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Identification of C-type Natriuretic Peptide as a novel target in age-related cognitive decline

A thesis submitted in partial fulfillment of the requirements for the degree of

Doctorate of Philosophy

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by

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Dedication

To my Grandparents:

Harold Maitland Paterson, who sadly lost his memory, but inspired my questions.

Barbara Jean Paterson, who taught me determination in the face of struggle.

Donald James Rapley, who wanted a Doctor in the family, and I hope will now be satisfied.

Winifred Mary Rapley, who alone will see the conclusion of this work. You have aged as gracefully as anyone can, and still have a memory to rival us all. Given different opportunities, at a different time, a doctorate would surely have been yours. This one is dedicated to you, for all the opportunities you have given to me.

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I declare that the work described in the current thesis has been done by myself except where indicated.

All statistical analyses were conducted and figures created using the base ‘R’ statistical software and packages (below) recommended in Field, Miles & Field (2012).

R packages

- ggplot2
- pastecs
- WRS2
- car
- compute.es
- multcomp
- ez
- nlme
- reshape2

This thesis uses the *Journal of Neuroscience* referencing format

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Abbreviations

ABC-DAB – Avidin-Biotin Complex Diaminobenzidine immunohistochemistry
ADHD – Attention Deficit Hyperactivity Disorder
ANP – A-type Natriuretic Peptide
BDNF – Brain Derived Neurotrophic Factor
BNP – B-type Natriuretic Peptide
CA1 – *Cornu Ammonis* 1 region of hippocampus
CA3 – *Cornu Ammonis* 3 region of hippocampus
cGMP – Cyclic Guanosine Monophosphate
CNP – C-type Natriuretic Peptide
cpm – Counts per minute
CSF – Cerebrospinal Fluid
d2 – Discrimination ratio
DG – Dentate Gyrus region of hippocampus
EPM – Elevated Plus Maze
fmol - Femtomoles
GABA – γ-Aminobutyric Acid
HPA axis – Hypothalamo-Pituitary Adrenal axis
icv – Intracerebroventricular
ip – Intraperitoneal
LTD – Long Term Depression
LTP – Long Term Potentiation
mRNA – messenger Ribonucleic Acid
ng - Nanogram
NGF – Nerve Growth Factor
NMDA – N-methyl D-aspartic Acid
NP – Natriuretic Peptide
NPR-A – Natriuretic Peptide Receptor A
NPR-B – Natriuretic Peptide Receptor B
NPR-C – Natriuretic Peptide Receptor C
NPPC – Natriuretic Peptide Precursor C
NTproCNP – N-terminal pro C-type Natriuretic Peptide (bioinactive fragment)
OR – Novel Object Recognition
pmol – Picomoles
RAM – Radial Arm Maze
RIA - Radioimmunoassay
SLR – Spontaneous Location Recognition
SM – Small separation condition of SLR
XS – Extra small separation condition of SLR

Abstract

C-Type Natriuretic Peptide (CNP) is an important neuroendocrine regulator, suggested to be involved with cognitive decline and pathological aging. From the literature, and based on pilot work contributing to a Master's Thesis, three questions were identified for investigation regarding a putative involvement of CNP in age-related cognitive decline. 1) Do CNP and its related signalling system vary in cerebral tissue during normal brain aging? 2) Could an involvement of CNP activity be identified in relation to a rodent model of spatial/episodic-like learning and memory? 3) What effects would administration of CNP have on rodent models of working, object and location recognition memory? During investigation of this final question, rodents were identified as cognitively impaired.

Question one was addressed by housing aged rodents (23 months old) in an enriched environment, previously indicated as useful for modifying endogenous CNP expression and degradative regulation in young (8 to 9-month-old) rats. Response of CNP and related markers to this manipulation was markedly different in aged rats compared with young rodents. Data acquired indicated CNP synthesis and regulation to be dysfunctional within hippocampus and retrosplenial cortex during aging, identifying CNP as a novel target in age-related cognitive decline.

A study using a radial arm maze task of spatial reference memory was designed to address question two. For rodents learning a spatial reference task, CNP synthesis was increased in retrosplenial cortex and mammillary bodies during memory consolidation. In a second group, interference was introduced to the reference memory component by use of a forced-arm choice, yoked to rodents learning the spatial reference memory task. Increases in CNP synthesis were restricted to mammillary bodies in this group. Rodents in both behaviourally trained groups and a third control group (accounting for effects of environmental conditions) had reduced CNP concentrations in hippocampus compared with previous reports, suggesting an effect of food-deprivation on central CNP activity. Findings provided the first indication that endogenous CNP activity is modified during spatial/episodic-like memory formation, commonly impaired in cognitive disorders.

As working memory, object recognition and memory 'pattern separation' are impaired during cognitive decline, the final question was addressed by investigating effects of infused CNP in rodent models of each. A working memory task (radial arm maze) was negatively affected by behavioural outcomes of CNP infusion, resulting in inclusion of a

rodent model testing anxiety (elevated plus maze). Object recognition was examined using a standard novel-object recognition paradigm, with pattern separation tested using a recent adaptation of spontaneous location recognition (Bekinschtein et al., 2014). Hypothesising that CNP would increase anxiety, decrease locomotion and interfere with recognition memory in a dose-dependent manner, doses ranging from 200 to 1000ng were administered (icv) prior to each task in a repeated-measures design. Contrary to the hypothesis, locomotion increased across increasing doses of CNP, an effect that was attenuated across multiple tasks, as were anxiogenic effects of the peptide. In line with the hypothesis, CNP interfered with recognition memory dose dependently in a spontaneous location recognition task with low pattern separation load. However, recognition memory was facilitated when pattern separation load was high. Dose-dependent effects on recognition were no longer apparent during novel object recognition, but recognition (measured by discrimination ratio) was overall improved compared with previous tasks. Immunohistochemistry for cyclic Guanosine Monophosphate (cGMP; directly stimulated by CNP) provided supporting evidence for attenuation of behavioural effects across chronic administrations, and indicated bidirectional effects of CNP on memory may relate to variable cGMP stimulation in frontal cortical regions. Taken together, results of this study provide the first experimental evidence that CNP can variably affect memory acquisition, but may overall improve memory deficits within a rodent model of cognitive decline.

Several novel findings of this thesis present interesting implications regarding the role of CNP and its signalling system in relation to aging, memory and anxiety. Additionally, identification of dysregulation of this system during aging, partly accounted for by modifications to the Natriuretic Peptide C receptor, introduces theoretical considerations for Natriuretic Peptide research at large. Combined, investigations presented here point to a special relationship between CNP and retrosplenial cortex. This suggests CNP not only as a novel target in cognitive decline, but also for ongoing research within intersecting neural systems for incorporating sensation and emotion into memory.

Keywords: C-type natriuretic peptide, CNP, NTproCNP, NPR-C receptor, aging, age-related cognitive decline, spatial memory, recognition memory, pattern separation, retrosplenial cortex, hippocampus, frontal cortex, rodents

Chapter 1. General Overview

C-type Natriuretic Peptide (CNP) is the primitive, but most recently discovered member of the Natriuretic Peptide family. Since its description, multiple biological actions have been identified for CNP, many of which are related to cellular growth and development. CNP is the major neuroactive peptide of the Natriuretic family, thus far exhibiting a critical role in nervous system development, neuroprotective activity, and modulatory actions in connection to food and fluid intake, response to drug administration and anxiety regulation. Recently, a contribution to memory formation has been posited, with several studies supporting this hypothesis. Though it has been nearly 30 years since its discovery, research regarding CNP still lags compared with other natriuretic peptides, A- and B-types. In particular, there is a paucity of research regarding memory and behavioural associations with CNP activity.

Relatively well established biological activity of the CNP signalling pathway has indicated an involvement in regulation of multiple neurological activities including various neuroendocrine systems. In turn, CNP has been identified as a potential therapeutic target for neurological and psychiatric disorders, but much about this peptide remains to be understood. Beyond a possible contribution to memory acquisition and consolidation, CNP has become of interest where these functions intersect with clinical conditions. In addition to a possible functional role for CNP in learning, contributions to memory dysfunction have been suggested in cases of neurodegenerative disorders and general age related cognitive decline. To date, no experimental work had examined CNP specifically in this context.

1.1 Aims of the current work

Given the scarcity of behavioural studies involving CNP, and its relationship to memory, the primary aim of this thesis was a novel contribution to this small body of research. To date, there are two major experimental findings: CNP improves the acquisition and consolidation of passive avoidance memory (Telegdy, Adamik, & Glover, 2000; Telegdy, Kokavszky, & Nyerges, 1999) and reductions in CNP signalling improve object recognition (Barmashenko et al., 2014). Building from these, and initial work towards a Master's Thesis (Rapley, 2012, unpublished Master's Thesis), this thesis originally intended to examine CNP in rodent models of spatial/episodic-like memory and working memory. Whereas examination of CNPs influence on working memory was negatively impacted by anxiogenic effects of CNP, a resulting study examined the peptide in relation to spontaneous location recognition and "pattern separation" of memory.

Localisation of CNP gene expression, and that of its specific receptor (Natriuretic Peptide Receptor-B; NPR-B) has been relatively well described in brain and central nervous system. Several studies have examined CNP concentrations within cerebrospinal fluid (CSF) and hypothalamus of multiple species, with remarkably consistent results (Jankowski, Reis, Wang, & Gutkowska, 2004; Komatsu et al., 1991; Pemberton, Yandle, & Espiner, 2002; Totsune et al., 1994; Wilson, McNeill, Barrell, Prickett & Espiner, 2017). Though not strictly part of central nervous system, the pituitary gland has also drawn special interest from CNP researchers, with high concentrations of CNP consistently found therein (Pemberton et al., 2002; Yandle, Fisher, Charles, Espiner, & Richards, 1993). Despite multiple reports outlining localisation of the CNP signalling system, there is a dearth of data regarding peptide or regulatory changes during specific behavioural conditions, including learning and memory. A secondary aim of this thesis was to provide these measures throughout select regions of rodent brain.

Though CNP has been well studied in the developing nervous system, no work has considered it within the aging brain. Given recent suggestions that CNP may provide a novel-target in cognitive decline and age-related pathologies (Mahinrad, de Craen, Yasar, van Heemst, & Sabayan, 2016), this presents a major gap in the literature. In addition to describing the CNP signalling system within aging cerebral tissue, this thesis also provides comparison of CNP response to an enriched environment between young and aged rodents.

The general aim of this thesis, was to investigate CNP as a novel target influencing learning and memory that may contribute to cognitive impairment.

1.2 Outline of Thesis

In Chapter Two, I review literature regarding CNP, describe its actions within central nervous system, and outline research in regarding potential roles in regulating behaviour. Special focus is then given to research examining CNPs relationship to memory and neurological disorder. A potential role in age-related cognitive decline is described, providing rationale for the thesis. Chapters Three, Four and Five describe experimental contributions. A general discussion outlines major contributions of the thesis, addresses theoretical considerations, general limitations and directions for future work.

Chapter 2. Literature Review

2.1 Natriuretic Peptide Signalling System

CNP was originally isolated from porcine brain tissue, and demonstrated similarity in structure to previously described A-type Natriuretic Peptide (ANP) and B-type Natriuretic Peptide (BNP; Sudoh, Minamino, Kangawa, & Matsuo, 1990). Specifically, the family shares a ring structure consisting of 17 amino acid residues, flanked by two cysteine residues which form a disulfide bond that is critical to biological activity (Pandey, 2005; Potter, Abbey-Hosch, & Dickey, 2006). ANP and BNP share a common binding site (Natriuretic Peptide Receptor A; NPR-A; (Koller et al., 1991; Potter, 2011; Potter et al., 2006) and all NPs bind to a common clearance receptor, Natriuretic Peptide Receptor-C (NPR-C) with similar affinity (Pandey, 2005; Potter, 2011; Potter et al., 2006). However, CNP represents a unique member of the Natriuretic family. CNP has a specific receptor, Natriuretic Peptide Receptor B (NPR-B) through which it exerts biological activity (Pandey, 2005; Potter, 2011; Potter et al., 2006). Additionally, CNP has been identified as the oldest member of the NP family, with duplications of early CNP genes resulting in those for ANP and BNP (Inoue & Takei, 2006). Whereas ANP and BNP genes are located on chromosome 1 in humans, the NPPC (Natriuretic Peptide Precursor C) gene is located on chromosome 2 (Tawaragi et al., 1990). CNP-22 (the 'mature' active form of CNP) is the most highly conserved NP, has been identified in primitive species, and is identical in rodents, pigs and humans (Inoue et al., 2003; Inoue & Takei, 2006; Pandey, 2005; Potter, 2011; Tawaragi et al., 1990).

Expression of the NPPC gene results in three forms of the peptide. NPPC codes for an initial 126 residue precursor, preproCNP (Tawaragi et al., 1990) with further processing generating a 103 residue proCNP. This is then cleaved intracellularly to produce CNP-53 which is secreted extracellularly with a biologically inactive amino terminal fragment, NTproCNP, in equimolar amounts (T C R Prickett & Espiner, 2012; Wu, Wu, Pan, Morser, & Wu, 2003). Further extracellular processing results in CNP-22. This process is summarised in Figure 2.1 (From Prickett & Espiner, 2012). CNP-22 is thought to be the fully active form of the peptide with CNP-53 proposed as a storage form (Barr, Rhodes, & Struthers, 1996). This is supported by high concentrations and differing regulation of the peptide within pituitary (Yandle et al., 1993; Wilson et al, 2017) and overlapping but distinct distribution patterns of CNP-22 and CNP-53 (Pemberton et al., 2002; Yandle et al., 1993). Regulation of NPPC expression has not been widely studied, but recent work from Professor Espiner's research group shows it is upregulated in several nervous system tissues by dexamethasone,

indicating endogenous stimulation by glucocorticoids (Wilson et al, 2017). Experimental work in this thesis focusses on CNP-22 as the assumed major neuroactive form.

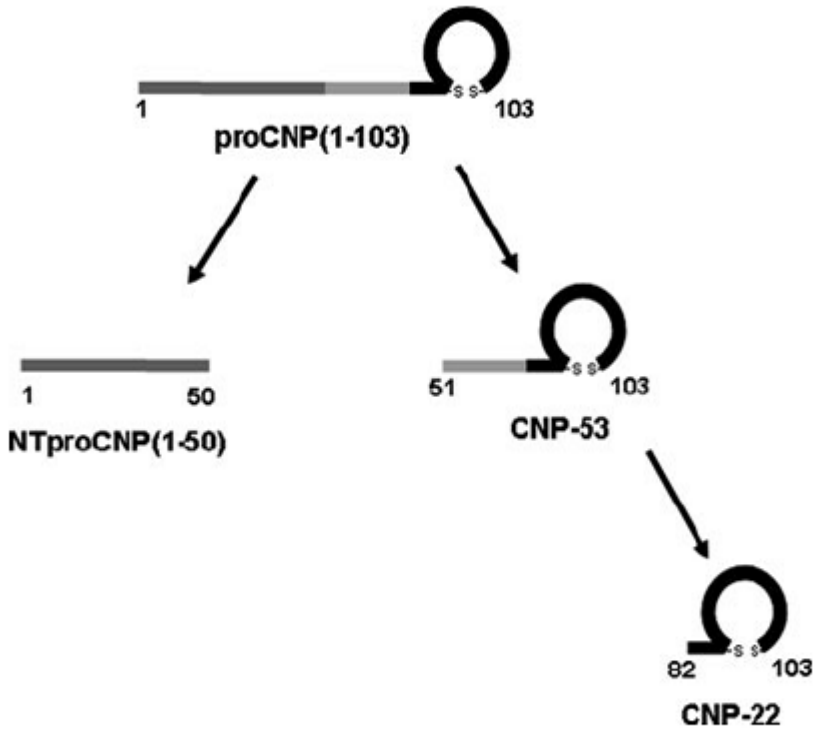


Figure 2.1: Processing of CNP prohormone (proCNP) resulting in an amino terminal fragment (NTproCNP) and two active forms of peptide. From Prickett & Espiner (2012).

Following expression and processing, NPs bind with NPR-A (ANP and BNP), NPR-B (CNP) or NPR-C (clearance) receptors. All three receptors exhibit strong homology, but NPR-C lacks intracellular domains (Potter, 2011). NPR-A and NPR-B consist of an extracellular ligand binding domain, a transmembrane region, and intracellularly, kinase homology and guanylyl cyclase catalytic domains (Potter, 2011; Potter et al., 2006). Binding with these receptors results in an increase of cyclic Guanosine Monophosphate (cGMP). NPR-C, the so-called "clearance" receptor, binds all NPs and clears them from the extracellular space via internalisation and degradation (Potter, 2011). Although nomenclature as the clearance receptor has persisted, some work indicates NPR-C may have a functional role beyond this, potentially acting through adenylyl cyclases to elevate cyclic adenosine monophosphate (Abdelalim et al., 2008; Anand-srivastava, 2005 for review; Rose et al., 2005).

All NPs and both active forms of CNP (22 and 53) stimulate cGMP (Potter, 2011; Potter et al., 2006; Yeung, Ho, Nicholls, & Cockram, 1996). As a second messenger, cGMP exerts biological activity by binding to cGMP gated ion channels, activating cGMP dependent protein kinases or regulating phosphodiesterases, each resulting in different cell signalling outcomes (Potter et al., 2006). Previous research implicates cGMP in multiple neural regulatory functions, including neurotransmitter release, long-term potentiation, neurodegeneration, and behaviour such as working memory and anxiety (see Boess et al., 2004; Wang & Robinson, 1997; Wincott et al., 2014). While most research relating cGMP with these functions has focussed on stimulation by nitric oxide, associations with CNP in these regulatory roles have also been identified, and are outlined in later sections.

The NP signalling system is additionally regulated by several proteolytic enzymes. Both CNP and ANP undergo proteolysis by neprilysin and insulin degrading enzyme (Potter, 2011). While both peptides can be degraded by both enzymes, neprilysin preferentially degrades CNP, and ANP is the preferred substrate for insulin degrading enzyme (Kenny, Bourne, & Ingram, 1993; Muller, Schulze, Baumeister, Buck, & Richter, 1992; Potter, 2011, p. 1812; Watanabe, Nakajima, Shimamori, & Fujimoto, 1997). Interestingly, human BNP is more resistant to neprilysin degradation and is degraded by another metalloendopeptidase, meprin A (Pankow et al., 2007; Smith, Espiner, Yandle, Charles, & Richards, 2000). Figure 2.2 provides a schematic of CNP binding sites and degradative activity.

Regulation of NP receptor activity may be influenced by several other molecules, but these have been little studied. Gö6976 (an indolocarbazole) inhibits both NPR-A and NPR-B activity, while the endogenous indole (which is not bound to a carbazole) isatin can inhibit NPR-A activity and interfere with behavioural effects of infused CNP (Medvedev, Sandler, & Glover, 1999; J. W. Robinson, Lou, & Potter, 2011; Telegdy et al., 2000). A microbial polysaccharide – HS-142-1 – inhibits NPR-A and NPR-B activity allotopically, and can abolish electrophysiological effects of CNP (J M Decker et al., 2009; Poirier, Labrecque, Deschenes, & DeLean, 2002). The bioactive lipid, Sphingosine-1-Phosphate, is reported to be a highly potent NPR-B inhibitor and interacts with CNPs actions in fibroblasts and vascular smooth muscle (Abbey-Hosch, Cody, & Potter, 2004). In addition, NPR-C has a recently discovered endogenous ligand, Osteocrin, which may regulate NP actions on bone growth in rodents, but is also present in primate neocortex where it restricts activity dependent dendrite growth (Ataman et al., 2016; Moffatt et al., 2007). Of these molecules, Osteocrin particularly

presents an important consideration for central nervous system tissues where CNP degradation and clearance is concerned, especially in human and primate studies.

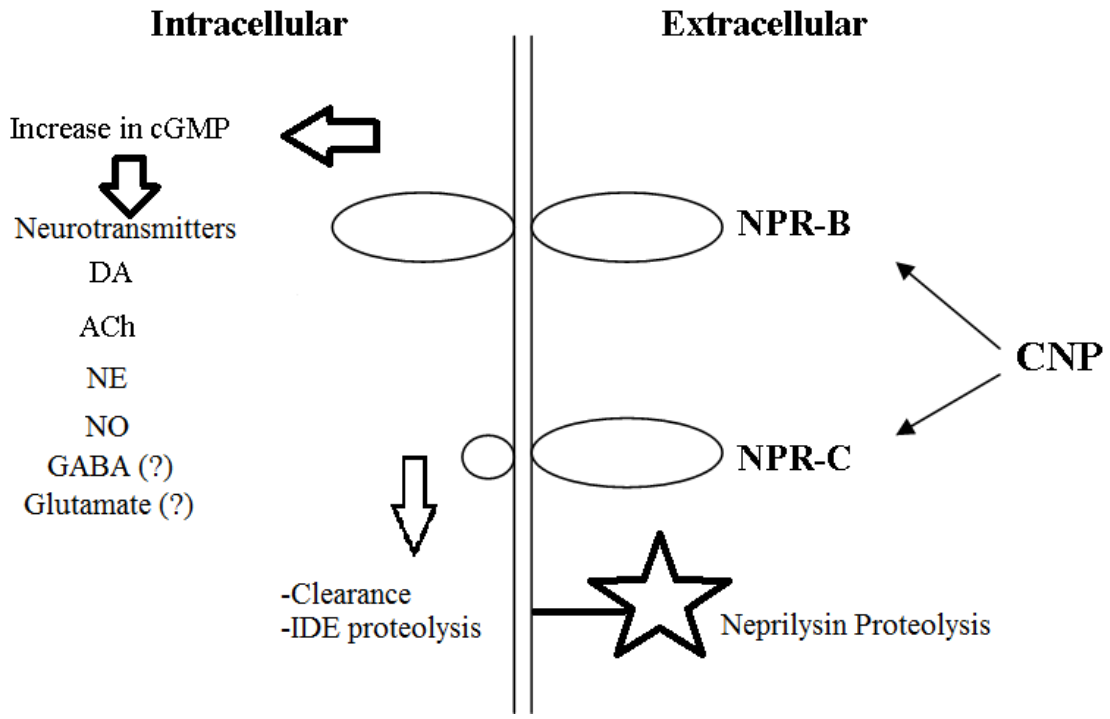


Figure 2.2: CNP binding sites, degradative activity, and eventually affected neurotransmitters resulting from increases in cGMP. Adapted from Potter (2011).

2.1.1 Functions and expression in bodily tissues

The starkest difference among the major NPs are found in their actions as endocrine peptides. Both ANP and BNP are primarily synthesised in the heart, with ANP predominant in the atrium, and BNP in the ventricle (Pandey, 2005). ANP and BNP then reach extra-cardiac targets via the circulatory system in an endocrine fashion and are readily measurable in plasma (Barr et al., 1996; Pandey, 2005; Prickett & Espiner, 2012; Sellitti, Koles, & Mendona, 2011). ANP and BNP generally regulate blood pressure and fluid homeostasis, earning the title of "cardiac natriuretic peptides" (Espiner, Richards, Yandle, & Nicholls, 1995; Prickett & Espiner, 2012; Scotland, Ahluwalia, & Hobbs, 2005). In contrast, highest concentrations of CNP are found in reproductive tissues, brain tissue and vascular endothelium (Pandey, 2005) with minimal expression in the heart (Cameron, Aitken, Ellmers, Kennedy, & Espiner, 1996; Langub, Watson, & Herman, 1995). Circulating CNP concentrations are low in human, and undetected in rodent (Komatsu et al., 1991; Toshio et al., 1996) but can become elevated following septic shock, and during pregnancy in ruminants (Hama et al., 1994; McNeill et al., 2009; Prickett et al., 2004; Wilson, Barrell,

Prickett, & Espiner, 2015). Low concentrations found in humans and other mammals likely follows in part from a short half-life in tissue and plasma (1.6 minutes in sheep and 2.6 minutes in humans; Charles, Espiner, Richards, Nicholls, & Yandle, 1995; Hunt, Richards, Espiner, Nicholls, & Yandle, 1994). Fortunately, NTproCNP (the amino terminal fragment of proCNP produced in tissues) is readily identifiable in the circulation and can be used to study changes in CNP production under specified conditions (Prickett, Yandle, Nicholls, Espiner, & Richards, 2001). However, since multiple tissues express the gene, which contributes to circulating NTproCNP remains to be firmly established.

It is important to note that the concentration of CNP at any time point is a function of both production and clearance or degradation rates. Since NTproCNP is not subject to rapid degradation or clearance (half-life of 30-40 mins compared with 2-3 minutes for CNP), it provides a reliable measure of NPPC gene expression and peptide production (Schouten et al., 2011; Woodward, Prickett, Espiner, & Anderson, 2017). Calculation of NTproCNP:CNP ratio (by taking NTproCNP/CNP concentrations) provides an index of CNPs clearance or degradation in the tissue sampled, either via NPR-C mediated internalisation or hydrolysis by neprilysin. Higher ratios reflect increases in the rate of loss of the bioactive form of the peptide. Combined measurement of CNP and NTproCNP, with calculation of the ratio, allows separate contributions of peptide production and metabolic clearance to be determined.

CNP expression has been described in multiple bodily tissues and is widely accepted as acting in a paracrine/autocrine fashion. An important site of CNP production is the vascular endothelium. Here, CNP acts locally to regulate vascular tone and blood pressure, is vasoprotective following ischaemia/reperfusion injuries, and has been identified as a likely endothelium derived hyperpolarising factor (Ahluwalia & Hobbs, 2005; Qian et al., 2002; Scotland et al., 2005). These effects may occur through modification of calcium ion concentrations and nitric oxide production (Andrade, Restini, Grando, Ramalho, & Bendhack, 2014; Qian et al., 2002). CNP additionally regulates circulating immune cells and presents a potential therapeutic target in cardiovascular diseases, particularly diabetic cardiomyopathy (Abbey-Hosch et al., 2004; Cabiati et al., 2013; Chrisman & Garbers, 1999; Christoffersen, Bartels, & Nielsen, 2006; Scotland et al., 2005; Walther et al., 2000).

Necessity for normal skeletal development is the best demonstrated of CNPs bodily functions with overexpression of CNP resulting in skeletal overgrowth, and interference with normal signalling leading to reduced stature (Prickett & Espiner, 2012; Tamura et al., 2004).

NTproCNP concentrations in plasma relate strongly to markers of bone formation and growth velocity in both young sheep and human children (Prickett et al., 2005, 2007; Prickett, Dixon, et al., 2008). Additionally, in growth hormone deficient rats, growth hormone treatment increases plasma NTproCNP and growth plate CNP, both of which also correlate with linear growth velocity (Prickett, Bothwell, Yandle, Richards, & Espiner, 2012). In humans, a loss of function mutation in NPR-B results in a specific form of dwarfism, acromesomelic dysplasia type Maroteaux (Bartels et al., 2004), and CNP is currently undergoing phase 2 trials as a treatment for children with Achondroplasia (clinicaltrials.gov NCT02055157).

CNP has additionally been implicated in the regulation of several other organ systems. In the female reproductive system, CNP is apparently critical to normal development (Tamura et al., 2004) and expression therein varies with the estrous cycle in rats (Jankowski et al., 1997). Oestradiol increases CNP in both plasma and uterine tissue (Acuff, Huang, & Steinhelper, 1997; Prickett, Barrell, et al., 2008). Additionally, in sheep, CNP exhibits a strong relationship to both placental and fetal maturation, with synthesis elevated in fetal tissues (Mcneill et al., 2009; Prickett, Barrell, et al., 2008). Actions in the male reproductive system involve CNP in the central control of penile erection (Tamura et al., 2004) and testis where the gene is expressed in Leydig cells (Middendorff, Muller, Paust, Davidoff, & Mukhopadhyay, 1996). Despite its name, CNP does not contribute to natriuresis or diuresis. Nevertheless, it is present in kidney tissue and presents a novel biomarker for identifying age-related renal changes (Sangaralingham et al., 2011). CNP not only regulates circulating immune cells, but is found throughout immune organs suggesting a general role in immune regulation (Vollmar, Gerbes, Nemer, & Schulz, 1993). Recently, CNP has been identified throughout the gastrointestinal tract and secretory glands of the digestive system, with the suggestion that NPs may participate in both central and local regulation of digestive secretion (Cho, Kim, Cho, & Park, 2000; Sabbatini, 2009 for review). Taken together, these findings indicate CNP and the NP family to be important regulators of multiple bodily functions.

Juxtaposed with the circulation, CNP is readily detectable in CSF where it is present in higher concentrations than either ANP or BNP (Kaneko et al., 1993). NTproCNP and the ratio of NTproCNP:CNP are also much higher in CSF compared to plasma (Schouten et al., 2011). The source of CNP present in the CSF may be closely related neural tissues, but is unlikely to be the systemic circulation as, in cases of elevated plasma CNP seen during ovine pregnancy, circulating concentrations do not affect those in CSF (Wilson et al., 2015). CNP is

reported to be the most potent NP in cerebral microvessels, with strong localisation of NPR-B receptors in choroid plexus and the ability to modify blood-brain barrier (Bohara et al., 2014; Herman, Dolgas, Rucker, & Langub, 1996; Vigne & Frelin, 1992). Overall, these findings indicate CNP to be an important molecule in regulation of both cerebrovascular and CSF environments.

All NPs are found in the central nervous system, but CNP is the most abundant and is the major neuroactive member of the family (Kaneko et al., 1993; Langub, Dolgas, Watson, & Herman, 1995; Pemberton et al., 2002). As outlined, CNP directly activates cGMP, known to have a role in multiple neurological functions. It is also widely accepted that CNP acts in an autocrine/paracrine fashion, indicating that production within cerebral tissue results in function locally. Within the body, CNP acts as a regulator of development and normal function of multiple systems, including the blood-brain barrier. In central nervous system, CNP also exhibits growth factor-like and regulatory actions. Although research of CNP in brain is still relatively sparse, many biological actions therein have been well described.

2.2 Biological Activity in Central Nervous System

CNP is present throughout the central nervous system from its inception. Gene transcripts for CNP emerge with the onset of neurogenesis in the dorsal ventricular zone, hindbrain and spinal cord (DiCicco-Bloom et al., 2004). Expression in the ventricular zone is notable as a location where neurons are undergoing differentiation (DiCicco-Bloom et al., 2004). In olfactory neuronal precursors, CNP inhibits proliferation stimulated by BDNF (Brain-Derived Neurotrophic Factor) and NGF (Nerve Growth Factor), and induces maturation (Simpson et al., 2002). Additionally, NPR-B was later shown to be essential for appropriate bifurcation of sensory axons within the spinal cord (Schmidt et al., 2007). CNP also stimulates axon outgrowth of dorsal root ganglia in culture, and CNP deficient mice lack bifurcated branches in sensory afferents (Zhao & Ma, 2009).

CNP continues to be important in nervous system development postnatally. PreproCNP mRNA expression increases throughout rodent brain postnatally, plateauing at levels seen in adulthood by postnatal day 28 (Ryan & Gundlach, 1998). Within the hypothalamus, CNP concentrations rise steadily postnatally until adulthood (postnatal day 60; (Jankowski et al., 2004). Mice lacking the NPR-B receptor demonstrate disorganisation of auditory circuits (Lu et al., 2014), and some exhibit tonic-clonic seizures, self-clasping, and priapism (Tamura et al., 2004).

By adulthood, CNP and NPR-B receptors are widely dispersed throughout central nervous system tissues (see Cao & Yang, 2008 for review). In rodents, both CNP and NPR-B mRNA occur throughout olfactory bulb, basal forebrain, basal ganglia, limbic cortices, thalamus, amygdala, mammillary nuclei, hippocampus, and cerebellum (Herman et al., 1996; Langub, Watson, et al., 1995). Within hippocampus, CNP mRNA predominates in CA1 through CA3, whereas NPR-B mRNA is largely expressed within DG (Herman et al., 1996; Langub, Watson, et al., 1995), leading to the interesting suggestion that within the hippocampus CNP is produced within CA1-3 but exerts action in DG, indicating a global influence on this important memory system. As previously mentioned, CNP-22 and CNP-53 coexist across various subregions, but CNP-53 is the only form in ovine pituitary (Yandle et al., 1993). In rodent and ovine brain, highest concentrations of CNP are consistently found in the pituitary, hypothalamus and pineal gland (Middendorff et al., 1996; Müller et al., 2009; Pemberton et al., 2002; Yandle et al., 1993; Wilson et al, 2017). Few studies have examined distribution in human brain tissue, but have shown that CNP is also widespread, with high concentrations in hypothalamus (Totsune et al., 1994). Taken together, these findings indicate CNP and its signalling system are crucial to the normal development of the nervous system, with dysfunctions in this system a potential contributor to neurological disorder.

Cellular targets of CNP in central nervous system are unclear. Within the brain, studies of ANP indicate a general process whereby NPs are produced by neurons and then target astrocytes which have prominent levels of NP receptors associated with them (Prado, Baltrons, Pifarré, & García, 2010; Simonnet et al., 1989; Yeung et al., 1996). However, reports vary as to whether this pattern consistently applies to CNP and the NPR-B receptor. For example, one study reported that NPR-B was less common than NPR-A in cultured astroglia, with CNP generating weaker stimulation of cGMP than ANP (Sumners & Tang, 1992). Others have reported a greater stimulatory action of CNP than ANP in cultured astroglia (Yeung et al., 1996). Yet another study reported that CNP had a lower stimulatory activity than ANP and primarily acted in non-glial components of brain tissue derived from the diencephalon (Goncalves, Grove, & Deschepper, 1995). This finding, from tissue as opposed to culture, may be better representative of normal function in intact brain, but of note is that tissue examined was immature, acquired at a maximum age of postnatal day 9. As previously outlined, NP expression in the developing brain exhibits temporal changes, with CNP less widely expressed in the immature diencephalon than in adulthood (Langub, Watson, et al., 1995; Ryan & Gundlach, 1998) whereas ANP is the predominant

hypothalamic peptide at this early developmental stage (Jankowski et al., 2004). As the presence of NPs change in this region over time, so may the proposed neuron-glia interaction with CNP, but this remains to be examined in tissue of adult rats.

Other than previously mentioned receptor mediation of CNP activity, brief half-life in plasma and known degradative regulation, relatively few studies have examined the pharmacokinetics of CNP within cerebral tissue. Arterial/Venous differences quantify the efficiency of molecular removal from organs, with those reported for CNP consistent with secretion from cerebral tissues (Potter, 2011). Though not directly studied for CNP, ANP is eliminated from cerebral tissues via brain-to-blood efflux with a half-life of approximately 20 minutes (Ito, Ohtsuki, Katuskura et al, 2011). A similar mechanism may occur for CNP, given the reported arterial/venous difference and the paracrine nature of the peptide. As CNP may be involved in regulation of BBB (Bohara et al., 2014) and appears to have differential processing in central versus pituitary tissue (Wilson, Barrell, Prickett, & Espiner, 2018), it would be safe to assume that it does not cross BBB from the systemic circulation. Administration of intravenous CNP-22 to sheep across a two-hour time period at 10pmol/kg/min resulted in a peak of cGMP between 60 and 90 minutes (Charles, Espiner, Richards, Nicholls, & Yandle, 1995b). In mouse astrocytes, 10nmol concentration of CNP-22 or CNP-53 resulted in peak cGMP concentrations between 10 and 20 minutes of incubation (Yeung et al, 1996). This faster response at a lower dose may be an effect of species, tissue or methodological difference. Behavioural effects of icv peptide administration have been reported between 20 and 1000ng doses, 30 minutes following administration (Bíró, Tóth, & Telegdy, 1996b; Gardi et al., 1997; Telegdy et al., 1999). Given the molecular weight of CNP (2197.6) the dose of CNP required to elicit these behavioural effects is approximately 20-fold less than that used by Yeung and colleagues (1996) to investigate the cGMP response. As such, these latter reports are used to determine the dose range applied in this thesis (see Chapter 5).

CNP can modify availability of, and exert action through, several neurotransmitters, primarily dopamine and norepinephrine. In tuberoinfundibular dopaminergic neurons of the dorsomedial arcuate nucleus, CNP inhibits neuron firing alone, and magnifies inhibitory effects of dopamine application (Pan, Lai, & Yen, 1996). In retinal amacrine cells, NPR-B is co-localised with the dopaminergic marker, tyrosine hydroxylase (Abdelalim, Masuda, & Tooyama, 2008) which is also indicative of norepinephrine. CNP modulates norepinephrine by increasing reuptake and decreasing release of the neurotransmitter in hypothalamus and

other encephalic nuclei, olfactory bulb and adrenal medulla (Fermepín, Vatta, Bianciotti, Wolovich, & Fernández, 2000; Vatta, Presas, Bianciotti, Zarrabeitia, & Fernfindez, 1996; Vatta, Presas, Bianciotti, Ambros, & Fernandez, 1997). In addition, NPR-C receptor is co-expressed with catecholaminergic nuclei in rat brainstem, indicating peptide clearance in these regions (Abdelalim et al., 2008).

Beyond the catecholamines, CNP has also been linked to modification of nitric oxide. Increases of nitric oxide production by CNP have been demonstrated in the context of its vasorelaxant, antithrombotic and anti-inflammatory effects in arteries (Andrade et al., 2014; Qian et al., 2002). Like CNP, nitric oxide also stimulates cyclic GMP, but acts via soluble guanylyl cyclase (Garthwaite, Goodwin, & Garthwaite, 1999) as opposed to the NPR-B membrane bound guanylyl cyclase. The complex relationship between CNP and nitric oxide as co-producers of cGMP remains to be elucidated and has not been investigated in brain tissue. However, inhibition of nitric oxide synthase by nitro-L-arginine can prevent behavioural effects of CNP (Telegdy et al., 1999), indicating a similar relationship may occur therein.

CNP has also been linked with acetylcholine, GABA (γ-Aminobutyric Acid) and NMDA (N-Methyl-D-Aspartic acid) signalling. NPR-B and NPR-C co-localise with cholinergic neurons in retinal amacrine cells, and multiple brainstem nuclei, respectively (Abdelalim et al., 2008; Abdelalim, Masuda & Tooyama, 2008). CNP may also modulate GABA-mediated inhibition and NMDA receptors within the hippocampus (Decker et al., 2009, 2010; Decker, Wojtowicz, Heinemann, & Braunewell, 2008). However, while antagonists of dopamine, norepinephrine, nitric oxide and acetylcholine activity all prevent actions of CNP on behavioural sequelae in the elevated plus maze and a passive avoidance learning paradigm, interference with GABA and NMDA receptors did not alter behavioural effects of CNP in either context (Bíró, Tóth, & Telegdy, 1996; Telegdy et al., 1999). Regardless, effects on multiple neurotransmitter systems indicate that CNP is a broad acting modifier of synaptic transmission.

CNP can also modify secretion of various other peptides, acting in opposition to ANP. Studies of ANP indicate it inhibits release of progesterone, testosterone, luteinising hormone, vasopressin, corticotropin, prolactin and growth hormone (Huang, Skala, & Samson, 1992; Pandey, 2005). For those that CNP can also modify (thus far, prolactin, growth hormone and corticotropin) a notable feature is opposition of ANP action, with CNP stimulating, and ANP inhibiting secretions (Charles, Espiner, Richards, & Donald, 1995; Huang et al., 1992;

Samson, Huang, & Fulton, 1995; Shimekake, Ohta, & Nagata, 1994). In magnocellular neurosecretory cells, known for secreting oxytocin and vasopressin, CNP via the NPR-C receptor selectively inhibits L-type calcium channel currents (Leng & Ludwig, 2008; Rose et al., 2005), suggesting it may additionally interact with these peptides. Abundance of CNP in both hypothalamus and pituitary, alongside effects on endocrine peptides has led to the suggestion it may be important in neuroendocrine regulation of multiple functions, including reproduction (Fowkes & McArdle, 2000).

As a potential neuroendocrine regulator, most research to date has examined CNP regulation of the HPA (hypothalamo-pituitary-adrenal) axis. CNP stimulates corticotropin releasing hormone, the key regulatory peptide of the HPA axis, which centrally regulates the peripheral anxiety response via release of adrenocorticotropin and cortisol. Central administration of CNP increases circulating levels of both adrenocorticotropin and cortisol in sheep, and magnifies the effects of the HPA-axis response to induced haemorrhage (Charles, Espiner, Richards, & Donald, 1995). CNP similarly magnifies adrenocorticotropin response in healthy males administered CNP prior to induction of panic attacks (Kellner et al., 2002). In rodents, centrally infused CNP increases corticotropin releasing factor-like immunoreactivity in the basal forebrain at low doses, but decreases it in hypothalamus at high doses (Gardi et al., 1997) and modification of anxiety by CNP is dependent on corticotropin releasing hormone (Jahn et al., 2001). Interestingly, the recent finding that NPPC expression in cerebral tissue is increased by dexamethasone, suggests that CNP and the endogenous glucocorticoids may form an important regulatory feedback loop (Wilson et al., 2017).

As in the periphery, CNP can modify multiple biological subsystems of central nervous system that are critical to development and normal function. Demonstrated effects on various other signalling systems imply that CNP should be involved with a broad range of behaviours. To date few have been investigated, but CNP can affect food and fluid consumption, locomotion and response to drug, emotionality and memory.

2.3 Central Regulation of Behaviour

Unsurprisingly, high concentrations and localisation of CNP within the hypothalamus has drawn much research attention. Known involvement of the hypothalamus in regulating fluid intake, alongside NP roles in natriuresis and diuresis suggested possible involvement in central regulation of fluid and salt balance. Prior to discovery of CNP, ANP had already been shown to inhibit both water drinking and salt appetite (Samson, Skala, & Huang, 1991).

Conversely, centrally administered CNP was found to stimulate water drinking and magnify the effects of Angiotensin II induced water drinking in hypertensive rats. (Goto, Shibata, & Furukawa, 1997; Samson et al., 1991). Challenges to fluid and salt balance are also able to affect expression of CNP in olfactory regions and medulla (Cameron, Cumming, Espiner, Nicholls, & Richards, 2001).

More recently, CNP has been considered as a potential regulator of food intake and energy expenditure in addition to its effects on fluid intake. CNP-null mice with targeted CNP expression in chondrocytes to reduce skeletal dysplasia, exhibited a reduction in food intake and modifications to multiple measures of metabolic function (Inuzuka et al., 2010). Furthermore, icv administration of either CNP-22 or CNP-53 reduced food intake in normal mice (Yamada-Goto et al., 2013). Complementing these findings, calorie restriction reduces plasma concentrations of CNP and NTproCNP (indicating reductions in peptide synthesis) in young lambs, and reduces plasma CNP via increases in degradative activity in adult sheep (Prickett et al., 2010; Prickett et al., 2007). These initial studies provide promising evidence that CNP contributes to the central control of both food and fluid intake.

Several studies indicate CNP participates in neurological and behavioural response to drugs. Ventricular infusion of CNP prior to ip administration of cocaine, inhibits cocaine-induced expression of the immediate early genes c-fos and egr-1 in nucleus accumbens and caudate-putamen (Thiriet et al., 2001). CNP can also attenuate cocaine-induced egr-1 expression throughout striatum, after infusion into either the ventral tegmental area or caudate-putamen (Jouvert et al., 2004). Moreover, CNP reduces both cocaine-induced extracellular dopamine rises and cocaine-induced locomotor activity (Thiriet et al., 2001). When infused into medial prefrontal cortex, CNP reduces self-administration and motivation to seek cocaine, and concomitantly down-regulates expression of epigenetic markers associated with synaptic plasticity (Deschatrettes, Romieu, & Zwiller, 2013). Additional findings that CNP administration can reduce alcohol intake in a rodent model of addiction (Romieu, Gobaille, Aunis, & Zwiller, 2008), reduce acute antinociceptive effects of morphine, and prevent development of both acute and chronic morphine tolerance (Babarczy, Vizi, Toth, & Telegdy, 1995) suggest that CNP is involved in modifying the response to drug in dopaminergic neural circuits, and can alter behavioural outcomes of their reinforcing properties.

Studies of CNP regulation of both food and fluid intake and response to drug have a pertinent common finding. In both cases, CNP was found to increase production of c-fos

alone (Thiriet et al., 2001; Yamada-Goto et al., 2013). In addition to modifying food intake, icv administration of CNP-53 increased immunoreactivity of c-fos in various nuclei of the hypothalamus (Yamada-Goto et al., 2013). CNP-22 administered icv also induced c-fos expression in frontal cortex (Thiriet et al., 2001). Another study did not find this effect for CNP (B. Zhu & Herbert, 1996), but used a lower dose range, indicating c-fos induction by CNP may be dose dependent. The c-fos gene, and its product Fos protein, are thought to be critical for translating short-term synaptic activity into long-term memory, because interference with its expression can impair memory (e.g. Seoane, Tinsley, & Brown, 2012; Tischmeyer & Grimm, 1999 for review). The mechanism by which CNP can induce c-fos expression is unknown, but this indicates a general role for CNP in information integration within central nervous system.

As previously mentioned, CNP may also participate in regulation of anxiety behaviour, via interaction with the HPA axis. CNP was originally shown to be anxiolytic at doses of 100 to 200ng, infused centrally (Bíró et al., 1996b), though two subsequent studies failed to replicate this effect, and instead found infused CNP to be anxiogenic (Jahn et al., 2001; Montkowski et al., 1998). Anxiogenic effects of CNP are better supported, but possible anxiolytic effects at low doses are not precluded, based on CNPs ability to increase corticotropin releasing factor-like immunoreactivity in basal forebrain in a similar dose range (Gardi et al., 1997). Moreover, as ANP is apparently consistently anxiolytic (Wiedemann, Jahn, & Kellner, 2000), and CNP action within the HPA axis opposes ANP, behavioural effects of CNP are more likely to be anxiogenic. This gains support from human studies where CNP given intravenously to healthy human males did not increase anxiety alone, but magnified anxiogenic effects of the panicogen cholecystokinin tetrapeptide (Kellner et al., 2002). Opposing effects of ANP and CNP on anxiety have implicated them as a possible therapeutic target in panic disorder (Kellner, Jahn, & Wiedemann, 2003). Furthermore, activity and expression of CNP throughout frontal and limbic cortices, alongside its effects on anxiety, suggest it may represent an important molecule in regulation and processing of emotion more generally, potentially of interest in multiple psychological disorders exhibiting mood dysregulation.

Alongside appetitive, locomotor, anxiety and drug-related behaviours, CNP may also participate in memory acquisition and consolidation. Aside from previously mentioned CNP-induced modifications of c-fos and other immediate early genes, a hypothesised role for CNP in regulation of memory formation comes from four lines of evidence: 1) Localisation of

mRNA expression and NPR-B receptors within central nervous system; 2) improvement of memory in passive avoidance paradigms; 3) modification of synaptic plasticity towards long term depression (LTD) in both CA1 and CA3 of the hippocampus; and 4) opposing hippocampal plasticity modifications subsequent to functional downregulation of NPR-B receptor signalling associated with improved object discrimination in rats. However, as with central regulation of other behaviours, contribution of CNP has received little research attention.

2.4 CNP and Memory

Regardless of any additional information, localisation of NPR-B receptors and CNP precursor mRNAs suggest involvement in systems for integrating sensory input, emotion and memory. As previously outlined, CNP precursor mRNAs are found throughout the central nervous system, but are most abundant in olfactory and amygdaloid nuclei, limbic cortices, hippocampus, hypothalamus, mammillary bodies, cerebellum and various brainstem regions (Langub, Watson, et al., 1995). NPR-B receptors overlap greatly with this distribution pattern, but are also found throughout the neocortex, basal ganglia, and the anterodorsal thalamic nucleus (Herman et al., 1996). Furthermore, and as noted, within hippocampus precursor mRNA and NPR-B receptor expression exhibit localisation to CA1-3 or DG, respectively. Overall, CNP and NPR-B locations directly compare with multiple works indicating these regions represent overlapping systems underlying the integration of emotion and visceral sensation (Catani, Dell'Acqua, & Thiebaut de Schotten, 2013) with various aspects of declarative memory (Aggleton & Brown, 1999; Aggleton & Brown, 2006).

Initial indication that CNP improves memory comes from studies of passive avoidance learning. Briefly, passive avoidance learning consists of an initial sample trial wherein a foot-shock is administered to a rat following entry to a dark compartment, which rats prefer to their brightly lit initial position. Memory for the aversive stimulus (foot-shock) is indicated by a subsequent increase in step-through latency following a delay period. Ventricular administration of 1µg (1000ng) CNP either 30 minutes before or immediately following a single acquisition trial, increased step-through latency when tested 24 hours later (Telegdy et al., 2000, 1999). The authors themselves identify that this finding may be complicated by CNPs aforementioned effects on anxiety (Telegdy et al., 1999). Doses used for passive avoidance studies were in the range demonstrated to be anxiogenic, potentially aiding memory for this task by increasing the salience of the aversive foot-shock. However, in both passive avoidance studies CNP had no effect on recall when administered prior to the

test trial, suggesting this was not solely an effect of anxiogenesis (Telegdy et al., 2000, 1999). Moreover, as anxiety tends to have negative impacts on learning and memory (Gülpınar & Yegen, 2004 for review; Izquierdo, Wellman, & Holmes, 2006), this indicates these studies demonstrate a genuine learning improvement.

Three studies have examined CNP signalling in relation with electrophysiological correlates of synaptic plasticity (Decker et al., 2009, 2010; Decker et al., 2008). Application of CNP was initially demonstrated to decrease population spike amplitude in stratum pyramidale of CA1, measured following high frequency stimulation originating in stratum radiatum of CA3 (Decker et al., 2008). Subsequently, Decker and colleagues showed that CNP application also decreased the power of induced γ-oscillations and reduced the frequency of sharp wave-ripple complexes in hippocampal areas CA1 and CA3 (Decker et al., 2009). Finally, CNP application was shown to impede long-term potentiation (LTP) induction while facilitating LTD in area CA1 (Decker et al., 2010). Each of these electrophysiological functions provide important contributions to the process of memory consolidation (see Bartos, Vida, & Jonas, 2007; Buzsáki, 2015; Connor & Wang, 2016 for recent reviews).

Identifying a behavioural connection between CNPs modulation of LTP/LTD was achieved by genetic modification of NPR-B receptors restricted to brain tissue. NPR-BΔKC rats exhibit a decrease (but not complete eradication) of CNP stimulation of cGMP via functional downregulation specific to NPR-B receptor activity, which can be isolated to central nervous system (Barmashenko et al., 2014; Langenickel et al., 2006). In hippocampal slices from these rats, LTP was facilitated at lower stimulus frequencies than wild type controls, complementing the opposite previous finding that CNP application inhibits LTP induction in hippocampal slices from wild-type rodents (Barmashenko et al 2014; Decker et al., 2010). NPR-BΔKC rats compared to wild-type also showed increased exploratory behaviour in an open field task, and better novel-object discrimination after a 24-hour delay, but performed no differently in a spatial variant of the object recognition task (Barmashenko et al., 2014).

In addition to effects on synaptic plasticity and recognition behaviour, hippocampal slices from NPR-BΔKC rats exhibited *metaplastic* changes (Barmashenko et al., 2014) indicating a complex contribution of CNP to learning and memory. Metaplasticity, or "plasticity of synaptic plasticity" (Abraham & Bear, 1996) is a broad term used to describe multiple mechanisms exerting longer-term regulatory control over the ability of neurons to

generate LTP or LTD (Abraham, 2008). Temporal and mechanistic overlap in metaplastic and conventional plasticity functions (LTP/LTD) make this a challenging phenomenon to study experimentally, and to interpret (Abraham, 2008). Additionally, contributions of LTD to memory formation have only recently gained experimental support (Goh & Manahan-Vaughan, 2013; Kemp & Manahan-Vaughan, 2004, 2007, 2008; Lemon & Manahan-Vaughan, 2012; Manahan-Vaughan & Braunewell, 1999; Connor & Wang, 2016 for review). Furthermore, stress responses, as modified by CNP, also modify synaptic plasticity and learning and memory outcomes (Gülpınar & Yegen, 2004; Howland & Wang, 2008; Kim & Diamond, 2002 for reviews). This study not only demonstrates the complex relationship between anxiety, exploration, memory, synaptic plasticity and metaplasticity, but also indicates an overarching role for CNP within each of these domains. Although this complex relationship will require much research to tease apart, these broad and interactive actions of CNP suggest it is critical to normal cognitive function and the integration of sensory and emotional experiences into memory.

2.5 CNP and Neurological Disorder

If CNP is critical to normal cognitive function, it follows that it may also be affected in multiple conditions of cognitive dysfunction. Although CNP has been implicated in multiple neurological conditions, research in this area is again sparse. As previously mentioned, interactions of ANP and CNP in modifying anxiety and HPA axis response has suggested them as a therapeutic target in panic disorder (Kellner et al., 2003). CNP is elevated in CSF immediately following subarachnoid haemorrhage, protects against cytotoxic injury of retinal cells in vitro and in vivo, and ANP contributes to the neuroprotective effects of cortical spreading depression (Ikeda, Ikeda, Onizuka, Terashi, & Fukuda, 2001; Ma et al., 2010; Wiggins, Shen, & Gundlach, 2003). One recent review, focusing heavily on ANP, outlines a potential role in addiction (Hodes & Lichtstein, 2014), which could also be posited for CNP based on involvement in dopaminergic and behavioural response to drug (Babarczy et al., 1995; Deschatrettes et al., 2013; Jouvert et al., 2004; Romieu et al., 2008; Thiriet et al., 2001). Midbrain dopaminergic signalling is also of interest in schizophrenia, ADHD (Attention Deficit Hyperactivity Disorder) and Parkinson's disease. Although the NPR-C receptor has recently been implicated in ADHD (Gong et al., 2017), CNP has only been specifically investigated in relation to Parkinson's disease (Espiner, Dalrymple-Alford, Prickett, Alamri, & Anderson, 2014; Koziorowski, Tomasiuk, Szlufik, & Friedman, 2012; Woodward et al., 2017).

Various aspects of CNP and its signalling system suggest an involvement in Parkinson's disease, with several studies now providing support for this notion. Expression of CNP and NPR-B receptors throughout dopaminergic pathways and structures, alongside interactions with dopamine signalling and effects on locomotion provide good indication that CNP may be modified in Parkinson's pathology (Barmashenko et al., 2014; Herman et al., 1996; Jouvert et al., 2004; Langub, Watson, et al., 1995; Tamura et al., 2004; Thiriet et al., 2001). One study has indicated increased serum levels of NTproCNP in Parkinson's patients, in association with elevated inflammatory factors, suggesting this indicates a contribution of CNP to neuroinflammation and development of the disorder (Koziorowski et al., 2012). However, a recent finding from Professor Espiner's group indicates plasma NTproCNP is reduced in Parkinson's disease (Woodward et al., 2017). In measures from CSF, NTproCNP is reduced in Parkinson's disease and correlates with more rapid functional decline, indicated by earlier need for treatment intervention (levodopa; Espiner et al., 2014). Moreover, treatment with deprenyl which can delay levodopa treatment, prevented CSF reductions in NTproCNP, and ameliorated further reductions for participants starting delayed deprenyl treatment (Espiner et al., 2014). While these findings are positive indicators that CNP activity is modified in Parkinson's disease, and may represent an additional treatment target to those already available, the authors highlight the need for ongoing research.

Two findings from Espiner et al (2014) are particularly relevant to this thesis. First, their study found no relationship between CSF peptide measures and cognitive status of either mild cognitive impairment or dementia. Reasons to believe CNP may be involved in cognitive decline are outlined below, although this finding indicates no relationship as such in Parkinson's disease at the level of CSF. They also report no relationship with participant age. However, the source of CNP and associated molecules in the CSF is not yet clarified, and CNP within cerebral tissues may exhibit a different profile.

