ROLE OF RELAXIN-3 IN

ADULT HIPPOCAMPAL NEUROGENESIS

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Declaration

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

Hong Jia Mei

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Summary

Relaxin-3, a member of the insulin/relaxin superfamily, is highly expressed in the brain, predominantly in a hindbrain region known as nucleus incertus. From nucleus incertus, and other smaller groups of neurons, relaxin-3 is transmitted widely to numerous forebrain regions, and has been proposed to represent an ascending arousal system. A variety of functions have been reported for relaxin-3, including circadian activity, stress responses, and feeding, cognitive, affective and addictive behaviour. However, the neural processes involved in relaxin-3-mediated actions are less studied and understood. Adult hippocampal neurogenesis is a multi-step process of generating new neurons in the dentate gyrus, and has been implicated in functions like stress responses, and cognitive and affective behaviour. As a form of structural plasticity, neurogenesis is tightly regulated by several brain systems. In particular, the dentate gyrus receives abundant relaxin-3 input, especially in the temporal pole. Therefore, relaxin-3 is hypothesized to modulate neurogenesis in the adult hippocampus. To establish the role of relaxin-3 in neurogenesis, a genetic loss-of-function approach was undertaken with relaxin-3 knockout (KO) mice, and to examine levels of neurogenesis, 5-bromo-2-deoxyuridine (BrdU) was administered to birth-date newborn cells. Quantitative analyses of BrdU revealed perturbed neurogenesis in the lifelong absence of relaxin-3, in an age-, sex- and septotemporal-dependent manner. Specifically, in the young adult stage, male KO mice displayed an attenuation in cell proliferation, in the temporal dentate gyrus. In addition, in the mature adult stage, male KO mice had less age-related reduction in proliferation, while female KO mice had decreased proliferation, in the temporal subregion when compared to wildtype controls. Furthermore, in the mature adult stage, neuronal maturation was also

dysregulated, with male KO mice showing more neuronal differentiation, and female KO mice having increased migration, also in the temporal dentate gyrus. These findings have demonstrated, for the first time, the necessity of relaxin-3 in the modulation of adult hippocampal neurogenesis.

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5-HT	5-hydroxytryptamine
BDNF	Brain-derived neurotrophic factor
BLBP	Brain lipid binding protein
BNST	Bed nucleus of the stria terminalis
BrdU	5-bromo-2-deoxyuridine
cAMP	Cyclic adenosine monophosphate
CRF-R1	Corticotropin releasing factor receptor 1
CRHR1	Corticotropin releasing hormone receptor 1
DG	Dentate gyru
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
FABP7	Fatty acid binding protein 7
FGF2	Fibroblast growth factor 2 (basic)
GABA	γ-aminobutyric acid
GCL	Granule cell layer
GDNF	Glial cell derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GPCR	G protein-coupled receptor
HPA	Hypothalamic-pituitary-adrenal
HPG	Hypothalamic–pituitary–gonadal
IDE	Insulin-degrading enzyme
IGF	Insulin-like growth factor
IL-1β	Interleukin-1 beta
INSL	Insulin-like
KO	Knockout
LGR7	Leucine-rich repeat-containing G-protein coupled receptor 7
NMDA	N-methyl-D-aspartate
NSE	Neuron specific enolase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A

PKC	Protein kinase C
PLC	Phospholipase C
RLN	Relaxin
RT-PCR	Reverse transcription polymerase chain reaction
RXFP	Relaxin/insulin-like family peptide receptor
SALPR	Somatostatin- and angiotensin-like peptide receptor
SEM	Standard error of mean
SGZ	Subgranular zone
SNP	Single nucleotide polymorphisms
SOX2	SRY (sex determining region Y)-box 2
TGF-β1	Transforming growth factor, beta 1
WT	Wildtype

Chapter 1 Introduction

"*If our brains were simple enough for us to understand them,*

we'd be so simple that we couldn't."

Ian Stewart, Mathematician.

The Collapse of Chaos: discovering simplicity in a complex world, 1994.

1. Introduction

1.1. Plasticity in the brain

Our brain is one of the most complex organs in the body. It is responsible for a wide range of functions, such as sensing the environment, processing of information, controlling motor movements, cognition, mood and emotions, and much more. To achieve this diverse range of activities, the adult human brain makes use of its 86 billions of neurons (Azevedo et al., 2009) and 600 trillions of synapses (Pakkenberg et al., 2003) to form the numerous complicated neuronal networks.

As impressive as the "hardware" is, a static neuronal network may not be sufficient for all the challenges we face. To cope with the computational demands, the brain has acquired plasticity, in the form of synaptic and cell-based plasticity. Plasticity allows the brain to alter and adapt its network structure based on the activity requirements.

Plasticity on the synaptic level may involve recruitment or removal of synaptic proteins and receptors (reviewed in Collingridge et al., 2004), increase or decrease of transmitter release (reviewed in Hawkins et al., 1993), or even formation (Engert and Bonhoeffer, 1999) or elimination (Trachtenberg et al., 2002) of entire synapses. This phenomenon has been observed in a few brain regions, such as the cortex (Grutzendler et al., 2002; Trachtenberg et al., 2002) and hippocampus (Sutula et al., 1988; Matsuzaki et al., 2004), in an experience-dependent manner.

On the other hand, cellular plasticity offers structural modification to neuronal networks on the whole cell level. New neurons can be created and, consequently, new synapses can be incorporated in existing circuitries in the adult brain, a process termed adult neurogenesis.

1.2. Adult neurogenesis

Adult neurogenesis is the physiological process of generating new functional neurons, from proliferation and differentiation of neural precursor cells, to integration of maturing neurons into existing networks in the adult brain. Neurogenesis in adulthood has been found to exist in many animal species, including non-mammalian vertebrates, such as lizards (Lopez-Garcia et al., 1988), zebrafish (Byrd and Brunjes, 2001) and birds (Goldman and Nottebohm, 1983), and mammals, like rodents (Altman, 1963; Altman and Das, 1965) and primates (Gould et al., 1999b; Kornack and Rakic, 1999), including humans (Eriksson et al., 1998).

Currently, in adult rodents and primates, including humans, two constitutive neurogenic regions in the brain have been established and widely accepted. Neural stem or progenitor cells, or collectively termed as neural precursor cells (Kempermann, 2011), are found in the subventricular zone of the lateral ventricles (Doetsch et al., 1999) and the subgranular zone of the hippocampal dentate gyrus (Seri et al., 2001). From the subventricular zone and subgranular zone, precursor cells supply newborn neurons to the olfactory bulb (Doetsch et al., 1999) and the granule cell layer of dentate gyrus (Seri et al., 2001), respectively.

There have been reports on neurogenesis in other adult brain regions such as the neocortex (Kaplan, 1981; Gould et al., 1999b; Gould et al., 2001; Dayer et al., 2005), striatum (Bedard et al., 2002; Dayer et al., 2005), substantia nigra (Zhao et al., 2003), hypothalamus (Kokoeva et al., 2007), amygdala (Bernier et al., 2002), and CA fields of hippocampus (Rietze et al., 2000). However, normal physiological adult neurogenesis in these regions are still debatable in the field as they have not been consistently and/or independently reproduced (reviewed in Gould, 2007). Only single descriptions, such as that for amygdala and CA fields of hippocampus, were presented; or conflicting pieces of evidence, like that for neocortex (Kornack and Rakic, 2001; Ehninger and Kempermann, 2003; Koketsu et al., 2003; Bhardwaj et al., 2006), striatum (Benraiss et al., 2001) and substantia nigra (Frielingsdorf et al., 2004; Chen et al., 2005b), were reported.

On the other hand, there have been more consistent reports for neurogenesis induced by damage or pathological conditions in the adult brain. For example, experimental ischemic stroke was shown to be able to generate newborn neurons in striatum, one of the areas of damage (Arvidsson et al., 2002; Parent et al., 2002; Darsalia et al., 2005; Tonchev et al., 2005).

1.2.1. Stages of adult hippocampal neurogenesis

Neurogenesis is a tightly-regulated multi-step process involving maintenance of stem cell pool by self-renewal, proliferation of progenitor cells, cell fate determination, differentiation, survival, migration, maturation and integration of newborn neurons into existing circuitries (Fig. 1.1).

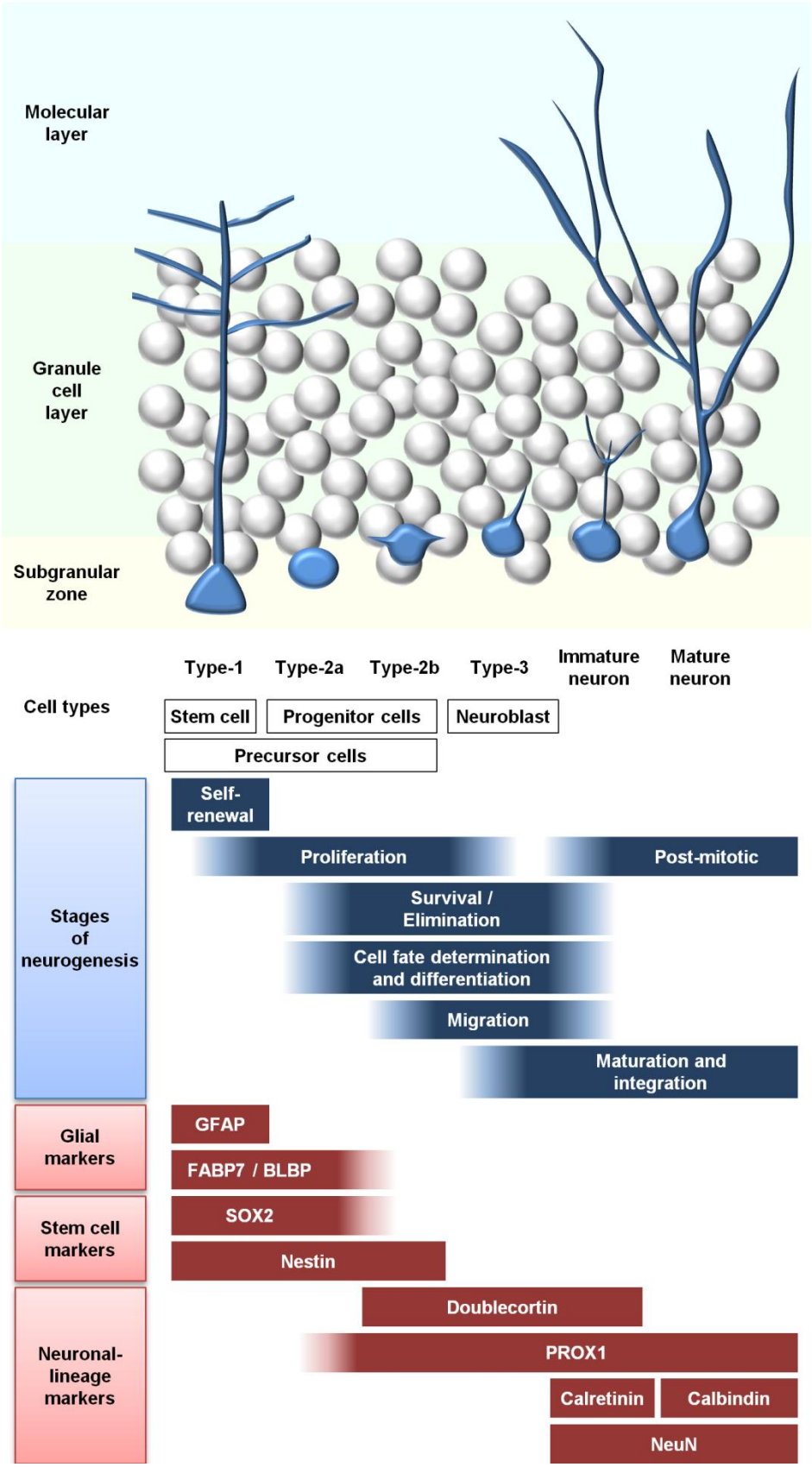

Figure 1.1. Stages of neurogenesis in the dentate gyrus of hippocampus.

1.2.1.1. Maintenance of stem cell pool

Putative stem cells (Seri et al., 2001; Garcia et al., 2004) of adult hippocampal neurogenesis are termed type-1 cells (Filippov et al., 2003; Kempermann et al., 2004a), also known as radial glia-like cells (Bonaguidi et al., 2011), or quiescent neural progenitors (Encinas et al., 2006). The morphology of type-1 cells is similar to that of radial glia, with a triangular cell body in the subgranular zone, and a thick long process across the granule cell layer that branches out in the molecular layer of dentate gyrus (Filippov et al., 2003; Fukuda et al., 2003; Mignone et al., 2004).

Type-1 cells express key protein markers similar to that of astroglia and stem cells, partially reflecting their overlapping properties. For example, type-1 cells express GFAP (glial fibrillary acidic protein) (Fukuda et al., 2003; Garcia et al., 2004; Mignone et al., 2004) but not S100β (Filippov et al., 2003; Suh et al., 2007). In addition, type-1 cells also express radial glia marker FABP7 (fatty acid binding protein 7, also known as BLBP, brain lipid binding protein) (Steiner et al., 2006). Common stem cell markers like nestin (Filippov et al., 2003; Fukuda et al., 2003; Mignone et al., 2004) and SOX2 (SRY (sex determining region Y)-box 2) (Suh et al., 2007) are also present in type-1 cells.

As putative stem cells, type-1 cells have low proliferative activity and are mostly quiescent (Filippov et al., 2003; Fukuda et al., 2003; Kronenberg et al., 2003; Tozuka et al., 2005; Suh et al., 2007; Encinas et al., 2011). Fulfilling the criteria of neural stem cells (Gage, 2000), these type-1 cells have been shown to be able to self-renew (Seri et al., 2001; Suh et al., 2007; Namba et al., 2009; Bonaguidi et al., 2011) and are multipotent, giving rise to neurons and astroglia, also called astrocytes (Suh et al., 2007; Bonaguidi et al., 2011).

Three modes of self-renewal of type-1 cells and, hence, the maintenance of stem cell pool have been reported (Bonaguidi et al., 2011). The first type of self-renewal is the asymmetric division of type-1 cells to produce one type-1 cell and one neural progenitor daughter cell, while an astroglia daughter cell is produced in the second type of asymmetric division (Bonaguidi et al., 2011). The last mode of self-renewal is a symmetric cell division generating two type-1 cells, as first reported by Bonaguidi and colleagues in 2011.

1.2.1.2. Proliferation of progenitor cells

Putative stem cells proliferate and give rise to progenitor cells, also termed as type-2 cells (Fukuda et al., 2003; Kempermann et al., 2004a), transiently amplifying progenitor cells (Kempermann et al., 2004a; Encinas et al., 2006), or intermediate progenitor cells (Bonaguidi et al., 2011). Type-2 cells have irregularly-shaped or round nucleus, with short tangential processes (Filippov et al., 2003; Fukuda et al., 2003). In contrast to type-1 cells, transiently-amplifying type-2 cells have high proliferative activity (Filippov et al., 2003; Fukuda et al., 2003; Kronenberg et al., 2003), and are responsible for most of the cell divisions in adult neurogenesis.

1.2.1.3. Cell fate determination and differentiation

Type-2 progenitor cells reflect a stage of transition from glial-like to neuronal-like and signal the start of neuronal-lineage determination. A subpopulation of type-2 cells, called type-2a cells, no longer expresses the astroglia marker GFAP, though the cells still retain radial glia marker FABP7 (Steiner et al., 2006). In contrast, type-2a cells start to express neuronal-lineage proteins, such as NeuroD1 (Steiner et al., 2006) and PROX1 (Steiner et al., 2008).

At this initial stage of development, synaptic inputs are beginning to be established in these progenitor cells of neuronal lineage (Tozuka et al., 2005; Wang et al., 2005; Ge et al., 2006). These functional synapses are receptive to the GABA neurotransmitter, which is inhibitory to mature neurons but excitatory to progenitor cells (Tozuka et al., 2005; Wang et al., 2005; Ge et al., 2006). This phenomenon is in parallel to neuronal development in the immature brain (reviewed in Ben-Ari, 2002). Possible sources of GABA in the adult hippocampus are the local interneurons (Tozuka et al., 2005), including parvalbumin-expressing interneurons (Song et al., 2013) and Ivy and neurogliaform interneurons (Markwardt et al., 2011).

1.2.1.4. Survival of new born cells

Several rounds of cell divisions rapidly amplify the neural progenitor cell population, producing a surplus of progenitor cells and immature neurons. However, not all cells will survive to the final integration into existing hippocampal networks (Kempermann et al., 2003), many excess cells are discarded by programmed cell death, or apoptosis (Biebl et al., 2000; Kuhn et al., 2005; Sierra et al., 2010).

Decision of survival of these newborn cells is made early in the process of neurogenesis, most cells are eliminated within two to five days after cell division (Sierra et al., 2010; Encinas et al., 2011), during the transition from progenitor cell stage to neuroblast stage (type-2 to type-3 cells) (Kuhn et al., 2005; Sierra et al., 2010; Encinas et al., 2011).

Survival rate of newborn cells has been found to be animal strain-specific. In a study of four common mouse strains, the survival rate was reported to be lowest in 129/SvJ (25%) and highest in CD1 (75%) mice (Kempermann et al.,

1997a), demonstrating the importance of genetic background in the baseline control of adult hippocampal neurogenesis.

1.2.1.5. Migration, morphological maturation and integration

Similar to the early decision of survival, localization of newborn cells is also determined early in the process of neurogenesis (Zhao et al., 2006). Roughly 60% of the newly generated cells stayed in the inner third of the granule cell layer, and this relative position has been reported to remain stable for at least 11 months (Kempermann et al., 2003).

Doublecortin, a protein associated with neuronal migration through its regulation of microtubules (Gleeson et al., 1999), was reported to be expressed as early as in type-2b cells, a subpopulation of type-2 neural progenitor cells (Kronenberg et al., 2003). Its expression is continued throughout till the immature neuron stage (Kempermann et al., 2004a). During this period of doublecortin expression, the newborn cells transit from a proliferative state to a post-mitotic immature neuron state, and undergo large morphological changes (Plumpe et al., 2006), building most of the dendritic tree.

Thus, following migration, newborn cells continue with morphological maturation, including axonal growth and dendritogenesis (Zhao et al., 2006). Studies of morphological stages of neuronal development in the adult hippocampus have found axonal growth to be initiated as soon as four days after mitosis (Hastings and Gould, 1999) and continued to grow and fully establish contact at CA3 by four to eight weeks post-viral injection (Zhao et al., 2006; Gu et al., 2012).

On the other hand, dendritogenesis was found to begin three days after injection of retroviral vector GFP (green fluorescent protein) (Zhao et al., 2006). However, the apical dendrites only reached the edge of the molecular layer 21 days post-cell division (Zhao et al., 2006). Dendritic spines were reported to be clearly present at the 21-day time-point but their spine density continued to increase until the 56-day time-point (Zhao et al., 2006). Therefore, in contrast to cell fate determination, survival and migration, morphological development of adult-born neurons takes a much longer time of roughly two months post-proliferation to reach a fully mature granule cell stage.

In parallel, during the post-mitotic immature neuron stage, neuronal development continues with the expression of calcium-binding proteins, firstly the transient expression of calretinin, followed by a switch to calbindin which is the calcium-binding protein present in mature hippocampal granule cells (Brandt et al., 2003). During the period of transient calretinin expression, newly generated immature neurons were found to express excitatory amino acid transporter (Brandt et al., 2003), demonstrating acquisition of glutamatergic phenotype at this stage. With reference to morphological development, it was found that doublecortin-positive cells with the most elaborate dendritic morphology were also calretinin positive (Plumpe et al., 2006). This suggests that the building of dendritic tree is largely completed at this immature neuronal stage, while dendritic spine development and fine-tuning continue into young neuronal stage.

In agreement to the timeline of dendritic development, synaptic plasticity is greatest in newborn immature neurons with a basic dendritic tree that has not reached the full complexity of a mature neuron (Schmidt-Hieber et al., 2004).

Also, at around this period, there was the first detection of glutamatergic inputs to newly born cells of two to four weeks old (Esposito et al., 2005; Ge et al., 2006), indicating the maturation of these adult-born cells into excitatory granule cells in hippocampal dentate gyrus.

Functional integration of adult-born neurons has been shown by the expression of immediate early genes in these newly generated cells, as well as mature granule cells, during hippocampal-dependent tasks (Jessberger and Kempermann, 2003; Ramirez-Amaya et al., 2006; Trouche et al., 2009). These reports support the idea of participation of newborn neurons in functional hippocampal circuitries.

1.2.2. Regulation and control of adult hippocampal neurogenesis

Formation of new neurons in a brain region is intuitively regulated by the same factors influencing that brain region. These factors regulate and control the level of plasticity in response to the region's computational activity. Activity-dependent regulation of neurogenesis may be conceptualised on several levels, such as external environment/stimuli, physiological state of animal, neurotransmitter systems, neuropeptide systems and others (Table 1.1). The regulators may affect one or more stages of adult neuronal development, to bring about the final outcome of generating the optimal quantity and quality of new neurons for integration into existing functional networks.

Table 1.1. Regulation and control of adult hippocampal neurogenesis.

Level of regulation	Factor	Manipulation		Effect	References
		↑	↓		
External environment/ stimuli	Enriched environment			Proliferation: - Survival: ↑	(Kempermann et al., 1997b, 1998; Nilsson et al., 1999)
	Hippocampal-dependent learning			Proliferation: - Survival: ↑	(Gould et al., 1999a)
	Stress			Proliferation: ↓ Survival: ↓ (in general)	(Gould et al., 1997; Gould et al., 1998; Malberg and Duman, 2003; Pham et al., 2003; Lee et al., 2006)
Physiological state of animal	Running			Proliferation: ↑ Survival: ↑	(van Praag et al., 1999a; van Praag et al., 1999b)
	Sleep		✓	Proliferation: ↓ Survival: ↓	(Roman et al., 2005; Guzman-Marin et al., 2007)
	Adrenal steroids (including glucocorticoids)	✓		Proliferation: ↓	(Gould et al., 1992; Cameron and Gould, 1994; Tanapat et al., 2001)
			✓	Proliferation: ↑ Survival: ↑	
	Estrogen	✓		Variable effects, dependent on other factors	(reviewed in Galea et al., 2013)
	Progesterone	✓		Proliferation: ↑ Survival: ↑	(Liu et al., 2010; Zhang et al., 2010; Bali et al., 2012)
	Androgens		✓	Proliferation: - Survival: ↓	(Spritzer and Galea, 2007; Carrier and Kabbaj, 2012)
	Testosterone	✓		Proliferation: - Survival: ↑	(Spritzer and Galea, 2007)

Physiological state of animal	DHEA	✓		Proliferation: ↑ Survival: ↑	(Karishma and Herbert, 2002)
	Ageing			Proliferation: ↓ Survival: ↓	(Kuhn et al., 1996; Kempermann et al., 1998)
Neurotransmitter systems	Glutamate	✓		Proliferation: ↓	(Cameron et al., 1995)
			✓	Proliferation: ↑	(Cameron et al., 1995)
	GABA	✓		Neuronal differentiation: ↑	(Tozuka et al., 2005)
			✓	Dendritogenesis: ↓ Survival: ↓	(Ge et al., 2006; Jagasia et al., 2009)
	Serotonin	✓		Proliferation: ↑	(Brezun and Daszuta, 2000)
			✓	Proliferation: ↓	(Brezun and Daszuta, 1999)
	Noradrenaline		✓	Proliferation: ↓	(Kulkarni et al., 2002)
	Dopamine	✓		Proliferation: - Survival: ↑	(Takamura et al., 2013)
			✓	Proliferation: ↓	(Hoglinger et al., 2004)
	Acetylcholine	✓		Proliferation: ↑ Survival: ↑	(Mohapel et al., 2005; Kaneko et al., 2006)
			✓	Proliferation: ↓ Survival: ↓	(Cooper-Kuhn et al., 2004; Mohapel et al., 2005)

Neuropeptide systems	Neuropeptide Y	✓		Proliferation: ↑ Neuronal differentiation: ↑ Survival: ↑	(Decressac et al., 2011)
			✓	Proliferation: ↓	(Howell et al., 2005)

1.2.2.1. External environment/stimuli

Since the discovery of neurogenesis in adult brain, one of the first reported factors regulating adult hippocampal neurogenesis was enriched environment (Kempermann et al., 1997b). Enriched environment was shown to have a positive effect on neurogenesis by promoting the survival of newly generated cells while having no effect on the proliferation rate (Kempermann et al., 1997b, 1998; Nilsson et al., 1999). Similarly, when adult rats underwent hippocampal-dependent learning tasks, but not hippocampal-independent learning tasks, the survival of newborn neurons was specifically increased without any alteration of the proliferation rate (Gould et al., 1999a). These reports demonstrated the regulation of adult neurogenesis in response to a more cognitive challenging environment, and hence supporting the functional relevance of adult-born neurons.

However, there is no simple correlation of stress to adult neurogenesis as some types of mild stress may be viewed as challenging or beneficial, such as enriched environment or learning, and can actually increase neurogenesis in hippocampus (as described earlier). To complicate matters, a few of the experimental stress paradigms could not be replicated by other researchers, and some even have conflicting data (Lee et al., 2006; Dagyte et al., 2009; Hanson et al., 2011). This highlights that the effect of stress is still poorly understood.

1.2.2.2. Physiological state of the animal

The physiological state of the animal, such as the level of physical activity, sleep, hormonal status and ageing, can also have an influence on the levels of neurogenesis in the adult hippocampus. For example, a high level of physical activity, in the form of voluntary running for laboratory animals, was

discovered to improve adult hippocampal neurogenesis, in both proliferation and survival stages (van Praag et al., 1999a; van Praag et al., 1999b).

Another physiological state, sleep, has also been found to be important in neurogenesis in the adult hippocampus (reviewed in Meerlo et al., 2009). In general, prolonged sleep disruption, introduced either as sleep deprivation or fragmentation, has been found to reduce cell proliferation and/or net neurogenesis in the hippocampus (Roman et al., 2005; Guzman-Marin et al., 2007).

Other physiological systems, like the endocrine system, have been studied too. One example is the hormones produced by the adrenal glands. Adrenal steroids, including glucocorticoids, were one of the first hormones found to regulate neurogenesis in the adult (Gould et al., 1992; Cameron and Gould, 1994). Level of adrenal steroids reciprocally correlated with the level of neuronal production in the adult dentate gyrus (Gould et al., 1992; Cameron and Gould, 1994). In fact, the increase in adrenal hormones was thought to be responsible for stress-induced suppression of cell proliferation in adult hippocampal neurogenesis (Tanapat et al., 2001).

Gonadal steroids have also been reported to regulate neurogenesis. The most well-known effect is that of estrogen, which has been shown to enhance the cell division rate of neurogenesis in adult female rats and female rats during proestrus (a stage during the estrous cycle with the highest levels of estrogen) (Tanapat et al., 1999). However, it was later found that the regulation by estrogen may not be as straight-forward as thought, and is dependent on many other factors like time, dose, age and sex (reviewed in Galea et al., 2013). Similarly, there are some reports of progesterone

increasing cell proliferation (Liu et al., 2010; Bali et al., 2012) and survival (Zhang et al., 2010) in adult rodents.

Additional studies on the regulation of adult hippocampal neurogenesis by other sex hormones, like testosterone (Spritzer and Galea, 2007; Hamson et al., 2013) and dehydroepiandosterone (DHEA) (Karishma and Herbert, 2002), have also shown enhancement in proliferation and/or survival of newborn cells. Though, again, like that of estrogen studies, other factors including duration of androgen treatment, may affect the upregulation effect of androgens. For example, a shorter period of androgen treatment revealed no effect on new neuron production in the hippocampus (Carrier and Kabbaj, 2012).

Physiological ageing has one of the strongest influences on adult hippocampal neurogenesis. Although neurons can be produced continually throughout life, the level of production dramatically decreases with age (Kuhn et al., 1996; Kempermann et al., 1998). Nevertheless, the aged brain is still be able to respond to external stimuli, such as an enriched environment, to produce an increase in the number of newborn neurons (Kempermann et al., 1998), illustrating the interplay of physiological state of animal and external stimuli on neurogenesis.

1.2.2.3. Neurotransmitter systems

Neurotransmitters are the form of chemical communications between neural cells. There are several types of neurotransmitter systems in the brain, namely the amino acid transmitters, glutamate and GABA; monoamine transmitters, serotonin or 5-HT (5-hydroxytryptamine), noradrenaline and dopamine; and other transmitters like acetylcholine. All of these innervate the

dentate gyrus (Leranth and Hajszan, 2007) and regulate neurogenesis in one way or another.

Glutamate is the main excitatory neurotransmitter in the brain. In hippocampus, dentate gyrus is the first stop of the trisynaptic circuit, collecting signals from the entorhinal cortex. This glutamatergic input from the pyramidal neurons of entorhinal cortex to the granule cells of dentate gyrus plays a role in hippocampal-dependent learning and memory, and also regulates neurogenesis in this region. Glutamatergic deafferentiation and glutamate receptor antagonism both resulted in an upregulation of neurogenesis in the adult hippocampus (Cameron et al., 1995). Conversely, activation of glutamate receptors suppressed generation of adult-born neurons in dentate gyrus (Cameron et al., 1995).

As for the main inhibitory neurotransmitter, there are several sources of GABA, including that from the medium septum, diagonal band of Broca, and local hippocampal interneurons, to the dentate gyrus. GABA is one of the first synaptic inputs to the neurogenic system and acts as an excitatory signal to the progenitor cells (Tozuka et al., 2005; Ge et al., 2006). Activation of GABA receptors by systemic administration of agonists was found to increase neuronal differentiation, and hence net neurogenesis (Tozuka et al., 2005). In addition, GABA also regulates the dendritogenesis (Ge et al., 2006; Jagasia et al., 2009) and survival of adult-born neurons in the hippocampus (Jagasia et al., 2009). Most of the studies investigated GABA inputs from the local hippocampal interneurons, and proposed that these interneurons in the neurogenic niche couple the activity of hippocampal circuitries to regulation of neurogenesis (Markwardt et al., 2011; Song et al., 2013), reflecting the activity-dependent nature of neuroplasticity.

For studies on the regulation by monoamine neurotransmitters, most efforts have been made in the field of serotonin or 5-HT. Serotonergic inputs in the dentate gyrus come from the raphe nuclei, and selective loss of serotonin could result in a decrease of cell proliferation in the hippocampus (Brezun and Daszuta, 1999). Conversely, increase in the local serotonin can upregulate the proliferation rate in hippocampal production of newborn cells (Brezun and Daszuta, 2000). Similarly, for other monoamine neurotransmitters, like noradrenaline (or norepinephrine) (Kulkarni et al., 2002) and dopamine (Hoglinger et al., 2004), selective depletion of each neurotransmitter resulted in a reduction of cell division in the dentate gyrus. In addition, pharmacological activation of dopamine receptors could stimulate an increase in survival of newborn cells while not altering the rate of proliferation in the dentate gyrus (Takamura et al., 2013).

Acetylcholine is an organic molecule neurotransmitter. In hippocampus, cholinergic inputs mainly come from the medial septum and modulate several hippocampal functions (Teles-Grilo Ruivo and Mellor, 2013), including neurogenesis. In addition, during neurogenesis, there are direct contacts of cholinergic fibres on newborn cells as early as seven days after cell division (Ide et al., 2008). Manipulation to reduce or increase levels of acetylcholine resulted in a reduction (Cooper-Kuhn et al., 2004; Mohapel et al., 2005) or increment (Mohapel et al., 2005; Kaneko et al., 2006) of proliferation and survival of newborn neurons, respectively.

In summary, most of the neurotransmitters, including GABA, serotonin, noradrenaline, dopamine and acetylcholine, with the exception of glutamate,

positively regulate proliferation and/or survival phases of neurogenesis in the hippocampus.

1.2.2.4. Neuropeptide systems

Neuropeptides are small protein molecules that form one class of chemical communication signals, and play modulatory roles in numerous functions of the brain. Currently, there are over 100 neuropeptides reported but only a selective few have been studied in the regulation of neurogenesis. One of the more commonly studied neuropeptides is neuropeptide Y. Neuropeptide Y is widely expressed in various brain regions, including the hypothalamus, cerebral cortex and hippocampal hilus (Morris, 1989). Increasing the levels of neuropeptide Y resulted in an enhancement of cell proliferation, neuronal differentiation and survival of newborn cells (Decressac et al., 2011), while lifelong loss of neuropeptide Y receptor, Y_1, had a decrease in proliferation (Howell et al., 2005).

1.2.2.5. Others

Many other neural systems regulate and control the level of neurogenesis in the adult hippocampus (reviewed in Lledo et al., 2006; Kempermann, 2011). Some of the important systems in the neurogenic niche are growth factors, neurotrophins, secreted proteins and immune system (Table 1.2). Another level of control would be within the cell. Some of the intracellular mechanisms include signalling pathways and activities of transcription factors (Table 1.3). All extracellular signals, like environmental stimuli, physiological activities, neurotransmitters, neuropeptides, autocrine and paracrine factors, converge to intracellular molecular mechanisms, resulting in the final cellular output of proliferation, differentiation, survival, morphological maturation or synaptic integration.

Table 1.2 Other regulators in the hippocampal neurogenic niche.

Type of regulator	Regulator	Manipulation		Main effect	References
		↑	↓		
Growth factor	EGF	✓		Proliferation: - Neuronal differentiation: ↓	(Kuhn et al., 1997)
	FGF2	✓		Proliferation: - Survival: -	
			✓	Proliferation: - Survival: -	(Yoshimura et al., 2001)
	IGF1	✓		Proliferation: ↑ Survival: ↑ Neuronal differentiation: ↑	(Aberg et al., 2000)
			✓	Proliferation: - Survival: ↓	(Lichtenwalner et al., 2006)
Neurotrophin	BDNF	✓		Survival: ↑	(Scharfman et al., 2005)
			✓	Proliferation: ↓ Survival: ↓	(Lee et al., 2002)
Secreted protein	Shh	✓		Proliferation: ↑	(Machold et al., 2003)
			✓	Proliferation: ↓	(Banerjee et al., 2005)
	Reelin		✓	Proliferation: ↓	(Won et al., 2006)
Immune system	Microglia			Complex effects	(Reviewed in Ziv and Schwartz, 2008)
	T cells				

Table 1.3. Intracellular mechanisms of adult hippocampal neurogenesis *in vivo*.

