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Investigation of C-type natriuretic peptide in the intact rat brain under formal and informal learning conditions

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Abbreviations

ACh	Acetylcholine
ANCOVA	Analysis of covariance
cGMP	Cyclic guanosine monophosphate
CNP	C-type natriuretic peptide
CNS	Central Nervous System
DA	Dopamine
D[N]MS	Delayed (Non-) Matching to Sample
EE	Enriched Environment
EE-14	Fourteen days enriched housing
EE-28	Twenty-eight days enriched housing
fmol/g	Femtomoles per gram of tissue
HpcLH	Left hemisphere of hippocampus
HpcRH	Right hemisphere of hippocampus
i.c.v	Intracerebroventricularly
HTh	Hypothalamus
LTD	Long term depression
LTP	Long term potentiation
mPFC	Limbic medial prefrontal cortex
MB	Mammillary bodies
N-K	Post-hoc Newman-Keuls test
NPR-A	Natriuretic peptide receptor A
NPR-B	Natriuretic peptide receptor B
NPR-C	Natriuretic peptide clearance receptor
NO	Nitric Oxide
NTproCNP	Amino-terminal pro-CNP fragment
Occ	Occipital cortex
rH	Relative humidity
RIA	Radioimmunoassay
Rgb	Retrosplenial cortex
SH-14	Fourteen days standard housing
SH-28	Twenty-eight days standard housing
SOR	Spontaneous object recognition

Abstract

C-type Natriuretic Peptide (CNP), a relatively new member of the natriuretic peptide family, is found throughout the central nervous system. Circumstantial evidence associates CNP with learning and memory, as its expression is highest in brain regions known to be involved in memory and associated with hippocampal physiology. Here, the first study housed rats in an enriched environment, regarded as providing an 'informal' learning experience, for either 14 or 28 days of housing in enrichment in six regions of interest, which was attributed to changes in the degradation of CNP. The second study examined a group of rats trained on object -recognition task – the bow-tie maze. A difference was found in CNP production in the limbic medial prefrontal cortex over repeated exposures to novel objects relative to controls that received 'yoked learning' an exposure only to the test room. CNP concentrations also tended to be lower in rats with better levels of discrimination between familiar objects. Together, these studies provide some initial evidence that CNP influences learning –induced plasticity in the intact brain.

Investigation of C-type natriuretic peptide in the intact rat brain under formal and informal learning conditions

C-type Natriuretic Peptide (CNP) is a relatively novel molecule that is the most recently identified of a family of peptides, the other members of which play an important role in cardiovascular and renal functions (Fowkes & McArdle, 2000). Of the natriuretic peptides, CNP is the most abundant in the CNS and exhibits the highest concentration of this family in human cerebrospinal fluid (Kaneko et. al, 1993). Furthermore, both CNP messenger ribonucleic acid (mRNA)[1] molecules and CNP receptors (natriuretic peptide receptor B; NPR-B) have been discovered extensively throughout the brain and spinal cord (Langub Jr., Watson Jr., & Herman, 1995; Herman, Dolgas, Rucker & Langub Jr., 1996).

CNP is a multitasking hormone, with various roles throughout the body, and within the nervous system. Much of the work to date on CNP's role in the central nervous system (CNS) has investigated this neuropeptide at the level of neurophysiology. CNP stimulates intra-cellular cyclic guanosine monophosphate (cGMP) production, which also plays a role in axon growth and guidance, and has a demonstrated involvement in the corticotropin-releasing hormone (CRH) system, and thus may also influence stress and anxiety (Gardi et.al., 1997). However, sufficient evidence has accrued to suggest a function for CNP beyond anxiety, and in particular in the mechanisms underlying neuroplasticity, learning and memory. The current study addresses this latter possibility.

[1] mRNA provides the genetic code for production of the peptide and as such indicates regions in which CNP is produced.

C-type Natriuretic Peptide

CNP is released in a paracrine/autocrine fashion, indicating that its action is largely limited to tissues secreting the hormone (Fowkes & McArdle, 2000) with the possible exception of actions of the peptide conveyed by circulation of cerebrospinal fluid. CNP is found in the endothelial cells of blood vessels and may be important in regulating tissue blood flow by inducing vasodilation. In addition, CNP has anti-prolifereative actions within the vasculature and exerts a protective effect against arteriosclerosis and myocardial fibrosis (Potter, Abbey-Hosch & Dickey, 2006). It is also found in chondrocytes (cartilage cells) wherein it promotes cell differentiation, and participates in placental and fetal growth (Potter et al., 2006; Tamura et al., 2004; McNeill et al., 2009). Collectively, these findings suggest that the peptide plays an important role in regulating cell growth and differentiation in a wide range of tissues.

In rodents, a knockout of the gene encoding CNP results in dwarfism. Animals die prematurely from maldeveloped organs (particularly those affecting breathing, chewing and ingestion) and a lack of bone ossification (Potter et al., 2006). Further, two rodent models with loss-of-function mutations in the NPR-B receptor likewise result in dwarfism and female sterility (Potter et al., 2006). Tonic-clonic seizures, self-clasping and priapism (in males)[2] occur as neurological sequelae in one model (Tamura et al., 2004) suggesting a role for CNP in regulation of neural functions. In humans, a rare form of dwarfism results from a loss of NPR-B function. However to date no neurological sequelae analogous to those in the rodent loss-of-function mutation models has been reported (Potter et al., 2006).

CNP and the NPR-B receptor are also important for proliferation and differentiation of sensory neurons during CNS development (Schmidt et al., 2007; Schmidt et al., 2009; Zhao & Ma, 2009). In addition, CNP has been shown to have neuroprotective qualities, dose dependently

[2] A prolonged erection of the penis which results from neurological dysfunction and/or obstruction of blood vessels.

protecting against excitotoxic insult and trophic factor withdrawal in retinal ganglion cells (Ma et al., 2010). This evidence suggests a critical role of CNP for neural growth and activity, and suggests that it may have actions similar to other neurotrophic factors (e.g. BDNF, NGF; see Simpson et al., 2002).

Biochemistry of CNP

Although the third of the natriuretic peptide family to be discovered, evolutionary studies indicate that ANP (Atrial) and BNP (B-type) have evolved from CNP (Potter et al., 2006). Both ANP and BNP bind to natriuretic peptide receptor A (NPR-A), whereas CNP has low affinity for NPR-A and is the sole ligand for NPR-B (Prickett & Espiner, 2012). All three peptides bind to the C receptor (NPR-C), which acts by internalising and degrading intracellular peptides (Prickett & Espiner, 2012).

CNP is synthesised in the body from a 103 amino acid pro-hormone (proCNP) which is cleaved intracellularly to release the biologically active peptide (CNP-53) and a biologically inactive amino-terminal fragment (NTproCNP) which is secreted in equimolar quantities to CNP (Potter et al., 2006, Prickett et al., 2004; See Figure 1; adapted from Prickett & Espiner, 2012). CNP-53 is the main bioactive form in tissues, including the brain, but further cleavage results in a smaller bioactive form (CNP-22) found at very low levels in systemic circulation (Potter et al., 2006). In addition to uptake by NPR-C and intra-cellular degradation, CNP is also degraded by the action of at least two enzymes – neprilysin (Herman et al., 1996, Potter et al, 2006., Prickett et al, 2004, Prickett & Espiner, 2012) and Insulin-degrading enzyme (Potter, 2011). The combined actions of proteolysis and receptor clearance result in a relatively short half-life for CNP-22 in blood plasma, which in humans is approximately two to three minutes (Prickett & Espiner, 2012). The half-life of CNP-53 is not known, but is likely to be longer than CNP-22.

CNP-53 has previously been successfully measured in ovine hypothalamus and pituitary with extraction times of 10 minutes (see Yandle, Fisher, Charles, Espiner & Richards, 1993) suggesting a half-life of at least this length of time. As NTproCNP is biologically inactive, measures of concentrations of this molecule alongside CNP allow stronger conclusions to be drawn regarding secretion of CNP (reflected by NTproCNP concentrations) compared with degradative actions on the peptide which are assessed using the ratio of NTproCNP toCNP concentrations. Both neprilysin and Insulin-degrading enzyme also degrade the amyloid-β protein, deposits of which form plaques in Alzheimer's disease (Walther et al., 2009; Cordes, Bennett, Siford, & Hamel, 2011). Interestingly, one recent study suggested that neprilysin deficiency facilitated learning and memory in aged mice with a knockout of the gene encoding the enzyme (Walther et al., 2009). The notion of neprilysin deficiency improving learning and memory in the context of propsed effects of CNP on mnemonic processes is intriguing.

Actions of CNP are mediated via increases of cGMP, an intracellular second messenger (Herman et al., 1996, Potter et al., 2006, Ma et al., 2010, Schmidt et al., 2007, Schmidt et al., 2009). cGMP regulates many neural functions including axon growth and guidance, long term potentiation and depression (LTP/LTD), neurotoxicity and neurodegenerative processes (Wang & Robinson, 1997). Neurotransmitters reportedly affected by this cascade appear to include dopamine (DA), acetylcholine (ACh) and nitric oxide (NO; Telegdy, Kokavszkey & Nyerges, 1999), although the relationship with NO is complex given that its action also influences cGMP. Telegdy and colleagues (1999) demonstrated that by pre-treating rats with receptor blockers of these transmitters, the action of CNP in aiding passive avoidance learning was nullified. See Figure 2 for visual representation summarising these actions and interactions.

Within the brain, both NPR-B receptors and CNP mRNA are abundant in the amygdala, hippocampus, limbic cortex and diencephalic-limbic regions, and the hypothalamus (Langub Jr et

al., 1995, Herman et al., 1996; See Figure 3). These localities for the concentration of CNP imply a role in learning and memory. These regions are richly interconnected and many are regarded as contributing to an extended hippocampal-diencephalic network involved in episodic-like learning and memory (Aggleton & Brown, 1999; 2006). As mentioned earlier, CNP also has a demonstrated involvement with CRH and the hypothalamo-pituitary-adrenal (HPA) axis, associated with stress and anxiety (Gardi et al., 1997, Jahn et al., 2001).

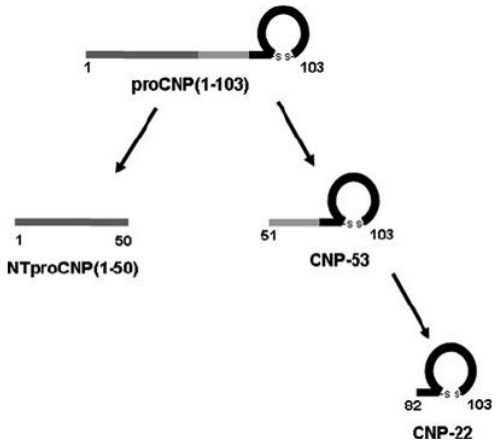


Figure 1: Cleavage of CNP from 103 amino acid pro-hormone results in two biologically active peptides, and biologically inactive amino terminal fragment (NTproCNP; Adapted from Prickett & Espiner, 2012).

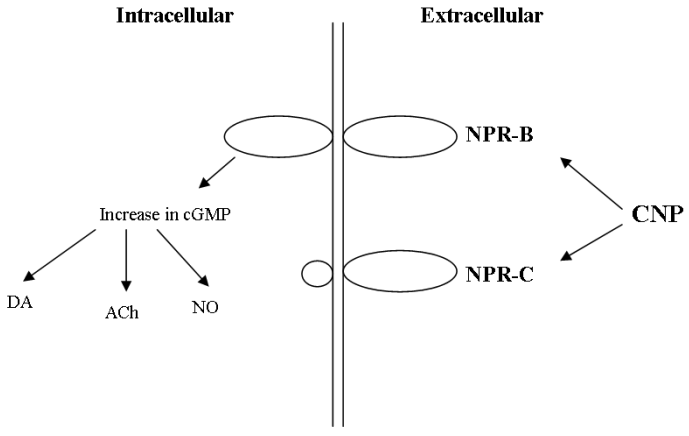
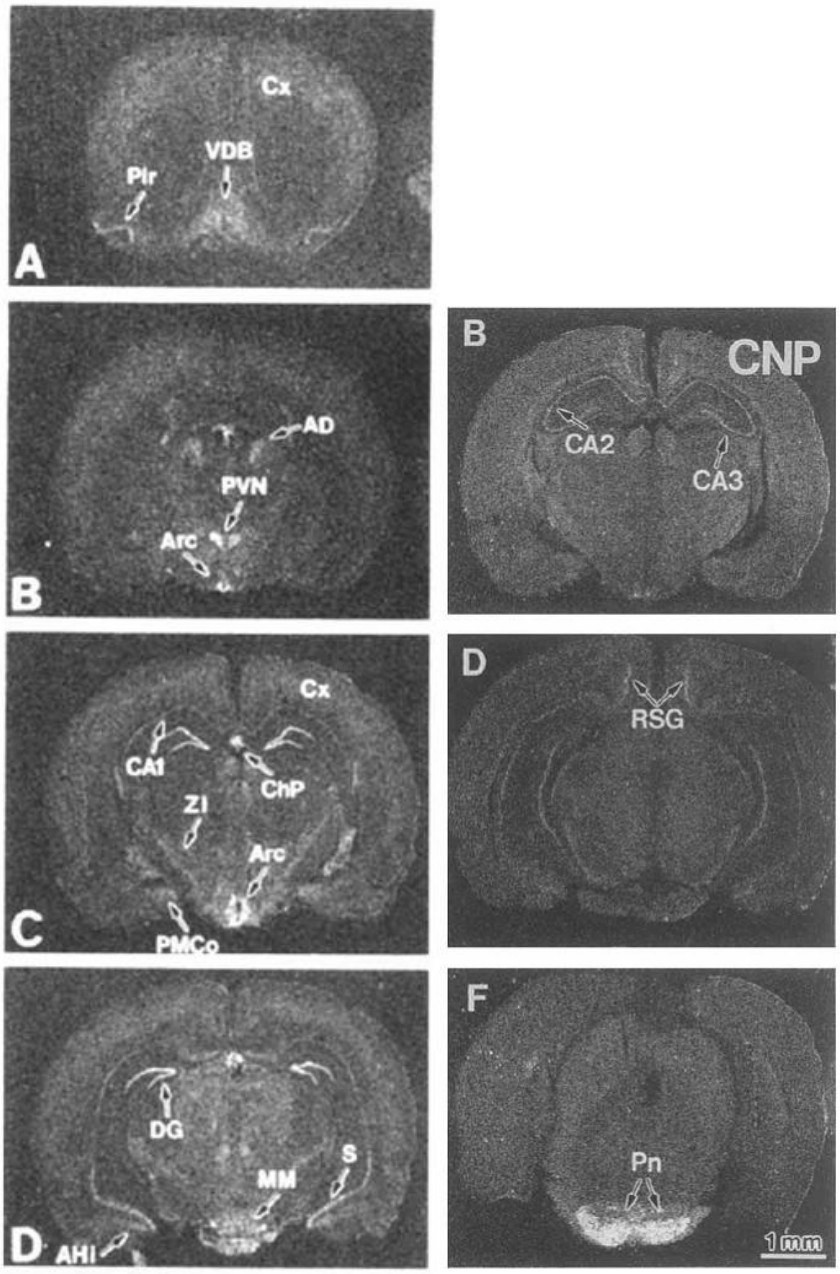


Figure 2: CNP binds with NPR-B receptor which then results in increases in cGMP. Neurotransmitters thought to be influenced by CNP include dopamine, acetylcholine and

nitric oxide, although the relationship with NO is more complex than that demonstrated here. CNP also binds with the clearance receptor (NPR-C) and is degraded by neprilysin and Insulin-degrading enzyme. (Adapted from Prickett & Espiner, 2012.)



