THE ROLE FOR NEDD4 AND NEDD4-2 IN THE CENTRAL NERVOUS SYSTEM

A Thesis Presented in Total Fulfilment of the Degree of Doctor of Philosophy

by

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Bongiorno, D., Boase, N.A., Kumar, S., & Poronnik, P. Nedd4 and Nedd4-2 E3 ubiquitin ligase – unravelling the physiological roles in the central nervous system. *International Brain Research Organisation, Florence, Italy,* 2011


Bongiorno, D., Petratos, S., Kumar, S., & Poronnik, P. DigiGait, a useful method assessing subtle gait abnormalities in Nedd4 transgenic mice. *Australian Neuroscience Society, Sydney, Australia,* 2010
Bongiorno, D., Boase, N.A., Kumar, S., & Poronnik, P. Nedd4 and Nedd4-2 heterozygosity leads to opposing anxiety behaviour in mice. *Australian Physiological Society, Adelaide, Australia*, 2010

Bongiorno, D., Boase, N., Kumar, S., & Poronnik, P. Long-term memory and learning impairments in Nedd4 heterozygous mice. *Ozbio, Melbourne, Australia*, 2010

DECLARATION

I, the candidate, Daria Camera, certify that:

a) Due to marriage, surname changed from Bongiorno to Camera
b) Except where due acknowledgment has been made, the work is that of the candidate alone;
   i. HPLC experiments were conducted by Doctor Clare Parish at the Florey Neuroscience Institute, Melbourne Australia
   ii. LTP recording were performed by Professor Helena Parkington at Monash University, Melbourne Australia

c) The work has not been submitted previously, in whole or in part, to qualify for any other academic award;

d) The content of the thesis is the result of work, which has been carried out since the official commencement date of the approved research programme.

Signature of candidate

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DEDICATION
My family, my daughter Mia and my loving and supporting husband Donny
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The author would like to acknowledge the people who have helped and guided me throughout these studies.

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CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

Figure 1.1. Schematic representation of the three-enzymatic steps involved in ubiquitination of a substrate. Step 1 involves the activation of ubiquitin by the activating enzyme (E1). During step 2, the activated ubiquitin molecule interacts with the conjugating enzyme (E2), and finally step 3, the E2 enzyme interacts with the ubiquitin ligase (E3), which in turn binds to recognition motif on the target substrates and results in the transfer of the ubiquitin molecule to the target. ................................................................. 34

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Figure 1.12. Multitude of known and potential targets for Nedd4 and Nedd4-2 highlight the important roles to regulate neuronal function. The in vivo phenotype of these targets remains unknown however is likely due to the integration of all of these targets shown here.

CHAPTER 2: NEDD4 IN MOTOR FUNCTION AND GAIT

Figure 2.1. Schematic diagram showing the connections between various brain regions in regulating voluntary movement. Motor cortex sends projections through the spinal cord (SC), which synapse onto motor neurons (MN). Axons from MN then synapse onto skeletal muscle (SKM) via neuromuscular junctions (NMJ). The basal ganglia and cerebellum receive input from motor cortex, which provides important information regarding the intended movements. In turn they project back to the motor cortex to fine tune motor cortex output. The cerebellum also receives inputs directly from the spinal cord that provide postural and sensory information to allow the cerebellum to send accurate feedback to the motor cortex about movement in space and time. Arrows indicate projections from one compartment to the other.

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Figure 2.19. At 6 months of age, stride length was significantly increased in the hind paws of Nedd4 heterozygous mice (+/-) compared to age-matched wild-type controls (+/+) at a speed of 15 cm/sec with no changes evident in the fore paws (A). No changes in stride length were evident at the higher speeds of 20 cm/sec (C) or 30 cm/sec (D). Stride length variation measures the step-step variability and was significantly increased in the hind paws only at 20 cm/sec (D), with no changes observed at 15 cm/sec (B) or 30 cm/sec (F). Data represented as mean±SEM and statistical comparisons made using unpaired Student’s t-test * < 0.05 was deemed significant. 

Figure 2.20. In the 12 month cohort, there was no difference between Nedd4 heterozygous (+/-) and age-matched wild-type controls (+/+) in stride length in both fore and hind paws at 15 cm/sec (A), 20 cm/sec (C) or 30 cm/sec (D). Stride length variability was not different at 15 cm/sec (B), 20 cm/sec (D), but at 30 cm/sec a significant decrease in variability was
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Figure 2.23. No change in stance width was observed in 12 month old Nedd4 heterozygous mice (+/-) compared to age-matched wild-type controls (+/+) at 15 cm/sec (A), 20 cm/sec (C) or 30 cm/sec (E). Similarly, no change was observed in stance width variation at 15 cm/sec (B), 20 cm/sec (D) or 30 cm/sec (F). Data represented as mean±SEM.

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ABBREVIATIONS

ACK (Tnk2) Activated Cdc42-associated tyrosine kinase
Akt (PKB) Protein Kinase B
ALS Amyotrophic lateral sclerosis
AMPA α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
BEAN Brain expressed associated with Nedd4
BFCN Basal forebrain cholinergic neurons
BDNF Brain derived neurotrophic factor
C2 Calcium binding domain
CA1 Cornu Ammonis 1
CA2 Cornu Ammonis 2
CA3 Cornu Ammonis 3
CalbB/C2 Calcium/lipid binding domain
Ca,v Voltage-gated calcium channel
Ca,β Voltage-gated calcium channel subunit β
Ca,α2δ Voltage-gated calcium channel subunit α2δ
CIC Voltage-gated chloride channels
CREB cAMP response element-binding protein
CNS Central nervous system
D1 Dopamine receptor 1
D2 Dopamine receptor 2
DG Dentate gyrus
DNA Deoxyribonucleic acid
EC Entorhinal cortex
E1 Activating enzyme
E2 Conjugating enzyme
E3 Ubiquitin ligase  
E6-AP E6-associated protein  
EAAT Excitatory amino acid transporter  
ENaC Epithelial sodium channels  
EGF Epidermal growth factor  
EGF-1R (ErbB-1) Epidermal growth factor-1 receptor  
EGF-R Epidermal growth factor receptor  
EGFR-2 Epidermal growth factor receptor 2  
(ErbB2/c-neu)  
EPSP Excitatory post-synaptic potential  
ErbB3 Epidermal growth factor receptor 3  
ErbB4 Epidermal growth factor receptor 4  
ERK Extracellular signal-regulated kinase  
GABA Gamma-aminobutyric acid  
GLAST Glutamate amino acid transporter  
GLT-1 Glutamate transporter 1  
GluR1 Glutamate receptor subunit 1  
GluR2 Glutamate receptor subunit 2  
GluR3 Glutamate receptor subunit 3  
GluR4 Glutamate receptor subunit 4  
GLUT1 Glucose transporter 1  
GLUT 4 Glucose transporter 4  
GPi Globus pallidus internal  
GPe Globus pallidus exerna  
Gpr10 Growth factor receptor-bound protein 10  
HECT Homologous to the E6-AP Carboxyl Terminus  
HG-EFG Heparin-binding EGF  
IGF-1 Insulin-like growth factor-1  
IGF-2 Insulin-like growth factor-2  
IGFBP3 Insulin binding protein 3  
IGF-1R Insulin-like growth factor-1 receptor  
Kv Voltage-gated potassium channels  
LTP Long-term potentiation
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ABSTRACT

Nedd4 and Nedd4-2 are E3 ubiquitin ligases that are important components of the ubiquitin proteasome system (UPS). Abnormal activity in the UPS has been implicated to underlie a number of neurological disorders and so there is great interest in understanding the role of specific ligases and their involvement in neuronal processes. Initial characterization of Nedd4 and Nedd4-2 found them to be most highly expressed during early neurodevelopment, with a subsequent decrease post-natally. Numerous studies have shown that the function of Nedd4 and Nedd4-2 are not restricted to development, with a number of targets now identified suggesting important functions in the mature brain. To date however, there is very little known in regards to the in vivo physiological implications within the CNS.

Nedd4 and Nedd4-2 knockout mice are not viable, with lethality observed during gestation or shortly after birth. Studies into the physiological phenotypes must therefore be conducted in heterozygotes. Nedd4 and Nedd4-2 heterozygous mice are viable and survive into adulthood, and the only overt phenotype is a significant growth retardation in the Nedd4 heterozygotes. Behavioural testing can be conducted to examine motor function, gait and cognitive aspects of neuronal function. This study used well characterized tests to assess behaviour in heterozygotes and age-matched wild-type controls. Testing was conducted primarily in mice of 2 and 6 months of age, to determine if there are any age-related changes in behaviour. A small cohort of 12 month old Nedd4 heterozygote mice were also assessed for motor function and gait.

Results from this study found that reductions in brain levels of Nedd4 and Nedd4-2 produced distinct behavioural outcomes. For instance, Nedd4 heterozygous mice show an age-dependent change in gait assessed using DigiGait analysis, with very little effect on motor capacity (assessed with RotaRod). The heterozygous mice showed changes in several gait parameters, with an overall extension to gait illustrated by increased stride duration and stride length. This extension to gait is likely due to the contribution of GluR1-containing AMPA receptors (AMPARs), as previous studies have shown that in the absence of GluR1 severe ataxia is produced. This study showed that in the 6 month old Nedd4 heterozygous mice a significant
reduction in GluR1 levels were evident in the cerebellum, which is likely to contribute to the extended gait observed.

*Nedd4-2* heterozygous mice do not show any alterations in gait but at 6 months of age have reduced motor capacity illustrated by the reduced latency on the RotaRod. Due to the known interaction between *Nedd4-2* and the dopamine transporter (DAT), dopamine mediated signalling was evaluated. Total levels of DAT were found to be unchanged in the heterozygotes compared to the age-matched controls, but immunohistochemistry showed increased staining intensity of DAT in striatal fibres. This suggests that membrane levels of DAT may be increased in the heterozygous mice due to reduced endocytosis by *Nedd4-2*. HPLC analysis also found reduced levels of striatal dopamine, with alterations in dopamine metabolism and turnover also evident. Further support for *Nedd4-2* in dopaminergic signalling, comes from the high levels of *Nedd4-2* expression in regions such as substantia nigra pars compacta (SNpc) and striatum.

In addition to gait abnormalities observed in the *Nedd4* heterozygous mice, cognitive abnormalities were also evident. Impaired spatial long-term learning and memory was accompanied by an increase in anxiety behaviours. GluR1-containing AMPARs were again investigated due to their importance during synaptic plasticity, LTP and memory. Although no change in total levels of GluR1 were observed in the hippocampus of the heterozygous mice, significant impairments were evident in long-term potentiation (LTP). This study did not identify whether changes in membrane levels of GluR1, or whether trafficking of GluR1-containing AMPARs is altered in the heterozygous mice. The impairment in LTP in the heterozygous mice supports a role for *Nedd4* in regulation of GluR1-containing AMPARs, and thus contribution to cognitive function. The contribution to anxiety is poorly understood, but there is evidence that GluR1-containing AMPARs may also be implicated.

Cognitive assessment of *Nedd4-2* heterozygous mice, showed subtle abnormalities in long-term spatial memory, and reduced levels of anxiety. This study did not investigate in depth the interaction between these behavioural outcomes and known targets, but highlights that important further work is necessary to elucidate the involvement of *Nedd4-2* in cognitive function especially reduced anxiety. Altered dopamine neurotransmission may also be underlying the anxiety findings, but is yet to be determined.
The expression of Nedd4 and Nedd4-2 within the central nervous system was also investigated, as previous studies have never addressed expression within the CNS. Both proteins were found to be relative ubiquitous in their expression within the brain including; motor cortex, striatum, motor neurons of the spinal cord, cerebellum, hippocampus and amygdala. One major difference in expression is in the striatum and dopaminergic regions such as SNpc. Nedd4-2 was highly expressed within the SNpc region, with strong co-localization with tyrosine hydroxylase. Nedd4 on the other hand showed low levels of expression with very little co-localization evident. Within the striatum, Nedd4-2 staining was highest in the cell bodies of medium spiny neurons, further suggesting a role for Nedd4-2 in dopaminergic system.

The findings from this study are the first to illustrate the physiological outcome of reduced brain levels of Nedd4 and Nedd4-2 in vivo. Nedd4 and Nedd4-2 now appear to have distinct targets, with distinct physiological functions. The ubiquitous expression of these two proteins within the CNS suggests a wide range of neuronal function, however the discrete expression within certain regions such as SNpc also suggests specific roles. Further work is necessary to further elucidate the precise mechanism of action, and how Nedd4 and Nedd4-2 contribute to motor function, gait and cognitive function.
CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION AND BACKGROUND

Ubiquitin Proteasome System

The removal of plasma membrane proteins is a crucial regulatory mechanism that provides a method of controlling the levels of specific proteins at the cell surface at any one time (Cho et al., 2005). Furthermore, within the cell, the degradation of misfolded and denatured proteins must also occur for normal physiological function. This regulatory mechanism is critical for cellular adaptation and controlling growth in response to environmental stimuli, which may be developmental, physiological or pathological (Lecker et al., 2006). Ubiquitin is a highly conserved 76 amino acid polypeptide that serves as a tag for the internalization and degradation of membrane proteins (Pickart et al., 2004a; Pickart et al., 2004b). The proteins controlled by ubiquitin proteasome system (UPS) are essential for cell cycle, DNA repair, cell signalling, gene transcription and apoptosis (Ciechanover et al., 2004), all responses of cellular niche stimuli. Ubiquitination of membrane proteins requires three enzymatic steps: first, ubiquitin activating enzyme (E1) which activates the ubiquitin molecule; second, the transfer of ubiquitin to the E2 conjugating enzyme; and finally, the interaction between E2 and E3 ligases leads to the transfer of ubiquitin to the target molecule (Figure 1.1).

Ubiquitination

Ubiquitin can attach to a target molecule via seven lysine residues (K6, K11, K27, K29, K33, K48 and K63), with the lysine residue involved determining the fate of the protein (David et al., 2011). For instance, whether the molecule is degraded by the proteasome or recycled and trafficked, largely depends on the lysine residue involved but also the type of ubiquitination (i.e. mono- versus poly-ubiquitination) (Sadowski et al., 2012) (Figure 1.2). The determining factor in the formation of mono- versus poly-ubiquitination remains largely unknown, however it appears that the combination of specific E2/E3 interaction may be important (David et al., 2011; van Wijk et al., 2010). The specificity of this system is integral, as uncontrolled protein degradation can be detrimental to a cell. It is therefore not surprising that there are thousands of E3 ligases, which can interact with the substrate to transfer ubiquitin, but also hundreds of E2
ligases capable of interacting with the E3 ligases (Handley et al., 1991; Lecker et al., 2006). It is clear then that E2/E3 combinations can have a profound effect on protein outcome within a cell.

**Figure 1.1.** Schematic representation of the three-enzymatic steps involved in ubiquitination of a substrate. Step 1 involves the activation of ubiquitin by the activating enzyme (E1). During step 2, the activated ubiquitin molecule interacts with the conjugating enzyme (E2), and finally step 3, the E2 enzyme interacts with the ubiquitin ligase (E3), which in turn binds to recognition motif on the target substrates and results in the transfer of the ubiquitin molecule to the target.
Figure 1.2. The type of ubiquitination determines the fate of a substrate. The conjugation of Ubiquitin (Ub) into a monomer or polymer can have a major impact on protein outcome. The formation of a single ubiquitin (Ub) monomer results in endocytosis and membrane trafficking of proteins (A). Ubiquitin (Ub) molecules contain seven lysine residues that can be involved in substrate interactions. The involvement of lysine 48 residues predominantly results in proteasomal degradation of the substrate (B), whereas lysine 63 linked chains results in trafficking and lysosomal degradation (C).

Abnormal ubiquitination can contribute to neurological disorders

Since ubiquitination plays a key role in the regulation of a broad array of cellular functions, such as the activation and silencing of transcription, signal transduction, apoptosis, immune and inflammatory response, cell cycle, receptor-mediated endocytosis, and autophagy it is not surprising that disruption of these systems can lead to pathological states and conditions (Harvey et al., 1999).

In neurons, impairment in the ubiquitin proteasome system (UPS) results in aggregation and accumulation of proteins ultimately disrupting normal cellular processes, and leading to neuronal cell death. Many neurodegenerative diseases have been implicated to arise in part due to abnormal function of the UPS, leading to abnormal protein folding and aggregation. These include Alzheimer’s disease (Lehman, 2009), Parkinson’s disease (Dusonchet et al., 2009) and Amyotrophic Lateral Sclerosis (or motor neuron disease) (Kim et al., 2009; Lowe et al., 1988;
Matsumoto et al., 1993). The underlying cause of these neurological disorders share a common disruption of the UPS leading to the accumulation of mutant protein, and thus cell death (Ciechanover et al., 2003; Dennissen et al., 2012).

There is also evidence that in some polyglutamine repeat diseases such as Huntington disease (Finkbeiner S et al., 2008; Iwata et al., 2009) and spinocerebellar ataxias (Hong et al., 2002; Riley et al., 2004), the UPS is unable to degrade the abnormal proteins again leading to accumulation of protein and thus neuronal cell death. Accordingly it is important to study the function of the ubiquitin proteasome system both under physiological conditions but also in pathological states to better understand the contribution to pathophysiology of these neurodegenerative disorders.

1.2 NEDD4 AND NEDD4-2 E3 LIGASES

Nedd4 and the closely related protein Nedd4-2 (neuronal precursor cell-expressed developmentally down-regulated protein 4 and 4-2) are E3 ubiquitin ligases. Nedd4 was first identified as a mouse gene that is highly expressed during early embryonic development and vital for the differentiation of the central nervous system (Kumar et al., 1992). Nedd4 is ubiquitously expressed whereas Nedd4-2 is restricted to the heart, kidney, brain, lung and liver (Kumar et al., 1997). Nedd4 proteins in different mammalian species have similar structures (Shearwin-Whyatt et al., 2006) and are highly conserved in eukaryotic cells (Kumar et al., 1992).

Nedd4 and Nedd4-2 Structure

Nedd4 and Nedd4-like proteins consist of a calcium/lipid binding (CaLB/C2) domain, three or four tryptophan-rich (WW) domains and an E6-AP C-terminal (HECT) domain that is similar to human papilloma virus (HPV) oncoprotein E6-associated protein (E6-AP) (Kumar et al., 1992) (Figure 3). The C2 (calcium-binding domain), is approximately 120 amino acids in length, and is believed to regulate the expression of proteins by translocating them to phospholipid membranes. This C2 domain has also been shown to mediate auto-inhibition in both Nedd4 and Nedd4-2, by binding to the HECT domain and rendering them inactive. Calcium can relieve the auto-inhibition by disrupting C2 and HECT domain binding and therefore may play a role in the
activation of Nedd4 and Nedd4-2 (Wang et al., 2010). The WW domains are composed of 35 to 40 amino acids, with the two conserved tryptophan residues being 21 amino acids apart. These domains consist of a hydrophobic core surrounded by β-sheets and interact with the PY motifs of substrate proteins (Kanelis et al., 2006), with the most common proline-rich sequence being the PPxY motif (Kay et al., 2000). However, WW domains are known to bind to alternative motifs; for example, Nedd4-2 is reported to bind to the amino acid sequence LPXY (Kumar et al., 1997). These studies show that Nedd4-like proteins interact with a number of different protein motifs via their WW domains. Finally, the HECT domain, which is approximately 350 residues in length, transfers the ubiquitin from a conserved cysteine residue located at the carboxyl end of the HECT domain, to a lysine residue in a target protein.

![Figure 1.3. Nedd4 and Nedd4-2 structural homology. Both contain a calcium/lipid binding domain (C2), 3-4 tryptophan rich WW domains, and a catalytic HECT domain.](image)

**Nedd4 family-interacting protein 1 and 2 (NDFIPs 1 and 2)**

The Nedd4 family-interacting proteins 1 and 2 (NDFIP 1 and 2) are small membrane proteins that contain a PY-motif. NDFIPs and can either promote or reduce interactions between membrane and Nedd4 family of proteins. Furthermore, they have been shown to regulate catalytic activity (Mund T et al., 2009). In the brain, NDFIP 1 is abundant and shows co-localisation with Nedd4 in neurons (Mund T et al., 2009). NDFIP 1 can induce the expression of both Nedd4 and Nedd4-2 into exosomes, thereby contributing to the rapid removal of proteins during neuronal stress and is important for survival of cortical neurons following injury and transient ischaemia (Lackovic et al., 2012; Sang Q et al., 2006). NDFIPs have also been implicated to play an important role in neuronal iron homeostasis. Studies have shown that Nedd4 and Nedd4-2 can regulate the divalent metal (ion) transporter (DMT1) (Garrick MD et al., 2012), with abnormal levels of iron resulting in significant protein aggregation, oxidative stress and ultimately neuronal death (Howitt et al., 2009). Finally, NDFIPs can associate with
EGF receptor and PTEN (phosphatase and tensin homolog deleted on chromosome 10), therefore exerting a physical and functional role in signalling cascades (Mund et al., 2010).

**Physiological roles for Nedd4 family of proteins**

The initial physiological role for Nedd4 family of proteins was identified in Liddle’s syndrome, a hereditary form of extreme hypertension (Botero-Velez et al., 1994). Liddle’s syndrome occurs due to a mutation in the proline rich region of the epithelial sodium channels (ENaCs), resulting in loss of Nedd4-2 mediated channel ubiquitination. ENaCs are therefore up-regulated, and sodium re-absorption increased in the kidney, giving rise to the hypertension observed in these patients (Fotia et al., 2002; Rauh et al., 2006). Nedd4 and Nedd4-2 are now known to regulate a large number of proteins, transporters and ion channels, with differential substrate specificity (Yang et al., 2010).

This thesis focuses on investigating the physiological role of Nedd4 and Nedd4-2 in the central nervous system; with the core biological processes investigated focusing on growth and development, memory and cognition and motor function.

**Cells that make up the CNS**

The central nervous system is composed of many cell types, principally neurons and glia. Neurons are responsible for neurotransmission, forming synapses with other neurons to form a complex network necessary for their physiological functions. The pre-synaptic compartment of a neuron allows for the synthesis and release of neurotransmitter. The post-synaptic compartment contains ion channels and receptors that enable the initiation and propagation of action potentials (Kandel et al., 2000).

Glia function as support cells and can be classified into many different types, each with an important role. Astrocytes are the most abundant glial cell and have a number of integral roles. Astrocytes are capable of maintaining a stable external environment by the rapid removal of neurotransmitters following release, and maintain stable level of ions. They can also provide structural support to neurons, and form an important barrier, the blood brain barrier. Oligodendrocytes are important for myelination of neurons (Kandel et al., 2000). Microglia are
specialised glial cells of the macrophage lineage that are capable of phagocytosis and are the first line of immune defence within the CNS (Kandel et al., 2000).

1.3 GROWTH AND DEVELOPMENT

Nedd4 and Nedd4-2 have an important role in the regulation of growth and differentiation in both peripheral and neural tissue. This is achieved due to interaction between Nedd4 and Nedd4-2 with receptors and/or adaptor proteins critical for normal growth signalling. Nedd4 seems to have a more significant role in overall growth and development, which is highlighted by the severe growth retardation evident in Nedd4 heterozygous and homozygous knockout mice, which is most likely due to, reduced IGF-1 signalling (Figure 1.5).

Figure 1.4. Reduced Nedd4 levels results in severe growth retardation. Growth retardation observed in Nedd4 knockout (-/-) and heterozygous (+/-) mice when compared to wild-type controls (+/+ ) at embryonic day 16.

Insulin-growth factor-1 Receptor (IGF-1R)

The insulin-like growth factor axis is comprised of three ligands; insulin like-growth factor 1 (IGF-1), insulin-like growth factor 2 (IGF-2) and insulin, that bind two closely related tyrosine kinase receptors; IGF-1 receptor and insulin receptor. The main role of the IGF-1 is in the regulation of fetal and postnatal growth in peripheral and neuronal tissues (Baker et al., 1993; Lupu et al., 2001). In the CNS, IGF-1 is produced by all cell types and found in most region of the brain including the cortex, hippocampus, cerebellum, hypothalamus, brain stem and spinal cord (Bach et al., 1991). Since IGF-1 has important roles in growth and development highest
expression levels are observed during the perinatal period. Conversely in the adult brain levels are substantially reduced (Bondy et al., 1993). IGF-1 acts with highest affinity with its cognate IGF-1 receptor (IGF-1R) to activate phosphoinositide 3-(PI-3) kinase and downstream Akt (Protein Kinase B) and extracellular signal-regulated kinase (ERK) signalling pathways required for cellular events such as gene transcription, protein synthesis, apoptosis and proliferation (Fernandez et al., 2012).

**Nedd4 promotes growth via IGF-1 mediated signaling**

Nedd4 has been shown to have a positive effect on IGF-1 signalling, by ubiquitinating the growth factor receptor-bound protein 10 (Grb10) (Monami et al., 2008; Morrione et al., 1999; Vecchione et al., 2003). When Grb10 is bound to IGF-1R, inhibition of tyrosine kinase activity occurs, preventing downstream signalling cascade. Therefore, by removing the Grb10 from IGF-1R, Nedd4 is able to oppose the actions of Grb10 and thus promotes IGF-1 mediated signaling (Monami, Emiliozzi et al. 2008). Indeed in Nedd4 knockout mice, body weights are reduced by 64-68% (as expected) and in the heterozygotes body weights are reduced by 15-20% (Cao et al., 2008).

The peripheral growth retardation observed in the Nedd4 knockout mice may also affect CNS growth. This may be due to direct actions of IGF-1 on receptors located on neurons, or indirect by mediation of blood supply and nutrient availability to neurons, however this has yet to be investigated.

**Epidermal growth factor receptor (EGFR)**

Epidermal growth family consist of structurally related ligands including epidermal growth factor (EGF), transforming growth factor-α (TGF-α), amphiregulin, heparin-binding EGF (HG-EGF) and epiregulin (EPR) (Lee et al., 1995). This family of proteins are essential for growth, differentiation, maintenance and repair of many tissues including the nervous system (Xian et al., 1999). They bind to the integral tyrosine kinase receptor, EGF-1R (or ErbB-1) (Prigent et al., 1992). The EGF-R forms heterodimers with 3 closely related receptors, ErbB2/c-neu, ErbB3 and ErbB4 (Lee et al., 1995). The ErbB tyrosine kinase receptors can be activated either by EGF or the neuregulin family of proteins. The neuregulin family, in particular Neuregulin-1 (NRG1)
acting via ErbB4 receptor, has been shown to be crucial for neural development and synaptic plasticity (Krivosheya et al., 2008; Mei et al., 2008).

**Expression of EGF ligands and receptors within the CNS**

The expression of EGF ligands and EGF-R receptor in the brain is variable, for instance TGF-α is most highly expressed in the CNS, and considered the primary ligand for EGF-R in the developing and adult brain. Highest expression of TGF-α is seen in the striatum, hippocampus, brainstem and olfactory bulb (Lazar et al., 1992; Seroogy et al., 1991). Messenger RNA (mRNA) expression for HB-EGF peaks in early postnatal period and is located in the cortical plate, hippocampus, cerebellar Purkinje neurons and thalamus in the developing brain (Kornblum et al., 1999). The regional expression of EGF-R is similar to TGF-α but temporally similar to HB-EGF-R (Kornblum et al., 1999). With the early and widespread expression of EGF-R and its ligands in the developing brain, it is not surprising that the EGF family are important for genesis, differentiation, migration and survival of neuronal populations in the embryonic brain (Kornblum et al., 1997). Evidence also exists showing the importance for EGF-R ligands in survival of post-mitotic neurons. For instance, mice lacking EGF-R show profound brain defects including the development of progressive neurodegeneration (Sibilia et al., 1998). This illustrates that modulation of the ligands or the receptor can have important physiological outcomes in the CNS.

**Nedd4 promotes growth via EGF mediated signaling**

In response to EGF, Nedd4 has been shown to cause the ubiquitination of endocytic proteins and targets them for proteasomal degradation, leading to the enhancement of EGFR mediated signalling. Like IGF-1 mediated signalling, the regulatory mechanism involves an indirect mechanism, that is Nedd4 and Nedd4-2 bind to an ubiquitin-binding protein ACK (activated Cdc42-associated tyrosine kinase, also know as Tnk2) (Chan et al., 2009; Lin et al., 2010). The C-terminus of ACK contains a PPxY motif allowing for interactions with Nedd4 and Nedd4-2, although both are able to bind, it is primarily Nedd4 that is responsible for ubiquitination of ACK in cells, having a 10 fold higher activity than Nedd4-2 (Lin et al., 2010). Whether Nedd4 has a role in the ErbB4/NRG1 pathway remains unknown, there is some evidence to show that ErbB4 is ubiquitinated by AIP4/Itch, a Nedd4 family member (Omerovic et al., 2007).
findings suggest that Nedd4 and to some extent Nedd4-2 may be pivotal for neuronal growth and differentiation.

*Transforming Growth Factor-β (TGF-β)*

So far, Nedd4 has shown a predominant role in the modulation of signalling necessary for growth and differentiation. However Nedd4-2 is a negative regulator of TGF-β intracellular signalling. The TGF-β family of proteins function to regulate cellular growth, differentiation and apoptosis (Roberts *et al.*, 1990). TGF-β can bind to either TGF-β type I and type II receptors, and upon binding induces the phosphorylation of Smad2 and Smad3. Once phosphorylated, Smad2 and 3 translocate to the nucleus where they bind to transcription factors and control transcription of target genes (Heldin *et al.*, 1997). Nedd4-2 can ubiquitinate TGF-β type I receptors directly (Kuratomi *et al.*, 2005) but can also ubiquitinate Smad 2 and 3 (Gao *et al.*, 2009). These data suggest that Nedd4-2 has a significant role in modulating the intracellular signalling of TGF-β, and can therefore play a role in growth of neuronal tissue, but also apoptosis.

*Neurotrophin Receptors*

The neurotrophins are a group of growth factors important for the developing and mature nervous system (Huang *et al.*, 2001). Neurotrophins comprise of nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), and neurotrophin 3 and 4 (NT-3 and 4). Neurotrophin signalling occurs through 2 main classes of receptors, the high affinity Trk (tyrosine kinase) and the low affinity p75NTR co-receptor (Allen *et al.*, 2006). All neurotrophins are able to signal through p75 NTR, however, each neurotrophin has a higher affinity for the different Trk receptors, TrkA, TrkB and TrkC. The mature form of nerve growth factor (NGF-β) signals through TrkA, BDNF through TrkB, and NT3/4 through TrkC preferentially. NGF provides trophic support to a major group of neurons, the basal forebrain cholinergic neurons (BFCN) (Hefti *et al.*, 1989). The BFCNs provide cholinergic input to the areas associated with memory, especially the hippocampus and amygdala, which contain both TrkA (Steininger *et al.*, 1993) and p75 NTR (Batchelor *et al.*, 1989).
**Nedd4-2 interaction with TrkA receptors**

The TrkA receptors contain a PPxY motif in the C-terminal domain that allows Nedd4-2 binding. Once bound, direct ubiquitination results in the regulation of receptor turnover (Georgieva et al., 2011). Nedd4-2 specifically targets TrkA, with no effect observed on TrkB, TrkC or p75$_{NTR}$ receptors (Arevalo et al 2006). As expected, when Nedd4-2 is deleted an accumulation of TrkA receptors is evident in the early and late endosomal stages suggesting the disruption of the trafficking to degradative pathways (Yu et al., 2011). Studies to date have shown that ubiquitination of neurotrophin receptors is indeed complex, but very little is known regarding the in vivo physiological implications of NGF-mediated modulation of Trk signalling by Nedd4-2.

**Nedd4 and Nedd4-2 growth and development**

Nedd4 and Nedd4-2 have important roles in regulating signalling pathways involved in growth and differentiation. The consequences of reduced Nedd4 levels for growth are evident in mice where levels of Nedd4 are reduced (heterozygous) or removed (knockout), with severe growth retardation evident. The in vivo effect within the central nervous system remains largely unknown and warrants investigation.

**1.4 MEMORY AND COGNITION**

Cognitive processes associated with learning, memory and anxiety are complex, with a multitude of proteins involved in maintaining neuronal integrity necessary for the changing needs of neurons during these processes. Ubiquitination is gaining interest as a mechanism to regulate proteins and receptors necessary for memory and cognitive processes (Chain et al., 1999; Fioravante et al., 2011a). Currently, very little is known regarding which E3 ligases are involved and indeed in Nedd4 or Nedd4-2 contribute to the mechanisms of learning and memory, and will be addressed in this thesis.

**The hippocampus is important for memory and cognition**

The hippocampus is well regarded to be central for memory and learning processes. However the hippocampus forms connections with other brain regions such as the amygdala and
hypothalamic-pituitary axis, and therefore plays a role in anxiety and stress responses (Dedovic et al., 2009). In addition, it is now accepted that the hippocampus does not function as a unitary structure, but it can be broadly subdivided into the dorsal and ventral portions, which have distinct roles (Dedovic et al., 2009; Fanselow et al., 2010). Evidence to support this view arose from a number of studies, including anatomical where the dorsal and ventral hippocampus have distinct inputs (Dedovic et al., 2009; Fanselow et al., 2010). Additionally, lesion studies in rodents show that a disruption in the dorsal hippocampus results in impaired spatial memory (Moser et al., 1995), whereas disruption in the ventral hippocampus alters stress and emotional behaviour (Moser et al., 1995). Finally, previous studies have demonstrated that there are two perforant paths from the entorhinal cortex, the ‘superior’ and ‘inferior’ that target the dorsal and ventral hippocampus respectively (Fanselow et al., 2010). This suggests that networks involving the dorsal and ventral portions of the hippocampus are also distinct.

**Subdivisions and connections of the hippocampus**

The hippocampus is multilayered with a densely packed pyramidal layer, where pyramidal neurons are located extending their apical and basal dendrites above and below this layer. The hippocampus can be subdivided into 4 main segments along its longitudinal axis, in accordance to the afferent inputs; CA1, CA2, CA3 and dentate gyrus (DG) regions. The hippocampus has connections to several subcortical areas, with the major cortical input arising from the perirhinal and postrhinal cortices through the entorhinal cortex (EC). The subiculum, which sits adjacent to CA1 regions is considered the major output region, and sends projections to the thalamus, hypothalamus, septum and EC (Figure 1.6). It is also important to consider that the hippocampus has projections to the amygdala, which enables the emotional component of memory.
Figure 1.5. Schematic illustration of the main regions of the hippocampus. The entorhinal cortex (EC) is intimately connected to the hippocampus, with projections to dentate gyrus (DG) and CA3 region through the perforant path (pp). The DG can send projections to the CA3 region through mossy fibres (mf) and CA1 regions via Schaffer collaterals (sc). The CA1 sends projections to the EC, and can receive input from the EC, DG and CA3 regions. The CA2 region lies in between CA1 and CA3 regions, where the pyramidal layer is not densely packed. D, dorsal; L, lateral. Adapted from (Grove et al., 1999).

Synaptic plasticity and long-term potentiation (LTP)

Synaptic plasticity is the change in strength between two neurons in response to an increase or decrease in activity. One of the best characterised and extensively studied example of synaptic plasticity is long-term potentiation (LTP) in the hippocampus, which is regarded as the cellular mechanism for memory and learning. LTP is an excitatory process requiring glutamate as the neurotransmitter, acting on NMDA (N-methyl-D-aspartate) and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors located on the post-synaptic compartment. Glutamate is released by the pre-synaptic compartment and binds to both AMPA and NMDA receptors to enable neurotransmission to occur. Glutamate binding to AMPA receptors (AMPAR) is necessary to initiate an important influx of Na\(^+\) ions required to remove the Mg\(^{2+}\) block at NMDA receptors. Once the Mg\(^{2+}\) block is removed, Ca\(^{2+}\) influx takes place in the post-synaptic compartment, which is essential for the initiation of gene transcription, signalling events such as the insertion of more AMPARs. Therefore the insertion of additional AMPARs at the cell membrane increases the strength of the synapse, as subsequent release of glutamate will have a greater and faster response, ultimately strengthening the excitatory post-synaptic potential (EPSP) (Figure 1.7).
Thus the regulated insertion but also removal of AMPA receptors is integral in the dynamic process of LTP and thus memory and learning, therefore, AMPA receptors are continuously trafficked (endocyotosed, recycled and re-inserted) into and out of the membrane. (Sprengel, 2006). Scaffolding complexes including post-synaptic density-95 (PSD-95) are implicated in this trafficking of AMPARs, indirectly via transmembrane AMPA regulatory proteins (TARPs) such as stargazin (Bats C et al., 2007; Bhattacharyya S et al., 2009; Elias et al., 2008; Stein et al., 2010). LTP is dependent upon glutamate and thus the density of AMPA receptors at the synapse determines the availability of glutamate binding sites and therefore the level at which they synapse can be excited. A wealth of evidence has provided strong support for density of AMPARs with LTP and memory formation (Makino et al., 2009; Malinow, 2003; Malinow et al., 2002).

Figure 1.6. Insertion of AMPA receptors (AMPAR) into post-synaptic compartment is necessary for LTP to occur. Upon glutamate binding to AMPAR, the influx of sodium ions (Na⁺) results in the removal of Mg^{2+} block from the NMDA receptors (1). This enables the influx of calcium ions to occur through NMDA receptors, with calcium initiating gene transcription and insertion of more AMPAR into the post-synaptic membrane (2). Continuous stimulations that occur during the process of LTP results in synaptic changes such as increased levels of AMPARs that are facilitated by PSD-95 scaffolding proteins via TARPs such as stargazin (3). This increases the synaptic plasticity by enhancing the signal between the pre- and post-synaptic compartments.
The role of ubiquitination in memory and learning

Synaptic activity and dynamic post-synaptic modifications such as dendritic growth and regulated turnover of molecules such as glutamate receptors underpin memory formation. It is now well established that consolidation of long-term memory requires de novo protein synthesis, as well as protein degradation for the fine tuning of synapses required for memory and learning (Artinian et al., 2008; Fonseca et al., 2006). The UPS is now well accepted to have a major function in LTP and memory formation, with proteasome inhibition resulting in the prevention of LTP initiation (Dong C et al., 2008) and therefore reducing capacity for memory formation (Fioravante et al., 2011b; Lopez-Salon et al., 2001). The underlying molecular determinants of LTP are complex, however as already discussed the trafficking of AMPA receptors at the cell surface is a critical component.

Regulation of glutamate receptors

AMPA receptors are ligand gated, and can either be tetrameric, homomeric or heteromeric and are assembled from four different subunits (Glu1-4). It is these subunits that differ in the C-terminal domain and allow for protein interactions with other molecules in the synapse such as scaffolding proteins. The combination of these subunits leads to the formation of distinct receptors subtypes, that are dependent on cell type, brain region and are developmentally specific (Sato et al., 1993). Glu4 only appears to be expressed during development, and in the mature brain AMPARs are predominantly composed of two distinct combinations (Glu1/2 and Glu2/3) (Martin et al., 1993). The C-terminus of the Glu1 subunit is able to interact with PDZ proteins important for exocytosis (Sheng, 2001). AMPA receptors are clustered in the postsynaptic density (PSD), which provides a structural and spatially restricted association with scaffolding and other adapter proteins. These protein interactions have a significant impact on the activity dependent alteration in dynamic cycling of AMPARs in the PSD (Man et al., 2000; Sprengel, 2006).

As already discussed, NMDA receptors are critical for initiating LTP. As with AMPA, NMDA receptors are also comprised of many different subunits (GluN1-3), which confer differences in opening specificity, CNS distribution and magnesium sensitivity (Monyer et al., 1994). NMDA receptors comprised of GluN2D subunit are likely to have a specific role in synapse
formation/elimination both during development and in the adult brain (Gautam et al., 2013). Recently, Nedd4 was shown to interact with GluRN2D subunit of NMDA receptors (Gautam et al., 2013), with currently unknown physiological outcomes. This provides further support for the potential importance of Nedd4 in memory, learning and synaptic plasticity.

*Ubiquitination as a method of regulating AMPA receptors*

For a long time ubiquitination has been suggested to be the mechanism that marks AMPA receptors to be endocytosed and/or degraded (Schwartz, 2003). Recently, Nedd4 was shown to target the Glu1 subunit of AMPA receptors in an activity dependent manner and thus plays an important role in the regulation of AMPA receptor densities in the post-synaptic membrane (Hou et al., 2011; Lin et al., 2011; Schwarz et al., 2010) (Figure 1.8). This has important implications in synaptic change, such as plasticity required for the fundamental processes of learning and memory, with Nedd4 having a potentially important role.

*Figure 1.7.* Nedd4 interacts with the GluR1 subunit of AMPARs in an activity dependent manner, and therefore can regulate the trafficking (insertion and removal) of AMPARs in the post-synaptic membrane.

*Nedd4 and Nedd4-2 targets implicated in synaptic plasticity and LTP*

*Serum and glucocorticoid inducible kinase 1 (SGK1)*
SGK-1 was originally identified in a rat mammary tumour cell line, where mRNA levels increase dramatically in cells following exposure to serum, glucocorticoids, or both (Lang et al., 2006; Lang et al., 2001; Webster et al., 1993). SGK-1 and its related isoforms SGK-2 and SGK-3, share 80% amino acid sequence homology, and are members of the “AGC” subfamily which include protein kinases A, G and C (Webster et al., 1993) and possess a PY motifs to which Nedd4-2 has been shown to bind (Lang et al., 2001). SGK-1 expression is also induced by a large spectrum of stimuli including aldosterone (Náray-Fejes-Tóth et al., 1999; Rozansky et al., 2009), neuronal injury (Imaizumi et al., 1994; Nishida et al., 2004), neuroexcitotoxicity (Hollister et al., 1997), psychophysiological stress (Murata et al., 2005) and following memory formation (Tsai et al., 2002). The activation of SGKs is dependent upon phosphorylation of Thr-256 and Ser-422 sites on the C-terminal domain by phosphoinisitide 3-kinase (PI3-K) (Alessi et al., 1996). SKG has also been shown to phosphorylate similar substrates to Akt, which share a common consensus site (RXRXXS/T). Nedd4-2 contains this consensus site, and SGK-1 has been shown to phosphorylate Nedd4-2 and result in the inactivation of the ligase activity (Debonneville et al., 2001).

