (immunoglobulin-α)	NCK1 and NCK2	B-cell antigen receptor signaling	(Castello et al., 2013)
CEACAM3	NCK1 and NCK2	293 cells (human kidney)	(Pils et al., 2012)
Cortactin	Not specified	Cytoskeletal reorganization	(Okamura & Resh, 1995)
Dab1	NCK2 selective (when phosphorylated)	Cultured forebrain neurons (mouse)	(Pramatarova et al., 2003)
EGFR	NCK1 and NCK2	Bacterial cultures/microarray	(R. B. Jones et al., 2006; Tu et al., 1998)
ELMO1	Only NCK1 tested	RAC1 activation (although paper has been withdrawn)	(Zhang et al., 2014) (withdrawn J Biol Chem., 2019)
EphA3	Only NCK1 tested	Process retraction and cell migration	(T. Hu et al., 2009)
EphA4	Only NCK2 tested	Blastomere adhesion	(Bisson et al., 2007)
EphB1	NCK2 selective (when phosphorylated)	NG108 cells (mouse neuroblastoma)	(Cowan & Henkemeyer, 2001)
EphB2	Not specified	Neuronal cell line (NG108)	(Holland et al., 1997)
ErbB2 (EGFR family)	NCK1 and NCK2	Bacterial cultures/microarray	(R. B. Jones et al., 2006)
ErbB3 (EGFR family)	NCK1 and NCK2	Bacterial cultures/microarray	(R. B. Jones et al., 2006)
Nephrin	NCK1 and NCK2	podocyte foot process formation/actin cytoskeletal rearrangement shown in MEFs	(N. Jones et al., 2006)
P130CAS	NCK1 and NCK2	Actin cytoskeletal rearrangement shown in MEFs (PDGF-B signalling)	(G. M. Rivera et al., 2006)
p62DOK 1	NCK2 2-3fold over NCK1	EGFR and PDGFR signalling	(M. Chen et al., 1998)
PDGFR(Y1009)	NCK2 selective	NIH 3T3 cells surprisingly NCK2 blocks PDGFR and rac1 activity	(M. Chen et al., 2000)
PDGFR(Y751)	NCK1 selective	Dog kidney epithelial cells (TRMP)	(Nishimura et al., 1993)
PERK (EIF2AK3)	NCK1 and NCK2	COS1 cells, MEFs, Mouse insulinoma (Min6) cells, ER stress related protein	(Yamani et al., 2014)
Tir	NCK1 and NCK2	E-coli	(Frese et al., 2006)

VEGFR2	NCK1 and NCK2	Mouse retina vascular endothelial	(Dubrac et al., 2016)

SH3(1) BINDING PARTNERS

PROTEIN	NCK SELECTIVITY	SYSTEM	REFERENCE
CBL	Not specified	HEL and U937IF cells, phosphorylation dependant interaction	(Izadi et al., 1998)
CD3ε	NCK1 and NCK2	Jurkat cells, interaction independent of tyrosine phosphorylation	(Gil et al., 2002)
DCC	Only tested NCK1	Commissural neurons, Cos cells, N1E-115 cells, promotes neurite outgrowth, Rac1 activation	(X. Li et al., 2002)
EIF2B2	Only tested NCK1	Transformed rat hepatocytes overexpressing human insulin receptor, involved in protein synthesis	(Kebache et al., 2002)
IRE1α	Only tested NCK1	FR3T3 fibroblasts, MEFs, NCK alters the ER stress mediated activation of ERK1	(Nguyên et al., 2004)

SH3(2) BINDING PARTNERS

PROTEIN	NCK SELECTIVITY	SYSTEM	REFERENCE
Abl1	Not specified	Human kidney 293 cells	(Ren et al., 1994)
Abl2	NCK1 and NCK2	NIH 3T3 cells (mouse embryonic fibroblast), actin rearrangement	(Antoku et al., 2008)
DOCK180	Only tested NCK2	Yeast two-hybrid screen	(Tu et al., 1998)
FAK	NCK1 and NCK2	Human kidney 293 cells	(Goicoechea et al., 2002)
FASLG (CD178)	NCK1 and NCK2	KFL9 and JFL39.1 cell lines (immune cells)	(Voss et al., 2009)
IRS1	NCK1 and NCK2	Yeast two hybrid binding assay	(Tu et al., 1998)
N-WASP	NCK1 and NCK2	293T cells (human kidney)	(Rajat Rohatgi et al., 2001)
NIK (MAP4K4)	Not specified	293 cells (human kidney)	(Su et al., 1997)
PAK1	NCK1 and NCK2	COS7 (monkey kidney), Swiss 3T3 cells (mouse embryo)	(Bokoch et al., 1996)
PAK3	NCK2 selective	Dissociated hippocampal neurons cultured E17, transfected DIV21	(Thévenot et al., 2011)
PRK2	Not specified	In vitro bacterial cultures	(Quilliam et al., 1996)
RRAS	Not specified	293 cells (human kidney)	(B. Wang et al., 2000)

SAM68 (KHDRBS1)	Only tested NCK1	Human embryonal kidney 293Tcells	(Asbach et al., 2012)
SOCS7	Not specified	TIG1 (human fetal lung fibroblast), BALB 3T3 (mouse embryo) cells	(Matuoka et al., 1997)
Synaptojanin	Not specified	Mouse brain lysate	(Fawcett et al., 2007)
WIP	Not specified	BJAB cells (human lymphoma)	(Antón et al., 1998)

SH3(3) BINDING PARTNERS

PROTEIN	NCK SELECTIVITY	SYSTEM	REFERENCE
α2-chimaerin	Only tested NCK1	COS-1, COS-7 (monkey kidney), and HeLa cells (human cancer)	(Gutierrez-Uzquiza et al., 2013)
β2-chimaerin	Only tested NCK1	COS-1, COS-7 (monkey kidney), and HeLa cells	(Gutierrez-Uzquiza et al., 2013)
DCC	Only tested NCK1	Commissural neurons, Cos cells, N1E-115 cells (mouse neuroblastoma), promotes neurite outgrowth, Rac1 activation	(X. Li et al., 2002)
DOCK1	Only tested NCK2	293 cells (human kidney)	(Tu et al., 2001)
FASLG (CD178)	NCK1 and NCK2	KFL9 (human leukemia) and JFL39.1 cell lines (human T lymphocyte)	(Voss et al., 2009)
IRE1β	Only tested NCK1	FR3T3 fibroblasts (rat), MEFs, NCK alters the ER stress mediated activation of ERK1	(Nguyên et al., 2004)
IRS1	NCK1 and NCK2	Yeast two hybrid binding assay	(Tu et al., 1998)
N-WASP	NCK1 and NCK2	293T cells (human kidney)	(Rajat Rohatgi et al., 2001)
p120-RasGAP	Only tested NCK1	Human hepatocellular carcinoma HepG2 cells	(Ger et al., 2011)
PINCH	NCK2 selective	Yeast two-hybrid binding assay	(Tu et al., 1998)
SAM68 (KHDRBS1)	NCK1 and NCK2	Human embryonal kidney 293Tcells	(Asbach et al., 2012)
WASP	Not specified	COS-7 (monkey kidney)	(Rivero-Lezcano et al., 1995)

SH3(not specified) BINDING PARTNERS

PROTEIN	NCK SELECTIVITY	SYSTEM	REFERENCE
Abi-1	NCK1 and NCK2	NG108 cells (mouse neuroblastoma)	(Cowan & Henkemeyer, 2001)

axin	NCK2 selective	NG108 cells (mouse neuroblastoma)	(Cowan & Henkemeyer, 2001)
CAP	NCK2 selective	NG108 cells (mouse neuroblastoma)	(Cowan & Henkemeyer, 2001)
Cdc42	NCK1 selective	Human dermal fibroblast, promotes filapodia formation	(Guan et al., 2009)
Dynamin	NCK1 and NCK2	NG108 cells (mouse neuroblastoma)	(Cowan & Henkemeyer, 2001)
hnPNPK	NCK2 selective	NG108 cells (mouse neuroblastoma)	(Cowan & Henkemeyer, 2001)
MINK1	Not specified	Phoenix-A cells (293T cell derivative)	(Y. Hu et al., 2004)
MTA1	Not specified	Balb/MK cells (mouse epithelial)	(Simpson et al., 2001)
MTA3	Not specified	Balb/MK cells (mouse epithelial)	(Simpson et al., 2001)
RhoA	NCK2 selective	Human dermal fibroblast, promotes stress fibre formation	(Guan et al., 2009)
ROBO1	NCK1 and NCK2	Cultured cortical neurons (Only NCK2 mediates slit-induced cortical neurite outgrowth)	(Round & Sun, 2011)
ROBO2	NCK1 and NCK2	Cultured cortical neurons	(Round & Sun, 2011)
TNK2 (Ack1)	Not specified	COS-7 (monkey kidney)	(Chan et al., 2011)
WAVE2	Only NCK1 tested	293 cells (human kidney)	(Pils et al., 2012)

Table 1.1: NCK1 and NCK2 protein binding partners.

1.3.3: NCK1 signaling leads to the activation of N-WASP, WAVE1, ARP2/3, actin branching, and polymerization

Although many studies have interchanged NCK1 and NCK2, a number of studies have specifically addressed NCK1 as a key regulator of actin dynamics. In 3D collagen matrices, silencing NCK1 leads to the disruption of F-actin organization (Chaki et al., 2015). NCK1 has been shown to form a complex with cortactin, WIP, and N-WASP that stimulates Arp2/3 actin branching and polymerization (Tehrani et al., 2007). Indeed, clustering of NCK1 SH3 domains at the plasma membrane is sufficient to induce localized actin polymerization (Gonzalo M. Rivera et al., 2004). NCK1 is recruited to the cellular membrane by binding its SH2 domain to phosphorylated tyrosine residues on membrane bound receptors, adhesion proteins, and other proline-rich or phosphorylated membrane proteins like cortactin (Oser et al., 2010). Simultaneously, autoinhibited N-WASP binds an activation motif within the linker region between the first two SH3 domains of NCK1, releasing N-WASP's VCA domain to activate the Arp2/3 complex and leads to localized actin branching and polymerization at the membrane (Banjade & Rosen, 2014; Okrut et al., 2015) (Fig. 1.3). VCA domains consist of a verprollin homology sequence (V), which binds G-actin, and central (C) and acidic sequences (A), which bind to Arp2/3 (A. E. Kelly et al., 2006). Similarly, NCK has been shown to interact with Rac1 and members of the WAVE1 inhibitory complex, leading to the dissociation of inhibited WAVE1, releasing active WAVE1, and stimulating actin nucleation also through the Arp2/3 complex (Eden et al., 2002).

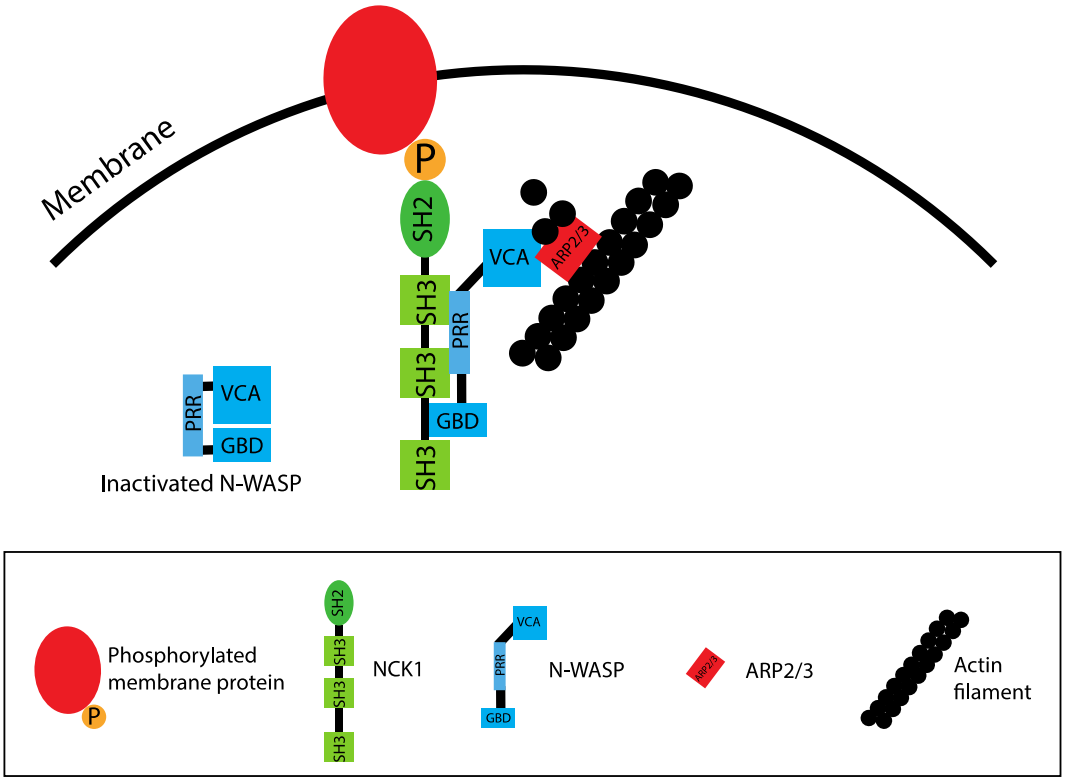

Figure 1.3: NCK1, N-WASP, Arp2/3 interaction promotes actin filament branching. N-WASP's verprolin, cofilin, acidic (VCA) domain is autoinhibited by binding to the GTPase-binding domain (GBD) domain. NCK1's Src homology 2 (SH2) domain binds phosphorylated tyrosine residues resulting in the ability of phosphorylated membrane proteins to recruit NCK1 to the membrane. The GBD domain of N-WASP associates with the linker region between the first two Src homology 3 (SH3) domains, while the proline rich region (PRR) of N-WASP binds to the second and third SH3 domains of NCK1. The associate of N-WASP with NCK1 releases N-WASP's VCA domain. The VCA domain is then free to activate the actin related proteins 2/3 (Arp2/3) complex and promote actin filament branching.

1.3.4: NCK1 regulates PAK signaling

A second distinct signaling cascade that links NCK1 to actin dynamics is through its association with p21-activated kinase (PAK). PAKs are characterized by their GTPase-binding domain/Cdc42- and Rac-interactive binding (GBD/CRIB) domain (which bind the Rho GTPases Cdc42 and Rac), their kinase domain (which initiates protein phosphorylation), and their autoinhibitory domain (Baskaran et al., 2012). There are six PAK proteins that can be divided into two families based on their sequence homology and mechanisms of activation (Rane & Minden, 2014). Group A (Type I) PAKs consists of PAK1, PAK2, and PAK3, and are all highly expressed in the nervous system (Civiero & Greggio, 2018). Group A PAKs form inactive dimers that inhibit their kinase domains. Group A PAK activation occurs by binding Cdc42 or Rac via its GBD/CRIB domain, which releases PAK from the dimer, disinhibiting the kinase domain resulting in autophosphorylation and kinase activation (Rane & Minden, 2014). Group B (Type II) PAKs, consisting of PAK4, PAK5, and PAK6, and are all also expressed in the nervous system (Civiero & Greggio, 2018). Unlike the Group A PAKs, Group B PAKs do not dimerize. Instead, Group B PAKs exist in a confirmation as a monomer that autoinhibits their own kinase domain (Rane & Minden, 2014). Two different mechanisms of Group B PAK activation have been proposed. The first is simply that Cdc42 binds to the GBD/CRIB domain, releasing the kinase domain to phosphorylate neighbouring proteins (Baskaran et al., 2012). The second proposed mechanism of activation is that inactivated Group B PAKs bind to a pseudosubstrate that helps stabilize it in the inactive form, and that both

Cdc42 and a SH3 containing protein (like NCK1) are required to release and activate the kinase domain (Ha et al., 2012).

Indeed, NCK1 has been shown to interact with PAKs. PAK1 binds to NCK1's second SH3 and this association allows for the localization of PAK kinase activity at the membrane, which can initiate RhoA signaling, and cytoskeletal changes leading to receptor endocytosis (Srivastava et al., 2013). PAKs are important effectors downstream of GTPases, like Rac and Cdc42, which have been implicated in regulating the actin cytoskeleton in NCK1-stabilized complexes (Howe, 2001; Zhao et al., 2000). Simultaneously, the NCK1-dependent recruitment and activation of PAK1 can also result in PAK1 phosphorylating LIM domain kinase (LIMK), which can then lead to the phosphorylation of cofilin on a specific serine residue (Edwards et al., 1999). Serine-phosphorylated cofilin results in a reduced affinity for cofilin to bind f-actin, thus preventing the breakdown of actin filaments (Arber et al., 1998; N. Yang et al., 1998) (Fig. 1.4). In this way, NCK1 is directly involved in bridging signaling events that result in intracellular actin modulation. Together, a number of studies have linked the importance of the SH3 domains of NCK1 with proteins that have direct roles in modulating actin dynamics. Unlike the SH3 domains, the SH2 domain of NCK1 and NCK2 have been more directly linked with their ability to engage with receptor tyrosine kinases (and soluble tyrosine kinases) suggesting the SH2 domain is important to link NCK proteins with receptor mediated signaling.

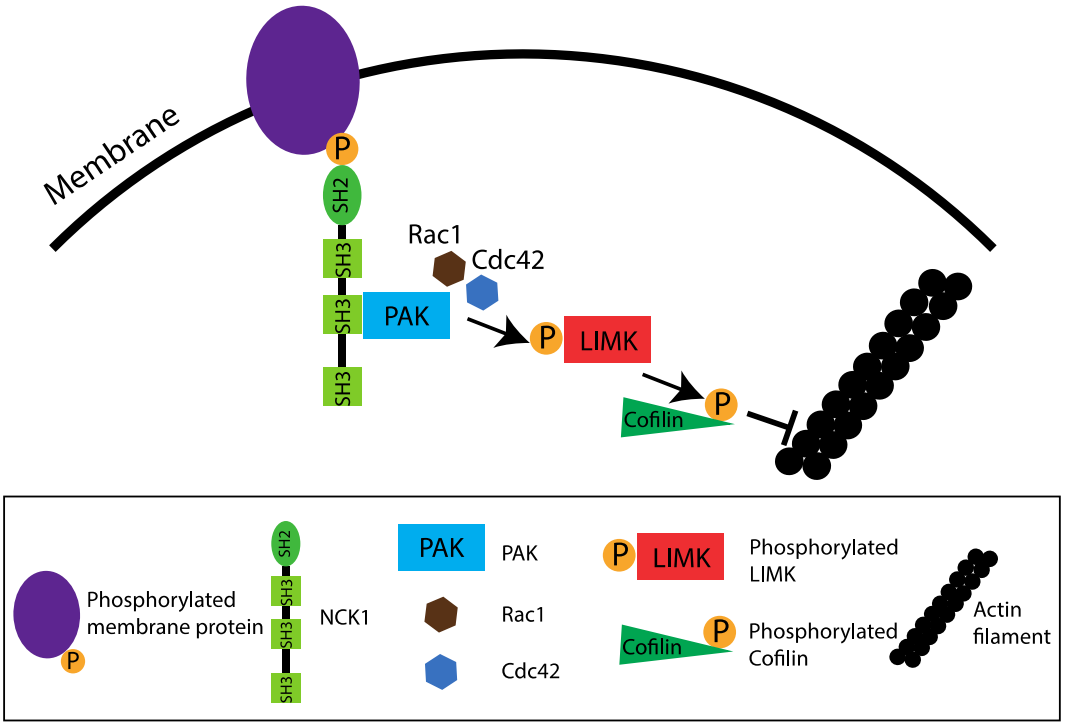

Figure 1.4: NCK1 associates with PAK leading to the phosphorylation of Cofilin and the inhibition of actin filament disassembly. NCK1 gets recruited to cell membranes by binding phosphorylated membrane proteins via its Src homology 2 (SH2) domain. p21-activated kinase (PAK) binds NCK1's second SH3 domain and promotes localized PAK activation by the Rho GTPases, Rac1 and/or Cdc42 leading to the phosphorylation of LIM kinase (LIMK), which goes on to phosphorylate Cofilin. Phosphorylated Cofilin can no longer associate with actin filaments and results in the inhibition of actin filament disassembly.

1.3.5: Upstream recruiters of NCK1

To date a number of cell-surface receptors have been shown to associate with NCK proteins, mainly by in vitro studies. These include receptor tyrosine kinases as well as unique interactions with receptors that do not have kinase activity. Below is a short description of receptors that have been linked with NCK proteins, to show the diverse nature of receptor mediated signaling that are attributed to the NCK proteins.

1.3.5.1: SH2-associating receptor tyrosine kinases

NCK1 has been shown to bind a number of growth factor activated receptor tyrosine kinases via its SH2 domain, including vascular endothelial growth factor receptor 2 (VEGFR2) (Dubrac et al., 2016), platelet-derived growth factor receptor (PDGFR) (Nishimura et al., 1993), epidermal growth factor receptor (EGFR) (R. B. Jones et al., 2006), as well as the Eph receptors (Holland et al., 1997; T. Hu et al., 2009; Stein et al., 1998). Recruitment of NCK1 by these receptor tyrosine kinases has been proposed as a potential mechanism of action that links the actin cytoskeleton to their function in growth and migration. Indeed, recent work has demonstrated that growth-factor activated VEGFR2 recruits NCK1 in a PAK-GTP bound Cdc42-GEF containing protein complex to influence the direction of growth in retinal angiogenesis (Dubrac et al., 2016).

1.3.5.2: Receptors that engage SH2 after soluble tyrosine kinase activation

Interestingly, in certain situations NCK proteins can interact with certain receptors that have been phosphorylated by intracellular kinases. One clear example of this occurs in kidney podocytes where NCK interacts with the nephrin receptor via NCKs SH2 domain only after nephrin has been phosphorylated by Src-family kinases (N. Jones et al., 2006).

1.3.5.3: SH3 associating docking proteins and non-receptor tyrosine kinases

NCK1 has also been shown to interact via it's SH3 domain with tyrosine phosphorylated docking proteins, such as $p62^{DOK-1}$ and $p130^{cas}$, in growth factor-stimulated actin remodeling (M. Chen et al., 1998; G. M. Rivera et al., 2006). Concurrently, interactions between NCK proteins and integrin regulators, such as the particularly interesting new cysteine-histidine-rich (PINCH)-integrin-linked kinase (ILK) protein complexes (Tu et al., 1998) and focal adhesion kinase (FAK) (Goicoechea et al., 2002), implicate the NCK proteins as bridges between integrin, growth factor receptors, and the actin cytoskeleton, and therefore potential regulators of adhesion dynamics.

1.3.5.4: SH3 associating receptors that are not kinases

NCK1 is recruited to the cell membrane via its SH3 domain by Slit2-activated roundabout homolog 1 (ROBO1) and roundabout homolog 2 (ROBO2). NCK1 is involved in ROBO1 signaling, in the context of cell polarity, as recruited NCK1 brings together phosphorylated-PAK, cdc42, and other GEFs that can

influence the orientation and rate of actin outgrowth and transport of organelle (Dubrac et al., 2016). ROBO receptors have no autocatalytic or intrinsic enzymatic activity, but instead function by clustering intracellular regulators of actin dynamics, like NCK1 with cytoplasmic kinases and Rho GTPases (Tong et al., 2019). In neurons, both NCK1 and NCK2 bind ROBO1 and ROBO2, however only NCK2 seems to play a role in Slit-induced cortical neurite outgrowth (Round & Sun, 2011).

NCK1 also interacts with the Netrin-1 receptor, deleted in colorectal carcinoma (DCC) to promote neurite outgrowth (X. Li et al., 2002). Indeed, Netrin-1 activation of DCC dimers promotes NCK1 interactions with downstream regulators of the actin cytoskeleton, including Rac1, Cdc42, PAK1, and N-WASP to alter and direct the actin cytoskeleton in the axonal growth cone (Shekarabi et al., 2005). Further, a dominant negative form of NCK1 inhibits DCC-induced actin outgrowth (X. Li et al., 2002).

Taken together, upstream recruiters of NCK1 appear to have evolved to rely on NCK1 to link their signaling to the manipulation of the actin-cytoskeleton important for cell polarity in the form of directional growth, a similar mechanism that is proposed to take place in dendritic spine formation.

1.3.6: NCK1 associates with clathrin-associating proteins

Intriguingly, NCK1 also associates with clathrin-associating proteins, such as activated Cdc42-associated kinase 1 (ACK1), which colocalizes with clathrin and adaptor protein complex 2 (AP-2) in clathrin coated vesicles (Buday et al.,

2002). Thus, NCK1 may function in clathrin mediated events such as receptor internalization. In fact, recent work has shown Nephrin receptor clustering in kidney podocytes recruits NCK1/2 to phosphorylated tyrosines via their SH2 domain. NCK recruitment results in actin rearrangement through an NCK SH3-N-WASP mediated mechanism. Additionally, dynamin, a GTPase important for clathrin-mediated endocytosis, is also recruited to the Nephrin clusters via NCK's SH3 domain and promotes Nephrin internalization (Martin et al., 2020). Therefore, hyperphosphorylation of the Nephrin receptor leads to receptor endocytosis through an NCK-dependent mechanism. All together, NCK1's signaling partners places it as a potentially important spatiotemporal node regulating protrusion dynamics, as well as adhesion and receptor turnover.

1.3.7: Bacteria and viruses have evolved to manipulate NCK1's actin-polymerizing activity as part of their infection strategies

Interestingly and solidifying NCK1's role as an actin regulator, at least two non-phylogenetically related species, one virus and one bacterium, have independently evolved to take advantage of cellular NCK1 and it's actin polymerizing abilities as part of their infection strategies. The vaccinia virus binds the SH2 domain of NCK1 through a tyrosine-phosphorylated motif on its A36R gene product, and through NCK1's SH3 interactions and N-WASP activation, actin polymerization is initiated, and the virus forms an actin tail that gives it motility and allows it to infect adjacent cells (Frischknecht et al., 1999). In the enteropathogenic bacteria *Escherichia coli*, the Tir protein becomes tyrosine

phosphorylated at the plasma membrane of infected cells and binds to the SH2 domain of NCK1, and again through NCK1-N-WASP-Arp2/3 complex activity produces a large rearrangement of the actin cytoskeleton and pedestal formation at the sites of bacterial infection (Gruenheid et al., 2001). Strikingly, both of these mechanisms result in the hijacking, via NCK1, of the host-cells own actin cytoskeleton and manipulating it in specific and predictable context dependent manners, again similar to what neurons may have evolved to do at synaptic junctions during dendritic spine formation.

1.3.8: NCK1 and NCK2 function in development

To address NCK1 and NCK2's functional roles *in vivo*, Dr. Tony Pawson's research group generated NCK1-, NCK2-, and NCK1 and NCK2-mutant mice that would be deficient in NCK1, NCK2, or both proteins (Bladt et al., 2003; Fawcett et al., 2007). They reported that in the mouse embryo NCK1 and NCK2 have broad overlapping expression patterns. They also found that NCK1 and NCK2 are likely functionally redundant in development since the single mutants were all viable but the double mutant, which were deficient in both NCK1 and NCK2, had profound defects in mesoderm-derived notochord development and embryonic lethality at embryonic day (E) 9.5. Furthermore, fibroblast cell lines that were derived from the double mutant embryos displayed defects in cell motility and in the organization of the lamellipodial actin network (Bladt et al., 2003).

To further explore the functional role of the NCK proteins and overcome the embryonic lethality, tissue specific conditional mutant mice were developed that were deficient in both NCK proteins in specific cell types (Aryal A.C et al., 2015; Chaki et al., 2015; Fawcett et al., 2007; N. Jones et al., 2006). These conditional mutant mice further confirmed NCKs role in the development of mesoderm-derived embryonic structures, including in the cardiovascular system (Chaki et al., 2015), bone development (Aryal A.C et al., 2015), and kidney podocyte formation (N. Jones et al., 2006). NCK1 and NCK2 function in endothelial cells through angiogenic factor-stimulated cytoskeletal remodeling and directional migration. Proper endothelial lumen formation requires NCK1 and NCK2 to properly link Cdc42-dependent polarity signals, junctional actin polymerization and vascular endothelial (VE)-cadherin adhesion dynamics (Chaki et al., 2015). Similarly but in bone tissue, deletion of NCK1 and NCK2 in preosteoblasts and osteoblasts causes osteopenia (Aryal A.C et al., 2015). The NCK proteins, through actin interactions, regulate preosteoblastic and osteoblastic migration and bone mass (Aryal A.C et al., 2015). Finally, mice with NCK-deficiency within kidney podocytes result in glomerular filtration defects due to failure of the podocyte foot processes to properly form (N. Jones et al., 2006). The dysregulated foot processes provide an *in vivo* example of how NCK1 and NCK2 interact in a complimentary manner with other cell-type specific and selectively expressed proteins to form an intricate actin-based cellular morphology. Interestingly, in all these contexts NCK1 and NCK2 seemed to be

functionally redundant as deficiency in both NCK1 and NCK2 was required to result in impaired actin regulation that lead to the developmental defects.

1.3.9: NCK1 and NCK2 in axon guidance

NCK1 and NCK2 have also been shown to be critical in the central nervous system (CNS) for proper axon guidance *in vivo*. In drosophila, NCK's functional homologue DOCK is required for growth cone expansion, axon guidance, and target recognition in the fly visual system (Rao, 2005). In the mammalian CNS, restriction of both NCK1 and NCK2 protein expression in the CNS leads to deficits in corticospinal tract axon guidance and a reduced anterior commissure in the spinal cord (Fawcett et al., 2007). Indeed, NCK1 is necessary for Netrin1-DCC growth cone expansion as NCK1 serves as a scaffold that forms a complex with PAK1, Cdc42, Rac1, N-WASP, and the DCC receptor leading to actin polymerization (Shekarabi et al., 2005). Previous work in Dr. Fawcett's lab demonstrated that NCK1 and NCK2 are expressed in the developing spinal cord, and that loss of both proteins leads to a reduction in axonal growth cone complexity in DCC-positive neurons, a decreased ventral commissure thickness, and a reduction in DCC mRNA levels (C. Lane et al., 2015). However, no significant differences were found between the NCK1 single mutants, NCK2 single mutants, and control mice, suggesting that NCK1 and NCK2 are functionally redundant in the developmental stages of DCC-mediated spinal cord axonal guidance. Despite this cursory examination, no in-depth studies of the

individual mutant mice have been done to further determine if more subtle defects in CNS function are apparent.