Type	Name	Main effect	References
Signalling pathway	ERK or MAPK pathway	Proliferation	(Kim et al., 2004; Choi et al., 2008)
	PI3K/Akt/mTOR pathway	Proliferation	(Palazuelos et al., 2012)
		Survival	(Bruel-Jungerman et al., 2009)
	Wnt pathway	Stem cell maintenance	(Reviewed in Varela-Nallar and Inestrosa, 2013)
		Proliferation	
		Neuronal differentiation	
Transcription factor	SOX2	Stem cell maintenance	(Ferri et al., 2004)
	STAT3	Stem cell maintenance	(Muller et al., 2009)
	CREB	Proliferation	(Nakagawa et al., 2002)
		Survival	(Jagasia et al., 2009)
		Dendritic maturation	(Jagasia et al., 2009)

1.2.3. Physiological functions of adult hippocampal neurogenesis

The exact physiological functions of neurogenesis in the adult hippocampus have been difficult to define precisely (Kempermann et al., 2004b; Lledo et al., 2006). Neuroplasticity in the form of adding whole new neurons, rather than simply enhancing synaptic strength, could have been acquired to address specific functional and computational needs. In hippocampus, dentate gyrus, where neurogenesis occurs, is the bottleneck of the trisynaptic neuronal circuitry (Kempermann et al., 2004b). Hence, having production of new neurons in this particular region may specifically boost the capacity of this limiting factor.

However, neurogenesis does not only alter neural computational power quantitatively, it also provides quality changes to the neuronal network. Generation of new cells in dentate gyrus does not simply add new granule cells to the region; during the multi-step process of production, a mixture of cell types with different cellular and electrophysiological properties are added to the repertoire of cells that can be utilised.

Also, neurogenesis is a relatively slow structural remodelling on the timescale of days to weeks, in contrast to synaptic transmission of milliseconds. Therefore, as new neurons are being added to the neuronal network quantitatively and qualitatively, they may not contribute to immediate functional demands that stimulated their production (Kempermann et al., 2004b). Instead, the roles of these new neurons may be to adapt the neuronal circuitries for future challenges of greater complexity. As such, the environment-interactive nature of neurogenesis allows development of individuality, even in laboratory animals with identical genetic background (Freund et al., 2013).

With the understanding of quantitative, qualitative and late contribution nature of neurogenesis, the functions of adult hippocampal neurogenesis may be better understood in its contribution to hippocampal functions in cognition and emotion.

1.2.3.1. Learning and memory

The most widely known and established function of hippocampus is learning and memory. Therefore, adult-born neurons are intuitively thought to play a role in learning and memory as well. Indeed, earlier studies have looked at the correlations between learning and memory performances and levels of adult hippocampal neurogenesis. One such study utilised natural differences in number of new neurons generated in ten different mouse strains, and showed a positive correlation to acquisition performance in water maze (Kempermann and Gage, 2002).

In addition, factors that increased generation of neurons in hippocampus generally also improved animals' cognition. For instance, enriched environment (Kempermann et al., 1997b) and voluntary running (van Praag et al., 1999b) both have been shown to increase survival of adult-born neurons in the hippocampus. This enhancement of neurogenesis has been positively associated with the improvement on hippocampal-dependent learning tasks like Morris water maze (Nilsson et al., 1999; van Praag et al., 1999a).

In contrast, when neurogenesis was suppressed by low dose irradiation or anti-proliferation toxin, their cognitive capabilities in water maze (Snyder et al., 2005) or hippocampal-dependent trace conditioning (Shors et al., 2001), respectively, were compromised. However, these experimental manipulations

of neurogenesis were usually not specific to the production of neurons only, cell birth of other cell types including glia cells could also be reduced, and thus confounding the effect on behavioural tasks. Nevertheless, such studies began to establish the causal link between adult hippocampal neurogenesis and learning and memory.

Despite many studies demonstrating the role of adult-born neurons in hippocampal-dependent learning and memory, other research failed to observe such simple positive association (Merrill et al., 2003). As such, the exact function of neurogenesis in cognition has been hard to define precisely. This could partly be due to the qualitative differences in the various cell types that arise in the process of neurogenesis, and the specific aspects of learning and memory tasks investigated (Dobrossy et al., 2003).

Therefore, in recent studies, attention has been shifted to understand neurogenesis in the context of dentate gyrus function. As newborn neurons are strategically positioned in the dentate gyrus, it has been proposed and shown that these young neurons play a role in pattern separation, a subset of hippocampal function in learning and memory (Clelland et al., 2009; Sahay et al., 2011; Nakashiba et al., 2012). Adult born neurons were decreased or increased, by X-ray irradiation or genetic manipulation, and were respectively found to impair or enhance ability in distinguishing similar contexts in radial arm maze, touch screen location discrimination and contextual fear conditioning tasks (Clelland et al., 2009; Sahay et al., 2011; Nakashiba et al., 2012).

Based on new understandings, review of experiments that included pattern separation in their behavioural testing has found a consistent evidence for

adult-born neurons supporting dentate gyrus function (reviewed in Aimone et al., 2011). Accordingly, it has now been accepted that one of the functions of adult hippocampal neurogenesis is pattern separation.

1.2.3.2. Emotion and stress

Hippocampus is not a homogenous structure. There are differences in the gene expression profiles and, afferent and efferent connections of septal (or dorsal) and temporal (or ventral) hippocampus (reviewed in Fanselow and Dong, 2010). Early reports showed region-specific lesions to either septal or temporal hippocampus correlated to behavioural impairments in Morris water maze spatial learning (Moser et al., 1993; Moser et al., 1995) or elevated plus maze anxiety test (Kjelstrup et al., 2002), respectively. However, cognitive and affective functions are not exclusively limited to septal and temporal hippocampus, respectively, both regions could function similarly, depending on the specific training and/or testing protocol, e.g. in Morris water maze (de Hoz et al., 2003; Ferbinteanu et al., 2003). Therefore, hippocampus may have both unitary and septotemporal-specific functions. In cases where septal and temporal hippocampus play differential functional roles, septal hippocampus appears to be more involved in cognition while temporal hippocampus in emotion and stress (reviewed in Bannerman et al., 2004; Fanselow and Dong, 2010).

Consequently, other than a role of adult hippocampal neurogenesis in cognition, recent studies have also started to investigate the role of new born neurons in emotion and stress. Specific reduction of neurogenesis in a transgenic mouse model resulted in an increase in anxiety-like behaviours while not affecting depressive-like behaviours (Revest et al., 2009). On the other hand, impairment of hippocampal neurogenesis may also induce

increased levels of despair, but this was only observed after stress treatment (Snyder et al., 2011). Though stress is a regulator of neurogenesis, stress response was found to be regulated by newborn cells in hippocampus. Abolishment of hippocampal neurogenesis resulted in an increase in the level of serum corticosterone (Snyder et al., 2011). Therefore, adult-born neurons could also contribute to the hippocampal function in the stress-induced regulation of hypothalamic-pituitary-adrenal (HPA) axis.

1.2.4. Clinical relevance of adult hippocampal neurogenesis

There is tremendous interest in adult neurogenesis, not only for its basic scientific understanding, but also for its clinical relevance. Impairment in neurogenesis may contribute to pathogenesis of disorders and diseases, while stimulation of neurogenesis may activate intrinsic regenerative mechanisms of repair. Therefore, research on adult neurogenesis could contribute to understanding of pathogenesis and offer new treatment methodologies for neurological conditions and disorders (Kempermann, 2011).

Specifically, clinical applications for adult hippocampal neurogenesis may be directly associated to its function in learning and memory, and emotion and stress, and hence may be relevant for Alzheimer's disease and depression.

1.2.4.1. Depression

In the last two decades of research, links have been established between adult hippocampal neurogenesis and a neuropsychiatric disorder, depression. In particular, it has been hypothesised that neurogenesis in the adult hippocampus could contribute to the pathogenesis of depression (Jacobs et al., 2000). This hypothesis arose from a few reasonings, one of them being that stress is one of the causal factors for depression (Kendler et al., 1999)

and stress generally impairs production of adult-born neurons (reviewed in McEwen, 1999). Indeed, in animal models of depression, unpredictable chronic mild stress has resulted in depressive-like behaviour and downregulation of neurogenesis (Surget et al., 2008).

In addition to possible involvement of adult hippocampal neurogenesis in pathogenesis, neurogenesis has also been implicated in the treatment of depression. One key report has found birth of new cells in the hippocampus to be necessary for the therapeutic effects of antidepressants (Santarelli et al., 2003). Also, almost all antidepressants stimulate adult neurogenesis (Malberg et al., 2000), and many compounds (Xu et al., 2007; Zhang et al., 2009; Jiang et al., 2012; Vithlani et al., 2013) and activities (Duman et al., 2008; Veena et al., 2009) that enhance production of neurons have antidepressant effect as well.

Despite the implication of adult hippocampal neurogenesis in etiology and treatment of depression, it is not fully necessary or sufficient for this neuropsychiatric disorder (Petrik et al., 2012). However, it is undeniable that there exists a component of neurogenesis in depression, and the translational potential of neurogenesis for treating depression (Eisch and Petrik, 2012).

1.2.4.2. Alzheimer's disease

Other than neuropsychiatric disorders, neurodegenerative diseases have also received attention in their relation with adult hippocampal neurogenesis. Specifically, Alzheimer's disease perhaps has the most direct link with the learning and memory function of adult-born neurons in the hippocampus. In the disease progression of Alzheimer's disease, hippocampus is one of the first regions to be affected (Braak et al., 1993), and within the hippocampus,

alterations in neurogenesis presumably occur earlier than neuronal loss (reviewed in Lazarov and Marr, 2010).

The link between Alzheimer's disease and adult hippocampal neurogenesis comes from the common pool of molecular players. For example, ApoE4 is an important genetic risk factor for Alzheimer's disease (reviewed in Bu, 2009) and is also involved in the maintenance of neural precursor cell population in the dentate gyrus (Yang et al., 2011). In addition, many transgenic animal models for Alzheimer's disease exhibit altered production of adult neurons in the hippocampus (reviewed in Mu and Gage, 2011), supporting an involvement of neurogenesis in the disease.

Although the quantitative scale of adult hippocampal neurogenesis may not be sufficient to replace massive neuronal loss in Alzheimer's disease, stimulating continual generation of neurons in the aged Alzheimer's brain has been proposed as one therapeutic strategy to help with preservation of cognitive capacity (Lazarov et al., 2010; Mu and Gage, 2011).

1.2.5. Adult neurogenesis in humans and animal models

Until half a century ago, adult neurogenesis was thought to be non-existent in the mammalian brains (reviewed in Ming and Song, 2005). It was only in the 1960s that neurogenesis was shown to be present in adult rats (Altman, 1963; Altman and Das, 1965). Since then, it took another three decades for evidence of adult-born neurons in the human hippocampus to be presented (Eriksson et al., 1998). The study demonstrated presence of a nucleotide analogue, BrdU (5-bromo-2-deoxyuridine), in cells expressing neuronal markers, including NeuN, calbindin, or NSE (neuron specific enolase) (Eriksson et al., 1998). As BrdU could only be incorporated during DNA

synthesis in cell cycle, neurons with BrdU must have been generated at the time of BrdU administration. However, it was a unique piece of evidence that could not be repeated as BrdU was administered in cancer patients for tumour staging, a procedure no longer in practice now.

Thereafter, further investigations of adult hippocampal neurogenesis in the human brain depended on indirect evidence of protein marker expression associated with the different stages of neurogenesis (Knoth et al., 2010). Recently, a new direct method using ^{14}C has shown neurogenesis in adult human hippocampus (Spalding et al., 2013). Similar to BrdU, ^{14}C was taken up by proliferating cells and served as a birth-dating marker. Unlike BrdU, ^{14}C was "administered" during a particular time period in history, nuclear bomb testing from 1955 to 1963, and could be retrospectively detected by accelerator mass spectrometry (Spalding et al., 2013).

Although there may be species-specific effects, some similarities may be shared across species, including rodents and humans. For example, there exists presence of neurogenesis in the hippocampus of both rodents and humans throughout life (Altman and Das, 1965; Kuhn et al., 1996; Eriksson et al., 1998), with approximately 0.05% (mouse), 0.2% (rat) and 0.004% (human) new neurons added to each dentate gyrus per day (Kempermann et al., 1997a; Cameron and McKay, 2001; Spalding et al., 2013). There is also age-related decline in hippocampal neurogenesis in laboratory animals and humans, though the rate and magnitude of decrease appears to be smaller in humans than in mice (Ben Abdallah et al., 2010; Spalding et al., 2013). As research opportunities on the adult human hippocampus are rare and limited, most studies have been conducted on rodents, in the hope that the functional implications and significance are translatable to humans.

1.3. Background of relaxin-3

Relaxin-3 (RLN3; or insulin-like 7 (INSL7)) was first discovered in a bioinformatics search for relaxin-related genes, and is a member of the insulin/relaxin superfamily (Bathgate et al., 2002). Currently, there are seven members in the relaxin family, namely relaxin 1, 2, 3 (RLN1-3) and insulin-like 3, 4, 5, 6 (INSL3-6). There is no relaxin-2 gene in the rodents, instead, relaxin-1 in rodents functionally corresponds to relaxin-2 of human, and they are generally referred to as relaxin (Wilkinson et al., 2005).

The protein structure of the relaxin family is similar to that of insulin, being produced as a pro-hormone peptide, which is then cleaved to produce B- and A-chains that are bridged by three characteristic disulphide bonds (Bathgate et al., 2002; Rosengren et al., 2006). Degradation of relaxin-3 is mediated by insulin-degrading enzyme (IDE; also known as insulysin) (Bennett et al., 2009) which also degrades most of other members in the insulin/relaxin superfamily, including insulin, IGF1 and IGF2 (insulin-like growth factors; Misbin et al., 1983), relaxin, and INSL3 (Bennett et al., 2009).

Relaxin-3 has a very highly conserved amino acid sequence (>90%) and protein structure, especially in the B- and A-chain peptides, between human, primate, pig, rat and mouse (Bathgate et al., 2002; Burazin et al., 2002; Silvertown et al., 2010). The high degree of conservation suggests its functional importance in evolution. Phylogenetically, relaxin-3 is the most ancestral gene among the relaxin family members (Bathgate et al., 2002; Hsu et al., 2005; Wilkinson et al., 2005).

Relaxin-3 is a ligand to several receptors, including RXFP1 (relaxin/insulin-like family peptide receptor 1; or LGR7 (leucine-rich repeat-containing G-protein coupled receptor 7)) (Bathgate et al., 2002; Sudo et al., 2003), RXFP3 (relaxin/insulin-like family peptide receptor 3; GPCR135 (G-protein coupled receptor 135); or SALPR (somatostatin- and angiotensin-like peptide receptor)) (Liu et al., 2003b) and RXFP4 (relaxin/insulin-like family peptide receptor 4; GPCR142 (G-protein coupled receptor 142) or GPR100) (Liu et al., 2003a), and none of the other RXFP2 (relaxin/insulin-like family peptide receptor 2) (Sudo et al., 2003), insulin, or IGF receptors . As the affinity to RXFP3 is the strongest, and has the most overlap in expression and distribution, relaxin-3's cognate receptor is thought to be RXFP3 (Liu et al., 2003b; Chen et al., 2005a; Ma et al., 2007; Smith et al., 2010). On the other hand, RXFP1 and RXFP4 are proposed to be cognate receptors for relaxin (Hsu et al., 2002) and INSL5 (Liu et al., 2005), respectively (Fig. 1.2).

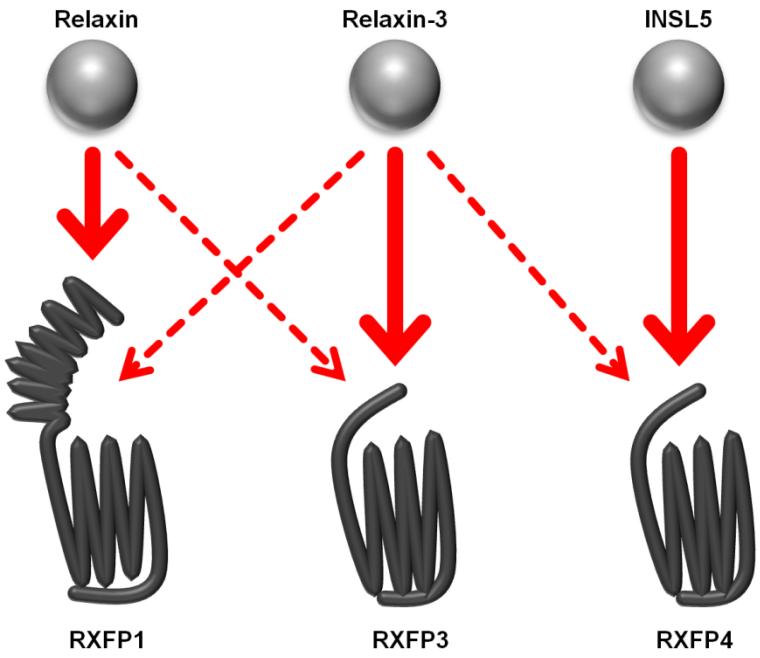

Figure 1.2. Diagrammatic representation of binding interactions of relaxin family members and RXFPs. The weight of arrow corresponds to the relative binding affinities, while the ligands and receptors are not drawn to scale.

1.3.1. Expression and distribution of relaxin-3

Developmentally, the mRNA expression of relaxin-3 started from embryonic day 18 of rats, and gradually increased into the young adulthood of eight weeks (last time-point investigated) (Miyamoto et al., 2008). As for the protein expression of relaxin-3, there was weak detection by fluorescent immunohistochemistry in postnatal day 1, and stronger adult-level detection by postnatal day 7 (Miyamoto et al., 2008).

Relaxin-3 expression is relatively restricted in the whole body, with expression found in the brain and testis only (Bathgate et al., 2002; Liu et al., 2003b; Silvertown et al., 2010). Within the brain, relaxin-3 is mostly expressed in the nucleus incertus, a region near the fourth ventricle (reviewed in Ryan et al., 2011; Smith et al., 2011), and has been reported in several species, including the mouse (Bathgate et al., 2002; Smith et al., 2010), rat (Burazin et al., 2002; Liu et al., 2003b; Tanaka et al., 2005; Ma et al., 2007; Brailoiu et al., 2009), primate (Ma et al., 2009b; Silvertown et al., 2010) and human (Silvertown et al., 2010).

In the rat nucleus incertus, relaxin-3 is present in roughly 30% of all neurons (Ma et al., 2013) and the relaxin-3 population size was reported to be roughly 2000 cells (Tanaka et al., 2005). Most, if not all, relaxin-3 positive cells express CRF-R1 (corticotropin releasing factor receptor 1; or officially termed as CRHR1 (corticotropin releasing hormone receptor 1)) (Tanaka et al., 2005; Ma et al., 2013). In addition, majority of the relaxin-3 neurons also express 5-HT_{1A} receptor (Miyamoto et al., 2008). Relaxin-3 neurons are likely to be inhibitory in nature as most of them express GAD65 (glutamic acid decarboxylase-65; or GAD2 (glutamic acid decarboxylase 2) (Ma et al., 2007), an enzyme synthesising the neurotransmitter GABA.

Other than the nucleus incertus, relaxin-3 is also expressed in subregions of periaqueductal gray, raphe nuclei and substantia nigra (Tanaka et al., 2005; Ma et al., 2007; Brailoiu et al., 2009; Ma et al., 2009b; Silvertown et al., 2010; Smith et al., 2010). However, relaxin-3 neurons are present in this regions at lower absolute cell counts, in the range of 300-500 cells, as compared to the 2000 cells in nucleus incertus of adult rat (Tanaka et al., 2005).

From these groups of neurons, relaxin-3 is transmitted to numerous brain regions via long projection fibres. Dense innervations have been reported in regions like the hypothalamus, medial septum, hippocampus and intergeniculate leaf (Tanaka et al., 2005; Ma et al., 2007; Brailoiu et al., 2009; Ma et al., 2009b; Smith et al., 2010), suggesting involvement of relaxin-3 in the functions of these regions. One region of interest is the hippocampus, which is differentially targeted by relaxin-3 along the septo-temporal axis. Septal hippocampus is only sparsely innervated with relaxin-3 projections while temporal hippocampus has a much higher density of relaxin-3 fibres (Tanaka et al., 2005; Ma et al., 2007; Brailoiu et al., 2009; Ma et al., 2009b; Smith et al., 2010).

In brain regions receiving relaxin-3 innervation, e.g. lateral hypothalamus and medial septum, ultrastructural localisation of relaxin-3 has found its presence in dense-cored vesicle-like structures in axonal synaptic terminals (Tanaka et al., 2005; Olucha-Bordonau et al., 2012), supporting its role in synaptic chemical transmission to targeted regions.

1.3.2. Regulations and functions of relaxin-3

Neuropeptides play modulatory roles in the central nervous system, and are involved in various brain functions including energy homeostasis, mood and motivation, drug addiction, arousal states, cognition, stress response, and regulation of neuroendocrinology (reviewed in van den Pol, 2012). Likewise, as a neuropeptide, relaxin-3 has been found to be implicated in a wide range of functions (Table 1.4 and Table 1.5), many of which are beginning to be unravelled in the field.

1.3.2.1. Regulations and functions of relaxin-3 at the cellular and molecular level

Regulation of relaxin-3 expression at the molecular level has not been investigated much. Currently, there is only one report on the regulation of mouse relaxin-3 gene promoter activity, which could be enhanced by forskolin and an analog of cAMP (cyclic adenosine monophosphate) (Tanaka et al., 2009). In addition, it was also found that these stimulations of relaxin-3 promoter activity were protein kinase A (PKA)-dependent (Tanaka et al., 2009).

For the cellular and molecular functions, relaxin-3 has been reported to be able to activate RXFP1, RXFP3 and RXFP4, which are G-protein coupled receptors (GPCRs) (reviewed in Bathgate et al., 2013a). In particular, RXFP3 and RXFP4 belong to class A GPCRs (Bathgate et al., 2013b), and they are coupled to the G_i/G_0 protein subunit that inhibits adenylate cyclase (Liu et al., 2003a; Liu et al., 2003b; van der Westhuizen et al., 2005). Upon activation by relaxin-3, forskolin-induced cAMP production was blocked in cells expressing RXFP3 or RXFP4 (Liu et al., 2003a; Liu et al., 2003b; van der Westhuizen et al., 2007).

Table 1.4. Regulations of relaxin-3.

Level	Regulator	Manipulation		Effect	References
		↑	↓		
Molecular	cAMP	✓		↑ relaxin-3 promoter activity	(Tanaka et al., 2009)
	CRF	✓		↑ relaxin-3 promoter activity	
Systems	Stress	✓		↑ relaxin-3 mRNA and protein	(Tanaka et al., 2005; Banerjee et al., 2010; Lenglos et al., 2013)
	Serotonin		✓	↑ relaxin-3 mRNA	(Miyamoto et al., 2008)
Behavioural	Alcohol and sucrose intake	✓		↑ relaxin-3 mRNA	(Ryan et al., 2014)

Table 1.5. Functions of relaxin-3

Level	Manipulation		Effect	References
	↑	↓		
Molecular	✓		↓ cAMP production (via RXFP3, RXFP4)	(Liu et al., 2003a; Liu et al., 2003b; van der Westhuizen et al., 2007)
	✓		↑ cAMP production (via RXFP1)	(Sudo et al., 2003)
	✓		↑ intracellular calcium concentration (via RXFP3, RXFP4)	(Liu et al., 2003a; Liu et al., 2003b)
	✓		↑ ERK pathway (via RXFP3)	(van der Westhuizen et al., 2005; van der Westhuizen et al., 2007)
	✓		↑ PI3K pathway (via RXFP3)	
Systems	✓		↑ CRF mRNA	(Watanabe et al., 2011a)
	✓		↑ HPA axis	(Watanabe et al., 2011a)
	✓		↑ HPG axis	(McGowan et al., 2008)
	✓		↑ hippocampal theta activity	(Ma et al., 2009a)
		✓	↓ hippocampal theta activity	
Behavioural	✓		↑ food intake	(McGowan et al., 2005; Hida et al., 2006; McGowan et al., 2006; McGowan et al., 2007; Ganella et al., 2013)
		✓	↓/no change in spatial memory	(Ma et al., 2009a; Callander et al., 2012)
	✓		↓ anxiety-like behaviour	(Nakazawa et al., 2013; Ryan et al., 2013a)
		✓	↓/no change in anxiety-like behaviour	(Watanabe et al., 2011b; Callander et al., 2012)
		✓	↑ fear-related behaviour	(Pereira et al., 2013; Lee et al., 2014)
		✓	↓ alcohol seeking/relapse	(Ryan et al., 2013b)
		✓	↓ circadian activity	(Smith et al., 2012)

In addition, activation of RXFP3 by relaxin-3 could lead to several downstream signalling cascades, including the ERK1/2 (extracellular signal-regulated kinase 1/2) and PI3K pathways (van der Westhuizen et al., 2005; van der Westhuizen et al., 2007). Induction of ERK1/2 pathway involved several signalling mechanisms and molecules, including RXFP3 receptor internalization, epidermal growth factor (EGF) receptor transactivation, protein kinase C (PKC), Src tyrosine kinase, and phospholipase C (PLC) (van der Westhuizen et al., 2007). As for PI3K pathway, it is implicated in cellular survival, and relaxin-3 has been found to be protective against apoptosis in human neuroblastoma cells (Silvertown et al., 2010).

On the other hand, relaxin-3's activation of RXFP1 led to stimulation of cAMP (Sudo et al., 2003), and induction of RXFP1-associated signalling pathways and functions, such as antifibrotic effect (Hossain et al., 2011).

1.3.2.2. Regulations and functions of relaxin-3 at the systems and behavioural level

Studies on the regulation of relaxin-3 have been based on the type of receptors co-expressed by relaxin-3 neurons in the rat nucleus incertus. The first receptor reported was CRF-R1 (corticotropin releasing factor receptor 1) (Tanaka et al., 2005).

Administration of CRF intracerebroventricularly was able to activate relaxin-3 neurons in the nucleus incertus, as shown by their expression of c-Fos (Tanaka et al., 2005). As endogenous CRF could be induced by stress, stressful conditions, like water-immersion restraint, and repeated forced swim, could also result in the expression of c-Fos in relaxin-3 or nucleus incertus

neurons (Tanaka et al., 2005; Banerjee et al., 2010). In addition, relaxin-3 mRNA and protein levels in the nucleus incertus were increased in the presence of stress (Tanaka et al., 2005; Banerjee et al., 2010; Lenglos et al., 2013), and this effect is CRF-R1 mediated as it could be blocked by CRF-R1 antagonist (Banerjee et al., 2010). Furthermore, enhancement of relaxin-3 gene promoter activity was also present *in vitro*, after CRF treatment on CRF-R1 expressing cells, via the cAMP-PKA pathway (Tanaka et al., 2009).

In 2008, 5-HT_{1A} receptor was the second receptor to be shown co-expressing in relaxin-3 positive cells (Miyamoto et al., 2008). Similar to CRF/CRF-R1 system, relaxin-3 neurons with 5-HT_{1A} receptor could be regulated by serotonin (5-HT). Specifically, when serotonin was depleted in the brain, there was an increase in the mRNA expression of relaxin-3 in rat nucleus incertus (Miyamoto et al., 2008), suggesting a negative regulation by serotonin.

While regulation of relaxin-3 is associated with receptors expressed on relaxin-3 neurons, functions of relaxin-3 correlate to its projections in the various forebrain regions. One of the regions targeted by relaxin-3 is the hypothalamus. Hypothalamus is the first part of the HPA (hypothalamus-pituitary-adrenal) axis, a neuroendocrine system important in stress responses. Intracerebroventricular injection of relaxin-3 could activate hypothalamic neurons, and increase CRF mRNA levels (Watanabe et al., 2011a). Downstream stress response, such as levels of adrenocorticotropic hormone in the plasma, were also elevated (Watanabe et al., 2011a), demonstrating the activation of HPA axis by relaxin-3.

Hypothalamus is also involved in the HPG (hypothalamic–pituitary–gonadal) axis that regulates the reproductive system. Stimulation with relaxin-3

resulted in the dose-dependent release of gonadotropin-releasing hormone (GnRH; also known as luteinizing-hormone-releasing hormone (LHRH)) from hypothalamic tissue *ex vivo*, and hypothalamic cells *in vitro* (McGowan et al., 2008). On the other hand, *in vivo* injection of relaxin-3 in the lateral ventricles and paraventricular nucleus upregulated the levels of luteinizing hormone in plasma (McGowan et al., 2008), supporting the role of relaxin-3 in HPG axis.

An additional function of hypothalamus is the control of appetite and food intake. In fact, the first reported function of relaxin-3 is the stimulation of food intake (McGowan et al., 2005). When relaxin-3 was administered into lateral ventricular and various hypothalamic nuclei, acutely (McGowan et al., 2005; McGowan et al., 2006; McGowan et al., 2007) and chronically (Hida et al., 2006; McGowan et al., 2006; Ganella et al., 2013), into rat brains, food intake was increased in satiated rats and weight gain was significantly higher. Relaxin-3-regulated changes in appetite was reported to be independent of hypothalamic neuropeptide Y, proopiomelanocortin or agouti-related peptide (McGowan et al., 2005; Ganella et al., 2013), a group of proteins involved in feeding behaviour. Instead, relaxin-3 induced changes in the levels of hypothalamic oxytocin, which may mediate the effects of relaxin-3 in feeding (Ganella et al., 2013; Nakazawa et al., 2013). Furthermore, in rats receiving relaxin-3 administration in the brain, plasma levels of leptin and insulin were increased, while plasma thyroid stimulating hormone was decreased (Hida et al., 2006; McGowan et al., 2006), suggesting a perturbed energy homeostasis.

Other than hypothalamus-mediated functions of relaxin-3, relaxin-3 may play a role via other brain regions. For example, relaxin-3 has dense projections in the medial septum which is involved in hippocampal theta rhythm and spatial memory. Indeed, when RXFP3 was selectively stimulated in the medial

septum, hippocampal theta rhythm activity was enhanced (Ma et al., 2009a). Conversely, when RXFP3 was pharmacological blocked, enriched environment-induced theta rhythm was decreased, so was spatial memory in a spontaneous alternation task (Ma et al., 2009a). However, when relaxin-3 gene expression was silenced in the nucleus incertus, spatial memory was not affected in the same task (Callander et al., 2012), suggesting that duration of blockade and/or localisation of blockade (intraseptal vs. whole brain) are important factors in the role of relaxin-3 in spatial memory.

In addition to cognitive functions, there is a role for relaxin-3 in the regulation of emotion and mood. Neuroanatomically, relaxin-3 fibres and/or RXFP3 are present in brain regions associated with anxiety/depression, such as the amygdala, raphe nuclei, hypothalamus and hippocampus (Smith et al., 2011). Infusion of relaxin-3 or RXFP3-specific agonist into the lateral ventricles of the brain attenuated anxiety-like and stress-induced depressive-like behaviour in rats (Nakazawa et al., 2013; Ryan et al., 2013a). However, loss-of-function studies of relaxin-3 in viral-mediated knockdown model and knockout mouse model found no differences and decreases, respectively, in anxiety-related behavioural paradigms (Watanabe et al., 2011b; Callander et al., 2012). Again, the findings so far suggest additional factors affecting relaxin-3's modulatory role in anxiety/depression-like behaviour.

Related to emotion and mood, relaxin-3 has also been found to be involved in fear-related behaviour. In a CRF-R1-selective nucleus incertus lesion model, in which levels relaxin-3 markedly decreased, there was increased freezing in cued fear conditioning (Lee et al., 2014). Furthermore, in another nucleus incertus lesion model, in which neuronal damage was induced electrolytically and relaxin-3 reduced, there was slower extinction of freezing behaviour in a

conditioned fear paradigm (Pereira et al., 2013). Together, these results demonstrated relaxin-3/nucleus incertus's contribution to at least two different aspects of fear-related behaviour and memory.

Recently, relaxin-3/RXFP3 system has been reported to be involved in reward seeking, in particular, alcohol intake and relapse behaviour (Ryan et al., 2013b). Central and local antagonism of RXFP3 in the brain and bed nucleus of the stria terminalis (BNST), respectively, was able to induce a reduction in alcohol self-administration and relapse behaviour (Ryan et al., 2013b). In contrast, natural reward seeking, e.g. sucrose, was not affected (Ryan et al., 2013b), supporting the importance of relaxin-3 in alcohol-specific addiction. However, both alcohol and sucrose intake positively correlated with the levels of relaxin-3 mRNA expression in the nucleus incertus (Ryan et al., 2014), suggesting an involvement of relaxin-3 in more general reward seeking behaviours too.

Other functions of relaxin-3 have also been shown, for example, a role in general arousal, wakefulness and/or motivation has been implicated in a behavioural phenotyping study of relaxin-3 deficient mice (Smith et al., 2012). Relaxin-3 knockout mice had reduced circadian activity on the running wheels during the dark/active phase, and spent an increased amount of time immobile (Smith et al., 2012). This circadian hypoactivity has been attributed to possible loss of function of relaxin-3 in the septohippocampal system (spatial memory, exploration and REM sleep), mesolimbic dopaminergic pathway (motivation and reinforcement), arousal centres, including the raphe nuclei, lateral hypothalamus, periaqueductal grey and intergeniculate leaflet (Smith et al., 2012; Blasiak et al., 2013).

In summary, after ten years of research into the functional roles, relaxin-3, like many other neuropeptides, has modulatory effects on multiple aspects of brain systems and behaviours, such as the stress and sex hormonal systems, food intake, cognition, emotion and mood, and general arousal behaviours. Based on the functions and neuroanatomical distribution, relaxin-3 has been proposed to be one of the ascending arousal systems (Smith et al., 2011), like that of serotonin from the raphe nuclei, noradrenaline from the locus coeruleus and orexin from the lateral hypothalamus (Saper et al., 2005). Interestingly, other than the locus coeruleus, relaxin-3 projects to most of other arousal centres, and could be reciprocally regulated by at least the serotonergic system (Miyamoto et al., 2008; Smith et al., 2011). However, with only a decade of studies, there is still a lot to discover and investigate.