1 *2*

Figure 3: *1* – Low power X-ray autoradiographs of coronal slices (A-D moves anterior to posterior) demonstrating distribution of NPR-B mRNA throughout rat brain (from Herman et al, 1996). *2* – X-ray photomicrographs of coronal slices (B-F moves anterior to posterior) demonstrating CNP mRNA presence in hippocampal fields CA2 and CA3, retrosplenial cortex and pontine nuclei (from Langub Jr et al, 1995). Abbreviations: Cx – neocortex; PIr – piriform cortex; VDB – ventral diagonal band of Broca; AD – anterodorsal thalamic nucleus; PVN – paraventricular nucleus; Arc – Arcuate nucleus; CA1 – subfield CA1 of hippocampus; ChP – choroid plexus; ZI – zona incerta; PMCo – posteromedial cortical amygdala; DG – dentate gyrus; S – subiculum; MM – medial mammillary nucleus; AHI – amygdalohippocampal area; RSG – retrosplenial granular cortex; Pn – pontine nuclei.

Evidence for the role of CNP in the brain

An early connection was made between CNP and dopaminergic pathways. As such, extensive research has investigated the effects of CNP in animal models of addiction. One early study (Babarczy, Vizi, Toth & Telegdy, 1995) provided evidence that CNP attenuated the analgesic effects of morphine, as well as the development of both acute and chronic tolerance to the drug. Although the authors do not discuss the potential mechanisms for this, later evidence suggests that these effects of CNP may be mediated by actions regulating and controlling dopamine release (Thiriet et al, 2001; Jouvert et al., 2004; Romieu, Gobaille, Aunis & Zwiller, 2008.).

In an animal model of alcohol addiction, CNP injected into dopaminergic brain structures (either the ventral tegmental area or nucleus accumbens) greatly reduced the alcohol intake of rats (Romieu et al., 2008). Further, when given prior to administration of cocaine, CNP inhibited increase in locomotor activity, expression of several immediate early genes and extracellular dopamine (Thiriet et al., 2001). Later these same effects were shown to be dose-dependently influenced by CNP, and that this action could be reversed by selective inhibition of cGMP dependent protein kinases (Jouvert et al., 2004). All studies suggested that inhibition of dopamine release by CNP occurs via the activation of the cGMP pathway, which has been well documented in controlling dopamine release, and also reducing reward in models of drug addiction (Romieu et al., 2008; Thiriet et al., 2001). CNP also inhibited the firing rates of dopamine dependent neurons taken from the hypothalamic arcuate nucleus *in vitro,* and this inhibition was amplified when CNP and dopamine are present together (Pan, Lai & Yen, 1996).

CNP alone is also able to increase the neuronal expression of the immediate early gene c-Fos in frontal cortex (Thiriet et al., 2001). It should be noted that immediate early genes, including c-Fos, are also associated with neural plasticity, and have been used for imaging neural

activiation associated with learning and memory throughout the brain (see Tischmeyer & Grimm, 1999, for review), but in particular in the hippocampus, perirhinal cortex, retrosplenial cortex and the mediodorsal thalamic nucleus (e.g. Zhu, Brown, McCabe & Aggleton, 1995; Albasser, Poirier, Warburton & Aggleton, 2007; Albasser, Poirier & Aggleton, 2010). Evidence from the perirhinal cortex suggests that expression of c-fos is necessary for the formation of stable recognition memory, in particular in perirhinal cortex (Seoane & Brown, 2007; Seoane, Tinsley & Brown, 2012). If the capability of CNP to increase c-fos in frontal cortex generalised to these regions, it is plausible it may also be important for plasticity and memory processes.

Although research as outlined above has expanded on the connection between CNP and DA, no reports have followed the suggested links between CNP and the neurotransmitters ACh or NO in the brain (but see Qian et al, 2002 for CNP enhancement of NO synthase in carotid arteries). ACh has long been associated with learning and memory, in particular encoding of new information (see Hasselmo, 2006 for review). Degradation of the cholinergic basal forebrain is a prominent pathology in Alzheimer's disease and together with parahippocampal degeneration may play a significan role in memory deficits in this disorder (Cassel, Mathis, Majchrzak, Moreau & Dalrymple-Alford, 2008). Relevant to the current study, ACh is also associated with object-recognition memory (Steckler, Sahgal, Aggleton & Drinkenburg, 1998c; and see below) and may have an important role in synaptic plasticity in the cortex and hippocampus (see Rasmusson, 2000 for review).

NO activates cGMP and is a likely candidate for retrograde signalling (from post- to pre-synapse) in LTP (Wang & Robinson, 1997; Lores-Arnaiz et al., 2004). Increases in NO contribute to increased synaptic plasticity and prevent age related cognitive declines including memory deficits (Lores-Arnaiz et al., 2004; 2006; 2007). Recently, NO has also been linked to an inactivation of Insulin-degrading enzyme, although no connections were investigated associated

with cognitive changes (Cordes et al., 2011). CNP also activates cGMP and as such the relationship between CNP, NO and cGMP, and the effects of the three in combination, is likely complex.

As previously mentioned, CNP plays a part in the CRH pathway and HPA axis, which are well established as part of the neurobiology of anxiety. CNP has itself been demonstrated to exert effects on anxiety, although the direction of these effects is unclear. Initially, CNP at doses of 100 and 200 nanograms given intracerebroventricularly (i.c.v) was shown to cause an anxiolytic state in rats in the elevated plus maze paradigm (Bíro, Tóth & Telegdy, 1996). It was subsequently shown that higher doses of CNP (greater than 0.5 micrograms) exerted anxiogenic effects (Montkowski et al., 1998, Jahn et al., 2001). Jahn and colleagues (2001) demonstrated that CNP administered i.c.v in rats not only increased anxiety-related behaviours in the elevated plus maze paradigm but also that the anxiogenic effects of CNP could be reversed by an antagonist of the CRH receptor. These findings suggest that CNP modulates anxiety via the CRH pathway (Jahn et. al., 2001). Subsequently, behavioural research on CNP has focussed on its effects on learning associated with anxiety.

Telegdy and colleagues demonstrated in several studies that CNP has a role in passive avoidance learning (e.g Telegdy et al., 1999; Telegdy, Adamik & Glover, 2000). Passive avoidance learning denotes a form of classical conditioning where the subject must learn to abstain from a particular response to avoid an aversive stimulus. Rats are placed on a lighted platform and allowed to enter into a dark box, due to their innate preference for the dark over light. Then, a footshock is applied to the rat upon entering the dark box and subsequent avoidance of the dark box is shown by greater entrance latency. CNP administered i.c.v was shown to improve acquisition when administered 30 minutes before footshock, and consolidation when administered 30 minutes after footshock (Telegdy et. al., 1999; 2000). No effect was found when

CNP was administered just prior to the recall trial, suggesting no effect on memory retrieval (Telegdy et. al., 1999). It is, however, unclear whether these findings are related to the processes involved in learning and memory or whether the results are influenced by the effects CNP has on anxiety (Telegdy et. al., 1999). However, the consolidation effect suggests the influence of CNP on anxiety versus memory may be separable.

Recent electrophysiology studies suggest that CNP does have a role in learning and memory processes. *In vitro* studies using hippocampal slices have shown that CNP modulates neuronal activity associated with LTP and LTD in hippocampal fields CA1 and CA3 (Decker et.al., 2009; 2010). Long-term potentiation refers to the increase, over time, in the excitability of a neuron following a particular synaptic input, caused by prior repeated high frequency activity input; Long-term depression is a decrease in excitability by prior repeated low frequency activity. The increases and decreases in synaptic strength via LTP/LTD are widely regarded as an important neuroplastic mechanism for learning and memory (Bear & Abraham, 1996). Decker and colleagues (2009) showed that in area CA3, CNP caused hyperpolarisation, increased input resistance and reduced inhibitory synaptic conductance. In the presence of CNP, the threshold for bidirectional plasticity was shifted to higher stimulus frequencies in area CA1 of the hippocampus (Decker et al., 2010). Whether thresholds for LTP/LTD induction were shifted similarly in CA3 to CA1 was not investigated in the second study. This evidence suggests that the induction of LTP in the presence of CNP is inhibited and LTD is facilitated in areas CA1and CA3 of the hippocampus (Decker et. al., 2009; 2010) suggesting that CNP modulates the potential for synaptic changes in these regions. The authors comment on a possible relationship between CA1 and passive avoidance memory, but a direct connection between this CNP effect and memory *in vivo* has yet to be shown.

These electrophysiological studies provide strong evidence for involvement of CNP in learning, memory and neuroplasticity. The hippocampus has long been associated with memory, especially declarative memory and perhaps specifically episodic memory (e.g. Squire, 1992; Kesner & Hopkins, 2006) although the concept of an extended hippocampal system is now widely accepted (Aggleton & Brown, 1999; 2006). Many regions associated with this extended system are those in which CNP receptors are prevalent, and hence where CNP exerts its influence. These areas have also been found to exhibit modifications at a neuronal level subsequent to Environmental Enrichment which is thought to provide an informal learning environment for animals, as opposed to training on a formal task such as continuous object recognition.

Environmental Enrichment

Environmental enrichment (EE) refers to conditions which provide enhanced physical and social interaction compared to standard animal housing conditions (Will, Galani, Kelche & Rosenzweig, 2004). Improvements in various cognitive functions including learning and memory, and changes to neural processes resulting in modified neural plasticity occur in intact rats following EE (see Van Praag, Kempermann & Gage, 2000 for review). Benefits of EE following a range of CNS insults are evidenced by recovery of behavioural function (see Will et. al., 2004 for review) and modifications in neural plasticity (see Nithianantharajah & Hannan, 2006 for review). Those effects related to neural plasticity are pertinent to putative functions of CNP in the intact brain. Neural plasticity is stimulated widely in the brain by EE, including the hippocampus.

A wide array of neurological changes has been demonstrated in EE. Enrichment promotes neurogenesis within the hippocampus, plus hippocampal synaptogenesis, neurite branching and

gliogenesis within the cortex and hippocampus (see Van Praag et al., 2000; Will et al., 2002 for reviews). Many of the hippocampal and cortical changes associated with EE, can occur in as little as 14 days (e.g. Bruel-Jungerman et al., 2005; Frick & Fernandez, 2003; Keyvani, Sachser, Witte & Paulus, 2004; Magalon, Cantarella, Monti, Cayre & Durbec, 2007; Wagner et al., 2002). However, many changes are still evident following prolonged periods of enrichment, with effects often reported following three weeks and up to five months of EE (e.g. Artola et al., 2006; Bardo et al., 1995; Bowling, Rowlett & Bardo, 1993; Fan , Liu, Weinstein, Fike & Liu, 2007; Francis, Diorio, Plotsky & Meaney, 2002; Hattori et al, 2007; Lazarov et al., 2005; Levi, Jongen-Relo, Feldon, Roses & Michaelson, 2003; Matsumori et al., 2005; Moncek, Duncko, Johansson & Jezova, 2004; Pham, Söderström, Winblad & Mohammed, 1999; Pinaud et al., 2002; Shum et al., 2007; Ueda, Sakakibara & Yoshimoto, 2005). One study showed increases in production of trophic factors after one year of enrichment (Pham et al., 1999).

Enrichment has been consistently shown to modify LTP in the hippocampus (e.g. Foster, Gagne & Massicote, 1996; Duffy, Craddock, Abel & Nguyen, 2001; Foster & Dumas, 2001; Artola et al., 2006; Davis, Jones & Derrick, 2010; Eckert, Bilkey & Abraham, 2010). This increase in neural plasticity is thought to be the mechanism responsible for learning and memory improvements after exposure to EE (Duffy et al., 2001; Van Praag et al., 2000). One study in particular has suggested that EE may not only enhance LTP, but also the range of synaptic plasticity seen in the hippocampus (Artola et al., 2006). This study showed that both the frequency of stimulation required to induce LTP in *in vitro* hippocampal slices was lowered post EE exposure, and more LTD occurred in hippocampal slices from enriched animals during low frequency stimulation (Artola et al., 2006). Together with evidence of abundant CNP transcripts in brain regions associated with learning and the evidence described earlier that CNP can

similarly modulate LTP/LTD (Decker et al., 2009; 2010), the possibility exists that changes in CNP activity may participate in these enrichment-induced changes.

Several other findings concerning the effects of EE can also be connected to CNP actions or interactions. CNP degradation occurs via the action of the enzyme neprilysin (as mentioned previously) which also degrades amyloid-β proteins (over expression of which is seen in Alzheimer's disease). Increase in neprilysin occurs in the brains of enriched mice, and this increase is associated with a marked reduction of amyloid-β deposition (Lazarov et al., 2005), although potential behavioural outcomes of these changes were not investigated. Additionally, EE stimulates NO production and, as previously noted, this increase ameliorates cognitive deficits seen in aging (Lores-Arnaiz et al., 2004; Lores-Arnaiz et al., 2006) and can improve cognitive performance in young rats (Lores-Arnaiz et al., 2007). The neuroprotective effects of EE exposure in animal models where housing occurs prior to some variety of neurological insult is another line of evidence that potentially connects with CNP effects. For example, positive effects of EE have been found in stroke (Gobbo & O'Mara, 2004; Matsumori et al., 2006), Parkinson's disease (Bezard et al., 2003; Faherty et al., 2005; Anastasia, Torre, de Erausquin & Mascó, 2009), Alzheimer's disease (Jankowsky, et al., 2005), Huntington's disease (Hockly et al., 2002) and Multiple Sclerosis (Magalon et al., 2007) models.