1.4: Studying gene and protein function in cognition and behaviour

1.4.1: Mouse models

Rodents (especially *Mus musculus* and *Rattus norvegicus*) have been the leading model organisms in biomedical research for over a century (Ellenbroek & Youn, 2016). Therefore, decades of research have gone into the basic understanding of rodent physiology and the validation of tools, techniques, and protocols to predictably probe cellular and system functioning and responses. Over the last three decades, a large genetic toolbox has been established for the *Mus musculus* starting with embryonic cell-based targeting technologies for gene disruption. The first mutant mouse was created in 1987 (Thomas & Capecchi, 1987) allowing for *in vivo* loss of function experiments. Indeed, 99% of genes are functionally shared between mice and humans, and similarities in nervous, cardiovascular, endocrine, immune, musculoskeletal, and other organ systems have been extensively documented (Rosenthal & Brown, 2007). Therefore, making the study of candidate genes for human diseases and disorders possible in a parallel system in the mouse. Here to examine the role of NCK1 in the brain and behaviour, the *NCK1* gene has been genetically disrupted resulting in NCK1 protein deficiency in the NCK1 mutant mice.

1.4.2: Evolutional and neurobiological understanding of behaviour

The triune brain model was first proposed by Dr. Paul MacLean to provide an evolutionary framework to the understanding of behaviour (Ploog, 2003). According this model the mammalian brain (including human and mouse) can be divided into three distinct regions, the protoreptilian brain, the paleomammalian formation, and the neomammalian formation (Anderzhanova et al., 2017). The protoreptilian brain is the evolutionarily oldest part of the brain that is made up of the brainstem (including the midbrain, the pons and the medulla), the basal ganglia, and the hypothalamus and is important for survival, arousal and homeostatic regulation. Therefore, nuclei and circuits in these brain regions are important for circadian rhythms, sleep, and wakefulness, but also instinctive behaviours like feeding, aggression, and reproductive behaviours (Lanciego et al., 2012; Nicholls & Paton, 2009; Watts, 2015). The paleomammalian brain consists of the limbic structures, including the hippocampus, amygdala and olfactory tubercles and is responsible for emotional and motivational responses that are important for modifying instinctive behaviours into learned adaptive behaviours (Morgane et al., 2005). Finally, the neomammalian region, which consists of the neocortex, is where actual conscious awareness of all incoming sensory information is represented (Briscoe & Ragsdale, 2018). Of course, the brain is not as neatly divided as described above, neither anatomically nor functionally, but instead all systems are integrated to maintain homeostasis. For example, although emotional and motivational valance is primarily encoded in the amygdala, the emotional tone set by the amygdala can directly influence both the

evolutionarily older instinctive parts of the brain and the higher order cognitive centers (Panksepp, 2005; J. Park et al., 2016; Waal, 2011). Simultaneously, properly developed higher order cortical networks provide a regulatory mechanism on the emotional and motivational brain circuits favouring learned behavioural responses (Adhikari et al., 2015; Comte et al., 2016). Indeed, disrupted communication between cortical networks and the limbic/instinctive systems in the brain can lead to the cognitive-emotional imbalances that define neuropsychiatric and neurodevelopmental disorders (Elliott et al., 2011).

1.4.3: Cognition

Cognition is defined by the Oxford dictionary as, “the mental action or process of acquiring knowledge and understanding through thought, experience, and the senses”. Similarly, Dr. John Kihlstrom defines cognition as, “the mental functions by which knowledge is acquired, retained, and used” (Kihlstrom, 2018). It refers to the ability to acquire, store, manipulate and retrieve internal representations of the external environment. Cognition can be broken into a number of evolutionarily conserved components including, social cognition, executive functioning, attention, psychomotor speed, and memory. Social cognition refers to an awareness of socially appropriate interactions, as well as recognizing, processing and properly responding to emotional cues (Frith, 2008). Executive functioning is defined as higher-level thought processing and decision making, which includes problem solving, mental flexibility or the ability to adapt behavioural responses, and the ability to suppress inappropriate responses

(Miller & Wallis, 2009). Attention refers to the ability to selectively focus on important information for appropriate amounts of time (Haladjian & Montemayor, 2015). Psychomotor speed is amount of time it takes to detect and react to the environment. Central to cognition is memory and the ability to learn, which can be thought of as the short-term or long-term storage of information. Proper cognitive functioning is critical for survival, as cognition is the brain's way of regulating and adapting behaviour for the benefit of the organism. Human neuropsychiatric disorders are often defined by impairments in cognition and a better understanding of the genetic, molecular, and circuit level influencers of cognition in the brain has been identified as a major frontier leading to the better understanding and management of these debilitating disorders (S. J. Bailey et al., 2017; Shackman et al., 2015).

1.4.4: Memory and the hippocampus

Memory can be broken down into components that include working memory, recognition memory, and episodic memory. Where working memory is defined as the ability to hold and use information in the mind (Chai et al., 2018). Recognition memory is the ability to recognize previously encountered objects, individuals, and environments (Davis et al., 2010). While, episodic memory is the ability to recall an experience in a particular context often associating it to a place and time (Tulving, 2002). The brain region most closely associated with these forms of memory formation is the hippocampus (Bird & Burgess, 2008). Original insights into hippocampal function were driven by observations of the lack of

ability to acquire new memories in patients and animal models with hippocampal lesions (Broadbent et al., 2004; Milner & Penfield, 1955; Scoville & Milner, 1957). These early observations led to the Declarative Theory of hippocampal function. The Declarative Theory suggests that the hippocampus is critical for initial memory formation, but that memories would then get consolidated into neocortical regions (Morris, 2007). Thus, the hippocampus is important for acquiring memories (a process that is not instantaneous and may take days, weeks, months, or years), but once consolidated, recollection of memories are independent of hippocampal function (Squire, 1986). More recent advances in neuroimaging, electroencephalography, single-cell recording, neuroanatomical tract tracing, and manipulation of gene expression has led to a more nuanced understanding of hippocampus function as a brain region important for the integration of newly acquired information from all the sensory cortical regions with previously encoded representations (B. Leuner & Gould, 2010; Rubin et al., 2014). Therefore, the hippocampus is especially important for combining information from multiple sources and making associations from current and previous experience to guide behaviour. Strikingly, specific hippocampal neuron populations have been shown to fire selectively to previously encountered places (O'Keefe & Nadel, 1978), spatial cues such as direction and distance (Gothard et al., 1996; Ravassard et al., 2013), temporal signatures (Eichenbaum, 2014), auditory tones (Aronov et al., 2017), social cues (Tavares et al., 2015), and other non-spatial cues (Komorowski et al., 2009; Lenck-Santini et al., 2008). Together, these findings have led to the theory that the hippocampus creates a cognitive

map where new experiences are anchored to previously networked neuronal circuits and this leads to understanding. Thus, the hippocampus is both important for acquiring new episodic memories, for recollecting past memories (especially those that have not yet been deeply consolidated), and for imagining the future.

1.4.5: The functional anatomy of the hippocampal formation

The hippocampal formation is made up of the hippocampus proper, which can be divided in the cornu ammonis 1 (CA1), cornu ammonis 2 (CA2), cornu ammonis 3 (CA3), cornu ammonis 4 (CA4), the dentate gyrus, the subiculum, and the entorhinal cortex (Schultz & Engelhardt, 2014). The perforant pathway, which originates in the entorhinal cortex, is the primary input pathway into the hippocampus and projects monosynaptically onto all other regions of the hippocampal formation. The perforant pathway is activated by a number of cortical areas including all the sensory cortices and thus is a point of convergence of all sensory stimuli. Although the perforant pathway projects onto all regions of the hippocampal formation, the majority of contacts are made onto the dentate gyrus (Witter, 2007). The dentate gyrus is made up of dense neuronal cell bodies called granule cells and functions in pattern separation (Leutgeb et al., 2007), which allows for similar signals from the sensory cortices to activate distinct populations of neurons. Perforant pathway activation of the dentate gyrus then leads to the activation of the so-called trisynaptic loop. The trisynaptic loop consists of neurons in the dendate gyrus activating neurons in the CA3 region that then activate neurons in the CA1 (Stepan et al., 2015).

Neurons of the CA1 then project to the subiculum, which is the main output region of the hippocampal formation. Neurons in the subiculum project to the entorhinal cortex, and neurons in the entorhinal cortex project back to higher cortical regions, including sensory cortices (J. Basu & Siegelbaum, 2015). Thus, the hippocampal formation forms a synaptic loop that both starts and finishes in the entorhinal cortex, and on its own can lead to the re-stimulation of all sensory cortical areas that were stimulated during an external experience, even if those external stimuli are no longer present. Importantly however, there are a number of regulatory mechanism and feedback loops that exists throughout the hippocampus, including local inhibitory interneurons (Pelkey et al., 2017). Also, the CA3 projects back onto the dentate gyrus and onto itself. The CA1 also receives direct input from dopaminergic, noradrenergic, and serotonergic neurons originating in the midbrain that can lead to synaptic changes, including enhanced long-term potentiation, and improves learning (Otmakhova & Lisman, 1999). Interestingly, the ventral CA1 (in rodents), or anterior CA1 (in humans), has reciprocal connections to a number of brain regions associated with emotional and stress responses, including the amygdalar complex, prefrontal cortex, and nucleus accumbens (Arszovszki et al., 2014), suggesting that multiple factors including the rewarding or aversive nature of an experience can influence hippocampal function and vice versa. Indeed, it has been shown that the value one attaches to a place affects one's perception of its distance from other places (O'Keefe & Nadel, 1978).

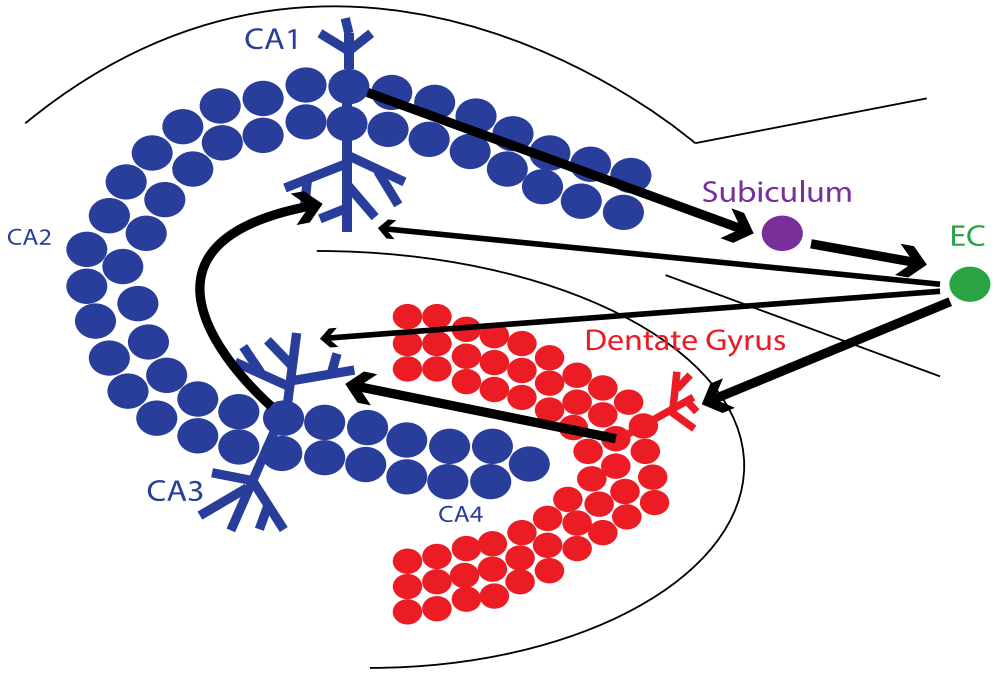

Figure 1.5: The trisynaptic loop, the primary neuronal circuit of the hippocampal formation. A pictorial representation of the major input and output circuit of the hippocampal formation depicting the granule neurons of the dendate gyrus (red) and the pyramidal neurons of the hippocampus (blue). The entorhinal cortex (EC) is the primary input center of the hippocampal formation and projects to the dentate gyrus via the perforant pathway, and to the CA3 and CA1 regions of the hippocampus. The granule neurons of the dendate gyrus project to the CA3 via the mossy fibers, and the CA3 projects to the CA1 via the Schaffer collaterals. CA1 is the major output pathway of the hippocampus proper and projects to the subiculum. Finally, neurons of the subiculum project to the entorhinal cortex (EC) completing the loop.

1.4.6: Emotional regulation and the amygdala

A second brain system critical for cognition that influences behaviour is the amygdaloid complex. The amygdaloid complex is an evolutionarily conserved set of interconnected brain nuclei (Janak & Tye, 2015). Human and animal lesion studies as well as studies that use molecular, electrophysiological, genetic, optogenetic, imaging including magnetic resonance imaging (MRI), pharmacological, and behavioural techniques have implicated the amygdaloid complex as a system critical for the detection of threats and rewards that modulates subsequent behavioural responses. The amygdaloid complex is seen as placing an emotional valance onto an experience thus promoting either avoidance or approach. Deregulated amygdalar circuits can promote excessive avoidance and anxiety-like behaviours, and/or excessive approach and increased risk-taking behaviours. Indeed, dysfunction in amygdalar activity has been implicated in numerous neuropsychiatric and neurodevelopmental disorders (Schumann et al., 2011).

1.4.7: The functional anatomy of the amygdaloid complex

Central to the functional role of the amygdaloid complex is the basolateral amygdala (BLA), which can be further divided into the lateral (LA), basal (BA) and medial (BM) amygdalar nuclei. The BLA consists of glutamatergic principal neurons and inhibitory interneurons (Babaev et al., 2018; Muller et al., 2005; Veres et al., 2017). The LA region of the BLA receives inputs from the sensory and pain cortices and the sensory thalamus. The LA projects locally onto other

BA and BM BLA neurons. The BLA also receives inputs from the prefrontal cortex and hippocampus, as well as a number of neuromodulatory centers, including dopaminergic projections from the ventral tegmental area and the substantia nigra, acetylcholinergic projections from the ventral pallidum, norepinephinergic projections from the locus coeruleus, and serotonergic projections from the dorsal raphe nucleus (Janak & Tye, 2015; Tovote et al., 2015).. Therefore, the BLA functions as a major point of integration between sensory experience, higher-order internal control and memory centers, as well as neuromodulatory centers. All these differing projections converge on two major mechanisms that influence BLA function, either through activating or inhibiting the regulatory gamma-aminobutyric acid (GABA)ergic interneurons in the BLA, or by directly altering the excitability of the BLA principal neurons including by inducing structural changes to dendritic spines (Babaev et al., 2018; Mantzur et al., 2009; Rehberg et al., 2010). The principal neurons of the BLA send glutamatergic projections back to the prefrontal cortex and hippocampus, as well as project to the central nucleus of the amygdala (CeA) and the nucleus accumbens. BLA control of the CeA is critical for increased vigilance and stress-responses (Janak & Tye, 2015). The CeA can be subdivided into two regions, lateral (CeL) and medial (CeM), both of which contain principal neurons that are GABAergic. The CeM provides inhibitory control over the hypothalamic nuclei involved in stimulating the hypothalamic-pituitary-adrenal (HPA) axis stress hormone release. The BLA projects onto the inhibitory neurons of CeL, which then project and disinhibit the inhibitory neurons of the CeM, and leads to the

activation of the HPA axis, stress hormone release, and increased anxiety-like behaviours (Janak & Tye, 2015). Conversely, optogenetic activation of specific projections from the ventromedial prefrontal cortex target the amygdala and suppress fear-related freezing and anxiety-like behaviours (Adhikari et al., 2015). Interestingly, the BLA also sends monosynaptic glutamatergic projections to the nucleus accumbens that can directly influence reward-seeking behaviours (Sharp, 2017). Taken together, BLA function is critical in integrating sensory experience with previously learned associations, assigning an emotional context to the situation, and promoting either approach or avoidant behaviours.

Despite a general understanding of the amygdala circuits, we still do not understand all the molecular signatures that are responsible for the establishment and function of these circuits to control anxiety. This is of importance given the wide spread nature of anxiety in humans and a better understanding of this system would help in the design and delivery of more specific and targeted therapeutics and intervention strategies.

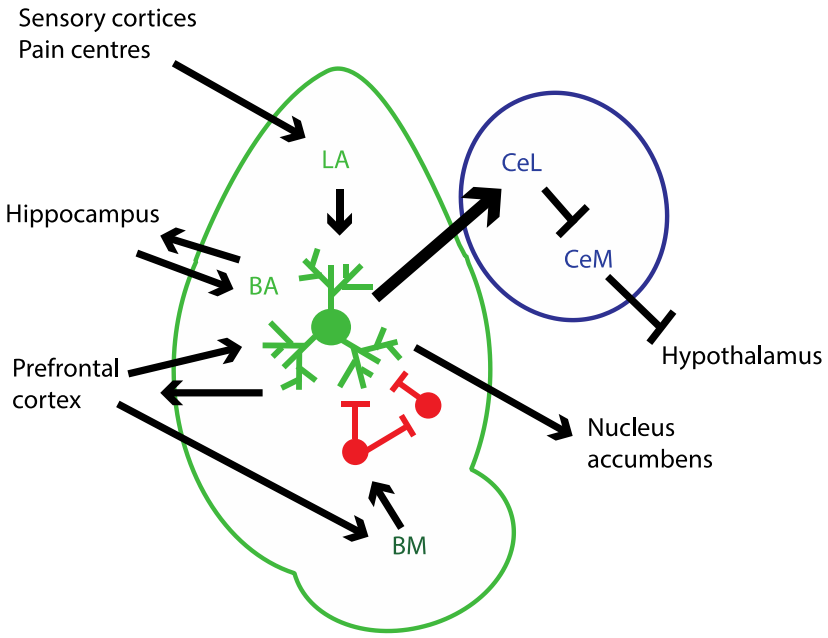

Figure 1.6: The primary input and output centers of the amygdaloid complex. A pictorial representation of the primary input and output centres of the amygdaloid complex depicting the basolateral amygdala (BLA, outlined in green) and the central nucleus of the amygdala (CeA, outlined in blue). The lateral amygdala (LA) receives inputs from the sensory cortices and pain centers and project onto the principal neurons (green) of the basal amygdala (BA). The BA contains local circuits of principal neurons (green) and inhibitory interneurons neurons (red). The BA also receives and sends reciprocal inputs from/to the hippocampus and prefrontal cortex. The prefrontal cortex also sends regulatory projections to the basomedial amygdala (BM). The BM projects onto the inhibitory interneurons of the BA and can promote feedforward inhibition. The primary output of the BA is to the lateral central amygdala (CeL). While the principal neurons of the BLA are excitatory, the principal neurons of the CeA are inhibitory. The CeL sends inhibitory projections to the central medial amygdala (CeM). The CeM sends inhibitory projections to the hypothalamus. Therefore, activation of the prinicipal neurons of the BLA results in the activation of the CeL and the disinhibition of the hypothalamus. Finally, the BA also sends projections to the nucleus accumbens.

1.4.8: Using mouse models to study neuropsychiatric traits

Human neuropsychiatric disorders are complex, multifactorial, and uniquely human. Characteristic symptoms, such as suicidality, internalization, hallucinations, delusions, guilt, and language impairments, cannot be assessed in mice. However, there are a number of intermediate traits, or endophenotypes, that can be modeled and studied in mice, including physiological and anatomical brain changes as well as certain behavioural traits. Human behavioural neuropsychiatric traits that can be studied in mice include behavioural responses to drugs, including drugs that are able to produce a typical therapeutic response or alter sedation or seizure thresholds in a disorder specific manner; motor function, including gait and balance defects; socialization, including social interaction times and responses; psychomotor agitation and measures of activity levels; cognitive deficits, such as impairments in learning and memory; and pleasure-seeking and anxiety-like behaviours, including approach and avoidance. Therefore, mice are valuable for evaluating the effect of changes in human candidate genes on the neuroanatomy of evolutionarily conserved brain regions, such as the hippocampus and amygdala, and a large range of behaviours. Decidedly, since rodents have been used for neuroanatomical and behavioural experiments for decades, a number of validated behavioural paradigms with predictable neurotypical behavioural responses have been established. Importantly, using the mouse as a model enables us to account for the full interplay between brain regions and body systems that produce complex behaviours. Furthermore, when loss of function of a conserved gene is shown to

impair behaviour in mice, all genes in the same signaling pathway become candidate genes in humans.

1.4.9: Synaptic actin dysregulation is associated with human neuropsychiatric disorders

One molecular point of convergence that has been associated with neuropsychiatric disorders is through the dysregulation of synaptic actin dynamics. Dysfunction of signaling pathways that reorganize synaptic actin is associated with a diverse range of developmental disorders, including autism spectrum disorders, schizophrenia, and intellectual disability (Yan et al., 2016). Human risk alleles for these disorders in genes such as, *SHANK3*, *GIT1*, *DISC1*, *SRGAP3*, *OPHN1*, *LIMK1*, *NRG1*, *CYFIP1*, *SYNGAP1*, *KALRN*, *NCKAP1*, and *CNKSR2* have been shown to regulate upstream signaling events that influence actin cytoskeleton dynamics in dendritic spines (Yan et al., 2016). Taken together, changes to the efficiency or ability of actin to remodel can lead to synaptic dysfunction that can result in behavioural impairment in mice, and dysfunction in these conserved mechanisms may be resulting in cognitive and behavioural impairments in humans.

1.5: Postsynaptic scaffolding and adaptor proteins in the regulation of synapse function, memory, and behaviour

1.5.1: The Shank family

Mutations to actin related adaptor proteins have been shown to impact synapse dynamics and behaviour in mouse models and have been associated with human behavioural disorders. The SH3 and multiple ankyrin repeat domains protein (Shank) family of proteins consists of well-studied large adaptor proteins, also known as scaffolding proteins, which are found in the post-synaptic density of many excitatory synapses (M. Sheng & Kim, 2000) (Fig. 1.7). Shank proteins bind Rho GTPases, RhoGEF proteins for Rac1 and cdc42, actin binding proteins, and actin modulators (E. Park et al., 2003). Dysregulation of Shank proteins lead to alterations in dendritic spine development, morphology, and function by altering actin dynamics (Sarowar & Grabrucker, 2016). Mice that have Shank deletions (Shank mutant mice) display increased anxiety-like behaviours, learning deficits and reduced sociability (Yoo et al., 2014). Further, mutations in human *SHANK* genes have been associated with autism spectrum disorders (Sala et al., 2015), schizophrenia (Peykov et al., 2015), and Alzheimer's disease (Gong et al., 2009).

1.5.2: The DLGAP (GKAP) family

A second family of scaffolding proteins, the Discs large associate protein/guanylate kinase associated protein (DLGAP/GKAP) family, also functions in the postsynaptic density and links, through its interaction with Shank

proteins, the actin cytoskeleton to both ionotropic and metabotropic glutamate receptors and has been proposed as an important regulator of glutamate receptor turnover (Rasmussen et al., 2017) (Fig. 1.7). Thus, DLGAP proteins are important for synaptic scaling, a process in which the synaptic strength is altered usually through the up-or down-regulation of glutamate receptors. Dysfunction in DLGAPs has been associated with schizophrenia, autism spectrum disorders, trichotillomania, obsessive compulsive disorder, cerebellar ataxia, post traumatic stress disorder, fragile X intellectual disability, Alzhiemer's disease, and major depressive disorder (Rasmussen et al., 2017).

1.5.3: The DLG (MAGUK) family

Similarly, the Disc large (DLG) (a subfamily of the membrane associated guanylate kinases (MAGUK)) family, of which postsynaptic density protein 95 (PSD95)(Disc large homolog 4 (DLG4)) is a well-studied member, function as an adaptor protein that brings the actin cytoskeleton together with receptors, ion channels, and intracellular effectors (Oliva et al., 2012) (Fig. 1.7). Deletion of PSD95 in mice results in enhanced LTP but complete absence of LTD, as well as increased anxiety-like behaviours, lack of sociability, and memory impairments (Coley & Gao, 2019; C. Gao et al., 2013).Genetic studies in humans have linked *DLG4* mutations to Alzheimer's disease, autism spectrum disorders, William's syndrome, and major depressive disorder (C. Gao et al., 2013; Good et al., 2011). Other synaptic large adaptor proteins that bind to the actin cytoskeleton that have been shown to influence behaviour in mouse models and have been

associated with human neuropsychiatric disorders include the Homer family of proteins, GIT1, densin, and intersectin (C. Gao et al., 2013; Good et al., 2011). Together, the data compellingly points towards actin-associating molecular scaffolds and adaptor proteins as essential regulators of the synaptic environment important for coordinating molecular responses and behavioural outputs. However, these same genes create a vulnerability to loss of function mutations that can disrupt complete signaling cascades and manifest as a spectrum of behavioural disorders.

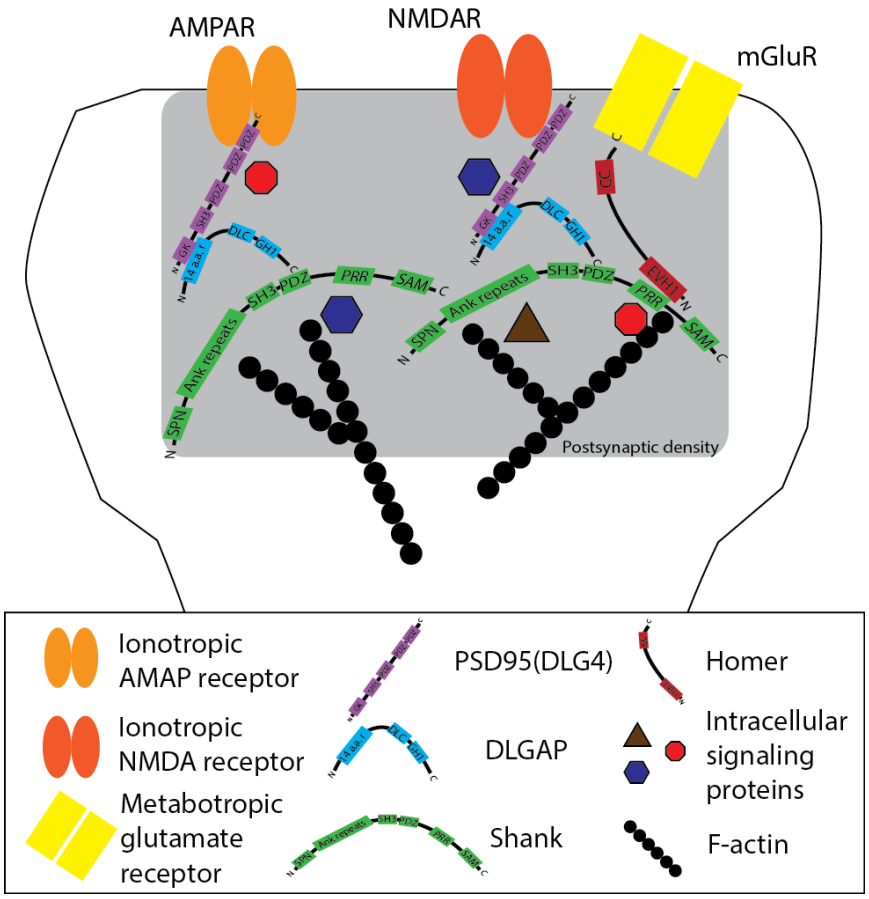

Figure 1.7: Scaffolding proteins in the postsynaptic density. Pictorial representation of Shank, DLGAP, PSD95, and Homer interactions depicting their protein binding domains. In the postsynaptic density, Shank anchors Homer to its proline rich region (PRR) by binding Homer's Ena/ vasodilator-stimulated phosphoprotein (VASP) Homology 1 domain (EVH1). Homer associates with metabotropic glutamate receptors via it's c-terminal. Shank anchors DLGAP to its PDZ domain by binding the c-terminal of DLGAP. DLGAP binds PSD95's guanylate kinase-like (GK) domain to its 14 amino acid repeat (14 a.a. r) region. PSD95 associates to AMPA and NMDA receptors through interactions with two of its three PDZ domains. The other protein binding domains are able to bind to a number of intercellular signaling proteins and complexes, including adaptor proteins, Rho GTPases, and actin regulators. Src homology-3 domain (SH3), Dynein light chain domain (DLC), GKAP homology domain 1 (GH1), Shank/ProSAP N-terminus domain (SPN), Ankyrin repeats region (Ank repeats), sterile alpha motif domain (SAM), coiled coil domain (CC).

1.6: Role of actin regulators in synapse function, memory, and behaviour

1.6.1: Formins and Profilins in synapse function and behaviour

The Formins and Profilins cooperate to produce actin filament elongation and have been shown to play an important role in synaptogenesis, especially in the context of the postsynaptic dendritic spine. The formin family in mammals is made up of 15 members and is thought to be important in early spinogenesis and filopodia formation. Indeed, direct deletion studies of the formin, mammalian Diaphanous-related formin (mDia2), demonstrate that mDia2 is important for the generation of filopodia during spine formation (Hotulainen et al., 2009). Also, loss of formin2 leads to a 32% reduction in dendritic spine numbers and age related learning and memory deficits (Law et al., 2014; Peleg et al., 2010). Similarly, two Profilin isoforms exist in the brain; profilin-1 (PFN1) and profilin-2α (PFN2α) that are also involved in filopodial elongation. PFN1 is important in early development for the development of dendritic filopodia. However, PFN1 is down regulated during aging and is not involved in adult hippocampal synaptic plasticity. Although, acute loss of PFN2α was shown to be critical for synaptogenesis, stability, and plasticity in mature adult neurons (Michaelsen-Preusse et al., 2016). Surprisingly however, Profilin1 and Profilin2 mutant mice did not display any defects to dendritic spine morphology or firing patterns (Görlich et al., 2012; Pilo Boyl et al., 2007), suggesting that compensatory mechanisms are able to overcome Profilin deficiencies. However, mice deficient in Profilin2 have increased levels of vesicle exocytosis and display hyperactive behaviour (Pilo

Boyl et al., 2007), suggesting that actin regulators have both pre- and postsynaptic effects and their dysregulation can result in changes in behaviour.

1.6.2: Cofilin and LIMK in synapse formation and behaviour

The ADF/ cofilin family members are important regulators of synapse formation and maintenance (Rust, 2015). Cofilin-1 (non-muscle (n)-cofilin) is found in the postsynaptic density and its actin-filament severing activity is required for LTP induced dendritic spine head enlargement (Rust et al., 2010). However, cofilin-1 then needs to be shut off to promote spine stabilization and LTP consolidation (Bosch et al., 2014). Cofilin-1 has also been shown to play a role in actin-related glutamate receptor recruitment (Gu et al., 2010; Rust et al., 2010) and synaptic vesicle exocytosis (Wolf et al., 2015). Cofilin is tightly regulated via serine-phosphorylation by upstream regulators such as LIMK1 (Y. Meng et al., 2004). Interestingly, loss of LIMK1 in neurons leads to decreased levels of phosphorylated cofilin-1, altered spine morphology, and enhanced LTP (Y. Meng et al., 2002, 2004). Conversely, active cofilin-1 is also required for dendritic spine pruning during LTD (Zhou et al., 2004). Genetic deletion of cofilin-1 in the forebrain of mice resulted in impaired memory and learning (Rust et al., 2010). Interestingly, ADF/cofilin gene variants are also associated with anxiety-like behaviours (Goodson et al., 2012).