1.3.3. Relaxin-3 in human

In humans, the relaxin-3 gene was shown to be expressed in the brain and testis (Liu et al., 2003b; Silvertown et al., 2010). As several of the earlier functional studies on relaxin-3 looked at appetite and food intake, research on relaxin-3 in humans has, thus far, focused on this area as well.

Serum levels of relaxin-3 was found to be significantly higher in patients with metabolic syndrome (Ghattas et al., 2013). However, specifically in diabetes patients, there was no difference in levels of relaxin-3 in their plasma samples (Zhang et al., 2013).

In addition, in a study on patients treated with antipsychotic drugs, one relaxin-3 SNP (single nucleotide polymorphisms) was found to be associated with hypercholesterolemia, and not other metabolic syndromes (obesity, diabetes, hypertriglyceridemia and hypertension) investigated (Munro et al.,

2012). Further associations were also found between two RXFP3 SNPs and metabolic syndromes like diabetes, hypercholesterolemia and obesity (Munro et al., 2012). However, it was not shown if these SNPs contribute to gain- or loss-of-functions of relaxin-3/RXFP3.

Generally, the studies in human have supported a role for relaxin-3 in some but not all metabolic syndromes, while no other studies have been reported for other relaxin-3 functions that were discovered in animal models.

1.4. Hypothesis

Relaxin-3 has been shown to be regulated by stressful stimuli, CRF and serotonin systems, which have been implicated in the regulation of adult hippocampal neurogenesis. In addition, relaxin-3 has a widespread of functions, influencing cognition, emotion and mood, motivation and addiction, circadian activity and appetite, many of which affect or are affected by levels of neurogenesis in the adult hippocampus. Furthermore, there are direct projections of relaxin-3 in the hippocampus, especially the temporal hippocampus. Therefore, we hypothesized that relaxin-3 will play a role in regulating the levels of adult hippocampal neurogenesis.

Specifically, to test the hypothesis, I aimed to establish:

1) the effects of lifelong deficiency of relaxin-3 and sex differences on the basal physiological levels of adult hippocampal neurogenesis.
2) the effects of age-related influence, lifelong deficiency of relaxin-3 and sex differences on the basal physiological levels of adult hippocampal neurogenesis.
3) the connections of relaxin-3 fibres in the hippocampus.

Chapter 2

Hippocampal neurogenesis in young WT and relaxin-3 KO adult mice

2. Hippocampal neurogenesis in young WT and relaxin-3 KO adult mice

2.1. Introduction

To investigate the role of relaxin-3 in adult hippocampal neurogenesis, a loss-of-function approach was utilised. Specifically, the strain of relaxin-3 knockout (KO) mice, *Rln3*$^{TM1/Lex}$, used in this study was generated on a mixed background of 129S5:B6 by Lexicon Pharmaceuticals, Inc. (TX, USA) (Sutton et al., 2009). Deficiency of relaxin-3 was verified in the homozygous KO while a reduction of *LacZ* reporter gene expression was shown in the heterozygote animals (Smith et al., 2009b).

Earlier studies on relaxin-3 KO mice were carried out on the mixed genetic background of 129S5:B6, and were sensitive to dietary conditions. Specifically, when the animals were maintained on a breeding diet with roughly 50% higher fat calories than the standard diet, the KO animals had reduced body weight, plasma insulin and leptin levels as compared to the WTs (Sutton et al., 2009). Such differences in metabolic symptoms were not observed when the animals were fed standard diet (Smith et al., 2009a).

Relaxin-3 KO mice may be sensitive to genetic background as well. In particular, there were subtle behaviour differences, in automated locomotor cells and weight loss response to stress, between animals on the mixed 129S5:B6 and backcrossed C57BL/6J background (Smith et al., 2009a; Smith et al., 2012). In addition, relaxin-3 KO mice had decreased anxiety-like behaviour, particularly in the elevated plus maze, a finding only present in the backcrossed C57BL/6N, and not backcrossed C57/BL6J background

(Watanabe et al., 2011b; Smith et al., 2012). However, the disparities in behavioural phenotype may not be limited to genetic background differences, the method of knocking out relaxin-3 gene and general housing conditions in different institutes could also contribute to the dissimilarities found.

In this study, a first attempt to examine levels of adult hippocampal neurogenesis in relaxin-3 KO mice was carried out. The strain of mice in study has been behaviourally phenotyped and reported (Smith et al., 2012). Generally, relaxin-3 KO animals were similar to their WT littermates physiologically in weight and general health, and behaviourally in cognition, anxiety- and depressive-related performance and stress responses (Smith et al., 2012). The main phenotypic difference was in their circadian activity, with the KO mice less active on running wheels in the dark/active phase (Smith et al., 2012). This finding supports the neuroanatomical basis of relaxin-3 as a possible ascending arousal system. Interestingly, many other arousal systems, such as serotonin, noradrenaline, dopamine and acetylcholine, could also regulate plasticity and neurogenesis in the adult hippocampus.

Historically, adult hippocampal neurogenesis was first demonstrated by the incorporation of tritiated thymidine in morphologically identified granule cells (Altman, 1963; Altman and Das, 1965). Presence of tritiated thymidine served as a direct evidence of uptake of nucleoside, due to DNA synthesis in cell proliferation, at the time of thymidine-H^3 administration.

In later studies, BrdU (5-bromo-2-deoxyuridine) became commonly used, as a nonradioactive alternative to tritiated thymidine. Detection of BrdU was immunohistochemical and could be combined with other protein marker immunolabelling to identify the phenotype of cell of interest. For example, in

1996, fluorescent immunolabelled BrdU was shown to be colocalised in NeuN-expressing cells by confocal scanning laser microscopy (Kuhn et al., 1996). The feasibility to be immunofluorescently co-localised with cell type markers and birth-dating nature of BrdU allowed it to become a popular technique for demonstrating neurogenesis in the adult brain. Indeed, the first study to report adult hippocampal neurogenesis in the human brain applied this method, BrdU in combination with one of the neuronal markers, NeuN, calbindin and neuron specific enolase (NSE) (Eriksson et al., 1998).

Subsequently, another technique was developed to study the generation of new cells in the brain in year 2001. As retroviruses selectively infect dividing cells, GFP (green fluorescent protein) retroviral vector could be injected into the dentate gyrus and specifically taken up by newly generated cells. GFP-labelled cells were then available for further analysis. The advantage of this technique is the ability to visualise newborn cells directly in living tissue slices, enabling electrophysiological studies to be carried out specifically on these GFP-positive cells (van Praag et al., 2002). In addition, as GFP fills up the entire cell, including the cell body and neurites, structural analysis of adult-born neurons could also be carried out (van Praag et al., 2002).

In this study, to establish the effects of lifelong depletion of relaxin-3 and sex differences on the basal physiological levels of adult hippocampal neurogenesis, BrdU was utilised to birth-date newborn neurons in young adult mice. BrdU was selected as an investigation tool for its nonradioactive and non-viral nature, and ease of administration systemically (c.f. intrahippocampal injection of retroviral vectors). The stages of neurogenesis examined were proliferation, cell fate determination, cell migration and survival of newly generated cells.

2.2. Materials and methods

2.2.1. Animals

Relaxin-3 (*Rln3*) knockout (KO)/*LacZ* knockin mouse strain was generated by Lexicon Pharmaceuticals, Inc. (TX, USA) on a mixed 129S5:B6 genetic background (Sutton et al., 2009), and was subsequently backcrossed to C57BL/6J background for 10 generations (Smith et al., 2012). Relaxin-3 KO and wildtype (WT) littermate controls were generated via heterozygous pairings and were housed in mixed genotype single-sex cages, with four to six mice per cage. The housing condition was maintained in a temperature and humidity-controlled environment, on a 12-hour light/dark cycle (lights on 0700-1900 h) with ad libitum access to food and water. All animal procedures were carried out in the Florey Institute of Neuroscience and Mental Health, Australia, were approved by the Howard Florey Institute Animal Welfare Committee, and in accordance with the ethical guidelines issued by the National Health and Medical Research Council of Australia.

Twenty eight mice of two months old were used in this study of basal physiological adult hippocampal neurogenesis in WT (male n = 7, female n = 8) and relaxin-3 KO (male n = 6, female n = 7) animals.

2.2.2. BrdU administrations

All animals were acclimatized to the experimental housing rooms for at least one week before the start of BrdU (5-bromo-2-deoxyuridine; Sigma-Aldrich) administration. Daily intraperitoneal injections of 10 mg/ml BrdU in sterile saline (Baxter, NSW, Australia) at a dose of 50 mg/kg body weight were administered to WT and relaxin-3 KO mice for seven consecutive days. Body

weight and general health was monitored throughout the experiment to ensure that no mice was adversely affected by the injection protocol. All mice were sacrificed four weeks after the final injection of BrdU.

2.2.3. Tissue preparation

The mice were sacrificed with an overdose of isoflurane (Abbott Laboratories, VIC, Australia) and immediately transcardially perfused with 0.1 M phosphate buffer, pH 7.4, followed by 4% paraformaldehyde (Merck, VIC, Australia) in 0.1 M phosphate buffer. Brains were post-fixed in 4% paraformaldehyde solution and then transferred into 20% sucrose in 0.1M phosphate buffer, sequentially overnight at 4^{o}C. The brains were then sagitally cut into two hemispheres, coated with OCT (Optimal Cutting Temperature) compound (Tissue-Tek, CA, USA), frozen on dry ice and stored at -80^{o}C until use.

Serial 40 µm coronal sections through the entire hippocampus (bregma -0.94 to -3.88 mm) were cut on a Leica CM3050 cryostat and stored in a cryoprotectant solution of 0.1 M phosphate buffer (pH 7.4), 30% (w/v) sucrose, 30% (v/v) ethylene glycol (modified from Watson et al., 1986) at -20^{o}C. Every sixth section cut was collected to the same series, such that each animal has six series of sections spaced 240 µm apart, from each of the left and right hemispheres.

2.2.4. Antibodies

All antibodies were diluted in PBS (phosphate buffered saline) containing 0.3% Triton X-100 and 5% goat serum (Life Technologies). The primary antibodies used in this study were: monoclonal rabbit anti-Ki67 (1:500; Abcam ab16667), monoclonal rat anti-BrdU (1:500; Accurate OBT0030), monoclonal mouse anti-NeuN (1:200; Millipore MAB377) and monoclonal mouse anti-

S100β (1:1000; Sigma-Aldrich S2532). For indirect immunofluorescent detection, the following secondary antibodies (Life Technologies), goat anti-rabbit conjugated with Alexa Fluor® 555, goat anti-rat conjugated with Alexa Fluor® 555, goat anti-mouse conjugated with Alexa Fluor® 488, were all used at a dilution of 1:500.

2.2.5. Immunohistochemistry

Hippocampal sections were rinsed in PBS after transferring out from cryoprotectant solution. For BrdU immunolabelling, sections were treated with 2 M hydrochloric acid for 30 min at 37^{o}C, followed by PBS rinses. Permeabilisation of the tissue sections were carried out in PBS with 0.3% Triton X-100 (PBST), followed by blocking in PBST with 5% goat serum. Immunofluorescent detection was accomplished by sequential overnight 4^{o}C incubation with primary antibodies, then fluorophore-conjugated secondary antibodies. Finally, sections were rinsed in PBS and mounted on glass slides with Prolong® Gold antifade mounting medium without or with DAPI (Life Technologies) depending on whether the sections were acid-treated or not, respectively.

2.2.6. Anatomical definition

The dentate gyrus was analysed septotemporally, with coronal sections 1.34 to 2.54 mm posterior to bregma, classified as septal dentate gyrus, and sections 2.92 to 3.88 mm posterior to bregma considered as temporal dentate gyrus.

For quantification of immunolabelled cells, every sixth section, a total of 12 sections per series on average, covering each of the left and right dentate gyrus in its entire rostro-caudal extension, were analysed. Cells were counted

in the subgranular zone (SGZ) and/or the granule cell layer (GCL). The subgranular zone was defined as the 25 µm three-cell layer at the boundary between GCL and hilus of dentate gyrus (Fig. 2.1) (Kempermann, 2006). For analysis of migration of newborn cells, the granule cell layer was divided into three regions, 1) the inner layer consisted of subgranular zone and inner third of granule cell layer, 2) the middle layer was the middle third of granule cell layer, while 3) the outer layer was representing the outer third of the granule cell layer (Fig. 2.1).

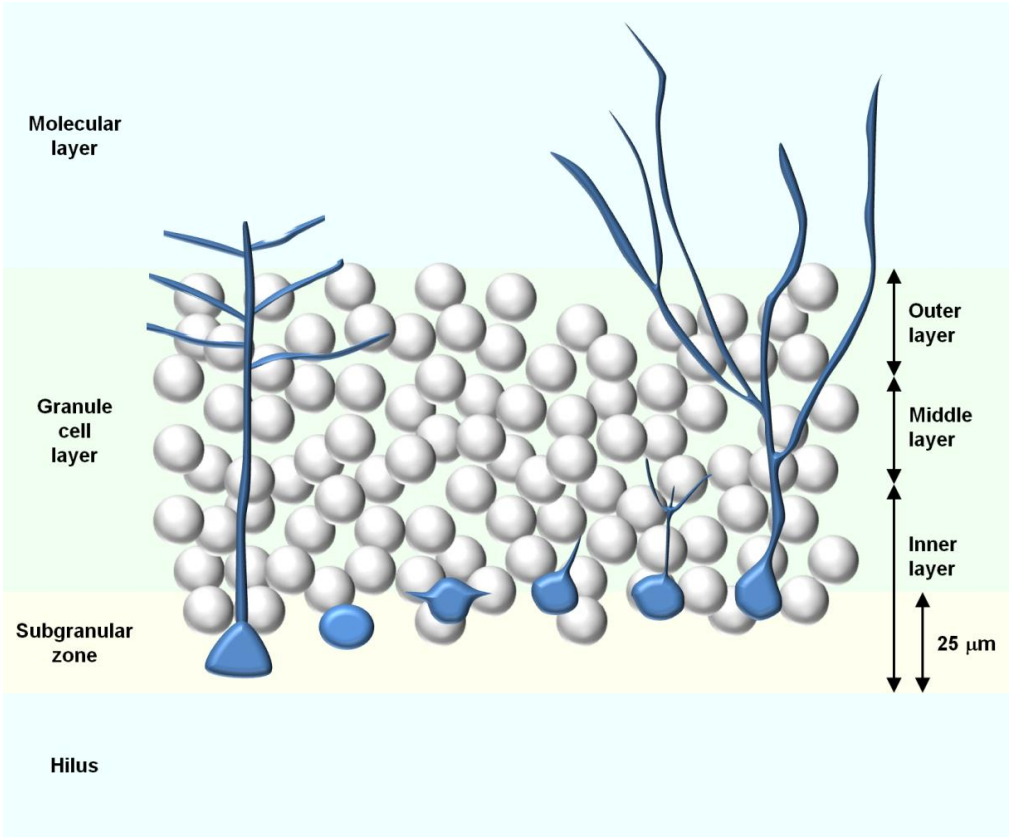

Figure 2.1. Anatomical definition of the subregions in dentate gyrus.

2.2.7. Quantification

To estimate the volume of granule cell layer in dentate gyrus, NeuN-immunostained granule cell layer was photomicrographed at 100X magnification with a digital camera (DP70; Olympus, Japan) equipped

fluorescence microscope (BX51; Olympus). Subsequently, the area of granule cell layer was measured using Fiji software (Schindelin et al., 2012) and multiplied by the section thickness of 40 μm to obtain granule cell layer volume in the section. Finally, the volume of granule cell layer in the entire series of hippocampal sections was summed up, multiplied by 6 (sampling factor), and averaged from bilateral dentate gyrus, for estimation of volume of the entire granule cell layer in one brain hemisphere.

Similarly, to calculate the reference volume of subgranular zone, DAPI-stained granule cell layer was photomicrographed, with the length of subgranular zone measured using Fiji software. Volume per section was determined by multiplication of the subgranular zone length, 25 μm width and 40 μm section thickness. Septal or temporal volume of subgranular zone were summed from the volume of sections classified as septal or temporal dentate gyrus, and averaged from bilateral hippocampi.

For Ki67 and BrdU stereological analysis, Ki67- and BrdU-immunolabelled cells were counted under 40X objective lens (NA 0.65) of a upright fluorescence microscope. Ki67- and BrdU-positive cells were exhaustively counted throughout the thickness of the sampled section, and along the entire subgranular zone or granule cell layer, respectively. Density of Ki67- and BrdU-labelled cells was obtained by the division of counted cell number by subgranular zone or granule cell layer volume, and averaged from the two hemispheres, for each septal and temporal subregion.

To determine neuronal and astroglial cell fate decision, BrdU-immunolabelled cells were checked for co-localisation with either neuronal marker, NeuN, or

astroglial marker, S100β, using a confocal laser scanning microscope LSM 510 (Carl Zeiss, Germany). Co-localisation with cell type marker was determined for every BrdU-positive cell throughout the thickness of the sampled section, and along the entire granule cell layer. The proportion of colabelled cells was expressed as a percentage of BrdU-positive cells, and averaged from the two hemispheres, for each septal and temporal subregion. At the same time, to evaluate migration of newborn neurons, the distribution of BrdU/NeuN-colabelled cells in each subdivision of granule cell layer (Fig. 2.1) was determined. The proportion of newborn neurons in each subdivision of granule cell layer was expressed as a percentage of BrdU/NeuN-coexpressing cells, and averaged from the two hemispheres, for each septal and temporal subregion.

All samples were coded and cell counting was conducted blind to the genotype of the sample.

2.2.8. Image acquisition

Representative photomicrographs of Ki67- and BrdU-immunolabelled cells were captured at 100X magnification with an upright fluorescence microscope (BX51; Olympus) with digital camera (DP70; Olympus). All images were imported into Fiji software, and cropped to the dentate gyrus region. Brightness and contrast were adjusted equally for the entire image, without over-saturation or losing any information. No specific feature within an image was enhanced, obscured, moved, removed, or introduced.

Representative immunofluorescent images of BrdU/NeuN- and BrdU/S100β-coexpressing cells were captured with a confocal laser scanning microscope LSM 510. Each fluorescent signal was acquired sequentially to avoid

simultaneous excitation and bleed-through of the two fluorochromes, Alexa Fluor® 488 and Alexa Fluor® 555. To demonstrate the co-localisation of BrdU and cell type marker, 10 μm z-stacks were scanned at 0.1 μm intervals at a pinhole setting of ~1 airy unit, corresponding to an optical slice of 0.8 μm. Images were collected under 63X oil immersion objective lens (NA 1.4) and 2.5X digital zoom, followed by reconstruction with orthogonal projections. To illustrate distribution of BrdU/NeuN-colabelled cells in granule cell layer, ~25 μm z-stacks were scanned at 1 μm intervals at a pinhole setting of ~1 airy unit, corresponding to an optical slice of 1 μm. Images were collected under 40X oil immersion objective lens (NA 1.3), followed by z-projection with maximum intensity.

2.2.9. Statistical analyses

All statistical analyses were performed with SPSS (version 21; IBM Corp, NY, USA). Two-way ANOVA was conducted for the analysis of volume of granule cell layer, while three-way repeated measures ANOVA was computed for volume of subgranular zone, density of Ki67-labelled cells, percentage of BrdU/NeuN-colabelled cells, percentage of BrdU/S100β-colabelled cells, distribution of BrdU/NeuN-colabelled cells in each GCL subdivision and density of BrdU-labelled cells analyses. Where appropriate, post hoc tests were carried out with Bonferroni corrections. All graphical representations were generated using GraphPad Prism 5 (GraphPad Software, CA, USA). The data are expressed as mean ± SEM (standard error of mean) and statistical significance is defined as P value < 0.05.

2.3. Results

2.3.1. Volume of dentate gyrus

As lifelong deficiency of a gene could result in structural changes in the hippocampal neurogenic region (or the brain) (Manning et al., 2012; Shi et al., 2012), the volume of granule cell layer in dentate gyrus was compared between WT and relaxin-3 KO animals (Fig. 2.2). Null mutation of relaxin-3 gene did not have an effect on the volume of granule cell layer (*P* value = 0.377, main effect of genotype, two-way ANOVA). Similarly, there was no significant effect of sex (*P* value = 0.237, main effect of sex, two-way ANOVA) and no interaction of genotype and sex (*P* value = 0.467, interaction effect, two-way ANOVA) of the animals.

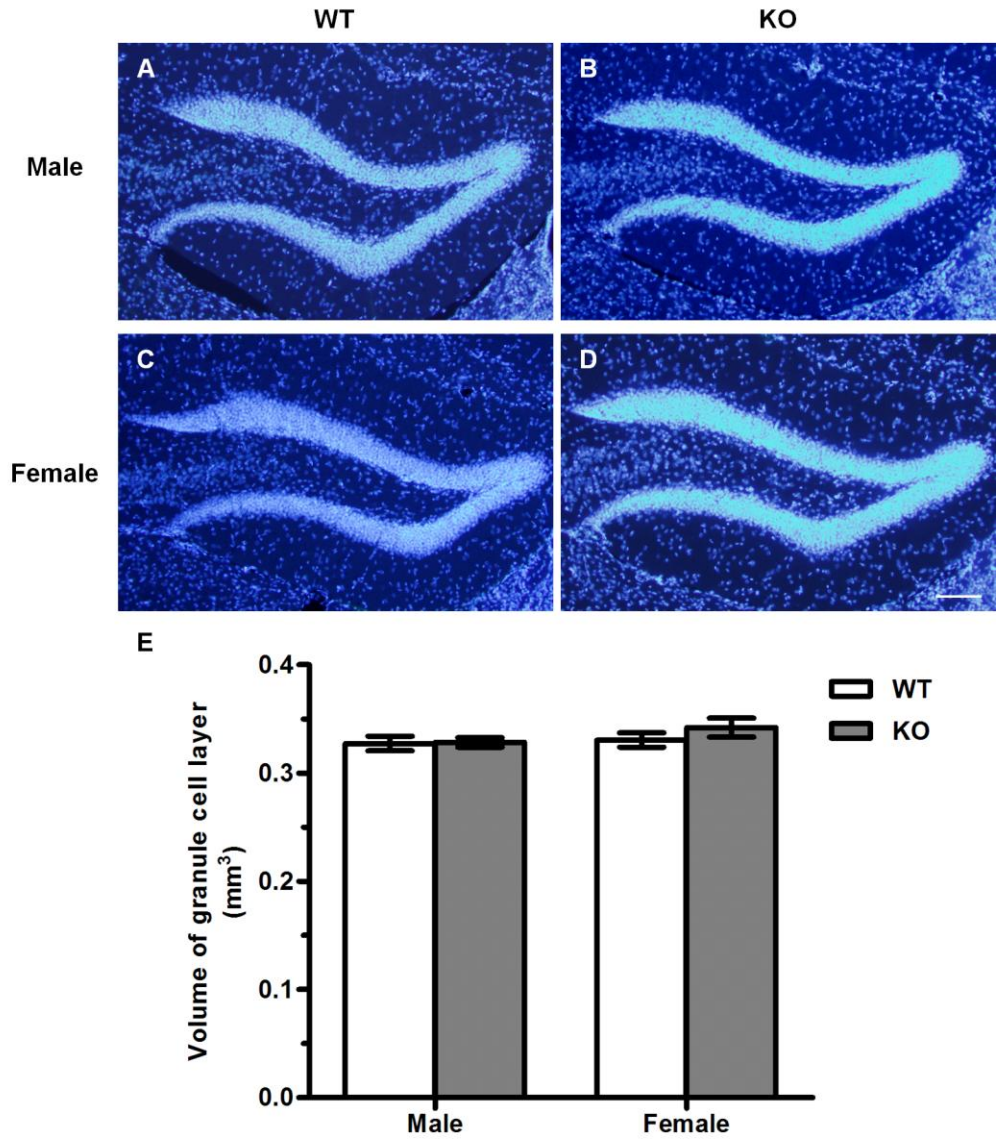

Figure 2.2. No significant differences in volume of granule cell layer (GCL) between WT and KO mice of male and female sex. Representative photomicrographs of DAPI-stained GCL in male WT (A) and KO (B), and female WT (C) and KO (D) mice. Scale bar corresponds to 100 μm. Volume of granule cell layer was estimated by one in six sampling of hippocampal sections, and statistically analysed with two-way ANOVA (all *P* values > 0.05; E). All values were averaged from bilateral hippocampi, with data expressed as mean ± SEM; n = 6-8 per group.

As there were more relaxin-3 projection fibres in the temporal than the septal dentate gyrus (Smith et al., 2010), analyses were performed according to their septal or temporal localisation, which were anatomically defined in the previous section (2.2.6). As a result of the septotemporal definition, roughly five to six coronal hippocampal sections were considered as septal while three to four sections were temporal. On average, the septal sections had 0.010 mm^3 subgranular zone, which was significantly different from the temporal sections' 0.012 mm^3 subgranular zone (*P* value < 0.001, main effect of septotemporal location, three-way repeated measures ANOVA; Fig. 2.3).

Knocking out relaxin-3 gene had a mild but significant effect on the volume of subgranular zone (*P* value = 0.035, main effect of genotype, three-way repeated measures ANOVA; Fig. 2.3), with the volume of subgranular zone in relaxin-3 KO animals 4.1 ± 1.8% larger than that in WT controls. In addition, male animals were found to have slightly larger (4.1 ± 1.8%) subgranular zone than female mice, a difference reported to be statistically significant (*P* value = 0.035, main effect of sex, three-way repeated measures ANOVA; Fig. 2.3).

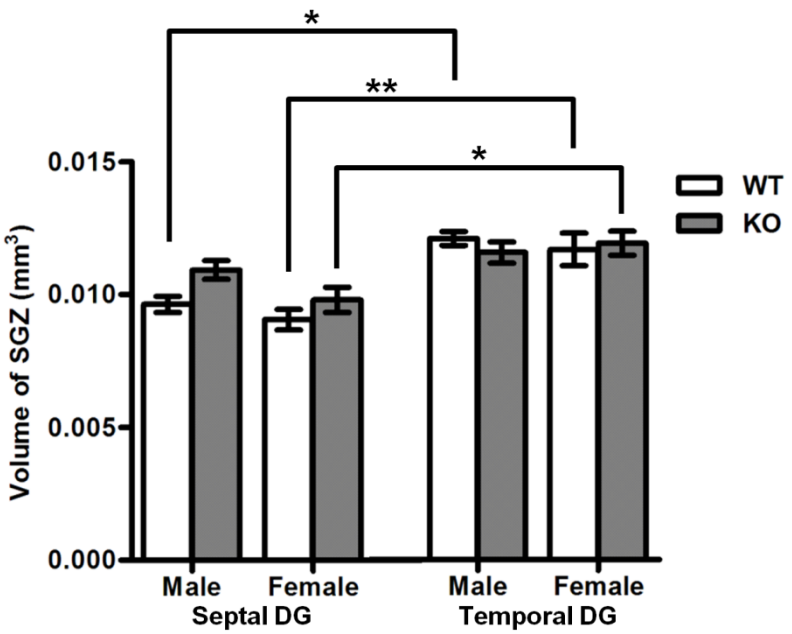

Figure 2.3. Significant effect of septotemporal location, genotype and sex on the volume of subgranular zone. Volumes of subgranular zone were measured and computed in sections defined as septal or temporal dentate gyrus. Main effects of septotemporal location, genotype and sex were statistically significant (all *P* values < 0.05) in three-way repeated measures ANOVA analysis. All values were averaged from bilateral hippocampi, with data expressed as mean ± SEM; n = 6-8 per group.

2.3.2. Proliferation

To investigate the loss-of-function effect of relaxin-3 on the proliferation phase in adult hippocampal neurogenesis, immunohistochemistry of an endogenous cell cycle-associated protein, Ki67, was carried out (Fig. 2.4). Expression of Ki67 could be found in proliferating cells from late G1 to M phase, and absent in quiescent (G0 phase) and early G1 phase cells (Scholzen and Gerdes, 2000). Therefore, Ki67 was utilised to identify the population of proliferating cells in dentate gyrus.

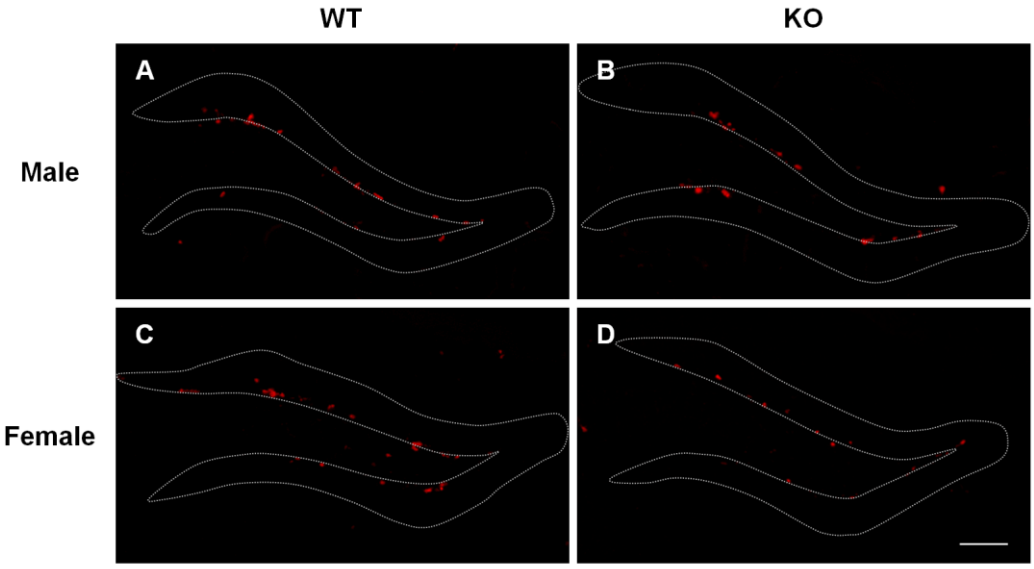

Figure 2.4. Ki67-expressing cells were mostly located in the subgranular zone of dentate gyrus. Representative photomicrographs of Ki67-immunolabelled (red) cells in male WT (A) and KO (B), and female WT (C) and KO (D) mice. Scale bar corresponds to 100 μm.

Due to the unequal volume sampled from septal and temporal dentate gyrus (Fig. 2.3), quantitative analysis of Ki67-labelled cells was conducted on its density values. Significant effect was found in the interaction of genotype and septotemporal location (*P* value = 0.032, interaction effect, three-way repeated measures ANOVA; Table 2.1). Further post hoc analyses revealed a significant difference between male WT and KO mice only in the temporal dentate gyrus (*P* value = 0.009, Bonferroni adjusted; Fig. 2.5), with KO having 19.3 ± 5.9% lesser Ki67-labelled cells/mm^3 than WT.

Additionally, significant sexual dimorphism was detected in the density of Ki67-labelled cells (*P* value < 0.001, main effect of sex, three-way repeated measures ANOVA; Table 2.1). Specifically, female KOs have an increase of 31.4 ± 9.7% and 30.3 ± 7.3% Ki67 density than male KOs, in septal and temporal dentate gyrus respectively (*P* values = 0.011 and 0.001, respectively, Bonferroni adjusted; Fig. 2.5).

Table 2.1. Results of three-way repeated measures ANOVA of Ki67 density by genotype, sex and septotemporal location.

Source of variation	$F_{1,24}$	*P* value	Significance
Genotype × sex × ST location	0.175	0.679	
Genotype × sex	3.988	0.057	
Genotype × ST location	5.172	0.032	*
Sex × ST location	1.280	0.269	
Genotype	0.480	0.495	
Sex	18.296	< 0.001	***
ST location	3.868	0.061	

ST = septotemporal; * *P* value < 0.05; *** *P* value < 0.001

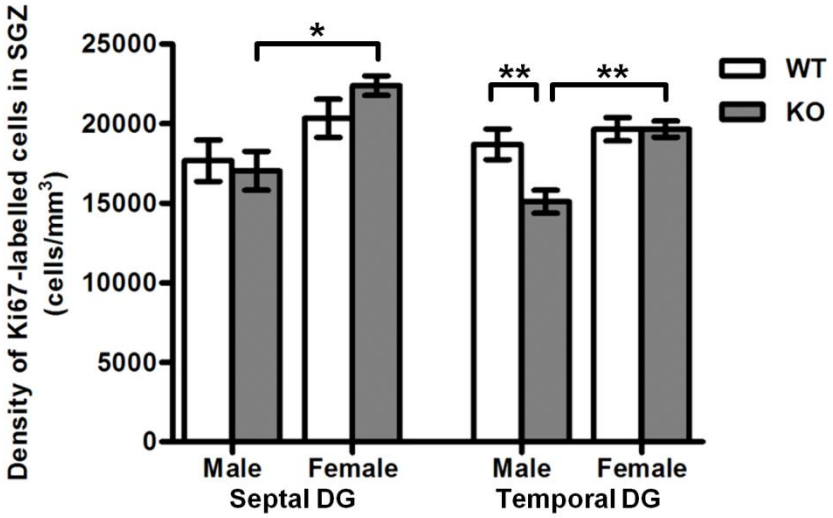

Figure 2.5. Male relaxin-3 KO mice have reduced proliferation specifically in the temporal dentate gyrus (DG). Density of Ki67-labelled cells was obtained from the division of counted cells by volume. There was a significant interaction effect of genotype and septotemporal location (*P* value < 0.05, three-way repeated measures ANOVA), and also a significant main effect of sex (*P* value < 0.001) on Ki67 density. All values were averaged from bilateral hippocampi, with data expressed as mean ± SEM; n = 6-8 per group. **P* value < 0.05, ***P* value < 0.01; Bonferroni post hoc tests.

2.3.3. Neuronal differentiation

Neuronal cell fate determination was analysed by comparing the fraction of differentiated neurons in the population of newborn cells. To identify newly generated neurons, double immunofluorescent labelling of BrdU and NeuN was carried out. NeuN is a nuclear marker for postmitotic neurons (Mullen et al., 1992), such that the presence of BrdU in NeuN-positive cells (Fig. 2.6) could serve as an evidence for the neuron having undergone cell division at the point of BrdU administration.