In summary, the action of CNP as a factor influencing cell growth and differentiation in the body periphery (chondrocytes and placental tissues in particular – see previously) suggests that it may have a similar action in the central nervous system. Additionally, the findings that CNP can be neuroprotective (Ma et al., 2010), is necessary for sensory axon bifurcation during development (Schmidt et al., 2007; 2009; Zhao & Ma, 2009) and modulates hippocampal plasticity (Decker et al., 2009; 2010) support the view that CNP may be altered under conditions which provide neuroprotective effects or stimulate similar neuronal changes and plasticity, such

as EE. The many changes stimulated by EE that may be linked with CNP now deserve closer scrutiny. An investigation of CNP in intact brain tissues following EE is the first needed and is therefore the subject of the first experiment reported in this thesis. The second study in this thesis addresses a formal rather than informal learning procedure, specifically object recognition memory.

Object-recognition memory

Long-term memory is generally accepted as comprising declarative and non-declarative memory elements (Squire, 1992; Squire & Zola, 1996; Winters, Saksida & Bussey, 2008). Recognition memory is a form of declarative memory where it can be indicated that a stimulus or event has previously occurred (Steckler, Drinkenburg, Sahgal & Aggleton, 1998a; Aggleton & Brown, 2006; Winters et al., 2008). It can be further delineated into a "remember-know" dichotomy, reflecting genuine recollection of the previous occurrence (remember) or simply familiarity (know) that the event or item has occurred before (Aggleton & Brown, 2006; Squire, Wixted & Clark, 2007). Recognition memory that involves genuine recollection is regarded as an example of episodic memory – memory for events which occur in a specific time and place (Aggleton & Brown, 1999; 2006).

Recognition memory is often impaired in human neurodegenerative diseases (e.g. Buffalo, Reber & Squire,1998; Laatu, Revonsuo, Jäykkä, Portin & Rinne, 2003; Lee, Rahman, Hodges, Sahakian & Graham, 2003; Holdstock, 2005; Hajilou & Done, 2007; Clark & Squire, 2010; Winters, Saksida & Bussey, 2010)). The use of animal models to study the neurological processes and structures underlying recognition memory has led to the development of many behavioural paradigms (see Steckler et al, 1998a for review of key paradigm classifications). The two most common recognition memory tasks in rodent models are Delayed (Non-) Matching to

Sample (D[N]MS) (e.g. Mishkin & Delacour, 1975, Aggleton, 1985; Mumby, Pinel & Wood, 1990; Prusky, Douglas, Nelson, Shabanpoor & Sutherland, 1994) and Spontaneous Object Recognition (SOR; Ennaceur & Delacour, 1988; Ennaceur, Neave & Aggleton, 1997; Dix & Aggleton, 1999).

Recognition memory based on familiarity engages the perirhinal and entorhinal cortices, association areas (TE1, 2 and 3; OC1 and 2; S1 and 2), the mediodorsal thalamic nucleus and prefrontal cortex (Steckler, Drinkenburg, Sahgal & Aggleton, 1998b; Aggleton and Brown 1999; 2006). Episodic memory, especially where there is a spatial task component in animal models (e.g. an object in place; Dix & Aggleton, 1999; Aggleton & Brown, 2006), hippocampus, mammillary bodies, anterior thalamic nucleus and retrosplenial cortex are probably collectively engaged as an extended hippocampal system (Aggleton & Brown, 1999; 2006). The latter system will also be engaged when recognition involves recollection. As previously outlined, CNP and associated molecules are widely expressed in these regions (Langub Jr. et al., 1995, Herman et al., 1996), suggesting it may have a role in the neurobiological cascade associated with declarative memory in general.

Although these circuits are widely accepted, the specific roles of different brain regions associated with memory are still under debate. In particular for recognition memory, discussion surrounds the relative involvement of hippocampus and perirhinal cortex and how these regions interact and contribute separately (e.g. compare Brown & Aggleton, 2001; Wixted & Squire, 2010; see also Prusky et al., 2004; Winters et al., 2008; Albasser, Poirier & Aggleton, 2010; Aggleton et al., 2010; Horne et al., 2010; Clark & Squire, 2010). Given the debate over the relative contribution of these two structures to recognition memory, related regions with rich interconnections to them are also of interest. For example, the medial prefrontal cortex, retrosplenial cortex, mammillary bodies and several thalamic nuclei have all been the focus of

extensive research on their role in memory (see Squire, 1992; Aggleton & Shaw, 1996; Van Der Werf, Jolles, Witter & Uylings, 2003; Harker & Whishaw, 2004; Vann & Aggleton, 2004; Vann, Aggleton & Maguire, 2009; Kesner & Churchwell, 2011 for reviews of contributions of individual regions). Given the strong research focus on hippocampal involvement in 'spatial' memory aspects, research has likewise implied an involvement for these connected regions in aspects of spatial/episodic memory.

As mentioned previously, two key paradigms – D[N]MS and SOR – are usually used to test object recognition in rats. D[N]MS tasks require subjects to learn either a matching or non-matching rule, and then apply this rule when indicating that a particular stimulus has been previously experienced. For example, in a non-matching paradigm, this would require selection of a novel object for a reward. However training rats in D[N]MS visual recognition tasks can prove difficult, which has restricted their utility (Prusky et al., 2004; Albasser, Chapman, Amin, Iordanova, Vann & Aggleton, 2010). More studies of rodent object recognition memory have thus employed SOR tasks. SOR tasks take advantage of a natural novelty preference in rodents, which results in a greater exploration time for novel rather than familiar objects. These tasks require minimal pre-training. But the general one-trial format can however, lead to greater non-experimental interference between trials (for example from animal handling, object handling) and accumulation of consistent data has been problematic (Steckler et al., 1998a; Albasser et al., 2010).

A new task has recently been developed which combines aspects of both DNMS and SOR visual recognition tasks – the bow-tie maze (for description see Methods – bow-tie maze; See also Aggleton et al., 2010; Albasser et al., 2010; Albasser, Poirier & Aggleton, 2010; Horne et al., 2010). The bow-tie gains several advantages by combining these two paradigms. First, in contrast to traditional DNMS tasks, all objects are rewarded. This removes influences of rule

learning, and instead encourages approach to, and exploration of, both novel and familiar objects. The critical indicator of recognition thus becomes the difference in exploration of the novel versus familiar object, as opposed to recognition based on the reward of a rule. This methodology creates greater ease in training by exploiting the rat's natural novelty preference. Second, non-specific interference from handling the rat or objects is minimised by having 20 trials continuously in one session. Third, as opposed to passive exposure to novel stimuli seen in other object recognition tasks, encouragement of active exploration of the objects provides distinct behavioural evidence of novelty discrimination, which in turn facilitates interpretation of any null results which may occur in such as in neurological measures of activation (e.g. Zhu, Brown, McCabe & Aggleton, 1995; Wan, Aggleton & Brown, 1999).

The bow-tie task has been shown to be appropriate for imaging of brain activity associated with the immediate-early gene c-Fos, which is associated with the formation of stable recognition memory (Tischmeyer & Grimm, 1999; Seoane & Brown, 2007; Albasser, Poirier & Aggleton, 2010). While the bow-tie is sensitive to lesions of perirhinal cortex (Horne et al., 2010; Aggleton et al., 2010) and unaffected by hippocampal lesions (Albasser et al., 2010), this task activates the hippocampus and perirhinal cortex in intact animals (Albasser, Poirier and Aggleton; 2010). Hence this task was regarded as a relevant one to examining CNP levels in the hippocampus and its related memory regions.

Summary rationale and aims

Sufficient evidence has accumulated to suggest CNP has a critical role in the brain, possibly as part of a neurological cascade responsible for neuroplasticity, learning and memory. While CNP is ubiquitous throughout the CNS, there are scant reports of direct measurements of CNP in brain (see Ueda et al., 1991; Minamino, Makino, Tateyama, Kangawa, & Matsuo, 1991; Komatsu et

al., 1991; Yandle et al., 1993). Moreover, no studies to date have investigated CNP concentrations in the intact brain in relation to behavioural manipulations. The current thesis used two studies to address this issue.

First, evidence that EE stimulates neuroplasticity and provides an 'informal' learning environment for animals suggests an excellent starting point for study of CNP's role in the intact brain. Briefly, animals were housed in EE for two different time periods (14 or 28 days), with concentrations of CNP measured in fresh (non-perfused) brain tissue taken from the medial prefrontal cortex, hypothalamus, mammillary bodies, hippocampus and retrosplenial cortex. The occipital cortex was also taken because this has traditionally shown the most reliable neurobiological effects of enrichment (Rosenzweig & Bennett, 1996). Directional changes in CNP were not hypothesised. The study also included an investigation of potential strain differences in standard housed animals.

To examine whether changes in CNP occur during a formal learning and memory task, as opposed to the widespread informal stimulation due to an EE, the second experiment used the bow-tie maze object recognition task. One group of rats was trained on the bow-tie task and compared to two control groups, one given a repeated sequence of the same objects (learning and general experience control) while the second was a home-cage control (no behavioural experience). CNP concentrations were measured in the same regions as those investigated in the EE study. It was proposed that there may be a difference in tissue concentrations of CNP in the experimental (trained) group. Again, no directional hypothesis was made about possible changes to CNP. The bow-tie task provided a memory task with minimal anxiety expected. The examination of CNP in the brain under both "informal" and "formal" learning conditions was anticipated as a first step towards future research on the role of CNP in memory processes.

Experiment 1 – Environmental Enrichment

Experiment one was undertaken to investigate potential changes in CNP during "informal learning" provided by an enriched environment.

Methods

Subjects

Subjects were 48 male rats bred in the Psychology Department at the University of Canterbury. Prior to enrichment, all rats were housed in groups of three or four in standard opaque plastic cages (45 cm x 27 cm x 22 cm high). During enrichment, 24 PVGc Hooded rats were re-housed with new cage mates in one of two enrichment cages (12 rats per cage). The EE cages are made of wire mesh with a solid metal floor covered with sawdust and measure 85 cm by 60 cm by 30 cm high. Twelve other PVGc rats were re-housed with new cage mates (three per cage) into standard cages. These PVGc rats weighed between 306 and 413g and were between 8 and 9 months old at the start of the experiment. To learn of potential strain differences, 12 male albino Wistar rats of the same age were re-housed in groups of three in standard opaque plastic cages (62 cm x 40 cm x 22cm high). The Wistar rats weighed between 589 and 737g. The colony room was maintained at 22°C and 48% rH under a reversed 12 hour light cycle (lights off 0800h to 2000h). Food and water was available *ad libitum* throughout experiment one. All protocols conformed to the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Animal Ethics Committee of the University of Canterbury.

Enrichment

A standardised enrichment protocol developed at the University of Canterbury was used (Harland & Dalrymple-Alford, 2012). The two groups of enriched rats were exposed to enrichment for a

period of either 14 or 28 days (EE-14 and EE-28, respectively). The protocol consisted of 40 possible configurations made from 93 different objects. Objects included items such as tunnels, metal chains, ceramic containers, metal racks, hardware components and other 'knick knacks' of varying size, shape, colour and texture. A different configuration was used daily, consisting of a combination of 13 of the objects plus two wooden blocks (to provide a favoured object to chew). No single object was repeated within five days. Every seventh day was denoted as a "tunnels only" day, with configurations for these days consisting of PVC spouting and junctions. Every eighth day was a "no objects" day with no objects (aside from wooden blocks) present in the cage. Additionally, positions of food and water were altered every day, and the cages moved in the colony room every four days. Configurations were changed early in the dark period of the reversed light cycle (between 0900h and 1000h) during which enriched rats were held in a typical standard cage.

Sacrifice and Tissue extraction

After either 14 or 28 full days of enrichment, the PVGc rats were removed from the enrichment cage and placed into a standard (holding) cage at the same time of day as previous configuration changes had taken place. Six standard-housed rats were compared to each duration of enrichment (SH-14 and SH-28 respectively), and were sacrificed on the same day as those in the enrichment conditions. The 12 albino Wistars housed in standard cages received a similar protocol. All animals were then taken to a room separate to the colony room prior to euthanasia with an overdose of Sodium Pentobarbitone (300mg/mL), and held there for the duration of the sacrifice and tissue extraction process.

Once the rat was fully unconscious and had stopped breathing the brain was rapidly removed from the skull whole and placed in a brain matrix (Ted Pella). An initial coronal cut was

made at the level of the optic tract, then cuts made 5mm anterior and posterior to this point, resulting in two large 'slabs' of fresh tissue. Figure 4 (A through C; adapted from Paxinos & Watson, 1998) shows the approximate level of the anterior face of the anterior slab and the posterior slab, and the posterior face of the posterior slab. Slabs were placed with the anterior side up on a 70% alcohol cleaned glass petri dish with a small amount of sterile saline coating the surface. Microdissection scissors were used to extract the required tissue regions into pre-weighed eppendorf tubes. Tissues were taken from the posterior slab first and order of extraction was: occipital cortex (Occ), retrosplenial cortex (Rgb), dorsal hippocampus (left and right hemisphere; HpcLH, HpcRH), mammillary bodies (MB) and hypothalamus (HTh). Finally, the limbic medial prefrontal cortex (mPFC) was taken from the anterior slab. Tissue samples were quickly weighed and snap frozen in liquid nitrogen. Frozen samples were kept on dry ice until transport to the Christchurch Cardio Endocrine Research Group laboratory for storage at -80°C.

The order of sacrifice for enriched and standard-housed rats was counterbalanced across the day (Table 2, Appendix). Although only the hippocampus was separated by hemisphere (each providing sufficient above minimum tissue necessary for analysis), the hemisphere approached first (left versus right) was counterbalanced such that half of the samples were approached from the right hemisphere first, and half from the left hemisphere. Issues in tissue processing resulted in the exclusion of data from one rat in the EE-14 group. Additionally, the CNP assay was unsuccessful in one EE-14 Occ sample and one SH-14 Rgb sample. Final sample sizes are summarised in Table 8 (see Appendix). All NTproCNP assays (conducted to determine relative levels of protein production and indicate degradation of CNP) were successful.

