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Title:
The role of ERK and p38 MAPK signalling in visual recognition memory

The role of ERK and p38 MAPK signalling in visual recognition memory

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Abstract

Recognition memory is the ability to recognise objects, events or people and make judgements based on their prior occurrence. There is considerable evidence that interference to synaptic plasticity processes in perirhinal cortex (PRH) disrupts recognition memory. Synaptic plasticity, in turn, is supported by dynamic interactions between intracellular proteins. Mitogen-activated protein kinases (MAPKs) are some of the most well characterised classes of signalling proteins, and their activity cascades are relatively well documented.

The roles of the MAPK subfamilies ERK and p38 in recognition memory were studied behaviourally using the preferential object recognition task designed to test the ability of rodents to recognize previously encountered stimuli. Intraperirhinal infusions of MEK (the intermediate kinase in the ERK pathway) inhibitor impaired consolidation of long-term memory when test at 24 h. Inhibition of the p38 pathway also disrupted long-term memory consolidation when tested at 24 h. No memory impairment following either inhibitor was found when tested at 20 min delay.

Populations of neurons in PRH and adjacent association cortex (area Te2) respond more strongly to novel than previously encountered stimuli. It is possible to measure this differential response using immunohistochemical staining as demonstrated in studies using the intracellular kinases, CaMKI and CaMKII, and the nuclear substrates, phosphorylated CREB and Fos protein, as markers of neuronal activation. The expression of phosphorylated ERK (pERK) and p38 (p-p38) was measured at varying delays following the paired-viewing procedure in which novel and familiar visual stimuli are simultaneously presented to different eyes. Levels of pERK in combined PRH and Te2 were higher 10 min after viewing novel images, indicating a role for ERK in the consolidation of new information. No changes in the levels of p-p38 expression were found at any time delay studied and this unexpected negative result is discussed. The time course of intracellular ERK activation in response to novel stimuli was earlier than that of nuclear substrates phosphorylated CREB and Fos, consistent with an upstream role for ERK in memory consolidation.

Author’s declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED: .....  ..... DATE:Oct 2011.....

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Finally, this thesis is dedicated in memory of Grandad.

Abbreviations

π = Pi = 3.14

μM – micromolar

ATF -1 – activating transcription factor-1

AMPA - (2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl)propanoic acid)

AMPAR – AMPA receptor

ANOVA – analysis of variance

AUD – auditory cortex

BDNF – brain derived neurotrophic factor

BSA – bovine serum albumin

CaMK - Calcium-calmodulin-dependent protein kinases

CaMKI/II/IV – CaMK

CaMK – Ca^{2+}- calmodulin dependent protein kinase

CaMKK – CaMK-kinase

CREB - cAMP responsive element (CRE) binding protein

dH_2O – distilled water

DR - discrimination ratio (2.2.10)

EGF - epidermal growth factor

ERK - extracellular signal regulated kinase

ENT - entorhinal cortex

FCM - fluorophore- conjugated muscimol

FOS - protein product of c-*fos*

GPCR - G-protein coupled receptors

JNK - c-Jun N-terminal kinase

IEG - immediate early gene

LTD - long-term depression

LTP - long-term potentiation

mAChR - muscarinic acetyl choline receptor

mGlu-R - metabotropic glutamate receptor

MAPK – mitogen activated protein kinases

MTL - medial temporal lobe

NaCl - sodium chloride

NGF- nerve growth factor

NMDA - N-Methyl-D-aspartate

NMDAR - NMDA receptor

NOPT - novel object preference task

OIP - object-in-place task

(p) - phosphorylated version of kinase

PKA - cAMP-dependent protein kinase

PKC - protein kinase C

P38 (p-p38) – MAPK isoform, p38 (phosphorylated p38)

PB – phosphate buffer

pERK - Phosphorylated ERK

PFA - paraformaldehyde

PKA- cAMP-dependent protein kinase

PKC - protein kinase C

PRH - perirhinal cortex

PoRH – postrhinal cortex

PVP - paired-viewing procedure

RS - rhinal sulcus

RTK - receptor tyrosine kinase

SB203580 - (p38 α, p38 β inhibitor) 4-[5-(4-Fluorophenyl)-2-[4-(methylsulphonyl)phenyl]-1H- imidazol-4-yl]pyridine hydrochloride

SEM - standard error of the mean

Te2 - associative visual area, Te2

TTBS - triton tris-buffered solution

U0124- (Control peptide) 1,4-Diamino-2,3-dicyano-1,4-bis(methylthio)butadiene

U0126 - (MEK inhibitor) 1,4-diamino-2,3-diacyano-1,4-bis 2-(aminophenylthio)butadiene

V1 - primary visual cortex

Contents

Chapter 1. General Introduction

Chapter 2. Materials and Methods

Chapter 3. The effect of intraperirhinal inhibition of ERK and p38 on novel-preference exploration behaviour

Chapter 4. Measurement of neuronal activation using phosphorylated ERK and p38 in response to novel and familiar visual stimuli

Chapter 5. General Discussion

Figures

Chapter 1. General Introduction

Chapter 2. Materials and Methods

Chapter 3. The effect of intraperirhinal inhibition of ERK and p38 on novel-preference exploration behaviour

Chapter 4. Measurement of neuronal activation using phosphorylated ERK and p38 in response to novel and familiar visual stimuli

Chapter 5. General Discussion

Tables

Chapter 1. General Introduction

Chapter 2. Materials and Methods

Chapter 3. The effect of intraperirhinal inhibition of ERK and p38 on novel-preference exploration behaviour

Chapter 4. Measurement of neuronal activation using phosphorylated ERK and p38 in response to novel and familiar visual stimuli

1. General Introduction

1.1 Thesis objectives

The *Society for Neuroscience* (S*f*N) recently celebrated 40 years of annual meetings that have attracted neuroscientists from around the world. Within this time, research has led to many advances in our understanding of the brain. However, one of the foremost challenges in the field of neuroscience remains to understand the neural basis of learning and memory.

The aim of this thesis is to elucidate the role of the mitogen-activated protein kinase (MAPK) signalling cascade in visual recognition memory, in particular familiarity discrimination, i.e. judgement concerning previous encounters with stimuli. Single-exposure familiarity discrimination is known to be dependent on the perirhinal cortex (Brown, Warburton et al. 2010). This thesis will focus on two of the MAPK subtypes: extracellular signal regulated protein kinases (ERKs) and the p38's (p38), both of which are associated with mechanisms of synaptic plasticity.

Chapter 3 will identity the roles of ERK and p38 in the acquisition and consolidation of recognition memory, using the spontaneous novel object preference task. Intraperirhinal inhibition of each kinase will be achieved using U0126 and SB203580, respectively. A variation of the spontaneous novel object preference task will be used to assess the effect of inhibition on associative object-in-place memory.

Chapter 4 will investigate the activation of ERK and p38 in response to visual stimuli using the paired-viewing procedure (PVP). The effect of MEK (an intermediate in the ERK cascade) inhibitor, U0126, on the early activation of ERK will be assessed using the PVP.

1.1.1 Clinical relevance

Alzheimer's disease is now the most prevalent form of dementia seen worldwide (Munoz and Ammit 2010) with over 400,000 sufferers in the UK alone (Alzheimer's society 2011). With an aging population, this degenerative disease results in huge financial impact on the NHS, and more poignant are the heart-breaking social consequences endured by the patient and his or her family. An early symptom of Alzheimer's disease is loss of memory for the on-going experiences of their lives (impaired acquisition of episodic memories) and a consequent impairment of the ability to judge recent occurrences as familiar (loss of recognition memory). Hence in the course of a single conversation, questions and statements are repeated.

1.2 Visual recognition memory

Visual recognition memory is the ability to correctly identify stimuli previously encountered and to associate the context or location in which this encounter took place (Brown and Aggleton 2001). Recognition memory is widely considered to be a dual process, in which both components are functionally distinct (Jacoby and Dallas 1981; Gardiner and Parkin 1990; Yonelinas 1994; Brown and Aggleton 2001; Eichenbaum, Yonelinas et al. 2007) though this view continues to be challenged (see below). It has been proposed that the initial, rapid identification ('knowing') that a stimuli has been encountered - *familiarity discrimination* - is dependent on the perirhinal cortex (PRH), a cortical region within the medial temporal lobe; whereas remembrance including further associations and/or the spatiotemporal context of occurrence of a stimulus or group of stimuli - *recollective matching* - requires the functional integrity of the hippocampus (Brown and Aggleton 2001; Eichenbaum, Yonelinas et al. 2007; Brown, Warburton et al. 2010; Warburton and Brown 2010).

In contrast to the qualitative dual-process hypothesis, a quantitative single-process model has been proposed to underlie episodic retrieval. The latter model proposes that familiarity discrimination represents a weaker memory trace than that when the contextual information associated with the stimuli is recalled. Thus the structures in the medial temporal lobe are collectively

concerned and any damage to this area affects both components, equally (Haist, Shimamura et al. 1992; Donaldson 1996; Hirshman and Master 1997; Squire, Stark et al. 2004; Squire, Wixted et al. 2007).

1.2.1 Functional integrity of MTL is required for episodic memory

Clinical findings reporting profound amnesic effects as a result of bilateral MTL resections have established a critical role for this brain region in episodic memory formation (Scoville 1954; Scoville and Milner 1957; Cohen and Squire 1980; Zola-Morgan, Squire et al. 1982; Corkin, Amaral et al. 1997; Buffalo, Reber et al. 1998). A critical requirement for the MTL in recognition memory has been supported by ablation studies in both non-human primates (Mishkin 1978; Zola-Morgan, Squire et al. 1982; Murray and Mishkin 1983; Murray and Mishkin 1986; Zola-Morgan, Squire et al. 1989; Meunier, Bachevalier et al. 1993; Gaffan 1994; Bussey, Muir et al. 1999) and the rodent (Mumby and Pinel 1994; Ennaceur, Neave et al. 1996; Aggleton, Keen et al. 1997; Winters, Forwood et al. 2004) Object recognition memory deficits were initially associated with damage to the hippocampus and amygdala (Mishkin 1978; Zola-Morgan, Squire et al. 1982); however, subsequent lesion studies discovered memory impairments resulted from damage to parahippocampal cortical regions immediately lateral to the initial site of interest (Murray and Mishkin 1986; Zola-Morgan, Squire et al. 1989; Gaffan and Murray 1992; Suzuki and Amaral 1994; Zola-Morgan, Squire et al. 1994; Murray and Mishkin 1998).

