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Neuropeptide Y signalling on Hippocampal Stem Cells in Health and Disease

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Summary:

Neuropeptides are emerging as key components in the hippocampal neurogenic niche in health and disease, regulating many aspects of neurogenesis and the synaptic integration of newly generated neurons. This review focuses on the role of Neuropeptide Y in the control of stem/precursor cells in the postnatal and adult hippocampus. It is likely that Neuropeptide Y releasing interneurons are key sensors of neural activity, modulating neurogenesis appropriately. This is likely to be a fruitful area of research for extending our understanding of the control of stem cells in the normal and diseased brain.

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1. Introduction / Outline

Over the last fifteen years there has been an explosion of interest in stem cell biology, particularly in the adult brain, since the realization of the importance of Joseph Altman's work demonstrating neurogenesis in the post natal dentate gyrus of the hippocampus (Altman & Das, 1965). The hippocampus is involved in the learning and memory of explicit information (Eichenbaum, 1997) and with the amygdalar formation, is important in the generation of behavioural responses to stress. Given the emerging roles of postnatal neurogenesis in hippocampal dependant learning and behaviour, much research has concentrated on elucidating the function of hippocampal (or more properly granule cell layer) neurogenesis as well as determining factors that influence it (Gage, 2002). More recently, there is an emerging awareness of the effect of pathological processes on dentate neurogenesis and on possible roles of abnormal neurogenesis in the pathophysiology of disease. Disease may alter the production and or integration of newly born neurons within the dentate gyrus and hippocampus and therefore may be viewed as perturbing a physiological function such as mood (Jacobs *et al.*, 2000) or hippocampal dependant learning (Shors, 2004), or create abnormal circuits that might subserve altered network activity, as in epilepsy (Parent, 2002; Scharfman & Gray, 2006). Central to these questions are the mechanisms whereby neurogenesis is linked to neural activity.

Although the principle neural inputs into the dentate gyrus have been well defined, there is much evidence to suggest that local networks, largely sub-served by neuropeptide releasing interneurons, significantly modulate activity, and are thus ideally placed to signal neuronal activity to the local stem cell niche. This review highlights the emerging role of neuropeptides in modulating hippocampal neurogenesis. It focuses particularly on

neuropeptide Y, which is both released by dentate interneurons under specific firing frequencies in the dentate, and also from the sympathetic nervous system into the circulation (Lundberg *et al.*, 1985; Pernow *et al.*, 1986), crossing the blood brain barrier (Kastin & Akerstrom, 1999) in the neurogenic subgranular zone (SGZ) of the dentate gyrus. The review first details relevant hippocampal anatomy and the principle neurogenic niches of the dentate gyrus, and caudal subventricular zones. It then briefly describes the putative roles of adult neurogenesis in the normal brain and summarises knowledge regarding its control. It then details the roles of neuropeptides, particularly NPY, in neurogenesis under normal conditions and those of pathology, mainly focusing on temporal lobe epilepsy, which is the main pathological state relevant to neuropeptides and neurogenesis investigated to date.

1.1 The dentate gyrus and adult hippocampus

Within the hippocampus, the adult dentate gyrus consists of a dense layer of glutamatergic granule cells, and many other types of neurons and glia that are dispersed throughout all strata (Fig. 1). The somata of granule cells are tightly packed, and form the so-called granule cell layer. Granule cells are oriented in a stereotypical fashion, with a spiny arbor of dendrites that begins at the cell body and extends unilaterally to the hippocampal fissure (Fig. 1). On the opposite pole of the granule cells from their dendrites lie the granule cell axons or “mossy fibers.” These axons are a major component of the large region just beneath the granule cell layer, called the hilar region or hilus (Fig. 1). The mossy fibers primarily target the proximal apical dendrites of CA3 pyramidal cells of hippocampus, but also form a complex array of collaterals that

terminate on hilar cells. These hilar cells are termed interneurons as they appear to modulate the local activity of granule cell neurons. They can be divided into the mossy cells, which innervate the inner third of the ipsilateral and contralateral granule cell layer, and hilar cells that are GABAergic, which are highly diverse in morphology and projection. The somata and axons of GABAergic neurons are located throughout the dentate gyrus. All of the cells in the dentate gyrus, both glutamatergic and GABAergic, contain secondary chemicals and proteins that are thought to be neuromodulators, and which are often cell-type specific. GABAergic neurons contain a variety of unique peptide neurotransmitters that are not normally expressed in other dentate gyrus neurons, such as neuropeptide Y, Galanin, PACAP, somatostatin, cholecystokinin, and vasoactive intestinal polypeptide, among others (Freund & Buzsaki, 1996). NPY is normally expressed very specifically, in a subset of GABAergic neurons in the dentate hilus and in the pyramidal cell layers of the hippocampus, and not other cell types (Kohler *et al.*, 1986; Deller & Leranth, 1990), except for noradrenergic projection fibres arising from the locus coeruleus and terminating in the dentate hilus (Xu *et al.*, 1998), suggesting that NPY can be released in the hilus and SGZ by the activation of largely intrinsic and some projection pathways. Many interneurons co-localize NPY with other peptides, including somatostatin and cholecystokinin. Most NPY-immunoreactive axon terminals appose molecular layer dendrites, of granule cells as well as other non-granular cells (Deller & Leranth, 1990; Milner & Veznedaroglu, 1992). NPY terminals also appose astrocytic processes (Pickel *et al.*, 1995), suggesting an interaction with glia (Ubink *et al.*, 2003). This may be especially relevant to neurogenesis as radial glia like astrocytes that extend

processes through the granule cell layer into the inner molecular layer are the stem cells that give rise to newly generated granule cell neurons (Seri *et al.*, 2001 and see below).

