The Role of Notch Signaling in Learning and Memory

by

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Abstract

The Notch receptor and its ligands constitute a ubiquitous signaling pathway found throughout all multicellular animal life. In addition to its highly conserved function in development, a growing body of evidence suggests Notch signaling has important roles to play in adult processes, including long-term memory (LTM) formation. Building on previous work showing a specific requirement for the Notch1 receptor in spatial memory in mice, I show here a similar requirement for the Notch ligand Jag1. Mice with mutations to Dll1 (another Notch ligand) and Lfng (a Notch regulatory protein) do not display such phenotypes. I propose a model in which signaling between Notch pathway components found in the adult mouse hippocampus (such as Notch1 and Jag1) is required for LTM encoding, with no requirement for pathway components not expressed in this tissue (such as Dll1 and Lfng).
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Abbreviations

α-CaMKII – α calcium-calmodulin
Abl - Abelson murine leukemia virus protein
AID – amyloid precursor protein intracellular domain
ANOVA – analysis of variance
APP – amyloid precursor protein
AR – ankyrin repeat
Arg – Abelson-related gene
ato – atonal
BET – brain specific, EGF-like transmembrane protein
CADASIL – cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
cAMP – cyclic adenosine monophosphate
CBF-1 – C promoter binding factor
CCN - connective tissue growth factor/cysteine-rich protein 61/nephroblastoma overexpressed
cDNA – complementary DNA
cGMP – cyclic guanosine monophosphate
cKO – conditional knockout
CNS – central nervous system
CR – cystein-rich
CREB – cAMP response element binding protein
CSL –CBF-1/RBP-J/Su(H)/ lag-1
CREB – cAMP response element binding protein
dH2O – distilled H2O
DNER – Delta/Notch-like EGF-related receptor
DII – Delta-like
DSL – Delta-Serrate-lag2
EBD – EGF-binding domain
EGF – epidermal growth factor
ELR – epidermal growth factor-like repeat
ER – endoplasmic reticulum
GFP – green fluorescent protein
glp-1 – abnormal germ line proliferation-1
HD – Huntington’s disease
Herp – Hes-related repressor protein
Hes – Hairy and enhancer of split
HET – heterozygous
Hip1 – huntingtin interacting protein 1
HSV – herpes simplex virus
Jag – Jagged
klg – klingon
kuz – kuzbanian
lag-1 – longevity assurance gene 1
Lfg – Lunatic fringe
lin-12 – abnormal cell lineage-12
LNR – lin-12/Notch repeat
LTM – long-term memory
LTP – long-term potentiation
Mam – Mastermind
Maml – Mastermind-like
mEPSPs – miniature excitatory post-synaptic potentials
mib – mind bomb
MS – multiple sclerosis
MWM – Morris water maze
NAS – Notch anti-sense
NCAM – neural cell adhesion molecule
neur – neutralized
NMDA – N-methyl-D-aspartic acid
NO – nitrous oxide
ORF – open reading frame
PBS – phosphate buffered saline
PDZ – post synaptic density protein (PSD95)-Drosophila disc large tumor suppressor (DlgA)-zonula occludens-1 protein (zo-1)
PEST – proline-glutamic acid-serine-threonine
PFA – paraformaldehyde
PS1 – presenilin1
PS2 – presenilin2
RBP-J – recombination signal binding protein for immunoglobulin kappa J region
RIP – regulated intramembrane proteolysis
S1, S2, S3 – site 1, site 2, site 3
Shh – Sonic hedgehog
siRNA – short interfering RNA
STM – short-term memory
Su(H) – suppressor of hairless
T-ALL - T lymphoblastic leukemia
TM – transmembrane
Wnt – Wingless/integration site of mouse mammary tumour virus
WT – wild-type
YFP – yellow fluorescent protein
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The Role of Irx3 and Irx5 in Behaviour

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1. Introduction

1.1 What is Notch signaling?

Surveying the literature on the Notch cell-cell signaling pathway provides an opportunity to retrace in miniature the history of modern genetics and cell biology, since the pathway has been studied with the most powerful biological techniques of the day for the better part of 100 years. The ‘notch’ trait (the presence of characteristic wing notches in female fruit flies and their female progeny) was originally described as a sex-linked inheritable trait of *Drosophila melanogaster* by T.H. Morgan (1917), and it provided some early experimental evidence for the theory of heritability as applied to model organisms such as the fruit fly. Even before the nature of genes was truly understood ‘notch’ mutants proved useful in the early mapping of the *Drosophila* genome through the study of chromosomal deletion and translocation mutants (Poulson, 1937).

An advanced understanding of what the ‘notch’ trait was had to wait for the latter half of the 20th century and the emergence of cell- and molecular-biological techniques. Several decades of genetic studies of various mutant *Drosophila* embryos placed the Notch locus in a group of “neurogenic” genes, homozygous mutations of which result in hyperproliferation of neural tissue at the expense of epidermis and embryonic lethality (Lehmann *et al.*, 1983). The neurogenic genes of *Drosophila* also include the canonical Notch ligand Delta (see section 1.3.1). The Notch gene was originally cloned by Wharton *et al.* (1985) who inferred that Notch was an integral membrane protein and described the characteristic presence of epidermal growth factor-like repeats (ELRs) in its extracellular domain.
While originating in *Drosophila* developmental biology, the inquiry into the role of the Notch locus and its gene product quickly spread to other organisms once it was appreciated that it had a conserved role in other species. The Notch protein (and its homologues in vertebrate systems and mammals) is now understood to sit in the middle of a large network of interacting proteins. Notch is a type I (single-pass) integral membrane protein. It functions as a receptor that interacts with various protein ligands. Unlike many signal transduction systems, the Notch receptor interacts with ligands in closely apposed or neighboring cells that are themselves integral membrane proteins. This unusual characteristic makes Notch signaling ideal for direct cell-to-cell communication.

Notch signaling is one of the most heavily studied developmental pathways in the scientific literature. As it permits cell to cell communication, its role is typically in lateral inhibition during developmental differentiation; as one cell adopts a certain fate it frequently instructs its neighbors to adopt a different fate by signaling through the Notch receptor, allowing different tissues to arise from a pool of largely identical stem cells (reviewed by Lewis, 1998). It is frequently described in relation to development of the central nervous system (CNS) (see section 1.6.1), although the correct formation of almost all major tissue types relies on Notch. In addition to its critical role in development, it is now understood that Notch signaling is active in important processes throughout adulthood, usually in the context of maintaining stem or other cell populations. Examples of this include regulation of melanocyte proliferation (Kumano *et al*., 2008) and in regulation of hematopoiesis (Burns *et al*., 2005) such as the maintenance of T-cell populations (reviewed by Laky and Fowlkes, 2008). The proliferative and pro-survival qualities of Notch signaling also make Notch pathway members genes of interest in diverse cancer types (reviewed by Roy *et al*., 2007).
A decline in Notch signaling may even explain certain age-related disorders, particularly in degeneration of tissue. In muscle, reduced expression of certain members of the Notch pathway impairs Notch signaling and the ability of myoblasts to proliferate to repair damage to muscle fibers (Conboy et al., 2003). This may also be true of many degenerative disease processes in the CNS (Lathia et al., 2008) (see section 1.6.3).

The Notch pathway is now believed to exist in all forms of multicellular animal life. Its existence in marine sponges (which exist at the evolutionary boundary between single-celled organisms and animals) certainly suggests as much (Nichols et al., 2006). It is found in radially symmetrical animals such as the sea urchin (Walton et al., 2001); however it does not exist in plants (Wigge and Wiegel, 2001). The study of Notch signaling has benefitted greatly from the use of model organisms such as Drosophila, Caenorhabditis elegans and Xenopus laevis, and many studies also use mice or human paradigms. In the next few sections I will briefly summarize the history of Notch research, in order to gain insight into the basic and conserved outline of the pathway, the biochemical properties of Notch pathway components, and an appreciation for the subtle differences which exist in the pathway from species to species. I will begin with a survey of Notch receptor homologues known to exist in various model organisms.

### 1.2.1 Notch homologues: from Caenorhabditis elegans to Homo sapiens

The first homologue of Notch described outside of Drosophila was abnormal cell lineage-12 (lin-12), originally identified in a screen for genes important in developmental cell fate decisions in C. elegans (Greenwald et al., 1983). Gain- and loss-of-function mutations showed that lin-12 acts in cell fate decisions during development. It was discovered soon
afterwards that lin-12 was an integral membrane protein and that its extracellular sequence contained homology to epidermal growth factor (EGF), a characteristic it shares with Drosophila Notch (see section 1.1) (Greenwald, 1985). Comparison of the Notch and lin-12 sequences suggested these two genes were homologues of one another, sharing several conserved domains including the extracellular ELRs (Yochem et al., 1988). A second C. elegans Notch homologue was discovered simultaneously by two groups (Yochem and Greenwald, 1989; Austin and Kimble, 1989). Termed abnormal germ line proliferation-1 (glp-1), its gene product was shown to share a “lin-12/Notch” domain as well as extracellular ELRs with both lin-12 and Notch. As we shall see, the lin-12/Notch repeats (LNRs) are found in all Notch receptor homologues. A Xenopus homologue, Xotch, was identified through sequence homology by Coffman et al. (1990), providing the first evidence that Notch signaling exists in vertebrates; its expression pattern in the Xenopus embryos suggests it plays a similar role in cell fate decisions during development as in C. elegans and Drosophila.

The first mammalian homologue of Drosophila Notch was identified by screening a rat cDNA library with the Xotch sequence, an effort which resulted in cloning and sequencing of Notch1 (Weinmaster et al., 1991; Del Amo et al., 1992). As for Drosophila Notch and Xotch, mammalian Notch1 contains ELRs (36 arranged in a conserved configuration), along with 3 LNRs in its extracellular domain. Weinmaster et al. (1991) showed that Notch1 is expressed transiently in the embryonic brain between days 12 and 14. As this is a time of expansion of the neuronal population, Notch1 was thought to be involved in neural proliferation during development, as for Drosophila Notch. Through in situ hybridization of rat embryos, the authors also showed that Notch1 is expressed in many other tissues such as the eyes, ears and thymus, particularly at times when these tissues are undergoing expansion of their own cell populations.
The human homologue of Notch1 was originally termed translocation-associated Notch homologue 1 (TAN-1), and was discovered during an investigation of the gain-of-function effects of a chromosomal translocation on a locus of unknown function which results in T lymphoblastic leukemia (T-ALL). Characterization of the locus showed that it had homology to *Drosophila* Notch (Ellisen *et al*., 1991).

The discovery of these first mammalian Notch receptors inspired efforts to clone other Notch homologues in mammalian species and more importantly to characterize their function. A second mammalian homologue, Notch2, was subsequently cloned from rat cDNA libraries (Weinmaster *et al*., 1992). The cloning and expression-profile characterization of Notch3 indicated that Notch1-3 have distinct but overlapping expression profiles in the developing nervous system, suggesting an opportunity for qualitatively distinct forms of Notch signaling depending on the combination of receptors present in a given tissue at a given developmental stage (Lardelli *et al*., 1994). A considerable amount of interest has since arisen in the Notch3 locus, at it has been shown to be a principle determinant of the genetic disorder known as cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) (reviewed by Ungaro *et al*., 2008). A final mouse homologue of Notch was discovered after an RNA species found to be overexpressed in mouse mammary tumour virus-infected cells was found to encode fragments from a Notch homologue, originally termed int-3 (Robbins *et al*., 1992). The gene encoding it eventually came to be known as Notch4 (sometimes referred to Notch4/int-3) (Uyttendaele *et al*., 1996).

Within 15 years of the cloning of *Drosophila Notch*, homologues of the gene had been discovered in many model organisms, including four in mammals. As discussed previously, it is now believed that Notch is conserved in all animal life. All Notch homologues so far identified
have critical roles in development. In mammals, embryonic lethality and severe developmental defects are associated with homozygous deletions of *Notch1* and *Notch2* (Swiatek *et al*., 1994; McCright *et al*., 2006). *Notch3*−/− mice are developmentally normal and viable (Krebs *et al*., 2003); however as mentioned above the locus is known to be important in the developmental syndrome CADASIL in humans (Ungaro *et al*., 2008). *Notch4* is involved in developmental hematopoiesis (Ye *et al*., 2004).

Even as Notch homologues were being discovered, an increasingly determined effort was made to understand the biochemistry behind Notch signaling. As mentioned above, Notch homologues are integral membrane receptors that interact with ligands on opposing cells. As discussed below, the binding of receptor and ligand produces an intramembrane cleavage of the receptor, releasing the intracellular domain of the receptor to the cytoplasm. This fragment translocates to the nucleus and is transcriptionally active. This sequence of events forms the core of Notch signaling (Fig. 1).

The following sections consist of an examination of the various conserved domains of Notch and the properties that they confer on the receptor. I will examine the various Notch ligands known to exist, and their function, as well as other molecules in the Notch pathway involved in regulation of Notch signaling.
Figure 1. The components of mammalian Notch signaling. (A) The canonical Notch pathway. (1) Before presentation on the signal-receiving cell surface, Notch receptor (in red – Notch extracellular domain=NECD, Notch intracellular domain=NICD) is cleaved by furin-like convertases in the NECD immediately adjacent to the plasma membrane to produce a heterodimeric molecule (referred to as the S1 cleavage). Notch receptors can also be modified in the Golgi apparatus through the activity of enzymes such as the glycosyltransferase lunatic fringe (Lfng) (a mammalian homologue of Drosophila Fringe) that affects the affinity of Notch for its ligands through the addition of carbohydrate moieties (black hexagons). Once on the surface (2), the Notch receptor can bind to one of its ligands (in blue – ligand extracellular domain=LECD, ligand intracellular domain=LICD) on the signal-sending cell (3). Ubiquitination (U) of the intracellular domain of the ligand by proteins such as Mind bomb (Mib) and Neuralized (Neur) promotes its endocytosis (4), a step necessary for the sequential cleavage (at S2 and S3 sites) of the receptor and the release of NICD into the cytoplasm (5). Once translocated to the nucleus, the NICD associates with co-factors C promoter binding factor (CBF-1)/Suppressor of hairless [Su(H)]/Lag-1 (CSL) and Mastermind (Mam) or Mastermind-like (Maml) to affect gene expression (6). (B) The prototypical Notch receptor contains both EGF-like domains (ELRs) and lin-12/Notch repeats (LNRs) in its extracellular domain. The intracellular domain contains ankyrin repeats (ARs) and a proline-glutamic acid-serine-threonine (PEST) sequence. Both mammalian Notch ligands, Jagged and Delta, contain an EGF-binding domain (EBD) in their extracellular domains that are composed of N-terminal (NT) and Delta-Serrate-lag2 (DSL) domains. Jagged ligands additionally have a cysteine-rich domain (CR). Both ligands have short, non-conserved intracellular domains which frequently contain lysine residues used as sites of ubiquitination and post synaptic density protein (PSD95)-Drosophila disc large tumor suppressor (DlgA)-zonula occludens-1 protein (zo-1) (PDZ) binding domains (not pictured).
1.2.2 The conserved regions of the Notch extracellular domain

Notch is functions primarily as a cell surface receptor. Binding of Notch to one of its cognate ligands induces cleavage and activation of the receptor. Comparative analysis of the extracellular region of the Notch receptor (NECD) shows conservation of various domains and their relative configuration across evolution (Lardelli et al., 1995) (Fig. 1B). As discussed in section 1.2.1, the NECD of most Notch receptors contains ELRs and LNRs, both of which mediate binding of the Notch receptor to appropriate ligands on opposing cells.

Early studies employing site-directed mutagenesis of the Drosophila NECD showed that two ELRs (ELRs 11 and 12) in particular were necessary and sufficient to mediate ligand binding to both Delta and Serrate, another Drosophila Notch ligand (Rebay et al., 1991). Similarly, ELRs 8-12 are necessary for ligand binding by mouse Notch1 (Ge et al., 2008). NECD is also a target of glycosyltransferases such as Fringe whose modifications to specific ELRs influence ligand affinity (see section 1.5.2). Certain LNR mutations in C. elegans lin-12 and glp-1 affect interaction of receptor and ligand, and therefore Notch signaling (Pepper et al., 2003), and it is believed that correct disulphide bond formation within LNRs is critical for correct folding of the receptor and presentation on the cell surface (Tien et al., 2008). During protein processing in the Golgi apparatus, Notch receptors are cleaved by a furin-like convertase in their extracellular domains, producing functional heterodimeric proteins on the cell surface (Blaumeuller et al., 1997; Logeat et al., 1998) (Fig. 1A). This first site of proteolytic cleavage is referred to as site 1 (S1); there are additional sites of proteolytic processing in the receptor that are associated with activation (see section 1.4). Therefore, conserved regions of the NECD
appear to be principally involved in localization of the protein on the cell surface and in binding to ligand.

1.2.3 The Notch intracellular domain and regulation of gene expression

As discussed above, the early sequence information on Notch receptors in multiple species showed a striking conservation of the NECD. However, a considerable amount of the Notch primary sequence resides within the cytoplasm, and conserved domains are to be found here as well. The Notch intracellular domain (NICD) is the signal-transmitting region. It is released from the rest of the protein and the plasma membrane after ligand-dependant site 2 and 3 (S2 and S3) proteolytic cleavages (Mumm et al., 2000) (see section 1.4). Once in the cytoplasm, NICD quickly translocates to the nucleus and associates with a DNA-binding protein known alternatively as C promoter binding factor (CBF-1 – human), recombination signal binding protein for immunoglobulin kappa J region (RBP-J – mouse), suppressor of hairless [Su(H) – Drosophila] or longevity assurance gene 1 (lag-1 – C. elegans). When spoken of in general terms this protein is referred to as CSL (Christensen et al., 1996). Both NICD and CSL bind to the co-activator Mastermind (Mam – Drosophila) or Mastermind-like (Maml – mammals) (Fig. 1A). This three-protein complex is transcriptionally active and regulates expression of a number of downstream transcription factors (reviewed by Kovall, 2008). The best known downstream effectors of Notch signaling directly controlled by transcriptional regulation by NICD are the basic helix-loop-helix transcription factor hairy and enhancer of split (Hes) and the Hes-related repressor protein (Herp) gene families (whose roles are reviewed in
Iso et al., 2003). Both Hes and Herp proteins are themselves transcription factors, placing Notch at the head of a cascade of gene regulation.

Several conserved intracellular domains have been described that bind to important mediators of Notch signaling (reviewed by Fleming et al., 1998). The ankyrin repeat (AR)/cdc10 domain is believed to facilitate binding of NICD to CSL and Mam/Maml co-activators (Nam et al., 2006). The proline-glutamic acid-serine-threonine (PEST) sequence at the C-terminus is believed to be involved in negative regulation of NICD through ubiquitination and degradation (Öberg et al., 2001). NICD is also the site of Deltex binding and regulation (see section 1.5.1). Therefore, NICD is involved in signal-transduction and attenuation of Notch signaling through protein degradation.