1.6.3: The Arp2/3 complex and its regulators in synapse function, memory, and behaviour

The Arp2/3 complex is a seven-subunit protein that has been shown to be necessary for actin filament branching. The direct deletion of the $ARPC_3$ subunit of the Arp2/3 complex results in disrupted structural plasticity of dendritic spines and associated synaptic and behavioural abnormalities (I. H. Kim et al., 2013). Similarly, genetic mutations leading to the to deletion of upstream effectors of Arp2/3 also impairs synapse function. The two best-studied effectors of Arp2/3 activation in neurons are WAVE1 and N-WASP. Ablating WAVE1 expression leads to irregularities in spine morphology and behavioural abnormalities (Y. Kim et al., 2006; Soderling et al., 2007). Similarly to Arp2/3 and WAVE1, N-WASP has also been shown to be an important regulator of dendritic spine formation and synapse complexity (Wegner et al., 2008). N-WASP is enriched in the postsynaptic terminals of 55-66% of excitatory synapses and loss of N-WASP in hippocampal neurons results in decreased dendritic spine and synapse density (Wegner et al., 2008), suggesting that interfering either directly or indirectly with Arp2/3 signaling can disrupt actin dynamics in dendritic spines and lead to behavioural impairment.

Interestingly, NCK1 has been shown to interact with both N-WASP and WAVE1. Since both N-WASP and WAVE1 are auto-inhibited, the NCK1 interaction is able to promote a conformational change that leads to N-WASP and WAVE1 activation by releasing their Arp2/3 activating domains to interact with the Arp2/3 complex and resulting in actin filament branching. Together, the

disruption of the NCK1/N-WASP/Arp2/3/actin and/or NCK1/WAVE1/Arp2/3/actin signaling pathways in neurons may disrupt brain functioning and result in behavioural abnormalities. However, NCK1's function in these pathways in neurons and in the brain remains underexplored.

1.6.4: PAK signaling in spine formation and behaviour

The NCK1 interactors PAK1 and PAK3 have been shown to play a role in hippocampal neurons, the brain, and behaviour (Civiero & Greggio, 2018; Rane & Minden, 2014). All six PAK proteins are expressed in the nervous system. PAK1 mutant mice have normal-appearing dendritic spines, however their dendritic spines have decreased levels of polymerized actin when compared to control mice (M. L. Kelly & Chernoff, 2012). NCK and PAK are both necessary for EphB-dependent cortical growth cone repulsion *in vitro* (Srivastava et al., 2013) and mutated PAK3 in hippocampal neurons results in abnormal dendritic spine morphology and long-term potentiation anomalies (Thévenot et al., 2011). Interestingly, similarly to NCK1 mutant mice, PAK3 mutant mice are viable, fertile, have a normal lifespan, and are outwardly indistinguishable from their wildtype littermates, including when comparing their overall brain size and structure. However, they have cognitive impairments defined by deficiencies in memory and learning (J. Meng et al., 2005). Furthermore, it has been shown in hippocampal neurons, *in vitro,* that PAK3 down-regulates synaptic transmission through its interaction with NCK2, where transfecting PAK3 into hippocampal neurons results in reduced miniature excitatory postsynaptic current (mEPSC)

amplitudes, but no reduction in amplitude occurs if you transfect a mutated form of PAK3 that cannot interact with NCK2 (Thévenot et al., 2011). Whether or not loss of NCK1 may lead to similar impairments in synaptic transmission and cognitive deficits, even if mice appear outwardly indistinguishable from their wildtype littermates, remains to be explored.

1.6.5: Rho GTPase activity in spine formation and behaviour

A critical function of NCK1 in both Arp2/3 and PAK activation is through its interactions with the Rho family of GTPases, namely Cdc42, which is known to activate N-WASP and PAK (Parsons et al., 2005; Royal et al., 2000), and Rac1, which can activate WAVE1 and PAK (B. Chen et al., 2017; Koronakis et al., 2011; Tahirovic et al., 2010). Importantly, Cdc42 and Rac1 have both been shown to be critical for normal synapse formation and brain function (Haditsch et al., 2009; I. H. Kim et al., 2014). *In vitro* two-photon fluorescence lifetime imaging microscopy (2pFLIM) of single dendritic spines after glutamate uncaging has demonstrated that structural plasticity of dendritic spines involves continuous activation of Cdc42 for more then 30minutes after glutamate stimulation, and that inhibiting the Cdc42-PAK signaling pathway resulted in blocked maintenance of the increased spine size (Murakoshi et al., 2011). Correspondingly, *in vivo* conditional deletion of Cdc42 in the forebrain postnatally also leads to decreased spine density in the CA1 region of the hippocampus, along with impaired LTP in Schaffer collateral synapses (projections from the CA3 to the CA1), and deficits in long-term memory recall (I. H. Kim et al., 2014). Likewise, a number of *in vitro*

studies have shown that Rac1 activity is also critical for activity-dependent spine enlargement and AMPA receptor clustering during synapse maturation and that blocking Rac1 function leads to decreased synapse density (Nakayama et al., 2000; A. Tashiro et al., 2000; A. Tashiro & Yuste, 2004; Wiens et al., 2005). Importantly, conditional *in vivo* deletion of Rac1 in postmitotic neurons of the hippocampus also results in a reduction in synaptic density and impaired spatial learning in the Morris water maze (Haditsch et al., 2009). Interestingly, Rac1's role in memory formation is not restricted to hippocampal-dependent memory tasks as conditional disruption of Rac1 in the basolateral amygdala also impaired fear memory (Q. Gao et al., 2015) suggesting a conserved mechanism of neuronal plasticity that is exploited by multiple brain regions and systems. Whether NCK1 is required for the effective and efficient functioning of Cdc42 and Rac1 in the brain and behaviour, and if loss of NCK1 results in similar abnormalities in synapse maintenance, memory formation, or recall remains to be addressed.

1.6.6: Netrin1-DCC signaling in dendritic spine formation and memory

Work out of Dr. Timothy E. Kennedy's lab has established Netrin1-DCC signaling as an important regulator of synaptogenesis, synapse maturation, and memory formation in the mammalian brain (Glasgow et al., 2018; Goldman et al., 2013; Horn et al., 2013; E. W. Wong et al., 2019). They have shown that DCC is enriched at excitatory PSDs in the mature mammalian brain and selective deletion of DCC in the forebrain reduces dendritic spine volume in the

hippocampus, attenuates hippocampal LTP, and impairs hippocampal-dependent learning and memory (Horn et al., 2013). Further, they show that Netrin-1, through DCC-signaling, is synaptogenic in mammalian cortical neurons during development, that Netrin-1 and DCC are enriched at synapses in developing cortical neurons, and that Netrin-1, through DCC signaling, promotes actin cytoskeletal rearrangement that leads to filopodial extension, recruitment, and clustering of synaptic proteins. Indeed, Netrin-1 increases the number and strength of excitatory synapses (Goldman et al., 2013). Interestingly, NMDA-receptor activation and neuronal depolarization in hippocampal dendritic spines results in Netrin-1 secretion from the spine head and autocrine activation of DCC receptors that can trigger the upregulation of GluA1-containing AMPA receptors, enhance LTP, and promote dendritic spine maturation (Glasgow et al., 2018). Finally, conditional deletion of Netrin-1 from glutamatergic neurons in the forebrain of mice results in impairments in spatial memory in the Morris water maze (E. W. Wong et al., 2019). Although a role of NCK proteins in DCC-meditated axon guidance and growth cone morphology has been established (C. Lane et al., 2015; Shekarabi et al., 2005), whether NCK proteins are required for Netrin-1-DCC mediated synaptogenesis and maturation remains to be addressed.

1.6.7: NCK1 is found in neurons and has been associated with neuropsychiatric disorders in humans

NCK1 is well positioned as a potential key player in actin-based neuronal structural plasticity and network wiring. The NCK adaptor proteins are present in neurons and play a role in both DCC- and EphB2-mediation axon guidance (C. Lane et al., 2015; Srivastava et al., 2013). *In vitro* studies have also shown that NCK1 is localized to dendritic branches with a strong affinity for spines and protrusions (Pilpel & Segal, 2005), that in neurons neurotrophic factors are able to promote the interaction of NCK1 with specific tyrosine kinase receptors (Suzuki et al., 2002), and that stimulation with reelin, a secreted glycoprotein that plays a role in dendrite and dendritic spine formation, maintenance and plasticity, redistributes NCK1 from the soma to neuronal processes through disabled-1 (Dab1) phosphorylation and can lead to actin remodeling at the plasma membrane (Pramatarova et al., 2003). However, if these processes happen *in vivo* and whether disruption in NCK1 signaling results in cognitive and behavioural changes remains to be addressed. Yet, it is interesting to note that two well-studied human psychiatric disease risk genes, *TNIK* and *NCKIPSD*, were named based on their original discovery as genes that code for NCK-associating proteins, TRAF2 and NCK-interacting protein kinase (TNIK) and NCK-interacting protein with SH3 domain/ SH3 protein interacting with NCK, 90kDa (NCKIPSD/SPIN90). These proteins have been shown to be critical in regulating proper synapse formation, function, and their deletion in mice results in cognitive and behavioural abnormalities (Coba et al., 2012; D. H. Kim et al.,

2017; Q. Wang et al., 2011). In fact, human genome wide association studies (GWAS) have linked NCK1 to schizophrenia (Ripke et al., 2014) and neuroticism (Luciano et al., 2018; Nagel et al., 2018), and NCK2 has been associated with addiction (Liu et al., 2013) and depression (Howard et al., 2018), again suggesting that NCK family signaling may be critical for regulating cognition and behaviour.

1.7: The aims and findings of this study

Given 1) the critical role the NCK proteins have been shown to play in regulating signaling cascades important for actin dynamics; 2) a number of *in vitro* studies have linked loss of NCK1 and NCK2 to a number of different aspects of neuronal function; and 3) NCK1 has been linked to behavioural defects in humans, we wanted to address the functional significance of loss of NCK1 in vivo using a murine model and whether any behavioural defects could be linked to changes in neuronal development or architecture.

Here, *I hypothesize that the NCK1 adaptor protein functions in CNS tissue to influence actin dynamics affecting dendritic spine development and morphology and NCK1 deficiency would lead to the dysregulation of neuronal circuits important for learning, memory, and behaviour.*

To address this, I had the four following aims -

1. Determine if there are behavioural deficits in the NCK1 deficient mice.
2. Determine the regional and cellular distribution of NCK1 in the adult mouse brain.

3. Determine if NCK1 plays a role in cell proliferation and/or migration in the mouse brain.
4. Determine if NCK1 plays a role in dendritic spine formation and/or normal synapse morphology.

These aims are addressed in two main data Chapters. Chapter 2 addresses the role of NCK1 in memory, learning, and the hippocampus. We find that loss of NCK1 results in impaired short term and working memory, as well as spatial learning. However, there are no defects in hippocampal embryonic neuronal proliferation or development. NCK1 is found in all postmitotic neurons in the hippocampus but not in the progenitor cell layer of the adult dentate gyrus. Although loss of NCK1 does not impair hippocampal pyramidal neuron dendritic branching or complexity, there is a significant decrease in dendritic spine and synapse number. Mechanistically, this is likely due to a role NCK1 has on the rate of actin turnover in dendritic spines. Together I conclude that NCK1 is critical for either synapse formation or maintenance and that loss of NCK1 results in impaired learning and memory.

Chapter 3 addresses the role of NCK1 in anxiety-like behaviours and in basolateral amygdala (BLA) functioning. I report that loss of NCK1 results in increased anxiety-like behaviours in elevated plus maze (EPM) and the light/dark box assays, as well as an increase in circulating stress-hormone levels when exposed to the EPM that is not present at baseline. The behavioural phenotype is reversible to control levels after treatment with diazepam, a positive allosteric

modulator of the GABA-A receptor. Loss of NCK1 does not impair neuronal proliferation at E12.5, the height of amygdala neurogenesis, cortical migration, or BLA axon targeting. We also find that NCK1 is found in neurons in the BLA and that exposure to the EPM results in decreased activation of parvalbumin-positive inhibitory interneurons neurons in the BLA. This effect is likely correlated to a reduction in the density of dendritic spines on spiny neurons within the BLA. Therefore, I conclude that NCK1 is an important regulator of inhibitory/excitatory balance in the BLA and that its loss results in hyperexcitability of BLA circuitry.

Chapter 2: NCK1 stabilizes neuronal actin dynamics to promote dendritic spine, synapse, and memory formation.

My contributions to this chapter include all of section *2.1: Introduction* and *2.4: Discussion*. All experiments in section *2.2: Methods and Materials* were carried out by me with the exception of (1) preparing the tissue for electron microscopy analysis (2.2.6) this was preformed by Mary Ann Trevors, facilities manager of the electron microscope facility at Dalhousie University, (2) *2.2.7:Hippocampal neuron extraction and in vitro analysis*, preparation of neuron cultures was done by Josee Normand in Dr. Fawcett's lab while immunohistochemistry and anaylsis was done by Dr. Dylan Quinn in Dr. Fawcett's lab, (3) *2.2.8: Fluorescence recovery after photobleaching (FRAP) assay* was carried out by Dr. Michael Wigarius, and (4) *2.2.9: Actin barbed end experiments and imaging* was carried out by Dr. Michael Wigarius. I authored all of *2.3: Results* with the exception of *2.3.7: Loss of NCK1 results in increased actin recovery in hippocampal dendritic spines after photobleaching* was primarily authored by Dr. Michael Wigarius. All figures were designed and created by me with the exception of Figure 2.7, which was created by Dr. Michael Wigarius. Additionally, the images in Figure 2.6A, 2.6D and 2.6F were taken by Dr. Dylan Quinn. Cell counts for results sections 2.3.3, 2.3.4 and dendritic spine counts (2.3.5) were done by Ibrahim Shahin in Dr. Fawcett's lab and myself. Finally, synapse counts and postsynaptic density measurements (2.3.5) were done by Julia Paffile in Dr. Fawcett's lab. The full chapter was additionally modified and edited by Dr. James Fawcett.

2.1: Introduction

Brain function is dependent on the ability of neuronal networks to integrate sensory information with previously established internal representations and to accurately and efficiently respond. It is becoming increasingly clear that this is not only true for the sensory modalities but also for higher cognitive functions such as memory and learning. Memory formation and maintenance has been shown to be modulated by changes in ionic channel function including NMDA and AMPA channels; however, there is also evidence that structural changes at synapses through the remodeling and enlargement of preexisting synapses and/or the elimination and/or addition of synaptic connections also plays an important role (C. H. Bailey et al., 2015; S. Basu & Lamprecht, 2018; Caroni et al., 2012). The actin cytoskeleton and its modulation is an essential constituent required for the structural changes associated with memory formation. Indeed, interfering with actin rearrangement by pharmacologically blocking actin polymerization during or directly after learning impairs memory formation (Mantzur et al., 2009; Nelson et al., 2012; Rehberg et al., 2010).

The majority of excitatory synapses in the brain occur on specialized actin-rich dendrite protrusions known as dendritic spines. Dendritic spines function to compartmentalize and control signaling cascades important for neuronal communication. Changes in dendritic spine morphology, stability, and number have been associated with memory formation (C. H. Bailey et al., 2015; Lamprecht & LeDoux, 2004; G. Yang et al., 2009). Indeed, studies have shown that new dendritic spines form following learning paradigms, and that the change

in spine density occurs in specific brain regions and circuits are recruited during learning for proper task performance (L. Wang et al., 2011; Xu et al., 2009). Further, these new spines are preferentially stabilized by subsequent training sessions (L. Wang et al., 2011; Xu et al., 2009), and motor task learning can be disrupted by optical shrinkage of task potentiated spines (Hayashi-Takagi et al., 2015). In addition, learning impairments correlate with abnormal dendritic spine morphologies, synapse function, and plasticity (Chazeau & Giannone, 2016; Sala & Segal, 2014). Finally, several neurological disorders, such as Alzheimer's disease, autism spectrum disorders, schizophrenia, and fragile X syndrome, present with both cognitive impairment, including deficits in memory and learning, and are correlated with abnormal dendritic spine formation and maintenance (Fiala et al., 2002; Herms & Dorostkar, 2016; Martínez-Cerdeño, 2017).

Over the past 15 years, a role for the actin cytoskeleton in brain development, function, and disease has been established, including in the context of learning and memory (S. Basu & Lamprecht, 2018; Chazeau & Giannone, 2016; Sala & Segal, 2014). Studies addressing the function of actin signaling and cytoskeletal dynamics in dendritic spine and synapse formation, maintenance, and elimination have highlighted the critical role played by actin-regulatory proteins in learning and memory formation (Grove et al., 2004; Haditsch et al., 2009; Rust et al., 2010; Soderling et al., 2007). Modulation of actin cytoskeletal dynamics is controlled by small GTPases, such as Rac1 and Cdc42, and their downstream effectors, like PAK, N-WASP, WAVE, and Arp2/3 (Chazeau & Giannone, 2016). Critically, these actin regulators are functionally

linked with synaptic receptors, such as glutamate receptors, Eph receptors, and adhesion molecules, and together they have been shown to participate in spine morphogenesis and memory formation (Woolfrey & Srivastava, 2016). Many *in vitro* studies have linked the NCK family of adaptor proteins, which consists of two members NCK1 and NCK2, with actin modulators including PAK, N-WASP, and Arp2/3 (Bokoch et al., 1996; Cowan & Henkemeyer, 2001; Rajat Rohatgi et al., 2001; Thévenot et al., 2011; Zhao et al., 2000). The NCK proteins have also been linked with a number of cell surface receptors, including from the Eph, ephrin family members (Bisson et al., 2007; Holland et al., 1997; T. Hu et al., 2009; Stein et al., 1998) as well as DCC (X. Li et al., 2002). Further, many of these NCK associating proteins have been shown to be important for learning and memory (Civiero & Greggio, 2018; Dines & Lamprecht, 2015; Horn et al., 2013; M. L. Kelly & Chernoff, 2012; I. H. Kim et al., 2013; J. Meng et al., 2005); however, whether these functions in the CNS are dependent on NCK1, NCK2, or both to regulate learning, memory, and complex behaviours remains unknown.

NCK1 and NCK2 are 47kDa proteins that primarily consist of four protein-binding domains, one SH2 domain and three SH3 domains. NCK1 and NCK2 share a 67-68% amino-acid identity, which rises to 80-85% identical when only comparing their protein binding domains (M. Chen et al., 1998). Indeed, the NCK proteins functionally compensate for each other during development since double deletion of NCK1 and NCK2 results in embryonic lethality, but mice with single gene deletions of NCK1 or NCK2 are viable and outwardly indistinguishable from their littermates (Bladt et al., 2003; Fawcett et al., 2007). Both NCK proteins are

expressed in a wide variety of adult tissues including the CNS and can functionally compensate for each other in axon guidance of spinal cord neurons (Fawcett et al., 2007; C. Lane et al., 2015), cardiovascular- (Chaki et al., 2015), bone- (Aryal A.C et al., 2015), and kidney podocyte- (N. Jones et al., 2006) development. However, although the NCK proteins have similar expression levels in many adult tissues, NCK2 is less abundant when compared to NCK1 in the brain (Bladt et al., 2003). Importantly, *in vitro* studies have shown NCK1 is localized to dendrites and enriched in dendritic spines and protrusions (Pilpel & Segal, 2005). Finally, in genome wide association studies, NCK1 has been linked with schizophrenia and in neurotism as an enhancer of worry (Luciano et al., 2018; Nagel et al., 2018; Ripke et al., 2014). Despite these studies, we still do not have a clear understanding of the role of NCK1 and/or NCK2 in CNS development and behaviours including learning and memory.

Here, we utilized mice lacking either NCK1 or NCK2 to determine whether loss of either was important for memory and/or learning. We find that mice lacking NCK2 (herein NCK2$^{-/-}$) have increased levels of activity in an open field, whereas mice lacking NCK1 (herein NCK1$^{-/-}$) are indistinguishable from control mice in this regard. Both NCK1$^{-/-}$ and NCK2$^{-/-}$ mice have impairments in short-term social recognition and in working memory; however, only NCK1$^{-/-}$ mice display impairments in spatial learning and memory as assessed in a Morris water maze paradigm. Analysis of the NCK1-/- mice revealed that NCK1 is expressed exclusively in postmitotic neurons, but is not necessary for neuronal migration or dendritic patterning. NCK1, however, is critical for dendritic spine

density and for spine head morphology. These defects are likely attributable to a role for NCK1 in regulating actin turnover in dendritic spines. Taken together, our data implicates NCK1 as an important regulator of actin stability in dendritic spines. This change in actin stability likely leads to the decrease in synapse density and synapse morphology and is correlated with defects in hippocampal-based memory and learning.

2.2: Methods and Materials

2.2.1: Animals

Both the NCK1 and NCK2 mutant mice used have previously been reported (Bladt et al., 2003). All mice were bred in-house. 2-4month old male adult mice were used for all experiments. For time-pregnant experiments, embryonic day was defined from vaginal plug detection as E0.5. Rooms were maintained at 21^0C under a 12h light-dark cycle (7am to 7pm lights on). Food and water were available *ad libitum*. The study was approved by the Dalhousie University Committee on Laboratory Animals (Dalhousie Animal Protocol #14-062) and was conducted according to the Canadian Council on Animal Care guidelines.

2.2.2: Behavioural Testing

Social interaction/social recognition test. Modified from (Moy et al., 2004), briefly, mice were placed into the middle chamber of a three-chambered apparatus and after a two-minute acclimatization period prior to testing, mice were allowed to freely explore the three chambers. One of the chambers contained a mouse (of the same sex and age from a different litter) in a plastic cage. The opposite chamber contains an empty cage. The time spent interacting (sniffing/touching) with each cage is recorded over a 5 minute trial (social interaction). The social recognition trial was a 5 minute trial run an hour after the social interaction trial, the subject mouse is returned to the center compartment, and the mouse that was previously present during the social interaction trial

(familiar) is in one cage, and in the other cage is a new mouse of the same age and sex from a different litter (unfamiliar). The time spent interacting with each cage is recorded.

Spontaneous alternations in a Y-Maze. Adapted from (Lalonde, 2002), briefly, the subject mouse is placed in the center of a symmetrical Y-maze and allowed to run freely for 5 minutes and each arm entry is recorded. Spontaneous alternations are scored as overlapping triplet sets in which three different arms are entered (eg. ABC, BCA, CAB, BAC, etc). A percent alternation score is determined by (number of spontaneous alternations (as defined above)/total number of arm entries – 2) x 100.

Morris water maze. Testing protocol was adapted from (Wong and Brown, 2007), briefly, mice were tested in a ~115cm diameter pool filled with water (room temperature) made opaque by the addition of non-toxic white liquid tempera paint (Schola, Marieville, Quebec). Escape platform consisted of a clear Plexiglass cylinder (10cm diameter). Mice were tested 4 trials/day for 8 consecutive days, followed by a single probe trial on day 9, and an additional 4 trials on day 10. Trials timed out when the mouse mounted the escape platform or at 60s if the mice did not escape, in which case mice were lead to the escape platform and left on it for 30s. For each trial, mice were released into the pool at a different randomly assigned start position (North, South, East, West). Testing occurred in four phases: acquisition, reversal, probe, and visible platform trials. During

acquisition (Day 1-4), the platform was hidden in the northwest quadrant. During reversal (Day 5-8), the platform was hidden in the southeast quadrant. During the probe trial (Day 9), there was no platform, mice were released from the South start position, and were left in the pool for the complete 60s. During the visible platform trials (Day 10), a visible platform was placed in the northeast quadrant. Swim paths were recoded using a video camera based tracking system and the WatermazeBeta software (Actimetrics, Wilmette, IL). Swim paths and percentage of time spent in each quadrant were recorded and analyzed.

2.2.3: Adult mouse brain histology

Mice were deeply anesthetized (pentobarbital sodium, 2.4mg/g i.p; Bimeda-MTC Animal Health Inc.), perfused transcardially with phosphate buffer saline (PBS) followed by 4% paraformaldehyde (PFA). Brains were dissected, fixed (4% PFA, 5 hours), washed (PBS 3X20mins), cryoprotected (30% sucrose, 48hours at 4^{o}C), embedded (Tissue Tek), and sectioned.

Free-floating sections were washed in a solution of 0.25% triton X100 in PBS for (t-PBS) 3X10mins, then transferred to 1% triton X100 in PBS 10mins). Sections were then blocked in 10% normal goat serum (NGS) t-PBS for 1h). Sections were then incubated in primary antibody solution containing 3%NGS in t-PBS overnight at 4^{o}C. The primary antibodies used in this study include: chicken anti-β-Galactosidase, 1:1000, abcam, ab9361; mouse anti-NeuN, Millipore, MAB377; rabbit anti-Iba1, Wako Chemicals, 019-19741, 1:1000; Sox2). Following overnight incubation in primary antbody, sections were then washed 3

x 10 minutes in t-PBS followed by incubation in appropriate secondary antibody. Alexa Fluor 488 goat anti-chicken, 1:500, Life Technologies, A11039; Alexa Fluor 594 goat anti-mouse, 1:500, Invitrogen, A11005; Alexa Fluor 647 goat anti-rabbit, 1:500, Life Technologies, A21246; Alexa Fluor 594 goat anti-rabbit, 1:500, Invitrogen, A11037 Alexa Fluor 488 goat anti-mouse, 1:500, Molecular Probes, A11029. Following incubation in secondary antibody, sections were washed extensively, incubated in Hoechst 33342, 1:500, Life Technologies, H3570, for 1min), washed again in PBS, 3X5mins. Sections were mounted in Fluoromount Aqueous Mounting Medium (Sigma, F4680) and left to dry overnight prior to imaging.

Images were acquired on a Zeiss LSM 710 laser scanning confocal microscope. Setting for laser power, gain and offset were kept constant for each experiment. All images for quantification were taken through the 20X objective (numerical aperture 0.40) in the hippocampus. Images were analyzed using the spots function on the Imaris8 (Bitplane) software. Each marker was counted independently before overlaying images to observe colocalization or to calculate as a percentage of total Hoechst labeled nuclei. Two separate experimenters analyzed 4 sections/mouse from 6 control and 6 NCK1$^{-/-}$ mice. Experimenters were blind to the genotype during every step of this process.

2.2.4: Ethynyl deoxyuridine (EdU) proliferation and migration assays

Two groups of pregnant NCK1 heterozygous dams received intraperitoneal injections of EdU (10ul/g) at E14.5. Group 1 was sacrificed by

cervical dislocation 30 minutes after injection, embryos were dissected, fixed (4% PFA, 45mins), washed (PBS, 3X20mins), cryoprotected (30% sucrose, 48hrs). Coronal sections ($20\mu m$) were cut and mounted onto charged slides (Fisher brand Superfrost Plus). Mice in Group 2 were returned to their cages after injection until E18.5 when the brains of the E18.5 embryos were dissected out and the same procedures as above were followed.

Slides of coronal sections were then treated according to manufacturer's instructions for the Click-iT EdU Alexa Fluor 647 Imaging Kit (Life Technologies, C10340). Images were acquired on a Zeiss LSM 710 laser scanning confocal microscope and displayed using ZEN2009 software. The setting for laser power, gain and offset were kept constant for each experiment. All images for quantification were taken through the 20X objective (numerical aperture 0.40) in the hippocampal formation at E14.5 and in the hippocampus at E18.5. Images were analyzed using the spots function on the Imaris8 (Bitplane) software. Each marker was counted independently before overlaying images to calculate the amount of EdU positive cells as a percentage of total Hoechst labeled nuclei. Two separate experimenters analyzed 4 sections/mouse from 6 control and 6 NCK1$^{-/-}$ mice at both E14.5 and E18.5 time points. Experimenters were blind to the genotype during every step of this process.

2.2.5: Golgi's Method and dendritic spine analysis

Mice received a lethal injection of pentobarbital sodium (2.4mg/g, i.p; Bimeda-MTC Animal Health Inc.), their brains were dissected and whole brains

we immersed in the Golgi impregnation solutions for two weeks according to the FD Rapid Golgi Stain kit (FD Neurotechnologies) and tissue preparation and staining procedure was done following the manufacturer's protocol.

Images were acquired on a Leica DM2000 microscope and displayed using QCapture Suite PLUS software. Images for Sholl analysis were taken with a 20X objective. Sholl analysis (Sholl, 1953) was conducted on ImageJ and consisted of a series of concentric circles 20μm apart where the center circle was placed over the neuron's soma. Images for dendritic spine quantification were taken from the basal dendrites of the CA1 Pyramidal neurons through the 100X objective. Images were analyzed and spines were counted using Image J software and the cell counter plugin. Two separate experimenters analyzed 20 dendrites/mouse from 5 control and 7 NCK1$^{-/-}$ mice. Experimenters were blind to the genotype during every step of this process.

2.2.6: Transmission electron microscopy

Mice received a lethal injection of sodium pentobarbital (2.4mg/g, i.p; Bimeda-MTC Animal Health Inc.), the dorsal CA1 region of their hippocampi were dissected. Samples were fixed (2.5% glutaraldehyde in 0.1M sodium cacodylate buffer, 2 hrs), rinsed (3X sodium cacodylate buffer, 10mins), fixed again (1% osmium tetroxide, 2 hrs), rinsed quickly (distilled water), incubated (0.25% uranyl acetate, 4°C overnight), dehyrdrated (graduated series of acetone, 50% acetone 10mins, 2X 70% acetone 10mins, 2X 95% acetone 10mins, 2X 100% acetone 10mins, dried 100% acetone 10mins), infiltrate (epon araldite

resin, 3:1 actone:resin 3hours, 1:3 actone:resin overnight, 2X 100% epon araldite resion 3hrs), embed (100% epon araldite resion, 60°C 48hrs). 100nm sections were cut using a Reichert- Jung Ultracut E Ultramicrotome with a diamond knife and placed on 300 mesh copper grids. Sections were stained (2% aquous uranyl acetate 10mins, 2X distilled water rinse 5mins, lead citrate 4minutes, quick rinse distilled water, air dried). Samples were viewed using a JEOL JEM 1230 Transmission Electron Microscope at 80kV. Images were captured using a Hamamatsu ORCA-HR digital camera. Images for synapse density counts were taken at 15000X magnification. 6 fields/animal were quantified using ImageJ software for 3 controls and 5 NCK1$^{-/-}$. For postsynaptic density area analysis, area was calculated using ImageJ for 10-15 synapses/animal. Experimenters were blind to the genotype during every step of this process.

2.2.7: Hippocampal neuron extraction and *in vitro* analysis (these experiments were done by two other members of the Fawcett Lab, Josee Normand and Dr. Dylan Quinn)

Immunocytochemistry. Dissociated neuronal cultures were fixed with 4% paraformaldehyde, 4% sucrose at room temperature for 3 min followed by methanol at 4 °C for 10 minutes. Coverslips were washed with PBS, transferred onto parafilm wax, and blocked with 1% bovine serum albumin in PBS for 1 hr. Primary antibodies were diluted in blocking solution and applied for 24 hrs at room temperature. Coverslips were then washed with PBS and blocked for 30 minutes. Fluorescently labeled secondary antibodies were diluted in blocking

solution and applied for 1 hr at room temperature. Coverslips were washed with PBS and mounted onto slides with Fluoromount (Sigma).