1.2 Progenitor niches in the hippocampus and peri-hippocampal areas

1.2.1 Granule Cell Layer of the Dentate gyrus

Unlike the rest of the hippocampus, which is generated from the hippocampal anlage at embryonic day 16 (E16), the granule cell layer of the dentate is largely generated postnatally. Cells of the second dentate migration form the tertiary matrix in the hilus of the developing dentate by postnatal day 3 (P3), which generates approximately 50% of the granule cell layer after P5, and from where precursor cells migrate to the inner border of the granule cell layer to form the adult neurogenic subgranular zone (SGZ) by P20 (Fig. 1). The granule cell layer therefore develops as an inside-out structure with the newer cells being added successively along the inner margin initially from the tertiary germinal matrix and later from the subgranular zone (Schlessinger *et al.*, 1975; Altman & Bayer, 1990) (See Fig. 1). This neurogenic niche is the site of ongoing neurogenesis throughout adult life in all mammals studied to date including humans (Eriksson *et al.*, 1998) and it has been estimated to generate 6% of the total granule cell population every month (Cameron & McKay, 2001). Within the neurogenic subgranular zone (SGZ) of the dentate gyrus, astrocytes with a radial glia like morphology extending a single main process into the granule cell layer are primary progenitor cells (Type B cells) (Seri *et al.*, 2004). Nestin is an intermediate filament protein which is expressed in progenitor/stem cells (Lendahl *et al.*, 1990) in the SGZ. Using nestin driven fluorescent protein expression, Kempermann and colleagues have classified stem/progenitor cells in the SGZ

as bipotent GFAP expressing Type 1 cells which have astrocytic properties and GFAP negative Type 2 and Type 3 lineage determined progenitor cells (for review see (Kempermann *et al.*, 2004)) (Fig. 2). These different cells form a functional unit referred to as the stem cell niche in which Type 2 cells are held in the SGZ within a basket of processes arising from the cell body of the radial Type 1 cell, while Type 3 cells lie without the SGZ but with an intimate relationship between its apical dendrite and the radial process of the Type 1 cell which is thought to guide its migration into the molecular layer (Seki & Arai, 1999; Seri *et al.*, 2004; Shapiro *et al.*, 2005).

1.2.2 Caudal Subventricular and Subcallosal Zones

Proliferating cells continue to be seen in the subventricular zone (SVZ), a remnant of the embryonic ventricular zones, which persists throughout life. *In vivo*, in rodents progenitors in the anterior SVZ give rise to chains of rostrally migrating cells called the rostral migratory stream that eventually reach the olfactory bulb where they differentiate into GABAergic and dopaminergic interneurons (Lois & Alvarez-Buylla, 1993). In humans a similar pathway has been recently described between the lateral ventricle and the olfactory bulb (Curtis *et al.*, 2007). The caudal subventricular zone of the lateral ventricle lines the superficial layer of the hippocampus over areas CA1 and CA3 and contains neural progenitors that develop into glia, giving rise to white matter astrocytes and oligodendrocytes in neonatal and juvenile rodents (Levison & Goldman, 1993; Levison *et al.*, 1999) as well as neurons that migrate to the olfactory bulb (Merkle *et al.*, 2007). The adjacent contiguous subcallosal zone lies in a potential space between the extraventricular hippocampus and the overlying corpus callosum and generates

oligodendrocytes that migrate radially to myelinate the major white matter tracts (Seri *et al.*, 2006). Using transient mitotic inhibition Doetch et al (Doetsch *et al.*, 1999) elegantly demonstrated a reconstitution of the SVZ neurogenic niche by the initial division of GFAP+ subependymal astrocytes (Type B cells), followed by GFAP negative nestin positive population of rapidly cycling cells (Type C cells) and lastly by proliferating neuroblasts (Type A cells). These A cells then continued proliferating whilst migrating towards the olfactory bulb surrounded by Type B and Type A cells in the rostral migratory stream (RMS). Dopaminergic periglomerular neurons originate predominantly from precursors in the RMS while granule interneurons arise from progenitors in the SVZ demonstrating specific regionalisation of olfactory bulb interneuron precursors (Hack *et al.*, 2005).

2. Function of Dentate Neurogenesis

The hippocampus has been repeatedly implicated in the modulation of cognition in association with learning and memory, and the demonstration of functional integration of these newly born dentate granule cells into hippocampal circuitry (Scharfman *et al.*, 2000; van Praag *et al.*, 2002) has led to the hypothesis that neurogenesis may be important in learning and memory formation (Gould *et al.*, 1999; Shors *et al.*, 2001; Snyder *et al.*, 2001). Hippocampal dependant associative learning tasks increase dentate neurogenesis (Gould *et al.*, 1999) and pharmacological reduction of newly born neurons in the dentate impairs certain types of hippocampal dependant learning such as trace conditioning (Shors *et al.*, 2001). Moreover, prenatal stress produces learning deficits associated with inhibition of dentate neurogenesis (Lemaire *et al.*, 2000) and the level of cell proliferation in the SGZ predicts the spatial memory performance of male rats in the water maze (Drapeau *et al.*, 2003). Exposure to a novel or enriched environment increases neurogenesis largely by increasing the proportion of cells that survive and integrate (Kempermann *et al.*, 1997) (for a critical review see (Prickaerts *et al.*, 2004)). In addition, several avenues of research have suggested that decreased hippocampal neurogenesis may be important in the pathogenesis of depression (D'Sa & Duman, 2002). Stress, which is often a requisite for depression, reduces hippocampal neurogenesis (Gould *et al.*, 1997), tri-cyclic antidepressants increase hippocampal neurogenesis (Malberg *et al.*, 2000) and the antidepressant induced increase in dentate neurogenesis appears to be necessary for their behavioural effects (Santarelli *et al.*, 2003). As newly born neurons functionally integrate into hippocampal circuitry 2-3 weeks after birth

(Hastings & Gould, 1999; Markakis & Gage, 1999), this may explain the puzzling 2-3 week delay in mood elevation after commencing antidepressants.