1.2.2 Perirhinal cortex and hippocampus are differentially required for recognition memory processes

Dissociations between the role of the rhinal cortices and hippocampus in recognition memory have been widely established. PRH is responsible for familiarity discrimination of single items, whereas the hippocampus is required for associations and recollective information of single or multiple items to be made (Brown and Aggleton 2001; Piterkin, Cole et al. 2008; Brown, Warburton et al. 2010). Non-human primates with lesions specific to PRH are severely impaired in recognition memory tasks compared to animals with lesions to the hippocampus or fornix (Meunier, Bachevalier et al. 1993; Gaffan

1994; Baxter and Murray 2001; Clark and Squire 2010). Further, PRH lesioned rats are severely impaired in tasks measuring spontaneous object recognition and not those that tax spatial memory. Conversely, hippocampal- or fornix- lesioned rats show deficits in spatial memory tasks and little or no impairment in object recognition tasks (Ennaceur and Aggleton 1994; Mumby and Pinel 1994; Ennaceur, Neave et al. 1996; Aggleton, Keen et al. 1997; Aggleton and Brown 1999; Bussey, Muir et al. 1999; Mumby 2001; Forwood, Winters et al. 2005).

1.2.3 Spontaneous novel object preference task

Visual recognition memory can be tested using innate or operant behavioural paradigms (Murray and Richmond 2001). In the rat, recognition memory is widely assessed using the spontaneous novel object preference (NOPT) task. NOPT was originally based on the visual paired comparison test designed to study early visual development in chicks (Frantz., 1956) and human infants (Fagan 1970). NOPT is a single-trial task that exploits rodents' natural propensity to preferentially explore novel stimuli over familiar stimuli, using visual, olfactory and tactile cues. Contrary to the reward-based delayed match to sample (DMS) and delayed non-match to sample (DNMS) tasks, the NOPT requires no training. Animals are habituated to the arena prior to commencement of the experiment in order to minimise stress levels. Due to the innate behavioural demands of the NOPT, no restrictions (such as food or water deprivation) need to be imposed on the animals (Dere, Huston et al. 2007). Further, the minimal stress levels associated with the task make the experimental conditions more comparable to those associated with human recognition memory paradigms (Ennaceur and Delacour 1988) . The task also benefits from low perceptual demands as the objects tested are designed to have low feature ambiguity (Warburton and Brown 2010).

NOPT is typically composed of three phases – sample phase, retention delay and test session (Figure 2.2.1).

1. Sample phase - Animal allowed to freely explore 3D objects being tested. In the NOPT this is 2 identical copies of the same object.

2. Retention delay – Intermediate phase in which information about the objects explored in the sample phase is withheld

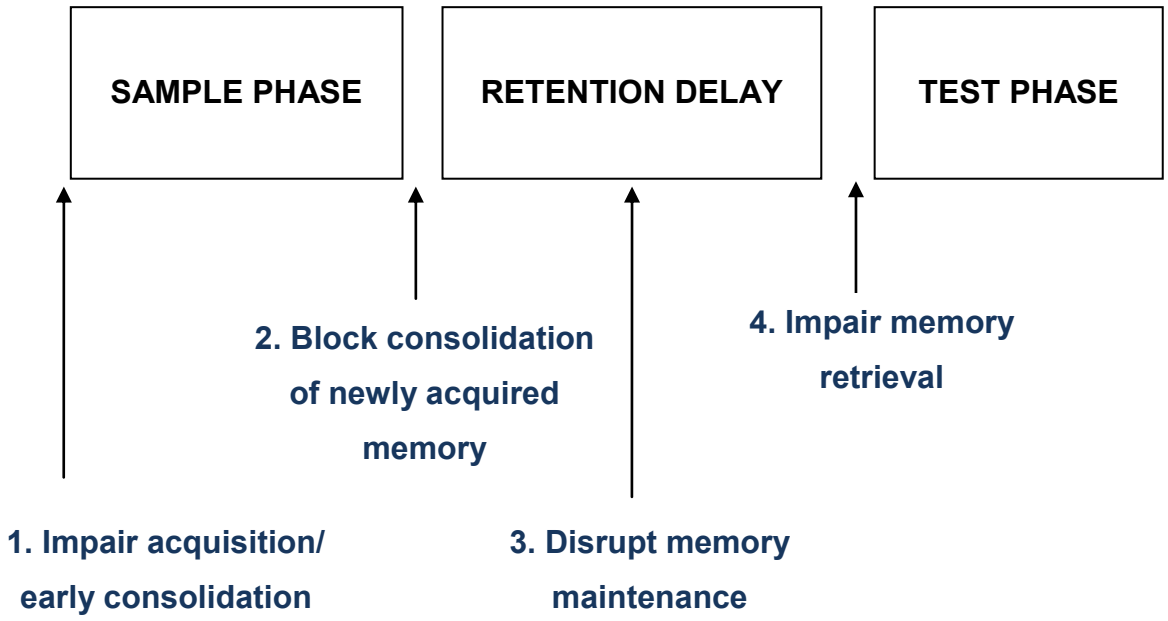
3. Test phase - Animal exposed to a novel object and a third copy of the previously investigated object.

Explorative behaviour is typically calculated as a ratio that makes a comparison between exploration levels of the novel object compared to that of the object previously encountered (Material and Methods, section 2.2.10).

Variations of the NOPT allow for the assessment of different forms of recognition memory. The spatial object location task tests the animal's ability to show preference for the familiar object that has changed location within the arena (Dix and Aggleton 1999). In the object-in-place (OIP) task, animals show increased levels of exploration for the pair of objects that have switched position, compared to those that remain in their original positions (Gaffan and Saunders 1985; Mumby, Gaskin et al. 2002). The recency task predicts novel preference will be in terms of the relative recency in which the object was encountered (Mitchell and Laiacona 1998; Hannesson, Howland et al. 2004). The object-in-context task, tests the preference for a familiar object presented in a novel environment (Mumby, Gaskin et al. 2002; Dere, De Souza-Silva et al. 2003; Norman and Eacott 2004; Dere, Kart-Teke et al. 2006). Details of spontaneous preferential exploration tasks used in this thesis are described in Materials and Methods (section 2.2.8).

Due to the design of the NOPT it is possible to administer reversible inhibitors at different stages of the task and assess the effect of selective effectors in specific brain regions during different memory processes, such as acquisition, consolidation, maintenance and retrieval.

Figure 1.2.3.1 Administering inhibitors at different stages during the NOPT can disrupt different memory process. 1). Infusions made prior to the sample phase of the test can be used to disrupt acquisition of information in the sample phase, and block early memory consolidation. 2). Infusions made post-sample can be used to determine the effect of drug on memory consolidation (infusions can be made at various delays to determine the time point at which the component is required). 3). Infusions made late-on in the retention delay can be used to determine if the compound can block the maintenance of the already consolidated memory. 4). Inhibition prior to the test phase can be used to test memory retrieval.



1.2.4 Altering learning conditions attenuates PRH-associated memory impairments

Although it is widely established that damage to the PRH results in recognition memory impairments, there are instances when lesioned rats are able to discriminate between novelty and previously encountered objects. PRH lesioned rats can discriminate between a novel item and one previously encountered, when multiple exposures of the familiar item have been made (Albasser, Amin et al. 2011). Further, lesioned animals exposed to repeat familiarization trials during the NOPT can perform familiarity discrimination at delays of 24 h though not 3 weeks (Mumby, Piterkin et al. 2007). Conversely, hippocampal- lesioned rats exposed to the same objects over multiple sessions are able to preferentially discriminate at delays up to 3 weeks (Mumby, Tremblay et al. 2005). The ability of lesioned animals to perform accurately in tasks following multiple exposure learning but not single exposures, suggests that mechanisms independent of the PRH can be employed (Mumby, Piterkin et al. 2007; Brown, Warburton et al. 2010; Albasser, Amin et al. 2011).

Additionally, when interference is minimized during the retention delay of the NOPT, PRH-lesioned animals are able to discriminate between novel and familiar objects. However, when the same animals are placed in their holding cage for the same retention delay, they are significantly impaired compared to the control group (McTighe, Cowell et al. 2010).

1.3 Anatomy and connections of the medial temporal lobe

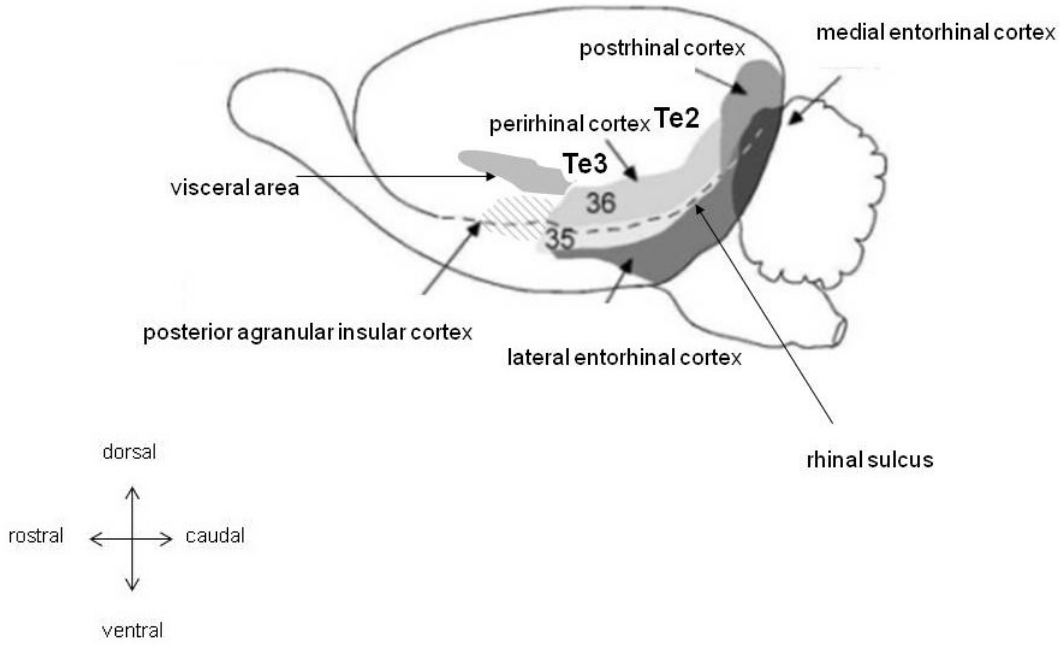
In the rat, the equivalent of the primate MTL anatomically comprises of the hippocampus, amygdala and adjacent cortices: entorhinal, perirhinal and postrhinal (Simons and Spiers 2003; Kealy and Commins 2011).