2.6 Proposed Role in Age-related Cognitive Decline

Previously mentioned roles in modifying synaptic plasticity, anxiety, memory and in nervous system development, suggested that the CNP signalling system may vary further with normal brain aging within cerebral tissue. Indeed, ours was not the only research group to expect some involvement of CNP in the effects of brain aging, cognitive impairment or neurological disorders associated with age. Mahinrad and colleagues (2016) review possible contributions of all NPs to cognitive impairment, focussing on pathological endpoints of dementia and Alzheimer's disease.

The process of 'normal' aging does not necessarily result in pathology, but is often accompanied by memory loss and accelerated cognitive decline (Mora, 2013). Across humans, non-human primates and rodents, this cognitive decline is typified by deficits in spatial and recognition memory (Erickson & Barnes, 2003 for review). Though these deficits are similar to those in Alzheimer's disease, age-related cognitive deficits are less severe, and exhibit variation in the underlying neurobiology (Fjell, McEvoy, Holland, Dale, & Walhovd, 2014 for review). While many memory deficits are hippocampal dependent, and extreme hippocampal neuronal loss occurs in typical Alzheimer's pathology, the same cannot be said for healthy aging. Nonetheless the normally aging hippocampus exhibits reductions in the persistence of LTP induction, alongside a loss of synaptic connectivity and integration (Burke & Barnes, 2010; Erickson & Barnes, 2003). In addition to hippocampal synaptic effects reminiscent of CNP-related modifications (cf Decker et al., 2009, 2010; Decker et al., 2008), proliferation of new DG neurons also decreases with age (Burke & Barnes, 2010; Kempermann, Kuhn, & Gage, 1998; Verret, Trouche, Zerwas, & Rampon, 2007). Moreover, those neurons that are generated exhibit normal migration and survival, but delayed maturation and poor dendrite outgrowth (Rao, Hattiangady, Abdel-Rahman, Stanley, & Shetty, 2005). This links to CNP as the peptide promotes both maturation of olfactory neuronal precursors (Simpson et al., 2002) and dendrite outgrowth (Schmidt et al., 2007, 2009). It therefore seems likely that CNP is implicated in age-related neurological changes, at least within the hippocampal formation.

Aside from a role in synaptic regulation, Mahinrad and colleagues (2016) identify seven additional contexts indicating a role for NPs in cognitive decline, six of which CNP has been demonstrated to contribute to, and have been mentioned previously. These are 1) neurovascular dysfunction (through association with blood-brain barrier modification); 2) neuro-inflammation (modifications of immune and inflammatory responses); 3) neuroprotection (demonstrated in retinal cells); 4) anxiety (section 2.3); 5) memory (section 2.4); and 6) fluid homeostasis of the central nervous system (through contributions to the regulation of CSF). The seventh context outlined by Mahinrad and colleagues (2016), a relationship with amyloid-beta and tau protein deposits, has not been demonstrated for CNP, and is more specifically relevant to proposed involvement in pathological aging. Currently, elevated levels of BNP in CSF are proposed as a novel biomarker for Alzheimer's diagnosis (Craig-Schapiro et al., 2011) and BNP concentrations are increased in plasma alongside diagnosis of mild cognitive impairment or Alzheimer's disease (Hu et al., 2012). Higher

serum levels of BNPs amino terminal fragment (NTproBNP) have also been associated with lower volumes of both gray and white matter, and poorer memory performance in an aging population (Sabayan, Buchem, Craen, Harris, & Launer, 2015). Relevance of these relationships between the least neuroactive NP and other markers of Alzheimer's disease pathology remains to be investigated. However, there is historical precedent within NP research of BNP effects later becoming attributed to cross-talk with CNP receptors (CNPs contribution to skeletal development was originally attributed to BNP – (Prickett & Espiner, 2012). As CNP is the major neuroactive NP, future investigation of its contribution in the context of pathological aging is critical.

A final indication that CNP may either contribute to, or be affected by both normal and pathological aging, lies in its degradation by neprilysin. Aside from degrading CNP, neprilysin is the most potent enzyme to degrade amyloid-beta peptide (Higuchi, Iwata, & Saido, 2005). While amyloid-beta plaques are a hallmark of Alzheimer's disease pathology, deposits have also been found in normally aging brains, although the density is much lower (Armstrong et al., 1996). Human and rodent studies indicate region specific, age-related reductions in neprilysin activity or expression, which correlate with plaque depositions, regardless of pathological status (Apelt, Ach, & Schliebs, 2003; Hellström-Lindahl, Ravid, & Nordberg, 2008; Iwata, Takaki, Fukami, Tsubuki, & Saido, 2002; Russo, Borghi, Markesbery, Tabaton, & Piccini, 2005; Yasojima, McGeer, & McGeer, 2001; Yasojima, Akiyama, McGeer, & McGeer, 2001). This has led some to suggest that the age-related downregulation of neprilysin may be a key factor in the switch from normal to abnormal amyloid deposition, and contribute to the development of Alzheimer's disease (Iwata et al., 2001; Yasojima et al., 2001; Yasojima et al., 2001). Reductions of neprilysin would suggest an accumulation of CNP with age, which would align with previously mentioned age-related reductions in LTP persistence and CNPs modification of synaptic plasticity toward LTD (Burke & Barnes, 2010; Decker et al., 2009, 2010; Decker et al., 2008). However, if higher concentrations of CNP improve memory, this suggests that increased CNP levels during aging may confer some benefit. One study has indicated such a benefit to learning and memory in aged neprilysin knock-out mice, seemingly conveyed by other peptides degraded by neprilysin (Walther et al., 2009). While a similar possibility is feasible for CNP, this remains untested.

Accumulating evidence, reviewed here, indicates CNP is a critical molecule to normal function and integration of information within the nervous system, which is additionally

modified in cases of nervous system dysfunction and disorder. A multitude of questions remain within various fields of study concerned with this peptide, with few able to be answered within a single thesis. With a view to providing behavioural support for CNPs role in memory, and potential participation in age-related cognitive impairment, three lines of question were identified.

2.7 Rationale for the Thesis

Following from restricted previous literature, this thesis was highly investigative. A primary question is whether CNP expression or degradation within brain tissue varies during normal aging. Although no relationship was previously found with age in CSF across a specific age-range (51-90 years; Espiner et al., 2014; Schouten et al., 2011), this does not exclude the possibility that regional variations may still be found within cerebral tissue. This remains plausible given paracrine/autocrine action of CNP and relationships with synaptic plasticity, memory, anxiety, neprilysin degradation and other functions outlined by Mahinrad and colleagues (2016). Investigation of CNP in the aging brain in the context of environmental enrichment is outlined in Chapter 3.

Secondly, at the outset of this thesis (in 2013), no published findings had demonstrated a relationship between CNP and recognition or spatial/episodic-like memory. Associations with each of these measures is a suitable starting point to address contributions of CNP to cognition, given all are affected during both normal and pathological aging (Erickson & Barnes, 2003). A preliminary study for my Master's thesis, showed statistically significant associations between endogenously measured CNP concentrations and novel object discrimination, wherein better novelty discrimination was associated with lower CNP concentrations in medial prefrontal cortex, retrosplenial cortex and dorsal hippocampus (Rapley, 2012, unpublished Master's Thesis). The report that NPR-BΔKC rodents exhibited improved recognition memory was published late in 2014, providing excellent support for this hypothesis, but no effect was identified in a spatial object recognition task (Barmashenko et al., 2014). Outside this thesis, no research had examined CNP in association with exclusively spatial, or episodic-like memory. Concurring with Barmashenko and colleagues (2014) in terms of recognition memory, spatial/episodic-like memory associations were examined by measuring endogenous CNP in tissue from rats following acquisition of a spatial reference memory task in the radial arm maze (RAM), outlined in Chapter 4.

Although multiple memory difficulties occur during aging, deficits in pattern separation of memory have been suggested to underpin the combined cognitive issues (Burke, Wallace, Nematollahi, Uprety, & Barnes, 2010). Initial investigation of CNP in association with working memory was unsuccessful, due to interference with the working memory paradigm by the anxiogenic effects of CNP and small final numbers for data analysis. A subsequent study was designed to quantify this anxiogenic effect, and examine effects of infused CNP on object recognition and a spontaneous location recognition variant which manipulates pattern separation (Bekinschtein et al., 2014; Kent, Beynon, et al., 2015). Though it was an unplanned aspect of this study, rodents used were cognitively impaired as demonstrated by poor discrimination performance in recognition tasks. This study is outlined in Chapter 5.

Regions of Interest (ROI) across all studies were selected following multiple considerations. Included were medial prefrontal cortex, occipital cortex, retrosplenial cortex, dorsal hippocampus, hypothalamus and mammillary bodies. Additional ROIs and subregional examination are used in Chapter 5 (outlined therein). This general selection was based on 1) demonstrated distribution of CNP and NPR-B receptors (Herman et al., 1996; Langub, Watson, et al., 1995); 2) Previously demonstrated effects of enrichment within these regions (relevant to Chapter 3; Mohammed et al., 2002 for review); and 3) involvement of many of these regions in a neurological system underpinning spatial/episodic and recognition memory, relevant to Chapters 4 and 5 (Aggleton & Brown, 1999; Aggleton & Brown, 2006). These regions also provided a basis for potential ongoing research linking CNP, enriched housing and amelioration of cognitive deficits seen after brain injury or during aging. In addition, many of these regions show age-related changes, particularly the hippocampus (Burke & Barnes, 2010; Erickson & Barnes, 2003), and/or involvement in regulating anxiety and the HPA axis (Jankord & Herman, 2008). Although the hypothalamus is usually not directly considered where memory is concerned, in terms of CNP it is important to include. Hypothalamus has been a consistent brain region for investigation of CNP within CNS tissues, thus provides an indicator of both cross-species and cross-experimental consistencies. Additionally, feedback regarding anxiety and bodily cues relating to food and fluid intake especially, are mediated by the hypothalamus. Inclusion of the hypothalamus thus represents an important neurological structure for consideration of CNP's affects related to memory, although it does not influence memory consolidation directly *per se*.

Chapter 3. Age, Environmental Enrichment and CNP

3.1 Rationale

Use-dependent nervous system plasticity, initially proposed by Donald Hebb in 1949, was demonstrated a decade later using enriched environments by Rosenzweig, Bennett, Diamond and colleagues (Rosenzweig & Bennett, 1996 for review). Their initial findings of modifications to cortical acetylcholinesterase, weight and thickness by environmental complexity (e.g. Rosenzweig et al., 1962) have inspired nearly 60 years of ongoing research into the neurobiological effects of enriched environments. Generally, an enriched environment refers to housing methods for laboratory rodents that provide additional social, physical and cognitive stimulation, and thus opportunities for 'informal' learning experiences (Clemenson, Deng, & Gage, 2015; Hannan, 2014; Nithianantharajah & Hannan, 2006; Rosenzweig & Bennett, 1996; van Praag, Kempermann, & Gage, 2000; Will, Galani, Kelche, & Rosenzweig, 2004). Enriched environments elicit a broad array of effects in terms of neurogenesis, synaptic plasticity and neuroprotection across the lifespan. I briefly outline below my previous research on the CNP response to enriched housing (Rapley, Prickett, Dalrymple-Alford, & Espiner, 2018; Abstract and link provided in Appendix A), and work regarding enriched environments and the aging brain.

Based on previously outlined works implicating CNP in neuroplasticity, neuroprotection and new neuron growth, we posited that an enriched environment would provide excellent context to study CNP within brain tissue. Using the same design as that reported here (see Methods), my initial Master's thesis study (published alongside results from this study; Rapley et al., 2018) demonstrated that in young adult rats (8-9months old) CNP was present in higher concentrations throughout all regions of interest (outlined below) following a brief period (14 days) of enrichment, and that this increase in CNP occurred via a decrease in either degradative or clearance activity on the peptide. However, increased concentrations of CNP were unsustained after a longer period of enrichment (28 days) and this effect was weakest in hippocampus. This difference across time since enrichment initiation, suggested CNP is part of the early cascade of neuroplastic changes due to enriched housing. In addition to the major pattern of results (elevated CNP with concurrent ratio drop following 14 days of enrichment), NTproCNP concentrations were also altered in retrosplenial cortex, hippocampus and hypothalamus. In retrosplenial cortex and hypothalamus this related to the rehousing period. NTproCNP increased across time for

rodents housed in standard cages within retrosplenial cortex, and decreased across time for all rodents within hypothalamus. Within hippocampus NTproCNP was reduced by enrichment, regardless of rehousing period. This suggested that within these regions production of CNP is also a factor in the response to enriched housing, but that overall, degradative or clearance rate is a key determining factor in tissue concentrations of the peptide.

Critical considerations in design of the original experiment were length of enrichment exposure (Clemenson et al., 2015; J. Simpson & Kelly, 2011) and regions of interest (ROI) for analysis. Time periods of 14 and 28 days of housing were selected to represent both a short- and longer-term exposure, typical of many enrichment studies (See Simpson & Kelly, 2011 for review of enrichment durations). ROIs selected were medial prefrontal cortex, occipital cortex, retrosplenial cortex, dorsal hippocampus, hypothalamus and mammillary bodies. This selection was based on 1) demonstrated distribution of CNP and NPR-B receptors (Herman et al., 1996; Langub, Watson, et al., 1995); 2) Previously demonstrated effects of enrichment within these regions (Mohammed et al., 2002 for review); and 3) involvement of many of these regions in a neurological system underpinning spatial/episodic and recognition memory (Aggleton & Brown, 1999; Aggleton & Brown, 2006). These regions also provided a basis for potential ongoing research linking CNP, enriched housing and amelioration of cognitive deficits seen after brain injury or during aging. In addition, many of these regions show age-related changes, particularly the hippocampus (Burke & Barnes, 2010; Erickson & Barnes, 2003), and/or involvement in regulating anxiety and the HPA axis (Jankord & Herman, 2008). The purpose of the current study was to investigate CNP in the aging brain in the context of differing housing experiences.

Most enrichment studies use young or young-adult rats introduced to enrichment soon after weaning. As of 2011 only 2% of the literature examined animals aged over a year, representing enrichment onset at mid-life or later (Simpson & Kelly, 2011). Nevertheless, life-long enrichment can benefit age-related spatial, working and recognition memory impairments, counter loss of synaptic plasticity, and reduce anxiety and stress-reactivity of aged rats both behaviourally and neurologically (Kobayashi, Ohashi, & Ando, 2002; Leal-Galicia, Castañeda-Bueno, Quiroz-Baez, & Arias, 2008; Lores-Arnaiz et al., 2006; Lores-Arnaiz et al., 2004; Mora, Segovia, & del Arco, 2007; Segovia, Arco, Blas, Garrido, & Mora, 2008; Segovia, Del Arco, Garrido, de Blas, & Mora, 2008). Furthermore, life-long enrichment can prevent memory deficits in mouse models of Alzheimer's disease (Jankowsky, 2005) and reduce amyloid deposition (Lazarov et al., 2005).

Whereas life-long-enrichment provides greater benefits, short-term enrichment can also improve memory performance in aged rats (Kobayashi et al., 2002; Winocur, 1999). Introduction of enrichment at an advanced age promotes new neuron survival in the dentate gyrus, although this has not been consistently linked to better memory performance (Burke & Barnes, 2010; Kempermann et al., 1998; Segovia, Yag, & Mora, 2006 but see Kempermann, Gast, & Gage, 2002; Speisman et al., 2013 for concurrent memory improvement). Improvements in spatial memory occur in both male and female aged mice (Bennett, McRae, Levy, & Frick, 2006; Frick & Fernandez, 2003; Harburger, Lambert, & Frick, 2007) but neurobiological effects of enriched housing can vary across sexes during aging (Bennett et al., 2006; Frick & Fernandez, 2003; Kolb, Gibb, & Gorny, 2003). In aged male rats, enrichment enhances Synapsin I, glucocorticoid receptor expression, and astrocytic density and complexity within the hippocampal formation, all of which are additionally associated with better spatial memory and greater control of anxious responses (Sampedro-Piquero, De Bartolo, et al., 2014; Sampedro-Piquero, Arias, & Begega, 2014; Sampedro-Piquero, Begega, & Arias, 2014). These studies collectively indicate enrichment also provides a valid approach for investigating CNP during brain aging.

Based on previous work indicating neprilysin concentrations decline with age in a region specific manner, reductions of CNP degradation measures (NTproCNP:CNP ratio) were expected in aged rats throughout fronto-temporal regions due to reductions in neprilysin (Apelt et al., 2003; Fjell et al., 2014; Iwata et al., 2002). As this is the first study of its kind, no predictions could be made about how age or enriched housing may affect concentrations of CNP, or synthesis of the peptide measured by NTproCNP concentrations

3.2 Methods

3.2.1 Subjects

Thirty-six male PVGc hooded rats were used, weighing between 293 and 440g, and aged 22-23 months old at the start of enrichment. Prior to enrichment, all rats were housed in standard opaque plastic cages (45 cm x 27 cm x 22 cm high) in groups of three or four until the start of this experiment. All rats received food and water *ad libitum* and were behaviourally naïve (no previous experimental participation) until this point. Rats were accustomed to handling from regular cleaning and general care procedures, and this increased in the two weeks prior to the experiment start to become accustomed to the experimenter. On the first day of enrichment, 24 rats were re-housed in two enrichment cages (12 per cage) for either 14 or 28 days (1 cage per time-period; Enriched-14-day and Enriched-28-day). Twelve remaining rats were re-

housed with new cage mates in standard cages (3 rats per cage) for the same time periods (2 cages per time-period; 6 rats total per time-period; Standard-14-day and Standard-28-day). All rats were rehoused with novel cage mates. Rats were maintained on a reversed light-dark cycle (lights off 0800 – 2000h) and colony rooms were maintained at 22°C and 48%rH. Food and water were available *ad libitum*. All procedures conformed to the NIH guide for the care and use of laboratory animals and were approved by the University of Canterbury Animal Ethics Committee.

3.2.2 Enrichment

A standardised enrichment protocol developed at the University of Canterbury was used (Harland, Collings, McNaughton, Abraham, & Dalrymple-Alford, 2014; and see http://www.psyc.canterbury.ac.nz/Standardized%20Enrichment.shtml for details of, objects, arrangements and procedures). Enrichment cages were made of wire mesh with a sawdust covered metal floor and measured 85 cm x 60 cm x 30 cm high. Each day of enrichment consisted of a different combination of "junk" objects such as ceramic figurines, metal chains, PVC pipes and junctions and other small items, along with an ever-present wooden block to discourage chewing of enrichment objects. Enrichment configurations differed over 40 different days (though a maximum of 28 was used here) and ensured no object was repeated within five days of itself. On every seventh day, PVC pipes and junctions were presented in a "tubing only" day, and on every eighth day, all objects (except the wooden blocks) were removed from the cage. Additionally, food and water positions were changed daily, and cages were rotated through one of four possible positions in the colony room every fourth day. Objects were changed at the start of the dark period (between 0900 and1000h), during which rats from one enrichment cage were held together in a large opaque plastic cage (62 cm x 40 cm x 22cm high).

3.2.3 Sacrifice and Tissue extraction

After either 14 or 28 full days of enrichment, or the same period of standard housing with new cage mates, rats were placed in standard cages at the same time enrichment objects were usually changed and held in a novel, dimly lit room separate to both the colony room and tissue dissection room. Rats from Enriched and Standard Housing were euthanised throughout a single day, with two Enriched rats sacrificed per one Standard Housed, and order of sacrifice randomised within these groups of three. Rats were deeply anaesthetised with an overdose of Sodium Pentobarbitone (1mL, 300mg/mL, ip). Once unresponsive to

both tail pinch and plantar reflex (approximately 5 minutes following injection), with no discernible heartbeat, rats were decapitated and brains rapidly removed from the skull and placed in a brain matrix (Ted Pella). An initial coronal cut was made at the level of the optic tract. Additional coronal cuts were made 5mm anterior and posterior to the initial cut, resulting in two 'slabs' of fresh tissue (see Figures 3.1 & 3.2 for approximate location of the anterior face of each slab, and 3.3 for posterior extent). These tissue slabs were placed anterior-face-upwards on a glass petri dish, previously sterilised with 70% ethanol and rinsed with saline. Sterilisation and rinsing was repeated between rats. Microdissection scissors were used to acquire tissue samples from seven regions of interest, starting in the posterior slab, in the order: occipital cortex, retrosplenial cortex (containing both dysgranular and granular B tissue), dorsal hippocampus (left and right hemispheres separately), mammillary bodies, hypothalamus and medial prefrontal cortex. Tissue samples were placed in pre-weighed Eppendorf tubes, weighed, and snap frozen with liquid nitrogen, within 15 minutes post-sacrifice, before long-term storage at -80°C.

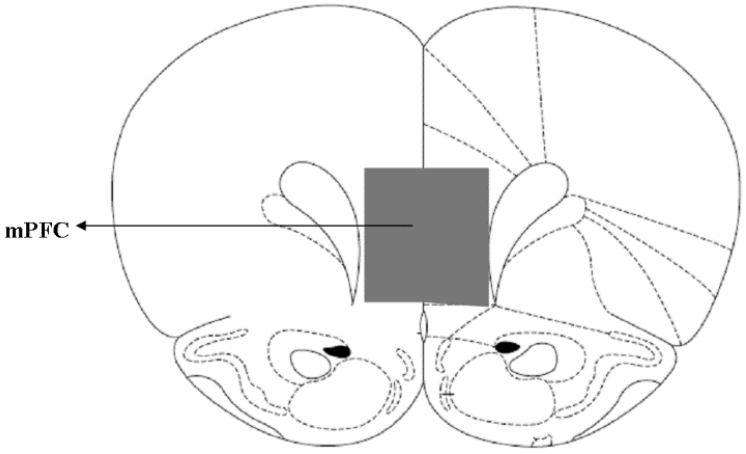


Figure 3.1: Approximate location of anterior face from anterior 'slab' of tissue. Tissue sampled for peptide measures in prefrontal cortex (mPFC) is indicated by gray box. All figures adapted from Paxinos & Watson (2009).

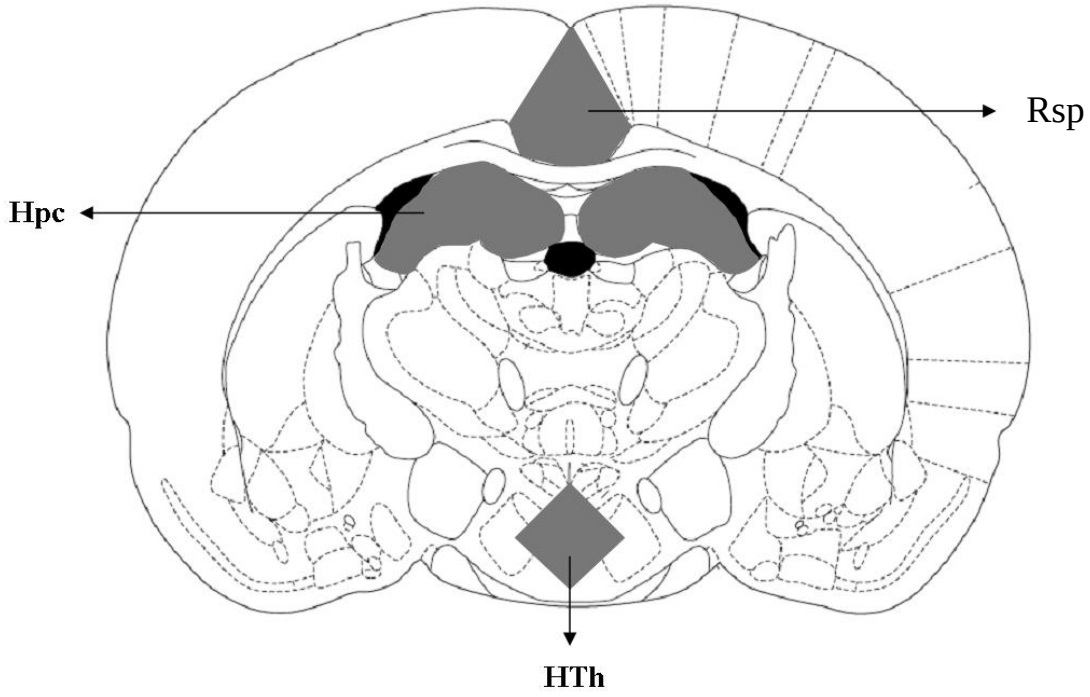


Figure 3.2: Approximate location of anterior face from posterior 'slab' of tissue. Tissue sampled for peptide measures in hypothalamus (HTh), dorsal hippocampus (Hpc) and retrosplenial cortex (Rsp) are indicated in gray.

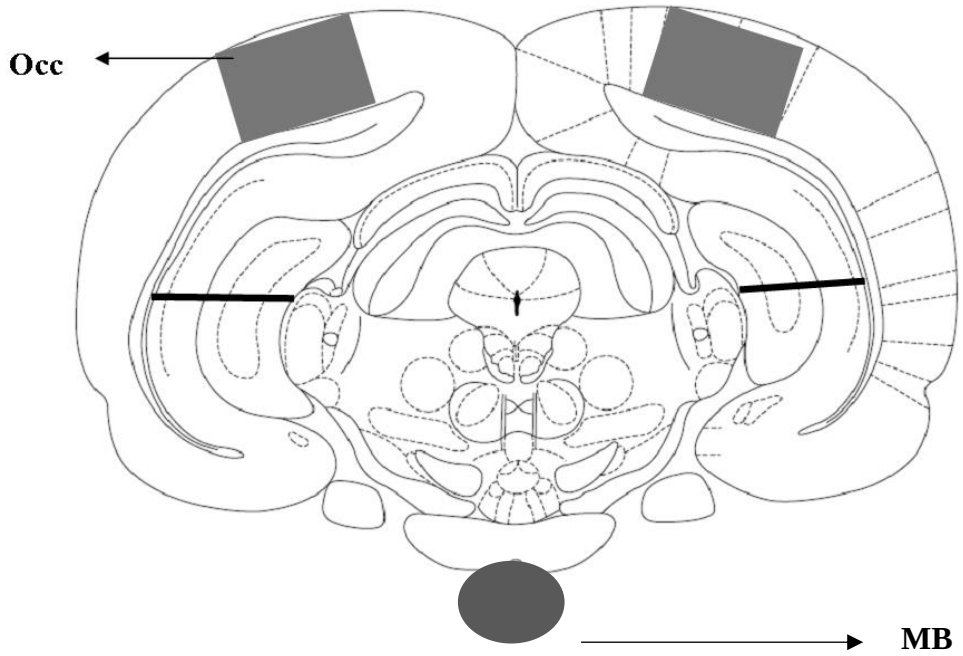


Figure 3.3: Posterior extent of poterior tissue slab. Furthest extent of tissues sampled from occipital cortex (Occ) and mammillary bodies (MB) are indicated in gray, and for dorsal hippocampus by black lines

3.2.4 Tissue preparation and peptide measurements

CNP and NTproCNP were measured by radioimmunoassay (RIA), described in detail in Yandle et al (1993; CNP) and Prickett et al (2012; NTproCNP). Prior to assay, frozen tissue samples were transferred to scintillation vials and kept frozen on dry ice, then held at 98°C in a water bath for 5 minutes in 10mL of distilled water containing 0.01% Triton X-100. Samples were acidified with 610µL glacial acetic acid and homogenised prior to extraction on Sep-Pak C18 cartridges (Waters Corporation, Milford, MA, USA). Following extraction, samples were dried under an air stream and frozen for later re-suspension in assay buffer for RIA. All tissues from an individual rat were processed in the same extraction, with tissues from housing groups counterbalanced across extractions.

3.2.5 CNP assay

As mentioned previously, CNP is identical in all mammals studied thus far. The amino terminal extended form CNP-53, containing the 17-amino acid ring essential for bioactivity, has 100% cross reactivity with CNP 22 in the assay used to measure CNP. Antiserum to CNP-22 (Phoenix pharmaceuticals, Belmont, CA) was diluted 1:2000 with assay buffer. Labelled CNP was prepared by chloramine-T iodination of [Tyr0] CNP-22 (Peninsula Labs), and purified by reverse-phase HPLC. Fifty microliters each of antiserum and CNP standard (0.7 – 235 pmol/L) or sample extract (all in duplicate) were mixed and incubated for 22-24h at 4°C, followed by addition of 50µL labelled CNP containing ~3000 cpm for 22-24h at 4°C. Bound and free labelled CNP were separated by a solid-phase secondary antibody method (Sac-cell, Rabbit-Anti Goat, IDS Ltd., England). CNP assays had a detection limit of 0.6 pmol/L and ED50 of 7.3 pmol/L; intra- and inter-assay coefficients of variation were 5.9 and 7.4%, respectively, at 17 pmol/L.

3.2.6 NTproCNP assay

For NTprocCNP an in-house 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 et al., 2012). Fifty microliters of sample extract or 0.5-372 pmol/L proCNP(38-50) standards (again in duplicate) were incubated with 50µL antiserum for 22-24h, followed by addition of 50 µL tracer solution (proCNP(38-50)-[125I]Tyr37) containing ~3000 cpm for 22-24h at 4°C. Bound and free labelled proCNP were separated by solid-phase second antibody method (Sac-cell, Donkey-Anti Rabbit, IDS Ltd., England). NTproCNP assays had a detection limit

of 0.4 pmol/L and ED50 9.9 pmol/L; intra- and inter-assay coefficients of variation were 6.8 and 7.5%, respectively, at 45 pmol/L.

3.2.7 Statistical Analyses

CNP and NTproCNP concentrations were expressed as femtomoles per gram of wet tissue (fmol/g). NTproCNP:CNP ratio was calculated by dividing NTproCNP concentration by CNP concentration. In aged rats, a portion of one sample from the hypothalamus in the Enriched-28-day group was lost, and data subsequently excluded. Two tissue samples had NTproCNP concentrations beyond the detection limit of the assay: one from mammillary bodies in the Enriched-14-day group and one from hypothalamus in the Enriched-28-day group. For these two samples, the NTproCNP:CNP ratio could not be calculated, and CNP concentrations were also excluded from analysis. Thus, sample Ns for aged rats are reduced for each of these regions (mammillary bodies and hypothalamus). For all other regions of interest, final Ns for aged rats were: Enriched-14-day, N = 12; Enriched-28 day, N = 12; Standard-14 day, N = 6; Standard-28 day, N = 6. Data from young rats is available in Rapley et al, 2018 (link provided in Appendix A) with reference made to these data for across-age comparisons. Initial analysis indicated there were no differences in any measure (CNP, NTproCNP or ratio) between left and right hemispheres of the dorsal hippocampus. Data was averaged across hemispheres for each rat and analysed as a single value. Because multiple effects were identified in young rats (Rapley et al, 2018), Null Hypothesis Significance Testing was inappropriate for data gathered from aged rats. Cohen's d effect sizes (±95% CI) were calculated on specific mean differences using 20% trimmed means to enable comparison with data from young rats (Rapley et al, 2018). This also accounted for outlying data points, which represented different rats in each case, meaning individual exclusions of outlier were unnecessary.

3.3 Results

3.3.1 NTproCNP

NTproCNP concentrations did not vary across conditions in occipital cortex, medial prefrontal cortex, retrosplenial cortex or hippocampus (Figures 3.4 – 3.7, top panels, all p's > .05). In the mammillary bodies (Figure 3.8), NTproCNP concentrations increased across the rehousing period, for rats housed in Standard cages only (Standard-14-days vs Standard-28-days d = 1.44 [0, 2.88], p = .05). In hypothalamus, NTproCNP concentrations were lower in

rats rehoused in Standard cages for 14 days than those rehoused in Enrichment for 14 days (Standard-14-days vs Enriched-14-days d = 1.66 [0.45, 2.87], p = .01).

3.3.2 CNP

CNP concentrations did not vary across conditions in occipital cortex, retrosplenial cortex, mammillary bodies, hippocampus or hypothalamus (Figures 3.4, 3.6 – 3.9, middle panels; all p's > .05). In medial prefrontal cortex, CNP concentrations were higher following 14 days of rehousing than at 28 days of rehousing, regardless of housing environment (Figure 3.6 middle panel; 14-days vs 28-days d = 0.68 [-0.01, 1.38] p = .05).

3.3.3 Ratio of NTproCNP:CNP

Ratios of NTproCNP:CNP did not vary in medial prefrontal cortex, retrosplenial cortex, hippocampus or hypothalamus (Figures 3.5 – 3.7, 3.9, all p's > .05). Ratio values increased across the rehousing period for rats housed in Standard cages only in both occipital cortex (Standard-14-days vs Standard-28-days d = 1.95 [0.39, 3.51] p = .02) and mammillary bodies (Standard-14-days vs Standard-28-days d = 2.13 [0.52, 3.74], p = .01).

3.3.4 Comparisons to Young rats

A key aspect in understanding data from aged rats, is a comparison to concentration measures from young rodents. These results are included here, from Rapley et al, 2018. All data used cohen's d [±95%CI] mean comparisons. For NTproCNP data from both Young and Aged rats did not vary substantially, so was pooled and compared directly. For CNP and NTproCNP:CNP ratio, pooled data from Aged rats was compared with either Young-Enriched-14-day rats, or a pooled mean of all other groups because of substantive differences in Young-Enriched-14-day rats. This was modified slightly for CNP measures in hippocampus and medial prefrontal cortex due to effects therein (see below).

NTproCNP concentrations were higher in Aged rats in occipital cortex (d = 0.62 [0.14, 1.11], p = .01) and lower than Young rats in medial prefrontal cortex (d = 0.62 [0.14, 1.10], p = .01). NTproCNP concentrations were equivalent across ages in all other ROIs (retrosplenial cortex, hippocampus, mammillary bodies and hypothalamus). For Aged rats overall, concentrations of CNP were lower than the reported peak in Young-Enriched-14-day rats in occipital cortex, retrosplenial cortex, mammillary bodies and hypothalamus (see Table 3.1 for between group comparisons). Of these regions, CNP concentrations were higher in Aged rats compared to other young rats in occipital and retrosplenial cortices (Table 3.1). In hippocampus of Young rats, CNP was affected by rehousing period, necessitating

comparisons reflecting this. In Aged rats overall, concentrations were equivalent to Young-14-day rats (d = 0.12 [-0.46, 0.47], ns) but higher than Young rats rehoused for 28 days (d = -1.01 [[-1.62, -0.4], p < .001). Because of the difference identified in medial prefrontal cortex across the rehousing period for Aged rats, data was pooled accordingly for this comparison. Concentrations of CNP in Aged medial prefrontal cortex were lower than the reported peak in Young-Enriched-14-day rats (Aged-14-day vs Young-Enriched-14-day d = -0.76 [-1.54, 0.03], p = .06; Aged-28-day vs Young-Enriched-14-day d = -1.46 [-2.31, -0.6], p < .001). Additionally, CNP concentrations in medial prefrontal cortex of Aged rats rehoused for 14 days was higher than all other Young rats (d = 0.89 [0.23, 1.55], p = .01), but there was no difference between Aged-28-day rats and other Young rats (d = 0.23 [-0.4, 0.86], p = .47). NTproCNP:CNP ratio was lower in Aged rats than Young rats (excluding Young-Enriched-14-day rats) in occipital, medial prefrontal and retrosplenial cortices and hypothalamus, but not in mammillary bodies or hippocampus (Table 3.1). However, this ratio reduction was higher than that reported in Young-Enriched-14-day rats in occipital cortex, retrosplenial cortex, hippocampus and mammillary bodies (Table 3.1)

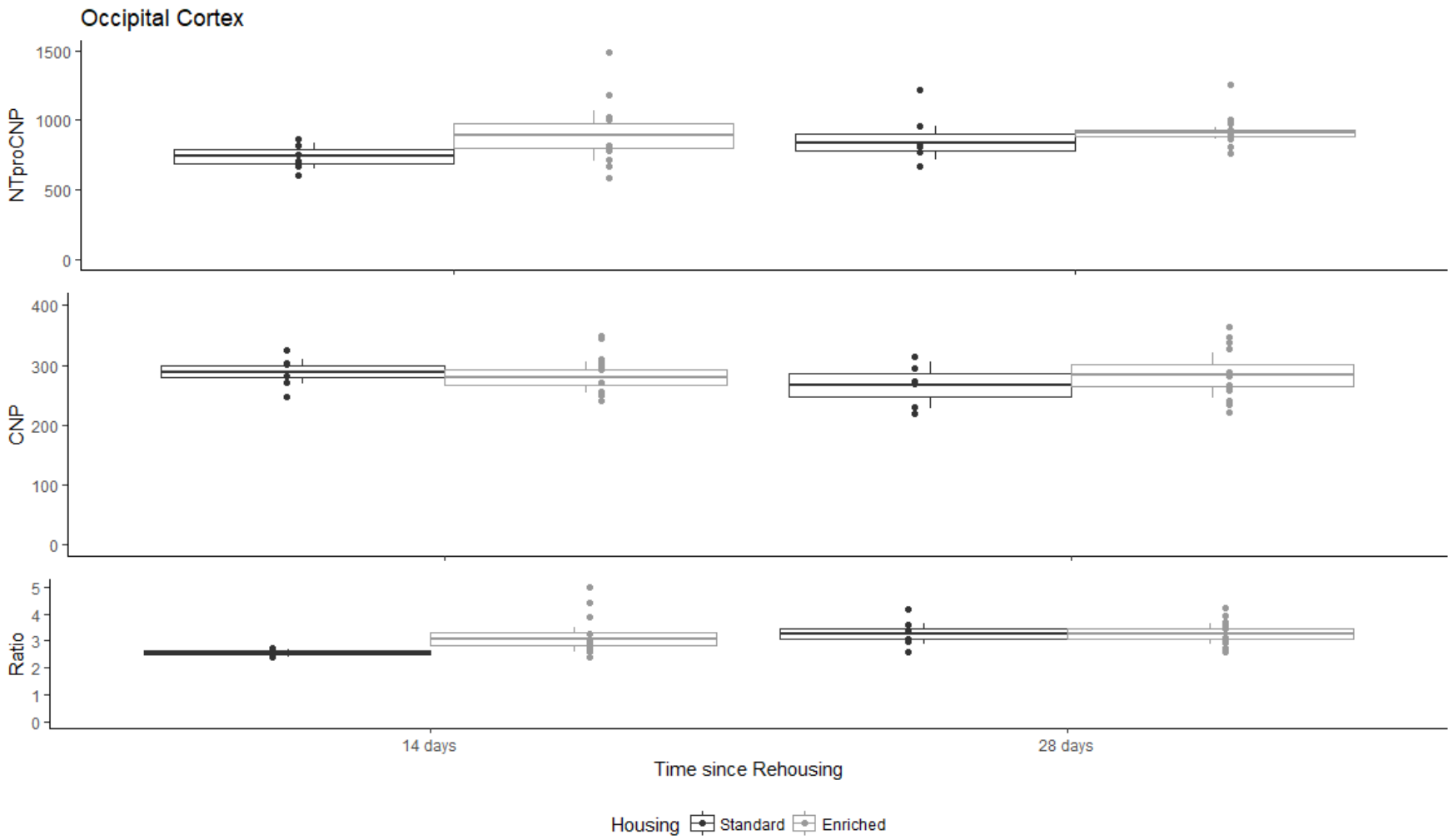


Figure 3.4: Concentration (femtomoles/g wet tissue) of NTproCNP (top), CNP (middle) and NTproCNP:CNP ratio (bottom) in occipital cortex. Summary data is 20% trimmed means (reduction of n = 2 for all groups), standard error (box) and 95% confidence interval (whisker) overlaid by individual data points. For NTproCNP:CNP Ratio Standard-14-days < Standard-28-days; All other comparisons ns.

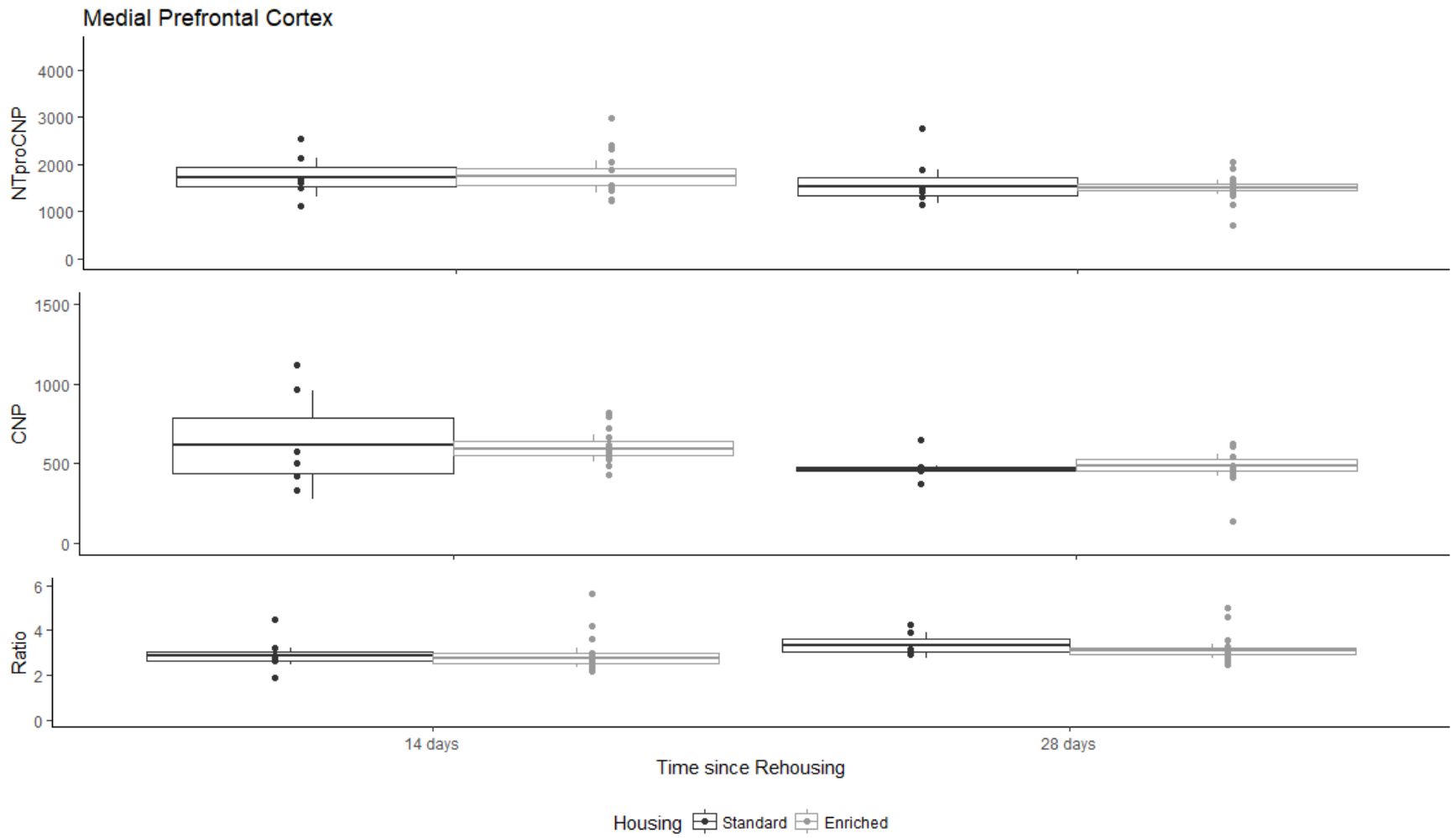


Figure 3.5: Concentration (femtomoles/g wet tissue) of NTproCNP (top), CNP (middle) and NTproCNP:CNP ratio (bottom) in medial prefrontal cortex. Summary data is 20% trimmed means (reduction of n = 2 for all groups), standard error (box) and 95% confidence interval (whisker) overlaid by individual data points. For CNP 14-days > 28-days; All other comparisons ns.

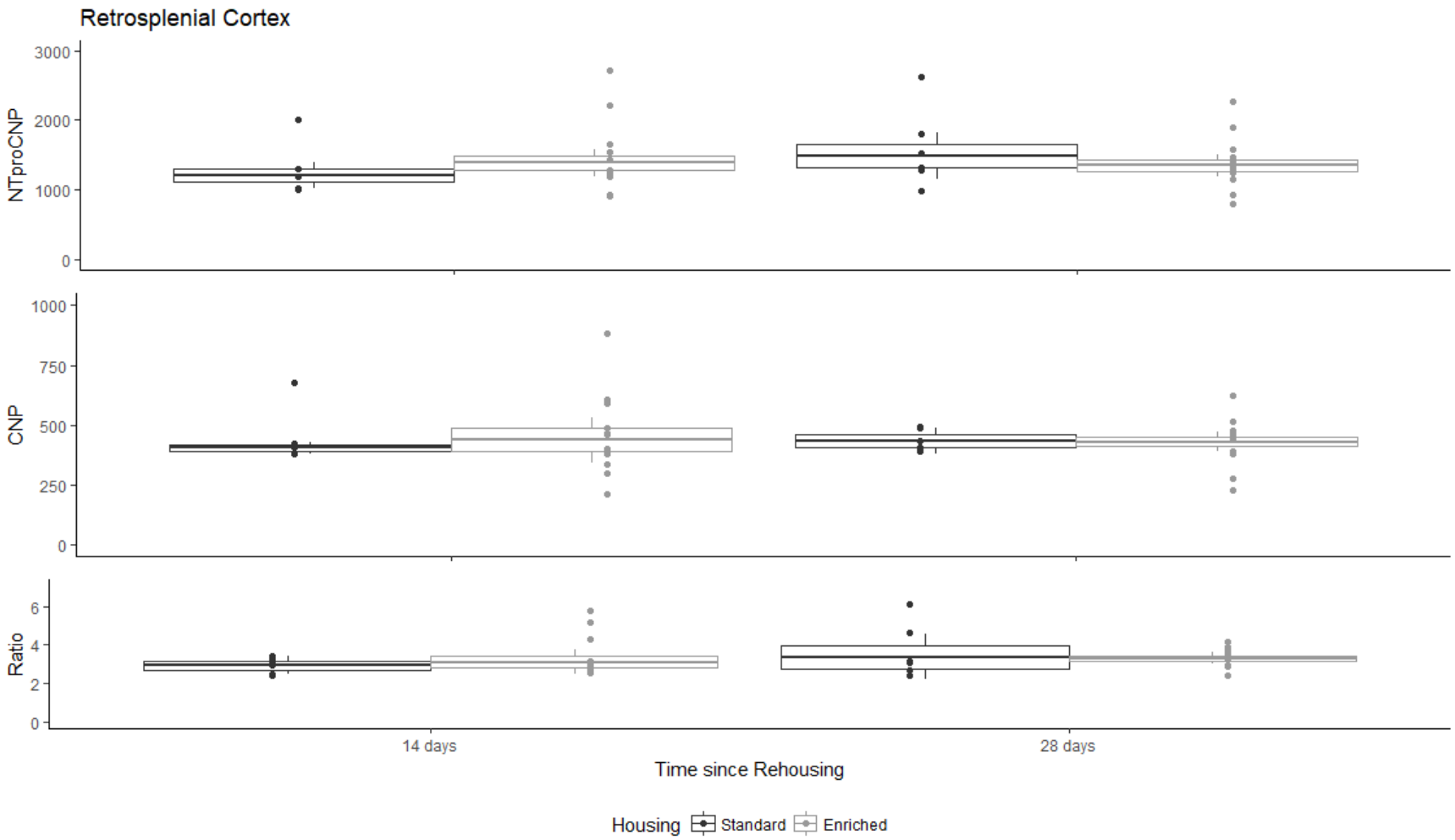


Figure 3.6: Concentration (femtomoles/g wet tissue) of NTproCNP (top), CNP (middle) and NTproCNP:CNP ratio (bottom) in retrosplenial cortex. Summary data is 20% trimmed means (reduction of n = 2 for all groups), standard error (box) and 95% confidence interval (whisker) overlaid by individual data points. All comparisons ns.

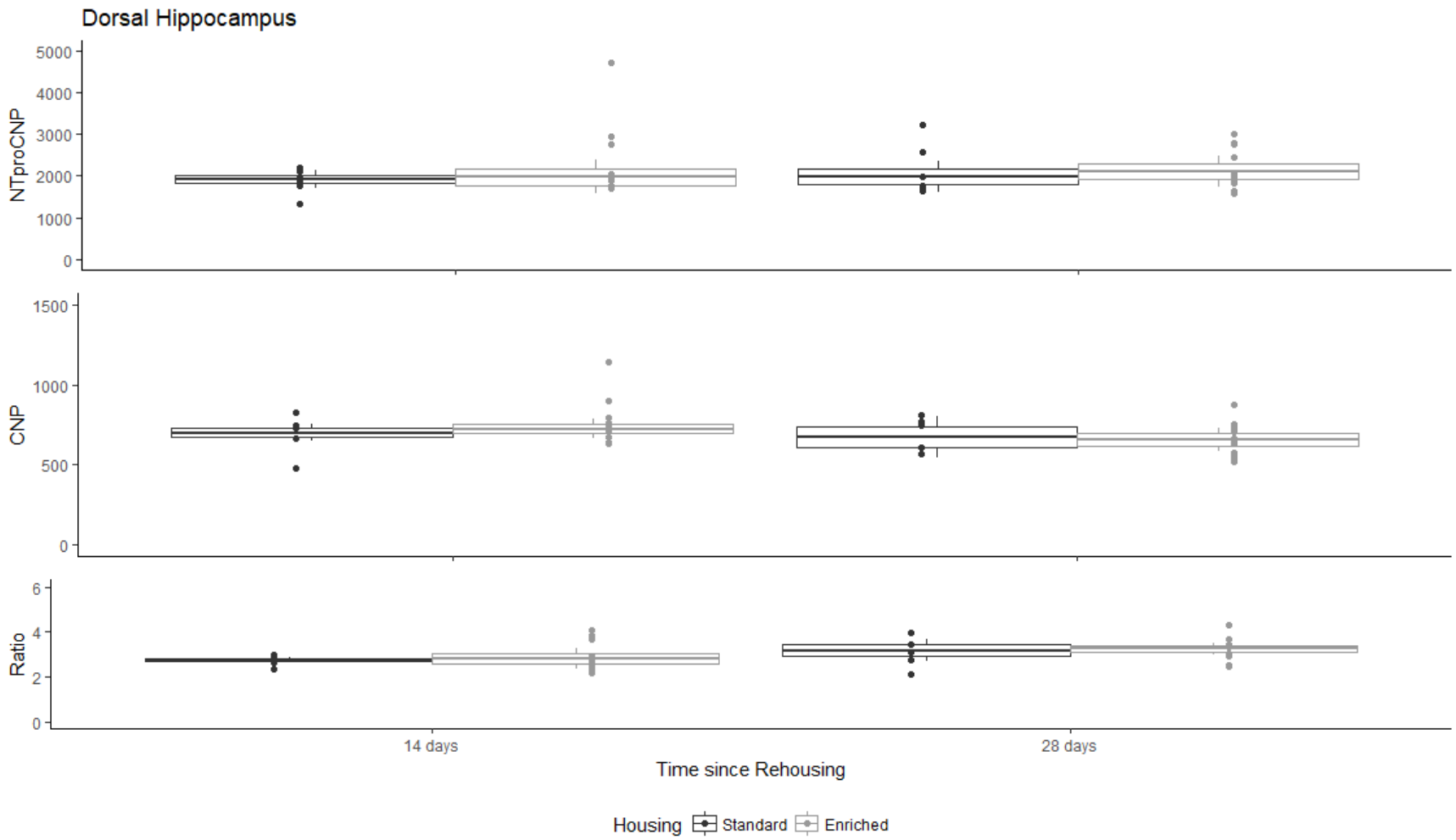


Figure 3.7: Concentration (femtomoles/g wet tissue) of NTproCNP (top), CNP (middle) and NTproCNP:CNP ratio (bottom) in dorsal hippocampus. Summary data is 20% trimmed means (reduction of n = 2 for all groups), standard error (box) and 95% confidence interval (whisker) overlaid by individual data points. All comparisons ns.

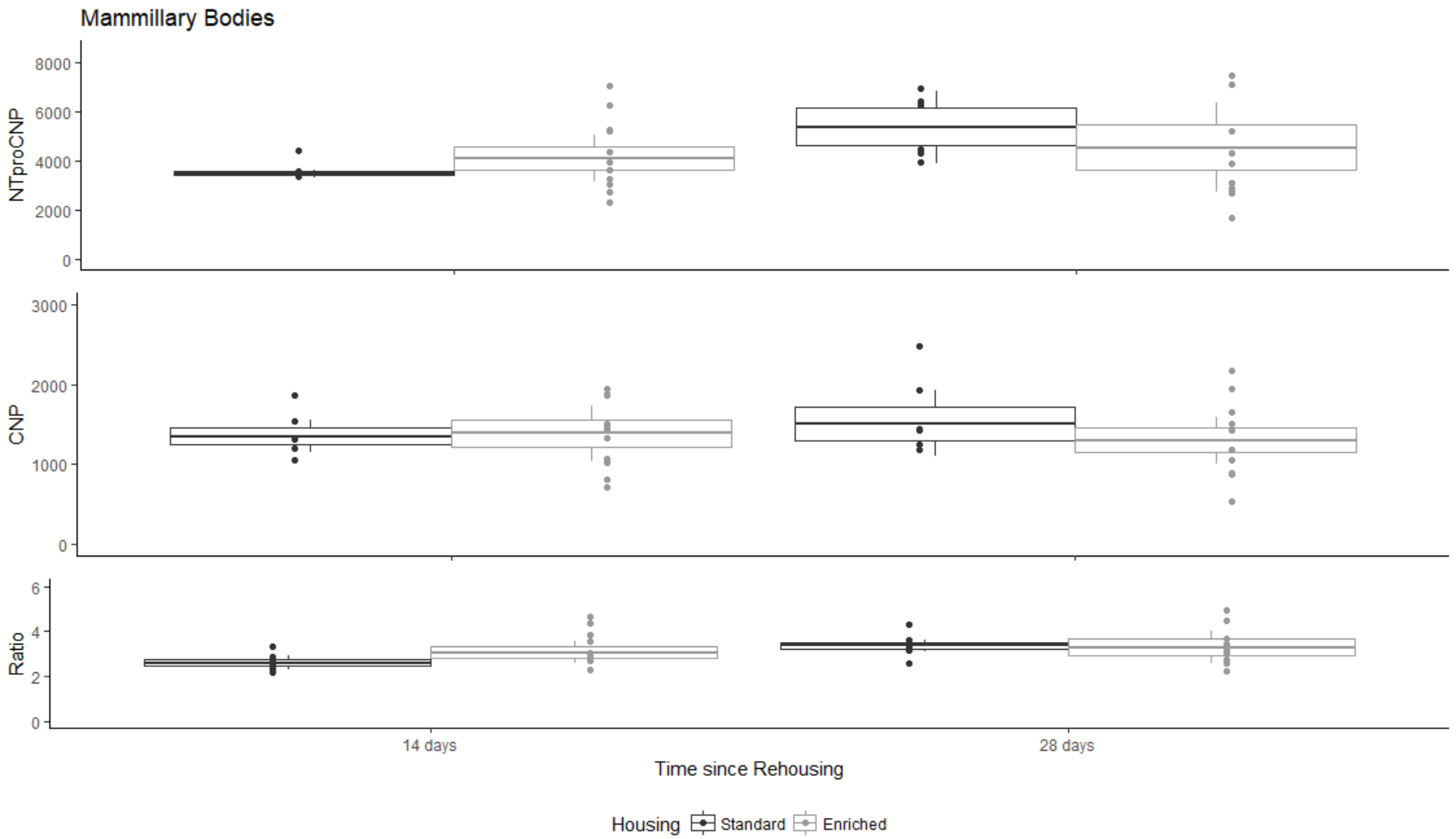


Figure 3.8: Concentration (femtomoles/g wet tissue) of NTproCNP (top), CNP (middle) and NTproCNP:CNP ratio (bottom) in mammillary bodies. Summary data is 20% trimmed means (reduction of n = 2 for all groups), standard error (box) and 95% confidence interval (whisker) overlaid by individual data points. For NTproCNP Standard-14-days < Standard-28-days. For NTproCNP:CNP ratio Standard-14-days < Standard-28-days. All other comparisons ns.