3. Control of Dentate neurogenesis

Despite an increasing knowledge of the structure of the SGZ neurogenic niche, our knowledge of the control mechanisms underlying hippocampal neurogenesis remains far from complete. Only a brief summary of the neuro-humoral control of dentate neurogenesis will be attempted here. For a more complete review the interested reader is referred to (Abrous *et al.*, 2005). Circulating growth factors such as IGF1 increase hippocampal neurogenesis (O'Kusky *et al.*, 2000) and may partly mediate the neurogenic effect of prolonged exercise, such as running, on dentate neurogenesis. Glucocorticoids depress cell proliferation and neurogenesis in the SGZ, while adrenalectomy has the opposite effect (Cameron & Gould, 1994). In contrast, neurosteroids such as DHEA increase SGZ cell proliferation (Karishma & Herbert, 2002). CNS inflammation has been shown to decrease neurogenesis in the SGZ (Ekdahl *et al.*, 2003; Monje *et al.*, 2003) as has the pro-inflammatory cytokine IL-6 (Vallieres *et al.*, 2002). Moreover, autoimmune T cells program microglia to make their phenotype supportive of neuronal survival (Butovsky *et al.*, 2005) and renewal (Butovsky *et al.*, 2006) and contribute to the maintenance of neurogenesis and spatial learning in adulthood (Ziv *et al.*, 2006). Although neurogenesis is clearly a neural activity driven process, precise details of the neuronal control of dentate neurogenesis remain to be elucidated. Glutamatergic afferent input negatively regulates dentate neurogenesis (Cameron *et al.*, 1995; Deisseroth *et al.*, 2004) and acts downstream of steroid control while serotonin increases SGZ cell

proliferation (Brezun & Daszuta, 2000). Input from the medial septum and forebrain also regulates hippocampal neurogenesis (Mohapel *et al.*, 2005; Van der Borgh *et al.*, 2005). Although the principle neural inputs into the dentate gyrus have been well defined, there is much evidence to suggest that local networks, largely sub-served by GABAergic releasing interneurons, significantly modulate activity. Thus GABA has emerged as another candidate for excitation neurogenesis coupling (for review see (Ge *et al.*, 2007)). Progenitors in the SVZ and dentate SGZ receive GABAergic input (Wang *et al.*, 2003; Overstreet Wadiche *et al.*, 2005; Wang *et al.*, 2005), and while non synaptic GABA controls the proliferation of GFAP-expressing progenitors in the postnatal SVZ (Liu *et al.*, 2005), it promotes neuronal differentiation and integration in the SGZ (Tozuka *et al.*, 2005; Ge *et al.*, 2006). In the dentate gyrus, GABAergic interneurons modulate granule cell excitability mainly via feedback circuits from mossy fibre collaterals (HIPP cells (Freund & Buzsaki, 1996)) but also via feed forward circuits from entorhinal cortex and contralateral hippocampal inputs (Basket Cell interneurons) (Freund & Buzsaki, 1996). They are thus ideally positioned to sample neuronal activity and signal it to the local stem cell niche. A subset of GABAergic interneurons co-release neuropeptides, usually under specific firing conditions. Principle among these is Neuropeptide Y, which is a powerful modulator of granule cell excitability (Colmers & Bleakman, 1994; Vezzani *et al.*, 1999b). A recent addition to the many physiological roles for NPY is as a proliferative factor for neural precursor cells, first demonstrated in the olfactory epithelium of adult mice (Hansel *et al.*, 2001a). Here we review the emerging role of NPY as a modulator of dentate and hippocampal SVZ stem/precursor cells.

4. NPY in the Hippocampus

Neuropeptide Y is a 36 amino acid polypeptide neurotransmitter widely distributed in the mammalian central nervous system. It has a very high sequence homology spanning a large evolutionary spectrum from invertebrates to humans (Larhammar, 1996) suggesting important functions for this peptide. Known physiological functions within the CNS include the regulation of social and feeding behaviour (de Bono & Bargmann, 1998; Sokolowski, 2003; Wu *et al.*, 2003; Kalra & Kalra, 2004), circadian rhythm (Albers & Ferris, 1984; Albers *et al.*, 1984) and central cardiovascular function (Pedrazzini *et al.*, 1998). NPY has a number of cognitive and behavioural effects on alcohol intake (Thiele *et al.*, 2004), learning and memory (Redrobe *et al.*, 1999), the anxiety response to stress and the maintenance of normal mood (for review see (Heilig, 2004). Neuropeptides are synthesised as prohormones, which undergo a number of cleavage steps initially in the endoplasmic reticulum and later in immature large core dense vesicles (LCDV) where they undergo proteolytic cleavage and post-translational modification to the final bioactive neuropeptide. The neuropeptide is released from LCDVs under specific firing conditions which are distinct from those for classical neurotransmitter release (Hokfelt *et al.*, 1984). NPY is one of the many neuropeptides that undergoes post-translational amidation, and in mammals, amidated peptides serve as growth factors mediating cell survival and neuronal proliferation (Krsmanovic *et al.*, 1993; Wettstein *et al.*, 1995; Waschek *et al.*, 1997). A recent addition to the many physiological roles for NPY is as a proliferative factor for neural precursor cells, first demonstrated in the olfactory epithelium of adult mice (Hansel *et al.*, 2001a). Recent studies have begun to identify a role for a number of amidated neuropeptides in neural stem cell biology both in the

developing and adult mammalian brain and particularly in the olfactory epithelium (for review see (Hansel *et al.*, 2001b)).

Of the 5 NPY receptors known to be expressed in the brain, only Y1, Y2, and Y5 mRNA is robust in the dentate gyrus (Parker & Herzog, 1999). mRNA for Y1, Y2, and especially Y5 receptors appear to be expressed primarily on hilar neurons and in granule cells (Parker & Herzog, 1999). Receptor protein appears to have a different expression pattern, with Y2 and Y5 receptors primarily on mossy fibres and Y1 receptors primarily in the molecular layer and neurogenic subgranular and peri-hippocampal subventricular zones (Kopp *et al.*, 2002a).