1.3.1 Perirhinal cortex neuroanatomy

PRH or 'periallocortex' is a strip of cortex found adjacent to the hippocampus and classified by its intermediate allocortical and neocortical morphology (Gibb and Kolb 1998). PRH is positioned equivalently in primates and rodents and has recently been characterised in the human brain (Ding and Van Hoesen 2010). In the rat, PRH is found approximately 2/3 along the rhinal sulcus (Shi and Cassell 1999) and encompasses Brodmann's areas 35 and 36 (Amaral, Insausti et al. 1987). Classically, Brodmann (1909) defined PRH as area 35; however, area 36 has subsequently been included due to its requirement in memory studies (Burwell, Witter et al. 1995; Burwell 2001; Furtak, Wei et al. 2007). The exact boundaries of PRH have been contentious (Krieg 1946; Deacon, Eichenbaum et al. 1983; Burwell, Witter et al. 1995; Burwell and Amaral 1998; Burwell and Amaral 1998); more conservative limits for this area have now been suggested, and are adopted in this thesis (Shi and Cassell 1999). The dorsal border of PRH is auditory cortex (Te3) and association cortex area Te2; ventrally positioned is the entorhinal cortex (Deacon, Eichenbaum et al. 1983; Burwell, Witter et al. 1995) (see Figure 1.3.1.1).

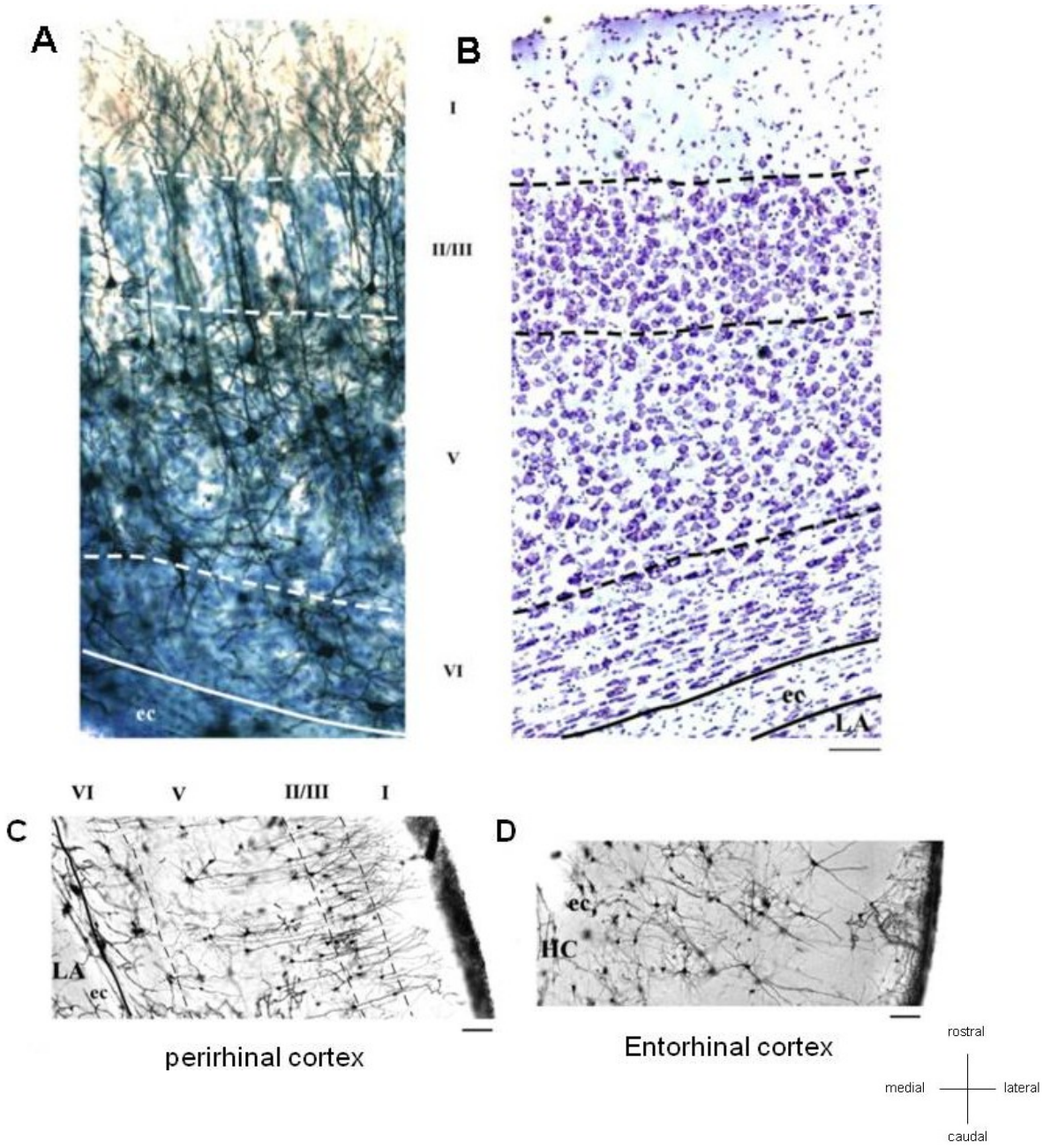
In the rat, PRH is considered to begin approximately - 4 mm from bregma and extend 2.5 mm caudally (Shi and Cassell 1999). Rostrally adjacent to areas 35 and 36 is the insular cortex; dorsal to area 36 is the visceral area (Shi and Cassell 1999).

Figure 1.3.1.1 Neuroanatomy of rat brain – lateral view (modified from Burwell et al (2009)). Perirhinal cortex (areas 35 and 36) is found 2/3 along the rhinal sulcus, adjacent to the hippocampus. Ventrally adjacent to PRH is entorhinal cortex; positioned dorsally are area Te2 and Te3.



Investigations into cytoarchitecture and connections of the cortex along the rhinal sulcus report significant differences along the rostrocaudal axis; this cortex is divided into a more rostral portion classified as PRH, and a caudal area known as postrhinal cortex (PoRh), suggested to be comparable to the parahippocampal region in primates (Deacon, Eichenbaum et al. 1983; Burwell, Witter et al. 1995; Furtak, Moyer et al. 2007; Furtak, Wei et al. 2007). PRH is further subdivided into Brodmann's areas 35 and 36; area 35 is ventral and area 36 is dorsal (Kealy and Commins 2011). PRH is distinguishable from surrounding areas by the absence (35) or meagreness (36) of layer IV (Burwell 2001). Areas 35 and 36 can be further recognised by their varying cytoarchitecture (Burwell 2001; Furtak, Moyer et al. 2007) (Figure 1.3.1.2).

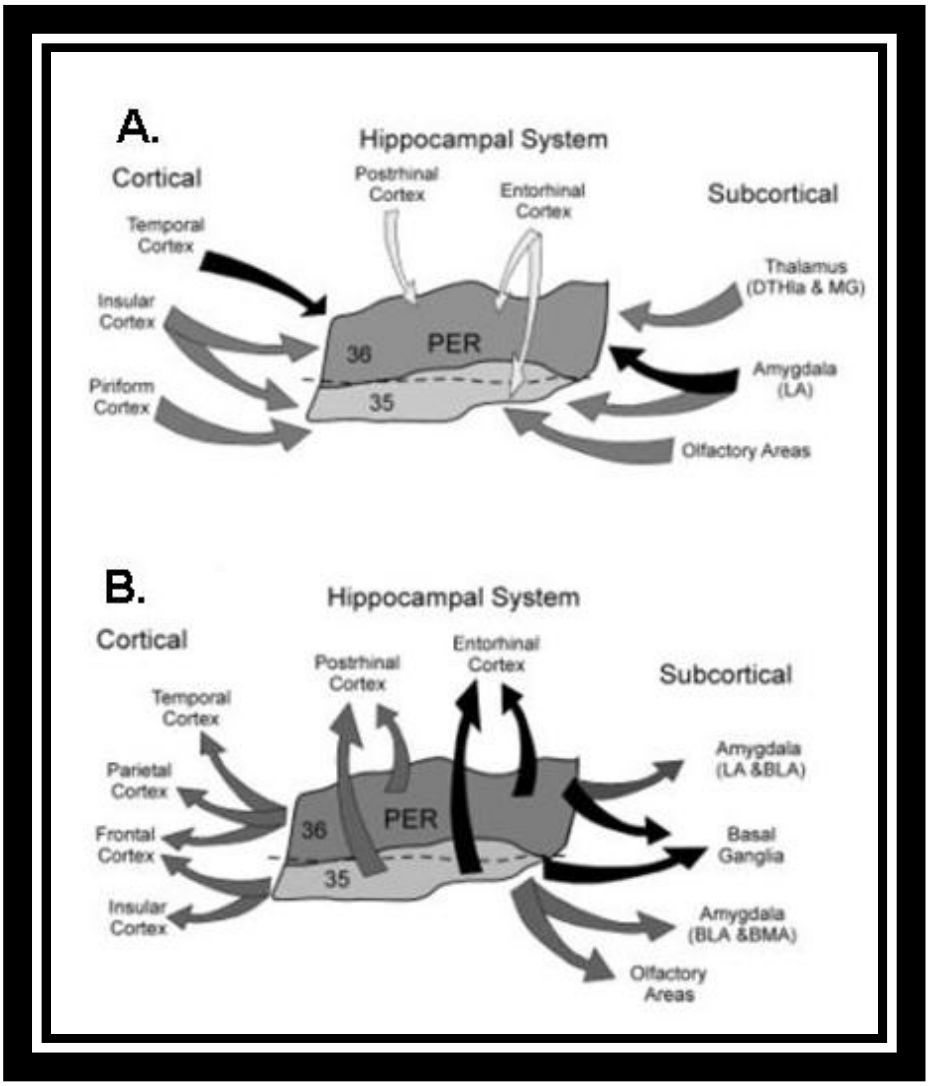
Figure 1.3.1.2 Laminar organisation of perirhinal cortex. A- Golgi-stained and Nissl-stained perirhinal tissue reveals no neuronal staining in layer I. B – Nissl -stained tissue exhibits varying cell arrangement and density across PRH layers. C/D – golgi-impregnated PRH and entorhinal tissue (respectively) from post natal 23 day rats (p23) demonstrating the laminar organisation of PRH compared to entorhinal. Cortical layers are represented by dashed lines. (ec – external capsule; LA – lateral nucleus of amygdala). Scale bar equals 100 μm. Figure reproduced from (Furtak, Moyer et al. 2007).