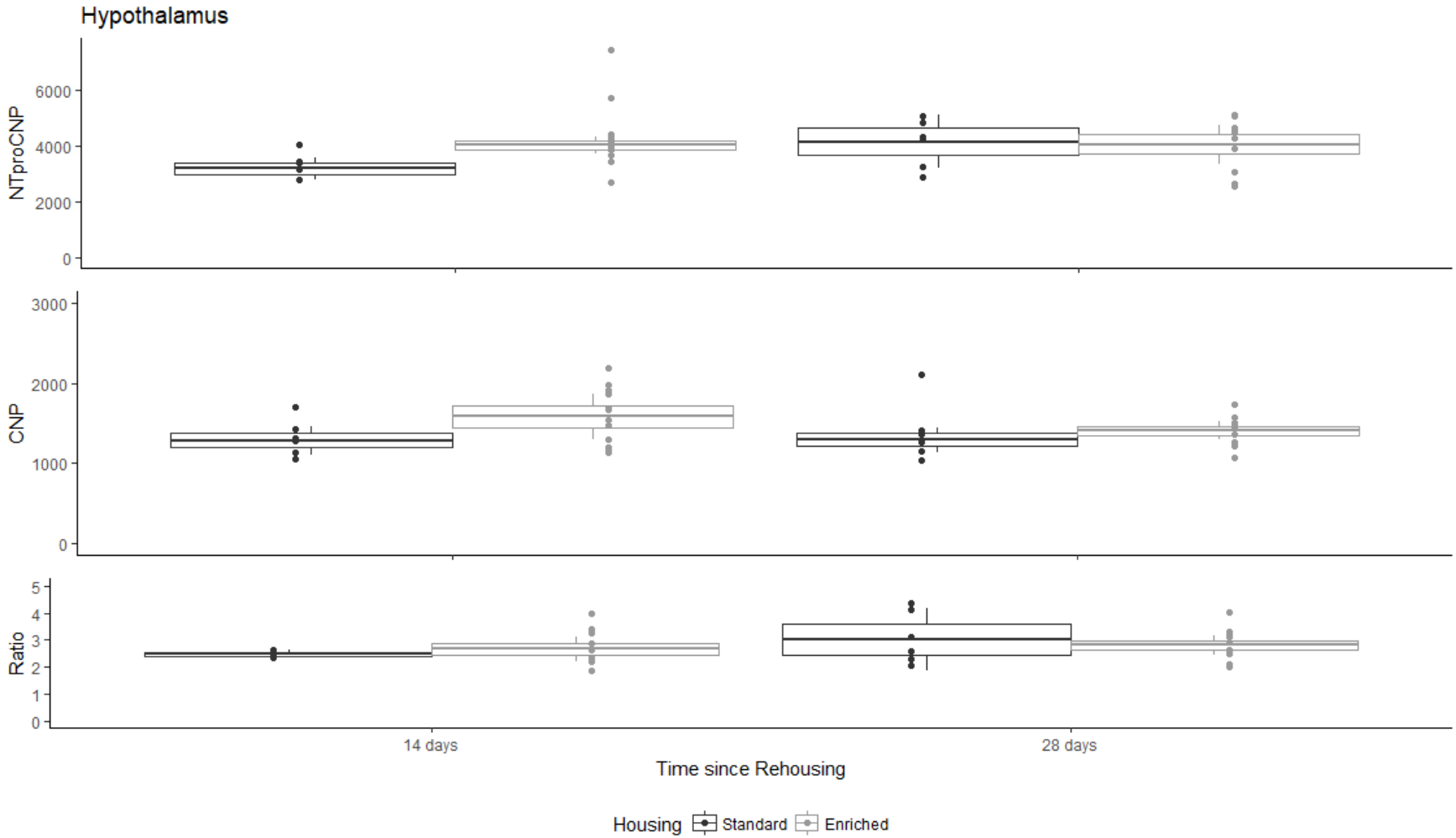


Figure 3.9: Concentration (femtomoles/g wet tissue) of NTproCNP (top), CNP (middle) and NTproCNP:CNP ratio (bottom) in hypothalamus. Summary data is 20% trimmed means (reduction of n = 2 for all groups), standard error (box) and 95% confidence interval (whisker) overlaid by individual data points. For NTproCNP Standard-14-days < Enriched-14-days. All other comparisons ns.

Table 3.1: Between group comparisons (cohen's d [±95% CI]) for CNP concentrations and NTproCNP:CNP ratio in aged rats overall versus Young-Enriched-14-day rats or all other Young groups within all regions of interest (see text for hippocampus and medial prefrontal cortex CNP). EE: Enriched Environment; SH: Standard Housed; Other Young indicates pooled Young data, excluding Young-Enriched-14-day rats. p < .001 *** p < .01 ** p < .05 *. Originally published in Rapley et al, 2018.

Region	Aged Rats Overall	CNP	NTproCNP:CNP ratio
Occipital Cortex	Young EE 14 vs	d = 1.22 [0.48, 1.96]***	d = -1.33 [-2.07, -0.58]***
	Other Young vs	d = -1.57 [-2.17, -0.97]***	d = 0.9 [0.34, 1.45]***
Medial Prefrontal Cortex	Young EE 14 vs		d = -0.63 [-1.32, 0.05] ns
	Other Young vs		d = 1.52 [0.92, 2.11]***
Retrosplenial Cortex	Young EE 14 vs	d = 1.31 [0.59, 2.03]***	d = -1.0 [-1.70, -0.30]**
	Other Young vs	d = -1.13 [-1.71, -0.56]***	d = 1.24 [0.66, 1.82]***
Hippocampus	Young EE 14 vs		d = -0.84 [-0.53, 0.15]*
	Other Young vs		d = 0.45 [-0.08, 0.99] ns
Mammillary Bodies	Young EE 14 vs	d = 1.75 [0.99, 2.52]***	d = -1.21 [-1.93, -0.49]***
	Other Young vs	d = 0.30 [-0.24, 0.83] ns	d = 0.15 [-0.39, 0.68] ns
Hypothalamus	Young EE 14 vs	d = 1.21 [0.49, 1.94]***	d = -0.13 [-0.81, 0.55] ns
	Other Young vs	d = -0.45 [-0.99, 0.09] ns	d = 0.77 [0.21, 1.32]**

3.4 Discussion

Recent work has suggested CNP may be an important target molecule with respect to age-related cognitive decline, and neurological disorders featuring cognitive impairment (Espiner et al., 2014; Koziorowski et al., 2012; Mahinrad et al., 2016). However, to date there has been no investigation of CNP in aging cerebral tissue. This study aimed to address this research gap by investigating CNP response to enriched housing conditions in aged rats and comparing with data previously acquired from young rats. A major aim was to provide a starting point for ongoing research testing the hypothesis that CNP is involved in neurological changes that occur during aging. This was also important given few previous studies have investigated tissue concentrations or degradative regulation of CNP within cerebral tissue. Previous work with young rats (8-9 months) showed that CNP was available in higher concentrations throughout six regions of interest, following a brief period of enriched housing (14 days), and that this greater availability of CNP occurred via reductions in either degradative (for example by neprilysin) or clearance (via NPR-C) activity (Rapley et al., 2018). This indicated that for young rats, CNP is likely to form part of the early cascade of molecular neuroplasticity effects previously demonstrated following enriched housing. Both studies used the same design, with the current study initiating enrichment at approximately 23 months of age.

For aged rats overall, concentrations of CNP in occipital cortex and hypothalamus were similar to previous reports in adult sheep (Pemberton et al., 2002; Wilson et al., 2017), rodents (Jankowski et al., 2004) and humans (Komatsu et al., 1991; Totsune et al., 1994). NTproCNP concentrations were approximately half of those reported in ovine cerebral tissue (Wilson, McNeill, Barrell, Prickett, & Espiner, 2017). Concentration of CNP in hippocampus and mammillary bodies was also similar to those reported, with similarly reduced NTproCNP in these regions (Wilson et al., 2017). Concurring with previous reports, highest CNP concentrations occurred in hypothalamus and mammillary bodies, with moderate concentrations in prefrontal, hippocampal and retrosplenial tissue, and lowest concentrations in occipital cortex. Minimal modifications to CNP, NTproCNP and the degradative ratio were identified in aged rats, all related to the rehousing period rather than enrichment *per se*. In hypothalamus, mammillary bodies and occipital cortex, modifications to NTproCNP and the NTproCNP:CNP ratio across the rehousing period were restricted to rats rehoused in standard cages. This indicates a reaction which may be ameliorated by enrichment within these regions. Modifications to CNP concentrations in aged medial prefrontal cortex occurred

regardless of type of rehousing. Most notable for aged rats was the markedly different response of CNP and its signalling system when compared to young rats.

3.4.1 Response to Enrichment

A subtle enrichment effect occurred in occipital cortex, mammillary bodies and hypothalamus of aged rats. In mammillary bodies, significantly increased peptide production (NTproCNP) across time was paired with significantly increased degradative activity, for rats housed in standard cages only. Within occipital cortex, the same pattern occurred, but variation in NTproCNP was non-significant (Figure 3.4). This pairing of increased production with increased degradative activity results in stable active peptide concentrations. Within hypothalamus, peptide production was decreased after an initial period of 14 days of rehousing, resulting in marginal (but again non-significant) variation in CNP, with no apparent variation in degradative ratio. Interestingly, this pattern in hypothalamus was the only mild similarity to the response to 14 days of enrichment seen in young rats. Certain hypothalamic nuclei are reported to be stable during brain aging, with some exhibiting increases in activity (Coleman & Flood, 1987; Hofman, 1997; Lucassen, Ravid, Gonatas, & Swaab, 1993; Roberts, Killiany, & Rosene, 2012; Swaab & Bao, 2011; Swaab, Goudsmit, Kremer, Hofman, & Ravid, 1992). That this was the only ROI in aged rats with slight elevations to CNP following 14 days of enriched housing suggests there may also be a certain amount of stability in the CNP signalling system within hypothalamus during aging. As multiple nuclei were included within the tissue sampled, this will require more focussed examination.

Subtle changes to peptide production and degradative activity in standard housed rats, which is apparently modified by enriched housing represents a stark contrast with the clear enrichment response seen in young rats, wherein higher concentrations of CNP following a brief period of enrichment were accounted for by reductions of degradation or clearance and were widespread (Rapley et al., 2018). This suggests that, while degradative activity continues to be an important contributor to regulating CNP concentrations within cerebral tissue across aging, peptide production (as measured by NTproCNP) also gains a degree of importance. Peptide production changes in response to external stimulus may represent a compensatory mechanism during aging to maintain peptide activity in the face of generally depleted degradative activity (see below). It is additionally possible that this initiation of enrichment later in life has been less effective than earlier initiation of enrichment for a

longer period in stimulating bioactive CNP for these rats – a concept supported by previous investigation of short-term, late-initiated enrichment which is expanded upon in Chapter 6.

3.4.2 Rehousing period and Anxiety

As in the regions outlined above, modifications to CNP concentrations in medial prefrontal cortex across the rehousing period were accompanied by non-significant modifications to NTproCNP, rather that degradative activity (Figure 3.5), but was not affected by enriched conditions. Whereas in aged rats, modifications due to enrichment were subtle – because above outlined modifications were restricted to rats in standard housing – the opposite was the case in young rats. For young rats, an effect on CNP concentrations following a brief rehousing period in enrichment was clear but more subtle effects on NTproCNP occurred across the rehousing period within hypothalamus and retrosplenial cortex (Rapley et al., 2018). Because the general pattern in aged rats relates more to the rehousing period, and CNP has been previously related to anxiety regulation and behaviours (Bíró et al., 1996b; Jahn et al., 2001; Montkowski et al., 1998; Telegdy et al., 2000, 1999) this indicates a relationship with anxiety induced by rehousing with new cage mates. Moreover, it is likely that this is an initial response to the rehousing stressor for rodents rehoused in standard cages for a brief period because in rodents given 28 days of adjustment to rehousing in standard cages, concentrations of CNP or NTproCNP more closely resemble those seen in enriched animals.

Though this was not explicitly tested here with behavioural tasks, several previous findings are supportive of this idea. Medial prefrontal cortex and limbic system structures participate in regulation of the HPA axis (Herman, Ostrander, Mueller, & Figueiredo, 2005). Changes to HPA axis signalling and glucocorticoid feedback are thought to contribute to neurological and behavioural changes during aging (Mizoguchi et al., 2009; Swaab & Bao, 2011). Additionally, environmental enrichment has been previously shown to modify neuroendocrine and neurotransmitter related stress responses in both young and aged rats (Moncek, Duncko, Johansson, & Jezova, 2004; Segovia, Del Arco, De Blas, et al., 2008; Segovia, Del Arco, Garrido, et al., 2008; Segovia, Del Arco, & Mora, 2009). The current observation of a response in the CNP system relative to a stressful event, within pertinent neurological regions (particularly hypothalamus and medial prefrontal cortex), which can be modified by a brief enrichment period is highly suggestive that effects in standard caged rats across the rehousing period relate to CNP regulation of the HPA axis and an initial anxiety response in rats rehoused for only 14 days.

Because of this notably different effect in aged rats this may reflect age-related HPA axis dysregulation associated with the CNP signalling system. Dysregulation of HPA axis occurs during aging and modifies feedback in the glucocorticoid system (Mizoguchi et al., 2009; Swaab & Bao, 2011). Moreover, increased concentrations of CNP seen in occipital, retrosplenial and medial prefrontal cortices of aged rats (see below) may be associated with a shift toward LTD in these regions (Decker et al., 2009, 2010; Decker et al., 2008) which occurs more readily during aging. This is also associated with stress impairment of hippocampal learning and memory functions (Burke & Barnes, 2010; J. J. Kim & Diamond, 2002) wherein some accumulation of CNP was also seen with age. A potential involvement of CNP with age related HPA axis dysregulation also deserves consideration as modifications in these circuits are associated with executive dysfunction during aging (Buckner, 2004). Some clarity in interpreting this relationship could have been provided here by including a "zero day" group – rats of the same age, sacrificed at the start of rehousing. This would have provided some indication whether overall effects in aged rats related to the rehousing period definitely represent an initial response due to the anxious event of rehousing, or continuing gradual modifications to the signalling system over time. Additionally, this would account for the limitation – in comparing to young rats – that aged rats were housed in standard conditions for a much longer period before initiation of enrichment. As stated above, this may contribute to the relative lack of response to enrichment in aged rats and is expanded on in Chapter 6. However, because the overall response generally relates to the period of rehousing, this now encourages further investigation into the relationship between CNP, anxiety and aging.

3.4.3 General effects of Age

Both NTproCNP and CNP concentrations increased with age in occipital cortex accompanied by reduced degradative activity. Peptide production was reduced in medial prefrontal cortex which was also accompanied by a general reduction in degradative activity. In this region, increased CNP compared with young rats only occurred following 14 days of rehousing. CNP concentrations were also higher in the aged retrosplenial cortex in combination with reduced degradative activity. Unlike young rats, no modifications were seen in retrosplenial NTproCNP concentrations in response to the rehousing period or environment. The reduction in degradative ratio within these regions was as hypothesised and of a different magnitude to the drop seen in young rats housed in enrichment for 14 days. However, this did not occur in either mammillary bodies or hippocampus. This hypothesis was based on prior work indicating the CNP proteolytic enzyme, neprilysin, reduces with normal brain aging in a

region-specific manner – notably fronto-temporal regions including cortex, hippocampal DG and thalamus (Hellström-Lindahl et al., 2008; Higuchi et al., 2005; Iwata et al., 2001; Iwata et al., 2002; Russo et al., 2005; Yasojima et al., 2001; Yasojima, McGeer & McGeer, 2001). It was therefore highly surprising that degradative regulation of CNP was maintained across aging in hippocampal tissue particularly.

Within hippocampus, this could be due to subregional differences in peptide degradation. Neprilysin activity is reportedly reduced in DG but maintained in CA1 and CA3 (Iwata et al., 2002). Feasibly, modifications within a single subregion may be masked by inclusion of all regions in a single tissue sample. This indicates identifying subregional hippocampal CNP distribution and concentrations in future studies will be critical to further understanding of the peptide. However, this is also suggestive of an increase in NPR-C receptors within hippocampus because NTproCNP:CNP ratio is influenced by both neprilysin (decreased with age) and NPR-C mediated internalisation and degradation. In combination with age-dependent changes to peptide production and availability in medial prefrontal, retrosplenial and occipital cortices, this provides evidence that variations in CNP synthesis, regulation, and availability contributes to age-related cognitive decline. As outlined above, these regions are implicated in many changes that occur with aging including facilitation of LTD, modifications of glucocorticoid feedback and executive dysfunction (Buckner, 2004; Burke & Barnes, 2010; J. J. Kim & Diamond, 2002; Mizoguchi et al., 2009; Swaab & Bao, 2011). Retrosplenial cortex in particular is thought to act as a key region underpinning functions such as episodic memory and navigation, and integration of these with executive function (Buckner, 2004; Fjell et al., 2014; Vann, Aggleton, & Maguire, 2009). It is also the first cortical region to develop hypometabolism in both mild-cognitive impairment and Alzheimer's disease, possibly contributing to age-related cognitive decline by disconnecting frontal and temporal cortical networks (Nestor, Fryer, Ikeda, & Hodges, 2003). It is specifically this network identified here, and strongly suggests a contribution of CNP within this circuit to age-related cognitive decline.

These data now encourage several continuing lines of question. Primarily, clarification of the interaction between CNP and glucocorticoids of the HPA axis. In combination with other recent findings from Professor Espiner's research group (Wilson et al., 2017), these findings suggest a complex interaction between central CNP and HPA axis regulation, and their combined influence on stress, memory, and aging. Understanding this interaction will likely require clarifying target cells of CNP within cerebral tissue. Neuron-glia interactions are indicated in a proposed involvement of CNP in cognitive decline

(Mahinrad et al., 2016) and additionally suggest CNP should be investigated in relation to amyloid-β deposition. Amyloid plaque deposition is associated with nearby astroglial and neprilysin upregulation (Apelt et al., 2003) which may in turn associate with local reductions in CNP. Additionally, neprilysin may increase with increased severity of Alzheimer's disease pathology (Miners, Baig, Tayler, Kehoe, & Love, 2009), suggesting continued modifications to the CNP signalling system in cases of pathological aging. Ongoing research with CNP may help clarify why all age-related cognitive decline with amyloid plaque deposition does not result in Alzheimer's disease. It will additionally be of interest to query how and whether CNP participates in neurogenesis within DG. Modifications to CNP clearance via NPR-C with age could potentially contribute to age-related reductions in neurogenesis and delayed maturation of new neurons (Kempermann et al., 1998; Lee, Clemenson, & Gage, 2012; Rao et al., 2005; Simpson et al., 2002). Additionally, while this provides initial evidence supporting a role for CNP in age-related cognitive decline, further attention should be given to other neurological factors wherein CNP may contribute to cognitive decline such as modifications to neurovascular function, synaptic regulation, anxiety and memory (Mahinrad et al., 2016).

3.4.4 Limitations and Summary of Findings

There are several caveats on the assertion that the CNP system contributes to age-related cognitive decline including that no inferences can be made about cause and effect (whether modifications to synthesis or clearance activity precede cognitive decline). First, it is not known whether these rats were cognitively impaired. However, rats of the same strain and age are used in Chapter 5 and are demonstrated to have spatial and recognition deficits. This suggests it is also safe to assume cognitive decline in rats used here. Second, whether there were behavioural effects of enrichment, such as improved memory or reduced anxiety, was not tested here. If enrichment altered neither behaviour, nor CNP response, this would better support the supposition that these changes represent solely age-related neurological changes associated with cognitive decline. Third, that this study has not explicitly examined concurrent changes in neprilysin, relevant NP receptors or NPPC gene expression in these tissues. This was not possible here because of constraints from tissue processing and amounts required for RIA. However, NTproCNP is considered a reliable indicator of NPPC expression (Schouten et al., 2011; Woodward et al., 2017). Moreover, there is increasing recognition that gene expression may not equate with protein concentration at source, which is the primary factor determining function. Further research should consider alternative methods, expanded upon in Chapter 6.

Some of these issues were to be addressed as part of this thesis, but time and financial constraints prevented completion of a complementary study. Additionally, that study was to include further ROIs including perirhinal cortex, amygdala and the anterodorsal thalamic nucleus. Perirhinal cortex receives multiple sensory inputs and, alongside post- and entorhinal cortices, represents the major cortical input to the hippocampal formation (Burwell, 2006). Additionally, perirhinal cortex is also suggested to be involved in working memory, is connected to reward systems, is critical to recognition memory, and may participate in age-related recognition deficits (Aggleton & Brown, 2006; Buffalo, Reber, & Squire, 1998; Burke et al., 2010; Burwell, 2006; Erickson & Barnes, 2003). The amygdala has long been accepted as involved in emotion, but is also critical for emotion based learning and memory (Phelps & LeDoux, 2005). Anterodorsal thalamic nucleus has a strong localisation of NPR-B receptors, and represents a key node within the extended hippocampal-diencephalic system, previously mentioned (Aggleton & Brown, 1999). Due to its size and location, this region was excluded by tissue acquisition methods and minimum RIA requirements. Because CNP and related receptors within these regions seem likely to contribute to dysregulation of sensory, emotional and mnemonic integration seen with age-related cognitive decline based on results observed here, any future research should ensure their consideration.

Although these data are generally limited by only having a single previous study in young rats for comparison (Rapley et al., 2018), the pattern of results suggest modifications to CNP synthesis and regulation within fronto-temporal networks contributes to age-related cognitive decline. This is proposed to occur via NPR-C receptor increases in hippocampus, alongside modifications to CNP synthesis and availability within retrosplenial cortex, medial prefrontal cortex, and occipital cortex. This latter may generalise to other regions related to sensory input. As a broad response to enrichment was not evident compared with young rats, this supports an age-related modification, or loss of sensitivity to external stimulus, of the CNP signalling system. Additionally, the assumed stress response based on observations across the rehousing period may be representative of dysfunction in HPA axis regulation. These observations also align with modifications to glucocorticoid feedback and executive function changes with age. As with all rodent models, this may not generalise to human research and if these generalisations are made, further investigation of the role of Osteocrin in relation to NPR-C regulation in primate and human-neocortex will be critical. Whilst CNP-22 is highly conserved, meaning rodents represent a reasonable model system in this context, it is still plausible that CNP-22 and CNP-53 may have a varying role in age-related cognitive

decline. Because there is 100% cross-reactivity for the active forms of peptide in the RIA used here, it is impossible to determine from these data whether varying effects on each active form of the peptide have occurred. This may be especially important to consider in future work, because distribution of these two peptide forms varies across neurological regions, and the conversion process from CNP-53 to CNP-22 is still not known (Wilson et al., 2018).

Chapter 4. CNP synthesis increases in retrosplenial cortex and mammillary bodies following spatial reference learning.

4.1 Rationale

This study was designed to investigate an association between endogenous measures of CNP in relevant cerebral tissues and spatial/episodic-like memory. Various models of rodent spatial memory are thought to be representative of an 'episodic-like' memory, although rodents cannot be claimed to experience episodic memory as in the human experience, *per se* (Aggleton & Pearce, 2001; Morellini, 2013; Morris, 2001; Paul, Magda, & Abel, 2009). Nevertheless, rodent models of spatial memory provide an excellent tool in our understanding of learning, memory and human degenerative diseases (Aggleton & Pearce, 2001; Morris, 2001). Aged rodents have deficits in various spatial tasks, which also align with age-related spatial learning deficits in humans (Rosenzweig & Barnes, 2003). Thus, in proposing a role for CNP as a novel target in cognitive decline, identifying some involvement in a rodent model of spatial, episodic-like memory is relevant. Aged rats are impaired in such tasks (Bennett et al., 2006; Kim et al., 2010; Lores-Arnaiz et al., 2004; Rosenzweig & Barnes, 2003), so this study provides an initial approach to determine associations between spatial memory and CNP from the perspective of young adult rats.

At the time of designing this study, no direct involvement of CNP in spatial/episodic-like memory had been identified. Participation of CNP in spatial memory acquisition and consolidation would be expected based on 1) distribution of CNP and NPR-B receptors throughout multiple structures generally accepted as underlying rodent spatial memory (Aggleton & Brown, 1999; Aggleton & Brown, 2006; Herman et al., 1996; Langub, Watson, et al., 1995); 2) previous evidence that CNP contributes to the acquisition and consolidation of memory in passive avoidance (Telegdy et al., 2000, 1999); and 3) effects on locomotor and exploration behaviours (Barmashenko et al., 2014; Jouvert et al., 2004; Thiriet et al., 2001). Seemingly negating these expectations, NPR-BΔKC rats performed no differently to wild-type counterparts in an object-location task (Barmashenko et al., 2014). Object recognition tasks that include a spatial component can be used to model aspects of 'episodic-like' memory in rodents. However, spatial memory itself is more commonly tested with radial arm mazes or the Morris water maze (Aggleton & Pearce, 2001; Hodges, 1996; Morellini, 2013; Paul et al., 2009; Vorhees & Williams, 2014)

One consideration in selection of the radial arm maze (RAM) for this study was to minimise any confound of anxiety in the context of CNP and memory (cf Telegdy et al, 2000,

1999). Because of its aversive nature (Paul et al., 2009). the water maze was not used. Stress levels for rodents are minor in the RAM compared to the water maze (Hodges, 1996). An additional advantage of the RAM is its combination of 'what?' and 'where?' components, of 'episodic-like' memory (Aggleton & Pearce, 2001) providing a long-term learning component (reference memory) across trials. Working memory (within trial avoidance of arms previously visited) is also required for optimal performance, whereas in the water maze generally only one of these aspects can be tested (Hodges, 1996). Reference memory in the RAM is often tested by having three to four arms always unbaited, requiring rodents to learn avoidance of these arms across successive trials (Vorhees & Williams, 2014). Here, a simpler reference memory paradigm used a single always-unbaited arm location (similar to Harland et al., 2014) to permit a clear criterion for reference memory learning.

At the time of design, little could be claimed about CNP and memory except that peptide activity contributes to acquisition and consolidation processes in passive avoidance (Telegdy et al., 2000, 1999). During acquisition and consolidation of memory, two periods are sensitive to inhibition of protein synthesis – immediately following training, and approximately four hours later (Abel & Lattal, 2001). In consideration of the process required for tissue acquisition, the latter window was selected. This timing most likely captures CNPs participation in consolidatory functions, which occur across minutes to days, rather than a contribution to memory acquisition, which occurs within the immediate learning context (Abel & Lattal, 2001).

A final consideration in study design was appropriate comparison groups. This required control for food restriction which could plausibly affect CNP concentrations (food effect citations from lit review), and a 'disruption' of the learning process to account for CNPs involvement in locomotor and exploratory behaviour (Barmashenko et al., 2014; Jouvert et al., 2004; Thiriet et al., 2001). In addition to the primary experimental group ("Active Learning" rats – see Methods), a control group of rats were similarly food-deprived and exposed to the testing environment, but received no exposure to the RAM nor any behavioural training (Methods). This provided baseline measures of CNP in context of food-deprivation and environmental influences. Learning of the reference component was disrupted in a second behaviour group ("Passive-Learning" rats – see Methods). Whereas the "Active Learning" rats experienced a standard RAM with free selection of arm choices, "Passive Learning" rats were given a forced choice. This was in a manner and with a reinforcement history that was yoked to an "Active" learner. Matched within-trial (working memory) errors from "Active" learners were intended to serve as interference for the always-

unbaited arm location across trials (reference memory) and disrupt representation of the reference memory location for "Passive" learners.

Regions of interest for CNP analysis were retained from previous studies (Chapter 3) to provide a suitable range of neural structures, many of which contribute to spatial memory in the RAM. Hippocampal dependence of the RAM and spatial episodic-like memory is widely accepted (Aggleton & Pearce, 2001). Frontal cortical regions (pre- and infralimbic cortices) elicit similar deficits in the RAM to hippocampal lesions, and participate in working memory and prospective coding aspects of the task (Kesner & Churchwell, 2011; Kolb, Pittman, Sutherland, & Whishaw, 1982; Paul et al., 2009; Vertes, 2006). Spatial memory deficits have also been described following lesions and disconnections of retrosplenial cortex or mammillary bodies (Aggleton, 2010; Aggleton & Vann, 2004; Cain, Humpartzoomian, & Boon, 2006; Neave, Nagle, & Aggleton, 1997; Pothuizen, Davies, Aggleton, & Vann, 2010; Pothuizen, Davies, Albasser, Aggleton, & Vann, 2009; Santín, Rubio, Begega, & Arias, 2000; Sziklas & Petrides, 1993, 1998; Troy Harker & Whishaw, 2004; Van Groen, Kadish, & Wyss, 2004; Vann & Aggleton, 2003, 2004, 2005; Vann et al., 2009; Vann & Nelson, 2015). Occipital cortex provides primary visual information input to several of these structures (Aggleton, 2010; Vann et al., 2009; Zhu, Brown, McCabe, & Aggleton, 1995). Retention of these ROIs thus provided several key nodes related to the integration of episodic-like memory and sensory input (Aggleton & Brown, 1999, 2006; Catani et al., 2013).

4.2 Methods

4.2.1 Subjects

Thirty-six naïve male PVGc rats, aged 7-8 months and weighing between 320 and 395g prior to training, were housed in groups of four in standard opaque laboratory cages (45 cm x 27 cm x 22 cm high). Prior to training, all rats were food deprived to 85% of their free-feeding weight, then maintained at 85-90% of this weight during behavioural testing. Twelve rats received standard training using a reference memory version of the RAM (see below; Active Learning group) and 12 rats experienced a forced choice version of this task, with their behaviour yoked to a rat from the Active Learning group (see below; Passive Learning). A final group of 12 rats (Control) did not experience any RAM training, but were exposed to the training room for the same length of time as both other groups. This group was to provide a baseline level of CNP and NTproCNP associated with food deprivation, handling and room exposure. Rats were maintained on a reversed light-dark cycle (lights off 0800 – 2000h) and colony rooms were maintained at 22°C and 48%rH. Water was available *ad libitum*. All

procedures conformed to the NIH guide for the care and use of laboratory animals and were approved by the University of Canterbury Animal Ethics Committee.

4.2.2 Radial Arm Maze

Apparatus and testing room

Active and Passive Learning groups were pre-trained in an eight-arm RAM to run for reward (chocolate piece). The RAM consisted of a central wooden platform 35cm in diameter, painted black, with eight equally spaced detachable aluminium arms (65cm long x 8.6cm wide) with Perspex barriers (25cm high) extended a partial distance (19cm) along the arm length to discourage jumping between arms. Food wells (2cm diameter, 1cm deep) were located at the end of each arm, and entrance to the arms was controlled by clear Perspex doors, operated by the experimenter. The maze was cleaned between rats to reduce odour cues within the maze with a disinfectant solution suitable for use with laboratory animals (4% ExpressSani). During the testing phase, individual arm positions were pseudorandomly ordered (based on order 8 latin square from Drake & Myrvold, 2004) and arm placement was changed daily to prevent strategies relating to specific arms as opposed to locations. The maze was centrally located in a windowless room (approximately 4m x 4m) with various extra-maze cues located on the walls, and the experimenter visible in a constant corner location. Additionally, rats were held within the maze room during testing, such that home cages on a trolley provided a further extra-maze cue. Figure 4.1 shows rats exploring the apparatus during pre-training.

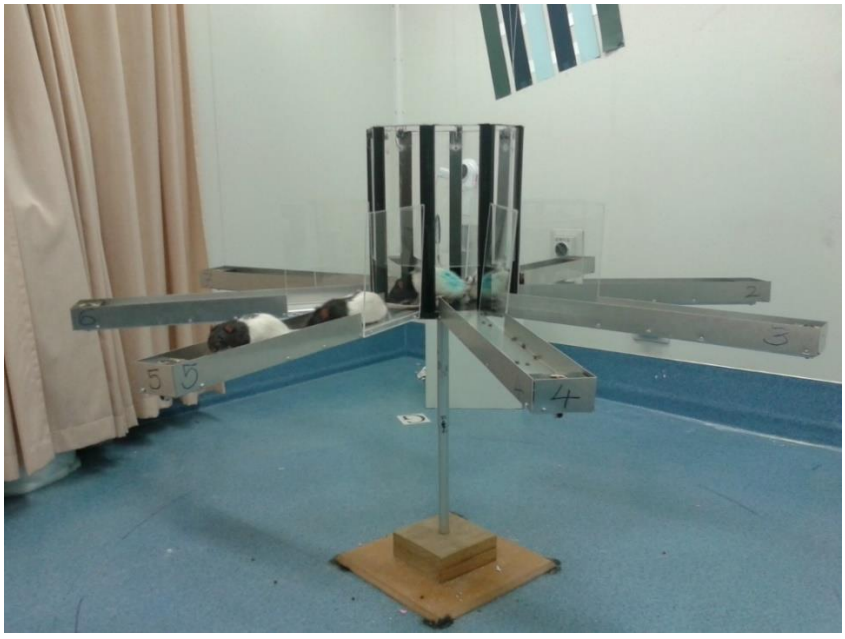


Figure 4.1: Rats exploring radial arm maze during pre-training

Pre-training

Rats in their home cages were familiarised to the maze room on the first day for four to five hours. On days two and three, rats freely explored the maze with their cage mates. All maze doors were held open and chocolate pieces were scattered liberally on the maze floor. Rats then foraged individually in the open maze for ten minutes on days four and five, with chocolate pieces restricted to four pieces on each arm and four in each food well. Operation of the doors was introduced on day six, and chocolate reduced to two pieces in each food well. Rats were placed on the centre platform and all doors opened to allow the rat to enter an arm. Doors were opened for approximately 30 seconds, and if the rat did not enter an arm, closed again for approximately 30 seconds. This was repeated until rats successfully entered an arm, after which all other doors were closed until the rat re-entered the centre platform. After a brief confinement (~3 seconds) all doors were opened again and the rat allowed another arm entry. This continued for 10 minutes, 20 arm visits or until all rewards were claimed (whichever occurred first). This procedure was repeated on days 7 and 8, by which time all rats were successfully running for reward.

Testing

For Active Learning rats, the procedure was the same as that from the final days of pre-training. Rats were placed on the central platform; all doors were opened and the rat allowed to freely choose an arm to gain reward (2 x 0.1g chocolate pieces) before returning to the central platform. The key difference during testing was that one arm location remained un-baited throughout the testing period (consistent for a given rat, but varied across rats) providing a reference memory component in the task. The same criteria as during pre-training dictated completion of a session on a given day – 10 minutes, 20 visits, or all rewards claimed. Repeating an arm already visited within a session constituted a working memory error and entering the un-baited arm constituted a reference memory error (and a working memory error if it was repeated within a session). Rats were tested for a minimum of 15 days (maximum 35) until reaching a criterion of 2 out of 3 sessions without visiting the un-baited arm and with no more than 5 working memory errors across these 3 sessions.

Maze experience for Passive Learning rats was yoked to an individual in the Active Learning group, such that the critical behavioural difference was that Active Learning rats made free choices of arm entries, and Passive Learning rats experienced forced choices, including repeats of arms where corresponding Active Learning rats made errors. In addition to matched arm entry, total time spent in the maze for Passive Learning rats was matched to

Active Learning counterparts as closely as possible. Control rats were held in the testing room for the same amount of time as Active and Passive Learning rats for each day of training and testing. For practical purposes, training of rats was staggered into three replications, with start dates for each replication separated by two weeks. Each replication constituted four rats from each group.

4.2.3 Sacrifice and tissue extraction

After reaching criterion, rats were returned to their colony room and fed per usual testing days. Sacrifices began approximately four hours after criterion and were conducted such that a "trio" of Active Learning, Passive Learning and Control rats were euthanized and tissues acquired in that order, to have approximately equivalent time for each rat between maze or room exposure and sacrifice (that is, based on the order in which they were tested earlier in the day). On days that multiple rats reached criterion, some variance was introduced to this interval, and the potential influence of this variation was examined in initial data analyses (4.2.5 Statistical Analyses). Sacrifice and tissue acquisition methods were the same as described in Chapter 3

4.2.4 Tissue preparation and peptide measurements

Tissue preparation, peptide extraction, CNP and NTproCNP assays were conducted as outlined in Chapter 3. During protein extraction, all tissues from individual rats and from a "trio" of rats were extracted together. CNP assays had a detection limit of 0.6 pmol/L and ED50 of 7.3 pmol/L; intra- and inter-assay coefficients of variation were 5.9 and 7.4%, respectively, at 17 pmol/L. NTproCNP assays had a detection limit of 0.4 pmol/L and ED50 9.9 pmol/L; intra- and inter-assay coefficients of variation were 6.8 and 7.5%, respectively, at 45 pmol/L. Protein extractions prior to assays in this experiment may have been less efficient than expected, indicated by reduced values acquired from QC standards in both CNP and NTproCNP assays. Although absolute values for both molecules were possibly reduced, relative concentrations for regions within single rats and between rats were likely maintained (T. Prickett, personal communication).

4.2.5 Statistical Analyses

CNP, NTproCNP and NTproCNP:CNP ratios were expressed and calculated as previously described (Chapter 3). Values from four tissues were excluded due to contamination or loss of tissue during processing: one retrosplenial cortex sample, one sample from left hippocampus and two samples from medial prefrontal cortex. For all other regions of interest, total N = 36, with N = 12 for each of the Active Learning, Passive Learning and Control

groups. Preliminary data analysis indicated that hippocampal concentrations were equivalent across hemispheres, so subsequent analysis used the average concentration from left and right hemispheres (except from one Passive Learning rat where only right hippocampus was available). Inspection of whether the use of three replication groups affected peptide measures indicated that replication had no effect, and was not considered further. Analyses of the effect of group on peptide measures used One-way ANOVA, followed up with robust one-way ANOVAs using medians to account for assumption violations and potential outlying values (see Wilcox & Keselman, 2003). Corresponding robust post-hoc comparisons between medians were conducted in the case of significant omnibus tests. As statistical tests were conducted on medians, data is presented as traditional boxplots

4.3 Results

Due to concern regarding peptide extraction efficiency, overall concentrations are first compared to previous reports. Generally, tissue concentrations of CNP were consistent with previous reports (Jankowski et al., 2004; Komatsu et al., 1991; Pemberton et al., 2002; Totsune et al., 1994; Wilson et al., 2017) and with concentrations reported in Chapter 3. NTproCNP concentrations were consistent with those reported in Chapter 3, with all ROIs exhibiting similar 50% reductions compared to the report of Wilson et al (2017). Overall, this suggests peptide extraction was of an acceptable efficiency. An exception was seen in hippocampal tissue. CNP concentrations were mildly reduced compared to those in Chapter 3 and the report of Wilson et al (between 350 and 700 fmol/g here, versus 500 to 1000 fmol/g in Chapter 3 and Wilson et al., 2017; Figure 4.2). NTproCNP concentrations were consistent with those in Chapter 3 (see Figure 4.3), but NTproCNP:CNP ratio values were mildly increased compared with those reported in Chapter 3 (ratios between 2 to 4 in Chapter 3 and between 3 and 5 here – see Figure 4.4). The combined increase in ratio with reduction in CNP is internally consistent, suggesting a true difference from previous data (i.e. presented in Chapter 3 and Wilson et al, 2017). Hippocampal concentrations were not affected by behaviour group (4.3.2: Peptide Measures; Figures 4.2 – 4.4). Overall this finding suggests that manipulations relating to food deprivation and handling may have influenced peptide regulation and availability within hippocampal formation.

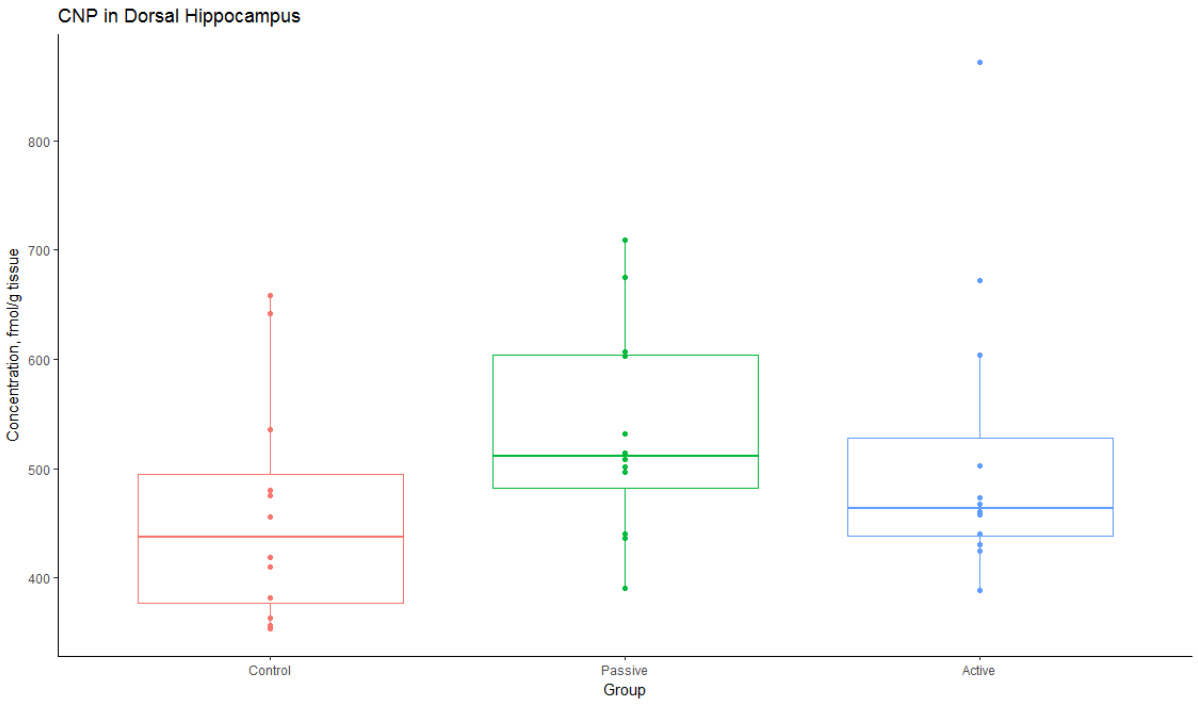


Figure 4.2: Boxplot with individual data points for CNP concentrations by behaviour group in dorsal hippocampus. All groups equal. Concentration reduced compared with Chapter 3.

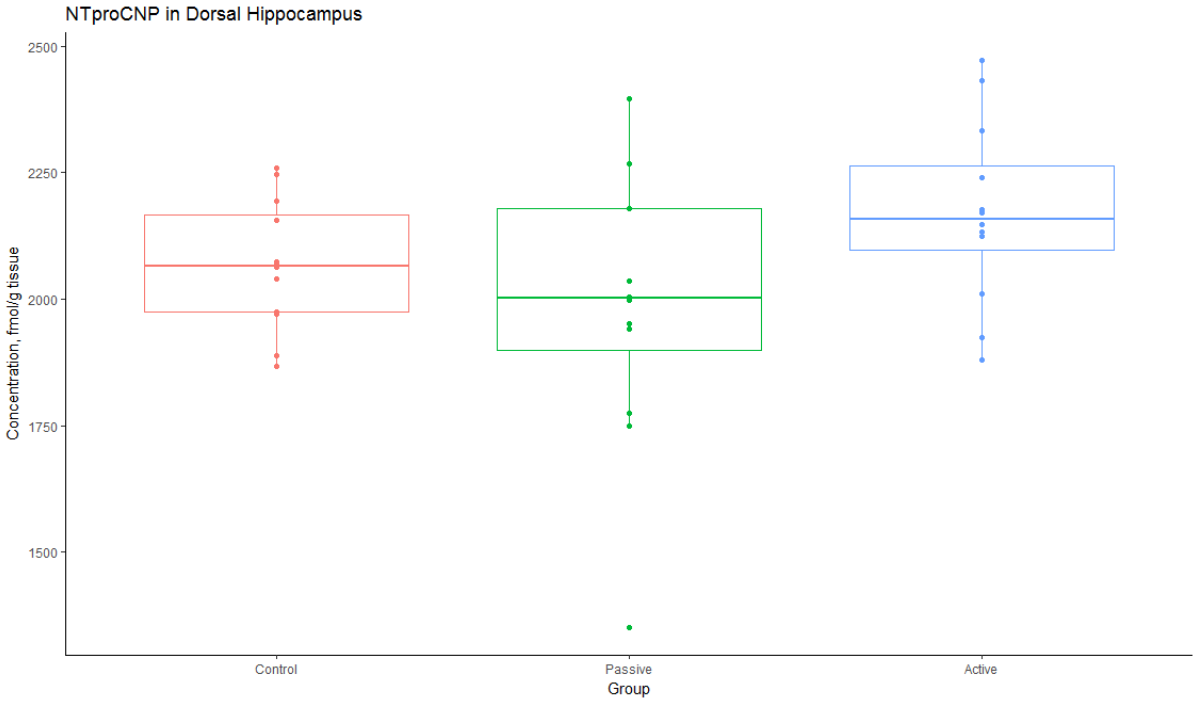


Figure 4.3: Boxplot with individual data points for NTproCNP concentrations by behaviour group in dorsal hippocampus. All groups equal. Concentrations equivalent to Chapter 3.

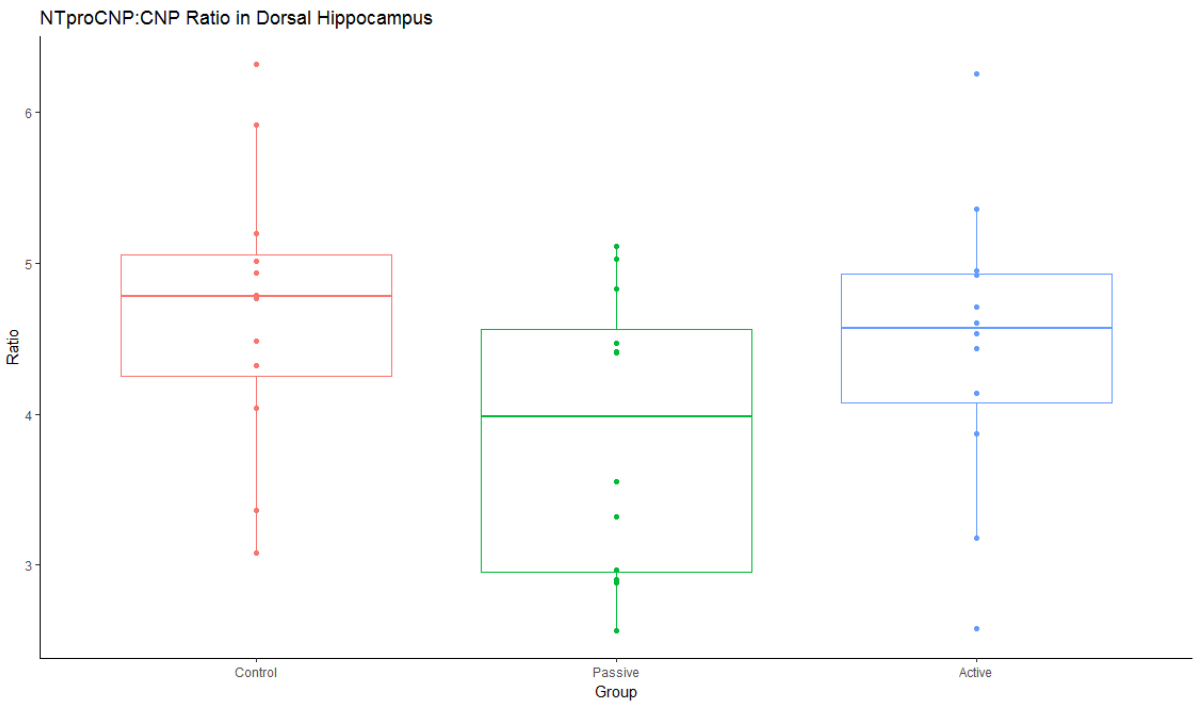


Figure 4.4: Boxplot with individual data points for NTproCNP:CNP ratio by behaviour group in dorsal hippocampus. All groups equal. Values increased compared to Chapter 3.

4.3.1 Acquisition by Active Learning group

Eight of 12 rats in the Active Learning group reached behavioural criterion (2/3 sessions with no reference memory error; <5 total errors across these sessions) in less than 20 days. Three rats reached criterion between 30 and 35 days, and one rat did not reach criterion by the 35-day end-point. Figures 4.5 and 4.6 show working and reference memory errors respectively for individual rats in the Active Learning group; Passive Learning rats' performance was matched to this via forced arm choice, and Control rats were not tested on the RAM. In all ROIs, number of days to reach criterion had no relationship with CNP (all $rs < .10$, all $ps > .562$), NTproCNP (all $rs < .27$, all $ps > .118$) or the NTproCNP:CNP ratio (all $rs < .17$, all $ps > .310$). As varying time to reach criterion had no apparent effect, all rats were retained for between group comparisons.

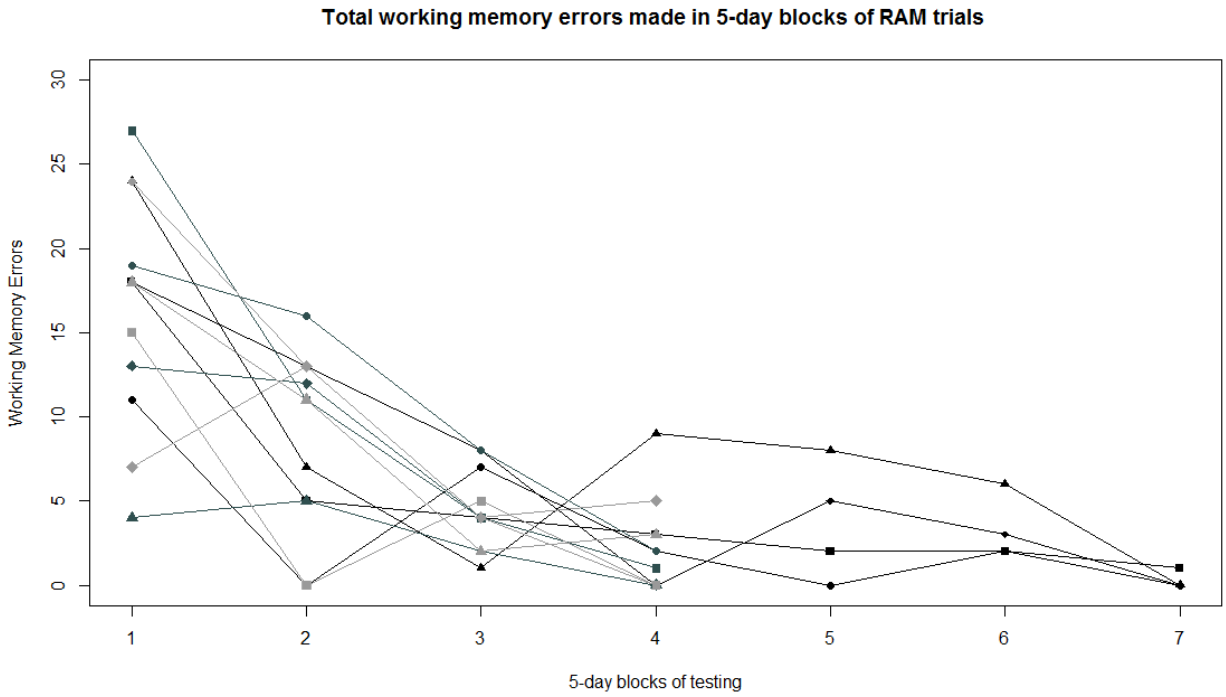


Figure 4.5: Total working memory errors summed across five-day blocks of trials for Active Learning group (12 rats) in the radial arm maze.

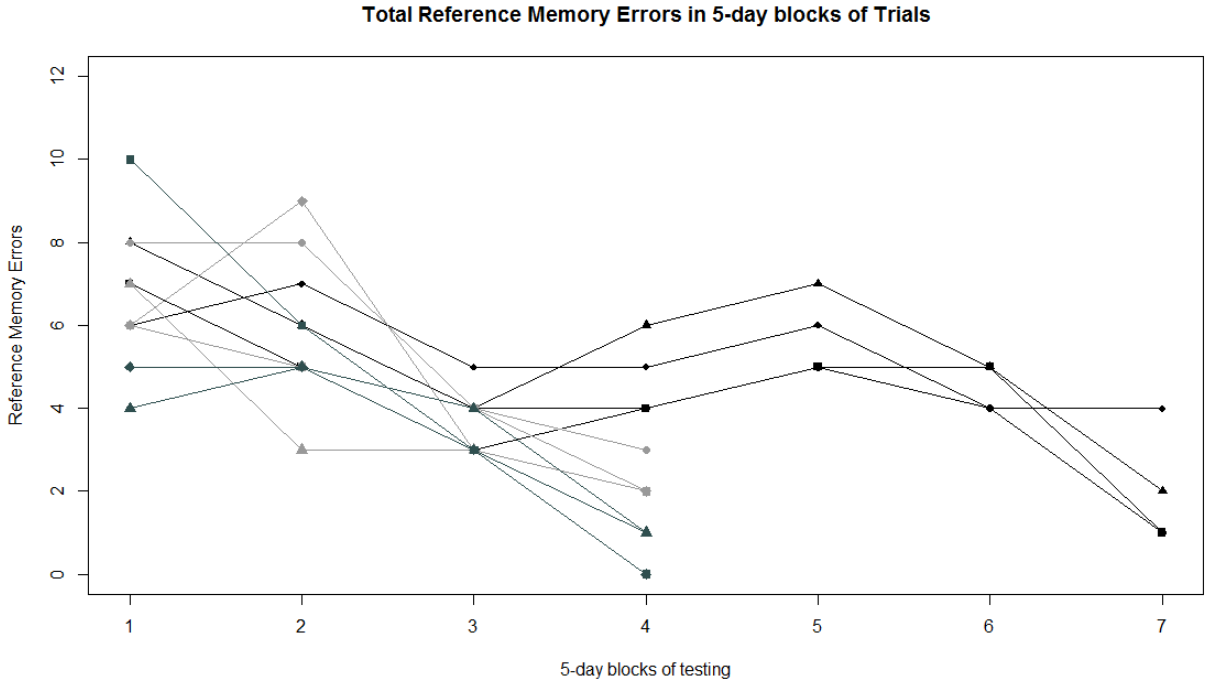


Figure 4.6: Total reference memory errors summed across five-day blocks of trials for Active Learning group (12 rats) in the radial arm maze.

4.3.2 Peptide measures

NTproCNP concentrations in retrosplenial cortex differed across groups (robust ANOVA: F (2, 11.49) = 5.20, p = .025; Figure 4.7). Examination of Figure 4.7 shows that NTproCNP was elevated in Active Learning rats compared to both Passive Learning and Control groups. Post-hoc comparisons confirmed a significant difference between Control and Active Learning rats (Median difference = 292.89, Ψ = -327.16, p = 0.010, CI of the estimate: [-620.03, -34.29]), but the difference with Passive Learning rats failed to reach significance (Median difference = 138.11, Ψ = 218.11, p = .086, CI of the estimate: [-101.74, 537.97]). Control and Passive Learning rats did not differ (Ψ = -109.05, p = .196, CI of the estimate: [-333.46, 115.36]. No effects were found for CNP concentrations or the NTproCNP:CNP ratio (Figures 4.8 and 4.9).

NTproCNP concentrations also differed across groups in mammillary bodies (robust ANOVA: F (2, 13.96 = 4.08, p = .040; Figure 4.10). NTproCNP concentrations were elevated in both Active Learning and Passive Learning rats compared to Controls (Control vs Active Learning: Median difference = 1133.89, Ψ = -1018.46, p = .036, CI of the estimate: [-2202.99, 166.08]; Control vs Passive Learning: Median difference = 1329.18, Ψ = -1096.76, p = .018, CI of the estimate: [-2199.87, 6.34]). Active and Passive Learning rats did not differ (Ψ = -78.31, p = .860, CI of the estimate: [-1251.81, 1095.20]). As in retrosplenial cortex, there were no effects for CNP or the NTproCNP:CNP ratio (Figures 4.11 and 4.12). All other effects were null and are provided with supplemental figures in Appendix A: Supplemental Figures for each peptide measure in all regions of interest.