1.3.1 Notch ligands: the “Delta-Serrate-lag-2” protein families and their molecular domains

The canonical transmembrane proteins that are known to bind to Notch receptors on opposing cells are referred to as “Delta-Serrate-lag-2” (DSL) ligands, so-named for the principle members of the group (Fig. 1B). DSL ligands have conserved N-terminus (NT) and “DSL domains” in their extracellular regions, domains that are collectively referred to as the EGF-motif binding domain (EBD). The two DSL ligands of Drosophila are Delta and Serrate. Serrate and Serrate-like (primarily known as Jagged, see section 1.3.3) ligands share an additional cysteine-rich (CR) motif immediately adjacent to the transmembrane domain (TM) (Fleming, 1998). DSL ligands also contain a variable number of ELRs in the extracellular domain, a trait they share with Notch receptors. The intracellular domains of DSL ligands are short with little conserved sequence among them; however many DSL ligands contain post
synaptic density protein (PSD95)-*Drosophila* disc large tumor suppressor (DlgA)-zonula
occludens-1 protein (zo-1) (PDZ) binding domains that may be involved in interactions between
DSL ligands and the cytoskeleton (Pintar *et al*., 2007) as well as lysine residues which are
thought to be important for ubiquitination and endocytosis (see section 1.4).

Evidence from cell-culture aggregation studies suggests that the EBD is necessary for
binding of DSL ligands and Notch receptors (reviewed in Fleming, 1998). Conflicting evidence
has emerged around the role of the ligand ELRs in ligand-receptor binding. The N-terminal 2
ELRs are required for binding of *Drosophila* Delta to Notch (Parks *et al*., 2006) and of mouse
Jagged-1 to Notch 2 (Shimizu *et al*., 1999). However, lag-2 mutants with all ELRs deleted retain
the ability to rescue *C. elegans* with non-functional lag-2 (Henderson *et al*., 1997). The theory
that has emerged is that DSL ligands require certain ELRs to bind correctly to Notch receptors in
some contexts but not others, and that many ELRs in a given ligand are not needed to mediate a
particular binding event. There are many possible receptor-ligand combinations and many
functional pairs have been suggested by comparing expression patterns in the developing embryo
(Lindsell *et al*., 1996).

Additionally, recent studies have identified non-canonical Notch ligands whose
expression is frequently CNS specific. Among them are the brain specific, EGF-like
transmembrane protein (BET) (Nishizumi *et al*., 2002) and Delta/Notch-like EGF-related
receptor (DNER) (Eiraku *et al*., 2005).
1.3.2 Delta and Delta-like (Dll) ligands

The *Drosophila Delta* gene was originally identified as one of several neurogenic loci required for correct development of the nervous system (Lehmann *et al*., 1981). Homozygous deletions of *Delta* are lethal, and as for other neurogenic genes they result in hyperplasia of the developing central nervous system (Muskavitch, 1994). Wing and eye defects are associated with *Delta* heterozygosity (Vässin *et al*., 1987). In *C. elegans*, the lag-2 locus shares homology with both *Drosophila* Notch-binding genes *Delta* and *Serrate* (see section 1.3.3), and it plays a similar role in Notch signaling. Following the discovery of vertebrate *Notch* homologues, interest arose in identifying *Serrate* and *Delta* homologues in mammalian genomes.

Bettenhausen *et al*. (1995) cloned the first *Delta* homologue in mice, *Delta-like1* (*Dll1*), and showed that it has high homology with *Delta*, particularly in the EGF-like repeat domains and in the DSL domain required for binding to Notch proteins. RT-PCR experiments demonstrated that *Dll1* mRNA is transiently expressed in development between E8.5 and E15.5. Whole-mount *in situ* hybridization of embryos showed that *Dll1* is expressed early in the presomitic mesoderm and later in the caudal halves of newly condensed somites, producing a characteristic banding pattern of expression (indicating a role for *Dll1* in somitogenesis). A role for *Dll1* in development of the CNS was also suggested by strong expression in the neural tube at E9.5 and E10.5. In *Dll1*−− embryos (Hrabé de Angelis *et al*., 1997) a familiar neurogenic phenotype is observed along with severe defects in somitogenesis (note this strain of mice is the same as that used in the current work – see Materials and Methods).

Other vertebrate *Delta* homologues have since been discovered; the *Xenopus laevis* genes *Delta-like1* and 2 (known as *X-Delta1* and 2), and the mammalian *Delta-like3* and 4 (*Dll3* and
Dll4). X-Delta1 is expressed in the developing Xenopus nervous system and promotes glial differentiation (Chitnis et al., 1995). A similar gene in chicks, C-Delta1, appears to have the same role (Henrique et al., 1995). The role of X-Delta2 in Xenopus is also nearly identical to that of Delta in Drosophila; it is strikingly expressed at somitic boundaries and dominant negative mutations of the gene severely disrupt segmentation during development of fish embryos (Jen et al., 1997). Similarly, loss of Dll3 function is involved in a developmental disorder known as spondylocostral dysplasia (which results from defects in segmentation during development) in humans (Sparrow et al., 2002) and Dll4 mutations produce severe defects in vascular development (Gale et al., 2004).

1.3.3 Serrate and Jagged (Jag) ligands

When the Drosophila Serrate gene sequence was first analyzed, it was discovered to share the ELRs also found in Notch and Delta (Fleming et al., 1990). Its expression pattern in Drosophila embryos along with sequence homology suggested it played a similar role in development as Notch and Delta (Thomas et al., 1991). The Xenopus homologue of Drosophila Serrate was discovered subsequent to X-Delta1 and X-Delta2, and was named X-Serrate1 (Kiyota et al., 2001).

The first mammalian homologue of Drosophila Serrate was found in rats and was termed Jagged1 (Jag1) (Lindsell et al., 1995). The authors demonstrated that Jag1 protein, when expressed in myoblasts co-expressing Notch, could activate Notch signaling and inhibit differentiation into muscle cells. A second Serrate homologue, Jag2, was described shortly thereafter (Luo et al., 1997).
Studies involving null mutations of Jag1 indicate it plays a critical role in development. Jag1−/− embryos do not survive to birth and suffer from severe defects in vascular remodeling during development (Xue et al., 1999). Specific loss of gene function in the cerebellum during development results in loss of Bergmann glia and severe defects in the migration of granule cells (Weller et al., 2006). Jag1 is also known to be implicated in the autosomal dominant development disorder Alagille syndrome (primarily a disease of the liver, it is also now believed to involve other areas of the body including the CNS as mental retardation is a hallmark of the disease) (Picolli and Spinner, 2001). Jag2−/− mice also die perinatally from developmental defects (Jiang et al., 1998).

1.4 Endocytosis of the DSL ligand and cleavage of the Notch receptor

The evidence described above from loss-of-function studies has conclusively demonstrated that mutations in Notch, Delta and Serrate/Jagged result in either abnormal neural expansion or severe patterning defects during development. This provides circumstantial evidence that these proteins are involved in the same pathway, but what is the nature of their interactions? I have already reviewed biochemical evidence that these proteins bind to each other on opposing cells and function as receptor and ligand. Therefore I will here briefly review the conserved mechanics of Notch signaling and roles played by Notch, its ligands and a number of other proteins (Fig. 1A).

As discussed in section 1.2.2, the Notch receptor is cleaved during processing in the Golgi apparatus in its extracellular region, at a site referred to as S1. This cleavage is only required for correct trafficking of the protein to the cell surface and is not involved in signaling.
As discussed in section 1.2.3, further proteolytic processing is required for translocation of NICD from the cell surface to the nucleus, and this processing only occurs upon binding of a Notch receptor with an appropriate DSL ligand. The release of NICD follows two events; the first involves internalization of the DSL ligand through endocytosis, and the second is subsequent receptor cleavage, with the final proteolytic processing generating NICD.

The E3 ubiquitin ligase mind bomb (mib) ubiquitinates DSL ligands such as Delta. This promotes endocytosis of the ligand; endocytosis of ligand once bound to Notch receptor is believed to be a critical step in proteolytic events that are necessary for Notch signaling (Le Borgne and Schweisguth, 2003). Mib is believed to be involved in the correct functioning of all mammalian DSL ligands (Koo et al., 2005). Another positive regulator of Notch signaling that functions through ubiquitination of ligands is neuralized (neur) (Lai, 2002).

It is now believed that the endocytosis of the ligand and cleavage of the receptor are temporally linked and constitute one event. One model for this event is that the mechanical stress of ligand internalization (on the signal-sending cell) produces conformational changes in the bound receptor (on the signal-receiving cell) that in turn lead to proteolytic cleavages. The second cleavage (and the first dependant on ligand binding) occurs at S2 and is induced by disintegrin-metalloprotease tumor necrosis factor α-converting enzyme (TACE) (Brou et al., 2000) in mammalian cells or kuzbanian (kuz) in Drosophila (Lieber et al., 2002). The final cleavage at the intramembrane S3 depends on presenilin1 (PS1) gene function and other members of the gamma-secretase complex (Berezovska et al., 1999a), or the PS1 C. elegans homologue sel-12 (Levitan and Greenwald, 1995). It is this cleavage which finally releases NICD, allowing it to translocate to the nucleus. Release of the intracellular domain upon ligand binding is a recurring motif in cell signaling biology that has been described in many cell types,
including bacteria. Notch is one of many proteins that undergo such regulated intramembrane proteolysis (RIP) (reviewed by Brown et al., 2000).

It should also be noted that DSL-Notch activity is not limited to trans-interactions (between opposing cells) but also includes cis-interactions (between ligand and receptor on the same plasma membrane). Such cis-interactions are often inhibitory, with Delta-Notch intracellular binding resulting in the retention of receptor in the endoplasmic reticulum (ER) (Sakamoto et al., 2001).

1.5.1 Modifiers of Notch signaling

It is now understood that Notch receptor-ligand binding is imbued with required complexity and context specificity through the action of a range of proteins which interact with and modify both receptors and ligands.

The connective tissue growth factor/cysteine-rich protein 61/nephroblastoma overexpressed (CCN) family of secreted cysteine-rich proteins binds to Notch receptors and modifies their activity (Katsube and Sakamoto, 2005). As is the case for Notch receptors themselves, many CCN proteins are important for tumour suppression, highlighting their importance (Tong et al., 2001). Certain proteins are involved in ubiquitination-dependant regulation of Notch such as Suppressor of Deltex [(Su(dx))/Itch and sel-10 (Lai, 2002), both of which negatively regulate Notch activation.

The Deltex gene is another key component of the Notch pathway known to positively and negatively regulate Notch signaling through interaction with Notch. Deltex and its homologues have E3 ubiquitin ligase activity (as for Mib and Neur) (Takeyama et al., 2003). Deltex can
activate Notch receptor independently of DSL ligands through a mechanism that involves endocytosis and trafficking of Notch to the endosomal compartment (Hori et al., 2004). It is believed that under certain circumstances NECD is degraded by lysosomal enzymes in late endosomes; this degradation functionally replaces S2 cleavage, allowing S3 cleavage and nuclear translocation of NICD to occur (Wilkin et al., 2008).

1.5.2 Fringe and lunatic fringe (Lfng)

One of the most important Notch modifiers is Drosophila Fringe and its homologues. When the Fringe locus in Drosophila was originally discovered, it was found to be important for dorsal/ventral cell interactions during wing development (Irvine and Wieschaus, 1994). Fringe localizes to the Golgi apparatus (Munro and Freeman, 2000) and has glycosyltransferase activity on Notch (Moloney et al., 2000). Modification of Notch by Fringe alters its affinity for various DSL ligands (Hicks et al., 2000), and therefore Fringe is a critical regulator of Notch signaling (Fig. 1A).

Several homologues of Fringe exist in the vertebrate genome, with lunatic fringe (Lfng) and radical fringe (Rfng) being originally cloned from Xenopus (Wu et al., 1996). Thereafter, three mammalian homologues were discovered; Lfng, Rfng, and additionally manic fringe (Mfng) (Johnston et al., 1997; Cohen et al., 1997). Precise maps of all three genes were produced by Moran et al., 1999. The on-and-off nature of Notch signaling in somitogenesis is believed to be regulated in some measure by cyclical Lfng expression during development (reviewed in Pourquié, 1999). Interestingly, evidence exists to suggest that Fringe proteins also act on DSL ligands (Panin et al., 2002).
1.6.1 Notch signaling in CNS development

The current work is concerned with the role of Notch signaling in cognition. The following sections will therefore begin by reviewing the expansive literature on the role of Notch in CNS development. We will then examine the emerging evidence that Notch signaling continues to function in the post-development brain, where it plays important roles in cognition, particularly in learning and memory.

A large body of evidence describes a role for Notch signaling in development of the CNS, particularly in maintenance of precursor cells and in dictating the correct ratio of differentiated cells originating from stem cell populations. During zebrafish (Danio rerio) development, a role for Notch has been described in maintenance of radial glia precursors (Kim et al., 2008). Radial glia maintenance is a key function of Notch signaling in mammals as well (Gaiano et al., 2000). Furthermore it is important in limiting the number of motor neurons that arise from radial glia and in promoting differentiation of oligodendrocytes. The activity of Notch1, Notch2 and CSF-1 (CSL) is important in the association of Bergmann glia with Purkinje cells in the cerebellum (Komine et al., 2007). Correct differentiation of olfactory receptor neurons in Drosophila to produce a diverse population of different receptor cells is controlled by Notch signaling (Endo et al., 2007). Terminally differentiated cortical neurons contain nuclear NICD (Sestan et al., 1999), unlike undifferentiated precursors, suggesting that Notch signaling functions in the final differentiation of cells in the neuronal lineage to mature neurons.

Notch signaling also plays a critical role in myelination during development which is known to be inhibited by Jag1-Notch1 signaling (Wang et al., 1998). It is believed that Notch signaling controls myelination by oligodendrocyte precursor cells (reviewed by Blaschuk and
ffrench-Constant, 1998). However, despite that fact that Notch1 and Jag1 are both upregulated during demyelinating models of multiple sclerosis (MS), oligodendrocyte precursor-specific deletion of Notch1 appears to have no effect on remyelination (Stidworthy et al., 2004), suggesting that Notch signaling is important only in developmental myelination, or that other Notch genes function in this context.

The role of Notch in neuronal differentiation is also bolstered by studies which demonstrate that presenilin mutations (a critical component of the gamma-secretase complex responsible for activation-dependant S3 cleavage of the Notch receptor) produce defects in developmental neurogenesis in Drosophila (Guo et al., 1999) and C. elegans (Wittenburg et al., 2000). Perhaps the most striking evidence in support of a role for Notch signaling in CNS development remains loss-of-function studies in various species. As we have previously seen, homozygous deletions of Notch and DSL ligands frequently result in severe defects in embryonic CNS development.

1.6.2 Notch signaling in adult brain function: a role in cognition?

Notch signaling also functions in the post-embryonic brain, particularly in dendritic and axonal arborization and neurite outgrowth. As in most other contexts, it does so with other signaling pathways (reviewed in MacFarlane, 2000; Frisén and Lendahl, 2001). The Notch pathway is one of several systems, including the Wingless/integration-1 (Wnt) and Sonic hedgehog (Shh) pathways, known to be involved in this process in the cortex (reviewed by Whitford et al., 2002) and elsewhere in the brain. The first evidence for such a role came from Berezovska et al. (1999b) who showed that neurite outgrowth of cultured mature neurons was
inhibited by Notch signaling. In transfected hippocampal and neocortical neurons, overexpression of NICD caused retraction of neurites in a CBF-1 (CSL) dependant manner. While neurite outgrowth in such neurons was inhibited by ectopic expression of NICD it was increased by transfection with Notch antagonists Numb, Numb-like and Deltex. In the same vein, conditional deletion of the Numb-like gene decreased axon arborization (Huang et al., 2005). Interestingly, activity of the Drosophila metalloprotease kuzbanian (kuz), which is known to cleave Notch and precipitate release of NICD, is necessary for correct dendrite outgrowth (Fambrough et al., 1996). This apparent contradiction (that a Notch pathway component positively regulates neurite outgrowth in this case) may be because kuz is acting on other integral membrane proteins in this context, or perhaps because Notch signaling can have different effects on neurite outgrowth depending on the context. It has also been shown that mammalian neuralized1 (neur1) mRNA is localized to dendrites in the CNS (Timmusk et al., 2002); neur1 is a regulator of Notch signaling through ubiquitination of DSL ligands (Fig 1A).

Notch may influence the branch characteristics (i.e., the number of branches and the complexity of the arbor) of neurites and not just their length. Redmond et al. (2000) showed that interference of NICD signaling through the use of a dominant negative receptor caused a decrease in dendritic branching and primary processes in cultured cortical neurons even while increasing Notch signaling resulted in decreased dendritic length as previously demonstrated.

Sestan et al. (1999) also showed that cultured neurons were capable of promoting Notch signaling when cultured at high density in the absence of other cell types, suggesting that both Notch receptors and DSL ligands are expressed on neurons (and that glial cell types are not necessarily involved in Notch signaling in mature neurons) and raising the possibility that Notch signaling regulates neurite growth through contact inhibition. Additional evidence for this
hypothesis comes from Franklin et al. (1999) who demonstrated a similar effect in neuroblastoma cells expressing Notch1 and Dll1. Parent et al. (2005) showed that knockdown of PS1 function, through mutation or gamma-secretase inhibitors leads to an increase in neurite outgrowth, spine density and in miniature excitatory post-synaptic potentials (mEPSPs). These authors suggested that inhibition of other RIP-dependent proteins, such as deleted in colorectal cancer (DCC), were perhaps responsible but no data in their study exclude the possibility that a knockdown of Notch signaling was responsible for the effects observed.

What are the downstream mechanisms that mediate the Notch pathway’s control over neurite outgrowth? Salama-Cohen et al. (2006) showed that Notch signaling repressed expression of Neurogenin3, a proneural gene that stimulates dendritic branching and the ratio of excitatory to inhibitory (GABAergic) synapses. Notch also inhibits atonal (ato) gene expression in Drosophila. Ato functions to stimulate dendritic outgrowth (Hassan et al., 2000). Additionally, Hes1 (a gene whose expression is controlled directly by the CSL/Mastermind/NICD complex) is also important in hippocampal dendritic modeling (Castella et al., 1999).

The Notch receptor may influence cytoskeleton remodeling through interactions with the Abelson murine leukemia virus protein (Abl), a non-receptor tyrosine kinase localized to axons (reviewed by Hoffmann, 1991). Interestingly, the interaction between Notch and Abl is believed to constitute an entirely separate pathway from the CSL/Mastermind/NICD axis. Homozygous deletion of Abl with reduced Notch expression in Drosophila results in severe defects in axon outgrowth, without a neurogenic phenotype typical of Notch pathway mutations (Giniger, 1998). Guidance of the Drosophila ISNb motor neurons similarly requires Delta, Notch and Abl, with no requirement for downstream canonical Notch effectors such as Suppressor of hairless (CSL)
(Crowner et al., 2003). The Abl and Abl-related gene (Arg) families are active in dendritic branching in mammals, with mutant mice displaying reduced dendritic arborization in adulthood (Moresco et al., 2005). Abl and Notch are known to interact genetically in mammalian contexts, where gain of function mutations in Abl and Notch1 synergistically produce acute leukemia (Mizuno et al., 2008); no studies have reported Notch/Alb interactions in dendritic arborization in mammals, although the above evidence suggests that one likely exists.