CNP and NTproCNP assay

CNP and NTproCNP concentrations were established by radioimmunoassay (RIA), carried out by Doctor Tim Prickett (Christhcurch Cardioendocrine Research Group). Initial tissue preparation was conducted by Susan Rapley. For full descriptions of RIA methods see Prickett, Yandle, Nicholls, Espiner & Richards (2001; NTproCNP) and Yandle et al. (1993; CNP). For the measurement of rat NTproCNP an antiserum was used that recognises the C-terminal epitope in the region of proCNP (38-50) which is identical in human, mouse and rat (Prickett, Bothwell, Yandle, Richards & Espiner, 2012). Briefly, tissue samples were boiled and homogenised, then extracted on Sep-Pak C_{18} cartridges. The assays were run over three days. All samples from one rat were run in the same assay, and groups were randomised across assays in an attempt to reduce any influence of "between assay" variations. Concentrations of NTproCNP and CNP are expressed as femtomoles per gram (fmol/g) of tissue. The CNP assay had a detection limit of 0.6 pmol/L and ED50 7.3 pmol/L. Intra- and inter-assay coefficients of variation were 5.9 and 7.7%, respectively, at 23 pmol/L. The NTproCNP assay had a detection limit of 0.4 pmol/L and ED50 9.9 pmol/L. Intra- and inter-assay coefficients of variation were 3.5 and 5.0%, respectively, at 21 pmol/L.

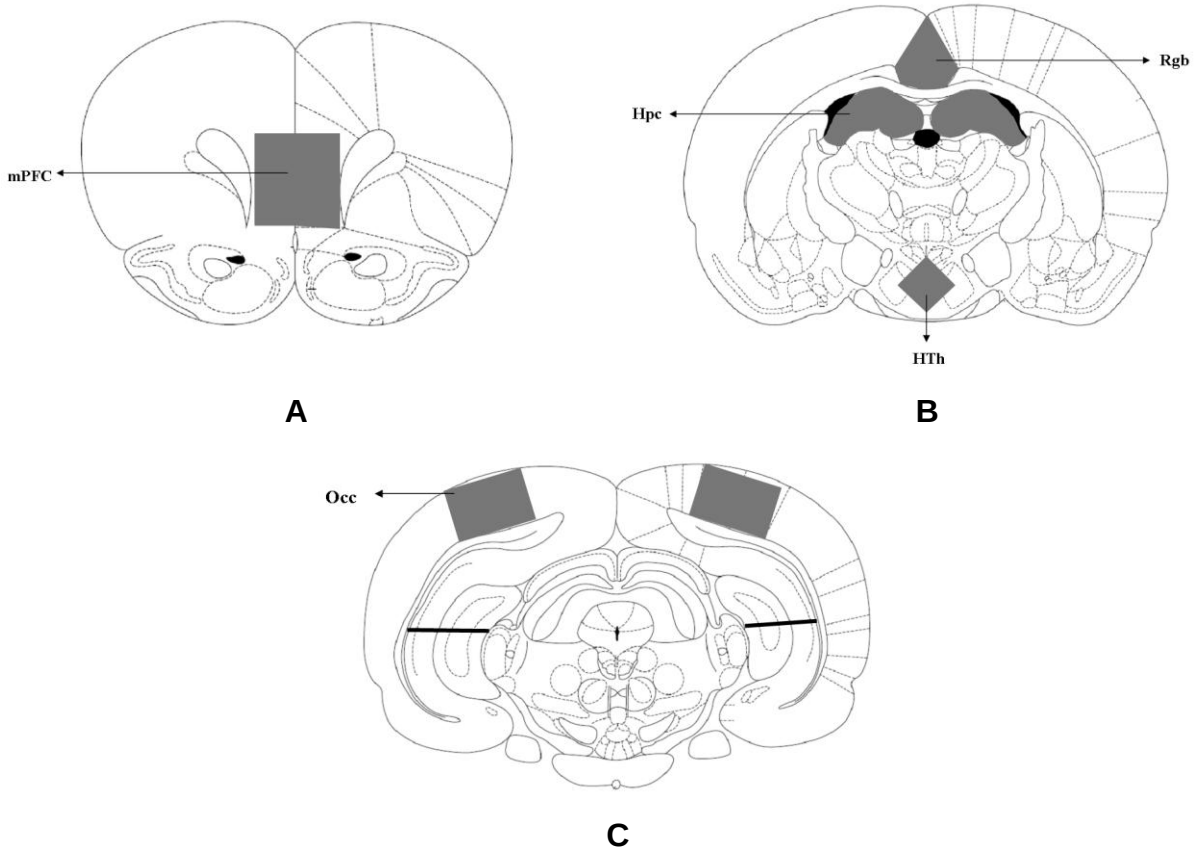


Figure 4: A – Approximate level of anterior face of anterior slab (~Bregma +3.20mm). B – Approximate level of anterior face of posterior slab (~Bregma -1.80mm). C – Extent of posterior slab of tissue (~Bregma-6.30mm). Gray areas indicate approximate samples taken from A and B. In C, gray indicates the extent to which tissue was captured from the posterior slab and thick black lines indicate the level to which hippocampus was captured moving toward the posterior region of the slab. Abbreviations: mPFC – limbic medial prefrontal cortex; Rgb – retrosplenial cortex; Hpc – dorsal hippocampus; HTh – hypothalamus; Occ – occipital cortex. Note: mammillary bodies are not illustrated as they were captured at approximately the centre third of the slab (~Bregma -5.30mm). Adapted from Paxinos & Watson (1998).

Results

Strain comparisons

The standard housed Albino Wistars were compared to the standard housed PVGc hooded rats. NTproCNP andCNP concentrations in plasma can be affected by caloric intake (see Prickett et al., 2007), which although not measured directly, was likely higher in Wistar rats given that they

are substantially larger than the PVGc strain. Independent means t-test confirmed that Wistars were significantly larger than PVGc's (Wistar M_{weight} = 666.45 grams, SD_{weight} = 37.72; PVGc: M_{weight} = 366.17 grams, SD_{weight} = 33.19; t(22) = -20.70, p <.001). Correlations between NTproCNP or CNP with animal weight were significant in several regions (NTproCNP in Occ, Rgb, HpcLH, r's = .49 to .68; CNP in Occ, Rgb, HpcRH, HTh, r's = .52 to .62; all p's <.05). In other regions correlations ranged between r = .07 (NTproCNP mPFC) and r = .33 (NTproCNP, HpcRH). In view of these findings, strain comparisons were conducted using Analysis of Covariance (ANCOVA), to control for animal weight. Repeated measures ANCOVA was used for the hippocampus.

Mean (+SE) concentrations of CNP and NTproCNP are shown in Figure 5A and B respectively. Mean (+SE) ratios of NTproCNP to CNP (i.e. NTproCNP concentration divided by CNP concentration) are shown in Figure 5C with lower ratios indicating lower degradation of CNP in tissue. No effects of Strain were found for CNP concentrations (all F's <3.15, all p's >.092; See Figure 5A). A significant Strain difference was found only in Occ for NTproCNP (F (1, 19) = 4.72, p = .043) with Wistars (M = 1041.67, SE = 97.47) having higher NTproCNP concentrations than PVGc's (M = 655.36 fmol/g, SE = 89.81; See Figure 5B). No other effects of Strain were found for NTproCNP concentrations (all F's < 1.70, all p's >.208; Figure 5B) and there were no significant effects of Strain for ratios of NTproCNP to CNP (all F's < 3.42, all p's > .080; Figure 5C). With no strain difference evident for most regions, standard housed Wistar data were combined with standard housed PVGc data for subsequent analyses (final N = 12 for SH-14 and SH-28) in all regions except Occ.

Effects of enriched environments

Analysis by 2 x 2 (Housing x Time) factorial ANCOVA (covariate: animal weight) was undertaken for each region separately. Again, the hippocampus was run as a repeated measure. Mean CNP and NTproCNP concentrations (+SE) are presented in Figure 6A and B, respectively; Figure 6C shows mean (+SE) NTproCNP/CNP ratios.

In mPFC, Rgb and MB regions, the overriding finding was one of higher CNP in EE-14 (see Figure 6A; Housing: F's > 6.85, p's<.012; Time: F's > 5.02, p's<.030; Housing x Time: F's > 4.20, p's < .047). However, within these regions, there were no substantial differences in NTproCNP concentrations (see Figure 6B; Housing: F's < 0.94, p's > .337; Time: F's < 1.52, p's > .224; Housing x Time: mPFC, MB, F's < .47, p's > .495; Rgb, $F(1,42) = 4.52$, $p = .039$), with the significant interaction in Rgb due to slightly lower concentrations in SH-28 compared to SH-14, and slightly higher concentrations in EE-28 compared to EE-14. However, in Rgb no group differed significantly from any other (post-hoc Newman-Keuls comparisons, all p's > .05). In mPFC and Rgb regions, ratios of NTproCNP to CNP in EE-14 were significantly lower than all other groups (see Figure 6C; Housing: F's > 20.05, p's < .001; Time: F's > 10.07, p's < .003; Housing x Time: F's > 17.89, p's < .001). The same pattern was seen in MB, however the effects of Time and the interaction effect failed to reach significance (Housing: $F(1,42) = 6.75$, $p = .013$; Time: $F(1,42) = 1.88$, $p = .177$; Housing x Time: $F(1,42) = 2.23$, $p = .143$).

In the hippocampus, concentrations of CNP, NTproCNP and the ratio between the two did not differ significantly across left and right hemispheres (Hemisphere: F's < 1.32, p's > .256). A similar pattern of an increase in CNP concentrations in the EE-14 group was seen in the hippocampus, although statistical support was weaker (see Figure 6A; Housing: $F(1,42) = 2.32$, $p = .135$; Time: $F(1,42) = 6.82$, $p = .012$; Housing x Time: $F(1,42) = 1.29$, $p = .263$). Concentrations of NTproCNP were lower in enriched rats in the hippocampus (Housing: F(1,42)

= 5.35, p = .026), with no other effects seen in NTproCNP concentrations (Time: F(1,42) = 2.23, p = .143; Housing x Time: F(1, 42) = 2.07, p = .157; Figure 6B). As in previous regions, ratios of NTproCNP to CNP were lower in enriched rats and at 14 days (Housing: F(1, 42) = 6.76, p = .013; Time: F(1,42) = 12.39, p = .001), although an interaction was not statistically significant (Housing x Time: F(1, 42) = 1.03, p = 0.316; Figure 6C). While the results here are somewhat weaker, they support the same picture as that seen in mPFC, Rgb and MB of reduced degradation of CNP in the EE-14 group.

In the hypothalamus, CNP concentrations were higher in enriched rats at both time points (Housing: F(1,42) = 12.19, p = .001; Time: F(1, 42) = 13.44, p = .001) and unlike other regions, this effect was not specific to the EE-14 group, evidenced by a non-significant interaction (Housing x Time: F(1, 42) = 2.01, p = .163; Figure 6A). The same pattern was apparent in NTproCNP concentrations in the hypothalamus, although the enrichment effect was non-significant (Housing: F(1, 42) = 1.17, p = .286; Time: F(1,42) = 6.34, p = .016; Housing x Time: F(1,42) = 0.12, p = .730; Figure 6B). However, ratios of NTproCNP to CNP here, demonstrated the same pattern to all other regions of reduced degradation in the EE-14 group (Housing: F(1, 42) = 11.19, p = .002; Time: F(1,42) = 4.07, p = .050; Housing x Time: F(1.42) = 4.56, p = .039; Figure 6C).

Occipital Cortex

Because of the strain difference in this region, it was analysed using only the PVGc rats. Analysis by 2 x 2 (Housing x Time) factorial ANCOVA (covariate: animal weight) was undertaken for Occ separately, without including data from Wistar rats. Figure 7 shows mean (+SE) concentrations for CNP, NTproCNP and the ratio of NTproCNP to CNP. As in mPFC, Rgb and MB regions, in Occ CNP was present in much higher concentrations in the EE-14 group than in

all other groups (Housing: $F(1,29) = 11.04$, $p = .002$; Time: $F(1,29) = 5.16$, $p = .031$; Housing x Time: $F(1,29) = 14.63$, $p < .001$). No effects were found for NTproCNP concentrations (all effects F's < 1.0, p's > .852). The ratios of NTproCNP to CNP indicated the higher EE-14 concnetration in CNP was attributable to lower levels of degradation in the EE-14 group compared with all others (Housing $F(1,30) = 14.07$, $p < .001$; Housing x Time: $F(1, 30) = 13.81$, $p < .001$) although the Time effect was not significant (Time: $F(1, 30) = 2.17$, $p = .151$).

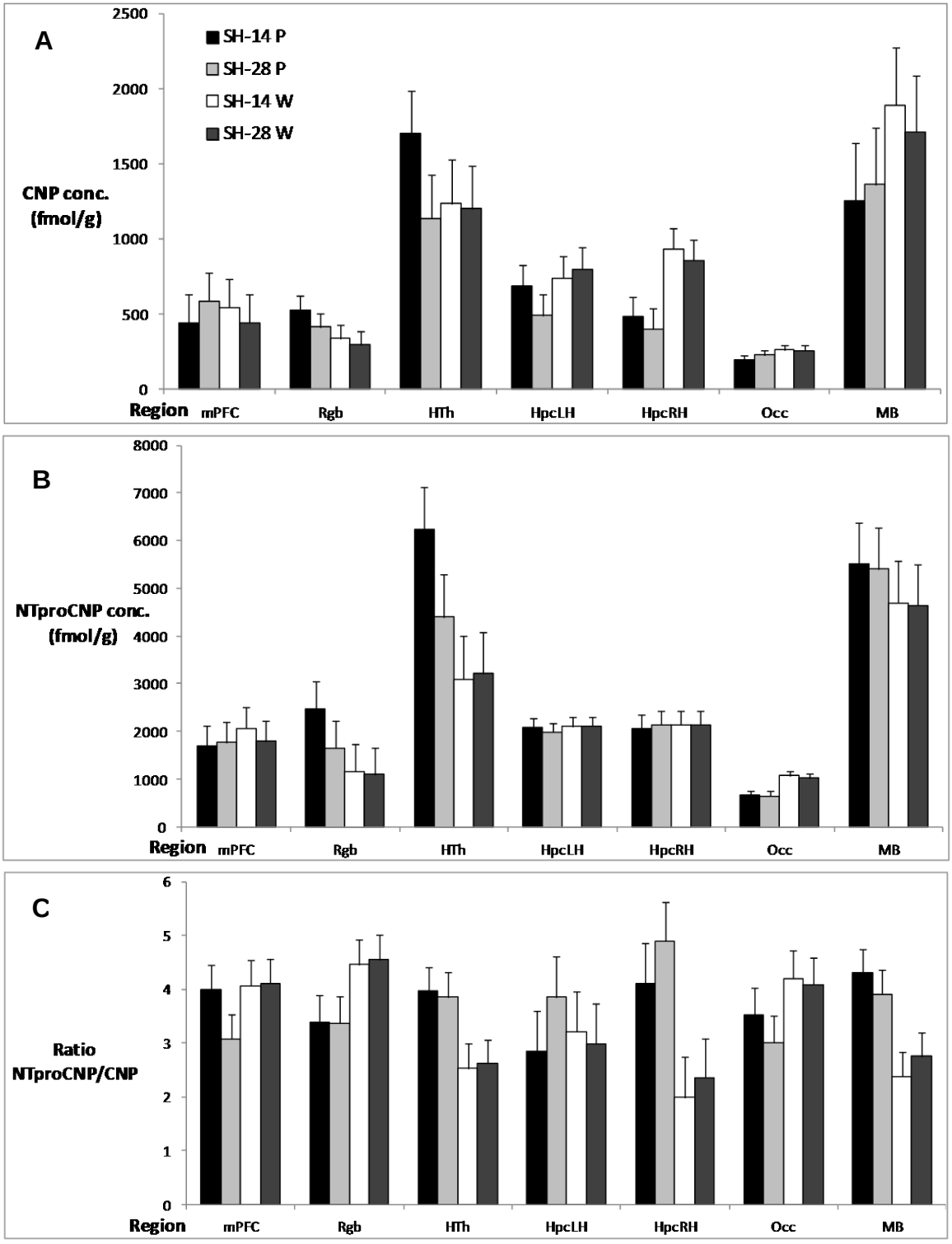


Figure 5: Strain comparisons for all regions. A - Mean (+SE) CNP concentration. B – Mean (+SE) NTproCNP concentration. All concentrations expressed as fmol/g of tissue. C – Mean (+SE) ratio of NTproCNP to CNP concentrations. Lower ratios indicate lower degradation. Key: SH-14 – 14 days standard housing; SH-28 – 28 days standard housing; P – PVGc rats; W – Wistar rats.