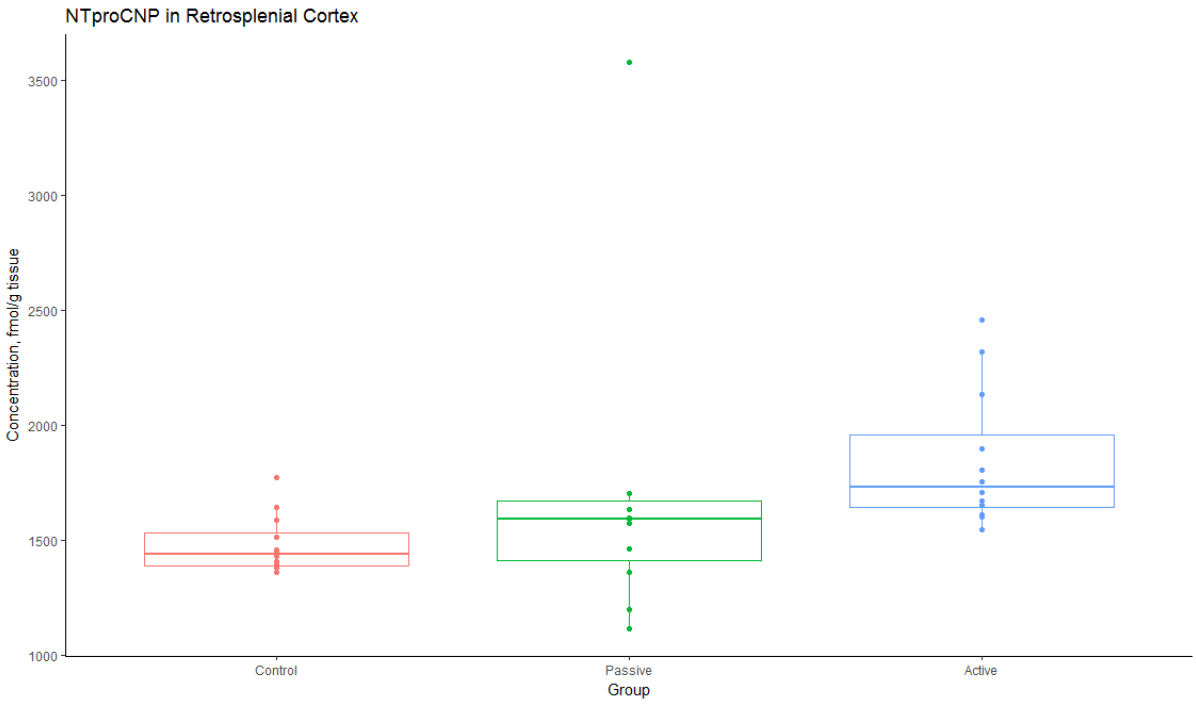


Figure 4.7: Boxplot with individual data points for NTproCNP concentrations by behaviour group in retrosplenial cortex. Active ≥ Passive = Control.

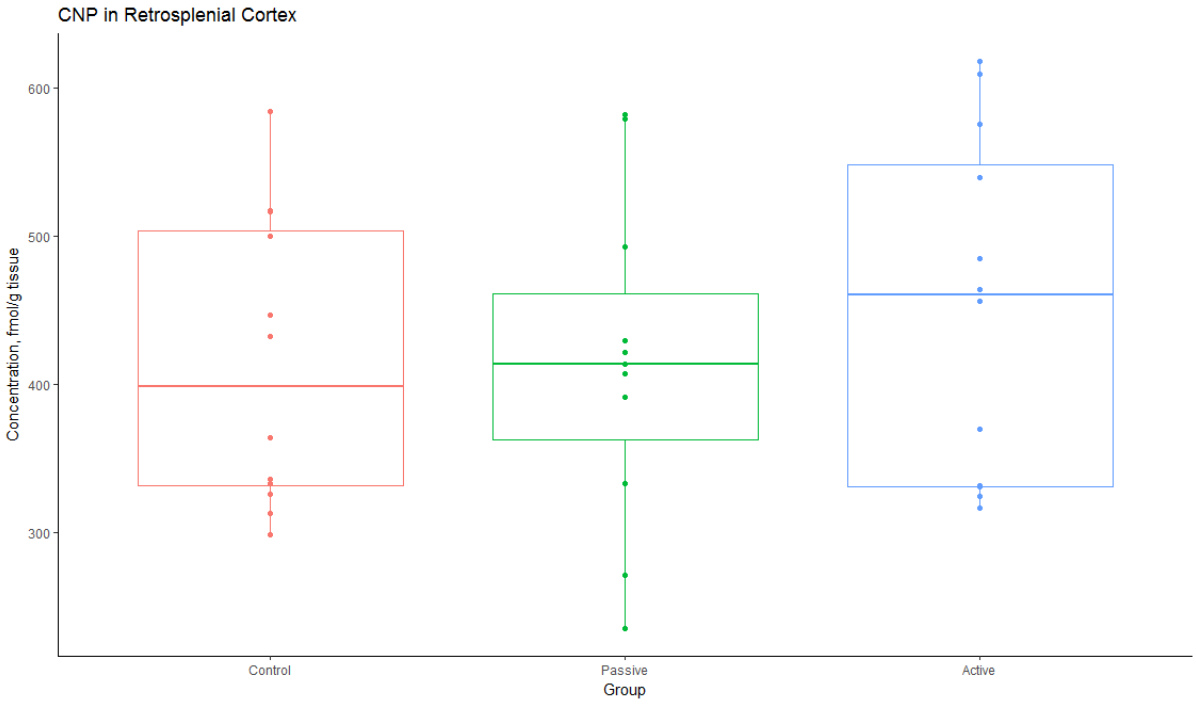


Figure 4.8: Boxplot with individual data points for CNP concentrations by behaviour group in retrosplenial cortex. All groups equivalent.

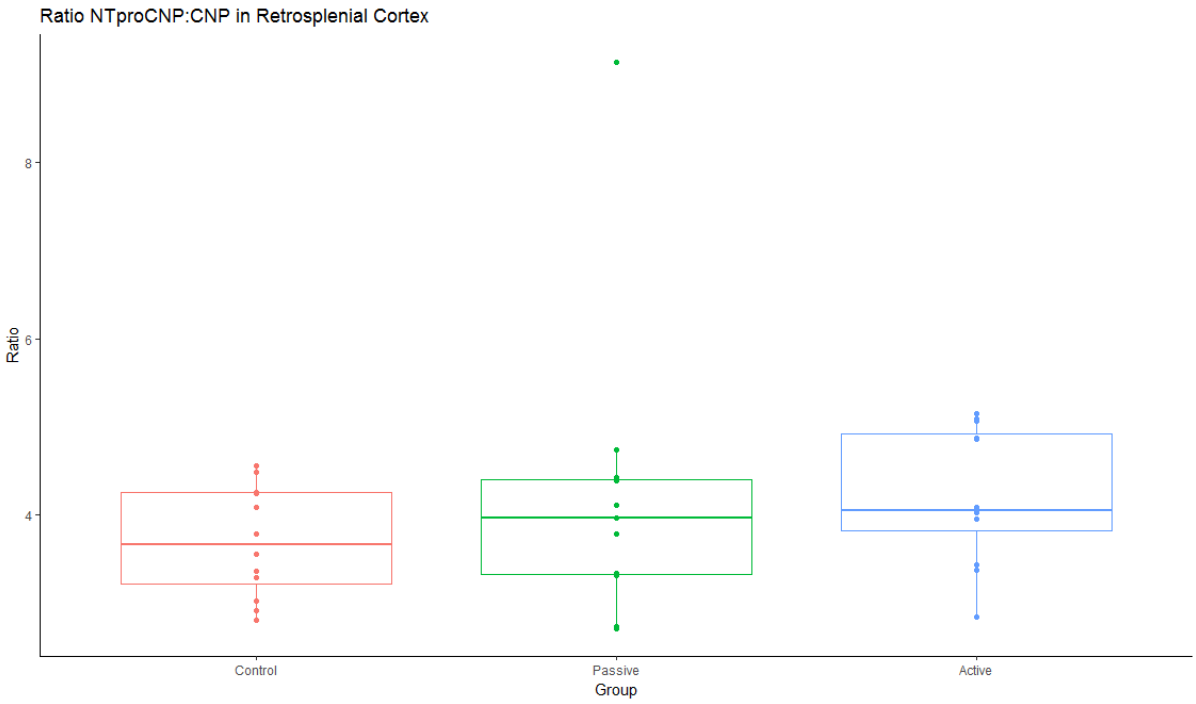


Figure 4.9: Boxplot with individual data points for NTproCNP:CNP ratio values by behaviour group in retrosplenial cortex. All groups equivalent.

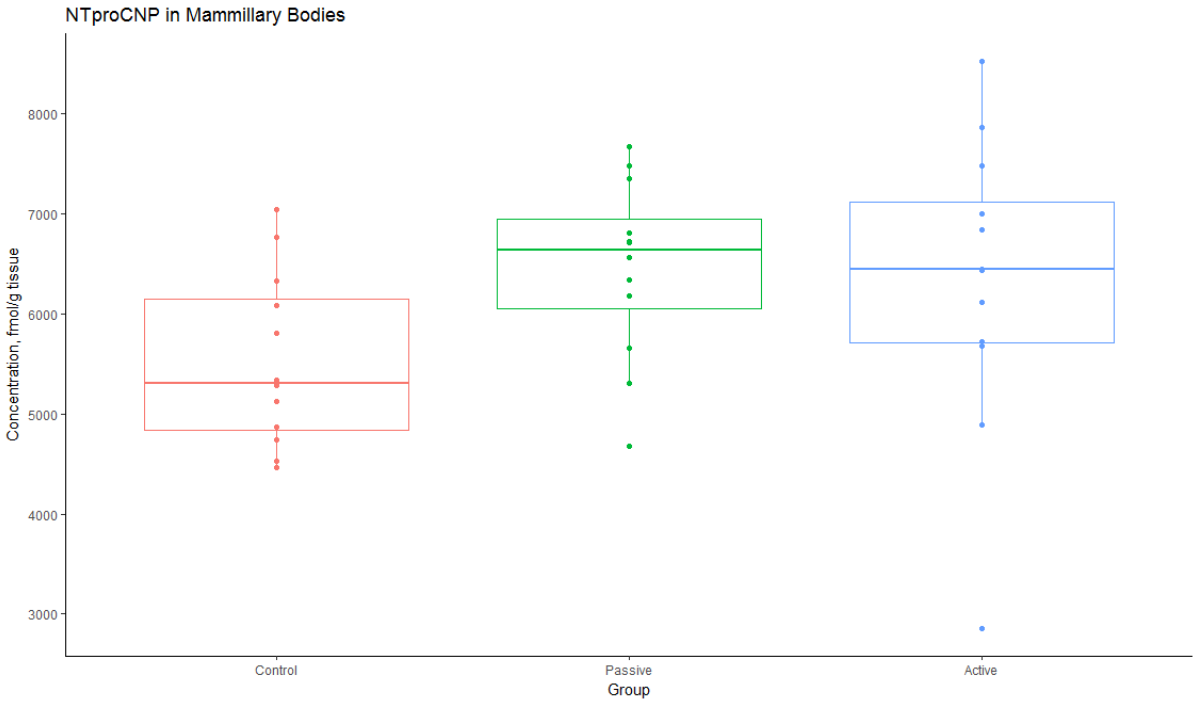


Figure 4.10: Boxplot with individual data points for NTproCNP concentrations by behaviour group in mammillary bodies. Active = Passive > Control.

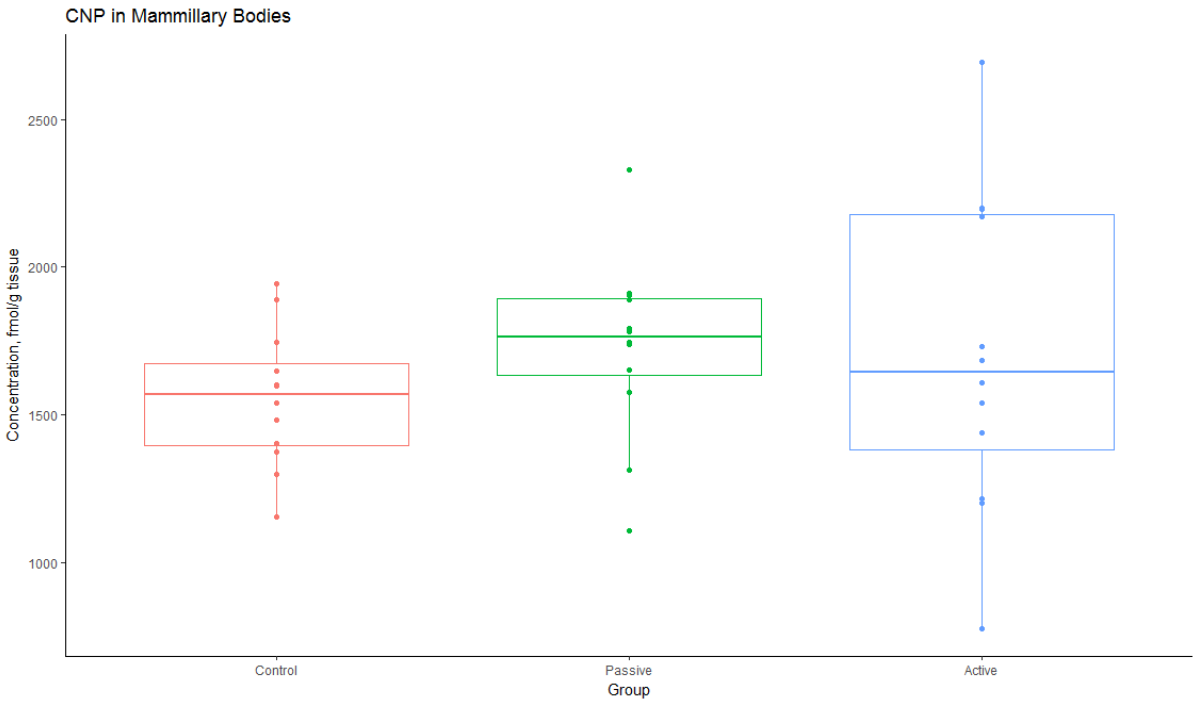


Figure 4.11: Boxplot with individual data points for CNP concentrations by behaviour group in mammillary bodies. All groups equivalent.

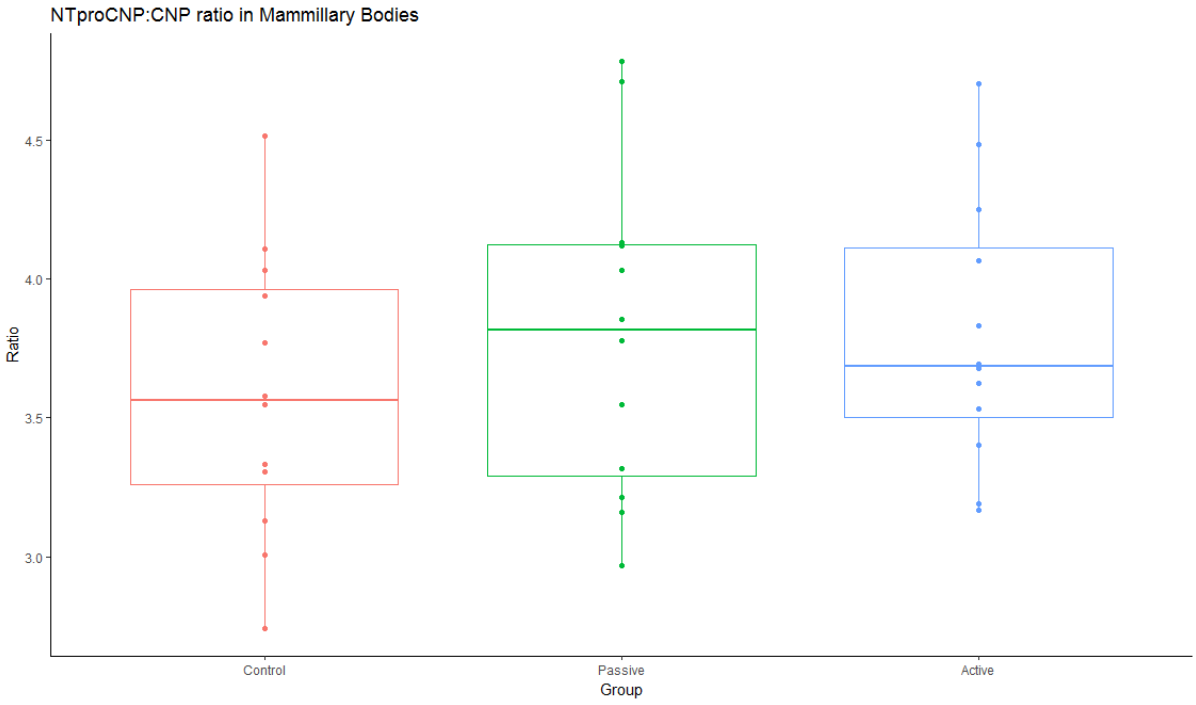


Figure 4.12: Boxplot with individual data points for NTproCNP:CNP ratio values by behaviour group in mammillary bodies. All groups equivalent.

4.4 Discussion

Only three previous studies have directly examined CNP's role in memory behaviourally (Barmashenko et al., 2014; Telegdy et al., 1999, 2000). Studies of alternative behavioural tasks, not directly influenced by an anxious response are necessary to broaden understanding of CNPs proposed role in memory formation. Additionally, identifying participation of CNP in spatial/episodic-like memory in adult rats without impairment is critical to fill a gap in research relating CNP with memory, and support the assertion that it may be involved in age-related cognitive decline at a later stage when this type of memory is known to be impaired. The current study used a typical RAM reference memory task, with a behavioural comparison group ("Passive Learners") in which interference was introduced to the reference memory component via forced arm choice, yoked to a freely navigating rat. Tissue samples were taken from six ROIs, approximately four hours after rats actively learning the task reached a criterion, indicating that memory for the reference location had been acquired. "Active Learning" rats had increased concentrations of NTproCNP (indicative of peptide production) in retrosplenial cortex. Additionally, both "Active Learning" and "Passive Learning" groups had increased NTproCNP in mammillary bodies. This is the first study to show modifications to endogenous CNP activity following a spatial memory task. Specifically, these data indicate that novel CNP synthesis occurs in both retrosplenial cortex and mammillary bodies during consolidation of spatial reference memory but is restricted to mammillary bodies when reference memory is disrupted.

Upon initial inspection, these data indicate that behavioural interference with the reference component of the task was successful. Lesions of retrosplenial cortex result in reference memory deficits in the Water Maze and Radial-Arm Maze, but lesion-induced deficits for working memory aspects of RAM tasks are inconsistent (Aggleton & Vann, 2004; Aggleton, 2010; Harker & Whishaw, 2004 for reviews). Mammillary body lesions result in deficits of spatial working memory in both the RAM and Morris Water Maze (Neave et al., 1997; Santín et al., 2000; Sutherland & Rodriguez, 1989; Sziklas & Petrides, 1993; Vann & Aggleton, 2003), but have no effect on reference memory (Santín et al., 2000). Furthermore, while the mammillary bodies are important for encoding allocentric cues, interactions between retrosplenial cortex and mammillary bodies are proposed to be responsible for translation of allocentric to egocentric cues and the separation of distinctive episodes (Burgess, 2002; Vann, 2010; Vann & Aggleton, 2004; Vann et al., 2009). Here, when rats could not separate distinctive reference location episodes (Passive Learners) no such interaction between these regions was apparent. Combined with the current observations, this

suggests CNP production contributes to both spatial and episodic-like memory encoding, and can be variably elicited in neurological subregions based on the type of memory encoded.

Elevations of NTproCNP indicate novel peptide synthesis within these regions during the four-hour delay between criterion and tissue acquisition. As a biologically inactive by-product of CNP synthesis and secretion, NTproCNP provides a more reliable marker of NPPC gene expression than CNP (Schouten et al., 2011; Woodward et al., 2017). Failure to detect a commensurate increase in CNP suggests other processes are involved in addition to NPPC expression. Reasons for this anomaly may include variable and/or increased loss of CNP at source due to activation of known degradative/clearance pathways, and variable loss during or after sample collection.

Possible effects of these experimental manipulations in hippocampal formation also deserve consideration. While reductions in hippocampal CNP, alongside slight increases in degradative ratio may be an effect of general handling and environmental exposure across all groups, this finding seems to support previous reports of a relationship between CNP, food intake and energy expenditure (Inuzuka et al., 2010; Prickett et al., 2010; Prickett et al., 2007; Yamada-Goto et al., 2013). Interestingly, the same pattern of CNP reduction in plasma due to increased degradation or clearance occurred during caloric restriction in sheep (Prickett et al., 2010). Data acquired here suggest a similar effect can occur centrally following manipulation related to a peripheral bodily system. Reductions of CNP would suggest a shift towards LTP within hippocampal tissues. These reductions may also align with previous reports indicating caloric restriction enhances cognitive performance and hippocampal neurogenesis (Kent, Oomen, Bekinschtein, Bussey, & Saksida, 2015). Though this study was not designed to examine effects of calorie restriction on central CNP, further investigation of the inter-relationship between cognition, food intake and energy expenditure related to CNP is warranted.

Also intriguing is a relationship between CNP, retrosplenial cortex and LTD. As previously mentioned, elevated CNP activity shifts synaptic plasticity towards LTD in hippocampus (Decker et al., 2008; 2009; 2010). In retrosplenial cortex slices, only LTD can be stimulated (Garden et al., 2009). This suggests that CNP may regulate a shift towards LTD in this region during memory consolidation. How this occurs and contributes to memory consolidation will require further elucidation. Initially it provides an interesting target for broadening our understanding of CNP, LTD and retrosplenial cortex in the neurological process of memory formation.

One concern of note regarding this data set is whether there may be an effect of variable learning rates. Although no relationship was identified between time to reach criterion and any CNP measure (Section 4.3), four rats (with their yoked counterparts) took a notably longer time to reach criterion than others, with one of these rats unable to reach criterion by the 35-day end-point. Exclusion of the rat unable to reach criterion from data analyses (data not shown) nullifies the statistical effect found in mammillary bodies for passive learners only, but not active learners. Effects identified in retrosplenial cortex remain. Exclusion of all slow learning rats nullifies all statistical effects. However, median values of NTproCNP in both mammillary bodies and retrosplenial cortex remain elevated beyond the interquartile range for active learners. This indicates loss of statistical effects arise due to a reduction in power to detect said effects, not a loss of the difference *per se.* Additionally, it suggests elevated concentrations of NTproCNP in mammillary bodies alone for passive learning rats may reflect an effect in these slow learning rats. Reasons for why this may be so are impossible to glean from this data. Mammillary bodies have been long known to contribute to memory processes, but have received renewed attention in recent years since their specific contribution is still unclear (for example, see Vann review "two memory systems"). Varying contributions of separate mamillary nuclei may be a contributing factor in this limitation here. In addition to further investigation of CNP in retrosplenial cortex indicated by these data, mammillary bodies should gain further consideration in future work.

Like much research, this study raises more questions than it answers. For example, does novel CNP synthesis occur in other regions relating to spatial and episodic-like memory during various stages of acquisition and consolidation? Extending on this, is CNP synthesis required for certain types of memory formation, or can stored peptide be released and used on an as needed basis? As the timing of sample acquisition and criterion used here may represent an occasion of re-consolidation, does peptide synthesis vary during an initial consolidatory episode? What is the time course of CNP activity within this interacting neurological network? Is this novel synthesis neuronal or glial in origin, and which cell type is targeted? Can other interactive effects of CNP be identified by extending ROIs and tasks tested? Of interest will be hippocampal subregions which serve differing roles in information processing (e.g. Gilbert, Kesner, & Lee, 2001; Hunsaker & Kesner, 2008) and wherein CNP can modify synaptic plasticity (Barmashenko et al., 2014; Decker et al., 2008; 2009; 2010). Although RIA provides a valuable tool as it is both highly sensitive and specific, regionally specific analysis within rodent brain is limited by tissue volume requirements. Continued use of RIA analysing CNP in rodent brain would be best complemented by microdialysis experiments.

Employment of both designs will allow insight into sub-regional CNP activity, and timing of events in the CNP signalling system.

Though limited in its conclusions, this study provides important supporting evidence for CNP as a novel target in age-related cognitive decline. Specifically, coinciding findings of possible age-related retrosplenial modification of CNP synthesis (Chapter 3), and retrosplenial CNP synthesis during episodic-like memory consolidation, indicate that the modification seen during age, may indeed contribute to age-associated episodic memory impairment.

Chapter 5. CNP-22 variably affects "pattern separation" of memory, based on stimulus overlap

5.1 Rationale

Rats for this study were initially cannulated to examine effects of infused CNP on a working memory task in the RAM. Additional complications arose during this initial study from difficulties in locating appropriate cannulation co-ordinates during pilots. This unfortunately led to a long delay before surgery could be conducted on these rats, resulting in cannulation at approximately 14 months old (see Methods). Figure 5.1 summarises the timing of this unforeseen delay, the initially chosen task (not reported here; below), and the eventual study design (expanded in Methods).

As previously mentioned, the RAM was selected in preference to the Morris water maze due to the aversive nature of the water-maze which makes it sensitive to stress and anxiety (Chapter 4; Paul et al., 2009). During RAM training, this cohort of rats were slow to learn at 14-19 months old compared with slightly younger rats (8-9 months old). This was unexpected given rats were "middle aged," but may have been an effect of surgery, or rats with poorly placed cannulae causing tissue damage. Moreover, infusion of CNP apparently increased anxiety as indicated by previous work (Jahn et al, 2001; Montkowski et al., 1998), and impacted RAM performance. Rats showed a tendency for greater arm-entry latencies, greater time spent on the central platform and more freezing behaviour. Eventual identification of adequate cannula placement greatly restricted data available for analysis of this behavioural task. Effects of CNP on working memory are thus not reported here, but this task and associated general difficulties are mentioned to account for prior behavioural experience. Instead, a study was designed examining effects of infused CNP on anxiety in the elevated plus maze (EPM), spontaneous location recognition (SLR) and novel object recognition (OR).

Although this thesis is focussed on CNP and memory, apparently increased anxiety following CNP infusions required quantification. This provided an opportunity to replicate reported anxiogenic effects of CNP (Jahn et al., 2001; Montkowski et al., 1998). Additionally, as CNP may be anxiolytic at low doses (Bíró et al., 1996) a dose range was selected to capture this possible bidirectional relationship. Based on these three previous works, and those investigating effects on passive avoidance learning (Bíró et al., 1996b; Jahn et al., 2001; Montkowski et al., 1998; Telegdy et al., 2000, 1999), doses of 0 CNP (Saline), 200ng, 500ng and 1000ng concentrations of CNP were selected (see Methods for detail). As

infusion procedures may also affect anxiety, a 'Baseline' condition (no infusion) was introduced for all tasks (see Methods). Infusions of CNP were known to be anxiogenic prior to this study, thus it was hypothesised that behavioural effects of elevated anxiety at 500 and 1000ng doses could be replicated in the EPM, and that anxiolytic effects may be evident in the low dose range (200ng).

The mechanism of action and consequences of ventricular administration of CNP are only able to be assumed currently based on previous works. Generally, one expects that following infusion into lateral ventricles any given infusate would diffuse throughout CSF and into cerebral tissue. As previously outlined, CNP can bind to both NPR-B and NPR-C receptors, with both accessible from lateral ventricle infusions based on their localisation (J. P. Herman, Dolgas, Marcinek, & Langub, 1996; J P Herman et al., 1996; Langub, Watson, et al., 1995). Consequences of CNP binding with NPR-B receptor have been previously outlined (intracellular increase of cGMP). This receptor can be desensitised via repeated exposure to CNP, but no evidence has been found for its internalisation or recycling (Fan, Bryan, Antos, Potthast, & Potter, 2005; Potter et al., 2006). Rather, NPR-B is hypothesised to release peptide into the extracellular space (Potter et al., 2006). In contrast, NPR-C is known to act via internalisation, degradation of peptide and recycling of the receptor into the cell membrane (Potter et al., 2006). As both receptors are accessible from ventricular administration, IHC is used here to ensure NPR-B related activity is generated by infusions (see below).

Several aspects of CNP indicate it may be involved in pattern separation functions of memory. Pattern separation is a term that has arisen from research in computational modelling to describe a mechanism whereby similar inputs are transformed into less similar outputs, resulting in non-overlapping representations (Bekinschtein et al., 2013; Bekinschtein, Oomen, Saksida, & Bussey, 2011; Kent, Beynon, et al., 2015; Sahay, Wilson, & Hen, 2011; Yassa & Stark, 2011). This mechanism is a crucial feature for separating complex memory representations in episodic memory and increases the likelihood for accurate encoding and subsequent retrieval of experiences that may be similar (Kent, Beynon, et al., 2015; Yassa & Stark, 2011). Pattern separation occurs during memory encoding, and experimental work indicates DG is critical (Aimone, Deng, & Gage, 2011 for review; Gilbert et al., 2001; Hunsaker & Kesner, 2008). Because CNP primarily influences memory encoding and not recall (Telegdy et al., 1999; 2000), and mRNA expression occurs in CA1 through CA3 whereas NPR-B receptors are isolated to DG (Herman et al., 1996; Langub, Watson, et al., 1995), an investigation of the peptide in "pattern separation" was indicated. Furthermore,

pattern separation deficits are now proposed to account for age-related changes in episodic and recognition memory in both rodents and humans (Burke et al., 2010; Yassa et al., 2011; Yassa & Stark, 2011). Hence, identifying a contribution of CNP to this process would provide valuable information regarding a role in age-related cognitive decline.

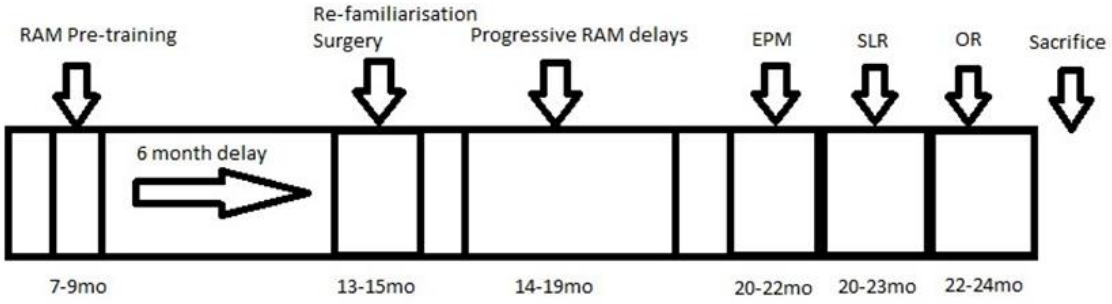
To test possible effects of CNP in pattern separation, a spontaneous location recognition task was employed. SLR provides a task of object recognition wherein objects themselves are identical, but rodents must identify when an object is presented in a novel location (Ennaceur, Neave, & Aggleton, 1997; Warburton, Baird, Morgan, Muir, & Aggleton, 2000). A recent modification to this paradigm has been used to test the ability to keep complex memory representations distinct (pattern separation) by manipulating distance between objects at the time of encoding (Bekinschtein et al., 2013). Additionally, this task was previously sensitive to the peptide acyl-ghrelin (Kent, Beynon, et al., 2015), suggesting it may also be appropriate for study of CNP.

Inclusion of SLR necessitated basic recognition testing to ensure rats could discriminate between objects generally. Inclusion of novel object recognition (OR) was also indicated by findings of Barmashenko and colleagues (2014) – that downregulation of CNP signalling improved recognition in the same task – and my own previous work showing correlations between better discrimination and lower concentrations of endogenous CNP (Rapley, unpublished Master's thesis). EPM testing was carried out first and as SLR was the task of greater interest, it was conducted before OR.

In addition to above outlined expected effects on anxiety, higher doses of CNP were predicted to decrease exploratory behaviour in all tasks (following from Barmashenko et al., 2014; Bíró et al., 1996; Jahn et al., 2001; Montkowski et al., 1998). Contrary to previous findings that administered CNP could improve memory in a passive avoidance task (Telegdy et al, 1999; 2000), here I hypothesised that increasing doses of CNP (500 and 1000ng) would interfere with memory acquisition during object recognition but that lower doses (200ng) may have a similar beneficial effect to that found by Barmashenko and colleagues (2014). This was hypothesised because reduction in CNP signalling improved recognition (Barmashenko et al., 2014) and my own work indicated improved recognition was associated with reduced endogenous CNP concentrations (Rapley, 2012, unpublished Master's thesis). According to Bekinschtein, Kent and colleagues (Bekinschtein et al., 2013, 2014; Kent, Beynon, et al., 2015), control animals discriminate at chance levels (indicated by discrimination ratio - $d2 \approx 0$) during SLR when pattern separation load is high (Extra-Small

separation), and show novelty preference when this load is reduced (Small separation – see 5.2.2 Behavioural Procedures). This was expected here during Baseline conditions. As SLR is fundamentally a task of recognition, the same hypothesis as for OR was applied – that increasing doses of CNP would interfere with recognition.

To test these several hypotheses (that high doses of CNP would increase anxiety on the EPM, decrease exploration overall, and interfere with object recognition in both OR and SLR tasks), all rats were tested on all possible tasks with all possible doses of CNP (see Methods) in a repeated measures design. Adequate cannula placement was achieved in nine rats. To ensure infused CNP generated neurological activity, brain tissue was stained for cyclic GMP using ABC-DAB immunohistochemistry. By the time rats performed the tasks reported here, cognitive impairment was apparent based on inability of most rats to discriminate novel locations in the spontaneous location recognition task (See 5.3 Results). While unintended, advanced age and cognitive deficits in these rats provides improved information for examining CNP during age-related cognitive decline.



5.1 Outline of timing for behavioural tasks and surgery for cohort of rats receiving cannulation surgeries. Data from RAM testing is unreported here due to interference with the task by CNP infusions (as outlined in text).

5.2 Methods

5.2.1 General Methods

Subjects

Male PVGc rats (initial N = 42; N at sacrifice = 28 – attrition due to age-related illness; Final N (adequate cannula placement) = 9) aged between 13 and 15 months at surgery, were initially housed in groups of two to four in standard opaque laboratory cages (45 cm x 27 cm

x 22 cm high). Following surgery rats were housed in the same cages individually. Nesting paper was provided in each cage and socialisation occurred daily during behavioural tasks in groups of two to four rats (also in standard laboratory cages) to ameliorate any effects of isolated housing. Wooden gnawing blocks were provided during socialisation to discourage chewing of cannulae. Rats were maintained on a reversed 12-hour light-dark cycle (lights off 0900-2100h), and colony rooms were kept at 22°C and 48%rH. Free feed was provided during surgical recovery and throughout all tasks comprising this study. Rats were also used to investigate whether infused CNP affected working memory in the RAM (data not reported) and experienced food deprivation during that task. Water was available *ad libitum*. All procedures conformed to the NIH guide for the care and use of laboratory animals and were approved by the University of Canterbury Animal Ethics committee.

Surgery

Rats were unilaterally implanted with a stainless-steel guide cannula (22ga; Plastics One, Roanoke) in either the left or right lateral ventricle under Ketamine / Domitor anaesthesia (Ketamine 85mg/kg ip followed by Domitor 0.35mg/kg ip) 30 minutes after Carprofen (5mg/kg sc) for pain relief. Rats were placed on a stereotactic frame (Stoelting) using atraumatic ear bars (Kopf) in a flat skull orientation, such that Bregma and Lambda DV coordinates were equivalent. Cannulae were attached to the skull with jewellery screws and dental cement. Dummy stylets (Plastics One, Roanoke) were inserted into guide cannulae to maintain patency. Location of the lateral ventricle was determined based on coordinates from Paxinos and Watson (1998), equivalent to: Bregma -.080 mm; Lateral +/- .14 mm; Ventral 3.5mm from skull surface (Figure 5.2). Local analgesia (2mg/ml Mepivicaine) was applied at the site of incision, followed by Emla analgesic cream after suturing, and anaesthesia reversed with Antisedan (2.5mg/kg ip) five minutes after Atropine (0.15mg/mL ip) to reduce respiratory distress. All rats recovered for a minimum of one week before behavioural testing began. Adequacy of cannula placement was determined by an infusion of methylene blue 15 minutes prior to sacrifice in combination with cresyl violet staining.

Peptide infusate and treatment

CNP was purchased from Bachem (CNP-22, Torrance, CA, USA) and infusate made in a stock concentration of 1.0 µg/2 µL (equivalent to 1000ng of CNP in 2µL) by dissolving in 0.9% Saline under sterile conditions. Aliquots of 50µL stock infusate were stored at -80°C, with quantities and dilutions required for this experiment made on a single occasion to reduce possible effects of thawing and refreezing. Infusions were made by use of internal cannulae

(Plastics One, Roanoke) which protruded 0.5mm from the stainless-steel guide cannula. Internal cannulae were attached to PE50 tubing (Plastics One) and a 10µL Hamilton syringe, with administration controlled by an infusion pump (Harvard apparatus). Following the two-minute infusion period, internal cannulae were left in position for a further two minutes, to allow diffusion of infusate away from the infusion site. All rats performed each behavioural task with no infusion initially (Baseline) followed by counterbalanced infusions of 2µL 0.9% Saline, or a 2µL volume of active infusate containing 200ng, 500ng or 1000ng CNP. Infusions occurred 30 minutes before being placed on the EPM or 30 minutes before sample trials of SLR and OR tasks. This timing was selected based on work by Bíró et al (1996), Montkowski et al (1998), and Jahn et al (2001) indicating effects on the EPM at this period post-infusion; and that CNP acts primarily during acquisition and consolidation of memory (Telegdy et al, 1999, 2000; SLR and OR tasks).

Sacrifice and Perfusion

Following completion of all behavioural tests, rats were given a final infusion of saline, 200ng, 500ng or 1000ng of CNP in a between-subjects fashion. Assignment to groups for final infusion was determined using stratified randomisation based on performance on the EPM (following the assumption that anxiety response should be stronger in rats with best cannula placement). Five additional rats (excluded due to malfunctioning cannulae) which had never received an infusion, provided non-infused comparisons for immunohistochemistry. Rats were anaesthetised 30 minutes following final CNP infusion (and 15 minutes after infusion of 2µL of methylene blue) with an overdose of sodium pentobarbitone (1mL at300mg/mL ip). Once unresponsive to tail pinch and plantar reflex, rats were transcardially perfused with 60mL 0.9% saline followed by 60mL of 4% paraformaldehyde. Fixed brains were removed from the skull and placed in 4% paraformaldehyde prior to transfer to a 0.1M phosphate buffered 20% glycerol solution. Forty µm coronal sections were gathered as a 1:5 series using a freezing microtome (Thermo Fisher) prior to staining with cresyl violet or storage at -20°C in 40% 0.1M phosphate buffer / 30% glycerol / 30% ethylene glycol for later immunohistochemistry for cyclic guanosine monophosphate (cGMP).

Histology

Cresyl Violet

One series of 40µm coronal sections was mounted on gelatine coated slides and stained with cresyl violet for assessment of cannula placement. Sections were delipidised in ascending

then descending concentrations (70%, 95%, 100%) of ethanol. Distilled water was used to hydrate sections before staining with 0.5% cresyl violet acetate solution, and subsequently rinsed in distilled water. Sections were dehydrated and differentiated by immersion in solutions of 70% ethanol, 95% ethanol, 95% acid-alcohol (ethanol / glacial acetic acid) then 100% ethanol, prior to cleaning in Xylene and mounting and coverslipping with Depex mounting medium.

cGMP immunohistochemistry

Cyclic GMP response was visualised using the ABC-DAB immunohistochemistry method. Free-floating sections were washed in phosphate buffered saline containing 0.3% Triton X-100, then endogenous peroxidase activity quenched with incubation in 3% H_2O_2 prior to incubation in 5% normal goat serum to block non-specific binding of secondary antibody. Subsequent incubations contained 1% normal goat serum, and tissues were washed with phosphate buffered saline / 0.3% Triton X-100 between each incubation. Tissues were incubated overnight in polyclonal Rabbit Anti-cGMP primary antibody (1:8000, Merck Millipore, California, USA) then in Biotinylated Goat Anti-Rabbit secondary antibody (1:400). Tissues were then incubated in Avidin-Biotin complex (ABC kit, Vector, CA, USA), washed with Tris buffer, and antigen binding revealed with Diaminobenzidine (DAB) substrate. After final washes in Tris buffer, sections were mounted on gelatine coated slides from ice-cold phosphate buffer and allowed to dry before dehydration in ascending alcohols, clearing with Xylene and coverslipping with Depex mounting medium.

Image Acquisition, Analysis and Regions of Interest

For examination of cannula placement, microscopy images of cannulae tracts were captured using a Nikon DS-Fi1 camera mounted on Nikon eclipse E800M microscope at 20x objective magnification. Adequately located cannulae are described in section 5.3.1 Cannula placement. Cyclic GMP images were obtained using a Leica DFC 7000T camera mounted on Leica DM6B microscope at 100x objective magnification. Regions of interest were: prelimbic, infralimbic and medial orbital cortices; cingulate gyrus; anterodorsal thalamic nucleus; retrosplenial dysgranular and retrosplenial granular B cortices; dorsal hippocampal regions CA1, CA3 and DG; hypothalamus sampled from medial arcuate and ventromedial nuclei; amygdala containing medial, central, basomedial and basolateral nuclei; perirhinal cortex and medial mammillary nucleus. Regions were selected based on a previous demonstration of NPR-B receptor localisation, and pertinence of these structures in relation to behavioural tasks investigated. For each region of interest, a maximum of three images

were captured from each hemisphere, to provide an average density value for a region within that hemisphere. All images were processed and neuron density data acquired using FIJI (Image J) software. After conversion to grayscale, image backgrounds were subtracted and brightness/contrast automatically adjusted. Image noise (introduced in some images by dust and/or microbubbles) was reduced by excluding dark outliers smaller than five pixels. Image thresholds were adjusted to contain the darkest 2% of pixels, based on the assumption that using the most extreme values on the grayscale distribution would most likely represent only neurons expressing cGMP, and exclude any background staining, which was widespread (Appendix B). Counts were acquired using the FIJI automatic particle analysis operation with a size threshold between 4 and 20 µm based on average feret size of neurons from Albasser, Poirier, & Aggleton (2010). Counts were expressed as particles per μm^2 by dividing particle count by total area sampled.

5.2.2 Behavioural Procedures

For all tasks, rats were initially tested with no infusion (Baseline) then with four possible infusion conditions: 0.9% Saline or 200, 500 or 1000ng CNP meaning each rat performed each task five times. The order in which infusions were administered was counterbalanced within a task, with several rats allocated to resulting infusion order conditions. Across tasks, no rat experienced the same repeated order of infusions. For example, a rat experiencing the infusion order 200ng, 500ng, Saline, 1000ng across exposures to the EPM, had different orders of infusions (e.g. 500ng, 1000ng, 200ng, Saline) during OR, which differed again for both versions of SLR (below).

Elevated Plus Maze

Apparatus

The EPM consisted of two closed and two open arms (50cm long x 10 cm wide), set perpendicular to each other around a central platform (15cm x 15cm), raised 1 metre above the floor, located in a dimly lit room. Closed arms were made of plywood painted black (24.5cm high), with the walls of open arms made of Perspex to prevent rats from falling. Previous work (Martínez, Cardenas, Lamprea, & Morato, 2002) has indicated the presence of Perspex does not affect aversion to the open arms. Occupancy of and entry to the open arms indicates lower levels of anxiety, with entries to closed arms indicative of general activity (Cruz, Frei, & Graeff, 1994).

Procedure

Thirty minutes after infusion, rats were placed on the central platform of the EPM facing an open arm. Rats were observed via a CCTV camera mounted above the EPM, by the experimenter located in a corner of the room opposite an open arm. Arm occupancy and entries were noted every three seconds for five minutes with entry to an arm or location on the central platform defined as having all four feet located within the arm or central platform. The EPM was cleaned between rats with a disinfectant solution suitable for use with rodents (4% ExpressSani). At least one day was interposed between each test to ensure adequate clearance time of the infused substance.

Spontaneous Location Recognition

This task was based on Bekinschtein et al (2014) and Kent et al (2015).

Apparatus

SLR used a black circular arena (90cm diameter, 50cm high) constructed of a Perspex wall covered with a black adhesive covering ('duraseal') on a wooden base covered with bedding (sawdust). The arena was located on the floor of a dimly lit room, with three proximal visual cues and varied distal furniture. All trials were filmed by a web camera mounted above the arena and videos stored digitally for later exploration quantification. Objects used for this task were cans and bottles of various shapes, colours and sizes (maximum 25cm high, 25cm circumference) in triplicate, filled with sand to prevent rats tipping the objects over. All objects were cleaned between rats with the same disinfectant as used for the EPM, to remove potential odour cues.

Procedure

All rats were familiarised to the arena across three 10-minute habituation sessions (one session per day for three days). SLR consisted of a 10-minute sample, and a five-minute trial, 24 hours later. Sample and trial phases for the entire cohort of rats ran across four-days, providing adequate infusate clearance within sample-trial delay and between individual trials. Object arrangement in sample and test phases is visualised in Figure 5.3. Objects were arranged in a triangular formation (one at the 'apex,' Object A; two at the 'base,' Objects B and C), located 15cm from the edge, and 30cm from the arena centre, with objects B and C equidistant from object A. Two versions of the task were used, where the major difference was distance between objects B and C during the sample phase. In the "small separation" condition (SM), objects B and C were separated by a 50° angle relative to object A (20.5 cm

apart); for the "extra-small separation" condition (XS) these objects were separated by 40° (15.4cm apart). All rats completed both separation conditions in a counterbalanced order (i.e. half experienced "small" condition first, and half experienced "extra small" first) at any given dose of peptide. For sample trials (30 minutes post-infusion), rats were placed centrally in the arena with three identical objects, and allowed to explore freely. During test phase, rats were placed in the arena with two object copies – one located as previously (object A; familiar location), and the other located centrally between object B and C locations (novel location). Recognition of the now centrally located object as novel was indicated by exploration preference for this object during the test trial. Exploration of an object included directing the nose or forepaws towards the object (<1cm) with vibrissae twitching, and excluded sitting on, chewing, or using the object to rear. Exploration was quantified by the experimenter from videos using a computer based stopwatch program which accumulated time based on keypress during exploration.

Object Recognition

Apparatus

OR used a wooden arena (80cm x 80cm x 80cm high) painted black, with a sawdust covered floor, elevated 72cm above the floor of a dimly lit room. Objects used were 'knick-knacks' (e.g. egg cup, coloured cube, espresso cup) of assorted colours, textures and sizes (maximum 15cm high, 8cm wide), held down by blu-tack to prevent rats from tipping them over. Objects were placed equidistant from each other and arena walls (see Figure 5.4 for configuration). All objects were cleaned between rats with disinfectant solution to reduce odour cues, and all trials were filmed with a web camera mounted above the arena. Videos were again stored digitally for later quantification of exploration.

Procedure

Rats were familiarised to the OR arena across three daily five-minute habituation sessions. OR consisted of sample (5 minutes; 30 minutes after infusions) and trial (5 minutes) phases, separated by a 24-hour delay, again allowing adequate infusate clearance. During the sample phase, rats were placed in the arena with two copies of a novel object (object A1 and object A2) and allowed to explore freely. Following a 24-hour delay, rats were returned to the arena which contained one copy of object A, plus another completely novel object (object B). Recognition of object A as familiar is indicated by a preference for exploring the novel object during the trial. Objects within a pair (A/B) were counterbalanced for use as the sample

object (half of rats experienced A in sample trial, half experienced B), as was novel object location within the trial phase. Exploration was classified and quantified as for SLR.

Order and timing of behavioural tasks

Prior to surgery, rats were pre-trained to run in a radial arm maze. Surgery occurred when rats were between 13 and 15 months old. Following a minimum one week surgical recovery, rats were trained on a working memory task in the RAM, before completing EPM, SLR and OR testing at between 19 and 23 months of age. At final sacrifice rats were between 21 and 23 months of age (see Figure 5.1).

5.2.3 Statistical Analyses

Following identification of adequately placed cannulae, final N for analyses was 9 rats (N=9). Three dependent variables were calculated for EPM – percent time spent in the open arm (as a proportion of time spent in either arm), percent entries to the open arm (as a proportion of total entries to either arm) and number of entries to the closed arm. Exploration times (in seconds) for individual objects in both OR and SLR tasks were used to calculate total exploration time within sample trials. Recognition in both tasks was quantified by calculating the discrimination ratio (d2) which varies between -1.0 and +1.0, with positive values indicating novelty preference. The ratio is calculated by taking the difference in exploration time for the novel and familiar object during the trial phase and dividing that difference by total exploration time for both objects within the trial (i.e. novel object exploration – familiar object exploration / total exploration time). Cyclic GMP expression was quantified as a density measurement, particles per μm^2, as outlined in 5.2.1.6 Image Acquisition, Analysis and Regions of Interest.

All data analyses included initial checks that assumptions were met for relevant statistical tests. Initial analyses for OR and SLR data ensured that object copies were explored for equivalent times during sample trials using dependent means t-tests for OR (object A1 vs object A2) and one-way repeated measures ANOVA (object A vs B vs C) for SLR, followed by dependent means t-tests (location A vs location B+C). For the EPM and OR tasks, one-way repeated measures ANOVAs were conducted on each dependent variable. SLR exploration time and d2 were analysed using 2 x 5 (Task x Infusion) repeated measures ANOVA in a linear mixed effects model framework. All models were tested using polynomial contrasts, given the ordered nature of infusion conditions (linear for effect of Task in SLR; linear, quadratic, cubic and quartic for effect of Infusion in all analyses). Effect sizes (Pearson's r) were calculated on significant trends.

As final infusions for cyclic GMP analysis were administered in a between-subjects fashion, this unfortunately resulted in small final Ns for varied infusion groups. As such, data were pooled into "Non-active" and "Active" infusions representing rats which received no infusion or Saline (N = 7; "Non-active"), versus rats which received final infusions of any CNP dose (N = 7; "Active"). This is recognised as problematic, but a preferable alternative to no analysis. Analyses were conducted by 2 x 2 (Infusion type x Hemisphere) repeated measures ANOVA for each region of interest. Medial mammillary nucleus was not separated by hemisphere, thus was analysed by independent means t-test. Effect sizes are reported as partial-eta squared for ANOVAs and Cohen's d within mammillary bodies.

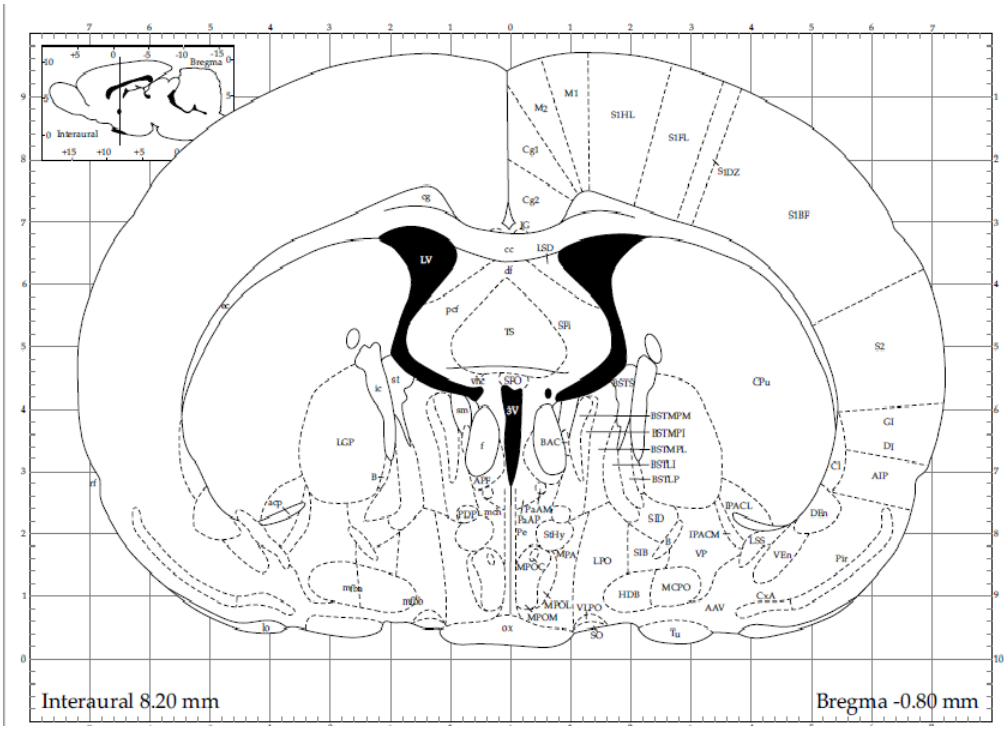


Figure 5.2: Location of cannulae target co-ordinates. From Paxinos & Watson (2009)

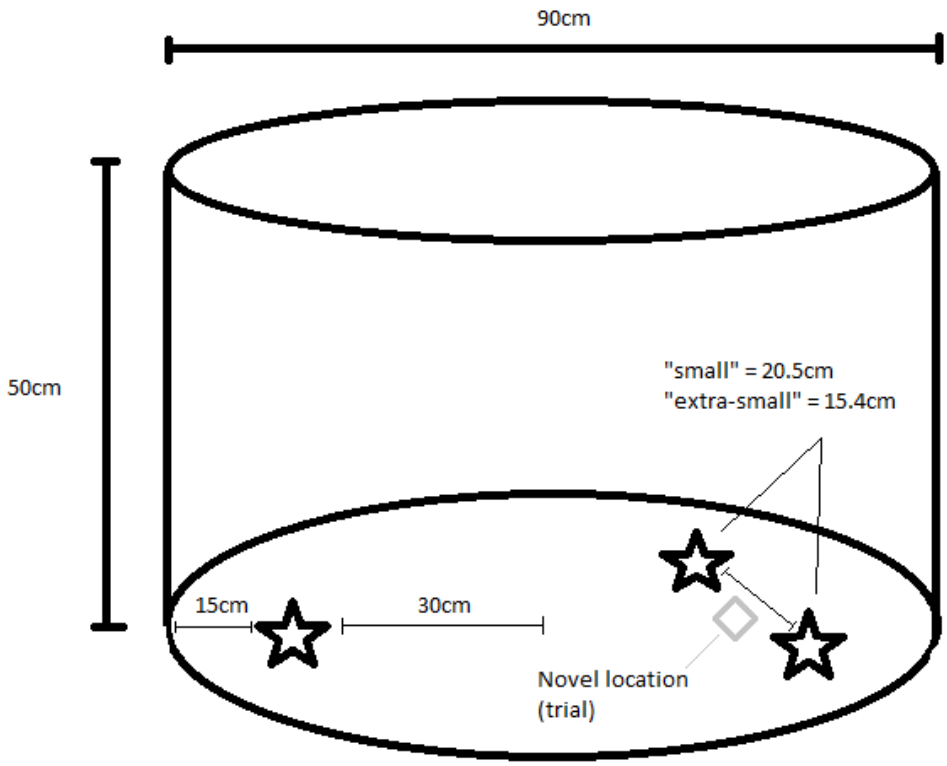


Figure 5.3: Visual representation of spontaneous location recognition arena. Black stars indicate object locations in sample phase. Gray diamond indicates central novel location during trial phase.