SGK-1 and LTP

SGK-1 is highly expressed in the hippocampus and important during long-term spatial memory formation. This is achieved by up-regulating expression of post-synaptic density 95 (PSD-95) protein and thus having an important impact on expression of long-term potentiation (LTP) (Ma et al., 2006). Previous studies have shown that SGK-1 expression in the hippocampus is stimulated following fear conditioning, elevated plus maze exposure and enrichment training (Lang et al., 2006). At the cellular level, SGK isoforms have been shown to up-regulate glutamate receptors such as AMPA, thereby enhancing the excitatory effects of glutamate (Lang et al., 2009; Lang et al., 2006) and thus contributing to enhancement of LTP. An increase in SGK-1 levels can also increases the activity and/or abundance of many proteins, including ion channels and carriers such as glucose (GLUT1 and 4) and glutamate (EAAT1-5) transporters, which also have important implications for neuronal function (Lang et al., 2010).

SKG-1 and glutamate clearance

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SGK-1 is known to stimulate the expression of glutamate transporters (EAATs) (Lang et al., 2006) to increase the re-uptake of glutamate and thus decrease excitation (Benarroch, 2010). Glutamate transporters, primarily located on astrocytes and some neurons maintain, extracellular glutamate levels by rapid re-uptake following glutamate release. The regulation of glutamate levels in the brain is crucial as it regulates normal glutamate levels to sustain appropriate signalling. In a pathological setting, glutamate transporters are also crucial as excess glutamate is neurotoxic and results in neuronal cell death (Rothstein et al., 1996). The evidence for SKG involvement in the brain and defects in SGK contributing to alterations in proteins responsible for excitation of neurons is expanding and complex. However since SGK has the potential to regulate the activity of Nedd4-2 the importance of this interaction in the CNS is profound and warrants further investigation.

Excitatory amino acid transporters (EAATs)

As already discussed, glutamate is the major excitatory neurotransmitter in the brain, with rapid clearance required for normal neuronal function. The re-uptake of glutamate is essential and occurs via excitatory amino acid transporters (EAATs). There are five types of glutamate transporters EAAT 1-5 with differential expression. EAAT1 (also known as glutamate amino acid transporter, GLAST) and EAAT2 (glutamate transporter 1, GLT-1) are the two most abundant of the main transporters in the brain and are primarily located on astrocytes (Lehre et al., 1995), but can also be found on oligodendrocytes (Pitt et al., 2003), microglia (López-Redondo et al., 2000) and macrophages (Rimaniol et al., 2000), with EAAT 3 being primarily neuronal.

Regulation of glutamate transporters

Tight regulation of EAAT1 and 2 is important as illustrated by neurological diseases, which in a large part arise due to alterations in transporter levels. For example, patients with amyloid lateral sclerosis have decreased EAAT2 in the brain and spinal cord (Maragakis NJ and JD. 2006(Bristol et al., 1996). In Alzheimer’s disease patients, EAAT1 levels are reduced, while with animal models, reductions in both EAAT1 and 2 protein levels are observed (Masliah et al., 2000). Furthermore, EAAT1 is found to be co-expressed with tau in cortical pyramidal neurons (Scott et al., 2002) and accumulation of tau observed in EAAT2 expressing neurons.
 Increases in glutamate levels are also evident in both people afflicted with Parkinson’s disease and animal models of Parkinson’s disease (Meredith et al., 2009). In a mouse model of Huntington’s disease, glial aggregates of mutant huntingtin are associated with a decrease in EAAT2 expression (Shin et al., 2005). These disorders all share in common a decrease in the clearance of glutamate which is suggested to contribute to the excitotoxicity and neurodegeneration (Maragakis NJ et al., 2006). Impaired glutamate clearance can also produce neuronal hyper-excitability associated with epilepsies and seizures. This is exemplified in EAAT2 (GLT-1) knockout mice, which have lethal spontaneous seizures due to uncontrolled extracellular glutamate (Tanaka et al., 1997).

Since glutamate transporters affect synaptic levels of glutamate, changes in expression and/or levels of these transporters can have a profound indirect effect on LTP. For instance, increased levels of glutamate transporters, by increasing levels of re-uptake, can substantially reduce glutamate levels able to act on NMDA and AMPA receptors, thereby reducing neurotransmission.

**Nedd4-2 regulation of glutamate transporters**

One important regulatory mechanism of glutamate transporters is ubiquitination. Nedd4-2 is able to regulate glutamate transport in *Xenopus* oocytes with Nedd4-2 found to be co-expressed with both EAAT1 and 2. Furthermore, when Nedd4-2 is overexpressed a substantial reduction in glutamate-induced currents is observed (Boehmer et al., 2003; Boehmer et al., 2006). These findings indicate that Nedd4-2 mediates the ubiquitination and down-regulation of both EAAT1 (GLT-1) (García-Tardón et al., 2012) and EAAT2 (GLAST) (Boehmer et al., 2006), and thus may be important in maintenance of adequate levels of glutamate transporters and thus regulating levels of glutamate at the synapse. This could have implications for glutamatergic neurotransmission required for processes such as LTP and cognitive functions.

**Growth Factors important for memory and cognition**

*IGF-1*

As already discussed IGF-1 is important for overall growth and differentiation, however IGF-1 is also important in the mature brain for cognitive processes. This is best illustrated in rodents where the *Igf1* gene is disrupted. These *Igf1* knockout mice have substantial cognitive
impairments including learning and memory deficits (Cheng et al., 2003). Neurons involved in these cognitive processes shown abnormalities such as reduced dendrite length, with the morphological abnormalities likely due to growth retardation in response to reduced IGF-1 signalling. Humans can also exhibit Igf1 deletions which can also produce cognitive impairments (Woods et al., 1997). Numerous studies have investigated the impact of IGF-1 mediated signalling in learning and memory, and found that not only is IGF-1 (acting via the IGF1-R) important during hippocampal development (Liu et al., 2009a), but it also increases neurogenesis (number of neurons) and synaptogenesis (number of synapses) in the postnatal dentate gyrus of the hippocampus (O'Kusky et al., 2000). Furthermore, IGF-1 exerts trophic effects on glutamatergic neurons, and contribute to hippocampal dependent LTP (Llorens-Martin et al., 2009; Trejo et al., 2007). Neural plasticity is important under normal physiological conditions such as the adaptation that occurs following memory and learning, in addition to changes due to injury and degeneration. Therefore any disruptions in IGF-1 signalling, as seen in Nedd4 heterozygous and knockout mice, can have an effect on learning and memory outcomes.

**Neurotrophins and memory and learning**

The basal forebrain cholinergic neurons (BFCNs) provide cholinergic input to the areas associated with memory, especially the hippocampus and amygdala, and contain both TrkA (Steininger et al., 1993) and p75NTR (Batchelor et al., 1989). Studies have shown that age-related cognitive decline is linked to atrophy or loss of these cholinergic neurons in the basal forebrain (Armstrong et al., 1993; Fischer et al., 1992). Furthermore, abnormalities in NGF transport to BFCN have been implicated to contribute to the neurodegeneration seen in Alzheimer’s disease (Calissano et al., 2010; Cuello et al., 2010; Whitehouse et al., 1981) and Downs syndrome patients (Delcroix et al., 2004). When NGF is infused the rescue of age dependent memory deficits and reduced degeneration of BFCN is evident (Fischer et al., 1991). Finally, mice heterozygous for the knockout of the NGF gene show a reduction in cholinergic neurons with accompanying spatial memory deficits (Chen et al., 1997). These studies all highlight the importance of NGF as a tropic factor both during development but also in the mature brain essential for normal cognitive function.
We know that Nedd4-2 interacts with TrkA receptor and therefore changes in levels of Nedd4-2 may have profound effects on cognitive processes associated with the hippocampus, such as learning and memory and to date have not been investigated.

Voltage-gated Calcium Channels (Ca_v)

The main pathway by which calcium enters excitable cells is by voltage-gated calcium channels (Ca_v) (Catterall et al., 2005). With intracellular calcium regulation crucial for LTP and thus learning and memory, the regulation of voltage-gated ion channels is important. There are three main types of Ca_v (Ca_v1-3) with differential expression in tissues and cell types (Catterall et al., 2005). Their composition is complex, and consists of four or five distinct subunits with the $\alpha_1$ subunit being the largest and important for pore conduction, voltage sensing and gating properties. The $\alpha_1$ subunit is organised into four homologous domains (I-IV), with six transmembrane regions within in each domain (Catterall et al., 2005). The Ca_v$\alpha_1$ subunit can also associate with accessory or auxiliary subunits such as Ca_v$\beta$ and Ca_v$\alpha_2\delta$, and thus can affect biophysical properties and trafficking of these channels (Dolphin, 2003; Dolphin, 2009). Previous studies have shown that phosphorylation of Ca_v$\beta_2$ subunit can regulate the expression of Ca_v1 and Ca_v2 channels at the plasma membrane (Viard et al., 2004). The L-type or Ca_v1 voltage gated ion channels are comprised of four distinct members: Ca_v1.1, Ca_v1.2, Ca_v1.3 and Ca_v1.4 (Ertel, 2000), of these Ca_v1.2 and Ca_v1.3 are primarily expressed in the nervous system (Calin-Jageman et al., 2008).

Interaction between Nedd4 and voltage-gated calcium channels

Recently, Nedd4 was shown to ubiquitinate Ca_v1.2 channels. The overexpression of Nedd4 results in reduced Ca_v currents, expression of Ca_v1.2$\alpha_1$ as well as the total expression of the accessory subunits Ca_v$\beta_2$ and Ca_v$\alpha_2\delta_1$ (Rougier et al., 2011). This study also highlighted that Ca_v$\beta$ is essential for Nedd4-mediated degradation of newly synthesized Ca_v channels. This finding is extremely important, as Ca_v channels have been shown to be important in regulating membrane excitability and intracellular signal transduction including gene transcription in the brain (Calin-Jageman et al., 2008). Furthermore, calcium has been shown to bind to the C2 domain of both Nedd4 and Nedd4-2 and releases the auto-inhibition (Rougier et al., 2011; Wang et al., 2010). This suggests that Nedd4 and possibly Nedd4-2 have important interactions with
calcium and can therefore regulate calcium signalling pathways which are important for neuronal excitability.

**Calcium and LTP**

As already discussed calcium influx through NMDA receptors is essential for the induction of LTP and thus memory formation (Moosmang S *et al.*, 2005). It is not surprising therefore that Cav1.2 is predominantly expressed in pyramidal neurons of the hippocampus (Di Biase V *et al.*, 2011; Lacinova *et al.*, 2008). In a mouse line where the CACNA1C (Cav1.2) gene was inactivated in the hippocampus and neocortex, a deficit in protein-synthesis dependent spatial memory was observed (Moosmang S *et al.*, 2005) as a result of Cav1.2 modulation of CA1 pyramidal neuron spiking patterns (Lacinova *et al.*, 2008). The increase in intracellular concentration of calcium during LTP stimulates the activation of ERK/CREB pathways as well as triggering the transcription of genes producing the long-lasting changes in synaptic plasticity (West *et al.*, 2001). For example, the rise in levels of intracellular Ca$^{2+}$ can activate CamKII producing a multitude of effects; protein synthesis, cytoskeletal changes, and changes in AMPAR receptor activity and density, all crucial processes for the maintenance of LTP, and thus contributing to synaptic plasticity ([Figure 1.9](#)).

It is therefore not surprising that alterations in neuronal excitability would result due to changes in intracellular calcium, either by increased or decreased density of NMDA receptors and (Cav)s channels. These changes will affect firing patterns of neurons and thus synaptic change (including plasticity) that can underlies cognitive behaviour. With Nedd4 being able to regulate the levels of newly synthesised Cav1.2 channels in the plasma membrane and thus affecting calcium signalling further investigation is required by the examination of behavioural outcomes.
Figure 1.8. Calcium plays a central role during synaptic plasticity, influencing gene transcription and thus protein synthesis, cytoskeletal changes such as MAP2 phosphorylation, signalling for GluR1-AMPAR insertion into the plasma membrane, and enhancing AMPAR activation by enhancing conductance. Calcium entry into the post-synaptic neuron is conducted via NDMA receptors, as well as voltage-gated calcium channels. Adapted from West, Chen et al. 2001. Although Nedd4 is not known to interact with NMDA receptors, their activation is necessary for Nedd4 mediated changes associated with synaptic plasticity.

Cytoskeletal changes that occur during synaptic plasticity

Orchestrated remodelling of the cytoskeleton is a feature of neuronal outgrowth involving both actin filaments and microtubules. MAP1 and MAP2 are microtubule-associated proteins, which have many functions in axons and dendrites, including stabilization of microtubule structure as well as acting as scaffolds for the growth of neurons (Gu J et al., 2008). The regulation of MAPs is important, not only during neuronal outgrowth and synaptogenesis but also in mature neurons where adaptive changes occur during plasticity required for memory, learning and following injury. MAP1 is primarily located in both axons and to a lesser extent in dendrites, however MAP2 is restricted to the cell bodies and dendrites. MAP2 comprises a large component of dendrites, and is associated with the synaptic changes that occur during memory and learning.

MAP2 can also anchor regulatory proteins such as protein kinase A (PKA), critical for CREB (cAMP response element-binding protein) signalling, and is thought to be the major PKA anchoring proteins in neurons (Zhong H et al., 2009). Using in silico analysis, data from our
laboratory show that MAP2 possesses a PY motif, and Nedd4-2 binding was demonstrated in PC12 cells (unpublished data). Furthermore, when the PC12 cells were treated with a proteasome inhibitor (MG 132) a significant increase in levels of MAP2 and α-tubulin was evident. A proteomic approach also supports these findings, with MAP2 identified as a target for both Nedd4 and Nedd4-2 (Persaud et al., 2009). There is currently no evidence to suggest that MAP1 is a target of either Nedd4 or Nedd4-2. However, Nedd4 and Nedd4-2, may play direct roles in cytoskeletal remodelling by regulating the levels of these proteins that modulate the structural stability of the cytoskeleton.

Proteins associated with neuronal growth and branching

Nedd4 involved in regulation of axonal growth and branching

Two important studies were recently published highlighting an important role for Nedd4 in axon growth and branching. The first study identified PTEN (phosphatase and tensin homolog deleted on chromosome 10) as a Nedd4 substrate which when down regulated, promotes axon branching in Xenopus retinal ganglion cells (Drinjakovic et al., 2010b). PTEN is an important regulator of PI-3 kinase signalling pathway, as mentioned previously crucial for growth, cell survival and differentiation. In neurons, the downstream signalling pathways of PI-3 kinase can function to regulate cytoskeletal dynamics and thus are important in neuronal morphology (Cosker KE et al., 2007). To test the functional role of Nedd4 in axonal growth, the HECT domain of Nedd4 was mutated, resulting in an E3 ligase that does not have the ability to transfer ubiquitin to target proteins. Retinal ganglion cells containing mutant Nedd4 produced axons lacking elaborate terminal arbours and axons lost their branching potential. Furthermore, down-regulating PTEN in these retinal ganglion cells rescued the Nedd4 loss of function defect (Drinjakovic et al., 2010b).

Nedd4 involved in regulation of dendrite growth and branching

The second study focused on the regulation of Nedd4 in dendrite development. A conditional Nedd4 knockout mouse was developed to examine dendrite development and synaptic function. Lack of Nedd4 was shown to result in impaired dendritogenesis, reduced dendrite arborisation,
reduced synaptic transmission and reduced synaptic numbers in vivo. Furthermore, Rap2A was identified as a novel Nedd4 substrate, important for the dendrite abnormalities observed in Nedd4 knockout mice (Kawabe et al., 2010).

This data shows that Nedd4 is able to regulate growth of both axons and dendrites and thus affect the pre and post-synaptic compartments. It is likely that such abnormalities in axons and dendrites observed in the Nedd4 knockout mice would have an effect on cognition but this has not yet been determined.

Voltage-gated ion channels

Plasma membrane voltage-gated ion channels are critical for the normal patterns of excitability in many cells including neurons. They are located along the axon and at synapses where they initiate and propagate action potentials, set resting membrane potential and control neurotransmitter release, and thereby regulate neuronal excitability. This large family of ion channels comprise of voltage-gated sodium (Na\textsubscript{s}), potassium (K\textsubscript{s}), chloride (Cl\textsubscript{C}) and calcium (Ca\textsubscript{s}), with many subtypes being differentially expressed. Voltage-gated ion channels contain several transmembrane domains, which are arranged around a central ion conducting pore and a C-terminal domain, which is integral for protein-protein interactions crucial for the regulation of cell surface expression. The levels at the plasma membrane are tightly regulated from a dynamic pool, which requires co-ordinated insertion, retention and removal to maintain appropriate levels of excitation. This trafficking of ion channels involves protein-protein interactions with a PPxY motif contained in most of the voltage-gated ion channels, which suggest Nedd4 and Nedd4-2 might be interacting (Table 1.1). As already discussed, Nedd4 can regulate Ca\textsubscript{1.2}, and thus may be implicated to have an essential role during learning and memory. In terms of the other voltage-gated channels, there is data showing both Nedd4 and Nedd4-2 interact with a number of channels and regulate channel density. However, there is no evidence showing direct interactions with neuronal subtypes, but it is likely that Nedd4 and/or Nedd4-2 may be integral in regulating these channels in neurons. This would suggest that Nedd4 and Nedd4-2 could be critical in the most basic neuronal function, excitability.
Table 1.1. PY motifs contained in voltage-gated ion channels.

<table>
<thead>
<tr>
<th>VGIC</th>
<th>Distribution</th>
<th>PY motifs in the C-terminal domain of VGIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Na_{v,1}$</td>
<td>CNS/PNS</td>
<td>S T A C P S T D R Y T K</td>
</tr>
<tr>
<td>$Na_{v,2}$</td>
<td>CH3</td>
<td>P S T T T P S T D S Y T K</td>
</tr>
<tr>
<td>$Na_{v,3}$</td>
<td>CNS</td>
<td>S S T T S P S Y D S Y T K</td>
</tr>
<tr>
<td>$Na_{v,5}$</td>
<td>CNS/heart</td>
<td>S S T S F P S Y T R</td>
</tr>
<tr>
<td>$Na_{v,6}$</td>
<td>CNS/PNS</td>
<td>S T S A L P S Y D S Y T K</td>
</tr>
<tr>
<td>$Na_{v,7}$</td>
<td>CNS/heart</td>
<td>S T S I S P S Y D S Y T K</td>
</tr>
<tr>
<td>$Na_{v,8}$</td>
<td>CNS</td>
<td>S T S F P S Y D S Y T R</td>
</tr>
<tr>
<td>$\gamma_1$</td>
<td>lymphocytes</td>
<td>I D I V A I P Y F I T L G</td>
</tr>
<tr>
<td>$\gamma_2$</td>
<td>heart/skin</td>
<td>L P S N T L P Y E Q L T V</td>
</tr>
<tr>
<td>$\gamma_3$</td>
<td>CNS/PNS</td>
<td>E T G P P E P P S F H G V</td>
</tr>
</tbody>
</table>

Nedd4 and Nedd4-2 may play a central role in synaptic plasticity, LTP, and therefore memory and learning.

There is now a large body of evidence for a multitude of targets for both Nedd4 and Nedd4-2 implicating them to play an integral role in the growth, and differentiation of neurons but also for synaptic plasticity. This forms a complex picture, and to date there is no evidence of the physiological effects for Nedd4 and Nedd4-2 in learning, memory other cognitive aspects such as anxiety. With the substantial interest in ubiquitination of molecular components necessary for learning and memory, it seems likely that changes in Nedd4 and Nedd4-2 levels may have profound effects and will be investigated in this thesis.

1.5 MOTOR FUNCTION

Movement (voluntary and involuntary) occurs due to a complex integration of several regions within the CNS that regulate spatial and temporal patterns of muscle contraction. Intentional or voluntary movements are largely initiated by the motor cortex, and descend through the spinal cord (via motor neurons) to innervate muscles that enable movement (Kandel et al., 2000). The interface between neurons and the muscle, the neuromuscular junction is also critical for the delivery of movement (Kandel et al., 2000). Cortical and subcortical regions including the basal ganglia and cerebellum can also modulate these descending motor pathways (Kandel et al., 2000). To date there is no data to shown whether Nedd4 or Nedd4-2 are expressed in these regions of the brain, however key possible targets regulated by Nedd4 and Nedd4-2 that are expressed in these locations and were investigated in this thesis.
**Motor Cortex**

The motor cortex is comprised of the primary, premotor and supplementary cortices, which together allow for the planning, control and final execution of voluntary movement (Kandel et al., 2000). Like the rest of the cortex, the motor cortex is comprised of layers formed during development. In layer V of the motor cortex, pyramidal neurons are found that descend through the spinal cord and synapse on motor neurons to form the corticospinal tract (Kandel et al., 2000).

**Spinal Cord**

The spinal cord contains tracts that descend to innervate, motor neurons that in turn innervate peripheral tissue including skeletal muscle required for voluntary movement. Motor neurons receive cortical input primarily from the motor cortex, and send projections to the skeletal muscle via the neuromuscular junction (Kandel et al., 2000). In Nedd4 knockout embryos, motor neuron numbers are reduced and abnormalities are evident in the neuromuscular junctions (NMJ), such as reduced capacity to depolarize (Liu et al., 2009b). Interestingly, Nedd4 was found not to be expressed in motor neurons, however this suggests that Nedd4 may be integral in the formation and/or stabilization and function of the NMJ and therefore have an important role in motor function.

**Basal Ganglia**

The basal ganglia are a group of nuclei including the striatum (caudate nucleus and putamen), globus pallidus, substantia nigra (pars reticulate and compacta), and subthalamic nucleus. The basal ganglia has many functions, with the regulation of voluntary motor control being particularly important. Overall, there is complex circuitry with motor cortical input, received by predominantly the striatum, is integrated and modulated before thalamic projections are sent back to the motor cortex (Kandel et al., 2000) (Figure 1.10). In order to understand, this complex circuitry it is important to briefly discuss each of the components, and their contribution to motor function.
Figure 1.9. Schematic representation of basal ganglia circuitry. The basal ganglia receives input from the motor cortex via the stratum, and is modulated by one of the many nuclei including globus pallidus (interna, GPi and externa, GPe), substantia nigra (pars compacta, SNpc and reticulata, SNr), and subthalamic nucleus that regulates feedback to the thalamus and thus back to the motor cortex.

The Striatum, Globus Pallidus and Subthalamic Nucleus

The striatum is the largest basal ganglia nuclei, composed of > 90% inhibitory GABAergic (gamma-aminobutyric acid) medium spiny interneurons. The striatum receives direct excitatory input from the motor cortex and provides major inhibitory output to substantia nigra pars reticulata (SNr) and globus pallidus (interna and externa; GPi and GPe). The striatum also receives dense innervation from the substantia nigra pars compacta (SNpc), where dopaminergic neurons project and release dopamine that binds to either dopamine receptors (D1 and D2), to inhibit or excite neurons of the striatum. It is important to note that neurons that have D1 or D2 receptors are segregated to the direct and indirect pathways, respectively (Thibault et al., 2013). Globus pallidus also contain predominantly inhibitory (GABAergic) and can be subdivided into the internal (GPI) and external (GPe) compartments. Both GPi and GPe receive afferent input from the striatum, but their targeted projections are distinct. For instance, GPi sends inhibitory projections to the thalamus, and GPe inhibitory projections to the subthalamic nucleus. The
main role of subthalamic nucleus is to provide excitatory projection to both substantia nigra pars reticulate (SNr) and GPi (Kandel et al., 2000).

**Substantia nigra**

Substantia nigra is composed of two segments, pars compacta (SNpc) and reticulata (SNr). SNr is largely composed of GABAergic inhibitory neurons that inhibit both the thalamus and SNpc. SNpc on the other hand contains dopaminergic neurons, which project to the striatum and can provide both inhibitory and excitatory input. These neurons degenerate in patients afflicted with Parkinson’s disease, where a loss in dopamine results in the classic symptoms: resting tremor, rigidity, bradykinesia and gait abnormalities (Dauer et al., 2003; Jankovic, 2008; Sulzer, 2007; Vernier et al., 2004). The interaction between SNpc and striatum is important as once dopamine is released into the striatum it is imperative that dopamine is rapidly taken up by dopamine transporters (DAT). Dopaminergic neurotransmission will be investigated in this thesis, due to the interaction between Nedd4-2 and DAT and discussed in more detail.

**Dopamine and motor function**

Dopamine is an important neurotransmitter in the brain involved in voluntary movement, but also for cognitive behaviours such as addiction and reward. Dopaminergic neurons originate in the SNpc, ventral tegmental areas and hypothalamus and project to various brain regions (Chinta et al., 2005). Dopamine is synthesized, packed into vesicles and upon depolarization of a neuron is released into the synaptic cleft. Dopamine acts on dopamine receptors (D1-5) and the termination of dopamine signalling occurs by the DAT, which enables dopamine reuptake back into the cytoplasm (Jaber et al., 1997; Torres, 2006).

**Dopamine Transporter**

DAT is expressed at the plasma membrane with a dynamic pool of DAT available implying that the regulation (insertion and removal from the plasma membrane) is under tight control. Alteration in the function of DAT will ultimately affect the level of dopamine at the synapse and thus have major implications in dopaminergic signaling. Evidence exists for polymorphisms in the DAT gene in patients suffering from hyperactivity disorders such as ADHD (Krause et al.,
Furthermore, mice containing a disrupted DAT gene display a distinct behavioural phenotype associated with hyperactivity due to the increased levels of synaptic dopamine (Giros et al., 1996; Spielewoy et al., 2009). Therefore, DAT regulation is imperative for normal dopamine signalling.

**Regulation of DAT**

Until recently, the mechanism of DAT regulation at the membrane surface remained poorly understood. Nedd4-2 was found to co-localize with DAT, and induce PKC-dependent endocytosis (Sorkina et al., 2006). Although DAT does not contain the typical PPxY motif, Nedd4-2 must be able to ubiquitinate DAT either through an atypical motif or by an indirect mechanism, such as an intermediate protein. This study also identified that both WW3 and WW4 domains are critical for this interaction. More recently, Nedd4-2 was shown to conjugate the ubiquitin via Lys63 site producing multi-monoubiquitination and/or poly-ubiquitination of DAT (Vina-Vilaseca et al., 2010). The effect of Nedd4-2 mediated DAT regulation on dopamine levels and/or behaviour has not been characterized *in vivo*. Nedd4-2 is likely to have profound effects on dopamine signalling, with decreased levels of Nedd4-2 in heterozygous mice perhaps resulting in increased cell surface levels of DAT, which may lead to decreased synaptic levels of dopamine.

**Cerebellum and motor co-ordination**

The cerebellum is an important region of the brain primarily responsible for fine-tuning of movements ensuring that voluntary movement occurs with accurate timing and specificity. Although the cerebellum does not initiate movement it makes a large contribution to motor co-ordination, postural control, motor learning and maintenance of gait (Kandel et al., 2000). The cerebellum is composed largely of two neuron types; granule cells and Purkinje neurons. Granule cells are located in the tightly packed granule cell layer. Granule cells send projections rising horizontally into the molecular layer where they synapse on Purkinje cell dendrites, and are termed parallel fibres (Manto et al., 2001). Parallel fibres are excitatory and use glutamate as a neurotransmitter. Granule (glutamatergic) cells receive input via mossy fibres, which carry information from the cerebral cortex (via pontine nucleus) and spinal cord, and therefore allow for the integration of stimuli from cortex, and sensory information to make adjustments.
necessary for accurate movement (Manto et al., 2001). Mossy fibres make excitatory synapses with granule cells and cells of the deep cerebellar nucleus (DCN) (Manto et al., 2001). Purkinje neurons, which are located in a single layer, are large projection neurons that have extensive and elaborate dendritic branches extending into the molecular layer (Manto et al., 2001). Purkinje neurons are inhibitory (GABAergic), and output from the cerebellum, by projecting to cells of the DCN. Climbing fibre also provide direct synaptic input to Purkinje neurons, with information from spinal cord, brainstem and cerebral cortex (via inferior olivary nucleus) (Manto et al., 2001) (Figure 1.11).

Figure 1.10. Schematic illustration of cells and fibres that comprise the complex cerebellar circuits. The cerebellum is composed of three principle layers; granule cell layer (GC), Purkinje cell layer (PC) and molecular layer (ML). The large Purkinje cells (neurons, PC) send extensive dendritic branches into the molecular layer, where they make excitatory synapses with parallel fibres of granule cells (GC). Purkinje neurons are the sole output of the cerebellum, sending inhibitory projections to the deep cerebellar nuclei (DCN). Dendrites from Purkinje neurons also receive climbing fibre input from the cerebral cortex, spinal cord and brainstem (via inferior olivary nucleus). Mossy fibres carry information from motor cortex and spinal cord and synapse onto granule cells. Purkinje cell dendrites also receive inhibitory input from interneurons located in the molecular layer.
**Purkinje cell function**

The main function of Purkinje neurons is to integrate motor and sensory information through two main synapses; parallel fibres and climbing fibres. These fibres are excitatory and release glutamate, which act predominantly on AMPA receptors (AMPARs) to stimulate neuronal excitability of Purkinje neurons (Evans, 2007). Central to this calcium has an important function both pre-synaptically, to control level of neurotransmitter (glutamate) release by climbing and parallel fibres, but also post-synaptically where the regulation of excitability and initiation of events necessary for synaptic plasticity is required for motor learning (Empson et al., 2012). The expression levels of AMPARs are also crucial in determining levels of neuronal excitability of Purkinje neurons (Evans, 2007).

**Calcium and Purkinje neurons**

The granule cell-Purkinje neuron synapse (via parallel fibres) is important as glutamate is released and acts on AMPA receptors to modulate level of Purkinje neuron excitability and thus extent of inhibition received by DCN. Purkinje neurons show a distinct electrophysiological activity, having both simple (when activated by parallel fibres) and complex (when activated by climbing fibres) spikes (De Schutter et al., 2009; Manto et al., 2001). Calcium is extremely important for Purkinje cell activity, and it is not surprising that voltage-gated calcium is present on Purkinje neurons to allow for the influx of calcium during neuronal activity (Kitamura et al., 2012). The regulation of calcium is therefore imperative for Purkinje neurons to maintain normal and adequate levels of neuronal excitability.

**Voltage-gated Ca\textsuperscript{2+} channels**

Voltage-gated Ca\textsuperscript{2+} channels are also essential for the maintenance of normal calcium levels both in the pre and post-synaptic compartment. In Purkinje neurons a deficiency in calcium signalling can have profound motor control deficits (Ito, 2002). As already discussed, Purkinje neurons are inhibitory GABAergic neurons with calcium signalling being important for the release of GABA at deep cerebellar nuclei terminals but also play a role in their spontaneous activity (Womack M et al., 2002). In *stargazer* mutant mice, which exhibit seizures and ataxic gait, a significant reduction in L-type Ca\textsubscript{1.2} channels are seen at the post-synaptic membrane.
This can affect excitatory neurotransmission when parallel fibres synapse onto Purkinje neurons, and is believed to contribute to ataxia observed in the *stargazer* mutant mice (Leitch *et al.*, 2009). Motor learning requires long-term depression (LTD), which underlies synaptic plasticity in the cerebellum. LTD is triggered by calcium signals in postsynaptic Purkinje neurons and enables motor learning to occur (Finch *et al.*, 2012; Lamont *et al.*, 2012). As already discussed, Nedd4 is able to regulate the levels of newly synthesised Ca,v,1.2 channels in the plasma membrane and may therefore play an essential role in Purkinje neuron and cerebellar function.

**AMPA Receptors and Purkinje cell function**

AMPA receptors (AMPARs) are located in the dendrites of Purkinje neurons where they allow the facilitation of glutamate by granule cell parallel fibres. The trafficking of AMPARs like in the hippocampus can have profound effects on neuronal function. Evidence from mutant mouse models (*stargazer*) show that when AMPARs are lost at the granule cell-Purkinje cell synapses severe ataxia can result (Shevtsova *et al.*, 2012). With evidence of Nedd4 mediated regulation of AMPARs, this further supports the potential role for Nedd4 in motor function and gait.

**Purkinje neurons involved in disorders of the cerebellum**

Abnormalities in Purkinje neuron function are seen in a multitude of movement disorders in both human diseases and rodent models. The most abundant class of movement disorders arising due to cerebellar dysfunction in humans are termed ataxias. There are many subtypes with different clinical outcomes and pathological causes that are beyond the scope of this thesis. Most of the molecular mechanisms are understood with the use of mouse models. What these disorders have in common however is an abnormality in cerebellar function, with Purkinje neuron degeneration being particularly prevalent.

**Spinocerebellar ataxias and motor function**

Spinocerebellar ataxias are autosomal-dominant cerebellar degenerative disorders, with neuropathology observed in the cerebellum, brainstem, spinal cord, and basal ganglia (Matilla-Dueñas *A et al.*, 2010; Owada K *et al.*, 2005). Spinocerebellar ataxias are a form of polyglutamine diseases characterized by CAG repeats in coding regions of genes. In patients
with spinocerebellar ataxia type 31, degeneration is seen in the cerebellar cortex, with particular susceptibility of the Purkinje cells (main projection neurons) (Owada K et al., 2005; Sakai et al., 2010), giving rise to gait and movement abnormalities. Recently, a novel Nedd4 binding partner BEAN (brain expressed associated with Nedd4) was found to be disrupted in a population of Japanese patients with spinocerebellar ataxia type 31 (Sato et al., 2009). These important findings suggest that Nedd4 may be implicated in disorders of the cerebellum such as spinocerebellar ataxias and may extend to motor function and capacity. The interaction between BEAN and Nedd4 is unknown; whether Nedd4 is unable to ubiquitinate and therefore degrade the mutant form of the protein in these patients requires investigation. To date, BEAN has only been identified as being highly expressed in the brain, but little is known about its role, function or distribution, in particular regulation of BEAN by Nedd4. In addition, whether Nedd4 contributes to the pathogenesis of SC31 disease remains unknown.

1.6 HYPOTHESIS AND AIMS

The potential substrates and roles for Nedd4 and Nedd4-2 are numerous and lead to a complex network of signalling pathways and systems that are affected including pre- and post-synaptic compartments (Figure 1.12). Although the literature is vast, one limitation is that these studies are mostly conducted in vitro utilizing cell systems and fail to address any in vivo physiological effect.
Figure 1.11. Multitude of known and potential targets for Nedd4 and Nedd4-2 highlight the important roles to regulate neuronal function. The *in vivo* phenotype of these targets remains unknown however is likely due to the integration of all of these targets shown here.

The central hypothesis of this thesis, is that Nedd4 and Nedd4-2 will have fundamental roles not only during the development but also in the mature adult CNS contributing to motor function, gait, and cognitive behaviours such as memory, learning and anxiety. It is becoming clearer that Nedd4 and Nedd4-2 have exclusive target substrates with no apparent redundancy and this would result different physiological outcomes.

This hypothesis of this thesis was assessed by the following aims:

**Aim 1:** Assess motor function and gait in Nedd4 heterozygous mice  
**Aim 2:** Assess motor function and gait in Nedd4-2 heterozygous mice  
**Aim 3:** Assess cognitive behaviours in Nedd4 heterozygous mice  
**Aim 4:** Assess cognitive behaviours in Nedd4-2 heterozygous mice
CHAPTER 2: NEDD4 IN MOTOR FUNCTION AND GAIT

2.1 BACKGROUND AND RATIONALE

Motor function

Motor function relies on a complex network of neurons to ensure that voluntary movements are precise and produce intended output. Motor function requires projections from motor cortex, which travel via the spinal cord (motor neurons) that innervate skeletal muscle, with the interface between motor neurons and skeletal muscle being the neuromuscular junction. The basal ganglia and cerebellum provide necessary fine-tuning to ensure timing, accuracy and spatial aspects of motor function are precisely executed (Figure 2.1).

Figure 2.1. Schematic diagram showing the connections between various brain regions in regulating voluntary movement. Motor cortex sends projections through the spinal cord (SC), which synapse onto motor neurons (MN). Axons from MN then synapse onto skeletal muscle (SKM) via neuromuscular junctions (NMJ). The basal ganglia and cerebellum receive input from motor cortex, which provides important information regarding the intended movements. In turn they project back to the motor cortex to fine tune motor cortex output. The cerebellum also receives inputs directly from the spinal cord that provide postural and sensory information to allow the cerebellum to send accurate feedback to the motor cortex about movement in space and time. Arrows indicate projections from one compartment to the other.
**Motor cortex**

The primary motor cortex is essential for the initiation and execution of voluntary movement. Pathways originating from the motor cortex descend through the spinal cord to finally innervate skeletal muscles resulting in voluntary movement. The motor cortex in turn receives feedback from the basal ganglia and cerebellum to fine tune motor output. Abnormalities in the motor cortex that occurs following brain damage or stroke, results in abnormal innervation of skeletal muscle and thus abnormal motor output (Cheung et al., 2012). The level of feedback from both the thalamus (via basal ganglia) and from the cerebellum is tightly regulated with abnormalities in these regions also contributing to motor disturbances. The expression of Nedd4 within the motor cortex has not been investigated.

**Basal ganglia**

The basal ganglia, is composed of a group of nuclei located within the brain that form complex neuro-circuitry, with overall modulation of motor output. Essentially, the basal ganglia determine the level of inhibition received by the thalamus that in turn regulates the level of excitatory feedback to the motor cortex. It was important to examine each of these regions more specifically in order to understand how abnormalities can result in motor dysfunction.

**Substantia nigra**

Substantia nigra is divided into two distinct regions; pars compacta and pars reticulata. Pars compacta (SNpc) neurons are predominantly involved in motor function. SNpc neurons produce dopamine and are identified using an antibody against tyrosine hydroxylase (rate-limiting enzyme for dopamine synthesis), and these neurons principally project to striatum. Pars reticulata (SNpr) on the other hand, does not contain dopaminergic neurons and are composed of predominantly GABAergic neurons, which project to areas such as thalamus and mediate output back to the motor cortex. SNpr, can also inhibit SNpc via collaterals. The loss of SNpc neurons is seen in Parkinson’s disease and contributes to the motor abnormalities associated with this debilitating disorder (Dauer et al., 2003).
**Striatum**

Dopaminergic neurons originating from substantia nigra pars compacta (SNpc), project to striatum where their terminate on medium spiny interneurons (comprise >90% of neurons in this region). Once dopamine is released in the striatum, it binds to dopamine receptors, whilst excess dopamine is rapidly removed by the dopamine transporter (DAT). Nedd4-2 is known to modulate DAT (Sorkina *et al.*, 2006; Vina-Vilaseca *et al.*, 2010), so it was necessary to determine whether DAT and Nedd4 are located in the same region and whether any changes were observed.

**Nedd4 and motor function**

There are a number of neuronal targets for Nedd4 that can have important implications for motor function, loco-motor activity and gait. The first piece of evidence came from embryonic *Nedd4* knockout mice, where abnormalities in the neuromuscular junctions were evident. These abnormalities included reduced capacity to depolarize and reduced number of motor neurons in the spinal cord that innervate the skeletal muscle at the neuromuscular junction (Liu *et al.*, 2009b). Since the neuromuscular junctions are at the interface of skeletal muscle and neurons, alterations may impact on motor capacity and thus interfere with voluntary movement.

**IGF-1**

The second piece of evidence revolves around the importance of Nedd4 in IGF-1 mediated signaling. Alterations in IGF-1 signaling have been identified in both patients’ and in rodent models of ataxias (Fernandez *et al.*, 2005; Torres-Aleman *et al.*, 1996). For example, patients with spinocerebellar ataxia type 3 (SCA3) have lower IGF-1 levels and a strong association between insulin binding protein (IGFBP3) levels and length of the CAG repeat (Saute *et al.*, 2011). Rodent models of cerebellar ataxias are well characterized, and represent excellent models for human disease. In two mutant mice, *weaver* and *lurcher*, IGF-1 levels were found to be significantly reduced (Vig *et al.*, 1994; Yao *et al.*, 2008). Furthermore, IGF-1 administered systemically has been shown to restore motor, gait abnormalities and delay and restore Purkinje neuron degeneration (Tolbert *et al.*, 2003; Vig *et al.*, 2006).
IGF-1 in the cerebellum

The cerebellum is a critical region of the brain highly involved in gait, motor control and motor learning. The ataxia diseases and models all share common characteristic of degeneration in Purkinje neurons, that ultimately affect output from cerebellum to motor cortex to fine-tune motor output. It is therefore not surprising that the cerebellum, a region critical for gait contains both IGF-1 and its receptors and the Purkinje neurons able to synthesize endogenous IGF-1 (Aguado et al., 1992; Bondy et al., 1992; Zhang et al., 1997).

In addition to ataxia diseases, IGF-1 has also been shown to be critical in other degenerative disorder of the central nervous system that can also impair motor function and gait. For example, in a mouse model of ALS (SOD\textsuperscript{G93A}), muscle specific IGF-1 is located at the neuromuscular junction and involved in the inflammatory response in spinal cord, and thus impacts on the survival of motor neurons, and more importantly delays the onset of movement deficits (Dobrowolny et al., 2005). When IGF-1 was delivered by adeno-virus into the spinal cord of SOD\textsuperscript{G93A} mutants, significant protection was observed (Lepore et al., 2007).

AMPA receptors

More recently Nedd4 was shown to mediate the endocytosis of AMPA receptors. AMPARs mediate the majority of fast excitatory (glutamate) neurotransmission, and thus are an important factor in regulating neuronal excitability. The importance of the insertion and removal of AMPARs at the post-synaptic membrane of neurons is crucial for motor learning and co-ordination. This is highlighted in the stargazer mutant mouse, where AMPARs are significantly reduced in cerebellar Purkinje neurons, and associated with the development of severe ataxias in these mice (Barad et al., 2012; Menuz et al., 2008).

Calbindin-D28k

The calcium buffering protein Calbindin-D28k, may also be an important Nedd4 target. Since, Nedd4 activity can be regulated by calcium, an important interaction with the proteins responsible for regulating intracellular calcium levels may exist. Calbindin-D28k heterozygous mice have profound motor control deficits that exist due to alterations in Purkinje neuron function (Ito, 2002).
**BEAN**

Finally, the novel Nedd4 interacting protein, BEAN (Brain expressed associated with Nedd4) was disrupted in a Japanese population of spinocerebellar ataxia type 31 patients. The BEAN protein contained of a complex penta-nucleotide repeat sequence the length correlating to age of onset and severity of disease (Sato et al., 2009). Very little is known about spinocerebellar ataxia type 31, however it is clear that this type of ataxia is late onset, purely cerebellar ataxia and the third most common adult onset cerebellar ataxia in Japan (Ishikawa et al., 2011).