Synapse density assay. Hippocampal neurons prepared from NCK1$^{+/-}$ (control) and NCK1$^{-/-}$ mice were fixed at 14-15 DIV and immunostained for Homer1 to label excitatory postsynaptic specializations and MAP2 to label dendrites. Image stacks comprising 5 images over a distance of 1.75 µm were acquired with a Zeiss Observer 2.1 inverted microscope using a 63x objective, Photometrics Coolsnap HQ2 camera and SlideBook 6 imaging software. Maximum intensity projection images were created and Homer1 puncta were detected with a custom script using IVision software. For each neuron, 2-4 primary dendrites were selected using the MAP2 image, and Homer1 puncta within 10-100 µm from the soma were counted. Homer1 puncta greater than 3 µm from primary dendrites were excluded from analysis. Images from 3 separate control and NCK1$^{-/-}$ cultures were analyzed. The experimenter was blinded to experimental conditions during image acquisition and analysis.

Primary abs: Homer1 - Rabbit polyclonal, Synaptic Systems, Lot # 160002, 1:3000*;* MAP2 - Guinea Pig polyclonal, Synaptic Systems, Lot # 188004, 1:400

Secondary abs: DyLight 549 (Donkey anti-rabbit, Thermo Fisher, 1:400); AMCA (Donkey anti-guinea pig, Jackson Labs, 1:400)

2.2.8: Fluorescence recovery after photobleaching (FRAP) assay (These experiments were done by Dr. Michael Wigerius in Dr. Fawcett's lab)

Photobleaching imaging. FRAP experiments were performed as described previously (Wigerius et al., 2018). Briefly, imaging was carried out on a spinning-disk microscope (ZEISS) using a 63x1.4 NA oil immersion lens with a stage incubator (37C and 5% CO_2). Dendritic spines on $Nck^{f/f}$ and $Nck1^{-/-}$ hippocampal neurons infected with lentiviral particles expressing GFP-actin (LV-GFP-□-actin) for 3-4 days were photobleached and imaged without or with Jasplakinolide (J4580; SIGMA-Aldrich). To monitor turnover, a ROI with a diameter of approximately 1 µm was photobleached at full laser power (100 % power and 100 % transmission) for 2.5 s. Fluorescence recovery was monitored by automatic scanning of the whole cell in 0.2-s intervals at low laser power. During image processing the mean fluorescence of an un-transfected area was measured as background and subtracted from the intensity of each frame to obtain recovery plots in Image J (NIH). The data were fit by nonlinear regression to an exponential one-phase association model in Prism (GraphPad software). The time for GFP-actin fluorescence to recover to 50% of its initial value (Halftime recovery) was estimated directly from the recovery plots and the mobile fraction (M_f), expressed as a percentage, was approximated by a photobleach correction equation (Feder et al., 1996; Lippincott-Schwartz et al., 1998). Results were presented by bar graphs made with Prism.

2.2.9: Actin barbed end experiments and imaging (These experiments were done by Dr. Michael Wigarius in Dr. Jim Fawcett's lab)

For the visualization of actin barbed ends a previously described protocol was used (Gu et al., 2010). Briefly, mouse hippocampal neurons infected with LV-GFP-actin were incubated with 0.45 μM rhodamine-conjugated G-actin (Cytoskeleton Inc.) for 2 min in saponin permeabilization buffer (20 mM Hepes, 138 mM KCl, 4 mM $MgCl_2$, 3 mM EGTA, 0.2 mg ml^{-1} saponin, 1 mM ATP and 1 % BSA, pH 7.5). Neurons on coverslips were immediately fixed in cytoskeleton buffer (4), mounted on microscope slides and prepared for imaging. Images were acquired as z stack series taken at optimal step intervals with an LSM710 confocal scanning microscope using a 63x 1.4 NA oil immersion lens and collected with a digital Axiocam camera controlled by ZEN software (ZEISS). To analyze incorporation of spine rhodamine-actin z stack images were collapsed by maximum-intensity projection using image J (NIH). Dendritic spines were identified by generating masks of regions of interest (ROIs) applied along GFP-actin expressing dendritic segments that were overlaid on dendritic segments expressing Rh-actin. Both channels were equally thresholded and actin in dendritic spines was assessed by measuring the ratio of spine to shaft fluorescence intensity of Rhodamine normalized to GFP, as previously described (Wigerius et al., 2018).

2.2.10: Statistical analysis

All data are expressed as means ± SEM and all statistics were analyzed using Prism 6 (GraphPad Software, inc). Independent t-tests were used for analysis of differences between two groups. When comparing more than two groups, ANOVA and Tukey's post hoc tests were used. Two-way ANOVA was used when multiple conditions were compared with multiple genotypes (social interaction, social recognition, visual platform task). To monitor changes over time, repeated measures ANOVA were run followed by Sidak's multiple comparisons test. A $P<0.05$ was considered statistically significant.

2.3: Results

2.3.1: NCK proteins are necessary for short-term and working memory

Given NCK1 and NCK2 are involved in actin cytoskeletal rearrangement, are abundant in the brain, and that the regulation of the actin cytoskeleton in neurons has been associated with cognitive function, we asked if loss of either NCK1 or NCK2 resulted in any behavioural defects that reflect global changes in cognition. Since a number of cognitive disorders are characterized by disruptions in social behaviour and short-term memory, we first tested the NCK1$^{-/-}$, NCK2$^{-/-}$, and their wild-type littermates (control) in the Crawley's three-chambered social interaction paradigm to test for socialization and social recognition (Moy et al., 2004). In the sociability test, all three groups showed a significant difference in time spent interacting with a caged mouse vs. an empty cage (P=0.0055, two-way ANOVA, Fig. 2.1A); however, there was no effect of genotype (P=0.8107, two-way ANOVA, Fig. 2.1A), and no interaction (P=0.8367, two-way ANOVA, Fig. 2.1A). Therefore, as a population both NCK1 and NCK2 mutant mice do not show deficits in sociability.

One hour following the social interaction task, mice were then tested for social recognition, a form of short-term memory. In the social recognition task, as expected, the control mice spend more time interacting with the unfamiliar mouse than with the familiar mouse (68.3± 7.9s vs. 40.5± 7.9s, P=0.0492, two-way ANOVA, Sidak's post-hoc comparisons test, n=8, Fig. 2.1B) (Moy et al., 2004); however, both the NCK1$^{-/-}$ and NCK2$^{-/-}$ mice failed to differentiate between the unfamiliar and familiar mice - spending an equal amount of time interacting with

both (43.0 ± 6.8s vs. 38.3 ± 6.8s, NCK1$^{-/-}$ n=11; 41.1 ± 5.5s vs. 52.6 ± 5.7s, NCK2$^{-/-}$ n=10; Interaction P=0.0037, Genotype P=0.0492, Interacting partner P=0.1170, two-way ANOVA; Fig. 2.1B). These data are consistent with defects in short-term memory in the NCK1 and NCK2 mutant mice (Moy et al., 2004; Richter et al., 2005).

To further assess for memory deficits, NCK1$^{-/-}$, NCK2$^{-/-}$, and control mice were challenged in the Y-maze, a test for spatial working memory. Although the control, NCK1$^{-/-}$ and NCK2$^{-/-}$ mice had similar exploration activity (control, 35.6 ± 3.3 arm entries, n=8; NCK1$^{-/-}$, 34.4 ± 2.8 arm entries, n=8; and NCK2$^{-/-}$ 43.3 ± 2.3 arm entries, n=10; P=0.0586, one-way ANOVA; Fig. 2.1C), both the NCK1$^{-/-}$ and NCK2$^{-/-}$ mice showed a decrease in their spontaneous alternations compared to control mice (58.1± 3.2% three-way alternations, NCK1$^{-/-}$, and 56.3 ± 1.4% three-way alternations, NCK2$^{-/-}$, vs. 65.9 ± 1.6% three-way alternations, P=0.0087, one-way ANOVA, Tukey's multiple comparison test, n=8 NCK1$^{-/-}$, 10 NCK2$^{-/-}$, 8 control, Fig. 2.1D). Thus, although all the mice showed similar total arm entries, both NCK1 and NCK2 mutant mice spend more time rotating between two adjacent arms relative to control mice. These data are consistent with a reduced working memory capacity (Kraeuter et al., 2019).

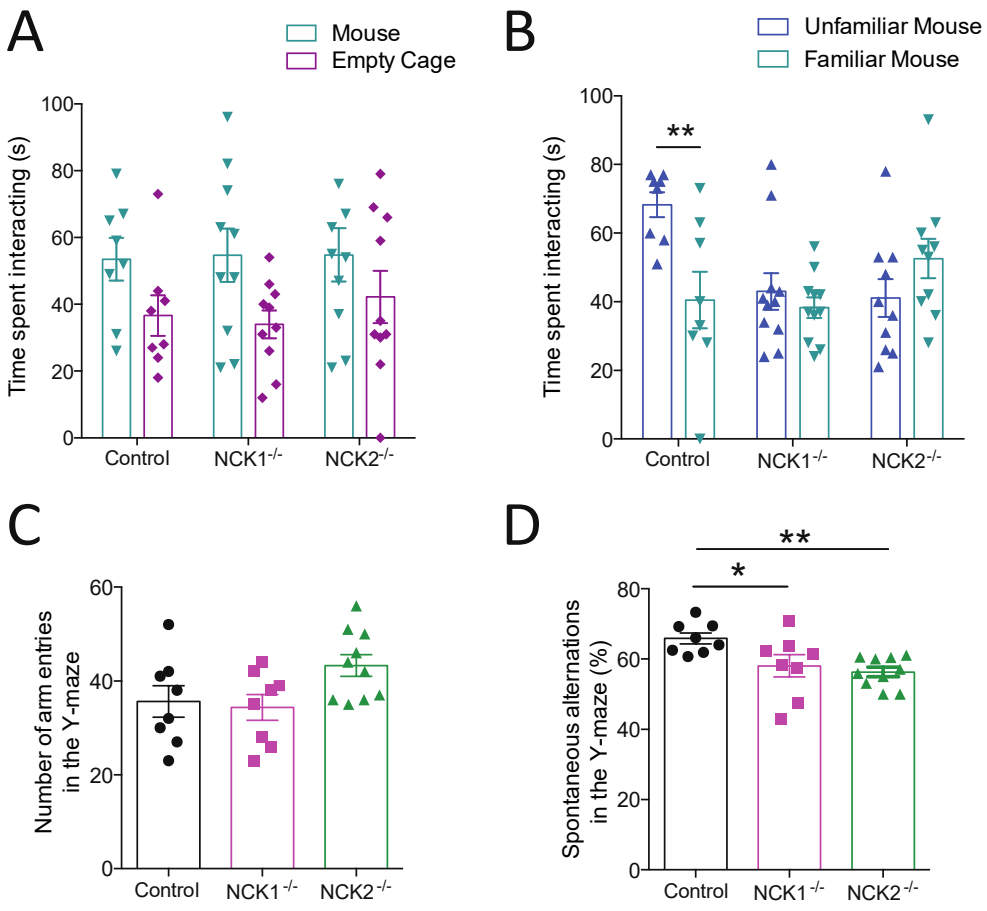

Figure 2.1: Sociability, short-term, and working memory assessment in mice deficient in NCK1, NCK2, and their wildtype littermates

A. Time spent interacting with an unfamiliar mouse and an empty cage during a three chambered social interaction task by control (n=8). NCK1$^{-/-}$ (n=10), and NCK2$^{-/-}$ (n=10) mice **p<0.01 (Two-way ANOVA). B. Time spent interacting with a familiar mouse and an unfamiliar mouse during a three chambered social recognition task by control (n=8), NCK1$^{-/-}$ (n=10), and NCK2$^{-/-}$ (n=10) mice **p<0.01 (Two-way ANOVA, Sidak's multiple comparisons test). C. Number of arm entries in the Y-maze of control (n=8), NCK1$^{-/-}$ (n=8), NCK2$^{-/-}$ (n=10) mice. D. Percentage of spontaneous alternations in the Y-maze of control (n=8), NCK1$^{-/-}$ mice (n=8), and NCK2$^{-/-}$ (n=10) mice *p<0.05, **p<0.01 (one-way ANOVA, Tukey's multiple comparisons test).

2.3.2: Loss of NCK1, but not NCK2, impairs spatial learning and memory formation

Given the reduced score on spatial working memory in the Y-maze, we next tested the mice in a more complex learning and memory paradigm using the Morris water maze, which tests their spatial memory and learning abilities. Testing consisted of a four-day acquisition phase, where the platform was hidden in the northwest quadrant, followed by four-day reversal learning phase, where the platform was hidden in the southeast quadrant, and finally a probe trial on the ninth day. During the acquisition phase, control and NCK2$^{-/-}$ were indistinguishable (Fig. 2.2A,C,E). However, the NCK1$^{-/-}$ mice were unable to learn the location of the hidden platform and did not differentiate between the platform containing quadrant and the opposing quadrant (Fig. 2.2B,E). This suggests that the NCK1 mutant mice are preferentially affected in spatial learning relative to the NCK2 mutant mice.

Next, cognitive flexibility and spatial memory extinction were assessed during the reversal-learning phase (days 5-9). Again, both, control and NCK2$^{-/-}$ mice were able to adapt during the reversal-learning, extinguish their memories of the escape platform in the northwest quadrant, and learn that the escape platform was re-located to the southeast quadrant of the water maze (Fig. 2.2A,C). The reversal learning is reflected in the decreased percentage of time the control and NCK2$^{-/-}$ mice spent in the northwest quadrant compared to southeast quadrant during the probe trial (Fig. 2.2A,C). Importantly, all mice were able to locate and mount a visible platform in under 30seconds, suggesting that

their visual ability was not impaired (Fig.2.2D). Together, this data suggests that the NCK1, but not NCK2, is necessary for spatial memory and learning in the Morris water maze.

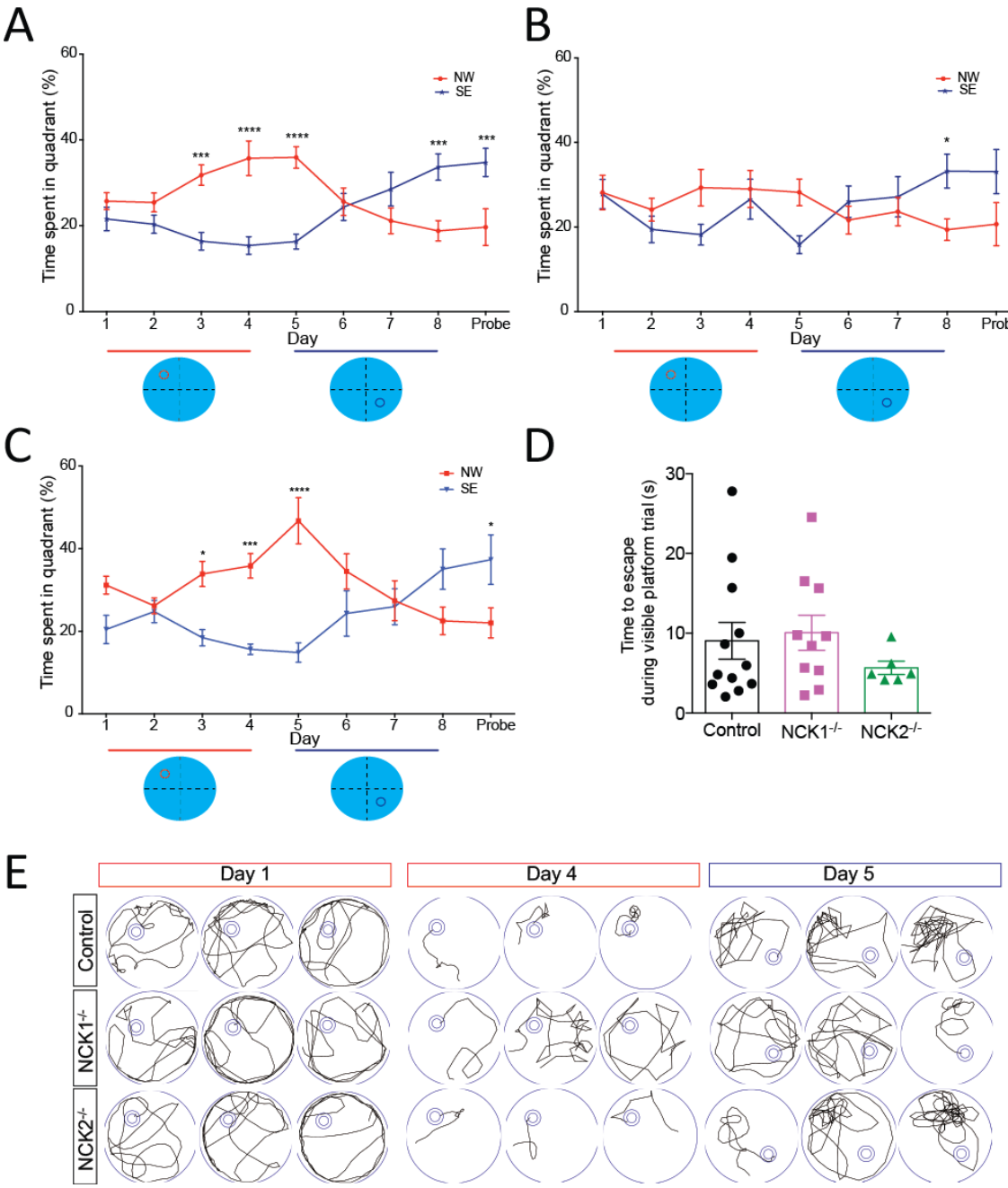

Figure 2.2: Assessment of spatial learning and memory in control, NCK1-deficient, and NCK2-deficient mice

A. Percentage of time spent by control mice in the northwest (NW, red) and southeast (SE, blue) quadrants over nine days of testing in the Morris water maze (MWM). B. Percentage of time spent by the NCK1$^{-/-}$ mice in the NW (red) and SE (blue) quadrants over nine days of testing. C. Percentage of time spent by the NCK2$^{-/-}$ mice in the NW (red) and SE (blue) quadrants over nine days of

testing. Hidden platform was placed in the NW quadrant on days 1-4 and in the SE quadrant on days 5-8. No platform was present on Day 9 (probe trial). *p<0.05, ***p<0.001, ****p<0.0001 (Two-way ANOVA, Tukey's post-hoc multiple comparisons test) D. Average time to locate and mount a visible platform in the MWM. E. Representative traces of swim-paths from three controls, three $NCK1^{-/-}$, and three $NCK2^{-/-}$ mice on Day 1, Day 4 and Day 5 in the MWM.

2.3.3: NCK1 is expressed in post-mitotic neurons in the adult hippocampus

Our behavioural studies revealed that loss of NCK1 has a much more profound effect on hippocampal dependent learning and memory than NCK2, thus we chose to focus on defects in the NCK1$^{-/-}$ mice that could contribute to these deficits. First, we asked whether NCK1 was present in the hippocampus, and in which cell types. Since NCK1 has a high degree of amino acid similarity to NCK2, we could not use specific antibodies to determine NCK1 expression alone due to cross reactivity (data not shown). Instead, we took advantage of the IRES-β-galactosidase (β-gal) cassette that was inserted into the first coding exon of NCK1 and used β-gal expression as a proxy of endogenous NCK1 expression (NCK1^{+ve}). β-gal expression revealed that NCK1 is expressed throughout the CNS including throughout the hippocampus (Fig. 2.3A). To determine which cells express NCK1 we co-stained sections with β-gal and NeuN (a neuronal marker), Iba1 (a microglial marker), Sox2 (a progenitor cell marker). We found that 98.8 ± 1.2% of the NeuN positive cells were β-gal (NCK1)$^{+ve}$ (Fig. 2.3B). However, none of the Iba1 positive cells were β-gal (NCK1)$^{+ve}$ (arrows, Fig. 2.3B), nor was there colocalization with the progenitor cell marker Sox2 (Fig. 2.3C). Together, these data suggest that NCK1 is predominantly expressed in postmitotic cells including neurons but restricted from microglia in the mature hippocampus.

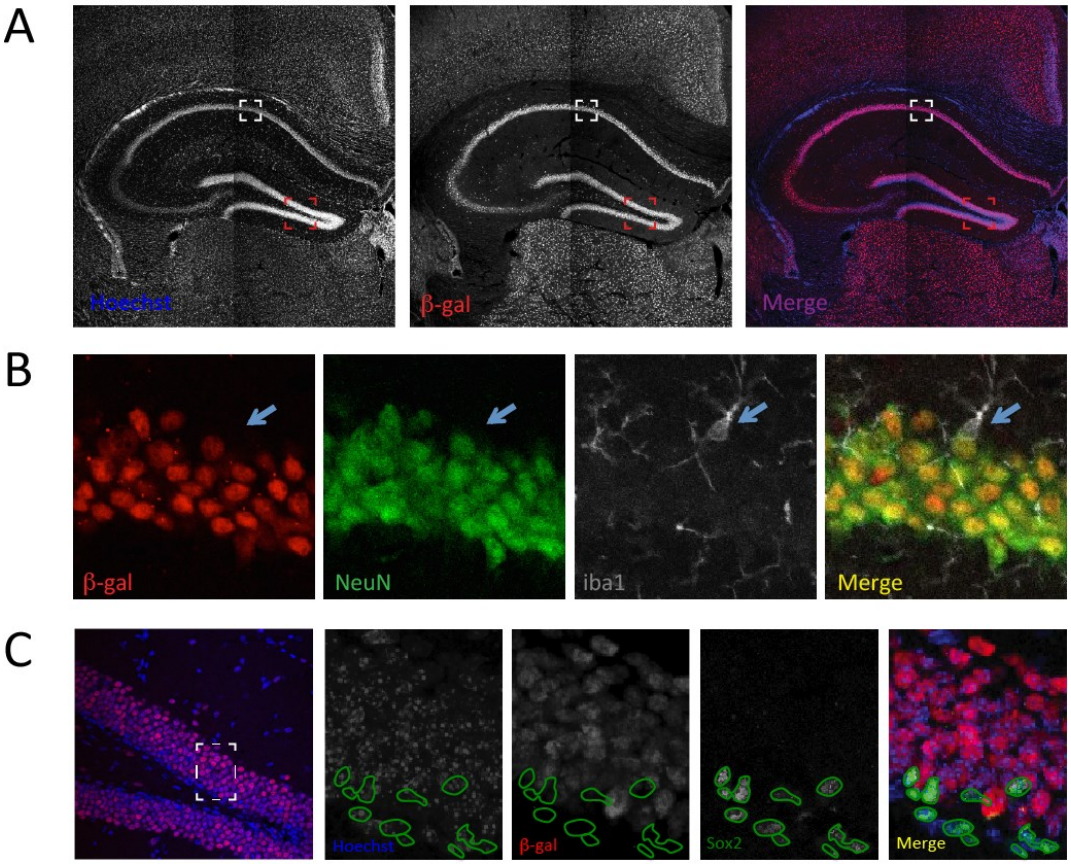

Figure 2.3: NCK1 expression in the hippocampus

A. Confocal image of a DNA marker Hoechst (blue) and β-gal (red) labeled coronal section through the hippocampus of an NCK1 heterozygous ($NCK1^{+/-}$) mouse. White box outlines the representative area of the CA1 shown in B. Red box outlines the representative area of the dentate gyrus shown in C. B. Confocal images of the CA1 from an $NCK1^{+/-}$ mouse. Immunostaining shows the NCK1 marker, β-gal (first panel, green), is colocalized with the neuron marker, NeuN (second panel, red), but not with the microglia marker, Iba1 (third panel, grey). Arrows indicate an Iba1 positive, β-gal negative cell. C. Confocal images of the dentate gyrus from an $NCK1^{+/-}$ mouse. Immunostaining shows the DNA marker, Hoechst (first, second, and fifth panels, blue), and that β-gal (first, third and fifth panels, red), is not colocalized with the progenitor cell marker, Sox2 (fourth and fifth panel, green). White broken line is a representative area of what was magnified in the next four panels. Green outlines Sox2 positive, β-gal negative cells.

2.3.4: Loss of NCK1 does not impair hippocampal embryonic neuronal proliferation or migration.

Although NCK1 was not detected in the Sox2 expressing cells in the adult dentate gyrus, others have implicated NCK proteins in cellular differentiation (Lu et al., 2015), thus we wanted to determine if NCK1 was important for neuronal proliferation. Pregnant dames were injected with EdU, a thymidine analogue that gets incorporated into the DNA of dividing cells, at E14.5, the height of hippocampal neurogenesis (Urbán & Guillemot, 2014). Embryos were then harvested thirty minutes later as a measure of proliferation. Both control and NCK1$^{-/-}$ embryos showed similar levels of proliferation in the hippocampal neuroepithelium (22.2 ± 0.85% vs. 24.3 ± 0.86%, P=0.1185, Student's t test, n= 6 control, 6 NCK1$^{-/-}$; Fig 2.4A). Therefore, NCK1 is not necessary for neuronal proliferation in the hippocampal neuroepitheliem.

Next, given that NCK1 has been implicated in cellular migration, we next asked whether loss of NCK1 affect hippocampal neuronal migration. We monitored this by assessing EdU incorporation at E14.5 then waiting 4 days and scoring whether there were differences in the number and patterning of cells that migrated to form the embryonic hippocampus. We find a similar percentage of EdU-labeled cells in the hippocampus of NCK1$^{-/-}$ and control embryos (77.1 ± 5.03% vs. 73.9 ± 4.36%, P=0.6419, Student's t test, n=8 control, 6 NCK1$^{-/-}$; Fig. 2.4B). Additional, EdU- labeled cells in the NCK1$^{-/-}$ embryos display characteristic hippocampal patterning, similar to control, with a visible hippocampal fissure and a dense CA layer (Fig. 2.4B).

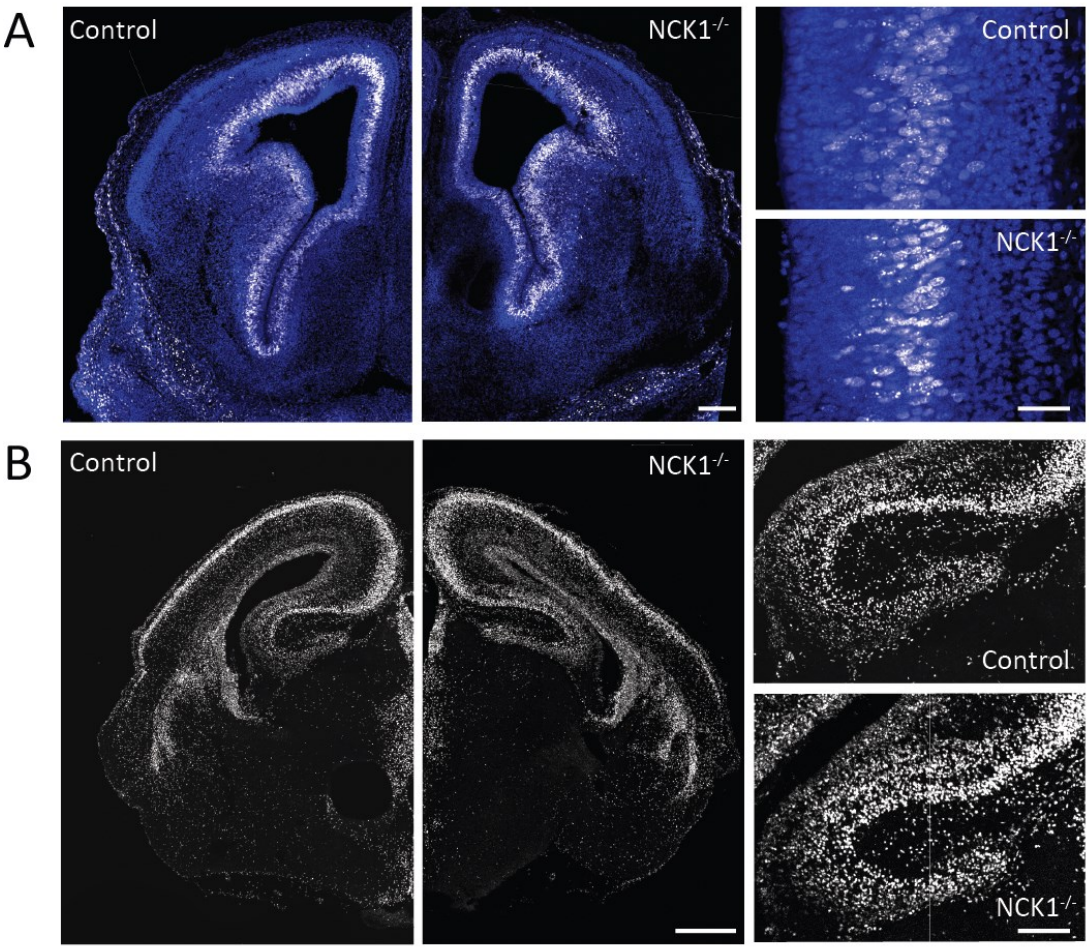

Figure 2.4: Embryonic neuronal proliferation, migration and hippocampal development in control and NCK1$^{-/-}$ mice

A. Confocal images of coronal sections of E14.5 brains and hippocampal formations that were EdU-labeled (white) at E14.5. Hoechst-labeled DNA is in blue. Second panel scale bar 200μm. Third panel scale bar 25μm. B. Confocal images of E18.5 brains and hippocampi that were EdU-labeled at E14.5. Second panel scale bar 500μm. Third panel scale bar 150μm.

2.3.5: Loss of NCK1 results in decreased hippocampal dendritic spine- and synapse-density

Given the impaired spatial memory and learning in the NCK1$^{-/-}$ mice and no gross abnormalities in embryonic development of the hippocampus, we next turned our attention to ask whether loss of NCK1 could affect neuronal morphology or synapse numbers of individual neurons in the adult hippocampus. We quantified neuronal morphology and synaptic density *in vivo* in the NCK1 mutant mice. In the CA1 pyramidal neurons, loss of NCK1 did not affect dendrite complexity, of either the apical or basal dendritic arbours (Fig. 2.5A,B). However, quantification of dendritic spine density revealed an 18% decrease in dendritic spine density in mutant relative to control mice (1.90 ± 0.07 vs. 1.56 ± 0.10spines/μm, P=0.0301, Student's t test, n=5 controls, 7 NCK1$^{-/-}$, Fig. 2.5C,D). Similarly, analysis of synaptic density from EM sections taken from the CA1 region of the dorsal hippocampus, showed an overall reduction in the NCK1$^{-/-}$ when compared to control (2.68 ± 0.24 vs. 4.60 ± 0.22 synapses/10μm^2, P<0.0001, Student's t test, n=29 NCK1$^{-/-}$, 18 control, Fig. 2.5E, F). Interestingly, we quantified the postsynaptic density area in the NCK1$^{-/-}$ synapses and found a significant increase compared to their control littermates (9.62 ± 0.76μm^2 vs. 6.19 ± 0.45μm^2, P=0.0002, Student's t test, n=59 NCK1$^{-/-}$, 53 control, Fig. 2.5G-I). Taken together, loss of NCK1 is resulting in decreased dendritic spine density and synapse number in the hippocampus, while synapses that are formed have larger PSD area.