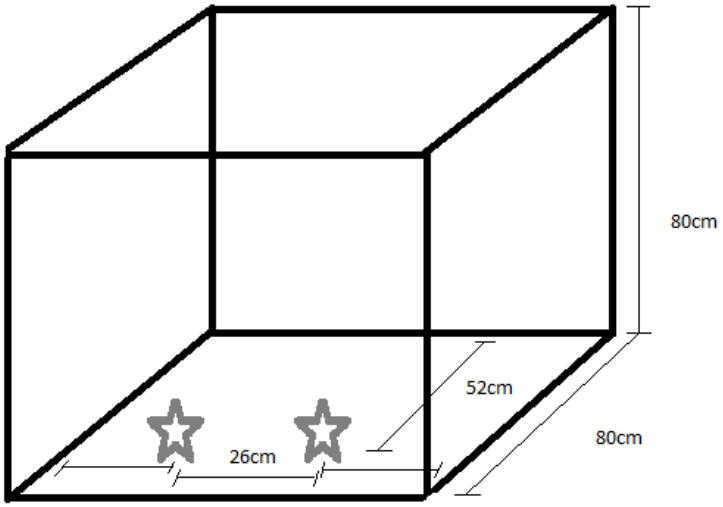


Figure 5.4: Visual representation of object recognition task. Gray stars indicate object locations within any given trial.

5.3 Results

5.3.1 Cannula placement

Figures 5.5 A, B and C provide approximate locations of centrally located cannulae in rats retained for behaviour analysis and rats used as non-infused immunohistochemistry controls. Examples of cGMP immunostaining are provided in Appendix B. Note that one rat had a cannula located within the corpus callosum and was not included in behavioural analysis. This rat was retained to increase numbers for “Inactive” cGMP immunohistochemistry and had never received an infusion of any kind.

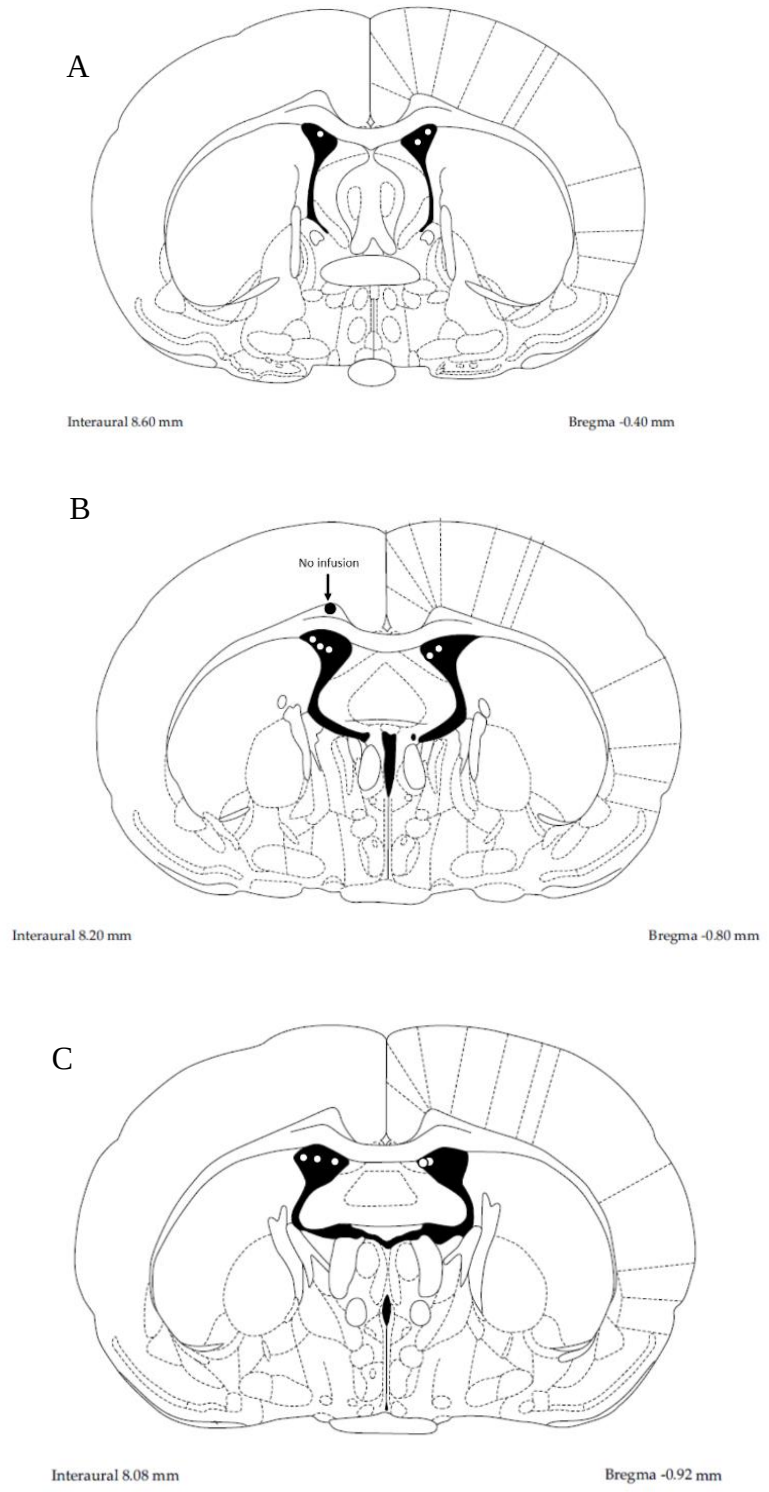


Figure 5.5A, B, C: Approximate locations of cannulae tips (white circles) within lateral ventricles of rats retained for analyses. Note: single rat with non-central cannula was a non-infused control and had never received any infusion. Modified from Paxinos & Watson, 2009.

5.3.2 Elevated Plus Maze

Infusions did not affect percent of time spent in the open arms of the EPM ($F(4, 32) = 0.68$, $p = .610$, generalized $\eta^2 = 0.044$) and no trends were evident across doses (all $ts < 1.17$, all p's $> .250$; Figure 5.6). Although statistical tests were non-significant, 1000ng infusion of CNP exhibited a trend towards a reduced percent of time spent in the open arm compared with all other infusion conditions (mean difference compared with Saline infusion = 9.78). Infusions also did not affect percent entries to the open arm in the EPM ($F(4, 32) = 0.83$, $p = 0.515$, generalized $\eta^2 = 0.068$), and no trends were evident (all $ts < 1.40$, all $ps > .171$; Figure 5.7).

Infusions did affect number of entries to the closed arm ($F(4, 32) = 2.79$, $p = .043$, generalised $\eta^2 = 0.196$; Figure 5.8) indicating an influence of CNP on general activity. A significant quadratic relationship across doses was identified ($t(32) = 3.13$, $p = .004$, $r = 0.484$). Inspection of Figure 5.8 shows any Infusion (Saline) decreased closed arm entries. Entries were further decreased by 200ng CNP then increased across 500ng and 1000ng dose conditions. This indicates that infused CNP alters motor activity of rats in a dose dependent manner.

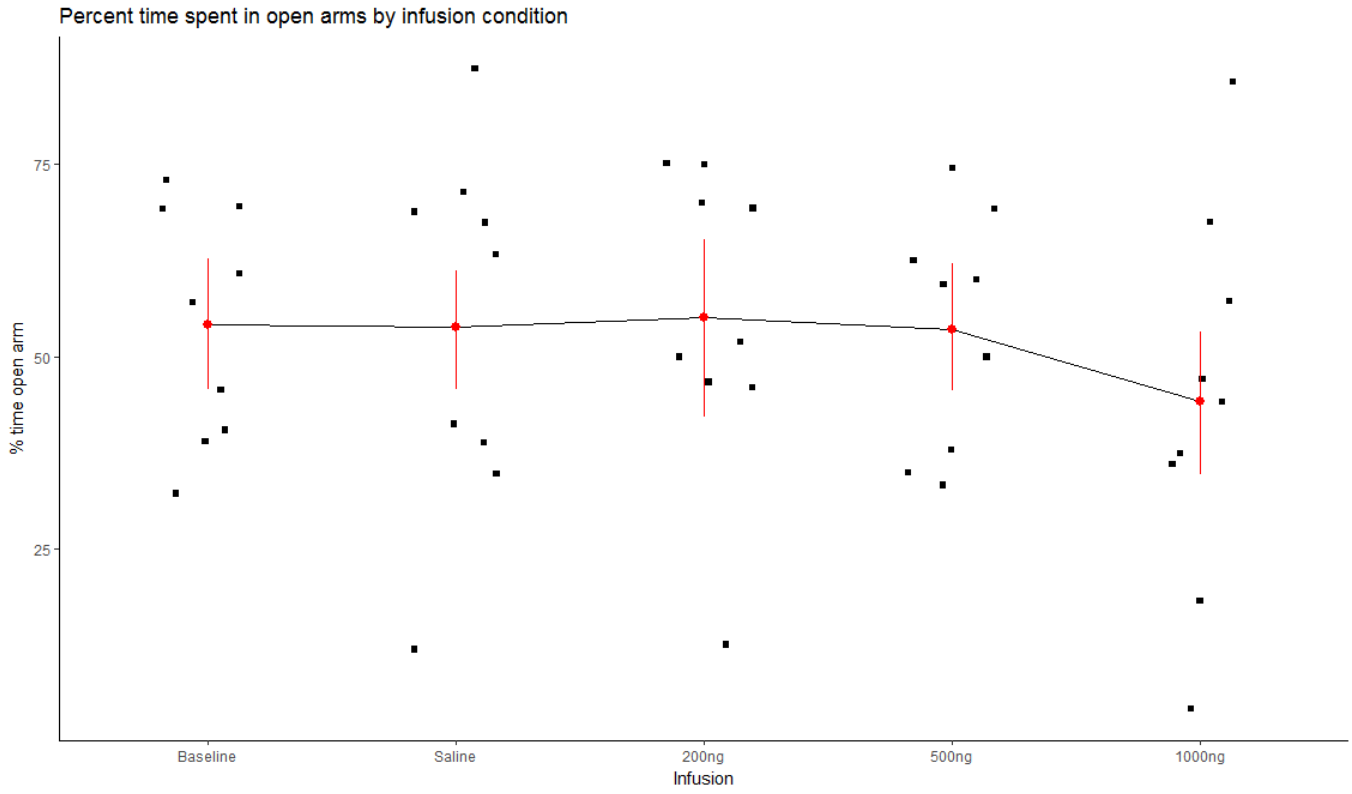


Figure 5.6: Mean [± 95% CI] and individual percent time spent in open arms of the elevated plus maze across infusion conditions. No significant effects.

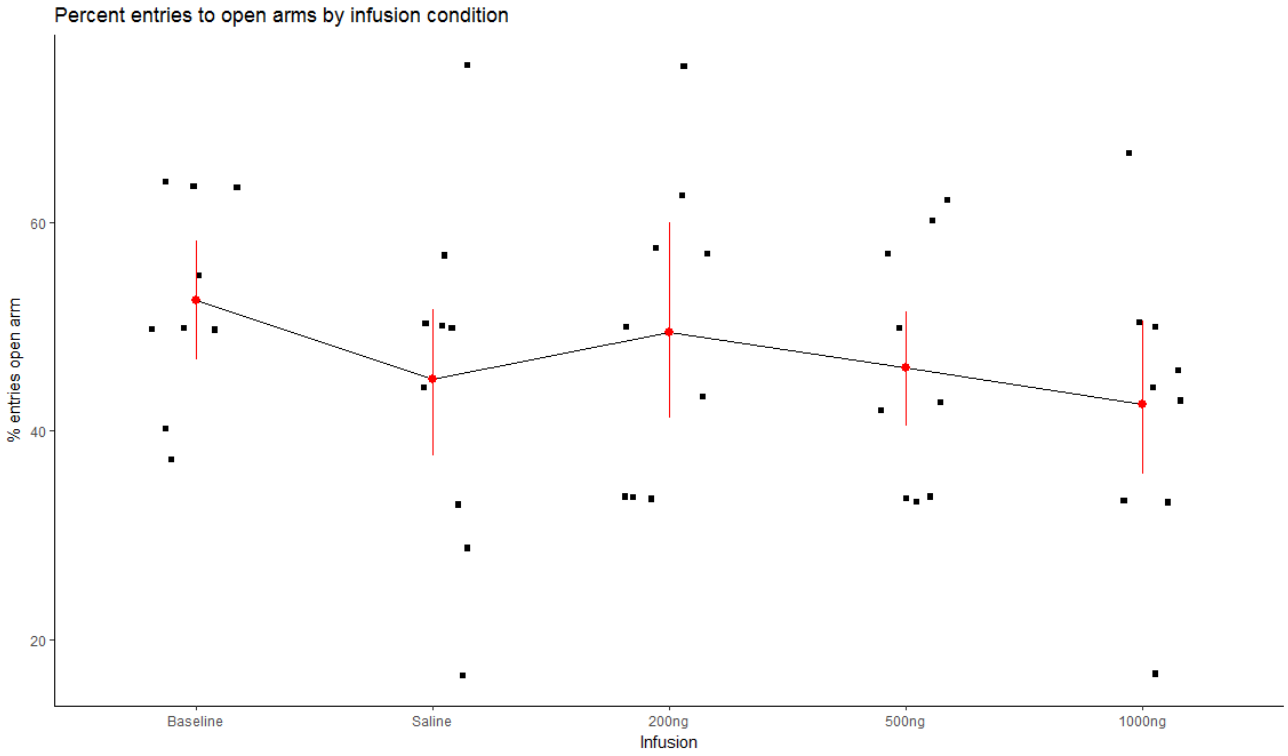


Figure 5.7: Mean [± 95% CI] and individual percent entries to open arms of the elevated plus maze across infusion conditions. No significant effects.

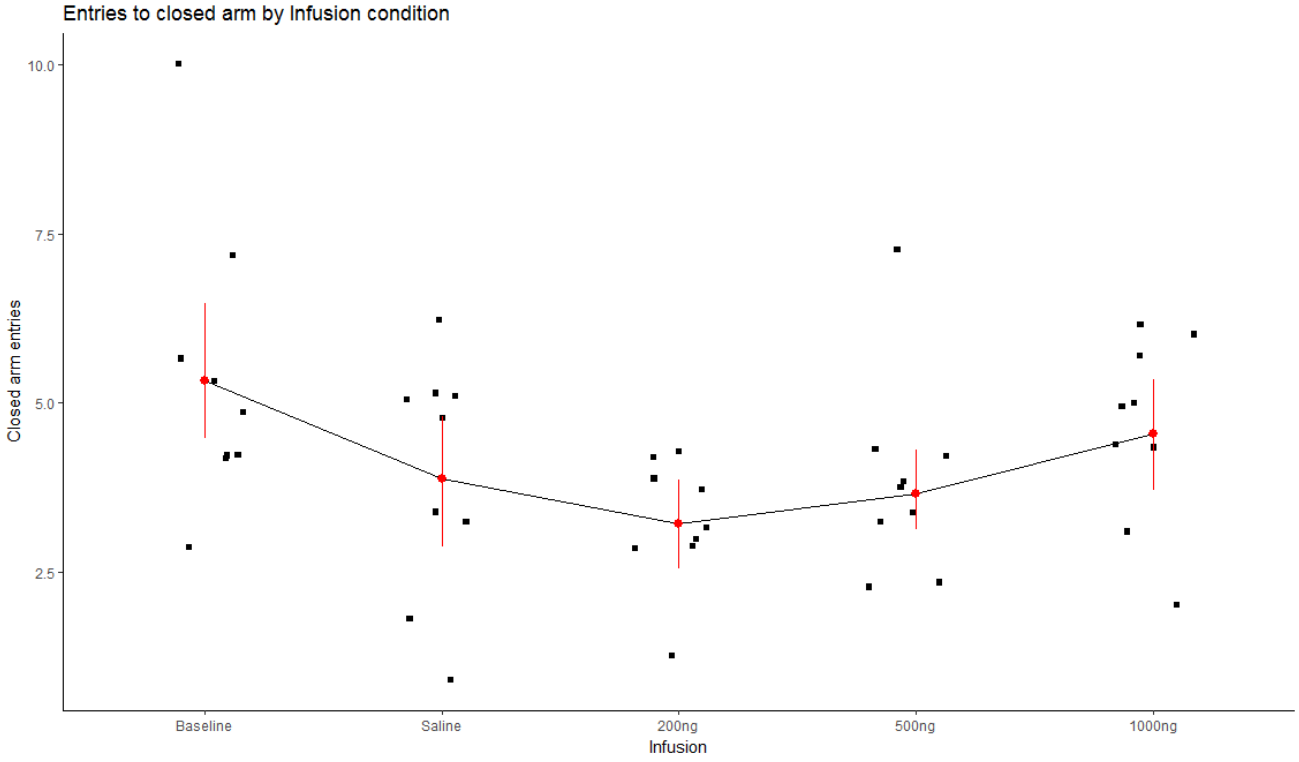


Figure 5.8: Mean [± 95% CI] and individual number of entries to closed arms of the elevated plus maze across infusion conditions. Significant quadratic trend – infusion decreases activity. Low dose CNP decreases further, then increases with increasing dosage.

5.3.3 Spontaneous Location Recognition

Initial analysis indicated that rats preferentially explored "Object A" compared with Objects "B" and "C" during the Sample trial for both the Extra-Small (XS) separation condition and the Small (SM) separation condition (Table 5.1; But note XS, 500ng condition and SM, 1000ng condition). As "Object A" was in a separate location to Objects "B" and "C," exploration times for objects B and C were combined, and dependent means t-tests indicated that exploration between the two maze locations was equivalent during sample trials for both tasks (Table 5.1)

Total exploration during SLR sample trials was affected by both the separation condition and CNP infusions (effect of Task (χ^2 (10) = 6.33, p = .012; F (1, 40) = 6.06, p = .018; effect of Infusion: χ^2 (9) = 15.26, p = .004; F (4, 32) = 4.18, p = .008) but there was no interaction between the two (Interaction effect: χ^2 (14) = 2.85, p = .583; F (4, 40) = 0.65, p = 0.634; Figure 5.9). Rats explored less during XS conditions than SM conditions (Linear effect of Task: t (44) = 2.48, p = .017, r = 0.35). The Infusion effect had significant linear (t (32) = -2.75, p = .010, r = 0.44) and quadratic (t (32) = 2.22, p = .033, r = .37) trends. Figure 5.9 indicates that the introduction of an infusion (Saline vs Baseline) reduced exploration, which was then reduced further by an infusion of 200ng CNP in both tasks. 500ng of CNP increased exploration compared with 200ng doses in both tasks, with exploration not affected further by a 1000ng Dose in the SM task. In the XS task, a 1000ng Dose decreased exploration. This effect in the XS task is mitigated by the exclusion of two outlying rats (see Figure 5.9) from analyses. When these outliers are excluded (data not shown) the same effects of significant linear and quadratic trends across dose are found. Exclusion of these data points accounts for a lack of significant interaction (Task x Dose) in the data, when Figure 5.9 indicates exploration varies by dose and task at 1000ng of CNP in the XS task. Mean values for this condition following this exclusion are no longer significantly different to 1000ng CNP dose in the SM condition (data not shown). Overall, this indicates CNP modifies exploratory behaviour and does so in a dose dependent manner.

One expectation for discrimination data was that rats would discriminate at chance levels ($d2 \approx 0$) in the XS task and show a novel location preference in the SM task at Baseline. Instead, rats performed at chance on average in the SM separation condition, and showed a familiarity preference during the XS separation condition (Figure 5.10). This suggests rats on average were impaired compared to control rats reported by Bekinschtein et al (2014) and Kent et al (2015). Discrimination was not affected by Infusions or Task type

overall (Effect of Infusion: χ^2 (9) = 1.68, p = .794; F (4, 32) = 0.57, p = .454; Effect of Task (χ^2 (10) = 0.54, p = .464; F (1, 8) = 0.29, p = .607). Although the Interaction between Task and Infusion was non-significant (F (4, 40) = 1.94, p = .123), its inclusion within the linear mixed-effects model introduced a substantial (non-significant) improvement in model fit (χ^2 (14) = 7.97, p = .093). Within this model, there was a significant Linear trend interaction (Linear trend of Infusion x Linear trend of Task; t (40) = -2.36, p = .023, r = 0.35). Inspection of Figure 5.10 indicates that this Interaction occurred via a tendency for d2 to increase across Infusions in the XS task, but decrease across Infusions in the SM task. CNP therefore modifies location discrimination in a dose dependent manner, and its effect varies with differing stimulus overlap.

Table 5.1: Mean [±95%CI] exploration time of each object (A, B, C) and exploration time of location (B+C) with accompanying repeated measures ANOVA (objects) and dependent t-tests (location) during spontaneous location recognition sample trials. Note: object A values are compared to B+C values for location comparison t-test.

Condition	A Mean [±95%CI]	B Mean [±95%CI]	C Mean [±95%CI]	ANOVA	B+C Mean [±95%CI]	t-test
Extra-Small						
Baseline	20.21 [11.88, 28.54]	11.12 [5.95, 16.29]	10.40 [3.89, 16.91]	F (2, 16) = 18.27, p < .001	21.52 [10.25, 32.79]	t (8) = -0.56, p = .591
Saline	14.41 [9.65, 19.17]	7.75 [4.94, 10.56]	6.90 [3.63, 10.17]	F (2, 16) = 16.68, p < .001	14.64 [8.82, 20.46]	t (8) = -0.12, p = .908
200ng	11.93 [9.28, 14.58]	6.52 [4.30. 8.74]	5.78 [3.47, 8.09]	F (2, 16) = 7.89, p = .004	12.30 [10.16, 14.44]	t (8) = -0.21, p = .840
500ng	11.78 [8.45, 15.11]	10.09 [5.66, 14.52]	7.35 [4.71, 9.99]	F (2, 16) = 2.27, p = .136	17.43 [11.61, 23.25]	t (8) = -1.90, p = .094
1000ng	11.54 [7.05, 16.03]	4.98 [3.16, 6.80]	5.47 [3.53, 7.41]	F (2, 16) = 10.20, p = .001	10.46 [7.32, 13.60]	t (8) = 0.56, p = .593
Small						
Baseline	17.78 [12.94, 22.62]	12.54 [9.28, 15.80]	12.39 [7.52, 17.26]	F (2, 16) = 4.56, p = .027	24.93 [17.22, 32.64]	t (8) = -2.16, p = .063
Saline	14.90 [10.88, 18.92]	11.74 [8.21, 15.27]	7.71 [4.08, 11.34]	F (2, 16) = 4.68, p = .025	19.45 [8.15, 30.75]	t (8) = -1.11, p = .301
200ng	13.63 [11.49. 15.77]	8.71 [4.16, 13.26]	6.51 [4.23, 8.79]	F (2, 16) = 8.12, p = .004	15.22 [9.24, 21.20]	t (8) = -0.56, p = .589
500ng	16.52 [12.01, 21.03]	10.93 [6.02, 15.84]	9.50 [5.01, 13.99]	*F (2, 16) = 7.42, p = .005	20.43 [11.51, 29.35]	t (8) = -1.12, p = .295
1000ng	16.01 [11.18, 20.84]	10.40 [4.54, 16.26]	9.31 [5.27, 13.35]	F (2, 16) = 2.96, p = .080	19.71 [13.04, 26.38]	t (8) = -1.17, p = .275

*Sphericity violated. GGe = 0.598, p = .019

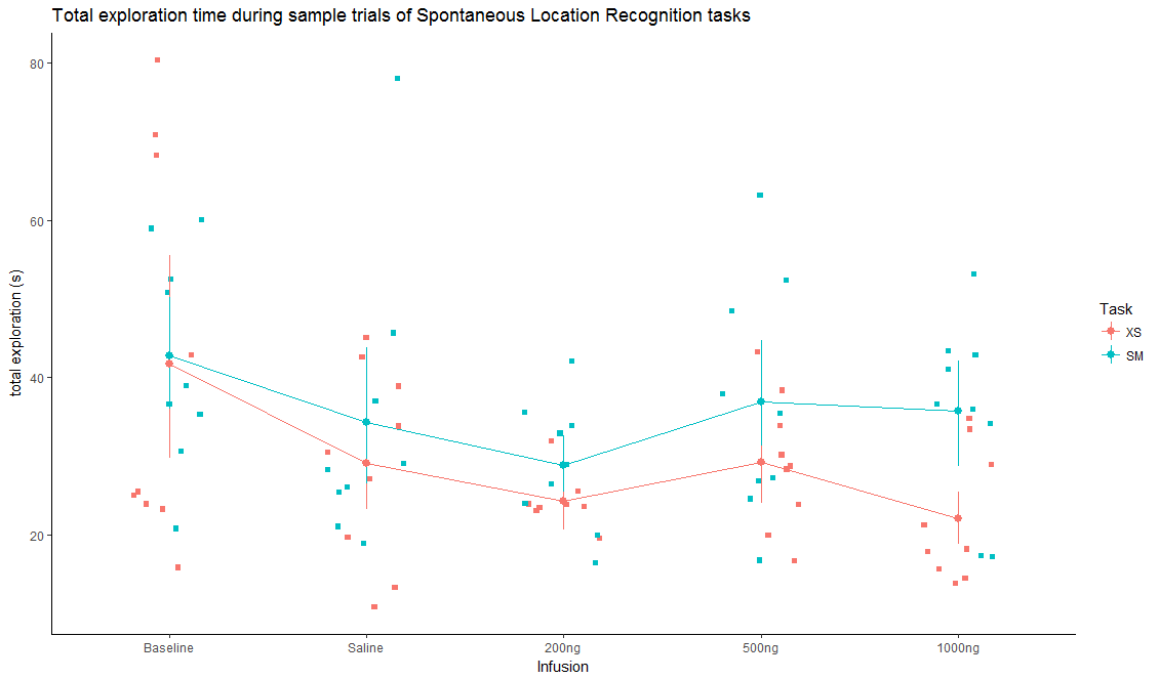


Figure 5.9: Mean (circles) ±95% CI exploration time (seconds) and individual data (squares) during spontaneous location recognition sample phase. XS = extra small separation; SM = small separation. Linear effect of Task: XS exploration < SM exploration. Linear and Quadratic effects of Infusion: Infusion decreases exploration; low dose CNP decreases further, then increases with increasing dose.

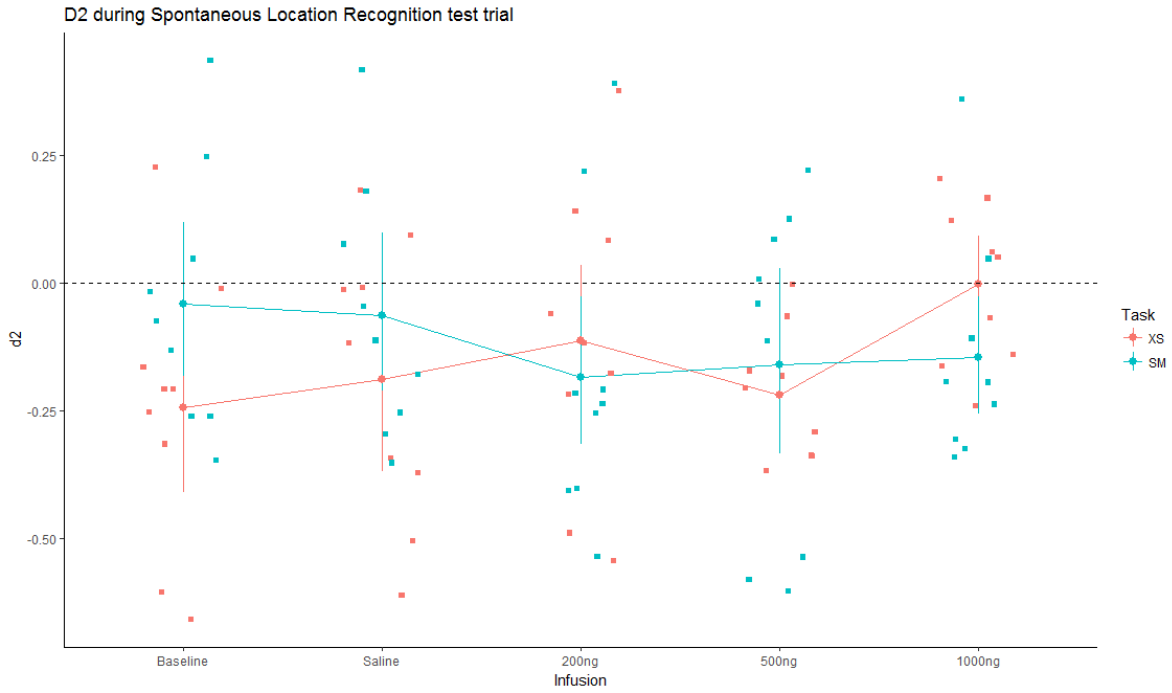


Figure 5.10: Mean (circles)±95% CI discrimination ratio (d2) and individual data (squares) during spontaneous location recognition trial phase. Horizontal line indicates equivalent exploration of novel and familiar object, with positive values corresponding to novelty preference. XS = extra small separation; SM = small separation. Linear trend interaction: Increasing dose increases recognition across XS task; Increasing dose decreases recognition across SM task.

5.3.4 Object Recognition

Both copies of objects used during sample trials were explored equivalently (descriptive statistics and t-tests provided in Table 5.2). Total exploration time during sample trials was unaffected by differing Infusions (Figure 5.11; $F(4, 32) = 0.32$, $p = 0.865$, generalised $\eta^2 = 0.021$) with no trends evident in the data (all $ts < 1.04$, all $ps > .305$). Three rats demonstrated a novelty preference ($d2 < 0$) during Baseline, indicating impairment, and were excluded from analysis. Analysis of discrimination from six remaining rats indicated infusions did not alter d2 ($F(4, 20) = 0.29$, $p = 0.878$, generalised $\eta^2 = 0.045$; Figure 5.12). Additionally, no trends were identified in the data (all $ts < 0.81$, all $ps > .429$). Although no statistical tests were significant, it should be noted that CNP infusion introduced a novelty preference in four cases, representing three rats (one demonstrating novelty preference in both 200ng and 500 ng conditions).

Table 5.2: Mean [+/- 95%CI], mean-difference and dependent means t-tests for exploration time of each object copy (A1 versus A2) during object recognition sample trials.

Infusion	A1 Mean [±95% CI]	A2 Mean [±95% CI]	Mean difference	t-test
Baseline	13.45 [9.99, 16.91]	13.56 [10.29, 16.83]	-0.11 [-5.28, 5.06]	t (8) = -0.05, p = .963
Saline	13.52 [8.08, 18.96]	14.45 [8.75, 20.15]	-0.93 [-2.34, 0.48]	t (8) = -1.52, p = .167
200ng	13.23 [8.76, 17.70]	11.77 [8.74, 14.80]	1.46 [-1.41, 4.33]	t (8) = 1.18, p = .273
500ng	16.69 [11.25, 22.13]	12.88 [9.44, 16.32]	3.82 [-0.47, 8.10]	t (8) = 2.06, p = .070
1000ng	13.95 [7.95, 19.95]	12.26 [7.19, 17.33]	1.69 [-0.81, 4.19]	t (8) = 1.56, p = .158

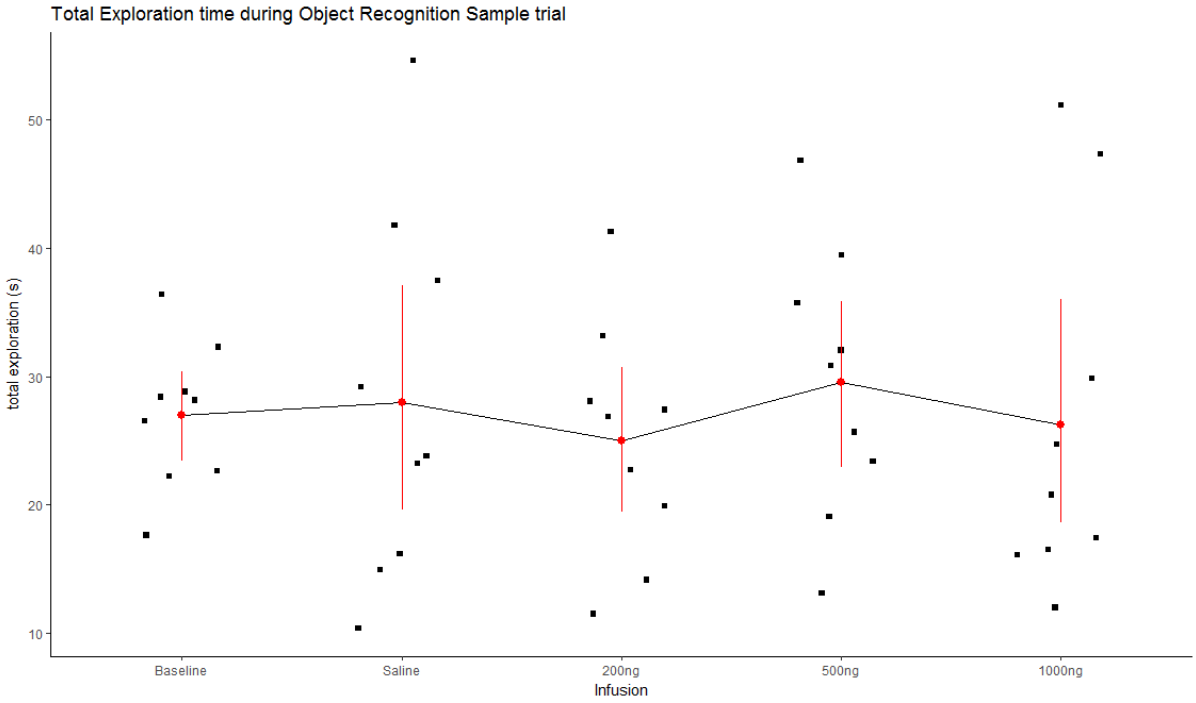


Figure 5.11: Mean [± 95% CI] and individual total exploration (seconds) for both objects during object recognition sample phase. No significant effects.

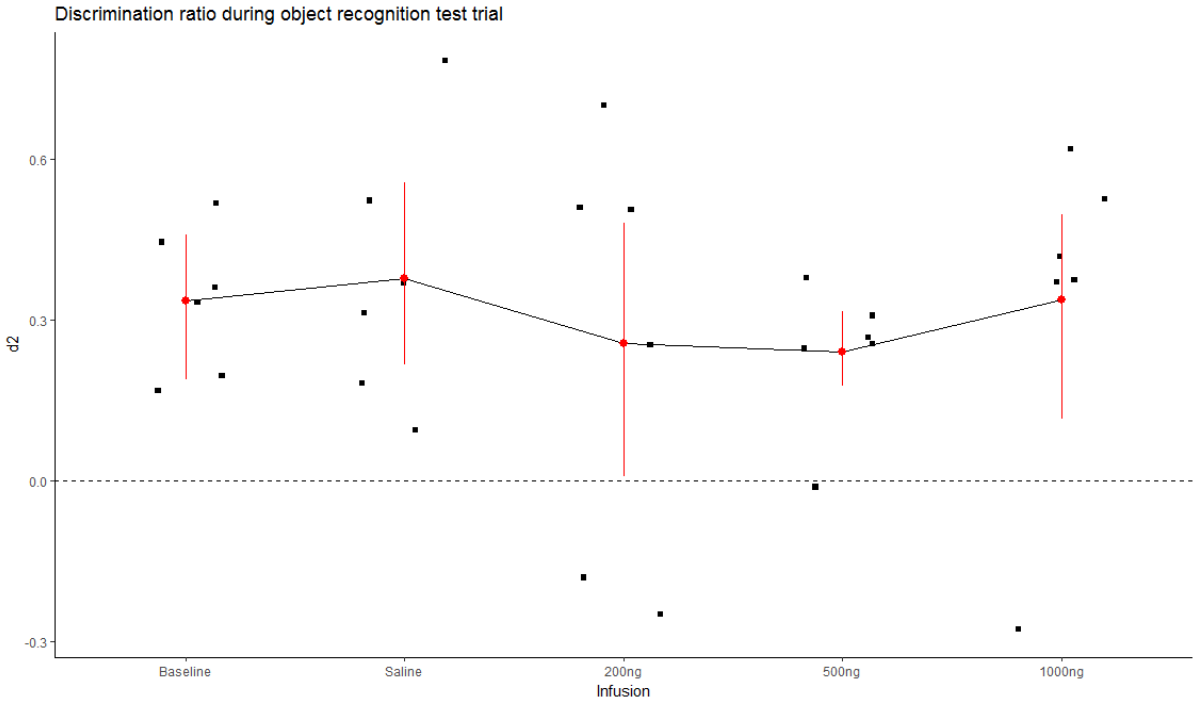


Figure 5.12: Mean [± 95% CI] and individual discrimination ratio (d2) during object recognition trial phase. Horizontal line indicates equivalent exploration of novel and familiar object, with positive values corresponding to novelty preference.

5.3.5 Cyclic GMP expression

There were no Main Effects of hemisphere in any ROI analysed and no effects of Infusion on density measurements in the anterodorsal thalamic nucleus, retrosplenial granular B cortex, amygdala, perirhinal cortex or the medial mammillary nucleus (Table 5.3). Active infusions of CNP increased cyclic GMP expressing neuron density in prelimbic and infralimbic cortices, cingulate gyrus, and hippocampal areas CA1 and CA3 (Main Effects of Infusion, Table 5.3, highlighted; Example images for these regions provided in Appendix B). Infusions also contributed a substantial (though non-significant) amount of variance to density measurements in dentate gyrus and hypothalamus (Table 5.3, highlighted). Cyclic GMP expression density was decreased by CNP infusions in medial orbital cortex (Table 5.3, highlighted).

Retrosplenial dysgranular cortex was the only ROI to exhibit an Interaction effect between Infusion type and Hemisphere (Table 5.3, highlighted; Figure 5.13). Calculations of Cohen's d effect sizes between individual mean differences indicated that density measures were equivalent across both hemispheres for "Inactive" infusions, and in "Active" ipsilateral hemisphere (ds between 0.25 and 0.51, ps > .37). Cyclic GMP was higher in contralateral than ipsilateral hemisphere of "Active" infusions ($d = 1.35$ [0.06, 2.64], $p = .04$), and elevated compared to contralateral hemisphere of "Inactive" infusions ($d = 1.43$ [0.12, 2.73], $p = .03$). This indicates that "Inactive" infusions did not affect cGMP expression, but within "Active" infusions, cGMP was only elevated in the hemisphere contralateral to infusion.

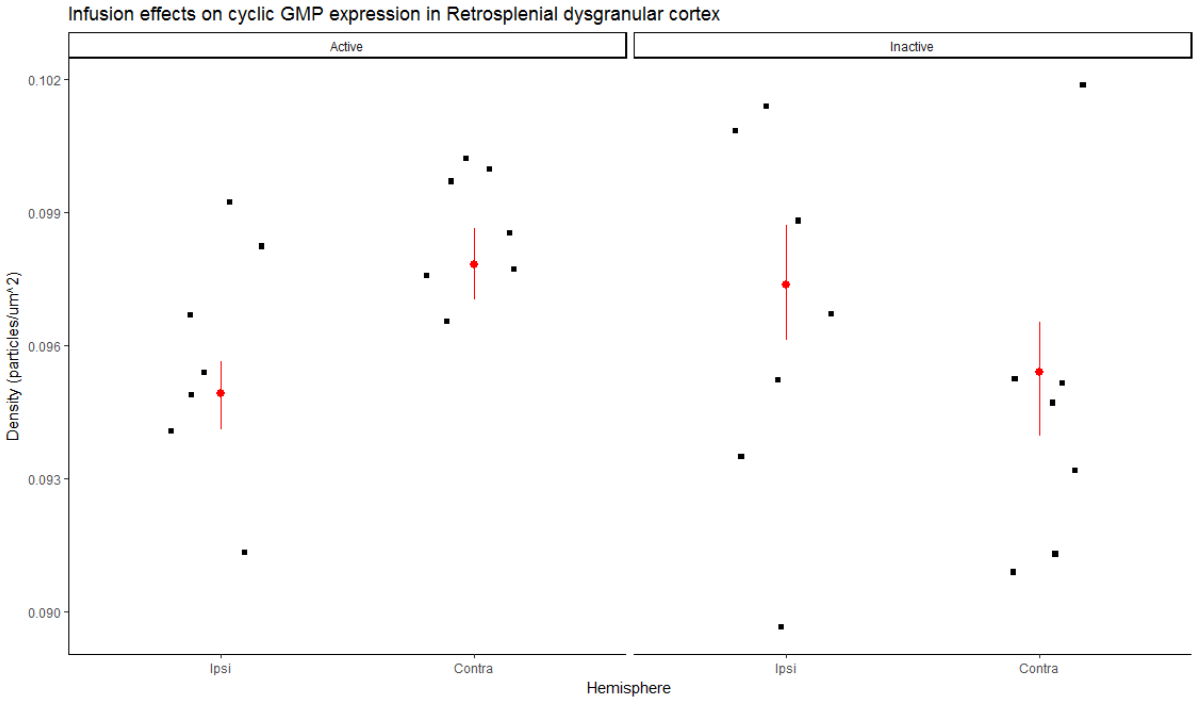


Figure 5.13: Mean [±95% CI] cGMP expressing neuron density in the retrosplenial dysgranular cortex by Infusion Type and Hemisphere. Active: Rats given final infusions of any dose of CNP; Inactive: Rats given no final infusion or a final infusion of 0.9% Saline; Ipsi: measures from hemisphere ipsilateral to cannula placement; Contra: measures from hemisphere contralateral to cannula placement. Active cGMP contralateral > Active cGMP ipsilateral.

Table 5.3: Mean [±95%CI] cyclic GMP expressing neuron density (particles/µm2) by infusion group and hemisphere, with repeated measures ANOVAs and effect sizes in previously outlined regions of interest. Statistical tests referred to in text are highlighted in gray. Note: Medial mammillary nucleus was analysed as a single region. Reported statistic is an independent means t-test across infusion groups with Cohen's d effect size.

ROI		Infusion: Inactive	Active	ANOVA effects		η^2
	Hemisphere					
Medial Orbital	Ipsi	.0968 [.0933, .1003]	.0947 [.0915, .0979]	Infusion	F (1, 12) = 16.62, p = .002	0.280
cortex	Contra	.0970 [.0940, .1000]	.0916 [.0894, .0938]	Hemisphere	F (1, 12) = 1.02, p = .332	0.058
				Infusion x Hemisphere	F (1, 12) = 1.29, p = .278	0.072
Prelimbic cortex	Ipsi	.0962 [.0938, .0986]	.0983 [.0956, .1010]	Infusion	F (1, 12) = 3.50, p = .009	0.191
	Contra	.0962 [.0938, .0986]	.0987 [.0971, .1003]	Hemisphere	F (1, 12) = 0.09, p = .774	0.001
				Infusion x Hemisphere	F (1, 12) = 0.09, p = .773	0.001
Infralimbic cortex	Ipsi	.0957 [.0920, .0994]	.0980 [.0949, .1011]	Infusion	F (1, 12) = 5.13, p = .043	0.248
	Contra	.0951 [.0929, .0973]	.0996 [.0970, .1022]	Hemisphere	F (1, 12) =0.32, p = .579	0.006
				Infusion x Hemisphere	F (1, 12) = 1.74, p = .212	0.032
Cingulate gyrus	Ipsi	.0946 [.0907, .0985]	.0995 [.0968, .1022]	Infusion	F (1, 12) = 6.12, p = .029	0.269
	Contra	.0960 [.0936, .0984]	.0983 [.0957, .1009]	Hemisphere	F (1, 12) = 0.00, p = .955	0.000
				Infusion x Hemisphere	F (1, 12) = 2.17, p = .166	0.048
Anterodorsal	Ipsi	.0974 [.0949, .0999]	.0986 [.0963, .1009]	Infusion	F (1, 12) = 0.18, p = .681	0.009
thalamic nucleus	Contra	.0985 [.0960, .1010]	.0982 [.0966, .0998]	Hemisphere	F (1, 12) = 0.16, p = .694	0.005
				Infusion x Hemisphere	F (1, 12) = 0.81, p = .385	0.024
Dorsal CA1	Ipsi	.0975 [.0948, .1002]	.1002 [.0988, .1016]	Infusion	F (1, 12) = 4.83, p = .048	0.200
	Contra	.0968 [.0939, .0997]	.0990 [.0964, .1016]	Hemisphere	F (1, 12) = 1.22, p = .292	0.037
				Infusion x Hemisphere	F (1, 12) = 0.05, p = .822	0.002

Table 5.3: Continued

ROI	Hemisphere	Infusion: Inactive	Active	ANOVA effects		η^2
Dorsal CA3	Ipsi	.0970 [.0955, .0985]	.0981 [.0965, .0997]	Infusion	F (1, 12) = 8.27, p = .014	0.285
	Contra	.0966 [.0944, .0988]	.0996 [.0984, .1088]	Hemisphere	F (1, 12) = 0.83, p = .381	0.028
				Infusion x Hemisphere	F (1, 12) = 2.20, p = .163	0.072
Dorsal DG	Ipsi	.0970 [.0953, .0987]	.0995 [.0959, .1031]	Infusion	F (1, 12) = 4.24, p = .062	0.211
	Contra	.0966 [.0947, .0985]	.0991 [.0972, .1010]	Hemisphere	F (1, 12) = 0.29, p = .601	0.006
				Infusion x Hemisphere	F (1, 12) = 0.00, p = .996	0.000
Retrosplenial dysgranular	Ipsi	.0966 [.0927, .1005]	.0957 [.0932, .0982]	Infusion	F (1, 12) = 1.13, p = .309	0.065
cortex	Contra	.0946 [.0912, .0980]	.0986 [.0973, .0999]	Hemisphere	F (1, 12) = 2.99, p = .594	0.006
				Infusion x Hemisphere	F (1, 12) = 8.06, p = .015	0.148
Retrosplenial granular B	Ipsi	.0943 [.0913, .0973]	.0960 [.0905, .1015]	Infusion	F (1, 12) = 2.68, p = .128	0.129
cortex	Contra	.0948 [.0916, .0980]	.0989 [.0966, .1012]	Hemisphere	F (1, 12) = 1.95, p = .187	0.052
				Infusion x Hemisphere	F (1, 12) = 0.92, p = .356	0.025
Amygdala	Ipsi	.0946 [.0928, .0964]	.0944 [.0922, .0966]	Infusion	F (1, 12) = 0.63, p = .443	0.036
	Contra	.0937 [.0913, .0961]	.0955 [.0939, .0971]	Hemisphere	F (1, 12) = 0.04, p = .854	0.001
				Infusion x Hemisphere	F (1, 12) = 2.47, p = .142	0.057
Hypothalamus	Ipsi	.0933 [.0914, .0952]	.0945 [.0908, .0982]	Infusion	F (1, 12) = 3.24, p = .097	0.126
	Contra	.0906 [.0881, .0931]	.0934 [.0916, .0952]	Hemisphere	F (1, 12) = 3.43, p = .089	0.118
				Infusion x Hemisphere	F (1, 12) = 0.55, p = .472	0.021
Perirhinal cortex	Ipsi	.0958 [.0937, .0979]	.0959 [.0931, .0987]	Infusion	F (1, 12) = 0.01, p = .923	0.000
	Contra	.0952 [.0902, .1002]	.0955 [.0873, .1037]	Hemisphere	F (1, 12) = 0.06, p = .811	0.003
				Infusion x Hemisphere	F (1, 12) = 0.00, p = .990	0.000
Medial mammillary nucleus		.0964 [.0949, .0979]	.0963 [.0921, .1005]		t (12) = 0.04, p = .966	0.020

5.4 Discussion

Previous work investigating behavioural effects of CNP indicate possible bidirectional relationships of CNP related to anxiety and memory. In contrast with the hypothesis, no effects of CNP were identified in the EPM (Figure 5.6). Generally, opposing effects to expected were evident for exploratory behaviours, wherein increasing doses of CNP also increased movement (in the EPM) and exploration (in SLR; Figure 5.8 and 5.9). Increasing doses of CNP were also expected to reduce discrimination in all recognition tasks. However, this effect only occurred across small SLR conditions, and discrimination was improved by increasing doses of CNP during extra-small SLR (Figure 5.10). This is the first study to show that infused CNP can modify location recognition, in a dose dependent manner, and that effects vary based on stimulus overlap ("pattern separation" load). Effects on both exploration and discrimination were not apparent during OR (Figure 5.11 and 5.12). Alongside anxiety measures from the EPM, this suggests effects of CNP can be attenuated across time, perhaps associated with concomitant experience. As rats exhibited initial cognitive impairment – compared with reports of Beckinschtein et al., (2014) and Kent et al., (2015) – this study provides promising avenues for research into CNP as a target in relation to cognitive decline. This observation also provides support for the idea that pattern separation deficits contribute to age-associated memory impairment (Burke et al., 2010; Yassa & Stark, 2011).

5.4.1 Failure to replicate anxiogenic effects of CNP

That anxiogenic effects of CNP were not evident in EPM behaviour is somewhat surprising, but likely due to previous experience in the RAM. Although Perspex enclosed arms in the EPM do not usually affect the validity of the task (Martínez et al., 2002), in this instance prior exposure to the RAM (the arms of which are open to the surrounding environment) has done so. Because of this previous experience, the EPM no longer presents an anxious situation for these rats. However, slightly increased anxiety at the highest CNP dose (Figure 5.6) supports previous findings (Jahn et al., 2001; Montkowski et al., 1998), and suggests task validity was not entirely negated.

An alternative possibility (alongside reduced exploration effects across tasks) is that CNP-induced behaviours are attenuated across multiple experiences as learning occurs. This may behaviourally support CNP as a metaplasticity regulator. Metaplastic processes (regulation of LTP and LTD) can prevent learning from occurring too readily when salience of stimuli is reduced (Hulme, Jones, & Abraham, 2013). Arguably, multiple exposures to situations of

anxiety and exploration has reduced the salience of emotional inputs and novel environments. Following these experiences, CNP no longer greatly modifies these behaviours.

5.4.2 Exploration Behaviour

It was unexpected that increasing doses of CNP would increase exploration behaviour (EPM and SLR small separation; Figures 5.7 and 5.8). Low doses of CNP were expected to represent a comparable situation to downregulation of CNP signalling in NPR-BΔKC rats, which showed increased exploration behaviours (Barmashenko et al., 2014). Additionally, administered CNP reduces the increased locomotion resulting from cocaine administration (Thiriet et al, 2001). This unexpected effect may be due to reductions in anxiogenic activity also seen here. Elevated anxiety is usually associated with decreased exploratory behaviours, and this association is exploited in measures such as the open-field test and EPM. As CNP no longer induced anxiety, its related effects on locomotor and exploration behaviour may have varied also.

Exploration was reduced generally, and dose-dependently, during extra-small SLR tasks compared with small separations (Figure 5.9). As these conditions were counterbalanced (half of rats performed extra-small first, and vice versa), this may not be an effect of CNP specifically. Perhaps the objects used during all extra-small occasions were less interesting than those for small separations. Additionally, as rats had difficulty discriminating the paired object location in SLR samples (Table 5.1), reduced proximity during extra-small conditions may have reduced interest for exploring the objects. However, this does not explain dose dependent reductions in exploration (as hypothesised) restricted to extra-small SLR. Combined with other findings here, this suggests behavioural effects of CNP are situationally dependent.

5.4.3 Spontaneous Location Recognition

Situation dependence of CNP effects is supported by data indicating the peptide can both facilitate and interfere with memory acquisition (Figure 5.10). Interference with memory acquisition was hypothesised based on improved recognition when CNP signalling was reduced (Barmashenko et al., 2014) and associations between lower endogenous CNP concentrations and better novel object discrimination (Rapley, 2012, unpublished Master's Thesis). Conversely, CNP facilitates acquisition and consolidation during passive avoidance (Telegdy et al., 1999; 2000). Combined, these findings indicate CNP bidirectionally affects memory acquisition. Moreover, as SLR manipulates pattern separation load during acquisition, this suggests CNP affects neurobiology contributing to this function.

The mechanism by which this occurs was not tested here, but endogenous, bidirectional modification of hippocampal plasticity by CNP is most likely. Application of CNP facilitates LTD (Decker et al., 2010) but in NPR-BΔKC rats LTP is facilitated (Barmashenko et al., 2014). Synaptic LTP is broadly accepted as contributing to memory acquisition. LTD occurs endogenously during novel spatial learning and is necessary for the encoding of spatial information (Ge et al., 2010; Goh & Manahan-Vaughan, 2013; Kemp & Manahan-Vaughan, 2004; Lemon & Manahan-Vaughan, 2006, 2012; Manahan-Vaughan & Braunewell, 1999). LTD occurrence within hippocampal subregions can also be dependent on the nature of spatial information (Kemp & Manahan-Vaughan, 2007; Rosenzweig & Barnes, 2003), aligning with varied CNP effects across SLR tasks. Alongside current behavioural observations, CNP seems to regulate the shift between LTP and LTD, variably contributing to memory acquisition based on the nature of stimuli.

5.4.4 Object Recognition

Although statistical tests of object recognition effects were non-significant, several aspects of the data support previous suppositions made here. Null effects on exploration and recognition supports the idea that CNP action is situationally dependent and modified by previous experience. Lack of effects on discrimination may be an effect of differing tasks, but CNP infusions introduced familiarity preference in several instances (Figure 5.12). This supports hypothesised interference with memory.

Performance on this task, combined with SLR observations, provides the first experimental evidence supporting CNP as a treatment target in cognitive decline. Two rats exhibited impaired recognition during baseline for this task but most rats were impaired during SLR. This suggests that across repeated administrations CNP has broadly ameliorated recognition deficits. However, as CNP also seems to interfere with memory, understanding of immediate versus long-term effects will be required to develop treatment paradigms.

However, it is also possible given the time taken to initiate this study following initially outlined difficulties, that rats were too old to establish reliable or valid effects of the peptide. Baseline measures in both SLR and OR tasks indicated likely senescence in these rats. These findings cannot be generalised to understanding the role of CNP during typical function. However, differing linear effects of CNP within the SLR task suggest it will be interesting to investigate using younger rats where typical function is intact. That these results cannot be generalised to broader knowledge of CNP does not invalidate the indication that CNP may improve age-related recognition deficits.

5.4.5 Cyclic GMP

Cyclic GMP analysis was used to ensure infused CNP promoted neurological activity. Expected increases of cGMP occurred in pre- and infralimbic cortices, cingulate gyrus, hippocampal areas CA1 and CA3, with statistically non-significant increases in DG and hypothalamus (Table 5.3). If infusions were followed by rapid clearance via NPR-C receptors from the ventricular space, cGMP would likely not be increased in these regions, and if this has occurred, it has not affected the ability of CNP to generate downstream neurological effects. Several observations were unexpected. cGMP was not increased by infusion in anterodorsal thalamic nucleus, retrosplenial granular B cortex, amygdala, perirhinal cortex and medial mammillary nucleus. In retrosplenial dysgranular cortex, cGMP was only elevated contralaterally to infusion. Combined these observations indicate a loss of cGMP stimulation by CNP which aligns with attenuation of behavioural effects on anxiety, exploration and recognition.

Involvement of the amygdala in emotion processing is widely accepted, and it additionally provides integration of emotional states with cognitive function (Catani et al., 2013; Phelps & LeDoux, 2005). Perirhinal cortex, anterodorsal thalamic nucleus, retrosplenial granular cortex and medial mammillary nucleus are critical nodes within an extended hippocampal-diencephalic network (Aggleton & Brown, 2006). These regions contribute to recognition processes, navigation, spatial memory encoding and normal hippocampal, prefrontal and retrosplenial function (Brown & Aggleton 2001; Aggleton & Brown, 2006; Vann & Aggleton, 2004; Vann 2010). Loss of CNP-stimulated cGMP activity in these regions indicates neurological effects accounting for long-term behavioural attenuation.