**Examination of motor function**

To determine the contribution of Nedd4 to motor function behavioural assessment can be used to examine many facets of motor function. Firstly it is important to determine basal locomotor activity and this can be accomplished with the use of locomotor cells. This test is useful in measuring explorative behavior, and can be a useful indicator of increased or decreased activity. For instance, increased activity is seen in rodent models of hyperactivity disorders associated with increased dopamine levels in the basal ganglia (Durieux et al., 2012; McCullough et al., 1992; Russell et al., 2005). Motor capacity, balance and co-ordination can also be examined using the Rota-Rod test. It assesses the ability of a mouse to remain on a rotating rod as the speed of rotation increases. Results from Rota-Rod can provide an indication of motor neuron function and basal ganglia dysfunction (Russell et al., 2005).

**Gait analysis**

Maintenance of normal gait patterns is largely controlled by the cerebellum, which ensures that movements are accurate, timely and balanced. As already discussed, IGF-1 and AMPAR have a strong contribution to normal cerebellar function, and due to the known interaction between Nedd4 with these targets, a potential role for Nedd4 in gait is likely. Gait analysis can be evaluated using the automated system DigiGait. Using the DigiGait system investigation into static (placement of paws) and dynamic (timing of movements) phases of gait can be assessed. One major advantage of DigiGait over other gait analysis systems is the ability to determine walking speed of the treadmill. Normal walking speed for mice ranges between 15-25 cm/sec, with changes in speed altering some gait parameters (Clarke et al., 1999), and as such different
walking speeds can be used to evaluate normal walking speed (15 cm/sec) and a more challenging speed (i.e. running, 30 cm/sec).

**Gait parameters**

Examination of gait parameters requires investigation of both spatial and temporal indices, including overall stride in duration and length. One stride can also be subdivided into the phases that comprise it including; stance, swing, brake and propel (Figure 2.2).

![Figure 2.2](image)

**Figure 2.2.** Stride parameters assessed by DigiGait. Braking phase of stride begins with initial paw contact with the belt, once maximum contact is reached; the propulsion phase begins and ends with the lift off by the toes (A). One complete stride is comprised of braking, propulsion and swing phases. Stance is defined by the duration of time the paws are in contact with the belt (B).

It is important to measure each of these phases of stride as they have important contributions to overall gait. For instance, the swing phase of stride measures the acceleration of motion and can be an indicator of joint mobility. Propulsion measures the initiation and acceleration of motion, with breaking measuring the deceleration of motion. Older methods such as footprint analysis can provide a measure of overall stride, but changes in stride could be due to alterations in one or more of the components of stride and cannot provide important insights into which aspect is affected. In addition, fore and hind paws are principally important for the different components of stride, for example the fore paws are predominantly used for propulsion and hind paws more effective for braking during rodent locomotion.
Alterations of one or more of these parameters can be associated with severe conditions. For instance, in arthritic mice and rodent pain models an increase in swing duration is observed (Plaas et al., 2011; Simjee et al., 2004). In addition, in animal models for Down syndrome and following ethanol exposure increases in propulsion duration (Hampton et al., 2004; Kale et al., 2004), and decrease in braking durations (Kale et al., 2004) are observed.

Stance duration measures the time that paws are in contact with the belt. In humans with cerebellar diseases such as ataxia, an increase in stance duration is observed which is believed to be a compensatory measure to maintain stability (Stolze et al., 2002). Other clinical signs of cerebellar ataxia include increases in stance width, irregularities in number of steps, increases in cadence (steps/min), increases in foot angle and increases duration of double limb support which will be discussed below (Stolze et al., 2002).

**Spatial gait parameters**

Spatial gait parameters include stride length and stance width (Figure 2.3), which measure the size of the stride and the width of the paws when in contact with the belt. The variability of stride length provides further information on ability to maintain consistency during successive strides. This is exemplified in patients and rodent models of Parkinson’s disease (PD), where a decrease in stride length is accompanied by increased variability stride to stride in affected individuals and rodent lesion models (Amende et al., 2005a; Hausdorff et al., 1998; Hausdorff et al., 2000). Stance width measures the distance between both fore and hind limbs. Increases in stance width and stance duration are clinical features of cerebellar ataxias. Increases in these parameters are thought to be a compensatory measure to improve stability during locomotion (Stolze et al., 2002).
As already mentioned, increases in cadence (steps/sec) is an important clinical hallmark in patients with cerebellar ataxia (Stolze et al., 2002). The DigiGait system can measure both the total number of steps taken but also cadence (number of steps per second) (Figure 2.3). Paw angle dynamics can also be investigated including; paw angle (that compares left and right paw angles), absolute paw angles (that compares paw angle in relation to the midline of the mouse), paw angle variability (measures consistency of paw angle placement), step angle (differences between left and right paws taken into account of stride length and width) and step angle variability (measures consistency of step angle between successive strides). There is some evidence of step angle changes in patients with cerebellar ataxia, where an increase is observed (Stolze et al., 2002). In rodents, paw angle parameters are poorly defined or understood, with interpretation into human gait limited. However, studies have identified changes in angle dynamics in many rodent disease models. For example, an increase in paw angle (or open) angles are present in hind paws of ataxic mice, rodent models of spinal cord injury and
demyelinating diseases (Powell et al., 1999) and following collagen-induced arthritis (Vincelette et al., 2007). In addition, increases in step angles, have been shown to reflect dysfunctional ataxia following prenatal alcohol exposure in rodents (Hannigan et al., 1988).

**Plantar placement**

Plantar placement is a measure of weight bearing abilities measured by paw area at peak stance. A decrease in paw area is an indicator of reduced weight bearing, with reductions evident in a mouse model of collagen-induced arthritis (Vincelette et al., 2007). Variability of paw area can also be measured with increases suggested to reflect a deficiency in co-ordination (Goldberg et al., 2010).

**Stance factor and gait symmetry**

Stance factor or the ratio between left and right fore and hind limb stance durations has been shown to be an accurate and consistent parameter in evaluating peripheral nerve function after nerve injury (Varejao AS et al., 2001). Gait can also be described as symmetrical or asymmetrical, with asymmetries often suggesting gait pathologies. This is evident in both human (Kahn et al., 2009; Sadeghi et al., 2000) and rodents (Domanskyi et al., 2011; Herbin et al., 2004). Symmetrical gait in rodents is determined when both limbs (on left and right side) behave identically, and as a pair alternate during locomotion, and as such the value should equal to 1. Asymmetry, when limbs move together, and as such the value measured using DigiGait deviates from 1.

**Hind limb shared and stance-swing ratio**

Hind paw shared stance is also known as ‘dual stance’ or ‘double support’, and is a measure of time that both hind paws are in contact with the belt. Studies have shown that shared stance times increase with stride time (Clarke KA et al., 1989), with carrageenan induced paw inflammation in rodents (Coulthard et al., 2002), in obese children (McGraw et al., 2000), Parkinson’s disease patients (O’Shea et al., 2002), and significant decreases observed in a mouse (R6/2) Huntington’s model (Pallier et al., 2009). Stance-swing ratio measures the ratio of durations between stance (contact with belt) and swing (no contact with belt). This parameter
can also be useful in examining ‘double support’. For instance in humans natural walking produces longer stance phase over swing-phase, and as speed increases so does the swing phase. During running, stance swing is reversed and double support disappears. There is some evidence suggesting that following stroke, alterations in stance swing-ratio is an useful indicator of motor capacity (Harris-Love et al., 2001; Roth et al., 1997).

The cerebellum largely contributes to the regulation of gait, in particular timing of movements and maintenance of balance. The basal ganglia however, also play an important role, which is evident in disorders arising due to damage to this region. Overall, gait and motor function require the contribution of all CNS regions involved in the planning, execution and fine-tuning of voluntary movement.

**Nedd4 in the CNS**

The expression of Nedd4 within the CNS has not been defined. Nedd4 is highly expressed in the brain and spinal cord during early neurodevelopment (Kumar et al., 1997). The expression in the adult brain has never been addressed; furthermore, regional and cellular expression has largely remained unknown. In order to determine the neuronal contribution of Nedd4 to motor function, Nedd4 must be expressed in regions of the brain such as cerebellum, motor cortex, spinal cord, and basal ganglia. The expression patterns may also refine the role, as motor function and co-ordination are complex processes requiring the integration of many brain regions. However, the cerebellum predominantly regulates gait and co-ordination.

These findings led to the main hypothesis of the project described in this Chapter; that Nedd4 would have a critical role in neuronal function necessary for motor control, co-ordination and gait and would show abundant expression in neurons necessary for these functional behaviours. To investigate this hypothesis, the following aims were set:

1) Conduct behavioural tests in Nedd4 heterozygous mice and assess basal locomotor activity, motor function and gait.

2) Determine cellular and regional expression of Nedd4 in the brain and spinal cord required for motor related behaviours.
3) Determine suitable targets that may be responsible for motor related behaviours

2.2 METHODS

Animals

A Nedd4 knockout line (Nedd4<sub>Gr(RES{Betageo})249Lex</sub>) was obtained from the Mutant Mouse Regional Resource Center (MMRRC). This line is derived by gene trap with a retroviral insertion disrupting the Nedd4 gene between exons 17 and 18 (www.mmrrc.org/strains/11742/011742.html). The following primers were used to genotype mice with the Nedd4 gene disruption. Primer genoN4 WT5’ (5’ GGA GTC TTT GGA TAT TGT AAG AGC 3’) and genoN4 WTandKO 3’ (5’ GAG CGT GCG CCT CAC AAG TAT GA 3’) amplify a 226bp fragment from the wild-type allele, whereas genoN4 KO5’ (5’ AAA TGG CGT TAC TTA AGC TAG CTT GC 3’) and genoN4 WTandKO 3’ amplify a 137bp fragment from the Nedd4 disruption allele.

Male Nedd4 heterozygotes and wild-type littermate controls were bred at the IMVS animal facility (Adelaide, Australia) and transported to Florey Neuroscience Institutes (FNI, Melbourne Australia) for behavioural assessment. At least one week acclimation prior to any testing was observed, during this period mice were handled daily to reduce stress related behaviours. Animals were singly housed a week prior to testing and maintained on a 12:12 h light/dark cycle. Animals were also given a day rest between each round of testing to ensure that variability due to fatigue was not a factor.

For motor function and gait analysis, locomotor activity, RotaRod and DigiGait were used in 2 and 6 month old Nedd4 heterozygous and age-matched controls were used (n = 8-13). The 2 and 6 month Nedd4 heterozygous mice are separate cohorts of mice. In order to test mice at twelve months of age, 5 Nedd4 heterozygous and 5 wild-type controls were kept from the 6 month old cohort. These mice were tested for RotaRod and DigiGait only. These mice were tested to establish that gait abnormalities are reproducible, and do continue with age.
**Motor function and gait analysis behavioural tests**

**Basal Locomotor Activity**

Prior to testing mice were habituated to low-light conditions in the testing room overnight whilst maintaining light/dark cycles as to not disturb circadian rhythms. Mice were placed in the photo-optic locomotor chambers (Coulbourn Instruments, Whitehall, PA) and spontaneous locomotor activity assessed for 30 minutes (Truscan 2.01). Activity was measured for both ventral and horizontal plane:

- Horizontal plane: distance traveled, number of moves, time spent moving
- Ventral plane: number of rearing events and time spent rearing

At the conclusion of the test, chambers were thoroughly cleaned with 70% ethanol. This is essential to remove foreign odour that can affect explorative behaviours.

**Rota-Rod**

A four-chamber mouse Rota-Rod (UgoBasile, Milan Italy) was used in this study. Mice were trained the day before the test trial.

Training protocol:

- Training session 1: Constant speed at the lowest speed (4 rpm) for 2 minutes
- Training session 2: Constant speed at the lowest speed (4 rpm) for 2 minutes
- Training session 3: Accelerating speed from 4 rpm for 2 minutes

On average, mice required 3-4 two minute trials and mice were deemed trained when they were able to maintain a time of 2 minutes at accelerating speed (up to 40 rpm) without falling off. Mice were also given 30 minute rest periods between each trial. One of the main reasons that mice need to be adequately trained is their innate desire to turn around on the rotating drum, which can lead to mice slipping off, irrespective of motor capacity. Some mice required more training trials, however there was no trend to suggest that heterozygous mice required more training than wild-type controls.
Testing was conducted the following day.

Testing protocol:

- Test 1: Accelerating speed from 4 rpm for 2 minutes
- Test 2: Accelerating speed from 4 rpm for 2 minutes
- Test 3: Accelerating speed from 4 rpm to 40 rpm for 5 minutes

The duration on the rotating drum from the third test (Test 3) was used for comparison.

_DigiGait Analysis_

Gait analysis was performed using computerized digital footprint analysis (DigitGait, Mouse Specifics Inc, USA). Parameters assessed allows for the investigation of static (placement of paws) and dynamic (timing of movements). The DigiGait chamber has a digital camera placed below a transparent belt of a motorized treadmill, and allows the capture and translation of all fore paw placements in the ventral plane. Spatial and temporal indices were quantified using the accompanying DigiGait Imaging software. Animals were placed on the treadmill and required to complete 4-6 strides that were used for analysis. Gait was assessed at 15 and 20 cm/sec in the 2 month cohort and 15, 20 and 30 cm/sec in the 6 and 12 month cohort. The 6 and 12 month cohorts were conducted at a later stage, and due to the limited change in gait at 2 months of age, the speed was increased to evaluate whether a more challenging speed would uncover changes in gait. DigiGait therefore has the capability of examining many facets of rodent gait, and makes this a powerful tool to determine even subtle gait anomalies. DigiGait indices are given for each paw; that is left and right fore paw, and left and right hind paws separately. Fore paw values represent an average from left and right fore paws, and the same for the hind paws.

_Tissue Collection_

_Western Blotting_

Following behavioural analysis tissue was collected for Western blotting. Mice (n = 3-5) were euthanized with sodium pentobarbital injection (100 mg/kg i.p.). Brains removed, weighed, and the two hemispheres separated. One hemisphere was homogenized to obtain whole brain lysates. The second hemisphere was dissected, and cerebellum, hippocampus and striatum were
removed and snap frozen. All samples were stored at -80°C until required. Tissue samples were homogenized in lysis buffer composed of 159mM NaCl, 50mM Tris-HCl, 1% Triton-X, pH 7.4 plus Complete lysis-M protease tablet (Roche USA) and processed according to manufacturers instructions. Protein quantification was determined using a BSA assay (Pierce), and samples resolved on 8% bis-glycine gels (Biorad) and transferred to nitrocellulose membrane (Pall Corporation, USA). For Nedd4 protein analysis, 150 μg of total protein was loaded, necessary due to low-level expression of Nedd4 in adult brain making it difficult to accurately measure the bands. For all other protein analysis, 50 μg of protein was loaded onto the gels. Adequate protein transfer was determined by staining with Ponceau-S. Membranes were then blocked with 5% milk powder in Tris-NaCl-Tween buffer (TBS-T) followed by overnight incubation at 4°C with primary antibodies. Blots were washed and incubated with fluorescently tagged Alexa secondary antibodies and visualized using FluoroChemQMultImage III. Blots were analyzed using Image J, with each gel containing both wild-type and heterozygous samples to allow direct measurement and comparison. β-actin was used as a loading control also measured on each gel. Protein bands were quantified, normalized to β-actin levels and then represented as % of control.

**Immunohistochemistry**

After behavioural analysis, mice were weighed (n = 3-7), deeply anesthetized with sodium pentobarbital (100 mg/kg i.p.) and perfused with 0.1M phosphate buffered saline (PBS, pH 7.2) followed by ice cold 4% paraformaldehyde (PFA) in 0.1M PBS. Brain and spinal cords were removed, weighed and placed in 4% PFA for 2 hours at room temperature, after which they were cryoprotected in 30% sucrose solution. Each brain was divided into two hemispheres, one sectioned in the sagittal plane and the other in a coronal plane and used for immunohistohemical analysis. A cryostat was used to obtain frozen 50μm free floating sections of whole brain, sequentially selecting 1 in 6 sections. Spinal cord sections were also collected at 50μm in 1 in 4 series. Sections were stored at -20°C in cryoprotectant (30% sucrose, 1% polyvinyl pyrolididone, and 30% ethylene glycol) solution. Brain and spinal cord sections were washed 4 times in PBS and blocked for 1 hour at RT in blocking buffer (3% goat serum and 0.03% Triton-X 100). Sections were washed 2 more times with PBS, then primary antibodies were applied in blocking buffer for 72 hrs at 4°C. The following primary antibodies were used:
Sections were washed 4 times in PBS and incubated with secondary antibody for 1 hr at RT. Secondary antibodies used were all conjugated to Alexa-Fluorophores (mouse-488 and rabbit-555), and raised in goat. Sections were washed again 4 times in PBS and labelled sections mounted using Prolong-Gold mounting medium (Invitrogen) according to manufacturer’s instructions. Fluorescence labeling was visualized using a Nikon C1 confocal microscope using a 488nm argon and 543nm HeNe laser (Nikon Group, Tokyo, Japan). Images were acquired using 20x, 40x and 60x Plan-Apochromat oil immersion objectives. All images were captured and analyzed using Nikon Elements software.

**Statistics**

For the behavioural and western blot analysis statistical comparisons were made between the *Nedd4* heterozygous mice and age-matched wild-type controls using unpaired Student’s t-test. Results were deemed significant when P < 0.05. Comparisons for the DigiGait analysis were conducted between the *Nedd4* heterozygous mouse and the age-matched wild-type control. Comparison between 2, 6 and 12 months mice were not conducted due to the testing being conducted at different times. The gait parameters examined using DigiGait are largely independent, and as such Student’s t-test was used to statistically compare gait parameters, with results deemed significant when P < 0.05.

2.3 **RESULTS**

**Body and brain weights**

Body and brain weights were measured. *Nedd4* heterozygous (+/-) mice had a significant reduction in body weight compared to wild-type (+/+ ) controls at 2 (wt: 20.3 ± 0.5g; n = 8 and het: 17.6 ± 0.7g; n = 14; P = 0.01) and 6 months (wt: 33.0 ± 1.3g; n = 10 and het: 27.4 ± 1.1g; n = 10; P = 0.004), with a trend also observed at 12 months (wt: 36.4 ± 2.3g; n = 5 and het: 31.4 ±
Brain weight was also reduced in Nedd4 heterozygous mice at all three time points relative to controls, with significance reached at 2 and 12 months of age (2 month wt: 0.45 ± 0.01g; n = 6 and het: 0.39 ± 0.01g; n = 13; P = 0.003, 6 month wt: 0.47 ± 0.01g; n = 4 and het: 0.45 ± 0.01g; n = 5; P = 0.08 and 12 month wt: 0.56 ± 0.003g; n = 5 and het: 0.51 ± 0.02g; n = 5; P = 0.03, Figure 2.4B). To determine whether brain size was smaller due to deficits in neuronal developmental we evaluated brain to body weight ratio and showed that brain weight was reduced in a pro rata manner (2 month, wt: 0.022 ± 0.001; n = 6 and het: 0.022 ± 0.001; n = 13; P = 0.94, 6 month, wt: 0.014 ± 0.001; n = 4 and het: 0.017 ± 0.001; n = 5; P = 0.35 and 12 month, wt: 0.016 ± 0.001; n = 5 and het: 0.017 ± 0.001, n = 5; P = 0.55, Figure 2.4C).
Figure 2.4. Body weight was significantly reduced in *Nedd4* heterozygous (+/-) mice compared to age-matched control (+/+) at both 2 and 6 months of age (A). Significant reduction in brain weight was observed at 2 and 12 months of age in *Nedd4* heterozygous mice compared to age-matched wild-type controls (B). Brain to body weight ratio was not different between *Nedd4* heterozygous and the age-matched controls at all three time points, thus showing a pro rata reduction in body and brain mass weight (C). Data represented as mean±SEM. * P < 0.05, ** P < 0.01 and *** P < 0.001 using unpaired Student’s t-test.
**Assessment of Nedd4 protein levels in the brain.**

Western Blotting was used to determine that Nedd4 levels were reduced by at least 50% in brains from *Nedd4* heterozygous mice when compared to wild-type controls at 2 and 6 months of age (2 month old mice: $49.0 \pm 11\%$ of control; $P < 0.01; n = 3-4$ and 6 month old mice: $33 \pm 8\%; P < 0.0001; n = 5$, Figure 2.5). Tissue from 12 month old mice was not obtained.

**Figure 2.5.** Nedd4 expression is reduced in whole brain lysates from 2 and 6 month old *Nedd4* heterozygous mice (+/-; $n = 4-5$) compared to age matched wild-type controls (+/+; $n = 4-5$). Representative western blots from two months (A) and six months cohort (B). Nedd4 levels in whole brain lysates represented as % of control (C). Data represented as mean±SEM. * $P < 0.05$, and *** $P < 0.001$ using unpaired Student’s t-test.
Activity and motor function

Locomotor activity

Basal locomotor activity was measured using locomotor cells and was not affected in Nedd4 heterozygous mice at either 2 or 6 months of age (Table 2.1).

Table 2.1. No differences were observed in basal locomotor activity between Nedd4 heterozygous (+/-) and wild-type (+/+) controls, at both 2 and 6 months of age. Data represented as mean±SEM. 2 months: wild-type n = 8 and heterozygous n = 14, 6 months: wild-type n=10 and heterozygous n=10.

<table>
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<th>2 month (+/-)</th>
<th>6 month (+/+)</th>
<th>6 month (+/-)</th>
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<td>6857±568</td>
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<td>41±10</td>
<td>101±14</td>
<td>88±13</td>
</tr>
</tbody>
</table>

Rota-Rod assessment of motor function

Rota-Rod is a widely used test of general co-ordination, balance and overall motor function based on the length of time mice can stay on a rotating drum. There was no significant difference in the latency to fall from the Rota-Rod between Nedd4 heterozygous mice compared to age-matched wild-type controls (2 month, wt: 253.9 ± 25.5 sec; n = 8 and het: 264.4 ± 16.8 sec; n = 13, 6 month, wt: 223.4 ± 18.0 sec; n = 10 and het: 203.4 ± 25.6 sec; n = 10 and 12 month, wt: 157.4 ± 22.0; n = 5 and het: 129.0 ± 15.3 sec; n = 5) (Figure 2.6). There was an age dependent decline in motor function, which is significantly decreased between 2 and 12 month Nedd4 heterozygous mice (P < 0.01).
Figure 2.6. Latency to fall from the RotaRod was not different between *Nedd4* heterozygous mice (+/-) and age-matched wild-type controls (+/+ ) at all three time points (2, 6 and 12 months of age). Data represented as mean±SEM. ** P < 0.01 using one way ANOVA with Newman-Keuls multiple comparison post hoc test.

**DigiGait analysis**

*Stride parameters*

*2-month cohort*

At the lower speed of 15 cm/sec, duration of stride and stance in the fore paw (Figure 2.7A) and hind paw (Figure 2.7B) were not affected in *Nedd4* heterozygous mice. The component of stride such as swing, brake and propulsion durations were also unaffected in both fore paws (Figure 2.7C) and hind paws (Figure 2.7D) in the *Nedd4* heterozygous mice compared to wild-type controls. Stride can also be examined as an overall % of stride, and again was not altered in both fore paws (Figure 2.7E) and hind paws (Figure 2.7F) of *Nedd4* heterozygous mice compared to wild-type controls. At the higher speed of testing, 20 cm/sec, when stride and stance was examined no change was observed in fore paw (Figure 2.8A) or hind paw (Figure 2.8B), and components of stride were also unaffected (Figure 2.8C and D), as was the percentage of time spent in each phase of stride (Figure 2.8E and F). Brake and propulsion as a percentage of stance also showed no differences in fore paws (Figure 2.9A and B) and hind paws (Figure 2.9C and D) at 15 and 20 cm/sec respectively.
Figure 2.7. At a speed of 15 cm/sec, the duration of stride and stance showed no difference between Ned4 heterozygous (+/-) and age-matched wild-type controls in both fore (A) and hind paws (B). The duration of swing, brake and propel components of stride were also unchanged in fore (C) and hind (D) paws. Finally, swing, brake and propulsion can be represented as a % of overall stride. No differences were observed in % of swing, brake and propulsion in fore (E) and hind (F) paws. Data represented as mean±SEM.
Figure 2.8. At a speed of 20 cm/sec, the duration of stride and stance showed no difference between Nedd4 heterozygous (+/-) and age-matched wild-type controls in both fore (A) and hind paws (B). The duration of swing, brake and propel components of stride were also unchanged in fore (C) and hind (D) paws. Finally, swing, brake and propulsion can be represented as a % of overall stride. No differences were observed in % of swing, brake and propulsion in fore (E) and hind (F) paws. Data represented as mean±SEM.
Figure 2.9. Stance begins with braking and ends with propulsion, and as such braking and propulsion can be examined as a percentage of stance duration. At 15 cm/sec, there was no difference between Nedd4 heterozygous (+/-) and age-matched wild-type controls (+/+) in brake and propulsion as a % of stance in fore (A) and hind (B) paws. Similarly, at 20 cm/sec no differences were observed in brake and propulsion as a % stance in fore (C) and hind (D) paws. Data represented as mean±SEM.
At 6 months significant changes become apparent which are dependent on speed of locomotion. At the lower speed of 15 cm/sec, a significant increase in stride duration was evident in the hind paws of the heterozygous mice (wt: 0.36 ± 0.008 sec and het: 0.38 ± 0.008 sec; P = 0.048, Figure 2.10B), with no changes in the fore paws (Figure 2.10A). Stance duration was not changed in both fore and hind paws (Figure 2.10A and B). When components of stride were examined, there was no change in the fore paws (Figure 2.10C), but significant increases were seen in swing (wt: 0.11 ± 0.004 sec and het: 0.13 ± 0.006 sec; P = 0.02) and braking (wt: 0.04 ± 0.002 sec and het: 0.05 ± 0.003 sec; P = 0.02, Figure 2.10D) in the Nedd4 heterozygous mice compared to wild-type controls (Figure 2.10D). When represented as a percentage of stride, an increase in fore paw propulsion was evident (wt: 25.7 ± 1.2%; and het: 32.4 ± 2.5%; P = 0.03, Figure 2.10E), with hind paws not affected (Figure 2.10F).

At a moderate speed of 20 cm/sec, no changes were observed in timing of stride in both fore and hind paws in Nedd4 heterozygous mice compared to wild-type controls (Figure 2.11A-E). At the highest speed of 30 cm/sec, no significant changes were evident (Figure 2.12A-E). When braking and propulsion as a percentage of stance was examined, there was a modest decrease in braking (wt: 62.4 ± 2.7 %; n = 9 and het: 52.3 ± 3.8 %; n = 10, P = 0.07) and increase in propulsion (wt: 35.6 ± 2.7 %; n = 9 and het: 46.8 ± 3.8; n = 10, P = 0.07) in fore paws of Nedd4 heterozygous mice compared to wild-type control at a speed of 15 cm/sec (Figure 2.13A). A similar trend was also observed at 30 cm/sec (Figure 2.13E), but no difference at 20 cm/sec (Figure 12C) or in hind paws at all three speeds tested (Figure 2.13B, D and F).
Figure 2.10. At 6 month of age and at a speed of 15 cm/sec, Nedd4 heterozygous mice (+/-) mice showed no difference in stride and stance duration in fore paws (A), however a significant increase is seen in the hind paws (B) when compared to age-matched wild-type controls (+/+). Duration of swing, brake and propulsion was not different in fore paws (C), but in the hind paws, a significant increase in swing and brake duration are evident in the Nedd4 heterozygous mice. Finally, when representing swing, brake and propulsion as a percentage of stride, a significant increase was observed in fore paws of Nedd4 heterozygous mice (E), with no differences in hind paws (F). Data represented as mean±SEM and statistical comparisons made using unpaired Student’s t-test. * < 0.05 and deemed significant.
Figure 2.11. At 6 months of age and at a speed of 20 cm/sec, no changes in stride or stance durations were evident between Nedd4 heterozygous (+/-) mice and age-matched wild-type controls (+/+ in both the fore (A) and hind (B) paws. Swing, brake and propulsion durations were also unchanged in fore (C) and hind (D) paws. When represented as a percentage of stride, no differences were found in swing, brake and propulsion in both fore (E) and hind (F) paws. Data represented as mean±SEM.
Figure 2.12. At 6 months of age and at a speed of 30 cm/sec, no alterations were evident between Nedd4 heterozygous mice (+/-) and age-matched wild-type controls (+/+) in fore (A) and hind (B) paw stride and stance durations. In addition, swing, brake and propel durations were not changed in both the fore (C) and hind paws (D). No differences were observed when swing, brake and propulsion were represented as a percentage of stride in both fore (E) and hind (F) paws. Data represented as mean±SEM.
Figure 2.13. When brake and propulsion were represented as a percentage of stance, there was no difference between 6 month old Nedd4 heterozygous (+/-) mice and age-matched controls (+/+) in fore paws at 15 cm/sec (A), 20 cm/sec (C), or 30 cm/sec (E) and hind paws at 15cm/sec (B), 20 cm/sec (D) or 30 cm/sec (F). Data represented as mean±SEM.
12-month cohort

No difference in stride parameters was evident at 15cm/sec (Figure 2.14A-E). At 20 cm/sec however, Nedd4 heterozygous mice had a significant increase in hind paw braking (wt: 0.04 ± 0.004 sec and het: 0.05 ± 0.005 sec; P = 0.04) and stance (wt: 0.18 ± 0.004 sec and het: 0.21 ± 0.01 sec; P = 0.049, Figure 2.15D) durations. A modest increase in overall stride (wt: 0.30 ± 0.01 sec and het: 0.32 ± 0.01 sec; P = 0.077) duration was observed (Figure 2.15B). As seen with the 6 month old cohort at 30cm/sec speed some gait parameters appeared to normalize (Figure 2.16). The percentage of stance spent in braking and propulsion were also not changed at either speeds tested (Figure 2.17). The data from this 12 months cohort can only be analyzed cautiously, due to the low numbers in each group but further supports that gait changes are evident in Nedd4 heterozygous mice.
Figure 2.14. At a speed of 15 cm/sec, stride and stance durations were unchanged between 12 month old Nedd4 heterozygous (+/-) mice and age-matched controls (+/+) in both fore (A) and hind (B) paws swing, brake and propulsion duration were also unchanged in both fore (C) and hind (D) paws. Finally, when swing, brake and propulsion were represented as a percentage of stride, no differences were in both fore (E) and hind (F) paws. Data represented as mean±SEM.
Figure 2.15. At a speed of 20 cm/sec, fore paw stride and stance durations were unchanged between 12 month old \textit{Nedd4} heterozygous (+/-) mice and age-matched controls (+/+). In the hind paws, stance duration is significantly increased in \textit{Nedd4} heterozygous mice, with increased time hind paws are in contact with the belt. Swing, brake and propulsion durations were also unchanged in the fore paws, in hind paws however, brake duration was significantly increased in the \textit{Nedd4} heterozygous mice, with no changes seen in swing and propulsion durations. When stride parameters were expressed as a percentage of stride, no differences were apparent in both fore and hind paws. Data represented as mean±SEM and statistical comparisons made using unpaired Student’s t-test * < 0.05 and deemed significant.
Figure 2.16. At a speed of 30 cm/sec, stride and stance duration were unchanged between 12 month old *Nedd4* heterozygous (+/-) mice and age-matched control (+/+), in fore (A) and hind (B) paws, as were swing, brake and propulsion duration in fore (C) and hind (D) paws. Even when swing brake and propulsion were represented as a percentage of stride, no differences were observed in fore (E) and hind paws (F). Data represented as mean±SEM.
Figure 2.17. In 12 months mice, brake and propulsion parameters represented as a percentage or stance were unchanged between Nedd4 heterozygous mice (+/-) and age-matched controls (+/+) in fore paws (A, C and E) and hind (B, D and F) paws, at the all three speeds tested (15 cm/sec; A and B, 20 cm/sec; C and D, and 30 cm/sec; E and F). Data represented as mean±SEM.
**Stride length and variability**

**2-month cohort**

Stride length was not different in *Nedd4* heterozygous mice (**Figure 2.18A and B**), which is consistent with no alterations in stride parameters. Stride length variability, on the other hand, was not changed at 15 cm/sec (**Figure 2.18C**) but significantly reduced at 20 cm/sec speed (wt: $0.72 \pm 0.1$ cm and het: $0.50 \pm 0.04$ cm; $P = 0.03$, **Figure 2.18D**). This suggests that at higher speeds *Nedd4* heterozygous mice are able to maintain stride length consistency better than the wild-type controls.

**6-month cohort**

At six months of age a significant increase in hind paw stride length is seen in *Nedd4* heterozygous mice ($5.7 \pm 0.1$ cm) compared to wild-type controls ($5.2 \pm 0.1$ cm; $P = 0.013$) at 15 cm/sec speed (**Figure 2.19A**). This is consistent with the results showing increased hind paw stride duration. In terms of variability, that is the maintenance of consistent length stride to stride, we observed a significant increase in variability of the hind paws of *Nedd4* heterozygous mice (wt: $0.53 \pm 0.06$ cm and het: $0.77 \pm 0.06$ cm; $P = 0.01$) at 20cm/sec (**Figure 2.19D**). This data shows that *Nedd4* heterozygous mice take on average larger strides an observation consistent with the increases stride duration, however are unable to maintain this consistently stride-to-stride.
Figure 2.18. The length of stride was evaluated in 2 month old Nedd4 heterozygous mice (+/-) and age-matched wild-type controls (+/+ ) at both speeds (15 and 20 cm/sec), no changes were observed when compared to age-matched wild-type controls (+/+ ) in both fore and hind paws (A and B). Variation in stride length in both fore and hind paws was not altered at 15 cm/sec (C). At a higher speed of 20 cm/sec, hind paw stride length variation was significantly decreased with no changes to fore paws (D). Data represented as mean±SEM and statistical comparisons made using unpaired Student’s t-test * < 0.05 was deemed significant.
Figure 2.19. At 6 months of age, stride length was significantly increased in the hind paws of Nedd4 heterozygous mice (+/-) compared to age-matched wild-type controls (+/+) at a speed of 15 cm/sec with no changes evident in the fore paws (A). No changes in stride length were evident at the higher speeds of 20 cm/sec (C) or 30 cm/sec (E). Stride length variation measures the step-step variability and was significantly increased in the hind paws only at 20 cm/sec (D), with no changes observed at 15 cm/sec (B) or 30 cm/sec (F). Data represented as mean±SEM and statistical comparisons made using unpaired Student’s t-test * < 0.05 was deemed significant.
Assessment of 12 months old Nedd4 heterozygous mice showed no changes in stride length at all three speeds tested (Figure 2.20A, C and E). Furthermore, stride length variability was also not changed at 15 cm/sec (Figure 2.20B) and 20 cm/sec (Figure 2.20D). At 30 cm/sec however, a significant decrease in stride length variability was seen compared to wild-type controls (wt: 1.05 ± 0.11 and het: 0.44 ± 0.08; P = 0.002) (Figure 2.20F). This, as in the 2 months cohort suggests that at higher speed Nedd4 heterozygous mice are able to maintain stride-to-stride consistency compared to the wild-types.
Figure 2.20. In the 12 month cohort, there was no difference between Nedd4 heterozygous (+/-) and age-matched wild-type controls (+/+) in stride length in both fore and hind paws at 15 cm/sec (A), 20 cm/sec (C) or 30 cm/sec (E). Stride length variability was not different at 15 cm/sec (B), 20 cm/sec (D), but at 30 cm/sec a significant decrease in variability was seen in the hind paws (F). Data represented as mean±SEM and statistical comparisons made using unpaired Student’s t-test ** < 0.01 was deemed significant.
Stance width and variability
Stance width measures the distance between both fore and hind limbs, and variability compares the distance between each successive stride.

2-month cohort
At two months of age, no changes were seen in the Nedd4 heterozygous mice at both speeds tested for either stance width or variation (Figure 2.21).

6-month cohort
At 6 months, there was no change in stance width (Figure 2.22A, C and E) but a significant increase in stance width variability in the hind paws only of Nedd4 heterozygous mice at 30 cm/sec (wt: 0.14 ± 0.01 cm and het: 0.18 ± 0.02 cm; P = 0.032) (Figure 2.22F). Since this parameter evaluates the distance between the hind paw from stride-to-stride, it reflects the inability to maintain consistent width within each stride.

12-month cohort
In the 12 month cohort, there are no differences in stance width at both 15 cm/sec (Figure 2.23A) and 20cm/sec (Figure 2.23C). At 30cm we see a marked increase of hind paw stance width in the Nedd4 heterozygous mice compared to wild-type controls (wt: 2.42 ± 0.13 and het: 2.74 ± 0.08; P = 0.06) (Figure 2.23E). No changes were evident in stance width variability at all three speeds tested (Figure 2.23B, D and E)
Figure 2.21. No difference in stance width between 2 month old Nedd4 heterozygous (+/-) mice and age-matched wild-type controls (+/+) at both 15 cm/sec (A) and 20 cm/sec (B). Variation in stance width stride-to-stride was also unchanged at both 15 cm/sec (C) and 20 cm/sec (D). Data represented as mean±SEM.
Figure 2.22. No difference in stance width between 6 month old *Nedd4* heterozygous mice (+/-) and age-matched wild-type controls (+/+) at 15 cm/sec (A), 20 cm/sec (C) or 30 cm/sec (E). Variation in stance width stride-to-stride was unchanged at 15 cm/sec (B) and 20 cm/sec (D), but significantly increased in the hind paws at 30 cm/sec (F). Data represented as mean±SEM and statistical comparisons made using unpaired Student’s t-test * P < 0.05.
Figure 2.23. No change in stance width was observed in 12 month old *Nedd4* heterozygous mice (+/-) compared to age-matched wild-type controls (+/+) at 15 cm/sec (A), 20 cm/sec (C) or 30 cm/sec (E). Similarly, no change was observed in stance width variation at 15 cm/sec (B), 20 cm/sec (D) or 30 cm/sec (F). Data represented as mean±SEM.
Step frequency and angle dynamics

The number of steps taken in all cohorts of mice was not significantly different (2 months; Figure 2.24A and B, 6 months; Figure 2.25A, C and E and 12 months; Figure 2.26A, C and E). This is important as keeping number of steps within range allows for consistency since each step is used for analysis of all aspects of gait. Step frequency however, allows us to examine the number of steps per second and therefore determine if stepping patterns are altered. At 2 and 12 months of age, step frequency was not significantly affected at all speeds tested (2 month; Figure 2.24C and D, and 6 month; Figure 2.26B, D and F). At 6 months of age, step frequency was significantly reduced in the hind paws of Nedd4 heterozygous mice (wt: 3.10 ± 0.17 steps/sec and het: 2.68 ± 0.06 steps/sec; P = 0.03) at 15 cm/sec (Figure 2.25B), with no difference at 20 cm/sec (Figure 2.25D) or 30 cm/sec (Figure 2.25F). This finding is consistent with increased stride length and duration in 6 month Nedd4 heterozygous mice. It therefore appears, that at six month Nedd4 heterozygous mice take longer and more frequent steps than wild-type controls.
Figure 2.24. The number of steps taken was similar between *Nedd4* heterozygous (+/-) and age-matched wild-type controls (+/+) at 2 months of age, at both speeds tested, 15 cm/sec (A) and 20 cm/sec (B). Step frequency translates to the number of steps taken per second, and was not changed at both speeds, 15 cm/sec (C) and 20 cm/sec (D). Data represented as mean±SEM.
Figure 2.25. The numbers of steps taken were no different between 6 month old Nedd4 heterozygous (+/-) mice and age-matched wild-type controls (+/+). The step frequency was significantly reduced in the hind paws of the heterozygous mice at 15 cm/sec (B), with no alterations observed at the higher speeds of 20 cm/sec (D), or 30 cm/sec (F). Data represented as mean±SEM and statistical comparisons made using unpaired Student’s t-test * P < 0.05 and deemed significant.
Figure 2.26. The numbers of steps taken were similar between 12 month old Nedd4 heterozygous (+/-) and age-matched wild-type controls (+/+) at 15 cm/sec (A), 20 cm/sec (C) and 30 cm/sec (D). Similarly, step frequency was also similar between heterozygous and wild-types at 15 cm/sec (B), 20 cm/sec (D), and 30 cm/sec (F). Data represented as mean±SEM.
Angle dynamics parameters such as; paw angle (which compares left and right paw angles within each mouse), absolute paw angle (compares angle changes in relation to midline of the mouse), step angle (takes into account differences between left and right paws within stride length and width), and variability of both paw and step angles (consistency between strides) were evaluated.

Paw angle and paw angle variation were not different at 2 months of age (Figure 2.27A and B). Absolute paw angle on the other hand was increased in the fore paws of the Nedd4 heterozygous mice compared to wild-type controls (wt: 4.6 ± 0.8 deg and het: 7.1 ± 0.9 deg; P = 0.06) (Figure 2.28A). Fore paw step angle was also increased (wt: 51.9 ± 3.1 deg and het: 61.0 ± 2.6 deg; P = 0.04) in Nedd4 heterozygous mice compared to wild-type controls (Figure 2.28C). No difference was evident in the hind paws (Figure 2.28B and D) or in terms of variation (Figure 2.28E and F).

At 6 months of age, paw angle was not different at all three speeds tested (Figure 2.29A, C and E). Paw angle variation was also unchanged at 15 cm/sec (Figure 2.29B) or 20 cm/sec (Figure 27D) but at 30 cm/sec fore paw showed a significant decrease (Figure 2.29F). No difference in absolute fore paw angle was observed in Nedd4 heterozygous mice at 15 cm/sec (wt: 5.5 ± 0.9 deg; n = 9 and het: 9.5 ± 2.3 deg; n = 10; P = 0.13) (Figure 2.30A), but a significant increase was observed at the higher speeds of 20 cm/sec (wt: 4.5 ± 0.9 deg; n = 10 and het: 9.3 ± 1.5 deg; n = 10; P = 0.01)(Figure 2.30B) and 30 cm/sec (wt: 4.5 ± 1.2 deg; n = 9 and het: 8.7 ± 1.1 deg; n = 10; P = 0.04)(Figure 2.30C). In terms of step angle only a significant decrease was seen in the hind paws of Nedd4 heterozygous mice at 20 cm/sec (wt: 54.8 ± 1.5 deg; n = 10 and het: 48.1 ± 2.7 deg; n = 10; P = 0.046)(Figure 2.31A-F).