1.3.2 Perirhinal connections

Situated at the end of the ventral visual pathway, PRH is positioned to support a role as a critical intermediary between the neocortex and hippocampal formation, both directly and via the entorhinal cortex (Deacon, Eichenbaum et al. 1983; Shi and Cassell 1999; Furtak, Wei et al. 2007). PRH is a multimodal association cortex and point of convergence for perceptual sensory information (Suzuki and Amaral 1994; Shi and Cassell 1999; Furtak, Wei et al. 2007). Anterograde and retrograde anatomical tracing studies have been used to demonstrate afferents and efferents of the rat PRH (Deacon, Eichenbaum et al. 1983; Burwell and Amaral 1998; Burwell and Amaral 1998; Shi and Cassell 1999; Agster and Burwell 2009). Area 36 receives its largest projections from frontal, parietal and temporal cortices; area 35 receives fewer temporal afferents (Furtak, Wei et al. 2007) (Figure 1.3.2.1). Rostral PRH receives afferents from somatosensory input, whereas more caudal PRH and PoRH receive visual input (Naber, Witter et al. 2000). Major PRH projections terminate in the lateral entorhinal cortex, amygdala, prefrontal cortex, thalamus and basal ganglia (Furtak, Wei et al. 2007). PRH has reciprocal connections with regions that have been demonstrated to be involved in mnemonic processes, in particular, medial thalamus, basal forebrain and medial prefrontal cortex; such areas have been associated with functions underlying recognition memory (Aggleton and Mishkin 1983; Aggleton and Mishkin 1983; Bachevalier and Mishkin 1986; Aigner, Mitchell et al. 1991).

Figure 1.3.2.1 Diagrammatic summary of afferents and efferents of PRH. A) afferents of area 35 and area 36. B) efferents of area 35 and area 36. The darker arrows represent stronger connections. Reproduced from (Furtak, Wei et al. 2007).

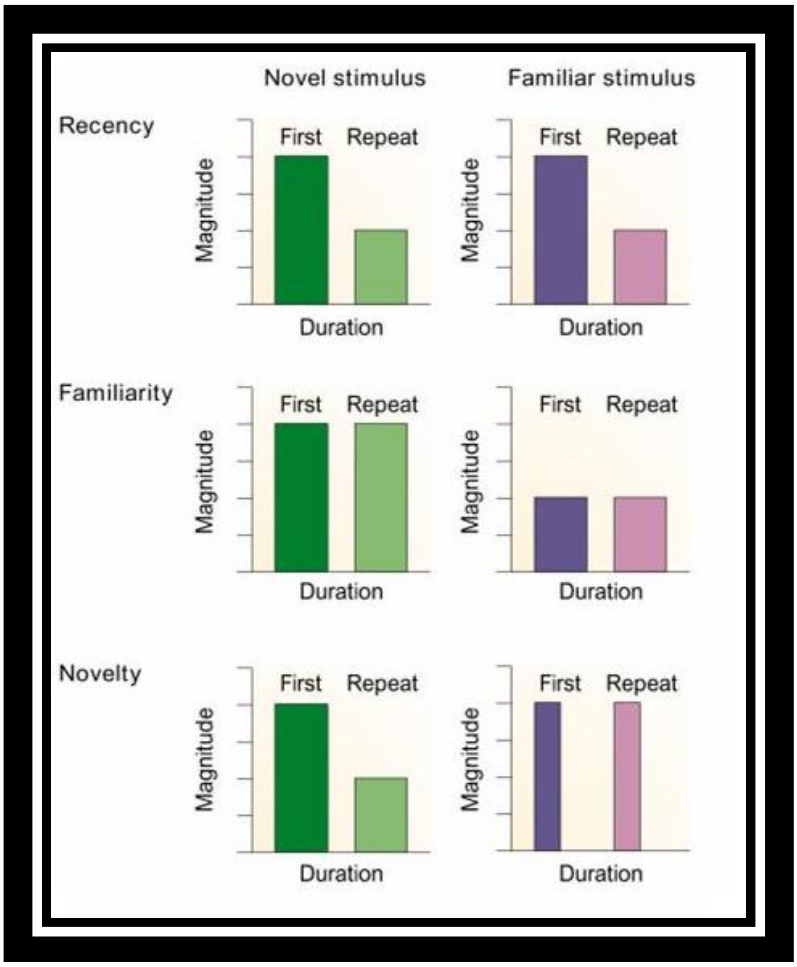


1.4 Populations of neurons in perirhinal cortex and adjacent association area Te2 respond to the relative familiarity of stimuli

In addition to lesion studies highlighting the role of the rhinal cortices in recognition memory, electrophysiological studies in behaviourally trained monkeys have established that populations of neurons signal information concerning the previous occurrence of visual stimuli (Xiang and Brown 1998). Such neuronal responses can signal the relative novelty, familiarity and recency of visual stimuli (Brown, Wilson et al. 1987; Fahy, Riches et al. 1993) (Figure 1.4.1). These responses are repetition-sensitive, thus a decrease in response is found on re-exposure (Brown, Wilson et al. 1987). Furthermore, these responses have been attributed to the familiarity discrimination and recency components of recognition memory (Brown and Xiang 1998).

Approximately 25% of PRH neurons respond more strongly to novel stimuli compared to stimuli previously encountered, even following delays > 24 h demonstrating a potentially high memory capacity (Brown and Aggleton 2001). Computational modelling has established that such reduced neuronal responsiveness has the capacity to store large volumes of information; supporting the possible role of this neural substrate as a mechanism underlying familiarity discrimination (Bogacz, Brown et al. 2001; Brown and Aggleton 2001; Bogacz and Brown 2003). Moreover, this novelty related increase elicits a signal large enough to detect, even in overall population measures of neuronal responsiveness (Li, Miller et al. 1993; Xiang and Brown 1998). Zhu et al. (1995) have also demonstrated such neuronal responses in the rat and further demonstrated that this increase can be visualised in PRH and Te2 using Fos, the protein product of immediate early gene, c-*fos* as summarised in section 1.4.2 (Zhu and Brown 1995; Zhu, Brown et al. 1995; Zhu, Brown et al. 1995; Warburton, Koder et al. 2003; Warburton, Glover et al. 2005; Seoane, Massey et al. 2009).

Figure 1.4.1 Populations of neurons in PRH and adjacent area Te2 signal the relative novelty, familiarity and recency of stimuli. Repetition-sensitive neurons elicit a weaker response when a stimulus is re-presented. Reproduced from (Brown and Aggleton 2001).



Conversely , fewer hippocampal neurons signal the relative familiarity of stimuli (< 1% neurons) (Brown, Wilson et al. 1987; Brown and Xiang 1998; Xiang and Brown 1998; Brown and Aggleton 2001). Neurons within the hippocampus are differentially responsive to the prior occurrence of spatial information, further supporting a double dissociation between the roles of PRH and hippocampus (Zhu, McCabe et al. 1997) (refer to section 1.2.2).

1.4.1 Perirhinal cortex is functionally distinct from adjacent Te2

The rat temporal association area Te2 is visually responsive (Sia and Bourne 2008) and lies more caudally in the ventral- visual- stream than adjacent PRH (Refer to section 1.3.1). Te2 is reciprocally interconnected to PRH; however, both the function and cytoarchitecture of these areas are distinct (Buckley, Gaffan et al. 1997; Buffalo, Ramus et al. 1999). Neurons in Te2 are arranged in laminar fashion, consistent with PRH, but unlike PRH, have a distinct layer IV (Sia and Bourne 2008). Neuronal changes in response to novel visual stimuli as seen in PRH are also evident in area Te2 (Zhu and Brown 1995; Brown and Xiang 1998; Xiang and Brown 1998; Brown and Aggleton 2001) (section 1.4). Average changes in activity of populations of recency and familiarity neurons occurred in Te2 earlier than PRH; but average memory spans were found to be longer in PRH (Xiang and Brown 1998), indicating that the neuronal response to novel stimuli in PRH is more than just a passive reflection of that observed in Te2. More recently, a role for Te2 in recognition memory at delays > 20 min has been established (Ho, Narduzzo et al. 2011).