Because cGMP activity was no longer stimulated, this suggests that NPR-B receptors have been either decreased or desensitised. Desensitisation is known to occur in this receptor following repeated exposure to CNP (Potter et al., 2006). That this has occurred in certain regions here gains some support from hemispheric effects in retrosplenial dysgranular cortex. Greater intensity of infusions near the cannula (ipsilateral hemisphere) have elicited this change more rapidly than at a distance (contralateral hemisphere). Also plausible is that potential age-related increases in NPR-C receptors (Chapter 3) have contributed to this effect by clearing CNP more rapidly and reducing cGMP stimulation. This possibility is addressed in Chapter 6 (General Discussion).

Medial orbital cortex exhibited decreased cGMP in response to CNP infusion. This is the first indication that this may occur and is intriguing given bidirectional behavioural effects. Within rodents, this region (and associated pre- and infralimbic cortices, constituting

ventral medial prefrontal cortex) mediates working and temporal order memory for objects and spatial locations, and paired associate learning for objects in place (Kesner & Churchwell, 2011 for review). These skills are clearly engaged for learning during both recognition tasks used here. Unfortunately, this study and thesis are not targeted toward analysing these specific aspects of recognition, nor executive control of such processes. However, this raises the possibility that variable effects of CNP within frontal-cortical regions mediates other bidirectional neurological and behavioural sequelae of the peptide. This unexpected finding suggests that CNP should now be considered in terms of a contribution to executive functions, and may be of interest for clinical conditions where these are compromised.

Chapter 6. General Discussion

6.1 Major contributions of the thesis

Accumulating evidence has indicated that CNP provides a novel target in neurological disorders featuring cognitive decline (Mahinrad et al., 2016). However, CNP has been little studied in relation to memory and neurological disorder, or in relation to brain aging where cognitive decline commonly occurs. Although much about this fascinating peptide remains elusive, this thesis has provided multiple findings contributing to our understanding of CNPs role in memory encoding, and confirms that it provides a novel target for study of, and intervention in cognitive decline. Novel contributions of this these are outlined in Table 6.1

Table 6.1: Novel contributions of the thesis

Chapter 3	First description of CNP, NTproCNP and degradative regulation of CNP in aged rodent cerebral tissue.
	Comparison of CNP and related measures in cerebral tissue between young and aged rodents
	Evidence that the CNP signalling system response to brief enrichment in young rats, is lost with aging
	Identification of CNP synthesis dysregulation in retrosplenial cortex during aging
	Proposal of the hypothesis that modifications to CNP regulation via NPR-C receptors in hippocampus occurs during brain aging. In combination with broader reported changes to synthesis, availability and peptide regulation within connected neurological regions, this is proposed to contribute to age-related cognitive decline
Chapter 4	First identification of increased CNP synthesis during spatial information consolidation in mammillary bodies
	First identification of increased CNP synthesis during spatial reference (episodic-like) memory consolidation in mammillary bodies and retrosplenial cortex
Chapter 5	Evidence that effects of infused CNP on behaviour and cGMP can be attenuated across chronic infusions in combination with concurrent experience
	Evidence that CNP can both facilitate and interfere with memory acquisition
	First evidence that CNP contributes to “Pattern Separation” of memory encoding
	Evidence that contribution of CNP to cognition is situationally dependent, varying by stimulus salience and (in memory) pattern separation load
	Indication that CNP may elicit both Long-Term Depression and Long-Term Potentiation *in vivo*, in line with its proposed role as metaplasticity regulator
	First experimental evidence that CNP may provide a treatment target in cases of cognitive decline
	Indication that CNP may mediate executive functions through variable cGMP stimulation in frontal cortical regions

Data from Chapter 3 is believed to provide initial experimental support for a proposed role for CNP in cognitive decline and generates initial hypotheses for ongoing research in this regard. This proposal is supported by findings from chapter 4, providing the first indication that CNP synthesis occurs during 'episodic-like' memory consolidation. Although this was evidenced in younger rats, because this type of memory is commonly impaired with age, this provides supporting evidence that the peptide contributes to these memory functions. Chapter 5 provides evidence within a single study of bidirectional behavioural effects at differing doses of the peptide. This indicates that previously demonstrated bidirectional effects on synaptic plasticity functions may also translate to behavioural outcomes. Moreover, the seemingly bidirectional effects on a "pattern separation" task lend additional credence to a proposed role for CNP in age-related cognitive decline, since this function is thought to contribute to age-related cognitive changes. When taken together, these experiments support the overall proposal that CNP and related receptors and regulatory enzymes contribute to age-related cognitive decline. Additionally, they indicate that, while CNP may contribute to the regulation of multiple cellular and molecular functions, an overarching theme is of a role in information integration across intersecting networks – including but perhaps not limited to, emotionality, learning and memory. CNP should thus be considered highly important to central nervous system integrity and function. Ongoing research considering CNP should not only benefit understanding of the peptide itself, but perhaps other areas of research interest, some of which these several experiments have drawn attention to.

6.2 General Considerations

One difficulty in the consideration of experiments using enrichment is that enrichment more closely replicates "normal life" for wild rats. In a recent review, Hannan (2014) identifies the assumption that standard housing is "standard" or typical as a violation of 'environmental construct validity.' Additionally, many human epidemiological factors such as lower socioeconomic status, less years in education or a more sedentary lifestyle, are known to be associated with increased incidence of cognitive decline and age-related neurological disorders. This may then represent a comparable situation to the declines seen in standard-housed rodents as compared with those exposed to enrichment, rather than enrichment being a special case, *per se.*

Given a particular interest in this research area for the hippocampus, many studies examining enrichment effects in aged rodents focus on outcomes for spatial memory tasks. Life-long enrichment rescues age-related declines in both spatial working and reference memory measured in the eight-arm RAM (Lores-Arnaiz et al., 2006; Lores-Arnaiz et al.,

2004). However, short-terms (two months) of enriched housing provide similar benefits when tested in the Radial-Arm Water Maze (Sampedro-Piquero, De Bartolo, et al., 2014; Sampedro-Piquero, Arias, et al., 2014). In mice, similar improvements to cognition seem to require continuous enrichment when compared with daily brief exposures (Bennett et al., 2006). Benefits to spatial memory as measured by the Morris Water Maze submerged platform task have been shown following either two (Speisman et al., 2013) or eight (Segovia et al., 2006) weeks of enrichment in aged rats, and similar time frames in aged mice (Frick & Fernandez, 2003; Harburger et al., 2007). For rodents at least, cognitive benefits of enriched housing in terms of spatial working and reference memory thus seem consistent and can be conveyed via either life-long or short-term interventions.

Though recognition memory is a key component of observed declines with aging, few studies have examined the effects of enrichment on recognition in aged rats. The most cited single study demonstrating a benefit in this context comes from Leal-Galicia and colleagues (2008). They demonstrate that intermittent, lifelong exposure to EE mitigates the declines in recognition memory seen in standard-housed rodents. Lifelong enrichment in the absence of exercise has also recently been reported to prevent age-related declines in recognition (Birch & Kelly, 2018). However, short-term enrichment beginning in late life (10-12 weeks in 20-22 month old rats) was reported to have no effect on novel object recognition, but exercise alone led to recognition improvements (Kumar, Rani, Tchigranova, Lee, & Foster, 2012). These more recent studies support earlier work indicating that life-long enrichment conveys benefits of greater magnitude related to aging than short-term exposure (Kobayashi et al., 2002; Soffie, Hahn, Terao, & Eclancher, 1999).

Just as the extent of "enriching" or cognitively beneficial factors across a human lifespan may vary greatly and lead to varying outcomes, the same seems to be true of rodents, as outlined. Any number of factors may contribute to the apparently more moderate or absent effects of enrichment when exposure begins in later life, as may have been the case here in Chapter 3. Changes to activity levels may result in lower engagement with enriching factors. Declines in sensory systems (for example, progressive vision or hearing loss) may result in lesser stimulatory effects of the same environmental factors during aging. Highly relevant to this thesis is age-related variance in hormonal systems (most notably, the ending of the oestrus cycle in females) which may influence other endocrine peptides and can contribute to variable incidence and outcomes in human neurological disorders for males and females (Galea et al., 2008). As seen here for CNP, the response in younger rodents following a brief period of enrichment was absent in aged rodents under the same conditions. Further research

is warranted into potential longer-term benefits of enrichment in the relationship between CNP and age-related cognitive decline. From the effects noted in chapter 5, a combination of enrichment and CNP administration in aging and the outcomes of this for age-related cognitive decline would also be of interest. Based on the loss of response to enrichment of CNP seen in chapter 3, would supplementation of the peptide result in better age-related outcomes when enrichment occurred with a late life exposure? The presence of cognitive benefits with a short-term of late life enrichment are encouraging for novel clinical research generalising 'enrichment' to humans and examining is protective contribution in clinical disorders. Greater understanding of how CNP contributes to the aging process and whether its presence may modify the benefits of enrichment could allow an application as treatment in cases of neurological decline.

Findings from both Chapter 3 and Chapter 4 (and Chapter 5 to a lesser extent) draw attention to retrosplenial cortex. This raises the concern that methods used within these studies may have targeted retrosplenial cortex in some fashion. However, considering emerging understanding of the role of retrosplenial cortex in supporting integration of differing sensory and contextual modalities into memory (Kinnavane et al., 2017; Pothuizen et al., 2010, 2009; Robinson, Adelman, Mogul, Ihle, & Davino, 2018; Vann et al., 2009) this 'targeting' may be unavoidable. Conditions used in each of the studies presented here are reliant on integration of sensory and environmental cues, as are many other paradigms throughout behavioural neuroscience. Thus, if this is the underlying function of retrosplenial cortex within the neurological circuits it provides interconnection for, then it will ultimately be targeted under any of these situations. More interesting is the consideration that, by virtue of the indication that retrosplenial cortex appears to be an important node for CNP activity within central nervous system, these findings lend support to the hypothesis that CNP is a critical molecule supporting said integratory processes. It may be that the identified functions of CNP in modifying LTP/LTD thresholds and contributing to metaplasticity, are intimately tied with integrative function of the retrosplenial cortex and contribute to further reaching modifications of broader neurological circuitry. As seen in hippocampal regions CA1-CA3 and DG, CNP and NPR-B expression demonstrate variable overlap with NPR-C receptors within granular and dysgranular retropslenial cortex (Herman, Dolgas, Marcinek, & Langub, 1996). This suggests that the differing distribution in this region should also be further investigated, as dysgranular and granular retrosplenial cortex apparently make variable contributions to spatial memory tasks (Pothuizen et al., 2009; Vann & Aggleton, 2005). Such research will be better served by more specific manipulation and sampling methods (outlined

below – General Limitations). Drawing together research regarding both CNP and retrosplenial cortex function is both indicated by this thesis, and will provide valuable insight in determining specific functions of both peptide and neuroanatomical region.

As retrosplenial cortex has drawn attention across these three studies, it is useful to compare effects of lesions to this region, and reciprocally interconnected hippocampus and anterior thalamic nucleus, with findings reported here. Retrosplenial cortex lesions do not affect novel object recognition (Ennaceur et al., 1997; Vann et al., 2009). Earlier lesion studies indicate retrosplenial cortex is important for spatial memory (Pothuizen et al., 2010; Vann & Aggleton, 2005), with more recent studies supporting the hypothesis that its importance lies in the integration of proprioceptive and contextual cues to support spatial and episodic-like memory (Kinnavane et al., 2017; Pothuizen et al., 2010; S. Robinson et al., 2018). Although a role in pattern separation has not been directly investigated, rodents with retrosplenial cortex lesions are impaired in object-in-place tasks (Vann et al., 2009). Findings from a recent study using contextual fear learning led the authors to suggest that further consideration should be given to retrosplenial cortex in contexts of both pattern separation and emotional learning (Robinson, Adelman, Mogul, Ihle, & Davino, 2018). This suggestion is supported by the convergent findings here of retrosplenial cortex as a key node for CNP activity, and a contribution of CNP to pattern separation (spontaneous location recognition task) in addition to previous work relating CNP with anxiety/emotionality. Based on the moderate success in replicating Bekinschtein and colleagues' (2016) "pattern separation" task here, providing a modicum of external validity, this suggests the task may also be useful in continued investigation of retrosplenial cortex.

Investigation of retrosplenial cortex contribution to 'pattern separation' is a logical next step given demonstration of its supporting role for traditionally hippocampal-dependent tasks. Like retrosplenial cortex, permanent lesions of hippocampus evidence no deficits in novel object recognition – though deficits are seen in temporary inactivation studies (reviewed by Cohen & Stackman, 2015). Hippocampal lesions are well known to produce deficits in spatial and episodic-like memory tasks. Additionally, research using hippocampal lesions has been the major contributor to pattern separation research, with the function thought to occur therein. Subregions CA1-CA3 and DG exhibit dissociable lesion effects in terms of their contribution to this function (Gilbert et al., 2001; Hunsaker & Kesner, 2008; Kesner, Gilbert, & Barua, 2002; Kesner, Hunsaker, & Ziegler, 2010; see (Hunsaker & Kesner, 2013; Kesner & Hopkins, 2006 for reviews). In part, these dissociable effects in pattern separation alongside variable expression of CNP mRNA and NPR-B receptors within

hippocampal formation were a motivating force for an examination of CNP infusions using the spontaneous location recognition task detailed here. Within those results, variable effects on cGMP expression were identified in CA1 and CA3 compared with DG. This is conclusive of very little, but once again highly suggestive for intersecting ongoing research related to CNP and its related regulatory system pertaining to a contribution to pattern separation within hippocampal formation.

A single anterior thalamic nucleus (anterodorsal) was examined in this thesis regarding cGMP expression in chapter 5. Further inclusion was limited by both amount of tissue required for RIA and location of this nuclei group. ATN lesions have similar effects to those of retrosplenial cortex and hippocampus. No impairments are evident in object recognition tasks (Dumont & Aggleton, 2013; Mitchell & Dalrymple-alford, 2005; Moran & Dalrymple-Alford, 2003). ATN lesions produce impairments in various spatial tasks, including object-in-place tasks (Warburton et al., 2000) which are the closest analogue to the spontaneous location recognition/pattern separation task used here. Because hippocampal function seems to be dependent on informational input from these nuclei, this provides another interesting ongoing target for understanding pattern separation – an initial query being, does disruption of the ATN also disrupt pattern separation? Alongside effects on memory, two studies indicate ATN lesions may reduce anxiety (Dupire et al., 2013; Suárez et al., 2004). This is interesting because, at the molecular level, hippocampal lesions increase stress responses (Herman et al., 2005), and stressors themselves have variable effects on memory (e.g. intense memories for extreme stressors vs later impairments in attention and memory; (Kim & Diamond, 2002). Here may exist a divergence in the support role of ATN for hippocampal function. A majority of research seems to consider either mnemonic functions or stress and anxiety. However, these comparisons, previous CNP research, and this thesis indicate that both should continue to be examined in combination. In addition, this suggests that CNP research will need to look beyond singular neurological regions and specific behaviours and consider the broader interacting neurological systems through which it is distributed. CLARITY is a highly novel technique allowing visualisation of entire rodent connectomes. Should it become possible to adapt such a technique for investigation of peptide or peptide gene expression, this would be highly valuable in understanding the nervous system-wide contribution of CNP to memory integration.

Although not traditionally considered as a contributor to memory formation, hypothalamus was included as a key ROI within this research given previous work indicating it as a major node for CNP activity and expression. Based on the above considerations, a

seemingly overall function for CNP as a regulator of information integration, and the known role of hypothalamus contributing to metabolism and emotional information, it is fortuitous that the region was retained. As outlined in sections 2.1.1 and 2.3, CNP has been implicated in both central and peripheral functions related to metabolism (central regulation of food/fluid intake, and peripheral regulation of digestive functions). Within chapter 3, hypothalamus was the only ROI to exhibit a mildly similar response to enriched conditions within aged rats when compared to young. Certain subnuclei of hypothalamus are relatively stable with aging, whereas others may be particularly vulnerable to neuroinflammatory and metabolic changes (Hofman, 1997; Maggi, Zasso, & Conti, 2015). This may account for the reduced effect of enrichment on hypothalamic response indicated by Chapter 3. A growing body of evidence supports the importance of metabolic processes (with a focus on caloric restriction and exercise) to positive cognitive outcomes, including beneficial effects during both typical and pathological aging (Cauwenberghe, Vandendriessche, Libert, & Vandenbroucke, 2016; Davies, 2017; Kent, Oomen, et al., 2015; Stranahan & Mattson, 2011 for reviews). Moreover, experimental studies indicate that caloric restriction enhances neurogenesis with hippocampal structures and is associated with improved memory (Hornsby et al., 2016; Lee, Duan, Long, Ingram, & Mattson, 2001). Evidence from Chapter 4 of this thesis indicates CNP concentrations were reduced in hippocampus alongside caloric restriction. Because increased CNP can switch BDNF-induced neuron proliferation to differentiation (Simpson et al., 2002), this observation of CNP reduction aligns with studies indicating enhanced BDNF expression alongside caloric restriction (Hornsby et al., 2016; Lee, Duan, Long, Ingram, & Mattson, 2001). Futhermore, as lower concentrations of CNP are associated with LTP facilitation, this would be assumed to align with a facilitation of memory as seen in the same studies. In combination with previous considerations here, this further supports the suggestion that CNP's contributions to cognitive function cannot be considered only in terms of memory, anxiety or metabolism, nor as it relates to only behaviours related to these processes. An integrative approach should be taken considering each of these aspects in future studies.

6.3 Regarding future investigation of NPR-C receptors

Generation of the hypothesis that NPR-C receptor modifications contribute to age-related cognitive decline was unexpected. This aspect of findings from Chapter 3 is given consideration here because loss of NTproCNP modification in retrosplenial cortex of aged rats was clear compared to young rats, whereas hypothesised modifications to NPR-C present additional implications for Chapter 5. Most in the NP field (including myself) have considered NPR-C to be a non-signalling receptor. As this thesis was targeted toward study

of CNP, little about this receptor has been outlined. These considerations are limited to speculation from the outset, in that age-related modifications to NPR-C have not been demonstrated directly. However, if confirmed, this finding may have important implications for understanding NP regulation and functional roles.

Like CNP, relatively little research attention has been given to NPR-C. NPR-C receptor mRNA is found in frontal cortex, retrosplenial granular cortices, medial preoptic nucleus, ventral cochlear nucleus, choroid plexus, deep layers of neocortex, limbic cortex, posterior cortical amygdala, ventral subiculum, amygdalohippocampal area, and dentate gyrus (summarised in Figure 6.1 from Herman, Dolgas, Marcinek, & Langub, 1996). NPR-C distribution overlaps with several key nodes of CNP activity indicated in this thesis (frontal cortex, retrosplenial cortex, DG, hypothalamic nuclei). The choroid plexus is also of note as the major site of CSF production, interaction with the circulation, and of special interest regarding NP regulation of CSF (Herman et al., 1996; Johanson et al., 2006).

Trachte (2005) outlines evidence that NPR-C provides signal transduction and is essential in regulating the neuromodulatory functions of NPs. Notably, CNP activity in rat magnocellular neurosecretory cells, and its inhibitory effects on dopamine release are dependent on activation of the NPR-C receptor (Rose et al., 2005; Trachte, 2000). Recently, NPR-C knockout mice were identified as having a phenotype likened to ADHD (Gong et al., 2011). Alongside the suggestion from this thesis that NPR-C increases in retrosplenial cortex contribute to age-related disconnection between frontal and temporal structures (Buckner, 2004), this implicates NPR-C in the executive control of CNP signalling.

Sabbatini (2009) suggests that NPR-C mediates peripheral responses to NPs in the digestive system, whereas CNP and NPR-B are likely to provide central control. However, in agreement with Trachte (2005), it may be more accurate to say that NPR-C mediates whole organ NP responses, whereas CNP and NPR-B regulate specific cellular responses. Within central nervous system, this gains some support from consideration of regions where NPR-C co-localises with NPR-A and NPR-B. For NPR-A, this occurs primarily in brainstem regions associated with cardiovascular control (Abdelalim et al., 2008), aligning with primarily cardiovascular roles of ANP and BNP. NPR-C and NPR-B co-localise in frontal and retrosplenial cortex, DG and periaqueductal gray of the hypothalamus (Herman et al., 1996), aligning with the suggestion that NPR-C provides executive control over CNP signalling and behavioural effects via these interconnected regions. This now encourages an expansion of research into NPR-C within central nervous system.

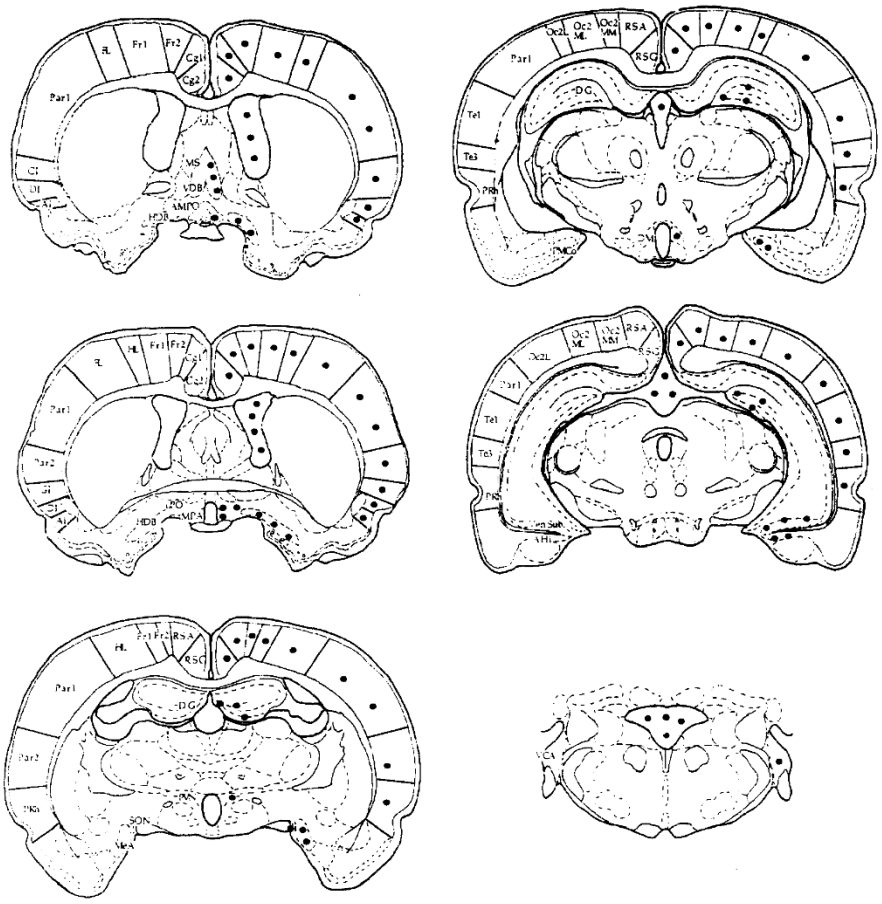


Figure 6.1: NPR-C mRNA expression (indicated by black dots) throughout rodent cerebral cortex. From Herman, Dolgas, Marcinek & Langub Jr (1996.)

6.3.1 Possible influence of NPR-C increases on loss of cGMP stimulation

Loss of NPR-C receptors has been shown to have no effect on CNP stimulated cGMP (Trachte, 2000). However, the clearance role of NPR-C acts to limit peptide availability which would attenuate cGMP generation (Herman et al, 1996). Hence, some consideration must be given to whether proposed age-related increases in NPR-C (Chapter 3) may have contributed to the attenuation of cGMP stimulation seen in Chapter 5. NPR-C increases are proposed to occur in hippocampus, but as NPR-C receptors are restricted to dentate gyrus (Herman, 1996), this consideration can be likewise restricted. It is also plausible that similar changes may occur in other regions identified in Chapter 3 within related neurological circuits (medial prefrontal, retrosplenial and occipital cortex). Medial prefrontal cortex and retrosplenial cortex both exhibited surprising effects of cGMP stimulation by CNP.

NPR-C receptors are also located in hypothalamic and amygdaloid nuclei (which contributed to ROIs in Chapter 5) and perirhinal cortex (analysed directly; Herman et al, 1996). Unfortunately, amygdala and perirhinal cortex were not included in investigation of age effects, so loss of cGMP activity in these regions may relate to either possible age-related

changes in NPR-C, or suggested modifications to NPR-B alongside behaviour (Chapter 5). CNP signalling within hypothalamus was identified as likely to be stable during aging in Chapter 3, indicating age effects have not contributed to attenuation of cGMP activity identified here. NPR-C receptors have not been described in anterodorsal thalamus nor medial mammillary nucleus (Herman, 1996). Thus, a loss of cGMP stimulation in anterodorsal thalamus, medial mammillary nucleus and hypothalamus cannot be attributed to possible NPR-C increases with age. This supports the postulation that loss of activity in most of these subregions aligns with attenuation of behavioural effects of CNP, and may occur through NPR-B receptor modifications. Loss of cGMP stimulation in retrosplenial granular cortex and DG cannot be definitively attributed to either chronic administration of CNP or possible age-related effects on NPR-C receptors with data available here. Reductions of cGMP in medial orbital cortex support postulations of a disconnect between frontal and medial temporal lobe structures centred on retrosplenial cortex which contribute to age-related cognitive decline (Buckner, 2004; Fjell et al., 2014; Nestor et al., 2003; Vann et al., 2009). Additional research of the CNP signalling system during aging, in combination with NPR-C interactions may prove valuable to our understanding of these neural systems and their sensitivity to age.

6.4 General Limitations and Future Directions

Several specific limitations and ongoing research suggestions have already been identified. In generating this thesis several limitations arose which require consideration for ongoing research design. First among them concerns recommendation that analysis of CNP activity within brain will benefit from immunohistochemical techniques. Use of ABC-DAB method here indicated a relatively prominent level of 'background' staining (Appendix B). This was not unexpected given CNP stimulates cGMP in both neuronal and glial components of brain tissue (Goncalves et al., 1995; Sumners & Tang, 1992; Yeung et al., 1996). Immunohistochemical analysis of CNP itself may result in similar issues, further highlighting a general need within this field to identify neuron-glia interactions of the peptide. Although immunohistochemical techniques are limited by these aspects of CNP, here they have provided valuable insight into subregional activity of CNP, not possible with RIA alone. However, alongside this valuable insight for the field regarding this technique, a drawback occurs for this thesis overall. Valuable analysis would be gained if tissue gathered for Chapter 5 could be compared directly with data gathered for Chapter 3. As tissue gathered in Chapter 5 was fixed during transcardial perfusion, it is no longer appropriate for RIA analysis. Access to additional antibodies (see below) would allow for further analysis in these

tissues of (for example) NPR-B or NPR-C receptors, neprilysin or IDE distribution, or regions of nppc gene expression. Any singular of these suggested examinations, or a combination, would be valuable in answering questions raised by this thesis.

Necessity for further examination within the field of the specific neuron-glia interactions of CNP is highlighted by a potential limitation comparing younger rats with aged rats (Chapter 3 and Chapter 4). Generally, it is now thought that substantial neuronal loss does not occur during typical aging (see Mora et al., 2007, p.79 for review), however in the aging rodent brain astrocyte hypertrophy occurs in specific regions including hippocampus (Soffie et al., 1999). Thus, neuronal versus glial components of the tissue gathered may vary across the ages studied here and within young rats reported by Rapley and colleagues (2018), although this was not directly examined. As outlined previously, studies of ANP indicate that NPs are produced by neurons then target astrocytes, but this has not been consistently shown for CNP. If CNP follows the same pattern as ANP, and neuronal components of aged tissue are reduced relative to total weight (i.e. are of a lesser volume) one would expect reduced production of CNP in similar weight of tissue (which was achieved). Tissue concentrations in aged rats would then be reduced in comparison to young rats. However, in comparisons within chapter 3, and across chapters 3 and 4 concentrations of both NTproCNP and CNP were strikingly consistent across age, and increased in aged occipital cortex. If a varying volume of neural tissue has been collected across young and aged rats, it has not affected tissue concentrations of the peptide. Alternatively, several of the age-related changes identified within chapter 3, including hypothesised increases in NPR-C receptors may represent compensatory mechanisms to maintain CNP activity as cellular changes occur.

Access to, and cost of antibodies and CNP-related antagonists also introduced general limitations to this thesis. As mentioned in Chapter 3, a complementary study was designed with the intention of identifying neprilysin, NPR-B receptor and CNP distribution within aged rodents in association with both enrichment and behaviour. Budget restrictions prevented acquisition of relevant antibodies. Additionally, design of the study for Chapter 5 underwent multiple iterations, several of which were rejected due to inaccessibility of an NPR-B receptor antagonist (HS-142-1). Gö6976 was similarly rejected following calculations indicating amounts required would be prohibitively expensive. These limitations will be common for many studying CNP and will need careful consideration as research moves forward.

Further to this limitation, the use of indwelling icv cannulae providing an assumed broad exposure to CNP may not be an ideal method in CNP research. To date, all studies

administering CNP centrally have used icv cannulation (e.g. Babarczy et al., 1995; Bíró et al., 1996b; Gardi et al., 1997; Telegdy et al., 2000, 1999). Hence, this was employed here for comparative purposes. All studies of this nature have the limitation that indwelling materials may cause inflammation of cerebral tissue. Additionally, as CNP has been proposed to mediate inflammatory processes (Qian et al., 2002) this may result in either additive or contrasting effects in this context. As mentioned in the rationale to chapter 5, the specific mechanism of action following ventricular administration of CNP can only be assumed at this juncture. Moreover, rapid degradation of the peptide at source, and modification of blood-brain barrier (Bohara et al., 2014) suggest targeted administration, manipulation or measurement procedures will be of greater value in understanding neurological functions of CNP. Microdialysis experiments would address several concerns noted herein, including targeted measurements (e.g. localised to hippocampal CA1, CA3 or DG), and can be used in combination with RIA to avoid background staining complications of IHC. Such methodology could also be applied in administering CNP to targeted regions – retrosplenial granular versus dysgranular suggested by the data gathered for this thesis. Optogenetic techniques can be used to target gene expression within specific nuclei and would provide interesting follow up to the results of Chapter 4 indicating increases in nppc expression in retrosplenial cortex and mammillary bodies. In a relatively nascent area of study, even the use of limited methodology may provide insight into ongoing research directions.

A final general limitation of this thesis is a reliance on solely male rodents. Because previous research of CNP and behavioural effects was limited, possible central interactions with reproductive hormones across the oestrous cycle (outlined in Chapter 2) could not be predicted. Gonadal hormones (estrogens and androgens) have varying effects neurologically and behaviourally, and can account for sex differences in both incidence and etiology of neurological disorders (see Galea et al., 2008 for review). Importantly, sex-differences occur during both healthy aging and Alzheimer's disease (Ishunina, Kamphorst, & Swaab, 2003; Kolb, Forgie, Gibb, Gorny, & Rowntree, 1998; Kolb et al., 2003; Swaab & Bao, 2011). In addition to previous suggestions regarding ongoing CNP research in contexts of normal aging and Alzheimer's disease (Chapter 3), integration of a consideration of sex-differences will be vital.

As research regarding central activity of CNP moves forward, it will benefit from consideration of the neurovisceral integration model (Thayer & Lane, 2000). Thayer & Lane (2000) focus on emotion regulation, dysregulation and the cardiovascular system to outline a model integrating "autonomic, attentional, and affective systems into a functional and

structural network" (Thayer & Lane, 2000, p.201) from a dynamic system perspective. This description could equally apply to roles and functions identified for CNP. Recent experimental work supports the neurovisceral integration model, with greater heart-rate variability in older adults linked to weakening of functional connections between amygdala and ventrolateral prefrontal cortex (Sakaki et al., 2016). A similar disconnect was identified here regarding CNP signalling. This suggestion is further supported by recent work indicating an influence of central CNP on heart-rate regulation related to both NPR-B and NPR-C receptors (Buttgereit et al., 2016; Moghtadaei, Langille, Rafferty, Bogachev, & Rose, 2017). As all natriuretic peptides could be described as participating in homeostatic regulation of multiple peripheral and central systems, they present an intriguing target system in the context of neurovisceral integration.

Appendix A: Work published from this thesis

frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
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Environmental Enrichment Elicits a Transient Rise of Bioactive C-Type Natriuretic Peptide in Young but Not Aged Rats

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Beneficial molecular and neuroplastic changes have been demonstrated in response to environmental enrichment (EE) in laboratory animals across the lifespan. Here, we investigated whether these effects extend to C-type Natriuretic Peptide (CNP), a widely expressed neuropeptide with putative involvement in neuroprotection, neuroplasticity, anxiety, and learning and memory. We determined the CNP response in 36 young (8–9 months) and 36 aged (22–23 months) male PVGc hooded rats that were rehoused with new cage mates in either standard laboratory cages or EE for periods of 14 or 28 days. Tissues were rapidly excised from four brain regions associated with memory formation (dorsal hippocampus, retrosplenial cortex, medial prefrontal cortex, and mammillary bodies) plus the occipital cortex and hypothalamus, and immediately frozen. Radioimmunoassay was used to measure bioactive CNP and the amino-terminal fragment of proCNP, NTproCNP. Because CNP but not NTproCNP is rapidly degraded at source, NTproCNP reflects CNP production whereas the ratio NTproCNP:CNP is a biomarker of CNP's local degradation rate. EE increased CNP at 14 days in all brain regions in young, but not old rats; this effect in young rats was lost at 28 days in all regions of interest. NTproCNP:CNP ratio, but not NTproCNP, was reduced in all regions by EE at 14 days in young rats, but not in old rats, which suggests a period of reduced degradation or receptor mediated clearance, rather than increased production of CNP in these young EE rats. Aged rats tended to show reduced NTproCNP:CNP ratios but this did not occur in dorsal hippocampus or mammillary bodies. This is the first study demonstrating modulation of CNP protein concentrations, and the effect of age, in response to environmental stimulation. Furthermore, it is the first to show that changes in degradation rate *in vivo* may be an important component in determining CNP bioactivity in neural tissues.

Keywords: C-type natriuretic peptide, environmental enrichment, aging, medial prefrontal cortex, hippocampus, retrosplenial cortex

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Appendix B: Supplemental Figures

Endogenous measures of CNP activity after RAM learning

CNP

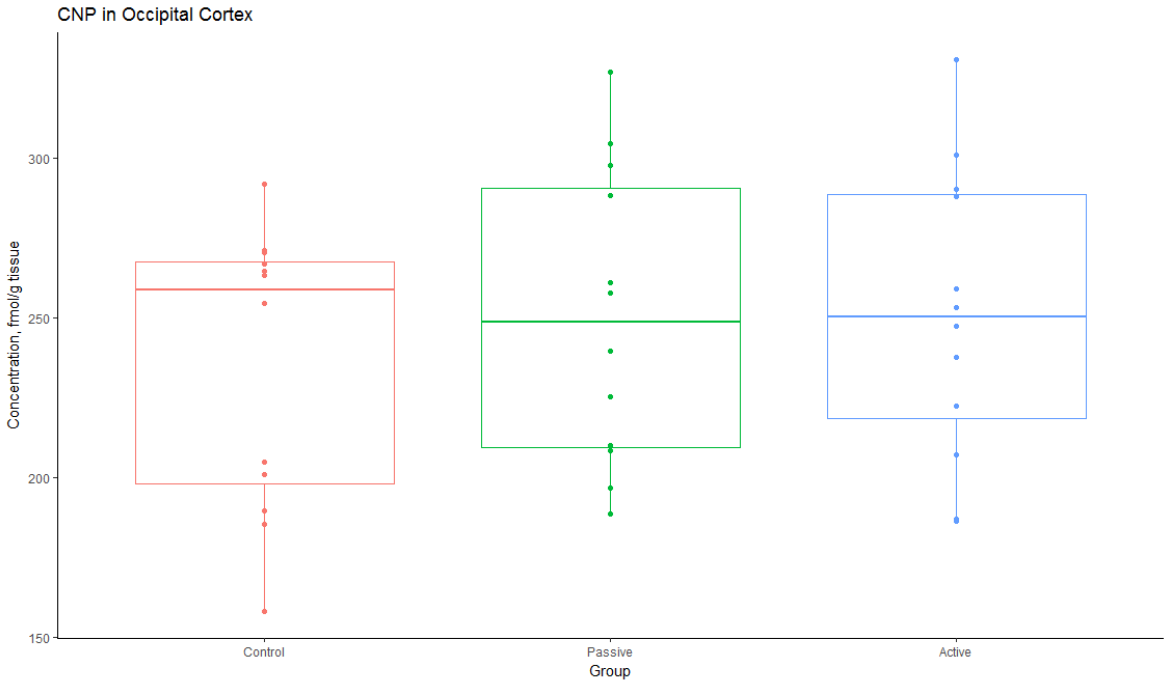

Figure A0.1: Boxplot and individual data points of CNP concentrations by behaviour group in occipital cortex.

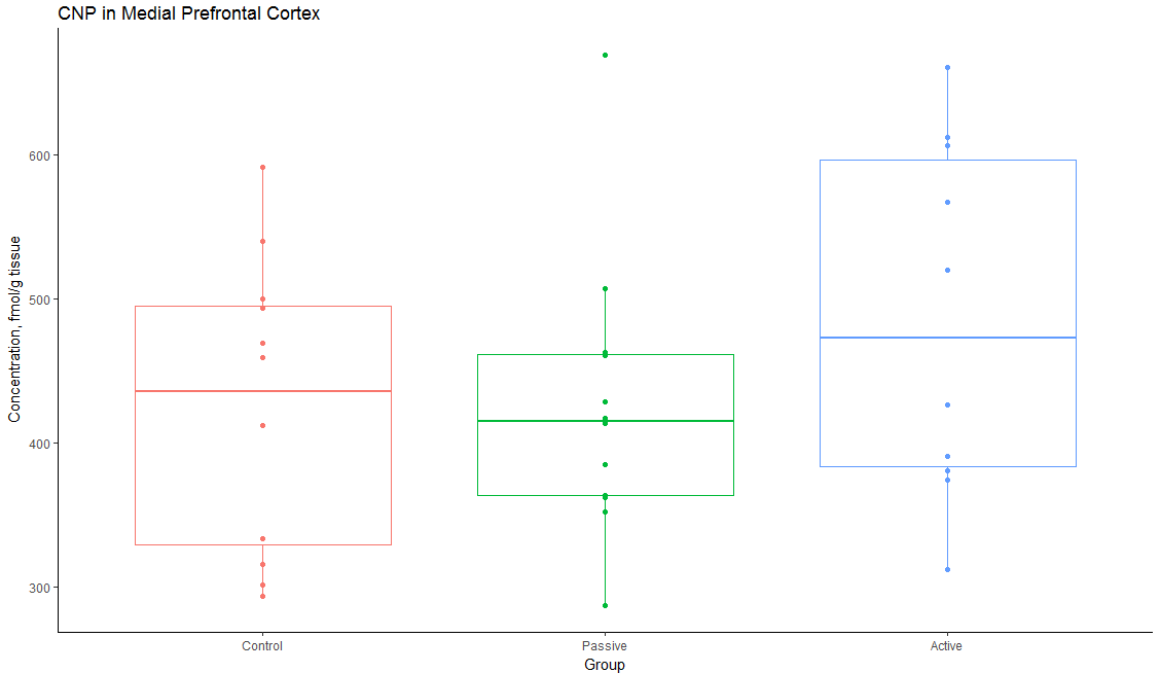

Figure A0.2: Boxplot and individual data points of CNP concentrations by behaviour group in medial prefrontal cortex.

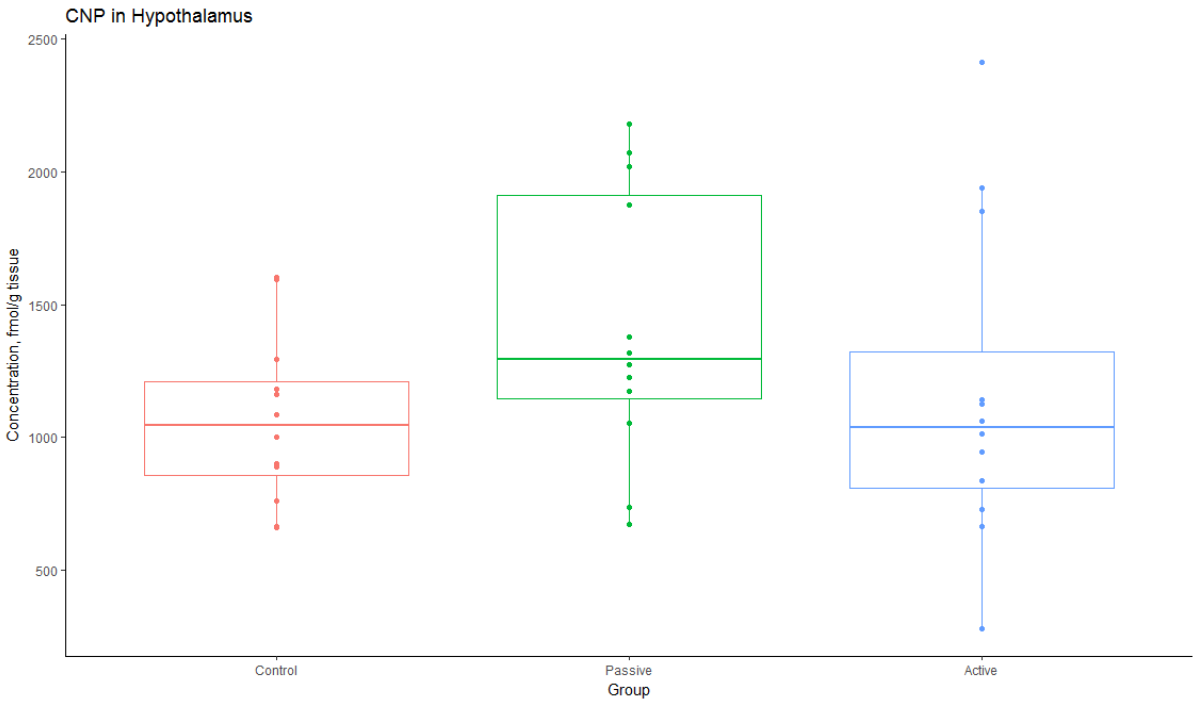

Figure A0.3: Boxplot and individual data points of CNP concentrations by behaviour group in hypothalamus.

NTproCNP

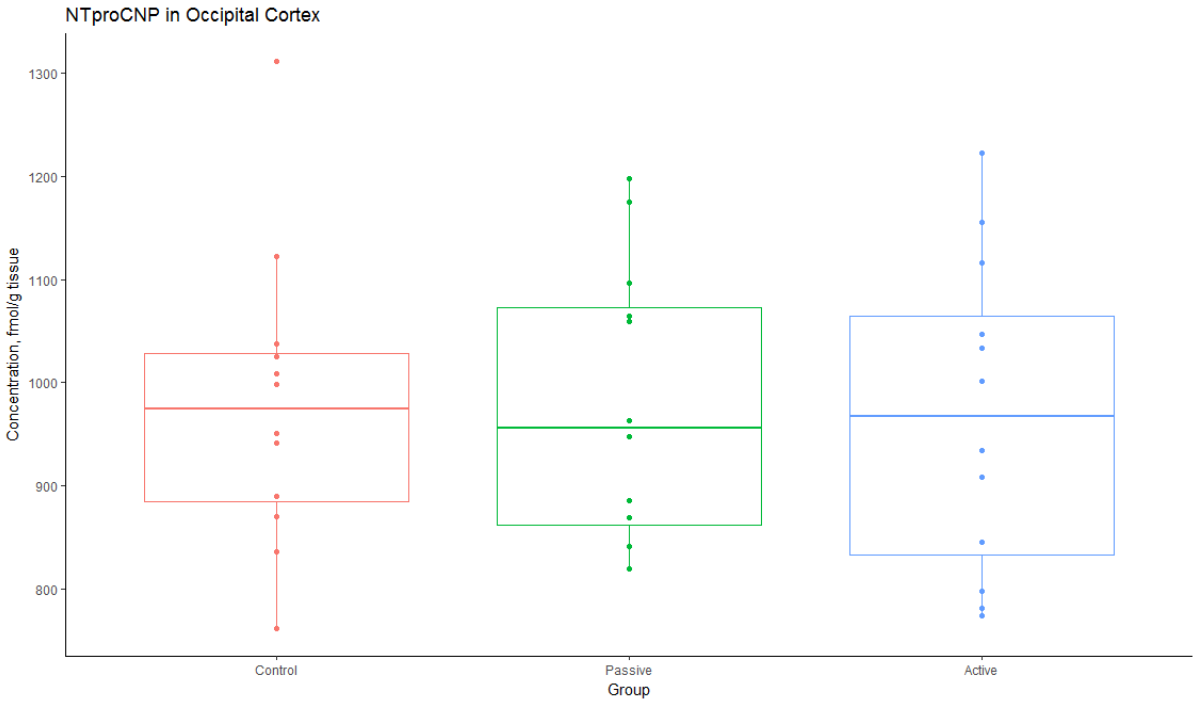

Figure A0.4: Boxplot and individual data points of NTproCNP concentrations by behaviour group in occipital cortex.

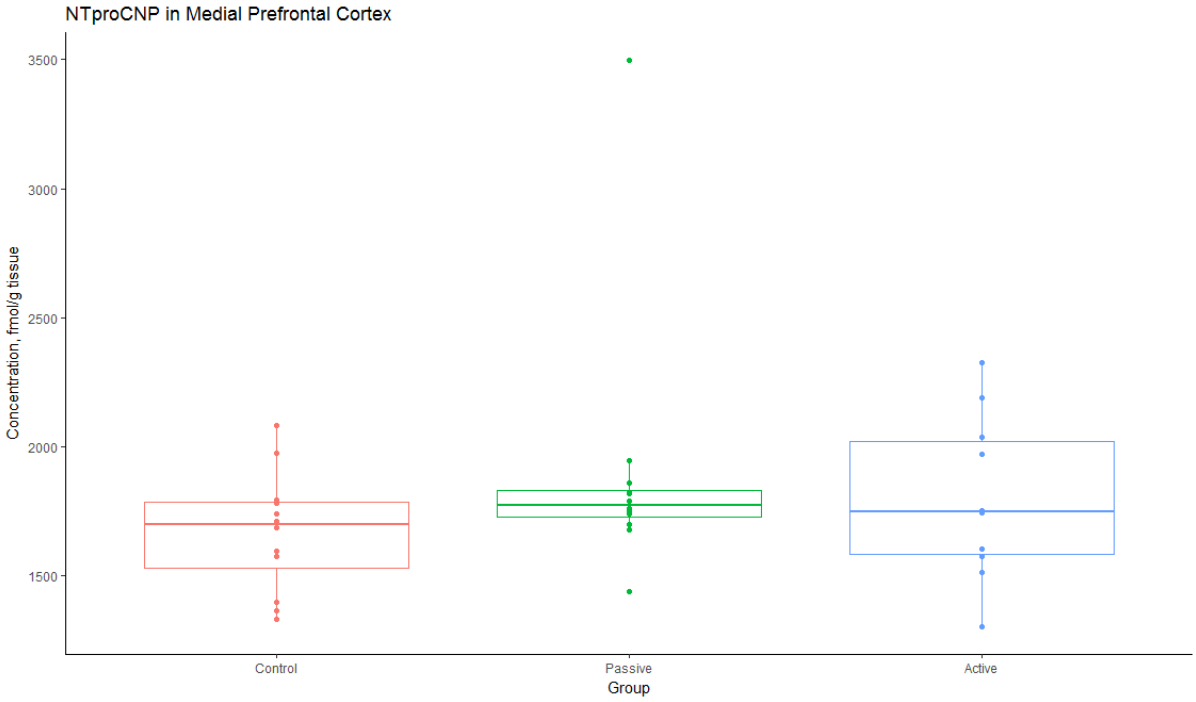

Figure A0.5: Boxplot and individual data points of NTproCNP concentrations by behaviour group in medial prefrontal cortex.

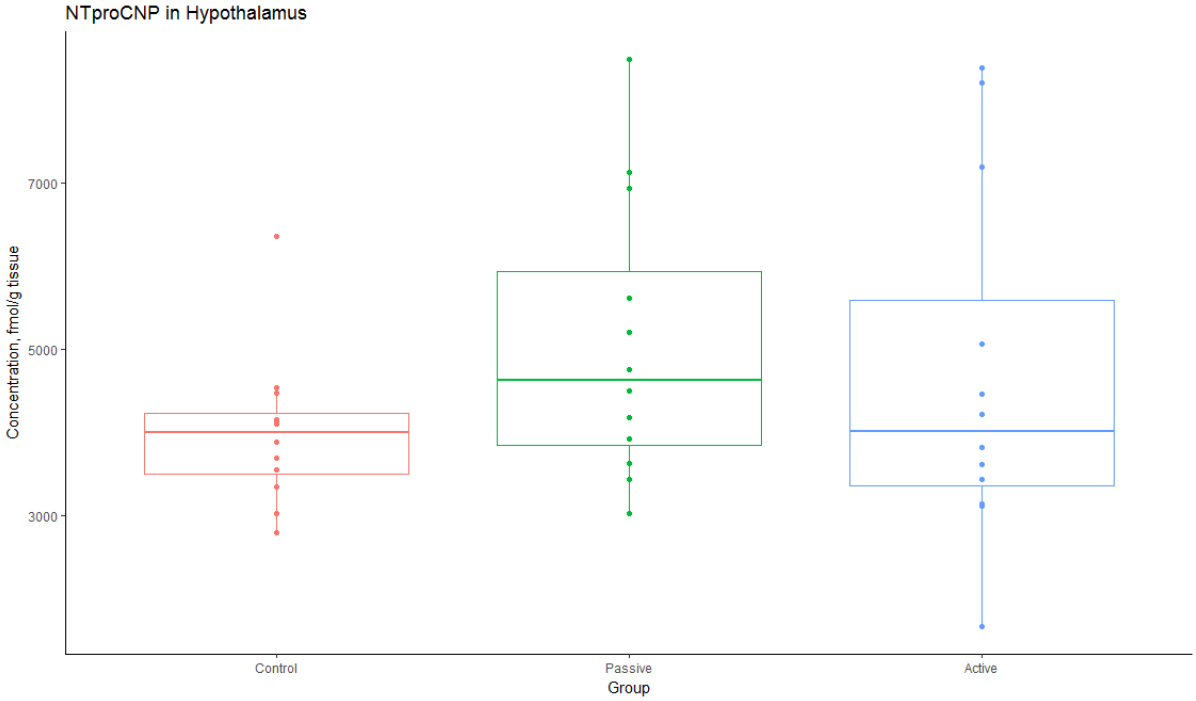

Figure A0.6: Boxplot and individual data points of NTproCNP concentrations by behaviour group in hypothalamus.

NTproCNP:CNP Ratio

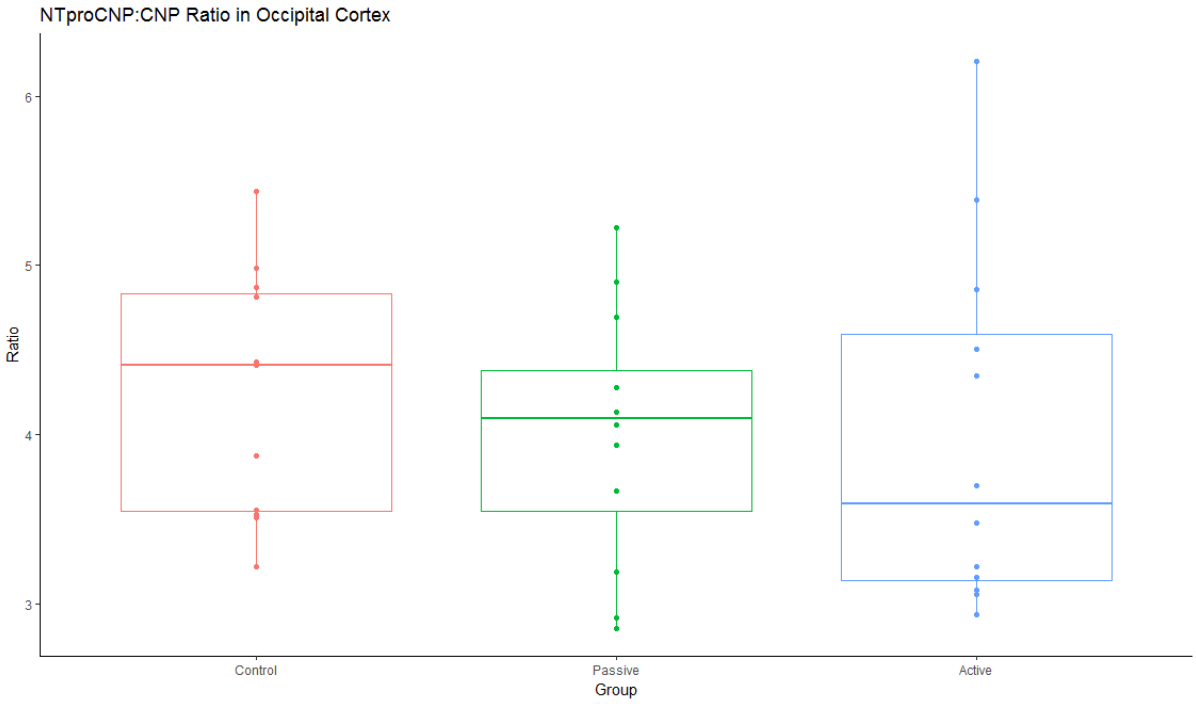

Figure A0.7: Boxplot and individual data points of NTproCNP:CNP ratio values by behaviour group in occipital cortex.

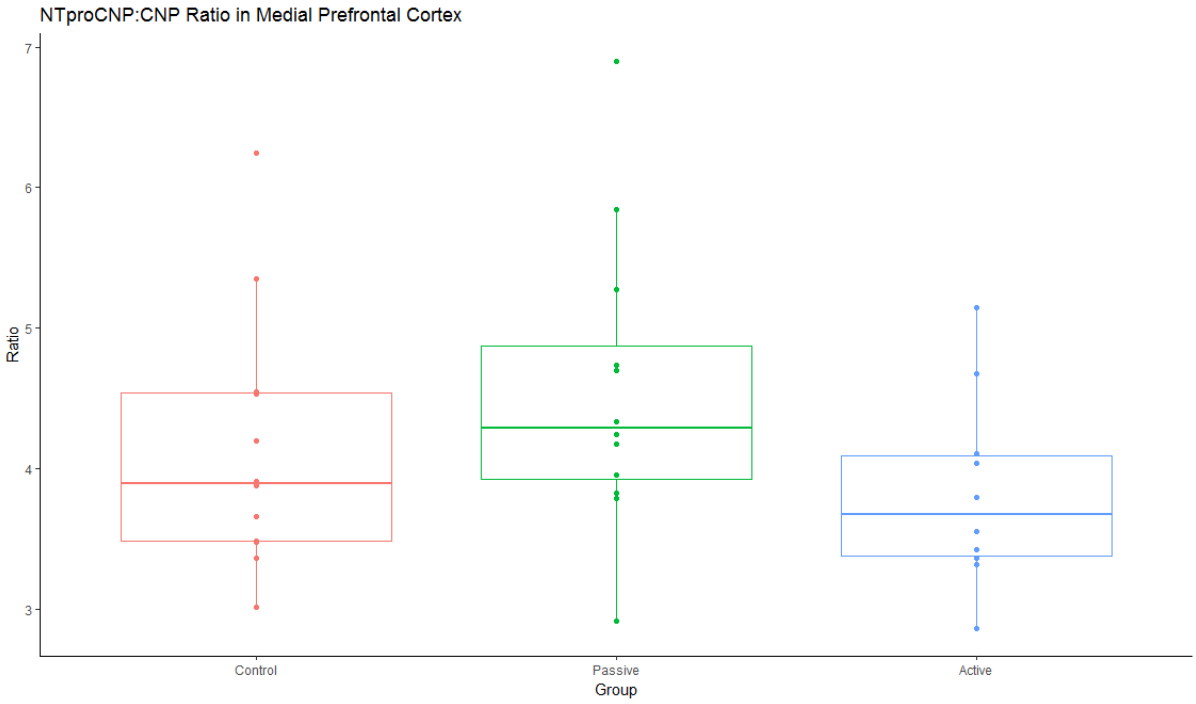

Figure A0.8: Boxplot and individual data points of NTproCNP:CNP ratio values by behaviour group in medial prefrontal cortex.