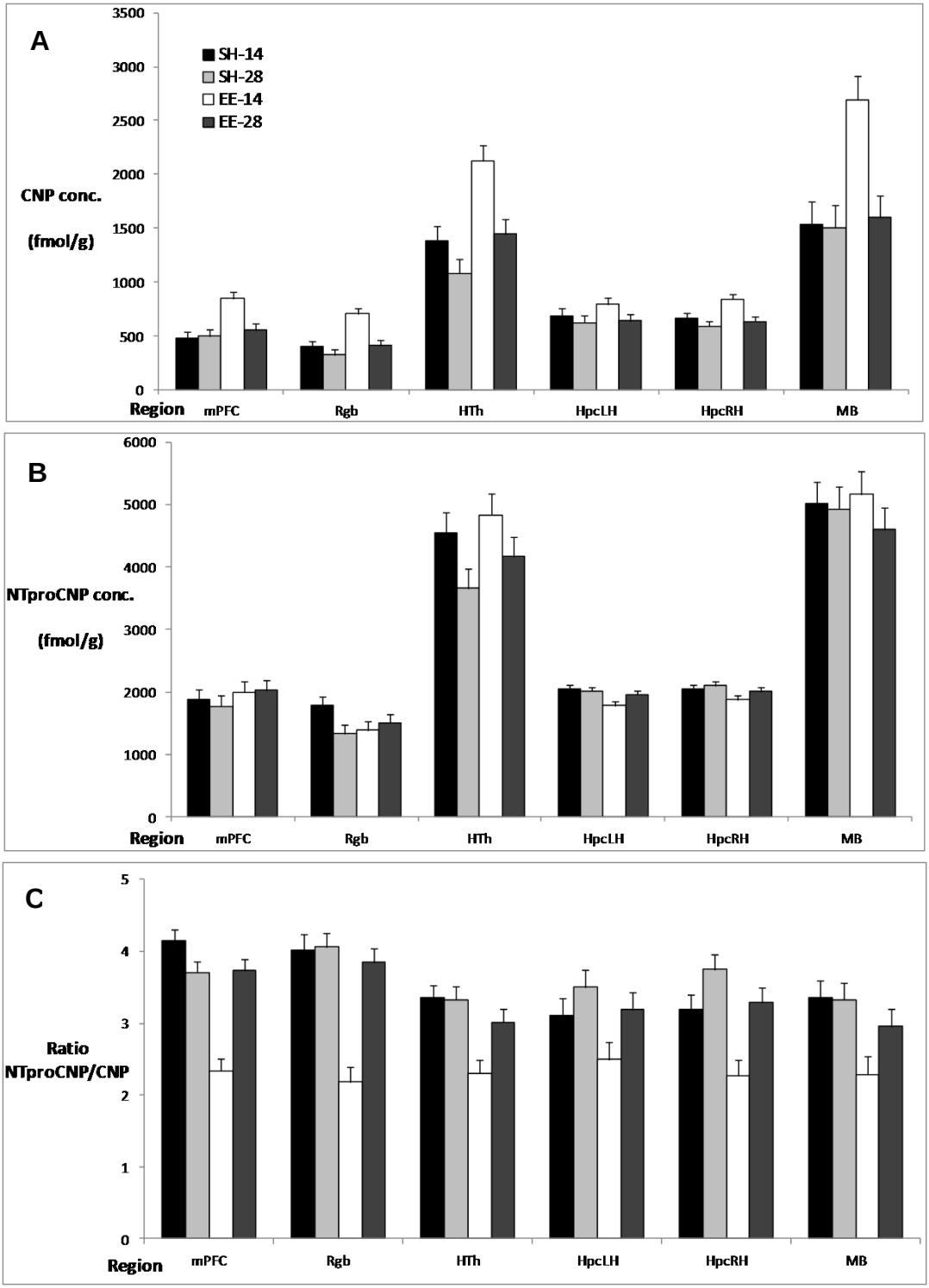


Figure 6: Comparisons of Enriched versus Standard housing. A – Mean (+SE) CNP concentration. B – Mean (+SE) NTproCNP concentration. All concentrations expressed as fmol/g of tissue. C – Mean (+SE) ratio of NTproCNP to CNP concentrations. Lower ratios indicate lower degradation. Key: SH-14 – 14 days standard housing; SH-28 – 28 days standard housing; EE-14 – 14 days enriched housing; EE-28 – 28 days enriched housing.

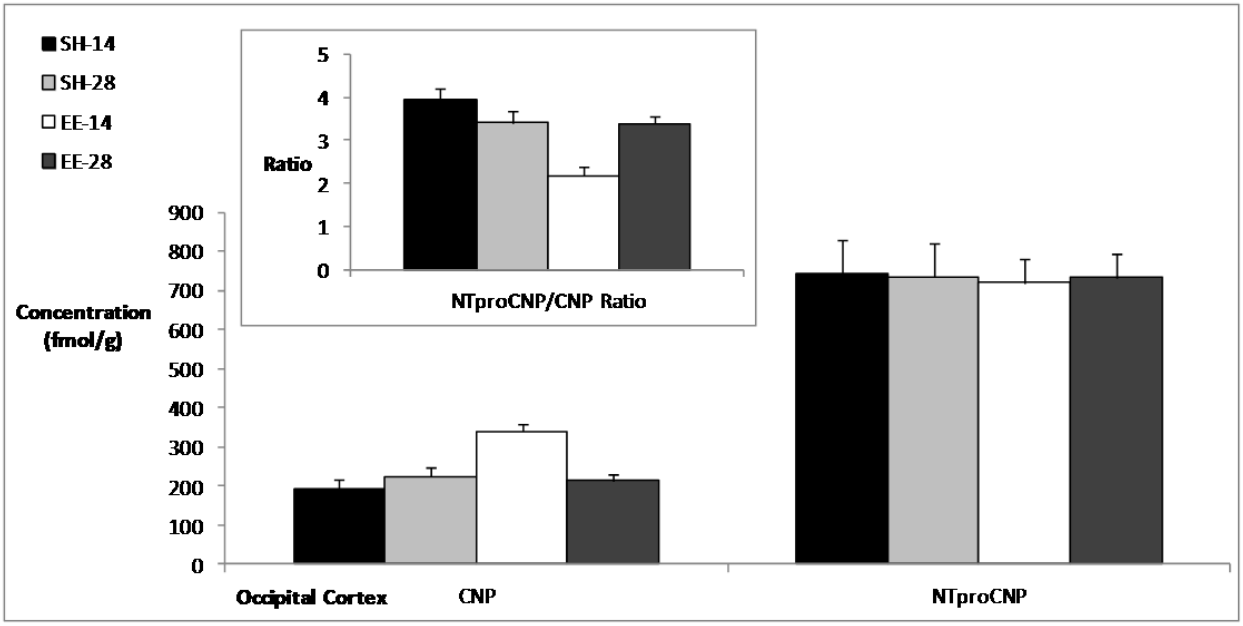


Figure 7: Data from occipital cortex, PVGc rats only. Main figure – Mean (+SE) concentrations of CNP and NTproCNP expressed in femtomoles (fmol) per gram of tissue. Inset – Mean (+SE) ratio of NTproCNP to CNP. Lower ratios indicate lower degradation. Key: SH-14 – 14 days of standard housing; SH-28 – 28 days of standard housing; EE-14 – 14 days of enriched housing; EE-28 – 28 days of enriched housing.

Summary – Environmental Enrichment

The global conclusion provided by the data is an indication of higher concentrations of CNP throughout the regions analysed following 14 days of enriched housing, which was usually not maintained by 28 days of enrichment. Lower ratios of NTproCNP to CNP and a lack of changes in NTproCNP throughout the brain indicated that this effect was due to a reduction in the degradation of CNP at the 14 day time point. These effects were clearly evident in mPFC, Rgb and MB regions. Statistical support for the same effects was somewhat weaker in the hippocampus and hypothalamus, but still provided evidence for the same pattern of higher CNP concentrations following 14 days of enrichment which was not attributable to an increase in the production of the protein.

The same effect was also evident in Occ, and in addition a strain effect was found in this region between the Albino Wistar and PVGc rats. This strain effect was such that there was a

higher production of CNP in Occ in Wistar rats, but no subsequent difference in the ratio of NTproCNP to CNP. The most likely explanation for a strain difference only in this region may lie in the decreased visual acuity in albino Wistars when compared to other pigmented strains of rat (Prusky, Harker, Douglas & Whishaw, 2002). A large proportion of the tissue sampled for the Occ region included the primary visual cortex. Previous work has demonstrated differences in evoked potentials in the visual cortex of albino Wistar rats when compared to pigmented strains (Heiduschka & Schraermeyer, 2008). This may provide initial explanation for the strain effect seen only in Occ.

The most likely explanation for the effects seen in the rats in this experiment is a reduction in the action of neprilysin, IDE and/or the NPR-C clearance receptor, allowing for higher concentrations of CNP to be present. Temporal changes to CNP which are not sustained suggest that this increased concentration may coincide with a developing cascade of changes that underlie longer term plasticity seen during enrichment. Implications of this are discussed further below (see general discussion).

Experiment Two - Bow-tie maze

Experiment two was undertaken to investigate potential changes to CNP under a "formal" learning task of object recognition.

Methods

Subjects

Subjects were 36 male PVGc hooded rats, bred in the Psychology Department at the University of Canterbury. All rats were housed in groups of four in standard opaque plastic cages (27 cm x 45 cm x 22 cm high). The colony room was maintained at 22°C and 48% rH under a reversed 12-

hour light cycle (lights off 0800h to 2000h). Behavioural testing occurred during the dark phase of the cycle. All rats were food deprived to 85% of their free feeding weight prior to training and maintained at 85-90% of free feeding weight during the testing phase. Water was available *ad libitum*. All rats were between 8 and 9 months old at the start of training and weighed between 292.8 grams and 363.4 grams. At the end of testing, animals were between 9 and 10 months old and weighed between 308.6 grams and 378.9 grams.

Thirty six rats were assigned to one of three groups, 'group novel,' or 'group familiar' (see below: behavioural procedure) – given different training on the bow-tie task - and a third group of 12 animals which provided home cage controls. The home cage control group provided evidence on CNP changes due to food-deprivation and exposure to the novel experimental room. All protocols conformed to the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Animal Ethics Committee of the University of Canterbury.

Bow-tie Maze

The bow-tie maze consisted of two triangular sections joined at their apex by a short central corridor (Figure 8). This apparatus consisted of a wooden base and aluminium walls. The base was painted grey with enamel based paint to allow cleaning of the maze between rats. The central corridor was 12 cm wide and 20 cm long. An opaque guillotine door (also painted grey) in the centre of this corridor was controlled by the experimenter. Two recessed food wells were present at each end of the apparatus (four wells total), and measured 3.5cm in diameter and 2 cm deep. The wells were 25 cm apart and separated by an opaque dividing wall, protruding 15 cm from the centre of the end wall of the maze (see Figure 8; Adapted from Albasser, Poirier & Aggleton, 2010).

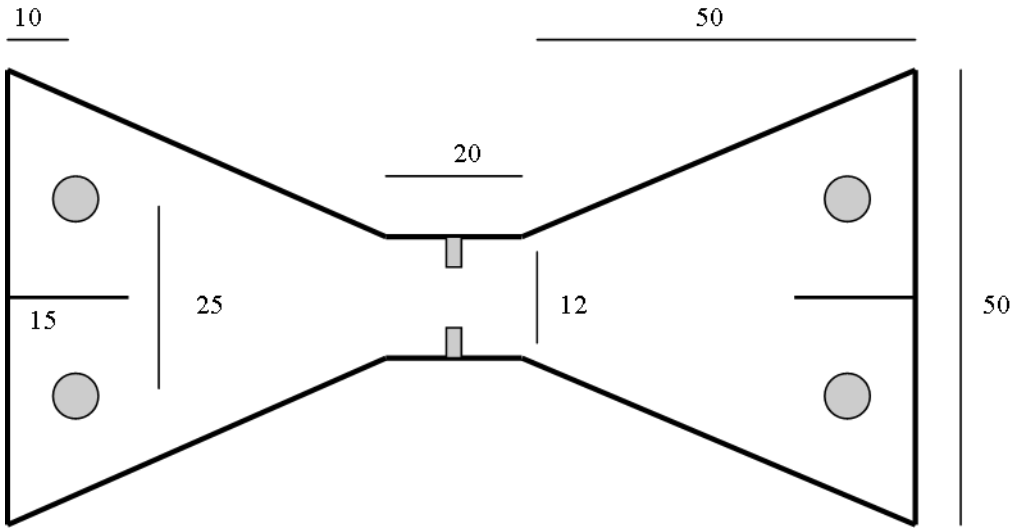


Figure 8: Dimensions of bow-tie maze (cm). Adapted from Albasser, Poirier & Aggleton (2010).