1.4.2 Gene expression

Gene expression studies support lesion studies in finding a double dissociation between the role of the PRH and hippocampus in object recognition memory (Wan, Aggleton et al. 1999). Further, Fos studies parallel electrophysiological studies in which changes are also seen in Te2 (Zhu, Brown et al. 1995; Zhu, McCabe et al. 1996; Wan, Aggleton et al. 1999). Immediate early genes (IEG) are activated in response to neuronal activation (Dragunow 1996; Herrera and Robertson 1996). Thus immunohistochemical staining of IEG products allows simultaneous comparisons of the activity of whole populations of neurons within multiple brain regions (Zhu, Brown et al. 1995). An advantage of gene expression compared to studying the effects of lesions is that activity is assessed in the fully functional brain, without complications arising from compensatory pathways (Aggleton and Brown 2005). It must, however, be noted that gene activation in any given region, in common with other types of activation, does not establish the necessity of that region for the task. Fos, the protein product of IEG c-*fos*, has been widely used as a marker of neuronal activation. In most cells the basal expression of

c-*fos* mRNA and levels of Fos protein are low (Morgan and Curran 1991); furthermore, mRNA levels peak between 30- 45 min following activation and the half life of Fos protein is ~ 2 h (Curran, Gordon et al. 1987). Fos expression is maximal ~60-90 min (Zangenehpour and Chaudhuri 2002) after activation, and has faded by ~4 h after stimulation. Fos expression has been implicated in processes underlying synaptic plasticity (Nikolaev, Tischmeyer et al. 1991; Nakazawa, Karachot et al. 1993; Fleischmann, Hvalby et al. 2003) and learning (Swank, Ellis et al. 1996; Albasser, Poirier et al. 2010)Seoane and Brown, BNA abstract 2007). Fos expression in PRH and Te2 is differentially increased following presentation of novel versus familiar visual stimuli and increased in the HPC in response to novel spatial arrangements (Zhu and Brown 1995; Zhu, Brown et al. 1995; Zhu, Brown et al. 1995; Zhu, McCabe et al. 1997; Wan, Aggleton et al. 1999; Warburton, Koder et al. 2003; Wan, Warburton et al. 2004; Warburton, Glover et al. 2005; Albasser, Poirier et al. 2010).

Further, the novelty-induced increase in Fos levels can be abolished by interfering with intraperirhinal receptor and intracellular signalling pathways (Warburton, Koder et al. 2003; Wan, Warburton et al. 2004Nadine Fontaine-Palmer PhD Thesis 2008; Warburton, Glover et al. 2005) (see Chapter 4).

1.4.3 Paired-viewing procedure

The paired-viewing procedure (PVP) has been used to passively present to rats novel and familiar images simultaneously, under the same state of alertness, motivation, hormones and motor activity (Zhu, McCabe et al. 1996; Aggleton and Brown 2005) (Materials & Methods, 2.4). Early studies of activation produced by novel and familiar stimuli used comparisons across subjects and were difficult to control for activations in response to non-visual stimuli, such as drinking (Zhu, Brown et al. 1995). As novel and familiar stimuli can be simultaneously presented to the opposite eyes of a rat, measurements of neuronal activation in response to novel or familiar stimuli can be made in the contralateral hemispheres allowing within-subject comparisons (Zhu, McCabe et al. 1996; Wan, Aggleton et al. 1999). The PVP requires an animal to learn to poke its head through an observation hole to obtain a juice reward;

once in position, 2D images are presented on two computer monitors positioned to be visible in the monocular field of each eye (Aggleton and Brown 2005) (refer to Materials and Methods, section 2.4).

1.5 Additional roles for perirhinal cortex

1.5.1 Perirhinal cortex in non-visual recognition memory

Imaging of immediate early genes (IEGs) has been widely established as a measure of neuronal activation (Zhu and Brown 1995; Zhu, Brown et al. 1995; Zhu, McCabe et al. 1997; Wan, Warburton et al. 2001). However, in the rat levels of Fos, the protein product of IEG- c-*fos*, were not differentially increased in response to novel and familiar sounds (Wan, Warburton et al. 2001). This finding is consistent with studies in monkeys (Saunders et al., 1998, SfN abstract) and dogs (Kowalska, Kusmierek et al. 2001).

The role of PRH has been assessed in cross-modal variants of the NOPT in which animals are required to discriminate using different sensory information to that previously used to explore the initial object. PRH-lesioned rats were found to be impaired in cross-modal variants in which tactile and visual information was tested; however, the ability to discriminate between objects using tactile information alone, was not impaired (Winters and Reid 2010). A novel behavioural paradigm - the 'bowtie maze' allows for multiple presentations of visual stimuli whilst simultaneously measuring novelty preference (Albasser, Poirier et al. 2010); PRH-lesioned rats can discriminate in the bowtie maze when tested in the dark (without visual information), but the same animals were impaired in the light condition at delay < 60 s (Albasser et al., 2010, Recognition Memory Mechanisms Symposium abstract). Further, PRH is required for olfactory recognition memory in the rat (Otto and Eichenbaum 1992).

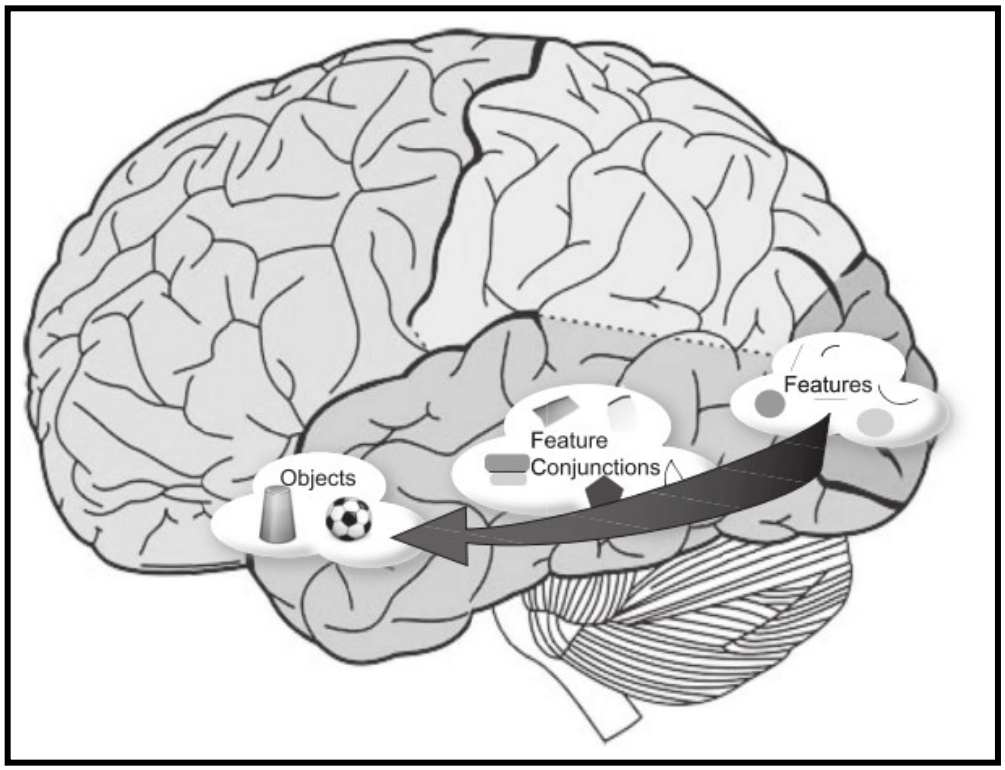
PRH-lesioned animals are able to discriminate in a test of object recognition when they are placed in a visually restrictive environment for the duration of the retention delay (in which the acquired memory is to be consolidated) (McTighe, Cowell et al. 2010). Lesioned animals, when returned to their home

cage (standard condition) are significantly impaired in their ability to discriminate between an item previously encountered and from one that is novel. Counter-intuitively, levels of exploration for the novel object were comparable to those of the familiar objects. This find supports the hypothesis that lesioned animals perceive the novel object as 'familiar', presumably as a result of sharing simple features with objects routinely encountered in the animal's home cage. According to this view, minimising interference following learning prevents features associated with the novel objects being encountered, and enables PRH-lesioned animals to discriminate based on simple stimulus features (McTighe, Cowell et al. 2010).

1.5.2 Roles for perirhinal cortex in perception

In addition to a critical role for PRH in mnemonic processes, a further role in perceptual judgements (identification) has been identified (Murray and Bussey 1999; Bartko, Winters et al. 2007; Bartko, Winters et al. 2007). The location of PRH serves as an ideal candidate for both processes. As well as strong hippocampal connections, via the entorhinal cortex (Suzuki 1996), PRH sits at the top of the ventral-visual-stream, 'what' pathway (Murray and Bussey 1999; Bussey and Saksida 2002; Bussey, Saksida et al. 2002). Caudal neurons in this pathway represent simple features, whereas more rostral neurons, such as those in Te2 carry information regarding complex conjunctions of features (Bussey and Saksida 2002; Bartko, Winters et al. 2007).

Figure 1.5.2.1 Visual stimuli features are organised in increasing complex conjunctions along the ventral visual stream. Early on in the ventral visual stream simple features are represented, whereas further down the stream (PRH) whole objects are represented. Taken from McTighe et al. (2010).



However, the contribution of PRH to visual perception has been disputed and suggested to result from inadvertent damage to adjacent area Te2, or more importantly, an inability to learn the task (Buffalo, Ramus et al. 1999; Stark and Squire 2000; Hampton 2005; Levy, Shrager et al. 2005; Clark, Reinagel et al. 2011). Levy et al. (2005) and Squire et al. (2006) maintain that in order to test for perception, no delay between the sample and test phase should be imposed. Single-trial zero delay recognition memory is widely used to assess perception, due to its minimal memory requirement (Eacott, Gaffan et al. 1994; Buffalo, Ramus et al. 1999; Levy, Shrager et al. 2005).

The functional integrity of PRH is considered imperative to solving visual object recognition when the objects tested share complex conjunctions of features, i.e. high feature-ambiguity (Eacott, Gaffan et al. 1994; Murray and Bussey 1999; Bussey, Saksida et al. 2002; Norman and Eacott 2004; Bartko,

Winters et al. 2007; Murray, Bussey et al. 2007). Selective perirhinal and postrhinal lesioned animals are increasingly impaired in an oddity task in which objects increasing in overlapped features (Bartko, Winters et al. 2007). Increasing the perceptual demands of visual recognition memory tasks results in behavioural deficits in monkeys with PRH and entorhinal damage, when tested immediately following the sample phase (Eacott, Gaffan et al. 1994) and when trained using large stimulus sets (Buckley and Gaffan 1997). It is argued that in the studies in which PRH lesioned animals are impaired at longer delays, the tasks are not providing specific measures of 'mnemonic' or 'perceptual' properties (Murray, Bussey et al. 2007). Further, the ability to perform in object matching tasks at short delays does not necessarily demonstrate the capacity to perceive objects under every circumstance; it is possible that impairments would be observed at even the shortest delays if the stimuli used was sufficiently complex and/or high in feature-ambiguity (Murray and Richmond 2001).