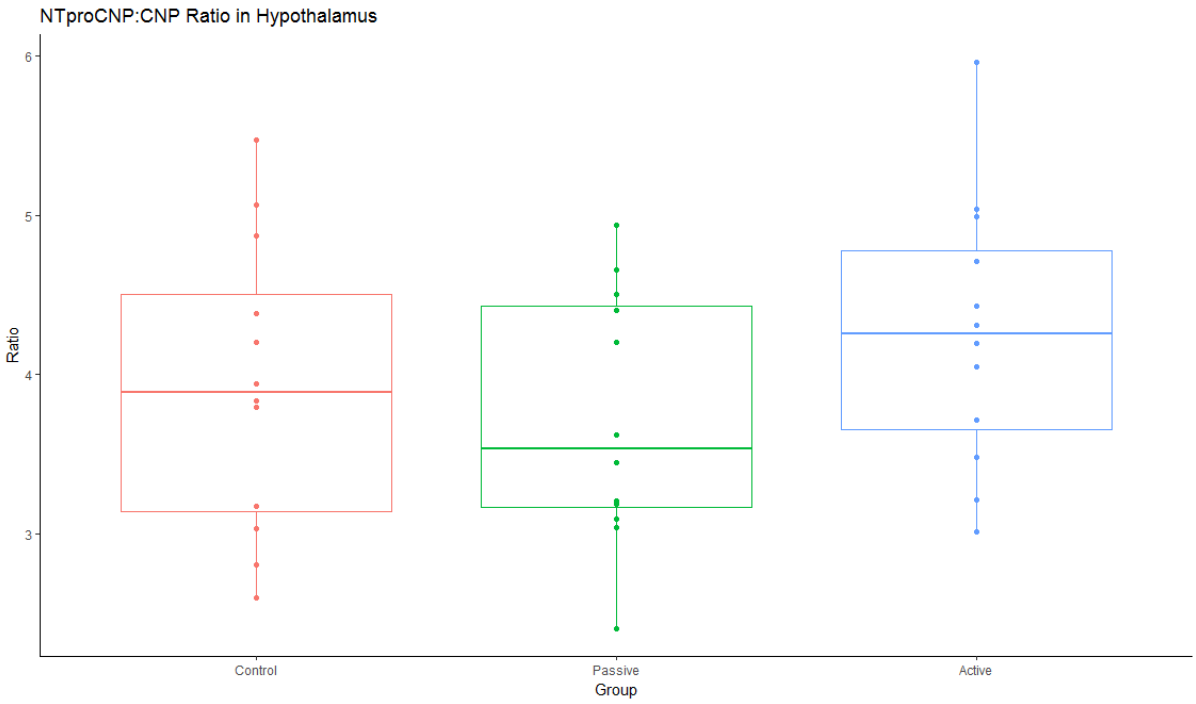

Figure A0.9: Boxplot and individual data points of NTproCNP:CNP ratio values by behaviour group in hypothalamus.

Statistical Tests

Table A0.1: Mean [±95%CI] CNP, NTproCNP and NTproCNP:CNP ratio for Control, Passive and Active Learning rats throughout six regions of interest, with accompanying statistical tests demonstrating generally null effects. Results reported in text are highlighted in gray.

	Mean [±95% CI]				
CNP	**Control**	**Passive Learning**	**Active Learning**	**ANOVA**	**Robust ANOVA**
Occipital Cortex	235.10 [207.11, 263.09]	250.40 [221.04, 279.76]	250.82 [221.74, 279.90]	F (2, 33) = 0.47, p = .630	F (2, 13.97) = 0.14, p = .876
Medial Prefrontal Cortex	420.85 [355.66, 486.04]	425.57 [364.16, 486.98]	484.76, [397.06, 572.46]	F (2, 31) = 1.19, p = .317	F (2, 9.83) = 0.55, p = .546
Retrosplenial Cortex	414.22 [352.00, 476.44]	414.52 [340.49, 488.55]	451.97 [378.54, 525.40]	F (2, 32) = 0.48, p = .625	F (2, 13.19) = 0.21, p = .816
Dorsal Hippocampus	461.16 [394.63, 527.69]	534.51 [473.04, 595.98]	516.26 [429.06, 603.46]	F (2, 33) = 1.34, p = .276	F (2, 13.99) = 1.89, p = .187
Mammillary Bodies	1557.16 [1408.94, 1705.38]	1728.61 [1532.97, 1924.25]	1704.53 [1365.21, 339.32]	F (2, 33) = 0.71, p = .497	F (2, 12.84) = 2.67, p = .107
Hypothalamus	1066.88 [863.79, 1269.97]	1414.98 [1091.47, 1738.49]	1167.19 [782.31, 1552.07]	F (2, 33) = 1.59, p = .220	F (2, 12.51) = 1.67, p = .228
NTproCNP					
Occipital Cortex	979.39 [887.96, 1070.82]	979.94 [894.41, 1065.47]	967.98 [871.17, 1064.79]	F (2, 33) = 0.03, p = .974	F (2, 13.08) = 0.00, p = .997
Medial Prefrontal Cortex	1670.39 [1522.71, 1818.07]	1899.62 [1570.61, 2228.63]	1802.21 [1571.64, 2032.78]	F (2, 31) = 1.09, p = .346	F (2, 9.07) = 1.10, p = .374
Retrosplenial Cortex	1479.47 [1398.25, 1560.69]	1683.89 [1242.33, 2125.45]	1846.01 [1655.95, 2036.07]	F (2, 32) = 2.36, p = .111	F (2, 11.49) = 5.20, p = .025
Dorsal Hippocampus	2066.70 [1984.83, 2148.57]	1982.98 [1808.37, 2157.59]	2170.32 [2054.41, 2286.23]	F (2, 33) = 2.45, p = .102	F (2, 13.35) = 1.72, p = .216
Mammillary Bodies	5531.72 [4979.90, 6083.54]	6458.11 [5888.99, 7027.23]	6322.52 [5380.07, 7264.97]	F (2, 33) = 2.40, p = .107	F (2, 13.96) = 4.08, p = .040
Hypothalamus	4007.33 [3423.71, 4590.95]	5071.20 [4964.36, 5178.04]	4696.44 [4560.89, 4831.99]	F (2, 33) = 1.28, p = .293	F (2, 10.83) = 1.15, p = .352
NTproCNP:CNP Ratio					
Occipital Cortex	4.25 [3.80, 4.70]	4.01 [3.53, 4.49]	3.99 [3.31, 4.67]	F (2, 33) = 0.35, p = .708	F (2, 13.81) = 0.53, p = .604
Medial Prefrontal Cortex	4.14 [3.55, 4.73]	4.56 [3.89, 5.23]	3.83 [3.34, 4.32]	F (2, 31) = 1.77, p = .187	F (2, 12.57) = 2.21, p = .150
Retrosplenial Cortex	3.70 [3.30. 4.10]	4.24 [3.06, 5.42]	4.23 [3.74, 4.72]	F (2, 32) = 0.87, p = .427	F (2, 13.21) = 1.17, p = .342
Dorsal Hippocampus	4.68 [4.09. 5.27]	3.87 [3.28, 4.46]	4.46 [3.85, 5.07]	F (2, 33) = 2.39, p = .107	F (2, 13.35) = 1.85, p = .196
Mammillary Bodies	3.58 [3.25, 3.91]	3.80 [3.43, 4.17]	3.80 [3.49, 4.11]	F (2, 33) = 0.67, p = .520	F (2, 13.94) = 0.36, p = .701
Hypothalamus	3.93 [3.35, 4.51]	3.72 [3.22, 4.22]	4.26 [3.72, 4.80]	F (2, 33) = 1.19, p = .316	F (2, 13.91) = 0.91, p = .426

Appendix C: Images of histology for cyclic Guanosine Monophosphate

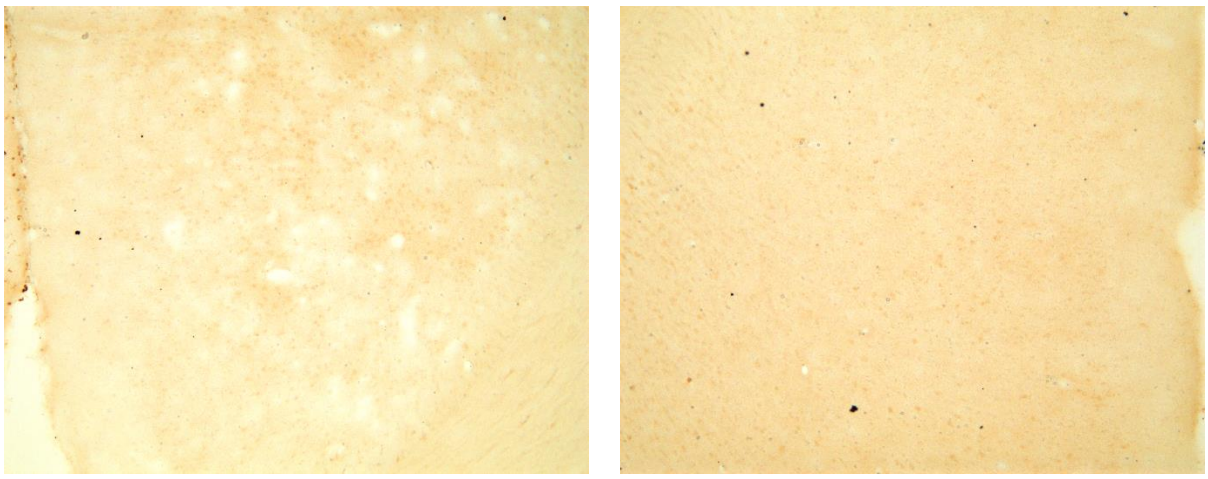

Figure C0.1: cGMP staining in cingulate gyrus following Saline (left) or CNP (right)

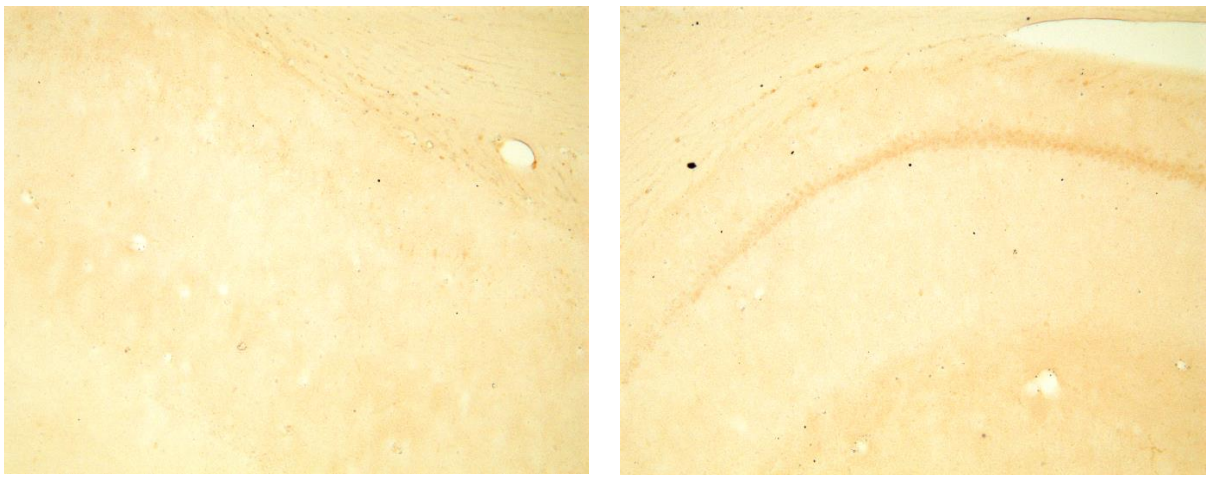

Figure C0.2: cGMP staining in dorsal CA1 following Saline (left) or CNP (right)

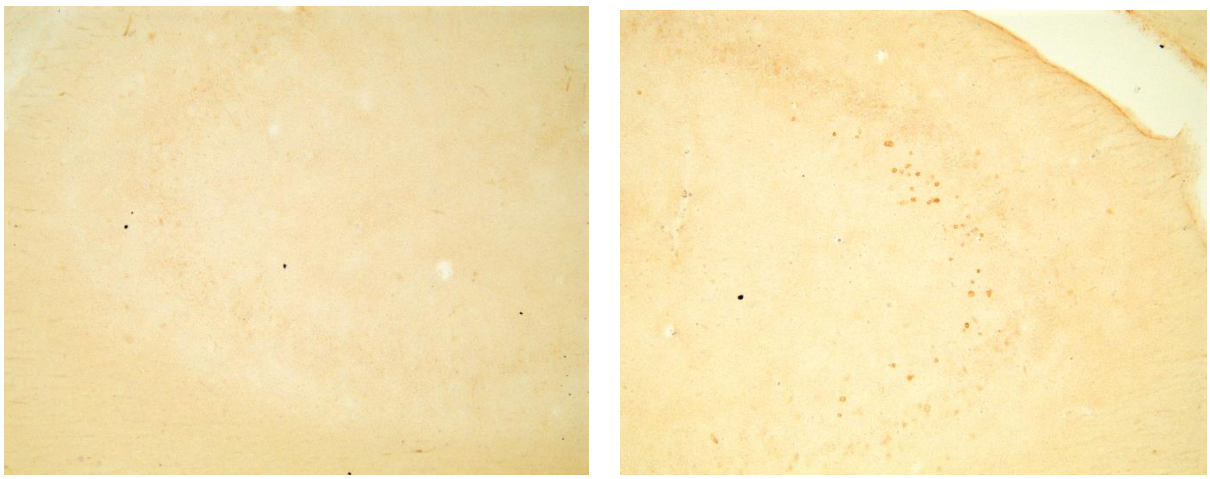

Figure C0.3: cGMP staining in dorsal CA3 following Saline (left) or CNP (right)

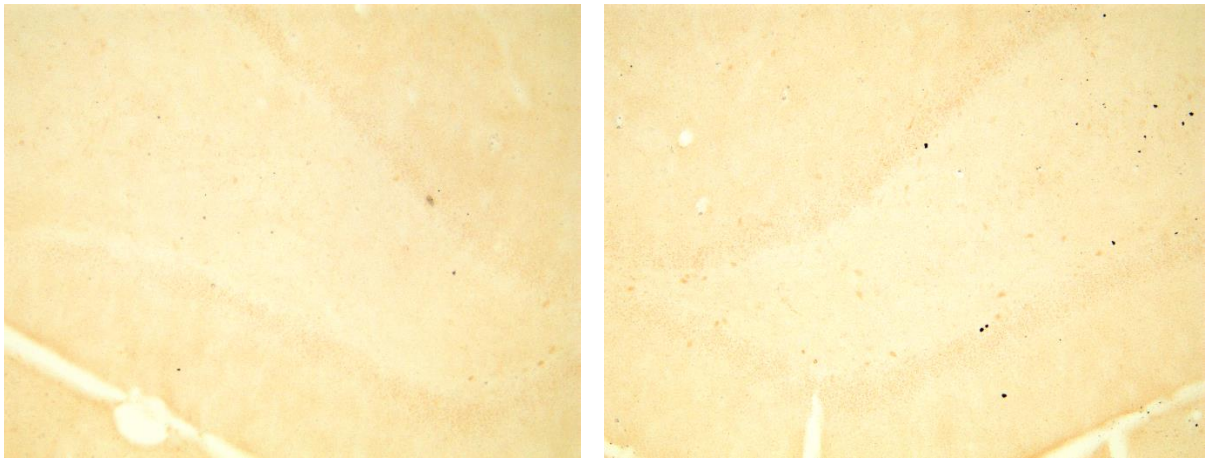

Figure C0.4: cGMP staining in dorsal dentate gyrus following Saline (left) or CNP (right)

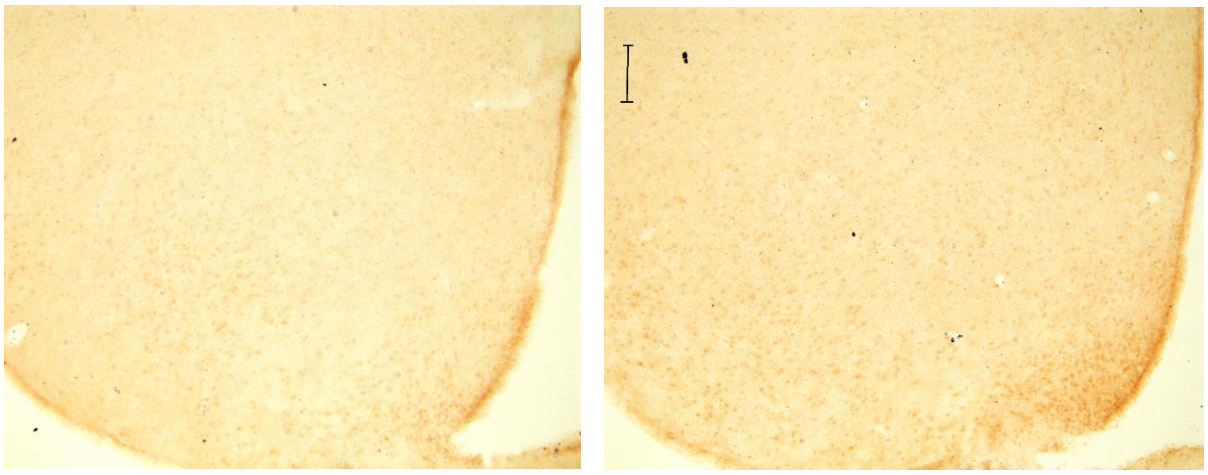

Figure C0.5: cGMP staining in hypothalamus following Saline (left) or CNP (right). Scale bar = 100µm, applies to all images.

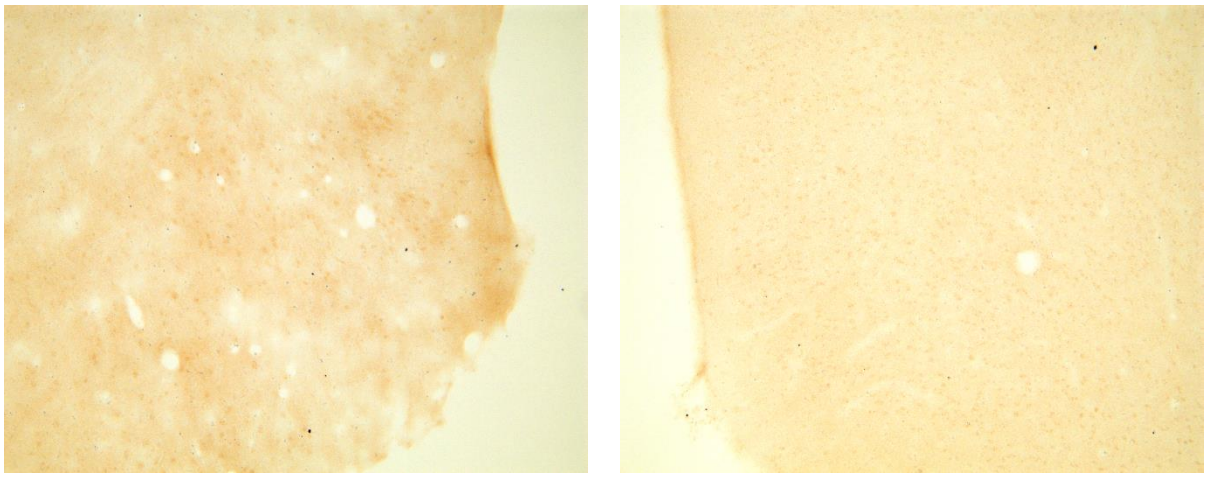

Figure C0.6: cGMP staining in hypothalamus following Saline (left) or CNP (right)

References

Abbey-Hosch, S. E., Cody, A. N., & Potter, L. R. (2004). Sphingosine-1-Phosphate Inhibits C-Type Natriuretic Peptide Activation of Guanylyl Cyclase B (GC-B/NPR-B). *Hypertension*, *43*(5), 1103–1109. https://doi.org/10.1161/01.HYP.0000124668.80811.d3

Abdelalim, E. M., Masuda, C., Bellier, J. P., Saito, A., Yamamoto, S., Mori, N., & Tooyama, I. (2008). Distribution of natriuretic peptide receptor-C immunoreactivity in the rat brainstem and its relationship to cholinergic and catecholaminergic neurons. *Neuroscience*, *155*(1), 192–202. https://doi.org/10.1016/j.neuroscience.2008.05.020

Abdelalim, E. M., Masuda, C., & Tooyama, I. (2008). Expression of natriuretic peptide-activated guanylate cyclases by cholinergic and dopaminergic amacrine cells of the rat retina. *Peptides*, *29*(4), 622–628. https://doi.org/10.1016/j.peptides.2007.11.021

Abel, T., & Lattal, K. M. (2001). Molecular mechanisms of memory acquisition, consolidation and retrieval. *Current Opinion in Neurobiology*, *11*(2), 180–187. https://doi.org/10.1016/S0959-4388(00)00194-X

Abraham, W. C. (2008). Metaplasticity: tuning synapses and networks for plasticity. *Nature Reviews. Neuroscience*, *9*(5), 387–399. https://doi.org/10.1038/nrn2356

Abraham, W. C., & Bear, M. F. (1996). Metaplasticity: the plasticity of synaptic plasticity. *Trends in Neurosciences*, *19*(4), 126–130. https://doi.org/10.1016/S0166-2236(96)80018-X

Acuff, C. G., Huang, H., & Steinhelper, M. E. (1997). Estradiol induces C-type natriuretic peptide gene expression in mouse uterus. *American Journal of Physiology - Heart and Circulation Physiology*, *42*, H2672–H2677.

Aggleton, J. P. (2010). Understanding retrosplenial amnesia: Insights from animal studies. *Neuropsychologia*, *48*(8), 2328–2338. https://doi.org/10.1016/j.neuropsychologia.2009.09.030

Aggleton, J. P., & Brown, M. W. (1999). Episodic memory, amnesia, and the hippocampal-anterior thalamic axis. *The Behavioral and Brain Sciences*, *22*(3), 425-444; discussion 444-489. https://doi.org/10.1017/S0140525X99002034

Aggleton, J. P., & Brown, M. W. (2006). Interleaving brain systems for episodic and recognition memory. *Trends in Cognitive Sciences*, *10*(10), 455–463. https://doi.org/10.1016/j.tics.2006.08.003

Aggleton, J. P., & Pearce, J. M. (2001). Neural systems underlying episodic memory: insights from animal research. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *356*(1413), 1467–1482. https://doi.org/10.1098/rstb.2001.0946

Aggleton, J. P., & Vann, S. D. (2004). Testing the importance of the retrosplenial navigation system: lesion size but not strain matters: a reply to Harker and Whishaw. *Neuroscience and Biobehavioral Reviews*, *28*(5), 525–31. https://doi.org/10.1016/j.neubiorev.2004.08.003

Ahluwalia, A., & Hobbs, A. J. (2005). Endothelium-derived C-type natriuretic peptide: More than just a hyperpolarizing factor. *Trends in Pharmacological Sciences*, *26*(3), 162–167. https://doi.org/10.1016/j.tips.2005.01.005

Aimone, J. B., Deng, W., & Gage, F. H. (2011). Perspective : Point / Counterpoint Resolving

New Memories : A Critical Look at the Dentate Gyrus , Adult Neurogenesis , and Pattern Separation. *Neuron*, *70*(4), 589–596. https://doi.org/10.1016/j.neuron.2011.05.010

Albasser, M. M., Poirier, G. L., & Aggleton, J. P. (2010). Qualitatively different modes of perirhinal-hippocampal engagement when rats explore novel vs. familiar objects as revealed by c-Fos imaging. *European Journal of Neuroscience*, *31*(1), 134–147. https://doi.org/10.1111/j.1460-9568.2009.07042.x

Anand-srivastava, M. B. (2005). Natriuretic peptide receptor-C signaling and regulation, *26*, 1044–1059. https://doi.org/10.1016/j.peptides.2004.09.023

Andrade, F. A., Restini, C. B. A., Grando, M. D., Ramalho, L. N. Z., & Bendhack, L. M. (2014). Vascular relaxation induced by C-type natriuretic peptide involves the Ca2+/NO-synthase/NO pathway. *PLoS ONE*, *9*(5), 1–9. https://doi.org/10.1371/journal.pone.0095446

Apelt, J., Ach, K., & Schliebs, R. (2003). Aging-related down-regulation of neprilysin, a putative ??-amyloid-degrading enzyme, in transgenic Tg2576 Alzheimer-like mouse brain is accompanied by an astroglial upregulation in the vicinity of ??-amyloid plaques. *Neuroscience Letters*, *339*(3), 183–186. https://doi.org/10.1016/S0304-3940(03)00030-2

Armstrong, R. A., Cairns, N. J., Myers, D., Smith, C. U. M., Lantos, P. L., & Rossor, M. N. (1996). A Comparison of β -Amyloid Deposition in the Medial Temporal Lobe in Sporadic Alzheimer ' s Disease , Down ' s Syndrome and Normal Elderly Brains, *5*(January), 35–41.

Ataman, B., Boulting, G. L., Harmin, D. A., Yang, M. G., Baker-Salisbury, M., Yap, E.-L., … Greenberg, M. E. (2016). Evolution of Osteocrin as an activity-regulated factor in the primate brain. *Nature*, *539*(7628), 242–247. https://doi.org/10.1038/nature20111

Babarczy, E., Vizi, Z., Toth, G., & Telegdy, G. (1995). C-type natriuretic peptide can modify the acute and chronic effects of morphine. *Neuropeptides*, *29*(3), 145–149.

Barmashenko, G., Buttgereit, J., Herring, N., Bader, M., Ozcelik, C., Manahan-Vaughan, D., & Braunewell, K. H. (2014). Regulation of hippocampal synaptic plasticity thresholds and changes in exploratory and learning behavior in dominant negative NPR-B mutant rats. *Frontiers in Molecular Neuroscience*, *7*(December), 95. https://doi.org/10.3389/fnmol.2014.00095

Barr, C. S., Rhodes, P., & Struthers, A. D. (1996). C-type Natriuretic Peptide. *Peptides*, *17*(7), 1243–1251.

Bartels, C. F., Bukulmez, H., Padayatti, P., Rhee, D. K., Ravenswaaij-Arts, C. Van, Pauli, R. M., … Warman, M. L. (2004). Mutations in the Transmembrane Natriuretic Peptide Receptor NPR-B Impair Skeletal Growth and Cause Acromesomelic Dysplasia , Type Maroteaux, 27–34.

Bartos, M., Vida, I., & Jonas, P. (2007). Synaptic mechanisms of synchronized gamma oscillations in inhibitory interneuron networks. *Nature Reviews. Neuroscience*, *8*(1), 45–56. https://doi.org/10.1038/nrn2044

Bekinschtein, P., Kent, B. A., Oomen, C. A., Clemenson, G. D., Gage, F. H., Saksida, L. M., & Bussey, T. J. (2013). BDNF in the dentate gyrus is required for consolidation of "pattern-separated" memories. *Cell Reports*, *5*(3), 759–768.

https://doi.org/10.1016/j.celrep.2013.09.027

Bekinschtein, P., Kent, B. A., Oomen, C. A., Clemenson, G. D., Gage, F. H., Saksida, L. M., & Bussey, T. J. (2014). Brain-derived neurotrophic factor interacts with adult-born immature cells in the dentate gyrus during consolidation of overlapping memories. *Hippocampus*, *24*(8), 905–911. https://doi.org/10.1002/hipo.22304

Bekinschtein, P., Oomen, C. A., Saksida, L. M., & Bussey, T. J. (2011). Effects of environmental enrichment and voluntary exercise on neurogenesis , learning and memory , and pattern separation : BDNF as a critical variable ? *Seminars in Cell and Developmental Biology*, *22*, 536–542. https://doi.org/10.1016/j.semcdb.2011.07.002

Bennett, J. C., McRae, P. A., Levy, L. J., & Frick, K. M. (2006). Long-term continuous, but not daily, environmental enrichment reduces spatial memory decline in aged male mice. *Neurobiology of Learning and Memory*, *85*(2), 139–152. https://doi.org/10.1016/j.nlm.2005.09.003

Birch, A. M., & Kelly, Á. M. (2018). Lifelong environmental enrichment in the absence of exercise protects the brain from age-related cognitive decline. *Neuropharmacology*. https://doi.org/10.1016/j.neuropharm.2018.03.042

Bíró, É., Tóth, G., & Telegdy, G. (1996b). Effect of receptor blockers on brain natriuretic peptide and C-type natriuretic peptide caused anxiolytic state in rats. *Neuropeptides*, *30*(1), 59–65. https://doi.org/10.1016/S0143-4179(96)90056-6

Bíró, É., Tóth, G., & Telegdy, G. (1996a). Effect of receptor blockers on brain natriuretic peptide and C-type natriuretic peptide caused anxiolytic state in rats. *Neuropeptides*, *30*, 59–65. https://doi.org/10.1016/S0143-4179(96)90056-6

Boess, F. G., Hendrix, M., Van Der Staay, F. J., Erb, C., Schreiber, R., Van Staveren, W., … Koenig, G. (2004). Inhibition of phosphodiesterase 2 increases neuronal cGMP, synaptic plasticity and memory performance. *Neuropharmacology*, *47*(7), 1081–1092. https://doi.org/10.1016/j.neuropharm.2004.07.040

Bohara, M., Kambe, Y., Nagayama, T., Tokimura, H., Arita, K., & Miyata, A. (2014). C-type natriuretic peptide modulates permeability of the blood-brain barrier. *Journal of Cerebral Blood Flow and Metabolism : Official Journal of the International Society of Cerebral Blood Flow and Metabolism*, *34*(4), 589–96. https://doi.org/10.1038/jcbfm.2013.234

Buckner, R. L. (2004). Memory and Executive Function in Aging and AD. *Neuron*, *44*(1), 195–208. https://doi.org/10.1016/j.neuron.2004.09.006

Buffalo, E. a., Reber, P. J., & Squire, L. R. (1998). The human perirhinal cortex and recognition memory. *Hippocampus*, *8*(4), 330–339. https://doi.org/10.1002/(SICI)1098-1063(1998)8:4<330::AID-HIPO3>3.0.CO;2-L

Burgess, N. (2002). The hippocampus, space, and viewpoints in episodic memory. *The Quarterly Journal of Experimental Psychology. A, Human Experimental Psychology*, *55*(4), 1057–1080. https://doi.org/10.1080/02724980244000224

Burke, S. N., & Barnes, C. A. (2010). Senescent synapses and hippocampal circuit dynamics. *Trends in Neurosciences*, *33*(3), 153–161. https://doi.org/10.1016/j.tins.2009.12.003

Burke, S. N., Wallace, J. L., Nematollahi, S., Uprety, A. R., & Barnes, C. A. (2010). Pattern separation deficits may contribute to age-associated recognition impairments.

Behavioral Neuroscience, *124*(5), 559–73. https://doi.org/10.1037/a0020893

Burwell, D. (2006). The Parahippocampal Region: Corticocortical Connectivity. *Annals of the New York Academy of Sciences*, *911*(1), 25–42. https://doi.org/10.1111/j.1749-6632.2000.tb06717.x

Buttgereit, J., Shanks, J., Li, D., Hao, G., Athwal, A., Langenickel, T. H., … Herring, N. (2016). C-type natriuretic peptide and natriuretic peptide receptor B signalling inhibits cardiac sympathetic neurotransmission and autonomic function. *Cardiovascular Research*, *112*(3), 637–644. https://doi.org/10.1093/cvr/cvw184

Buzsáki, G. (2015). Hippocampal sharp wave-ripple: A cognitive biomarker for episodic memory and planning. *Hippocampus*, *25*(10), 1073–1188. https://doi.org/10.1002/hipo.22488

Cabiati, M., Sabatino, L., Caruso, R., Verde, A., Caselli, C., Prescimone, T., … Del Ry, S. (2013). C-type natriuretic peptide transcriptomic profiling increases in human leukocytes of patients with chronic heart failure as a function of clinical severity. *Peptides*, *47*, 110–114. https://doi.org/10.1016/j.peptides.2013.07.019

Cain, D. P., Humpartzoomian, R., & Boon, F. (2006). Retrosplenial cortex lesions impair water maze strategies learning or spatial place learning depending on prior experience of the rat. *Behavioural Brain Research*, *170*(2), 316–325. https://doi.org/10.1016/j.bbr.2006.03.003

Cameron, V. A., Aitken, G. D., Ellmers, L. J., Kennedy, M. A., & Espiner, E. A. (1996). The Sites of Gene Expression of Atrial, Brain, and C-Type Natriuretic Peptides in Mouse Fetal Development: Temporal Changes in Embryos and Placenta. *Endocrinology*, *137*(3), 817–824.

Cameron, V. A., Cumming, S. A., Espiner, E. A., Nicholls, G., & Richards, M. (2001). C-type natriuretic peptide expression in olfactory regions of rat ... *Neuroendocrinology*, *73*, 46–53.

Cao, L.-H., & Yang, X.-L. (2008). Natriuretic peptides and their receptors in the central nervous system. *Progress in Neurobiology*, *84*(3), 234–248. https://doi.org/10.1016/j.pneurobio.2007.12.003

Catani, M., Dell'Acqua, F., & Thiebaut de Schotten, M. (2013). A revised limbic system model for memory, emotion and behaviour. *Neuroscience & Biobehavioral Reviews*, *37*(8), 1724–1737. https://doi.org/10.1016/j.neubiorev.2013.07.001

Cauwenberghe, C. Van, Vandendriessche, C., Libert, C., & Vandenbroucke, R. E. (2016). Caloric restriction : beneficial effects on brain aging and Alzheimer ' s disease. *Mammalian Genome*, *27*(7), 300–319. https://doi.org/10.1007/s00335-016-9647-6

Charles, C. J., Espiner, E. A., Richards, A. M., & Donald, R. A. (1995). Central C-Type Natriuretic Peptide Augments the Hormone Response to Hemorrhage in Conscious Sheep. *Peptides*, *16*(1), 129–132.

Charles, C. J., Espiner, E. A., Richards, A. M., Nicholls, M. G., & Yandle, T. G. (1995a). Biological actions and pharmacokinetics of C-type natriuretic peptide in conscious sheep. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*, *268*(1). Retrieved from http://ajpregu.physiology.org/content/268/1/R201.short

Charles, C. J., Espiner, E. A., Richards, A. M., Nicholls, M. G., & Yandle, T. G. (1995b). Biological actions and pharmacokinetics of C-type natriuretic peptide in conscious sheep. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology*, *268*(1 37-1).

Cho, E. S., Kim, S. Z., Cho, K. W., & Park, B. K. (2000). Immunohistochemical localization of C-type natriuretic peptide in the rat submaxillary salivary gland. *Archives of Oral Biology*, *45*(5), 425–430.

Chrisman, T. D., & Garbers, D. L. (1999). Reciprocal antagonism coordinates C-type natriuretic peptide and mitogen-signaling pathways in fibroblasts. *Journal of Biological Chemistry*, *274*(7), 4293–4299. https://doi.org/10.1074/jbc.274.7.4293

Christoffersen, C., Bartels, E. D., & Nielsen, L. B. (2006). Heart specific up-regulation of genes for B-type and C-type natriuretic peptide receptors in diabetic mice. *European Journal of Clinical Investigation*, *36*(2), 69–75. https://doi.org/10.1111/j.1365-2362.2006.01596.x

Clemenson, G. D., Deng, W., & Gage, F. H. (2015). Environmental enrichment and neurogenesis: From mice to humans. *Current Opinion in Behavioral Sciences*, *4*, 56–62. https://doi.org/10.1016/j.cobeha.2015.02.005

Cohen, S. J., & Stackman, R. W. (2015). Assessing rodent hippocampal involvement in the novel object recognition task . A review. *Behavioural Brain Research*, *285*, 105–117. https://doi.org/10.1016/j.bbr.2014.08.002

Coleman, P. D., & Flood, D. G. (1987). Neuron numbers and dendritic extent in normal aging and Alzheimer's disease. *Neurobiology of Aging*, *8*(6), 521–545. https://doi.org/10.1016/0197-4580(87)90127-8

Connor, S. A., & Wang, Y. T. (2016). A Place at the Table: LTD as a Mediator of Memory Genesis. *The Neuroscientist*, *22*(4), 359–371. https://doi.org/10.1177/1073858415588498

Craig-Schapiro, R., Kuhn, M., Xiong, C., Pickering, E. H., Liu, J., Misko, T. P., … Holtzman, D. M. (2011). Multiplexed immunoassay panel identifies novel CSF biomarkers for alzheimer's disease diagnosis and prognosis. *PLoS ONE*, *6*(4). https://doi.org/10.1371/journal.pone.0018850

Cruz, A. P. M., Frei, F., & Graeff, F. G. (1994). Ethopharmacological analysis of rat behavior on the elevated plus-maze. *Pharmacology, Biochemistry and Behavior*, *49*(1), 171–176. https://doi.org/10.1016/0091-3057(94)90472-3

Davies, J. S. (2017). Less is more : Caloric regulation of neurogenesis and adult brain function, (July), 1–11. https://doi.org/10.1111/jne.12512

Decker, J. M., Wójtowicz, a M., Bartsch, J. C., Liotta, a, Braunewell, K. H., Heinemann, U., & Behrens, C. J. (2010). C-type natriuretic peptide modulates bidirectional plasticity in hippocampal area CA1 in vitro. *Neuroscience*, *169*(1), 8–22. https://doi.org/10.1016/j.neuroscience.2010.04.064

Decker, J. M., Wójtowicz, a M., Ul Haq, R., Braunewell, K.-H., Heinemann, U., & Behrens, C. J. (2009). C-type natriuretic peptide decreases hippocampal network oscillations in adult rats in vitro. *Neuroscience*, *164*(4), 1764–75. https://doi.org/10.1016/j.neuroscience.2009.09.036

Decker, J. M., Wojtowicz, A., Heinemann, U., & Braunewell, K. H. (2008). C-Type natriuretic peptide modulates pre- and postsynaptic properties in hippocampal area CA1 in vitro. *Biochemical and Biophysical Research Communications*, *377*(3), 820–825. https://doi.org/10.1016/j.bbrc.2008.10.070

Deschatrettes, E., Romieu, P., & Zwiller, J. (2013). Cocaine self-administration by rats is inhibited by cyclic GMP-elevating agents: involvement of epigenetic markers. *The International Journal of Neuropsychopharmacology / Official Scientific Journal of the Collegium Internationale Neuropsychopharmacologicum (CINP)*, *16*(7), 1587–97. https://doi.org/10.1017/S1461145712001630

DiCicco-Bloom, E., Lelièvre, V., Zhou, X., Rodriguez, W., Tam, J., & Waschek, J. a. (2004). Embryonic expression and multifunctional actions of the natriuretic peptides and receptors in the developing nervous system. *Developmental Biology*, *271*(1), 161–75. https://doi.org/10.1016/j.ydbio.2004.03.028

Drake, D. A., & Myrvold, W. (2004). The non-existence of maximal sets of four mutually orthogonal Latin squares of order 8. *Designs, Codes, and Cryptography*, *33*(1), 63–69. https://doi.org/10.1023/B:DESI.0000032607.03637.1c

Dumont, J. R., & Aggleton, J. P. (2013). Dissociation of Recognition and Recency Memory Judgments After Anterior Thalamic Nuclei Lesions in Rats, *127*(3), 415–431. https://doi.org/10.1037/a0032750

Dupire, A., Kant, P., Mons, N., Marchand, A. R., Coutureau, E., Dalrymple-alford, J., & Wolff, M. (2013). A Role for Anterior Thalamic Nuclei in Affective Cognition : Interaction With Environmental Conditions, *404*, 392–404. https://doi.org/10.1002/hipo.22098

Ennaceur, A., Neave, N., & Aggleton, J. P. (1997). Spontaneous object recognition and object location memory in rats: The effects of lesions in the cingulate cortices, the medial prefrontal cortex, the cingulum bundle and the fornix. *Experimental Brain Research*, *113*(3), 509–519. https://doi.org/10.1007/PL00005603

Erickson, C. A., & Barnes, C. A. (2003). The neurobiology of memory changes in normal aging. *Experimental Gerontology*, *38*(1–2), 61–69. https://doi.org/10.1016/S0531-5565(02)00160-2

Espiner, E. A., Dalrymple-Alford, J. C., Prickett, T. C. R., Alamri, Y., & Anderson, T. J. (2014). C-type natriuretic peptide in Parkinson's disease: reduced secretion and response to deprenyl. *Journal of Neural Transmission (Vienna, Austria : 1996)*, *121*(4), 371–8. https://doi.org/10.1007/s00702-013-1123-9

Espiner, E. A., Richards, A. M., Yandle, T. G., & Nicholls, M. G. (1995). Natriuretic hormones. *Endocrinology and Metabolism Clinics of North America*, *24*(3), 481–509. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8575406

Fan, D., Bryan, P. M., Antos, L. K., Potthast, R. J., & Potter, L. R. (2005). Down-regulation does not mediate natriuretic peptide-dependent desensitization of natriuretic peptide receptor (NPR)-A or NPR-B: guanylyl cyclase-linked natriuretic peptide receptors do not internalize. *Molecular Pharmacology*, *67*(1), 174–183. https://doi.org/10.1124/mol.104.002436.receptors

Fermepín, M. R., Vatta, M. S., Bianciotti, L. G., Wolovich, T. J., & Fernández, B. E. (2000). B-type and C-type natriuretic peptides modify norepinephrine uptake in discrete

encephalic nuclei of the rat. *Cellular and Molecular Neurobiology*, *20*(6), 763–771. https://doi.org/10.1023/A:1007011127409

Fjell, A. M., McEvoy, L., Holland, D., Dale, A. M., & Walhovd, K. B. (2014). What is normal in normal aging? Effects of aging, amyloid and Alzheimer's disease on the cerebral cortex and the hippocampus. *Progress in Neurobiology*, *117*, 20–40. https://doi.org/10.1016/j.pneurobio.2014.02.004

Fowkes, R. C., & McArdle, C. A. (2000). C-type natriuretic peptide: An important neuroendocrine regulator? *Trends in Endocrinology and Metabolism*, *11*(8), 333–338. https://doi.org/10.1016/S1043-2760(00)00288-5

Frick, K. M., & Fernandez, S. M. (2003). Enrichment enhances spatial memory and increases synaptophysin levels in aged female mice. *Neurobiology of Aging*, *24*(4), 615–626. https://doi.org/10.1016/S0197-4580(02)00138-0

Galea, L. a M., Uban, K. a, Epp, J. R., Brummelte, S., Barha, C. K., Wilson, W. L., … Pawluski, J. L. (2008). Endocrine regulation of cognition and neuroplasticity: our pursuit to unveil the complex interaction between hormones, the brain, and behaviour. *Canadian Journal of Experimental Psychology = Revue Canadienne de Psychologie Experimentale*, *62*(4), 247–260. https://doi.org/10.1037/a0014501

Garden, D. L. F., Massey, P. V., Caruana, D. A., Johnson, B., Warburton, E. C., Aggleton, J. P., & Bashir, Z. I. (2009). Anterior thalamic lesions stop synaptic plasticity in retrosplenial cortex slices: expanding the pathology of diencephalic amnesia. *Brain*, *132*(7), 1847–1857. https://doi.org/10.1093/brain/awp090

Gardi, J., Biro, E., Vecsernyes, M., Julesz, J., Nyari, T., Toth, G., & Telegdy, G. (1997). The Effects of Brain and C-Type Natriuretic Peptides on Corticotropin-Releasing Factor in Brain of Rats. *Life Sciences*, *60*(23), 2111–2117.

Garthwaite, G., Goodwin, D. A., & Garthwaite, J. (1999). Nitric oxide stimulates cGMP formation in rat optic nerve axons, providing a specific marker of axon viability. *European Journal of Neuroscience*, *11*(12), 4367–4372. https://doi.org/10.1046/j.1460-9568.1999.00874.x

Ge, Y., Dong, Z., Bagot, R. C., Howland, J. G., Phillips, A. G., Wong, T. P., & Wang, Y. T. (2010). Hippocampal long-term depression is required for the consolidation of spatial memory. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(38), 16697–702. https://doi.org/10.1073/pnas.1008200107

Gilbert, P. E., Kesner, R. P., & Lee, I. (2001). Dissociating hippocampal subregions: A double dissociation between dentate gyrus and CA1. *Hippocampus*, *11*(6), 626–636. https://doi.org/10.1002/hipo.1077

Goh, J. J., & Manahan-Vaughan, D. (2013). Spatial object recognition enables endogenous LTD that curtails LTP in the mouse hippocampus. *Cerebral Cortex*, *23*(5), 1118–1125. https://doi.org/10.1093/cercor/bhs089

Goncalves, J., Grove, K. L., & Deschepper, C. F. (1995). Generation of cyclic guanosine monophosphate in brain slices incubated with atrial or C-type natriuretic peptides : comparison of the amplitudes and cellular distribution of the responses. *Regulatory Peptides*, *57*, 55–63.

Gong, R., Ding, C., Hu, J., Lu, Y., Liu, F., Mann, E., … Luo, M. (2017). Role for the

Membrane Receptor Guanylyl Cyclase-C in Attention Deficiency and Hyperactive Behavior Published by : American Association for the Advancement of Science Linked references are available on JSTOR for this article : Role for the Membrane Recepto, *333*(6049), 1642–1646.

Goto, E., Shibata, K., & Furukawa, T. (1997). POTENTIATION OF DIPSOGENIC ACTIONS BY CENTRALLY ADMINISTERED TYPE-C NATRIURETIC PEPTIDE IN SPONTANEOUSLY HYPERTENSIVE BUT NOT WISTAR-KYOTO RATS, 927–934.

Gülpınar, M. A., & Yegen, B. Ç. (2004). The Physiology of Learning and Memory: Role of Peptides and Stress. *Current Protein and Peptide Science*, *5*(JANUARY 2005), 0–0. https://doi.org/10.2174/1389203043379341

Hama, N., Itoh, H., Shirakami, G., Suga, S., Komatsu, Y., Yoshimasa, T., … Nakao, K. (1994). Detection of C-Type Natriuretic Peptide in Human Circulation and Marked Increase of Plasma CNP Level in Septic Shock Patients. *Biochemical and Biophysical Research Communications*, *198*(3), 1177–1182. https://doi.org/10.1006/bbrc.1994.1166

Hannan, A. J. (2014). Review: Environmental enrichment and brain repair: Harnessing the therapeutic effects of cognitive stimulation and physical activity to enhance experience-dependent plasticity. *Neuropathology and Applied Neurobiology*, *40*(1), 13–25. https://doi.org/10.1111/nan.12102

Harburger, L. L., Lambert, T. J., & Frick, K. M. (2007). Age-dependent effects of environmental enrichment on spatial reference memory in male mice. *Behavioural Brain Research*, *185*(1), 43–48. https://doi.org/10.1016/j.bbr.2007.07.009

Harland, B. C., Collings, D. A., Mcnaughton, N., Abraham, W. C., & Dalrymple-Alford, J. C. (2014). Anterior thalamic lesions reduce spine density in both hippocampal CA1 and retrosplenial cortex, but enrichment rescues CA1 spines only. *Hippocampus*, *24*(10), 1232–1247. https://doi.org/10.1002/hipo.22309

Hellström-Lindahl, E., Ravid, R., & Nordberg, A. (2008). Age-dependent decline of neprilysin in Alzheimer's disease and normal brain: Inverse correlation with Aβ levels. *Neurobiology of Aging*, *29*(2), 210–221. https://doi.org/10.1016/j.neurobiolaging.2006.10.010

Herman, J. P., Dolgas, C. M., Marcinek, R., & Langub, M. C. (1996). Expression and glucocorticoid regulation of natriuretic peptide clearance receptor (NPR-C) mRNA in rat brain and choroid plexus. *Journal of Chemical Neuroanatomy*, *11*(4), 257–265. https://doi.org/10.1016/S0891-0618(96)00170-6

Herman, J. P., Dolgas, C. M., Rucker, D., & Langub, M. C. J. (1996). Localization of natriuretic peptide-activated guanylate cyclase mRNAs in the rat brain. *J Comp Neurol*, *369*(2), 165–187. https://doi.org/10.1002/(SICI)1096-9861(19960527)369:2<165::AID-CNE1>3.0.CO;2-1

Herman, J. P., Ostrander, M. M., Mueller, N. K., & Figueiredo, H. (2005). Limbic system mechanisms of stress regulation: Hypothalamo-pituitary- adrenocortical axis. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *29*(8), 1201–1213. https://doi.org/10.1016/j.pnpbp.2005.08.006

Higuchi, M., Iwata, N., & Saido, T. C. (2005). Understanding molecular mechanisms of proteolysis in Alzheimer's disease: progress toward therapeutic interventions.

Biochimica et Biophysica Acta, *1751*(1), 60–7. https://doi.org/10.1016/j.bbapap.2005.02.013

Hodes, A., & Lichtstein, D. (2014). Natriuretic hormones in brain function. *Frontiers in Endocrinology*, *5*(NOV), 1–13. https://doi.org/10.3389/fendo.2014.00201

Hodges, H. (1996). Maze procedures: The radial-arm and water maze compared. *Cognitive Brain Research*, *3*(3–4), 167–181. https://doi.org/10.1016/0926-6410(96)00004-3

Hofman, M. A. (1997). LIFESPAN CHANGES IN THE HUMAN HYPOTHALAMUS, *32*, 559–575.

Hornsby, A. K. E., Redhead, Y. T., Rees, D. J., Ratcliff, M. S. G., Reichenbach, A., Wells, T., … Davies, J. S. (2016). Short-term calorie restriction enhances adult hippocampal neurogenesis and remote fear memory in a Ghsr-dependent manner. *Psychoneuroendocrinology*, *63*, 198–207.

Howland, J. G., & Wang, Y. T. (2008). Synaptic plasticity in learning and memory: Stress effects in the hippocampus. *Progress in Brain Research*, *169*(February 2008), 145–158. https://doi.org/10.1016/S0079-6123(07)00008-8

Hu, W. T., Holtzman, D. M., Fagan, A. M., Shaw, L. M., Perrin, R., Arnold, S. E., … Trojanowski, J. Q. (2012). Plasma multianalyte profiling in mild cognitive impairment and Alzheimer disease, 897–905.

Huang, F-L, S., Skala, K. D., & Samson, W. K. (1992). C-Type Natriuretic Peptide Stimulates Prolactin Secretion by a Hypothalamic Site of Action. *Journal of Neuroendocrinology*, *4*(5), 593–597.

Hulme, S. R., Jones, O. D., & Abraham, W. C. (2013). Emerging roles of metaplasticity in behaviour and disease. *Trends in Neurosciences*, *36*(6), 353–362. https://doi.org/10.1016/j.tins.2013.03.007

Hunsaker, M. R., & Kesner, R. P. (2008). Evaluating the differential roles of the dorsal dentate gyrus, dorsal CA3, and dorsal CA1 during a temporal ordering for spatial locations task. *Hippocampus*, *18*(9), 955–964. https://doi.org/10.1002/hipo.20455

Hunsaker, M. R., & Kesner, R. P. (2013). The operation of pattern separation and pattern completion processes associated with different attributes or domains of memory. *Neuroscience and Biobehavioral Reviews*, *37*(1), 36–58. https://doi.org/10.1016/j.neubiorev.2012.09.014

Hunt, P. J., Richards, A. M., Espiner, E. A., Nicholls, M. G., & Yandle, T. G. (1994). Bioactivity and metabolism of C-type natriuretic peptide in normal man. *The Journal of Clinical Endocrinology & Metabolism*, *78*(6), 1428–1435. https://doi.org/10.1210/jcem.78.6.8200946

Ikeda, K., Ikeda, T., Onizuka, T., Terashi, H., & Fukuda, T. (2001). C-type natriuretic peptide concentrations in the plasma and cerebrospinal fluid of patients with subarachnoid hemorrhage. *Critical Care (London, England)*, *5*(1), 37–40. https://doi.org/10.1186/cc976

Inoue, K., Naruse, K., Yamagami, S., Mitani, H., Suzuki, N., & Takei, Y. (2003). Four functionally distinct C-type natriuretic peptides found in fish reveal evolutionary history of the natriuretic peptide system. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(17), 10079–84.

https://doi.org/10.1073/pnas.1632368100

Inoue, K., & Takei, Y. (2006). Molecular evolution of the natriuretic peptide system as revealed by comparative genomics. *Comparative Biochemistry and Physiology, Part D*, *1*, 69–76. https://doi.org/10.1016/j.cbd.2005.10.002

Inuzuka, M., Tamura, N., Yamada, N., Katsuura, G., Oyamada, N., Taura, D., … Nakao, K. (2010). C-type natriuretic peptide as a new regulator of food intake and energy expenditure. *Endocrinology*, *151*(8), 3633–3642. https://doi.org/10.1210/en.2010-0141

Ishunina, T. A., Kamphorst, W., & Swaab, D. F. (2003). Changes in metabolic activity and estrogen receptors in the human medial mamillary nucleus: Relation to sex, aging and Alzheimer's disease. *Neurobiology of Aging*, *24*(6), 817–828. https://doi.org/10.1016/S0197-4580(03)00009-5

Iwata, N., Takaki, Y., Fukami, S., Tsubuki, S., & Saido, T. C. (2002). Region-specific reduction of A??-degrading endopeptidase, neprilysin, in mouse hippocampus upon aging. *Journal of Neuroscience Research*, *70*(3), 493–500. https://doi.org/10.1002/jnr.10390

Iwata, N., Tsubuki, S., Takaki, Y., Shirotani, K., Lu, B., Gerard, N. P., … Saido, T. C. (2001). Metabolic regulation of brain Aβ by neprilysin. *Science*, *292*(5521), 1550–2. https://doi.org/10.1126/science.1059946

Izquierdo, A., Wellman, C. L., & Holmes, A. (2006). Brief Uncontrollable Stress Causes Dendritic Retraction in Infralimbic Cortex and Resistance to Fear Extinction in Mice. *Journal of Neuroscience*, *26*(21), 5733–5738. https://doi.org/10.1523/JNEUROSCI.0474-06.2006

Jahn, H., Montkowski, a, Knaudt, K., Ströhle, a, Kiefer, F., Schick, M., & Wiedemann, K. (2001). Alpha-helical-corticotropin-releasing hormone reverses anxiogenic effects of C-type natriuretic peptide in rats. *Brain Research*, *893*(1–2), 21–8. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11222988

Jankord, R., & Herman, J. P. (2008). Limbic regulation of hypothalamo-pituitary-adrenocortical function during acute and chronic stress. *Annals of the New York Academy of Sciences*, *1148*, 64–73. https://doi.org/10.1196/annals.1410.012

Jankowski, M., Reis, A. M., Mukaddam-Daher, S., Dam, T. V, Farookhi, R., & Gutkowska, J. (1997). C-type natriuretic peptide and the guanylyl cyclase receptors in the rat ovary are modulated by the estrous cycle. *Biol Reprod*, *56*(1), 59–66. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9002633

Jankowski, M., Reis, A. M., Wang, D., & Gutkowska, J. (2004). Postnatal ontogeny of natriuretic peptide systems in the rat hypothalamus. *Developmental Brain Research*, *152*(1), 39–46. https://doi.org/10.1016/j.devbrainres.2004.05.011

Jankowsky, J. L. (2005). Environmental Enrichment Mitigates Cognitive Deficits in a Mouse Model of Alzheimer's Disease. *Journal of Neuroscience*, *25*(21), 5217–5224. https://doi.org/10.1523/JNEUROSCI.5080-04.2005

Johanson, C. E., Donahue, J. E., Spangenberger, A., Stopa, E. G., Duncan, J. A., & Sharma, H. S. (2006). Atrial natriuretic peptide: Its putative role in modulating the choroid plexus-CSF system for intracranial pressure regulation. *Acta Neurochirurgica, Supplementum*, (96), 451–456. https://doi.org/10.1007/3-211-30714-1-92

Jouvert, P., Revel, M.-O., Lazaris, A., Aunis, D., Langley, K., & Zwiller, J. (2004). Activation of the cGMP Pathway in Dopaminergic Structures Reduces Cocaine-Induced EGR-1 Expression and Locomotor Activity. *Journal of Neuroscience*, *24*(47), 10716–10725. https://doi.org/10.1523/JNEUROSCI.1398-04.2004

Kaneko, T., Shirakami, G., Nakao, K., Nagata, I., Nakagawa, O., Hama, N., … Hirai, O. (1993). C-type natriuretic peptide (CNP) is the major natriuretic peptide in human cerebrospinal fluid. *Brain Research*, *612*, 104–109.