5. NPY and hippocampal Neurogenesis

We began to investigate a possible role for NPY in neurogenesis in the hippocampus using dissociated monolayer and neurosphere cultures harvested from the hippocampi of perinatal rat pups when the level of neurogenesis is at its highest, in order to optimise the number of stem/precursor cells in culture (for detailed methods see Howell *et al.* 2003 (Howell *et al.*, 2003) as well as in adult NPY (Howell *et al.*, 2007) and NPY Y1 null mice (Howell *et al.*, 2005).

5.1 NPY is proliferative for nestin positive precursor cells and committed neuroblasts isolated from the entire hippocampus.

We followed the progression of cell number, rates of division and phenotypic fate (Class III β -tubulin (neuronal), GFAP (glial) and nestin (stem/precursor cell)) over 7 days in culture. BrdU was given for the final 24 hours before each time point as a marker of S-phase entry. Analysis from 72 h revealed significant increases in cell number, BrdU incorporation, nestin immunopositivity and Class III β tubulin immunopositivity in NPY

treated cultures relative to controls (Fig. 3 A-D). Nestin positive proliferating cells showed an exponential growth curve (Howell *et al.*, 2003) (Fig. 3C). These were small unipolar or bipolar cells, with phase bright cell bodies typical of hippocampal stem/precursor cells previously described by Palmer *et al.* (Palmer *et al.*, 1997) (Fig. 3E). The time course of increases in β -tubulin expressing cells was different with a peak at 3 and 5 days in-vitro (DIV) after which the numbers plateaued (Fig. 3D), characteristic of terminally committed progenitors or Type 3 cells (Howell *et al.*, 2003). There was no evidence of a trophic effect of NPY since cell death was not altered by NPY (Fig. 3F). Using short pulse BrdU experiments we confirmed a proliferative effect of NPY on both nestin positive precursors and committed β -tubulin positive neuroblasts (Fig 3 G&H). Sphere cultures showed that the nestin positive cells that were NPY responsive were sphere forming and when grown under clonal conditions gave rise to neurons, astrocytes and oligodendrocytes (Howell *et al.*, 2003; Howell *et al.*, 2007) consistent with their categorisation as multipotent precursors. These cells were GFAP negative and rapidly cycling, consistent with Type 2 cells (*vide supra*).

5.2 NPY is proliferative for nestin positive precursors from both the Dentate gyrus and Hippocampus/SVZ but is selectively proliferative for neuroblasts from the dentate.

The hippocampus is not uniform in its neurogenic structure (Fig. 1). Precursor cells cultured from the caudal subventricular zone cSVZ are true stem cells capable of indefinite self renewal and are multipotent, generating neurons, astrocytes and

oligodendrocytes, while precursor cells in the dentate gyrus are not easily passaged in culture, i.e. display a limited capacity for self-renewal (Seaberg & van der Kooy, 2002). In order to ascertain which neurogenic population of precursor cells responds to NPY, we generated region specific microcultures from the dentate gyrus and the hippocampus proper (including the cSVZ) after Seaberg and van der Kooy (Seaberg & van der Kooy, 2002).

The number of neurons (ClassIII β -tubulin expressing cells) was greater in control dentate vs hippocampus-only cultures as expected. However dentate cultures showed a selective increase in the number of neurons in response to 1 μ M NPY whereas hippocampal cultures did not (Howell *et al.*, 2005). This was not due to a neurotrophic effect as both control and NPY treated dentate cultures had similar proportions of cell death. NPY had a proliferative effect only on cycling β -tubulin positive neuroblasts derived from the dentate gyrus and not from the hippocampus. This regional specificity was in contrast to a proliferative effect on nestin positive cells from both regions (Howell *et al.*, 2005).

5.3 The proliferative effect of NPY on nestin and β -tubulin cells is Y1 receptor mediated.

A direct effect of NPY on cell proliferation is inferred from *in-vitro* experiments showing that fluorescently tagged NPY binds to proliferating BrdU positive cells in culture (Howell and Gray, unpublished data), and that both nestin and β -tubulin positive cells stain for the NPY Y1 receptor (Howell *et al.*, 2003). Pharmacological studies using specific Y1, 2 and 5 receptor agonists and antagonists showed that the proliferative effect

of NPY was mediated entirely by the Y1 receptor, which was confirmed by showing an absence of any proliferative effect of NPY on cultures from germ line Y1 $-/-$ mice compared to wild-type controls (Howell *et al.*, 2003). This Y1 receptor specificity was confirmed on both proliferating β -tubulin cells and nestin cells from the dentate gyrus (Howell *et al.*, 2005; Howell *et al.*, 2007) in culture. Studies in germ-line Y1 receptor knockout mice revealed significantly reduced BrdU incorporation in proliferating cells in the SGZ of the dentate (Howell *et al.*, 2005) as well as the caudal subventricular zones and subcallosal zones (Howell *et al.*, 2007; Laskowski *et al.*, 2007). Germline Y1 deletion did not affect BrdU bioavailability, as BrdU incorporation in the hilus and areas CA1 and CA3 of the hippocampus showed no change between wild-type and Y1 KO.

5.4 NPY mediated proliferation in the dentate gyrus requires ERK1/2 activation.

The ERK kinases (extracellular signal-regulated kinases) are a group of mitogen-activated protein kinases (MAPK) important in control of cellular proliferation (Lopez-Illasaca, 1998) including neural progenitors (Li *et al.*, 2001). Inhibition of ERK 1/2 phosphorylation by UO126 abolished the neuroproliferative effect of NPY on proliferating cells cultured from the dentate gyrus (Howell *et al.*, 2005). This finding was in agreement with studies from the olfactory epithelium where ERK phosphorylation was necessary for NPY mediated proliferation of neuroblasts (Hansel *et al.*, 2001a)

5.5 NPY has a pure proliferative effect and does not instruct differentiation.

When clonal spheres were generated from nestin positive progenitors, NPY significantly increased sphere number and size (Howell *et al.*, 2007). Analysis of the phenotype of

cells within the spheres was unchanged from those grown under control conditions and had a higher proportion of GFAP phenotype than those grown under combined FGF/EGF conditions. When these spheres were replated under differentiating conditions the well-described neurogenic effect of FGF/EGF was confirmed with a significantly increased proportion of neurons generated. In contrast NPY did not instruct differentiation and was purely proliferative for hippocampal precursors.