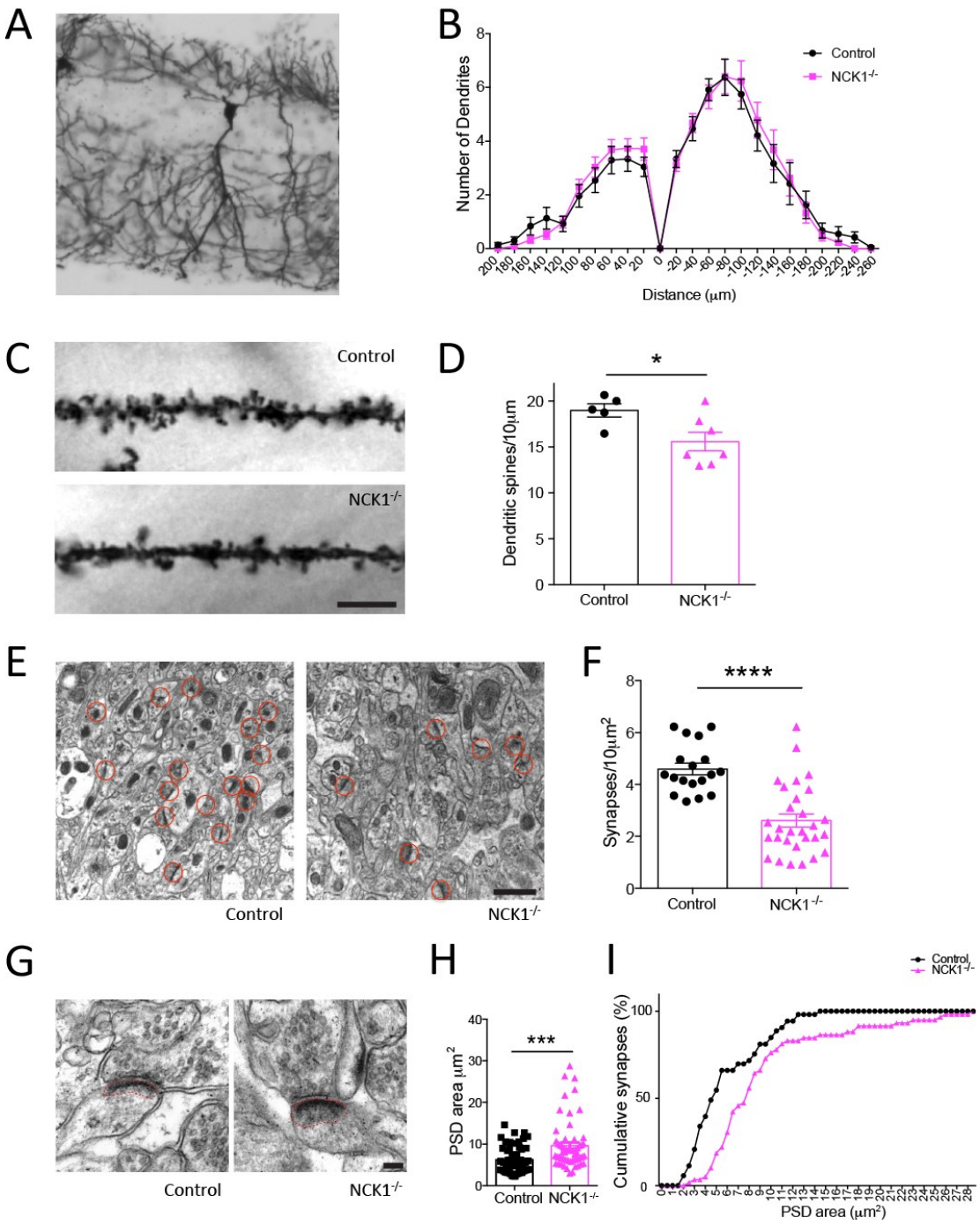

Figure 2.5. Analysis of control and NCK1$^{-/-}$ hippocampal pyramidal neurons' dendritic complexity, dendritic spine density, synapse number, and postsynaptic density area

A. Representative image of Golgi stained CA1 hippocampal pyramidal neurons. B. Sholl analysis of control and NCK1$^{-/-}$ CA1 hippocampal pyramidal neurons. C. Representative images of *in vivo* Golgi stained CA1 basal dendrites in control

and NCK1$^{-/-}$ mice. Scale bar = 5μm D. Quantification of dendritic spine density in the CA1 region in control (n=5) and NCK1$^{-/-}$ (n=7) mice. Each individual point represents average density of 20 dendrites per animal. E. Representative images of transmission electron micrographs from control and NCK1$^{-/-}$ hippocampi. Red circles surround synapses. Scale bar=1μm. D. Quantification of hippocampal synapse density. G. Representative electron micrograph of a hippocampal synapse. Broken red line outlines the postsynaptic density (PSD). Scale bar= 100nm. H. Quantification of the average area of PSD in the hippocampus. I. Cumulative percentage of total synapses by PSD area.

2.3.6: Loss of NCK1 results in decreased dendritic spine density in cultured hippocampal neurons

Given the decreased synapse density in the hippocampus of NCK1$^{-/-}$ mice, we next asked if this loss was due to a neuron intrinsic mechanism. To assess NCK1's function at the level of individual neurons, Dr. Dylan Quinn in Dr. Fawcett's lab first overexpressed GFP-NCK1 in dissociated hippocampal neurons. We found that GFP-NCK1 localized to axons, dendrites and was enriched in dendritic spines along with the PSD protein Homer (Fig2.6A). Given the localization to dendrites, we quantified the dendritic complexity and our Sholl analysis revealed no differences in the complexity between control and NCK1-/- neurons (Fig. 2.6B, C). Next, we quantified the density of synapses, and found a 23.5% reduction in the density of Homer positive puncta in the NCK1$^{-/-}$ cultures compared to controls (7.20± 0.41 vs. 5.51± 0.26 Homer puncta/10μm, P=0.0008, Student's t test, n=48 control, 47 NCK1$^{-/-}$, Fig. 2.6D,E), In addition to the decrease in density, we found an overall increase in fluorescent intensity of Homer puncta in the NCK1$^{-/-}$ neurons compared to control (Fig. 2.6F,G).

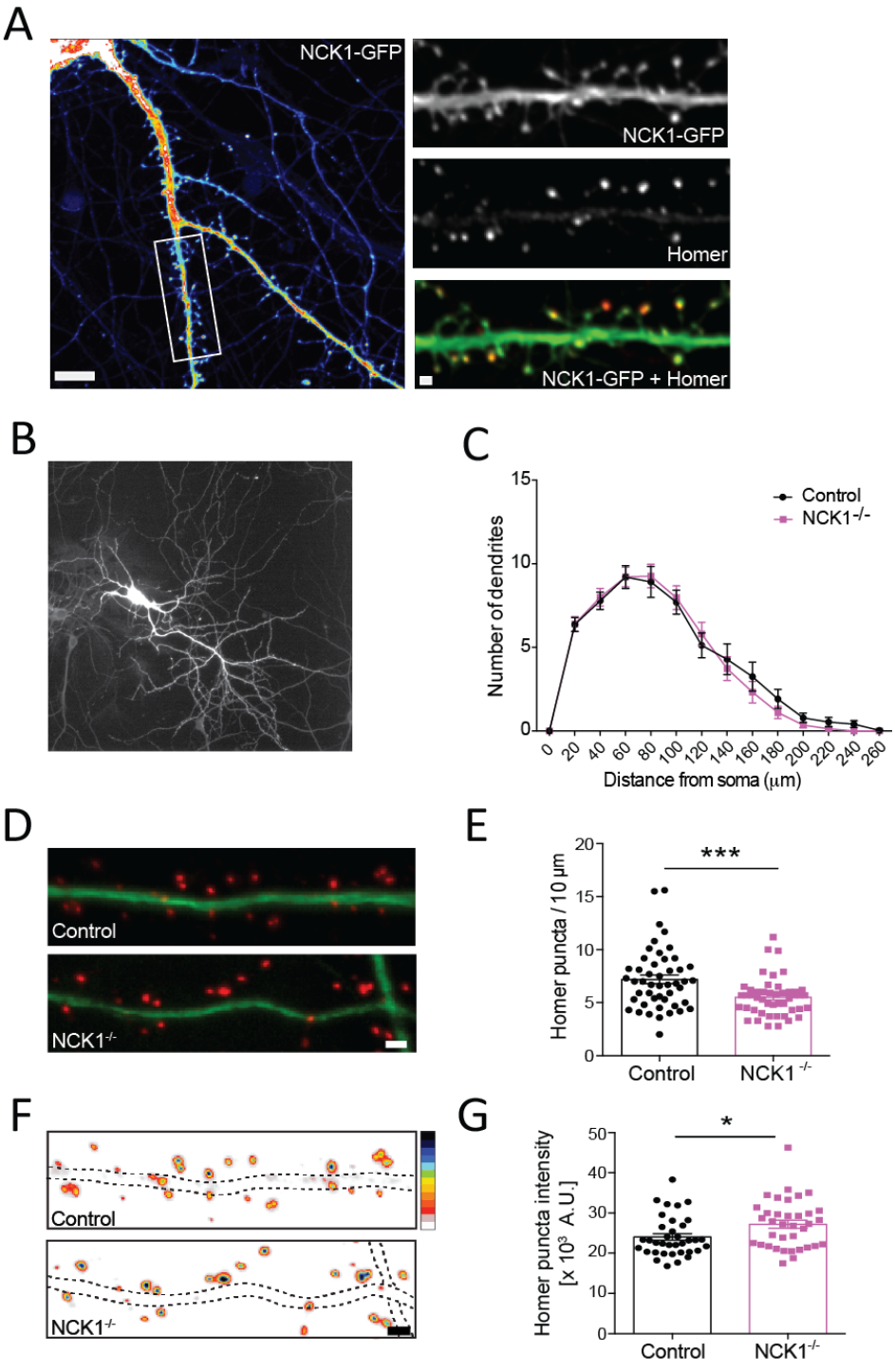

Figure 2.6. Analysis of control and NCK1$^{-/-}$ cultured hippocampal neurons' dendritic complexity and postsynaptic density density.

A. Left panel, heat map image of NCK1-GFP expression in dendrites of an NCK1-GFP transfected hippocampal neuron. Scale bar 10μm. Right panel,

NCK1-GFP and homer expression and colocalization in dendritic spines of an NCK1-GFP transfected hippocampal neuron. Scale bar =1μm. White box outlines region magnified and shown in the right panel. B. Representative image of a cultured hippocampal neuron. C. Sholl analysis of control and NCK1$^{-/-}$ cultured hippocampal neurons. D. Representative images of homer-labeled (red) cultured hippocampal neurons from control and NCK1$^{-/-}$ mice. Dendrites are labeled by MAP2 in blue. E. Quantification of homer puncta density on control and NCK1$^{-/-}$ cultured hippocampal neuron dendrites. F. Representative images of heat maps of homer puncta fluorescence intensity of control and NCK1$^{-/-}$ dendrites. G. Quantification of homer puncta fluorescence intensity. *$p<0.05$, ***$p<0.001$ Student's t-test.

2.3.7: Loss of NCK1 results in increased actin recovery in hippocampal dendritic spines after photobleaching

Given the decrease in spine density was linked to a cell instric event, we next turned our focus on whether loss of NCK1 affected actin dynamics in the postsynaptic region of dendritic spines. To directly assess whether the effect on spine morphogenesis from loss of NCK1 is linked to actin cytoskeleton dynamics, Dr. Michael Wigerius in Dr. Fawcett's lab examined the mobility of GFP-actin in fluorescence recovery after photobleaching (FRAP) experiments. Here, GFP-actin was transduced in either control or NCK1$^{-/-}$ hippocampal neurons and FRAP of GFP-actin labeled dendritic spines was used as a means to detect whether actin stability or mobility was affected. The mobile GFP-actin pool in spines from mutant cells (NCK1$^{-/-}$) was identical to the control and recovered within 60 seconds of photobleaching (Fig. 2.7A,B). Treatment with the actin stabilizing compound Jasplakinolide inhibited recovery completely in control cells (Fig. 2.7B; Control and NCK1$^{-/-}$ vs control + Jasp; $P < 0.01$) demonstrating that the mobility of GFP-actin reflected exchange of new actin filaments. However, actin turnover in NCK1 deficient neurons showed a ~ 40 % faster half-time recovery in spines (Fig. 2.7C; control vs NCK1$^{-/-}$; $P < 0.05$). This indicated that loss of NCK1 influences normal actin dynamics consistent with previous observations in heterologous cells (Buvall et al., 2013; Garg et al., 2007). Since NCK1 controls proteins such as the Arp2/3 complex that favor actin nucleation (Buday et al., 2002), Dr. Wigerius investigated whether increased actin turnover observed in FRAP is caused by changes in polymerization rate. Using monomeric actin

conjugated to rhodamine, barbed ends in actin filaments were labeled and assessed by imaging. A marked elevation in rhodamine-actin incorporation was observed in NCK1-depleted neurons and quantification of the ratio of Rh-actin to GFP-actin confirmed the significance of the enhanced labeling at barbed ends in spines (Fig. 2.7E; control versus NCK1$^{-/-}$; $P < 0.01$). Altogether, these results demonstrate that loss of NCK1 in hippocampal neurons enhances actin turnover implicating that this defect might underlie the behavioral effects in NCK1$^{-/-}$ mice.

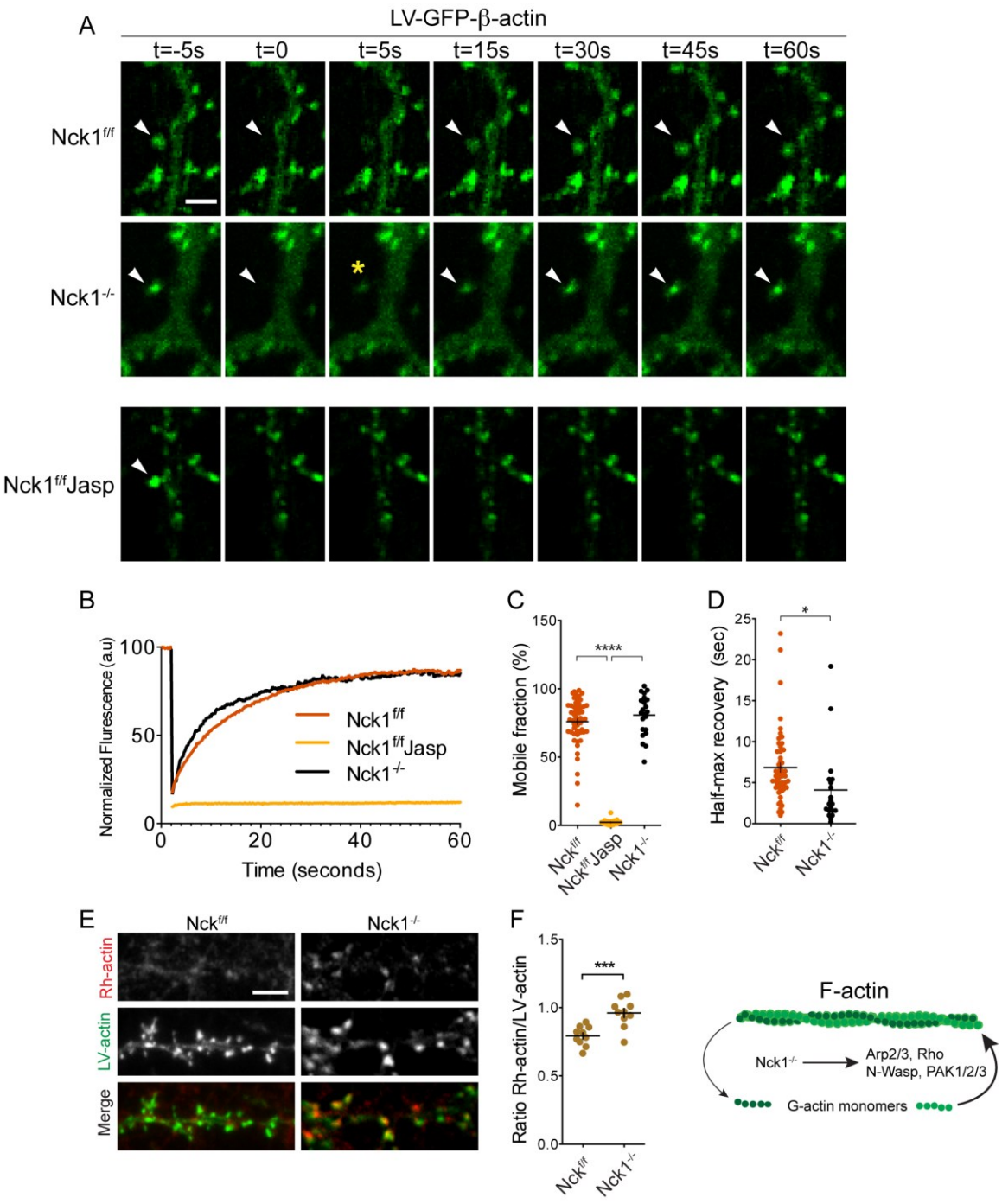

Figure 2.7. **Destabilized actin turnover in NCK1 deficient neurons.**

A. Time lapse images show GFP-actin (LV-GFP-actin) turn-over without or with Jasplakinolide (Jasp, 30 min pre-treatment, 1 μM) at the indicated time points in control (NCK1$^{f/f}$) and mutant (NCK1$^{-/-}$) neurons. Arrowheads point to bleached spines. Asterisk indicate enhanced kinetics of GFP-actin at t=5 seconds in

NCK1-deficient cells. Scale bar 5 μm. B. Time course of normalized GFP-actin fluorescence recovery in control and NCK1-deficient neurons. C. Bar graphs show percentage mobility of GFP-actin and D. half-maximum recovery (in seconds) in photobleached spines over time. Error bars represent mean ± SEM of mobile fraction and half-maximum recovery after photobleaching. ***, $P < 0.01$; one-way ANOVA followed by Tukey's post-hoc test. *, $P < 0.05$; Student's *t* test. E. Visualization of monomeric Rh-actin incorporated at barbed ends in actin filaments. F. Bar graphs show quantification of Rh- to GFP-actin ratio in dendritic spines. Error bars represent mean ± SEM. ***, $P < 0.01$; Student's *t* test.

2.4: Discussion

Genetic deletion of the adaptor protein NCK1 in the mouse results in deficits in memory formation in three hippocampal function dependent tasks; social recognition (Fig. 2.1B), spontaneous alterations in the Y-maze (Fig. 2.1D), and learning and remembering the location of the escape platform in the Morris water maze (Fig. 2.2B), implicating NCK1 as an important regulator of hippocampal function in the adult brain. Interestingly however, although NCK2 mutant mice displayed deficits in social recognition (Fig. 2.1B), and working memory in the Y-maze (Fig. 2.1D), they did not have learning and memory deficits in the Morris water maze (Fig. 2.2C). This would suggest that NCK1 and NCK2 play non-redundant roles in cognition and that NCK1, but not NCK2, is critical for spatial learning in the Morris water maze. Further analysis of the $NCK2^{-/-}$ mice, and whether NCK2 is expressed in these neurons, will be necessary to identify the underlying defect contributing to the short-term memory defects. Nonetheless, given the defect in the Morris water maze in the NCK1 mutant mice, our analysis was focused on this mutant.

Loss of NCK1 did not impact over all activity levels in any of the tests, nor did it result in impairments in sociability, which suggests that general sensory and motor processes seem to be intact. In fact, the $NCK1^{-/-}$ mice have no apparent olfactory, visual, or motor deficits (Fig. 3.1), suggesting that sensory and motor neuronal circuitry is functional in these mutant mice. Likewise, no differences were found in brain size or shape between $NCK1^{-/-}$ mice and control mice. Correspondingly, no differences in embryonic neuronal proliferation or in

hippocampal development were found (Fig 2.4), further suggesting that basic neuronal development is not impaired in the NCK1$^{-/-}$ mice. Together, these data suggest that the memory and learning impairment may be due to deficiencies in adult hippocampal function.

Analysis of NCK1 expression revealed that it is present in all postmitotic neurons in the hippocampus, but not in microglia nor in the progenitor cell layer of the dentate gyrus (Fig. 2.3). Therefore, NCK1 is likely playing a neuron-autonomous role in hippocampal function in mature differentiated neurons. Given the recent report that NCK2, but not NCK1, affects cellular proliferation (Jacquet et al., 2018), this is consistent with our data and implicates that NCK1 is likely not affecting proliferation as we find little to no expression in proliferating progenitor cells (Fig. 2.3). Whether NCK2 mice are affected remains to be determined; however, we do know that they do survive and have no learning defects (Fig 2.2C,E).

We next examined the overall morphology of CA1 pyramidal neurons in the NCK1 mutant mice and found that NCK1 deficiency did not affect overall branching of either the apical or basal dendritic arbourization (Fig. 2.5A). This was supported by the *in vivo* culture experiments (Fig. 2.6B,C). However, we did see a decreased in dendritic spine density in Golgi stained sections (Fig. 2.5C,D). The effect of NCK1 deficiency on dendritic spine density was also supported by Homer staining in dissociated culture studies (Fig. 2.6D,E), and the overall decreased synaptic density in our EM analysis (Fig 2.5F). Interestingly, we saw an increased Homer staining intensity in our dissociated cultures (Fig.

2.6F,G) that was supported by the increase in PSD density seen from the EM analysis (Fig. 2.5G-I). The larger PSD area is consistent with activity-induced turnover of postsynaptic proteins (Dosemeci et al., 2001, 2016; Meyer et al., 2014; M. Sheng & Kim, 2011), and thus more dynamic and potentially less stable synapses. Indeed, our FRAP and rhodamine actin incorporation studies this as we detect an increased rate of actin turnover and polymerization in NCK1 deficient neurons (Fig. 2.7). The larger PSD area *in vivo* in the NCK1-deficient synapses (Fig. 2.5G-I) is possibly due to increased actin turnover and polymerization as the dissociation of actin regulators from F-actin has been shown to destabilize the actin cytoskeleton and results in the expansion of the pallium and total PSD area (Dosemeci et al., 2016; K. Kim et al., 2015). Together, this data suggests that NCK1 plays a role in actin stability in dendritic spines and is important for the formation or maintenance of synapses in the adult hippocampus critical for memory formation and learning. Future work looking at dendritic spine stability in neurons deficient in NCK1 will allow us to more directly associate the changes in actin dynamics to the reduction in dendritic spine density.

Multiple signaling pathways converge on actin-cytoskeletal rearrangement to regulate activity-dependent spine morphogenesis, maintenance, and elimination (S. Basu & Lamprecht, 2018; Caroni et al., 2012). Indeed, the netrin-1 receptor DCC, a known upstream recruiter of NCK1 (X. Li et al., 2002), has been shown to be critical for synaptogenesis, synapse maturation, and memory formation in the mammalian brain (Glasgow et al., 2018; Goldman et al., 2013;

Horn et al., 2013; E. W. Wong et al., 2019). Interestingly, aged mice that have conditional deletion of DCC or netrin-1 from the forebrain develop deficits in spatial and recognition memory similar to what is seen in the NCK1-mutant mice (Horn et al., 2013; E. W. Wong et al., 2019). Recent work suggests that netrin-1 functions in hippocampal dendritic spine maturation by promoting GluA1-contain AMPA receptor insertion into the PSD through a DCC-mediated mechanism (Glasgow et al., 2018). NCK1 may be playing a role downstream of DCC to influence actin dynamics in the spine as actin regulations has been implicated in postsynaptic receptor anchoring and trafficking (Cingolani & Goda, 2008). However, the role of actin cytoskeletal reorganization in the DCC-mediate receptor insertion remains to be addressed, this will be important as NCK proteins in DCC-meditated actin rearrangement in axon guidance and growth cone morphology has been established (C. Lane et al., 2015; Shekarabi et al., 2005).

NCK1 has also been shown to function upstream of Rho-GTPases. The Rho-GTPases, including RhoA, Rac1, and Cdc42, are well known regulators of the actin cytoskeleton that have been shown to influence dendritic spine formation, maintenance, and elimination (Tada & Sheng, 2006). Interestingly, where RhoA activation inhibits spine morphogenesis and promotes spine elimination, Cdc42 and Rac1 activation promotes spine formation (A. Tashiro et al., 2000; A. Tashiro & Yuste, 2004). Indeed, NCK1 has been shown to be involved in Rac1 and Cdc42 activity (Guan et al., 2009; X. Li et al., 2002), but not in RhoA activity (Guan et al., 2009). Loss of NCK1 may be impairing Rac1 and/or

Cdc42 activation and their association with upstream and downstream modulators of the actin cytoskeleton and thus resulting in the decreased spine density observed in the NCK1$^{-/-}$ mice by preventing dendritic spine formation. Similarly to the NCK1 mutant mice, *in vivo* conditional deletion of Rac1 or Cdc42 in the forebrain postnatally results in decreased spine density in the CA1 region of the hippocampus, and deficits in spatial memory and learning in the Morris water maze (Haditsch et al., 2009; I. H. Kim et al., 2014). Future work looking at Rho-GTPase activity in NCK1-deficient neurons will provide insight into NCK1's role in Rho-GTPase activation in dendritic spines. This is critical, as NCK proteins have been linked with chimaerin dependent signaling to affect Rho-GTPase activity (Gutierrez-Uzquiza et al., 2013). Whether this activity is linked to either NCK1 or NCK2 remains to be established, and whether there are cell/tissue specific modulation also remains unknown.

Here, we demonstrate that NCK1 is necessary for social recognition, working memory capacity, and spatial learning. These effects are independent of developmental influences, as loss of NCK1 has no effect on progenitor cell proliferation or migration in the hippocampus. The behavioural defects are likely linked to impaired actin dynamics within the dendritic spines of hippocampal neurons. Finally, although we and others have suggested that NCK1 and NCK2 can functionally compensate for one another, our data support that NCK1 plays a unique role in cognition, specifically in learning and memory, by modulating actin turnover within dendritic spines. Further work will help elucidate a specific role for NCK2 in the CNS.

Chapter 3: NCK1 regulates amygdala activity to control context-dependent stress responses and anxiety-like behaviours

My contributions to this chapter include all of section *3.1: Introduction* and *3.4: Discussion*. All experiments in section *2.2: Methods and Materials* were carried out by me with the exception of (1) *3.2.4: Liquid chromatography and tandem mass spectrometry (LC-MS/MS) conditions* were optimized by Dr. Alejandro Cohen, Scientific Director of the Proteomics and Metabolomics Core Facility at Dalhousie University, and (2) *3.2.10: Western blotting* was preformed by Dr. jiansong Qi in Dr. Fawcett's lab. I authored all of *3.3: Results*. All figures were designed and created by me. However, western blot images (Figure 3.5) were taken by Dr. Jiansong Qi. Finally, cell counts for result sections 3.3.3, 3.3.4 and dendritic spine counts (3.3.7) were done by Ibrahim Shahin in Dr. Fawcett's lab and myself. The full chapter was additionally modified and edited by Dr. James Fawcett.

3.1: Introduction

Anxiety is an adaptive, evolutionarily conserved internal state defined by a sense of apprehension and avoidance behaviours. However, dysregulation of anxiety is often debilitating and characterizes the largest group of mental disorders (Craske et al., 2017). Although anxiety disorders are considered to be moderately heritable, and first-degree relatives are 4-6 times more likely to suffer from an anxiety disorder, specific genetic markers remain elusive (Otowa et al., 2016; Savage et al., 2017; Shimada- Sugimoto et al., 2015; Smoller, 2016). Nonetheless, anxiety disorders are characterized by changes in the functioning of neuronal circuits affecting excitatory/inhibitory (E/I) balance in the basolateral amygdala (BLA) (Babaev et al., 2018; B. Leuner & Shors, 2013; Marín, 2012; Meunier et al., 2017). The BLA consists of heterogeneous cell populations and circuits that code for both aversive and rewarding perceptions and responses (Beyeler et al., 2018). Importantly, disruption of local GABAergic signaling within the BLA results in hyperexcitability of the principle output neurons leading to the dysregulation of these circuits (Babaev et al., 2018; Prager et al., 2016; Sharp, 2017). This hyperexcitability contributes to the increased release of corticosterone from downstream targets in the hypothalamic-pituitary-adrenal axis, changes in emotional valance, and increased anxiety-like behaviours (Janak & Tye, 2015; Myers et al., 2012; Tovote et al., 2015). It is therefore important to identify genes involved in the regulation of anxiety-like responses that are linked to regulating excitatory/inhibitory balance, in order to develop selective therapeutics to relieve these mood disorders.

Advances in genomics and proteomics have implicated a number of genes involved in actin dynamics in psychiatric and cognitive disorders (Sala & Segal, 2014; Yan et al., 2016). For example, Fragile X syndrome (FXS), the most frequently inherited single-gene cause of intellectual disability, presents with impairments in neuronal actin dynamics and increased levels of anxiety (Crawford et al., 2001; Michaelsen-Preusse et al., 2018). Interestingly, mouse models of FXS display defects in GABA transmission and resulting excitatory/inhibitory (E/I) imbalances (Centonze et al., 2008; J.-Y. Kang et al., 2017). Combined, a number of genetic deletion studies implicate the importance of maintaining appropriate protein-protein complexes within the synapse to detect differences in neuronal firing patterns, establish proper E/I balance, and produce typical behaviours (Cuthbert et al., 2007; Grant, 2019; Migaud et al., 1998; Nithianantharajah et al., 2013). Recent genome-wide association studies (GWAS) in humans have implicated mutations in *NCK1* in schizophrenia, neuroticism, and more specifically as an enhancer of worry (Luciano et al., 2018; Nagel et al., 2018; Ripke et al., 2014). *NCK1* encodes the non-catalytic region of tyrosine kinase adaptor protein 1 (NCK1), which regulates the actin cytoskeleton and is involved in cellular remodeling via changes in actin dynamics through its SH3 domains (Bladt et al., 2003; Buday et al., 2002; Fawcett et al., 2007). In addition to *NCK1*, a second gene *NCK2*, has been shown to regulate actin dynamics downstream of tyrosine kinase signaling to promote cell polarization and directional migration (Bladt et al., 2003; Chaki et al., 2013). Given the high degree of amino acid similarity within the signaling domains of NCK1 and NCK2,

there is evidence that they can functionally compensate for the loss of one or the other in the nervous system (Fawcett et al., 2007); however recent studies suggest that in other cellular contexts these proteins can have unique functions within the cell (Jacquet et al., 2018; Ngoenkam et al., 2014). Given NCK1 has been implicated as an important regulator of worry and mood from GWAS, and that there are no studies to date examining the contribution of NCK1 alone on neuronal development and behaviours *in vivo*, we assessed the role of NCK1 in anxiety-like behaviours and neuronal development.