1.5.3 A role for perirhinal cortex in reward

PRH has strong reciprocal connections with areas associated with motivation and reward (Aggleton, Burton et al. 1980; Witter, Room et al. 1986) and it is innervated with dopamine fibres (Goldsmith and Joyce 1996; Liu, Richmond et al. 2004). A role for PRH in reward-association learning has been established in monkeys (Spiegler and Mishkin 1981; Liu and Bilkey 1999). Further, neurons in PRH respond to visual cues relating to reward schedules in DMS learning (Liu and Richmond 2000). Liu et al. (2004) has demonstrated in primates that reward associated cue learning is D2 receptor dependent.

1.6 Synaptic plasticity – a cellular candidate for neural changes associated with learning and memory

Learning is the process by which information and skills are acquired; newly acquired memories are susceptible to disruption by interference and trauma (Bartko, Cowell et al. 2010; McTighe, Cowell et al. 2010). Memory consolidation is critical for long-term storage of newly obtained information and is widely believed to be dependent on transcription and translational processes that result in molecular and structural synaptic modifications (Davis and Squire 1984; McGaugh 2000). Long-term memory (LTM) described as a 'dialogue between genes and synapses' (Kandel 2001) differs in underlying mechanisms from those of short-term memory (STM) (Izquierdo, Barros et al. 2002) in that STM can be maintained without protein transcription (Impey, Obrietan et al. 1999; Kandel 2001; Izquierdo, Barros et al. 2002). Consolidated memories can be rendered labile following reactivation during a process known as reconsolidation (Duvarci and Nader 2004; Tronson and Taylor 2007); There is still much debate as to whether the neural properties underlying consolidation and reconsolidation are the same; however, there is increasing evidence that these processes share at least some common features (Kelly, Laroche et al. 2003; Duvarci and Nader 2004; Duvarci, Nader et al. 2005; Cestari, Costanzi et al. 2006; Nader and Einarsson 2010).

It is widely considered that long lasting neural changes underlying learning and memory share properties with synaptic plasticity mechanisms as demonstrated *in vitro* (Bliss and Collingridge 1993; Malenka and Nicoll 1999; Kemp and Bashir 2001; Martin and Morris 2002; Abraham and Robins 2005). Learning has been associated with both mechanisms underlying long-term potentiation (LTP) - an enhancement in synaptic strength (Rioult-Pedotti, Friedman et al. 2000; Maren 2005) and long-term depression (LTD) - characterised by a decrease in the amplitude of a synaptic response following low frequency stimulation (Bear and Malenka 1994; Bear and Abraham 1996; Xiong, Kojic et al. 2006; Massey and Bashir 2007; Griffiths, Scott et al. 2008; Massey, Phythian et al. 2008). Neural modifications following induction of LTP allow for alterations in synapse strength, as proposed by Hebb's Postulate:

'When an axon of cell A is near enough to excite cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells so that A's efficacy as one of the cells firing B, is increased' (Hebb 1949).

1.6.1 Mechanisms underlying LTP and LTD

LTP was first identified in the hippocampus following repetitive stimulation (Bliss and Lomo 1973). Occurrence of a long-lasting change in synaptic efficacy in a region involved in memory has attracted much research. The study of NMDA receptor-dependent LTP and LTD in the hippocampus has been facilitated by the laminar neuronal arrangement allowing ready interpretation of electrophysiological recordings (Anderson, Bliss et al. 1971). It has since been established that alternative types of synaptic plasticity exist throughout multiple brain regions (Malenka and Bear 2004).

Neuronal response changes putatively underlying familiarity discrimination in PRH and adjacent area Te2, are consistent with a role for synaptic weakening and hence for LTD mechanisms (Brown and Bashir 2002; Warburton, Koder et al. 2003). As in any network, overall excitability must be balanced over the longer term and disruption to mechanisms underlying LTP could still impair familiarity discrimination (Warburton, Glover et al. 2005).

In vitro, LTD, similarly to LTP, has temporally distinct stages: initial LTD (induction); early-LTD (expression) and late-LTD (maintenance) (Kauderer and Kandel 2000). Transient early-LTD requires the activation of protein kinases and is characterised by the internalization of AMPAR (Xiong, Kojic et al. 2006). AMPA receptors are inserted into the postsynaptic neurons during LTP and removed following LTD-inducing stimuli (Luscher, Nicoll et al. 2000; Malinow, Mainen et al. 2000; Scannevin and Huganir 2000; Sheng and Lee 2001; Derkach, Oh et al. 2007). Late-LTD requires *de novo* gene expression and is sensitive to protein synthesis inhibitors (Frey, Krug et al. 1988; Huang and Kandel 1994; Kandel 2001).

1.6.2 Synaptic plasticity and familiarity discrimination mechanisms in perirhinal cortex

Glutamate is the main excitatory neurotransmitter in PRH, as elsewhere in the cerebral cortex (Fonnum 1984; Lodge and Collingridge 1990). PRH contains both ionotropic glutamatergic ligand-gated ion channels (NMDA, AMPA, kainate) that mediate fast excitatory neurotransmission (Dingledine, Borges et al. 1999) and metabotropic glutamate receptors (m-GluRs) coupled to GTP-binding proteins that connect to intracellular signalling pathways (Sladeczek, Pin et al. 1985; Nicoletti, Iadarola et al. 1986; Cho, Kemp et al. 2000). *In vitro* studies have demonstrated that it is possible to induce LTP and LTD in PRH slices following suitable stimulation (Bilkey 1996; Ziakopoulos, Tillett et al. 1999; Cho, Aggleton et al. 2001; Park, Jo et al. 2006); both mechanisms are associated with increased levels of intracellular calcium (Ca^{2+}) in postsynaptic neurons (Bear and Malenka 1994; Cho, Aggleton et al. 2001). Further, the requirement for several membrane receptor subtypes, and intracellular substrates in mechanisms underlying both mnemonic and plastic processes in PRH have been demonstrated and are summarised below.

1.6.2.1 Ionotropic receptors

1.6.2.1.1 NMDA-receptors

A role for ionotropic glutamate receptors has been identified in plasticity and behavioural mechanisms in PRH. Inhibition of NMDA receptors (NMDAR) blocks LTP and LTD and results in behavioural familiarity discrimination impairments at delays > 1 h (Ziakopoulos, Tillett et al. 1999; Cho, Kemp et al. 2000; Winters and Bussey 2005; Barker, Warburton et al. 2006; Barker and Warburton 2008). NMDAR dependent LTD is more readily induced in juvenile brain slices compared to adult tissue (Bear and Abraham 1996; Kemp and Bashir 2001) ; this difference is considered to result from changes in the NR2A-2B subunit ratio and the location of these specific receptor subunits at or near the synapse (Massey, Johnson et al. 2004). PRH LTD is dependent on activation of extrasynaptic NR2B-containing NMDARs, whereas LTP and depotentiation require activation of NR2A-containing NMDARs found at synaptic sites (Stocca and Vicini 1998; Tovar and Westbrook 1999; Massey, Johnson et al. 2004). Selective antagonism of both NR2A and NR2B containing NMDA receptors is required to impair recognition memory suggesting the possibility that more than one mechanism underlies long-term familiarity discrimination (Barker, Warburton et al. 2006).

1.6.2.1.2 Kainate receptors

A role for kainate receptors in short-term (20 min delay) but not longer term familiarity discrimination (Barker, Warburton et al. 2006; Barker and Warburton 2008) and in the induction of LTD (Park, Jo et al. 2006) has been established. The differential temporal role for kainate and NMDA receptors at short (20 min) and longer delays (> 1 h), respectively, supports the hypothesis that at least two distinct recognition memory subtypes exist, as previously indicated by the discovery of ‘fast change’ recency and novelty neurons (which could be kainate receptor dependent) and ‘slow, long lasting’ familiarity neurons (which could be NMDAR dependent) (Fahy, Riches et al. 1993; Xiang and Brown 1998).

1.6.2.1.3 AMPA-receptors

Intraperirhinal infusions of AMPA receptor (AMPAR) antagonist CNQX block recognition memory when active during encoding or retrieval (Winters and Bussey 2005). Furthermore, PRH LTD is known to be dependent on AMPA receptor internalisation (Griffiths, Scott et al. 2008). The interaction between AMPAR GluR2 subunits and AP-2 (a clatherin-coated pit adaptor protein, known to be required for endocytosis) is critical for this process. Viral disruption of this interaction in PRH results in both PRH plasticity and recognition memory impairments (Griffiths, Scott et al. 2008).

1.6.2.2 mGlu-receptors

mGlu-receptors (mGlu-R) are G-protein coupled receptors (GPCR) that are subdivided into 3 groups (GpI-III) and are coupled to intracellular signalling mechanisms (Harris, Gallyas et al. 2004). MGluR-LTD is more readily induced in the PRH than the NMDAR dependent equivalent. Additionally, a class of PRH mGlu-R LTD has been demonstrated in which Gp II mGlu-Rs mediate Ca^{2+} influx via Gp I mGlu-Rs and NMDAR activation (Cho, Kemp et al. 2000; Cho and Bashir 2002). *In vivo*, co-application of mGluR I/II inhibitors MPEP and LY341495, respectively blocks long-term familiarity discrimination but not memory tested at 20 min (Barker, Bashir et al. 2006).

1.6.2.3 Non-glutamatergic receptors

PRH LTD can be induced by non-glutamatergic receptors such as muscarinic receptors (Massey, Bhabra et al. 2001). Antagonism of cholinergic muscarinic receptors by the broad spectrum antagonist, scopolamine, prevents the induction of LTD but not LTP in PRH slices (Massey et al., 2003). Further, the induction of LTD and depotentiation are prevented in brain slices corresponding to the hemisphere to which familiar stimuli were presented during the PVP; this inhibition occurs in a muscarinic dependent manner (Massey, Phythian et al. 2008). *In vivo* studies support a role for cholinergic neurotransmission in the acquisition of short-term familiarity discrimination in PRH tested at a 15 min delay (Warburton, Koder et al. 2003; Tinsley, Fontaine-Palmer et al. 2011). However, memory impairments at longer delays (24 h) have been reported following pre-sample intraperirhinal infusions of

scopolamine (Winters and Bussey 2005). Administration of scopolamine prior to proactive or retroactive interference in the NOPT abolished the memory impairment associated with control animals (Winters, Bartko et al. 2007). Tinsley et al. (2011) have established that in PRH muscarinic and nicotinic antagonism are doubly dissociated, with nicotinic antagonism resulting in object recognition memory impairments when tested at 24 h delay.