It is therefore possible that Notch influences neurite outgrowth and dendritic arborization through multiple mechanisms, some of which are controlled by CSL/Mastermind/NICD complex-dependent gene expression and some of which may be transcription-independent. Evidence for such complexity comes from the observation by Levy et al. (2002) that neurite outgrowth mediated by Notch signaling can be both dependent and independent of CBF-1 (CSL). The authors furthermore present evidence that the length of neurites is dependent on CBF-1 signaling but that the absolute number of neurites is independent of canonical Notch gene expression, suggesting that the various mechanisms of Notch signaling in neurite outgrowth may produce functionally different results.

In contrast, little evidence currently exists to suggest a role for Notch signaling in dendritic spine development or plasticity. One of the only tangential links with Notch signaling and spine biology is the observation that the Notch antagonist Numb is involved in spine morphology in mature neurons (Nishimura et al., 2006). Although it was observed that Numb localizes to mature spines and that alterations in Numb protein levels cause alterations in spine morphology, no evidence as yet exists tying this function of Numb to its role in Notch signaling.

The literature is increasingly convincing in assigning a role(s) to Notch in dendritic arborization. However it is unclear whether regulation of neurite outgrowth or remodeling by
Notch is involved in memory formation. The Josselyn laboratory is chiefly focused on molecular mechanisms of memory, and therefore we are particularly interested in a potential role for such Notch-dependent mechanisms on learning.

It has been shown in simpler memory paradigms, such as the *Aplysia* gill-withdrawal reflex, that intensive training can induce changes in neurite outgrowth (Wainwright *et al.*, 2002). Dendritic growth in the mushroom bodies of honeybees has been shown to correlate with learning, with experienced forager bees displaying more dendritic arborization than age-matched non-forager bees (Farris *et al.*, 2001). Furthermore, proteins other than Notch, such as the neurotrophic factor leptin, have been shown to influence dendritic remodeling and play a role in learning and memory (O’Malley *et al.*, 2007). Leptin-dependent dendritic outgrowth requires excitatory synaptic input through NMDA receptors to occur, suggesting that leptin plays a dynamic part in neuronal plasticity required for learning and memory. The neural cell adhesion molecule (NCAM) is another example; NCAM is known to mediate neurite outgrowth necessary for learning through nitrous oxide (NO)/cyclic guanosine monophosphate (cGMP) signaling (Ditlevsen *et al.*, 2007). Another regulator of learning is the cell-adhesion protein klingon (klg), which is involved in memory formation in *Drosophila* and whose expression is controlled by Notch (Matsuno *et al.*, 2009). Mutations to klg produce deficits in long-term memory (LTM) but not short-term memory (STM). It is conceivable that Notch satisfies a similar requirement during memory consolidation and storage. It is also possible that Notch-dependent dendritic arborization that occurs during development is necessary for correct memory encoding (by allowing for optimal connectivity between neurons and underlying phenomena such as long-term potentiation [LTP] – discussed below).
Notch also affects the activity of downstream transcription factors believed to be involved in memory. For example, Notch1 affects the DNA-binding activity of NF-κB in hematopoietic stem cells (Cheng et al., 2001) as well as in differentiated neurons of the CNS (Wang et al., 2004). Interestingly, NF-κB is involved in learning and memory (reviewed by Romano et al., 2006). NF-κB is known to be involved in memory extinction in crabs (Merlo and Romano, 2008) and consolidation and reconsolidation of memories in mice (Boccia et al., 2007). The NF-κB family member c-Rel is important for learning and memory in mice, with c-Rel−/− mice displaying impaired performance in hippocampal-dependent tasks such as contextual fear conditioning and novel object recognition (Ahn et al., 2008). There is also circumstantial evidence that Notch interacts with the cAMP response element binding protein (CREB) pathway, an important mediator of neuronal plasticity and memory (Wang et al., 2006). Notch1 has been shown to interact with the CREB pathway co-activator CBP (CREB binding protein) by yeast two-hybrid screening (Nakajima et al., 2004). Cross-talk between the Notch, NF-κB and CREB signaling systems has also been suggested by Espinosa et al. (2003). Therefore, Notch interacts with transcription factors known to be involved in learning and memory.

There is also direct evidence for a role of Notch in learning and memory. Presente et al. (2004) used heat-sensitive Notch alleles in Drosophila to show that Notch is required for LTM but not STM (as is the case for klg – see above). In their study they employed flies that were raised at the permissive temperature to minimize the effect of developmental dysfunction on behavior. Such flies, once transferred to the restrictive temperature, showed impaired LTM in both a conditioned courtship task and a Pavlovian avoidance task. A similar study (Ge et al., 2004) demonstrated that flies expressing either a temperature sensitive Notch protein (Nts2) or a dominant negative Notch allele under the control of a heat-shock promoter (NΔcdc10rpm) displayed
impaired learning in a Pavlovian olfactory conditioning paradigm similar to that of Presente et al. (2004). The authors showed that learning is unaffected but (as for the work of Presente et al., 2004) LTM is impaired. These authors measure learning through a test of odour avoidance delivered immediately after training, whereas Presente et al. (2004) allowed flies to rest for 30 min before testing for what they refer to as STM. One could argue that Ge et al. (2004) are measuring STM as well with their test, but the salient point is that LTM is the behavior affected. Ge et al. (2004) provide further proof for this thesis through use of flies carrying a WT Notch allele that is overexpressed from a heat-sensitive promoter (hs-N\textsuperscript{+}). Strikingly, such flies demonstrate enhanced LTM (with no effects observed on learning/STM), particularly during a challenging undertraining protocol where flies were given only 1 or 2 training trials (as opposed to the 10 trials used elsewhere in this study). Furthermore, Ge et al. (2004) show that this enhancement is blocked by the protein synthesis inhibitor cycloheximide, suggesting this phenomenon is indeed protein synthesis-dependent LTM.

Emerging evidence has suggested that Notch function in learning and memory is conserved in mammals (reviewed by Costa et al., 2005). A seminal paper in this new field is that of Costa et al. (2003), which reports on the role of Notch in learning and memory in mice. This study examined the effects of a mutation to Notch\textsubscript{1}. Homozygous deletion of the Notch\textsubscript{1} gene results in early embryonic lethality (Swiatek et al., 1994); therefore the behavioral profiles of Notch\textsubscript{1}\textsuperscript{+/−} mice were examined. Costa et al. (2003) found that Notch\textsubscript{1}\textsuperscript{+/−} mice were impaired in the hidden version of the Morris water maze (MWM) as revealed by a poorer performance on a Day 3 probe test, although the mutant mice caught up to their WT littermates by a Day 5 probe tests (see Materials and Methods for a more complete explanation of the MWM). Notch\textsubscript{1}\textsuperscript{+/−} mice also show a deficit during a reversal protocol (where the platform is moved to a new location in
the pool) after the initial training phase. Costa et al. (2003) are furthermore able to suggest a possible mechanism for Notch-mediated learning and memory by showing more severe MWM deficits in RBP-J$^+/-$ mice. Since RBP-J (or CSL – see section 1.2.3) is required for the control of gene expression by Notch, the authors argue Notch function in learning and memory must by transcription- and protein synthesis-dependent.

More indirect evidence for a role of Notch in learning in memory comes from studies involving mutations in presenilin genes. A conditional knockout (cKO) of the PS1 gene (restricted to the postnatal forebrain) in mice produced a measurable deficit in a MWM acquisition protocol; however since this was not accompanied by changes in Notch-dependent gene expression at Hes1, Hes5 and Dll1 loci it is debatable whether the behavioral effect is due to alterations in Notch signaling (Yu et al., 2001). Mice with conditional mutations in both PS1 and PS2 genes (PS2$^{-/-}$ mice crossed with floxed fPS1/fPS1 mice and α-CaMKII-Cre mice) display more severe deficits in both the MWM and in contextual fear conditioning (Saura et al., 2004).

One study has also posited a role for Notch signaling in long-term potentiation (LTP) of synaptic transmission. Wang et al. (2004) employed a Notch anti-sense (NAS) mouse line in which Notch anti-sense RNA was expressed from a constitutively active promoter. The authors found that LTP in NAS mice was significantly reduced compared to WT controls and disappeared after 10-20 min. Wang et al. (2004) also show that NAS mice display enhanced LTD after low-frequency stimulation. Basal synaptic transmission and pair-pulse facilitation were found to be normal in NAS mice. Interestingly, addition of a peptide corresponding to the DSL domain of the Jag1 gene (see section 1.3.1) produced an enhancement of LTP in NAS mice and, strikingly, in WT controls as well. A similar result has been obtained in PS1 heterozygous
mice which display impaired LTP, with baseline synaptic transmission and paired-pulse facilitation being comparable between PS1^{+/-} mice and WT controls (Morton et al., 2002).

1.6.3 Notch signaling and diseases of the CNS

The theory that Notch signaling is important in adult brain function and cognition is bolstered by emerging evidence that posits a role for Notch in several cognitive disorders of the CNS. In some cases, the link is controversial, as is the case with Notch4 and schizophrenia. Notch4 was originally thought to play a role in this disease by linkage disequilibrium analysis (Wei and Hemmings, 2000), however this has since been challenged (Ivo et al., 2006). Evidence from a single study also suggests a possible role for Notch signaling in Huntington’s disease (HD). Moores et al. (2008) have demonstrated that full-length huntingtin-interacting protein 1 (Hip1) positively regulates Notch signaling in Drosophila to produce neurogenesis. It remains to be seen whether such an interaction exists in humans, and whether this dynamic is important for disease progression in HD.

Several diseases discussed above (CADASIL and Alagille syndrome) are known to arise directly from mutations in Notch pathway members; while these diseases are associated with mental retardation it is believed that this results principally from developmental defects in the cerebrovasculature.

Stronger evidence exists to implicate Notch signaling in prion disease. The levels of NICD derived from Notch1 are increased in prion disease, with an accompanying loss of dendrites and dendritic spines in prion-infected mice (Ishikura et al., 2005). Since NICD signaling is known to decrease dendritic arborization, the correlation between NICD levels and
loss of dendrites during prion disease is suggestive. Ishikura et al. (2005) hypothesize that accumulation of prion protein increases the expression of Notch1, as increased levels of Notch1 mRNA are observed with disease progression, as well as membrane-localized cleavage of Notch1. As further proof of a potential role for Notch signaling in prion disease, neuroblastoma cells transfected with prion protein show a loss of neurites, a defect that can be rescued by knockdown of Notch1 expression using siRNA.

Another important disorder in which Notch signaling may play a role is Alzheimer’s disease (AD), one of the underlying mechanisms of which is believed to be gamma-secretase complex dysfunction. As noted above, gamma-secretase is responsible for cleaving Notch and the amyloid precursor protein (APP). It has been shown that Notch1 protein levels are increased in the hippocampus of sporadic AD brains, suggesting a compensatory mechanism if the pathway is disrupted during disease progression (Berezovska et al., 1998). However, evidence exists to suggest that Notch signaling actually increases in AD hippocampi; an increase in the level glycogen synthase kinase 3β (GSK3β), a kinase whose expression is controlled by Notch1 and which is involved in hyperphosphorylation of tau protein, has been observed in human AD brain samples (Figueroa et al., 2002). Furthermore, the tyrosine kinase Abl whose main function is in cytoskeleton remodeling (including in axon outgrowth – see section 1.6.3) and which is known to interact with Notch has also been shown to phosphorylate tau (Derkinderen et al., 2005). A final connection between Notch signaling and AD comes from interaction of the intracellular domain of APP with NICD. The APP intracellular domain (AID) has been shown to bind to Numb and to downregulate Notch signaling through interactions with NICD (Roncarati et al., 2002). Since the rate of APP cleavage is elevated in AD, particularly in familial AD
(FAD), it is conceivable that Notch signaling is altered during disease progression through this mechanism as well.

1.7 The current project

With emerging evidence that Notch signaling is involved in learning and memory in both Drosophila and mammals, we have undertaken a study of the effect of mutations of Notch pathway members on memory tasks in mice. The genetic loci investigated are two of the principle DSL ligands in the mammalian genome, Dll1 and Jag1, as well as a critical modulator of Notch receptor activity, Lfng. Here we present evidence that heterozygous deletion of one of these genes, Jag1, produces measurable deficits in spatial memory, while mutations to Dll1 and Lfng do not produce such phenotypes. We propose here a new model to describe the role of Notch signaling in learning and memory, namely that Notch pathway components play an important role in LTM if they are expressed at relatively high levels in the adult hippocampus (see Discussion). Our hypothesis suggests that mutations to either Dll1 or Lfng do not result in spatial memory phenotypes since neither gene meet the above criteria (Stump et al., 2002; Breunig et al., 2007; the current project) whereas Jag1 and Notch1 do (the current project; Costa et al., 2003). Table 1 summarizes this model with reference to both spatial memory phenotypes and expression patterns in Dll1+/-, Jag1+/-, Lfng+/- and Notch1+/- mice.

In consideration of evidence supporting a role for Notch in neurite outgrowth and dendritic branching, we have also begun to study the effect of these mutations on the cellular morphology of a brain region particularly important for memory, the CA1 subfield of the hippocampus.
<table>
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<th>Gene</th>
<th>$Lfn1$</th>
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<td>No phenotypes</td>
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<td><strong>MWM deficits (reversal training)</strong></td>
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<td>Expression pattern</td>
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<td>Expression levels</td>
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Table 1. Comparison of behavioural and expression profiles of *Lfng, Dll1, Notch1*, and *Jag1*. The behavioural results are summarized from the current studies and from Costa *et al.* (2003) for *Notch1*+/− mice. CNS expression profiles of *Dll1, Jag1*, and *Notch1* are derived from *in situ* hybridization measurements of mRNA level by Stump *et al.* (2002) and Breunig *et al.* (2007). *Lfng* expression patterns are derived from protein levels measured using immunohistochemistry as part of the current project.
2. Materials and Methods

2.1 Mice

Breeders of three strains of mice used in the current study (\textit{Dll1}^{+/-}, \textit{Jag1}^{+/-} and \textit{Lfn}^{+/-}) were acquired from the laboratory of Dr. Sean Egan (Hospital for Sick Children). \textit{Dll1}^{+/-} mice were originally bred at the Jackson Laboratory (Bar Harbor, Maine) by Hrabě de Angelis \textit{et al.} (1997). These mice were generated through replacement of the first two exons of \textit{Dll1} with a \textit{LacZ/PGK-neo} cassette. \textit{Jag1}^{+/-} and \textit{Lfn}^{+/-} mice were produced in the Egan laboratory (unpublished data). Briefly, \textit{Jag1}^{-} alleles are produced through replacement of a portion of the extracellular domain and the entirety of the intracellular domain with a transmembrane domain and \textit{β-geo} cassette (which includes both a \textit{LacZ} gene and geomycin resistance gene) followed by a stop codon, and \textit{Lfn}^{-} alleles were generated through deletion of exon 2 of the gene. See Fig. 2 for a full description of the nature of the mutations.

Mice were crossed into a C57B/6 background (Taconic) and the F1 offspring were used in behavioural experiments. Litters of \textit{Jag1}^{+/-}, \textit{Dll1}^{+/-} and \textit{Lfn}^{+/-} mice (with WT littermate control mice) were produced by breeding a HET male with a C57B/6 female. Mice were weaned and segregated by gender at P21. Breeding of double heterozygotes was carried out by breeding male and female mice (taken from the above litters) with different heterozygous mutations to \textit{Dll1}, \textit{Jag1}, and \textit{Lfn}.
Figure 2. Mutations to the Dll, Jag1 and Lfng loci. For Dll1 and Lfng mutations, the genomic organization of the alleles is given, with gray bars representing exons and black lines representing introns. For Jag1, the protein sequence is given (NT=N-terminal domain, DSL=DSL domain, ELRs=EGF-like repeats, CR=cysteine-rich domain, TM=transmembrane domain). (A) To generate the Dll’ allele, the first two exons (comprising amino acids 2-116) of the Dll1 locus were replaced with a construct which includes a functional domain of the β-galactosidase gene and the PGK-neo neomycin resistance cassette, functionally disrupting the expression of the protein (Hrabě de Angelis et al., 1997). (B) The Jag1 mutant allele used in this study has a transmembrane domain (TM) and β-geo cassette (which contains both LacZ and geomycin resistance genes) inserted after the first 13 ELRs in the extracellular domain. (C) To generate the Lfng’ allele, exon 2 of the Lfng locus was deleted. This results in a frameshift and nonsense translation of the majority of the protein (Egan laboratory, unpublished data).
2.2 Behavioral Experiments

All mice were housed in 28 x 17 cm cages with between 2 and 5 animals per cage. Mice were provided with food pellets and water *ad libitum* and maintained on a 12h light-dark cycle. All mice used in behavioral training were between 2-3 months of age, and all mice were handled twice daily for 7 days immediately before training began. In order to minimize the interference of one behavioral test on another, tests were performed in the order listed below (from least stressful to most stressful).

2.2.1 Open Field

Open field arenas consisted of 45 x 45 x 20 cm featureless Plexiglas boxes evenly illuminated from above (Fig. 3). Naïve mice were placed singly in the middle of odour-neutral boxes and allowed to explore the environment for 15 min. Cameras placed above the boxes recorded the position of the animal over time. The LimeLight 2 program (ActiMetrics Software) was used to divide the arena into three zones (Zone 1 consisted of the outer perimeter of the box, Zone 2 an inner ring with dimensions of 36 x 36 cm on the outside and 18 x 18 cm on the inside, and Zone 3 an 18 x 18 cm square in the middle of the box). The program was also used to assess the percent time spent in each of the three zones (providing a measure of the animal’s basal anxiety levels as rodents will prefer the outer perimeter of the box to the more exposed center) and total distance travelled (a measure of baseline activity).
Figure 3. The open field experiment. Animals are placed singly in odour-neutral boxes and allowed to freely explore the new environment for 15 min. The position of the animal is tracked in real time using an overhead camera, and two measurements are derived from the data. (1) Total distance travelled, which provides a measure of the baseline activity of the animal. (2) The percent time spent in three zones imposed on the area of the box using the LimeLight 2 software, which provides a measure of the basal anxiety of the animal. Rodents instinctively feel safer near the edge of the box (zone 1) and spend progressively less time in the innermost zones (zones 2 and 3).
2.2.2 Morris Water Maze (MWM)

The Morris water maze (MWM) is a water-filled pool in which animals are trained to find a hidden platform and is a frequently used tool to assess spatial memory in rodents. The MWM used in the current studies measures 124 cm in diameter and is filled with water (made opaque by the addition of white non-toxic tempera paint) to a level approximately 15 cm below the rim of the pool (Fig. 4). The pool is given directionality with north, east, south and west ends arbitrarily assigned to different edges of the pool. A 10 cm diameter platform is placed 0.5 cm below the surface of the water during training trials, where it is hidden from the view of the mice. While in the pool, mice are tracked using an overhead camera and the WaterMaze program (ActiMetrics Software).

Training trials consisted of starting mice facing the wall of the pool in one of four randomly assigned start positions (north, east, south or west). Trials were terminated after mice successfully found and mounted the platform; if unsuccessful after 1 min mice were rescued and placed on the platform.

Probe tests were conducted by removing the platform and allowing mice to swim for 1 min before being rescued. The swim profiles of the mice (captured by the overhead camera) were subjected to a zone analysis using the WaterMaze program; the percent time spent in a 20 cm target zone centered over the original position of the platform was compared to the average time spent in three equivalent 20 cm zones in the other three quadrants of the pool. Note that the zones are larger than the platform itself (20 cm compared to 10 cm); this is to increase the resolution of the test for subtle memory effects.
A. Training Trial, Probe Test, Zone Analysis

B. 