Behavioural Procedure

Pre-training

Pre-training occurred across eight days for the group novel and group familiar rats. The control group received no behavioural training but were exposed to the experiment room for the same amount of time each day as the two trained groups.

On day one, pairs of animals were placed in the bow-tie maze for 20 minutes and allowed to explore freely. Chocolate chips were scattered on the floor and in food wells. On days two and three, single rats were placed in the maze for 10 minutes each and allowed to explore freely. Chocolate chips were now restricted to the food wells only. Control of movement back and forth in the maze by the central door was introduced on days four and five. Single rats were placed in the maze for 10 minutes with a single piece of chocolate in each food well. The door was opened every minute for a maximum of 20 seconds. If the rat did not go through the door, it was closed until a further minute had passed. Food wells were re-baited for the course of the ten minutes

provided the rat successfully shuttled and gained the reward. Objects were introduced on day six. Three pairs of objects were used, which were not included in later testing. Objects were placed behind the food well on day six, and progressively moved forward until they covered the well over the course of days seven and eight. Training sessions on these days lasted 10 minutes. By the end of training on day eight, all rats were able to shuttle back and forth and displace objects to gain the food reward.

Testing

Testing started on day nine, for nine days. Again, control animals were exposed to the experiment room for the same length of time as the two trained groups. For trained groups, rats were placed singly at one end of the maze with one object covering a food reward (object A) and allowed to explore for one minute. The guillotine door was then opened to allow the rat to enter the other side of the maze where it had free choice between object A (now familiar) and object B (novel – trial one). Note that all pairs of objects each covered a food well with a reward to encourage approach to either object, and that the behavioural test of recognition relies on the difference of exploration between novel and familiar objects. After a further minute, the rat was allowed to go back to the other side of the maze where it found object B (now familiar) and object C (novel – trial two). This continued for 20 trials until the end of the session and so used 21 pairs of objects (see Figure 9 for visual representation). The critical difference between the two training groups was that 'group novel' was exposed to a new set of 21 objects each day (for nine days, total 189 different objects), whereas 'group familiar' was exposed to the same set of 21 objects every day for the nine days of testing. Object order and placement was varied pseudo-randomly within a session, between rats, based on placement sequences for discrimination tasks outlined by Fellows (1976). Note that for the final session (session 9) the same set of objects was used for all rats in

both ‘group novel’ and ‘group familiar’. This procedure in session 9 was to control for any effects exposure to these objects themselves may have on CNP. For ‘group familiar’ the order of presentation of objects on session 9 was matched to a rat from ‘group novel,’ in order to control for whether CNP was altered merely by exposure the objects presented.

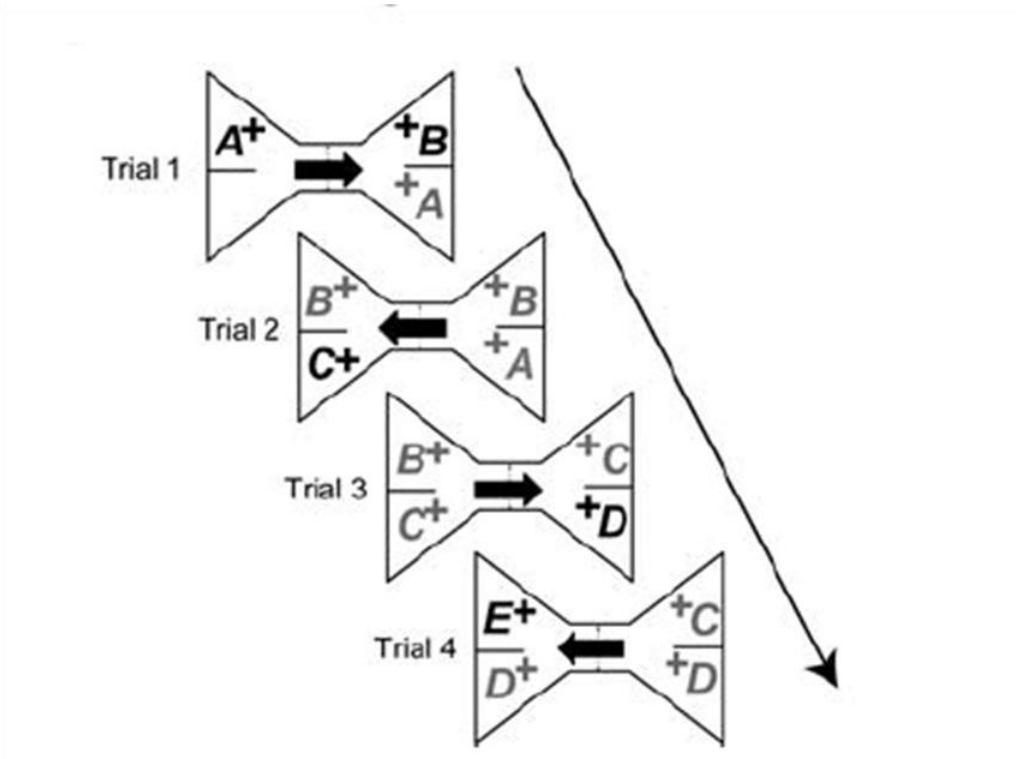


Figure 9: Diagram showing procedure for object recognition task in bow-tie maze. All objects are rewarded (+). Rat movements are shown by arrows. Black letters are novel objects, gray are familiar objects. Adapted from Albasser et al. (2010).

Sacrifice and Tissue Extraction

Procedures were as in the EE study. The sole difference lay in the order of animal sacrifice and tissue extraction. Sacrifice and extraction in this case occurred in the same order as testing, to ensure that each animal was sacrificed with the same time delay from the final testing session (five hours). Three sequential cohorts of rats were used with the start of testing staggered over time, with four rats from each behavioural group being included in each. Cohorts, extraction

order, and hemisphere first approached for tissue extractions are summarised in Table 3 (see Appendix).

Behaviour measures

Exploration time for each object was quantified by timing exploration of the novel and familiar objects using a computer. 'Exploration' includes the rat placing forepaws on the object or directing the nose at the object at a distance of <1cm with the vibrissae twitching; sitting on the object, using the object to rear up or chewing the object were not included as exploration.

As previously mentioned, the critical measure is the difference in exploration time between novel and familiar objects in a given trial. This difference is D1. A ratio of exploration time is preferable to compensate for any differences in absolute exploration time, which is called D2, calculated by taking the difference in time spent exploring the novel rather than the familiar object for one trial (i.e. D1) and dividing by the total exploration time within that trial. D2 ranges between 1 and -1, with positive numbers indicating a preference for the novel object. Cumulative D1 and D2 were calculated with each additional trial. The final measure of performance was overall D2 for each day of testing, with D2 on day 9 (when rats were sacrificed) being a critical measure.

The object rats first approached in each trial was also recorded. This 'first choice preference' indicates on how many trials the animal selected the novel object, expressed as a percentage.

Results

Behaviour

A 2 x 9 (Group x Day) Repeated-Measure ANOVA found no differences between 'group novel' and 'group familiar' in terms of average total exploration time (main effect of Group; $F<1$).Total

exploration time reduced over time in both groups (main effect of Day; $F(8, 176) = 5.41$, $p<.001$) and was significantly different between groups on day 8 (group novel M = 158.11sec, SD = 31.48; group familiar M = 122.57sec, SD = 38.89; $t(22) = 2.46$, $p = .022$). Independent means t-test revealed no differences between the groups in terms of average first-choice preference for objects (group novel M = 48.87%, SD = 2.85; group familiar M = 50.56%, SD = 3.70; $t(22) = -1.25$, $p = .224$). Furthermore, single sample t-tests revealed that neither group performed differently to chance (50%) in selecting novel objects first (group novel: $t(11) = -1.37$, $p = .197$; group familiar: $t(11) = 0.52$, $p = .611$). These measures are not considered further.

Although groups did not differ significantly overall on total exploration time, this did differ significantly on day 8 (see above). Figure 10A shows mean D1 (±SE) across nine days of testing. However, D2 was used preferentially for subsequent analyses as the ratio controls for differences in exploration time. A 2 x 9 (Group x Day) Repeated Measure ANOVA showed that group novel had a greater preference for novel objects than group familiar (effect of Group: $F(1, 176) = 19.67$, $p < .001$; Figure 10B). The effects of Day, and the interaction effect (Day x Group) were not significant (F's <1.61, p's $> .124$). A comparison of D2 between groups on day 9 (day of sacrifice for CNP analysis) showed that group novel had a significantly higher novelty preference on this day (independent means t-test; $t(22) = 2.56$, $p = .018$). Group novel's significant preference for exploring novel objects was evident by day three of testing and continued through day six (all t's > 2.26, all p's $<.034$).

CNP and NTproCNP.

Analysis in all regions was conducted using a one-way ANCOVA (covariate: animal weight), with the left and right hemispheres of the hippocampus run as a repeated measure for that region. No effect was found in the overall ANCOVAs for any region in CNP concentrations (all F's <

2.03, all p's > .149; Figure 11A) or NTproCNP concentrations (all F's < 1.43, all p's > .254), although in mPFC this approached significance with a higher NTproCNP concentration in group novel ($F(2,32) = 2.79$, $p = .077$; Figure 11B). Furthermore, no effects were found based on the NTproCNP/CNP ratio (all F's < 1.20, all p's > .314), although in MB there was a reduced ratio in group novel and the overall ANCOVA approached significance ($F(2, 32) = 2.90$, $p = .071$).

A priori it was hypothesised that there may be differences between group novel and pooled group familiar and the untrained control group. In mPFC a higher concentration of CNP and NTproCNP was evident in group novel and this was significant when compared with the pooled data from the other two groups (CNP: $M_{novel} = 677.42$ fmol/g, $SD_{novel} = 363.51$; $M_{controls} = 485.53$ fmol/g, $SD_{controls} = 157.85$; $t(34) = 2.22$, $p = .033$; NTproCNP: $M_{novel} = 2282.39$ fmol/g, $SD_{novel} = 1108.53$; $M_{controls} = 1680.85$, $SD_{controls} = 376.91$; $t(34) = 2.42$, $p = .021$).

In Rgb and MB regions, contrary to the hypothesis, CNP concentrations were not changed in group familiar. CNP concentrations appeared to be somewhat lower in group familiar than both other groups (see Figure 11A). No pair-wise comparisons of CNP concentrations reached significance for Rgb or MB regions, although in Rgb a comparison between group familiar and the untrained control group tended towards significance ($F(1, 32) = 3.28$, $p = .079$).

Concentrations of NTproCNP also appeared to be lower in group familiar than both other groups in Rgb and MB. Again, pair-wise comparisons were non-significant (all F's < 2.16, all p's > .151). However, the NTproCNP/CNP ratio comparison between group novel and group familiar suggested that there was less degradation of CNP in MB region for group familiar compared to both other groups (familiar versus novel: $F(1, 30) = 4.65$, $p = .039$; familiar versus control: $F(1, 30) = 3.57$, $p = .068$; see Figure 11C).

No differences were evident in the hippocampus for comparisons between group novel and pooled group familiar and control (all F's < 2.31, all p's > .138) or any other pair-wise

comparison (all F's < 1.48, all p's >.113). In HTh, mean CNP and NTproCNP concentrations and the NTproCNP/CNP ratio were higher in group familiar, but no pair-wise comparisons between group familiar and either group novel or the untrained control group were significant (all F's < 2.61, all p's > .145). No differences were found in Occ (group novel versus pooled controls: all t's < .80, p's > .428; pair-wise comparisons all F's <.50, all p's > .483).

Correlations between D2 and CNP/NTproCNP.

Correlations between final D2 and CNP and NTproCNP concentrations were calculated for both trained groups (novel and familiar) separately, and in combination (see Table 7 for summary correlations). No significant relationships were found between CNP or NTproCNP and D2 for group novel. Although these correlations were non-significant, all suggested that with greater novelty discrimination (higher D2), CNP and NTproCNP concentrations reduced. This same trend was found for group familiar, and in Rgb and Occ regions, where these correlations were statistically significant, and tending towards significance in mPFC (see Table1). Although the same trend was evident in MB, the hippocampus and HTh, only one correlation reached significance (HpcRH NTproCNP concentrations; see Table 1).Overall, with a greater preference for the novel of a pair of objects, CNP and NTproCNP concentrations were decreased, but this was particularly evident in group familiar.

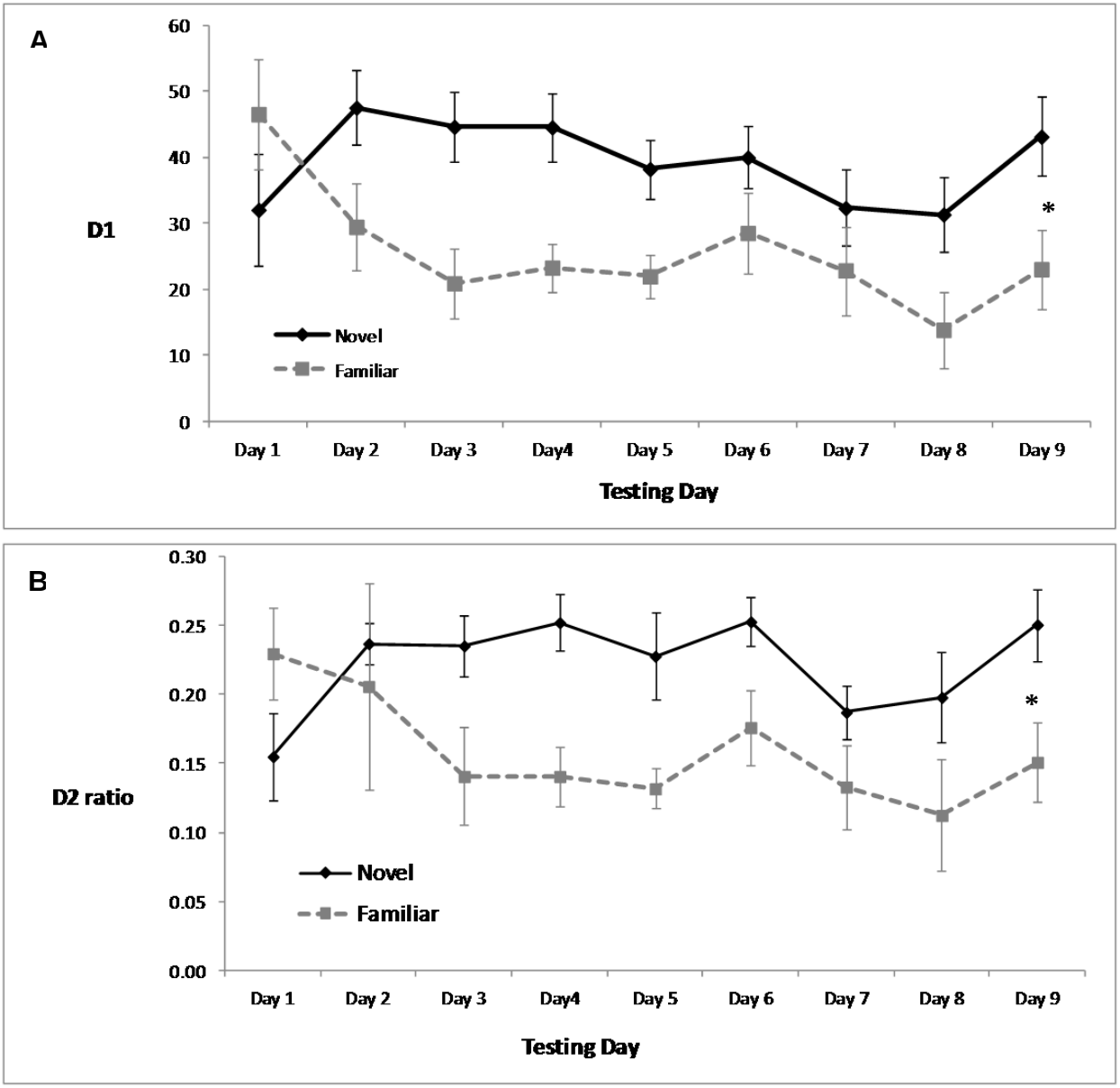


Figure 10: Exploration performance of rats in the bow-tie maze. A - Mean (±SE) D1 for group novel versus group familiar across nine days of testing. D1 is the exploration time of familiar objects subtracted from exploration time of novel objects, with greater numbers indicating novelty preference. B – Mean (±SE) D2 ratio for group novel versus group familiar across nine days of testing. D2 ratio is calculated by dividing D1 by total exploration time with positive values indicating novelty preference. Significantly different discrimination performance on day 9 is indicated by asterisk.