5.6 Germ line Y1 KO and NPY KO mice confirm a proliferative role for NPY via the Y1 receptor in-vivo.

Short survival (2 hours) and pulse and chase (21 days) BrdU experiments in adult germ line Y1 KO mice confirmed a reduction in S-phase BrdU incorporation two hours after intraperitoneal BrdU administration, consistent with a reduction in progenitor proliferation in the SGZ of the dentate (Howell *et al.*, 2005). Sacrifice 21 days later showed no difference in the survival of BrdU labelled cells between $Y_1^{-/-}$ and $Y_1^{lox/lox}$ wild type controls, again supporting the hypothesis that Y1 receptors had no effect on cell survival. Total cell counts in the granule cell layer in p35 and p90 mice showed no difference between $Y_1^{-/-}$ and $Y_1^{lox/lox}$ controls, implying a compensatory increase in survival of newly generated neurons in constitutive $Y_1^{-/-}$. Indeed reducing precursor proliferation using methylazoxymethanol (MAM) enhances the survival of newly born granule cells (Ciaroni *et al.*, 2002).

Labelling proliferating progenitors for 5 days with daily doses of BrdU before sacrifice in $Y_1^{-/-}$ adult mice, confirmed a SGZ effect, but also showed a significant reduction in the cSVZ and SCZs between the hippocampus and the ventricle and overlying corpus

callosum (Howell *et al.*, 2007; Laskowski *et al.*, 2007). Finally a similar BrdU labelling paradigm in adult NPY *-/-* mice again showed a significant reduction in BrdU incorporation in the dentate gyrus SGZ compared to wild-type controls (Howell *et al.*, 2007). These studies have demonstrated that germ line deletion of both the ligand and its receptor reduces proliferation in the neurogenic niche of the SGZ. Pharmacological experiments and germ line NPY deletion have also demonstrated a proliferative role for NPY via its Y₁ receptor in neuroblasts of the olfactory epithelium of adult mice (Hansel *et al.*, 2001a).

6. NPY control of dentate neurogenesis

The most likely origin of NPY for modulating dentate neurogenesis is from NPY releasing interneurons in the dentate hilus, as granule cell production of NPY is only induced after seizures (Gruber *et al.*, 1994) (*vide infra*) or perforant path stimulation (Causing *et al.*, 1996). The interneurons in the dentate gyrus that express NPY are heterogeneous, receiving diverse inputs and innervating a variety of dentate gyrus cell types, including axon projections to the SGZ, where they could release NPY. The neurogenic SGZ and the molecular layer of the dentate gyrus are rich in Y₁ receptors (Kopp *et al.*, 2002b), the functional significance of which has heretofore been unclear. NPY interneurons are thus ideally positioned to sample patterns of afferent and efferent activity and thus modulate neurogenesis via NPY release on Y₁ receptor positive subgranular zone precursors. Volume transmission by released NPY acting via rapidly internalising Y₁ receptors (Fabry *et al.*, 2000) is a well recognised mechanism of action of NPY and is particularly attractive as a means of orchestrating the proliferation of

populations of precursors in the dentate SGZ. Noradrenergic projection fibres from the locus coeruleus also release NPY, and this might be one pathway for NPY to stimulate proliferation after physical exercise and electroconvulsive shock therapy for depression, as both involve noradrenergic activation and elevate NPY and proliferation in the hilus and dentate gyrus (Modigh, 1976; Mikkelsen *et al.*, 1994; Bjornebekk *et al.*, 2006). The other possible origin of NPY that could modulate SGZ precursors is from the sympathetic nervous system, which releases NPY into the circulation (Lundberg *et al.*, 1985; Pernow *et al.*, 1986) crossing the blood brain barrier (Kastin & Akerstrom, 1999) to the vascular neurogenic SGZ. Indeed exercise induced systemic NPY release (Lundberg *et al.*, 1985) may also play a role in mediating the exercise induced increase in dentate neurogenesis (van Praag *et al.*, 1999).

7. NPY, Neurogenesis and cognitive function

Dentate neurogenesis also appears to be necessary for some forms of hippocampal dependant learning (Shors, 2004). NPY appears to have a role in learning and memory, albeit less established than in anxiety. Intracerebroventricular injections of NPY fragments have been shown to enhance memory retention, recall and prevent scopolamine induced amnesia (Flood *et al.*, 1987) especially when injected into the rostral hippocampus (Flood & Morley, 1989). NPY attenuates learning impairments induced by MK-801 (Bouchard *et al.*, 1997) and the Y2 receptor also appears to be important in learning and memory processing (Redrobe *et al.*, 2004). Although we found no *in-vitro* evidence for a direct Y2 receptor mediated effect on neurogenesis, Redrobe *et al.*'s Y2 knockout study is consistent with our data supporting a Y1 mediated effect on SGZ proliferation since Y2 receptors are considered to be autoreceptors that negatively

feedback to NPY-ergic terminals to reduce NPY release (Colmers & Bleakman, 1994) and so Y₂ receptor null mice are predicted to have increased endogenous NPY expression. Studies in hippocampal NPY over expressing rats have shown impaired spatial learning associated with decreased NPY- Y₁ binding in young animals (Thorsell *et al.*, 2000) but normal spatial learning in old animals (Carvajal *et al.*, 2004). It is interesting to note that NPY levels are reduced in the dentate gyrus of aged animals (Higuchi *et al.*, 1988) as is neurogenesis and so neurogenesis in aged animals may be resistant to changes in NPY expression.