Acquisition:

- Green triangle: Training Trials (2 x 3)
- Blue triangle: Probe Test

Day 1 2 3 4 5 6 7 8 9 10

C. Extinction

- Blue triangle: Probe Test

Day 1 2

D. Working Memory

- Green triangle: Training Trials (5)

Day 1 2 3 4 5

N W E N W W E W S S
Figure 4. The Morris water maze (MWM) tasks. By convention, the pool is marked with north (N), east (E), south (S) and west (W) directions. (A) Animals are subjected to two varieties of trials in the MWM, the training trial and probe trial. The training trial involves placing the animal in the MWM from a random start position (in either the N, E, S or W corners of the pool) and allowing it to explore until it finds the platform (animals are rescued and placed on the platform if they fail to escape after 1 min). The probe trial is conducted with the platform removed and animals are given 1 min to explore the pool. The performance of the animal in a probe trial is evaluated by zone analysis, by comparing the amount of time the animal spends in the target zone (green – centered around where the platform was before it was removed) to the other zones (red – in equivalent positions in the other three quadrants of the pool). (B) The acquisition phase of the experiment involves giving the animals 6 training trials a day (in 2 sessions of 3 trials) for 10 days, with the platform in the SE quadrant. The baseline performance of the animals is measured by a probe trial on day 1 before any training, and memory is assessed with probe trials before training on days 4, 7 and 10. (C) Memory for the platform location is extinguished with repeated probe trials, 6 per day for 2 days. (D) Working memory is assessed by training the animals to find a new platform location (in either the SW, NW, NE or SE quadrants of the pool) every day for 5 days. Animals were given 5 training trials per day.
Acquisition phase

During the acquisition (initial training) phase of the MWM experiment, mice performed 6 training trials per day (in two sessions of 3 trials each, with mice left on the platform for a 15 s interval between each trial) with the platform in the southeast quadrant for 10 consecutive days. Mice were also probed while naive on day 1, and also before training on days 4, 7, and 10. Memory was assessed by examining the amount of time taken to find and mount the platform during training trials on a per day basis (so-called escape latency) and by zone analysis of the probe tests. Note that earlier litters of mice were only given a 7 day acquisition protocol with no subsequent extinction and working memory tests.

Extinction and working memory

Immediately following acquisition, mice were given extinction tests (in which the animals ‘unlearn’ their preference for the target zone) and then working memory tests (i.e., tests of short-term as opposed to long-term memory). Extinction tests involved 2 days of 6 probe trials a day (i.e., with no platform). These probe tests were given one at a time, with an intertrial interval of approximately 30 min. Working memory tests were conducted over five days, with each day consisting of five training trials with the platform in a new and randomized position (northeast, northwest, southeast or southwest).
2.2.3 Fear conditioning (contextual and cued)

Animals were fear conditioned in Plexiglas and metal boxes measuring 24 x 30 x 21 cm located in a soundproof room with low-level white noise played to mask outside disturbances (Fig. 5). Boxes were washed with 70% ethanol and a small pool of ethanol (as an olfactory cue) was left in waste trays underlying the electric grids before animals were introduced into the boxes. Conditioning consisted of allowing animals to freely explore the novel environment for 2 min, following which they were exposed to an 85 dB tone for 30 s and then a 0.5 mA shock of 1 second duration. Contextual fear memory was assessed by placing animals in the same boxes (washed and scented with 70% ethanol as before) in which they were trained. Cameras located above each box recorded movement for a period of 5 min and freezing behavior was assessed using the FreezeFrame and FreezeView programs (ActiMetrics Software). To assess cued fear memory white Plexiglas inserts washed with water were added to boxes to produce a triangular environment with different olfactory, visual and textural cues than that used during training. Animals were allowed to explore the environment for 2 min before being exposed to the same 85 dB tone used during training for 3 min. The amount of freezing for each animal was calculated in one minute intervals.
Figure 5. The fear conditioning experiment. (A) Animals are trained on day 1 after being placed in an ethanol-scented box with an electric-grid floor. Animals are habituated to their environment for 2 min, exposed to an 85 dB tone for 30 s, then a 0.5 mA shock for 1 s. (B) Contextual fear memory (fear memory associated with a particular environment) is assessed by recording the percent time spent freezing after the animal is replaced in the same environment used during training (identical boxes and ethanol scent) for a 5 min period. (C) Cued fear memory (fear memory associated with an auditory cue, in this case the tone preceding the shock on training day) is assessed by placing the animals in a novel environment (with different shape, tactile and odour cues). Measurements are made of percent freezing during a 2 min pre-tone habituation period (which determines baseline freezing) and a 3 min tone period (during which most animals freeze progressively less during each time interval, providing a measure of memory extinction).
2.2.4 Pre-pulse inhibition (PPI) and acoustic startle threshold

To measure pre-pulse inhibition (PPI) and startle threshold, mice were placed in small tubes to constrain their movement. These tubes are fitted with sensors to detect muscular contractions elicited by acoustic startle pulses. During testing, tubes (with mice) were placed in startle boxes (SR Lab Startle Response System); the Startle Response Testing System (San Diego Instruments) software was used to record startle responses (Fig. 6). Mice were habituated to the startle apparatus on day 1 by subjecting them to a train of 80 startle pulses of 120 dB each (15 s inter-trial interval). PPI was measured on day 2 through randomized trials with 15 s intervals between trials; 120 dB pulse alone trials, and 70, 75 and 80 dB pre-pulse trials (with the pre-pulse preceding the startle pulse by 100 ms). The percent PPI (i.e., the percent reduction in startle response due to sensory gating provoked by the pre-pulse) was calculated per mouse for each of the three pre-pulse conditions. Startle threshold was assessed on day 3 through randomized startles of 0, 75, 80, 85, 90, 95, 100, 105, 110, 115, and 120 dB.
A  Habituation – Day 1
(80 startle pulses)

B  Prepulse inhibition – Day 2
Habituation (20 startle pulses)  0 dB prepulse  70 dB prepulse  75 dB prepulse  80 dB prepulse
120 dB startle  120 dB startle  120 dB startle  120 dB startle

C  Threshold – Day 3
Startle intensity
Figure 6. The pre-pulse inhibition (PPI) and startle threshold experiments. Both experiments are conducted with the animal placed inside a small Plexiglas tube which constrains the movement of the animals (within the startle box and near the speaker used to generate the sound pulses). On the underside of the tube a sensor measures the magnitude of the reflexive muscular contractions of the animal to a given acoustic startle pulse. (A) Day 1 consists of habituating the animals to the apparatus through exposure to a train (80 pulses) of 120 dB startles (yellow arrowheads) at the end of which the animal will have settled to a baseline startle response (black curve). (B) PPI is measured on day 2. Animals are first re-habituated to the apparatus through exposure to a shorter train (20 pulses) of 120 dB startles. To assess PPI, animals are subjected sequentially to one of four conditions; 1) 120 dB startle pulses (yellow arrowheads) with no pre-pulse or 2) 70 dB, 75 dB and 80 dB pre-pulses followed by 120 dB startle pulses. The pre-pulses (green arrowheads) reduce the magnitude of the startle response (black curve) due to sensory gating. Several trials are conducted for each condition, with all trials presented in a randomized manner. (C) Startle threshold (an assessment of the auditory and motor components of the startle response) is measured by exposing the animals to startles of various intensities (0, 75, 80, 85, 90, 100, 105, 110, and 120 dB). Each condition is presented in several trials, with trials given in a randomized manner.
2.3.1 Immunohistochemistry

*Lfng* expression has never been examined in the mature CNS; therefore we employed immunohistochemistry to study the expression pattern of this protein in this context. WT and HET animals were perfused with 50 ml of ice-cold PBS, followed by 50 ml ice-cold 4% PFA in 1 X PBS. Brains were post-fixed in PFA overnight, and then transferred to 30% sucrose for 2 days for dehydration. Excess tissue (olfactory bulbs, spinal cord, etc.) was then removed and brains were prepared for sectioning on a Leica CM1850 cryostat using TissueTek medium (Sakura). Coronal sections of 50 µm were taken and stored in PBS + 0.02% sodium azide until ready for staining. After brief washes in PBS to remove traces of sodium azide, sections were exposed to 1º antibody (goat anti-*Lfng*, Santa Cruz sc-8239) at a dilution of 1:500 in blocking buffer (0.1% bovine serum albumin, 0.2% Triton X-100 and 1% donkey serum in 0.1 M phosphate buffer) for 2 day at 4°C with gentle shaking. Sections were then washed 4 X in PBS + 0.05% Tween-20 (PBS-T), and then exposed to 2º antibody (biotinylated rabbit anti-goat) in blocking buffer at a dilution of 1:500, overnight at 4°C with gentle shaking. The following morning, sections were washed 4 X in PBS-T, exposed to 0.3% H$_2$O$_2$ in PBS for 30 min to quench endogenous peroxidase activity, washed 4 X in PBS-T, exposed to ABC solution (VectaStain ABC kit) for 60 min, washed 4 X in PBS-T, exposed to 3,3’-Diaminobenzidine (DAB) solution (Sigma, prepared according to manufacturer’s instructions) for 1-2 min and finally washed 4 X in PBS-T. Sections were then mounted on gelatin-coated slides, dehydrated, and coverslipped.
2.3.2 Golgi-Cox staining

Pairs of WT and HET littermates (age and gender-matched) were selected for Golgi-Cox staining. Golgi-Cox solution was prepared from a kit (FD Neurotechnologies) according to the manufacturer’s directions 24 h before animals were sacrificed. Animals were perfused with 50 ml of ice-cold PBS and brains were extracted and submerged in 2.5 ml of staining solution for 24 h in the dark. Solution was removed and brains were incubated in the dark for a further 2 weeks in 2.5 ml of fresh solution. Brains were then washed 2 X 24 h in 50 ml of 30% sucrose with agitation. Samples were sectioned on a Leica VT1200S vibratome at 120 µm. Free-floating sections were mounted on gelatin-coated slides. To allow proper adherence to slides, sections were firmly pressed by hand with bibulous paper and then incubated O/N in a humid chamber. To develop the stain, slides were washed with agitation in dH2O for 1 h, submerged in 0.5 N ammonium hydroxide for 30 min, washed in dH2O for 1 min, and treated with 1% sodium thiosulfate for 30 min. After a second wash in dH2O for 1 min, sections were dehydrated in 50, 70, 95, and 100% ethanol, and finally in xylene. Slides were mounted with CytoSeal (Richard Allan Scientific) and pressure was applied to slides during one week to avoid lifting of coverslips during drying. Once blind with respect to genotype, the examiner scanned sections for neurons with completely stained dendritic arbors in the CA1 hippocampal region. Branch and spine density analyses were conducted using the Neurolucida software (MBF Bioscience).
3. Results

3.1 Dll1+/− mice

Dll1+/− mice of at least two months of age were subjected to a battery of behavioural tests that included the open field, Morris water maze (MWM), auditory and contextual fear conditioning, and auditory startle prepulse inhibition (PPI) and acoustic startle threshold. Figure 7 shows our measurements of both distance travelled (a measure of baseline activity) and a zone analysis (a measure of basal anxiety levels) from the open field experiment. The upper panel shows that both WT and Dll1+/− mice are active in the open field, covering greater than 5000 cm over 15 min. A one-way analysis of variance (ANOVA) of the effect of Genotype (WT, HET) shows there is no genotypic interaction for this measure \((F(1,35) = .26, p > .05)\), suggesting that heterozygous mutations to Dll1 do not affect activity levels or gross motor skills. The lower panel of Figure 7 shows that all animals tend to spend more time in the periphery of the box (i.e., Zone 1) but that genotype does not affect performance in the open field. A two-way ANOVA with a between group factor Genotype (WT, HET) and within group factor Zone (Zone1-3) showed a significant effect of Zone, indicating that all animals exhibit (as expected) a preference for the outside of the box \((F(2,70) = 1.86, p < .001)\) but no Zone x Genotype interaction \((F(2,70) = 1.44, p > .05)\) or main effect of Genotype \((F(1,35) = .22, p > .05)\), indicating that heterozygous mutations to Dll1 do not produce a change in anxiety-like phenotypes in mice.

We initially characterized spatial memory in Dll1+/− mice in the MWM using a standard 7 day acquisition protocol (Fig. 8). We examined escape latencies during training and zone occupancy during probe tests. The upper panel of Figure 8 shows that both groups of mice
decreased in escape latencies over training days, indicating the acquisition of a memory of the platform location. This observation was confirmed with a two-way ANOVA with a between group factor Genotype (WT, HET) and within group factor Day (Days 1-6) showing a significant effect of Day (F(5,115) = 66.83, p < .001) but not of Day x Genotype (F(5,115) = 0.32, p > .05). There is, however, a significant main effect of Genotype (F(1,23) = 7.59, p < .05), although Newman-Keuls post-hoc analysis shows that WT and Dll1+/− mice do not differ significantly in escape latencies on any day.

Turning to probe test data, performance on Day 1 indicates that animals do not display a preference for the target zone before training, since animals spend equal time in the target and other zones. This is expected since the Day 1 probe test takes place before training. Turning to statistical analysis, a two-way ANOVA with a between group factor Genotype (WT, HET) and within group factor Zone (Target, Other) indicated no significant effect of Zone (F(1,23) = 5.33, p > .05), Zone x Genotype (F(1,23) = 3.57, p > .05) or main effect of Genotype (F(1,23) = 1.23, p > .05). Day 4 and Day 7 probe test data suggest that all animals acquire a preference for the target zone with training, indicating learning (Fig. 8, lower panel). A two-way ANOVA of the Day 4 probe test data with a between group factor Genotype (WT, HET) and within group factor Zone (Target, Other) showed a significant effect of Zone, indicating learning (F(1,23) = 13.67, p < .01) but no Zone x Genotype interaction (F(1,23) = 0.84, p < .05) or main effect of Genotype (F(1,23) = .55, p > .05). These relationships hold true for the day 7 probe test, where there is a significant effect of Zone (F(1,23) = 59.41, p < .001) but not Genotype x Zone (F(1,23) = 1.04, p > .05) or main effect of Genotype (F(1,23) = .32, p > .05). Therefore, the only indication that Dll1+/− mice display spatial learning deficits is the significant effect of Genotype observed for the escape latency data. The probe test data suggest that heterozygous mutations to Dll1 do
not affect spatial memory, and since the probe test is generally regarded as a more stringent measure of spatial memory than escape latencies we conclude that WT and *Dll1*+/– mice both display learning and memory in the 7 day MWM acquisition protocol.

In order to examine the performance of *Dll1*+/– mice in the MWM in greater detail we employed an extended protocol involving 10 days of acquisition (with probe tests on Days 1, 4, 7, and 10), extinction trials, and a working memory paradigm (Fig. 9). *Dll1*+/– mice appeared to lag behind WT littermates in escape latencies during training (Fig. 9, upper left panel), but performed normally during acquisition probe tests (upper right panel), extinction (lower left panel), and working memory tests (lower right panel). A two-way ANOVA conducted on the escape latency data with a between group factor Genotype (WT, HET) and within group factor Day (Days 1-6) produced significant effect of Day (F(9,288) = 53.14, p < .001), indicating that animals form a spatial memory of the platform location, as well as a significant effect of Day x Genotype (F(9,288) = 2.07, p < .05) and main effect of Genotype (F(1,32) = 8.92, p < .01). Newman-Keuls post-hoc analysis shows that this interaction is only significant on Days 5 (p < .05) and 7 (p < .05), with WT mice finding the platform more quickly than *Dll1*+/– mice. Turning to probe test data, as before no group displays a preference for the target zone on the Day 1 probe test (before training) although WT mice actually appear to avoid the target zone. A two-way ANOVA with a between group factor Genotype (WT, HET) and within group factor Zone (Target, Other) showed no Zone x Genotype interaction (F(1,32) = 1.04, p > .05) although curiously there was a significant effect of Zone (F(1,32) = 6.20, p < .05) and main effect of Genotype (F(1,32) = 5.42, p < .05). Newman-Keuls post-hoc analysis showed that WT mice occupied the target zone significantly less than the other zones during the Day 1 probe test (p < .05); this is unusual since animals typically occupy all zones equally before training. Animals
begin to display a clear preference for the target zone on Day 4, and this observation is
confirmed with a two-way ANOVA with a between group factor Genotype (WT, HET) and
within group factor Zone (Target, Other) showing significant effect of Zone (F(1,32) = 24.81, p < .001) but not of Zone x Genotype (F(1,32) = 3.53, p > .05) or main effect of Genotype (F(1,32) = 1.62, p > .05). Similar relationships hold on Day 7 for the effect of Zone (F(1,32) = 56.84, p < .001), Zone x Genotype (F(1,32) = 3.49, p > .05) and main effect of Genotype (F(1,32) = 2.34, p > .05). Finally, on Day 10, a two-way ANOVA with a between group factor Genotype (WT, HET) and within group factor Zone (Target, Other) showed a significant effect of Zone (F(1,32) = 73.51, p < .001) with no significant Zone x Genotype interaction (F(1,32) = 2.26, p > .05) or main effect of Genotype (F(1,32) = .99, p > .05). Because of the increased specificity of the zone analysis for measuring spatial memory, we conclude (as for the 7 day acquisition experiment) that our day 10 acquisition protocol confirms that heterozygous mutations to Dll1 do not affect acquisition of spatial memory in the MWM. We also employed extinction and working memory protocols in order to examine more subtle memory phenotypes in Dll1+/− mice. Visually, Dll1+/− mice appear to extinguish and perform the working memory task normally. A two-way ANOVA with a between group factor Genotype (WT, HET) and within group factor Zone (Target, Other) showed no significant Zone x Genotype interaction during extinction trials (F(11,352) = 1.07, p > .05). Similarly, a two-way ANOVA with a between group factor Genotype (WT, HET) and within group factor Day (Day 1-5) showed a significant effect of Day (F(4,28) = 9.72, p < .001), suggesting animals become more adept at the working memory task over time, but no significant Day x Genotype interaction (F(4,128) = .15, p > .05) or main effect of Genotype (F(1,32) = 3.84, p > .05). However, the profiles of both WT and Dll1+/− mice during the working memory test suggest that the animals may not have been learning the platform
location anew on each day, but rather finding it by chance (see Discussion). At any rate, the only indication that $Dll1^{+/−}$ mice have spatial memory deficits is the escape latency data during the 10 day acquisition protocols, an interaction which is contradicted by the probe tests from this same experiment, the 7 day acquisition protocol, the extinction test, and the working memory test. We therefore conclude that $Dll1^{+/−}$ mice have unaffected spatial memory.