Moreover, at 12 months of age no changes in any angle parameters were seen at 15cm/sec (Figure 2.32A and B, Figure 2.33A), 20 cm/sec (Figure 2.33B) or 30 cm/sec (Figure 2.33C). No change in step angle was evident in Nedd4 heterozygous mice compared to wild-type controls (Figure 2.34A-F).
Figure 2.27. No significant differences in paw angle were observed in 2 month old *Nedd4* heterozygous (+/−) compared to age-matched wild-type controls (+/+ ) at 15 cm/sec (A) or 20 cm/sec (B). Although paw area variations was not different at the lower speed of 15 cm/sec (C), at 20 cm/sec, a significant decrease in hind paw angle variations was seen in the heterozygous mice (D). Data represented as mean±SEM and statistical comparisons made using unpaired Student’s t-test * P < 0.05 and deemed significant.
Figure 2.28. Absolute paw angle was not different in 2 month old *Nedd4* heterozygous mice (+/-) compared to age-matched wild-type control (+/+)) at either 15 cm/sec (A) or 20 cm/sec (B). Step angle differences become apparent at 15 cm/sec where a significant increase in the fore paws of the heterozygous mice was seen (C), with no changes at 20 cm/sec (D). Step angle variation was not different at 15 cm/sec (E) or 20 cm/sec (F). Data represented as mean±SEM and statistical comparisons made using unpaired Student’s t-test * P < 0.05 and deemed significant.
Figure 2.29. Paw angle was unchanged in 6 month old *Nedd4* heterozygous mice (+/-) compared to age-matched wild-type controls (+/+ ) at all three speeds tested; 15 cm/sec (A), 20 cm/sec (C), and 30 cm/sec (E). Variance in paw angle was significantly reduced in heterozygotes at 30 cm/sec (F), with no changes apparent at 15 cm/sec (B) and 20 cm/sec (D). Data represented as mean±SEM and statistical comparisons made using unpaired Student’s t-test * P < 0.05.
Figure 2.30. Absolute paw angles were not different in 6 month old $Nedd4$ heterozygous mice (+/-) when compared to age-matched wild-type controls (+/+) at 15 cm/sec (A), but significantly increased in fore paws only at 20 cm/sec (B) and 30 cm/sec (C). Data represented as mean±SEM and statistical comparisons made using unpaired Student’s t-test * $P < 0.05$. 
Figure 2.31. Step angle was significantly reduced in the hind paws of 6 month old *Nedd4* heterozygous mice (+/-) compared to age-matched wild-type controls (+/+) at a speed of 20 cm/sec (C), with no changes observed at 15 cm/sec (A), or 30 cm/sec (E). The variation in step angle was no different at all three speeds tested; 15 cm/sec (B), 20 cm/sec (D) and 30 cm/sec (F). Data represented as mean±SEM and statistical comparisons made using unpaired Student’s t-test * P < 0.05.
Figure 2.32. No changes in paw angle were observed in 12 month old Nedd4 heterozygous mice (+/-) compared to age-matched wild-type controls (+/+) at all three speeds tested; 15 cm/sec (A), 20 cm/sec (C), and 30 cm/sec (E). Similarly, variation in paw angle was also unchanged at all three speeds tested; 15 cm/sec (B), 20 cm/sec (D), and 30 cm/sec (F). Data represented as mean±SEM.
Figure 2.33. Absolute paw angle was not different between 12 month old Nedd4 heterozygous mice (+/-) and age-matched wild-type controls (+/+), at all three speeds tested; 15 cm/sec (A), 20 cm/sec (B), and 30 cm/sec (C). Data represented as mean±SEM.
Figure 2.34. Step angle was not different between 12 month old Nedd4 heterozygous mice (+/-) and age-matched wild-type controls (+/+) at all three speeds tested; 15 cm/sec (A), 20 cm/sec (C) and 30 cm/sec (E). Similarly, variation in step angle was also not different at all three speeds tested; 15 cm/sec (B), 20 cm/sec (D) and 30 cm/sec (F). Data represented as mean±SEM.
Plantar placement

Extent of fore and hind limb plantar placement was significantly reduced in 2 month old *Nedd4* heterozygous mice illustrated by reduced paw area at peak stance in fore paws at 15 cm/sec (wt: $0.42 \pm 0.002$; $n = 8$ and het: $0.36 \pm 0.02$ cm$^2$; $n = 10$; $P = 0.055$) (Figure 2.35A) and 20 cm/sec (wt: $0.45 \pm 0.02$ and het: $0.36 \pm 0.02$ cm$^2$; $P = 0.002$) (Figure 2.35C). Paw area at peak stance was also reduced in the hind paws at both 15 cm/sec (wt: $0.71 \pm 0.03$; $n = 8$ and het: $0.60 \pm 0.03$ cm$^2$; $n = 10$; $P < 0.0001$) (Figure 2.35A) and 20 cm/sec (wt: $0.72 \pm 0.03$ and het: $0.58 \pm 0.02$ cm$^2$; $P = 0.001$) (Figure 2.35C).

As *Nedd4* heterozygous mice are significantly smaller than their wild-type littermates at both ages studied, a Pearson’s correlation was conducted (Pearson, 15 cm/sec $r=0.1827$; 20 cm/sec $r=0.6168$) to determine if reduced paw area is due to reduced body weight. We determined that this was not the case, and furthermore found that although 6 month mice were also significantly smaller than their age matched wild-type controls, there was no alteration in their paw area at peak stance (discussed below).

At 6 months of age paw area at peak stance was not different at all three speeds tested (Figure 2.36A, C and E) however a modest decrease was observed in the hind paws at 20 cm/sec (wt: $0.67 \pm 0.03$ cm$^2$; $n = 10$ and het: $0.60 \pm 0.01$ cm$^2$; $n = 10$; $P = 0.06$) (Figure 2.36C). At 12 months of age, with a slight reduction at 20 cm/sec (wt: $0.75 \pm 0.08$ cm$^2$; $n = 5$ and het: $0.58 \pm 0.03$ cm$^2$; $n = 5$; $P = 0.08$) (Figure 2.37C) however at 30 cm/sec significant reductions were evident in both the fore (wt: $0.43 \pm 0.04$ cm$^2$; $n = 5$ and het: $0.31 \pm 0.02$ cm$^2$; $n = 5$; $P = 0.03$) and hind (wt: $0.71 \pm 0.06$ cm$^2$; $n = 5$ and het: $0.51 \pm 0.01$ cm$^2$; $n = 5$; $P = 0.01$) (Figure 2.37E) paws of *Nedd4* heterozygous mice compared to wild-type controls.

At 2 month of age, variation of paw area at peak stance was not different at 15 cm/sec (Figure 2.35B). At 20 cm/sec however, hind paw area variance was significantly reduced (wt: $0.07 \pm 0.001$ cm$^2$; $n = 8$ and het: $0.04 \pm 0.007$ cm$^2$; $n = 10$; $P = < 0.01$) (Figure 2.35D). There was no change in paw area variability in the 6 month cohort at three speeds tested (Figure 2.36B, D and F). This was also the case in the 12 month cohort (Figure 2.37 B and D), with no decrease in fore paw area variability at 30 cm/sec (wt: $0.07 \pm 0.03$; $n = 5$ and het: $0.02 \pm 0.002$; $n = 5$; $P = 0.075$) (Figure 2.37F).
Figure 2.35. Paw area at peak stance was significantly reduced in hind paws and modestly reduced in the fore paws of 2 month old Nedd4 heterozygous mice (+/-) compared to age-matched wild-type controls (+/+) at 15 cm/sec (A). At 20 cm/sec, both fore and hind paws paw area at peak stance was significantly reduced in the heterozygous mice (C). Variation in paw area stride-to-stride was not different at 15 cm/sec (B), however at a speed of 20 cm/sec, a significant reduction in variation was observed in hind paws of heterozygous mice (D). Data represented as mean±SEM and statistical comparisons made using unpaired Student’s t-test * P < 0.05, ** P < 0.01, *** P < 0.001 and deemed significant. # P = 0.055
Figure 2.36. No significant difference was observed in paw area at peak stance in 6 month old Nedd4 heterozygous mice (+/-) compared to age-matched controls (+/+) at a speed of 20 cm/sec (C), with no differences evident at 15 cm/sec (A), or 30 cm/sec (E). Variation in paw area at peak stance showed no differences at all three speeds tested; 15 cm/sec (B), 20 cm/sec (D), or 30 cm/sec (F). Data represented as mean±SEM and statistical comparisons made using unpaired Student’s t-test # P = 0.06.
Figure 2.37. Paw area at peak stance was not different between 12 month old Nedd4 heterozygous mice (+/-) and age-matched wild-type controls (+/+) at 15 cm/sec (A) and at 20 cm/sec (C). At 30 cm/sec, a significant decrease in paw area is observed in both fore and hind paws of the heterozygotes (E). Variation in paw area was not different at 15 cm/sec (B), 20 cm/sec (D), or 30 cm/sec (F). Data represented as mean±SEM and statistical comparisons made using unpaired Student’s t-test * P < 0.05 and deemed significant # P = 0.06.
Stance factor, gait symmetry, hind limb shared and stance-swing ratio

At 2 months of age, Nedd4 heterozygous mice showed no difference in stance factor in the fore paws at 15 cm/sec (Figure 2.38A) or 20 cm/sec (Figure 2.38B). At 6 months, we see a significant increase in stance factor affecting the fore paws only at both 15 cm/sec (wt: 0.98 ± 0.02; n = 9 and het: 1.06 ± 0.03; n = 10; P = 0.03) (Figure 2.39A) and 30 cm/sec (wt: 0.93 ± 0.03; n = 9 and het: 1.05 ± 0.04; n = 8; P = 0.01) (Figure 2.39C) and no difference at 20 cm/sec (Figure 2.39B). Finally, at 12 months of age even with small numbers in the cohort, a significant increase in stance factor of the fore paws at 20 cm/sec (wt: 0.91 ± 0.03; n = 5 and het: 1.02 ± 0.03; n = 5; P = 0.04) was evident (Figure 2.40B). Overall, a consistent increase in fore paw stance factor duration with no changes in the hind paws was observed in Nedd4 heterozygous mice relative to controls.

Gait symmetry was not affected in 2 (Figure 2.41A), 6 (Figure 2.41B) or 12 (Figure 2.41C) month old Nedd4 heterozygotes compared to wild-type controls.
Figure 2.38. No changes in stance factor were observed in 2 month old Nedd4 heterozygous mice (+/-) compared to age-matched wild type controls (+/+) at both 15 cm/sec (A) and 20 cm/sec (B). Data represented as mean±SEM.
Figure 2.39. At 6 months of age a significant increase in stance factor was observed in the fore paws only of Nedd4 heterozygous mice (+/-) compared to age-matched wild-type controls (+/+) at 15 cm/sec (A) and 30 cm/sec (C), with no difference observed at 20 cm/sec (B). Data represented as mean±SEM and statistical comparisons made using unpaired Student’s t-test * P < 0.05 and deemed significant.
Figure 2.40. At 12 months of age, a significant increase in stance factor was evident in the fore paws of Nedd4 heterozygous mice (+/-) compared to age-matched wild-type controls (+/+ ) at 20 cm/sec (B), with no differences observed at 15 cm/sec (A) or 30 cm/sec (C). Data represented as mean±SEM and statistical comparisons made using unpaired Student’s t-test * P < 0.05 deemed significant.
Figure 2.41. No differences in gait symmetry were observed at all speeds tested in 2 month (A), 6 month (B) or 12 month (C) Nedd4 heterozygous (+/-) mice compared to age-matched wild-type controls (+/+). Data represented as mean±SEM.
Hind paw shared stance, which is a measure of time that both hind paws are in contact with the belt was also unaffected in both 2 (Figure 2.42A) and 6 (Figure 2.42B) months old Nedd4 heterozygotes. At 12 months however, an increase in hind limb shared stance was evident although significance was not reached (20 cm/sec, wt: 0.06 ± 0.01 ms; n = 5 and het: 0.09 ± 0.03 ms; n = 5; P = 0.15 and 30cm/sec, wt: 0.02 ± 0.01 ms; n = 5 and het: 0.04 ± 0.01 ms; n = 5; P = 0.2) (Figure 2.42C).

No difference in stance-swing ratio between Nedd4 heterozygous and wild-type controls was evident at 2 (Figure 2.43), 6 (Figure 2.44) and 12 (Figure 2.45) months of age at all speeds tested.
Figure 2.42. Hind paw shared stance were not different between Nedd4 heterozygous (+/-) mice and age-matched controls (+/+) at all three speeds tested at 2 (A), 6 (B) or 12 months of age (C). Data represented as mean±SEM.
Figure 2.43. Ratio of stance to swing was not altered in 2 month old Nedd4 heterozygous (+/-) mice compared to age-matched controls (+/+ ) at both 15 cm/sec (A) or 20 cm/sec (B). Data represented as mean±SEM.
Figure 2.44. Ratio of stance to swing was not altered in 6 month old Nedd4 heterozygous mice (+/-) compared to age-matched wild-type controls (+/+) at all three speeds tested; 15 cm/sec (A), 20 cm/sec (B), and 30 cm/sec (C). Data represented as mean±SEM.
Figure 2.45. Ratio of stance to swing was not altered in 12 month old Nedd4 heterozygous mice (+/-) compared to age-matched wild-type controls (+/+). Data represented as mean±SEM.
Table 2.2. Summary of gait parameters. Arrows represent an increase or decreases in the Nedd4 heterozygous mice compared to age-matched wild-type controls. NS, not significant.

<table>
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<th>Parameter</th>
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<td>↑ Hind paw</td>
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<td>↑ Hind paw</td>
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<td>20 cm/sec (P &lt; 0.05)</td>
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<td>↑ Hind paw</td>
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<td>variability</td>
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<td>30 cm/sec (P &lt; 0.05)</td>
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<td>Step angle variation (deg)</td>
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<td>Paw area at peak stance (cm³)</td>
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<td>Stance Swing ratio</td>
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- **Hind paw** indicates a significant decrease compared to the baseline.
- **Fore paw** indicates a significant increase compared to the baseline.
- **NS** indicates no significant difference compared to the baseline.

P-values are given as follows: (P < 0.05) and (P < 0.001) respectively.
Cellular expression of Nedd4 in the CNS associated with motor function and gait control

Motor cortex

Nedd4 was ubiquitously expressed in all six layers of the motor cortex. Nedd4 was mainly localized to the plasma membrane, surrounding the NeuN (nuclei marker) stained neurons. Nedd4 staining appears most abundant in the layer IV pyramidal neurons (Figure 2.46). Pyramidal neurons of the motor cortex are principally involved in sending axons to innervate motor neurons of the spinal cord.

**Figure 2.46.** Confocal images taken from coronal brain section obtained from a 2 month old wild-type mouse showing ubiquitous Nedd4 (red) expression in the motor cortex. Low levels of Nedd4 was observed surrounding NeuN (green) positive neurons in layer IV pyramidal neurons. Scale bar represents 50μm.
Basal ganglia

Substantia nigra

Nedd4 was found ubiquitously expressed within the SNpc with very little co-localization evident between Nedd4 and Tyrosine hydroxylase (TH) (Figure 2.47). In SNpr, Nedd4 expression was modest with no obvious cellular localization (Figure 2.48). Overall, within the SN low levels of Nedd4 expression were found.

**Figure 2.47.** Nedd4 (red) was expressed in low levels within the substantia nigra pars compacta (SNpc), with very little co-localization between dopaminergic tyrosine hydroxylase positive neurons (TH, green). Scale bar represents 50µm.

**Figure 2.48.** Moderate and ubiquitous expression of Nedd4 (red) in substantia nigra pars reticulata (SNpr). SNpr lies adjacent to SNpc where no tyrosine hydroxylase (green) staining is observed. Scale bar represents 50µm.
**Striatum**

Nedd4 showed ubiquitous expression within the matrix (grey matter) of the striatum. No expression was evident in the striatosomes (white matter) (Figure 2.49).

![Figure 2.49](image)

*Figure 2.49.* In the striatum Nedd4 (red) was expressed within the matrix, surrounding neuronal nuclei (NeuN) with no staining in the striatosomes (white matter). Scale bar represents 50µm.

**Spinal cord**

Low levels of Nedd4 were found in motor neurons of the spinal cord. Moderate ubiquitous staining was evident in the axons or fibres of the grey matter in the lumbar portion of the spinal cord (Figure 2.50).

![Figure 2.50](image)

*Figure 2.50.* In the lumbar portion of the spinal cord, Nedd4 (red) was found to be ubiquitously expressed in the grey matter. Low level of co-localisation was observed between Nedd4 and motor neurons (NeuN, green). Scale bar represents 50µm.
Cerebellum

Nedd4 was abundantly expressed in Purkinje neurons of the cerebellum, extending to the dendrites (Figure 2.51). In Nedd4 heterozygous mice Nedd4 expression appeared reduced particularly in the 6 month group. There did not appear to be any cell loss or morphological changes in the cerebellum in Nedd4 heterozygous mice at both time points.

Figure 2.51. Nedd4 (red) was highly expressed in Purkinje neurons of the cerebellum. In addition, Nedd4 was shown to extend into the dendrites of these Purkinje neurons as shown by the co-localisation with a marker for dendrites (MAP2, green). Scale bar represents 50µm.
<table>
<thead>
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<td>Striatum</td>
<td>++</td>
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<tr>
<td>Cerebellum</td>
<td>+++</td>
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</table>

**Table 2.3.** Qualitative analysis of Nedd4 staining intensity in regions of the brain associated with motor function. + low, ++ medium and +++ high. SNpc, substantia nigra pars compacta, SNpr, substantia nigra pars reticulata.
GluR1 subunit of AMPA receptors

GluR1 was examined both qualitatively (immunohistochemistry) and quantitatively (Western blotting) in the cerebellum from Nedd4 heterozygous and wild-type mice. Immunohistochemistry showed an increase in GluR1 staining around the Purkinje neuron cell bodies in the heterozygous mice compared to age matched wild-type controls (Figure 2.52A). When protein levels from whole cerebellar lysates were measured by Western blotting, a significant increase in GluR1 levels was found in the 6 month old Nedd4 heterozygous mice compared to wild-type controls (wt: 97.9 ± 36.4%; n = 3 and het: 262.8 ± 23.1%; n = 4, P = 0.01) (Figure 2.52B and C). There was no change in levels observed in the 2 month heterozygous mice however, which correlates with severity of gait abnormalities.
Figure 2.52. Immunohistochemistry showed an increase in GluR1 (red) staining surrounding soma of Purkinje neurons (PN) stained with neuronal marker, NeuN (green) in the cerebellum of Nedd4 heterozygous mice (+/-) relative to wild-type controls (+/+) at both time points (A). Representative Western blots from cerebellar lysates obtained from 2 and 6 month old Nedd4 heterozygous mice (+/-) and wild-type controls (+/+) (B). Quantification of Western blot showed a significant increase in GluR1 levels in cerebellum from 6 month old Nedd4 heterozygous (+/-) mice compared to age-matched controls (+/+), with no change observed in the 2 month cohort (C). * P < 0.05, unpaired Student’s t-test. Scale bar represents 10μm.
Calbindin-D28k levels were unchanged in cerebellar lysates from Nedd4 heterozygous mice at both 2 (wt: 100.0 ± 4.7%; n = 4 and het: 87.6 ± 12.4%; n = 3) and 6 (wt: 88.6 ± 27.0%; n = 4 and het: 68.6 ± 3.4%; n = 5) months of age (Figure 2.53).

**Figure 2.53.** Representative Western blots of Calbindin-D28k in cerebellum of 2 and 6 month old Nedd4 heterozygous mice (+/-) and age-matched wild-type controls (+/+/). No difference was seen in levels of in Calbindin-D28k from cerebellum obtained from 2 and 6 month Nedd4 heterozygous mice (+/-) compared to wild-type controls (+/+). Data represented as mean±SEM.
2.4 DISCUSSION

This study has made an important contribution to the understanding of the function of Nedd4 within the central nervous system, including behavioural outcomes, identification of regional and cellular expression as well as investigation of novel targets necessary for the maintenance of gait and motor control. Due to the lethality of the Nedd4 homozygous knockout mice any studies examining phenotypical changes in vivo had to be performed in heterozygous animals. Other groups have successfully used heterozygous animals (e.g. ataxic Moonwalker mutant) in identifying motor deficits when homozygous models are not viable (Becker et al., 2009). Conditional Nedd4 knockout mice are now available and can be used to examine the effect of complete removal of Nedd4 within the CNS. These knockout mice, will be extremely beneficial in determining in vivo effects and may indeed be superior in identifying behavioural and morphological outcomes.

Nedd4 heterozygous mice had at least a 50% reduction in brain Nedd4 levels at both 2 and 6 months of age. Closer inspection of Western blots showed an increase in Nedd4 levels in the 6 month group relative to the 2 month cohort, however statistical comparison did not reveal significant changes. There is no evidence to suggest an age-dependent increase in Nedd4 levels. One of the first physiological characteristics that became evident in the Nedd4 heterozygous mice was a significant reduction in size. This has previously been reported by our collaborators, and is due to reduced IGF-1 mediated signaling (Cao et al., 2008). Growth disruption was evident in overall body and brain mass and occurred in a pro-rata manner. It is likely that these reductions in growth are largely mediated by reduced IGF-1 signaling in the heterozygous mice. IGF-1 levels were not directly measured in this study due to difficulty in obtaining a reliable IGF-1 assay for mouse brain, however work by our collaborator Prof. Kumar (Cao et al., 2008) showed that IGF-1 signaling is indeed disrupted when Nedd4 was knocked-down. The use of heterozygous mice suggests that although IGF-1 signaling would be affected it is unlikely to be at the same extent as IGF-1 knockout mice or even Nedd4 knockout mice. There was also no evidence that growth and differentiation of neurons were impacted, or developmental delays in the Nedd4 heterozygous mice, supported by morphological examinations where no obvious cell loss, reduced volume or reduced cell size (data not shown) was evident.
First basal loco-motor activity was assessed and no difference between *Nedd4* heterozygous and age matched wild-type controls were found, showing no signs of hyper or hypo activity were evident that may confound other tests conducted. Next, motor capacity was assessed using the RotaRod and no changes present at all time points tested (2, 6 and 12 months of age). This is not surprising, as Nedd4 is only expressed in very low levels in motor neurons in the spinal cord and therefore unlikely to have a large contribution to motor output. Nedd4 is expressed abundantly in skeletal muscle, with a significant decrease observed in the heterozygotes at both 2 and 6 months of age (data not shown), however morphological and structural examinations need to be conducted to examine the impact a reduced level of Nedd4 has on skeletal muscle fibres. At least in the heterozygous setting, it does not appear that the loss of Nedd4 in skeletal muscle has a profound effect, as both heterozygous and wild-type mice showed very similar motor capacity even at 12 months of age. An age-dependent decline in motor capacity was observed in both *Nedd4* heterozygous mice and wild-type controls, which is not surprising, as motor function has been shown to normally decline with ageing in both rodents (Shukitt–Hale *et al.*, 1999) and humans (Volkow *et al.*, 1998).

Although *Nedd4* heterozygous mice have normal loco-motor activity and motor function, analysis of gait provided early and subtle changes that perhaps may not be obvious in a heterozygous setting or indeed may allow us to evaluate changes in gait patterns independent of motor function. That is, the *Nedd4* heterozygous mice may never develop gross motor function deficits that are routinely assessed by RotaRod, but may in fact have changes in gait indicative of cerebellar fine motor control abnormalities. Alternatively, changes assessed by RotaRod may manifest at a much later stage where degenerative changes in the cerebellum are more profound.

When gait was evaluated, significant alterations were found in *Nedd4* heterozygous mice, which became more pronounced with age. In the context of this study, Nedd4 was shown to be important for gait and thus cerebellar function. In interpreting these results, previous published data can be used where alterations in similar gait parameters are seen in rodent models of disease and can therefore allow for some comparison. However, the main aim is to highlight the importance of Nedd4 in gait, and regions of the brain associated with gait controls.

One of the first gait changes observed was the alteration in the timing of motion in the heterozygotes, suggesting abnormalities in the initiation, acceleration and deceleration of
motion. This is likely to occur due to altered Purkinje neuron signaling to the deep cerebellar nuclei, which can then affect the level of cortical inhibition (Kandel et al., 2000). This is supported by the abundant expression of Nedd4 in Purkinje neurons of the cerebellum. The Purkinje neurons are the sole neuronal output from the cerebellum, and send projections to the deep cerebellar nuclei. Cell loss or altered function of Purkinje neurons therefore, can have profound effects on gait (Diener et al., 1992). This is exemplified in the lurcher mutant mice where the loss of Purkinje neurons leads to ataxic gait as the feedback mechanism to the motor cortex is lost (Armstrong et al., 2010). The Nedd4 heterozygous mice did not show any Purkinje cell loss, suggesting instead that alterations in Purkinje neuron activity and/or function may contribute to gait abnormalities observed.

The first target investigated was GluR1 subunit of AMPARs. The GluR1 subunit of AMPARs are highly expressed in Purkinje neurons with studies in stargazer mutant mouse, showing an almost complete loss of AMPARs at the granule cell synapses that produces severe ataxia and epilepsy (Shevtsova et al., 2012). Recently, Nedd4 was shown to ubiquitinate the GluR1 subunit of AMPARs in an activity dependent manner (Schwarz et al., 2010). Therefore, in Nedd4 heterozygous mice, GluR1 levels should be elevated due to reduced levels of ubiquitination. When levels of GluR1 levels were measured in cerebellar lysates, there was a significant up-regulation in Nedd4 heterozygous mice at 6 months of age. This is important as no differences were observed at 2 months of age where very subtle changes in gait were evident, but at 6 months the alteration in timing of motion was evident and so were levels of GluR1. Although there is no evidence that Purkinje neuron activity is altered, it seems likely that this elevation in GluR1 levels can alter the level of excitatory feedback to the motor cortex that can have profound implications on gait.

In normal (wild-type) mouse inhibitory Purkinje neuron output project to the deep cerebellar nuclei and the level of excitatory feedback is normalized to the motor cortex to produce normal gait. Previous studies have demonstrated the loss of AMPARs in the stargazer mutant results in a loss of inhibitory projections to the deep cerebellar nuclei, increasing the extent of excitatory projections to the motor cortex, resulting in ataxia, or gait deficits (Shevtsova et al., 2012). The results from this work provide novel evidence that the increase in AMPARs in the Nedd4 heterozygous mice, potentiate the inhibitory output to the deep cerebellar nuclei, and thus reduce the level of excitation to motor cortex, resulting in extended or exaggerated gait (Figure
Future studies should examine Purkinje neuron activity, and determine whether AMPAR mediated currents are altered.

No obvious changes in the numbers of Purkinje neurons observed in Nedd4 heterozygous mice further supports this view. In addition, subtle changes in dendrite structure is not unexpected given the effects on dendrite branching reported in Nedd4 KO mice (Kawabe et al., 2010), and may in part account for alterations in gait parameters.

**Figure 2.54.** Schematic illustration of the hypothesized effect that Nedd4 could have on GluR1 subunit of AMPA receptors (AMPARs) and the contribution to gait. Deep cerebellar nuclei (DCN).

The contribution of voltage-gated ion channels can not be excluded as neuronal excitability can be altered by voltage gated Na⁺ channels (Na,s), which are responsible for the initial depolarization of the action potential. For instance, deletion of Na,1.1 results in deep cerebellar hyper-excitability, ataxic responses and shortening of stride length (Kalume et al., 2007). This could be another potential target as Na,1.1 contains a functional Nedd4 binding motif (Fotia et al., 2004), and therefore a decrease in Nedd4 may lead to a significant increase in levels of Na,1.1 and thus hypo-excitability of deep cerebellar nuclei which would produce extended gait.
The examination of Na,1.1 channels levels is difficult due to lack of specific antibodies, and would certainly warrant further investigation. Voltage-gated calcium channels have also been recently shown to be degraded by Nedd4 (Rougier et al., 2011). This is important as anomalies in calcium signaling and levels of voltage-gated calcium channels are also characteristic in ataxic models such as stargazer (Leitch et al., 2009), ducky (Brodbeck et al., 2002) and in human spinocerebellar ataxias (Pietrobon, 2002).

Calcium signaling is crucial for normal Purkinje neurons function, so in this study a target important for calcium homeostasis was investigated. Calbindin-D28k was chosen as a potential target, as is it highly expressed in the Purkinje neurons of the cerebellum and a member of a larger family of calcium buffering protein. In the cerebellum, Calbindin-D28k comprises approximately 15% of total cellular protein (Baimbridge KG et al., 1992). It is not surprising therefore that Calbindin-D28k knockout mice have ataxia and abnormal calcium signaling in the Purkinje neurons (Airaksinen et al., 1997). In the Nedd4 heterozygous mice we did not observe any changes in Calbindin-D28k levels in the cerebellum at both time points tested. It is therefore unlikely that Calbindin-D28k is a target of Nedd4 and may not have significant interactions. The hypothesis was that if alterations in Calbindin-D28k existed in the Nedd4 heterozygous mice, then alterations in intracellular calcium levels could have significant effects on Nedd4 activation, however this does not appear to be the case.

Calcium influx plays a crucial role in excitability of neurons, with Purkinje neurons requiring calcium for their complex spike patterns. With Nedd4 able to regulate newly synthesized Ca,1.2 channels, and therefore the reduced levels of Nedd4 in the heterozygotes may impact levels of Ca,1.2 channels and thus Purkinje neuron excitability. Although, this very important target was not evaluated in this study, future studies should examine Ca,1.2 channels in Nedd4 heterozygous to determine the consequences of Nedd4 level reduction or absence. My view is that both GluR1-containing AMPARs and Ca,1.2 channel densities on Purkinje neuron cell membrane are likely to be altered in the Nedd4 heterozygous mice, with both sodium and calcium currents affected. Future studies, could examine both sodium and calcium currents in whole brain cerebellar slices to determine the extent of contribution of each of these two important targets to Purkinje neuron function.
Aside from the cerebellum, which has a major role in gait control, there are other regions of the brain that can also contribute. For instance alterations in stride length variability, which is a measure of consistency between strides, is considered a reliable index of motor decline related to basal ganglia dysfunction (Fernagut et al., 2002). This is illustrated by increased stride length variability observed in patients with Parkinson’s disease as well as rodent models of Parkinson’s disease, Huntington’s disease and ALS (Amende et al., 2005b; Hausdorff et al., 1998; Hausdorff et al., 2000; Kurz et al., 2007). In this study, Nedd4 heterozygotes showed alterations in stride length variability, however no basal ganglia abnormalities were evident. Furthermore, Nedd4 was expressed at relative low levels in regions of the basal ganglia such as SNpc, SNpr and striatum. These data suggest that rather than basal ganglia defects, changes in accuracy of movement are likely as a result of abnormal firing patterns in Purkinje neurons.

Nedd4 heterozygous mice also showed an increase in paw angle i.e. ‘splayed phenotype’ which has been associated with ataxia, demyelinating diseases and spinal cord injuries (Powell et al., 1999) as well as a mouse model of lissencephaly (a disorder arising due to abnormal neuronal migration) (Yamada et al., 2009). Furthermore, a significant increase in step angle in fore paws of Nedd4 heterozygous mice was evident. Increased step angle has also been reported following cerebellar dysfunction due to neonatal ethanol exposure, where rats display more open or increased step angles with less gait symmetry (Hannigan et al., 1988). Similarly, in a mouse model of infantile Batten disease, which occurs due to abnormal lysosomal storage leading to subsequent axonal degeneration and widespread CNS neurodegeneration, an increased step angle is observed and occurs in conjunction with a loss of Purkinje neurons and reduced dendritic branching (Sleat et al., 2004). To date we have no evidence for neuronal migration or demyelination abnormalities in Nedd4 heterozygous mice. Furthermore, Nedd4 was not expressed in white matter, and therefore is unlikely to be located in oligodendrocytes and type II astrocytes. Some of the parameters described could also be associated with cerebellar dysfunction and potential involvement of AMPAR signaling.

Finally, alterations in paw area at peak stance and stance factor were found in Nedd4 heterozygous mice. These parameters are changed following neuronal injury and inflammatory pain models (Piesla et al., 2009). One possible cause may be due to enhanced motor mediated nociception, supported by previous reports that the voltage-gated Na+ channel Na,1.7 important for the sensation of pain is a target of Nedd4 (Fotia et al., 2004; Ghelardini et al., 2009). A test
to assess responses to nociception (hotplate test), was conducted and found that there was no difference to thermal nociception between Nedd4 heterozygous and wild-type controls (data not shown). However, the hotplate test is a crude method of nociception and subjective so it may not allow the identification subtle changes, therefore other more sensitive tests are required such as von Frey.

Previous gait analysis tools such as footprint analysis examined gait where speed of locomotion was not controlled or measured. It is difficult to draw comparisons between DigiGait and older methods for assessing gait, due to the fact that speed can be controlled. What has been established in both rodents and humans is that gait changes with varying speed. In some instances increasing speed provides a challenge to normal walking speed, however in other instances it may mask some gait changes by providing compensatory measures. Whether these points apply to the gait observed in this study remains to be determined and conclusions are difficult to draw. In this study gait changes became more pronounced with age. The reason for this remains unknown as no morphological changes such as neuronal cell loss was evident in regions of the brain that control gait (eg cerebellum). Further investigation is necessary to examine molecular changes that may exist for perhaps in Purkinje neurons that changes their signalling properties that may occur in an age-dependent manner.

In this study the changes in gait identified suggest an abnormality in the function of cerebellar circuits, most likely to involve Purkinje neurons. It is difficult to speculate about the type of cerebellar dysfunction that may be present in the Nedd4 heterozygous mice based purely on changes in gait. Further studies are necessary to examine perhaps with electrophysiology any changes in calcium or sodium currents in Purkinje neurons of the heterozygous mice that may affect firing patterns. Furthermore, examination in brain slices could be conducted to evaluate GluR1-mediated AMPA receptor currents and determine if any changes exist as are suggested with this data. Findings for these future experiments will provide much needed mechanism to better understand the gait changes identified in the Nedd4 heterozygous mice.

In conclusion, results from this chapter have identified a novel role for Nedd4 in gait control involving Purkinje neurons of the cerebellum. This is important as to date very little was known about the physiological role for Nedd4, and whether reduced levels of Nedd4 would lead to in vivo outcomes. Furthermore, data from this study has shown that a current known target of
Nedd4, GluR1 subunit of AMPA receptors, was significantly altered in the cerebellum from *Nedd4* heterozygous mice, which may contribute to gait abnormalities observed. Finally, a novel protein target linked to a human disorder may be a crucial link in understanding the complex role of Nedd4 in neurons of the brain. Further work is required to build a better understanding of the complex interactions of Nedd4 with its many neuronal targets, in particular the calcium-induced activation of Nedd4.
CHAPTER 3: NEDD4-2 IN MOTOR FUNCTION AND GAIT

3.1 BACKGROUND AND RATIONALE

This chapter explores the possible relationship between Nedd4-2 expression, the dopamine system and motor function.

_Dopamine transporter (DAT)_

DAT is a member of the neurotransmitter sodium symporter protein superfamily (SLC6 gene family) (Gether \textit{et al.}, 2006). DAT is expressed at the plasma membrane; however a dynamic pool of DAT is available implying that insertion and removal from the plasma membrane must be tightly regulated (Mortensen \textit{et al.}, 2003). Alteration in function of DAT will ultimately affect the level of dopamine at the synapse and thus have major implications in dopaminergic signaling. The importance of dopamine in motor function is best highlighted in Parkinson’s disease (PD), where dopamine levels are reduced due to degeneration of SNpc (dopamine producing) neurons. There is evidence that in Parkinson’s disease, that along side reduced dopamine nerve terminals there is also a reduction in levels of DAT (Booij \textit{et al.}, 1997; Frost \textit{et al.}, 1993).

_Abnormalities in levels ofDAT are associated with many neurological disorders_

Abnormalities in DAT level and function are observed in disorders such as depression (Amsterdam \textit{et al.}, 2012; Pinsonneault \textit{et al.}, 2011), attention deficit-hyperactivity disorder (ADHD) (Hoogman \textit{et al.}, 2012; Roman \textit{et al.}, 2004), Parkinson’s disease (PD) (Ravina \textit{et al.}, 2012) and addiction (Hanlon \textit{et al.}, 2012; Tammimaki \textit{et al.}, 2011). DAT gene polymorphisms are observed in patients suffering from hyperactivity disorders such as ADHD (Krause \textit{et al.}, 2003), with most current treatments for ADHD targeting DAT. Experiments in DAT knockout mice also show a distinct behavioural phenotype associated with hyperactivity, due to the increased extracellular levels of dopamine (Giros \textit{et al.}, 1996; Spielewoy \textit{et al.}, 2009), as well as depression with reduced anhedonia (Perona \textit{et al.}, 2008)
Dopamine regulation and trafficking

The de novo synthesis of DAT and export to the membrane surface of the dopamine neurons is a slow process with an approximate half life of 2-3 days (Kimmel et al., 2000), so neurons must use a range of post-translational regulatory strategies to control DAT function (Schmitt et al., 2010). One way to modulate DAT function rapidly is to alter trafficking by redistributing DAT between the plasma membrane and intracellular endosomal compartments (Mortensen et al., 2003). Phosphorylation is one example of a post-translational strategy for DAT regulation that can alter protein-protein interactions. It is therefore not surprising that the phosphorylation state of DAT can influence intrinsic activity, such as the affinity and responsiveness to ligands (eg. dopamine) but also membrane distribution of DAT (Mortensen et al., 2003). There are several protein kinases postulated to be involved in affecting DAT function including; PKC, PKA, PI3K, protein tyrosine kinase, Ca2+/calmodulin kinase, protein phosphatase 1, and mitogen-activated protein kinase (MAPK) family (Foster et al., 2006; Hoover et al., 2007; Melikian, 2004). Of these protein kinases, PKC has been most highly investigated and shown to be integral for endocytic trafficking of DAT to early and recycling endosomes (Sorkina et al., 2003; Sorkina et al., 2005). Furthermore, the PKC-induced loss of DAT at the plasmalemmal compartment is most likely due to combination of increased clathrin-dependent endocytosis and decreased recycling from endosomal compartments (Loder et al., 2003).

Ubiquitination, Nedd4-2 and Dopamine Transporters

Aside from phosphorylation, there is now mounting evidence that ubiquitination may contribute to post-translational modification of DAT to regulate cell surface expression and membrane trafficking (Miranda et al., 2005). More importantly, Nedd4-2 was shown to be necessary for the ubiquitination and endocytosis of DAT, which is mediated by PKC using lysine-63 (K63) linked mono-ubiquitin chains (Sorkina et al., 2006). Knock down of Nedd4-2 resulted in suppression of ubiquitination and endocytosis of DAT in human and porcine cells. Interestingly, DAT does not contain the ‘typical’ PPxY motif that is recognized by the WW domain of Nedd4-2, and therefore Nedd4-2 may either recognize other motifs not yet identified, use of intermediate or adapter proteins or not interact at all (Vina-Vilaseca et al., 2010).
The involvement of Nedd4-2 in motor function and gait

Dopamine is a major neurotransmitter in the brain, produced by neurons located in the SNpc, ventral tegmental area (VTA) and arcuate nucleus (Marek et al., 1996; Seibyl et al., 1995). Dopamine neurotransmission is involved in many processes including; motor activity, emotion, motivation and cognition (Le Moal, 1995) using several pathways. Of these pathways, the nigrostriatal pathway which connects SNpc with the striatum and forms an important feedback loop necessary for the regulation of locomotion and motor function (Figure 3.1) (Chinta et al., 2005).

![Figure 3.1. Schematic illustration of striatonigral pathway necessary for motor feedback to the motor cortex for motor function and gait.](image)

The removal of dopamine from the synapse by DAT is essential for the maintenance of normal dopamine levels, effectively terminating dopamine signaling (Jaber et al., 1997; Torres, 2006). Due to the potential role for Nedd4-2 in dopamine neurotransmission, it was necessary to evaluate the consequences of reduced Nedd4-2 levels with the use of heterozygous mice and motor function and gait. As already discussed in Chapter 2 RotaRod and DigiGait are excellent tests in identifying motor dysfunction and abnormalities in gait.

DAT regulation is imperative for normal dopamine signalling. Until recently, the mechanism of DAT regulation at the membrane surface remained poorly understood. The effect of Nedd4-2...
mediated DAT regulation on dopamine levels and/or behaviour have not been characterized in vivo.

The findings from these studies led to the main hypothesis of this study that a 50% reduction in Nedd4-2 levels (using heterozygous mice) levels is sufficient to alter dopamine transporter and subsequent dopamine levels which may contribute to motor and gait abnormalities. To investigate this hypothesis, the following aims were set:

1) Conduct behavioural tests in Nedd4-2 heterozygous mice and assess basal loco-motor activity, motor function and gait.
2) Determine cellular and regional expression of Nedd4-2 in the brain and spinal cord required for motor related behaviours
3) Measure DAT and dopamine levels in striata from Nedd4-2 heterozygous mice relative to wild-type controls
4) Examine brain morphology to determine if any changes are present in dopaminergic regions of the brain

3.2. MATERIAL AND METHODS

Mice

Our collaborator Professor Sharad Kumar generated Nedd4-2 heterozygotes and wild-type littermate controls, and were a generous gift. Mice bred at the IMVS animal facility (Adelaide, Australia) and transported to Florey Neuroscience Institutes (FNI, Melbourne Australia) for behavioural assessment. At least one week acclimation prior to any testing was observed, during this period mice were handled daily to reduce stress related behaviours. Animals were singly housed a week prior to testing and maintained on a 12:12 h light/dark cycle. Animals were also given a day rest between each round of testing to ensure that variability due to fatigue was not a factor.

For motor function and gait analysis, locomotor activity, RotaRod and DigiGait was used in 2 and 6 month old Nedd4-2 heterozygous and age-matched controls were used (n = 7-15). Basal locomotor activity, motor capacity and gait were assessed (as described in Chapter 2).
completion of behavioural assessment mice were weighed and deeply anaesthetized with sodium pentobarbital injection (100 mg/kg i.p.), and tissue removed for Western blot analysis or immunohistochemistry. A separate cohort of mice was used for HPLC analysis at both 2 (wt: n = 8 and het: n = 7) and 6 (wt: n = 5 and het: n = 5) months of age.