Here we show that mice lacking NCK1 display increased anxiety-like behaviours in the elevated plus maze (EPM) and the light/dark box assays. At rest both control and NCK1$^{-/-}$ mice showed normal circulating corticosterone levels. However, upon exposure to the EPM, the NCK1$^{-/-}$ mice had a significant increase in circulating serum corticosterone compared to control mice. Further, treatment with diazepam was able to reduce the anxiety-like behaviour in the elevated plus maze (EPM). Since disruption of local GABAergic circuitry within the BLA has been shown to modulate anxiety-like behaviours (Felix-Ortiz et al., 2016; Möhler, 2012), we focused our analysis on this region. Interestingly, the activity of GABAergic parvalbumin-positive inhibitory interneurons within the BLA was significantly reduced relative to controls following exposure to the EPM. Finally, we show a reduction in spine density in the principal neurons of the BLA. Together, our work contributes a novel function of the adaptor protein NCK1 in the control of approach and avoidant behaviours.

3.2: Methods and Materials

3.2.1: Animals

The NCK1 and NCK2 mutant mice used have previously been reported (Bladt et al., 2003). For time-pregnant experiments, embryonic day was defined from vaginal plug detection as E0.5. NCK1$^{-/-}$ thymocyte differentiation antigen 1 (Thy1)- yellow fluorescent protein (YFP) mice were generated by crossing the NCK1$^{-/-}$ with the Thy1-YFP mice obtained from Jax labs (B6.Cg-Tg(Thy1-YFP)HJrs/J). Rooms were maintained at 21^{0}C under a 12h light-dark cycle (7am to 7pm lights on). Food and water were available *ad libitum*. The study was approved by the Dalhousie University Committee on Laboratory Animals (Dalhousie Animal Protocol #14-062) and was conducted according to the Canadian Council on Animal Care guidelines.

3.2.2: Behavioural Testing

Olfactory habituation/dishabituation testing was adapted from (M. Yang & Crawley, 2009). Briefly, 8-14 week old male control (wild-type littermates) and NCK1$^{-/-}$ mice were placed in a clean cage with a cotton tip applicator inserted through the water bottle hole and were left to acclimatize for 30minutes. The mice were then presented with three different odors, applied to cotton tip applicators, sequentially, with 2-mins intervals in the following order, waterX3, lemonX3, social odorX3). The amount of time sniffing or interacting with the cotton tip applicator was recorded.

Hidden platform/visible platform in a pool task was adapted from (A. A. Wong & Brown, 2007). Mice were tested in a pool (90cm diameter), on two days, 4 trials/day, with a 10-minute inter-trial interval. For each trial, mice were placed at a different, randomly determined start point. Total swim path/time to locate the goal platform was recorded. Day 1, the goal platform was hidden (clear Plexiglas just under the water surface) in the northwest quadrant of the pool. Day 2, the goal platform was visible (opaque cover and flagpole) in the northeast quadrant. Trials timed-out at 60 seconds, or when the mice located and mounted the platform. Swim paths were recorded using a video camera based tracking system and the WatermazeBeta (Actimetrics, Wilmette, IL) software and the average swim latency (time to locate and mount the escape platform) over the four trials was measured.

Open Field. Mice were place in a corner of an open field (72 x 72 cm x 50 cm) facing the center and allowed to explore for 5 minutes. The open field was superimposed with a 5x5 grid; the number of line crossing (by crossing the line with all four paws), time spent in the center, periphery, and corners were scored. Mice were recorded and tracked using LimeLight software (Actimetrics, Wilmette, IL).

Elevated plus maze (EPM). Mice were placed onto the central platform facing a closed arm and allowed to explore the apparatus for 5 min. Mice were tracked and recorded using LimeLight software (Actimetrics, Wilmette, IL). Time

spent in the open arms, closed arms and the central square after all four paws crossed into/out of an arm were recorded.

Light/dark box. Mice were placed in a Plexiglas box with two compartments separated by an opaque wall with an opening. One compartment was opaque/covered (dark); the second was open and brightly lit (light). Mice were initially placed in the dark compartment and recorded for 5mins. The amount of time spent in each compartment and number of entries into the light compartment were scored.

For all behavioural testing, the experimenter was blind to the genotype throughout testing and scoring.

3.2.3: Collection of serum and mass spectrometry analysis of corticosterone levels

Serum collection for corticosterone analysis was adapted from (L. Kang et al., 2013). Control or mutant were taken either directly from their home cage (baseline), or following a 5min exposure to the EPM, then anesthetized (pentobarbital sodium, 0.09mg/g i.p., Bimeda-MTC Animal Health Inc.), and blood was collected by cardiac puncture. Samples were left to incubate for 2hrs at room temperature, spun (1900g, 4^{o}C, 10mins), and serum was collected. 10ul serum was then mixed with 5ul of d-8-corticosterone (100ng/mL, Cambridge Isotopes Laboratories, DLM-7347-0.01)), then suspended into 160ul of a 1:1:1 mixture of methanol, ethanol and acetone, and centrifuged (18000g, 5mins). The

resulting supernatant was transferred, evaporated under vacuum and reconstituted in 80uL of 20% acetonitrile.

3.2.4: Liquid chromatography with tandem mass spectrometry (LC-MS/MS) conditions

Blood serum digests were analyzed by LC-MS/MS operated in positive Selected Reaction Monitoring (SRM) mode. The liquid chromatograph (Ultimate 3000 RSLCnano, Thermo Scientific, Rochester, USA) was coupled to a triple quadrupole linear ion trap mass spectrometer (Qtrap 5500, Sciex) via a heated assisted electrospray ionization source (Turboionspray, Sciex) using a 25micron ESI electrode. Ionization parameters (software defined arbitrary units) were set as following: Curtain gas (20), Gas 1 (15), probe temperature (400C), ionization voltage (4500V).

The chromatographic separation was carried out on a reverse phase Luna C18(2)100A 150x0.3mm capillary column (Phenomenex, Torrance, CA, 00F-4251-AC) placed in column oven (50°C). Injection volume (5uL, using the micro-pickup technique to reduce sample loss). Chromatographic separation (5ul/min flow rate, 50 minute protocol with a linear gradient (Mobile Phase A: 0.1% formic acid in MS-grade water, mobile phase B: 0.1% formic acid in MS-grade acetonitrile) from 20% B to 95% B over 23 minutes, then holding at 95% B over 5 minutes). The remaining time of the run, mobile phase composition was restored to initial conditions for column equilibration

Selected Reaction Monitoring parameters were optimized for both a pure unlabeled sample of corticosterone (Cambridge Isotope Laboratories, Andover, MA, ULM-9988-PK) and for deuterated corticosterone (Cambridge Isotope Laboratories, Andover, MA, DLM-7347-0.01). Parent masses were identified for both the unlabeled and deuterated corticosterone and five fragment masses were identified for each compound. A total of 10 transitions were chosen and optimized. Data was acquired with Analyst 1.6.2 software (Sciex, Concord, ON, Canada), and SRM results analyzed using SKYLINE ver. 4.0 software.

3.2.5: Pharmacological testing

Drug naive mice received either diazepam injection USP (0.9mg/kg i.p., Sandoz, DIN 00399728) or an injection of saline (0.9mg/kg i.p., 0.9% sodium chloride, Hospira, Montreal, QC, 00037842) 5 minutes prior to a 5-minute exposure to the elevated plus maze. Prior to testing, optimal (non-sedative) dose was determined by testing control mice in the EPM at multiple doses of diazepam and scoring total distance travelled. Dose of 0.9mg/kg i.p. resulted in control levels of activity (~1400cm) and was selected.

3.2.6: Adult mouse brain histology

Mice were deeply anesthetized (pentobarbital sodium, 2.4mg/g i.p; Bimeda-MTC Animal Health Inc.) 2 hrs following EPM exposure, perfused transcardially with phosphate buffer saline (PBS) followed by 4% paraformaldehyde (PFA). Brains were dissected, fixed (4% PFA, 5 hours),

washed (PBS 3X20mins), cryoprotected (30% sucrose, 48hours at 4°C), embedded (Tissue Tek), and sectioned.

Free-floating sections were washed in a solution of 0.25% triton X100 in PBS (t-PBS) for 3X10mins, then transferred to 1% triton X100 in PBS 10mins). Sections were then blocked in 10% normal goat serum (NGS) t-PBS for 1h. Sections were then incubated in primary antibody solution containing 3%NGS in t-PBS overnight at 4°C. The primary antibodies used in this study include: chicken anti-β-Galactosidase, 1:1000, abcam, ab9361; mouse anti-NeuN, Millipore, MAB377; rabbit anti-Iba1, Wako Chemicals, 019-19741, 1:1000; rabbit anti-c-Fos, 1:1000, Synaptic Systems, 226 003; mouse anti-parvalbumin,1:1000, Millipore, MAB1572, rabbit anti-GFP, 1:1000, abcam, ab290. Following overnight incubation in primary, sections were then 3 x 10 minutes in t-PBS followed by incubation in appropriate secondary antibody. Alexa Fluor 488 goat anti-chicken, 1:500, Life Technologies, A11039; Alexa Fluor 594 goat anti-mouse, 1:500, Invitrogen, A11005; Alexa Fluor 647 goat anti-rabbit, 1:500, Life Technologies, A21246; Alexa Fluor 594 goat anti-rabbit, 1:500, Invitrogen, A11037 Alexa Fluor 488 goat anti-mouse, 1:500, Molecular Probes, A11029. Following incubation in secondary, sections were washed extensively, incubated in Hoechst 33342, 1:500, Life Technologies, H3570, for 1min), washed again in PBS, 3X5mins. Sections were mounted in Fluoromount Aqueous Mounting Medium (Sigma, F4680) and left to dry overnight prior to imaging.

Images were acquired on a Zeiss LSM 710 laser scanning confocal microscope (β-galactosidase/NeuN) or Zeiss Axio Observer.Z1 automated,

Marianas System from 3I (c-Fos/parvalbumin). Setting for laser power, gain and offset were kept constant for each experiment. All images for quantification were taken through the 20X objective (numerical aperture 0.40) in motor cortex, and basolateral amygdala. Images were analyzed using the spots function on the Imaris8 (Bitplane) software. Each marker was counted independently before overlaying images to observe colocalization or to calculate as a percentage of total Hoechst labeled nuclei. Experimenter was blind to the genotype during every step of this process.

3.2.7: Ethynyl deoxyuridine (EdU) proliferation and migration assays

Three groups of pregnant NCK1 heterozygous dams received an intraperitoneal injection of EdU (10ul/g). Group 1 received the i.p injection at E11.5, Group 2 received i.p injection at E14.5, the dams were sacrificed by cervical dislocation 30 minutes after injection, embryos were dissected, fixed (4% PFA, 45mins), washed (PBS, 3X20mins), cryoprotected (30% sucrose, 48hrs). Coronal sections (20um) were cut and mounted onto charged slides (Fisher brand Superfrost Plus). Group 3 received EdU injection at E14.5 but pregnant dams were then returned to their cages until E18.5 when the brains of the E18.5 embryos were dissected out and the same procedures as above were followed. Slides of coronal sections were then treated according to manufacturer's instructions for the Click-iT EdU Alexa Fluor 647 Imaging Kit (Life Technologies, C10340).

Images were acquired on a Zeiss LSM 710 laser scanning confocal microscope and displayed using ZEN2009 software. The setting for laser power, gain and offset were kept constant for each experiment. All images for quantification were taken through the 20X objective (numerical aperture 0.40) in the ventricular zone at E11.5, in cortical neuroepithelium at E14.5 and in the cortex at E18.5. Images were analyzed using the spots function on the Imaris8 (Bitplane) software. Each marker was counted independently before overlaying images to calculate the amount of EdU positive cells as a percentage of total Hoechst labeled nuclei. Experimenter was blind to the genotype during every step of this process.

3.2.8: Axonal targeting analysis

NCK1, Thy1-YFP mice were deeply anesthetized, perfused and processed as outlined above. YFP signal was enhanced using anti-GFP antibody staining as outlined above. Images were acquired on Leica DMI 6000 B microscope and analyzed using SlideBook6 (3I) software in a manner previously described (Richier et al., 2010; Zhu et al., 2016).

3.2.9: Golgi's Method and dendritic spine analysis

Mice received a lethal injection of pentobarbital sodium (2.4mg/g, i.p; Bimeda-MTC Animal Health Inc.), their brains were dissected and whole brains were immersed in the Golgi impregnation solutions for two weeks according to

the FD Rapid Golgi Stain kit (FD Neurotechnologies) and tissue preparation and staining procedure was done following the manufacturer's protocol.
Images were acquired on a Leica DM2000 microscope and displayed using QCapture Suite PLUS software. Images for dendritic spine quantification were taken from dendrites of the principal spiny neurons of the BLA through the 100X objective. Images were analyzed and spines were counted using Image J software and the cell counter plugin. Two separate experimenters analyzed 20 dendrites/mouse from 8 control and 9 $NCK1^{-/-}$ mice. Experimenters were blind to the genotype during every step of this process.

3.2.10: Western blotting

Amygdalae from control and $NCK1^{-/-}$ mice were dissected and homogenized in NP-40 lysis buffer (10% glycerol, 1% NP-40, 20 mM Tris [pH 8.0], 37.5 mM NaCl) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 10g/ml aprotinin, and 10g/ml leupeptin. The NP-40 lysis buffer was combined with tissue at a 1/10 (wt/vol) ratio as previously reported (Richier et al., 2010). Protein bands were normalized by comparing total protein levels in each lane and quantified using Image Lab 6.0.1 (Bio-Rad Laboratories) software.

Antibodies used for Western bloting: Anti-Gephyrin (1:1000, Cedarlane, 147011(SY)), Anti-GAD67 (1:5000, Millipore, MAB5406), Anti-Calbindin-D-28K (1:5000, Sigma-Aldrich, C9848), Anti-Parvalbumin (1:2000, Millipore, MAB1572), Anti-phospho-Tyr^{1252} NMDA receptor (1:1000, PhosphoSolutions, p1516-1252),

Anti-PSD95 (1:5000, Abcam, ab2723), Anti-VGluT2 (1:2000, Millipore, AB2251-I), Anti-Synaptophysin (1:500, Sigma, S 5768).

3.2.11: Statistical analysis

All data are expressed as means ± standard error of the mean (SEM) and all statistics were analyzed using Prism 6 (GraphPad Software, inc). Independent t-tests were used for analysis of differences between two groups. When comparing more then two groups, ANOVA and Tukey's post hoc multiple comparisons tests were used. To measure to assess the effect of two independent variables two-way ANOVA were run followed by Sidak's multiple comparisons test. A $P<0.05$ was considered statistically significant.

3.3: Results

3.3.1: Loss of NCK1 or NCK2 does not impair gross sensory or motor function

Previous studies have shown an important role for NCK proteins in the regulation of actin-dependent cellular processes through its association with actin regulatory proteins in axon guidance (Fawcett et al., 2007) kidney- (N. Jones et al., 2006), vascular system- (Clouthier et al., 2015), and bone-development (Aryal A.C et al., 2015). However, these *in vivo* studies were dependent on tissue specific conditional deletion of both NCK1 and NCK2 as global loss of both proteins leads to an embryonic lethality (Bladt et al., 2003). Despite this, little is known about the role for the individual NCK proteins on brain development and behaviour. Given NCK1/2's role in linking extracellular signals to actin cytoskeletal rearrangement (Blasutig et al., 2008; Buday et al., 2002; Chaki et al., 2013; C. Lane et al., 2015; W. Li et al., 2001), the increasing evidence that actin regulation is critical for the structural plasticity that underlies cognitive functioning (Bernardinelli et al., 2014; Borovac et al., 2018; Tejada-Simon, 2015), and that impairing actin dynamics can lead to reduced synaptic efficacy and E/I imbalances that present as increased anxiety-like behaviours (Deguchi et al., 2016; Mucha et al., 2011), therefore we asked if loss of NCK1 or NCK2 results in increased anxiety-like behaviours.

As behavioural assessment in mice is dependent on motor and sensory function, we first tested whether mice deficient in NCK1 ($NCK1^{-/-}$) or NCK2 ($NCK2^{-/-}$) had any gross sensory or motor defects. We find that $NCK1^{-/-}$ and $NCK2^{-/-}$ mice are able to discriminate between odors in an olfactory

habituation/discrimination task (Fig 3.1A) and have no visual deficits in a visual water maze task (Fig. 3.1B). In an open field test, NCK1$^{-/-}$ mice have similar levels of overall activity as control mice (2030 ± 277.7cm travelled, NCK1$^{-/-}$ vs. 1785 ± 214.7cm travelled, control). However, the NCK2$^{-/-}$ mice are significantly more active (2802 ± 146.6cm travelled; P=0.0178, one-way ANOVA, Tukey's multiple comparisons test; Fig 3.1C,D). Further, while both the control and NCK1$^{-/-}$ mice spent similar amounts of time in all regions of the open field, the NCK2$^{-/-}$ mice spent significantly more time in the center (32.8 ± 4.9s, NCK2$^{-/-}$ vs. 17.7 ± 2.9s, control vs. 19.2 ± 4.0s, NCK1$^{-/-}$; P=0.0245, one-way ANOVA, Tukey's multiple comparison test; Fig 3.1E) and less time in the periphery (267.1 ± 4.9s, NCK2$^{-/-}$ vs. 285.8 ± 3.2s, control vs. 280.7 ± 4.0s, NCK1$^{-/-}$; P=0.0094, one-way ANOVA, Tukey's multiple comparison test; Fig 3.1F) and corners (122.2 ± 8.0s, NCK2$^{-/-}$ vs. 184.2 ± 12.7s, control vs. 122.2 ± 8.0s, NCK1$^{-/-}$; P=0.0083, one-way ANOVA, Tukey's multiple comparison test; Fig 3.1G) of the open field. Suggesting that loss of NCK2 results in hyperactivity in the open field, while loss of NCK1 does not alter activity or exploration levels. These findings are consistent with previous reports in which mice lacking NCK1 or NCK2 had no profound defects in their ability to walk (Fawcett et al., 2007).

Interestingly, NCK1$^{-/-}$ mice have a 1.6-fold increase in the number of defecations during open field exposure when compared to control mice, while NCK2$^{-/-}$ mice defecate at control levels (5.7 ± 0.6, NCK1$^{-/-}$ vs. 3.6 ± 0.7, control vs. 2.9 ± 0.4, NCK2$^{-/-}$; P=0.0101, one-way ANOVA, Tukey's multiple comparison test; Fig 3.1H). The increased number of defecations in the open field is

consistent with an elevated stress and anxiety-like response in rodents (Babu et al., 2008; C. S. Hall, 1934; Taché & Million, 2015; D. V. Wang et al., 2011), suggesting that perhaps mice deficient in NCK1 were having an increased emotional response when exposed to the open field.

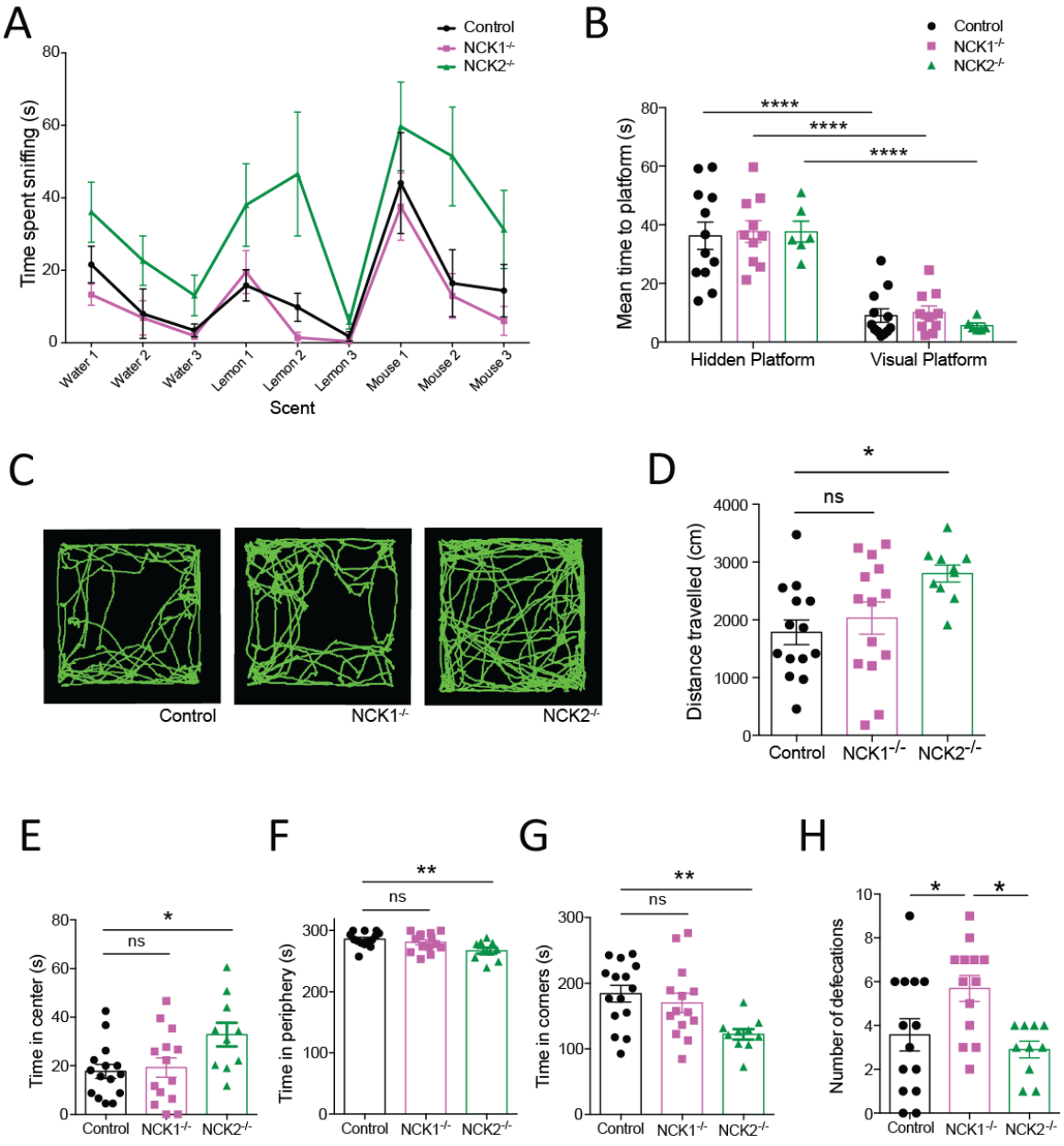

Figure 3.1: Control, NCK1 and NCK2 mutant mice olfactory-ability, visual-ability, and exploration activity in the open field

A. Time spent sniffing an applicator by NCK1 mutant mice (NCK1$^{-/-}$, n=5), NCK2 mutant mice (NCK2$^{-/-}$, n=5) and their wildtype littermates (control, n=5) in a olfactory habituation/dishabituation task B. Average time to escape of control (n=10), NCK1$^{-/-}$ (n=8), and NCK2$^{-/-}$ (n=6) in a hidden platform/visible platform watermaze task. ****p<0.0001 (Two-way ANOVA, Sidak's multiple comparisons test). C. Representative traces of levels of activity and exploration in an open

field (OF). D. Exploration activity of NCK1$^{-/-}$ mice (n=14), NCK2$^{-/-}$ mice (n=10) and their wildtype littermates (Control, n=14) in the OF. E-G. Time spent in center (E), time spent in the periphery (F), or time spent in corners (G) of the OF. H. Number of defecations during OF exposure. ns=not significant, *p<0.05, **p<0.01 (One-way ANOVA, Tukey's multiple comparison test).

3.3.2: NCK1 mutant mice have a context-dependent increase in anxiety-like behaviours and serum corticosterone that can be attenuated with diazepam treatment

To further evaluate if the hyperactivity in the NCK2$^{-/-}$ mice or the increased defecations by the NCK1$^{-/-}$ mice in the open field were indicative of altered emotional response to a novel environment, we next challenged the mutant mice in an elevated plus maze and a light/dark box, two well-established paradigms to assess context-dependent anxiety-like behaviours. In the elevated plus maze, the NCK2$^{-/-}$ mice behave similarly to control mice and spend a control-like amount of time in the closed and open arms (Fig 3.2A-C). However, the NCK1$^{-/-}$ mice spend significantly less time exploring the open arms than their wild-type littermates, with 6 of the 14 animals tested failing to enter the open arms (10.4 ± 4.5s, NCK1$^{-/-}$ mice vs. 30.6 ± 4.9s, control mice vs. 34.6 ± 8.8s; P=0.0128, ANOVA, Tukey's multiple comparisons test; n=14 NCK1$^{-/-}$, 10 control, 10 NCK2$^{-/-}$; Fig. 3.2A,B). The control and mutant mice spend similar amounts of time in the closed arms (204.7 ± 10.2s, control vs. 211.6 ± 20.5s, NCK1$^{-/-}$ vs. 200.5 ± 7.8s, NCK2$^{-/-}$; P=0.8802, ANOVA; n=10 control, 14 NCK1$^{-/-}$, 10 NCK2$^{-/-}$; Fig. 3.1A,C), and all but one mouse explored the full extent of the closed arms confirming that analogous to our observations in the open field there were no overt motor defects preventing the mutant mice from entering the open arms. Similarly, in the light/dark box assay the NCK1$^{-/-}$ mice showed a significant reduction in the number of entries in the light compartment, but the NCK2$^{-/-}$ entered the light compartment at control levels (2.9 ± 0.9, NCK1$^{-/-}$ vs. 7.5 ± 0.8, control vs. 4.8 ±

0.9; P=0.0027, ANOVA, Tukey's multiple comparisons test; n= 11 NCK1$^{-/-}$, 11 control, 10 NCK2$^{-/-}$; Fig. 3.2E). Together, these data reveal that mice deficient in NCK1 display increased anxiety-like avoidant behaviour in both paradigms while mice deficient in NCK2 behave like control mice.

Anxiety-like behaviours are linked to increased activation of the amygdala-hypothalamic-pituitary-adrenal axis (Herman et al., 2003; Holsboer, 1989), with increased risk assessment in mice correlated to increased levels of serum corticosterone (Rodgers et al., 1999). To determine whether the NCK1$^{-/-}$ mice had higher levels of circulating corticosterone at rest, we next measured the baseline levels in serum corticosterone in control and NCK1$^{-/-}$ directly out of their home-cages using Multiple Reaction Monitoring (MRM) tandem mass spectrometry. No difference in serum corticosterone was detected between the control and NCK1$^{-/-}$ mice (30 ± 2ng/ml, control vs. 32 ± 4ng/ml, NCK1$^{-/-}$; P=0.6303, *t* test; n= 10 control, 9 NCK1$^{-/-}$; Fig. 3.2G). We next measured corticosterone levels immediately following a 5-minute exposure to the elevated plus maze, a time-point when the NCK1$^{-/-}$ mice showed a robust behavioural phenotype in the elevated plus maze. Here, both control and NCK1$^{-/-}$ mice showed significant increases in serum corticosterone when exposed to the elevated plus maze; however, the NCK1$^{-/-}$ mice showed a 27 % increase above the serum corticosterone levels of the control mice (66 ± 4ng/ml, NCK1$^{-/-}$ vs. 52 ± 2ng/ml, control; P=0.0121; *t* test; Fig. 3.2H). Together, these data implicate NCK1 in the regulation of context-dependent stress responses and anxiety-like

behaviours and suggest that NCK1 may play a role in the regulation of the amygdala-hypothalamic-pituitary-adrenal axis.

To further validate the loss of NCK1 as anxiogenic, we treated the mouse with the anxiolytic diazepam. We first used control mice to establish a dose of diazepam that was not sedative and did not affect gross motor function or activity levels (data not shown). Once an adequate dose was established, control or mutant mice were injected 5-minutes prior to testing with either saline or diazepam and then subjected to the EPM. Unlike saline treated NCK1$^{-/-}$ mice, diazepam treated mutant mice were able to overcome their anxiety-like phenotype, entering, and exploring the open arms of the elevated plus maze (6.8 ± 2.2s, NCK1$^{-/-}$ saline injected vs. 26.5 ± 8.3s, NCK1$^{-/-}$ diazepam injected, Fig. 3.2J). Diazepam treatment had a significant effect on the amount of time spent in the open arms in both control and mutant mice (P=0.0127, two-way ANOVA, Sidak's multiple comparisons test; Fig. 3.2J).

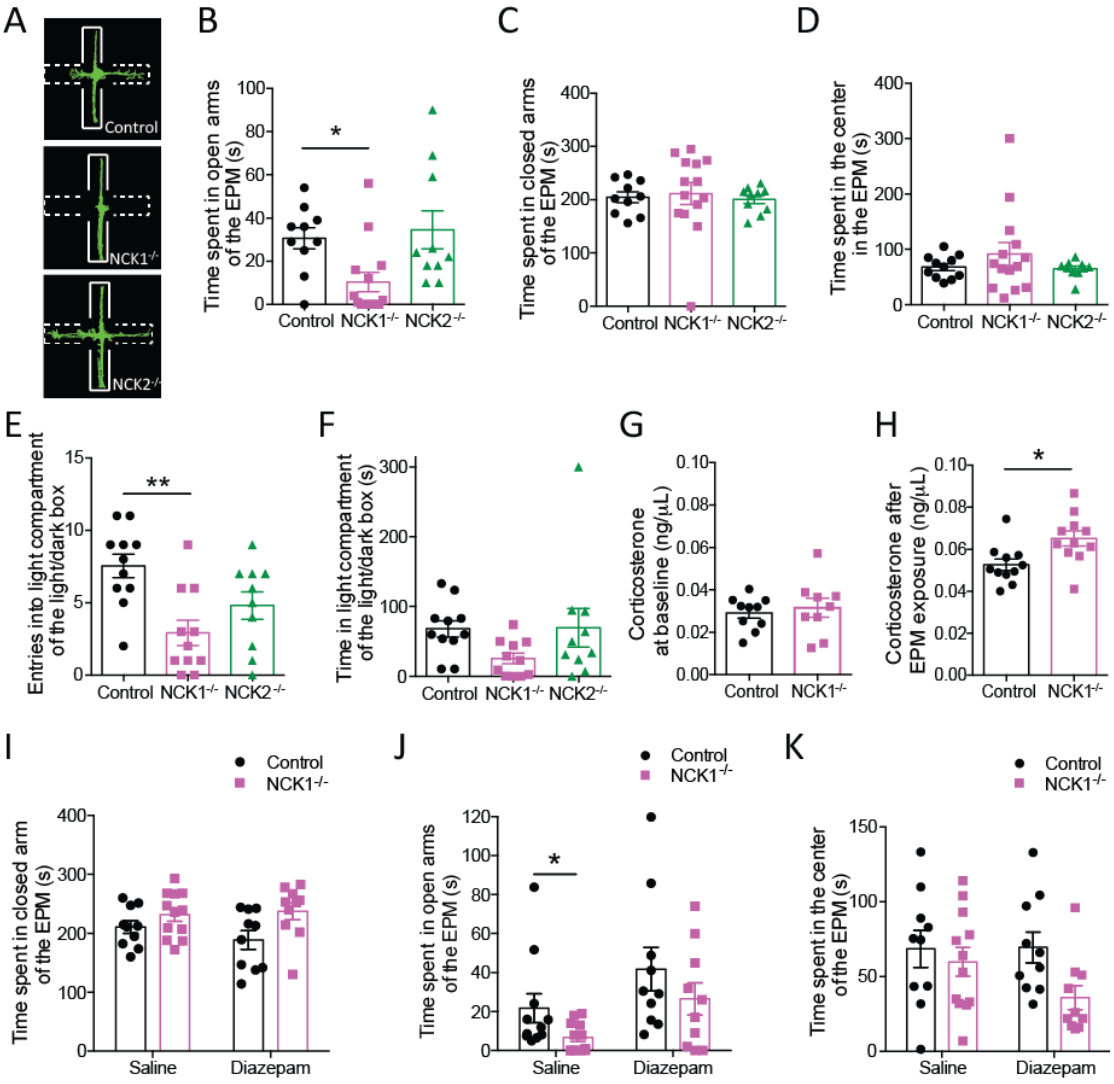

Figure 3.2: NCK1 is necessary for normal suppression of anxiety-like behaviours.