8. NPY, behaviour and mood control

Both laboratory (Heilig *et al.*, 1989) and clinical studies (Morgan *et al.*, 2000; Morgan *et al.*, 2002) have implicated NPY in the regulation of anxiety related behaviours and the central anxiolytic effect of NPY appears to be Y₁ receptor mediated (Wahlestedt *et al.*, 1993; Sajdyk *et al.*, 1999). Clinical studies have also reported reduced NPY in the cerebrospinal fluid and plasma from depressed patients (Westrin *et al.*, 1999). Animal models of depression (Caberlotto *et al.*, 1999; Husum & Mathe, 2002) have reduced hippocampal levels of NPY and altered Y₁ binding levels, antidepressants increase brain levels of NPY (Heilig *et al.*, 1988) and the antidepressant-like activity of NPY appears to be mediated via the Y₁ receptor (Redrobe *et al.*, 2002). NPY has been shown to be involved in the antidepressant actions of lithium, electroconvulsive therapy (ECT) and Citalopram (Husum *et al.*, 2000), all of which increase neurogenesis (Chen *et al.*, 2000; Madsen *et al.*, 2000; Malberg *et al.*, 2000) and the NPY gene is one of the few genes whose expression is increased only with chronic ECT (Altar *et al.*, 2004). For a comprehensive discussion of the role of the NPY system in stress, anxiety and

depression, the reader is referred to a review by Marcus Heilig (Heilig, 2004). More recently this group have also shown that the Y1 receptor is necessary for the anxiolytic effects of NPY, but not for the antidepressant effects of fluoxetine (Karlsson *et al.*, 2007).

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9. NPY and Stem Cells in the pathological / epileptic Hippocampus

9.1 NPY and Temporal lobe epilepsy

NPY expression in the rat dentate gyrus dramatically increases after seizures (Sperk *et al.*, 1992; Vezzani *et al.*, 1996; Vezzani *et al.*, 1999a), and the upregulation of NPY in the dentate gyrus has become an accepted marker of seizure activity. After acute seizures (lasting minutes), there is an elevation of expression in many non-granule cells, particularly those in the hilus and granule cell layer. After more severe seizures, such as status epilepticus following electrical stimulation, kainic acid or pilocarpine administration (Sperk *et al.*, 1992), this acute upregulation also occurs, but in addition some of the NPY cells die due to excitotoxicity (Mitchell *et al.*, 1997). When seizures occur chronically, there continues to be an increase in NPY expression in non-granule cells, but in addition, NPY is apparent in the granule cells and their axons (Marksteiner *et al.*, 1990; McCarthy *et al.*, 1998) and NPY immunolabeling becomes distributed throughout the mossy fiber axon plexus. Axonal sprouting of NPY-immunoreactive GABAergic neurons that survive seizures (Vezzani & Sperk, 2004) may compensate for the loss of some of the original NPY-containing neurons. There are substantial changes in NPY receptor expression after seizures. Y2/Y5 receptors mainly increase and Y1 receptors appear to decrease. These changes in NPY and Y2/5 receptor upregulation are consistent with a compensatory reaction that serves to increase seizure threshold and which may be neuroprotective. This follows logically from studies showing that NPY depresses mossy fiber transmission. For a comprehensive review see (Vezzani *et al.*, 1999b).

The majority of studies support the concept that increased neuronal activity increases neurogenesis in the dentate gyrus. Therefore, it is not surprising that seizures also increase neurogenesis. One of the first studies of this kind showed that a single after-discharge was sufficient to increase proliferation (Benzon *et al.*, 1997). Subsequent studies illustrated that seizures produced by many different methods could increase neurogenesis. For example, status epilepticus initiated by the systemic administration of the chemoconvulsant pilocarpine i.p. (Parent *et al.*, 1997) or intracerebroventricular kainic acid (Gray & Sundstrom, 1998) led to an increase in neurogenesis. Subsequent studies have provided even more evidence for the robust nature of seizure-induced neurogenesis using laboratory animals (Covolan *et al.*, 2000; Nakagawa *et al.*, 2000; Ferland *et al.*, 2002; Jiang *et al.*, 2003), and the ability of seizures to influence proliferation of the radial glia-like precursors (Huttmann *et al.*, 2003) and type 2 doublecortin positive cells (Jessberger *et al.*, 2005) that become granule cells. More recently studies have shown that in addition to increasing the rate of neurogenesis, they facilitate the maturation and assimilation of new cells into hippocampal circuitry (Overstreet-Wadiche *et al.*, 2006), generate ectopic granule cells (Scharfman *et al.*, 2000) which may contribute to hyperexcitability, and permanently alter the connectivity of subsequently born granule cell neurons (Jessberger *et al.*, 2007). The relevance of these findings to the development of the epileptic state after a signal episode of status epilepticus as occurs in children after complex febrile convulsions, is an exciting area of translational research, central to which is an understanding of the mechanisms subserving seizure induced neurogenesis.

These mechanisms underlying seizure-induced neurogenesis/ gliogenesis are incompletely understood, but clearly involve an increase in precursor cell proliferation. Seizures increase the proliferation of neuroblasts in the rostral SVZ (Parent *et al.*, 2002) and of both radial glia-like precursor cells (Huttmann *et al.*, 2003) and doublecortin-positive cells in the SGZ of the dentate gyrus (Jessberger *et al.*, 2005). One of the aspects of seizures that is perhaps as robust as their ability to increase neurogenesis of granule cells, is their induction of increased NPY expression in the dentate gyrus. It is therefore possible that the increase in NPY at least partly mediates the increase in neurogenesis of granule cells. In support of this hypothesis, the experimental seizure models that have demonstrated increased neurogenesis are models that have also shown an increase in NPY. Second, the increase in NPY is rapid (Marksteiner *et al.*, 1989), and occurs before neurogenesis begins, as would be necessary if neurogenesis is dependent on NPY. Thirdly, as reviewed above, NPY is proliferative for precursor cells in the dentate SGZ and also in the caudal SVZ where seizures also induce precursor proliferation.

9.2 NPY and seizure induced stem cell proliferation

To examine if NPY could be a modulator of proliferation in response to seizures, we examined SGZ cell proliferation following kainic acid-induced seizures in 3 month-old adult male NPY $-/-$ mice and controls. NPY $-/-$ animals are more sensitive to seizure induction (Erickson *et al.*, 1996) and required a “low dose” of kainic acid to display prominent equivalent seizures to wild-type mice.