1.6.2.4 GABA-receptors

The benzodiazepine lorazepam is known to enhance the activity of $GABA_A$ receptors and thus result in hyperpolarization of the cell membrane (Izquierdo and Medina 1991; Wan, Warburton et al. 2004). Intraperirhinal administration of lorazepam results in familiarity discrimination impairments at 20 min and disrupts the normal increase in Fos counts for novel over familiar stimuli when administered prior to PVP training sessions (Wan, Warburton et al. 2004). *In vitro,* lorazepam blocks LTP maintenance > 30 min following induction; whereas LTD potentials return to baseline within 20 min (Wan, Warburton et al. 2004).

1.6.2.5 L-type voltage-dependent calcium channels

Investigations into the role of L-type voltage-dependent calcium channels (LVCC) in mechanisms underlying plasticity and behaviour in PRH have demonstrated inhibitors of these channels within PRH block LTD and depotentiation. Further, LVCC antagonists impaired object recognition memory acquisition and retrieval when tested at 24 h delay (Seoane, Massey et al. 2009).

1.6.3 Protein kinases in synaptic plasticity & memory

Following receptor activation, complex intracellular protein kinase networks are required to integrate extracellular signals into neuronal modifications (Pearson, Robinson et al. 2001). Protein phosphorylation is one of the most prevailing post-translational cellular modifications in response to extracellular stimuli (Sharma and Carew 2004). A role for protein kinases in memory was first demonstrated in studies of Aplysia in which a requirement for PKA in 5-HT-induced plasticity was identified (Kandel 2001). Protein kinases are enzymes that covalently attach phosphate to proteins substrates (Johnson and Lapadat 2002). Intracellular protein kinases phosphorylate nuclear substrates and result in gene expression required for long-term synaptic plasticity and mechanisms underlying learning and memory (Soderling and Derkach 2000). Protein kinases have been identified throughout the cerebral cortex in mechanisms underlying the induction, expression and maintenance of LTP and LTD, in addition to processes underlying memory. More specifically, several protein kinases have been found to be critical in such processes in PRH.

1.6.3.1 Calcium-calmodulin dependent protein kinases

Calcium-calmodulin-dependent protein kinases (CAMKs) include CaMK-kinase (CaMKK), CaMKI, CaMKII and CaMKIV and are activated by $Ca2^{+}$-calmodulin (Tinsley, Narduzzo et al. 2011). Once activated, CaMKII can autophosphorylate, resulting in persistent activation in the absence of $Ca2^{+}$ (Miller and Kennedy 1986)**.** CaMKK activates both CaMKI and CaMKIV enzymes (Soderling 1999). The roles of CAMKs in synaptic mechanisms in PRH have yet to be investigated; however, the role of CAMKs in hippocampal LTP and LTD have been widely established (Malinow, Schulman et al. 1989; Silva, Stevens et al. 1992; Mayford, Wang et al. 1995; Lisman, Malenka et al. 1997; Giese, Fedorov et al. 1998; Oomura, Hori et al. 2006; Redondo, Okuno et al. 2010; Mockett, Guevremont et al. 2011; Molnar 2011).

Downstream substrates of CaMK include targets identified to be involved in recognition memory. CAMKII phosphorylates NMDAR and AMPAR

(Omkumar, Kiely et al. 1996; Barria, Muller et al. 1997) and results in an increase in the conductance of AMPARs (Derkach, Barria et al. 1999). Cytoplasmic substrates of CaMKs include the ERK cascade (Illario, Cavallo et al. 2003; Oh, Manzerra et al. 2004) and nuclear targets such as CREB (1.6.4.1) and activating transcription factor 1 (ATF1) (Sheng, Thompson et al. 1991; Sun, Enslen et al. 1994).

CaMKs are associated with mechanisms underlying learning and memory *in vivo* (Giese, Fedorov et al. 1998; Lisman, Schulman et al. 2002; Wei, Qiu et al. 2002; Peters, Mizuno et al. 2003; Blaeser, Sanders et al. 2006; Mizuno, Ris et al. 2006). Intraperirhinal administration of CaMKK (STO-609) or CaMKII (KN-62 or AIP) inhibitors, results in long-term recognition memory impairments when tested at a 24 h retention delay (Tinsley, Narduzzo et al. 2009; Tinsley, Narduzzo et al. 2011). Levels of phosphorylated CaMKII (p-CAMKII) are increased in response to novelty in PRH and area Te2, 70 min following presentation of visual stimuli in the PVP (Tinsley, Narduzzo et al. 2009). Conversely, levels of p-CAMKI are increased in response to familiar stimuli 10 and 40 min following the PVP (Tinsley, Narduzzo et al. 2011), demonstrating a possible role for this kinase in reconsolidation memory (4.5.4).

1.6.3.2 cAMP-dependent protein kinase and protein kinase C

cAMP-dependent protein kinase (PKA) and protein kinase C (PKC) are associated with mechanisms underlying synaptic plasticity (Frey, Huang et al. 1993; Weisskopf, Castillo et al. 1994; Qi, Zhuo et al. 1996; Salin, Malenka et al. 1996; Abel, Nguyen et al. 1997; Chain, Casadio et al. 1999; Sanderson and Dell'Acqua 2011) and learning and memory (Giralt, Saavedra et al. 2011; Guerra, Mello et al. 2011). Secondary messenger, cAMP is increased via adenylate cyclase and is responsible for both activation and inactivation of PKA (Zhang, Okutani et al. 2003). PKC is activated in response to diacylglycerol (DAG) or increased levels of intracellular Ca^{2+} (Roberson, English et al. 1999). Critical roles for PKA and PKC in long-term familiarity discrimination have been established within the PRH (Nadine Fontaine-Palmer, unpublished PhD thesis, 2008); however, their roles in PRH synaptic

plasticity have not been studied. Downstream targets of PKA and PKC signalling are known to include MAPK and CREB (section 1.6.4.1) (Brindle, Nakajima et al. 1995; Xie and Rothstein 1995).

The autonomously active PKC isoform, PKM zeta maintains LTP via N-ethylmaleimide-sensitive factor (NSF) /glutamate receptor subunit 2 dependent AMPA-receptor signalling (Sacktor, Osten et al. 1993; Nishimune, Isaac et al. 1998; Hernandez, Blace et al. 2003; Yao, Kelly et al. 2008). A role for PKM zeta in maintenance of recognition memory has also been established (Alexandra Outram, unpublished PhD Thesis, 2011).

1.6.3.3 Brain-derived neurotrophic factor

PRH brain-derived neurotrophic factor (BDNF) is a neurotrophin identified to underlie mechanisms of LTP and LTD (Pang, Teng et al. 2004; Bramham and Messaoudi 2005; Lu, Christian et al. 2008; Mei, Nagappan et al. 2011). In addition to roles in synaptic plasticity, BDNF has been associated with mechanisms underlying learning and memory (Alonso, Vianna et al. 2002; Alonso, Vianna et al. 2002; Rattiner, Davis et al. 2004; Alonso, Bekinschtein et al. 2005; Bekinschtein, Cammarota et al. 2007; Bekinschtein, Cammarota et al. 2008). More specifically, increased levels of PRH BDNF are associated with exercise-induced improvements in recognition memory (Hopkins, Nitecki et al. 2011), and is necessary for recognition memory consolidation tested at 24 h delays, demonstrated using antisense oligodeoxynucleotides (Seoane, Tinsley et al. 2011). Downstream targets of BDNF are thought to include transcription factor, CREB (refer to section 1.6.4.1) (Alonso, Bekinschtein et al. 2005; Bekinschtein, Cammarota et al. 2008; Seoane, Tinsley et al. 2011) activation of which results in Fos protein expression (refer to section 1.6.4.2) (Silva, Kogan et al. 1998).

1.6.4 Nuclear targets

Gene expression is essential for long-lasting neuronal changes associated with synaptic plasticity and learning (Sun, Mercado et al. 2005; Tabuchi 2008). Transcription factors located within the nucleus are responsible for gene expression and thus regulate protein synthesis (Zhang, Okutani et al. 2003). The most widely studied nuclear targets associated with familiarity discrimination in PRH are the transcription factor, cAMP responsive element binding protein (CREB), and immediate early gene (IEG), c-*fos*.

1.6.4.1 cAMP responsive element binding protein (CREB)

The transcription factor, cAMP responsive element (CRE) binding protein (CREB) is a nuclear substrate that modulates gene transcription by binding to the promoter CRE- sites of genes (Silva, Kogan et al. 1998; Ahn, Ginty et al. 1999). Increases in cAMP and intracellular levels of Ca^{2+} are associated with CREB phosphorylation on Ser133 (Gonzalez and Montminy 1989; Silva, Kogan et al. 1998). CREB is a critical component of intracellular signalling and has been widely associated with memory processes (Dash, Hochner et al. 1990; Bourtchuladze, Frenguelli et al. 1994; Liu and Graybiel 1996; Silva, Kogan et al. 1998; Dalley, Thomas et al. 1999; Kogan, Frankland et al. 2000; Genoux, Haditsch et al. 2002; Zhang, Okutani et al. 2003) and synaptic plasticity (Deisseroth, Bito et al. 1996; Ahn, Ginty et al. 1999; Dalley, Thomas et al. 1999). CREB is considered to be critical for regulating gene expression dependent late-phase synaptic plasticity (Sweatt 2001). Dominant negative adenovirus transfection of PRH and Te2, preventing the actions of phosphorylated CREB (pCREB) within the nucleus, blocks long-term familiarity discrimination and LTP induction (Warburton, Glover et al. 2005).