A. Representative traces of a control and an NCK1$^{-/-}$ mouse exploration in the EPM (open arms, x-axis; closed arms, y-axis). B-D. Time spent by NCK1$^{-/-}$(n=14) and their control littermates (n=10) in the closed arms (B), open arms (C), and center (D) of the EPM. E, F. Entries (E) and time spent (F) in the light compartment during the light/dark box test. G. Baseline levels of serum corticosterone levels as measured by LC-MS/MS of control (n=10) and NCK1$^{-/-}$ (n=9) mice. H. Serum corticosterone levels of NCK1$^{-/-}$ (n=11) or control (n=11) following a 5-minute exposure to the EPM. I-K. Time spent in closed arms (I), open arms (J) and center (K) of the EPM by saline injected-control (n=11) and -

NCK1$^{-/-}$ (n=12), and diazepam injected-control (n=10) and -NCK1$^{-/-}$ (n=10) mice. *p<0.05, **p<0.01 (One-way ANOVA, Tukey's multiple comparison test (B-F), Student's t-test (G,H), two-way ANOVA, posthoc one-tailed Student's t-test (J)).

3.3.3: NCK1 is expressed in neurons in the amygdala and cortex

The avoidant behaviour of the NCK1$^{-/-}$ mice in the elevated plus maze and the light-dark box, as well as the increased levels of circulating corticosterone, are consistent with dysregulation in the basolateral amygdala (BLA) circuitry (Herman et al., 2003; Mitra et al., 2009; D. V. Wang et al., 2011). Further, a number of mouse models with impairments in cortical developmental show defects in anxiety-like behaviours and increased serum corticosterone (Fukuda & Yanagi, 2017; S. Li et al., 2018), therefore we next examined whether loss of NCK1 contributed to defects in cortical and BLA development. First, we assessed the expression profile of NCK1 in the brain. Since NCK1 has a high degree of amino acid similarity to NCK2, we could not use specific antibodies to determine NCK1 expression alone. Instead, we took advantage of the IRES-β-galactosidase (β-gal) cassette, which was inserted into the first coding exon of NCK1, and used β-gal expression as a proxy for endogenous NCK1 expression (Bladt et al., 2003).Therefore, β-gal expressing cells in NCK1 heterozygous mice are referred to here as NCK1-positive (NCK1^{+ve}). NCK1 was expressed throughout the entire rostral-caudal axis of the adult brain including the BLA (Fig. 3.3A).

To determine the cell type(s) NCK1 was expressed in we utilized the neuronal marker NeuN, and the microglial marker Iba1, and co-stained with β-gal antibodies. We found that in the motor cortex over 81.0 ± 0.7% of the NCK1^{+ve} cells were NeuN positive, while 94.9 ± 0.4% of the NeuN positive cells were

NCK1^{+ve} (Fig. 3.3B closed arrows, E). Similarly, in the BLA and medial prefrontal cortex (mPFC), regions known to modulate anxiety-like behaviours (Felix-Ortiz et al., 2016; Janak & Tye, 2015; Tovote et al., 2015), we found that in the BLA 81.4 ± 6.6% of NCK1^{+ve} cells were NeuN positive, while 91.2 ± 1.5% of the NeuN positive cells were NCK1^{+ve} (Fig. 3.3C closed arrows, E), while in the mPFC 85.3 ± 2.2% of NCK1^{+ve} cells were NeuN positive, and 89.4 ± 4.0% of the NeuN positive cells were NCK1^{+ve} (Fig. 3.3D closed arrows, E). Interestingly, in all regions analyzed none of the 14-20% of NeuN negative, NCK1^{+ve} cells were Iba1^{+ve}, indicating that NCK1 is not expressed in microglia (Fig 3.3B-D open arrows, E). The remaining NCK1^{+ve} positive cells are likely to be expressed in neurons that do not express NeuN, like the cortical Cajal-Retzius cells, and in a subset of astrocytes as NCK1 has been implicated in human astrocytomas (Deshpande et al., 2019). Nevertheless, taken together this data suggests that NCK1 is primarily expressed in neurons throughout the brain and that 93.8 ± 4.2% off all neurons express NCK1.

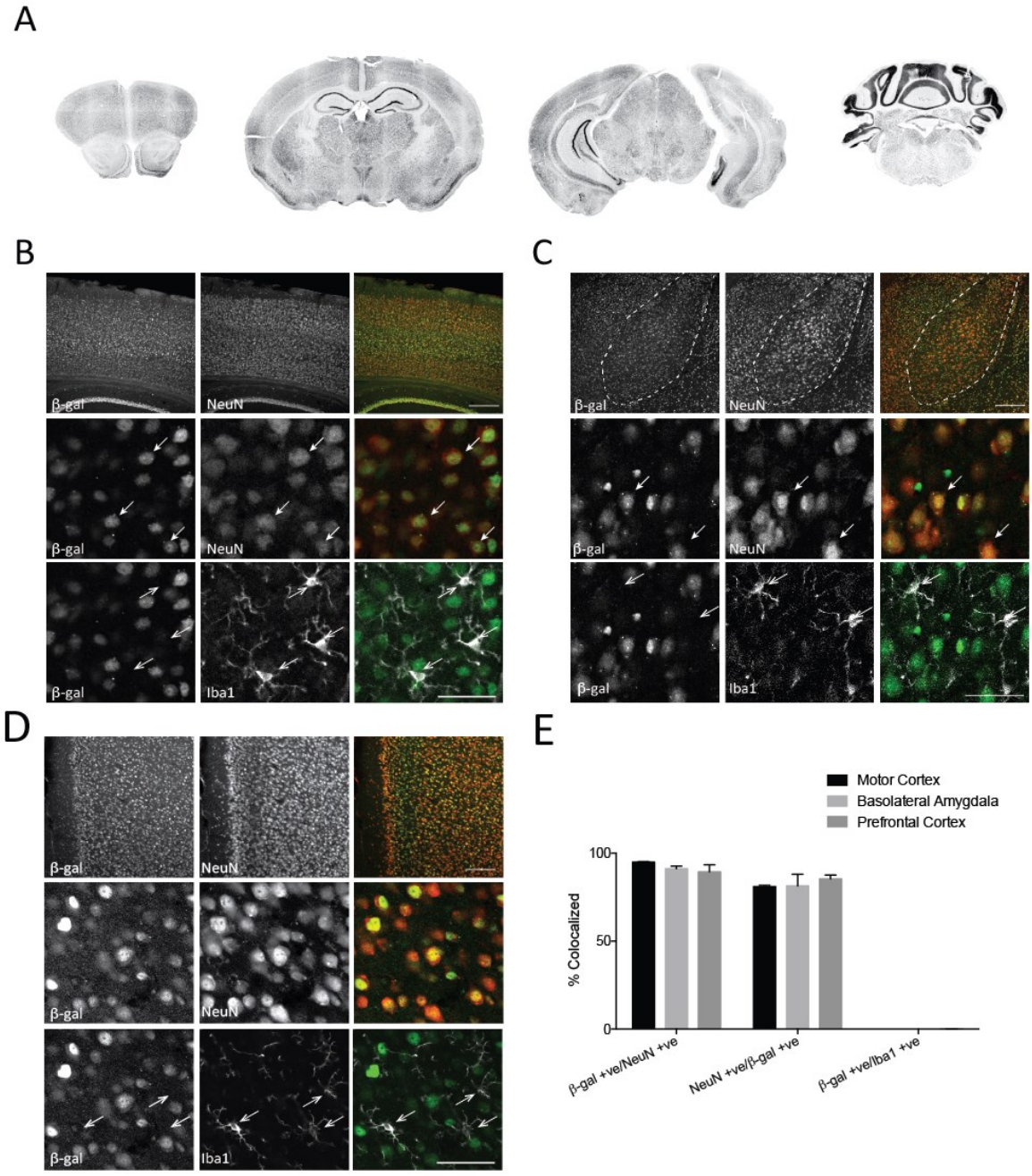

Figure 3.3: NCK1 is expressed in neurons throughout the brain

A. Confocal images of coronal sections of an adult NCK1$^{+/-}$ mouse brain. β-gal positive cells are labeled black. B, C, D. Immunostaining of β-gal (left panel; green) and NeuN (middle panel; red), in the motor cortex (B) basolateral amygdala (C), and prefrontal cortex (D) (upper panels, lower magnification scale bar = 150μm; middle panels higher magnification, scale bar = 50μm). Closed arrows in reveal examples of co-localization. Lower panels, immunostaining of β-

gal (left panel; green) and Iba1 (middle panel; red (open arrows reveal Iba1 positive, β-gal negative cells. E. Quantification of the percentage of colocalized cells expressing β-gal and NeuN, and β-gal and Iba1.

3.3.4: Loss of NCK1 does not impair cortical or amygdalar neuronal development

Given that NCK proteins are important for both cellular division and migration (Bladt et al., 2003; Jacquet et al., 2018), we next asked whether loss of NCK1 affected neuronal proliferation or migration during CNS development. Here we injected the thymidine analogue, 5-ethynl-2'-deoxyuridine (EdU), into pregnant NCK1 heterozygous dames at either E11.5 or E14.5, the height of neurogenesis of the developing basolateral complex of the amygdala and layer II/III of the cortex respectively (Caviness, 1982; Soma et al., 2009). Embryos were harvested 30 minutes post-EdU-injection to monitor effects of loss of NCK1 on proliferation or at E18.5 to assess effects on neuronal migration. No differences were found between mutant and their control littermates in terms of proliferation at E11.5 (49.2 ± 2.2% vs. 50.0 ± 2.5% of cells are EdU positive; P=0.8288; *t* test; n=17 NCK1$^{-/-}$, 12 control; Fig. 3.4A,B), or at E14.5 (21.8 ± 1.6% vs. 17.5 ± 1.2% of cells are EdU positive; P=0.1433; *t* test; n=6 NCK1$^{-/-}$, 6 control; Fig. 3.4C-E), or in migration patterning at E18.5 (P=0.1729; two-way ANOVA; n=6 NCK1$^{-/-}$, 8 control; Fig 3.4F,G). Together, this data suggests that loss of NCK1 does not impair neuronal proliferation or migration during embryonic development.

Since *in vitro* and *in vivo* studies have implicated NCK proteins in the regulation of axonal outgrowth in the spinal cord (Fawcett et al., 2007; C. Lane et al., 2015), we wanted to determine if the anxiety-like behaviours were associated with misguided axons into the basolateral amygdala. To address this, we crossed the NCK1$^{-/-}$ mice with the Thy1-YFP reporter mice. This enabled us to visualize

the stria terminalis, a major fasciculated axon track connecting the amygdala with numerous other brain regions (Shinonaga et al., 1994; Zhu et al., 2016). Interestingly, no differences in tract size were found between control and NCK1$^{-/-}$ mice (P=0.2729; *t* test; n= 10 control, 12 NCK1$^{-/-}$; Fig. 3.4H, I). Further, no defasciculation defects of these tracts were noted therefore, loss of NCK1 does not grossly disrupt axon targeting towards the amygdala via the stria terminalis. Taken together when combined with the apparent typical adult brain size and structure found in NCK1$^{-/-}$ mice, these data suggest that loss of NCK1 likely does not affect the gross development of the brain, or the establishment of critical axonal tracks to the amygdala.

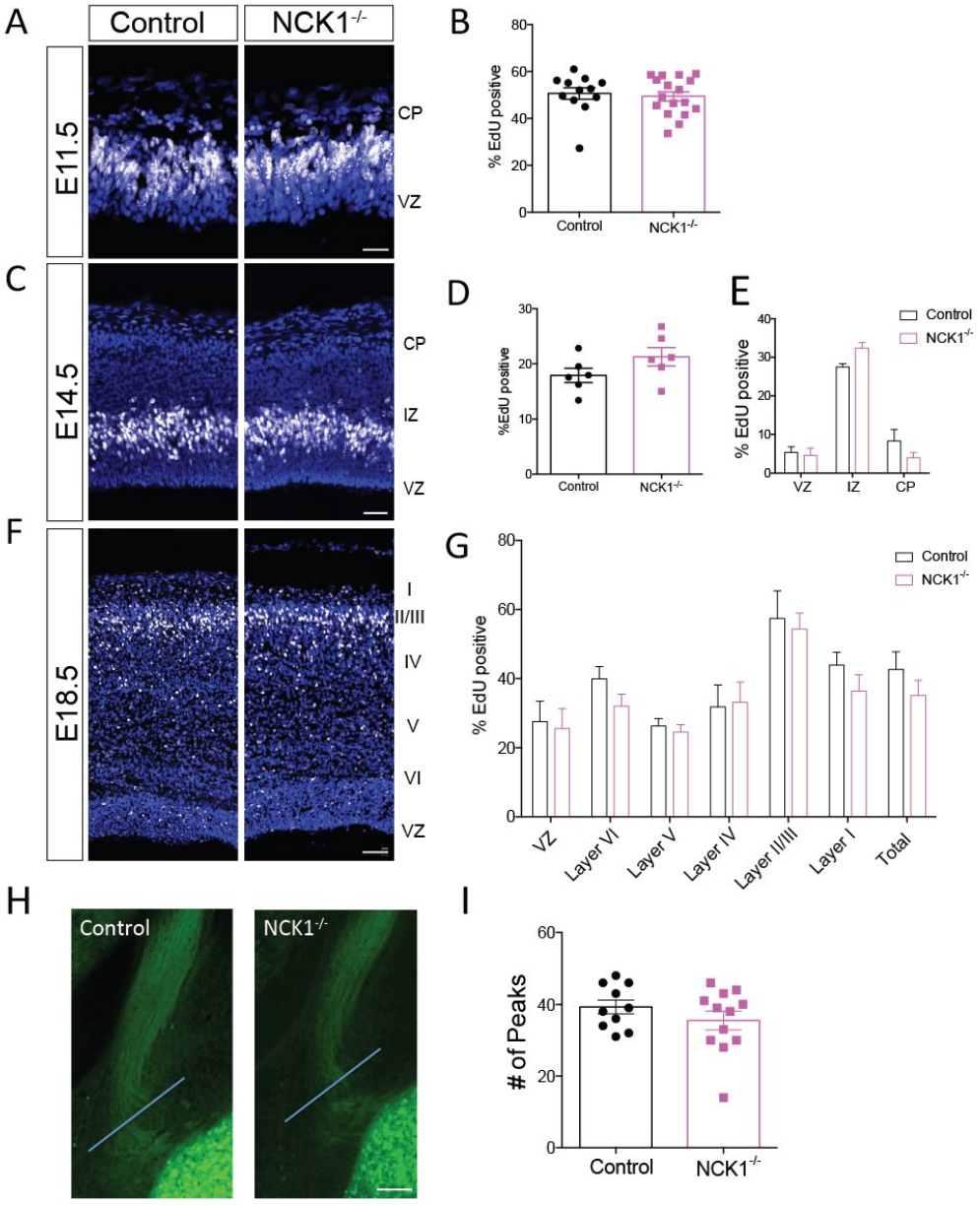

Figure 3.4: Loss of NCK1 does not affect neuronal proliferation, migration, axon targeting, or overall protein expression.

A. Coronal sections from the developing cortex of a control and NCK1$^{-/-}$ E11.5 embryo 30mins following injection of EdU. Sections are labeled with Hoechst (blue) and EdU (white). Scale bar = 25μm B. Quantification of EdU positive. C. Coronal sections of control and NCK1$^{-/-}$ embryo brains harvested 30 minutes following injection of dames with EdU at E14.5. Sections are labeled with

Hoechst (blue) and EdU (white). Scale bar = 25μm D. Quantification of total EdU positive cells. E. Percentage of EdU positive cells in the VZ, IZ, and CP. F. Coronal sections of control and NCK1$^{-/-}$ E18.5 embryo brains following injection of dames at E14.5 with EdU. Scale bar = 100μm G. Quantification of EdU positive cells labeled at E14.5 that have migrated throughout the cortex by E18.5. H. Representative images of Thy1-YFP positive stria terminalis tracts projecting towards the amygdala of wildtype-Thy1-YFP (control) and NCK1 mutant-Thy1-YFP (NCK1$^{-/-}$) mice. Line indicates where fluorescence intensity was measured. Scale bar = 150μm I. Quantification of number of florescence intensity peaks along a line intersecting the stria terminalis. CP= cortical plate, VZ= ventricular zone, IZ= intermediate zone.

3.3.5: NCK1 mutant mice have control levels of synaptic proteins and parvalbumin-positive interneurons in the basolateral amygdala

Our anatomical and morphological work suggested that loss of NCK1 does not adversely affect the gross development of the brain. Nonetheless, a number of studies have shown that dysregulation of local GABAergic circuits within the BLA contribute to the hyperexcitability of principle output neurons of the BLA resulting in anxiety-related behaviours (Babaev et al., 2018; Möhler, 2012; Prager et al., 2016). As such we assessed whether there were gross changes in either inhibitory or excitatory synaptic markers in the BLA or in the prefrontal cortex (PFC). Protein was extracted from control or NCK1$^{-/-}$ BLA and PFC tissue and the levels of a number of excitatory or inhibitory synaptic-associated proteins were assessed. No significant differences were found in the levels of vesicular glutamate transporter 2 (VGluT2), PSD95, glutamate decarboxylase 67 (GAD67), calbindin, parvalbumin, or gephyrin, proteins; nor were there differences in synaptophysin levels or in a phosphorylated N-methyl-D-aspartate receptor (NMDA-R) subunit that has been implicated in NCK dependent signaling (Levy et al., 2018) in the BLA (Fig. 3.5A) or PFC (Fig. 3.5B).

As the majority of inhibitory interneurons in the BLA are parvalbumin-positive and over 40% synapse directly onto the soma of principal output neurons and function to mediate feedback and feedforward inhibition (Babaev et al., 2018), we next focused on the activity of these neurons. Consistent with the overall levels of parvalbumin protein (Fig. 3.5A), the NCK1$^{-/-}$ mice had a similar number of parvalbumin positive neurons in the BLA as their wild type littermates

(8.1 ± 0.8% vs. 9.2 ± 1.0%; P=0.4077, *t* test; n= 12 $NCK1^{-/-}$, 12 control; Fig. 3.5C,D). Together, these data suggest that loss of NCK1 is not altering expression levels a number of proteins known to be involved in synaptic signaling.

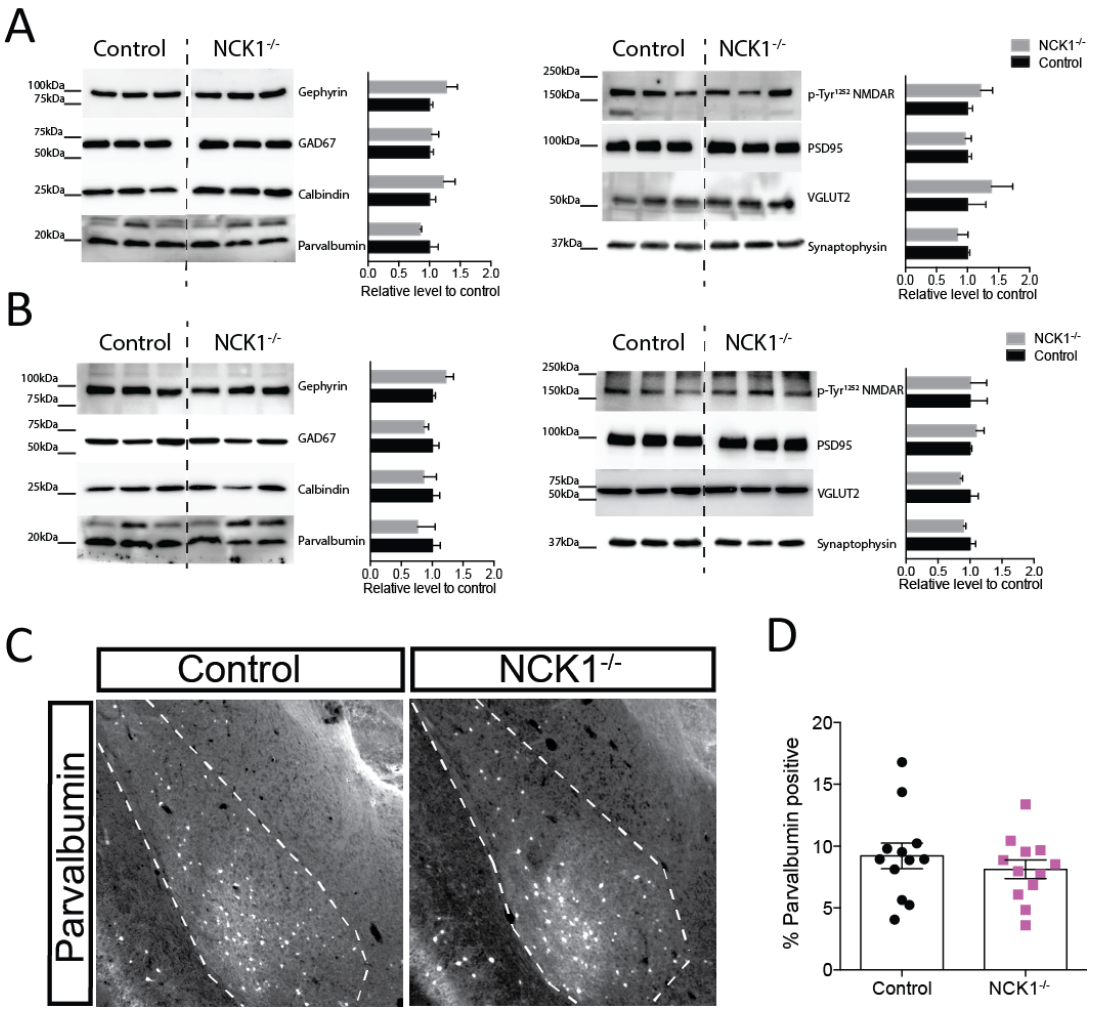

Figure 3.5: NCK1 mutant mice have control levels of synaptic proteins and parvalbumin-positive inhibitory interneurons. A-B. Western analysis and quantification of protein expression in the amygdala (A) and prefrontal cortex (B) of control and NCK1$^{-/-}$ mice. C. Representative images of parvalbumin positive neurons in the basolateral amygdala of control and NCK1$^{-/-}$ mice. Broken white lines outline the basolateral amygdala. D. Quantification of parvalbumin positive cells as a percentage of all Hoechst labeled nuclei.

3.3.6: NCK1 mutant mice have decreased activation of medial prefrontal cortex neurons and parvalbumin-positive interneurons in the basolateral amygdala during exposure to the elevated plus maze

Next, naïve control and NCK1$^{-/-}$ mice were exposed to the elevated plus maze, their brains were perfused, sectioned, stained and c-Fos expression was quantified as a measure of neuronal activation. Consistent with the similar levels of motor activity seen in our mice, mutant mice had control levels of c-Fos activation in the motor cortex (26.7 ± 1.6% vs. 26.9 ± 3.1%; P=0.9477, *t* test; n= 9 control, 9 NCK1$^{-/-}$; Fig. 3.6A, B). Surprisingly, the number of neurons expressing c-fos in the BLA was also similar in both the control and NCK1$^{-/-}$ mice (15.1 ± 2.4%, control vs 20.3 ± 3.1%, NCK1$^{-/-}$; P=0.1929, *t* test; n= 9 control, 10 NCK1$^{-/-}$; Fig. 3.6C, D). However, there was a 22.0% reduction in the number of c-Fos positive cells in the mPFC of NCK1$^{-/-}$ mice compared to wildtype controls (19.6 ± 2.2%, NCK1$^{-/-}$ vs 25.2 ± 1.5%, control; P=0.0481, *t* test; n= 6 NCK1$^{-/-}$, 10 control; Fig. 3.6E, F). Interestingly, although the overall number of c-Fos expressing cells in the BLA was unchanged between control and NCK1$^{-/-}$ mice, the percentage of parvalbumin-positive (PV^{+ve}) interneurons which were activated during EPM exposure was reduced by 44% in NCK1$^{-/-}$ mice when compared to their wild-type littermates (10.7 ± 2.3% vs. 19.5 ± 3.1%; P=0.0391, *t* test; n= 8 NCK1$^{-/-}$, 6 control; Fig. 3.6G, H) suggesting that mice deficient in NCK1 had differential activation of BLA cell populations from control mice. Therefore, together these data demonstrate that NCK1$^{-/-}$ mice have a reduction in neuronal activation in the mPFC and in PV^{+ve} inhibitory interneurons in the BLA during

EPM exposure that suggests NCK1-deficiency results in a reduction in inhibitory control of the BLA when challenged with an approach-avoidance conflict paradigm.

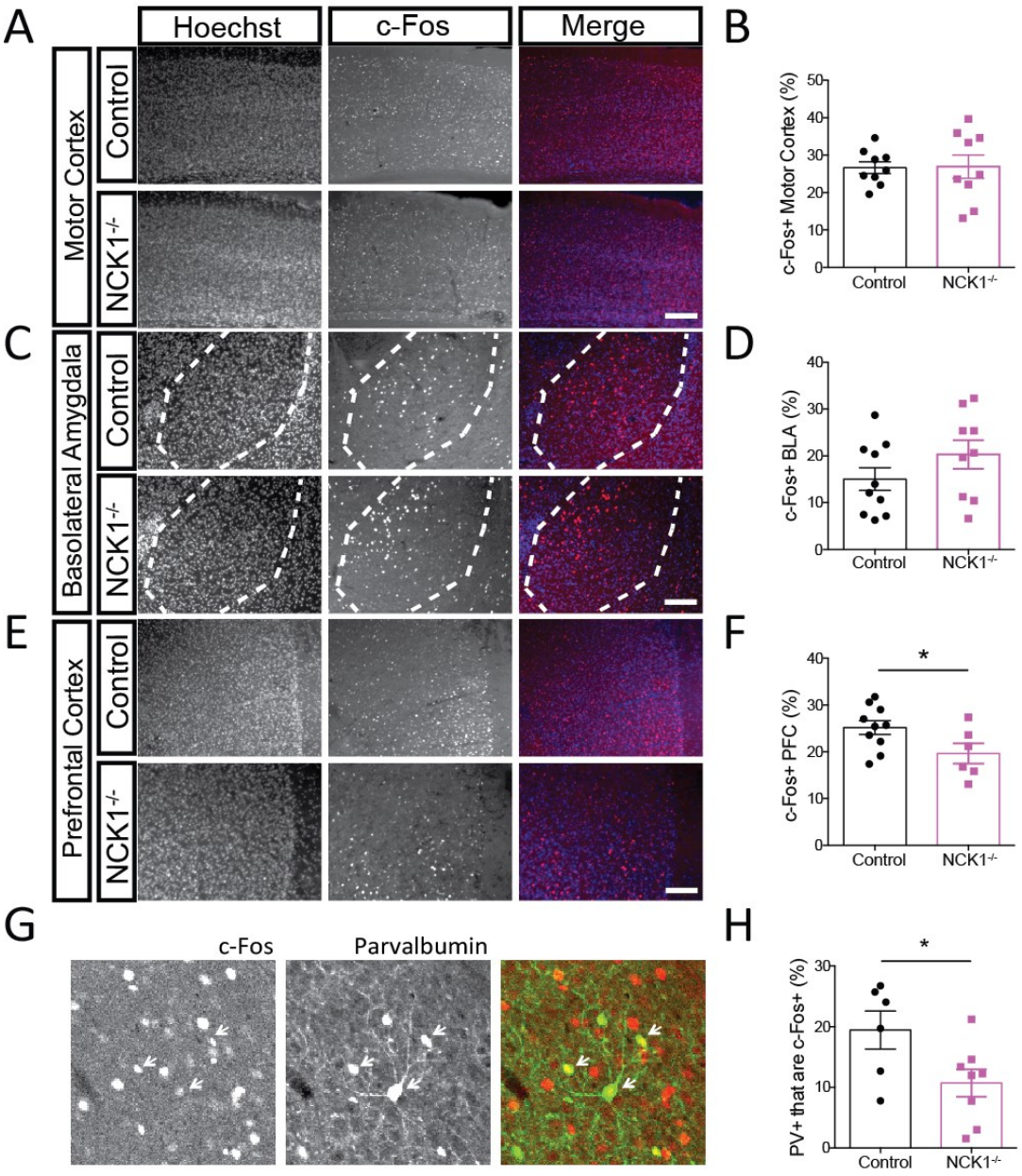

Figure 3.6: c-Fos expression analysis in the motor cortex, BLA, and mPFC during elevated plus maze exposure in NCK1$^{-/-}$ and control mice

A, C, E. Representative images of coronal sections of control and NCK1$^{-/-}$ motor cortex (A), basolateral amygdala (C), and prefrontal cortex (E) after elevated plus maze exposure. All nuclei are labeled with Hoechst (blue) and c-Fos expressing neurons are shown in center panels (red). Broken lines outline the basolateral amygdala. Scale bars = 150μm. B, D, F. Quantification of c-Fos expression as a percentage of total cells of control and NCK1$^{-/-}$ mice in the motor cortex (B),

basolateral amygdala (D), and prefrontal cortex (F). G. Representative image of a c-Fos positive (first panel, red) and parvalbumin positive (second panel, green) neurons. White arrows point to neurons that are both parvalbumin and c-Fos positive. Scale bar = 25μm H. Quantification of percentage of parvalbumin positive cells which are c-Fos positive. *$p<0.05$ (Student's t-test).