**Western Blotting**

Whole brain (2 month, wt: n = 4 and het: n = 5; 6 month, wt: n = 5 and het: n = 4) and striata (2 months, wt: n = 8 and het: n = 7; 6 months, wt: n = 4 and het: n = 4) were homogenized as described in Chapter 2. Briefly, after protein separation on SDS-page gels, membranes were probed with, rabbit anti-Nedd4-2 (1:1000; generated in Kumar laboratory), DAT (1:1000; Merck-Millipore), and chicken anti-β-actin (1:1000; Abcam) used as a loading control. Blots were washed and incubated with mouse-Alexa 488 (1:1000, Invitrogen) and chicken-Alexa 647 (1:1000, Invitrogen). Blots were visualized using FluoroChemQ Multimage III (Alpha Innotech, USA), and analysed using Image J Software.

**Immunohistochemistry**

For immunohistochemistry, 2 (n = 5) and 6 (n = 5) month old Nedd4-2 heterozygous and wild-type littermates (n = 4, n = 5 and n = 3, respectively) were used. Frozen 50μm thick free-floating coronal sections were incubated with primary antibodies. The following antibodies were used:

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Function</th>
<th>Dilution</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse anti-NeuN</td>
<td>Neuronal marker</td>
<td>1/1000</td>
<td>Merck-Millipore</td>
</tr>
<tr>
<td>Mouse anti-MAP2</td>
<td>MAP2 protein (dendritic marker)</td>
<td>1/1000</td>
<td>Sigma</td>
</tr>
<tr>
<td>Rabbit anti-Nedd4-2</td>
<td>Nedd4 protein</td>
<td>1/1000</td>
<td>Gift from Prof Sharad Kumar</td>
</tr>
<tr>
<td>Mouse anti-Tyrosine</td>
<td>The rate limiting enzyme in dopamine</td>
<td>1/1000</td>
<td>Merck-Millipore</td>
</tr>
<tr>
<td>hydroxylase (TH)</td>
<td>synthesis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat anti-Dopamine transporter (DAT)</td>
<td></td>
<td>1/1000</td>
<td>Merck-Millipore</td>
</tr>
</tbody>
</table>
Primary antibodies were visualized with Alexa-conjugated secondary antibodies, mounted using Prolong-Gold with DAPI mounting medium (Invitrogen). Stained sections were visualized using a Nikon C1 confocal microscope using a 488nm argon and 543nm HeNe laser (Nikon Group, Tokyo, Japan). Images were acquired using a 40x Plan-Apochromat oil immersion objective with identical gain settings applied for all groups. All images were captured and analyzed using Nikon Elements software.

*High Performance Liquid Chromatography (HPLC)*

*Isolation of brain tissue*

For HPLC, mice were euthanized with sodium pentobarbital injection (100 mg/kg i.p.). The brain was removed, placed on ice and the striatal region rapidly dissected on a chilled plate. Striatal tissue obtained was weighed so that 3-5mg of tissue was transferred to a glass homogenizer containing 200μl of extraction buffer (4M Perchloric acid, 0.008M sodium metabisulphate, 0.002M disodium ethylenediaminetetra-acetic acid (EDTA), MilliQ water to bring volume to 100ml). Samples were homogenized until a homogenous solution is produced and sonicated to rupture vesicular membranes. Samples were then spun at 10,500g for 5 minutes, and the supernatant transferred to a fresh tube. The samples were spun a further two times, to ensure all debris was eliminated. Samples were stored at -80°C until required.

*HPLC*

For HPLC analysis, 40μl of sample was transferred to a HPLC recovery vial. Standards for dopamine and L-DOPA were made in the same extraction buffer used for sample preparation. The mobile phase was composed of 70mM monopotassium phosphate, 0.5mM EDTA di-sodium salt, 8mM octane sulfonic acid sodium salt, 170ml HPLC grade methanol, to a final volume of 1000ml and pH 3. The flow rate was 500μl/min with reverse phase C18 columns. HPLC analysis was conducted on striatal samples from both 2 and 6 month *Nedd4-2* heterozygous mice and wild-type control for total (intracellular and extracellular) dopamine and DOPAC levels, to indicate whether dopamine levels and metabolism is affected.
Statistics

For the behavioural and western blot analysis statistical comparisons were made between the Nedd4 heterozygous mice and age-matched wild-type controls using unpaired Student’s t-test. Results were deemed significant when P < 0.05.

3.3. RESULTS

Body and Brain weights

Body weight of both 2 (24.1 ± 0.7; n = 16) and 6 month (30.6 ± 1.1; n = 10) Nedd4-2 heterozygous mice was not changed when compared to wild-type controls (26.0 ± 0.4; n = 8 and 29.8 ± 0.8; n = 10, P = 0.09 and P = 0.56 respectively) (Figure 3.2A). Furthermore, brain weight was also similar between 2 (0.43 ± 0.007; n = 13) and 6 month (0.44 ± 0.01; n = 10) Nedd4-2 heterozygous mice and wild-type controls (0.45 ± 0.004; n = 8 and 0.44 ± 0.005; n = 9; P = 0.18 and P = 0.60 respectively) (Figure 3.2B).

Figure 3.2. No difference in average body weight was evident between Nedd4-2 heterozygous mice (+/-) compared to age-matched wild-type controls (+/+ ) at both 2 and 6 months of age (A). Similarly, no differences in brain weights were evident (B). Data represented as mean±SEM.
**Nedd4-2 whole brain levels**

Nedd4-2 levels were significantly reduced in whole brain from 2 (wt: 100.0 ± 19.1%; n = 4 and het: 33.3 ± 6.9%; n = 7, P = 0.003) and 6 (wt: 100.1 ± 12.4%; n =5 and het: 48.1 ± 10.6%; n =4, P = 0.018) months old Nedd4-2 heterozygous compared to wild-type controls (Figure 3.3).

![Figure 3.3](image)

**Figure 3.3.** Representative whole brain Western blots showing Nedd4-2 expression from 2 (A) and 6 (B) month old Nedd4-2 heterozygous mice (+, -); n = 4-7) and age matched wild-type controls (+/+; A). Nedd4-2 levels were significantly reduced by at least 50% in Nedd4-2 heterozygous mice (+/-; n = 4-7) compared to age-matched wild-type controls (+/+; n = 4-5) (C). Nedd4-2 levels in whole brain lysates represented as % of control. Data represented as mean±SEM. * P < 0.05, and ** P < 0.01 using unpaired Student’s t-test.

**Locomotor activity**

Basal loco-motor activity assessment with locomotor activity cells showed no difference between Nedd4-2 heterozygous mice and their wild-type littermates at both 2 and 6 months of age (Table 3.1).
No change was observed in basal loco-motor activity between *Nedd4-2* heterozygous (+/-) and age matched wild-type (+/+ ) controls at both 2 and 6 months of age. Data represented as mean±SEM. 2 months: wild-type n = 8 and heterozygous n = 15, 6 months: wild-type n = 10 and heterozygous n = 10.

<table>
<thead>
<tr>
<th></th>
<th>2 months</th>
<th>6 months</th>
<th>P value</th>
<th>2 months</th>
<th>6 months</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(+/+ )</td>
<td>(+/-)</td>
<td></td>
<td>(+/+ )</td>
<td>(+/-)</td>
<td></td>
</tr>
<tr>
<td><strong>Floor Plane</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moves</td>
<td>583±10</td>
<td>563±7</td>
<td>0.09</td>
<td>551±6</td>
<td>573±11</td>
<td>0.09</td>
</tr>
<tr>
<td>Time (sec)</td>
<td>1164±27</td>
<td>1160±19</td>
<td>0.90</td>
<td>1219±36</td>
<td>1202±40</td>
<td>0.75</td>
</tr>
<tr>
<td>Distance (cm)</td>
<td>5131±230</td>
<td>5414±259</td>
<td>0.48</td>
<td>6167±442</td>
<td>5667±381</td>
<td>0.40</td>
</tr>
<tr>
<td><strong>Ventral Plane</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Rears</td>
<td>73±12</td>
<td>77±8</td>
<td>0.75</td>
<td>89±9</td>
<td>96±14</td>
<td>0.68</td>
</tr>
<tr>
<td>Time (sec)</td>
<td>68±12</td>
<td>71±8</td>
<td>0.85</td>
<td>74±8</td>
<td>83±10</td>
<td>0.49</td>
</tr>
</tbody>
</table>
**Motor capacity**

Motor capacity was measured with the RotaRod test where an age-dependent decline in motor capacity was observed in *Nedd4-2* heterozygous mice compared to age-matched controls. This was illustrated by a significant decrease in latency to fall in the 6 month *Nedd4-2* heterozygous mice (230.4 ± 12.3; n = 10) compared to wild-type controls (278.9 ± 10.6; n = 10, P = 0.008). Latency to fall at two months showed no differences (wt: 249.6 ± 16.4; n = 8 and het: 235.1 ± 13.5; n = 16, P = 0.52) (Figure 3.4).

![Figure 3.4. Motor function capacity was assessed with RotaRod with no changes observed between 2 month old *Nedd4-2* heterozygous mice (+/-) and age-matched wild-type controls (+/+). A significant reduction was observed in 6 month heterozygous mice compared to the age-matched controls. Data represented as mean±SEM. * < 0.05 using unpaired Student’s t-test.](image)

**DigiGait**

**Stride parameters**

At 2 months there was no alterations in stride parameters (dynamic phase) between *Nedd4-2* heterozygous and wild type controls, including no overall difference in stride duration, or in swing, brake or propulsion phases. Stance duration where the paws are in contact with the belt was also not different (Table 3.2). No differences in stance parameters were evident when the percentage of time the paws were in braking or propulsion phase were examined (Table 3.3). Similarly at 6 months, stride parameters (Table 3.4) and stance parameters (Table 3.5) were not
different in *Nedd4-2* heterozygous mice relative to wild-type controls. No differences were evident at both speeds tested (15 and 20 cm/sec). For details of parameters examined please refer to Figure 2.2 (Chapter 2).

**Table 3.2.** Stride parameters in *Nedd4-2* heterozygous (+/-) and wild-type (+/+) control mice at 2 months of age at speeds of 15 and 20 cm/sec. Data represented as Mean±SEM.

<table>
<thead>
<tr>
<th>Indices</th>
<th>15 cm/sec</th>
<th>20 cm/sec</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(+/+ n = 7)</td>
<td>(+/- n = 15)</td>
<td>P value</td>
<td>(+/+ n = 7)</td>
<td>(+/- n = 15)</td>
<td>P value</td>
</tr>
<tr>
<td><strong>Fore paw</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Swing</td>
<td>0.11±0.01</td>
<td>0.10±0.004</td>
<td>0.59</td>
<td>0.09±0.003</td>
<td>0.09±0.003</td>
<td>0.90</td>
</tr>
<tr>
<td>Brake</td>
<td>0.12±0.01</td>
<td>0.12±0.01</td>
<td>0.62</td>
<td>0.09±0.01</td>
<td>0.09±0.01</td>
<td>0.91</td>
</tr>
<tr>
<td>Propel</td>
<td>0.11±0.01</td>
<td>0.10±0.01</td>
<td>0.54</td>
<td>0.07±0.01</td>
<td>0.08±0.01</td>
<td>0.47</td>
</tr>
<tr>
<td>Stance</td>
<td>0.23±0.01</td>
<td>0.23±0.004</td>
<td>0.79</td>
<td>0.17±0.04</td>
<td>0.17±0.01</td>
<td>0.32</td>
</tr>
<tr>
<td>Stride</td>
<td>0.34±0.02</td>
<td>0.33±0.01</td>
<td>0.63</td>
<td>0.26±0.01</td>
<td>0.26±0.01</td>
<td>0.50</td>
</tr>
<tr>
<td><strong>Hind paw</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Swing</td>
<td>0.18±0.1</td>
<td>0.09±0.002</td>
<td>0.56</td>
<td>0.08±0.03</td>
<td>0.08±0.003</td>
<td>0.42</td>
</tr>
<tr>
<td>Brake</td>
<td>0.05±0.01</td>
<td>0.05±0.003</td>
<td>0.76</td>
<td>0.05±0.003</td>
<td>0.04±0.003</td>
<td>0.26</td>
</tr>
<tr>
<td>Propel</td>
<td>0.17±0.03</td>
<td>0.19±0.01</td>
<td>0.33</td>
<td>0.13±0.01</td>
<td>0.14±0.01</td>
<td>0.24</td>
</tr>
<tr>
<td>Stance</td>
<td>0.22±0.03</td>
<td>0.25±0.01</td>
<td>0.34</td>
<td>0.18±0.003</td>
<td>0.18±0.01</td>
<td>0.52</td>
</tr>
<tr>
<td>Stride</td>
<td>0.40±0.05</td>
<td>0.34±0.01</td>
<td>0.11</td>
<td>0.25±0.003</td>
<td>0.26±0.01</td>
<td>0.35</td>
</tr>
<tr>
<td><strong>Fore paw</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Propel</td>
<td>33.8±3.5</td>
<td>31.5±1.9</td>
<td>0.54</td>
<td>28.1±3.2</td>
<td>31.0±2.1</td>
<td>0.44</td>
</tr>
<tr>
<td>% Swing</td>
<td>31.9±0.6</td>
<td>31.3±0.7</td>
<td>0.69</td>
<td>35.4±0.8</td>
<td>34.2±0.9</td>
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</tr>
<tr>
<td>% Brake</td>
<td>34.4±3.4</td>
<td>37.2±1.9</td>
<td>0.44</td>
<td>36.6±3.3</td>
<td>34.8±2.1</td>
<td>0.64</td>
</tr>
<tr>
<td>% Stance</td>
<td>68.2±1.6</td>
<td>68.8±0.7</td>
<td>0.69</td>
<td>64.6±0.8</td>
<td>65.8±0.9</td>
<td>0.45</td>
</tr>
<tr>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>% Propel</td>
<td>48.6±7.4</td>
<td>56.6±1.3</td>
<td>0.14</td>
<td>50.1±2.0</td>
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</tr>
<tr>
<td>% Swing</td>
<td>36.7±9.6</td>
<td>27.2±0.7</td>
<td>0.16</td>
<td>30.6±1.1</td>
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<td>0.70</td>
</tr>
<tr>
<td>% Brake</td>
<td>14.7±2.5</td>
<td>16.1±1.1</td>
<td>0.54</td>
<td>19.3±1.1</td>
<td>16.5±1.0</td>
<td>0.12</td>
</tr>
<tr>
<td>% Stance</td>
<td>63.3±9.6</td>
<td>72.8±0.7</td>
<td>0.16</td>
<td>69.4±1.1</td>
<td>68.8±0.9</td>
<td>0.70</td>
</tr>
</tbody>
</table>
Table 3.3. Stance parameters in Nedd4-2 heterozygous (+/-) and wild-type (+/+) control mice at 2 months of age at speeds of 15 and 20 cm/sec. Data represented as Mean±SEM.

<table>
<thead>
<tr>
<th>Indices</th>
<th>15 cm/sec</th>
<th>20 cm/sec</th>
<th></th>
<th>P value</th>
<th></th>
<th>P value</th>
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<tbody>
<tr>
<td></td>
<td>(+/+) n = 7 (+/-) n = 15</td>
<td>(+/+) n = 7 (+/-) n = 15</td>
<td>P value</td>
<td>(+/+) n = 7 (+/-) n = 15</td>
<td>P value</td>
<td></td>
</tr>
<tr>
<td>Fore paw</td>
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<td></td>
</tr>
<tr>
<td>% Brake</td>
<td>50.4±5.0</td>
<td>54.0±2.7</td>
<td>0.50</td>
<td>56.5±5.1</td>
<td>52.8±3.1</td>
<td>0.52</td>
</tr>
<tr>
<td>% Propel</td>
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<td>0.50</td>
<td>43.5±5.1</td>
<td>47.2±3.1</td>
<td>0.52</td>
</tr>
<tr>
<td>Hind paw</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>% Brake</td>
<td>23.3±1.5</td>
<td>22.2±1.6</td>
<td>0.68</td>
<td>27.8±1.9</td>
<td>23.9±1.5</td>
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</tr>
<tr>
<td>% Propel</td>
<td>76.7±1.5</td>
<td>77.8±1.6</td>
<td>0.68</td>
<td>72.2±1.9</td>
<td>76.1±1.5</td>
<td>0.14</td>
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</tbody>
</table>
Table 3.4. Stride parameters in *Nedd4-2* heterozygous (+/-) and wild-type (+/+) control mice at 6 months of age at speeds of 15, 20 and 30 cm/sec. Data represented as Mean±SEM.

<table>
<thead>
<tr>
<th>Indices</th>
<th>15 cm/sec</th>
<th>20 cm/sec</th>
<th>30 cm/sec</th>
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<th>P value</th>
<th>P value</th>
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<td>(+/-) n = 10</td>
<td>(+/) n = 10</td>
<td>(+/-) n = 9</td>
<td>(+/) n = 10</td>
<td>(+/) n = 10</td>
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<tr>
<td></td>
<td>Stride duration (sec)</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Swing</td>
<td>0.12±0.01</td>
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<td>0.12±0.01</td>
<td>0.95</td>
</tr>
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<td>Brake</td>
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<td>0.11±0.01</td>
<td>0.22</td>
<td>0.07±0.01</td>
<td>0.09±0.01</td>
<td>0.35</td>
</tr>
<tr>
<td>Propel</td>
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<td>0.42</td>
<td>0.09±0.01</td>
<td>0.08±0.01</td>
<td>0.55</td>
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<td>0.36±0.01</td>
<td>0.16</td>
<td>0.28±0.01</td>
<td>0.29±0.01</td>
<td>0.64</td>
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<tr>
<td>Hind paw</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Swing</td>
<td>0.10±0.01</td>
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<td>0.09±0.04</td>
<td>0.10±0.02</td>
<td>0.32</td>
</tr>
<tr>
<td>Brake</td>
<td>0.04±0.002</td>
<td>0.05±0.006</td>
<td>0.20</td>
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<td>0.04±0.003</td>
<td>0.87</td>
</tr>
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<td>0.21±0.01</td>
<td>0.99</td>
<td>0.15±0.01</td>
<td>0.16±0.01</td>
<td>0.27</td>
</tr>
<tr>
<td>Stance</td>
<td>0.25±0.01</td>
<td>0.26±0.1</td>
<td>0.64</td>
<td>0.19±0.01</td>
<td>0.20±0.01</td>
<td>0.21</td>
</tr>
<tr>
<td>Stride</td>
<td>0.35±0.01</td>
<td>0.38±0.01</td>
<td>0.23</td>
<td>0.28±0.01</td>
<td>0.30±0.01</td>
<td>0.14</td>
</tr>
<tr>
<td>Fore paw</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Propel</td>
<td>37.1±2.0</td>
<td>30.8±3.2</td>
<td>0.11</td>
<td>32.5±2.8</td>
<td>29.1±2.9</td>
<td>0.40</td>
</tr>
<tr>
<td>% Swing</td>
<td>36.3±2.3</td>
<td>39.6±4.4</td>
<td>0.52</td>
<td>41.7±3.3</td>
<td>40.7±2.5</td>
<td>0.85</td>
</tr>
<tr>
<td>% Brake</td>
<td>26.6±2.9</td>
<td>29.7±3.5</td>
<td>0.51</td>
<td>25.8±2.2</td>
<td>30.0±4.2</td>
<td>0.39</td>
</tr>
<tr>
<td>% Stance</td>
<td>63.7±2.3</td>
<td>60.5±4.4</td>
<td>0.52</td>
<td>58.3±3.3</td>
<td>59.1±2.5</td>
<td>0.85</td>
</tr>
<tr>
<td>Hind paw</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Propel</td>
<td>60.1±1.6</td>
<td>56.2±3.0</td>
<td>0.27</td>
<td>52.8±1.8</td>
<td>54.0±1.5</td>
<td>0.60</td>
</tr>
<tr>
<td>% Swing</td>
<td>28.0±1.6</td>
<td>30.3±2.6</td>
<td>0.45</td>
<td>33.0±1.0</td>
<td>32.7±1.5</td>
<td>0.94</td>
</tr>
<tr>
<td>% Brake</td>
<td>12.0±0.2</td>
<td>13.5±1.5</td>
<td>0.32</td>
<td>14.2±1.4</td>
<td>13.1±1.0</td>
<td>0.52</td>
</tr>
<tr>
<td>% Stance</td>
<td>72.1±1.6</td>
<td>69.7±2.6</td>
<td>0.45</td>
<td>67.0±1.0</td>
<td>67.1±1.5</td>
<td>0.94</td>
</tr>
</tbody>
</table>
Table 3.5. Stance parameters in *Nedd4-2* heterozygous (+/-) and wild-type (+/+) control mice at 6 months of age at speeds of 15, 20 and 30 cm/sec. Data represented as Mean±SEM.

<table>
<thead>
<tr>
<th>Indices</th>
<th>15 cm/sec</th>
<th>20 cm/sec</th>
<th>30 cm/sec</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(+/+) n = 10</td>
<td>(+/-) n = 10</td>
<td>(+/+) n = 10</td>
</tr>
<tr>
<td>Fore paw</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stance</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Brake</td>
<td>41.3±3.5</td>
<td>49.1±3.9</td>
<td>0.15</td>
</tr>
<tr>
<td>% Propel</td>
<td>58.7±3.5</td>
<td>50.9±3.9</td>
<td>0.15</td>
</tr>
<tr>
<td>Hind paw</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stance</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Brake</td>
<td>16.7±0.5</td>
<td>19.5±2.2</td>
<td>0.21</td>
</tr>
<tr>
<td>% Propel</td>
<td>83.3±0.5</td>
<td>80.5±2.2</td>
<td>0.21</td>
</tr>
</tbody>
</table>
Stride length, stance width and variability

Stride length and variability was not different in the 2 or 6 months old Nedd4-2 heterozygous mice compared to wild-type controls (Table 3.6 and 3.7, respectively). Stance width determines the distance between right and left paws and was not altered in Nedd4-2 heterozygous mice at 2 months of age (Table 3.6). In the 6 month old Nedd4-2 heterozygous mice however, although stance width is not different, stance width variation was significantly increased in the both fore (at 15 cm/sec) and hind paws (at 30 cm/sec) (Table 3.7). Stance width variation measures consistency of stance width stride-to-stride.

Table 3.6. Stride length parameters in Nedd4-2 heterozygous (+/-) and wild-type (+/+) control mice at 2 months of age at the speeds of 15 and 20 cm/sec. Data represented as Mean±SEM.

<table>
<thead>
<tr>
<th>Indices</th>
<th>15 cm/sec</th>
<th>20 cm/sec</th>
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<th></th>
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</thead>
<tbody>
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<td>(+/+) n = 7</td>
<td>(+/-) n = 15</td>
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<td>(+/+) n = 7</td>
</tr>
<tr>
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<td>P value</td>
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<td>P value</td>
</tr>
<tr>
<td>Fore paw</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stride Length (cm)</td>
<td>5.0±0.23</td>
<td>4.9±0.10</td>
<td>0.58</td>
<td>5.1±0.13</td>
</tr>
<tr>
<td>Stride Length Variance</td>
<td>0.76±0.11</td>
<td>0.85±0.10</td>
<td>0.61</td>
<td>0.89±0.10</td>
</tr>
<tr>
<td>Hind paw</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stride Length (cm)</td>
<td>6.0±0.82</td>
<td>5.0±0.11</td>
<td>0.11</td>
<td>5.1±0.07</td>
</tr>
<tr>
<td>Stride Length Variance</td>
<td>0.60±0.20</td>
<td>0.76±0.10</td>
<td>0.37</td>
<td>0.75±0.20</td>
</tr>
<tr>
<td>Fore paw</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stance Width (cm)</td>
<td>1.7±0.20</td>
<td>1.7±0.10</td>
<td>0.81</td>
<td>1.7±0.12</td>
</tr>
<tr>
<td>Stance Width Variance</td>
<td>0.30±0.02</td>
<td>0.32±0.04</td>
<td>0.80</td>
<td>0.28±0.03</td>
</tr>
<tr>
<td>Hind paw</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stance Width (cm)</td>
<td>2.6±0.10</td>
<td>2.5±0.10</td>
<td>0.31</td>
<td>2.5±0.05</td>
</tr>
<tr>
<td>Stance Width Variance</td>
<td>0.20±0.06</td>
<td>0.24±0.03</td>
<td>0.58</td>
<td>0.17±0.02</td>
</tr>
</tbody>
</table>
Table 3.7. Stride length parameters in *Nedd4-2* heterozygous (+/-) and wild-type (+/+) control mice at 6 months of age at speeds of 15, 20 and 30 cm/sec. Data represented as Mean±SEM. * P < 0.05 using unpaired Student’s t-test.

<table>
<thead>
<tr>
<th>Indices</th>
<th>15 cm/sec</th>
<th>20 cm/sec</th>
<th>30 cm/sec</th>
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<td>(+/+), n = 10</td>
<td>(+/-), n = 10</td>
<td>(+/+), n = 10</td>
</tr>
<tr>
<td><strong>Fore paw</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stride Length (cm)</td>
<td>5.1±0.18</td>
<td>5.4±0.16</td>
<td>5.6±0.15</td>
</tr>
<tr>
<td>Stride Length Variance</td>
<td>1.1±0.12</td>
<td>0.87±0.12</td>
<td>0.76±0.10</td>
</tr>
<tr>
<td><strong>Hind paw</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stride Length (cm)</td>
<td>5.3±0.19</td>
<td>5.6±0.16</td>
<td>5.5±0.21</td>
</tr>
<tr>
<td>Stride Length Variance</td>
<td>0.83±0.09</td>
<td>0.73±0.13</td>
<td>0.79±0.10</td>
</tr>
<tr>
<td><strong>Fore paw</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stance Width (cm)</td>
<td>1.9±0.09</td>
<td>2.0±0.10</td>
<td>2.0±0.06</td>
</tr>
<tr>
<td>Stance Width Variance</td>
<td>0.41±0.04</td>
<td>0.29±0.04</td>
<td>0.40±0.05</td>
</tr>
<tr>
<td><strong>Hind paw</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stance Width (cm)</td>
<td>3.0±0.07</td>
<td>2.9±0.13</td>
<td>3.1±0.08</td>
</tr>
<tr>
<td>Stance Width Variance</td>
<td>0.23±0.02</td>
<td>0.24±0.03</td>
<td>0.29±0.07</td>
</tr>
</tbody>
</table>
Step frequency and angle dynamics

Number of steps taken and stride frequency were not different between Nedd4-2 heterozygous mice relative to wild-type controls at both ages (Table 3.8 and 3.9, respectively). When paw angle dynamics were examined, no differences were observed at 2 months of age (Table 3.8). At 6 months of age however, a subtle but significant decrease in fore paw angle was observed (at 20 cm/sec), and paw angle variation significantly increased (at 30 cm/sec) (Table 3.9).

Table 3.8. Stride frequency, number of steps and angle dynamics in Nedd4-2 heterozygous (+/-) and wild-type (+/+) control mice at 2 months of age at speeds of 15 and 20 cm/sec. Data represented as Mean±SEM.

<table>
<thead>
<tr>
<th>Indices</th>
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<th>15 cm/sec (+/- n = 15)</th>
<th>P value</th>
<th>20 cm/sec (+/+ n = 7)</th>
<th>20 cm/sec (+/- n = 15)</th>
<th>P value</th>
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<tbody>
<tr>
<td>Fore paw</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stride Frequency</td>
<td>3.0±0.14</td>
<td>3.1±0.06</td>
<td>0.76</td>
<td>3.9±0.09</td>
<td>3.9±0.10</td>
<td>0.76</td>
</tr>
<tr>
<td># Steps</td>
<td>4.5±0.12</td>
<td>4.4±0.10</td>
<td>0.77</td>
<td>4.4±0.07</td>
<td>4.5±0.06</td>
<td>0.77</td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>Hind paw</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Stride Frequency</td>
<td>2.7±0.31</td>
<td>3.1±0.07</td>
<td>0.10</td>
<td>4.0±0.07</td>
<td>3.9±0.12</td>
<td>0.17</td>
</tr>
<tr>
<td># Steps</td>
<td>3.9±0.45</td>
<td>4.3±0.12</td>
<td>0.34</td>
<td>4.6±0.17</td>
<td>4.4±0.08</td>
<td>0.47</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fore paw</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paw angle (°)</td>
<td>-0.9±1.44</td>
<td>-2.0±0.70</td>
<td>0.44</td>
<td>-1.4±1.00</td>
<td>-2.4±1.78</td>
<td>0.59</td>
</tr>
<tr>
<td>Paw angle Variance</td>
<td>17.5±3.94</td>
<td>19.3±2.46</td>
<td>0.69</td>
<td>25.1±6.90</td>
<td>26.6±6.73</td>
<td>0.90</td>
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<tr>
<td>Absolute paw angle</td>
<td>7.4±1.58</td>
<td>6.2±0.67</td>
<td>0.43</td>
<td>4.9±1.99</td>
<td>6.6±0.99</td>
<td>0.25</td>
</tr>
<tr>
<td>Step angle (°)</td>
<td>54.8±4.17</td>
<td>52.5±2.73</td>
<td>0.65</td>
<td>55.8±3.23</td>
<td>55.9±2.32</td>
<td>0.99</td>
</tr>
<tr>
<td>Step Angle Variance</td>
<td>15.1±2.54</td>
<td>14.2±1.70</td>
<td>0.75</td>
<td>12.9±1.79</td>
<td>12.4±1.23</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hind paw</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paw angle (°)</td>
<td>-2.8±2.59</td>
<td>-2.3±1.31</td>
<td>0.84</td>
<td>-2.2±0.99</td>
<td>1.3±1.63</td>
<td>0.18</td>
</tr>
<tr>
<td>Paw angle Variance</td>
<td>18.6±5.64</td>
<td>27.4±4.28</td>
<td>0.25</td>
<td>17.7±2.79</td>
<td>26.6±5.66</td>
<td>0.31</td>
</tr>
<tr>
<td>Absolute paw angle</td>
<td>16.2±2.97</td>
<td>13.1±0.98</td>
<td>0.23</td>
<td>15.8±1.32</td>
<td>12.3±1.15</td>
<td>0.08</td>
</tr>
<tr>
<td>Step angle (°)</td>
<td>57.9±4.67</td>
<td>55.4±1.91</td>
<td>0.56</td>
<td>57.0±1.25</td>
<td>58.5±1.11</td>
<td>0.38</td>
</tr>
<tr>
<td>Step Angle Variance</td>
<td>12.1±2.11</td>
<td>15.9±1.03</td>
<td>0.09</td>
<td>15.2±0.90</td>
<td>15.4±0.81</td>
<td>0.93</td>
</tr>
</tbody>
</table>
Table 3.9. Stride frequency, number of steps and angle dynamics in *Nedd4-2* heterozygous (+/-) and wild-type (+/+) control mice at 6 months of age at speeds of 15, 20 and 30 cm/sec. Data represented as Mean±SEM. * P < 0.05 using unpaired Student’s t-test.

<table>
<thead>
<tr>
<th>Indices</th>
<th>15 cm/sec</th>
<th>20 cm/sec</th>
<th>30 cm/sec</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(+/+) n = 10</td>
<td>(+/-) n = 10</td>
<td>(+/+) n = 10</td>
</tr>
<tr>
<td>Fore paw</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stride Frequency (steps/sec)</td>
<td>2.9±0.12</td>
<td>2.7±0.09</td>
<td>3.0±0.11</td>
</tr>
<tr>
<td># Steps</td>
<td>4.5±0.17</td>
<td>4.1±0.17</td>
<td>4.4±0.17</td>
</tr>
<tr>
<td>Paw angle (°)</td>
<td>0.4±1.90</td>
<td>-6.3±3.45</td>
<td>-0.02±1.44</td>
</tr>
<tr>
<td>Paw angle Variance</td>
<td>38.9±8.15</td>
<td>37.2±7.32</td>
<td>23.7±2.61</td>
</tr>
<tr>
<td>Absolute paw angle</td>
<td>6.1±1.03</td>
<td>15.3±3.78</td>
<td>23.7±2.61</td>
</tr>
<tr>
<td>Step angle (°)</td>
<td>55.3±3.03</td>
<td>57.4±2.71</td>
<td>13.6±1.54</td>
</tr>
<tr>
<td>Step Angle Variance</td>
<td>13.9±1.15</td>
<td>13.8±1.12</td>
<td>23.7±2.61</td>
</tr>
<tr>
<td>Hind paw</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stride Frequency (steps/sec)</td>
<td>3.0±0.11</td>
<td>2.7±0.09</td>
<td>3.0±0.11</td>
</tr>
<tr>
<td># Steps</td>
<td>4.4±0.17</td>
<td>4.1±0.17</td>
<td>4.4±0.17</td>
</tr>
<tr>
<td>Paw angle (°)</td>
<td>-0.02±1.44</td>
<td>-1.9±0.89</td>
<td>-0.02±1.44</td>
</tr>
<tr>
<td>Paw angle Variance</td>
<td>23.7±2.61</td>
<td>22.8±3.39</td>
<td>13.6±1.54</td>
</tr>
<tr>
<td>Absolute paw angle</td>
<td>13.6±1.54</td>
<td>15.3±1.33</td>
<td>47.8±1.77</td>
</tr>
<tr>
<td>Step angle (°)</td>
<td>47.8±1.77</td>
<td>47.8±2.50</td>
<td>13.9±1.10</td>
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<tr>
<td>Step Angle Variance</td>
<td>13.9±1.10</td>
<td>13.4±0.95</td>
<td>13.9±1.10</td>
</tr>
</tbody>
</table>

* P < 0.05 using unpaired Student’s t-test.
Plantar placement and Stance Factor

There was no difference in the extent of plantar placement in fore and hind paws of Nedd4-2 heterozygous mice relative to age-matched wild-type controls at both 2 and 6 months of age. This was exemplified by no changes in paw area at peak stance and stance factor (Table 3.10 and 3.11, respectively).

Table 3.10. Stride frequency, number of steps and angle dynamics in Nedd4-2 heterozygous (+/-) and wild-type (+/+) control mice at 2 months of age at speeds of 15 and 20 cm/sec. Data represented as Mean±SEM.

<table>
<thead>
<tr>
<th>Indices</th>
<th>15 cm/sec</th>
<th>20 cm/sec</th>
<th>P value</th>
</tr>
</thead>
<tbody>
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<td>(+/+) n = 7 (+/-) n = 15</td>
<td>(+/+) n = 7 (+/-) n = 15</td>
<td></td>
</tr>
<tr>
<td>Fore paw</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paw area at peak stance (cm$^2$)</td>
<td>0.49±0.04</td>
<td>0.44±0.01</td>
<td>0.09</td>
</tr>
<tr>
<td>Paw area at peak stance Variance</td>
<td>0.06±0.02</td>
<td>0.04±0.01</td>
<td>0.16</td>
</tr>
<tr>
<td>Stance Factor</td>
<td>1.0±0.05</td>
<td>1.0±0.02</td>
<td>0.34</td>
</tr>
<tr>
<td>Hind paw</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paw area at peak stance (cm$^2$)</td>
<td>0.75±0.02</td>
<td>0.71±0.02</td>
<td>0.26</td>
</tr>
<tr>
<td>Paw area at peak stance Variance</td>
<td>0.08±0.01</td>
<td>0.07±0.01</td>
<td>0.84</td>
</tr>
<tr>
<td>Stance Factor</td>
<td>0.9±0.10</td>
<td>1.0±0.03</td>
<td>0.08</td>
</tr>
</tbody>
</table>
Table 3.11. Stride frequency, number of steps and angle dynamics in Nedd4-2 heterozygous (+/-) and wild-type (+/+) control mice at 6 months of age at speeds of 15, 20 and 30 cm/sec. Data represented as Mean±SEM.

<table>
<thead>
<tr>
<th>Indices</th>
<th>15 cm/sec</th>
<th>20 cm/sec</th>
<th>30 cm/sec</th>
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<tbody>
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<td></td>
<td>(+/+) n = 10</td>
<td>(+/-) n = 10</td>
<td>P value</td>
</tr>
<tr>
<td>Fore paw</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paw area at peak stance (cm²)</td>
<td>0.51±0.02</td>
<td>0.46±0.02</td>
<td>0.06</td>
</tr>
<tr>
<td>Paw area at peak stance</td>
<td>0.05±0.01</td>
<td>0.05±0.007</td>
<td>0.74</td>
</tr>
<tr>
<td>Variance</td>
<td>1.0±0.06</td>
<td>1.1±0.08</td>
<td>0.75</td>
</tr>
<tr>
<td>Stance Factor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hind paw</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paw area at peak stance (cm²)</td>
<td>0.91±0.05</td>
<td>0.87±0.04</td>
<td>0.52</td>
</tr>
<tr>
<td>Paw area at peak stance Variance</td>
<td>0.08±0.01</td>
<td>0.07±0.02</td>
<td>0.83</td>
</tr>
<tr>
<td>Stance Factor</td>
<td>1.0±0.03</td>
<td>0.95±0.06</td>
<td>0.18</td>
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</table>
Gait symmetry, hind limb shared and stance-swing ratio

Gait symmetry, hind limb shared and stance-swing ratio were not changed in *Nedd4-2* heterozygous mice compared to age-matched wild-type controls at both 2 and 6 month of age (Table 3.12 and 3.13, respectively).

**Table 3.12.** Stride frequency, number of steps and angle dynamics in *Nedd4-2* heterozygous (+/-) and wild-type (+/+) control mice at 2 months of age at speeds of 15 and 20 cm/sec. Data represented as Mean±SEM.

<table>
<thead>
<tr>
<th>Indices</th>
<th>15 cm/sec</th>
<th></th>
<th>20 cm/sec</th>
<th></th>
</tr>
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<tr>
<td></td>
<td>(+/+) n = 7</td>
<td>(+/-) n = 15</td>
<td>(+/+) n = 7</td>
<td>(+/-) n = 15</td>
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<tr>
<td>Gait Symmetry</td>
<td>1.4±0.43</td>
<td>1.0±0.01</td>
<td>1.0±0.03</td>
<td>1.0±0.01</td>
</tr>
<tr>
<td>P value</td>
<td>0.16</td>
<td>0.21</td>
<td>0.16</td>
<td>0.99</td>
</tr>
<tr>
<td>Hind limb shared</td>
<td>0.2±0.02</td>
<td>0.2±0.01</td>
<td>0.1±0.02</td>
<td>0.1±0.01</td>
</tr>
<tr>
<td>Fore paw</td>
<td>2.2±0.15</td>
<td>2.2±0.08</td>
<td>1.9±0.06</td>
<td>2.0±0.08</td>
</tr>
<tr>
<td>Stance/Swing</td>
<td>0.85</td>
<td>0.31</td>
<td>0.31</td>
<td>0.70</td>
</tr>
<tr>
<td>Hind paw</td>
<td>2.4±0.44</td>
<td>2.7±0.10</td>
<td>2.4±0.18</td>
<td>2.3±0.10</td>
</tr>
</tbody>
</table>
Table 3.13. Stride frequency, number of steps and angle dynamics in *Nedd4-2* heterozygous (+/-) and wild-type (+/+ ) control mice at 6 months of age at speeds of 15, 20 and 30 cm/sec. Data represented as Mean±SEM. * P < 0.05 using unpaired Student’s t-test.

<table>
<thead>
<tr>
<th>Indices</th>
<th>15 cm/sec</th>
<th>20 cm/sec</th>
<th>30 cm/sec</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(+/+) n = 10 (+/-) n = 10 P value</td>
<td>(+/+) n = 10 (+/-) n = 10 P value</td>
<td>(+/+) n = 10 (+/-) n = 9 P value</td>
</tr>
<tr>
<td>Gait Symmetry</td>
<td>1.0±0.03</td>
<td>1.0±0.01</td>
<td>0.54</td>
</tr>
<tr>
<td>Hind limb shared</td>
<td>0.17±0.02</td>
<td>0.17±0.02</td>
<td>0.81</td>
</tr>
<tr>
<td>Fore paw</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stance/Swing</td>
<td>1.9±0.17</td>
<td>1.8±0.21</td>
<td>0.67</td>
</tr>
<tr>
<td>Hind paw</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stance/Swing</td>
<td>2.7±0.19</td>
<td>2.5±0.26</td>
<td>0.65</td>
</tr>
</tbody>
</table>
**Nedd4-2 expression in CNS regions associated with motor function**

The expression of Nedd4-2 was examined in the motor cortex, spinal cord, cerebellum, substantia nigra pars compacta (SNpc), substantia nigra pars reticulate (SNpr) and striatum. These regions are all necessary for initiation, control and execution of voluntary movement.

**Motor Cortex**

Nedd4-2 expression was abundant in the motor cortex with highest levels observed in pyramidal neurons of layer V. These large neurons were identified based on morphology and stained with neuronal marker, NeuN. As expected a decrease in Nedd4-2 expression was evident in the heterozygous mice compared to wild-type controls. Qualitatively, it appeared there was a decrease in the number of neurons in the cortex of the heterozygous mice, however quantification was not performed.

![Figure 3.5. Nedd4-2 (red) is abundantly expressed in motor cortex from 2 month old wild-type mouse, particularly in cell body of layer V pyramidal neurons (NeuN, green). (+/-) Nedd4-2 heterozygous mice and (+/+ ) wild type controls. Scale bar represents 50μm.](image)

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**Spinal cord**

Nedd4-2 expression was examined in the ventral portion of the lumbar spinal cord, and showed high expression within NeuN positive motor neurons. Nedd4-2 was predominantly expressed in the cell body, with no expression seen in the nucleus or projections of these motor neurons. In the Nedd4-2 heterozygous mice there appears to be a reduction in the numbers of motor neurons, although numbers have not been quantified.

**Figure 3.6.** Nedd4-2 (red) is abundantly expressed in motor neurons (arrow) in the ventral horn of the lumbar spinal cord. Motor neurons were stained with the neuronal marker NeuN (green), and co-localization was evident in the merged image between Nedd4-2 and NeuN (arrow). (+/-) Nedd4-2 heterozygous mice and (+/+) wild-type controls. Scale bar represents 50μm.
Cerebellum

Nedd4-2 was expressed in all three layers of the cerebellum. Interestingly it appeared that Nedd4-2 is predominantly found in the cell body (or soma) of Purkinje neurons with very little co-localization evident between the dendrite marker MAP2 and Nedd4-2.