We next examined both contextual and cued fear conditioning in $Dll1^{+/−}$ mice. Visually our data suggest that $Dll1^{+/−}$ mice have intact contextual and cued fear conditioning responses, with mice showing high levels of freezing during re-exposure to the training context (Fig. 10, upper panel) and a clear, tone-induced increase in freezing during the cued fear probe (lower panel). A one-way ANOVA of the effect of Genotype (WT, HET) on contextual fear conditioning performance indicated no interaction ($F(1,28) = 3.10, p > .05$). A two-way ANOVA of the cued fear memory data with a between group factor Genotype (WT, HET) and within group factor Time (Minutes 1-5) showed a significant effect of Time ($F(4,112) = 39.92, p < .001$) proving that animals respond significantly to the tone, but no Time x Genotype interaction ($F(4,112) = .48, p > .05$) or main effect of Genotype ($F(1,28) = .72, p > .05$). Taken together, our data indicate that heterozygous mutations to $Dll1$ do not affect fear memory encoding or retrieval. However, a trend towards more freezing was observed in $Dll1^{+/−}$ mice in both tests; this trend is similar to results obtained by Rui Costa during his investigation of $Notch1^{+/−}$ mice (unpublished data).

We also examined both PPI and acoustic startle threshold of $Dll1^{+/−}$ mice using our startle apparatus. $Dll1^{+/−}$ mice appear to habituate normally to the startle stimulus (Fig. 11, upper panel), display normal PPI responses (middle panel) but a slightly higher acoustic startle threshold profile (lower panel); in other words, $Dll1^{+/−}$ mice seem to startle more to a given
stimulus than WT littermates. A two-way ANOVA of the habituation data with a between group factor Genotype (WT, HET) and within group factor Trial (Trials 1-20, 21-40, 41-60, 61-80) showed a significant effect of Trial (F(3,72) = 13.69, p < .001), indicating that animals decrease in startle amplitude over habituation, but no Time x Genotype interaction (F(3,72) = .02, p > .05) or main effect of Genotype (F(1,24) = .00, p > .05). All animals display increasing PPI in response to increasing prepulse level, confirmed with a two-way ANOVA with a between group factor Genotype (WT, HET) and within group factor Pulse (prepulses of 70, 75, and 80 dB) showing a significant effect of Pulse level (F(2,48) = 9885.26, p < .001) but no Pulse x Genotype interaction (F(2,48) = .28, p > .05) or main effect of Genotype (F(1,24) = 2.74, p > .05), despite an apparent slight increase in PPI response in Dll1+/− mice. Dll1+/− mice appear to startle more during the acoustic startle threshold experiment. This observation is confirmed with a two-way ANOVA with a between group factor Genotype (WT, HET) and within group factor Pulse (0, 75, 80, 85, 90, 95, 100, 105, 110, 115, and 120 dB) showing a significant effect of Pulse (F(10,240) = 162.3667, p < .001) and also of Pulse x Genotype (F(10,240) = 1.94, p < .05) but no main effect of Genotype (F(1,24) = 2.08, p > .05). Newman-Keuls post-hoc analysis shows that this interaction is significant for startle pulses of 100 dB (p < .01) and 105 dB (p < .05) only, suggesting that Dll1+− mice only have impaired acoustic startle responses at very select pulse levels.

In order to explain the effect of Notch pathway mutations on hippocampal-dependent behaviours at the cellular level, we attempted to undertake a study of neuron morphology of CA1 pyramidal neurons in all three strains of mice. As Dll1+/− mice do not exhibit spatial memory phenotypes, they were to serve as controls for those strains that do (see section 3.2). No obvious morphological phenotypes appear either in terms of number of dendritic intersections or branch
length in either basal or apical dendrites (Fig. 12). A two-way ANOVA of the basal dendrite intersection data with a between group factor Genotype (WT, HET) and within group factor Radius (40, 80, 120, 160, 200 µm) showed a significant effect of Radius (F(4,28) = 345.37, p < .001) but no Radius x Genotype interaction (F(4,28) = 1.40, p > .05) or main effect of Genotype (F(1,7) = 1.95, p > .05). A similar two-way ANOVA with a between group factor Genotype (WT, HET) and within group factor Radius (40, 80, 120, 160, 200 and 240 µm) of branch length showed a significant effect of Radius (F(5,35) = 319.73, p < .001) but no Radius x Genotype interaction (F(5,35) = 1.01, p > .05) or main effect of Genotype (F(1,7) = 1.30, p > .05). For apical dendrite intersections, a two-way ANOVA with a between group factor Genotype (WT, HET) and within group factor Radius (40, 80, 120, 160, 200 and 240, 280, 320, 360, and 400 µm) showed a significant effect of Radius (F(9,63) = 74.03, p < .001) but no Radius x Genotype interaction (F(9,63) = 1.15, p > .05) or main effect of Genotype (F(1,7) = 1.22, p > .05). A two-way ANOVA with a between group factor Genotype (WT, HET) and within group factor Radius (40, 80, 120, 160, 200 and 240, 280, 320, 360, 400 and 440 µm) of apical dendritic branch length showed a significant effect of Radius (F(5,35) = 20.47, p < .001) but no Radius x Genotype interaction (F(9,63) = 1.57, p > .05) or main effect of Genotype (F(1,7) = 1.97, p > .05).

Therefore, we conclude that heterozygous mutations to Dll1 do not affect dendritic morphology in the CA1 region of the hippocampus. Examination of the data for spine density in CA1 neurons of Dll1+/− mice shows no obvious perturbations in the basal dendrites, but a slight change in the density profile in apical dendrites (Fig. 13). For basal dendrites, a two-way ANOVA with a between group factor Genotype (WT, HET) and within group factor Branch order (1-7) showed no significant effect of Branch order (F(6,36) = 1.94, p > .05). A Branch order x Genotype ANOVA of spine densities in basal dendrites is not significant (F(6,36) = .99,
p > .05) nor is there a main effect of Genotype (F(1,6) = .44, p > .05). In apical dendrites, there is a significant effect of Branch order (F(18,108) = 10.91, p < .001), but no significant effect of Branch order x Genotype (F(18,108) = 1.25, p > .05) or main effect of Genotype (F(1,6) = 1.51, p > .05). ANOVA in the apical dendrites is also not significant (F(9,54) = .26, p > .05) suggesting the trend mentioned above is not meaningful. We therefore conclude that heterozygous mutations to Dll1 do not affect spine densities in the CA1 region of the hippocampus.
Figure 7. **Open field behavioural results for Dll1<sup>+/−</sup> mice.** A 15 min open field experiment with Dll1<sup>+/−</sup> mice and WT littermates shows that Dll1<sup>+/−</sup> mice do not exhibit changes in baseline activity level (as measured by total distance travelled in the open field – upper panel) or in baseline anxiety level (as examined by a zone analysis of each animal’s path during the experiment – lower panel). See text for statistical analysis.
Figure 8. Morris water maze training results for Dll1+- mice (7 day acquisition protocol).
Spatial memory acquisition in the MWM was assessed by examining both escape latencies (i.e.,
the average time required to find the platform) on a per day basis (upper panel) and target zone
occupancy on days 1, 4, and 7 (lower panel). A slight (but statistically non-significant) lag in
Dll1+/- mice compared to WT littermates is observed. However, zone analysis of probe tests
show normal performance for Dll1+/- mice, indicating this strain does not have spatial memory
impairments in the MWM. See text for statistical analysis.
Figure 9. Morris water maze (MWM) training results for Dll1+/− mice (10 day acquisition, extinction, and working memory protocol). In order to examine the spatial memory profile of Dll1+/− mice in greater detail, Dll1+/− mice (with WT littermates) were subjected to a 10 day acquisition protocol in the MWM where both escape latencies across training days (upper left panel) and probe tests on days 1, 4, 7, and 10 (upper right panel) were used to assess spatial memory acquisition. All mice were then extinguished with repeated (unreinforced) probe trials (lower left panel) and working memory was assessed across five days (lower right panel). See text for statistical analysis.
Figure 10. Fear conditioning results for Dll1+/− mice. Both contextual (upper panel) and cued (lower panel) fear conditioning experiments were performed. Arrow in lower panel marks moment of tone onset during cued fear conditioning (between 2nd and 3rd minute). Dll1+/− mice display slight (not statistically significant) trend towards greater fear memory during contextual fear probe, and perform comparably to WT littermates during cued fear memory probes. See text for statistical analysis.
Figure 11. Prepulse inhibition (PPI) and acoustic startle threshold behavioural results for Dll1+/− mice. Dll1+/− mice habituate normally to the startle stimulus (upper panel). Dll1+/− mice display a slight (not statistically significant) trend towards greater PPI than WT littermates (middle panel). The acoustic startle response is intact in Dll1+/− mice, as demonstrated by the threshold test of startle magnitude elicited by a spectrum of pulse levels (lower panel). See text for statistical analysis.
Fig. 12. CA1 neuron morphology in Dll1+/− mice is normal. Dll1+/− mice display normal numbers of intersections in a Scholl analysis of both basal and apical dendrites of the CA1 region as well as normal dendritic length in both apical and basal dendrites. See text for statistical analysis.
Figure 13. Spine density in the CA1 region of $DII^{+/}$ appears normal. We have examined spine density by branch order in both basal and apical dendrites of CA1 pyramidal neurons, and we have observed no significant differences with WT controls. See text for statistical analysis.
3.2 Jag1+/− mice

Adult Jag1+/− mice were subjected to the same series of behavioural tests as for Dll1+/− mice (open field, MWM, fear conditioning, PPI and acoustic startle threshold). We began by examining performance in the open field in order to assess basal activity and anxiety levels (Fig. 14). Both WT and Jag1+/− mice were active in the open field (covering approximately 5000 cm), and displayed a clear preference for the outside of the box. A one-way ANOVA of the distance travelled data indicated that there is no effect of Genotype (WT, HET) (F(1,53) = .34, p > .05). A two-way ANOVA on zone occupancy data with a between group factor Genotype (WT, HET) and within group factor Zone (Zone 1-3) showed a significant effect of Zone (F(2,106) = 4.47, p < .001) but no Zone x Genotype interaction (F(2,106) = 1.80, p > .05) or main effect of Genotype (F(1,53) = .33, p > .05). We therefore conclude that heterozygous mutations to Jag1 do not affect activity, gross motor skills, or anxiety levels.

The standard 7 day acquisition MWM training protocol demonstrated a remarkable deficit in spatial memory in Jag1+/− mice. Jag1+/− mice appear to lag behind WT littermates in escape latencies during training (Fig. 15, upper panel), and display a clear deficit in target zone occupancy, particularly during the Day 7 probe test (lower panel). A two-way ANOVA of the escape latency data with a between group factor Genotype (WT, HET) and within group factor Day (Day 1-6) showed a significant effect of Day (F(5,140) = 29.87, p < .001), indicating the formation of a spatial memory of the platform location in all mice, but no Day x Genotype interaction (F(5,140) = 1.64, p > .05) or main effect of Genotype (F(1,28) = 3.40, p > .05). All mice display no preference for the target zone on Day 1 (before training), confirmed with a two-way ANOVA with a between group factor Genotype (WT, HET) and within group factor Zone.
(Target, Other) that showed no significant effect of Zone (F(1,28) = 2.09, p > .05), no Zone x Genotype interaction (F(1,28) = .94, p > .05), nor a main effect of Genotype (F(1,28) = .00, p > .05). A similar ANOVA of Day 4 probe test data showed a significant effect of Zone (F(1,28) = 41.44, p < .001) but no Zone x Genotype interaction (F(1,28) = .32, p > .05) or main effect of Genotype (F(1,28) = 2.31, p > .05). On Day 7, a two-way ANOVA with a between group factor Genotype (WT, HET) and within group factor Zone (Target, Other) showed a significant effect of Zone (F(1,28) = 120.30, p < .001) but also a significant Zone x Genotype interaction (F(1,28) = 8.06, p > .01) and main effect of Genotype (F(1,28) = 6.42, p < .05). Newman-Keuls post-hoc analysis showed that during the day 7 probe test, Jag1+/− mice occupied the target zone less than WT mice, indicating spatial memory dysfunction (p < .001).

In order to determine if Jag1+/− mice can eventually catch up with WT littermates if given more training, subsequent litters were given a 10 day training protocol (Fig. 16). The extinction and working memory paradigms were not performed with Jag1+/− mice since these tests assume that all groups included perform normally in an acquisition protocol (i.e., Jag1+/− mice would be starting at a disadvantage to their WT littermates, having a weaker memory for the existence of the platform before extinction and working memory protocols even begin). The preliminary data collected for this experiment suggested that Jag1+/− mice lag behind WT mice both in escape latencies (Fig. 16, upper panel) and during the extended probe tests (lower panel). However, due to low n-values we were unable to conduct statistical analysis. The reason so few animals were included in this study is because the Josselyn laboratory encountered difficulties with breeding Jag1+/− mice and time constraints prevented us from increasing the n-values. We would predict that statistical significance could be achieved with the addition of more animals.
We next tested \textit{Jag1\textsuperscript{+/−}} mice in a different memory task, contextual and cued fear conditioning. Interestingly, despite the deficit in the hippocampal-dependent MWM task, \textit{Jag1\textsuperscript{+/−}} mice do not appear to exhibit a deficit in contextual fear memory which also critically depends on the hippocampus (Fig. 17, upper panel) which we prove with a one-way ANOVA of the effect of Genotype (WT, HET) (F(1,47) = 1.78, p > .05). \textit{Jag1\textsuperscript{+/−}} mice do, however, display the characteristic (statistically non-significant) increase in freezing behaviour in this test (as for \textit{Dll1\textsuperscript{+/−}} mice and \textit{Notch1\textsuperscript{+/−}} mice – see section 3.1). \textit{Jag1\textsuperscript{+/−}} mice display normal behaviour in the cued fear memory task (Fig. 17, lower panel), proven by a two-way ANOVA with a between group factor Genotype (WT, HET) and within group factor Time (Minutes 1-5) showing a significant effect of Time (F(4,188) = 43.45, p < .001) but no Time x Genotype interaction (F(4,188) = .67, p > .05) or main effect of Genotype (F(1,47) = 2.29, p > .05). We therefore conclude that heterozygous mutations to \textit{Jag1} do not affect either contextual or cued fear memory encoding or retrieval.

Finally, we examined PPI and acoustic startle threshold in \textit{Jag1\textsuperscript{+/−}} mice. \textit{Jag1\textsuperscript{+/−}} mice appear to be normal in habituating to the startle stimulus, have a more pronounced increase in the PPI response than \textit{Dll1\textsuperscript{+/−}} mice, and have a normal acoustic startle threshold response. \textit{Jag1\textsuperscript{+/−}} mice habituate normally to the startle apparatus (Fig. 18, upper panel) as indicated by a two-way ANOVA with a between group factor Genotype (WT, HET) and within group factor Trial (Trials 1-20, 21-40, 41-60, 61-80) showing a significant effect of Trial (F(3,156) = 16.40, p < .001) but no Time x Genotype interaction (F(3,156) = .54, p > .05) or main effect of Genotype (F(1,52) = .02, p > .05). \textit{Jag1\textsuperscript{+/−}} mice display a more pronounced increase in the PPI response than \textit{Dll1\textsuperscript{+/−}} mice. Turning to statistical analysis, a two-way ANOVA with a between group factor Genotype (WT, HET) and within group factor Pulse (prepulses of 70, 75, and 80 dB), shows (as expected)
a significant effect of Pulse (F(2,106) = 165.24, p < .001) but no Pulse x Genotype interaction (F(2,106) = .17, p > .05). There is, however, a main effect of Genotype (F(1,53) = 5.37, p < .05). Newman-Keuls post-hoc analysis shows that Jag1+/− mice show increased PPI at prepulses of 70 dB (p < .05) and 75 dB (p < .05), indicating a dysfunction in the PPI response at those prepulse levels. For acoustic startle threshold, Jag1+/− mice appear to be completely normal. A two-way ANOVA with a between group factor Genotype (WT, HET) and within group factor Pulse (0, 75, 80, 85, 90, 95, 100, 105, 110, 115, and 120 dB) showed a significant effect of Pulse (F(10,530) = 102.05, p < .001) but no Pulse x Genotype interaction (F(10,530) = .16, p > .05) or main effect of Genotype (F(1,53) = .74, p > .05). We therefore conclude that heterozygous mutations to Jag1 have no effect on this measure.
Distance travelled

Zone analysis

% time in zone

WT (n=39)

HET (n=16)
Figure 14. Open field behavioural results for \textit{Jag}I\textsuperscript{+/-} mice. A 15 min open field experiment with \textit{Jag}I\textsuperscript{+/-} mice and WT littermates shows that \textit{Jag}I\textsuperscript{+/-} mice do not exhibit changes in baseline activity level (as measured by total distance travelled in the open field – upper panel) or in baseline anxiety level (as examined by a zone analysis of each animal’s path during the experiment – lower panel). See text for statistical analysis.
Figure 15. Morris water maze training results for Jag1<sup>+/−</sup> mice (7 day acquisition protocol). Spatial memory acquisition in the MWM has been assessed by examining both escape latencies (i.e., the average time required to find the platform) on a per day basis (upper panel) and target zone occupancy on days 1, 4, and 7 (lower panel). Jag1<sup>+/−</sup> mice lag behind WT littermates during training as revealed by escape latencies, and furthermore display a marked deficit in target zone occupancy during the day 7 probe tests. See text for statistical analysis.
Figure 16. Morris water maze (MWM) training results for Jag1+/- mice (10 day acquisition). In order to assess the spatial memory deficit of Jag1+/- mice in greater detail, Jag1+/- mice (with WT littermates) were subjected to a 10 day acquisition protocol in the MWM where both escape latencies across training days (upper left panel) and probe tests on days 1, 4, 7, and 10 (upper right panel) were used to assess spatial memory acquisition. See text for statistical analysis.
Figure 17. Fear conditioning results for Jag1<sup>−/−</sup> mice. Both contextual (upper panel) and cued (lower panel) fear conditioning experiments were performed. Arrow in lower panel marks moment of tone onset during cued fear conditioning (between 2<sup>nd</sup> and 3<sup>rd</sup> minute). Jag1<sup>−/−</sup> mice display slight (statistically non-significant) trend towards greater fear memory during contextual fear probe, and perform comparably to WT littermates during cued fear memory probes. See text for statistical analysis.
Figure 18. Prepulse inhibition (PPI) and acoustic startle threshold behavioural results for *Jag1*+/− mice. *Jag1*+/− mice habituate normally to the startle stimulus (upper panel). As for *Dll1*+/− mice, *Jag1*+/− mice display a slight greater PPI than WT littermates (middle panel); this effect is statistically significant at prepulse levels of 70 and 75 dB. The acoustic startle response is intact in *Jag1*+/− mice, as demonstrated by the threshold test of startle magnitude elicited by a spectrum of pulse levels (lower panel). See text for statistical analysis.
3.3 \textit{Lfng}^{+/−} mice

\textit{Lfng}^{+/−} mice (as for \textit{Dll1}^{+/−} and \textit{Jag1}^{+/−} mice) were characterized at the behavioural level with the open field, MWM, fear conditioning, PPI and acoustic startle threshold tests. We also examined the expression of \textit{Lfng} in the adult mouse brain, since to our knowledge this has never been undertaken. We show here that \textit{Lfng} is expressed at relatively low levels throughout the adult cortex, and at extremely sparse levels in the CA1 region of the hippocampus (Fig. 19).