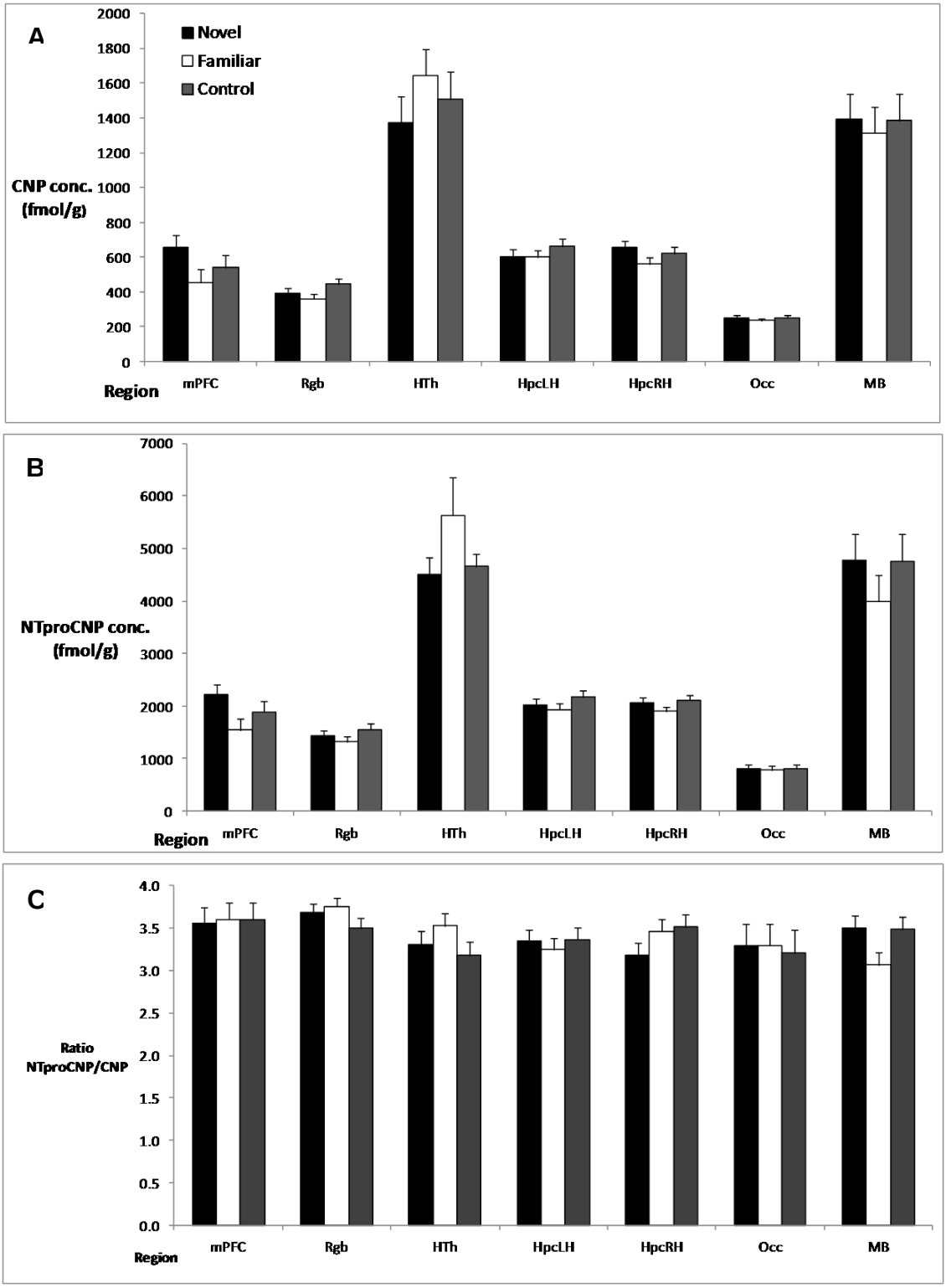


Figure 11: Bow-tie maze data. A - Least squares mean (+SE) CNP concentration. B - Least squares mean (+SE) NTproCNP concentrations. All concentrations expressed as femtomoles per gram (fmol/g) of risse. C – Least squares mean (+SE) ratio of NTproCNP to CNP concentrations. Lower ratios indicate lower degradation. Key – Novel: group novel; Familiar: group familiar; Control: untrained control group.

Table 1: Correlations of NTproCNP and CNP concentrations with final D2 for each region sampled. Significant correlations are indicated by asterisk, with p-values in brackets. Correlations approaching significance are highlighted in gray.

	mPFC	Rgb	HTh	HpcLH	HpcRH	Occ	MB
Groups combined							
CNP	-.025	-.384	-.335	-.382	-.093	***-.449**	-.357
	(.912)	(.078)	(.128)	(.079)	(.682)	**(.036)**	(.103)
NTproCNP	.014	-.340	-.283	-.316	-.249	-.324	-.230
	(.950)	(.122)	(.202)	(.151)	(.264)	(.122)	(.303)
Group Novel							
CNP	-.013	-.300	-.068	-.345	-.109	-.477	-.418
	(.971)	(.370)	(.843)	(.299)	(.751)	(.138)	(.201)
NTproCNP	.008	-.124	-.070	-.384	-.191	-.115	-.335
	(.981)	(.715)	(.839)	(.243)	(.574)	(.736)	(.314)
Group Familiar							
CNP	-.541	***-.607**	-.357	-.516	-.477	***-.667**	-.457
	(.086)	**(.048)**	(.281)	(.105)	(.138)	**(.025)**	(.281)
NTproCNP	-.540	***-.635**	-.230	-.319	***-.652**	***-.623**	-.438
	(.087)	**(.036)**	(.497)	(.338)	**(.030)**	**(.041)**	(.178)

Summary – Bow-tie Maze

Behavioural results indicated that group novel successfully discriminated novel objects from familiar. Although group familiar still demonstrated a preference for a novel object of a pair on average (positive D2), the more important issue is a clear group difference on day 9 when brain regions were harvested.

There was a clear difference in CNP in mPFC region in group novel, compared to the aggregated controls, and this was attributable to increases in production of the protein. In Rgb and MB regions, there was a tendency for a lower level of CNP in group familiar compared to the other two groups, although these trends were not statistically significant. Interestingly, the lower ratio of NTproCNP/CNP in MB region indicated that there is a reduced degradation of CNP in this region during this task. No differences were found between any groups in the hippocampus or Occ.

Relationships between D2 and CNP or NTproCNP concentrations revealed that, throughout the brain there was a tendency for a decrease of CNP with higher levels of discrimination between novel and familiar objects. This relationship was much weaker (and non-significant) in group novel than group familiar. This suggests that CNP may be of importance in recency aspects of recognition memory (group familiar) as opposed to novel object recognition per se. In particular, there may be a greater potential for plasticity in these regions when object discrimination is low (see general discussion – below).

General Discussion

The two experiments conducted herein have provided some evidence that CNP may play a role in learning and memory and the plastic processes by which these occur. Environmental enrichment which provides an "informal" learning environment for rats produced widespread changes in CNP, owing to a decrease in degradation activity after 14 days in the highly stimulating environment. In contrast to this, training on the bow-tie maze did not result in widespread changes in CNP. An increase in CNP was evident in mPFC region only in group novel, and this was due to increased production of the protein. Group familiar had a somewhat reduced concentration of CNP and NTproCNP in Rgb and MB, although these differences were not supported by statistical tests. However, there was relatively less degradation of CNP in MB in group familiar, as evidenced by significantly lower NTproCNP/CNP ratios. No differences were found for the hippocampus, HTh or Occ. One conclusion from the bow-tie task was that with greater discrimination for novel objects from familiar objects, CNP and NTproCNP concentrations had a tendency to decrease, and this was more evident in group familiar. Most notable was that in the bow-tie task, any changes that did occur were attributable to changes in production of CNP, whereas under EE conditions, changes in the 14 day group were due to

reduced degradative actions on CNP. Most likely this latter effect was due to a lessening of the activity of neprilysin, Insulin-degrading enzyme or the NPR-C clearance receptor.

Lazarov and colleagues (2005) demonstrated that neprilysin activity was increased in enriched transgenic mice expressing dysfunctional amyloid-β protein. However, it should be noted that their studies housed mice in enrichment for five months, whereas the results here suggest a reduction in neprilysin activity primarily at two weeks of enrichment, with a return to activity comparable to standard animals by 28 days of enrichment. It cannot, however, be excluded that further temporal effects may have occurred with longer periods of enrichment. Additionally, the dysfunction of amyloid-β protein in the transgenic mice in their study may also affect the function of neprilysin activity. For example, neprilysin is reduced in brain regions susceptible to amyloid plaque formations, and chemical inhibition of neprilysin results in an increase in plaque formation (e.g. see Marr et al., 2003). Rats used in this study had no known or obvious pathologies, and could not yet be considered aged as they were between eight and nine months old. An increase in neprilysin under conditions of dysfunction in amyloid-β would be beneficial to amyloid degradation. Whereas in this study, the reduction in activity resulting in increased CNP concentrations presumably results in some benefit under normative neurological conditions.

A deficiency in neprilysin has also been suggested to have beneficial cognitive effects in aged mice (Walther et al., 2009). In this study, mice with a knockout for the gene encoding neprilysin at 21 months of age performed better than wild-type mice in the Morris water maze, indicated by a shorter latency to find a hidden platform and were no different in terms of their cognitive performance compared to 6-month old mice. Wild-type aged mice had a greater latency to find the platform when compared to young mice and those mice deficient in neprilysin. The authors themselves suggest that the better cognitive performance of the aged mice deficient in

neprilysin may be due to increases in various other neuropeptides degraded by neprilysin which have been shown to improve learning and memory (e.g. oxytocin, neuropeptide-Y and cholecystokinin; Walther et al., 2009). Although not mentioned by those authors, CNP is also degraded by neprilysin and it may participate similarly to the neuropeptides referred to in cognitive improvements seen after exposure to enrichment. This point is interesting to consider given the speculation from the current study of a possible reduction in neprilysin activity at two weeks of enrichment. It may be that a transient reduction in enzyme activity allows for higher concentrations of CNP to be present and subsequently stimulate longer term processes associated with plasticity and cognitive improvements. The comparison with Walther et al.'s findings also elicits intriguing suppositions about how CNP may change in normal and degenerative aging. If a reduction in neprilysin is responsible for the increases seen in CNP concentrations in this study, then it may be that the greater availability of CNP contributes to these cognitive improvements. As a next step, investigating whether similar results can be found in aged rats may provide further support to a role for CNP in learning and memory improvements. However, the changes in CNP that have been shown with exposure to EE would also need to be related to some kind of improvement in learning or memory.

This last comment unfortunately highlights a limitation of the current study. Although widespread changes to CNP concentrations at 14 days of enrichment were observeds, this has not been linked to any cognitive benefits in these rats compared to those housed in standard cages, or in a longer period of enrichment. Further, the CNP changes were not specifically linked to any potential difference in neuroplasticity in the regions investigated. As such, proposed explanations are merely postulations. The indication that there is in fact a difference in CNP concentrations under these conditions suggests that further research to identify such connections is warranted.

Previous work has shown that two weeks of enrichment is sufficient to improve cognitive performance in intact animals (e.g. Tang et al., 2001; Frick & Fernandez, 2003; Bruel-Jungerman et al., 2005) and ameliorate cognitive deficits in animals with neurological insults (e.g. Passineau et al, 2001; Wagner et al., 2002; Hicks et al., 2002). Further, two weeks is also an adequate length of enrichment to stimulate alterations in response strength and response threshold in cortical neurons (e.g. Engineer et al., 2004), neural progenitor cell mobilisation (Magalon et al., 2007), and to upregulate multiple genes associated with neuroplasticity in the hippocampus (Keyvani et al., 2004). The changes in CNP concentrations seen at the same time point encourages further investigation into possible associations with improvements in cognitive performance and changes in plastic processes evident in previous studies using EE.

Both LTP and LTD are modified in the hippocampus after housing in enriched conditions (e.g. Duffy et al., 2001; Foster & Dumas, 2001; Artola et al., 2006). In the earlier two examples, LTP occurred at lower stimulus frequencies than that required in non-enriched animals (Duffy et al., 2001; Foster & Dumas, 2001). The latter study also demonstrated enhancement of LTD (Artola et al., 2006). Our finding that there is a higher concentration of CNP available in the hippocampus at 14 days of enrichment is interesting to consider alongside these studies. CNP has been demonstrated *in vitro* to modify hippocampal plasticity in a similar fashion. CNP increased the threshold for LTP to occur, and facilitated LTD (Decker et al., 2009; 2010). However, under enrichment conditions, modifications in LTP and LTD apparently did not occur by two weeks, although data for this time period were not published (see Duffy et al., 2001), and the minimum time period for enrichment in these studies was 25 days (Foster & Dumas, 2001). The higher CNP concentrations at 14 days in the current study may coincide with the developing cascade of neuroplasticity over a longer term. However, these increases were more evident in non-hippocampal regions so the connection with hippocampal LTP/LTD requires further study.