Figure 3.7. Nedd4-2 (red) was expressed in the cerebellum in all three layers; granule cell layer (GCL), Purkinje neuron (PN) layer and molecular layer (ML). Nedd4-2 is mainly localized in cell bodies of neurons, with no staining observed in dendrites from Purkinje neurons, highlighted by the lack of co-localization between Nedd4-2 and MAP2. (+/-) Nedd4-2 heterozygous mice and (+/+) wild-type controls. Scale bar represents 50μm.
**Substantia nigra**

The expression of Nedd4-2 in the substantia nigra was evaluated in both the pars compacta (SNpc) and reticulata regions (SNpr). The figure below indicates the boundaries between SNpc and SNpr, with SNpc neurons expressing tyrosine hydroxylase (TH), which was not present in SNpr (Figure 3.8).

**Figure 3.8.** Nedd4-2 (red) expression was found in both the SNpc (substantia nigra pars compacta) shown by tyrosine hydroxylase staining (TH, green) and SNpr (substantia nigra pars reticulata). Scale bar represents 100 μm.
Substantia nigra pars compacta (SNpc)

Higher magnification images show that Nedd4-2 was abundantly expressed in neurons located in SNpc, with co-localization between Nedd4-2 and tyrosine hydroxylase (TH). Nedd4-2 and TH expression were evaluated in brain slices from 2 and 6 month old Nedd4-2 heterozygous mice and age-matched wild-type controls. Quantitative measurements of SNpc positive neurons were not conducted, but no evidence of cell loss was seen (Figure 3.9).

Figure 3.9. Nedd4-2 (red) was abundant in high levels in dopaminergic neurons (TH, green) of substantia nigra pars compacta (SNpc). No morphological changes were evident at both 2 and 6 months of age in the Nedd4-2 heterozygous mice (+/-) relative to wild-type controls (+/+). Scale bar represents 50 µm.
Substantia nigra pars reticulata (SNpr)

Nedd4-2 expression was observed within the SNpr, in neurons negative for tyrosine hydroxylase. This suggests that Nedd4-2 is located in inhibitory neurons of this compartment. No morphological differences were observed at 2 and 6 months of age when comparing Nedd4-2 heterozygous mice and wild-type controls (Figure 3.10).

Figure 3.10. At 2 and 6 months of age, Nedd4-2 (red) was abundantly expressed in SNpr, in particular in neurons that do not express tyrosine hydroxylase (TH, green). (+/-) Nedd4-2 heterozygous and (+/+ ) wild-type controls. Scale bar Scale bar represents 50 µm.
**Striatum**

Nedd4-2 was primarily expressed in the cell bodies of medium spiny interneurons of the striatum. No Nedd4-2 staining was evident in the striatosomes (ST) indicating that Nedd4-2 is absent in white matter tracts (Figure 3.11).

**Figure 3.11.** Nedd4-2 (green) appears mainly localized around the cell bodies of what appears to be medium spiny interneurons located within the striatum. No Nedd4-2 was found in the striatosomal region (ST). Dopamine transporter (DAT) (red). Scale bar represents 50 μm.
**Dopamine transporter levels (DAT)**

DAT is primarily located in the striatum where it facilitates the re-uptake of dopamine. DAT levels appear up regulated in *Nedd4-2* heterozygous mice at 2 and 6 months of age (Figure 3.12).

![DAT immunohistochemistry](image)

**Figure 3.12.** DAT immunohistochemistry in the striatum showed an up-regulation in staining intensity in 2 and 6 month old *Nedd4-2* heterozygous (+/-) compared to wild-type controls (+/+). Scale bar represents 10 μm.

However in comparison to Figure 3.12, when striatal DAT levels were measured by Western blot, no difference was found in the total level of DAT between the *Nedd4-2* heterozygous mice and wild-type controls at both 2 (wt: 100.1 ± 17.6%; n = 8 and het: 94.6 ± 16.3%; n = 7; P = 0.83) and 6 (wt: 100.0 ±11.8%; n = 4 and het: 67.5 ± 19.8%; n = 4, P = 0.21) month of age (Figure 3.13).
Figure 3.13. Representative Western blot of dopamine transporter (DAT) in striatum of 2 and 6 month old Nedd4-2 heterozygous mice (+/−, n = 4-7) and age-matched wild-type controls (+/+, n = 4-8). No significant difference was observed in DAT levels at both 2 and 6 months of age. DAT levels represented as % of wild-type control. Data represented as mean±SEM.

Dopamine levels and dopamine turnover

Dopamine levels were significantly reduced in striatum from both 2 (1629 ± 439; n = 7) and 6 month old (2010 ± 461; n = 5) Nedd4-2 heterozygous mice compared to wild-type controls (2 month: 3101 ± 272; n = 8, P = 0.01 and 6 month: 3682 ± 432; n = 5, P = 0.03) (Figure 3.14). DOPAC, the main metabolite of dopamine, was also significantly reduced in 2 month Nedd4-2 heterozygotes (319 ± 108; n = 7) compared to wild-type controls (952 ± 102; n = 8, P = 0.0009). Although we see a modest reduction in the 6 month heterozygous (wt: 645 ± 76; n = 5 and het: 405 ± 118; n = 5, P = 0.13) mice, this did not reach significance. Dopamine turnover is significantly decreased in 2 month Nedd4-2 heterozygous mice (0.20 ± 0.03; n = 6) compared to wild-type controls (0.31 ± 0.02; n = 8, P = 0.02), with no significant difference apparent in the 6 month cohort (wt: 0.18 ± 0.02; n = 5 and het: 0.19 ± 0.02; n = 5, P = 0.67) (Figure 3.16).
Figure 3.14. HPLC analysis showed a significant decrease in dopamine levels in *Nedd4-2* heterozygous mice (+/-, n = 5-7) and age-matched wild-type control (+/+, n = 5-8) at both 2 and 6 months of age. Data represented as mean±SEM. Statistical analysis was conducted using unpaired Student’s t-test, with * P < 0.05 and deemed significant.

Figure 3.15. HPLC analysis showed a decrease in DOPAC from striatum in *Nedd4-2* heterozygous mice (+/-, n = 7) at 2 months of age compared to age-matched wild-type controls (+/+, n = 8). No difference was seen in the 6 month age group. Data represented as mean±SEM. Statistical analysis was conducted using unpaired Student’s t-test, with *** P < 0.0001 and deemed significant.
Figure 3.16. HPLC analysis of DOPAC to dopamine ratio showed a significant decrease in 2 month old *Nedd4-2* heterozygous mice (+/-) compared to age-matched wild-type controls (+/+), with no change evident at 6 months of age. Data represented as mean±SEM. Statistical analysis was conducted using unpaired Student’s t-test, with * P < 0.05 and deemed significant.

3.4 DISCUSSION

The interaction between Nedd4-2 and dopamine transporter (DAT) is important for motor function and control, however the *in vivo* consequences remain unknown. Dopamine is largely responsible for regulatory feedback to the motor cortex from the basal ganglia, which when disrupted results in symptoms associated with Parkinson’s disease. These symptoms include rigidity, resting tremor, gait and motor disturbances due to decreased levels of dopamine. Since Nedd4-2 was shown to facilitate DAT trafficking, it was essential to investigate DAT and dopamine levels, as well as motor function and gait behaviours in *Nedd4-2* heterozygous mice.

This study is the first to characterize *Nedd4-2* heterozygous mice in relation to motor function and gait.

Unlike Nedd4, the *Nedd4-2* heterozygous mice showed no growth phenotype with both body and brain weight similar between the heterozygotes and wild-type controls all both time points tested. Whole brain levels of Nedd4-2 were found to be decreased by at least 50% in the heterozygotes compared to the wild-type controls confirming that we are indeed examining the effects of a single allele deletion of Nedd4-2.
Analysis of basal loco-motor activity showed no differences between the \textit{Nedd4-2} heterozygous mice at both time points when compared to wild-type controls. This was somewhat surprising, as the hypothesis suggested that dopamine levels may be altered in these mice and should have resulted in alterations in basal activity. For instance, an increase in striatal dopamine transporter is evident in adults afflicted with ADHD (Krause \textit{et al.}, 2000). Furthermore, in DAT knockdown mice, a reduction in DAT (90\% reduction) produces hyperactivity due to increased dopamine levels in the striatum (Zhuang \textit{et al.}, 2001). Due to these findings it would have been expected to observe a decrease in basal locomotor activity in the \textit{Nedd4-2} heterozygous mice, due to increased levels of DAT and decreased dopamine levels. However, since we are using a heterozygous model perhaps the altered levels of both DAT and dopamine was not severe enough to produce behavioural changes.

Next motor function was examined and although no difference was observed at 2 months of age, by 6 months a significant reduction in motor capacity became evident. This suggests that at 6 months of age, \textit{Nedd4-2} heterozygous mice have impaired motor function. In addition, gait was also analyzed, as gait abnormalities are often present in patients and rodent models of Parkinson’s disease. Interestingly, no differences in gait were apparent at both 2 and 6 months of age in the \textit{Nedd4-2} heterozygous mice. The parameter that is particularly relevant to dopamine activity and function was stride length and stride length variability, which have both been suggested to be accurate indicators of basal ganglia abnormalities (Fernagut \textit{et al.}, 2002). Nevertheless, the significant reduction in motor capacity warranted investigation into expression of Nedd4-2 in regions of the CNS involved in motor function and gait.

The expression of Nedd4-2 was examined to determine what neuronal compartments shows high expression. Firstly, Nedd4-2 was highly expressed in layer V pyramidal neurons of the motor cortex, with these neurons synapsing onto spinal cord motor neurons, and therefore integral for the initiation of motor function. Nedd4 was found in the cell bodies with no staining evident in the nucleus of these pyramidal neurons. Nedd4-2 expression is not restricted to layer V pyramidal neurons with expression evident in other layers of the cortex with varying intensity. The number of neurons within the motor cortex also appeared reduced in the \textit{Nedd4-2} heterozygotes but was not quantified. Further work is necessary to determine whether the numbers of neurons are decreased, and to determine whether any age-related changes such as enhanced neurodegeneration is observed in older time points in the heterozygotes compared to the wild-
type controls. In addition examination of other cortical regions such as the visual and sensory cortex also requires further investigation.

Since pyramidal neurons of the motor cortex synapse on the motor neurons of the spinal cord, Nedd4-2 expression was also assessed there. Nedd4-2 was expressed in high levels in motor neurons in the ventral horn of the lumbar spinal cord. These neurons innervate skeletal muscle to execute voluntary movement. Interestingly, neuron number also appeared decreased here in the Nedd4-2 heterozygous mice. In order to better understand the role of Nedd4-2 in initiation and execution of motor function, further analysis is required to determine perhaps if any developmental delays occur, or whether early neuronal cell death is observed in the Nedd4-2 heterozygotes compared to wild-type controls in these two important neuronal compartments integral for motor function.

In terms of motor control and regulation, the cerebellum is integral for ensuring of timing, accuracy and precision of movement (Kandel et al., 2000). Unlike the Nedd4 heterozygous mice, no gait abnormalities were evident in Nedd4-2 heterozygous mice compared to wild-type controls at both time points. Although some changes were evident for example, significant changes to stance width variance, paw angle and paw angle variance, the data shows changes the wild-type mice in rather than heterozygotes. This indicates that these findings may be artifacts rather than true changes in gait. While there was no evidence for alteration in timing, or co-ordination of precision of movements, Nedd4-2 was present in all three layers of the cerebellum, in granule cells, Purkinje neurons and the molecular layer. This suggests that Nedd4-2 may play a role in cerebellar function, but any changes associated with reduced Nedd4-2 levels did not manifest into a physiological outcome. This may be due to the small or redundant role for Nedd4-2 in the cerebellum, or perhaps greater levels of Nedd4-2 loss are necessary before abnormalities become evident.

Due to the reduced motor capacity observed in the Nedd4-2 heterozygotes and the known interaction between Nedd4-2 and DAT, the expression of Nedd4-2 was evaluated in the substantia nigra pars compacta (SNpc) and reticulata (SNpr), as well as the striatum. Nedd4-2 was abundant in dopaminergic neurons of SNpc with strong co-localization observed with tyrosine hydroxylase positive neurons. This finding is important, as it supports the role for Nedd4-2 in dopamine neurotransmission. Neurons of SNpc degenerate in patients afflicted with
Parkinson’s disease, reducing the level of dopamine produced, and indeed these patients have compromised motor function. There was no evidence for reduced levels of tyrosine hydroxylase, cell numbers of any morphological changes within the SNpc, which suggests that the motor decline in the heterozygotes is unlikely due to degeneration of SNpc. So next, the expression of Nedd4-2 within SNpr was evaluated since neurons here provide inhibitory innervation to SNpc, to modulate dopaminergic neurotransmission. Interestingly, Nedd4-2 was also expressed in high levels of neurons within SNpr, which may therefore suggest that Nedd4-2 can also regulate dopamine release by the SNpc to the striatum, but again no morphological changes were evident. This data suggests that Nedd4-2 may play a role in dopaminergic neurotransmission.

Since dopamine is largely released in the striatum, most of the DAT is located in this compartment, so determining the expression of Nedd4-2 here was imperative. The striatum receives excitatory glutamatergic inputs from the cerebral cortex and the thalamus. Striatal neurons are largely composed of medium spiny neurons (approximately 95%) with the remainder composed of inhibitory interneurons (Kreitzer, 2009; Tepper et al., 2010). The dendrites of medium spiny neurons are covered in spines, which receive corticospinal and thalamostriatatal inputs as well as inhibitory inputs from the GABAergic interneurons and cholinergic interneurons (Tepper et al., 2010). Medium spiny neurons are GABAergic large projection neurons, which form both ‘direct’ and ‘indirect’ pathways. The direct pathway projects to the SNpr and globus pallidus interna (GPi), and thus modulate the output of the basal ganglia, whereas the ‘indirect’ pathway projects globus pallidus externa (GPe) which in turn projects to the subthalamic nucleus, and thus regulates excitatory projections to SNpr and GPi. In effect the direct and indirect pathways have functionally opposing effects; with direct pathway disinhibiting thalamocortical targets and the indirect reinforces this inhibition. Therefore the balance between these two pathways is fundamental to basal ganglia function in regulating motor behaviours. Another important factor of medium spiny interneurons is that dopamine receptors are expressed among the two different neuronal populations involved in direct and indirect pathways (Kreitzer, 2009).

Neurons involved in the direct pathway are enriched with D1 receptors, whereas neurons involved in the indirect pathway are enriched with D2 receptors (Crittenden et al., 2011). Dopamine can therefore regulate the balance between these two pathways, since D1 receptors have an overall excitatory effect and D2 receptors inhibitory. Importantly, it appears that D1 and D2 receptors contribute differently to functional behaviours, for example D1 receptors facilitate
dopaminergic contribution to learning, where as D2 are most important for motor behaviours (Berke et al., 2000). Nedd4-2 was found in the cell bodies of medium spiny neurons, however currently whether Nedd4-2 is present in D1, D2 expressing neurons or localized in both remains unknown. Interestingly, Nedd4-2 does not co-localize with DAT in terminals of the striatum, which is supported by previous studies which have shown that it is unlikely that there is a direct interaction between Nedd4-2 and DAT (Sorkina et al., 2006). DAT does not contain a typical PPXY motif and did not co-immunoprecipitate with Nedd4-2 (Sorkina et al., 2006), therefore it is suggested that an indirect mechanism is involved, perhaps with an intermediate or via adapter proteins. When DAT immunofluorescence was examined in the striatum from heterozygous mice, a substantial increase was observed in terminals. However, when levels of DAT were measured by Western blot, no differences were apparent. This is not entirely surprising as the total levels of DAT are unlikely to change in the heterozygous mice, but the level of endocytosis may be reduced, highlighted by the increased surface expression in the terminals.

The effect of increased staining of DAT in terminals of the striatum may translate to increased levels of DAT present at the membrane surface, which may affect dopamine levels, due to increased re-uptake. HPLC analysis uncovered significant decreases in levels of dopamine in striatum from both 2 and 6 month old Nedd4-2 heterozygous mice compared to the age-matched wild-type controls. Although this is an exciting result, this is a measure of total dopamine levels and cannot provide conclusive evidence for intracellular versus extracellular levels. Further work is necessary such as biotinylated dopamine measurements to determine rate of re-uptake by DAT. Pharmacological tools could also be used, such as cocaine which blocks DAT to determine if membrane levels of DAT are indeed up-regulated due to reduced endocytosis by Nedd4-2. When the main metabolite of dopamine metabolism, DOPAC was measured a significant reduction was observed in striatum from 2 month old Nedd4-2 heterozygous mice, with a modest decrease also observed at 6 months of age. This may suggest that dopamine is not metabolized to the same extent in Nedd4-2 heterozygous mice and so that instead of metabolism, the dopamine that is taken up by DAT is largely packaged back into vesicles for subsequent release. Dopamine turnover can also be determined by examining the ratio of DOPAC to dopamine levels. A significant decrease was observed in the 2 month old Nedd4-2 heterozygous mice, suggesting that dopamine turnover is reduced, again indicating that dopamine that is taken up by DAT is largely re-packaged and not metabolized. No differences were observed at 6 months of age, which
suggests that the fate of dopamine once taken up, is repackaged and metabolized in the same extent between the Nedd4-2 heterozygotes and age-matched wild-type controls.

These results can also be explained by reduced firing rate by dopaminergic neurons, which would result in decreased levels of dopamine released, therefore accounting for the reduced dopamine levels observed in the striatum from Nedd4-2 heterozygous mice. Due to the decreased levels of dopamine being released less dopamine is metabolized, and therefore DOPAC levels are also reduced, thereby affecting dopamine turnover (Grace, 1991). Data from these studies do not allow for discrimination between these two possibilities, and as such further work is necessary to explore these two scenarios.

Overall, the HPLC data shows that Nedd4-2 heterozygous mice have reduced dopamine levels at both time points, but only significant indications that dopamine metabolism is affected at 2 months of age. This is surprising, as the motor deficits are observed at the 6 month time point. Drawing conclusions from the HPLC data is difficult, as further experiments are required to verify whether there is evidence of increased DAT at the plasma membrane of dopaminergic terminals in the striatum, and if this translates to altered dopamine re-uptake. As well as examining dopamine metabolism, dopamine synthesis also requires investigation since Nedd4-2 is abundant in tyrosine hydroxylase positive neurons of SNpc. This study focused on dopamine levels, and the potential role between Nedd4-2 and DAT and implications for dopamine levels due to re-uptake, but future studies should also examine whether Nedd4-2 plays a role in synthesis of dopamine which may also result in reduced dopamine levels observed in the striatum of both 2 and 6 month old Nedd4-2 heterozygous mice.

Findings from this study are the first to show the in vivo consequence in Nedd4-2 heterozygous mice where Nedd4-2 levels are reduced by at least 50%, in particular the effects on motor function and gait. Behavioural testing has shown a reduced motor capacity at 6 months of age, with no alterations in gait or basal locomotor activity. Nedd4-2 is also highly expressed in many neuronal compartments associated with initiation, execution and regulation of voluntary movement.
CHAPTER 4: NEDD4 IN COGNITIVE BEHAVIOUR

4.1 BACKGROUND AND RATIONALE

The role for Nedd4 in cognitive function

The multitude of targets for Nedd4 involved in synaptic plasticity suggest that it may play an integral role in complex neuronal interactions including; cognitive processes such as learning, memory and anxiety. To date, the expression of Nedd4 in regions of the brain associated with these processes remains largely unknown. It was important to assess what role Nedd4 plays in cognitive function, determine patterns of expression and highlight targets that could be involved. As already discussed, Nedd4 heterozygous mice have significant growth abnormalities attributed to decreased IGF-1 mediated signaling. In addition, Nedd4 heterozygous mice have gait abnormalities; in part likely due to abnormal levels of GluR1-containing AMPA receptors (AMPARs) at granule cell-Purkinje neuron synapses. The evidence that Nedd4 is also involved in axonal and dendritic growth further supports a role for Nedd4 in cognition.

Nedd4 regulation of axonal and dendritic growth

The growth and development of axons and dendrites is essential during development of the CNS but also in the mature brain where adaptive changes must occur for synaptic plasticity to take place. Nedd4 has been shown to be important for both axonal and dendritic growth. For instance, in Nedd4 knockout mice a significant impairment of dendrite growth results due to loss of ubiquitination of a serine/threonine kinase TNIK and the small GTPase Rap2A (Kawabe et al., 2010). The function of Rap2A reduces the activity of the TNIK family of kinases which act to promote dendrite growth (Kawabe et al., 2010). So in Nedd4 knockout mice, there is the accumulation of Rap2A resulting in inhibition of TNIK and thus loss of dendrite growth (Kawabe et al., 2010). The reduced complexity of dendrite arbours limits the extent of synapses that can form and this may have profound implications for cognition as well as other important CNS functions such as motor function and gait.

In axons, Nedd4 was found to regulate levels of PTEN, and in Xenopus retinal ganglion cells knockdown of Nedd4 resulted in reduced axonal terminal branching (Drinjakovic et al., 2010a; Kawabe et al., 2010). As with dendrite branches, reduced levels of axons branches can have profound effect on CNS function, by reducing the potential for formation of synapses.
**AMPA Receptors and Synaptic plasticity**

Aside from growth and development of neurons, axons and dendrites, studies have shown that Nedd4 is also important in the mature synapse. For example, Nedd4 can cause AMPAR endocytosis via the ubiquitination of glutamate subunit 1 (GluR1) occurring in an activity dependent manner. (Lin et al., 2011; Schwarz et al., 2010). The regulation of GluR1-containing AMPAR is important as modification at the synapse allows for plasticity during learning and memory (Boehm et al., 2006). The synaptic insertion and removal of AMPA receptors lead to the activity-induced glutamatergic enhancement that occurs during LTP and plasticity in vivo (Bredt et al., 2003; Makino et al., 2009). With AMPA receptor trafficking important for long-term potentiation (LTP) and thus long-term memory and learning therefore Nedd4 may have important in vivo role in learning and memory related behaviours.

**Behavioural assessment of cognitive function**

The molecular targets and mechanisms discussed so far contribute to cognitive behaviours, however behavioural tests must be used to determine the in vivo consequences of alterations in these molecular mechanisms. Such tests include assessment of memory, learning, anxiety and sensory motor gating. These tests are robust and well defined, and are well supported by the literature. Most importantly, these tests can translate in understanding similar processes in the human brain.

**Memory and Learning**

Learning can be defined as the acquisition of new information, while memory is the expression of the acquired information. There are several types of memory, such as spatial and working memory. This study is particularly interested in spatial memory, as this is largely hippocampal based (Good, 2002). Due to the known interaction with AMPARs necessary for LTP, and LTP predominantly contributing to hippocampal based learning and memory it was important to examine spatial memory in the Nedd4 heterozygous mice. Spatial memory can be short and long-term, with distinctive tests to examine each type. The importance of short and long-term memory in context of this study arise due to the emerging importance of protein degradation (by ubiquitination) necessary for the consolidation of short to long-term memory (Figure 4.1). Part of
this consolidation process requires synaptic changes to occur, such as regulation of AMPAR crucial for LTP and strengthening of the synapse.

Figure 4.1. Cognitive processing of incoming information. Incoming information is initially stored as short-term memory but with consolidation can be converted to long-term memory. Ubiquitin proteasome system (UPS).

Short-term spatial memory

Y-maze is a test to assess memory, explorative behavior and short-term spatial memory performance. The test is a two-trial recognition memory test and importantly it does not rely on learning, instead it relies on the innate tendency of mice to explore novel environments. The Y-maze uses spatial cues, where mice are allowed to explore two familiar arms with no access to the third ‘novel’ arm. Following a short period of time (between 1 to 4 hours), the novel arm becomes accessible and rodents with intact spatial short-term memory recognize that this arm is novel as indicated by the increased exploration activity in this arm (Dellu et al., 1992).

Long-term spatial memory

Morris water maze (MWM) was used to investigate long-term spatial memory and learning, often following the spatial learning component (Morris, 1984). This test relies on innate rodent behaviours based on the high motivation to escape the pool during the learning phase. Rodents
use spatial cues to navigate the shortest distance to the hidden platform. Following adequate display of learning, the platform can be removed, and rodents with intact long-term spatial memory display behaviours indicative of searching for the platform in the quadrant of the pool where the hidden platform was located during learning.

**Anxiety**

The elevated plus maze is routinely used to assess anxiety related behaviours in rodents. The elevated plus maze uses natural characteristics innate to mice for exploration of novel environments, and the aversion to bright and open spaces (Walf AA et al., 2007). The elevated plus maze measures unconditioned responses to potentially dangerous environment, and the degree to which the mouse avoids the open spaces (open arms of the maze) can determine anxiety related behaviours.

**Pre-pulse startle responses**

Startle responses to auditory stimuli is a normal physiological response in both humans and rodents. Reduced startle response occurs when a lower frequency auditory stimuli is placed prior to the test auditory stimuli. This reduction or pre-pulse inhibition shows the ability to filter or ‘gate’ environmental information. The test used to measure pre-pulse startle responses are similar for rodents and humans, and so some level of translation is possible between rodent studies and human conditions (Swerdlow et al., 2001). The brain circuitry used to regulate pre-pulse startle responses is complex, and involves the hippocampus (both dorsal and ventral portions) (Swerdlow et al., 2000), prefrontal cortex (Yee, 1999), basolateral amygdala (Decker et al., 1995), striatum (Kodsi et al., 1995), substantia nigra pars reticulata (Koch et al., 2000) and many others (Swerdlow et al., 2001).

Pre-pulse inhibition abnormalities are observed in people with attention deficit disorders and people afflicted with schizophrenia (Braff et al., 2001; Parwani et al., 2000). The use of PPI assessment especially in transgenic mice is useful for understanding the relationship between the gene of interest and ability to process and filter sensory information. Alterations in PPI can be important indicators of altered striatal or limbic circuitry, which can form a strong basis for
further investigation, and characterization of the gene, regions of the brain involved and neurochemistry.

**Hippocampus and amygdala are important for cognitive behaviour**

The hippocampus can be divided into the dorsal and ventral portions, with these regions having distinct roles and contributions to cognition (Fanselow et al., 2010). As already discussed in Chapter 1, lesion studies have provided supporting evidence for the distinct roles of the dorsal and ventral portions of the hippocampus. Reciprocal connections with the amygdala are necessary for the processing of emotive behavior and regulation of anxiety related behaviours. Both the hippocampus and amygdala contain GluR1 containing AMPARs, with studies showing that levels of GluR1 in the amygdala can correlate with performance in elevated plus maze. In the hippocampus, GluR1 containing AMPARs are essential for LTP and thus memory formation. In terms of PPI, there is also evidence that mice lacking GluR1 AMPARs showed ‘schizophrenia-related behaviours’ including reduced PPI response (Wiedholz LM et al., 2008).

**LTP essential for synaptic changes during long-term memory**

Long-term memory requires synaptic changes to occur to produce long lasting enhancement between neurons. LTP is the molecular mechanism by which this enhancement can occur, especially in the hippocampus. The involvement of AMPARs in LTP is well established, in particular the trafficking of AMPARs in and out of the membrane (Sprengel, 2006). As already discussed Nedd4 has been shown to target GluR1 containing AMPARs in an activity dependent manner (Schwarz et al., 2010) and may therefore play an important role in LTP and long-term memory.

**The involvement of calcium during cognitive processes**

Calcium is essential for LTP and thus learning and memory, therefore the regulation of voltage-gated calcium channels is important. Given that Nedd4 is able to ubiquitinate and thus regulate newly synthesised Ca,1.2 channels (Rougier et al., 2011) it may exert important effects in regulating intracellular calcium levels and thus impact LTP. Furthermore, calcium has been shown to release the auto-inhibition of Nedd4 by releasing the C2 (calcium binding domain) from
the HECT (catalytic domain), and thus levels of intracellular calcium can in turn affect Nedd4 activity (Wang et al., 2010). During LTP, increases in intracellular calcium regulate membrane excitability and initiate intracellular signal transduction pathways necessary for maintenance of LTP (Calin-Jageman et al., 2008). The impact of reduced levels or loss of Nedd4 on LTP has never been investigated.

Memory and learning are fundamental cognitive processes with complex molecular component. Given the critical function of Nedd4 during the development of the nervous system, as well as the multitude of targets important in the synapses of the mature brain it was appropriate to investigate what roles Nedd4 might play in cognitive behavior. These previous studies led to the main hypothesis of this study; that Nedd4 would have an essential role in neuron function necessary for cognitive behaviours and may regulate AMPARs to allow for synaptic plasticity associated with these behaviours. To investigate this hypothesis, the following aims were set:

4) Conduct behavioural tests in Nedd4 heterozygous mice to assess short and long-term memory and learning
5) Behavioural assessment of anxiety behaviours
6) Evaluation of sensory motor gating
7) Determine cellular and regional expression of Nedd4 in the brain associated with cognitive behaviours
8) Determine whether changes in AMPARs are altered in Nedd4 heterozygous brains
9) Assess molecular mechanism of plasticity by assessing long-term potentiation in brain slices from Nedd4 heterozygous mice

Tests were conducted in 2 and 6 month old Nedd4 heterozygous mice.

4.2 MATERIALS AND METHODS

Behavioral assessment

Mice used for this study were previously described in Chapter 2. Cognitive behaviour followed the completion of the motor function and gait analysis. Testing was conducted in designated mouse behaviour rooms (3 x 3.5 m) at the Florey Neuroscience Institute. Tests were conducted
between 0900 and 1800 hours to minimize disturbance of light : dark cycle of the mice. Lighting levels varying upon each tests and are described for each test. Ethovision XT (Noldus) video tracking software was used to collect and analyze parameters in Y-maze, MWM and elevated plus maze.

**Y-maze**

The Y-maze apparatus was composed of three identical arms (30 cm length and 14 cm sides).

**Figure 4.2.** Set up of Y-maze test, where during trial 1 mice were allowed to explore the familiar arms, with access to the novel arm blocked. After 2 hours, access to the novel arm was allowed and mice explored all three arms of the Y-maze. Extra-maze cues were placed at the end of each arm as shown in the schematic.

Prior to testing, mice were habituated overnight in the testing room with low lighting (10 Lux). For trial one, the partition is placed so that the novel arm is inaccessible. A handful of bedding from the home cage of the mouse is placed in the Y-maze and mouse allowed 10 minutes to explore the familiar arms. Two hours later, trial two is performed where the partition is removed, and mouse placed back into the Y-maze, with access to the novel arm for 5 minutes (**Figure 4.2**). The Y-maze was thoroughly cleaned with 70% ethanol after each mouse to eliminate foreign odours. Ethovision XT was used to track and collect the following data:

- Duration time spent in novel versus familiar arms
- Latency to enter the novel arm
• Number of entries in novel versus familiar arms

These parameters can be used to evaluate explorative and spatial recognition memory.

**Morris Water Maze**

**Apparatus Set up**

The MWM pool used in this study was 1.44m in diameter.

![Figure 4.3](image)

**Figure 4.3.** Schematic representation of Morris Water maze set up. The hidden platform is placed in the home quadrant of the pool. Extra-maze cues are placed at each point; North, West, East and South, with three cues being two-dimensional, and the fourth a three-dimensional cue.

**MWM Testing**

The MWM test is comprised of two components:

• Learning trial (spatial reference memory)
• Probe trial (memory)
The pool is spatially separated so that the Ethovision software recognized four quadrants (NW, SW, SE and NE). Four extra-maze cues were used; with 3x A3 posters (2D) cues and one 3D cue also placed externally to the pool (Figure 4.3). These spatial cues are necessary for the mice to spatially navigate. The pool is filled with water (24 ± 1°C), and water based paint added in order to make the water opaque. The hidden platform is randomly assigned a quadrant location (‘home quadrant’), so that each mouse will have the hidden platform in the same location during each day of testing.

**Learning Trial**

The learning trial is conducted over 6 consecutive days, where mice learn to swim to the correct platform location showing over time decreased escape latencies and direct swim paths. Each day consists of four trials (max 120 sec) where the mouse is introduced into the pool from each of the four quadrants, and the latency to find the hidden platform recorded. On the first day however, since they have not encountered the hidden platform, mice are guided to the platform and allowed 30 sec to identify the cues in relation to their location. The four trials are repeated daily for six days, with wild-type mice typically showing a progressive decline in the time taken to locate the hidden platform indicating spatial learning.

**Probe Trial**

After completion of the learning trials, the probe trial was conducted (day 7). The platform was removed, and the mouse introduced from the opposite quadrant to their ‘home quadrant’, and allowed to swim for 60 sec. A mouse that has learned and remembered the location of the hidden platform, will swim to the ‘home quadrant’ quickly and show a low latency to enter the hidden platform quadrant. They will also repeatedly enter the home quadrant, and spend more time in the target quadrant providing evidence for spatial memory. The platform zone can also be analyzed, which is the area of the platform location within the home quadrant.

During testing, it was imperative that control procedures are strictly adhered to including ensuring that noise levels were kept to a minimum, maintaining one operator handling, and general maintenance of a stable environment ensures that stress related behaviours were kept to a minimum. Mice were also monitored for behaviours such as thigmotaxis, where mice tend to
remain close to the walls of the pool which is a sign of anxiety (Simon et al., 1994). Mice can also refuse to swim, whereby the float and is another indicator of anxiety. Any mice that exhibited any of these behaviours were excluded from testing. One wild-type and one heterozygous mice were excluded from testing due to abnormal learning profiles as a result of thigmotaxis.

_Elevated Plus Maze_

Prior to testing, mice were habituated in the testing room overnight in low light level conditions (9-10 Lux). On testing day, mice were placed in the center of the maze facing the closed arm and allowed to explore the maze for 5 minutes (Figure 4.4). Elevated plus maze was thoroughly cleaned with 70% ethanol between mice to remove foreign odours.

![Elevated Plus Maze schematic](image)

**Figure 4.4.** Elevated Plus Maze schematic, showing the location of open and closed arms

Ethovision software was used to collect and analyze the following parameters:

- Duration (time spent) in open versus closed arm
- Number of entries in the open versus closed arm
- Latency to enter the open arms
- Distance and velocity traveled
- Duration and entries into the central arm
Ethological assessment was also conducted where manual scoring of number of additional parameters that allow for breakdown of more specific indices such as anxiety itself, locomotion, risk assessment, decision making, vertical activity and exploration:

- Total arm entries
- Stretch attend postures which can be identified by two criteria:
  - Protected (mouse was located in the closed arm and central arm but explored the open arms)
  - Unprotected (mouse was in open arm and stretch to further explore)
  - Both protected and unprotected
- Closed arm returns (when the mouse backs into the closed arm without committing to leaving the closed arm)

These parameters can provide insight into the following behaviours:

- Anxiety: Activity in open and closed arms including duration and entries, and total arm entries
- Risk assessment: stretch attend postures, protected, unprotected and both.
- Decision making: time spent in center zone of the maze and closed arm returns
- Exploration and motivation: total stretch attend postures, distance and velocity

**Pre-pulse inhibition**

Mice were habituated in the test room for at least 30 minutes prior to placement in the pre-pulse chambers (SR Labs). These chambers measure startle of the mice that are motor based responses measured in milliVolts (mV). In this study we used 115 dB test stimulus (loud sound), with three softer sounds; 4 dB, 8 dB and 16 dB run over 4 consecutive blocks. Mice were initially exposed to background noise to establish base line responses, after which they were subjected to a preset protocol where the loud stimuli (115 dB for 40 msec) is delivered on its own or preceded by a weak non-startle stimuli (4, 8 or 16 dB, for 20 msec) or no stimuli at all (Figure 4.5). Raw data was collected by SR LAB Startle response system software, and then calculated as percentage of inhibition to a pre-pulse stimulus using the following formula: %PPI = (SR (115 dB) – SR (4, 8 or 16 dB) / SR (115 dB)*100. This protocol was used in accordance to Standard Operating Procedures of FNI.
Figure 4.5. Schematic representation of pre-pulse inhibition (PPI) protocol. Startle response was measured (mV) in response to 115 dB auditory sound alone, and following a pre-pulse tone of 4, 8 and 16 dB. The percentage difference between the startle response to the 115 dB tone alone and following pre-pulse was calculated.

**Tissue collection**

*Western Blotting*

A separate cohort of mice was used to obtain hippocampal tissue for Western blot analysis. Briefly, mice (n = 3-5) were euthanized with sodium pentobarbital injection (100 mg/kg i.p.). Brains were removed, and the hippocampus dissected on ice, and stored at -80°C until required. Tissue samples were homogenized and processed as previously described (Chapter 2). Blots were incubated with an antibody against GluR1 (1/1000, Merck-Millipore) overnight at 4°C. Blots were washed and incubated with fluorescently tagged Alexa secondary antibodies and visualized using FluoroChemQMultImage III. Blots were analyzed using Image J, with each gel containing both wild-type and heterozygous samples to allow direct measurement and comparison. β-actin was used as a loading control also measured on each gel. Protein bands were quantified, normalized to β-actin levels and then represented as % of control.
Immunohistochemistry

For immunohistochemistry, sections obtained from mice described in Chapter 2 were stained and assessed in this study. For immunohistochemistry, 2 (n = 5) and 6 (n = 5) month old *Nedd4* heterozygous and wild-type littermates (n = 4, n = 5 and n = 3, respectively) were used. Frozen 50μm thick free-floating coronal sections were incubated with primary antibodies. The following antibodies were used:

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Function</th>
<th>Dilution</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse anti-NeuN</td>
<td>Neuronal marker</td>
<td>1/1000</td>
<td>Merck-Millipore</td>
</tr>
<tr>
<td>Mouse anti-MAP2</td>
<td>MAP2 protein (dendritic marker)</td>
<td>1/1000</td>
<td>Sigma</td>
</tr>
<tr>
<td>Rabbit anti-Nedd4</td>
<td>Nedd4 protein</td>
<td>1/1000</td>
<td>Abcam</td>
</tr>
<tr>
<td>Rabbit-anti GluR1</td>
<td>GluR1 subunit of AMPARs</td>
<td>1/1000</td>
<td>Merck-Millipore</td>
</tr>
</tbody>
</table>

Primary antibodies were visualized with Alexa-conjugated secondary antibodies, mounted using Prolong-Gold with DAPI mounting medium (Invitrogen). Stained sections were visualized using a Nikon C1 confocal microscope using a 488nm argon and 543nm HeNe laser (Nikon Group, Tokyo, Japan). Images were acquired using a 40x Plan-Apochromat oil immersion objective. All images were captured and analyzed using Nikon Elements software. Images were acquired from the hippocampus and amygdala. The hippocampus was further subdivided into the dorsal and ventral portions using published delineations (Fanselow *et al.*, 2010) (Figure 4.6).
Figure 4.6. Schematic illustration of delineation between dorsal and ventral portions of the hippocampus. Adapted from (Franklin et al., 2007).

**Long-term potentiation (LTP) recordings**

Electrophysiological recordings of LTP were conducted in whole brain slices. Brain slices at 300μm thickness were obtained using a vibratome. LTP was determined by recording fEPSPs in the stratum radiatum layer of the CA1 region of the hippocampus. A glass electrode filled with artificial cerebrospinal fluid (125 mM NaCl, 3 mM KCl, 1.25 mM NaH₂PO₄, 1 mM MgCl₂, 25 mM NaHCO₃, 10 mM glucose and 2.5 mM CaCl₂) was placed in the CA1 region with an Ag/AgCl stimulating electrode placed in the Shaffer collateral positioned toward the CA3 region. The potentials were amplified using an Axoclamp 2B, were pass-filtered at 1 Hz-3 kHz and digitized at 10 kHz (Digidata 1322A, Clampex 9.2 software, Axon instruments USA). Stimuli were delivered very 20 sec at varying amplitudes to produce an input-output (I/O) curve per slice. To determine LTP, fEPSPs that were approximately one-third of the spiking amplitude of the I/O curve were used and a single fEPSP recorded very 20 sec for at least 20 min in order to obtain a stable baseline. Four tetanic bursts (each consisting of 100 Hz stimulation) were applied for 1 sec followed by a 20 sec rest period between bursts, after which a single stimulus was applied very 20 sec for 2 hours. LTP responses were normalized to the average of the baseline responses, and the fEPSPs analyzed in terms of their maximum rate of rise using seven-point smoothing.
For Morris water maze, the learning curve was analyzed using one-way repeated measures ANOVA comparing latencies to enter the home quadrant between *Nedd4* heterozygous mice and wild-type controls. For memory probe parameters of MWM, Y-maze, elevated plus maze, PPI, Western blot analysis and LTP recordings an unpaired-Student’s t-test was used to statistically compare the *Nedd4* heterozygous mice to their age-matched wild-type controls. Results were deemed significant when P < 0.05.

### 4.3 RESULTS

**Y-maze**

Short-term spatial memory was assessed with the Y-maze where duration and entries into the novel during the second trial were measured. If short-term memory is preserved then no changes in novel arm parameters should be observed. No difference was found in duration spent in the novel (2 months, wt: 83.0 ± 3.3 sec; n = 8 and het: 85.4 ± 4.9 sec; n = 14; P = 0.74 and 6 months, wt: 88.0 ± 4.3 sec; n = 10 and het: 81.6 ± 5.2 sec; n = 10; P = 0.77) or familiar arms (2 months, wt: 123.7 ± 5.3 sec; n = 8 and het: 129.9 ± 8.2 sec; n = 14 sec; P = 0.60 and 6 months, wt: 156.5 ± 5.2 sec; n = 10 and het: 161.7 ± 5.6 sec; n = 10; P = 0.83) ([Figure 4.7A and B](#), respectively). The percentage of time spent in the novel over the 5 minutes trial was also assessed, and no difference was found between *Nedd4* heterozygous mice and wild-type controls at both 2 and 6 months of age (2 months, wt: 50.1 ± 1.8 %; n = 8 and het: 52.8 ± 2.8 %; n = 14; P = 0.51 and 6 months, wt: 29.3 ± 1.4 %; n = 10 and het: 27.2 ± 1.7 %; n = 10; P = 0.77) ([Figure 4.7C and D](#), respectively).