\textit{Lfng}^{+/−} mice were first tested in the open field experiment. Both WT and \textit{Lfng}^{+/−} mice are active in the open field, covering approximately 5000 cm (Fig. 20, upper panel), and display the characteristic preference for the outside of the box in a zone occupancy analysis (lower panel). A one-way ANOVA of the effect of Genotype (WT, HET) on distance travelled showed that heterozygous mutations to \textit{Lfng} have no effect on basal activity levels ($F(1,23) = 2.38$, $p > .05$). A two-way ANOVA with a between group factor Genotype (WT, HET) and within group factor Zone (Zone 1-3) shows a significant effect of Zone ($F(2,46) = 9.25$, $p < .001$) but no Zone x Genotype interaction ($F(2,46) = 1.25$, $p > .05$) or main effect of Genotype ($F(1,23) = .97$, $p > .05$). We therefore conclude that heterozygous \textit{Lfng} mutations do not affect basal anxiety levels.

We initially characterized \textit{Lfng}^{+/−} mice for spatial memory using the 7 day MWM acquisition protocol showed. As for the other strains of mice studied, animals take less time to find the platform over training days as evidenced by escape latency data, and in this case \textit{Lfng}^{+/−} mice appear to perform the task equally as well as WT littermates (Fig. 21, upper panel). A two-way ANOVA with a between group factor Genotype (WT, HET) and within group factor Day (Day 1-6) showed a significant effect of Day ($F(5,55) = 44.68$, $p < .001$) but no Day x Genotype interaction ($F(5,55) = .33$, $p > .05$) or main effect of Genotype ($F(1,11) = 1.56$, $p > .05$). During
the Day 1 probe test (before training), mice do not appear to display a preference for the target zone, as confirmed by a two-way ANOVA with a between group factor Genotype (WT, HET) and within group factor Zone (Target, Other) showing no significant effect of Zone (F(1,11) = 3.65, p > .05), Zone x Genotype (F(1,11) = .58, p > .05) or main effect of Genotype (F(1,11) = .75, p > .05). Both groups appear to have a similar memory for the platform location on Day 4 (i.e., both groups display a similar preference for the target zone), confirmed with a two-way ANOVA with a between group factor Genotype (WT, HET) and within group factor Zone (Target, Other) showing a significant effect of Zone (F(1,11) = 33.25, p < .001) but no Zone x Genotype interaction (F(1,11) = 167.80, p > .05) or main effect of Genotype (F(1,11) = 1.13, p > .05). Similarly, on Day 7 both groups appear to learn equally well, as demonstrated by a similar ANOVA showing a significant effect of Zone (F(1,11) = 3134.96, p < .001) but no Zone x Genotype interaction (F(1,11) = 16.52, p > .05) or main effect of Genotype (F(1,11) = .01, p > .05). Therefore, the escape latency and probe test data lead us to conclude that heterozygous mutations to \textit{Lfng} do not affect spatial memory. Due to time constraints, only a small number (n=2) of mutant animals were subjected to the extended MWM protocol. Despite the fact that no standard errors could be calculated and the highly stochastic nature of the data, our preliminary results appear to indicate that \textit{Lfng}^+/− mice have no dysfunctions in acquisition, extinction, or working memory in the MWM (Fig. 22), with the caveat that the animals may not have been learning the platform location during the working memory test but rather finding it by chance (see section 3.1 and Discussion).

As for \textit{Jag1}^+/− and \textit{Dll1}^+/− mice, we examined both contextual and cued fear memory in \textit{Lfng}^+/− mice. Our data appear to show that \textit{Lfng}^+/− mice are normal in both these measures. A one-way ANOVA of the effect of Genotype (WT, HET) on time spent freezing during the
contextual fear memory probe was not significant (F(1,23) = .017, p > .05). For cued fear memory data, a two-way ANOVA with a between group factor Genotype (WT, HET) and within group factor Time (Minutes 1-5) showed a significant effect of Time (F(4,92) = 43.78, p < .001) but no Time x Genotype interaction (F(4,92) = .77, p > .05) or main effect of Genotype (F(1,23) = 3.08, p > .05). We therefore conclude that heterozygous mutations to Lfng do not affect fear memory encoding or retrieval.

We also measured PPI and acoustic startle threshold in Lfng+/− mice (Fig. 24). Lfng+/− mice appear to habituate normally and have normal PPI profiles, but have blunted acoustic startle threshold profiles. Statistically, Lfng+/− mice habituate normally to the startle stimulus as shown by a two-way ANOVA with a between group factor Genotype (WT, HET) and within group factor Trial (Trials 1-20, 21-40, 41-60, 61-80) showing a significant effect of Trial (F(3,69) = 7.64, p < .001) but no Time x Genotype interaction (F(3,69) = 1.07, p > .05) or main effect of Genotype (F(1,23) = 3.02, p > .05). Lfng+/− mice also have normal PPI profiles as measured by a two-way ANOVA with a between group factor Genotype (WT, HET) and within group factor Pulse (70, 75, and 80 dB) showing a significant effect of Pulse (F(2,46) = 59.46, p < .001) but no Pulse x Genotype interaction (F(2,46) = .12, p > .05) or main effect of Genotype (F(1,23) = .20, p > .05). Interestingly, we found that Lfng+/− mice have a blunted profile during acoustic startle threshold tests (Fig. 24, lower panel), confirmed with a two-way ANOVA with a between group factor Genotype (WT, HET) and within group factor Pulse (0, 75, 80, 85, 90, 95, 100, 105, 110, 115, and 120 dB) showing a significant effect of Pulse (10,230) = 47.34, p < .001), a significant Pulse x Genotype interaction (F(10,230) = 4.11, p < .001) and a significant main effect of Genotype (F(1,23) = 4.50, p < .05). Newman-Keuls post-hoc analysis shows that this interaction is significant at pulse levels of 110 dB (p < .01), 115 dB (p < .01) and 120 dB (p < .01). Such
deficits indicate either problems with either the ‘input’ component of this behaviour (i.e., hearing difficulties), the ‘output’ component (i.e., the muscular contractions measured by the sensor), or with the various levels of neural control regulating the behaviour. Additional experiments would be required to determine where the dysfunction occurs in this case.
Figure 19. **Lunatic fringe (Lfng) expression is chiefly cortical.** Expression of Lfng is shown at (A) Bregma -1.20 mm and (B) Bregma -1.60 mm. Position of sections relative to Bregma is estimated based on gross anatomy and with reference to *The Mouse Brain in Stereotaxic Coordinates* (Academic Press). Lfng expression as detected by immunohistochemistry is predominantly restricted to cortical areas. The staining pattern of Lfng is exclusively perinuclear, as expected for a Golgi-resident protein. Extremely sparse cell-specific staining is also occasionally observed in the CA1 region but nowhere else in the hippocampus.
Figure 20. Open field behavioural results for \textit{L fing}^{+/-} mice. A 15 min open field experiment with \textit{L fing}^{+/-} mice and WT littermates shows that \textit{L fing}^{+/-} mice do not exhibit changes in baseline activity level (as measured by total distance travelled in the open field – upper panel) or in baseline anxiety level (as examined by a zone analysis of each animal’s path during the experiment – lower panel). See text for statistical analysis.
Figure 21. Morris water maze training results for $Lfng^{+/}$ mice (7 day acquisition protocol).
Spatial memory acquisition in the MWM has been assessed by examining both escape latencies (i.e., the average time required to find the platform) on a per day basis (upper panel) and target zone occupancy on days 1, 4, and 7 (lower panel). $Lfng^{+/}$ mice show no obvious deficits in either escape latencies during training or in target zone occupancy during probe tests. See text for statistical analysis.
Figure 22. Morris water maze (MWM) training results for Lfng−/+ mice (10 day acquisition, extinction, and working memory protocol). Lfng−/+ mice (with WT littermates) were subjected to a 10 day acquisition protocol in the MWM where both escape latencies across training days (upper left panel) and probe tests on days 1, 4, 7, and 10 (upper right panel) were used to assess spatial memory acquisition. All mice were then extinguished with repeated (unreinforced) probe trials (lower left panel) and working memory was assessed across five days (lower right panel). See text for statistical analysis.
Figure 23. Fear conditioning results for $Lfn^{+/-}$ mice. Both contextual (upper panel) and cued (lower panel) fear conditioning experiments were performed. Arrow in lower panel marks moment of tone onset during cued fear conditioning (between $2^{nd}$ and $3^{rd}$ minute). $Lfn^{+/-}$ mice display slight (not statistically significant) trend towards greater fear memory during contextual fear probe, and perform comparably to WT littermates during cued fear memory probes. See text for statistical analysis.
Figure 24. Prepulse inhibition (PPI) and acoustic startle threshold behavioural results for $Lfng^{+/}$ mice. $Lfng^{+/}$ mice settle to a baseline startle level during habituation, although they have a lower average startle magnitude than WT littermates (upper panel). $Lfng^{+/}$ mice display entirely normal PPI levels (middle panel). The acoustic startle response is intact in $Lfng^{+/}$ mice is quite compromised (lower panel). See text for statistical analysis.
4. Discussion

Peer-reviewed scientific articles on the Notch pathway currently number in the thousands and the literature on this critical biological mechanism continues to expand daily. A smaller (though still large) number of studies have begun to posit a role for the pathway in post-development physiological processes, predominantly in hematopoiesis or immune cell function. However, only a handful of studies have addressed the role of Notch signaling in cognition, and the current project has sought to address some of the many questions waiting to be answered in this regard.

4.1 Summary of the behavioural results

In this project, I have examined the behavioural profiles of three strains of mice, each of which has a unique heterozygous null mutation in a canonical Notch pathway member (Dll1, Jag1 or Lfng).

A heterozygous null mutation to Jag1 results in specific deficits in spatial memory while leaving intact baseline activity, anxiety, acoustic startle threshold, and even other memory-based behaviours such as fear conditioning. The specificity of our effect suggests that the memory deficit is hippocampal-dependent. Interestingly, the memory dysfunction in Jag1+/− mice is reminiscent of the behavioural phenotype of Notch1+/− mice reported by Costa et al. (2003). Notch1+/− mice display specific spatial memory deficits without perturbations of any other behaviour. Interestingly, the deficit measured in Jag1+/− mice is arguably more marked than that in Notch1+/− mice, as Notch1+/− mice show impairment in a standard MWM protocol during early
probe tests but catch up to WT levels after 5 days of training, while \( \text{Jag1}^{+/} \) mice appear unable to catch up to WT levels even with more extended training (7 or 10 days). We also observe a disruption of the PPI response in \( \text{Jag1}^{+/} \) mice, an effect that is likely due to dysfunctions in brain areas other than the hippocampus.

Our results show that heterozygous null mutations to \( \text{Dll1} \) and \( \text{Lfng} \) do not affect any behavioural measure we have examined (although \( \text{Lfng}^{+/} \) mice do appear to have compromised hearing based on our acoustic startle threshold measurements). Specifically, both \( \text{Dll1}^{+/} \) and \( \text{Lfng}^{+/} \) mice have normal baseline activity and anxiety profiles as measured by the open field test, intact spatial memory as assessed by the MWM, normal fear memory encoding and retrieval, and intact PPI responses. In order to assess learning and memory as stringently as possible in both \( \text{Dll1}^{+/} \) and \( \text{Lfng}^{+/} \) mice, we employed additional acquisition training, extinction, and working memory tests. Both strains of mice appear to extinguish normally. However, the escape latency profile across trials of \( \text{Dll1}^{+/} \) (WT), \( \text{Dll1}^{+/} \) and \( \text{Lfng}^{+/} \) (WT) mice during the working memory task was generally flat, whereas one would predict a ‘saw tooth’ pattern in the curve with an increase in latency at the beginning of each day if the mice were genuinely learning the new platform location progressively during each day’s training. Because of this, we suspect that mice subjected to our working memory paradigm were not in fact learning the new platform location on each day but were instead only finding the platform by chance. The question of whether mutations to \( \text{Dll1} \) and \( \text{Lfng} \) result in working memory defects therefore remains open. Additionally, too few \( \text{Lfng}^{+/} \) mice were run through the extended MWM to be able to conclude anything about their extinction or working memory profiles.

At any rate, the finding of seemingly intact spatial memory in both \( \text{Dll1}^{+/} \) and \( \text{Lfng}^{+/} \) mice reinforces the novelty of the deficit we have discovered in \( \text{Jag1}^{+/} \) mice. Disruptions to the
pathway do not seem to cause anatomical abnormalities, and only in the case of \( \text{Jag1}^{+/-} \) mice do we observe the specific deficit in spatial memory (without deficits in closely related tasks such as fear conditioning).

This study represents therefore only the second instance of a direct implication of the Notch pathway in learning and memory in mammals, and it is also one of only a handful of studies that purport to show such a role in any species (see Introduction).

### 4.2 Correlating behaviour, expression levels and patterns, and neuron morphology

An obvious question that arises when considering these results is why do mutations to certain Notch pathway components (i.e., \( \text{Jag1} \) and \( \text{Notch1} \)) result in behavioural phenotypes while others do not? This is a complex question and considerable additional work would be needed to produce a satisfactory answer. However, one approach to analyzing the issue is to attempt to correlate behaviour with expression levels and patterns. Bearing in mind the hippocampal-dependent nature of the behavioural results described above, I will briefly summarize what is currently known about the expression levels of \( \text{Dll1} \), \( \text{Jag1} \), \( \text{Lfng} \), and \( \text{Notch1} \) in the adult mouse hippocampus.

Stump \textit{et al.} (2002) examined mRNA levels by \textit{in situ} hybridization of sagittal mounted sections for several Notch pathway components and found that \( \text{Dll1} \) is expressed at low levels in P4 mouse hippocampus and is undetectable in adult hippocampus (the age of ‘adult’ animals is never defined in this study, but in mice this term is typically applied to 2 month-old individuals). Breunig \textit{et al.} (2007) performed a similar study (again employing \textit{in situ} hybridization of sagittal sections) and found \( \text{Dll1} \) mRNA is detectable up to P24 in the hippocampus (they did not
examine later time points). Stump et al. (2002) showed that Jag1 mRNA is detected at higher levels than Dll1 in the P4 hippocampus, and continues to be detected in the adult, and Breunig et al. (2007) showed as expected that Jag1 is indeed expressed at a higher level than Dll1 in the hippocampus at P24. The literature is therefore consistent in finding that Jag1 is expressed at relatively high levels all the way to adulthood, while Dll1 is expressed at relatively low levels in juvenile animals, and probably ceases altogether by adulthood. Breunig et al. (2007) found levels of Notch1 mRNA in a similar pattern but a lower level than Jag1 mRNA in the P24 hippocampus, and Stump et al. (2002) find consistent levels of Notch1 mRNA in both P4 and adult hippocampi, in both cases at a lower level than Jag1 mRNA. To our knowledge, no study has yet addressed the issue of Lfng expression in the adult mouse forebrain. I have examined Lfng protein expression levels (as distinct from the above studies which employed in situ hybridization to examine mRNA transcription) through immunohistochemistry, and found little to no hippocampal expression in the adult, and only sparse expression throughout the cortex. Table 1 summarizes current knowledge of brain expression patterns for Dll1, Jag, Lfng and Notch1.

A hypothesis can therefore be formulated that a given Notch pathway component is critical in spatial memory formation if it is expressed in the adult hippocampus at relatively high levels. In this model, mutations to Lfng do not cause deficits in the MWM since Lfng expression is mostly restricted to the cortex. Similarly, mutations to Dll1 do not cause measurable deficits since Dll1 mRNA is detected at low levels in the hippocampi of P4 and P24 animals, and its expression appears to taper off completely in the adult. Conversely, mutations to Jag1 do result in spatial memory impairments, since Jag1 mRNA is detected at high levels at all post-development time points so far examined. There is also the milder behavioural phenotype
described by Costa et al. (2003) of Notch1+/− mice (i.e., where Notch1+/− mice catch up to WT levels after 5 days of acquisition) and the observations of Stump et al. (2002) and Breunig et al. (2007) that Notch1 is expressed in the adult hippocampus, but at slightly lower levels than Jag1. The comparison between Jag1 (higher expression, more pronounced behavioural deficit) and Notch1 (lower expression and milder dysfunction) therefore suggests a possible dose-dependent effect from impairment of Notch signaling on learning and memory. It is also worth considering whether functional redundancy is playing a role, particularly in the case of Dll1+/− mice; since both Dll1 and Jag1 are capable of binding to Notch1, it is possible that mutations in Dll1 do not result in spatial memory deficits since Jag1 can functionally compensate for its loss of function in the hippocampus.

We should additionally consider whether or not the behavioural effect we observe in the MWM for Jag1+/− mice is due to developmental defects (e.g. loss of neurons due to defects during development) or is truly due to loss of function of Jag1 in the adult brain. It would be difficult to speculate, given the current data, about which category of effect is responsible for the observed deficit. It is also possible (since these effects are not mutually exclusive) that a combination of the two is at play. At any rate, the exertion of temporal control over mutations to Notch pathway components would be a straightforward way of answering this question, since one could compare the behaviour of an adult animal both before and after deletion (see section 4.3.2).

In order to examine this hypothesis, I elected to study neuron morphology in adult CA1 hippocampal slices. The rationale for such an approach derives from the well-established role for Notch signaling in neurite outgrowth (see section 1.6.2). If indeed the behavioural phenotypes I have found are due to incorrect dendritic branching in this area of the brain, then
Golgi-Cox staining and neuron tracing should reveal this. Unfortunately, as is often the case for transgenic strains of mice, breeding of \(Jag1^{+/−}\) mice in the Josselyn laboratory began to slow down at a point after the initial behavioural characterization of the mice but before I had the opportunity to subject sections from these animals to the above analysis. Therefore, this study could not be conducted for \(Jag1^{+/−}\) mice. I was able to take some preliminary measurements of neuron morphology as well as spine densities for \(Dll1^{+/−}\) mice and have observed no obvious deficiencies in any measure examined.

It should be noted in passing that cellular or molecular mechanisms other than neuron morphology or spine density could be postulated to explain the behavioural effect observed in \(Jag1^{+/−}\) mice. As discussed in the Introduction, electrophysiological effects might be important, since impaired Notch signaling in the hippocampus negatively affects long-term potentiation (LTP) (Wang et al., 2004). Similarly, it is possible that loss of Notch signaling in the \(Jag1^{+/−}\) mouse hippocampus results in deficiencies in spine morphology or molecular characteristics (such as membrane receptor densities, etc.). However, to date there is no evidence for Notch-dependent control of such spine properties in the literature.

### 4.3.1 Future directions

The experiments described above make a contribution to the hypothesis that Notch signaling is important in hippocampal-dependent spatial memory formation. The bolstering of this new model will certainly require the elucidation of the underlying cellular and molecular mechanisms responsible for the behavioural phenotypes shown here (for \(Jag1^{+/−}\) mice) and by Costa et al. (2003) (for \(Notch1^{+/−}\) mice). There are several possibilities for approaching this
problem. Examination of neuron morphology by Golgi-Cox staining and computer-aided tracing is a logical starting point owing to the described role for Notch signaling in neurite outgrowth. Although this effort was begun over the course of this project, work remains to be done (as described above, particularly with regards to $Jag1^{+/}$ sections). It is also possible to imagine many other avenues to take, and I will briefly summarize such possible endeavours below.