From the analysis of percentage of BrdU/NeuN-colabelled cells, loss of relaxin-3 had no effect on neuronal cell fate determination (*P* value = 0.977, main effect of genotype, three-way repeated measures ANOVA; Table 2.2; Fig. 2.7). On the other hand, sex and septotemporal location had a significant interaction effect (*P* value = 0.039, interaction effect, three-way repeated measures ANOVA; Table 2.2) on the percentage of BrdU/NeuN-colabelled cells. In particular, female animals had significantly increased percentage of newly differentiated neurons than males in all groups, with the largest difference of 28.9 ± 4.8% (*P* value < 0.001, Bonferroni adjusted) in the temporal dentate gyrus of WT mice. Also, there was significantly less neuronal differentiation in temporal as compared to septal dentate gyrus in all groups, especially with the male WT group having the biggest reduction of 20.4 ± 2.7% (*P* value < 0.001, Bonferroni adjusted).

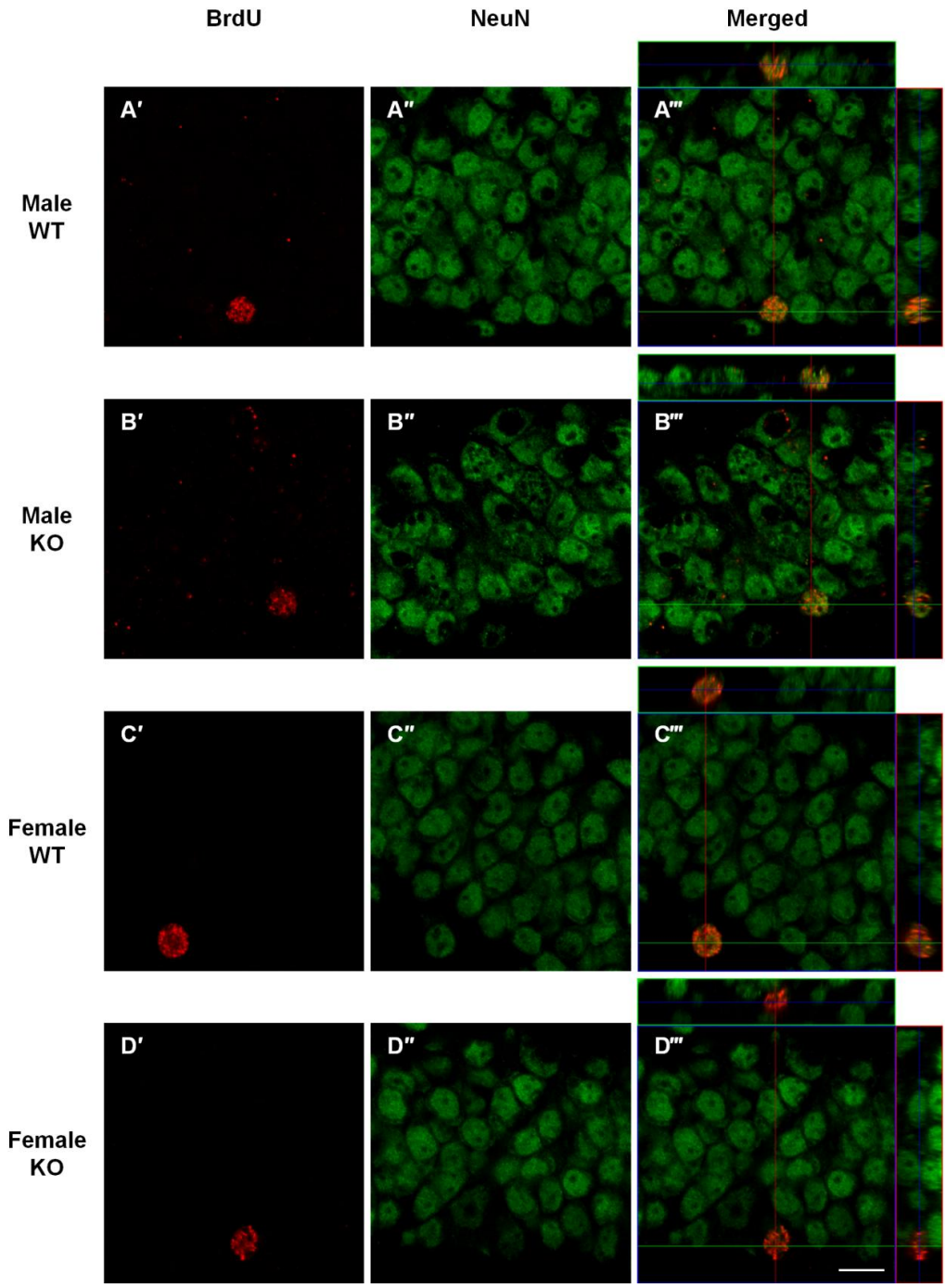

Figure 2.6. BrdU/NeuN-coexpressing cells were located in the dentate gyrus. Representative photomicrographs of BrdU/NeuN-colabelled cells (BrdU in red, NeuN in green) in male WT (A) and KO (B), and female WT (C) and KO (D) mice. Scale bar corresponds to 10 μm.

Table 2.2. Results of three-way repeated measures ANOVA of % BrdU/NeuN-colabelled cells by genotype, sex and septotemporal location.

Source of variation	$F_{1,24}$	*P* value	Significance
Genotype × sex × ST location	2.769	0.109	
Genotype × sex	1.098	0.305	
Genotype × ST location	0.234	0.633	
Sex × ST location	4.785	0.039	*
Genotype	0.001	0.977	
Sex	61.704	< 0.001	***
ST location	109.092	< 0.001	***

ST = septotemporal; * *P* value < 0.05; *** *P* value < 0.001

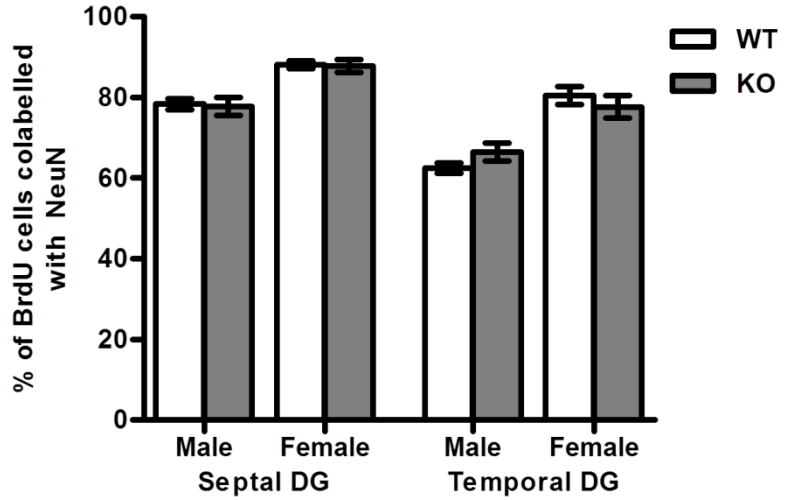

Figure 2.7. No effect of relaxin-3 on neuronal cell fate determination. Percentage of BrdU/NeuN-colabelled cells among total BrdU-labelled cells was calculated in septal and temporal dentate gyrus (DG). There was a significant interaction effect of sex and septotemporal location (*P* value < 0.05, three-way repeated measures ANOVA) on the percentage of BrdU/NeuN-colabelled cells. All values were averaged from bilateral hippocampi, with data expressed as mean ± SEM; n = 6-8 per group.

2.3.4. Astroglial differentiation

Similarly, proportion of newly differentiated astrocytes was analysed for astroglial fate choice decision. To identify newborn astrocytes, double labelling was performed for BrdU and S100β (Fig. 2.8), as S100β is selectively expressed in mature astrocytes (Cocchia, 1981; Steiner et al., 2004).

In general, WT and relaxin-3 KO mice have similar percentage of newly generated astrocytes (*P* value = 0.940; main effect of genotype, three-way repeated measures of ANOVA; Table 2.3; Fig. 2.9). On the other hand, sex and septotemporal location have significant main effects on astroglial differentiation (*P* values = 0.013 and < 0.001, respectively, three-way repeated measures ANOVA). Further post hoc analyses reported significant septotemporal differences in male KOs (*P* value = 0.006, Bonferroni adjusted), and both male (*P* value = 0.012, Bonferroni adjusted) and female WTs (*P* value = 0.012, Bonferroni adjusted). Specifically, there was an increase in astroglial differentiation in the temporal dentate gyrus, with the largest percentage increase of 104.5 ± 32.7% observed in female WT mice.

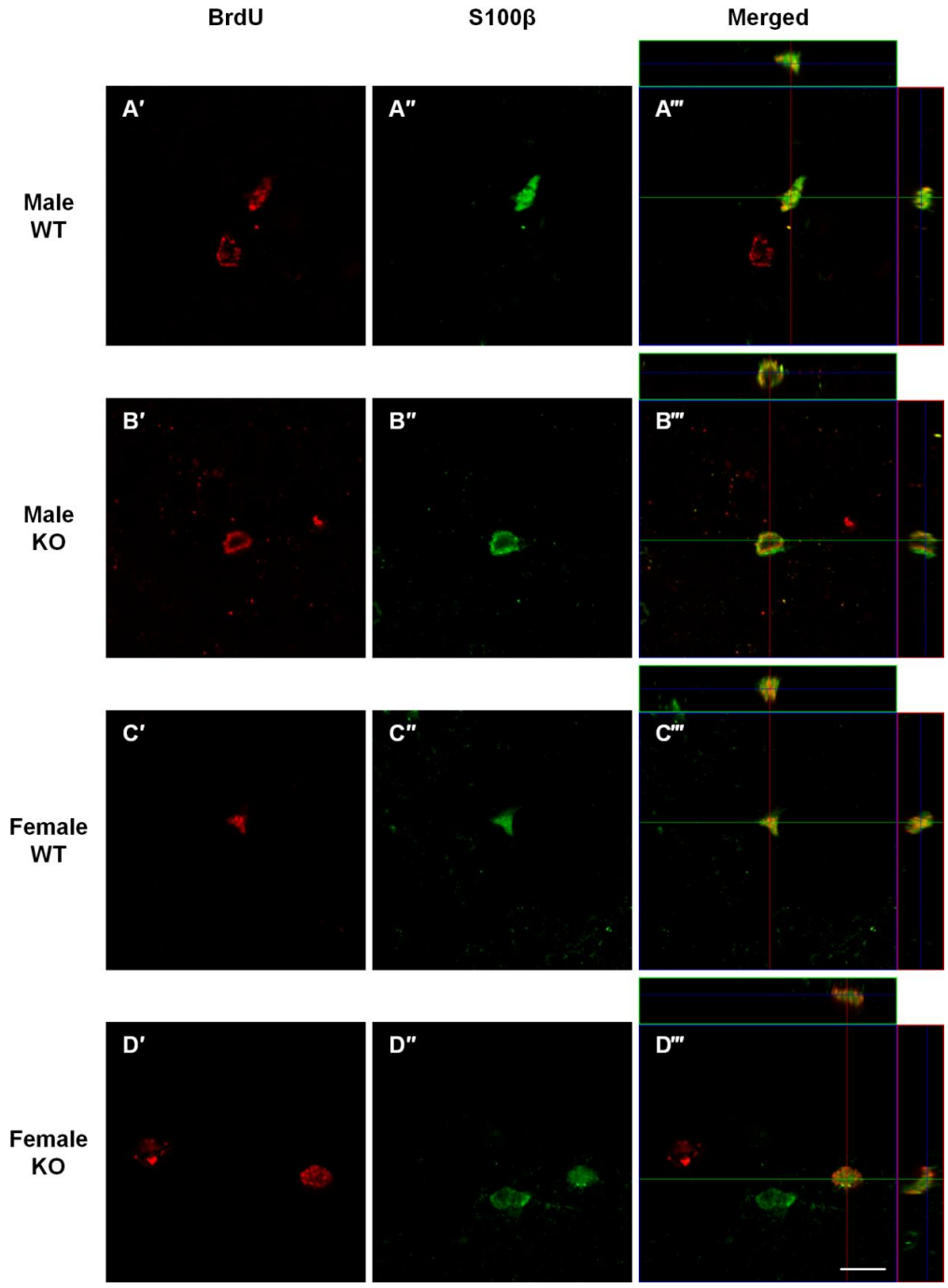

Figure 2.8. BrdU/S100β-coexpressing cells were located in the dentate gyrus. Representative photomicrographs of BrdU/S100β-colabelled cells (BrdU in red, S100β in green) in male WT (A) and KO (B), and female WT (C) and KO (D) mice. Scale bar corresponds to 10 μm.

Table 2.3. Results of three-way repeated measures ANOVA of % BrdU/S100β-colabelled cells by genotype, sex and septotemporal location.

Source of variation	$F_{1,24}$	*P* value	Significance
Genotype × sex × ST location	0.935	0.343	
Genotype × sex	0.480	0.495	
Genotype × ST location	0.116	0.736	
Sex × ST location	1.335	0.259	
Genotype	0.006	0.940	
Sex	7.252	0.013	*
ST location	33.576	< 0.001	***

ST = septotemporal; * *P* value < 0.05; *** *P* value < 0.001

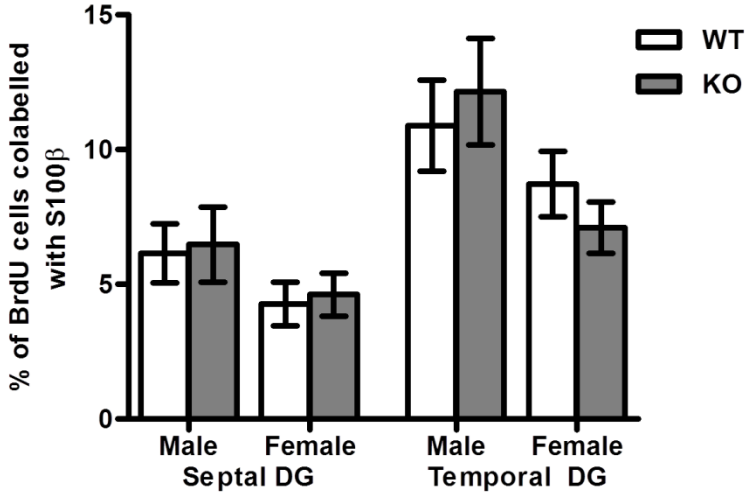

Figure 2.9. Relaxin-3 KO mice did not differ in astroglial differentiation as compared to WT animals. Percentage of BrdU/S100β-colabelled cells in total BrdU-labelled cells were calculated in septal and temporal dentate gyrus (DG). There were significant sexual and septotemporal dimorphisms (*P* values < 0.05 and < 0.001, respectively, main effects, three-way repeated measures ANOVA) in the percentage of BrdU/S100β-colabelled cells. All values were averaged from bilateral hippocampi, with data expressed as mean ± SEM; n = 6-8 per group.

2.3.5. Migration of newborn neurons

To assess migration of newborn neurons, the distribution of BrdU/NeuN-colabelled cells was examined (Fig. 2.10). BrdU/NeuN-colabelled cells were categorised according to their position within the granular cell layer (GCL). The GCL was approximately divided into three regions, with the inner layer being subgranular zone and inner third of GCL, middle layer being the middle third of GCL and outer layer being the outer third of GCL (Fig. 2.1). During adult neurogenesis, newly born cells migrate in the direction from subgranular zone towards the outer layer of GCL (Kempermann et al., 2003).

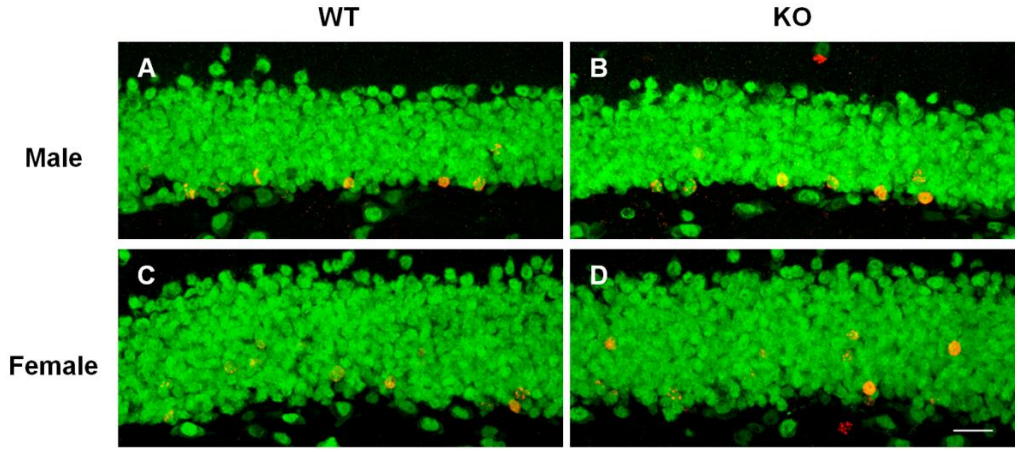

Figure 2.10. Distribution of BrdU/NeuN-colabelled cells in the dentate gyrus. Representative photomicrographs of BrdU/NeuN-coexpressing cells (BrdU in red, NeuN in green) in male WT (A) and KO (B), and female WT (C) and KO (D) mice. Scale bar corresponds to 20 μm.

The majority (80 – 90%) of the adult-born neurons migrated very little and remained in the inner GCL for all mice (Fig. 2.11). However, there were still subtle but significant differences in the distribution of BrdU/NeuN-colabelled cells. Specifically, there were significant interaction effects of sex and septotemporal location in the percentage of newborn neurons in the inner (*P* value = 0.036, three-way repeated measures ANOVA; Table 2.4), middle (*P* value < 0.001) and outer GCL (*P* value = 0.025).

In particular, female relaxin-3 KO mice have 6.0 ± 1.9% less newborn neurons in the inner GCL of septal DG as compared to male KO animals (*P* value = 0.013, Bonferroni adjusted). Also, within female KO mice, there was a significant decrease by 4.5 ± 1.7% in the percentage of newborn neurons in the inner GCL in septal as compared to temporal dentate gyrus (*P* value = 0.041, Bonferroni adjusted).

Conversely, in the middle GCL, female relaxin-3 KO mice have 62.0 ± 15.0% more newborn neurons in the middle GCL of septal dentate gyrus as compared to male KO animals (*P* value = 0.001, Bonferroni adjusted). The increase (51.3 ± 13.6%) of newborn neurons in the middle GCL of septal dentate gyrus is also significant when compared to that in temporal DG within the same group of female KO mice (*P* value = 0.003, Bonferroni adjusted). In addition, the 37.0 ± 11.8% increase in migration of newborn neurons to the septal middle GCL in female KO animals was significant when compared to female WT controls (*P* value = 0.013, Bonferroni corrected).

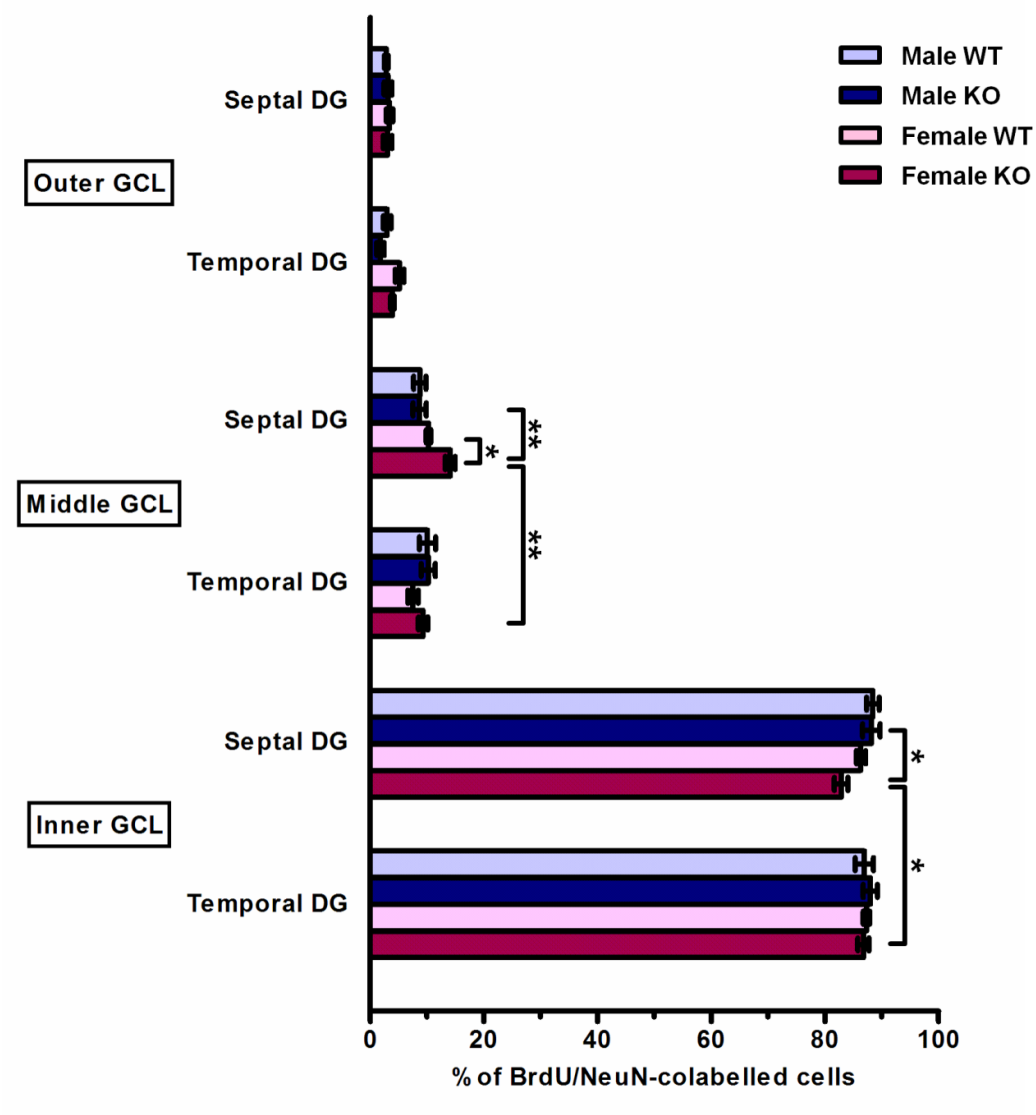

Figure 2.11. Female relaxin-3 KO mice have an increased migration of adult-born neurons into the middle layer of GCL in septal DG. Percentage of BrdU/NeuN-colabelled cells in each of inner, middle and outer GCL was calculated for each group of animals in both septal and temporal DG. Significant interaction effect of sex and septotemporal locations was detected for each subdivision of GCL (*P* value < 0.05, three-way repeated measures ANOVA). All values were averaged from bilateral hippocampi, with data expressed as mean ± SEM; n = 6-8 per group. **P* value < 0.05, ***P* value < 0.01; Bonferroni post hoc tests.

Table 2.4. Results of three-way repeated measures ANOVA of % BrdU/NeuN-colabelled cells in each GCL subdivision.

GCL subdivision	Source of variation	$F_{1,24}$	*P* value	Significance
Inner	Genotype × sex × ST location	0.299	0.590	
	Genotype × sex	1.890	0.182	
	Genotype × ST location	2.071	0.163	
	Sex × ST location	4.910	0.036	*
	Genotype	0.855	0.364	
	Sex	5.617	0.026	*
	ST location	1.187	0.287	
Middle	Genotype × sex × ST location	0.760	0.392	
	Genotype × sex	3.090	0.092	
	Genotype × ST location	0.517	0.479	
	Sex × ST location	16.690	< 0.001	***
	Genotype	3.326	0.081	
	Sex	1.243	0.276	
	ST location	3.380	0.078	
Outer	Genotype × sex × ST location	0.140	0.712	
	Genotype × sex	0.124	0.728	
	Genotype × ST location	2.307	0.142	
	Sex × ST location	5.716	0.025	*
	Genotype	1.558	0.224	
	Sex	5.823	0.024	*
	ST location	0.820	0.374	

ST = septotemporal; * *P* value < 0.05; *** *P* value < 0.001

2.3.6. Survival of newborn cells

For analysis on the survival of newly generated cells, BrdU was injected for birth-dating of cells in young adulthood, and examined four weeks post-final BrdU injection. Immunohistochemical detection of BrdU found its presence mostly in subgranular zone and granule cell layer of dentate gyrus (Fig. 2.12).

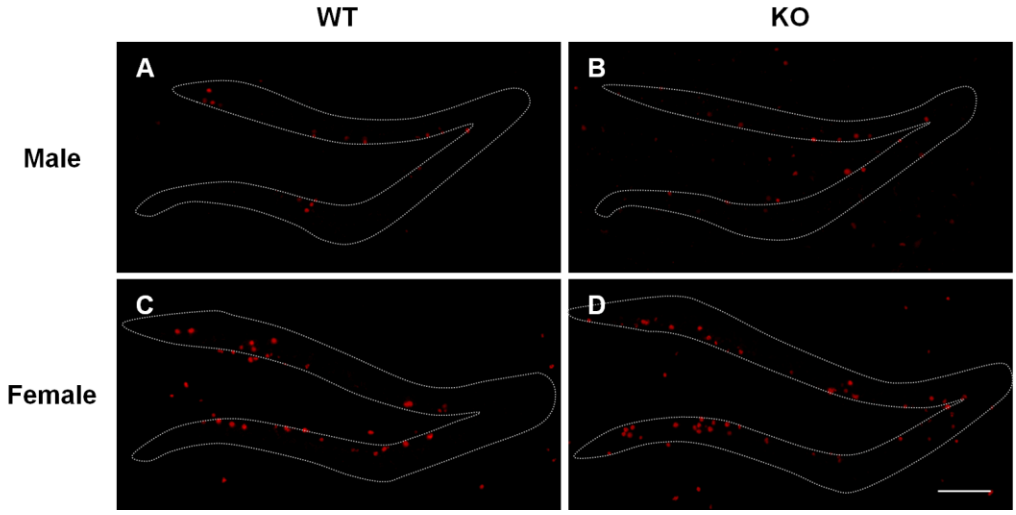

Figure 2.12. BrdU-expressing cells were mostly located in subgranular zone and granule cell layer of dentate gyrus. Representative photomicrographs of BrdU-immunolabelled (red) cells in male WT (A) and KO (B), and female WT (C) and KO (D) mice. Scale bar corresponds to 100 μm.

Quantitative analysis of BrdU cell density revealed a lack of relaxin-3 effect on the survival phase in adult neurogenesis (*P* value = 0.950, main effect of genotype, three-way repeated measures ANOVA; Table 2.5; Fig. 2.13). On the other hand, an interaction effect of sex and septotemporal location was found to be statistically significant (*P* value < 0.001, three-way repeated measure ANOVA; Table 2.5).

Subsequent post hoc analyses found that females had significant increases in BrdU cell density as compared to males, in septal dentate gyrus of KO mice, and in both septal and temporal dentate gyrus of WT mice. In particular, the

greatest increase of 66.2 ± 14.7% was detected in the septal dentate gyrus of female WT mice as compared to that of males (*P* value < 0.001, Bonferroni adjusted). Furthermore, there were significant reductions in the density of BrdU-labelled cells in temporal as compared to septal dentate gyrus, in each animal group. The largest percentage decrease (59.9 ± 4.6%) was found in female relaxin-3 null mice (*P* value < 0.001, Bonferroni adjusted).

Table 2.5. Results of three-way repeated measures ANOVA of BrdU density by genotype, sex and septotemporal location.

Source of variation	$F_{1,24}$	*P* value	Significance
Genotype × sex × ST location	0.071	0.792	
Genotype × sex	0.450	0.509	
Genotype × ST location	1.292	0.267	
Sex × ST location	25.537	< 0.001	***
Genotype	0.004	0.950	
Sex	29.617	< 0.001	***
ST location	392.311	< 0.001	***

ST = septotemporal; * *P* value < 0.05; *** *P* value < 0.001

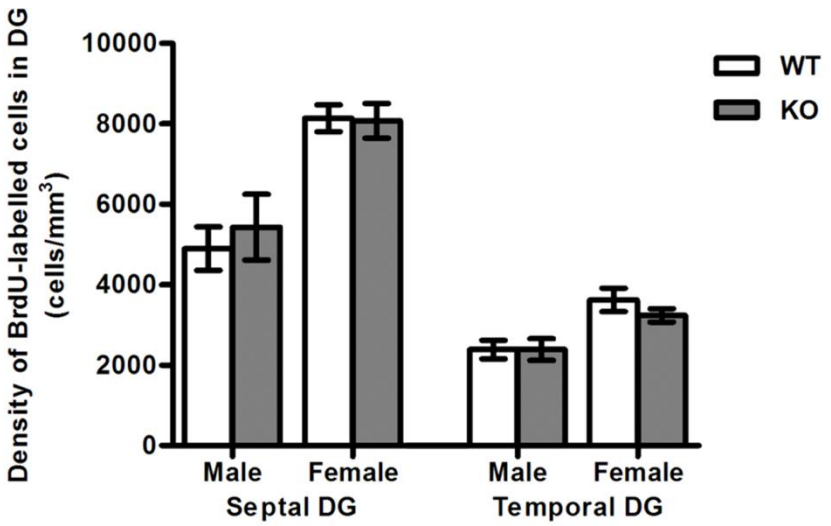

Figure 2.13. Sex- and septotemporal-dependent survival of newly born cells in the adult dentate gyrus. Density of BrdU-labelled cells was obtained from the division of counted cells by volume. There was a significant interaction effect of sex and septotemporal location (*P* value < 0.001, three-way repeated measures, ANOVA) on BrdU density. All values were averaged from bilateral hippocampi, with data expressed as mean ± SEM; n = 6-8 per group.

2.4. Discussion

In the present study, the role of relaxin-3 in adult hippocampal neurogenesis was investigated using a genetic knockout model. Similar to the general physiology and behaviour, the gross hippocampal structure, in terms of volume of granule cell layer, was comparable between relaxin-3 KO mice and their WT controls. On the other hand, there was a small increment in the volume of subgranular zone in the KO animals. Despite the increase in subgranular zone, a reduction in cell proliferation was detected specifically in the temporal dentate gyrus of male KO mice. This suggests that relaxin-3 is indispensible in the hippocampal subregion which is normally heavily innervated with relaxin-3 projections. However, the necessity of relaxin-3 in proliferation is sex-dependent, with loss of relaxin-3 having no effect in female mice.

In fact, sexual dimorphism was detected in the density of Ki67-labelled cells. However, post hoc analyses found further statistical significance of sex only in relaxin-3 KO mice, and not their WT littermates. Indeed, there was a trend for interaction between relaxin-3 and sex (*P* value = 0.057; Table 2.1), supporting a possible interplay between the neuropeptide and sex hormones. On the other hand, sexual dimorphic proliferation was not seen in the WT animals, an observation in agreement with literature, which has mostly reported proliferation to be sex-independent in mice (Lagace et al., 2007; Ben Abdallah et al., 2010; Manns et al., 2010; Ransome and Hannan, 2013).

Other stages of neurogenesis, including cell fate determination, migration and survival, were also examined, and found to have similar levels for both WT

and relaxin-3 KO mice. Despite some reduction in proliferation, these results hint that relaxin-3 does not play a major role in the homeostatic maintenance of net adult hippocampal neurogenesis, and/or that there are other compensatory mechanisms in the lifelong deficiency of relaxin-3. This is perhaps not surprising, as permanent depletion of another ascending arousal system, serotonin, also had no effect on baseline neurogenesis in the adult hippocampus (Klempin et al., 2013).

As relaxin-3 was reported to regulate sex-related hormones (via hypothalamic-pituitary-gonadal axis (McGowan et al., 2008)), and innervate dentate gyrus differentially in septal and temporal subregions (Tanaka et al., 2005; Ma et al., 2007; Smith et al., 2010), adult hippocampal neurogenesis was also analysed according to sex and septotemporal location in this study. Moreover, sex and septotemporal differences are two factors having been reported to play a role in the modulation of adult hippocampus.

While there were no differences between WT and relaxin-3 KO mice in cell fate determination, there were significant effects of sex and septotemporal localisation. Specifically, female mice have more neuronal and less astroglial differentiation than male mice, and there was less neuronal and more astroglial differentiation in the temporal than septal dentate gyrus.

There are few reports in literature on the sex differences in neuronal differentiation in mice. A couple of studies compared number or percentage of doublecortin cells (immature neurons) and found no sex differences (Ben Abdallah et al., 2010; Klaus et al., 2012; Ransome and Hannan, 2013). One report with analysis on percentage of BrdU/NeuN-colabelled cells found significantly higher neuronal differentiation in the female mice (Manning et al.,

2012), the same result as the present study. However, the exact mechanism of regulation by sex hormones and the functional significance of sexual dimorphic neuronal differentiation are still unknown. Similarly, very little is known about sex differences on astroglial differentiation in adult hippocampal neurogenesis.

Attention on septotemporal differences in adult hippocampal neurogenesis only began in recent years. Data from the present study were in agreement with the published literature in both mice and rats, that there were less neuronal differentiation in the temporal dentate gyrus, as compared to the septal pole (Jinno, 2011b; Piatti et al., 2011; Snyder et al., 2012). However, time-course studies showed that it was not a reduction in neuronal differentiation but rather delayed neuronal maturation in the temporal dentate gyrus (Piatti et al., 2011; Snyder et al., 2012). At later time points (4-10 weeks post cell division; depending on species and parameter investigated), both septal and temporal poles of dentate gyrus exhibited similar level of neuronal differentiation or maturation (Piatti et al., 2011; Snyder et al., 2012).

As for septotemporal effect on astroglial differentiation, although there has not been much documentation, the current results are in line with one other report (Jinno, 2011a). In the report, progenitor cells committed to astroglial lineage were identified by triple immunolabelling of SOX2, GFAP and S100β, with their density was significantly higher in the temporal dentate gyrus as compared to the septal pole (Jinno, 2011a). In addition, in a study of mature astrocytes, similar septotemporal gradient was observed for S100β-positive cells (Ogata and Kosaka, 2002). Therefore, collectively, these findings showed that both astroglial differentiation and eventual density of mature astrocytes are more in the temporal dentate gyrus.