We found that NPY strongly contributes to baseline and seizure-induced precursor proliferation in the SGZ, as proliferation was significantly reduced in NPY $-/-$ mice under both conditions. Seizure-induced proliferation in NPY $-/-$ animals was significantly

reduced compared to both kainate-dose matched and seizure-severity matched controls. There was no significant difference between the levels of proliferation when wild-type animals were given low or high doses of kainate, suggesting that the level of limbic seizure activity was similar in each. The greater intensity of hilar and granule cell layer NPY immunostaining was consistent with a seizure-induced increase in NPY expression as previously reported (Marksteiner *et al.*, 1989) and we found no immunostaining for NPY in NPY^{-/-} tissue. Interestingly, although the absolute levels of proliferation were lower in the NPY^{-/-} animals in control conditions and after kainate, the relative increase after kainate was the same in the wild type and NPY^{-/-} groups (4.4 fold and 4.7 fold respectively), implying that SGZ proliferation under control conditions and after seizures is not mediated entirely via NPY, and that at least in the constitutive knock out, other mechanisms were able to induce a seizure-induced response but were unable to fully compensate for loss of the NPY-mediated effect. Both FGF2 (Yoshimura *et al.*, 2001), serotonin via its 5HT1A receptor (Radley & Jacobs, 2003) and Galanin via its GalR2 receptor (Mazarati *et al.*, 2004) have also been implicated in seizure induced proliferation and cell survival in the dentate.

The caudal SVZ and SCZ contain constitutively active oligodendrocyte precursors, a proportion of which arise from SVZ Type B stem cells, which generate postnatal oligodendrocytes that contribute to the myelination of white matter tracts in the corpus callosum and fimbria fornix (Menn *et al.*, 2006). The cSVZ and SCZ also contain a prominent band of cellular Y1 receptor staining (Kopp *et al.*, 2002b) and the proliferation of these stem/precursor cells in the cSVZ is increased after seizures (Parent *et al.*, 2006). We confirmed an effect of NPY on proliferation in the adult cSVZ and SCZ under control

conditions in NPY Y1^{-/-} mice (Howell *et al.*, 2007). We also found that proliferation in the SCZ under control conditions and after seizures was mediated by NPY (Laskowski *et al.*, 2007) consistent with the hippocampal nestin positive cell population identified in our *in-vitro* culture system. Our results raise the intriguing possibility that some of the NPY-responsive β -tubulin-negative population in our postnatal HSVZ cultures are oligodendrocytes precursors, such that NPY modulates cSVZ and SCZ oligodendroglialogenesis as well as SGZ neurogenesis.

10. Conclusions

The significance of our results lies in the identification of NPY, and therefore NPY-releasing interneurons, as important modulators of the proliferation of precursor cells in both the dentate SGZ and caudal SVZ and SCZ under normal conditions and after seizures. Given that hilar and hippocampal interneuron loss is a prominent and early feature of mesial temporal lobe epilepsy, which is associated with cognitive decline, then targeting this population for neuroprotection, or pharmacological replacement, may ameliorate or reverse cognitive deficits by preventing or restoring the impaired neuropeptide drive on adult precursor cells.

These findings also support the concept that NPY and other neuropeptides have a ubiquitous role across all of the stem cell niches in the adult brain. It remains to be seen whether different neuropeptides have distinct roles in the recruitment, proliferation, differentiation of precursor cells as well as the survival of their newly generated progeny. Lastly, understanding the role of neuropeptides in the stem cell niche should enhance its

pharmacologically modification in an effort to effect repair of the damaged niche after acute or chronic brain injury.

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Figure Legends

Figure 1.

Schematic diagram of a cross section of adult hippocampus showing a granule cell neuron (Black) with its dendrites extending up to the hippocampal fissure (HF), its cell body in the granule cell layer (GCL) and its axon or mossy fiber extending out into area CA3 of the hippocampus. The neurogenic niche that produces granule cell neurons lies between the GCL and the hilus and is termed the subgranular zone (SGZ). Interneurons (IN) that release neuropeptides are located in the SGZ and hilus. The other stem/precursor niches in or around the hippocampus are the subcallosal zone (SCZ) lying in the potential space between the hippocampus and overlying corpus callosum and the caudal subventricular zone (cSVZ) lying on the ventricular surface of the dorsal hippocampus.

Figure 2.

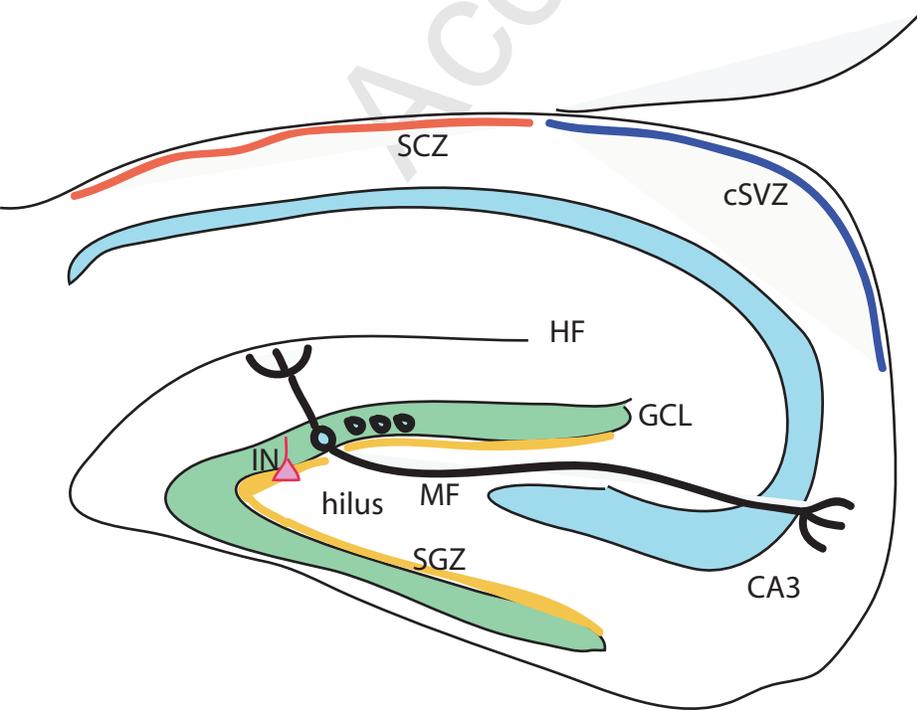
Schematic diagram of the different precursor/progenitor cells in the subgranular zone (SGZ) and their phases of maturation, together with the markers expressed and the timescale of their differentiation. Bold lettering indicates positive immunostaining. An attempted translation between different classifications as detailed in the main text is also shown.