4.3.2 Reimagining our genetic model

My current behavioural results, as well as those of Costa et al. (2003), provide compelling preliminary evidence of a role for Notch signaling in the processes of the adult mammalian brain, particularly in learning and memory. However, one could argue that both studies would present even stronger evidence if the genetic models employed were more refined. In both cases, the mutant animals compared to WT littermates have a heterozygous deletion of the given locus. In some cases, exons are simply removed, in others they are replaced with marker genes such as LacZ (see Materials and Methods for details); either approach results in functional disruption of the gene (a so-called ‘null allele’). A fundamental drawback of such an approach is that impact of the mutation is felt throughout the entire organism, from conception to adulthood, in all tissues where the gene is expressed. We are confident in ascribing our behavioural phenotype, as well as that of Costa et al. (2003), to hippocampal dysfunction because of the many control experiments (demonstrating normal behaviour in activity, anxiety, and even other forms of memory such as fear conditioning) that both groups undertook. However, a stronger case could be made in favour of our hypothesis if temporal and/or spatial control over the genetic deletion of a given locus could be exerted. Several experiments in this
vein suggest themselves, all variations on the conditional gene deletion system known as Cre-loxP (Fig. 25).

The Cre-loxP approach has been developed for use in many tissues, including the brain (reviewed by Gavériaux-Ruff and Kieffer, 2007). Briefly, a strain of mice is developed in which a gene of interest is flanked by so-called loxP sites, which act as recognition sites for the bacterial Cre recombinase. Genes that are flanked by loxP are said to be ‘floxed’. This strain is then crossed with a line of mice expressing Cre under the control of a particular promoter. Any cells in which Cre is expressed will have their copies of the gene of interest deleted through recombination at the loxP sites. In all other cells (where Cre is not expressed), no recombination can occur because both Cre and loxP are bacterial in origin and have no mammalian homologues. It should be apparent that the selection of the promoter governing expression of Cre is critical, since this ultimately determines where recombination (and deletion of the floxed gene) occurs. In studies involving cognition one would presumably select a forebrain-specific promoter to drive Cre expression, and several forebrain-specific Cre lines of mice have been developed in recent years. However, this would typically also involve deletion from other forebrain areas such as the cortex and amygdala (Gavériaux-Ruff and Kieffer, 2007). Such lines, though they cause deletions in other brain regions, frequently act in restricted portions of the hippocampus, for example the CA1 region in the α-CaMKII-Cre line (Xu et al., 2000).

Such an approach has been frequently used to address the role of genes where conventional homozygous deletion is lethal, beginning with seminal work on the NMDA receptor (McHugh et al., 1996). Since most of the Notch pathway components described above are homozygous lethal (with the exception of Lfng where homozygous null mutants are viable), use of the Cre-loxP system would allow us to homozygously delete a given locus in the tissue we
A simple way to enhance the specificity of the deletion of a gene of interest is through use of the Cre-loxP system. In this case, a mouse strain with a floxed Notch1 locus is crossed with a strain expressing the Cre recombinase under the control of an inducible, forebrain-specific promoter, e.g., the αCaMKII promoter. Upon tamoxifen treatment, the normally cytoplasmic ERiCreER protein translocates to the nucleus and excises floxed sequences through recombination (Casanova et al., 2002).
are interested in (i.e., the forebrain). Such an approach would result in a considerable improvement in the spatial resolution of our deletion, effectively confining recombination to brain areas implicated in the behaviours we are interested in. Additionally, one might expect an enhancement of the behavioural phenotypes currently described since one would be effecting homozygous (and not a heterozygous) deletions of genes of interest.

As mentioned above, we would be principally interested in Cre lines which allow both spatial and temporal control over deletions. Several variations on the Cre-\textit{loxP} system have been developed which allow temporal control to be wedded to spatial specificity, notably the tamoxifen- or doxycyclin-inducible Cre systems (Garcia and Mills, 2002). Briefly, a strain of mouse is developed where a Cre molecule with an ERT\textsubscript{2} tamoxifen-binding domain fused onto both the N- and C-termini of the molecule is expressed from a particular promoter. This recombinant ER\textit{i}CreER molecule remains cytoplasmic in cells where it is expressed until, upon tamoxifen treatment, the molecule translocates to the nucleus and produces excision of floxed elements. A tamoxifen-inducible \(\alpha\text{-CaMKII}\)-Cre line (ERiCreER) has already been developed that allows forebrain-specific deletion of any floxed gene upon tamoxifen treatment (Casanova \textit{et al}., 2002).

One major consideration in the use of this technology is the possibility of incomplete deletion. In the original study describing ERiCreER, use of a Southern blot-based reporter assay showed that only an estimated 5-10% of ERiCreER\textsuperscript{+} cells demonstrated recombination (Casanova \textit{et al}., 2002). Recent refinements of the system have increased recombination efficiency in the forebrain (Erdmann \textit{et al}., 2007), however it is likely impossible to achieve 100\% recombination in any context.
An additional consideration would be the identity of the genes selected for conditional deletion. Many Notch pathway components have been floxed over the last decade, including Notch1 (Radtke et al., 1999), Notch2 (Schouwey et al., 2007), Jag1 (Loomes et al., 2007) and Dll1 (Hozumi et al., 2004). Targeting Notch1 or Jag1 would make intuitive sense due to the behavioural phenotypes now reported for heterozygous mutants of both of these loci (Costa et al., 2003; the current study). The simplest study that could be devised to take advantage of this technology would be to simply delete either Notch1 or Jag1 from the hippocampi of adult animals and repeat the battery of behavioural tests we have performed on our current mutants, in order to see if the phenotypes are reproducible and whether homozygous (as opposed to heterozygous) deletion from the forebrain would actually increase the robustness of the effects. The Golgi-Cox staining and neuron tracing performed here could also be repeated in this instance, with the same objectives (reproducibility and/or increased robustness).

### 4.3.3 Other directions

As discussed above, stronger evidence for the specificity of our behavioural phenotype might be gained if we exerted temporal and spatial control over the deletion. If we gained such control through the use of inducible Cre-loxP systems, our first step could be to repeat our experiments with this new genetic paradigm. However, are there other approaches that we might take to explain our behavioural effects?

A current technology that we might take advantage of is the use of viral vectors to introduce transgenes into select populations of neurons through direct infusion of virus suspensions into distinct brain areas (Fig. 26). The Josselyn laboratory has an established
Figure 26. Use of viral vectors and Cre-loxP technology to examine the effect of the Notch pathway on neuron morphology in a single animal. Introduction of a suspension bearing infectious virions expressing Cre recombinase and a fluorescent marker (such as GFP) would allow accurate selection of genetically modified neurons (i.e., neurons in which a floxed gene of interest has been deleted) for neuron tracing/spine density analysis. Use of control vectors (containing only a fluorescent marker) would allow for within-animal controls.
expertise in this approach, and has used it to produce measurable changes in learning and memory in mice through introduction of the transcription factor CREB expressed from a construct packaged into a recombinant herpes simplex virus (HSV) (Han et al., 2007). If indeed Notch signaling is crucial for hippocampal-dependent learning and memory, than overexpression of certain Notch pathway components through such an approach could lead to enhanced performance in the MWM, providing positive evidence of the pathway’s role. One could overexpress native Notch pathway components, such as Jag1 or Notch1, or even constitutively active Notch signaling molecules, such as free NICD. Jag1 or Notch1 mutants with truncated intracellular domains could also be introduced as dominant negatives (Taylor et al., 2002).

One drawback to the use of the Cre-\textit{loxP} system to delete a gene of interest from a large portion of a living animal (e.g., the forebrain) is the lack of complete Cre expression penetrance – i.e., not all target cells will correctly express Cre recombinase when induced by drug treatment and therefore delete the gene of interest. For this reason, the Golgi-Cox staining and neuron tracing experiment described above (a comparison of sections from mice in which the gene of interest has been deleted with floxed littermates) could suffer from the confound that neurons selected for tracing in the experimental group could still be expressing the floxed gene. The use of viral vectors offers a potential way around this problem. Using a viral construct encoding Cre and a fluorescent marker (e.g., green fluorescent protein – GFP), one would select specifically for cells expressing the marker, which should also be correctly expressing Cre (and therefore have the gene of interest deleted). Use of a control vector expressing only a marker of a different emission wavelength (e.g., yellow fluorescent protein – YFP), one could compare morphology of neurons which either do or do not express the gene of interest, with greater assurance that the identity of a given neuron has been correctly assigned. An additional advantage of this approach
is that such comparisons could be made within the same animal, reducing the impact of animal-to-animal variations.
References


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Appendix – The roles of Irx3 and Irx5 in behaviour

Abstract

The Irx family of mammalian pre-pattern regulators are important actors in both neuronal and non-neuronal tissue specification. Their function in central nervous system (CNS) development, from formation of the neural tube through to differentiation of particular types of mature neurons, has been extensively studied in recent years. However, little effort has been made towards an understanding of the results of Irx deregulation on post-development adult brain physiology. We have undertaken a behavioural screen of Irx3+/-, Irx3-/-, Irx5+/-, and Irx5-/- mice. Our principle observation is a profound disruption of the prepulse inhibition (PPI) response in Irx3+/- and Irx3-/- mice, an effect which is partly rescued by the typical antipsychotic haloperidol. We also observe minor defects in Morris water maze performance in Irx3-/- mice and mild anxiety-like behaviour and hearing loss in Irx5-/- mice.

Introduction

Understanding the mechanisms that control differentiation of precursor cells into mature tissue types is a fundamental pursuit of modern biology with important clinical applications. The Iroquois (Iro) family of genes and its homologues are critical developmental regulators that control such cell fate decisions. One of the principle functions of the Iro chromosomal complex of genes (Iro-C) in Drosophila is to direct the expression of members of the proneural achaete-scute complex (AS-C). Regulation by Iro-C produces clusters of AS-C expressing cells among
larger populations of epithelial tissue. These clusters mark sites of future sensory bristles in the 
dorsal thoracic region. Bristle formation in the notum is one developmental model in which Iro-
C control over proneural processes has been extensively mapped [1]. The three known members
of the Iro-C (araucan, caupolican, and mirror) are also instrumental in the establishment of the
dorsoventral equator of symmetry in the Drosophila eye [2] and defining the dorsal portion of
the imaginal wing disc [3]. In general terms, Iro-C members direct (frequently proneural) gene
expression and define broad areas in developing embryos which are then subsequently
differentiated into diverse cell types to produce mature structures.

Numerous roles have been assigned to Iro homologues in vertebrate development that are
reminiscent of their function in fruit flies. In Xenopus embryos, the Iro homologue Xiro1 is
important in the early differentiation of the neural plate [4], and Xiro1-3 are later involved in
subdivision of neural tissue into mature brain areas. In mammals, six Iro-related (Irx) genes
have been described that are organized into two conserved chromosomal groupings [5]. Irx
genes are also implicated in CNS development [6] in particular through influence of the
mammalian achaete-scute homolog 1 (Mash-1) during brain patterning [7]. The control of Irx
gene expression is in turn believed to involve epigenetic mechanisms [8] and regulation by the
Wnt and Sonic hedgehog (Shh) pathways [9, 10]. In addition to the CNS, mammalian Irx
proteins have numerous other developmental roles, such as the differentiation of the principle
areas of the heart [11, 12] and lung [13, 14]. Sequence analysis has shown that Iro and Irx
members are three amino acid loop extension (TALE) homeobox domain proteins [15]. The
homeobox proteins as a family have also been studied for their role in brain development [16].

One of the more well-characterized Irx genes is Irx3. Irx3 is involved in specification of
certain neuronal subtypes during neural patterning [17], and is detected throughout the
developing spinal cord [6]. The Fused toes (Ft) chromosomal deletion eliminates one of the two Irx gene clusters, including Irx3, and produces severe defects in neural tube development [18]. As for most Iro and Irx family members, Irx3 influences the development of non-neuronal tissue as well, such as the kidney [19] and gonads [20].

Another Irx gene of interest is Irx5. As for Irx3, Irx5 is expressed in the developing brain [21]. It is also believed to be important in retinal development [22, 23] and is involved in establishing the cardiac repolarization gradient through control of potassium channel expression [24] and in cardiac development [12]. The Ft chromosomal deletion also eliminates Irx5 [18], suggesting (as for Irx3) that Irx5 may be important in neural tube development.

The majority of research into the mammalian Irx pathway to date, including of Irx3 and Irx5, has focused on the immediate developmental roles played by Irx family members. Loss-of-function studies have restricted themselves to the embryonic consequences of perturbations of Irx function. While the development of mature certain neuronal populations is known to critically rely on Irx proteins [17, 25], the post-development effects of disturbed Irx function have not been widely probed in adult animals.

We present here the results of a battery of behavioural tests of Irx3+/− and Irx5+/− (heterozygous) and Irx3−/− and Irx5−/− (homozygous null) mice, with comparisons made with wild-type (WT) littermate controls. We show that Irx3+/− and Irx3−/− mice have normal profiles in open field and contextual and auditory fear conditioning tests and that Irx3+/− mice have only mild defects in Morris water maze (MWM) performance. Irx5−/− mice display anxiety-like behaviour in the open field, but Irx5+/− and Irx5−/− animals perform normally in the MWM and in both fear conditioning paradigms.
Our salient finding is that $\text{Ir}{\textit{x}}_3^{+/\cdot}$ and $\text{Ir}{\textit{x}}_3^{+/\cdot}$ mice have severe deficits in a test of sensory gating ability (prepulse inhibition – PPI). Mouse models showing PPI deficits are of clinical interest since PPI dysfunction is a hallmark of several mental disorders including schizophrenia [26]. The defects observed in $\text{Ir}{\textit{x}}_3^{+/\cdot}$ and $\text{Ir}{\textit{x}}_3^{+/\cdot}$ mice are comparable to those typically observed in either human schizophrenic patients or in genetically- or pharmacologically-induced animal models commonly used to study antipsychotic agents. These deficits are partially rescued through the use of the standard antipsychotic drug haloperidol. $\text{Ir}{\textit{x}}_5$ mice appear to exhibit hearing difficulties as measured by the acoustic startle threshold method, but have largely normal PPI responses.

**Materials and Methods**

**Animals**

Homologous recombination of a vector containing a $\text{tau-LacZ}$ element replacing the first exon of $\text{Ir}{\textit{x}}_3$ was used to produce the $\text{Ir}{\textit{x}}_3^{\cdot}$ allele and a similar vector containing an $\text{EGFP}$ and $\text{puro}$ cassette replacing the first exon of $\text{Ir}{\textit{x}}_5$ produced the $\text{Ir}{\textit{x}}_5^{\cdot}$ allele (Hui laboratory, unpublished data); in both cases functional disruption of the WT alleles is achieved. $\text{Ir}{\textit{x}}_3^{+/\cdot}$ and $\text{Ir}{\textit{x}}_5^{+/\cdot}$ (WT), $\text{Ir}{\textit{x}}_3^{+/\cdot}$ and $\text{Ir}{\textit{x}}_5^{+/\cdot}$ (heterozygous) and $\text{Ir}{\textit{x}}_3^{+/\cdot}$ and $\text{Ir}{\textit{x}}_5^{+/\cdot}$ (homozygous null) mice were bred from heterozygous parents in a CD1 background. Mice were group housed (3-5 mice per cage) on a 12 h light/dark cycle. Behavioural experiments were conducted during the light-phase. All mice used in behavioural training were between 2-3 months of age. Food and water were available *ad libitum.* Procedures were approved by the Hospital for Sick Children Animal
Care and Use Committee. Before behavioural testing, mice were handled for 2 min/d for 1 week.

*Open Field*

Open field arenas (45 x 45 x 20 cm, white Plexiglas) were evenly lit from above. Individual mice were placed in the middle of boxes and allowed to explore the environment for 15 min. The distance each mouse traveled was determined by a camera connected to a tracking program (ActiMetrics, Wilmette, IL). Anxiety was measured by assessing the amount of time mice spent in each of three zones (Zone 1 consisted of the outer perimeter of the box, Zone 2 an inner ring with dimensions of 36 x 36 cm on the outside and 18 x 18 cm on the inside, and Zone 3 an 18 x 18 cm square in the middle of the box).

*Morris Water Maze (MWM)*

The circular water maze tank (120 cm diam, 50 cm deep) was located in a dimly lit room [see [27]]. The pool was filled to a depth of 40 cm with water made opaque by nontoxic white paint. Water temperature was maintained at 28 ± 1°C. A circular escape platform (10 cm diameter) was submerged 0.5 cm below the water surface and located in a fixed position throughout training. The pool was surrounded by white curtains painted with distinct cues, 1 m from the pool perimeter.

Mice were given 6 training trials per day (2 blocks of 3 trials, with an inter-block interval of 45-60 min) for 7 days. Each trial lasted a maximum of 60 s. To begin each trial, mice were
placed in the pool, facing the wall in one of four start locations (varied pseudorandomly). The trial was complete once the mouse found the platform or 60 s had elapsed. If the mouse failed to find the platform on any trial, the experimenter guided the mouse onto the platform. After each training trial, the mouse was allowed 15 s on top of the platform. Spatial memory was assessed in a probe test during which the platform was removed from the pool and the mouse allowed to search for 60 s. Probe trials were given before training on days 1, 4 and 7.

Behavioural data from the training and testing phases were acquired and analyzed using an automated tracking program (Actimetrics, Wilmette, IL). Using this software, we recorded a number of variables during training, including escape latency and swim speed. In probe tests we quantified spatial memory by measuring the amount of time mice searched the target zone (20 cm radius, centered on the location of the platform during training; 11% of the pool surface) versus the average of three other equivalent zones in other areas of the pool [28-30]. Thigmotaxic behaviour during training or the probe test was quantified by calculating the amount of time mice spent in the peripheral region of the pool (within an area of 5 cm from the wall) [31].

**Fear conditioning**

Mice were trained and tested for contextual and auditory (cued) Pavlovian fear conditioning in Plexiglas and metal boxes (24 x 30 x 21 cm, Med Associates, St. Albans, VT) located in a soundproof room with low-level white noise played to mask outside disturbances. Training consisted of placing mice in a conditioning chamber (Context A) and two min later presenting a tone (2800 Hz, 85 dB, 30 sec) that co-terminated with a footshock (0.7 mA, 1 sec).
Mice remained in the chamber for an additional 30 sec. Testing for contextual fear conditioning occurred 24 hr later when mice were placed back into the same context (Context A) for 5 min and the amount of time spent freezing was recorded. Twenty-four hr following this, mice were placed in a novel chamber (Context B) and tested for auditory (cued) fear conditioning. Two min after placement in the novel environment, the tone conditioned stimulus (CS) was presented for 3 min. Our index of memory, freezing (the cessation of all movement except for respiration) elicited either by context or by the tone CS, was assessed via automated procedures (Actimetrics, Wilmette, IL).

*Prepulse inhibition (PPI) and acoustic startle threshold*

*Auditory startle response.* Startle testing was conducted using a SR-LAB startle testing system (San Diego Instruments, CA, USA). Mice were placed in a Plexiglas testing cylinder (3.2 cm internal diam). Acoustic startle stimuli and prepulse stimuli were delivered via a high-frequency speaker, placed 15 cm from the testing cylinder. Background white noise was generated by a standard speaker. The testing cylinder was mounted on a sensor platform. A piezoelectric accelerometer, attached to the base of the sensor platform, detected and transduced cage movements that were then digitized by and stored in a computer. The startle amplitude was taken to be the maximal response that occurred in the 100 ms after presentation of the startle stimulus. The sound levels for background noise and startle/prepulse stimuli were calibrated with a digital sound level meter. The speakers, testing cylinder and sensor platform were housed within a sound-attenuated chamber.