Other than cell fate choices, there was also an interaction effect of sex and septotemporal location on the migration of adult-born neurons. In particular, female KO mice displayed an increase in migration from inner to middle granule cell layer in the septal dentate gyrus, such that statistical significances were detected when compared to male or temporal dentate gyrus. The septal dentate gyrus is not densely innervated with relaxin-3 fibres (Tanaka et al., 2005; Ma et al., 2007; Smith et al., 2010), hence the increase in migration in female KO mice may be due to an indirect sex-dependent pathway of relaxin-3.

Exact physiological significance of the positioning of newborn cells in granule cell layer remains uncertain, such that it is difficult to predict the functional effect of increased migration specifically in the septal dentate gyrus. Aberrant increase in migration of adult-born cells has been known to be associated with epilepsy (Jessberger et al., 2005; Heinrich et al., 2006; Parent et al., 2006; Gong et al., 2007; Jessberger et al., 2007) and schizophrenia (Duan et al., 2007; Manning et al., 2012). However, the level of increment in migration observed in the present study was only 4-6%, a magnitude much smaller than that in studies mentioned earlier. Therefore, the increase in migration in septal dentate gyrus of female KO mice may not be pathological, though it may confer vulnerability in the integrity of hippocampal neuronal circuitries.

For the survival phase of neurogenesis, there was similarly an interaction effect of sex and septotemporal location, in which female mice have an increased survival as compared to males, and that there was less survival in temporal than septal dentate gyrus. However, most studies, thus far, have reported a lack of sex-specific influence on survival in mice (Lagace et al.,

2007; Ma et al., 2012; Ransome and Hannan, 2013), though occasional reports showed sex differences (Mineur et al., 2007; Roughton et al., 2012). The effect of sex differences on survival of adult-born cells is perhaps equivocal at the moment.

Similarly, the results are conflicting for the effect of septotemporal location for cell survival in adult neurogenesis. In the present study, temporal dentate gyrus has a lower level of survival than the septal pole, a finding in agreement with that published by Snyder and colleagues in 2009. However, there exist several other reports showing no septotemporal differences for survival (Felice et al., 2012; Hawley et al., 2012; Xia et al., 2012). As investigations on the septotemporal effect only started in recent years, more studies are needed for the field to come to a consensus on survival in the different hippocampal subregions.

In summary, this chapter has established the effect of permanent depletion of relaxin-3 on the different stages of adult hippocampal neurogenesis. Specifically, there was reduced proliferation in the temporal dentate gyrus of male KO mice, while other phases of neurogenesis remain similar to WT littermates. Collectively, these results demonstrated, for the first time, sex- and septotemporal-dependent effect of relaxin-3 in the proliferation stage. On the other hand, relaxin-3 has less influence and/or compensable effect on net neurogenesis. In addition, several sex and septotemporal differences were observed in cell differentiation, migration and survival of adult-born cells. However, the effects of sex and septotemporal location are still not fully established in the field, such that the data in the current study were in agreement with some but not all reports. Nevertheless, this is the first investigation that analysed the effect of sex and septotemporal location in

combination, and could contribute to further understanding of these two factors in neurogenesis in the adult hippocampus.

Chapter 3

Hippocampal neurogenesis in mature WT and relaxin-3 KO adult mice

3. Hippocampal neurogenesis in mature WT and relaxin-3 KO adult mice

3.1. Introduction

Ageing is a natural process that is associated with progressive functional decline of physiological condition of organisms. In the brain, several changes take place with ageing, including age-associated memory impairment and disruption in synaptic plasticity (reviewed in Morrison and Hof, 1997). Similarly, cellular plasticity in the hippocampus also declines with ageing (reviewed in Couillard-Despres, 2013), and may have a role in the deterioration of hippocampal-dependent memory (Drapeau et al., 2003).

In ageing, multiple stages of adult hippocampal neurogenesis are affected to result in reduction of net neurogenesis (Kuhn et al., 1996; Kempermann et al., 1998; McDonald and Wojtowicz, 2005). One of the first stages, cell proliferation, is strongly decreased with age (Seki and Arai, 1995; Kuhn et al., 1996; Kempermann et al., 1998; Nacher et al., 2003; McDonald and Wojtowicz, 2005; Ben Abdallah et al., 2010), which could be due to a depletion of precursor cell pool (Nacher et al., 2003; Olariu et al., 2007; Lugert et al., 2010; Encinas et al., 2011; Jinno, 2011a) and/or increase in stem cell quiescence (Hattiangady and Shetty, 2008; Lugert et al., 2010).

Decline in net neurogenesis may also be contributed by a reduction in neuronal differentiation (Kempermann et al., 1998; Lichtenwalner et al., 2001; Nacher et al., 2003; Bondolfi et al., 2004). However, depending on the time point(s) investigated, such reduction in newborn neuronal phenotype may instead be due to a delayed neuronal development (Rao et al., 2005). In

addition, neuronal maturation, in terms of dendritic growth, was observed to be diminished in middle-aged and aged dentate gyrus (Rao et al., 2005). Similarly, delayed/reduced migration of newly born cells was reported (Heine et al., 2004; Rao et al., 2005), supporting a delayed/reduced neuronal development of newborn cells in ageing.

To control the net production of newly born cells, surplus of progenitor cells and immature neurons is eliminated. In ageing, the levels of elimination and survival have been found to be varying with different animal species and/or experimental conditions. Some studies documented a decrease in cell death (Heine et al., 2004; Ben Abdallah et al., 2010), while others found an increase (Lemaire et al., 2000). Similarly, there is a range of age-related effects on the survival rate of adult-born cells, from age-dependent decrease (McDonald and Wojtowicz, 2005), to no change (Rao et al., 2005), to an increase (Bondolfi et al., 2004), to fluctuations at different ages (Ben Abdallah et al., 2010). These findings probably reflect a dynamic regulation of elimination and survival to maintain the net levels of cells born into the aged dentate gyrus.

In humans, investigations of age-specific effects on hippocampal neurogenesis are difficult and rare. In 2010, a study, utilising a panel of neurogenesis-related markers, found coexpression of immature neuronal marker, doublecortin, and key progenitor markers like SOX2 and nestin even at the oldest age of 100 years (Knoth et al., 2010). Semi-quantitative analysis of hippocampal neurogenesis, with density of doublecortin-positive cells, demonstrated an exponential decrease with age (Knoth et al., 2010). Recently, another study, utilising carbon-14, demonstrated presence of adult-born neurons in the human hippocampus up to 92 years of age (Spalding et al., 2013). Mathematical modelling suggested that the relative age-related

decline of adult hippocampal neurogenesis is smaller in humans than mice, with the rate comparable to a middle-aged mouse (Spalding et al., 2013). These two studies showed that, like rodents, there is age-related reduction of newborn neurons in the hippocampus. The low but significant level of neurogenesis remaining, even in 90-100 years old humans, implies continual relevance of newly born neurons with age.

To explain for the age-related decline of hippocampal neurogenesis, several hypotheses have been proposed. Most of these hypotheses come from age-accompanied level changes in biochemical messengers. For example, one of the more well-studied causes suggested was elevated glucocorticoids with age, shown in rodents (Landfield et al., 1978; Sapolsky, 1992; Montaron et al., 2006). Furthermore, experimentally manipulated levels of glucocorticoids have been inversely correlated with levels of neurogenesis (Cameron and Gould, 1994; Montaron et al., 2006; Wong and Herbert, 2006), supporting the link between increased glucocorticoids and decreased newborn neurons in aged hippocampus. However, a few other studies did not observe the increased levels of glucocorticoids in aged animals (Sonntag et al., 1987; Heine et al., 2004). One report in 2004 also showed no correlation between natural varying basal levels of corticosterone and neurogenesis (Heine et al., 2004), suggesting that relationship may not be as straight-forward as proposed.

In human studies, there were no age-related changes in basal glucocorticoid levels (West et al., 1961; Sherman et al., 1985; Waltman et al., 1991). Nevertheless, a range of glucocorticoid levels is present in humans, and was shown to have a negative relationship with hippocampal volume (Lupien et al., 1998) and cognitive performances (Lupien et al., 1994; Lupien et al., 1998).

However, a direct measure of glucocorticoids and neurogenesis levels has not been conducted in humans. Therefore, despite indirect pieces of evidence associating glucocorticoids and age-related decline in hippocampal neurogenesis, causal relationships have not been firmly established.

A few other hypotheses loosely linked age-related changes of molecular factors with their effect on neurogenesis. For instance, factors like FGF2, IGF1, BDNF and VEGF are positive regulators of neurogenesis (reviewed in Section 1.2.2), and were documented to be decreased in ageing (Bhatnagar et al., 1997; Hattiangady et al., 2005; Shetty et al., 2005). Conversely, negative regulators, such as IL-1β (Koo and Duman, 2008) and TGF-β1 (Buckwalter et al., 2006), were shown to be increased in aged hippocampus (Nichols, 1999; Casolini et al., 2002). Together, age-related changes of these molecular factors could contribute to a anti-neurogenic environment for generation of newborn neurons.

Neurotransmitter systems may also be involved in age-accompanied reduction in neurogenesis. In ageing, serotonin system was reported to be decreased, in terms of serotonin levels (Nishimura et al., 1995; Stemmelin et al., 2000; Keuker et al., 2005), and also serotonin receptor binding sites, e.g. 5-HT_{1A} and 5-HT_{2A} receptors (reviewed in Meltzer et al., 1998). As serotonin is a positive regulator of neurogenesis, age-associated decline in the neurotransmitter system may play a role in the diminished levels of newborn neurons.

In summary, there is a whole array of changes taking place with increasing age. These changes may occur at any of the molecular, cellular, systems and functional levels. Hence, age-related decrease in adult neurogenesis was

proposed to be the by-product of age-related changes, and not truly "regulated" by ageing (Klempin and Kempermann, 2007). As such, ageing is a global covariate for baseline control and regulation of adult neuron generation.

In the previous chapter, it was demonstrated that relaxin-3 has a sex-dependent effect on proliferation in the temporal dentate gyrus. However, with ageing as a covariate for modulation of neurogenesis, the interaction effect of age and relaxin-3 is currently unknown. Therefore, in this chapter, to establish the age-specific role of relaxin-3 in hippocampal neurogenesis, levels of neurogenesis were examined in eight-month old mature adult mice with relaxin-3 gene knocked out.

3.2. Materials and methods

3.2.1. Animals

Eight months old relaxin-3 knockout (KO) mice and wildtype (WT) littermate controls were generated (described previously in Section 2.2.1). To examine basal levels of neurogenesis, twenty animals (male WT n = 4, KO n = 5, and female WT n = 6, KO n = 5) were intraperitoneally injected with BrdU (5-bromo-2-deoxyuridine; 50 mg/kg body weight) once daily for seven consecutive days. All mice were sacrificed four weeks after the final administration of BrdU. All animal procedures were carried out in the Florey Institute of Neuroscience and Mental Health, Australia, were approved by the Howard Florey Institute Animal Welfare Committee, and in accordance with the ethical guidelines issued by the National Health and Medical Research Council of Australia.

3.2.2. Immunohistochemistry

To investigate proliferation, differentiation, migration and survival of newborn cells, immunohistochemical labelling of various markers was performed as previously described (Sections 2.2.3 to 2.2.5). Briefly, mice were overdosed with isoflurane and transcardially perfused with 0.1 M phosphate buffer, pH 7.4, followed by 4% paraformaldehyde solution. Brains were post-fixed in 4% paraformaldehyde solution, cryoprotected with 20% sucrose, and stored in -80^{o}C with OCT compound. Serial 40 µm hippocampal sections were collected for each left and right hemispheres and stored in a cryoprotectant at -20^{o}C. Sections were rinsed in PBS after transferring out from cryoprotectant solution. For BrdU immunolabelling, sections were treated with 2 M hydrochloric acid for 30 min at 37^{o}C, followed by PBS rinses.

Permeabilisation of the tissue sections were carried out in PBS with 0.3% Triton X-100 (PBST), followed by blocking in PBST with 5% goat serum. Immunofluorescent detection was accomplished by sequential overnight 4^{o}C incubation with primary antibodies, then fluorophore-conjugated secondary antibodies. Primary antibodies were used in the following dilutions: monoclonal rabbit anti-Ki67 (1:500; Abcam ab16667), monoclonal rat anti-BrdU (1:500; Accurate OBT0030), monoclonal mouse anti-NeuN (1:200; Millipore MAB377) and monoclonal mouse anti-S100β (1:1000; Sigma-Aldrich S2532). For immunofluorescence, the following secondary antibodies (Life Technologies), goat anti-rabbit conjugated with Alexa Fluor® 555, goat anti-rat conjugated with Alexa Fluor® 555, goat anti-mouse conjugated with Alexa Fluor® 488, were all used at a dilution of 1:500. Immunolabelled sections were rinsed in PBS and mounted on glass slides with Prolong® Gold antifade mounting medium without or with DAPI (Life Technologies) depending on whether the sections were acid-treated or not, respectively.

3.2.3. Quantification

For quantification of immunolabelled cells, every sixth section, an average total of 12 sections per series, covering each left and right dentate gyrus in its entire rostro-caudal extension, were analysed as previously described (Sections 2.2.6 and 2.2.7). To evaluate levels of proliferation and survival, respective Ki67- and BrdU-immunolabelled cells were counted bilaterally and density obtained for each dentate gyrus in each septal and temporal subregion. To determine neuronal and astroglial cell fate decision, BrdU-immunolabelled cells were checked for co-localisation with either neuronal marker, NeuN, or astroglial marker, S100β, using a confocal laser scanning microscope LSM 510. Percentage of BrdU-positive cells colabelled with either marker was obtained and analysed. To establish migration of newborn

neurons, percentage of BrdU/NeuN-colabelled cells in each one-third subdivision of granule cell layer (GCL) was determined. All samples were coded and cell counting was conducted blind to the genotype of the sample.

To establish age-specific changes in the various aspects of neurogenesis, the individual animal value of each parameter in the mature adult was expressed as ratio of the mean of the same parameter in young adult mice.

3.2.4. Image acquisition

Representative photomicrographs of immunofluorescence were acquired as previously described (Section 2.2.8). Briefly, Ki67- and BrdU-immunolabelled cells were captured with an upright fluorescence microscope (BX51; Olympus) with digital camera (DP70; Olympus). Orthogonal and z-projections of BrdU/NeuN- and BrdU/S100β-coexpressing cells were obtained from confocal laser scanning microscope LSM 510 (Carl Zeiss).

3.2.5. Statistical analyses

All statistical analyses were performed with SPSS (version 21; IBM Corp, NY, USA). Two-way ANOVA was conducted for the analysis of volume of granule cell layer, while three-way repeated measures ANOVA was used for rest of the parameters studied. Where appropriate, post hoc tests were carried out with Bonferroni corrections. All graphical representations were generated using GraphPad Prism 5 (GraphPad Software, CA, USA). The data are expressed as mean ± SEM (standard error of mean) and statistical significance is defined as P value < 0.05.

3.3. Results

3.3.1. Volume of dentate gyrus

To establish the role of relaxin-3 in adult hippocampal neurogenesis, relaxin-3 KO mice were used as a genetic loss-of-function tool for investigation. In the previous chapter, there were no gross alterations found in the volume of dentate gyrus for young adult mice. In this chapter, mature adult mice were examined for their levels of neurogenesis in hippocampus. The granule cell layer (GCL) volume was found to be significantly different between WT and KO mice (*P* value = 0.032, main effect of genotype, two-way ANOVA) when they were nine months old (Fig. 3.1A). Particularly, in the female mice, WTs have larger volume than KOs by 6.2 ± 2.2% (*P* value = 0.028, Bonferroni adjusted). In addition, sexual dimorphism was detected in the volume of granule cell layer (*P* value = 0.004, main effect of sex, two-way ANOVA), especially with WT females having larger volume than WT males by 7.9 ± 2.5% (*P* value = 0.009, Bonferroni adjusted). No further interaction between genotype and sex was detected (*P* value = 0.196, interaction effect, two-way ANOVA).

Age-specific change in volume of GCL was analysed by the ratio of mature adult to young adult GCL volume. The ratios showed that age-related changes in volume were relatively small, with an average difference of 4.6% (values range from -10.4% to 9.5%). In particular, only female WT mice had an increase in GCL volume with age, all other groups displayed a decrease. Furthermore, statistical analysis revealed a significant interaction effect between relaxin-3 and sex (*P* value = 0.036, interaction effect, two-way ANOVA), with female WT mice having larger volume ratio than female KO

and male WT mice (*P* values = 0.001 and 0.021, respectively, Bonferroni adjusted).

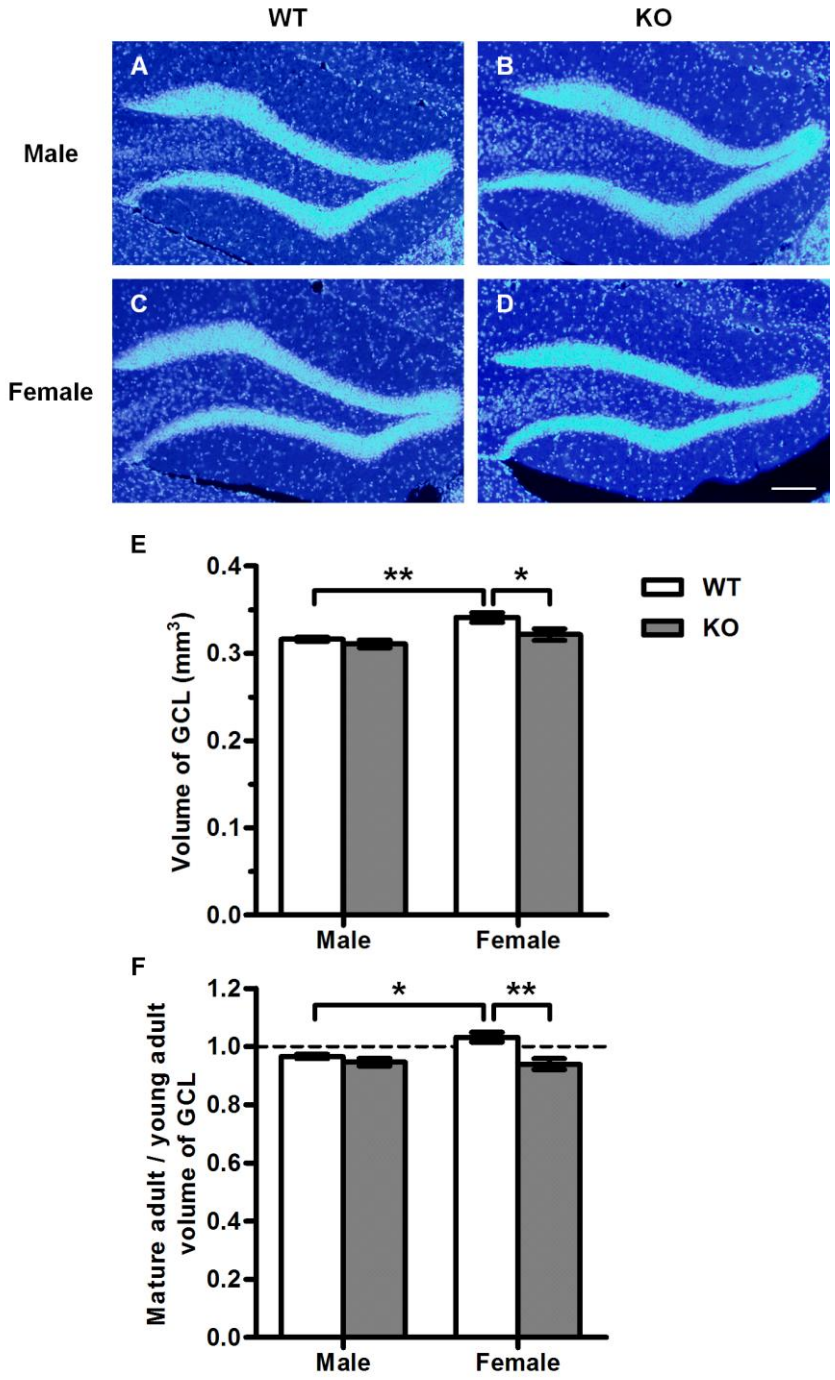

Figure 3.1. Female WT mice have largest volume of granule cell layer (GCL). Representative photomicrographs of DAPI-stained GCL in male WT (A) and KO (B), and female WT (C) and KO (D) mice. Scale bar corresponds to 100 μm. Volume of GCL was estimated by one in six sampling of hippocampal sections. Main effects of genotype (*P* value < 0.05) and sex (*P* value < 0.01) were statistically significant in two-way ANOVA analysis (E). Age-related change in GCL volume was analysed by expressing mature adult GCL volume as a ratio of young adult GCL volume. There was a significant interaction effect of genotype and sex (*P* value < 0.05, two-way ANOVA; F). All values were averaged from bilateral hippocampus, with data expressed as mean ± SEM; n = 4-6 per group. **P* value < 0.05, ***P* value < 0.01; Bonferroni post hoc tests.

3.3.2. Proliferation

To examine levels of proliferation in mature adult mice, density of Ki67-immunolabelled cells (Fig. 3.2) was acquired and analysed (Table 3.1A and Fig. 3.3A). Significant interaction between genotype and sex was detected (*P* value = 0.014, interaction effect, three-way repeated measures ANOVA). Specifically, proliferation in relaxin-3 KO mice, and not in littermate WT controls, was sexually dimorphic. Density of Ki67-positive cells was lower in female than male KOs in both septal and temporal dentate gyrus (*P* values = 0.027 and < 0.001, respectively, Bonferroni adjusted). Furthermore, in the temporal subregion of dentate gyrus, Ki67 cell density was found to be significantly lower in female KO than female WT animals (*P* value = 0.015, Bonferroni adjusted).

Effect of ageing on permanent deletion of relaxin-3 was also analysed by comparing proliferation in mature adult mice to that in young adults (Table 3.1B and Fig. 3.3B). At nine-month old, Ki67 cell density has decreased by 87.4%, on average, compared to that in three-month old mice. Statistical analysis of age-related changes in proliferation revealed a significant interaction between genotype and sex (*P* value = 0.003, interaction effect, three-way repeated measures ANOVA). Interestingly, density of Ki67 was significant different in relaxin-3 KO compared to WT controls, only in the temporal dentate gyrus (male *P* value = 0.039, female *P* value = 0.039, Bonferroni adjusted), where dense innervation of relaxin-3 fibres has been reported. However, KO animals showed less age-specific reduction than WT in males (WT: 86.1 ± 1.2% vs. KO: 80.2 ± 2.2% mean difference) but more age-specific decrease than WT in females (WT: 88.7 ± 0.7% vs. KO: 94.1 ± 1.2%).

In addition, sexual dimorphism was also found to be present only in the relaxin-3 KO mice, in both septal and temporal subregions of dentate gyrus (*P* values = 0.003 and < 0.001, respectively, Bonferroni adjusted). In both subregions, female KOs demonstrated larger age-related decline in Ki67 cell density than male KO mice. These results suggest that age-related effect of relaxin-3 is sex-dependent.

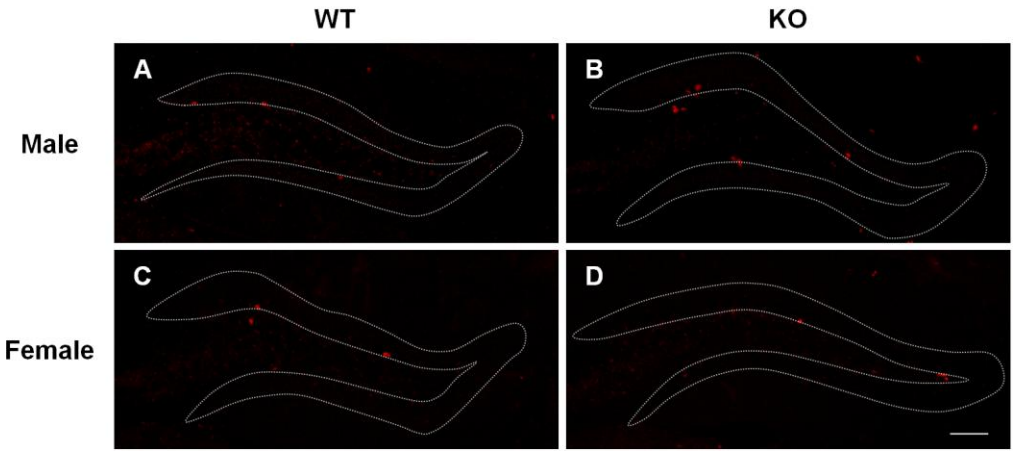

Figure 3.2. Ki67-expressing cells were located in the subgranular zone of dentate gyrus. Representative photomicrographs of Ki67-immunolabelled (red) cells in male WT (A) and KO (B), and female WT (C) and KO (D) mice. Scale bar corresponds to 100 μm.

Table 3.1. Results of three-way repeated measures ANOVA of Ki67 density by genotype, sex and septotemporal factor.

A. Density of Ki67 in mature adult mice

Source of variation	$F_{1,16}$	*P* value	Significance
Genotype × sex × ST location	0.033	0.858	
Genotype × sex	7.648	0.014	*
Genotype × ST location	1.150	0.299	
Sex × ST location	0.599	0.450	
Genotype	0.318	0.581	
Sex	15.498	0.001	**
ST location	0.760	0.396	

ST = septotemporal; * *P* value < 0.05; ** *P* value < 0.01

B. Ratio of mature adult to young adult mice Ki67 density

Source of variation	$F_{1,16}$	*P* value	Significance
Genotype × sex × ST location	0.510	0.485	
Genotype × sex	11.955	0.003	**
Genotype × ST location	0.010	0.922	
Sex × ST location	0.482	0.498	
Genotype	0.070	0.794	
Sex	28.815	< 0.001	***
ST location	0.007	0.934	

ST = septotemporal; ** *P* value < 0.01; *** *P* value < 0.001

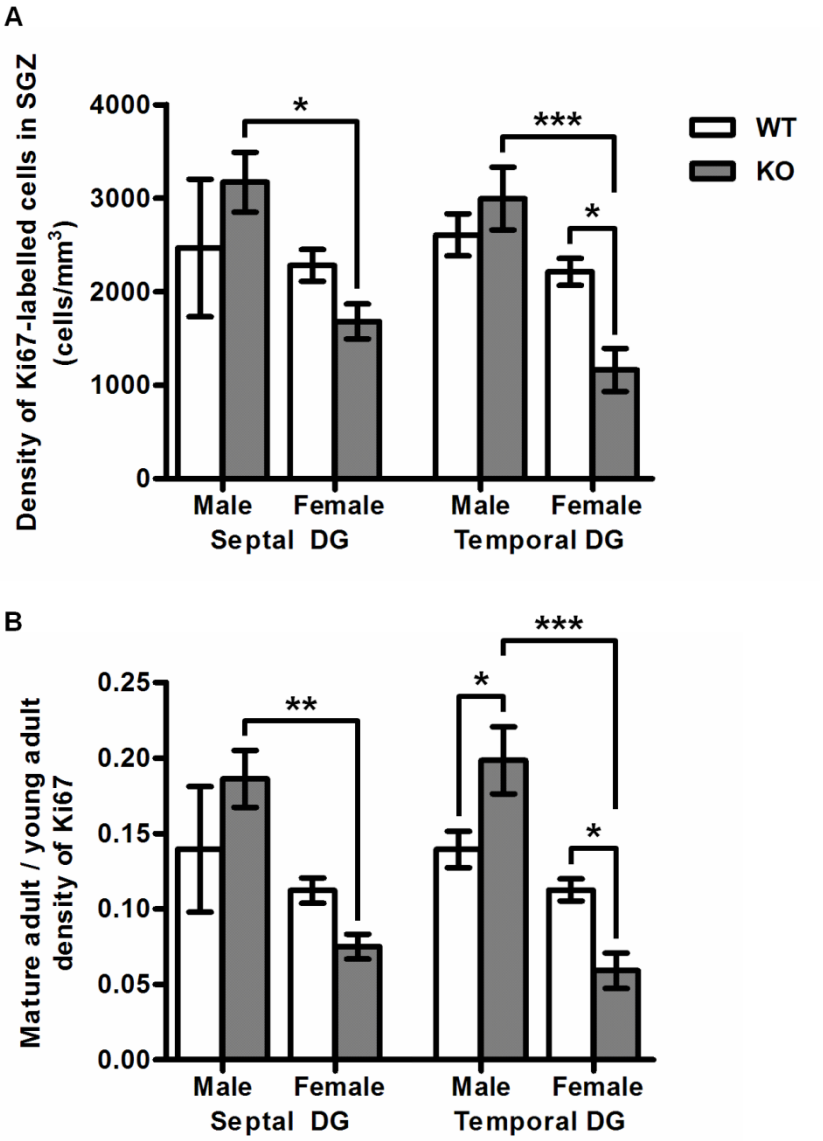

Figure 3.3. Sexual dimorphic proliferation in relaxin-3 KO mice at nine months of age. (A) Density of Ki67-labelled cells was obtained from the division of cell counts by volume of subgranular zone. Interaction effect of genotype × sex (*P* value < 0.05) and main effect of sex (*P* value < 0.01) were statistically significant in three-way repeated measures ANOVA analysis. (B) Age-related changes in Ki67 density was analysed by expressing mature adult Ki67 density as a ratio of young adult Ki67 density. Similarly, interaction effect of genotype × sex (*P* value < 0.01) and main effect of sex (*P* value < 0.001) were statistically significant in three-way repeated measures ANOVA analysis. All values were averaged from bilateral hippocampus, with data expressed as mean ± SEM; n = 4-6 per group. **P* value < 0.05, ***P* value < 0.01, ****P* value < 0.001; Bonferroni post hoc tests.

3.3.3. Neuronal differentiation

To assess the neuronal phenotype of newly born cells, double fluorescent immunolabelling for BrdU and NeuN was carried out (Fig. 3.4). Neuronal cell fate determination was analysed by comparing proportion of BrdU-positive cells coexpressing NeuN (Table 3.2A and Fig. 3.5A). Significant interaction effect between genotype and septotemporal localisation was found (*P* value = 0.046, interaction effect, three-way repeated measures ANOVA). Specifically, post hoc tests revealed that male relaxin-3 KO mice had greater percentage of BrdU/NeuN-colabelled cells than WTs in the temporal dentate gyrus (*P* value = 0.024, Bonferroni adjusted). In addition, except for male relaxin-3 KO mice, all other groups of animals displayed a lower degree of neuronal differentiation in the temporal subregion than septal dentate gyrus (*P* values for male WT < 0.001, female WT = 0.001 and female KO = 0.015, Bonferroni adjusted).

Longitudinal effect of ageing was investigated by analysing the percentage of BrdU/NeuN-coexpressing cells in eight- and two-month old mice (Table 3.2B and Fig. 3.5B). Age-related decrease in neuronal differentiation was found in both septal and temporal subregions of dentate gyrus, with reductions by one-third and half, respectively. Furthermore, statistical analysis showed significant main effect of sex and interaction effect between genotype and septotemporal localisation (*P* values = 0.018 and 0.037, respectively, three-way repeated measures ANOVA). Subsequent post hoc multiple comparisons revealed significant sex-specific difference in the temporal dentate gyrus of relaxin-3 KO mice (*P* value = 0.006, Bonferroni adjusted). Specifically, male KO animals have smaller age-related decrease of 37.8% as compared to female KO's 64.1% decrease in neuronal differentiation.

In addition, genotype-specific effect was also found in the temporal subregion of male mice, with KOs having a smaller decrement with ageing (*P* value = 0.033, Bonferroni adjusted). The genotype and septotemporal localisation interaction effect was also demonstrated with significant septotemporal differences in WT controls (*P* values for male = 0.003, female = 0.006, Bonferroni adjusted) but not relaxin-3 KO mice. These results illustrated age-related effects of relaxin-3 on neuronal differentiation in a dentate gyrus subregional-specific manner.

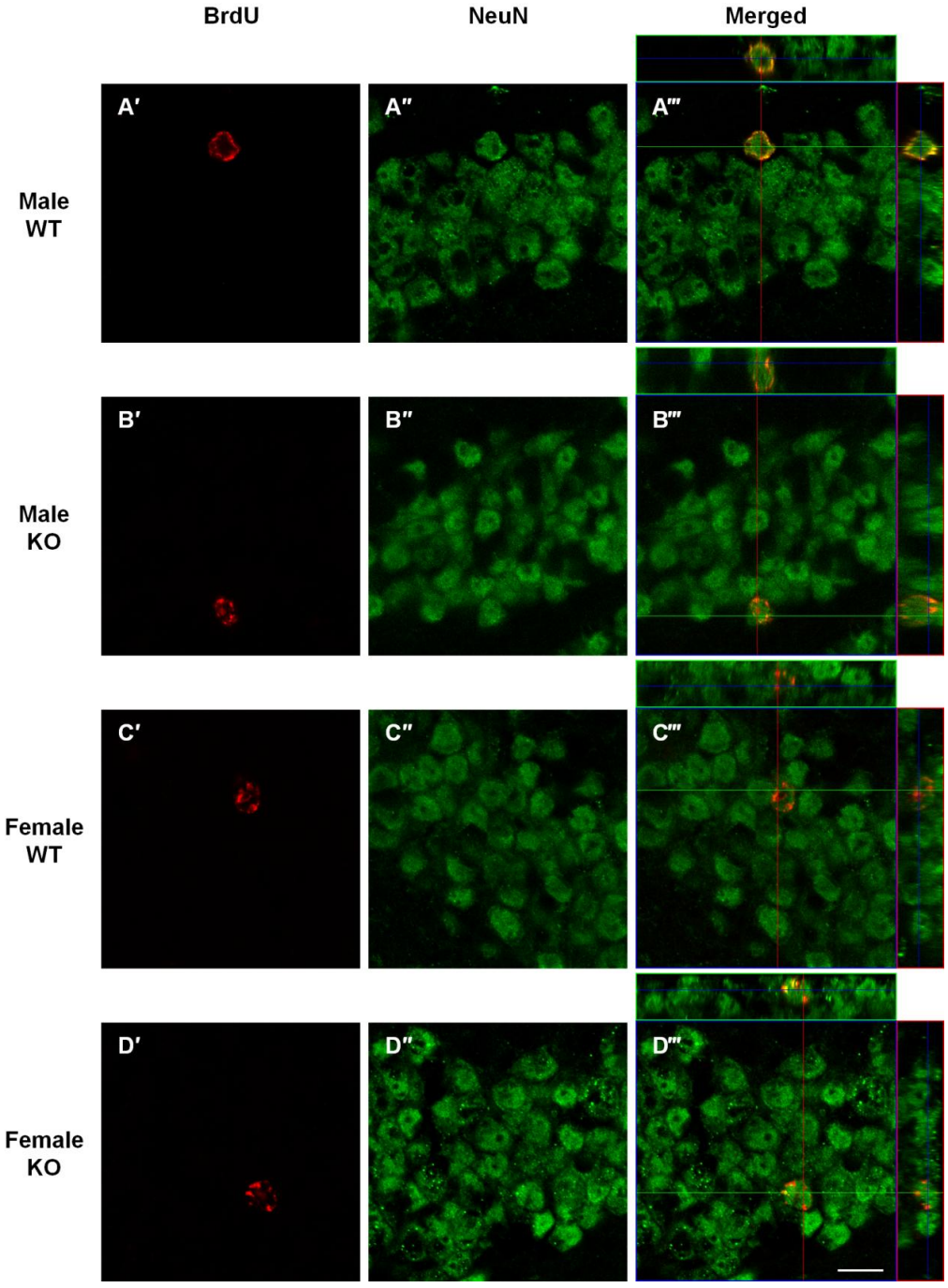

Figure 3.4. BrdU/NeuN-coexpressing cells were located in the dentate gyrus. Representative photomicrographs of BrdU/NeuN-colabelled cells (BrdU in red, NeuN in green) in male WT (A) and KO (B), and female WT (C) and KO (D) mice. Scale bar corresponds to 10 μm.