The number of entries into novel and familiar arms at 2 months of age, showed a decrease in the familiar arms by *Nedd4* heterozygous (wt: 27 ± 2; n = 8 and het: 22 ± 2; n = 14, P = 0.05) mice compared to their wild-type controls, and no difference observed in the novel arm (wt: 17 ± 2; n = 8 and het: 16 ± 1; n = 14; P = 0.61) ([Figure 4.8A](#)). At 6 months old no difference was seen in the number of entries between *Nedd4* heterozygous mice and wild-type controls in both novel (wt: 20 ± 3; n = 10 and het: 18 ± 3; n = 10; P = 0.94) and familiar arms (wt: 33 ± 4; n = 10 and het: 32 ± 3; n = 10; P = 0.81) ([Figure 4.8B](#)).
The latency to enter the novel arm can also be a useful indicator of short-term spatial integrity. In 2 months old *Nedd4* heterozygous mice the latency to enter the novel arm (wt: 5.2 ± 1; n = 8 and het: 10.0 ± 4; n = 14 sec; P = 0.05) was increased (Figure 4.9A). At 6 months of age, there was no difference in the latency to enter the novel arm in *Nedd4* heterozygous mice compared to their age matched wild-type controls (wt: 9.8 ± 5.4; n = 10 and het: 14.7 ± 5.4 sec; n = 10: P = 0.53) (Figure 4.9B).

Overall data obtained from the Y-maze showed that no impairment in short-term memory was evident in *Nedd4* heterozygous mice at both 2 and 6 months of age.

![Figure 4.7.](image-url) Y-maze showing duration in novel and familiar arms in 2 month and 6 month *Nedd4* heterozygous mice (+/-) and age-matched wild-type controls (+/+). Data represented as mean±SEM.
Figure 4.8. No difference in number of entries between *Nedd4* heterozygous mice (+/-) and age-matched wild-type controls (+/+) at both 2 (A) and 6 (B) months of age. Data represented as mean±SEM.

Figure 4.9. Latency to enter the novel arm was not different between *Nedd4* heterozygous (+/-) mice and age-matched wild-type controls (+/+) at both 2 (A) and 6 (B) months of age. Data represented as mean±SEM.

**Morris Water Maze**

*Learning Phase*

The first phase involves the evaluation of time spent locating a hidden platform and can determine whether learning is affected. With time, normal learning process results in a decrease
in time to reach the platform. In *Nedd4* heterozygous mice at 2 months of age, a significant increase in time taken to find the hidden platform was observed (Figure 4.10). This was evident at day 2 (wt: 76 ± 8; n = 7 and het: 79 ± 7 sec; n = 13, P = 0.01), day 4 (wt: 41 ± 11; n = 7 and het: 75 ± 9 sec; n = 13, P = 0.03) and day 6 (wt: 27 ± 5; n = 7 and het: 65 ± 10 sec; n = 13, P = 0.007), with a modest increases on days 3 (wt: 44±9; n=7 and het: 66±9 sec; n=13) and day 5 (wt: 35±7; n=7 and het: 58±11 sec: n=13) and no difference at day 1 (wt: 76 ± 8; n = 7 and het: 79 ± 7 sec; n = 13), and. This data, suggests that *Nedd4* heterozygous mice display a significant learning impairment at 2 months of age.

At 6 months of age, no impairment in learning was evident. In fact, there was no significant difference in time taken to locate the platform, with only a modest impairment at day 6 (wt: 35.7 ± 8.7; n = 7 and het: 56.0 ± 7.4 sec; n = 10, P = 0.06) (Figure 4.11). The data did suggest however that the learning profile of wild-type was affected in particular during day 4 and 5. Path length measured by distance travelled was unchanged between *Nedd4* heterozygous mice and age-matched wild-type controls at both 2 and 6 months of age (Figure 4.12).

**Figure 4.10.** Learning phase showing a significant increase in the latency to find hidden platform in 2 month old *Nedd4* heterozygous (closed, +/-) mice relative to wild-type controls (open, ++/) mice over six trial days. Data represented Mean ± SEM of average of four trials per day. * < 0.05 One way RM ANOVA.
Figure 4.11. No difference in latency to find the hidden platform between 6 month old Nedd4 heterozygous (closed, +/-) mice relative to wild-type controls (open, +/+ ) mice over six trial days. Data represented Mean ± SEM of average of four trials per day.

Figure 4.12. Distance was not changed in Nedd4 heterozygous mice (+/-) compared to wild-type controls (+/+) at both 2 and 6 months of age. Data represented as Mean ± SEM.

Probe Trial

2 month cohort

In the 2 months old cohort, latency to enter the home quadrant was significantly increased in the Nedd4 heterozygous mice (wt: 6.4 ± 1.3 sec; n = 8 and het: 23.0 ± 5.4 sec; n = 13, P = 0.03) suggesting that long-term spatial memory was impaired (Figure 4.13A). Nedd4 heterozygous mice also showed a significant decrease in the number of entries in the home quadrant (wt: 8 ± 1;
n = 8 and het: 5 ± 1; n = 13, P = 0.051 (Figure 4.13B) accompanied with a decrease in the time spent in the home quadrant that did not reach significance (wt: 16 ± 2; n = 8 and het: 11 ± 2; n = 13, P = 0.1 (Figure 4.13C). When the platform zone was examined there were no differences in the latency to enter (wt: 20.9 ± 7.2; n = 8 and het: 33.4 ± 5.6 sec; n = 12), number of entries (wt: 2.0 ± 1; n = 8 and het: 2 ± 1; n = 12) or duration (wt: 1.2 ± 0.4; n = 8 and het: 1.1 ± 0.3; n = 12) (Figure 4.13D, E and F, respectively).

6 month cohort

In the 6 months old cohort, there was no difference in the latency to enter the home quadrant between the Nedd4 heterozygous and their age matched wild-type controls (wt: 19.3 ± 6.8; n = 7 and het: 27.7 ± 6.9 sec; n = 10) (Figure 4.14A). Furthermore, there was no difference in the number of entries (wt: 6 ± 1; n = 7 and het: 3 ± 1; n = 10) (Figure 4.14B) or duration (wt: 13.0 ± 2.2; n = 7 and het: 12.3 ± 3.1 sec; n = 10) (Figure 4.14C). When the platform zone parameters were examined, again no difference was found in the latency to enter (wt: 39.1 ± 7.7; n = 7 and het: 40.0 ± 7.0 sec; n = 10) (Figure 4.14D), number of entries (wt: 2 ± 1; n = 7 and het: 1 ± 0.3; n = 10) (Figure 4.14E) or duration (wt: 1.0 ± 0.4; n = 7 and het: 0.5 ± 0.2; n = 10) (Figure 4.14F). Overall there did not appear to be any impairment in the capacity to develop and maintain long-term spatial memory in the Nedd4 heterozygous mice compared to their age-matched wild-types at 6 months of age.
**Figure 4.12.** At 2 months of age, a significant increase in latency to enter the platform quadrant was seen in Nedd4 heterozygous (+/-) mice compared to wild-type controls (+/+). The number of entries into the home quadrant was also significantly increased in Nedd4 heterozygotes compared to wild-type controls. No significant difference was observed in the time spent in the home quadrant. No differences were seen in latency to enter, number of entries or time spent in the platform zone. Data represented as Mean ± SEM. * P < 0.05, assessed by unpaired Student’s t-test.
Figure 4.13. At 6 months of age, no difference was seen in latency to enter the platform quadrant between \textit{Nedd4} heterozygous (+/-) mice compared and wild-type controls (+/+) (A). The number of entries into the home quadrant was significantly decreased in \textit{Nedd4} heterozygotes compared to wild-type controls (B). No significant difference was observed in the time spent in the home quadrant (C). No differences were seen in latency to enter (D), although a small reduction was observed in the number of entries (E) and time spent in the platform zone (F) however this did not reach significance. Data represented as Mean ± SEM. P < 0.05, assessed by unpaired Student’s t-test.
When anxiety measures were assessed, *Nedd4* heterozygous mice at 2 month of age showed a significant increase in time spent in the closed (safe) arm (wt: 119.1 ± 8.7; n = 8 and het: 153.9 ± 10.9; n = 14, P = 0.04) and reduced time spent in the open (unsafe) arm (wt: 28.3 ± 7.2; n = 8 and het: 17.5 ± 6.5; n = 14, P = 0.30) of the maze (Figure 4.15A). This indicates that *Nedd4* heterozygous mice have an increase in anxiety related behaviours compared to wild-type controls. *Nedd4* heterozygous mice entered the open arm less (wt: 6 ± 1; n = 8 and het: 4 ± 1; n = 14; P = 0.31) frequently than wild-type controls, however this did not reach significance (Figure 4.15B). Time spent in the centre zone of the maze was also assessed, and no differences were evident (P = 0.11) (Figure 4.15C). In addition, distance traveled (wt: 1392 ± 83; n = 8 and het: 1309 ± 69; n = 14) and velocity (wt: 4.7 ± 0.3; n = 8 and het: 4.4 ± 0.2; n = 14) was unchanged (Figure 4.15D and E).

At 6 months of age, no differences in duration, number of entries, time spent in centre zone, distance or velocity was apparent between *Nedd4* heterozygous mice and wild-type controls (Figure 4.16). These findings suggest that anxiety related behaviours were absent in the *Nedd4* heterozygous mice at this age.
Figure 4.14. A significant increase in time spent in the closed arm was observed in 2 month old Nedd4 heterozygous (+/-) mice compared to wild-type controls (+/+), accompanied with a marked decrease in the open arm (A). No significant difference was evident in number of entries in the closed or open arms (B). Time spent in the centre zone (C), distance travelled (D) and velocity (E) were also unchanged between the heterozygotes and wild-type controls. Data represented Mean ± SEM. *, P < 0.05 unpaired Student’s t-test.
Figure 4.15. No difference was observed in 6 month old Nedd4 heterozygous mice (+/-) compared to wild-type controls (+/+) in time spent in open versus closed arm (A), number of entries in open versus closed arms (B), time spent in the centre zone (C), distance travelled (D) or velocity (E). Data represented Mean ± SEM.
Assessment of ethological parameters

Due to the observed change in anxiety in the 2 month cohort, ethological parameters were also investigated. These include; locomotor and vertical activity such as total number of entries (closed and open), and number of rearing events during the testing period. A slight reduction was observed in overall number of entrances in both open and closed arms of the maze (wt: 28.3 ± 2.0; n = 8 and het: 22.8 ± 1.8; n = 14; P = 0.07) (Figure 4.17A) but no differences in number of rearing events (wt: 15 ± 2; n = 8 and het: 14 ± 2; n = 14) were evident (Figure 4.17B).

Risk assessment and decision making can also provide further information into thought processing that may underlie anxiety behaviours and to do this manual scoring of the number protected, unprotected and total (protected and unprotected) stretch attend postures was assessed. The number of protected (wt: 26 ± 3; n = 8 and het: 23 ± 2; n = 11), unprotected (wt: 7 ± 1; n = 8 and het: 5 ± 2; n = 11) and total (protected and unprotected) stretch attend postures (wt: 33 ± 3; n = 8 and het: 28 ± 2; n = 11) (Figure 4.17C) were not different between the Nedd4 heterozygous mice and their wild-type controls. Closed arm returns can also provide important information on explorative decision making in mice, where they explore the open arms however do not commit to entry in the open arms. A modest decrease in the number of closed arm returns in Nedd4 heterozygous (wt: 5 ± 2; n = 8 and het: 2 ± 1; n = 11, P = 0.065) was observed (Figure 4.17D). Interestingly, three Nedd4 heterozygous mice never entered the open arm at all. Overall, analysis from ethological scoring revealed that risk making and decision-making was not altered in Nedd4 heterozygous mice. Ethological parameters were not assessed in the 6 month cohort as there was no change in anxiety behaviours.

This along with no change in distance travelled and velocity shows that general activity in exploring the maze was not affected in the Nedd4 heterozygous mice compared to wild-type controls which is comparative to locomotor activity measurements in Chapter 2.
Figure 4.16. A marked reduction in total arm entries was evident in the 2 month old *Nedd4* heterozygous (+/-) mice compared to wild-type controls (+/+) (A). No difference in rearing events (B), or number of stretched attenuated responses in protected, unprotected or total (C). A slight reduction in closed arm returns was also present in *Nedd4* heterozygous mice (D). Data represented Mean ± SEM. #, P = 0.07 unpaired Student’s t-test.
Pre-pulse inhibition

First, demonstration of auditory responses at each of the different auditory stimuli was conducted in the mice to determine that hearing was not impaired in both 2 month (NS wt: 8 ± 3; and het: 5 ± 2, 4dB wt: 29 ± 5 and het: 28 ± 4, 8dB wt: 41 ± 3 and het: 55 ± 9, 16dB wt: 77 ± 9 and het: 78 ± 12 and 115dB wt: 95 ± 10 and het: 109 ± 16; with n= 7 and n = 10 respectively) (Figure 4.18A) and 6 month (NS wt: 18.4 ± 3.6 and het: 13.7 ± 1.8, 4dB wt: 82.7 ± 24.9 and het: 52.2 ± 8.9, 8dB wt: 74.9 ± 21.0 and het: 44.2 ± 7.1, 16dB wt: 51.7 ± 18.0 and het: 30.0 ± 4.9, n = 10 and n = 9 respectively). At 6 months of age Nedd4 heterozygous showed a significant decrease in startle response to the test stimuli of 115dB (wt: 133.8 ± 23.0 and het: 76.1 ± 14.6, P = 0.05) (Figure 4.18B). Mice that did not respond to a startle response were assumed to have an auditory deficit as they did not respond beyond base line levels and were excluded from analysis. A total of two Nedd4 heterozygous and two wild-type littermates at 2 months of age, and one Nedd4 heterozygous mouse at 6 months of age were therefore excluded from analysis.

Figure 4.17. No difference in startle was observed in response to no startle (NS), 4 dB, 8 dB, 16 dB and 115 dB between 2 month old Nedd4 heterozygous (+/-) mice and wild-type controls (+/+) (A). At 6 months of age, no significant differences were observed to all stimuli, however a modest reduction was present in Nedd4 heterozygotes to the 115 dB stimulus (B). Data represented as Mean ± SEM. * P = 0.05 unpaired Student’s t-test.
Next the startle responses to the test stimulus within the four block trials (response to pre-pulse stimulus, 115dB) were evaluated. In the 2 month cohort there was a similar response with decreased with time as expected between Nedd4 heterozygous and wild-type controls (block 1 wt: 107 ± 10 and het: 141 ± 26, block 2 wt: 103 ± 15 and het: 112 ± 18, block 3 wt: 84 ± 9 and het: 86 ± 11 and block 4 wt: 86 ± 13; and het: 97 ± 17; n=7 and n=10 respectively) (Figure 4.19A). In the 6 month cohort however, a significant decrease in baseline responses were seen within the four block trials in the Nedd4 heterozygous mice compared to wild-type controls (block 1 wt: 143 ± 28 and het: 92 ± 17, P = 0.15, block 2 wt: 132 ± 23 and het: 69 ± 13, P = 0.03, block 3 wt: 133 ± 24 and het: 73 ± 18, P = 0.07 and block 4 wt: 128 ± 20 and het: 70 ± 13, P = 0.03; n = 10 and n = 9 respectively) (Figure 4.19B).

Figure 4.18. Startle responses over four block of 115 dB stimuli were not different between 2 month old Nedd4 heterozygous (+/-) mice compared to wild-type controls (+/+). Startle responses in 6 month old Nedd4 heterozygous mice were significantly reduced compared to wild-types. Data represented as Mean ± SEM. # P = 0.07 and * P < 0.05 unpaired Student’s t-test.

To determine the percentage of inhibition when a pre-pulse stimulus was also applied we used the following formula as previously described. \( \%\text{PPI}= \frac{\text{SR (115dB)}-\text{SR (4,8 or 16dB)}}{\text{SR(115dB)}\times 100} \). When we examined \% PPI to the test stimulus (115 dB) following each of the different pre-pulse auditory stimuli (i.e 4, 8 and 16dB) no difference between Nedd4 heterozygous and wild-type controls at 2 months of age (4dB wt: 19 ± 3 and het: 29 ± 4, 8dB wt: 54 ± 5 and het: 49 ± 6 and 16dB wt: 68 ± 6 and het: 71 ± 5; n=7 and n=10 respectively) was apparent (Figure 4.20A). In the 6 month cohort, no significant difference was observed when %
PPI was compared between *Nedd4* heterozygous and wild-type controls (4dB wt: 41 ± 10 and het: 31 ± 8, 8dB wt: 47 ± 8 and het: 35 ± 6 and 16dB wt: 63 ± 6 and het: 50 ± 6) (Figure 4.20B).

**Figure 4.19.** Percentage of pre-pulse inhibition (%PPI) was not significantly different between *Nedd4* heterozygous (+/-) mice and wild-type controls (+/+) at both 2 (A) and 6 months of age (B). Data represented as Mean ± SEM.

This data suggests that *Nedd4* heterozygous mice at both 2 and 6 months of age had no alterations in filtering of sensory information.

**Nedd4 expression in the CNS associated with cognitive behaviours**

**Hippocampus**

*Nedd4* expression in the hippocampus was assessed including the following subdivision; CA1, CA2, CA3 and DG regions. Hippocampus was captured at location of -1.22 from Bregma and mouse brain atlas used as a guide (Franklin *et al.*, 2007). *Nedd4* was ubiquitously expressed in all layers of the hippocampus with highest level evident surrounding the cell bodies of pyramidal neurons, which were stained using the neuronal marker NeuN. Furthermore, all subdivisions showed similar patterns of expression, with a reduction in staining intensity seen in the heterozygous mice relative to controls in CA1, CA2, CA3 and DG (Figure 4.21, 4.22, 4.23, and 4.24, respectively). Within the 6 month brains, *Nedd4* staining intensity appeared higher, which may be due to differing levels of fixation during the perfusion process. The brains from the 2 and 6 months cohorts were perfused and processed at different time, and although the
immunohistochemical method were identical, differences in fixative cannot be excluded and may explain the increased brightness in these sections. Nevertheless, all comparisons were made between Nedd4 heterozygous mice relative to age-matched wild-type controls.

Due to the prominence of Nedd4 in dendrites of Purkinje neurons, MAP2 staining was used to determine whether Nedd4 is also highly expressed in the dendrites of pyramidal neurons of the hippocampus. Surprisingly, this did not appear as pronounced as dendrites of Purkinje neurons of the cerebellum (Figure 4.25).

**Figure 4.20.** Within the CA1 region of the hippocampus, Nedd4 (red) was found to be most highly expressed surrounding cell bodies of pyramidal neurons (NeuN, green). (+/-) Nedd4 heterozygous and (+/+) wild-type controls. Scale bar represents 50μm.
Figure 4.21. Within the CA2 region of the hippocampus, Nedd4 (red) was found to be most highly expressed surrounding cell bodies of pyramidal neurons (NeuN, green). (+/-) Nedd4 heterozygous and (+/+) wild-type controls. Scale bar represents 50μm.
Figure 4.22. Within the CA3 region of the hippocampus, Nedd4 (red) was found to be most highly expressed surrounding cell bodies of pyramidal neurons (NeuN, green). (+/-) Nedd4 heterozygous and (+/+ ) wild-type controls. Scale bar represents 50μm.
**Figure 4.23.** Within the DG region of the hippocampus, Nedd4 (red) was found to be most highly expressed surrounding cell bodies of pyramidal neurons (NeuN, green). (+/-) Nedd4 heterozygous and (+/+) wild-type control. Scale bar represents 50μm.
Figure 4.24. MAP2 (green) staining of dendrites within the hippocampus, showed some co-localization with Nedd4 (red), in the CA1, CA2, CA3 and DG regions of a 2 month wild-type mouse. Scale bar represents 50\(\mu\)m.
**Nedd4 expression within the dorsal and ventral compartments of the hippocampus**

**Dorsal Hippocampus**

In the CA1 region, the pyramidal layer showed disorganization and neurons appeared dispersed in *Nedd4* heterozygous mice compared to wild-type controls, which was evident at both 2 and 6 months of age (Figure 4.26). Although this observation was also made in the CA3 region, it was less prominent (Figure 4.27).

![Image](image.png)

**Figure 4.25.** Within the dorsal CA1 (CA1) region of the hippocampus, Nedd4 (red) was found to be most highly expressed surrounding cell bodies of pyramidal neurons (NeuN, green). A dispersion of the pyramidal layer neurons (arrow) was evident in the *Nedd4* heterozygous mice (+/−) at both 2 and 6 months of age relative to wild-type controls (+/+). Scale bar represents 50 μm.
Figure 4.26. Within the dorsal CA3 (dCA3) region of the hippocampus, Nedd4 (red) was found to be most highly expressed surrounding cell bodies of pyramidal neurons (NeuN, green). (+/-) Nedd4 heterozygous and (+/+) wild-type controls. Scale bar represents 50µm.
Ventral Hippocampus

In the ventral hippocampus the pyramidal layer thickness appeared reduced in the Nedd4 heterozygous mice compared to wild-type controls in the CA1 region (Figure 4.28), and increased in the CA3 region (Figure 4.29).

Figure 4.27. Within the ventral CA1 (vCA1) region of the hippocampus, Nedd4 (red) was found to be most highly expressed surrounding cell bodies of pyramidal neurons (NeuN, green). A reduction in the thickness pyramidal layer (arrow) was evident in the Nedd4 heterozygous mice (+/-) at both 2 and 6 months of age relative to wild-type controls (+/+). Scale bar represents 50μm.
**Figure 4.28.** Within the ventral CA3 (vCA3) region of the hippocampus, Nedd4 (red) was found to be most highly expressed surrounding cell bodies of pyramidal neurons (NeuN, green). The pyramidal layer appeared thicker (arrow) in the Nedd4 heterozygous mice (+/-) at both 2 and 6 months of age relative to wild-type controls (+/+). Scale bar represents 50μm.
Since the amygdala is an important region crucial for anxiety related behaviours, Nedd4 expression was examined. Nedd4 was ubiquitously expressed and found to surround NeuN positive neurons (Figure 4.30).

Figure 4.29. In the central nucleus of the amygdala Nedd4 (red) was ubiquitously expressed, with no co-localization with neurons stained with the neuronal marker NeuN (green). (+/-) *Nedd4* heterozygous compared and (+/+) wild-type controls. Scale bar represents 50µm.
**Hippocampal Long-term potentiation (LTP)**

Whole brain recordings showed a non significant effect of group $F_{(1,5)} = 0.82$, $P = 0.41$ or group by time $F_{(1,5)} = 0.912$, $P = 0.38$. However, when individual time points were assessed a decrease in the LTP potentiation from the hippocampus of 2 month old *Nedd4* heterozygous mice relative to the wild-type controls by 120 minutes post tetanic stimulus application (wt: $1.19 \pm 0.07$; $n = 3$ and het: $0.80 \pm 0.07$; $n = 4$, $P = 0.01$) (**Figure 4.31**).

![Figure 4.30. Fold potentiation response to tetanic stimuli (TS at time = 0) were significantly reduced in *Nedd4* heterozygous (+/-) mice compared to wild-type controls (+/+). Data represented as Mean ± SEM. * P < 0.05 unpaired Student’s t-test.](image-url)
Molecular targets associated with cognition

GluR1 containing AMPARs
Due to the abnormalities seen in the 2 month old Nedd4 heterozygous mice such as impaired long-term spatial learning and memory levels of GluR1 were measured by Western blot, and no differences were seen (Figure 4.32). GluR1 levels were not measured in 6 month old cohorts, as no differences in cognitive behaviours became evident.

![Western blot analysis](image)

**Figure 4.31.** Western blot analysis showed no differences in GluR1 levels in hippocampal lysates obtained from 2 month old Nedd4 heterozygous (+/-) mice and wild-type controls (+/+). Data represented as Mean ± SEM.

GluR1 expression in the hippocampus
GluR1 expression was assessed in the hippocampus from 2 and 6 month Nedd4 heterozygous mice and age-matched wild-type controls. Importantly, GluR1 staining showed similar pattern of expression as Nedd4, in that it is ubiquitously expressed and shows high expression surrounding the cell bodies of neurons within the pyramidal cell layer. No obvious change in staining intensity was evident in the heterozygous mice relative to wild-type controls at both time points, supporting the findings that GluR1 levels are not changes measured by Western blot. Similar pattern of expression was evident in the CA1, CA2, CA3 and DG regions (Figure 4.33, 4.34, 4.35 and 4.36, respectively).
Figure 4.32. In the CA1 region of the hippocampus, GluR1 (red) was found to be most highly expressed surrounding cell bodies of pyramidal neurons (NeuN, green). No difference in staining intensity was evident between *Nedd4* heterozygous mice (+/-) at both 2 and 6 months of age relative to wild-type controls (+/+). Scale bar represents 50μm.
Figure 4.33. In the CA2 region of the hippocampus, GluR1 (red) was found to be most highly expressed surrounding cell bodies of pyramidal neurons (NeuN, green). No difference in staining intensity was evident between \textit{Nedd4} heterozygous mice (+/-) at both 2 and 6 months of age relative to wild-type controls (+/+). Scale bar represents 50\mu m.
**Figure 4.34.** In the CA3 region of the hippocampus, GluR1 (red) was found to be most highly expressed surrounding cell bodies of pyramidal neurons (NeuN, green). No difference in staining intensity was evident between *Nedd4* heterozygous mice (+/-) at both 2 and 6 months of age relative to wild-type controls (+/+). Scale bar represents 50μm.
Figure 4.35. In the DG region of the hippocampus, GluR1 (red) was found to be most highly expressed surrounding cell bodies of pyramidal neurons (NeuN, green). No difference in staining intensity was evident between Nedd4 heterozygous mice (+/-) at both 2 and 6 months of age relative to wild-type controls (+/+). Scale bar represents 50µm.
4.4 DISCUSSION

Nedd4 is important during development of axons and dendrites, but recently evidence emerged supporting a role for Nedd4 in the mature brain. Nedd4 was shown to regulate AMPA receptors (AMPAR) at the synapse in an activity dependent manner (Schwarz et al., 2010), however, the in vivo implications remained unknown. As already shown, the regulation of GluR1 played an important role in gait and motor co-ordination, with Nedd4 heterozygous mice showing significant gait abnormalities. AMPAR are also crucial at synapses within the hippocampus and amygdala to regulate cognitive behaviours.

This study investigated cognitive behaviours in Nedd4 heterozygous mice and examined regional and cellular expression within brain regions necessary for cognition. Learning and memory are important functions in everyday life and understanding the molecular machinery that underlie these processes are fundamental. Short-term memory was found to be intact in Nedd4 heterozygous mice at both 2 and 6 months of age. Long-term learning and memory however were significantly impaired in the 2 month old Nedd4 heterozygous mice relative to age-matched wild-type controls. The fact that short term is intact but long term memory is affected is supported by studies showing that ubiquitination regulates the consolidation of short into long-term memories (Artinian et al., 2008). Part of the consolidation process requires changes at the synapse where strengthening occurs between the pre and post-synaptic compartments, including de novo protein synthesis but also protein degradation (Artinian et al., 2008).

Long-term spatial learning and memory were found not to be significantly impaired in the 6 month old Nedd4 heterozygous mice. Although these findings were initially surprising, closer inspection of the data indicated that the wild-type mice did not display normal learning levels that were seen in the 2 month cohort. Nevertheless, by day 6 a modest increase in latency to find the hidden platform became evident, however significance was not reached.

Initial interpretation was that a delay in hippocampal development may have occurred and this contributed to the difference in long-term spatial learning and memory between the 2 and 6 month Nedd4 heterozygotes relative to the age-matched wild-type controls. Although Nedd4 was found to be ubiquitously expressed in all layers of the hippocampus, and within the four main regions; CA1, CA2, CA3 and DG including the dorsal and ventral segments. No evidence for
delayed development was found. For example, an increase in the thickness of the pyramidal layer was found in dCA1 and vCA3 regions with a reduced layer thickness found in vCA1 region in Nedd4 heterozygous mice relative to wild-type controls. These differences however were apparent at both 2 and 6 months of age, suggesting that developmental issues are unlikely to explain the behavioural differences observed. Nedd4 expression was not quantified from immunohistochemical sections, as it is widely accepted that immunohistochemistry is semi-quantitative (Taylor et al., 2006). Hippocampal tissue will be collected for Western blot analysis and future studies should measure Nedd4 levels in this important brain region. Layer thickness was not quantified due to the inability to accurately match sections from respective anatomical regions for section stained with Nedd4.

On a molecular level, the combination of GluR containing subunits may be modified at this later stage of adulthood that may allow learning and memory to occur as an adaptive mechanism. To better understand the contribution of GluR subunits to AMPAR function and thus synaptic plasticity, it is important to consider how the different subunit combinations can affect the synapse. AMPAR are composed of four subunits (GluR1, GluR2, GluR3 and GluR4), forming tetramers comprised of different combinations. The composition can affect AMPAR permeability, for instance the presence or absence of GluR2 subunit can alter permeability to calcium ions. The lack of GluR2 makes the AMPARs permeable to cations including sodium, calcium and potassium, with the presence of GluR2 resulting in impermeability to calcium. The modulation of AMPARs by trafficking (including endocytosis, recycling and re-insertion) to the plasma membrane is also dependent upon subunit composition. Although GluR1 and GluR2 both play an important role in synaptic plasticity, the trafficking of AMPAR containing these subunits are undertaken by two pathways; the regulated and constitutive (Malinow et al., 2000; Parwani et al., 2000).

In the regulated pathway, GluR1 containing AMPARs are trafficked to the plasma membrane in an activity dependent manner, and require NMDA receptor activation (Hayashi Y et al., 2000). This pathway then is crucial for long-term potentiation (LTP), and is inactive under basal conditions (Kessels et al., 2009). This regulatory pathway is important for the formation of new memories (Malinow et al., 2000). The constitutive pathway involves AMPAR that lack GluR1 subunit, usually composed of GluR2/3 combination. These AMPARs replace GluR1 containing receptors in an activity independent manner, under basal conditions that function to preserve total
numbers of AMPARs. This pathway is important for the maintenance of new memories (Malenka, 2003).

It may be possible that in the 6 month old *Nedd4* heterozygous mice, synapses are saturated with GluR1 containing AMPARs due to decreased ubiquitination and thus endocytosis. This reduction in ubiquitination could be as a result of decreased Nedd4 levels but needs to be investigated. Changes in ubiquitination and GluR1 plasma levels could allow for the formation of new memories but disrupt the maintenance of these memories due to the loss of GluR2/3 replacement, which was not assessed in these mice. Future studies could examine the longevity of memory formation by repeating the probe trial several times too examine how well the maintenance of memory is preserved. Furthermore, examination of GluR2 and GluR3 levels may also shed light onto whether trafficking is altered. Finally, LTP recordings should also be performed in older mice (i.e 6 months) to examine whether changes to synaptic plasticity occurs.

Due to the importance of GluR1 containing AMPARs for long-term memory, LTP was examined in whole brains slices from 2 month old *Nedd4* heterozygous mice and age-matched wild-type controls. LTP is the molecular mechanism that allows for synaptic changes to occur and long-term memories to develop. LTP was significantly reduced in *Nedd4* heterozygous mice relative to wild-type controls. This provides strong evidence that a reduction in Nedd4 levels is not only sufficient to produce disruption on long-term learning and memory, but indicates that GluR1 level mediated processes are also altered. Total GluR1 levels were measured from the hippocampi obtained from 2 month *Nedd4* heterozygous mice and were not different relative to wild-type controls. Immunohistochemical analysis of GluR1 within the hippocampus showed similar patterns of expression between GluR1 and Nedd4, which is expected since these two proteins are known to directly interact. GluR1 staining intensity did not appear to have changed in the *Nedd4* heterozygous brains compared to wild-type controls supported by the Western blot findings. This is not surprising, as GluR1 levels are altered in an activity dependent manner and thus total levels indicates that overall GluR1 levels are not changed. Future studies could examine the extent of trafficking, during LTP to confirm the contribution and impact of Nedd4 level reduction.

*Nedd4* heterozygous mice show an increase in anxiety behaviours at 2 months of age compared to wild-type controls, with no alteration observed in 6 month old heterozygotes. The interaction
between Nedd4 and GluR1 may also underlie the anxiety changes observed. Previous studies have shown a correlation between GluR1 levels anxiety measured by elevated plus maze (Xiang et al., 2011). The contribution of GluR1 to anxiety is controversial with discrepancies. For example, some studies have shown that deletions in GluR1 results in increased anxiety (Bannerman DM et al., 2004; Mead et al., 2006), other studies however suggest the opposite (Das et al., 2008). Further investigation is required to understand fully what contribution GluR1-containing AMPARs to anxiety like behaviours. Nedd4 was found in both the ventral portion of the hippocampus and amygdala, two regions of the brain necessary for the processing of emotive behaviours. Within the amygdala, Nedd4 was found to be ubiquitously expressed surrounding NeuN positive neurons. Further work is necessary to examine anxiety behaviours further, which may include other behavioural tests such as open field, light dark test and potentially fear based learning. Data from this study simply suggests that Nedd4 may be important for the regulation of anxiety behaviour which may be due to altered GluR1 containing AMPARs.

Sensory motor gating assessed using pre-pulse inhibition (PPI) was investigated to determine if any schizophrenia like behaviours were evident in these mice. Although deregulation of dopamine seems to be a main contributor to pathophysiology of schizophrenia, glutamate has also been suggested to be extremely important. Evidence for this mainly arise from animal studies where NDMA (glutamate) receptor antagonists such as phencyclidine or ketamine that stimulates psychosis (Krystal et al., 2005; Lahti et al., 1995). Due to the involvement of Nedd4 in regulation of glutamatergic AMPA receptors, it was necessary to investigate these behaviours. No difference PPI was evident in Nedd4 heterozygous mice at either 2 and 6 months of age. One subtle observation however, was the decrease in basal startle responses in the 6 month Nedd4 heterozygous mice. Since acoustic startle is a motor response to an unexpected stimulus, the decrease in basal startle responses may be due to underlying motor deficiency in the Nedd4 heterozygous mice. Further studies need to address whether any changes in the neuromuscular junction can impact on startle responses, such as reduced depolarization and reduced number of motor neuron that innervate skeletal muscle can have an impact on movement. Finally, hearing and changes in auditory pathways may also need to be investigated to determine if any changes are evident in the Nedd4 heterozygotes. Overall data from PPI testing showed no abnormal behaviours relating to sensory motor gating in Nedd4 heterozygous mice at both time points tested.
This study is the first to examine cognitive behaviours in *Nedd4* heterozygous mice and determine expression within regions of the CNS associated with these behaviours. *In vivo* implication of Nedd4 loss include long-term spatial memory and learning deficits and increased anxiety behaviours. GluR1-containing AMPARs may again be a pivotal target contributing to these findings. Since LTP is considered the molecular mechanism for memory, and dependent on GluR1 subunits of AMPARs, LTP recordings from brain slices provided further evidence for Nedd4 in cognitive behavior, with significant reduction in LTP observed in the *Nedd4* heterozygous mice.
CHAPTER 5: NEDD4-2 IN COGNITIVE BEHAVIOUR

5.1 RATIONALE AND BACKGROUND

Dopamine related motor behaviours were assessed in *Nedd4-2* heterozygous mice due to the known interaction between Nedd4-2 and dopamine transporter (DAT). Nedd4-2 was expressed in medium spiny neurons of the striatum, which are enriched with D1 or D2 receptors. With D1 receptors mediated signaling implicated to be involved in a range of cognitive functions, assessment in *Nedd4-2* heterozygous mice was warranted. Furthermore, Nedd4-2 is able to regulate the neurotrophin receptor TrkA, which binds to nerve growth factor also essential for cholinergic neurotransmission affecting hippocampal function.

*Dopamine and cognition*

*Learning and memory*

As already discussed the dopaminergic system is crucial for motor function and control, however dopaminergic pathways have also been implicated in cognitive function. These include learning and memory, hedonic reactions and motivation. For instance, associative learning involves the dorsal striatum and removal of dopamine can affect striatal based learning (Berke *et al.*, 2000). As already discussed (Chapter 3), D1 receptors within the striatum are responsible for learning behaviours. Dopamine is largely involved in short-term memory, which integrates moment-to-moment information with previous experiences (Packard *et al.*, 1994). Studies have shown that antagonism of D1 receptors in the prefrontal cortex, can impair memory (Sawaguchi *et al.*, 1994).

*Schizophrenia*

Dysregulation of dopamine also plays an important role in the pathophysiology of schizophrenia, with all antipsychotics targeting dopamine (D2) receptors. The original ‘dopamine hypothesis’ suggests that increases in dopamine contribute to the cause of schizophrenia (Packard *et al.*, 1994). Further evidence support this theory include exposure to amphetamines exacerbating symptoms of schizophrenia, but also inducing psychotic symptoms in healthy individuals (Connell, 1958), and drugs that deplete dopamine alleviate the symptoms (Connell, 1958). Although in *Nedd4-2* heterozygous mice dopamine levels are reduced, it is unlikely that these
mice will display any schizophrenia like symptoms. PPI testing could provide some information on whether potential changes in dopamine manifest into sensory motor gaiting abnormalities.

**Dopamine and synaptic plasticity**

There is evidence showing that activation of glutamate and dopamine receptors in the striatum can produce long lasting modification of synaptic excitability. This is supported by morphological feature of the striatum where glutamatergic and dopaminergic boutons are in close proximity to dendrites of medium spiny neurons (Smith *et al.*, 1990; Starr, 1995). Dopamine acting on striatal spiny neurons can affect synaptic plasticity in other brain areas including the hippocampus (Bourtoulade *et al.*, 1994) and cerebral cortex (Law-Tho *et al.*, 1995).

Intracellular recordings of cortical activation produced excitatory post-synaptic potentials (EPSPs) that are largely mediated by AMPA receptors (Jiang *et al.*, 1991). Repetitive stimulation of corticospinal fibres can induce both long-term potentiation (LTP) and long-term depression (LTD), and during this repetitive stimulation a dramatic release of both dopamine and glutamate is observed in producing striatal synaptic plasticity (Calabresi *et al.*, 1992; Calabresi *et al.*, 1990), with D1 and D2 receptors playing an essential role. By regulating DAT, Nedd4-2 may affect synaptic dopamine levels that can have an important role in striatal synaptic plasticity associated with a range of cognitive functions, which to date has not been investigated.

**Glutamate Transporters**

Glutamate as already discussed (Chapter 4) is essential for synaptic plasticity, underlying learning and memory. Nedd4-2 is able regulate membrane levels of glutamate transporters (EAAT1 and 2), and thus may be important in maintenance of normal glutamate levels. Although glutamate transporters are predominantly located on astrocytes, alterations in levels of these transporters may have an impact on levels of synaptic glutamate. For example, a decrease in transporter levels can result in reduced re-uptake in glutamate, leading to excessive levels of synaptic glutamate and vice versa.
**Nedd4-2 and TrkA receptors**

The neurotrophins are growth factors important for the developing and mature nervous system (Huange et al., 2001). The basal forebrain cholinergic system, which is composed of medial septum, horizontal and vertical diagonal bands of Broca, and nucleus basalis of Meynert provide the major cholinergic input to the hippocampus and cerebral cortex (Schliebs et al., 2011). The degeneration of this group of neurons underlies cognitive decline associated with ageing and diseases such as Alzheimer’s disease (Schliebs et al., 2011; Schliebs et al., 2006). The mechanism is thought to involve a decrease in NGF mediated trophic support, where it is required for protecting and maintaining cholinergic neurons (Dekker et al., 1991; Hefti et al., 1985). Furthermore, studies have shown that disruptions in NGF mediated trophic support in terms of trafficking and ability to interact with the high affinity TrkA and low affinity p75NTR receptors are the main contributors to the degeneration and morphological changes associated with cognitive decline in both normal ageing and pathological states such as Alzheimer’s disease (de Lacalle et al., 1996; Delcroix JD et al., 2004). Although Nedd4-2 is known to interact with TrkA, cognitive behaviours or expression of Nedd4-2 in the cholinergic neurons remained largely unknown.

**Nedd4-2 expression in CNS regions associated with cognitive function**

As discussed in Chapter 4, the hippocampus is essential for learning and memory, divisions into dorsal and ventral portions showing different roles in cognition (Fanselow et al., 2010). The hippocampus also has important reciprocal connections with the amygdala necessary for processing of emotive behavior and regulation of anxiety related behaviours. Cholinergic input to the hippocampus from the medial septal regions is important for hippocampal based learning and memory, with contribution to the induction and expression of LTP (Kanju et al., 2012). Due to the known interaction between Nedd4-2 and TrkA receptors, the expression of Nedd4-2 should also be assessed in the medial septal region.
**Behavioural assessment of cognition**

As discussed in Chapter 4, a range of behavioural tests can be used to measure short and long-term learning and memory, anxiety and sensory motor gating. A number of important targets already discussed suggest that some of these behaviours are likely to be affected in mice with reduced Nedd4-2 levels. In particular, the interaction between Nedd4-2 and TrkA receptors suggests that any alterations in Nedd4-2 levels are likely to alter TrkA receptor trafficking and thus expression in the membrane. This does not necessarily imply that there would be a memory deficit; it may in fact increase memory formation.

**Unknown targets of Nedd4-2 that may be implicated for cognitive function**

It is important to consider that there may also be currently unknown targets for Nedd4-2 in regions such as the hippocampus to regulate other types of memory including spatial short and long-term memory, and learning. Furthermore, the expression of Nedd4-2 in the CNS has not been investigated, and as such may provide further clues to its potential roles and novel targets.

Since Nedd4-2 has been shown to interact with DAT and TrkA receptors, both of which may have important implications in cognitive function lead the main hypothesis of this study; *that Nedd4-2 would have a essential role in neuronal function necessary for cognitive behaviours which may be due to reduced striatal dopamine levels or disrupted NGF mediated signaling via TrkA receptor*. To investigate this hypothesis, the following aims were set:

1) Conduct behavioural tests in *Nedd4-2* heterozygous mice to assess short and long-term memory and learning
2) Behavioural assessment of anxiety behaviours
3) Evaluation of sensory motor gating
4) Determine cellular and regional expression of Nedd4-2 in the brain associated with cognitive behaviours

Tests were conducted in 2 and 6 month old *Nedd4-2* heterozygous mice.
5.2 MATERIALS AND METHODS

Animals and Behavioural testing

The same mice were used as those described in Chapter 3 ‘Nedd4-2 in motor function’. Behavioural tests conducted have been thoroughly described in Chapter 4 ‘Nedd4 in cognitive behaviours’.