Habituation. Mice were placed in the testing cylinder and 5 min later, presented with 80 startle pulses of 120 dB each (15 s interstimulus interval, ISI).

Prepulse inhibition. The next day, mice were tested for prepulse inhibition of the startle response. Following a 5-min acclimation period where no stimuli were delivered, mice were presented with 20 habituation trials (120 dB, ISI 15 s). In the prepulse inhibition phase, mice were presented with a total of 90 trials. Three prepulse intensities were tested: 70, 75 and 80 dB. Prepulses were 20 ms in duration with a rise/fall time of less than 1 ms. For each prepulse intensity, there were three types of trial: prepulse alone, prepulse/startle stimulus and startle stimulus alone. In the prepulse/startle stimulus trial, the onset of the prepulse preceded the onset of the startle stimulus by 100 ms. All startle stimuli were presented in a pseudorandom sequence with the constraint that each stimulus intensity occur only once in each consecutive four-trial block. The % PPI was calculated per mouse for each of the three prepulse conditions.

Startle threshold. The following day, mice were given a startle threshold test session. Following an acclimation period of 5 min, mice were presented with a total of 99 trials (15 s ISI). There were 11 trial types: no stimulus (0 dB), and 10 types of startle trials in which the intensity of the startle stimulus varied from 75–120 dB (in 5 dB increments). The startle stimuli were 40 ms noise bursts with a rise/fall time of less than 1 ms. The 11 trial types were presented in a pseudorandom order such that each trial type was presented once within a block of 11 trials. Startle threshold was defined as the minimal intensity at which responding was significantly greater than in the NS trials. Because Irx5+/− and Irx5−/− mice may have hearing impairments (Hui laboratory, unpublished data), we used only those mice that showed intact startle to the 120 dB stimulus (above 100 mV produced in the piezoelectric sensor).
Rescue of PPI deficits with haloperidol. Haloperidol was dissolved in dH$_2$O acidified to pH 4.0 with sodium acetate, solutions were brought to pH 7.3 with NaOH and then diluted to 0.01 or 0.03 mg/ml. All vehicle and drug dose volumes were equivalent to 0.1 ml/kg. All mice received each dose of drug (vehicle, 0.1 and 0.3 mg/kg) in a counterbalanced fashion with 3 days in between tests to allow for drug clearance. Mice were administered the drug 30 min before prepulse inhibition was measured as above. Threshold was also measured as above both before and after drug treatments to assess any effects on the drug on hearing.

Results

Mice with mutations in Irx3 show normal locomotion and anxiety-like phenotypes while mice with mutations in Irx5 have normal locomotion but appear more anxious. To examine if mice with mutations in Irx3 or Irx5 show impaired locomotor activity or anxiety, we tested mice in the open field. As shown in Fig. 1A mice with mutations in Irx3 show normal levels of locomotion. The results of an ANOVA examining the average distance traveled showed no significant effect of Genotype (F(2,32) = 0.265, p > .05). As anxious rodents avoid exposed spaces, the relative amount of time an animal spends near the wall of an open field box provides a measure of their baseline anxiety levels [32]. Therefore, we examined the percentage time mice spent in each of the zones. Mice with mutations in Irx3 showed no difference in time spent in each of the zones (Fig 1A). This is supported by the results of a Genotype x Zone ANOVA which showed a significant effect of Zone only (F(2,64) = 252.0, p < .001) but no interaction between Zone and Genotype (F(4,64) = 0.129  p > .05) or main effect of Genotype.
Figure 1. Behavioural tests on Irx3+/− and Irx3−/− mice. (A) Results from the open field experiment for distance travelled (a measure of basal activity) and zone occupancy (a measure of basal anxiety levels). (B) Results from the MWM experiment for probe tests (a measure of preference for the target zone and therefore spatial memory) and escape latencies (average time to find the platform). (C) Results from contextual and cued fear memory conditioning. Arrowhead marks time of tone onset in cued fear conditioning experiment. (D) Results from PPI and acoustic startle threshold experiments.
Figure 2. Behavioural tests on \( Irx5^{+/} \) and \( Irx5^{-/-} \) mice. (A) Results from the open field experiment for distance travelled (a measure of basal activity) and zone occupancy (a measure of basal anxiety levels). (B) Results from the MWM experiment for probe tests (a measure of preference for the target zone and therefore spatial memory) and escape latencies (average time to find the platform). (C) Results from contextual and cued fear memory conditioning. Arrowhead marks time of tone onset in cue fear conditioning experiment. (D) Results from PPI and acoustic startle threshold experiments.
(F(2,32) = .125, p > .05). Together these findings indicate that *Ir3* is not critically involved in locomotor behaviour or anxiety.

Mice with mutations in *Ir5* show similar levels of locomotor behaviour as WT mice (F(2,37) = 25.34, p > .05) (Fig 2A). However, mice with a homozygous disruption in *Ir5* show increased levels of anxiety, as shown by an increase in time spent in the outer zone of the open field. The results of an ANOVA showing a significant interaction between Zone and Genotype (F(4,56) = 2.68, p < .05) and a significant effect of Zone (F(2,56) = 291.9 p < .05) but no significant effect of Genotype (F(2,28) = 0.9, p < .05) supports this conclusion. Post-hoc Newman-Keuls tests on the significant interaction shows that *Ir5*−/− mice spend greater time in the outer zone than WT or mice with a heterozygous mutation in *Ir5*, which did not differ. Therefore, a homozygous mutation in *Ir5* increases anxiety-like behaviour in the open field.

*Ir3* mutations cause impairments in spatial memory while *Ir5* mutations have no effect on spatial memory. To examine the effects of disruptions of *Ir3* and *Ir5* in spatial memory, we trained mice in a hidden platform version of the Morris water maze. This task depends on intact hippocampal function [33].

Over training trials, mice tend to adopt more spatially-based search strategies to locate the platform, and this change to a spatial strategy is reflected by a decrease in latency to locate the platform. Over the 7 training days, WT littermate mice as well as *Ir3*+/− mice required progressively shorter times to locate the hidden platform, but the escape latencies of *Ir3*−/− mice were longer (Fig. 1B). The results of a Genotype x Day ANOVA supports this conclusion, showing a significant Vector x Block interaction (F(12,246) = 1.87, p < .05) as well as a significant effect of Day (F(6,246) = 63.31, p < .001) and Genotype (F(2,42) = 8.65, p > .001). Neuman-Keuls post-hoc analysis revealed that on the first training day all groups required the
same time to locate the platform but that over training days the $\text{Irx3}^{+/+}$ group failed to locate the platform as quickly as WT and $\text{Irx3}^{+/-}$ mice. This result suggests that $\text{Irx3}^{+/-}$ mice failed to adopt a more effective spatial search strategy and therefore have an impairment in the acquisition of spatial memory.

To more thoroughly examine spatial memory in $\text{Irx3}$ mutant mice, we tested mice in probe tests on day 1, 4, and 7. We quantified spatial bias by comparing the amount of time mice spent in the target zone versus the average time spent in equivalent zones in the other three quadrants of the pool. Figure 1B shows that, as expected, all mice displayed no spatial bias (i.e., all mice spent equal times in the area of the pool where the platform was located during training) on the first probe test (conducted before any training), and that $\text{Irx3}^{+/-}$ mice lag behind WT littermates on day 4 but catch up by day 7. An ANOVA of day 1 probe data with a between-subjects variable Genotype and within-subjects variable Zone (Target, Others) revealed no significant interaction between Genotype and Zone ($F(2,41) = 0.41, p > .05$) or main effect of Zone ($F(1,41) = 2.43, p > .05$) or Genotype ($F(2,41) = 1.34, p > .05$). Therefore, before training, no group of mice showed a spatial bias. The results of an ANOVA comparing the time spent in the target zone between the three groups confirmed this ($F(2,41) = 1.08, p > .05$). On the day 4 probe test all groups of mice showed a preference for the target zone of the pool, indicating spatial memory. The results of an ANOVA showing a significant effect of Zone ($F(1,41) = 25.2, p < .001$) and Genotype ($F(2,41) = 4.71, p < .05$) but no Genotype by Zone interaction ($F(2,41) = 1.31, p > .05$) supports this conclusion. The ANOVA performed on the amount of time spent in the target zone by the 3 groups showed a significant effect of Genotype ($F(2,41) = 3.23, p < .05$). A Newman-Keuls post-hoc analysis showed a significant difference between $\text{Irx3}^{+/-}$ and $\text{Irx3}^{+/-}$ mice on day 4 target zone occupancy ($p < 0.01$). The day 7 probe produces a similar effect of
Zone (F(1,41) = 86.78, p < .001) but no significant effect of Genotype (F(2,41) = 2.93, p > .05) or Genotype x Zone interaction (F(2,41) = 2.30, p > .05). There is also no difference in time spent in target zone on day 7 by Genotype (F(2,41) = 2.74, p > .05). Therefore homozygous mutations to *Irx3* produce impairments in a day 4 probe test, but the animals appear to catch up by day 7. Together with the training latency phenotype, this data leads us to conclude that *Irx3*/* mice are impaired in the MWM but that these mice can achieve WT performance levels with sufficient training.

Mice with mutations in *Irx5* show intact acquisition of spatial memory, as measured by latency to find the platform during training. An effect of Day ANOVA of the training data from *Irx5* mice confirmed that all animals learn (F(6,258) = 49.537, p < .001), and Genotype (F(2,43) = .45756, p > .05) and Genotype x Day (F(12,258) = .92582, p > .05) ANOVAs demonstrated that there is no effect of genotype on escape latencies during training. Our results from the probe tests of *Irx5* animals validate this conclusion, with an ANOVA of the effect of Zone (F(5, 215) = 21.509, p < .001) suggesting that the animals do learn, but with Genotype x Zone (F(10,215) = 1.7089, p > .05) and main effect of Genotype (F(2,43) = .11799, p > .05) ANOVAs indicating no interaction between genotype and probe test performance in *Irx5* mice.

*Irx3* and *Irx5* mice show intact conditioned fear memory. We tested the effects of mutations in *Irx3* on Pavlovian fear conditioning (Fig. 1C and 2C). This task is known to depend on both the amygdala and hippocampus. Despite the high shock intensity (0.7 mA), all genotypes tested (including WT) showed low context fear conditioning freezing in the contextual fear memory test (approximately 10%), suggesting an insensitivity of the background used (the CD1 mouse) to the test. An ANOVA of the effect of Genotype confirmed there is no interaction at the *Irx3* locus (F(2,38) = 1.3201, p > .05) in this measure. In the auditory (cued) fear memory
paradigm, an ANOVA of the effect of the Time interval showed that Irx3 mice show increased freezing due to the tone (F(4,152) = 34.162, p < .001), but an ANOVA of the main effect of Genotype (F(2,38)=.64840, p>.05) and of Time x Genotype (F(8,152)=1.1703, p>.05) showed that Irx3 does not influence auditory (cued) fear memory in mice.

An ANOVA of the effect of Genotype showed no interaction for Irx5 in the contextual fear memory paradigm (F(2,19)=1.4716, p>.05); as for Irx3 mice we believe the CD1 background renders the mice insensitive to the task. In auditory (cued) fear conditioning, an ANOVA of the effect of the Time interval showed that Irx5 mice display higher levels of freezing due to the tone (F(4,116) = 15.956, p < .001), but an ANOVA of the main effect of Genotype (F(2,29)=1.4239, p>.05) and of Time x Geotype (F(8,116)=2.0023, p>.05) demonstrate the Irx5 locus plays no role in this form of fear memory.

Prepulse inhibition in Irx3 mutant mice is markedly attenuated but is unaffected in Irx5 mice despite hearing loss. We next examined the effects of the Irx3 and Irx5 mutations on acoustic startle responding and prepulse inhibition, a behaviour dependent on a large number of brain areas including the hippocampus, ventral tegmental area and thalamus [34-37]. A commonly used measure of sensory gating is PPI, where the magnitude of a startle reflex is blunted by presentation of a prepulse shortly before the main sensory pulse used to evoke the response. Using a standard auditory PPI protocol (where startles are elicited by 120 dB sound pulses), we found as expected that all mice (Irx3+/+, Irx3+/-, and Irx3−/− mice) displayed increasing PPI with increasing prepulse levels, demonstrated statistically by an ANOVA of the effect of Prepulse (F(2,82) = 55.716, p < .001). However, comparison of the PPI profiles of Irx3+/- mice to WT controls showed a striking deficit at the two highest prepulse levels (75 and 80 dB), an effect which is confirmed by an ANOVA of Prepulse x Genotype (F(4,82) = 3.6586, p < .01)
Neuman-Keuls post-hoc analysis showed that the interaction between WT and $Irx3^{+/}$ was statistically significant at a prepulse of 80 dB (p<.05) and between WT and $Irx3^{-/-}$ at prepulses of 75 and 80 dB (p<.05 for both). Little to no PPI is observed at the 70 dB prepulse level in all animals tested. ANOVAs of both the effect of Genotype on habituation trials ($F(2,41) = .83366, p > .05$) and habituation Trial x Genotype ($F(6,123) = .27620, p > .05$) confirmed that all animals habituate to the startle apparatus normally (Fig. 1D). Mutant $Irx3$ mice furthermore display no differences in acoustic startle threshold measurements when compared to WT littermates as demonstrated by ANOVAs of both the effect of Genotype ($F(2,41) = .57745, p > .05$) and Pulse level x Genotype ($F(20,410) = .41814, p > .05$) (Fig. 1D), eliminating trivial explanations such as impaired habituation or hearing loss for the observed deficits.

We attempted to examine PPI in $Irx5$ mice, but found through our preliminary startle threshold tests (data not shown) that $Irx5^{+/}$ mice appear to have hearing difficulties (confirming observations from the Hui laboratory, unpublished data). This produced aberrant and widely variable values in the PPI test, confounding our measurements. We thus set an arbitrary minimum of 100 mV for the baseline startle amplitude measured by the piezoelectric sensor at 120 dB and eliminated animals that fell below this cut-off. This exclusion parameter removed 1 WT animal (out of 11 tested), 2 $Irx5^{+/}$ animals (out of 22 tested) and 6 $Irx5^{-/-}$ animals (out of 18 tested). Once unresponsive animals were eliminated, we obtained a typical linear relationship between prepulse level and % PPI, as well as normal habituation and acoustic startle threshold profiles (Fig. 2D). Slight defects were detected in $Irx5^{+/}$ and $Irx5^{-/-}$ animals at 75 and 80 dB prepulses, but these were not statistically significant as shown by ANOVAs of the main effect of Genotype ($F(2,39) = 1.5601, p > .05$) and Prepulse x Genotype ($F(4,78) = .54854, p > .05$).
The PPI deficits of Irx3 mutant mice are partly rescued by the typical antipsychotic haloperidol. We attempted to rescue the deficit in Irx3 mice through administration of the typical antipsychotic haloperidol, with mixed results (Fig. 3). An ANOVA of the main effect of Genotype (F(2,28) = 9.0462, p < .001) confirmed there were still differences between genotypes throughout the experiment. Neuman-Keuls post-hoc analysis showed that the difference between WT and Irx3+/− mice which exists with vehicle treatment was eliminated at the low dose (0.1 mg/kg), although this appears to be because of an unexplained decrease in WT PPI (from approximately 70% to 50%) observed in this condition only. Further evidence for this comes from the observation that the difference between WT and Irx3+/− mice is once again significant at 80 dB prepulse at the high dose (p<.01). Examination of the magnitude of PPI elicited from Irx3+/− mice confirms that it remains essentially unaltered over drug dosages at both 75 and 80 dB prepulses (at approximately 25% and 45% respectively). Conversely, at the high dose (0.3 mg/kg) Irx3+/− mice enjoy a rescue of their deficit back to Irx3+/− levels (from approximately 25% to 40%), eliminating the statistical difference between Irx3+/− and Irx3−/− animals in this condition. We therefore conclude that haloperidol partly rescues Irx3+− animals. We additionally tested the acoustic startle threshold of animals both before and after drug treatments to assess the effects of haloperidol on hearing, but found no differences (data not shown).

Discussion

A burgeoning field has emerged around the TALE homeobox domain Iro family of proneural and pre-pattern developmental regulators and their homologues in vertebrate genomes. An increasingly concise and detailed picture of the role of these proteins has emerged in recent
Fig 3. Haloperidol only partly rescues pre-pulse inhibition deficit in Irx3+/− and Irx3−/− mice. Shown are PPI measurements under vehicle treatment as well as two doses of haloperidol (0.1 mg/kg and 0.3 mg/kg).
years, with Iro and vertebrate Irx genes now being known to control the expression of proneural
AS-C complex members (*Drosophila*) or mammalian downstream regulators such as Mash-1
(mammals) to influence patterning and differentiation decisions during development. Little
headway has been made, however, in assessing the impact of deregulation of Irx function in
adult mammals in post-development physiology. To our knowledge this study represents the
first effort to examine the cognitive effects of mutation of members of the *Irx* gene family. We
have examined the behavioural profiles of mice carrying either heterozygous or homozygous null
mutations of the *Irx3* and *Irx5* locus.

*Irx3*+/− and *Irx3*−/− animals performed normally in physical tasks such as the open field and
*Irx3*+/− mice display only mild impairment in the MWM, an effect which may be explained by
decreased swim speeds and increased anxiety-like thigmotaxic behaviour. *Irx5*+/− animals display
increased anxiety-like behaviour in the open field, but both *Irx5*+/− and *Irx5*−/− animals perform
normally in the MWM. No differences in either *Irx3* or *Irx5* mice were detected in both
contextual and an auditory fear memory paradigms. Despite impaired hearing, *Irx5* mice also
exhibit normal PPI responses.

Our principle observation is the severe deficit of the PPI response in *Irx3*+/− and *Irx3*−/− mice.
Many genetic, environmental and pharmacological mouse models displaying deficits in PPI have
been developed in recent years [38-40]. The deficits of auditory PPI we have observed here are
comparable to that typically measured in schizophrenic patients [41]. The attenuation of the PPI
response in *Irx3*−/− mice appears to be partly rescued with the typical antipsychotic haloperidol,
although the drug is of no benefit to *Irx3*+/− mice.

The dependence of the PPI response on diverse areas of the brain [34-37] and the importance of
*IrX3* in the early patterning of large parts of the CNS [42] results in a large number of possible
explanations for the observed deficits. One possible explanation is disruption of serotonergic signaling. Deletion of the serotonin$_{1B}$ receptor ($5$-HT$_{1B}$) is known to perturb PPI [43] and the zebrafish Irx homologue Irx1a is known to be critical for differentiation of serotonergic neurons in the raphe nuclei [25]. However, deletion of $5$-HT$_{1B}$ in mice causes an increase in the PPI response as opposed to the decrease we observe with $Irx3^{+/}$ and $Irx3^{-/-}$ mice. PPI is also known to be dependent on dopamine signaling [44] and our partially successful rescue of $Irx3^{-/-}$ mice with the dopamine receptor antagonist haloperidol suggests that dopaminergic signaling may partly underlie our result; to our knowledge no evidence yet exists to posit a role for Irx genes in dopaminergic neuron differentiation. As additional efforts are made to delineate neuronal populations dependent on Irx3 for their terminal differentiation and incorporation into the CNS during development, new mechanisms may be proposed.