Table 3.2. Results of three-way repeated measures ANOVA of % BrdU/NeuN-colabelled cells by genotype, sex and septotemporal factor.

A. % of BrdU cells colabelled with NeuN in mature adult mice

Source of variation	$F_{1,16}$	*P* value	Significance
Genotype × sex × ST location	1.765	0.203	
Genotype × sex	2.003	0.176	
Genotype × ST location	4.701	0.046	*
Sex × ST location	0.022	0.885	
Genotype	0.017	0.899	
Sex	1.215	0.287	
ST location	56.028	< 0.001	***

ST = septotemporal; * *P* value < 0.05; *** *P* value < 0.001

B. Ratio of mature adult to young adult mice % BrdU/NeuN-colabelled cells

Source of variation	$F_{1,16}$	*P* value	Significance
Genotype × sex × ST location	1.978	0.179	
Genotype × sex	1.778	0.201	
Genotype × ST location	5.200	0.037	*
Sex × ST location	0.009	0.927	
Genotype	0.117	0.736	
Sex	6.952	0.018	*
ST location	31.157	< 0.001	***

ST = septotemporal; * *P* value < 0.05; *** *P* value < 0.001

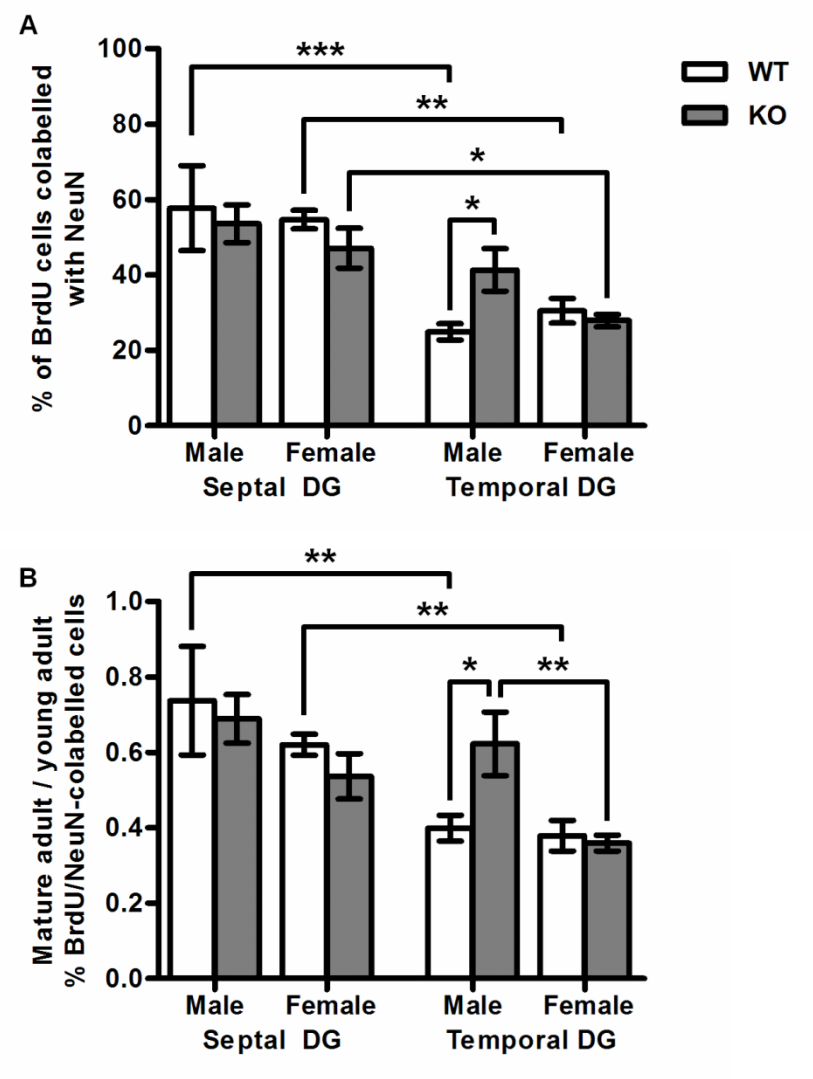

Figure 3.5. Male relaxin-3 KO mice displayed an attenuation of age-related reduction of neuronal differentiation specifically in temporal dentate gyrus. (A) Percentage of BrdU-positive cells colabelled with NeuN was calculated in septal and temporal dentate gyrus (DG). There was a significant interaction effect of genotype and septotemporal localisation (*P* value < 0.05, three-way repeated measures ANOVA). (B) Age-related changes in neuronal differentiation were analysed by expressing mature adult % BrdU/NeuN-colabelled cells as a ratio of young adult values. Interaction effect of genotype and septotemporal localisation (*P* value < 0.05) and main effect of sex (*P* value < 0.05) were statistically significant in three-way repeated measures ANOVA analysis. All values were averaged from bilateral hippocampus, with data expressed as mean ± SEM; n = 4-6 per group. **P* value < 0.05, ***P* value < 0.01, ****P* value < 0.001; Bonferroni post hoc tests.

3.3.4. Astroglial differentiation

In addition, to identify astroglial phenotype of newborn cells, BrdU and S100β were fluorescently labelled by double immunostaining (Fig. 3.6). Astroglial cell fate determination in mature adult mice was then studied by analysing percentage of BrdU-positive cells colabelled with S100β (Table 3.3A and Fig. 3.7A). Lifelong deficiency of relaxin-3 did not affect astroglial differentiation as both WT and KO mice had similar proportions of newborn cells with S100β marker. Of the factors investigated, only septotemporal location had a significant effect on astroglial fate decision (*P* value < 0.001, main effect, three-way repeated measures ANOVA). Higher level of astroglial differentiation was observed in the temporal dentate gyrus with 29.7 ± 2.7% newborn cells coexpressing S100β, as compared to 20.3 ± 2.2% in the septal subregion. Further post hoc pairwise comparisons revealed significant septotemporal difference particularly in female WT animals (*P* value = 0.015, Bonferroni adjusted).

Comparing with astroglial differentiation in young adult mice, age-specific changes were analysed (Table 3.3B and Fig. 3.7B). In contrast to age-related reduction in neuronal differentiation, there was an age-related increase in astroglial differentiation by an average of 3.49 fold. The changes were independent of relaxin-3 and sex factors; instead, they were septotemporal specific (*P* value = 0.034, main effect, three-way repeated measures ANOVA). Septal dentate gyrus had a slightly higher age-related increase of 3.81 ± 0.37 fold, while temporal subregion 3.16 ± 0.28 fold. These results suggested that, unlike neuronal differentiation, astroglial differentiation in ageing was independent of relaxin-3's actions.

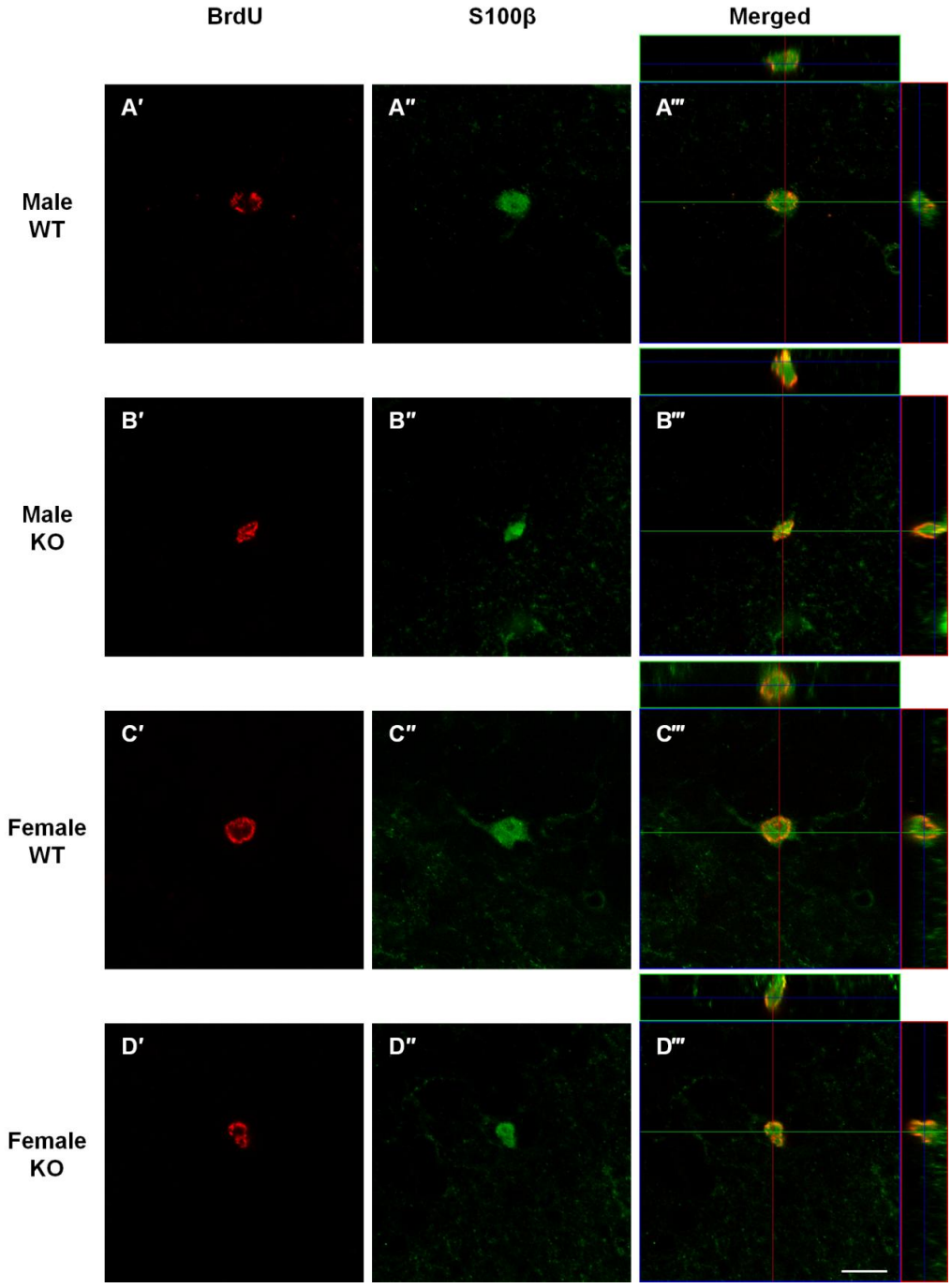

Figure 3.6. BrdU/S100β-coexpressing cells were located in the dentate gyrus. Representative photomicrographs of BrdU/S100β-colabelled cells (BrdU in red, S100β in green) in male WT (A) and KO (B), and female WT (C) and KO (D) mice. Scale bar corresponds to 10 μm.

Table 3.3. Results of three-way repeated measures ANOVA of % BrdU/S100β-colabelled cells by genotype, sex and septotemporal factor.

A. % of BrdU cells colabelled with S100β in mature adult mice

Source of variation	$F_{1,16}$	*P* value	Significance
Genotype × sex × ST location	0.057	0.814	
Genotype × sex	0.033	0.858	
Genotype × ST location	0.128	0.725	
Sex × ST location	0.266	0.613	
Genotype	0.476	0.500	
Sex	2.658	0.123	
ST location	23.046	< 0.001	***

ST = septotemporal; *** *P* value < 0.001

B. Ratio of mature adult to young adult mice % BrdU/S100β-colabelled cells

Source of variation	$F_{1,16}$	*P* value	Significance
Genotype × sex × ST location	0.490	0.494	
Genotype × sex	0.113	0.741	
Genotype × ST location	0.009	0.925	
Sex × ST location	1.829	0.195	
Genotype	0.536	0.475	
Sex	0.048	0.829	
ST location	5.407	0.034	*

ST = septotemporal; * *P* value < 0.05

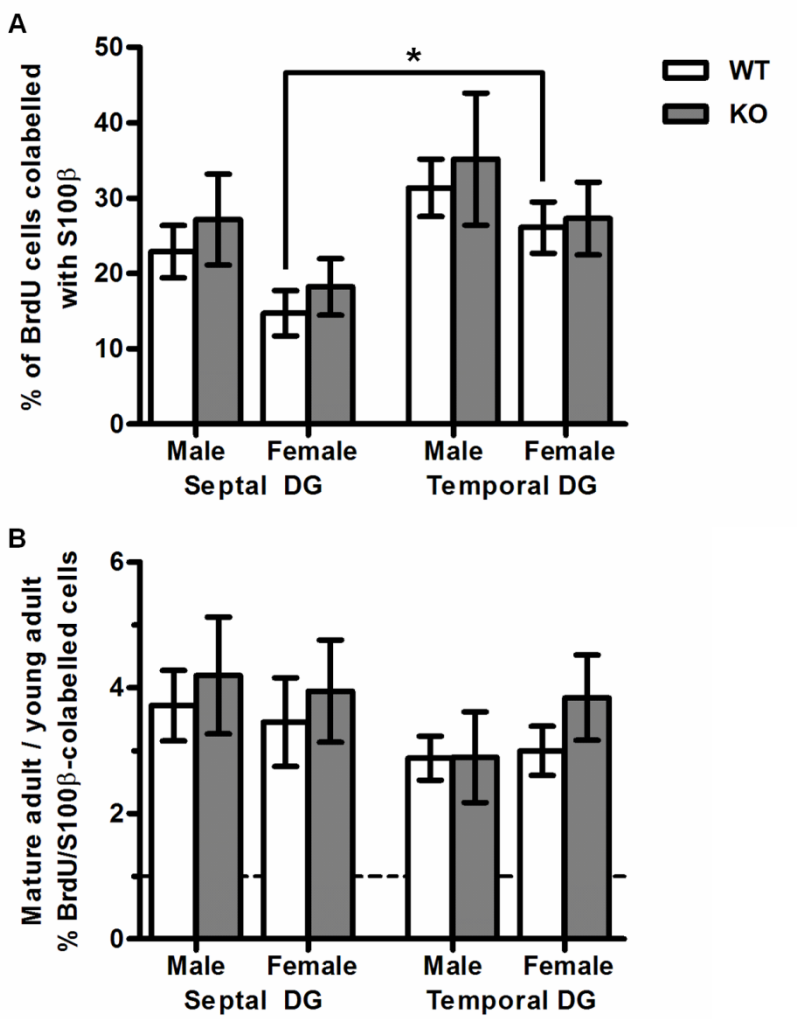

Figure 3.7. No difference between relaxin-3 KO mice and WT littermates in astroglial differentiation with ageing. (A) Percentage of BrdU-positive cells colabelled with S100β was calculated in septal and temporal dentate gyrus (DG). There was a significant main effect of septotemporal localisation (*P* value < 0.001, three-way repeated measures ANOVA). (B) Age-related changes in astroglial differentiation were analysed by expressing mature adult % BrdU/S100β-colabelled cells as a ratio of young adult values. Similarly, a significant main effect of septotemporal localisation (*P* value < 0.05, three-way repeated measures ANOVA) was detected. All values were averaged from bilateral hippocampus, with data expressed as mean ± SEM; n = 4-6 per group. **P* value < 0.05; Bonferroni post hoc test.

3.3.5. Migration of newborn neurons

Migration of newborn neurons in mature adult mice was investigated by quantifying proportion of BrdU/NeuN-colabelled cells in each one-third zone of the granule cell layer (GCL) (Table 3.4A and Fig. 3.8A). Permanent deletion of relaxin-3 gene had sex- and septotemporal-dependent effects on the distribution of newly born neurons, particularly in the inner and outer GCL (*P* values < 0.05, three-way interaction effect, three-way repeated measures ANOVA). The percentage of BrdU/NeuN-coexpressing cells residing in the temporal inner GCL of female KO mice was significantly lower than that in same-location same-sex WT controls (*P* value = 0.027, Bonferroni adjusted). Furthermore, in the same location, sexual dimorphism in the KO mice was detected, with female having lesser percentage of BrdU/NeuN-colabelled cells than male mice (*P* value = 0.002, Bonferroni adjusted). In addition, within the female KO group, the proportion of newborn neurons was also lower in the temporal inner GCL when compared to the septal subregion (*P* value = 0.003, Bonferroni adjusted). Conversely, when compared to the septal outer GCL, the temporal proportion of newborn neurons in the same group of female KO mice was four-fold higher (*P* value = 0.003, Bonferroni adjusted).

When compared to young adult mice, migration in mature adult animals was increased. The changes in ageing were analysed by the ratio of mature adult to young adult percentage of BrdU/NeuN-colabelled cells in each one-third subdivision of GCL (Table 3.4B and Fig. 3.8B). On average, the proportion of newborn neurons in the inner GCL decreased by 16.6% in septal and 23.1% in temporal dentate gyrus. In the middle GCL, there were 1.92-fold and 1.73 fold increase in septal and temporal dentate gyrus, respectively; and in the outer GCL, 2.55-fold and 5.37-fold increase in septal and temporal dentate

gyrus, respectively. Therefore, ageing from two months old to eight months old resulted in an altered distribution of newborn neurons, with less proportion staying in the inner GCL and more migrating to the middle and outer layers.

Statistical analyses revealed significant interaction effect of genotype, sex and septotemporal localisation in the inner GCL (*P* value = 0.030, three-way repeated measures ANOVA). Post hoc comparisons in the inner GCL reported an age-related decrease in percentage of BrdU/NeuN-colabelled cells in the temporal dentate gyrus of female KO mice, and is significantly higher when compared pairwise with temporal female WT (*P* value = 0.030, Bonferroni adjusted), temporal male KO (*P* value = 0.003, Bonferroni adjusted), and septal female KO mice (*P* value < 0.001, Bonferroni adjusted).

In the middle GCL, there was a significant main effect of genotype (*P* value = 0.030, three-way repeated measures ANOVA), especially in the septal dentate gyrus of male mice (*P* value = 0.016, Bonferroni adjusted). As for the outer GCL, main effect of septotemporal location was found (*P* value = 0.004, three-way repeated measures ANOVA), particularly in the female KO animals (*P* value = 0.025, Bonferroni adjusted).

These results showed that, in the permanent absence of relaxin-3, there was a female-specific upregulation of migration of newborn neurons in the temporal dentate gyrus of mature adult mice.

Table 3.4. Results of three-way repeated measures ANOVA of % BrdU/NeuN-colabelled cells in each GCL subdivision.

A. Distribution of BrdU/NeuN-colabelled cells in mature adult mice

GCL subdivision	Source of variation	$F_{1,16}$	*P* value	Significance
Inner	Genotype × sex × ST location	5.009	0.040	*
	Genotype × sex	4.663	0.046	*
	Genotype × ST location	6.915	0.018	*
	Sex × ST location	1.449	0.246	
	Genotype	0.193	0.667	
	Sex	7.686	0.014	*
	ST location	3.348	0.086	
Middle	Genotype × sex × ST location	0.158	0.696	
	Genotype × sex	2.760	0.116	
	Genotype × ST location	0.829	0.376	
	Sex × ST location	0.162	0.693	
	Genotype	2.752	0.117	
	Sex	3.935	0.065	
	ST location	1.802	0.198	
Outer	Genotype × sex × ST location	6.796	0.019	*
	Genotype × sex	0.702	0.414	
	Genotype × ST location	1.894	0.188	
	Sex × ST location	0.420	0.526	
	Genotype	0.348	0.564	
	Sex	1.350	0.262	
	ST location	11.407	0.004	**

ST = septotemporal; * *P* value < 0.05; ** *P* value < 0.01

Table 3.4. (Continued)

B. Ratio of mature adult to young adult mice distribution of BrdU/NeuN-colabelled cells

GCL subdivision	Source of variation	$F_{1,16}$	*P* value	Significance
Inner	Genotype × sex × ST location	5.679	0.030	*
	Genotype × sex	3.621	0.075	
	Genotype × ST location	8.706	0.009	**
	Sex × ST location	2.867	0.110	
	Genotype	0.331	0.573	
	Sex	5.575	0.031	*
	ST location	4.299	0.055	
Middle	Genotype × sex × ST location	0.187	0.671	
	Genotype × sex	0.861	0.367	
	Genotype × ST location	1.135	0.303	
	Sex × ST location	2.715	0.119	
	Genotype	5.660	0.030	*
	Sex	2.158	0.161	
	ST location	0.495	0.492	
Outer	Genotype × sex × ST location	3.018	0.102	
	Genotype × sex	0.284	0.601	
	Genotype × ST location	3.513	0.079	
	Sex × ST location	0.951	0.344	
	Genotype	0.986	0.335	
	Sex	0.032	0.861	
	ST location	10.917	0.004	**

ST = septotemporal; * *P* value < 0.05; ** *P* value < 0.01

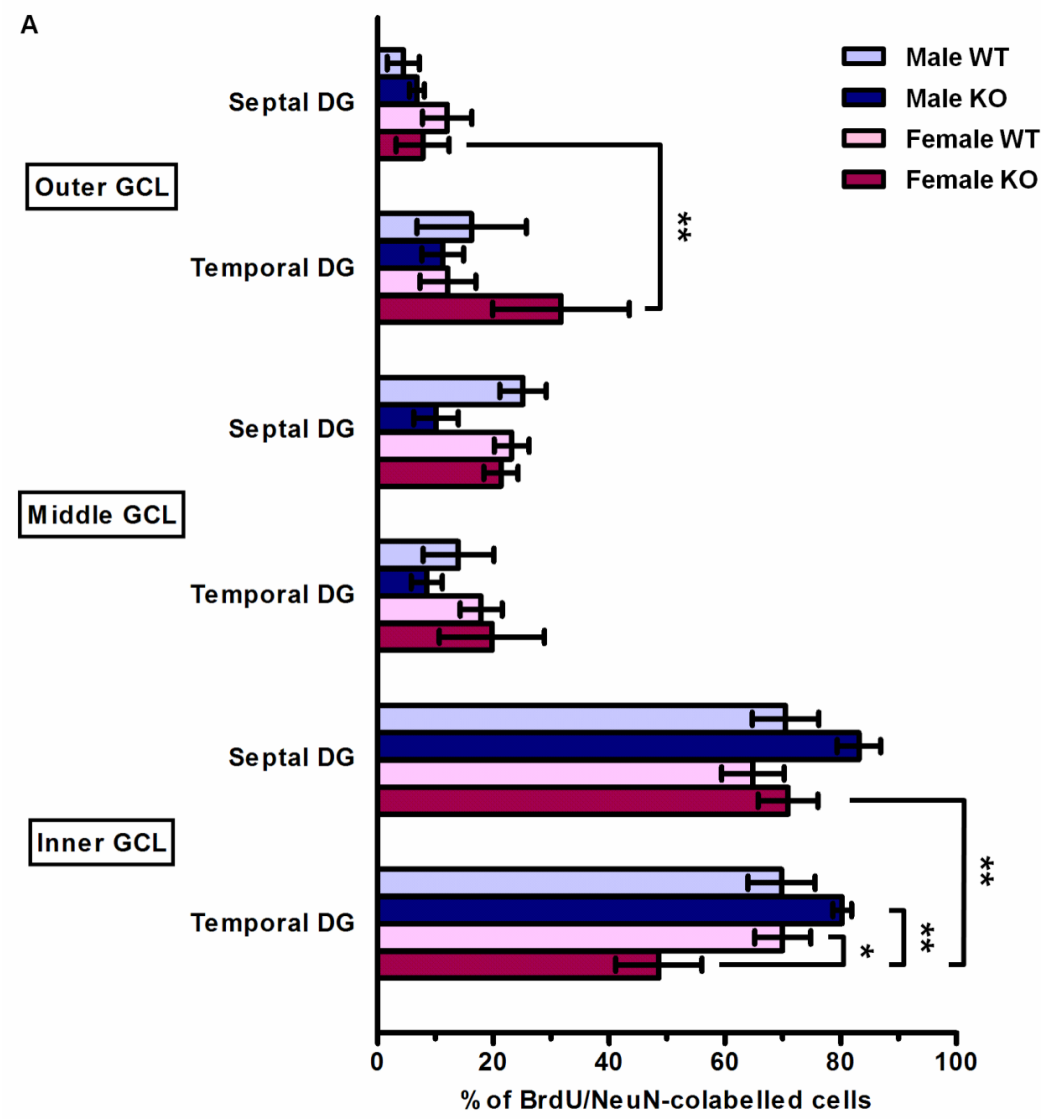

Figure 3.8. Female relaxin-3 KO mice displayed an increase in migration of newborn neurons, specifically in the temporal dentate gyrus. (A) Percentage of BrdU/NeuN-colabelled cells in each of inner, middle and outer granule cell layer (GCL) was calculated for each group of animals, in both septal and temporal dentate gyrus (DG). Significant interaction effect of genotype, sex and septotemporal localisation was detected for inner and outer GCL (*P* values < 0.05, interaction effect, three-way repeated measures ANOVA). (B) Age-related changes in distribution of newborn neurons were analysed by expressing mature adult % BrdU/NeuN-colabelled cells as a ratio of young adult values. Interaction effect of genotype, sex and septotemporal localisation for inner GCL (*P* value < 0.05), main effect of genotype for middle GCL (*P* value < 0.05) and main effect of septotemporal location (*P* value < 0.01) were statistically significant in three-way repeated measures ANOVA analysis. All values were averaged from bilateral hippocampus, with data expressed as mean ± SEM; n = 4-6 per group. **P* value < 0.05, ***P* value < 0.01, ****P* value < 0.001; Bonferroni post hoc tests.

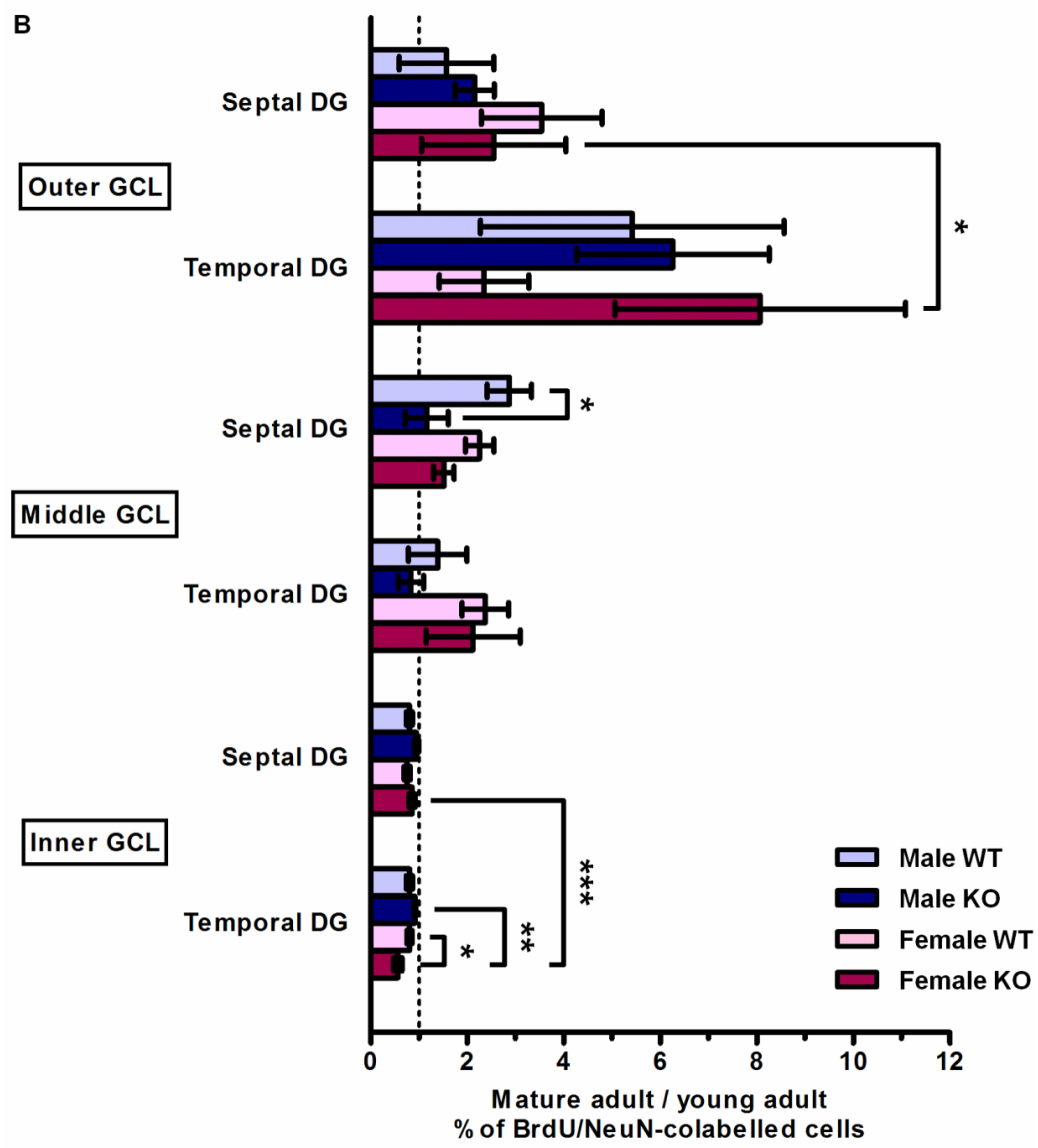

Figure 3.8. (Continued)

3.3.6. Survival of newborn cells

In adult neurogenesis, a surplus of progenitor cells is produced, and undergoes elimination to keep only those selected few. The survival of newborn cells in mature adult mice (labelled by BrdU, Fig. 3.9) was examined by analysing the density of BrdU-positive cells remaining four weeks after BrdU administration (Table 3.5A and Fig. 3.10A). Constitutive deletion of relaxin-3 gene was not found to play a role in the survival process. Instead, sex and septotemporal localisation interact and significantly affect density of BrdU-labelled cells (*P* value = 0.048, three-way repeated measures ANOVA).

Post hoc comparisons found significant septotemporal differences only in the female mice (*P* values for WT = 0.003, KO < 0.001, Bonferroni adjusted), but not the male animals. Temporal BrdU density was 30.6 ± 7.6% and 39.4 ± 8.2% lesser than that in septal dentate gyrus for WT and KO female mice, respectively. In addition, within each septal and temporal subregions, sexual dimorphic survival was detected significant for WT mice in septal dentate gyrus (*P* value = 0.009, Bonferroni adjusted), and for both WT and KO mice in the temporal pole (*P* values < 0.001 and = 0.026, respectively, Bonferroni adjusted). For each of these comparisons, female have higher density of BrdU-positive cells, by 1.24 to 1.80 fold, than male mice.

To investigate the effect of ageing on survival of newborn cells, the ratio of mature adult to young adult BrdU density was computed and analysed (Table 3.5B and Fig. 3.10B). Ageing from two-month old to eight-month old resulted in a strong reduction of survival, with an average of 17.2 ± 4.0% and 26.8 ± 3.5% remaining in the mature adult septal and temporal dentate gyrus, respectively. Similar to the levels of survival in mature adult mice, relaxin-3 did not have a significant effect on the age-specific changes in survival.

Instead, the septotemporal location was the only factor found to be significant (*P* value < 0.001, main effect, three-way repeated measures ANOVA). Further post hoc tests confirmed septotemporal differences in each group of animals (*P* values ≤ 0.001, Bonferroni adjusted). Therefore, although there were sex and septotemporal localisation interaction in both young and mature adult mice, sex differences did not contribute to proportional changes in the ageing process.

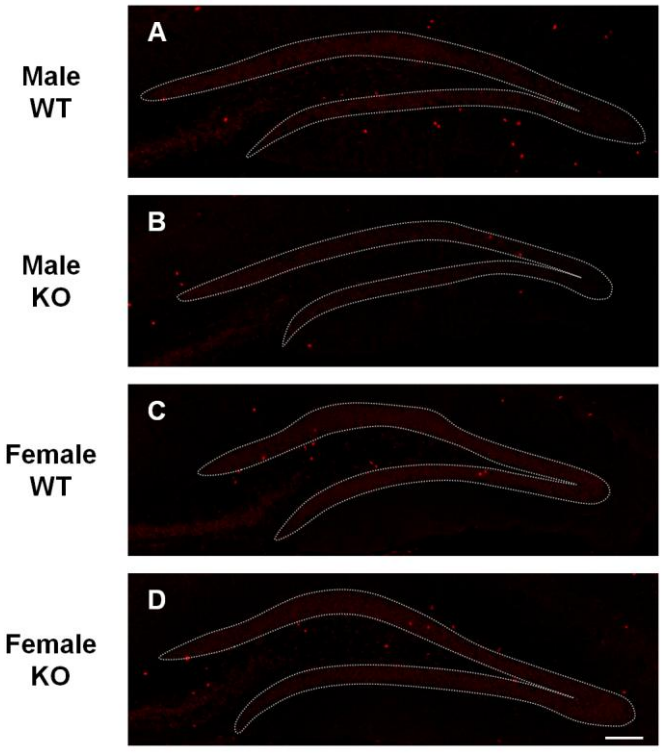

Figure 3.9. BrdU-expressing cells were located in the granular cell layer of dentate gyrus. Representative photomicrographs of BrdU-immunolabelled (red) cells in male WT (A) and KO (B), and female WT (C) and KO (D) mice. Scale bar corresponds to 100 μm.