Figure 3.

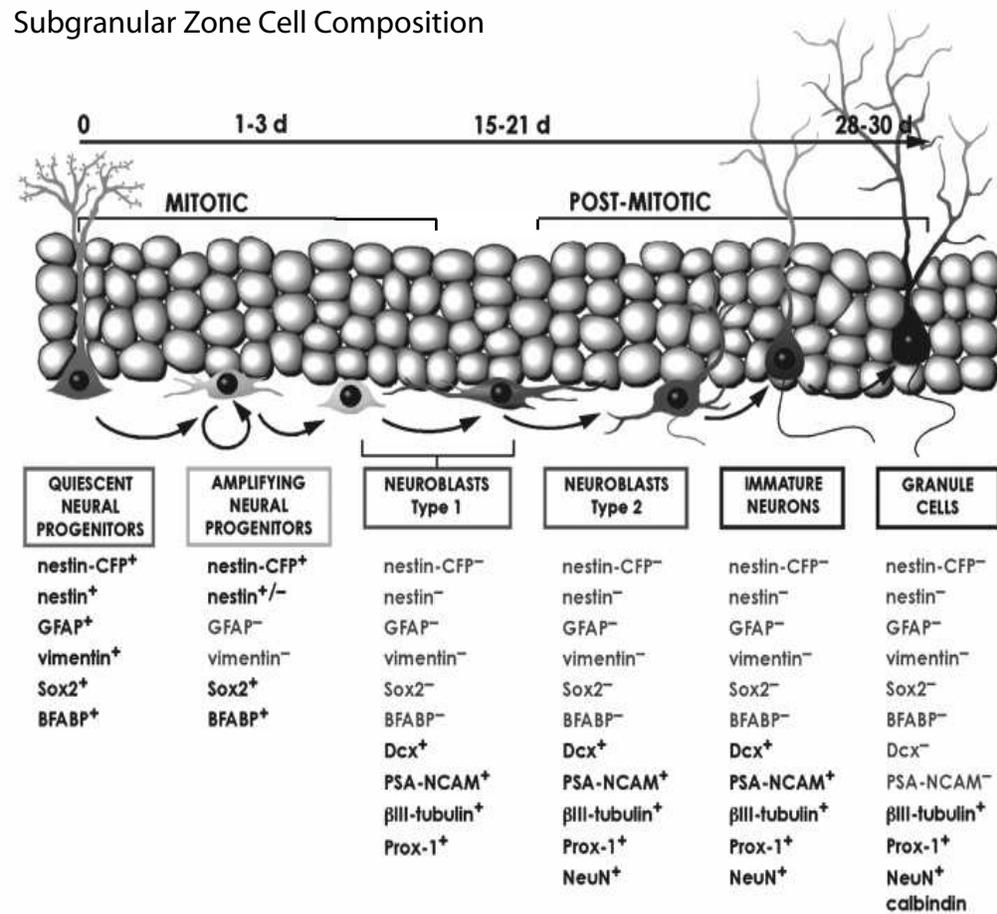
NPY enhances the total number of cells, BrdU positive cells, β tubulin positive cells and nestin immuno-positive cells with time in culture.

Hippocampal cell cultures were grown for the indicated number of days in standard control conditions, or in the presence of 1mM NPY. BrdU was added for the final 24hrs of each time-point. (A), total cell counts increase under standard conditions, and are further significantly increased in the presence of NPY. (B), the number of BrdU incorporating cells is increased between day 1 and 7 in control conditions, and are increased relative to controls at each of day 3, 5, 7 in the presence of NPY. (C), nestin counts increase with days *in vitro*, and are significantly different from controls at days 3, 5 & 7. (D), β -tubulin counts are maximal at day 3, and in control and NPY conditions fall with time in culture. (Comparing control counts to control counts at day 1, +, $p < 0.05$, ++, $p < 0.01$; Comparing NPY to respective time point controls; **, $p < 0.01$). (E) Representative phase contrast images of cells cultured in 1mM NPY. Cells were imaged at 3, 5 and 7 div. Note the large increase in cell numbers, and in particular the high proportion of immature, phase bright cells seen on days 5 & 7. It should be noted that other and often diverse cell types are present on all days. Scale bar; 100 μ m. (F) NPY does not affect cell survival. Cells were cultured in control or 1mM NPY conditions for the indicated times. Cell death was assessed by quantifying chromatin condensation (ie: cells displaying a pyknotic nucleus) and expressed as a proportion of total cells counted per field of view. Cell death was similar under control or NPY culturing conditions. NPY increases the proportion of mitotic nestin and beta-tubulin positive cells. 3 day-old cultures exposed to a 4hr pulse of BrdU and 1mM NPY have an increased proportion of β -tubulin/ BrdU (G) and nestin / BrdU (H), cells relative to cells in control media indicative of a proliferative effect of NPY on these cell phenotypes. *, $p < 0.05$.

BrdU = bromodeoxyuridine,



Subgranular Zone Cell Composition



Type 1 Type 2a Type 2b Type 2c Type 3 (Kempermann et al 2004)

Type A Type D1 Type D2 Type D3 (Seri et al 2004)

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Figure 3