Kellner, M., Jahn, H., & Wiedemann, K. (2003). Natriuretic peptides and panic disorder : therapeutic prospects, 381–387.

Kellner, M., Yassouridis, A., Hua, Y., Wendrich, M., Jahn, H., & Wiedemann, K. (2002). Intravenous C-type natriuretic peptide augments behavioral and endocrine effects of cholecystokinin tetrapeptide in healthy men. *Journal of Psychiatric Research*, *36*(1), 1–6. https://doi.org/10.1016/S0022-3956(01)00042-5

Kemp, A., & Manahan-Vaughan, D. (2004). Hippocampal long-term depression and long-term potentiation encode different aspects of novelty acquisition. *Proceedings of the National Academy of Sciences*, *101*(21), 8192–8197. https://doi.org/10.1073/pnas.0402650101

Kemp, A., & Manahan-Vaughan, D. (2007). Hippocampal long-term depression: master or minion in declarative memory processes? *Trends in Neurosciences*, *30*(3), 111–118. https://doi.org/10.1016/j.tins.2007.01.002

Kemp, A., & Manahan-Vaughan, D. (2008). The hippocampal CA1 region and dentate gyrus differentiate between environmental and spatial feature encoding through long-term depression. *Cerebral Cortex*, *18*(4), 968–977. https://doi.org/10.1093/cercor/bhm136

Kempermann, G., Gast, D., & Gage, F. H. (2002). Neuroplasticity in Old Age : Sustained Fivefold Induction of Hippocampal Neurogenesis by Long-term Environmental Enrichment, (Mdc), 135–143. https://doi.org/10.1002/ana.10262

Kempermann, G., Kuhn, H. G., & Gage, F. H. (1998). Experience-induced neurogenesis in the senescent dentate gyrus. *J Neurosci*, *18*(9), 3206–3212. https://doi.org/10.1016/S0149-7634(97)00008-0

Kenny, A. J., Bourne, A., & Ingram, J. (1993). Hydrolysis of human and pig brain natriuretic peptides, urodilatin, C-type natriuretic peptide and some C-receptor ligands by endopeptidase-24.11. *The Biochemical Journal*, *291*, 83–88.

Kent, B. A., Beynon, A. L., Hornsby, A. K. E., Bekinschtein, P., Bussey, T. J., Davies, J. S., & Saksida, L. M. (2015). The orexigenic hormone acyl-ghrelin increases adult hippocampal neurogenesis and enhances pattern separation. *Psychoneuroendocrinology*, *51*, 431–439. https://doi.org/10.1016/j.psyneuen.2014.10.015

Kent, B. A., Oomen, C. A., Bekinschtein, P., Bussey, T. J., & Saksida, L. M. (2015). Cognitive enhancing effects of voluntary exercise, caloric restriction and environmental enrichment: A role for adult hippocampal neurogenesis and pattern separation? *Current Opinion in Behavioral Sciences*, *4*, 179–185. https://doi.org/10.1016/j.cobeha.2015.06.009

Kesner, R. P., & Churchwell, J. C. (2011). An analysis of rat prefrontal cortex in mediating executive function. *Neurobiology of Learning and Memory*, *96*(3), 417–431.

https://doi.org/10.1016/j.nlm.2011.07.002

Kesner, R. P., Gilbert, P. E., & Barua, L. A. (2002). The Role of the Hippocampus in Memory for the Temporal Order of a Sequence of Odors, *116*(2), 286–290. https://doi.org/10.1037//0735-7044.116.2.286

Kesner, R. P., & Hopkins, R. O. (2006). Mnemonic functions of the hippocampus : A comparison between animals and humans, *73*, 3–18. https://doi.org/10.1016/j.biopsycho.2006.01.004

Kesner, R. P., Hunsaker, M. R., & Ziegler, W. (2010). Neurobiology of Learning and Memory The role of the dorsal CA1 and ventral CA1 in memory for the temporal order of a sequence of odors. *Neurobiology of Learning and Memory*, *93*(1), 111–116. https://doi.org/10.1016/j.nlm.2009.08.010

Kim, J. J., & Diamond, D. M. (2002). The stressed hippocampus, synaptic plasticity and lost memories. *Nature Reviews. Neuroscience*, *3*(6), 453–62. https://doi.org/10.1038/nrn849

Kim, S. E., Ko, I. G., Kim, B. K., Shin, M. S., Cho, S., Kim, C. J., … Jee Yong-Seok, Y. S. (2010). Treadmill exercise prevents aging-induced failure of memory through an increase in neurogenesis and suppression of apoptosis in rat hippocampus. *Experimental Gerontology*, *45*(5), 357–365. https://doi.org/10.1016/j.exger.2010.02.005

Kinnavane, L., Davies, M., Amin, E., Powell, A. L., Vann, S. D., Olarte-s, C. M., … Nelson, A. J. D. (2017). The retrosplenial cortex and object recency memory in the rat, *45*, 1451–1464. https://doi.org/10.1111/ejn.13577

Kobayashi, S., Ohashi, Y., & Ando, S. (2002). Effects of enriched environments with different durations and starting times on learning capacity during aging in rats assessed by a refined procedure of the Hebb-Williams maze task. *Journal of Neuroscience Research*, *70*(3), 340–346. https://doi.org/10.1002/jnr.10442

Kolb, B., Forgie, M., Gibb, R., Gorny, G., & Rowntree, S. (1998). Age, experience and the changing brain. *Neuroscience and Biobehavioral Reviews*, *22*(2), 143–159. https://doi.org/10.1016/S0149-7634(97)00008-0

Kolb, B., Gibb, R., & Gorny, G. (2003). Experience-dependent changes in dendritic arbor and spine density in neocortex vary qualitatively with age and sex q, *79*, 1–10.

Kolb, B., Pittman, K., Sutherland, R. J., & Whishaw, I. Q. (1982). Dissociation of the contributions of the prefrontal cortex and dorsomedial thalamic nucleus to spatially guided behavior in the rat. *Behavioural Brain Research*, *6*(4), 365–378. https://doi.org/10.1016/0166-4328(82)90018-3

Koller, K., Lowe, D., Bennett, G., Minamino, N., Kangawa, K., Matsuo, H., & Goeddel, D. (1991). Selective activation of the B natriuretic peptide receptor by C-type natriuretic peptide (CNP). *Science*, *252*(5002), 120–123. https://doi.org/10.1126/science.1672777

Komatsu, Y., Nakao, K., Suga, S., Ogawa, Y., Mukoyama, M., Arai, H., … Imura, H. (1991). C-TYPE NATRIURETIC PEPTIDE (CNP) IN RATS AND HUMANS. *Endocrinology*, *129*(2), 1104–1106. https://doi.org/10.1210/endo-129-2-1104

Koziorowski, D., Tomasiuk, R., Szlufik, S., & Friedman, A. (2012). Inflammatory cytokines and NT-proCNP in Parkinson's disease patients. *Cytokine*, *60*(3), 762–766. https://doi.org/10.1016/j.cyto.2012.07.030

Kumar, A., Rani, A., Tchigranova, O., Lee, W., & Foster, T. C. (2012). Influence of late-life exposure to environmental enrichment or exercise on hippocampal function and CA1 senescent physiology. *NBA*, *33*(4), 828.e1-828.e17. https://doi.org/10.1016/j.neurobiolaging.2011.06.023

Langenickel, T. H., Buttgereit, J., Pagel-langenickel, I., Lindner, M., Monti, J., Beuerlein, K., … Bader, M. (2006). Cardiac hypertrophy in transgenic rats expressing a dominant-negative mutant of the natriuretic peptide receptor B, *103*(12).

Langub, M. C., Dolgas, C. M., Watson, R. E., & Herman, J. P. (1995). the C-Type Natriuretic Peptide Receptor Is the Predominant Natriuretic Peptide Receptor Messenger-Rna Expressed in Rat Hypothalamus. *Journal of Neuroendocrinology*, *7*(4), 305–309.

Langub, M. C., Watson, R. E., & Herman, J. P. (1995). Distribution of Natriuretic Peptide Precursor Messenger-Rnas in the Rat-Brain. *Journal of Comparative Neurology*, *356*(2), 183–199.

Lazarov, O., Robinson, J., Tang, Y.-P., Hairston, I. S., Korade-Mirnics, Z., Lee, V. M.-Y., … Sisodia, S. S. (2005). Environmental Enrichment Reduces Aβ Levels and Amyloid Deposition in Transgenic Mice. *Cell*, *120*(5), 701–713. https://doi.org/10.1016/j.cell.2005.01.015

Leal-Galicia, P., Castañeda-Bueno, M., Quiroz-Baez, R., & Arias, C. (2008). Long-term exposure to environmental enrichment since youth prevents recognition memory decline and increases synaptic plasticity markers in aging. *Neurobiology of Learning and Memory*, *90*(3), 511–518. https://doi.org/10.1016/j.nlm.2008.07.005

Lee, J., Duan, W., Long, J. M., Ingram, D. K., & Mattson, M. P. (2001). Dietary Restriction Increases the Number of Newly Generated Neural Cells , and Induces BDNF Expression , in the Dentate Gyrus of Rats, *15*, 99–108.

Lee, S. W., Clemenson, G. D., & Gage, F. H. (2012). New neurons in an aged brain. *Behavioural Brain Research*, *227*(2), 497–507. https://doi.org/10.1016/j.bbr.2011.10.009

Lemon, N., & Manahan-Vaughan, D. (2006). Dopamine D1/D5 receptors gate the acquisition of novel information through hippocampal long-term potentiation and long-term depression. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *26*(29), 7723–9. https://doi.org/10.1523/JNEUROSCI.1454-06.2006

Lemon, N., & Manahan-Vaughan, D. (2012). Dopamine D1/D5 receptors contribute to de novo hippocampal LTD mediated by novel spatial exploration or locus coeruleus activity. *Cerebral Cortex*, *22*(9), 2131–2138. https://doi.org/10.1093/cercor/bhr297

Leng, G., & Ludwig, M. (2008). Neurotransmitters and peptides: whispered secrets and public announcements. *The Journal of Physiology*, *586*(Pt 23), 5625–5632. https://doi.org/10.1113/jphysiol.2008.159103

Lores-Arnaiz, S., Bustamante, J., Arismendi, M., Vilas, S., Paglia, N., Basso, N., … Lores Arnaiz, M. R. (2006). Extensive enriched environments protect old rats from the aging dependent impairment of spatial cognition, synaptic plasticity and nitric oxide production. *Behavioural Brain Research*, *169*(2), 294–302. https://doi.org/10.1016/j.bbr.2006.01.016

Lores-Arnaiz, S., D'Amico, G., Paglia, N., Arismendi, M., Basso, N., & Arnaiz, M. D. R. L.

(2004). Enriched environment, nitric oxide production and synaptic plasticity prevent the aging-dependent impairment of spatial cognition. *Molecular Aspects of Medicine*, *25*(1–2), 91–101. https://doi.org/10.1016/j.mam.2004.02.011

Lu, C. C., Cao, X. J., Wright, S., Ma, L., Oertel, D., & Goodrich, L. V. (2014). Mutation of Npr2 Leads to Blurred Tonotopic Organization of Central Auditory Circuits in Mice. *PLoS Genetics*, *10*(12). https://doi.org/10.1371/journal.pgen.1004823

Lucassen, P. J., Ravid, R., Gonatas, N. K., & Swaab, D. F. (1993). Activation of the human supraoptic and paraventricular nucleus neurons with aging and in Alzheimer's disease as judged from increasing size of the Golgi apparatus. *Brain Res.*, *632*(0006-8993), 105–113.

Ma, J., Yu, W., Wang, Y., Cao, G., Cai, S., Chen, X., … Pang, I.-H. (2010). Neuroprotective effects of C-type natriuretic peptide on rat retinal ganglion cells. *Investigative Ophthalmology and Visual Science*, *51*(7), 3544–3553. https://doi.org/10.1167/iovs.09-5049

Maggi, R., Zasso, J., & Conti, L. (2015). Neurodevelopmental origin and adult neurogenesis of the neuroendocrine hypothalamus. *Frontiers in Cellular Neuroscience*, *8*(January), 1–7. https://doi.org/10.3389/fncel.2014.00440

Mahinrad, S., de Craen, A. J. M., Yasar, S., van Heemst, D., & Sabayan, B. (2016). Natriuretic peptides in the central nervous system: Novel targets for cognitive impairment. *Neuroscience and Biobehavioral Reviews*, *68*, 148–156. https://doi.org/10.1016/j.neubiorev.2016.05.022

Manahan-Vaughan, D., & Braunewell, K.-H. (1999). Novelty acquisition is associated with induction of hippocampal long-term depression. *Proceedings of the National Academy of Sciences*, *96*(15), 8739–8744. https://doi.org/10.1073/pnas.96.15.8739

Martínez, J. C., Cardenas, F., Lamprea, M., & Morato, S. (2002). The role of vision and proprioception in the aversion of rats to the open arms of an elevated plus-maze. *Behavioural Processes*, *60*(1), 15–26. https://doi.org/10.1016/S0376-6357(02)00102-X

Mcneill, B. A., Barrell, G. K., Wellby, M., Prickett, T. C. R., Yandle, T. G., & Espiner, E. A. (2009). C-Type Natriuretic Peptide Forms in Pregnancy : Maternal Plasma Profiles during Ovine Gestation Correlate with Placental and Fetal Maturation, *150*(October), 4777–4783. https://doi.org/10.1210/en.2009-0176

Medvedev, A., Sandler, M., & Glover, V. (1999). The influence of isatin on guanylyl cyclase of rat heart membranes. *European Journal of Pharmacology*, *384*(2–3), 239–241. https://doi.org/10.1016/S0014-2999(99)00688-3

Middendorff, R., Maronde, E., Paust, H., Muller, D., Davidoff, M., & Olcese, J. (1996). Expression of C-Type Natriuretic Peptide in the Bovine Pineal Gland.

Middendorff, R., Muller, D., Paust, H. J., Davidoff, M. S., & Mukhopadhyay, A. K. (1996). Natriuretic Peptides in the Human Testis: Evidence for a Potential Role of C-Type Natriuretic Peptide in Leydig Cells. *Journal of Clinical Endocrinology and Metabolism*, *81*(12), 4324–4328.

Miners, J. S., Baig, S., Tayler, H., Kehoe, P. G., & Love, S. (2009). Neprilysin and insulin-degrading enzyme levels are increased in Alzheimer disease in relation to disease severity. *Journal of Neuropathology & Experimental Neurology*, *68*(8), 902–914.

Mitchell, A. S., & Dalrymple-alford, J. C. (2005). Dissociable memory effects after medial thalamus lesions in the rat, *22*, 973–985. https://doi.org/10.1111/j.1460-9568.2005.04199.x

Mizoguchi, K., Ikeda, R., Shoji, H., Tanaka, Y., Maruyama, W., & Tabira, T. (2009). AGING ATTENUATES GLUCOCORTICOID NEGATIVE FEEDBACK IN RAT BRAIN. *NSC*, *159*(1), 259–270. https://doi.org/10.1016/j.neuroscience.2008.12.020

Moffatt, P., Thomas, G., Sellin, K., Bessette, M.-C., Lafreniere, F., Akhouayri, O., … Lanctot, C. (2007). Osteocrin Is a Specific Ligand of the Natriuretic Peptide Clearance Receptor That Modulates Bone Growth. *The Journal of Biological Chemistry*, *282*(50), 36454–36462. https://doi.org/10.1074/jbc.M708596200

Moghtadaei, M., Langille, E., Rafferty, S. A., Bogachev, O., & Rose, R. A. (2017). Altered heart rate regulation by the autonomic nervous system in mice lacking natriuretic peptide receptor C (NPR-C). *Scientific Reports*, *7*(1), 1–13. https://doi.org/10.1038/s41598-017-17690-7

Mohammed, A. H., Zhu, S. W., Darmopil, S., Hjerling-Leffler, J., Ernfors, P., Winblad, B., … Bogdanovic, N. (2002). Environmental enrichment and the brain. *Progress in Brain Research*, *138*, 109–133.

Moncek, F., Duncko, R., Johansson, B. B., & Jezova, D. (2004). Effect of Environmental Enrichment on Stress Related Systems in Rats, *16*(22), 423–431.

Montkowski, a, Jahn, H., Ströhle, a, Poettig, M., Holsboer, F., & Wiedemann, K. (1998). C-type natriuretic peptide exerts effects opposing those of atrial natriuretic peptide on anxiety-related behaviour in rats. *Brain Research*, *792*(2), 358–60. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9593994

Mora, F. (2013). Successful brain aging: plasticity, environmental enrichment, and lifestyle. *Dialogues in Clinical Neuroscience*, *15*(1), 45–52. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3622468&tool=pmcentrez&rendertype=abstract

Mora, F., Segovia, G., & del Arco, A. (2007). Aging, plasticity and environmental enrichment: Structural changes and neurotransmitter dynamics in several areas of the brain. *Brain Research Reviews*, *55*(1), 78–88. https://doi.org/10.1016/j.brainresrev.2007.03.011

Moran, J. P., & Dalrymple-Alford, J. C. (2003). Perirhinal Cortex and Anterior Thalamic Lesions: Comparative Effects on Learning and Memory. *Behavioral Neuroscience*, *117*(6), 1326–1341. https://doi.org/10.1037/0735-7044.117.6.1326

Morellini, F. (2013). Spatial memory tasks in rodents: What do they model? *Cell and Tissue Research*, *354*(1), 273–286. https://doi.org/10.1007/s00441-013-1668-9

Morris, R. G. (2001). Episodic-like memory in animals: psychological criteria, neural mechanisms and the value of episodic-like tasks to investigate animal models of neurodegenerative disease. *Philosophical Transactions of the Royal Society of London Series B*, *356*(1413), 1453–1465. https://doi.org/10.1098/rstb.2001.0945

Müller, D., Hida, B., Guidone, G., Speth, R. C., Michurina, T. V., Enikolopov, G., & Middendorff, R. (2009). Expression of guanylyl cyclase (GC)-A and GC-B during brain development: Evidence for a role of GC-B in perinatal neurogenesis. *Endocrinology*,

150(12), 5520–5529. https://doi.org/10.1210/en.2009-0490

Muller, D., Schulze, C., Baumeister, H., Buck, F., & Richter, D. (1992). Rat insulin-degrading enzyme: Cleavage pattern of the natriuretic peptide hormones ANP, BNP, and CNP revealed by HPLC and mass spectrometry. *Biochemistry*, *31*(45), 11138–11143. https://doi.org/10.1021/bi00160a026

Neave, N., Nagle, S., & Aggleton, J. P. (1997). Evidence for the Involvement of the Mammillarv Bodies and Cingulum Bundle in Allocentric Spatial Pricessing by Rats. *European Journal of Neuroscience*, *9*.

Nestor, P. J., Fryer, T. D., Ikeda, M., & Hodges, J. R. (2003). Retrosplenial cortex (BA 29/30) hypometabolism in mild cognitive impairment (prodromal Alzheimer's disease). *European Journal of Neuroscience*, *18*(9), 2663–2667. https://doi.org/10.1046/j.1460-9568.2003.02999.x

Nithianantharajah, J., & Hannan, A. J. (2006). Enriched environments, experience-dependent plasticity and disorders of the nervous system. *Nature Reviews. Neuroscience*, *7*(September), 697–709. https://doi.org/10.1038/nrn1970

Pan, J.-T., Lai, C.-J., & Yen, S.-H. (1996). Effects of natriuretic peptides and dopamine on single-unit activity of dorsomedial arcuate neurons in rat brain slices. *Brain Research*, *737*, 78–82.

Pandey, K. N. (2005). Biology of natriuretic peptides and their receptors. *Peptides*, *26*(6 SPEC. ISS.), 901–932. https://doi.org/10.1016/j.peptides.2004.09.024

Pankow, K., Wang, Y., Gembardt, F., Krause, E., Sun, X., Krause, G., … Walther, T. (2007). Successive action of meprin A and neprilysin catabolizes B-type natriuretic peptide. *Circulation Research*, *101*(9), 875–882. https://doi.org/10.1161/CIRCRESAHA.107.153585

Paul, C.-M., Magda, G., & Abel, S. (2009). Spatial memory: Theoretical basis and comparative review on experimental methods in rodents. *Behavioural Brain Research*, *203*(2), 151–164. https://doi.org/10.1016/j.bbr.2009.05.022

Pemberton, C. J., Yandle, T. G., & Espiner, E. A. (2002). Immunoreactive forms of natriuretic peptides in ovine brain : response to heart failure, *23*, 2235–2244.

Phelps, E. A., & LeDoux, J. E. (2005). Contributions of the amygdala to emotion processing: From animal models to human behavior. *Neuron*, *48*(2), 175–187. https://doi.org/10.1016/j.neuron.2005.09.025

Poirier, H., Labrecque, J., Deschenes, J., & DeLean, A. (2002). Allotopic antagonism of the non-peptide atrial natriuretic peptide (ANP) antagonist HS-142-1 on natriuretic peptide receptor NPR-A. *Biochemical Journal*, *362*, 231–237.

Pothuizen, H. H. J., Davies, M., Aggleton, J. P., & Vann, S. D. (2010). Effects of selective granular retrosplenial cortex lesions on spatial working memory in rats. *Behavioural Brain Research*, *208*(2), 566–575. https://doi.org/10.1016/j.bbr.2010.01.001

Pothuizen, H. H. J., Davies, M., Albasser, M. M., Aggleton, J. P., & Vann, S. D. (2009). Granular and dysgranular retrosplenial cortices provide qualitatively different contributions to spatial working memory: Evidence from immediate-early gene imaging in rats. *European Journal of Neuroscience*, *30*(5), 877–888. https://doi.org/10.1111/j.1460-9568.2009.06881.x

Potter, L. R. (2011). Natriuretic peptide metabolism, clearance and degradation. *FEBS Journal*, *278*(11), 1808–1817. https://doi.org/10.1111/j.1742-4658.2011.08082.x

Potter, L. R., Abbey-Hosch, S., & Dickey, D. M. (2006). Natriuretic Peptides, Their Receptors, and Cyclic Guanosine Monophosphate-Dependent Signaling Functions. *Endocrine Reviews*, *27*(1), 47–72. https://doi.org/10.1210/er.2005-0014

Prado, J., Baltrons, M. A., Pifarré, P., & García, A. (2010). Glial cells as sources and targets of natriuretic peptides. *Neurochemistry International*, *57*(4), 367–374. https://doi.org/10.1016/j.neuint.2010.03.004

Prickett, T. C. R., Barrell, G. K., Wellby, M., Yandle, T. G., Richards, A. M., & Espiner, E. A. (2007). Response of plasma CNP forms to acute anabolic and catabolic interventions in growing lambs. *American Journal of Physiology. Endocrinology and Metabolism*, *292*(5), E1395-400. https://doi.org/10.1152/ajpendo.00469.2006

Prickett, T. C. R., Barrell, G. K., Wellby, M., Yandle, T. G., Richards, A. M., & Espiner, E. A. (2008). Effect of sex steroids on plasma C-type natriuretic peptide forms: Stimulation by oestradiol in lambs and adult sheep. *Journal of Endocrinology*, *199*(3), 481–487. https://doi.org/10.1677/JOE-08-0267

Prickett, T. C. R., Bothwell, J. C., Yandle, T. G., Richards, A. M., & Espiner, E. A. (2012). Pharmacodynamic responses of plasma and tissue C-type natriuretic peptide to GH: Correlation with linear growth in GH-deficient rats. *Journal of Endocrinology*, *212*(2), 217–225. https://doi.org/10.1530/JOE-11-0387

Prickett, T. C. R., Dixon, B., Frampton, C., Yandle, T. G., Richards, A. M., Espiner, E. A., & Darlow, B. A. (2008). Plasma amino-terminal pro C-type natriuretic peptide in the neonate: Relation to gestational age and postnatal linear growth. *Journal of Clinical Endocrinology and Metabolism*, *93*(1), 225–232. https://doi.org/10.1210/jc.2007-1815

Prickett, T. C. R., & Espiner, E. A. (2012). C-Type Natriuretic Peptide (CNP) and Postnatal Linear Growth. In V. R. Preedy (Ed.), *Handbook of Growth and Growth Monitoring in Health and Disease* (pp. 2789–2809). Springer. https://doi.org/10.1007/978-1-4419-1795-9

Prickett, T. C. R., Kaaja, R. J., Nicholls, M. G., Espiner, E. A., Richards, A. M., & Yandle, T. G. (2004). N-terminal pro-C-type natriuretic peptide, but not C-type natriuretic peptide, is greatly elevated in the fetal circulation. *Clinical Science*, *106*(5), 535–40. https://doi.org/10.1042/CS20030307

Prickett, T. C. R., Lynn, A. M., Barrell, G. K., Darlow, B. A., Cameron, V. A., Espiner, E. A., … Yandle, T. G. (2005). Amino-Terminal proCNP : A Putative Marker of Cartilage Activity in Postnatal Growth, *58*(2), 334–340. https://doi.org/10.1203/01.PDR.0000169964.66260.4B

Prickett, T. C. R., Ryan, J. F., Wellby, M., Barrell, G. K., Yandle, T. G., Richards, A. M., & Espiner, E. A. (2010). Effect of nutrition on plasma C-type natriuretic peptide forms in adult sheep: evidence for enhanced C-type natriuretic peptide degradation during caloric restriction. *Metabolism: Clinical and Experimental*, *59*(6), 796–801. https://doi.org/10.1016/j.metabol.2009.09.026

Prickett, T. C. R., Yandle, T. G., Nicholls, M. G., Espiner, E. A., & Richards, A. M. (2001). Identification of Amino-Terminal Pro-C-Type Natriuretic Peptide in Human Plasma. *Biochemical and Biophysical Research Communications*, *286*(3), 513–517.

https://doi.org/10.1006/bbrc.2001.5419

Qian, J. Y., Haruno, A., Asada, Y., Nishida, T., Saito, Y., Matsuda, T., & Ueno, H. (2002). Local expression of C-type natriuretic peptide suppresses inflammation, eliminates shear stress-induced thrombosis, and prevents neointima formation through enhanced nitric oxide production in rabbit injured carotid arteries. *Circulation Research*, *91*(11), 1063–1069. https://doi.org/10.1161/01.RES.0000043631.25915.E6

Rao, M. S., Hattiangady, B., Abdel-Rahman, A., Stanley, D. P., & Shetty, A. K. (2005). Newly born cells in the ageing dentate gyrus display normal migration, survival and neuronal fate choice but endure retarded early maturation. *European Journal of Neuroscience*, *21*(2), 464–476. https://doi.org/10.1111/j.1460-9568.2005.03853.x

Rapley, S. A., Prickett, T. C. R., Dalrymple-Alford, J. C., & Espiner, E. A. (2018). Environmental Enrichment Elicits a Transient Rise of Bioactive C-Type Natriuretic Peptide in Young but Not Aged Rats. *Frontiers in Behavioral Neuroscience*, *12*(July), 1–12. https://doi.org/10.3389/fnbeh.2018.00142

Roberts, D. E., Killiany, R. J., & Rosene, D. L. (2012). Neuron numbers in the hypothalamus of the normal aging rhesus monkey: stability across the adult lifespan and between the sexes. *Journal of Comparative Neurology*, *520*(6), 1181–1197. https://doi.org/10.1002/cne.22761.Neuron

Robinson, J. W., Lou, X., & Potter, L. R. (2011). The indolocarbazole, Gö6976, inhibits guanylyl cyclase-A and -B. *British Journal of Pharmacology*, *164*(2b), 499–506. https://doi.org/10.1111/j.1476-5381.2011.01291.x

Robinson, S., Adelman, J. S., Mogul, A. S., Ihle, P. C. J., & Davino, G. M. (2018). Neurobiology of Learning and Memory Putting fear in context : Elucidating the role of the retrosplenial cortex in context discrimination in rats ☆. *Neurobiology of Learning and Memory*, *148*(September 2017), 50–59. https://doi.org/10.1016/j.nlm.2017.12.009

Romieu, P., Gobaille, S., Aunis, D., & Zwiller, J. (2008). Injection of the neuropeptide CNP into dopaminergic rat brain areas decreases alcohol intake. *Annals of the New York Academy of Sciences*, *1139*, 27–33. https://doi.org/10.1196/annals.1432.050

Rose, R. A., Anand-srivastava, M. B., Giles, W. R., Bains, J. S., Robert, A., Anand-srivastava, M. B., … Ca, L. (2005). C-type Natriuretic Peptide Inhibits L-type Ca 2 ϑ Current in Rat Magnocellular Neurosecretory Cells by Activating the NPR-C Receptor, 612–621. https://doi.org/10.1152/jn.00057.2005.

Rosenzweig, E. S., & Barnes, C. A. (2003). *Impact of aging on hippocampal function: Plasticity, network dynamics, and cognition*. *Progress in Neurobiology* (Vol. 69). https://doi.org/10.1016/S0301-0082(02)00126-0

Rosenzweig, M. R., & Bennett, E. L. (1996). Psychobiology of plasticity: Effects of training and experience on brain and behavior. *Behavioural Brain Research*, *78*(1), 57–65. https://doi.org/10.1016/0166-4328(95)00216-2

Rosenzweig, M. R., KRECH, D., Bennett, E. L., Diamond, M. C., Rosenzweig, M. R., Bennett, E. L., … Diamond, M. C. (1962). Effects of environmental complexity and training on brain chemistry and anatomy: a replication and extension. *Journal of Comparative and Physiological Psychology*, *55*(4), 429–437. https://doi.org/10.1037/h0045402

Russo, R., Borghi, R., Markesbery, W., Tabaton, M., & Piccini, A. (2005). Neprylisin decreases uniformly in Alzheimer's disease and in normal aging. *FEBS Letters*, *579*(27), 6027–6030. https://doi.org/10.1016/j.febslet.2005.09.054

Ryan, M. C., & Gundlach, A. L. (1998). Ontogenic expression of natriuretic peptide mRNAs in postnatal rat brain : Implications for development ?

Sabayan, B., Buchem, M. A. Van, Craen, A. J. M. De, Harris, T. B., & Launer, L. J. (2015). N-terminal pro – brain natriuretic peptide and abnormal brain aging.

Sabbatini, M. E. (2009). Natriuretic peptides as regulatory mediators of secretory activity in the digestive system. *Regulatory Peptides*, *154*, 5–15. https://doi.org/10.1016/j.regpep.2009.02.009

Sahay, A., Wilson, D. A., & Hen, R. (2011). Pattern Separation: A Common Function for New Neurons in Hippocampus and Olfactory Bulb. *Neuron*, *70*(4), 582–588. https://doi.org/10.1016/j.neuron.2011.05.012

Sakaki, M., Yoo, H. J., Nga, L., Lee, T.-H., Thayer, J. F., & Mather, M. (2016). Heart rate variability is associated with amygdala functional connectivity with MPFC across younger and older adults. *NeuroImage*, *139*, 44–52. https://doi.org/10.1016/j.neuroimage.2016.05.076

Sampedro-Piquero, P., Arias, J. L., & Begega, A. (2014). Behavioral testing-related changes in the expression of Synapsin I and glucocorticoid receptors in standard and enriched aged Wistar rats. *Experimental Gerontology*, *58*, 292–302. https://doi.org/10.1016/j.exger.2014.09.004

Sampedro-Piquero, P., Begega, A., & Arias, J. L. (2014). Increase of glucocorticoid receptor expression after environmental enrichment: Relations to spatial memory, exploration and anxiety-related behaviors. *Physiology and Behavior*, *129*, 118–129. https://doi.org/10.1016/j.physbeh.2014.02.048

Sampedro-Piquero, P., De Bartolo, P., Petrosini, L., Zancada-Menendez, C., Arias, J. L., & Begega, A. (2014). Astrocytic plasticity as a possible mediator of the cognitive improvements after environmental enrichment in aged rats. *Neurobiology of Learning and Memory*, *114*, 16–25. https://doi.org/10.1016/j.nlm.2014.04.002

Samson, W. K., Huang, F. S., & Fulton, R. J. (1995). Opposing Neuroendocrine Actions of the Natriuretic Peptides: C-Type and A-Type Natriuretic Peptides do not Interact with the same Hypothalamic Cells Controlling Prolactin Secretion, *7*, 759–763.

Samson, W. K., Skala, K. D., & Huang, F.-L. S. (1991). CNP-22 stimulates, rather than inhibits, water drinking in the rat: evidence for a unique biological action of the C-type natriuretic peptides. *Brain Research*, *568*, 285–288.

Sangaralingham, S. J., Heublein, D. M., Grande, J. P., Cataliotti, A., Rule, A. D., McKie, P. M., … Burnett, J. C. (2011). Urinary C-type natriuretic peptide excretion: a potential novel biomarker for renal fibrosis during aging. *American Journal of Physiology. Renal Physiology*, *301*(5), F943-52. https://doi.org/10.1152/ajprenal.00170.2011

Santín, L. J., Rubio, S., Begega, A., & Arias, J. L. (2000). Effects of chronic alcohol consumption on spatial reference and working memory tasks, *20*, 149–159.

Schmidt, H., Stonkute, A., Jüttner, R., Koesling, D., Friebe, A., & Rathjen, F. G. (2009). C-type natriuretic peptide (CNP) is a bifurcation factor for sensory neurons. *Proceedings*

of the National Academy of Sciences of the United States of America, *106*(39), 16847–16852. https://doi.org/10.1073/pnas.0906571106

Schmidt, H., Stonkute, A., Jüttner, R., Schäffer, S., Buttgereit, J., Feil, R., … Rathjen, F. G. (2007). The receptor guanylyl cyclase Npr2 is essential for sensory axon bifurcation within the spinal cord. *Journal of Cell Biology*, *179*(2), 331–340. https://doi.org/10.1083/jcb.200707176

Schouten, B. J., Prickett, T. C. R., Hooper, A. A., Hooper, G. J., Yandle, T. G., Richards, A. M., & Espiner, E. A. (2011). Central and peripheral forms of C-type natriuretic peptide (CNP): Evidence for differential regulation in plasma and cerebrospinal fluid. *Peptides*, *32*(4), 797–804. https://doi.org/10.1016/j.peptides.2011.01.013

Scotland, R. S., Ahluwalia, A., & Hobbs, A. J. (2005). C-type natriuretic peptide in vascular physiology and disease. *Pharmacology and Therapeutics*, *105*(2), 85–93. https://doi.org/10.1016/j.pharmthera.2004.08.011

Segovia, G., Del Arco, A., De Blas, M., Garrido, P., & Mora, F. (2008). Effects of an enriched environment on the release of dopamine in the prefrontal cortex produced by stress and on working memory during aging in the awake rat, *187*, 304–311. https://doi.org/10.1016/j.bbr.2007.09.024

Segovia, G., Del Arco, A., Garrido, P., de Blas, M., & Mora, F. (2008). Environmental enrichment reduces the response to stress of the cholinergic system in the prefrontal cortex during aging. *Neurochemistry International*, *52*(6), 1198–1203. https://doi.org/10.1016/j.neuint.2007.12.007

Segovia, G., Del Arco, A., & Mora, F. (2009). Environmental enrichment, prefrontal cortex, stress, and aging of the brain. *Journal of Neural Transmission*, *116*(8), 1007–1016. https://doi.org/10.1007/s00702-009-0214-0

Segovia, G., Yag, A. G., & Mora, F. (2006). Environmental enrichment promotes neurogenesis and changes the extracellular concentrations of glutamate and GABA in the hippocampus of aged rats, *70*, 8–14. https://doi.org/10.1016/j.brainresbull.2005.11.005

Sellitti, D. F., Koles, N., & Mendona, M. C. (2011). Regulation of C-type natriuretic peptide expression. *Peptides*, *32*(9), 1964–1971. https://doi.org/10.1016/j.peptides.2011.07.013

Seoane, A., Tinsley, C. J., & Brown, M. W. (2012). Interfering with Fos expression in rat perirhinal cortex impairs recognition memory. *Hippocampus*, *22*(11), 2101–2113. https://doi.org/10.1002/hipo.22028

Shimekake, Y., Ohta, S., & Nagata, K. (1994). C-type natriuretic peptide stimulates secretion of growth hormone from rat-pituitary-derived GH3 cells via a cyclic-GMP mediated pathway. *European Journal of Biochemistry*, *222*, 645–650.

Simonnet, G., Allard, M., Legendre, P., Gabriont, J., Vincent, J. D., Neurobiologie, L. De, … Ua, C. (1989). CHARACTERISTICS AND SPECIFIC LOCALIZATION OF RECEPTORS FOR ATRIAL NATRIURETIC PEPTIDES AT NON-NEURONAL CELLS IN CULTURED MOUSE SPINAL CORD CELLS, *29*, 189–199.

Simpson, J., & Kelly, J. P. (2011). The impact of environmental enrichment in laboratory rats—Behavioural and neurochemical aspects. *Behavioural Brain Research*, *222*(1), 246–264. https://doi.org/10.1016/j.bbr.2011.04.002

Simpson, P. J., Miller, I., Moon, C., Hanlon, A. L., Liebl, D. J., & Ronnett, G. V. (2002). Atrial natriuretic peptide type C induces a cell-cycle switch from proliferation to differentiation in brain-derived neurotrophic factor- or nerve growth factor-primed olfactory receptor neurons. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *22*(13), 5536–51. https://doi.org/20026586

Smith, M. W., Espiner, E. A., Yandle, T. G., Charles, C. J., & Richards, a M. (2000). Delayed metabolism of human brain natriuretic peptide reflects resistance to neutral endopeptidase. *The Journal of Endocrinology*, *167*(2), 239–46. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11054637

Soffie, M., Hahn, K., Terao, E., & Eclancher, F. (1999). Behavioural and glial changes in old rats following environmental enrichment. *Behavioural Brain Research*, *101*, 37–49.

Speisman, R. B., Kumar, A., Rani, A., Pastoriza, J. M., Severance, J. E., Foster, T. C., & Ormerod, B. K. (2013). Environmental enrichment restores neurogenesis and rapid acquisition in aged rats. *NBA*, *34*(1), 263–274. https://doi.org/10.1016/j.neurobiolaging.2012.05.023

Stranahan, A. M., & Mattson, M. P. (2011). Bidirectional metabolic regulation of neurocognitive function. *Neurobiology of Learning and Memory*, *96*(4), 507–516. https://doi.org/10.1016/j.nlm.2011.01.004

Suárez, M. M., Rivarola, M. A., Molina, S. M., Levin, G. M., Enders, J., & Paglini, P. (2004). The role of the anterodorsal thalami nuclei in the regulation of adrenal medullary function, beta-adrenergic cardiac receptors and anxiety responses in maternally deprived rats under stressful conditions. *Stress*, *7*(3), 195–203. https://doi.org/10.1080/10253890400010705

Sudoh, T., Minamino, N., Kangawa, K., & Matsuo, H. (1990). C-Type natriuretic peptide (CNP): A new member of natriuretic peptide family identified in porcine brain. *Biochemical and Biophysical Research Communications*, *168*(2), 863–870. https://doi.org/10.1016/0006-291X(90)92401-K

Sumners, C., & Tang, W. (1992). Atrial natriuretic peptide receptor subtypes in rat neuronal and astrocyte glial cultures. *American Journal of Physiology - Cell Physiology*, *262*(5). Retrieved from http://ajpcell.physiology.org/content/262/5/C1134.short

Sutherland, R. J., & Rodriguez, A. J. (1989). The role of the fornix/fimbria and some related subcortical structures in place learning and memory. *Behavioural Brain Research*, *32*(3), 265–277. https://doi.org/10.1016/S0166-4328(89)80059-2

Swaab, D. F., & Bao, A. (2011). (Re-) activation of neurons in aging and dementia : Lessons from the hypothalamus. *EXG*, *46*(2–3), 178–184. https://doi.org/10.1016/j.exger.2010.08.028

Swaab, D. F., Goudsmit, E., Kremer, H. P. H., Hofman, M. A., & Ravid, R. (1992). The human hypothalamus in development, sexual differentiation, aging and Alzheimer's disease. *Progress in Brain Reseach*.

Sziklas, V., & Petrides, M. (1993). Memory Impairments Following Lesions to the Mammillary Region of the Rat. *European Journal of Neuroscience*, *5*(5), 525–540. https://doi.org/10.1111/j.1460-9568.1993.tb00518.x

Sziklas, V., & Petrides, M. (1998). Memory and the region of the mammillary bodies.

Progress in Neurobiology, *54*(1), 55–70. https://doi.org/10.1016/S0301-0082(97)00064-6

Tamura, N., Doolittle, L. K., Hammer, R. E., Shelton, J. M., Richardson, J. A., & Garbers, D. L. (2004). Critical roles of the guanylyl cyclase B receptor in endochondral ossification and development of female reproductive organs. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(49), 17300–17305. https://doi.org/10.1073/pnas.0407894101

Tawaragi, Y., Fuchimura, K., Nakazato, H., Tanaka, S., Minamino, N., Kangawa, K., & Matsuo, H. (1990). Gene and precursor structure of porcine C-type natriuretic peptide. *Biochemical and Biophysical Research Communications*, *172*(2), 627–632. https://doi.org/10.1016/0006-291X(90)90720-8

Telegdy, G., Adamik, a, & Glover, V. (2000). The action of isatin (2,3-dioxoindole) an endogenous indole on brain natriuretic and C-type natriuretic peptide-induced facilitation of memory consolidation in passive-avoidance learning in rats. *Brain Research Bulletin*, *53*(3), 367–70. https://doi.org/S0361-9230(00)00359-2 [pii]

Telegdy, G., Kokavszky, K., & Nyerges, A. (1999). Action of C-type natriuretic peptide (CNP) on passive avoidance learning in rats: involvement of transmitters. *The European Journal of Neuroscience*, *11*(May), 3302–3306. https://doi.org/10.1046/j.1460-9568.1999.00750.x

Thayer, J. F., & Lane, R. D. (2000). A model of neurovisceral integration in ER and dysregulation. *Journal of Affective Disorders*, *61*(201–216), 201–216.

Thiriet, N., Jouvert, P., Gobaille, S., Solov'eva, O., Gough, B., Aunis, D., … Zwiller, J. (2001). C-type natriuretic peptide (CNP) regulates cocaine-induced dopamine increase and immediate early gene expression in rat brain. *European Journal of Neuroscience*, *14*, 1702–1708. https://doi.org/10.1046/j.0953-816X.2001.01791.x

Tischmeyer, W., & Grimm, R. (1999). Activation of immediate early genes and memory formation, *55*, 564–574.

Toshio, I., Hiroshi, I., Suga, S.-I., Norio, H., Ogawa, Y., Komatsu, Y., … Sugawara, A. (1996). C-type natriuretic peptide in chronic renal failure and its action in humans. In *Kidney International Supplement* (pp. pS144-S-147). Retrieved from http://web.b.ebscohost.com/abstract?direct=true&profile=ehost&scope=site&authtype=crawler&jrnl=00986577&AN=48499935&h=iDWAivGphgRFcQ1ifxTL7%2BVUKCGnHaDh7nJJe0UBtC1EUwyOHEIHv6sehtwGwBkmnNAT9oJqMBC2QlCd2kanSg%3D%3D&crl=c&resultNs=AdminWebAuth&resultLocal=Er

Totsune, K., Takahashi, K., Ohneda, M., Itoi, K., Murakami, O., & Mouri, T. (1994). C-type natriuretic peptide in the human central nervous system: distribution and molecular form. *Peptides*, *15*(1), 37–40. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8015979

Troy Harker, K., & Whishaw, I. Q. (2004). A reaffirmation of the retrosplenial contribution to rodent navigation: reviewing the influences of lesion, strain, and task. *Neuroscience & Biobehavioral Reviews*, *28*(5), 485–496. https://doi.org/10.1016/j.neubiorev.2004.06.005

Van Groen, T., Kadish, I., & Wyss, J. M. (2004). Retrosplenial cortex lesions of area Rgb (but not of area Rga) impair spatial learning and memory in the rat. *Behavioural Brain*

Research, *154*(2), 483–491. https://doi.org/10.1016/j.bbr.2004.03.016

van Praag, H., Kempermann, G., & Gage, F. H. (2000). Neural consequences of environmental enrichment. *Nature Reviews. Neuroscience*, *1*(December), 191–198. https://doi.org/10.1038/35044558

Vann, S. D. (2010). Re-evaluating the role of the mammillary bodies in memory. *Neuropsychologia*, *48*(8), 2316–2327. https://doi.org/10.1016/j.neuropsychologia.2009.10.019

Vann, S. D., & Aggleton, J. P. (2003). Evidence of a spatial encoding deficit in rats with lesions of the mammillary bodies or mammillothalamic tract. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *23*(8), 3506–3514. https://doi.org/23/8/3506 [pii]

Vann, S. D., & Aggleton, J. P. (2004). The mammillary bodies: two memory systems in one? *Nature Reviews. Neuroscience*, *5*(1), 35–44. https://doi.org/10.1038/nrn1299

Vann, S. D., & Aggleton, J. P. (2005). Selective dysgranular retrosplenial cortex lesions in rats disrupt allocentric performance of the radial-arm maze task. *Behavioral Neuroscience*, *119*(6), 1682–1686. https://doi.org/10.1037/0735-7044.119.6.1682

Vann, S. D., Aggleton, J. P., & Maguire, E. A. (2009). What does the retrosplenial cortex do? *Nature Reviews Neuroscience*, *10*(11), 792–802. https://doi.org/10.1038/nrn2733

Vann, S. D., & Nelson, A. J. D. (2015). The mammillary bodies and memory: More than a hippocampal relay. In *Progress in Brain Research* (Vol. 219). https://doi.org/10.1016/bs.pbr.2015.03.006

Vatta, M. S., Presas, M., Bianciotti, L. G., Zarrabeitia, V., & Fernfindez, B. E. (1996). B and C types natriuretic peptides modulate norepinephrine uptake and release in the rat hypothalamus, *65*, 175–184.

Vatta, M. S., Presas, M. F., Bianciotti, L. G., Ambros, R., & Fernandez, B. E. (1997). B and C Types Natriuretic Peptides Modify Norepinephrine Uptake and Release in the Rat Adrenal Medulla, *18*(10), 1483–1489.

Verret, L., Trouche, S., Zerwas, M., & Rampon, C. (2007). Hippocampal neurogenesis during normal and pathological aging. *Psychoneuroendocrinology*, *32*(SUPPL 1), 26–30. https://doi.org/10.1016/j.psyneuen.2007.04.014

Vertes, R. P. (2006). INTERACTIONS AMONG THE MEDIAL PREFRONTAL CORTEX , HIPPOCAMPUS AND MIDLINE THALAMUS IN EMOTIONAL AND COGNITIVE PROCESSING IN THE RAT, *142*, 1–20. https://doi.org/10.1016/j.neuroscience.2006.06.027

Vigne, P., & Frelin, C. (1992). C-type natriuretic peptide is a potent activator of guanylate cyclase in endothelial cells from brain microvessels. *Biochemical and Biophysical Research Communications*, *183*(2), 640–644. https://doi.org/10.1016/0006-291X(92)90530-X

Vollmar, A. M., Gerbes, A. L., Nemer, M., & Schulz, R. (1993). Detection of c-type natriuretic peptide (Cnp) transcript in the rat heart and immune organs. *Endocrinology*, *132*(4), 1872–1873. https://doi.org/10.1210/endo.132.4.8462485

Vorhees, C. V., & Williams, M. T. (2014). Assessing spatial learning and memory in rodents.

ILAR Journal, *55*(2), 310–332. https://doi.org/10.1093/ilar/ilu013

Walther, T., Albrecht, D., Becker, M., Schubert, M., Kouznetsova, E., Wiesner, B., … Siems, W.-E. (2009). Improved learning and memory in aged mice deficient in amyloid beta-degrading neutral endopeptidase. *PloS One*, *4*(2), e4590. https://doi.org/10.1371/journal.pone.0004590

Walther, T., Heringer-Walther, S., Tschöpe, R., Reinecke, A., Schultheiss, H. P., & Tschöpe, C. (2000). Opposite regulation of brain and C-type natriuretic peptides in the streptozotocin-diabetic cardiopathy. *Journal of Molecular Endocrinology*, *24*(3), 391–395.

Wang, X., & Robinson, P. J. (1997). Cyclic GMP-dependent protein kinase and cellular signaling in the nervous system. *Journal of Neurochemistry*, *68*(2), 443–456. https://doi.org/10.1046/j.1471-4159.1997.68020443.x

Warburton, E. C., Baird, A. L., Morgan, A., Muir, J. L., & Aggleton, J. P. (2000). Disconnecting hippocampal projections to the anterior thalamus produces deficits on tests of spatial memory in rats. *European Journal of Neuroscience*, *12*(5), 1714–1726. https://doi.org/10.1046/j.1460-9568.2000.00039.x

Watanabe, Y., Nakajima, K., Shimamori, Y., & Fujimoto, Y. (1997). Comparison of the hydrolysis of the three types of natriuretic peptides by human kidney neutral endopeptidase 24.11. *Biochemical and Molecular Medicine*, *61*(1), 47–51. https://doi.org/10.1006/bmme.1997.2584

Wiedemann, K., Jahn, H., & Kellner, M. (2000). Effects of natriuretic peptides upon hypothalamo-pituitary-adrenocortical system activity and anxiety behaviour. *Experimental and Clinical Endocrinology and Diabetes*, *108*, 5–13.

Wiggins, A. K., Shen, P. J., & Gundlach, A. L. (2003). Atrial natriuretic peptide expression is increased in rat cerebral cortex following spreading depression: Possible contribution to SD-induced neuroprotection. *Neuroscience*, *118*(3), 715–726. https://doi.org/10.1016/S0306-4522(03)00006-X

Wilcox, R. R., & Keselman, H. J. (2003). Modern Robust Data Analysis Methods: Measures of Central Tendency. *Psychological Methods*, *8*(3), 254–274. https://doi.org/10.1037/1082-989X.8.3.254

Will, B., Galani, R., Kelche, C., & Rosenzweig, M. R. (2004). Recovery from brain injury in animals: Relative efficacy of environmental enrichment, physical exercise or formal training (1990-2002). *Progress in Neurobiology*, *72*(3), 167–182. https://doi.org/10.1016/j.pneurobio.2004.03.001

Wilson, M. O., Barrell, G. K., Prickett, T. C. R., & Espiner, E. A. (2015). Sustained increases in plasma C-type natriuretic peptides fail to increase concentrations in cerebrospinal fluid: Evidence from pregnant sheep. *Peptides*, *69*, 103–108. https://doi.org/10.1016/j.peptides.2015.04.016

Wilson, M. O., Barrell, G. K., Prickett, T. C. R., & Espiner, E. A. (2018). Molecular forms of C-type natriuretic peptide in cerebrospinal fluid and plasma reflect differential processing in brain and pituitary tissues. *Peptides*, *99*(July 2017), 223–230. https://doi.org/10.1016/j.peptides.2017.10.007

Wilson, M. O., McNeill, B. A., Barrell, G. K., Prickett, T. C. R., & Espiner, E. A. (2017).

Dexamethasone increases production of C-type natriuretic peptide in the sheep brain. *The Journal of Endocrinology*, *235*(1), 15–25. https://doi.org/10.1530/JOE-17-0148

Wincott, C. M., Abera, S., Vunck, S. a., Choi, Y., Titcombe, R. F., Antoine, S. O., … Ziff, E. B. (2014). CGMP-dependent protein kinase type II knockout mice exhibit working memory impairments, decreased repetitive behavior, and increased anxiety-like traits. *Neurobiology of Learning and Memory*, *114*, 32–39. https://doi.org/10.1016/j.nlm.2014.04.007

Winocur, G. (1999). Environmental influences on cognitive decline in aged rats. *Neurobiology of Aging*, *19*(6), 589–97. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10192219

Woodward, Z., Prickett, T. C. R., Espiner, E. A., & Anderson, T. J. (2017). Central and systemic C-type Natriuretic Peptide are both reduced in Parkinson's Disease. *Parkinsonism & Related Disorders*. https://doi.org/10.1016/j.parkreldis.2017.06.019

Wu, C., Wu, F., Pan, J., Morser, J., & Wu, Q. (2003). Furin-mediated Processing of Pro-C-type Natriuretic Peptide. *The Journal of Biological Chemistry*, *278*(28), 25847–25852. https://doi.org/10.1074/jbc.M301223200

Yamada-Goto, N., Katsuura, G., Ebihara, K., Inuzuka, M., Ochi, Y., Yamashita, Y., … Nakao, K. (2013). Intracerebroventricular Administration of C-Type Natriuretic Peptide Suppresses Food Intake via Activation of the Melanocortin System in Mice. *Diabetes*, *62*(5), 1500–1504.

Yandle, T. G., Fisher, S., Charles, C., Espiner, E. A., & Richards, A. M. (1993). The Ovine Hypothalamus and Pituitary Have Markedly Different Distributions of C-Type Natriuretic Peptide Forms. *Peptides*, *14*, 713–716.

Yasojima, K., Akiyama, H., McGeer, E. G., & McGeer, P. L. (2001). Reduced neprilysin in high plaque areas of Alzheimer brain: A possible relationship to deficient degradation of ??-amyloid peptide. *Neuroscience Letters*, *297*(2), 97–100. https://doi.org/10.1016/S0304-3940(00)01675-X

Yasojima, K., McGeer, E. G., & McGeer, P. L. (2001). Relationship between β amyloid peptide generating molecules and neprilysin in Alzheimer disease and normal brain. *Brain Research*, *919*(1), 115–121.

Yassa, M. A., Lacy, J. W., Stark, S. M., Albert, M. S., Gallagher, M., & Stark, C. E. L. (2011). Pattern separation deficits associated with increased hippocampal CA3 and dentate gyrus activity in nondemented older adults. *Hippocampus*, *21*(9), 968–979. https://doi.org/10.1002/hipo.20808

Yassa, M. A., & Stark, C. E. L. (2011). Pattern separation in the hippocampus. *Trends in Neurosciences*, *34*(10), 515–525. https://doi.org/10.1016/j.tins.2011.06.006

Yeung, V. T. ., Ho, S. K. ., Nicholls, M. ., & Cockram, C. . (1996). Binding of CNP-22 and CNP-53 to Cultured Mouse , Astrocytes and Effects on Cyclic GMP. *Peptides*, *17*(I), 101–106.

Zhao, Z., & Ma, L. (2009). Regulation of axonal development by natriuretic peptide hormones. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(42), 18016–21. https://doi.org/10.1073/pnas.0906880106

Zhu, B., & Herbert, J. (1996). Central antagonism of atrial natriuretic peptides on behavioral

and hormonal responses to angiotensin II: mapping with c-fos. *Brain Research, 734*, 55–60.

Zhu, X. O., Brown, M. W., McCabe, B. J., & Aggleton, J. P. (1995). Effects of the novelty or familiarity of visual stimuli on the expression of the immediate early gene c-fos in rat brain. *Neuroscience, 69*(3), 821–829. https://doi.org/10.1016/0306-4522(95)00320-I