Table 3.5. Results of three-way repeated measures ANOVA of BrdU density by genotype, sex and septotemporal factor.

A. Density of BrdU in mature adult mice

Source of variation	$F_{1,16}$	*P* value	Significance
Genotype × sex × ST location	0.027	0.871	
Genotype × sex	1.978	0.179	
Genotype × ST location	0.974	0.338	
Sex × ST location	4.575	0.048	*
Genotype	0.811	0.381	
Sex	31.678	< 0.001	***
ST location	39.368	< 0.001	***

ST = septotemporal; * *P* value < 0.05; *** *P* value < 0.001

B. Ratio of mature adult to young adult mice BrdU density

Source of variation	$F_{1,16}$	*P* value	Significance
Genotype × sex × ST location	0.761	0.396	
Genotype × sex	1.218	0.286	
Genotype × ST location	0.313	0.583	
Sex × ST location	0.070	0.795	
Genotype	1.604	0.223	
Sex	0.008	0.930	
ST location	123.522	< 0.001	***

ST = septotemporal; *** *P* value < 0.001

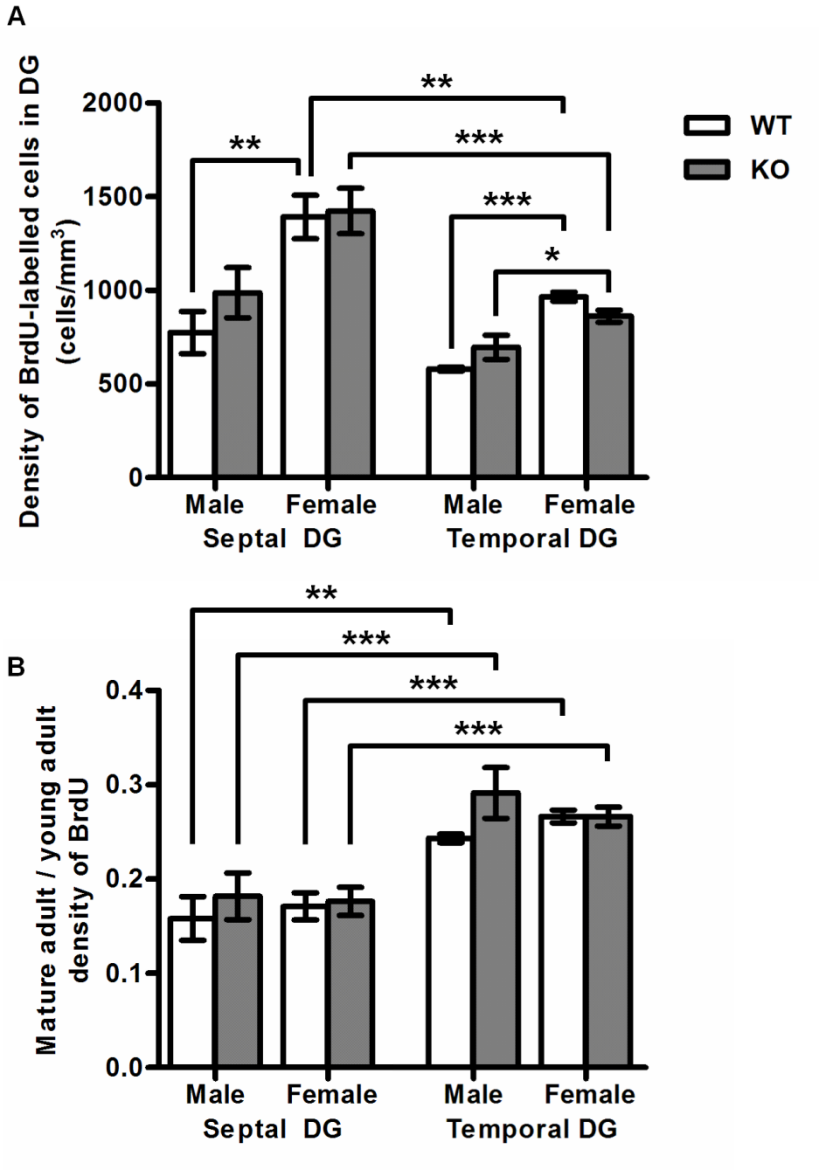

Figure 3.10. Survival of newborn cells in ageing was dependent on sex and/or septotemporal localisation, but not relaxin-3. (A) Density of BrdU-positive cells was obtained from the division of cell counts by GCL volume. There was a significant interaction effect of sex and septotemporal location (*P* value < 0.05, three-way repeated measures ANOVA). (B) Age-related changes in BrdU density was analysed by expressing mature adult BrdU density as a ratio of young adult BrdU density. Only the main effect of septotemporal localisation was detected significant in three-way repeated measures ANOVA (*P* value < 0.001). All values were averaged from bilateral hippocampus, with data expressed as mean ± SEM; n = 4-6 per group. **P* value < 0.05, ***P* value < 0.01, ****P* value < 0.001; Bonferroni post hoc tests.

3.4. Discussion

In this chapter, the role of relaxin-3 in hippocampal neurogenesis was investigated in the process of ageing. Constitutive knockout of relaxin-3 gene was found to have sex-specific effects in eight-month-old mice. Particularly, in the temporal dentate gyrus of male knockout mice, there was less age-related reduction in proliferation when compared to that in wildtype littermates. Also, male knockout male exhibited a higher level of neuronal differentiation in the same temporal subregion. On the other hand, female knockout mice had a lower temporal dentate gyrus proliferation than controls. In addition, female knockout mice also displayed an altered neuronal development in the temporal pole of dentate gyrus, with increased migration of newborn neurons from the subgranular zone into granule cell layer. As relaxin-3 innervates temporal dentate gyrus more densely than the septal pole, the effects of lifelong deficiency of relaxin-3 appear to be localised in the temporal subregion in mature adult mice. These findings suggest that relaxin-3 may act locally to regulate stages of neurogenesis in temporal dentate gyrus.

Cell proliferation in adult neurogenesis decreases exponentially with age (Seki and Arai, 1995; Kuhn et al., 1996; Kempermann et al., 1998; Nacher et al., 2003; McDonald and Wojtowicz, 2005; Ben Abdallah et al., 2010). In this study, there were 85 – 90% reductions in Ki67 cell density from three to nine month old wildtype C57BL/6J mice. This magnitude of age-associated decline is in consistent with the literature, such as the 85% reduction for same age difference in the same mouse strain reported by Ben Abdallah and colleagues in 2010. Interestingly, relaxin-3 deficiency resulted in male-specific decrease in young adult but female-specific decrease in mature adult temporal dentate

gyrus. The age-dependent sex differences were also reflected in the analysis of mature adult to young adult ratio, whereby male knockouts had reduced while female knockouts had enhanced age-related decline in proliferation, in the temporal pole of dentate gyrus.

In addition, sexual dimorphism existed in both septal and temporal subregions of dentate gyrus, at both time points in the knockout mouse adulthood. However, there was a reverse in sex differences from young adult to mature adult stages. In young adult knockout mice, female animals had higher levels of proliferation than male animals; while, in mature adult knockout mice, male animals had higher levels of proliferation than female animals. This reversal in sexual dimorphism with ageing suggests an intricate interaction between relaxin-3, sex differences and age differences in the modulation of cell proliferation.

One possible explanation could be the age-dependent differences in levels of gonadal hormones. In mammals, including humans and rodents, levels of sex steroids decrease with age (reviewed in Gillies and McArthur, 2010). Estrogen level in females drastically drops after menopause in humans, while testosterone level in males declines more gradually (Lamberts et al., 1997). In addition to the different hormonal profile in the two sexes, gonadal hormones may play sex-specific roles in each sex as well. For example, estrogen can induce an upregulation of cell proliferation in female but not male rats (reviewed in Galea et al., 2013), Therefore, due to the differences in the levels and actions of gonadal hormones, the two sexes may utilise different modulatory mechanisms for homeostatic control of cell proliferation. As deletion of relaxin-3 gene resulted in age-specific sexual dimorphisms in mice,

relaxin-3 may be a sex-specific modulator playing different roles in young adult and mature adult stages.

Neuronal differentiation has been observed to be reduced in the course of ageing (Kempermann et al., 1998; Lichtenwalner et al., 2001; Nacher et al., 2003; Bondolfi et al., 2004; van Praag et al., 2005). In male C57BL/6 mice, the percentage of BrdU-positive cells coexpressing NeuN was found to be reduced from ~70% in two month old mice to ~40% in twelve month old mice (Bondolfi et al., 2004). The reported range is in agreement to the same sex group in this study – an average of 70% septal and temporal neuronal differentiation in two-month-old mice, and an average of 41% in eight month old mice.

As with proliferation, the effect of relaxin-3 depletion on neuronal differentiation was septotemporal-, sex- and age-dependent. There were no knockout-induced differences in young adult mice for both sex groups in both septal and temporal dentate gyrus. However, in mature adult mice, male knockout mice exhibited higher degree of neuronal differentiation than wildtype controls, specifically in the temporal pole of dentate gyrus. As delayed neuronal fate choice has been demonstrated in older animals (Rao et al., 2005), the effect of relaxin-3 knockout could be due to an increased rate of neuronal development and/or final neuronal fate decision. Also, as there is a lack of understanding on the age- and sex-specific regulations of neuronal differentiation, the mechanism of relaxin-3's action is therefore uncertain at the moment.

Another stage of newborn neuron development is migration from subgranular zone into granule cell layer. There have been few studies looking at the effect

of ageing on migration of newborn cells. One study found a reduction in migration of four week old BrdU-labelled cells in 12 and 24 month old rats, as compared to that in 6 weeks old rats (Heine et al., 2004). Similarly, another study reported 65% of cells less than a month old in the granule cell layer in older rats, as compared to 82% of that in young adult rats (Rao et al., 2005). However, the distribution became similar for newborn cells of five months old in all three groups of young, middle-aged and aged rats, hence, it was a delay in migration in older animals instead of reduction of final migration (Rao et al., 2005).

In contrast to literature, there was an increase in migration of one month old newborn neurons in eight month old mice. This discrepancy could be due to examination of newborn neurons (double labelled with BrdU and NeuN), instead of newborn cells (singly labelled with BrdU) in previous reports. However, brief analysis of the distribution of BrdU-positive cells in the current study revealed similar increase in migration as BrdU/NeuN-coexpressing cells (results not shown). An alternative explanation could the different species used, mice in the current study and rats in the mentioned literature, suggesting species-dependent effect in age-related migration of newborn cells.

In the previous chapter, relaxin-3 deficiency, specifically in the female, exhibited an increased migration of newborn neurons in the septal dentate gyrus. In this chapter, ageing from young adult stage to mature adult stage similarly saw an increased migration in the female knockout mice. However, the localisation of this effect changed from septal pole to temporal pole of dentate gyrus. As there is a higher density of relaxin-3 fibres in the temporal dentate gyrus, the altered migration in septal pole in young adult mice was

attributed to possible indirect pathways. Therefore, one possibility for an age-dependent change in localisation of knockout effect could be an age-dependent change from indirect to direct relaxin-3 modulation of migration in hippocampal neurogenesis.

Two other neurogenesis stages investigated in this chapter are astroglial differentiation and survival of newborn cells. In both literature and current chapter, astroglial differentiation increases (Kempermann et al., 1998; Bondolfi et al., 2004; van Praag et al., 2005), while survival of newborn cells decreases with age (Seki and Arai, 1995; Kuhn et al., 1996; Kempermann et al., 1998). Lifelong depletion of relaxin-3 did not have an effect on these two processes in mature adult mice, suggesting a more compensable role.

While there was a lack of relaxin-3 effect in astroglial fate choice, septotemporal localisation was found to have a significant role. In particular, there was more astroglial differentiation in the temporal dentate gyrus than septal. This septotemporal gradient is similar in both young adult and mature adult mice. In agreement, Jinno (2011a) has reported comparable findings by studying density of astroglial lineage-committed progenitor cells in 10 month old mice. As astroglia have regulatory role in neurogenesis (Song et al., 2002), a septotemporal gradient of newly born astroglia could possibly contribute to a septotemporal-dependent regulation. Furthermore, the age-related increase in astroglial differentiation may also mean a larger share of astroglia regulation with ageing.

As for the survival phase of neurogenesis, sex and septotemporal localisation were detected to have significant interaction effect. As a result of the interaction between the two factors, septotemporal difference in survival of

newly born cells was only present in the female and not male mice. In particular, less newborn cells remained in the temporal dentate gyrus four weeks after cell division. To the best of our knowledge, this is the first report on sex and septotemporal interaction effect on survival of newborn cells in neurogenesis. This finding suggests a vulnerability in functions of temporal dentate gyrus for females in older age.

In summary, this chapter has examined the role of relaxin-3 in hippocampal neurogenesis in the mature adult stage. In contrast to that in young adults, constitutive knockout of relaxin-3 gene resulted in sex- and/or septotemporal-dependent alterations in several neurogenic phases. All knockout effects were specifically localised in the temporal pole of dentate gyrus, where dense relaxin-3 projections normally exist. Therefore, there is probably an increased age-related importance of relaxin-3 as a local modulator for cell proliferation and development of newborn neurons. However, the modulatory actions of relaxin-3 are sex-specific, with the absence of relaxin-3 having less proliferation in female and less age-related reduction in proliferation in male mice, increased neuronal differentiation in male and increased migration of newborn neurons in female mice. Together, these findings demonstrated sex- and septotemporal-dependent effect of relaxin-3 in various neurogenesis stages in the mature adult mouse hippocampus.

Chapter 4

Relaxin-3 innervation in the mouse dentate gyrus

4. Relaxin-3 innervation in the mouse dentate gyrus

4.1. Introduction

Since the discovery of relaxin-3 gene in 2002, study of its spatiotemporal expression has been carried out. Gene expression was first detected in rat brain at embryonic day 18, and the expression was shown to increase with development until the young adult stage in rats (Miyamoto et al., 2008). Similarly, protein expression of relaxin-3 was weakly detected after birth, and more strongly detected by the end of the neonatal period (Miyamoto et al., 2008). In humans, peripheral relaxin-3 could be detected in the middle age and elderly, and its level was not statistically correlated with age (Ghattas et al., 2013; Zhang et al., 2013). Collectively, these studies suggest a gradual increase in relaxin-3 expression from late embryonic stage to young adulthood, and stable levels in later adult stages.

Examination of relaxin-3 expression was typically performed in male rodents (Tanaka et al., 2005; Ma et al., 2007; Miyamoto et al., 2008; Smith et al., 2010), a phenomenon commonly observed in neuroscience research (reviewed in Beery and Zucker, 2011). The main rationale for using male animals is to avoid possible variations in experimental responses due to estrous cycle in females (Beery and Zucker, 2011). However, with regards to relaxin-3, this limits understanding of its roles and functions in female. In particular, knocking out relaxin-3 gene was found to have sexually dimorphic effects on different stages of adult hippocampal neurogenesis. In addition, stress and food restriction were found to have sex-specific regulation of relaxin-3 expression in the nucleus incertus (Lenglos et al., 2013). Therefore,

these two studies suggest possible sex differences in regulations and functions of relaxin-3.

Detailed spatial expression of relaxin-3 has been studied and reported. Restricted expression of relaxin-3 was found mostly in the nucleus incertus, and lesser in periaqueductal grey, pontine raphe nucleus and dorsal area around substantia nigra (Tanaka et al., 2005; Ma et al., 2007; Smith et al., 2010). From these neuronal groups, relaxin-3 is then projected extensively to numerous brain regions, including, but not limited to, the dentate gyrus, medial septum, and lateral hypothalamus (Tanaka et al., 2005; Ma et al., 2007; Smith et al., 2010). As distribution of relaxin-3 largely overlaps with projections from nucleus incertus (Goto et al., 2001; Olucha-Bordonau et al., 2003), the major source of relaxin-3 in the brain is likely to be from nucleus incertus.

While expression and distribution of relaxin-3 have been thoroughly mapped out, targets of relaxin-3 have been less investigated. One brain region that has been examined in detail is the medial septum (Olucha-Bordonau et al., 2012). In the rat medial septum, relaxin-3 was reported to project to cholinergic and GABAergic neurons, and parvalbumin-, calbindin-, and calretinin-expressing neurons (Olucha-Bordonau et al., 2012). In addition, relaxin-3 was also detected to make contacts with calcium-binding proteins-expressing neurons in the hippocampus and entorhinal cortex (García Enguídanos et al., 2008).

Therefore, this chapter aims to establish the distribution and targets of relaxin-3 innervation in the dentate gyrus, in male and female mice, at two and eight months of age, previously investigated in adult hippocampal

neurogenesis chapters. Specifically, fluorescent immunolabelling of relaxin-3 will be conducted for neuroanatomical analyses.

4.2. Materials and methods

4.2.1. Relaxin-3 antibody production

Mouse monoclonal antibody was raised against N-terminal of relaxin-3 A-chain (DVLAGLSSSC), which is 100% conversed in human, rat and mouse, by Takeda Chemical Industries Ltd., Japan (Kizawa et al., 2003). The hybridoma clone HK4-144-10 was obtained from the International Patent Organism Depository (IPOD), National Institute of Technology and Evaluation (NITE), Japan, and cultured in GIT medium (Wako Pure Chemical Industries, Ltd., Japan) supplemented with 5% FBS (fetal bovine serum; Life Technologies), 50 U/ml penicillin (Sigma-Aldrich) and 50 μg/ml streptomycin (Sigma-Aldrich), in a humidified 95% air / 5% CO_2 incubator at 37^{o}C. Culture supernatant was collected, filtered through 0.22 μm polyethersulfone membrane (Merck Millipore), followed by purification of IgG with Protein A agarose (Thermo Scientific), and protein concentration with 50 kDa nominal molecular weight limit (NMWL) centrifugal filter unit (Merck Millipore). Purified mouse IgG was stored as 1 mg/ml stock in PBS with 0.05% sodium azide. Characterization of HK4-144-10 has been carried out in rats by Tanaka and colleagues (2005), and in rhesus and human by Silvertown and colleagues (2010). In particular, specificity was demonstrated in these two studies with preabsorption with relaxin-3 peptide (Tanaka et al., 2005; Silvertown et al., 2010). Further validation of HK4-144-10 specificity will be shown in this chapter with relaxin-3 KO mice.

4.2.2. Immunohistochemistry

To analyse distribution of relaxin-3 fibres in wildtype and knockout mice, of male and female sex, at two and eight months old, immunofluorescent

labelling of relaxin-3 was performed on 1-in-6 series of coronal hippocampal sections. 40 μm free-floating tissue sections were transferred from cryoprotectant storing solution, and rinsed in PBS, followed by antigen retrieval in 10 mM sodium citrate, pH 8.5, at 65^{o}C for 30 min (Jiao et al., 1999). Permeabilisation and blocking of hippocampal sections were then carried out in PBS with 0.3% Trition X-100 (PBST) and 5% normal goat serum in PBST, respectively. Immunofluorescent detection was accomplished by sequential incubation with mouse anti-relaxin-3 (1:500; HK4-144-10) and Alexa Fluor® 488-conjugated goat anti-mouse (1:500; Life Technologies). Finally, tissue sections were rinsed in PBS and mounted on glass slides with Prolong® Gold antifade mounting medium with DAPI (Life Technologies).

For double immunolabelling of relaxin-3, and either calretinin or parvalbumin antibodies, the immunostaining procedure was same as above, except for sequential antibody incubation with mouse anti-relaxin-3 (1:500) and Alexa Fluor® 488-conjugated goat anti-mouse (1:500), followed by mouse anti-calretinin (1:2000; Millipore MAB1568) or anti-parvalbumin (1:2000; Millipore MAB1572) and Alexa Fluor® 555-conjugated goat anti-mouse (1:500). For double immunolabelling of relaxin-3 and doublecortin, blocking was carried out in 5% normal donkey serum, followed by antibody incubation with mouse anti-relaxin-3 (1:500) and Alexa Fluor® 488-conjugated donkey anti-mouse (1:500), then goat anti-doublecortin (1:500; Santa Cruz Biotechnology sc-8066) and Alexa Fluor® 555-conjugated donkey anti-goat (1:500).

4.2.3. Image acquisition

Representative photomicrographs of relaxin-3 immunoreactive fibres were captured with 10X objective lens (NA 0.30) on an upright fluorescence microscope (BX51) equipped with digital camera (DP70). All images were

imported into Fiji software, and cropped to the dentate gyrus region. Brightness and contrast were adjusted equally for the entire image, without over-saturation or losing any information. Background intensity was kept similar for tissue sections from different animal samples. No specific feature within an image was enhanced, obscured, moved, removed, or introduced.

Representative immunofluorescent images of double immunolabelled relaxin-3/ calretinin, relaxin-3/parvalbumin, and relaxin-3/doublecortin dentate gyrus were captured with a confocal laser scanning microscope LSM 510. Each fluorescent signal was acquired sequentially to avoid simultaneous excitation and bleed-through of the two fluorochromes, Alexa Fluor® 488 and Alexa Fluor® 555. To demonstrate the juxtapositioning of relaxin-3 and cell type marker, z-stacks were scanned at 0.05 μm intervals at a pinhole setting of ~1 airy unit, corresponding to an optical slice of 0.8 μm. Images were collected under 63X oil immersion objective lens (NA 1.4) and 5.2X digital zoom with a pixel size of 0.054 μm, followed by reconstruction with orthogonal projections. Z-projection with maximum intensity was created with Fiji software to provide a top-down view of the image.

4.3. Results

4.3.1. Verification of relaxin-3 depletion in relaxin-3 KO mice

Constitutive relaxin-3 knockout mice were used in the study of hippocampal neurogenesis at two adult stages – young (two months old) and mature (eight months old), in both male and female sexes. To verify the depletion of relaxin-3 in hippocampus, immunofluorescent labelling of relaxin-3 was performed (Fig. 4.1). In young wildtype male mice, abundant relaxin-3 innervation could be observed in the temporal dentate gyrus, specifically in the hilus (Fig. 4.1A). On the other hand, lifelong deficiency of relaxin-3 was indeed achieved in the knockout mouse model, with absence of relaxin-3 immunolabelling at both young adult and mature adult stages, in both male and female mice (Fig. 4.1B-E).

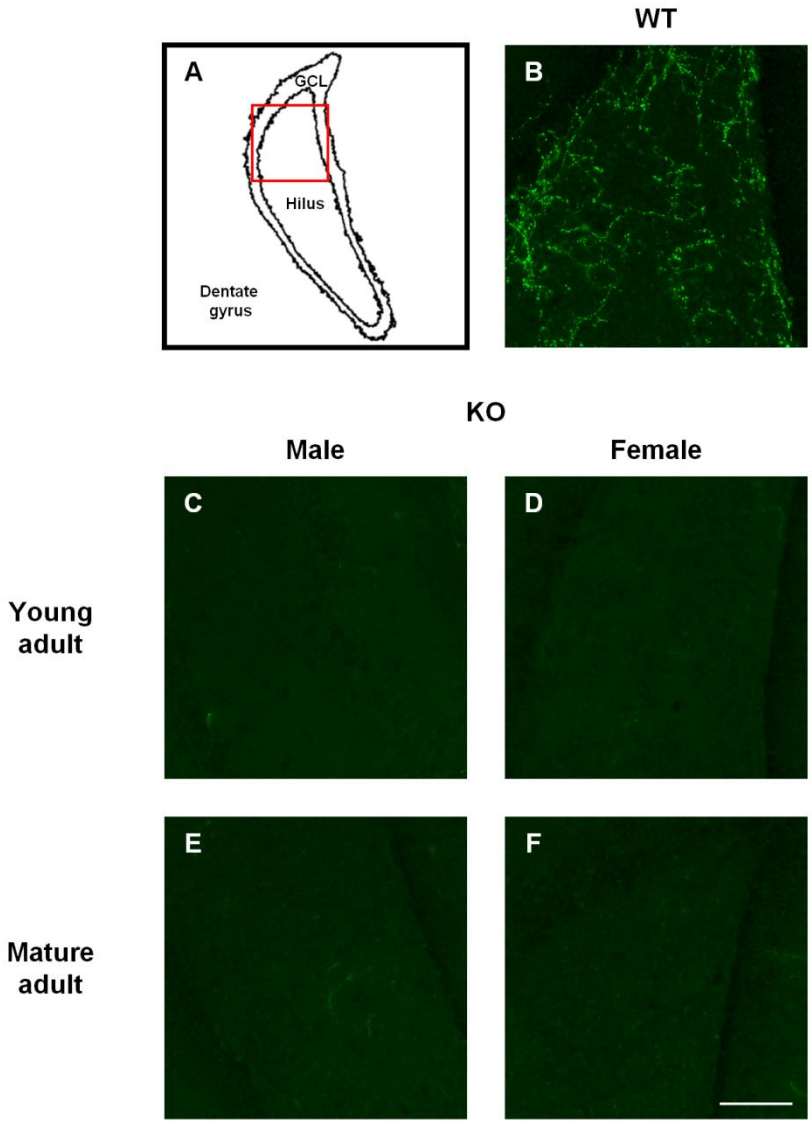

Figure 4.1. Relaxin-3 immunolabelling in the hilus of temporal dentate gyrus. Schematic diagram of temporal dentate gyrus with red box representing the image field for following photomicrographs (A). Representative photomicrographs of relaxin-3-immunolabelled (green) fibres in young wildtype male mice (B), and lack of relaxin-3 immunoreactive fibres in young male (C) and female (D), and mature male (E) and female (F) knockout mice. Scale bar corresponds to 100 μm.

4.3.2. Distribution of relaxin-3 fibres in mouse dentate gyrus

Relaxin-3 fibres could be immunohistochemically detected in the entire dentate gyrus, from its septal to temporal extents. However, there is a strong septotemporal gradient of relaxin-3 projections with sparse immunoreactive fibres in the septal (Fig. 4.2) and intermediate (Fig. 4.3) hilus, and dense innervations in the temporal hilus (Fig. 4.4). In addition, immunohistochemical examination of relaxin-3 in young adult and mature adult mice revealed continued innervation in ageing. Similarly, relaxin-3 innervation in dentate gyrus was observed in both male and female mice with comparable densities. Therefore, no apparent age-related or sex-related differences were found in the innervation of relaxin-3 in the mouse dentate gyrus.

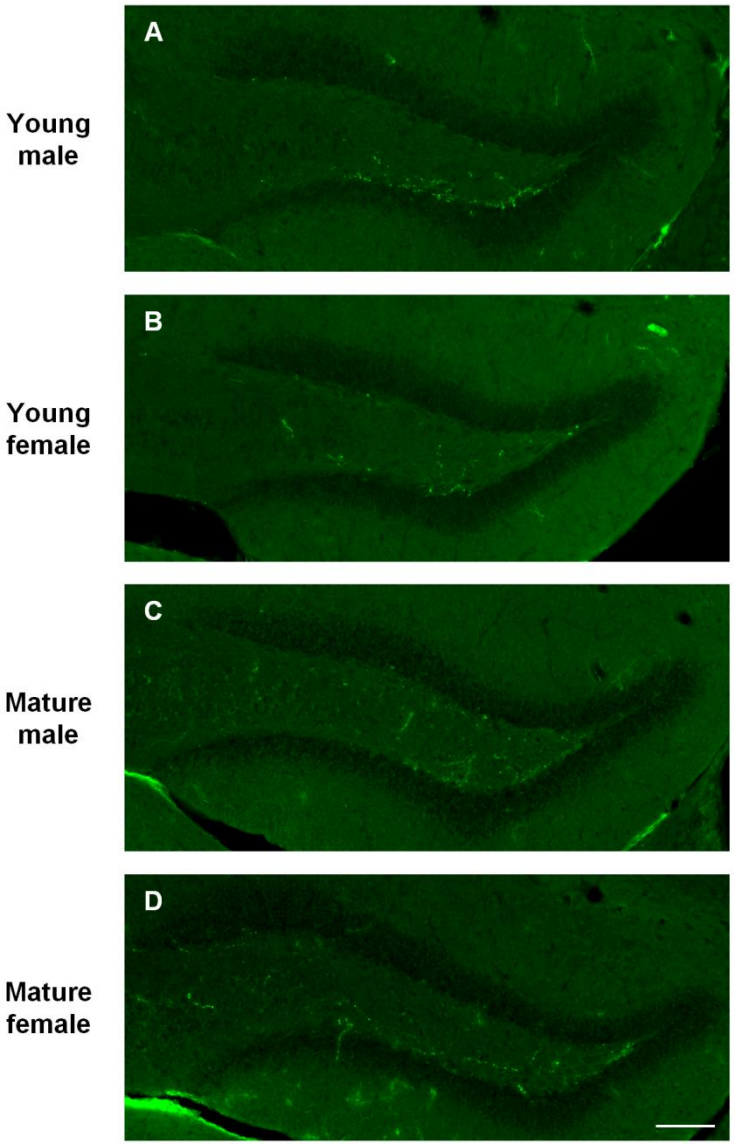

Figure 4.2. Immunofluorescence of relaxin-3 fibres in the septal dentate gyrus (~AP -1.5 mm). Representative photomicrographs of relaxin-3 immunolabelled (green) projections in young adult male (A) and female (B) mice, and mature adult male (C) and female (D) mice. Scale bar corresponds to 100 μm.

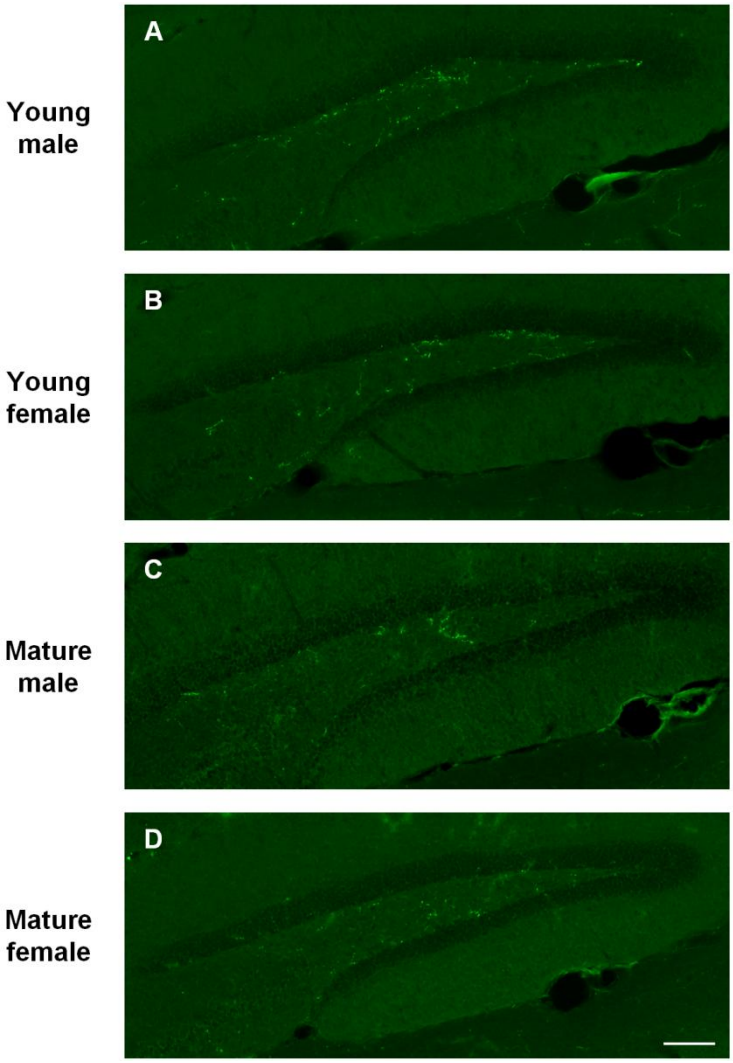

Figure 4.3. Immunofluorescence of relaxin-3 fibres in the intermediate dentate gyrus (~AP -2.5 mm). Representative photomicrographs of relaxin-3 immuno-labelled (green) projections in young adult male (A) and female (B) mice, and mature adult male (C) and female (D) mice. Scale bar corresponds to 100 μm.