Tissue Collection

Immunohistochemistry

For immunohistochemistry, sections obtained from mice described in Chapter 3 were stained and assessed in this study. For immunohistochemistry, 2 (n = 5) and 6 (n = 5) month old Nedd4-2 heterozygous and wild-type littermates (n = 4, n = 5 and n = 3, respectively) were used. Frozen 50μm thick free-floating coronal sections were incubated with primary antibodies. The following antibodies were used:

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Function</th>
<th>Dilution</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse anti-NeuN</td>
<td>Neuronal marker</td>
<td>1/1000</td>
<td>Merck-Millipore</td>
</tr>
<tr>
<td>Mouse anti-MAP2</td>
<td>MAP2 protein (dendritic marker)</td>
<td>1/1000</td>
<td>Sigma</td>
</tr>
</tbody>
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Primary antibodies were visualized with Alexa-conjugated secondary antibodies, mounted using Prolong-Gold with DAPI mounting medium (Invitrogen). Stained sections were visualized using a Nikon C1 confocal microscope using a 488nm argon and 543nm HeNe laser (Nikon Group, Tokyo, Japan). Images were acquired using a 40x Plan-Apochromat oil immersion objective. All images were captured and analyzed using Nikon Elements software.

Statistics

For Morris water maze, the learning curve was analyzed using one-way repeated measures ANOVA comparing latencies to enter the home quadrant between Nedd4-2 heterozygous mice and wild-type controls. For memory probe parameters of MWM, Y-maze, elevated plus maze, and PPI an unpaired-Student’s t-test was used to statistically compare the Nedd4-2 heterozygous mice to their age-matched wild-type controls. Results were deemed significant when P < 0.05.
5.3 RESULTS

**Y-maze**

Short-term spatial memory was assessed in both 2 and 6 month old *Nedd4-2* heterozygous mice. At 2 months of age, the time spent (wt: 150.8 ± 10 sec; n = 7 and het: 148.2 ± 6.5 sec; n = 16, P = 0.8) in the novel arm was not different between *Nedd4-2* heterozygous mice and wild-type controls (Figure 5.1A). Similarly, at 6 months of age time spent (wt: 86.0 ± 3.9 sec; n = 10 and het: 88.4 ± 2.4 sec; n = 10, P = 0.6) (Figure 5.1B) was not different. When duration spent in the novel arm was represented as a percentage of total duration time, no differences were evident in both 2 (wt: 29.8 ± 2.4%; n = 10 and het: 28.9 ± 1.6%; n = 10, P = 0.77) and 6 months (wt: 28.7 ± 1.3%; n = 7 and het: 29.5 ± 0.8%; n = 16, P = 0.61) (Figure 5.1C and D, respectively).

The number of entries into the novel arm were also not significantly different between *Nedd4-2* heterozygous mice and age-matched wild-type controls at 2 (wt: 12.7 ± 0.9; n = 7 and het: 12.8 ± 0.8; n = 16, P = 0.9) and 6 (wt: 17.7 ± 0.7; n = 10 and het: 18.3 ± 1.0; n = 10) months of age (Figure 5.2A and B, respectively).
Figure 5.1. No difference was apparent in time spent in the novel or familiar arms between Nedd4-2 heterozygous (+/-) mice and age-matched wild-type controls (+/+ at both 2 (A) and 6 month old (B) cohorts. Similarly, the percentage of time spent in the novel arm was also not different at both 2 (C) and 6 months of age (D). Data represented as mean±SEM.

Figure 5.2. No difference in the number of entries was evident between Nedd4-2 heterozygous (+/-) mice compared to age-matched wild-type controls (+/+ at both 2 (A) and 6 months of age (B). Data represented as mean±SEM.
A non-significant increase in the latency to enter the novel arm was observed in 2 month old *Nedd4-2* heterozygous mice compared to the wild-type controls (wt: 4.5 ± 1.9 sec; n = 7 and het: 9.5 ± 2.1 sec; n = 16, P = 0.15), with no difference evident at 6 months of age (wt: 4.7 ± 1.0 sec; n = 10 and het: 5.9 ± 1.4 sec; n = 10, P = 0.49) (Figure 5.3A and B, respectively).

![Figure 5.3](image)

**Figure 5.3.** A non-significant increase in latency to enter the novel arm was seen in 2 month old *Nedd4-2* heterozygous (+/-) mice compared to age-matched wild-type controls (+/+) (A). No difference in latency to enter the novel arm was observed at 6 months of age (B). Data represented as mean±SEM.

This data shows that short-term spatial memory was intact in *Nedd4-2* heterozygous mice at both ages.

**Morris Water Maze**

Latency to find the hidden platform is an indicator of learning capacity, with a decrease in latency observed with progression of the trial days. In both the 2 and 6 month cohorts, there was no difference in latencies to find the hidden platform between the *Nedd4-2* heterozygotes and wild-type controls (Figure 5.4 and 5.5, respectively).
Figure 5.4. No difference in latency to find the hidden platform between 2 month old *Nedd4-2* heterozygous (+/-) mice compared to wild-type controls (+/+) at all trial days. Data represented as mean±SEM.

Figure 5.5. No difference in latency to find the hidden platform between 6 month old *Nedd4-2* heterozygous (+/-) mice compared to wild-type controls (+/+) at all trial days. Data represented as mean±SEM.
At 2 months of age, path length (distance travelled) was not different between $Nedd4-2$ heterozygous mice compared to wild-type controls during the learning trial (Figure 5.6). Due to an unforeseen error on the Ethovision set up, distance was not recorded for the 6 month old cohort during learning phase of the test.

Figure 5.6. In the 2 months cohorts, no difference was observed between $Nedd4-2$ heterozygous (+/-) mice and wild-type controls (+/+) in distance travelled. Data represented as mean±SEM.

Probe Trial

2 month

Following the learning phase of the test, evaluation of the latency to enter, time spent and number of entries in the home quadrant and platform zone can be used to determine memory. At 2 months of age, latency to enter the home quadrant (wt: 10.0 ±2.7 sec; n = 12 and het: 20.5 ± 6.7 sec; n = 12, P = 0.16), number of entries (wt: 8.6 ± 1.6; n = 12 and het: 8.1 ± 1.9; n = 12, P = 0.8) or duration (wt: 17.3 ± 2.5 sec; n = 12 and het: 12.7 ± 2.6 sec; n = 12, P = 0.2) were not affected in $Nedd4-2$ heterozygous mice compared to wild-type controls (Figure 5.7A, B and C, respectively). When platform zone parameters were assessed; latency to enter (wt: 28.4 ± 6.6 sec; n = 12 and het: 31.3 ± 7.0 sec; n = 12, P = 0.76), number of entries (wt: 3.3 ± 1.2; n = 12 and het: 3.9 ± 1.5; n = 12, P =0.4) and duration (wt: 1.9 ± 0.6 sec; n = 12 and het: 1.4 ± 0.4 sec; n = 12, P
= 0.55) were also not different in the Nedd4-2 heterozygotes compared to the wild-type controls (Figure 5.7D, E and F).

**Figure 5.7.** No difference was observed in home quadrant parameters of the probe trial including; latency to enter (A), number of entries (B) or time spent (C) between Nedd4-2 heterozygous (+/-) mice and wild-type controls (+/+) Similarly, parameters assessed in the platform zone showed no differences in latency (D), number of entries (E) or time spent (F). Data represented as mean±SEM.

6 months

At 6 months of age, latency to enter (wt: 19.5 ± 6.3 sec and het: 16.4 ± 2.9 sec; P = 0.7), number of entries (wt: 4.8 ± 0.8 and het: 6.6 ± 0.9; P = 0.15) and time spent (wt: 10.6 ± 2.0 sec and het: 12.9 ± 1.1 sec; P = 0.3) in the home quadrant were not different between the heterozygotes relative to wild-type controls (Figure 5.8A, B and C, respectively). When platform zone parameters were investigated, latency to enter the platform zone was significantly increased (wt: 21.7 ± 4.9 sec and het: 44.6 ± 5.5 sec; P = 0.006) (Figure 5.8D). The heterozygotes also showed a modest decrease in number of entries (wt: 2.1 ± 0.5 and het: 1.0 ± 0.3; P = 0.08) (Figure 5.8E), and a significant decrease in duration in the platform zone (wt: 1.2 ± 0.3 sec and het: 0.5 ± 0.1 sec; P = 0.02) and relative to wild-type controls (Figure 5.8F).
Figure 5.8. No difference was observed in home quadrant parameters of the probe trial including; latency to enter (A), number of entries (B) or time spent (C) between Nedd4-2 heterozygous (+/-) mice and wild-type controls (+/+). When platform zone parameters were evaluated, a significant increase in the latency to enter the platform zone was seen in Nedd4-2 heterozygotes compared to wild-type controls (D). A non-significant decrease in the number of entries was seen in the Nedd4-2 heterozygotes (E). Finally, a significant decrease in time spent in the platform zone was seen in the Nedd4-2 heterozygous mice (F). Data represented as mean±SEM. Statistical analysis was conducted using an unpaired Student’s t-test with * P < 0.05 deemed significant.

Findings from the Morris water maze suggest that at 2 months of age no effect was observed in Nedd4-2 heterozygotes in relation to long-term spatial learning and memory. At 6 months of age, although learning was not affected, we did observe significant changes in parameters associated with the platform zone suggesting that subtle changes in memory in the heterozygotes compared to the wild-type controls.
Anxiety was assessed in the *Nedd4-2* heterozygous mice using the elevated plus maze. In the 2 month cohort, a significant decrease in duration spent in the closed arm (wt: 167.9 ± 6.3 sec; n = 7 and het: 136.8 ± 8.6 sec; n = 15, P = 0.03) which was associated with an increase in open arm duration (wt: 20.1 ± 5.3 sec; n = 7 and het: 38.0 ± 8.4 sec; n = 15, P = 0.18) (Figure 5.9A). In addition, there was an almost significant decrease in number of entries in the closed (wt: 21 ± 2; n = 7 and het: 18 ± 1; n = 15, P = 0.056) versus open arms (wt: 4 ± 1; n = 7 and het: 7 ± 1; n = 17, P = 0.09) (Figure 5.9B), and no alteration in centre duration time (wt: 111.9 ± 7.3 sec; n = 7 and het: 125.2 ± 6.6 sec; n = 15, P = 0.23) (Figure 5.9C). Finally, distance (wt: 1410 ± 96 cm; n = 7 and het: 1351 ± 69 cm; n = 15, P = 0.63) and velocity (wt: 4.7 ± 0.3 cm; n = 7 and het: 4.5 ± 0.2 cm; n = 15, P = 0.61) were also unchanged (Figure 5.9D and E, respectively).

**Figure 5.9.** In the 2 month cohort a significant decrease of time spent in the closed arm was observed in *Nedd4-2* heterozygous (+/-) mice compared to wild-type controls (+/+), which was accompanied by a modest increase in time spent in the open arms (A). A non-significant increase in the number of entries in the open accompanied by decreased entries in closed arm was evident in the *Nedd4-2* heterozygous mice (B). No difference was observed in time spent in the centre (C), or distance (D) and velocity (E). Data represented as mean±SEM.
6 months

In the 6 month cohort, no differences in time spent in open (wt: 42.7 ± 9.4 sec; n = 10 and het: 29.8 ± 11.1 sec; n = 10, P = 0.39) versus closed (wt: 100.8 ± 18.2 sec; n = 10 and het: 116.6 ± 13.6 sec; n = 10, P = 0.50) arms was observed when Nedd4-2 heterozygous mice were compared to age-matched wild-type controls (Figure 5.10A). The number of entries into the open (wt: 8.2 ± 2.0; n = 10 and het: 5.7 ± 1.7; n = 10, P = 0.34) and closed arms (wt: 10.8 ± 1.4; n = 10 and het: 13.7 ± 1.4; n = 10, P = 0.16) was also not different between the heterozygous mice relative to wild-type controls (Figure 5.10B). Time spent in the centre zone was also not different (wt: 156.5 ± 11.7; n = 10 and het: 153.6 ± 12.3; n = 10, P = 0.87) (Figure 5.10C).

Figure 5.10. At 6 months of age no significant differences were seen between Nedd4-2 heterozygous (+/-) mice compared to wild-type controls (+/+). Data represented as mean±SEM.

Ethological Parameters

Ethological parameters were only assessed in the 2 month old mice due to changes in anxiety. When total number of entries into both the open and closed arm (wt: 24.7 ± 1.4; n = 7 and het: 24.9 ± 1.5; n = 15, P = 0.95) were evaluated no differences were observed between Nedd4-2 heterozygous mice and age-matched wild-type controls (Figure 5.11A). The number of rearing events was also unchanged (wt: 13.9 ± 3.4; n = 7 and het: 16.3 ± 2.1; n = 15, P = 0.54) (Figure 5.11B). Furthermore, the stretched attenuated responses were also not different, including protected (wt: 18.3 ± 1.6; n = 7 and het: 19.1 ± 1.4; n = 15, P = 0.74), unprotected (wt: 4.7 ± 1.3; n = 7 and het: 7.9 ± 1.6; n = 15, P = 0.23) and total (wt: 23.0 ± 2.2; n = 7 and het: 26.9 ± 1.7; n = 15, P = 0.19) (Figure 5.11C). Finally, the number of closed arm returns (wt: 1.0 ± 0.4; n = 7 and
het: 1.5 ± 0.3; n = 15, P =0.32) was also unchanged in the Nedd4-2 heterozygotes (Figure 5.11D). Overall, ethological parameters did not show any significant differences between the Nedd4-2 heterozygous mice compared to wild-type controls.

**Figure 5.11.** Ethological parameters assessed in 2 month old Nedd4-2 heterozygous (+/-) and wild-type controls (+/+ ) showed no differences in total number of entries (A), number of rearing events (B), number of stretched attenuated responses (SAPs) (C), or closed arm returns (D). Data represented as mean±SEM.
**Pre-Pulse Inhibition**

*Response to auditory stimuli*

2 month cohort

Responses to the different auditory stimuli were assessed to confirm that mice were able to hear the stimuli. No difference was observed in the absence of stimuli (wt: 17.7 ± 2.2 mV; n = 8 and het: 20.2 ± 2.4 mV; n = 13, P = 0.12), and to the various pre-pulse stimuli including; 4 dB (wt: 108.8 ± 20.8 mV; n = 8 and het: 109.8 ± 25.8 mV; n = 13, P = 0.98), 8 dB (wt: 76.4 ± 16.3 mV; n = 8 and het: 93.1 ± 24.6 mV; n = 13, P = 0.63), 16 dB (wt: 56.5 ± 11.8 mV; n = 8 and het: 82.2 ± 24.6 mV; n = 13, P = 0.45) or to the startle stimuli 115 dB (wt: 152.0 ± 31.5 mV; n = 8 and het: 132.7 ± 32.1 mV; n = 13, P = 0.69) (Figure 5.12A). Due to lack of response to the auditory stimuli, two *Nedd4-2* heterozygous mice were excluded from analysis. These mice were excluded blindly.

6 month cohort

Similarly, responses to the auditory stimuli were not different between *Nedd4-2* heterozygous mice compared to the age-matched controls; no stimulus (wt: 18.9 ± 2.3 mV; n = 10 and het: 18.3 ± 2.9 mV; n = 10, P = 0.89), 4 dB (wt: 85.7 ± 11.1 mV; n = 10 and het: 107.4 ± 26.0 mV; n = 10, P = 0.45), 8 dB (wt: 61.6 ± 7.9 mV; n = 10 and het: 73.1 ± 14.8 mV; n = 10, P = 0.50), 16 dB (wt: 41.1 ± 4.3 mV; n = 10 and het: 53.9 ± 8.3 mV; n = 10, P = 0.20) or 115 dB (wt: 133.8 ± 11.6 mV; n = 10 and het: 142.7 ± 23.4 mV; n = 10, P = 0.74) (Figure 5.12B).
Figure 5.12. No differences in startle responses to the different auditory stimuli in *Nedd4-2* heterozygous (+/-) mice compared to age-matched wild-type controls at both 2 (A) and 6 (B) months of age. Data represented as mean±SEM.

Startle Response

2 months

Startle responses to the 115 dB stimulus was analyzed over the 4 block trials, with no difference observed between *Nedd4-2* heterozygous mice and age-matched wild-type controls (Block 1, wt: 214.1 ± 46.1 mV; n = 10 and het: 164.6 ± 38.5 mV; n = 10, P = 0.43, Block 2, wt: 163.4 ± 35.7 mV; n = 10 and het: 132.1 ± 33.3 mV; n = 10, P = 0.55, Block 3, wt: 120.7 ± 26.0 mV; n = 10 and het: 118.3 ± 31.5 mV; n = 10, P = 0.96, and Block 4, wt: 109.9 ± 26.6 mV; n = 10 and het: 115.8 ± 27.2 mV; n = 10, P = 0.89) (Figure 5.13A).

6 month cohort

At 6 months of age, no differences in responses to the startle stimulus was observed between *Nedd4-2* heterozygous mice and age-matched wild-type controls (Block 1, wt: 134.5 ± 13.8 mV; n = 10 and het: 149.1 ± 23.6 mV; n = 10, P = 0.60, Block 2, wt: 138.3 ± 13.7 mV; n = 10 and het: 140.7 ± 24.5 mV; n = 10, P = 0.93, Block 3, wt: 137.8 ± 11.4 mV; n = 10 and het: 136.8 ± 24.0 mV; n = 10, P = 0.97, and Block 4, wt: 124.8 ± 12.3 mV; n = 10 and het: 144.1 ± 25.5 mV; n = 10, P = 0.50) (Figure 5.13B).
Figure 5.13. Startle responses over four blocks of 115 dB stimuli were not different between 2 month old *Nedd4-2* heterozygous (+/-) mice compared to wild-type controls (+/+) (A). Similarly, startle responses in 6 month old *Nedd4-2* heterozygous mice were also not significantly different (B). Data represented as Mean ± SEM.

**Percentage of PPI**

**2 month cohort**

The percentage of pre-pulse inhibition (%PPI) was not different between the *Nedd4-2* heterozygous mice and age-matched wild-type controls at all pre-pulse stimuli; 4 dB (wt: 26.8 ± 6.8 %; n = 8 and het: 14.0 ± 4.6 %; n = 13, P = 0.12), 8 dB (wt: 45.8 ± 7.0 %; n = 8 and het: 31.5 ± 6.0 %; n = 13, P = 0.15) and 16 dB (wt: 58.4 ± 5.9 %; n = 8 and het: 42.8 ± 5.8 %; n = 13, P = 0.09) (Figure 5.14A).

**6 month cohort**

Similarly, at 6 months of age there was also no difference between *Nedd4-2* heterozygous mice and age-matched controls at all pre-pulse stimuli; 4 dB (wt: 36.3 ± 5.9 %; n = 10 and het: 30.4 ± 7.2 %; n = 10, P = 0.53), 8 dB (wt: 53.6 ± 5.1 %; n = 10 and het: 49.2 ± 4.9 %; n = 10, P = 0.54) and 16 dB (wt: 68.4 ± 2.7 %; n = 10 and het: 57.4 ± 7.6 %; n = 10, P = 0.19) (Figure 5.14B).
Figure 5.14. Percentage of pre-pulse inhibition (%PPI) was not significantly different between Nedd4-2 heterozygous (+/-) mice and wild-type controls (+/+). Data represented as Mean ± SEM. 

Expression of Nedd4-2 in regions of the brain associated with memory, learning and anxiety behaviours

Hippocampus

Nedd4-2 expression was investigated to determine cellular expression. Nedd4-2 was most highly expressed in the pyramidal cell layer, surrounding cell bodies of these pyramidal neurons in all regions including; CA1, CA2, CA3) and DG (Figures 5.15, 5.16, 5.17 and 5.18, respectively). Furthermore, Nedd4-2 expression was found not to extend in the dendrites of pyramidal neurons shown by low level of co-localization with MAP2 dendritic marker (Figure 5.19). Interestingly upon closer inspection Nedd4-2 was found to stain neurons that did not express NeuN. This suggests that these neurons may be immature neurons or neuronal progenitors contained within the subventricular zone of the DG (Figure 5.21).
Figure 5.15. Nedd4-2 (red) expression was highly expressed in the cell bodies of pyramidal neurons (NeuN, green) in the CA1 region of the hippocampus. (+/-) Nedd4-2 heterozygous and (+/+) wild-type controls. Scale bar represents 50µm.

Figure 5.16. Nedd4-2 (red) expression was highly expressed in the cell bodies of pyramidal neurons (NeuN, green) in the CA2 region of the hippocampus. (+/-) Nedd4-2 heterozygous and (+/+) wild-type controls. Scale bar represents 50µm.
Figure 5.17. Nedd4-2 (red) expression was highly expressed in the cell bodies of pyramidal neurons (NeuN, green) in the CA3 region of the hippocampus. (+/-) Nedd4-2 heterozygous and (+/+) wild-type controls. Scale bar represents 50μm.

Figure 5.18. Nedd4-2 (red) expression was highly expressed in the cell bodies of pyramidal neurons (NeuN, green) in the DG region of the hippocampus. Within the subventricular zone, Nedd4-2 positive neurons were evident that did not express NeuN (arrow). This suggests that Nedd4-2 is present in immature neurons, which are more prevalent in the Nedd4-2 heterozygous (+/-) mice compared to wild-type controls (+/+). Scale bar represents 50μm.
Figure 5.19. Nedd4-2 (red) expression in the CA1, CA2, CA3 and DG regions of the hippocampus showed very little co-localization with the dendritic marker MAP2 (green) in brain sections obtained from 2 month old wild-type controls (+/+). Scale bar represents 50μm.
Amygdala

Due to the decrease in anxiety observed in the 2 month old \textit{Nedd4-2} heterozygous mice, expression was examined in the amygdala. \textit{Nedd4-2} was expressed at high levels in the cell body of neurons located within the basolateral nucleus of the amygdala (\textbf{Figure 5.20}). As expected, \textit{Nedd4-2} staining intensity was reduced in the heterozygous mice relative to controls. A slight decrease in the number of \textit{Nedd4-2} positive neurons was evident in the heterozygous mice, however this requires further investigation such as cell counting.

\textbf{Figure 5.20.} \textit{Nedd4-2} (red) expression in the basolateral amygdala, showing high expression in the cell bodies of the NeuN positive neurons (green). As expected a reduction in \textit{Nedd4-2} staining intensity was evident in the \textit{Nedd4-2} heterozygous mice (+/-) compared to wild-type controls (+/+), with a slight reduction in the number of \textit{Nedd4-2} positive neurons. Scale bar represents 50\(\mu\)m.
Medial septal region

Cholinergic neurons of the forebrain provide crucial hippocampal projections required for normal cognitive function. Due to the known interaction between Nedd4-2 and TrkA, it was expected that Nedd4-2 would be highly enriched in these neurons. At least within the medial septal (MS) region, Nedd4-2 was found not to be highly expressed (Figure 5.21), which was a surprising finding. NeuN staining was not expected within these neurons, as other markers such as choline acetyltransferase (ChAT) antibodies could be used to characterize the expression and co-localization with Nedd4-2.

Figure 5.21. Nedd4-2 (red) was not highly expressed in the medial septal (MS) region of the forebrain. (+/+) wild-type. Scale bar represents 100µm.
5.4 DISCUSSION

Behavioural assessment of cognitive function in *Nedd4-2* heterozygous mice was conducted investigating short and long-term memory, learning, anxiety and pre-pulse inhibition. Due to the important modulatory role for Nedd4-2 in regulating dopamine transporters (DATs), glutamate transporters (GLAST/GLT-1) and TrkA neurotrophin receptors, it seemed likely cognitive behaviours would be impacted.

No difference in short-term memory was found when *Nedd4-2* heterozygous mice were compared to age-matched wild-type controls at both 2 and 6 months of age. Like with Nedd4, there is strong evidence showing that ubiquitination contributes largely to the consolidation of short to long-term memory (Artinian *et al*., 2008). Long-term spatial learning was also assessed and no differences were found between *Nedd4-2* heterozygous mice relative to wild-type controls at both 2 and 6 months of age. Long-term spatial memory however uncovered subtle changes in 6 month old *Nedd4-2* heterozygous mice, with significant changes evident when platform zone. This data suggest that Nedd4-2 may have some role in long-term memory formation, however the extent of this contribution remains to be determined.

Since the learning and memory assessed here is largely hippocampal dependent the expression of Nedd4-2 within the hippocampus was investigated. Nedd4-2 was expressed predominantly in the pyramidal cell layer, surrounding cell bodies, with very little co-localization within dendrites. Interestingly, the CA3 region of the hippocampus contained the highest level of Nedd4-2, followed by the DG. The CA3 region is essential for encoding new short-term memories requiring interaction between the CA3 and DG (Kesner, 2007). The CA3 regions is also the main site of projection from the Ammon’s horn, with evidence showing high levels of input to the lateral and medial septal region and vertical band of Broca, with these regions providing important cholinergic and GABAergic feedback to the hippocampus (Amaral *et al*., 1995; Gaykema *et al*., 1991). Nedd4-2 expression therefore becomes important, as both medial septal and vertical band of Broca, are cholinergic neurons that are enriched with TrkA receptors, and mediate NGF signaling.

Preliminary tests shown here found that Nedd4-2 was not expressed in neurons of the medial septal nucleus, which is one of the main regions of cholinergic input to the hippocampus.
This was unexpected as TrkA is abundant in the medial septal region (Steininger et al., 1993), and the absence of Nedd4-2 in this region is surprising. TrkA however is also present in the striatum, where Nedd4-2 is abundantly expressed (Steininger et al., 1993). In this study, other important regions of the basal forebrain cholinergic neurons were not assessed, and they may in fact be enriched in Nedd4-2, to regulate NGF mediated signaling via TrkA receptor. Overall, Nedd4-2 heterozygous mice show strong levels of expression in the hippocampus, suggesting an important role for memory and learning. It is likely perhaps that in the heterozygous setting, there is still enough Nedd4-2 present to preserve neuronal functions necessary for memory and learning behaviours.

Another interesting finding was the increase in number of cells that did not stain for NeuN in the Nedd4-2 heterozygous mice, which was evident in the subventricular zone (SVZ) of the dentate gyrus. These cells expressed Nedd4-2, suggesting that Nedd4-2 may be important for neurogenesis, as the SVZ is site of neurogenesis (Steininger et al., 1993). Future studies could examine whether these cells are indeed neurons, and if so are they also doublecortin positive, a protein abundant in migrating and immature neurons. If this is indeed the case, then reduction in Nedd4-2 levels may increase neurogenesis within the SVZ of the hippocampus, which is potentially a very exciting result.

Although no significant effects were observed in cognitive function in the Nedd4-2 heterozygous mice, the high expression of Nedd4-2 in the striatum warrants further investigation into more specific tests. The current tests used to evaluate cognitive function are spatial learning and memory tests that are largely hippocampal based. Alternatively, striatal based tests such as radial arm maze (Floresco et al., 1997), holeboard task (Kuc et al., 2006) and delayed spatial win-shift tasks (Richter et al., 2013) could be used to expand the behavioural profile tested.

Anxiety was also investigated and at 2 months of age Nedd4-2 heterozygous mice show a decrease in anxiety related behaviours assessed by elevated plus maze. This finding may in part be due to abnormal dopamine levels and/or signaling in Nedd4-2 heterozygous mice relative to wild-type controls. Data from Chapter 3, showed that striatal dopamine level are reduced in both 2 and 6 month old heterozygotes and may affect anxiety behaviours. There is some evidence for the involvement of dopamine, for example mice lacking D3 receptors show decreases in anxiety behaviours including increased time spent in open versus closed arm of the elevated plus maze.
(Steiner et al., 1997). Dopamine afferents arising from the ventral tegmental area also innervate nuclei of the amygdala activating both D1 and D2 dopamine receptors (de la Mora et al., 2010). Nedd4-2 was shown (in Chapter 3) to be expressed in medium spiny neurons of the striatum, which are enriched with D1 and D2 receptors. Nedd4-2 expression within the amygdala was found in the cell bodies of neurons, with a slight reduction in the number of neurons. The contribution of Nedd4-2 in anxiety related behaviours warrants further investigation to understand the mechanism that regulates these behaviours. Furthermore, no changes in anxiety was observed in the 6 month old Nedd4-2 heterozygotes which also requires further investigation.

As discussed in depth in Chapter 4, the dorsal and ventral portions of the hippocampus have distinct afferents and efferent and therefore contribute to the different aspects of cognition. Further characterization of Nedd4-2 expression in these regions may be conducted, however due to the subtle changes in long-term memory were not conducted in this study.

Pre-pulse startle responses were not affected in Nedd4-2 heterozygous mice compared to age-matched wild-type controls at both 2 and 6 months of age. Auditory responses were also unaffected, with mice producing similar startle responses to each of the startle stimuli. Pre-pulse inhibition (PPI), was also unchanged, which is not entirely surprising, as PPI is normally enhanced when there is an increase in dopamine. There was a slight (non-statistical) decrease in % of PPI in the 2 month old Nedd4-2 heterozygotes compared to age-matched wild-type controls, which may be due to decreased dopamine levels. In addition, impairment in D1/D2 receptor levels may also contribute to changes in PPI. Evaluation of D1/D2 receptor levels is necessary, as changes may also be important for motor function deficits observed in Chapter 3.

Findings from this study provide important new insights into the role and possible functions for Nedd4-2 in cognitive functions. It also raises many new questions, and highlights the need to further investigate in more detail, how known targets may affect cognitive function. Unlike the Nedd4 heterozygotes, Nedd4-2 heterozygotes show very little difference in cognitive function, with the only alteration evident is a decrease in anxiety. Nedd4-2 is highly expressed in regions of the brain essential for these cognitive functions, especially the hippocampus, suggesting that it must have some target and role in hippocampal neuronal function. However, it seems as though Nedd4-2 may have some redundancy as the 50% loss of did not having a substantial effect, or
Nedd4-2 may be more important for neuronal development with no substantial role at the mature synapse. Support for the neuronal development arises from the TrkA association, which is a trophic receptor essential for growth and development of neurons. In addition, the expression of Nedd4-2 in neurons that lie within the subventricular zone of the hippocampus, which is the main site of adult neurogenesis also strengthens the role for Nedd4-2 for development.

One important limitation in this study however is that the cognitive tests used do not necessarily assess effect of dopamine on cognition. Future studies should utilize other cognitive tests that better assess dopamine-mediated behaviours. For example instrumental conditioning, such as goal directed learning assesses the association between a response and the incentive value of the outcome (Balleine et al., 1998; Valentin et al., 2007). The 5-choice serial reaction time task allows the assessment of learning, attention and vigilance and requires normal dopaminergic signaling (Robbins, 2002). Dopamine is also important for reward based and motivational behaviours (Wise, 2004), and so the use of operant boxes where rodents learn to push a lever to obtain a sugar pellet can be and be used to evaluate both reward based learning and motivational behaviours (Wise, 2004). The outcomes of these tests will be extremely valuable and may show changes in cognition that have simply not been tested here.
CHAPTER 6: DISCUSSION AND CONCLUSIONS

6. Discussion

The studies undertaken in this thesis were designed to investigate the contribution of Nedd4 and Nedd4-2 in the CNS. More specifically, to determine what effect reduced levels of Nedd4 or Nedd4-2 would have to in vivo physiological responses. This was undertaken by examining behavioural outcomes of Nedd4 and Nedd4-2 heterozygous mice and to determine cellular expression of these two proteins within the CNS. There are a large number of targets for both Nedd4 and Nedd4-2, with very little understanding of the contribution to physiological processes such as motor function, gait and cognition.

When Nedd4 and Nedd4-2 were first characterized, they were found to share structural homology and target proteins containing PPXY motif, and therefore suggested that these proteins may have similar physiological effects. Furthermore, it was suggested they may share functional redundancy. What is becoming clear is that these two proteins have distinct targets and data in this thesis show that they also have distinct physiological phenotypes.

The first chapter (Chapter 2) showed that Nedd4 plays an important role in gait control, with the heterozygous mice showing significant gait impairments. These gait changes could in part be due to alterations in GluR1 containing AMPA receptors, which were found to be up-regulated in the cerebellum from the heterozygous mice relative to wild-type controls. Although further experiments are required to provide more conclusive evidence, increases in AMPAR levels at the synapse between granule cells and Purkinje neurons could affect the level of feedback to the motor cortex resulting in the extended gait observed. In order to verify this hypothesis, recordings from whole brain slices can be conducted to measure Purkinje neuron output in response to stimulation. Furthermore, blocking GluR1-containing AMPARs would provide confirmation whether increased levels of GluR1 in the cerebellum translates to signaling abnormalities.

Calcium makes a large contribution to Purkinje neuron signaling, and Nedd4 has been shown to target voltage-gated calcium channels. It cannot be excluded that alterations in intracellular calcium levels may exist and contributed to gait abnormalities observed in the Nedd4 heterozygous mice. It would be important to examine intracellular calcium within Purkinje
neurons, and determine whether plasma levels of voltage-gated calcium channels are altered in response to a reduction in Nedd4 levels.

Assessment of gait and motor function in Nedd4-2 heterozygous mice (Chapter 3) showed that gait was not changed, but a significant deficit in motor function was evident in 6 month old heterozygotes relative to wild-type controls. Due to the interaction between Nedd4-2 and DAT, dopamine levels were measured and Nedd4-2 expression assessed in regions of the brain associated with dopamine signaling and motor function. Dopamine levels measured in the striatum were reduced in the Nedd4-2 heterozygous mice relative to the age-matched wild-type controls. This study measured total dopamine levels (both intra- and extracellular) and there is a need to examine dopamine re-uptake with labeled dopamine to examine whether intracellular levels are reduced.

DAT immunohistochemistry showed increased intensity of staining in striatal fibres suggesting that membrane levels of DAT may be increased. Western blot measurements showed no overall (total) DAT levels, suggesting that although total levels remained unchanged there may still be increased membrane levels. Further experiments are required to elucidate whether there are changes at the membrane. For example, experiments can be performed using cocaine, which binds to DAT to elicit behavioural responses. These experiments would provide a qualitative method of determining whether there membrane levels of DAT are indeed increased.

Nedd4-2 was highly expressed in neuronal compartments associated with motor function. Nedd4-2 was expressed within neurons of the motor cortex, and motor neurons of the spinal cord. Within dopaminergic compartments, Nedd4-2 was abundant in dopaminergic neurons of SNpc, with strong co-localization with tyrosine hydroxylase, the rate-limiting enzyme for dopamine synthesis. What role Nedd4-2 plays in these neurons is unknown, but future studies should examine whether there are any contributions to dopamine synthesis, which may in part explain the reduced levels of dopamine in the striatum. Nedd4-2 was also found in neurons within SNpr, which can provide important input to SNpc to regulate dopamine release, but also to other regions of the basal ganglia to regulate motor output. The large proportion of dopamine is released in the striatum, where dopamine receptors facilitate dopamine neurotransmission. Dopamine receptors are located on the medium spiny neurons, which comprise over 90% of neurons within the striatum. Nedd4-2 was strongly expressed in the cell bodies of these neurons,
suggesting that some interaction with dopamine receptors is likely. Further experiments are required to determine whether Nedd4-2 interacts with dopamine receptors, and whether any changes in dopamine receptors are observed in the heterozygous mice.

Overall data from Chapter 3 show an important role for Nedd4-2 for motor function with evidence that dopaminergic neurotransmission was affected. Further studies are necessary to determine the exact mechanism of Nedd4-2 that mediates the motor changes observed.

Assessment of gait using the DigiGiat system is a suitable behavioural assay in analyzing rodent gait changes in transgenic and rodent models of human disease. Some parameters can be translated to changes observed in human conditions. One example is in Parkinson’s disease, where decreases in stride length, increases in stride length variability and abnormalities with the initiation and termination of voluntary movement is observed. Similarly, this is also observed in rodent models of Parkinson’s disease. Although the gait changes observed were small and subtle they were significant. In comparison with gait changes seen in rodent ataxia models they are not as overt, however these gait changes not readily discernable without gait measurements being applied.

The contribution of Nedd4 to cognitive function was investigated in Chapter 4, with a focus on learning, memory, anxiety and sensory motor gating. The interest in cognitive function arose from numerous studies showing novel targets for Nedd4 that are implicated including growth and branching of axons and dendrites, but also GluR1 subunit of AMPARs. When short-term spatial memory was assessed in Nedd4 heterozygous mice, no differences were apparent. Long-term spatial learning and memory were found to be significantly impaired in 2 month old Nedd4 heterozygous mice, with very little difference observed at 6 months of age. Previous studies have shown that ubiquitination is necessary for the consolidation of short into long-term memories, and evidence from this study support this notion. The limited change observed in the 6 month Nedd4 heterozygous mice were surprising, however upon closer inspection of the data it became apparent that the wild-type mice did not display normal learning patterns, and therefore may confound the findings.

Synaptic plasticity is necessary for long-term memory to occur, with LTP the molecular mechanism underlying synaptic changes. LTP is dependent on the insertion and removal of
AMPAR, with the balance producing strengthening or weakening of a synapse. GluR1-containing AMPARs are crucial for the activity dependent changes in synaptic plasticity involved in LTP. When LTP was investigated in whole brain slices from Nedd4 heterozygous mice, a significant impairment in LTP was evident, providing further support for Nedd4 in modulating synaptic plasticity necessary for consolidation of short to long-term memories. Although GluR1-containing AMPARs were not investigated in the LTP recordings, future studies could pharmacologically block these receptors to determine if levels are altered and whether this translates to changes in LTP that was evident.

*Nedd4* heterozygous mice showed an increase in anxiety behaviours at 2 months of age. GluR1-containing AMPARs may also be contributing to anxiety behaviours, with previous studies showing a correlation between GluR1 levels and anxiety assessed by elevated plus maze. Further studies are necessary to evaluate what contribution Nedd4 has to anxiety. When Nedd4 expression was investigated in the brain, Nedd4 was found in brain regions associated with learning, memory and anxiety. For instance, Nedd4 was expressed in the hippocampus, with the dorsal portion largely responsible for memory and learning and the ventral portion that has reciprocal connections with the hippocampus functions to mediate anxiety behaviours. Nedd4 was expressed in the dorsal, ventral hippocampus and amygdala, areas necessary for learning, memory and anxiety behaviours. Subtle changes in morphology were evident, such as changes in pyramidal layer thickness in hippocampal regions; the extent of change needs to be more thoroughly investigated.

Overall, data from Chapter 4 show that Nedd4 is important for LTP and thus synaptic changes associated long-term memory consolidation. It is possible that the effects of Nedd4 in cognitive function are largely due to the important interaction with GluR1 subunit of AMPARs. Alterations in AMPAR trafficking and/or levels at the membrane can impact on a number of behaviours including learning, memory and anxiety. Further work is necessary to better understand the contribution of Nedd4 to these cognitive behaviours.

As with Nedd4, there were several key targets identified for Nedd4-2 to suggest an important role for cognitive function. These include TrkA neurotrophin receptor, DAT and glutamate transporters, with very little known with respect to physiological outcomes. The *Nedd4-2* heterozygous mice showed a subtle but significant deficit in long-term spatial memory and
reduced anxiety. The expression of Nedd4 was high in all compartments of the hippocampus, but especially in the CA3 region as well as the amygdala. No gross morphological changes became apparent when hippocampal or amygdala sections were examined. Due to the interaction between Nedd4-2 and TrkA, the medial septal regions was assessed, as this region gives rise to the bulk of the cholinergic efferent that innervate the hippocampus. Since TrkA is expressed in this region, however little or no Nedd4-2 expression was observed in the medial septal region. TrkA is expression in many other regions including the striatum, where Nedd4 is abundant. Although the focus of this study was not to address the effect of Nedd4-2 reduction on cholinergic innervation and contribution of TrkA, future studies should investigate this relationship as loss of neurons in this region is thought to contribute to cognitive decline in Alzheimer’s disease.

Findings from Chapter 5 show that Nedd4-2 is highly expressed within the hippocampus and amygdala, with decreased anxiety and subtle deficits in long-term spatial memory in Nedd4-2 heterozygous mice.

The main findings from this thesis show for the first time the physiological outcomes of reduced levels of Nedd4 and Nedd4-2 in terms of motor, gait and cognitive function. Data obtained for this thesis also highlight that although Nedd4 and Nedd4-2 share similar structural homology, and interact with targets with a similar PPXY motifs, the functional outcomes are distinct. For instance, Nedd4 heterozygous mice show increases in anxiety whereas Nedd4-2 heterozygous mice have decreased anxiety behaviours. An attempt was made to investigate known targets for both Nedd4 and Nedd4-2 in relation to the behavioural outcomes, but it is likely that no single target underlies the physiological outcomes. It is likely that any alterations in behaviour are as a result of changes in a multitude to targets that together contribute to altered physiological outcomes in the heterozygous mice.

Behavioural analysis using heterozygous mice has some important limitations. One such limitation is the fact that some Nedd4 or Nedd4-2 is still present and may be sufficient to maintain most of the neuronal function and as such the true extent of the role for Nedd4 and Nedd4-2 in the CNS can’t be identified. The neuronal targets for both Nedd4 and Nedd4-2 are numerous, and although appear discrete may involve some redundant roles. That is, compensatory effects may mask the phenotype. Conditional knockouts are now available and
future studies could utilize these mice to obtain are more comprehensive assessment of Nedd4 and Nedd4-2 function in the CNS.

The data presented here are also limited in that we conducted basic behavioural screens that identified important phenotypical changes however testing should be extended to focus on each type of cognitive behaviour. For instance, other cognitive behaviours could also be examined such as working memory, fear based learning, motivation based learning paradigms and social recognition/interaction test. With Nedd4-2 in particular due to the potential affects on the dopamine system, addictive behaviours should also be examined. Aside from cognitive test, the motor function test can also be extended to examine other components such as neuromuscular strength (using grip strength test), other balance and co-ordination test such as ledged beam and grid walking. Data obtained from this study show important roles for Nedd4 and Nedd4-2 but also highlight the need for further testing to gain a clearer understanding into the mechanism that underlie these changes. Finally, comparisons with other studies are not possible as there are no published studies that have assessed behavioural phenotypes in Nedd4 or Nedd4-2 transgenic mice.

The interest in ubiquitination in the CNS is growing with many neurological disorders now considered to be caused in part due to a disruption in UPS. Data presented here provide exciting and novel roles for these two E3 ligses within the CNS and highlight the many future experiments required to better understand how Nedd4 and Nedd4-2 function with their many neuronal targets.
REFERENCES


Gautam V, Trinidad JC, Rimerman RA, Costa BM, Burlingame AL, Monaghan DT (2013). Nedd4 is a specific E3 ubiquitin ligase for the NMDA receptor subunit GluN2D. *Neuropharmacology* 74(0): 96-107.


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References