Perhaps increased CNP at 14 but not 28 days suggests CNP's involvement may only be at the initial stages of a more widespread neuroplasticity cascade. CNP concentrations may not continue to change subsequent to 28 days of enrichment; but future study would be needed to examine this. Subsequent research could be conducted to measure CNP concentrations more frequently, and over longer time courses of enrichment, producing a "time-response" curve. Additionally, the finding of increased concentration in CNP at 14 days of enrichment indicates that further research connecting this change directly with changes in plasticity and behaviour is required.

The medial prefrontal cortex exhibited the most consistent results across both experiments. In the bow-tie task, group novel exhibited higher concentrations of CNP than the two control groups (group familiar and the untrained). Unlike the EE experiment, however, changes in CNP in mPFC associated with the bow-tie task were a result of increased production of the protein. The medial prefrontal cortex is generally thought to be responsible for processes associated with strategy memory encoding and retrieval (Aggleton & Brown, 1999; 2006; McDermott, Jones, Petersen, Lageman & Roedigerl, 2000). In terms of recognition it is thought to be more important for familiarity and recency than novelty discrimination (Aggleton & Brown, 2006; Barker, Bird, Alexander & Warburton, 2007). However, our results indicate that CNP production in mPFC is increased during novel object discrimination. If the suggestion is correct that CNP is associated with modulation in plasticity processes, this result indicates an increase in the potential for plasticity in mPFC over repeated presentations of novel objects. The most likely explanation, given previous research disseminating the role of mPFC in recognition memory, is that as more objects are presented, more information must be encoded, and potentially retrieved in the future, requiring greater potential for LTP/LTD in this region. If CNP is involved in adaptations in neuroplasticity as proposed, and suggested by results of the EE study,

enhancements of these functions in mPFC (i.e. alterations in LTP/LTD) may contribute to novel object recognition.

A surprising finding of the bow-tie experiment was that in group familiar, higher CNP production was generally associated with lower levels of discrimination in mPFC. This same result was found in the Rgb and Occ. Retrosplenial cortex lesions have been widely demonstrated not to effect novel object recognition (Steckler et al., 1998b) and as such research into its function in the neurological system responsible for memory has focussed on the contribution to spatial/episodic type memory (see Harker & Whishaw, 2004 for review) Occipital cortex is one of the earliest regions for visual stimulus perception (e.g. see Steckler et al., 1998b). The negative relationship between D2 and CNP in these regions may have a similar explanation to the increased concentrations of CNP in mPFC in group novel: if higher levels of CNP are associated with greater neuroplasticity potential, then as repeated exposure to the objects occurs and memory of them is consolidated (thus they are more familiar and discrimination is less) that the potential for future plastic changes is increased in these regions. In other words, if the objects are less familiar (higher D2), these regions have less plastic potential, and are therefore not engaged in novelty discrimination. These propositions support previous work for 'non-involvement' of these regions in object recognition, but expand previous work by suggesting that these regions are not necessarily 'non-involved' but rather there is a 'disengagement' of these neural processes in these regions.

The method used for the bow-tie maze in this experiment closely replicated that used in Albasser, Poirier & Aggleton (2010). However, several differences between this study and theirs should be noted, and may be responsible for some of the limitations in the results gained. Firstly, all group novel objects were novel across nine sets of 21 objects, whereas in Albasser et al., six sets of 21 objects were used and repeated across two blocks of six test sessions, with a

completely novel set of objects for a thirteenth session of testing. The modification used in this study therefore provides a truly trial-unique object recognition paradigm and as such, direct comparisons from their study to ours in terms of group novel may be limited. It is possible that upon the second repeat of an object in their study, rats were better able to distinguish the novelty of it due to a mismatch from a previous pairing, allowing for others strategies besides novelty discrimination to come into play.

Additionally, Albasser et al's animals received two testing sessions per day. Steckler et al. (1998b) note that in terms of object recognition, increased exposure to objects allows for better recognition of their familiarity. Especially in terms of group familiar, in Aggleton et al. this group was exposed to more test sessions (13 compared with nine in the current study), and hence more exposures to the familiar objects in a contracted amount of time (seven days compared to nine). This could explain the D2 for their group familiar falling to chance levels rapidly (see. Aggleton et al., 2010; novel behavioural protocols) – the time between testing sessions, and hence exposure to the 'familiar' objects, was much shorter than that employed in this study. As a result, it could be that the animals in group familiar for this study were still demonstrating sufficient discrimination between objects that this condition did not provide a stark enough contrast in discrimination, compared with group novel, for differences in CNP and NTproCNP between the conditions to emerge in regions other than mPFC. For example, differences in CNP and NTproCNP concentrations were observed between groups in the mamillary bodies and the hypothalamus, although statistical comparisons did not reach significance. Perhaps a greater contrast in discrimination between group novel and group familiar would lead to greater differences in CNP and NTproCNP concentrations, allowing further hypotheses to be proposed regarding CNPs involvement in learning and memory. This idea is also supported by the evidence that, in the MB degradation of CNP was decreased. This suggests there is a change in

CNP activity in this region, but restricted differences in behavioural performance between differently trained groups may have limited the capability to observe concentration differences in the MB region. Finally, the use of a different strain of rats to Albasser et al. (who used Dark Agouti rats) may have restricted behavioural effects. It has been demonstrated previously that PVGc rats have poorer performance in DNMS paradigms compared to Dark Agouti rats (Aggleton, 1996) and this may have influenced the smaller differences in D2 seen in this study when compared to Albasser et al. (2010).

Other studies using the bow-tie note that the hippocampus is recruited during this task (Aggleton et al., 2010; Albasser, Poirier & Aggleton, 2010; Horne et al., 2010). Although largely a non-spatial task, objects are presented in either the same or displaced position on their second presentation according to a pseudorandom schedule. Albasser et al. (2010) note this brief judgement may be sufficient to activate the hippocampus – most commonly found to be involved in tasks with a spatial component in animals (e.g. see Aggleton & Brown, 1999; 2006; Kesner & Hopkins, 2006; Squire, 2007; Clark & Squire 2010). While no differences were found in CNP in the hippocampus in this study, the relationship between greater object discrimination and lower CNP concentrations (although not statistically significant) was present, and this relationship was stronger in group familiar. The correlations suggest that there may be a similar action in the hippocampus to that seen in mPFC, Rgb and Occ during recency judgements, of a 'disengagement' of plastic processes here as memory is consolidated.

Results in the hippocampus may have been limited however, by potential for sub-regional differences. Albasser et al. were able to analyse sub- regional differences in activation (using c-fos) in CA1, CA3 and DG of hippocampus subsequent to training on the bow-tie task. Relative amounts of c-fos were higher in group novel in CA1 and CA3, but counts were increased in DG in group familiar. It is plausible that any sub-regional differences such as these in CNP may have

'averaged out' to render the null results seen in this study. This may also explain the weaker results in the hippocampus in the EE experiment. While all care was taken to ensure tissue was taken from the same regions in each animal, and across hemispheres in individual animals, if there were sub-regional differences, varied capture of those regions could also lead to the weaker effects seen in the hippocampus generally.

Similar issues of sub-regional differences may have limited results in MB in particular. The mammillary bodies have long been associated with memory, with degeneration of the region seen consistently in Korsakoff's amnesia in particular. It has been recently suggested that the issues with resolving a definitive role for the mammillary bodies in mnemonic processes may be that different nuclei make different contributions (Vann & Aggleton, 2004). The samples taken from MB in this study have inevitably included tissue from both the medial and lateral mammillary nuclei, and the proportion of each nuclei sampled may have varied between rats. CNP may be differentially expressed in each nucleus. However, reduced degradation of CNP was found in group familiar. CNP changes may occur in at least one of the nuclei during object recognition, particularly in that engaging recency decisions. This suggests, as others have, that the mammillary bodies have a part to play in memory as a whole, but unfortunately suffers from similar limitations to previous research, that the main nuclei may act independently.

By increasing the sample size in a future study, it may be possible to examine, the data for CNP in each experiment using structural equation modelling to further explore relationships across the regions sampled. Structural equation modelling allows the complex analysis of multiple related variables as well as comparison between differing proposed models of the relationships between said variables. Neural connections between regions of the brain are now quite well understood, and as such can provide an underlying model for analysis of relationships between CNP concentrations in these regions. Beyond differences found in a single region of

interest in CNP concentrations, SEM could allow investigation into relationships in CNP throughout the network as a whole. In particular, a comparison of differing expression of CNP for group novel versus group familiar, given some of the differences between these two groups in correlations with D2, may shed further light on CNPs activity associated with object recognition. Albasser et al. (2010) have already applied this technique to demonstrate differing engagement of various regions between group novel and group familiar in the context of c-fos changes.

Also of interest will be research investigating CNP during tasks thought to reflect different types of memory. For example, with the possible connection of changes in CNP to Alzheimer's disease pathology due to its degradation by neprilysin, it would be of value to study CNP concentrations during tasks that represent other aspects of episodic memory. Severe deficits in episodic memory are characteristic of Alzheimer's disease (e.g. Cassel et al., 2007) and thus, it would be of benefit to investigate CNP changes subsequent to a task with spatial components, thought to be better representative of episodic memory in animal models (e.g. Morris Water Maze, Radial-arm maze).

Concluding Remarks

Results of the two different studies support the proposal that, in the intact brain, CNP has some role in learning and memory processes. A consistent pattern was found in all regions of interest in the EE experiment, with transiently higher levels of CNP evident after 14 days of enriched housing, and that this was due to alterations in degradation of CNP. Further, evidence from explicit memory training using the bow-tie maze also suggest that CNP is altered during learning in the intact brain, although findings were far less dramatic than those seen during exposure to enrichment. The suggestion arising from the bow-tie study is that CNP production is altered

during a formal object recognition task and that engagement of CNP may be more important during judgements of recency when objects are familiar, rather than in novel object recognition.

Several suggestions for ongoing research have already been outlined. Subsequent to the EE study an investigation of potential changes to CNP during both normal and degenerative aging and an attempt to connect CNP changes to changes in plasticity *in vivo* as opposed to *in vitro* is proposed. Following from the bow-tie study, experiments using different paradigms representative of episodic memory will be of interest to investigate whether CNP may be expressed differently during other memory tasks. To investigate whether CNP changes might be responsible for differences in behaviour, future research should also examine the administration or inhibition of the protein and subsequent effects on tasks such as the bow-tie maze are recommended.

Overall, this study has provided initial evidence that CNP has some association with learning and memory. Furthermore, these data constitute the first measures of CNP and its stable by-product, NTproCNP in intact rat brain, subsequent to behavioural manipulations. Although continued investigation is needed, CNP may to be an early marker in a neurological cascade responsible for neuroplasticity processes underlying learning and memory.

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Appendix

Table 2: Order of extraction for animals in 14 and 28 days of enrichmed or standard housing including hemisphere approached first for tissue extraction. Note: Animal P1 was subsequently excluded due to issues in tissue processing.

Animal	Extraction order	1st hemisphere	Condition
R	1	Left	EE-14
G	2	Right	EE-14
P7	3	Left	SH-14
B	4	Right	EE-14
R7	5	Left	SH-14
P	6	Right	EE-14
R5	7	Left	SH-14
R1	8	Right	EE-14
G1	9	Left	EE-14
B1	10	Right	EE-14
P1	11	Left	EE-14
G5	12	Right	SH-14
R2	13	Left	EE-14
B5	14	Right	SH-14
R6	15	Left	EE-14
P5	16	Right	SH-14
B6	17	Left	EE-14
B8	18	Right	EE-14
Final group N	**EE-14 = 11**	**SH-14 = 6**	
G2	1	Left	EE-28
B2	2	Right	EE-28
B4	3	Left	SH-28
P2	4	Right	EE-28
P4	5	Left	SH-28
R3	6	Right	EE-28
G7	7	Left	SH-28
G3	8	Right	EE-28
B3	9	Left	EE-28
P3	10	Right	EE-28
R4	11	Left	EE-28
B7	12	Right	SH-28
G4	13	Left	EE-28
P6	14	Right	SH-28
G6	15	Left	EE-28
R8	16	Right	SH-28
G8	17	Left	EE-28
P8	18	Right	EE-28
Final group N	**EE-28 = 12**	**SH-28 = 6**	

Table 3: Order of extraction for animals in bow-tie maze experiment, including hemisphere approached first for tissue extraction, behavioural training received, and training cohort.

Animal	Extraction order	1st hemisphere	Group	Cohort
3RED	1	Left	Group Novel	1
3GREEN	2	Right	Group Novel	1
3BLUE	3	Left	Group Novel	1
3PURPLE	4	Right	Group Novel	1
5RED	5	Left	Control	1
5GREEN	6	Right	Control	1
5BLUE	7	Left	Control	1
5PURPLE	8	Right	Control	1
4RED	9	Left	Group Familiar	1
4GREEN	10	Right	Group Familiar	1
4BLUE	11	Left	Group Familiar	1
4PURPLE	12	Right	Group Familiar	1
6RED	1	Left	Group Novel	2
6GREEN	2	Left	Group Novel	2
6BLUE	3	Right	Group Novel	2
6PURPLE	4	Right	Group Novel	2
8RED	5	Left	Control	2
8GREEN	6	Right	Control	2
8BLUE	7	Left	Control	2
8PURPLE	8	Right	Control	2
7RED	9	Left	Group Familiar	2
7GREEN	10	Right	Group Familiar	2
7BLUE	11	Left	Group Familiar	2
7PURPLE	12	Right	Group Familiar	2
9RED	1	Left	Group Novel	3
9GREEN	2	Right	Group Novel	3
9BLUE	3	Left	Group Novel	3
9PURPLE	4	Right	Group Novel	3
11RED	5	Left	Control	3
11GREEN	6	Right	Control	3
11BLUE	7	Left	Control	3
11PURPLE	8	Right	Control	3
10RED	9	Right	Group Familiar	3
10GREEN	10	Left	Group Familiar	3
10BLUE	11	Right	Group Familiar	3
10PURPLE	12	Left	Group Familiar	3