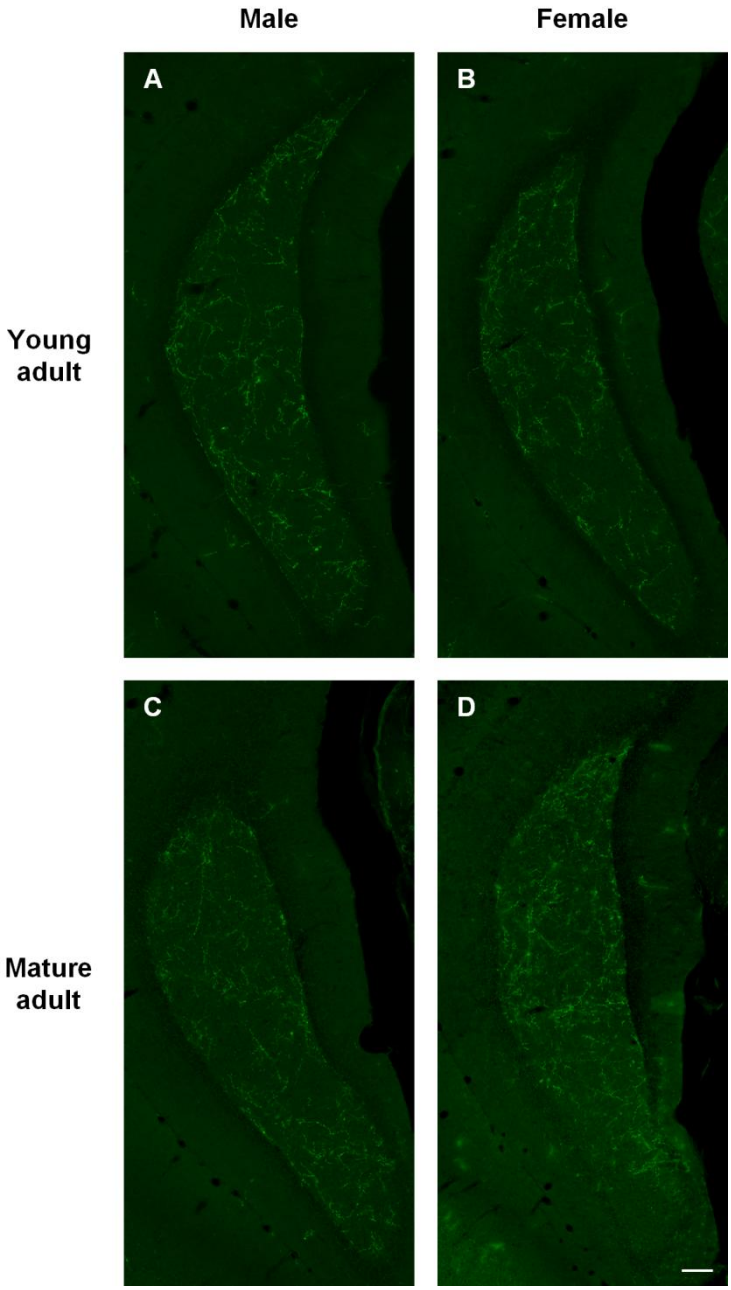

Figure 4.4. Immunofluorescence of relaxin-3 fibres in the temporal dentate gyrus (~AP -3.8 mm). Representative photomicrographs of relaxin-3 immuno-labelled (green) projections in young adult male (A) and female (B) mice, and mature adult male (C) and female (D) mice. Scale bar corresponds to 100 μm.

4.3.3. Possible targets of relaxin-3 projections in dentate gyrus

To identify possible targets of relaxin-3 innervation in the mouse dentate gyrus, double immunohistochemistry of relaxin-3 and cell type markers were conducted (Fig. 4.5 and 4.6). As it has been previously reported that relaxin-3 contact on calcium-binding protein-expressing neurons in the hippocampus (García Enguídanos et al., 2008), and calretinin- and parvalbumin-expressing neurons in the medial septum (Olucha-Bordonau et al., 2012), calretinin and parvalbumin were two cell type markers investigated (Fig. 4.5A and B, and Fig. 4.6A and B). Due to the antibodies against relaxin-3, calretinin and parvalbumin were all from mouse host species, sequential double immunostaining with relaxin-3 and calcium-binding proteins resulted in relaxin-3 being labelled with both secondary antibodies while calcium-binding protein was labelled with single secondary antibody. Nevertheless, relaxin-3 (labelled in yellow) could be observed to make putative contacts with the soma of calretinin- (Fig. 4.6A"") and parvalbumin-expressing (Fig. 4.6B"") neurons in the dentate gyrus.

In addition, to determine whether relaxin-3 projections target cells in neurogenic process, double immunohistochemistry of relaxin-3 and doublecortin was performed (Fig. 4.5C and 4.6C). Doublecortin is expressed in late progenitor cells and immature neurons, with its expression discontinued in mature neurons. Juxtapositioning of relaxin-3 immunoreactive fibre and doublecortin-expressing cell was detected in the temporal dentate gyrus (Fig. 4.6C""), suggesting possible direct regulation of relaxin-3 on neurogenesis process.

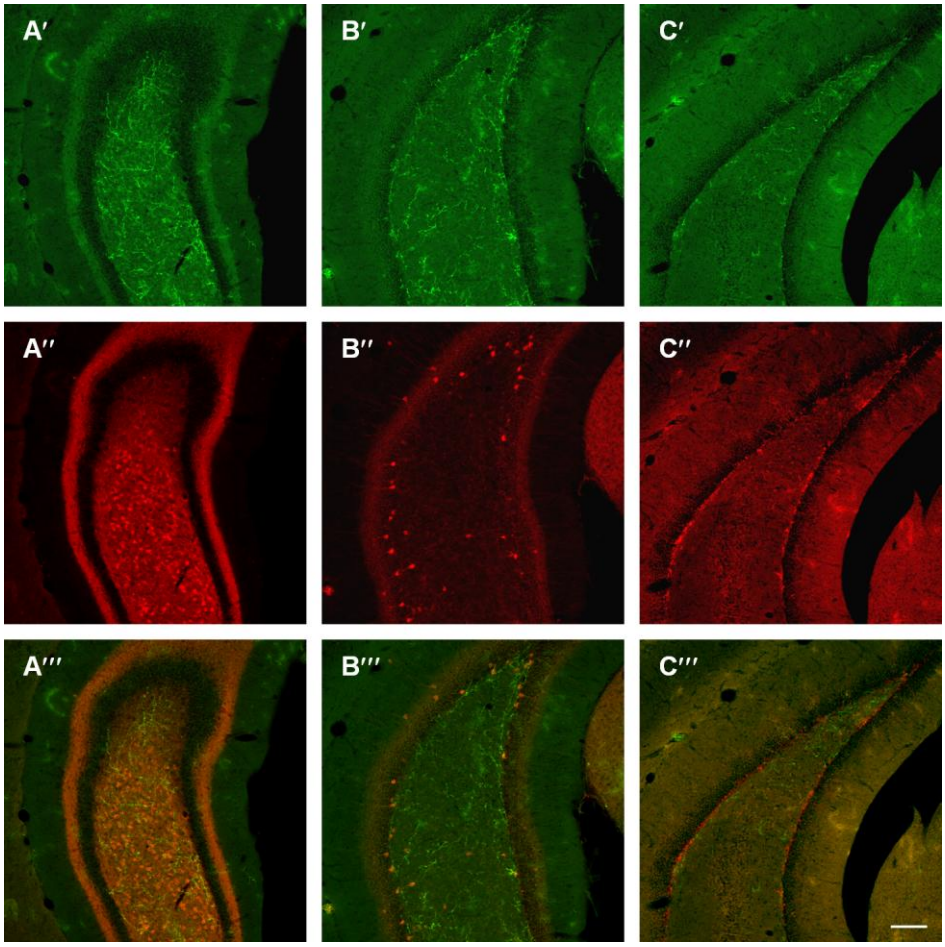

Figure 4.5. Double immunofluorescent labelling of relaxin-3 and cell type markers in the temporal dentate gyrus. Representative photomicrographs of relaxin-3 (A', B' and C') and calretinin (A"), parvalbumin (B") and doublecortin (C") in young adult male WT mice. Corresponding merged images of relaxin-3 and cell type markers were also shown (A‴ – C‴). Scale bar corresponds to 100 μm.

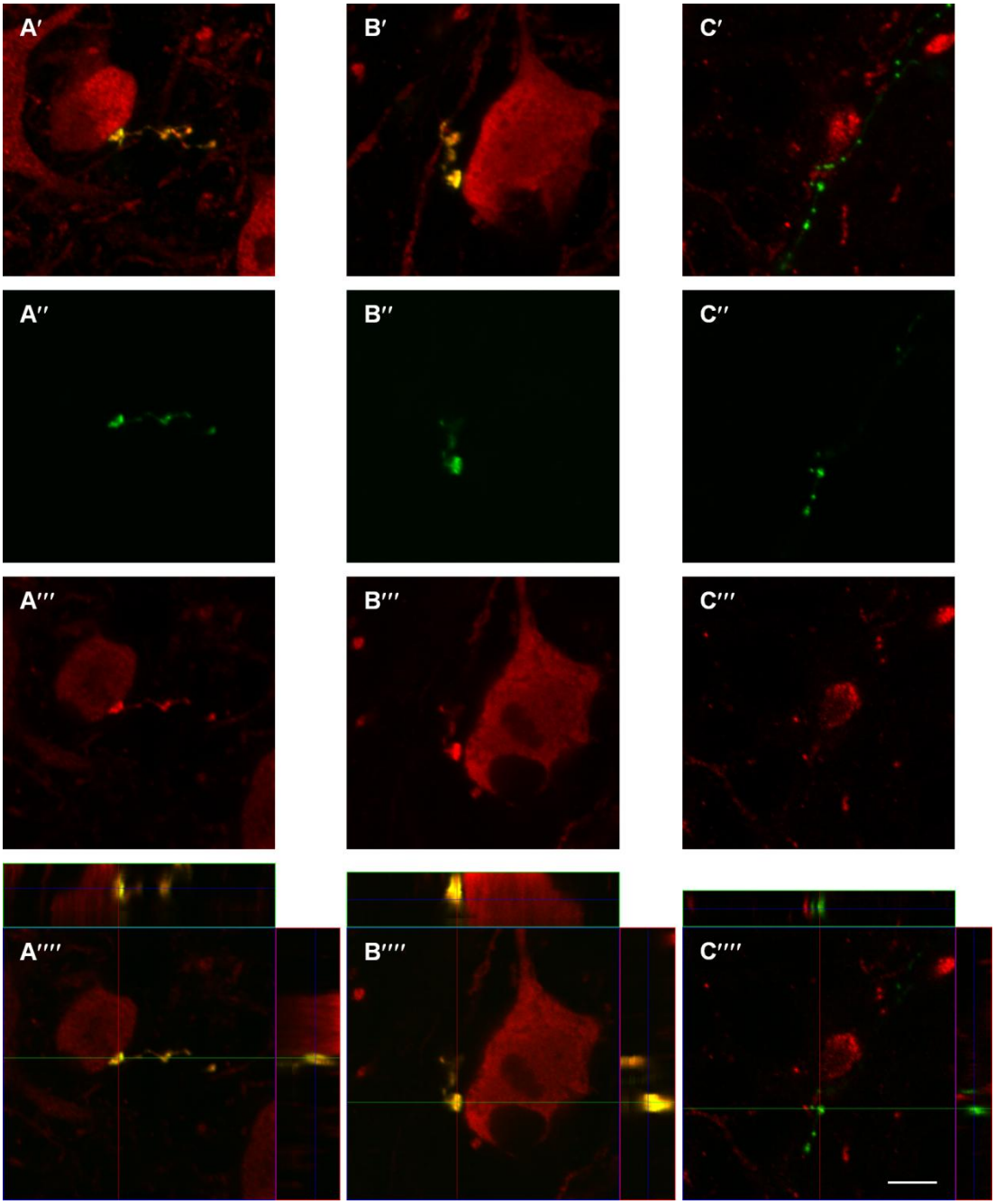

Figure 4.6. Possible targets of relaxin-3 projections in the temporal dentate gyrus. Representative confocal images with z-projection were shown for relaxin-3 and calretinin (A'), parvalbumin (B') and doublecortin (C'). Relaxin-3 fibres were immuno-labelled with Alexa Fluor 488 (A'' – C'') and Alexa Fluor 555 (A''' and B'''). Calretinin, parvalbumin and doublecortin were each immuno-labelled with Alexa Fluor 555 (A''' – C'''). Orthogonal projections of putative contacts between relaxin-3 and cells in dentate gyrus were also shown (A'''' – D''''). Scale bar corresponds to 5 μm.

4.4. Discussion

In this chapter, distribution and targets of relaxin-3 innervations were examined specifically in the dentate gyrus where adult neurogenesis occurs. First, relaxin-3 depletion was verified in knockout mice at the two adult stages – young and mature, in both male and female animals. Subsequently, in wildtype mice, septotemporal gradient of relaxin-3 projections in the dentate gyrus was shown, and that there were no apparent age- or sex-related differences in the degree of innervation. Lastly, putative contacts of relaxin-3 projections were preliminarily investigated, with calretinin-, parvalbumin- and doublecortin-expressing cells found to be possible target candidates.

Verification of relaxin-3 deficiency in knockout mice has been previously reported in mice of mixed genetic background (Smith et al., 2009b). In this study, to investigate the role of relaxin-3 in adult hippocampal neurogenesis, relaxin-3 knockout mice of backcrossed C57BL/6J background were used as lifelong deficiency model. Genotyping was PCR-based (polymerase chain reaction-based) and was conducted at weaning age. Therefore, in this chapter, immunohistochemical detection of relaxin-3 was further carried out to confirm permanent loss of relaxin-3 at experimental ages of two and eight months. Lack of relaxin-3 immunoreactive signals also served as validation for specificity of mouse HK4-144-10 antibody clone.

Examination of relaxin-3 immunohistochemistry in dentate gyrus revealed a strong septotemporal gradient of relaxin-3 innervation, with temporal dentate gyrus having higher density of relaxin-3 fibres. This was similarly reported in a

study of relaxin-3 distribution in mouse, which had utilised a rabbit polyclonal antibody against propeptide relaxin-3 C-chain (Smith et al., 2010). Such septotemporal gradient of relaxin-3 is correlated with dysregulation of neurogenesis processes specifically in the temporal dentate gyrus of relaxin-3 knockout mice, thus suggesting a direct relationship between relaxin-3 and hippocampal neurogenesis.

In addition, in the previous chapters, age- and sex-related differences were found in different phases of neurogenesis in relaxin-3 knockout mice, indicating possible age- and sex-specific roles of relaxin-3. In the current analysis of relaxin-3 immunoreactive fibres in dentate gyrus, there were no substantial differences in the degree of relaxin-3 innervation. Therefore, the age- and sex-specific roles of relaxin-3 in hippocampal neurogenesis are less likely to be attributed by the density of relaxin-3 projections. However, immunohistochemical investigation is limited to understanding of anatomical distribution, there may possibly be age- and/or sex-related differences in the amount of relaxin-3 peptide in dentate gyrus and/or the control in relaxin-3 peptide release.

This chapter also began a preliminary investigation on the targets of relaxin-3 projections in the dentate gyrus. Calretinin-, parvalbumin- and doublecortin-expressing cells were found to be possible target cells with relaxin-3 immunoreactive axonal terminals juxtaposition to potential targets . Of these possible relaxin-3 targets, calretinin- and parvalbumin-expressing cells have also been reported as targets cells for relaxin-3 in the medial septum (Olucha-Bordonau et al., 2012). In this study, as antibodies against relaxin-3, calretinin and parvalbumin were from mouse host species, sequential double immunolabelling with these antibodies resulted in double immunofluorescent

signals for relaxin-3, of which antibody was applied first. Therefore, it would be important to repeat the double immunostaining with antibodies from different host species for validation of this preliminary finding. In addition, to confirm synaptic contact of relaxin-3 projections on putative target cells, electron microscopy would be more superior in providing ultrastructural resolution.

The preliminary finding of calretinin-, parvalbumin- and doublecortin-expressing cells as targets for relaxin-3 innervation suggests that receptors for relaxin-3 are present on multiple cell populations. The spatial expression for the endogenous receptor for relaxin-3, RXFP3, has been examined by *in situ* hybridisation, and RXFP3 mRNA expression was shown to be restricted to selective cells in the mouse dentate gyrus (Smith et al., 2010). Many of these cells are positioned between the granular cell layer and hilus (Smith et al., 2010), also known as the subgranular zone, where the progenitor cells are. In addition, in the hilus of temporal dentate gyrus where there was a high density of relaxin-3 fibres, RXFP3 mRNA expression could also be found in occasional hilar cells (Smith et al., 2010). One possible interpretation would be that RXFP3 is expressed in multiple cell types, including those found to be putative targets for relaxin-3.

In addition to RXFP3, RXFP1 has also been reported to be a receptor for relaxin-3 (Bathgate et al., 2002; Sudo et al., 2003) and is expressed in the brain (Burazin et al., 2005; Piccenna et al., 2005). However, expression of RXFP1 in the dentate gyrus has not been conclusively demonstrated as *in situ* hybridisation showed a lack of RXFP1 mRNA expression while β-galactosidase positive neurons were detected in the RXFP1 knockout/*LacZ* knockin mice (Piccenna et al., 2005). Furthermore, there could be a yet

unknown additional receptor for relaxin-3, such that each target cell type expresses different receptor profile for relaxin-3.

Though there is a current lack of understanding of the type of receptors expressed on the target cells for relaxin-3, there is information on calretinin-, parvalbumin- and doublecortin-expressing cells in the dentate gyrus.

Calretinin, a calcium-binding protein, is expressed in glutamatergic principal neurons, also called mossy cells, in the hilus (reviewed in Scharfman and Myers, 2012) and immature postmitotic newborn neurons in the subgranular zone and granule cell layer (Brandt et al., 2003). Septotemporal difference in the expression of calretinin has been reported in hilar mossy cells, with calretinin present in the temporal dentate hilus and absent in the septal pole (Fujise et al., 1998), sharing similarities with relaxin-3 distribution in dentate gyrus.

In relation to hippocampal neurogenesis, constitutive knockout of calretinin gene resulted in impairments of neurogenic processes including proliferation, migration and survival (Todkar et al., 2012). Although the effect on neurogenesis was not investigated septotemporally, dysregulation of neurogenesis could be observed in the septal dentate gyrus (Todkar et al., 2012). In addition, the downregulation of neurogenesis in calretinin-deficient mice was proposed to be due to the loss of calretinin in the early postmitotic adult-born neurons (Todkar et al., 2012). On the other hand, mossy cells in the temporal dentate gyrus were also found to be associated with neurogenesis. In particular, hilar mossy cells were demonstrated to provide first excitatory glutamatergic input to newborn neurons in adult mice (Chancey et al., 2014). Age-related decrease was also report for calretinin cell in mouse

dentate gyrus (Han et al., 2006). Therefore, both maturing newborn granule cells and hilar mossy cells could contribute to modulation of adult hippocampal neurogenesis in an age-dependent manner.

The second putative target for relaxin-3 is parvalbumin-expressing cells. Parvalbumin, also a calcium-binding protein, is expressed in basket cells, or GABAergic interneurons, in dentate gyrus (Kosaka et al., 1987; Ribak, 1992). Within the dentate gyrus, many parvalbumin-positive cells are positioned in the subgranular zone with no clear septotemporal differences. In addition, parvalbumin cell population did not appear to be affected by ageing process (de Jong et al., 1996; Yamada and Jinno, 2013).

Parvalbumin-expressing interneurons have been reported to regulate hippocampal neurogenesis in two opposing directions for different neurogenesis stages. First, stimulation of parvalbumin interneurons was shown to increase quiescence of neural stem cells, while inhibition of parvalbumin interneurons resulted in activation of quiescent neural stem cells (Song et al., 2012). However, in subsequent neurogenesis stages, stimulation of parvalbumin interneurons was demonstrated to enhance cell survival and dendritic maturation of adult-born cells (Song et al., 2013). Parvalbumin interneurons were also found to be a source of early GABAergic synaptic inputs for neural progenitor cells, and could act as an activity-dependent regulator of neurogenesis (Song et al., 2013).

The last putative target found for relaxin-3 is doublecortin-positive cells in dentate gyrus. Doublecortin is a microtubule-associated protein (Gleeson et al., 1999) and is expressed in late progenitor cells and immature neurons (Brown et al., 2003). Positioning of relaxin-3 terminal next to doublecortin

cells suggest possible direct regulation of relaxin-3 on newborn and maturing cells in hippocampus. Therefore, it may be of interest to further investigate if relaxin-3 make contacts on other cell types in the process of neurogenesis, for example, type-1 putative stem cells and type-2a early progenitor cells.

In this study, three cell types were found to be potential relaxin-3 targets in the dentate gyrus, it remains to be determined if only one or all of them are functionally relevant in the regulation of neurogenesis. If only a single cell type is the function target, then it would be interesting to further investigate how a single functional target leads to regulation of multiple neurogenesis processes. If there are multiple cell types as functional targets, the proportion of each cell type may be of interest. In addition, mapping of each cell type to the stage(s) of neurogenesis regulated may be carried out to understand the exact mechanism of relaxin-3's effects on neurogenesis.

To identify functional targets of relaxin-3, one possible experiment would be to infuse exogenous relaxin-3 into (temporal) dentate gyrus, followed by double immunohistochemistry of an immediately early gene product (e.g. c-fos) and one of calretinin, parvalbumin or doublecortin. In neurogenesis, cells expressing doublecortin and/or calretinin are involved in the migration process (Fig. 1.1). Therefore, if doublecortin-expressing cells and/or calretinin immature newborn neurons were found to be responsive to relaxin-3. the effects of relaxin-3 on migration, as an example, may be due to this direct functional connection.

In summary, this chapter has examined distribution of relaxin-3 innervations in the mouse dentate gyrus. Constitutive relaxin-3 knockout mice were verified with lack of relaxin-3 immunoreactive fibres at both ages investigated.

In wildtype C57BL/6J mice, septotemporal, age and sex factors were considered in the analysis of relaxin-3 distribution. While there was a strong septotemporal difference in the degree of relaxin-3 innervations, age and sex did not seem to contribute to any differential distribution. In addition, connections of relaxin-3 projections were investigated and several putative targets were found. These relaxin-3 target candidates include calretinin-, parvalbumin- and doublecortin-positive cells. Each of these cell types is involved in the neurogenesis process and may be part of relaxin-3's modulatory mechanism.

Chapter 5 General discussion and conclusion

5. General discussion

5.1. General discussion

This thesis aimed to establish the role of relaxin-3 in adult hippocampal neurogenesis. A genetic loss-of-function approach was undertaken, with levels of neurogenesis examined in constitutive relaxin-3 knockout mice. As there was differential distribution of relaxin-3 projections in septal and temporal dentate gyrus, there were corresponding septotemporal effects of relaxin-3 deficiency in neurogenesis. Furthermore, when age and sex factors were included in the analyses, dysregulations of neurogenic processes in relaxin-3 knockout mice were in an age- and sex-dependent manner. In particular, in the temporal dentate gyrus, reductions in cell proliferation were found in male knockout mice at young adult stage, but female knockout mice at mature adult stage. Similarly, there were sex-specific differences in newborn neuron development in knockout mice, with increased neuronal differentiation in males and increased migration in females of eight months old.

Permanent loss of relaxin-3 resulted in altered neurogenesis specifically in the temporal dentate gyrus where a high density of relaxin-3 innervations normally exist. In recent years, there have been an increased awareness for the heterogeneity of hippocampus, with septal, or dorsal, hippocampus commonly associated with functions in cognition, and temporal, or ventral, hippocampus more associated with functions in emotions (reviewed in Bannerman et al., 2004; Fanselow and Dong, 2010). Furthermore, adult-born neurons in the dentate gyrus could also contribute to the different hippocampal functions according to its position along the septotemporal axis

(Wu and Hen, 2014). Thus far, functions of relaxin-3 have been shown to involve both cognitive and affective behaviours (reviewed in Chapter 1, Section 1.3.2.2). Therefore, specific role of relaxin-3 in temporal dentate gyrus suggests neurogenesis in this subregion could partially contribute to relaxin-3's functions in mood and emotions.

In addition, relaxin-3 has recently been proposed to have therapeutic potential for neuropsychiatric disorders, such as depression and anxiety (reviewed in Smith et al., 2014). The relaxin-3 system responds to stress (Tanaka et al., 2005; Banerjee et al., 2010; Lenglos et al., 2013), which in turn has intricate relationship with depression (reviewed in Lucassen et al., 2014). Furthermore, stress modulates adult hippocampal neurogenesis (reviewed in Lucassen et al., 2010), and neurogenesis has been proposed to be involved in depression and/or its treatment (Santarelli et al., 2003; reviewed in Sahay and Hen, 2007). Hence, together with the finding in this current study that relaxin-3 regulates temporal dentate neurogenesis, relaxin-3 may contribute to depression therapy in part via its modulatory properties in neurogenesis. Also, the age- and sex-dependent effects of relaxin-3 could provide additional dimensions to the understanding of relaxin-3-related affective functions and disorders.

Other than establishing the role of relaxin-3 in adult hippocampal neurogenesis, putative targets in dentate gyrus were also investigated. Preliminarily, calretinin-, parvalbumin- and doublecortin-expressing cells were found to be possible target cells for relaxin-3 innervations. Of which, calretinin-positive mossy cells and parvalbumin-positive interneurons could act as relaxin-3 downstream regulators on neurogenesis, as mossy cells and parvalbumin interneurons have been described to be involved in the

neurogenesis process (Song et al., 2012; Song et al., 2013; Chancey et al., 2014). Alternatively, relaxin-3 could possibly modulate neurogenesis via its putative direct contacts on doublecortin-expressing precursor cells and/or calretinin-expressing immature newborn neurons. These cell types could undergo cell proliferation and/or neuronal maturation, which were the processes found to be dysregulated in relaxin-3 knockout mice.

Investigation of the direct contacts of relaxin-3 projections in the dentate gyrus is in line with the idea that relaxin-3 modulates neurogenesis locally, e.g. in the temporal dentate gyrus. However, it is possible that relaxin-3 could exert its modulatory actions indirectly through other brain regions. For example relaxin-3 innervates medial septum heavily (Tanaka et al., 2005; Ma et al., 2007; Smith et al., 2010), and medial septum has inputs to the dentate gyrus (Mosko et al., 1973), with reports on its regulation on neurogenesis as well (Cooper-Kuhn et al., 2004). Therefore, more studies will be required to understand the mechanism of relaxin-3's modulation on neurogenesis.

In this thesis, constitutive knockout mouse model was utilised to study the effect of lifelong loss of relaxin-3 on adult hippocampal neurogenesis. This approach is commonly used to study gene functions and is useful to evaluate the necessity of the gene throughout life. However, it has its limitations as well. For example, there may be compensatory mechanisms especially when there are several other genes with overlapping functions. In addition, the effect of the deletion of gene is cumulative over the lifetime, and would not be useful if the interest is to examine the role of the gene at a particular stage in life.

In the investigation of age-related process on hippocampal neurogenesis in relaxin-3 knockout mice, eight-month-old animals (which were nine month old at end of experiment) were examined. A typical lifespan of mouse is between two to three years (Flurkey et al., 2007), therefore eight to nine month old mice may not seem to be old enough for effect of ageing. However, a study on the first nine months of mice has found age-related reduction in cell proliferation to remain stable by nine months of age (Ben Abdallah et al., 2010), demonstrating that age-related impairment in neurogenesis occurs long before other ageing symptoms in older mice.

To quantify the levels of neurogenesis in this study, BrdU-labelling method was employed. BrdU assay is one of the most common techniques used in neurogenesis studies as it is well-established and relatively straightforward with administration, immunohistochemistry and stereology. One feature of BrdU assay is its birth-dating property. As BrdU is synthetic, presence of BrdU in cells implies its uptake only during BrdU administration. On the other hand, there are concerns on the specificity of BrdU in newborn cells as cells undergoing DNA repair may incorporate BrdU too. However, several neurogenesis studies with radiation-induced DNA damage, and therefore DNA repair, did not observe an increase in BrdU labelling (Parent et al., 1999; Santarelli et al., 2003). Moreover, in this current study, neurogenesis was investigated under basal physiological conditions where high levels of DNA repair are not expected.

Nevertheless, there are alternatives to BrdU, such as endogenous markers associated with specific stages of neurogenesis. In this study, Ki67, a nuclear protein expressed in late G1 phase to M phase of cell cycle (Scholzen and Gerdes, 2000), was used to identify the proliferating population in dentate

subgranular zone. Use of Ki67 has been demonstrated in neurogenesis, and was compared to proliferation usage of BrdU (Kee et al., 2002). Although the absolute numbers for Ki67- and BrdU-positive were different, both markers could detect relative increase and decrease in experimental conditions (Kee et al., 2002). However, one shortfall of endogenous markers is the lack of birth-dating ability, which is important to demonstrate newly generated cells.

Another method commonly used in neurogenesis studies is retroviral-mediated labelling of newborn cells. This technique utilised the ability of retrovirus targeting diving cells to detect neurogenesis among postmitotic neurons (van Praag et al., 2002). The key advantage of this technique is the expression of inserted gene, usually green fluorescent protein (GFP), throughout the entire cell, including neurites. GFP-filled newborn cells allow visual identification in electrophysiological studies and morphological studies for dendritic development. These two types of study are not able to be accomplished with BrdU or endogenous markers. However, the disadvantage is additional viral preparation and stereotaxic surgical injection of viruses. Therefore, retroviral-mediating labelling is usually done when electrophysiology and/or morphology aspects are of interest in the study.

5.2. Future studies

In this study, a lifelong loss-of-function approach was used to examine role of relaxin-3 in adult hippocampal neurogenesis. To supplement the study, rescue experiments with exogenous relaxin-3 will help to demonstrate the role of specific loss of relaxin-3 in the observed phenotype. This could be particularly important in constitutive relaxin-3 knockout mouse model as

dysregulated neurogenesis could be a result of other changes in response to relaxin-3 depletion, and not direct loss of relaxin-3 function. In addition, other loss-of-function approaches such as pharmacological inhibition of receptors for relaxin-3 (e.g. RXFP3), immunological blocking of relaxin-3 with antibodies, optogenetical inhibition of relaxin-3 neurons, viral-mediated knockdown of relaxin-3, and conditional knockout of relaxin-3 could be considered These techniques allow manipulation of relaxin-3 deficiency in various time span, ranging from milliseconds in optogenetics to more chronic conditional knockout.

Conversely, gain-of-function studies will greatly complement current loss-of-function findings. Some gain-of-function approaches include application of exogenous relaxin-3, pharmacological activation of receptors for relaxin-3 (e.g. RXFP3), optogenetical stimulation of relaxin-3 neurons, viral-mediated overexpression of relaxin-3 and transgenic mice overexpressing relaxin-3.

These loss- and gain-of-function studies could contribute to the understanding of relaxin-3 in modulation of adult hippocampal neurogenesis. However, for research interest in downstream molecular mechanisms, other approaches should be considered. One prerequisite knowledge required is whether relaxin-3 acts in the neurogenic niche locally or indirectly via other brain regions, which could be answered by carefully designed loss- and/or gain-of-function experiments. As there are direct inputs of relaxin-3, especially in the temporal dentate gyrus, there should be local modulation of neurogenesis. Though preliminary investigated in the current study, target cell types of relaxin-3 should be further confirmed with additional experiments, such as electron microscopy. In addition, the phenotype of RXFP3-expressing cells in dentate gyrus is also unknown at the moment. Knowing which, downstream

molecular mechanism can then begin to be elucidated. One known signalling pathway of relaxin-3 is activation of ERK1/2 through RXFP3 (van der Westhuizen et al., 2007), and activation of ERK1/2 has been reported to upregulate cell proliferation in neurogenesis (Zhao et al., 2014). Therefore, ERK1/2 pathway is one possible downstream molecular mechanism of relaxin-3, though more studies are required.

To further investigate age- and sex-dependent roles of relaxin-3 in hippocampal neurogenesis, several approaches could be considered. One, in addition to the distribution of relaxin-3 innervation, the amount of relaxin-3 available in different age and sex groups could be studied by quantitating the amount of relaxin-3 peptide by western blot or ELISA (enzyme-linked immunosorbent assay), or amount of peptide release by *in vivo* microdialysis. Two, specific actions of relaxin-3 in different ages and sexes could be examined concurrently with loss- and/or gain-of-function experiments described previously. Sex-specific actions of molecules have been previously reported, and one example is estrogen, which could increase proliferation in female rats but not have an effect in male rats (reviewed in Galea et al., 2013). Therefore, inherent age- and/or sex-specific actions of relaxin-3 could result in the age- and sex-dependent differences in neurogenesis. Third, there could also be age- and/or sex-specific differences in the density of target cells and/or amount of receptors for relaxin-3 in the neurogenic niche, which could then be quantitatively compared with respective mRNA and/or protein expression. Therefore, additional studies of age- and sex-dependent roles of relaxin-3 would be on age- and/or sex-related differences in regulations, functions, target cells and/or receptors of relaxin-3.

Relaxin-3 is regulated by stress and CRF, which also regulate adult hippocampal neurogenesis. In addition, one common clinical interest for relaxin-3, adult hippocampal neurogenesis and stress is depression. Therefore, one future direction is to examine the role of relaxin-3 in adult hippocampal neurogenesis in the context of stressful environment, and how relaxin-3 contributes to stress-induced affective behaviour through neurogenesis in the dentate gyrus.

5.3. Conclusion

This study has demonstrated, for the first time, the necessity of relaxin-3 in adult hippocampal neurogenesis. Specifically, lifelong depletion of relaxin-3 resulted in dysregulation of neurogenesis, in an age-, sex- and septotemporal-dependent manner. In particular, in the temporal dentate gyrus, cell proliferation was downregulated in young adult male mice and mature adult female mice, while neuronal differentiation and migration were upregulated in mature adult male and female mice, respectively. While there were more relaxin-3 innervations in the temporal pole of dentate gyrus, there were no apparent age- and sex-related differences in the distribution of relaxin-3 fibres, suggesting other sources of age- and sex-related differences. Nevertheless, the findings reported in this study could contribute to future research on relaxin-3 and adult hippocampal neurogenesis.

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Appendix

Oral Presentations

Hong, J.M. (2010). Role of relaxin-3 in adult hippocampal neurogenesis. In 2010 Postgraduate Student Review Presentations at The Florey Institute of Neuroscience and Mental Health (Melbourne, Australia).

Hong, J.M. (2011). The role of relaxin-3 in neurogenesis. In miNI Symposium on the Nucleus Incertus/Relaxin-3 at National University of Singapore (Singapore).

Poster presentations

Hong, J.M., Smith, C.M., Ransome, M.I., Gundlach, A.L., and Dawe, G.S. (2012a). Adult hippocampal neurogenesis and migration in young adult relaxin-3 knockout mice. In 8th FENS Forum of Neuroscience (Barcelona, Spain).

Hong, J.M., Smith, C.M., Ransome, M.I., Gundlach, A.L., and Dawe, G.S. (2012b). Effect of ageing on adult hippocampal neurogenesis and migration in relaxin-3 knockout mice. In 6th International Conference on Relaxin and Related Peptides (Florence, Italy).

Hong, J.M., Smith, C.M., Ransome, M.I., Gundlach, A.L., and Dawe, G.S. (2013). Sexual dimorphism in hippocampal neurogenesis of mature adult relaxin-3 null mice. In International Conference on Pharmacology and Drug Development 2013 (Singapore).