3.3.7: NCK1 mutant mice have a loss of dendritic spine density in the basolateral amygdala

Given the anxiety-like phenotype in the NCK1 mutant mice was linked with changes in c-Fos expression of parvalbumin-positive cells, we next asked whether there were changes in synaptic integrity within the amygdala region. Indeed, anxiety behaviours have been linked to the disruption of excitation/inhibition balance within the amygdala including defects associated with synaptic function in both inhibitory (Babaev et al., 2018) and excitatory (Fragale et al., 2016; B. Leuner & Shors, 2013; Shonesy et al., 2014) neurons. The principal excitatory neurons of the basolateral amygdala are spiny and function to integrate information coming from various inputs involved in sensory processing, executive functioning, and memory (Janak & Tye, 2015; Tovote et al., 2015). The principal spiny neurons then project to the central nucleus of the amygdala. The central nucleus of the amygdala is a region known to regulate hypothalamic nuclei involved in the further regulation of hormone release and other aspects of the stress response (Janak & Tye, 2015; Tovote et al., 2015). To assess synaptic integrity, we quantified dendritic spine density in the BLA, as dendritic spine structure depends on dynamic actin and given NCK1's role as an actin regulator. Loss of NCK1 resulted in a 22.8% decrease in dendritic spine density in the principal spiny neurons of the basolateral amygdala (1.33 ± 0.09 spines/μm, control vs. 1.03 ± 0.07 spines/μm, NCK1$^{-/-}$, P=0.0178, *t* test, n=8 control, 9 NCK1$^{-/-}$; Fig. 3.7B). However, there were no differences in the ratio of dendritic spine morphology between the mutants and their wildtype littermates

when grouped as filapodia-like (P=0.2064, *t* test; Fig. 3.7C), mushroom shaped (P=0.7211, *t* test; Fig. 3.7D), thin (P=0.8581, *t* test; Fig. 3.7E), or stubby (P=0.1751, *t* test; Fig. 3.7F). Together, this data suggests that loss of NCK1 is leading to a general decrease in spine density of the principal excitatory neurons in the BLA.

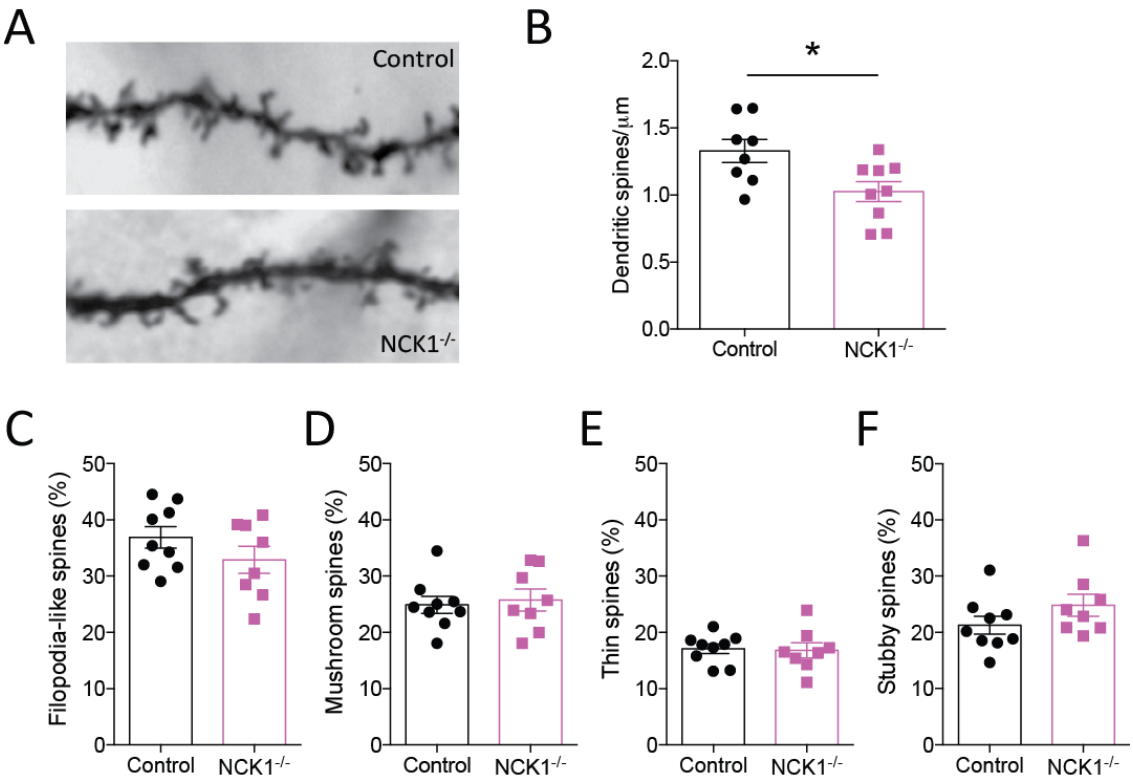

Figure 3.7: NCK1 mutant mice have decreased dendritic spine density in the basolateral amygdala. A. Representative images of control and NCK1 mutant dendrites of the principal spiny neurons of the basolateral amygdala. B. Quantification of dendritic spine number per 1 μm length of dendrite in the basolateral amygdala. C-F. Quantification of the percentage of total dendritic spines that are filapodia-like (C), mushroom shaped (D), thin (E), or stubby (F). *p<0.05 (student's t-test).

3.4: Discussion

Here we find that deficiency in the adaptor protein NCK1, but not NCK2, leads to anxiety-like behaviours when mice are challenged with approach-avoidance conflict-based tasks (Fig 3.2A-F). The increase in avoidance behaviour is also reflected in a significant increase in the levels of serum corticosterone, following exposure to the elevated plus maze (EPM), above those achieved in similarly treated control mice (Fig 3.2G,H). Further, treatment with diazepam a positive allosteric modulator of GABA-A receptors was able to attenuate the increased anxiety-like response in the NCK1$^{-/-}$ mice and control mice (Fig 3.2I-K), suggesting that the anxiety behaviour is unlikely due to gross defect in neuronal circuitry in the mutant mice. Dysregulation of neuronal activity within the BLA has been shown to result in increased activation of the hypothalamus-pituitary-adrenal axis (Kolber et al., 2008; Shinonaga et al., 1994) and is consistent with the increase in circulating corticosterone and anxiety-like behaviours we observe in the NCK1$^{-/-}$ mice during EPM exposure. Interestingly, c -Fos analysis in control and NCK1$^{-/-}$ mice revealed differential activation in inhibitory interneurons in the BLA during EPM exposure (Fig 3.6G,H), while loss of NCK1 also resulted in decreased dendritic spine density in the principal spiny neurons of the BLA (Fig 3.7A,B) pointing to a novel function for NCK1 in influencing the normal excitatory/inhibitory balance within the CNS.

The genetic disruption of *NCK1* results in a context dependent presentation of anxiety-like avoidant behaviours and hormonal stress response. The mutant mice are not chronically stressed and in their home cage

environments have control levels of circulating corticosterone (Fig 3.2G). They also do not display increased anxiety-like thigmotaxis or freezing behaviours in the open field (Fig 3.1C-G). Instead, the NCK1$^{-/-}$ mice are over-responding to the perceived threat of the unknown open environments when faced with a choice to leave the safe known environment for the unknown open environment. Importantly, no deficits were observed when the mutant mice were assessed for gross sensory (olfaction and vision) and locomotor abilities, including general activity levels (Fig 3.1A-D). Together this would suggest that the increased stress response and accompanying anxiety-like behaviours are due to differences in internal state between the NCK1$^{-/-}$ mice and their control littermates resulting in increased risk assessment and an increased drive towards avoidant behaviour.

The increased risk assessment, avoidant behaviours and stress hormone levels in NCK1$^{-/-}$ mice are associated with differential activation of neuron populations in the BLA. The BLA functions as a point of convergence of external sensory and pain processing with internal memory and higher order decision making processing and gives emotional valance to the current situation resulting in either approach or avoidance behaviours (Janak & Tye, 2015; O'Neill et al., 2018). Interestingly, overall BLA activation is similar in NCK1$^{-/-}$ mice and control (Fig 3.6C,D). However, loss of NCK1 results in decreased activation of the regulatory parvalbumin-positive inhibitory interneurons and therefore different populations of neurons are being activated in the mutant and control mice (Fig. 3.6C,G,H).

Interestingly, NCK1$^{-/-}$ mice have reduced activation of medial prefrontal cortex (mPFC) neurons during EPM exposure (Fig 3.6E,F). The mPFC and BLA share reciprocal projections and have been shown to regulate each other's activity leading to changes in behaviour (Felix-Ortiz et al., 2016). Consist with our findings others have reported that pharmacological inactivation of the mPFC leads an anxiogenic effect in the EPM and light dark box (de Visser et al., 2011; Lisboa et al., 2010). However, given that NCK1 is expressed throughout the brain (Fig 3.3A), we cannot rule out that the decreased activation of the inhibitory interneurons is not due to impaired activation from other associated brain nuclei that have also been shown to regulate BLA activity, or whether NCK2 can functionally compensate for the loss of NCK1 in certain pathways and brain nuclei but not others. However, loss of NCK1 does not affect the levels of NCK2 transcript within CNS tissues (C. Lane et al., 2015), therefore there does not appear to be a rebound upregulation of NCK2 in neurons deficient of NCK1. Interestingly, we also find that NCK2-deficient mice do not display increased anxiety-like behaviour and behave similarly to control mice in the EPM and light/dark box (Fig 3.2A-F) and they have hyperactivity in the open field (Fig 3.1C,D). Therefore, NCK1 and NCK2 have some non-redundant roles in the adult brain.

Previous work has shown that NCK1 controls the translation of proteins involved in ER stress, implicating NCK1 in translation dependent events (Kebache et al., 2002). However, we find that NCK1 is not an important regulator of either excitatory or inhibitory synaptic proteins (Fig 3.5A,B) suggesting that the

differences in GABAergic interneuron activation are unlikely due to changes in overall synaptic protein translation. Further, given that a GABA-A positive allosteric modulator is effective in reducing the anxiety-like response back to control levels (Fig 3.2J) would suggest that major circuitry involved in regulating anxiety-like behaviours are present in the NCK1$^{-/-}$ mice. Therefore, loss of NCK1 is likely leading to decreased efficiency in synaptic circuitry and induces either a hyperactive anxiogenic response or conversely a hypoactive anxiolytic response.

Although NCK1 is widely expressed throughout the CNS, the increase in anxiety-like behaviours are unlikely attributable to gross developmental or morphological defects given that NCK1-deficient mice have normal adult brain structure and there are no differences in embryonic neuronal proliferation or migration (Fig 3.4A-G). Again, pointing to NCK1-deficiency as leading to improper activation of the avoidance promoting brain circuitry. Interestingly, our findings parallel findings in humans that suggest that although anxiety disorders are associated with neurodevelopmental disorders and the dysregulation of amygdala activity (Dichter et al., 2012; Schumann et al., 2011), other studies fail to detect developmental or anatomical defects within the amygdalae (Schumann et al., 2011; Warnell et al., 2018).

Nonetheless, anxiety behaviours are linked to the disruption of excitation/inhibition balance within the amygdala (Prager et al., 2016; Schumann et al., 2011). In this context, NCK proteins have been shown to bind the GluN2B subunit of NMDA-R's, and are important for their function (Levy et al., 2018). Thus, loss of NCK1 may affect the frequency of NMDA-R currents, which may

contribute to the decreased activation of the inhibitory BLA neurons we observe in the NCK1$^{-/-}$ mice. In addition to the potential defects in excitatory function, NCK1 may have a direct effect on GABAergic synaptic transmission by regulating endocannabinoid signaling. Recently, p21 activated kinase 1 (PAK1), an NCK1 associating protein, has been shown to affect GABA release by regulating cyclooxygenase-2 (Cox-2) levels and endocannabinoid signaling (Xia et al., 2018). Future work will help resolve these issues.

Finally, the reduction in dendritic spine density seen in the mutant mice, implicates NCK1 in synaptic function and/or synaptic plasticity, which have been implicated in mood disorders (Yan et al., 2016). NCK proteins have been shown to function downstream of Eph receptor signaling and B-type ephrin signaling and have been implicated in axon guidance and spinogenesis to establish neuronal circuits. Mice lacking Ephrin-B3 (Zhu et al., 2016), Ephrin-A5 (Sheleg et al., 2017) or EphB2 (Attwood et al., 2011) show abnormal behaviours in the elevated plus maze implicating Eph/ephrin signaling as an important regulator of anxiety-like behaviours. However, unlike the NCK1$^{-/-}$ mice, Eph/ephrin mutant mice show more entries and time spent in open arms suggesting that Eph/ephrin signaling contributes to a hyperactive state favouring approach over avoidance. Also, NCK proteins have been shown to be important in DCC signaling (C. Lane et al., 2015; X. Li et al., 2002), and up-regulation of neuronal DCC has been shown to cause vulnerability to stress-induced disorders in humans and stress-induced social avoidance and anhedonia in mice (Torres-Berrío et al., 2017). Thus, it is possible that loss of NCK1 results in aberrant DCC dependent

signaling, which contributes to the anxiety-like phenotype. Further dissection of DCC dependent signaling in the BLA may be informative in this regard.

Together, our work contributes a novel role for NCK1 in controlling anxiety-like behaviours by regulating the excitatory/inhibitory balance of amygdala circuits and is consistent with GWAS pointing to NCK1 as a modulator of worry in humans. Further, our work points to this animal model as a useful preclinical tool for the study of novel anxiolytics.

Chapter 4: General Discussion

I hypothesized that the NCK1 adaptor protein functions in CNS tissue to influence actin dynamics affecting dendritic spine development and morphology and NCK1 deficiency would lead to the dysregulation of neuronal circuits important for learning, memory, and behaviour.

To address this, I had the four following aims -

1. Determine if there are behavioural deficits in the NCK1-deficient mice.
2. Determine the regional and cellular distribution of NCK1 in the adult mouse brain.
3. Determine if NCK1 plays a role in cell proliferation and/or migration in the mouse brain.
4. Determine if NCK1 plays a role in dendritic spine formation and/or normal synapse morphology.

In Chapter 2, I report that NCK1 stabilizes neuronal actin dynamics to promote dendritic spine, synapse and memory formation. Using NCK1-mutant mice and comparing them to their wildtype littermates, I find that loss of NCK1 results in deficits in social recognition, spontaneous alterations in the Y-maze, and spatial learning and memory in the Morris Water maze. Therefore, mice deficient in NCK1 have memory impairments in three tasks that assess three different forms of memory (short term, working, and spatial learning, respectively), but that have all been associated with hippocampal function. Consequently, further analysis was focused on the hippocampus. In the adult hippocampus, I find that NCK1 is predominately expressed in neurons. Indeed,

99% of hippocampal neurons are NCK1-positive, while all iba1-positive microglia and Sox2-positive progenitor cells of the dentate gyrus are NCK1-negative. Interestingly, this expression pattern suggests that in the granule cells of the dentate gyrus NCK1 is turned on post-mitotically and therefore only after neuronal differentiation has occurred. Similarly, in the embryo we find that loss of NCK1 has no effect on neuronal proliferation or early neuronal migration and embryonic hippocampal formation. Further, given that NCK1 is not present in microglia and predominately present in neurons, the memory deficits present in the NCK1 mutant mice are likely due to a neuronal-mediated mechanism. Indeed, mice deficient in NCK1 have decreased hippocampal dendritic spine and synapse density. Although interestingly, in the synapses that do form, they have thicker post-synaptic densities. Similarly, cultures of dissociated hippocampal neurons from NCK1-deficient embryos also show a reduction in dendritic spine density with larger post-synaptic densities in the dendritic spines that do form. Again, this would suggest NCK1 acts in dendritic spine formation or maintenance by some neuronal intrinsic mechanism. Finally, we show that loss of NCK1 results in increased actin turnover and polymerization in dendritic spines, suggesting that NCK1 plays a role in maintaining actin network stability in dendritic spines of hippocampal neurons.

In Chapter 3, I report that NCK1 regulates amygdala activity to control context-dependent stress response and anxiety-like behaviours. Again using NCK1-mutant mice and comparing them to their wildtype littermates, I find that loss of NCK1 does not impair gross olfactory or visual ability, nor does it alter

general activities levels in an open field. However, mice deficient in NCK1 display increased anxiety-like avoidant behaviours in the elevated-plus maze (EPM) and light/dark box. NCK1-deficient mice also have increased serum corticosterone after EPM exposure but not at baseline when the mice are taken directly from their homecages, suggesting that they are not chronically stressed but are instead over-responding in the EPM. Interestingly, treatment of NCK1-deficient mice with the anxiolytic diazepam reduces the anxiety-like behaviour and promotes control-levels of exploration in the open arms, further validating the anxiety-like phenotype in the mutant mice and suggesting that basic GABAergic circuitry is still functional with NCK1 deficiency. Next, I report that NCK1 is ubiquitously expressed in neurons throughout the brain and that NCK1 deficiency does not impair embryonic neuronal proliferation, migration or the formation of the primary axon tract targeting the amygdala. Similarly, NCK1 mutant mice have control levels of synaptic protein expression in the prefrontal cortex (PFC) and basolateral amygdala (BLA), and control levels of parvalbumin-positive inhibitory interneurons in the BLA. However, during EPM exposure NCK1 mutant mice show decreased neuronal activation in the PFC and in the parvalbumin positive inhibitory interneurons of the BLA, suggesting a reduction in inhibitory control of the principal glutamatergic neurons of the BLA. Interestingly, in mice deficient of NCK1 the principal glutamatergic neurons of the BLA have reduced dendritic spine density. Given the control levels of synaptic proteins, it is possible that loss of NCK1 is impairing the organization of synaptic proteins into dendritic spines.

Altogether, and to directly address my initial aims, I find that 1. NCK1 mutant mice do have behavioural deficits resulting from impairments in memory, learning, and neuronal activation, 2. NCK1 is ubiquitously and predominately expressed in neurons throughout the brain, 3. NCK1 is not necessary for embryonic neuronal proliferation, migration, and brain formation, and 4. NCK1 deficiency results in decreased dendritic spine density in both the hippocampus and basolateral amygdala.

4.1: Non-redundant roles of NCK1 and NCK2 in memory and anxiety-like behaviour

Although many studies interchange NCK1 and NCK2 due to the high degree of similarity in terms of amino acid identity in their protein binding domains (M. Chen et al., 1998) and their functional redundancy in embryonic development (Bladt et al., 2003), here I report that NCK1 and NCK2 are not functionally redundant in spatial learning, memory formation, activity levels, and anxiety-like behavioural responses. Loss of NCK1 or NCK2 results in impaired social recognition and a decreased percentage of spontaneous alternations in Y-maze (Fig 2.1). However, in the Morris water maze, NCK1, but not NCK2, is necessary for proper spatial learning and memory, as mice deficient in NCK1 do not learn which quadrant the escape platform is located in, while NCK2 deficient mice are able to learn and remember which quadrant the platform is located in by the third day of testing (Fig 2.2). Interestingly, while $NCK1^{-/-}$ mice have control levels of activity in the open field, $NCK2^{-/-}$ mice are hyperactive (Fig 3.1). Further,

NCK2 deficiency does not lead to increased avoidant behaviours (Fig 3.2). Therefore, although both NCK1 and NCK2 deficiency results in impaired short-term and working memory, only NCK1 deficiency impairs spatial learning and increases avoidant behaviours, while NCK2 deficiency results in hyperactivity. This would suggest that genetic deletion of NCK1 or NCK2 are leading to two distinct endophenotypes.

The differences in behavioural output between NCK1 and NCK2 mutant mice suggest that these proteins are functioning in distinct signaling pathways or brain circuits. Indeed, early protein expression studies suggested that NCK2 was less abundant in the adult mammalian brain when compared to NCK1 (Bladt et al., 2003), and others suggest that while NCK1 is equally abundant in the embryo and adult brain, NCK2 is slightly more abundant in the embryonic brain than in the adult (Thévenot et al., 2011). Future work focusing on the regional expression of NCK2 in the brain will provide more insight into the main driver of these behavioural differences.

Similarly, future work better elucidating neuronal specific signaling partners of NCK1 and NCK2 will also help refine our understanding of the mechanisms driving the behaviours they regulate. Indeed, although NCK1 and NCK2 have been shown to interact with many of the same proteins (Table 1), neuronal specific binding partners have also been documented. For example, in cultured neurons from mouse forebrains NCK2, but not NCK1, was shown to interact with phosphorylated disabled-1 (Dab-1), downstream of reelin signaling (Pramatarova et al., 2003). Reelin signaling is important in embryonic neuronal

migration during development and in neurotransmission, memory formation and synaptic plasticity in the adult brain (Fatemi, 2005). Reelin stimulation redistributes NCK2 from the soma to neuronal processes, while Dab1 phosphorylation recruits NCK2 to the membrane and leads to actin remodeling (Pramatarova et al., 2003). Interestingly, evidence also suggests that even though NCK1 and NCK2 may interact with the same protein, they can bind with different affinities and mediate different cellular responses. For example, although in neurons both NCK1 and NCK2 can bind PAK3, NCK2 preferential binds to PAK3 (Thévenot et al., 2011). Interestingly, NCK2 binds PAK3 independent of its kinase activity, and the PAK3-NCK2 complex does not influence dendritic spine or synapse formation, but instead regulates excitatory postsynaptic currents in the cortex and spontaneous synaptic transmission leading to decreased miniature excitatory postsynaptic currents (mEPSCs) in hippocampal neurons (Thévenot et al., 2011). Future studies looking at the different roles played by NCK1 and NCK2 to regulate neuronal excitability *in vivo* may provide greater insight into how genetic deletion of two similar proteins can lead to such distinct behavioural phenotypes as increased avoidant behaviours versus hyperactivity.

4.2: Genetic influences on actin dynamics can alter cognitive function

Here, I find that genetic disruption of *NCK1* leads to changes in cognition and altered behaviour, more specifically, memory and learning deficits, as well as increased avoidant behaviour. Interestingly, NCK1 deficiency also leads to

decreased dendritic spine density of principal output neurons of the hippocampus, a brain region important for memory formation, and the basolateral amygdala (BLA), a region important for modulating avoidant behaviours. Further, the loss of dendritic spine density on the hippocampal neurons is likely cell-autonomous since *in vitro* cultures of disassociated hippocampal neurons also had a reduction in dendritic spine density. Therefore, genetic disruption of *NCK1* is directly able to alter neuronal structure and these structural changes are likely contributing to the changes in cognition and altered behaviour. I propose that the mechanism by which NCK1 functions in the CNS is to stabilize actin-dynamics in dendritic spines (Fig. 2.7), consistent with previous observations in heterologous cells (Buvall et al., 2013; Garg et al., 2007). Importantly, actin stabilization is critical for synapse formation as well as for the trafficking and localization of AMAP receptors into the postsynaptic membrane (S. Basu & Lamprecht, 2018; Hanley, 2014; O. L. Johnson & Ouimet, 2006; Korobova & Svitkina, 2010). Indeed, NCK1 deficiency results in overall decreased synapse density in the hippocampus, as well as decreased activation of the prefrontal cortex and inhibitory interneurons in the BLA during exposure to the elevated plus maze. Interestingly, however, genetic disruption of *NCK1* does not impair gross motor or sensory function, nor does it impair activity levels or result in any outwardly visible changes in the mice, including weight or their ability to breed. This would suggest that NCK1-deficiency in mice is heritable and could be spread through a population adding to the variability in cognitive performance seen across the population.

It has become clear that genetic differences between individuals account for the variation in cognitive abilities in a population. In modern human populations, genetic differences are thought to account for 50-70% of the variance on tests of cognitive performance (Davies et al., 2011; Tucker-Drob et al., 2013). However, the mechanisms by which genes influence cognition and its disorders remain obscure (Chabris et al., 2012; Payton, 2009). Genome wide association studies (GWAS) have identified the polygenic nature of cognitive ability and predisposition to psychiatric disorder. Therefore, it is likely that a number of variations, sometimes in hundreds of different genes, are converging to produce changes in behaviour. Thus, focus should be placed on the functional roles of these loci and more specifically on network and pathway analysis at points of molecular convergence. Indeed, NCK1 function falls into two potential nodes of molecular convergence that have been identified by GWAS as associated with cognitive function and neuropsychiatric disorders, one, as an actin regulator in neurons and, two, as a potential synapse organizer (Grant, 2019; Lima Caldeira et al., 2019; Ribic & Biederer, 2019; van Spronsen & Hoogenraad, 2010). Human GWAS have associated *NCK1* variants with schizophrenia and as an enhancer of worry (Luciano et al., 2018; Nagel et al., 2018; Ripke et al., 2014). Although the NCK1 deficient mice are not chronically stressed, future work assessing their behaviour and well-being over their lifespan and into senescence, as well as in different enriched or stressful environments, will better address if NCK1 deficiency predisposes the CNS to more rapid decline or severe disorder.

4.3: The relationship between memory and anxiety

NCK1 deficiency results in both memory impairment and increased anxiety-like behaviours. Although a link between anxiety and memory has long been proposed, several inconsistent findings have accumulated in the literature (Moran, 2016). For example, a number of studies have shown that anxiety impairs working memory capacity (Ashcraft & Kirk, 2001; Darke, 1988; Maloney et al., 2010; Shackman et al., 2006), while others have shown no association between working memory capacity and anxiety (Moritz et al., 2002; Salthouse, 2012), and yet others have found that anxiety improves working memory capacity (Moriya & Sugiura, 2012). Some discrepancies in the literature are likely due to differences in anxiety-assessment in participants including in the behaviours and severity studies chose to include. Therefore, properly defining anxiety-like behaviours is essential.

Importantly, anxiety is not fear (Janak & Tye, 2015; Tovote et al., 2015). Fear is a learned response requiring memory formation, maintenance, and storage (Janak & Tye, 2015; Tovote et al., 2015). Anxiety is defined as a sense of apprehension to the unknown, realized in the NCK1 mutant mice as the avoidant behaviour produced in both the elevated-plus maze (EPM) and the light/dark box (Fig. 3.2). Therefore, anxiety is produced before learning occurs and it is possible that memory deficits may prolong anxiety-like behaviours. In the case of mice in the EPM, control mice are initially apprehensive of the open arms but as they explore the maze and repeatedly encounter the entrance to the open arms they quickly overcome their apprehension and enter the open arms.

However, the NCK1 mutant mice remain apprehensive of the open arms. It is possible that the memory deficits in the NCK1 mutant mice prevent them from habituating to the entrance of the open arms, and therefore the unknown opening continues to promote a stress response resulting in avoidance. The inability of the NCK1 mutant mice to remember that they have previously encountered the open arms may be reflected in the decreased neuronal activation in the prefrontal cortex, and of the inhibitory interneurons in the BLA, two neuronal populations that typically fire based on previous experience to regulate BLA output (Janak & Tye, 2015; Tovote et al., 2015). Indeed, in humans low working memory capacity can promote anxiety-related deficits in performance (D. R. Johnson & Gronlund, 2009). Alternatively, increased anxiety may impair learning, especially if avoidant behaviors are preventing sufficient engagement with the task. However, this does not seem to be occurring in the NCK1 mutant mice as they have control levels of exploration activity in all tasks assessed, including in interaction time in the social interaction task, arm entries in the Y-maze, and exploration activity in both the Morris water maze and open field. Future work assessing the neuronal circuit mechanisms leading to the regulation of anxiety-like behaviours and their relationship with memory circuitry will provide greater insight into the relationship between memory and anxiety and the role NCK1 plays in these processes.

4.4: Strengths and limitations of global mutant mice

To assess NCK1 function in the CNS and on behaviour, I took advantage of NCK1 mutant mice. Importantly, the NCK1 mutant mice were generated from

genetically modified embryonic stem (ES) cells. Inserting an IRES-β-galactosidase cassette into the first coding exon inactivated the gene encoding NCK1 and mutant ES cells were generated by homologous recombination. The mutated ES cells were then injected into recipient blastocysts and used to generate chimeric mice (Bladt et al., 2003). Importantly, since ES cells contribute to the generation of all embryonic tissues, mice homozygous for the mutated *NCK1* gene (NCK1$^{-/-}$) would have no NCK1 expressed in all tissues, resulting in a "global" mutant mouse. Additionally, since these NCK1 mutant mice were generated with an IRES-β-galactosidase cassette 29 nucleotides downstream of the start codon (Bladt et al., 2003), β-galactosidase gets transcribed and translated instead of NCK1 and can be used as a reporter that labels all cells and tissues that would normally be expressing NCK1.

The basic principal behind using homologous recombination to generate mutant mice is that you replace a functional gene with a non-functional gene and then using this loss of function approach can assess gene function on development and physiology by looking for impairments and deficits in the mutant mouse. Therefore, the use of global mutant mice is advantageous when studying gene function on developmental processes, on physiological processes at a systems level, or on processes that likely involve the integration of multiple systems. Behaviour falls into all of the above categories as it is heavily influenced by developmental processes and is a product of the integration of numerous systems (including sensory and motor systems, the central nervous system, and the endocrine system) that can each influence behavioural drives and

motivations, perceptions to pain, responses to stress, and ability to learn. Indeed, global NCK1 deficiency results in impaired learning, and altered motivational drive reflected in the increased avoidant behaviours in the elevated plus maze and light/dark box, as well as an increased stress response. Importantly, it is becoming increasingly clear in humans that many neurodevelopmental and neuropsychiatric disorders are heavily influenced by gene mutations, many of which were likely germline, therefore global mutant mice may be more translational valid when creating model organisms to study human neurodevelopmental and neuropsychiatric disorders.

However, a major limitation of the global mutant mice is that great care must be taken in the interpretation of results, especially when addressing cellular mechanisms, as multiple additive-effects of gene disruption in numerous tissues and cell types may be combining to produce a phenotype. Indeed, although I can conclude that loss of NCK1 is resulting in impairments in memory, learning, increased anxiety-like behaviours, loss of dendritic spine density in the hippocampus and basolateral amygdala (BLA), and decreased neuronal activation in the prefrontal cortex and in inhibitory interneurons in the BLA, it remains uncertain on how these deficits are related and if there is a principal driver behind the behavioural changes seen in the NCK1 deficient mice. However, recently established alterations to gene modification techniques have made it possible to generate conditional and inducible mutant mice that can help overcome this limitation. Conditional mutants allow for gene modification in specific tissues or cell types, while inducible mutants allow for the gene to be

disrupted at a specific time point. Together, these gene mutagenesis techniques allow for greater confidence when making conclusion about specific cellular mechanisms leading to the phenotype observed. Indeed, future work conditionally deleting NCK1 from only the hippocampus, only the basolateral amygdala, only the prefrontal cortex, only from spiny neurons, or only from parvalbumin-positive neurons, and assessing them for the presence of memory deficits and anxiety-like behaviours, will be necessary to address if there is a regional or cell specific driver of the behavioural impairments produced by NCK1 deficiency. As we continue to narrow down the precise mechanism by which NCK1 deficiency is leading to deficits in learning, memory, and promoting anxiety-like behaviours, it provides us with not only a better understanding of the neurological basis and relationship of these processes, but will also give us a mechanism in which to target new therapies to alter actin regulation in the brain to promote euthymia.

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