1.6.4.2 c-*fos*

The expression of the protein product of immediate early gene (IEG) c-*fos*, Fos, increases with neuronal activity (Herrera and Robertson 1996) and has been associated with learning (Tischmeyer and Grimm 1999; Aggleton and Brown 2005; Spencer, Eckel et al. 2011). Fos levels in PRH and adjacent area Te2 increase following the presentation of novel visual stimuli in the PVP (Zhu, Brown et al. 1995; Zhu, McCabe et al. 1996; Wan, Aggleton et al. 1999; Warburton, Koder et al. 2003; Seoane, Massey et al. 2009). Inhibition of VGCC, PKC and disruption to CREB phosphorylation in PRH abolished this novelty-induced increase (Nadine Fontaine-Palmer, unpublished PhD thesis 2008;(Warburton, Glover et al. 2005; Seoane, Massey et al. 2009). Fos protein expression has been established as a marker of LTD in the cerebellum (Nakazawa, Karachot et al. 1993) and hippocampus (Lindecke, Korte et al. 2006) and is required for learning (Swank, Ellis et al. 1996).

As described above, multiple receptors, protein kinases and nuclear substrates have been identified as critical for long-term familiarity discrimination. However, the mechanisms of intracellular signalling in PRH are still far from understood. Among the intracellular signalling kinases are the MAPK signalling cascades, identified to have critical roles in synaptic plasticity mechanisms *in vitro* and established roles in learning and memory (English and Sweatt 1996; English and Sweatt 1997; Adams and Sweatt 2002; Thomas and Huganir 2004; Peng, Zhang et al. 2010). MAPK signalling relays extracellular signals to the nucleus (Treisman 1996). However, the roles of the MAPK signalling pathways in familiarity discrimination have yet to be investigated.

1.7 MAPK signalling cascades

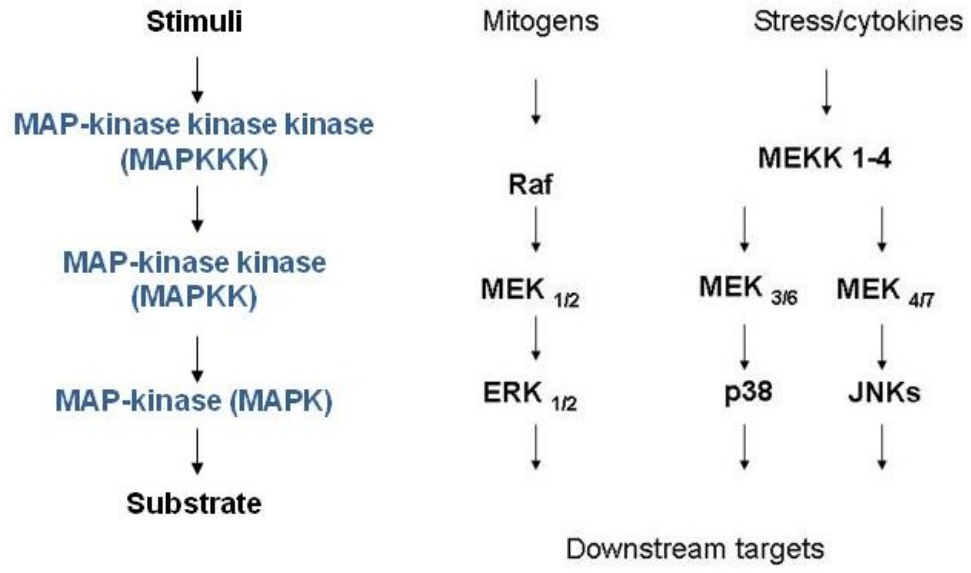
This section will characterise two of the MAPK signalling cascades, ERK and p38, and overview roles for these proteins in synaptic plasticity. Downstream targets of ERK will be considered in terms of their potential roles in learning and memory. In addition, evidence to suggest ERK as a point of convergence for other intracellular signalling will be discussed.

Downstream of the Ras family GTP-ases, Raf and Rap, are the MAPK signalling cascades identified to be important for mediating AMPA-R trafficking during synaptic plasticity (Zhu, Qin et al. 2002). MAPKs are critical regulators of cell proliferation, differentiation, development, apoptosis and inflammation (Seger and Krebs 1995). The abundant expression of MAPKs in post-mitotic neurons suggests an additional role for these kinases initially associated with processes underlying mitosis (Boulton, Nye et al. 1991; Fiore, Bayer et al. 1993; Thomas and Huganir 2004). Further, the role of MAPK in inducible gene expression supports the hypothesis that these kinases regulate synaptic plasticity and long-term memory (Impey, Obrietan et al. 1999; Thiels and Klann 2001). The best characterised of the MAPK subgroups are the extracellular-signal regulated protein kinases (ERKs), the c-Jun N-terminal kinases/stress-activated protein kinases (JNKs/SAPKs) and the p38 kinases (Belelovsky, Maroun et al. 2007).

1.7.1 Characterisation of the MAPK signalling cascade

MAPK cascades can be distinguished by a highly conserved cytosolic serially linked 3-kinase core, in which each protein is sequentially phosphorylated (Cobb and Goldsmith 1995; Widmann, Gibson et al. 1999; Sweatt 2001).

Figure 1.7.1.1 Simplified schematic representation of typical MAPK pathway arrangement. Serial arrangement of the highly characterised ERK, JNK and p38 MAPK pathways.



The initial protein kinase in the cascade is a MAP-kinase kinase kinase (MAPKKK) which dually phosphorylates the substrate specific MAP-kinase kinase (MEK) on serine and either threonine or tyrosine residues (Pearson, Robinson et al. 2001). The final step in the cascade is the activation of the downstream MAPK (ERK, p38 or JNK) which requires dual phosphorylation of both threonine and tyrosine residues by upstream MEK (Peng, Zhang et al. 2010). Scaffolding proteins are known to mediate the specificity of MAPK signalling (Roux and Blenis 2004). Although it has been reported that activation of these pathways is mutually exclusive (Roux and Blenis 2004), there is evidence to suggest that crosstalk between these pathways does exist (Dong and Bode 2003). Furthermore, the 3- tier arrangement of the kinase cascade allows MAPKs to be enhanced or suppressed by signals

received from other intracellular enzymes, e.g. PKA and PKC (Roberson, English et al. 1999).

1.7.2 Extracellular signal regulated protein kinases (ERKs)

The first identified and most widely studied MAPK are the ERKs (Seger and Krebs 1995).The ERKs exist in 2 isoforms: ERK-1 and ERK-2 (also known as p44 and p42, respectively) and share ~ 84% sequence homology (Pearson, Robinson et al. 2001). ERK-1/-2 have a threonine -glutamic acid- tyrosine (Thr-Glu-Try) motif in their activation loop and thus require dual phosphorylation on both threonine and tyrosine residues by the upstream MEK (Pearson, Robinson et al. 2001; Sweatt 2001). MEK 1 (the predominant MEK isoform in neurons) phosphorylates ERK 1 and 2 with the same kinetics (Zheng and Guan 1993; Adams and Sweatt 2002). MEK is phosphorylated on serine and threonine residues by the first kinase in the cascade, Raf (Raf-1 or B-Raf) (Pearson, Robinson et al. 2001). B-Raf is present in dendrites, whereas Raf-1 is most prominent in neuronal bodies (Morice, Nothias et al. 1999).

ERKs regulate their own activity by increasing transcription of phosphatases, required for their dephosphorylation (Sun, Charles et al. 1993; Muda, Theodosiou et al. 1996). However, there are circumstances in which ERK can remain persistently active. Activity-dependent modifications in dendritic spines, associated with long-term synaptic changes are dependent on prolonged ERK activation (Wu, Deisseroth et al. 2001), suggesting that both positive and negative feedback regulates ERK signalling (Adams and Sweatt 2002).

1.7.2.1 Activation of the ERK cascade

The ERK cascade can be elicited by a diverse range of stimuli, ranging from mitogenic factors such as epidermal growth factor (EGF) and nerve growth factor (NGF), to neurotransmitter receptors, such as dopamine receptors, mGluRs, muscarinic acetylcholine receptors and β-adrenergic receptors (Roberson, English et al. 1999; Sweatt 2001; Adams and Sweatt 2002). Activation can also result from activation of a small G-protein, Ras, which recruits Raf to the membrane and activates the Raf-ERK pathway (Adams et al., 2000).

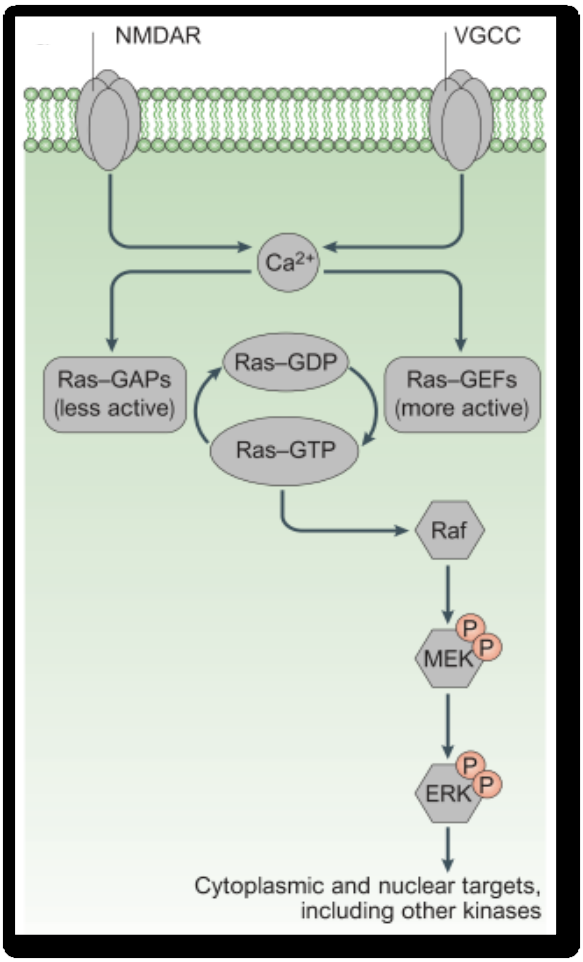
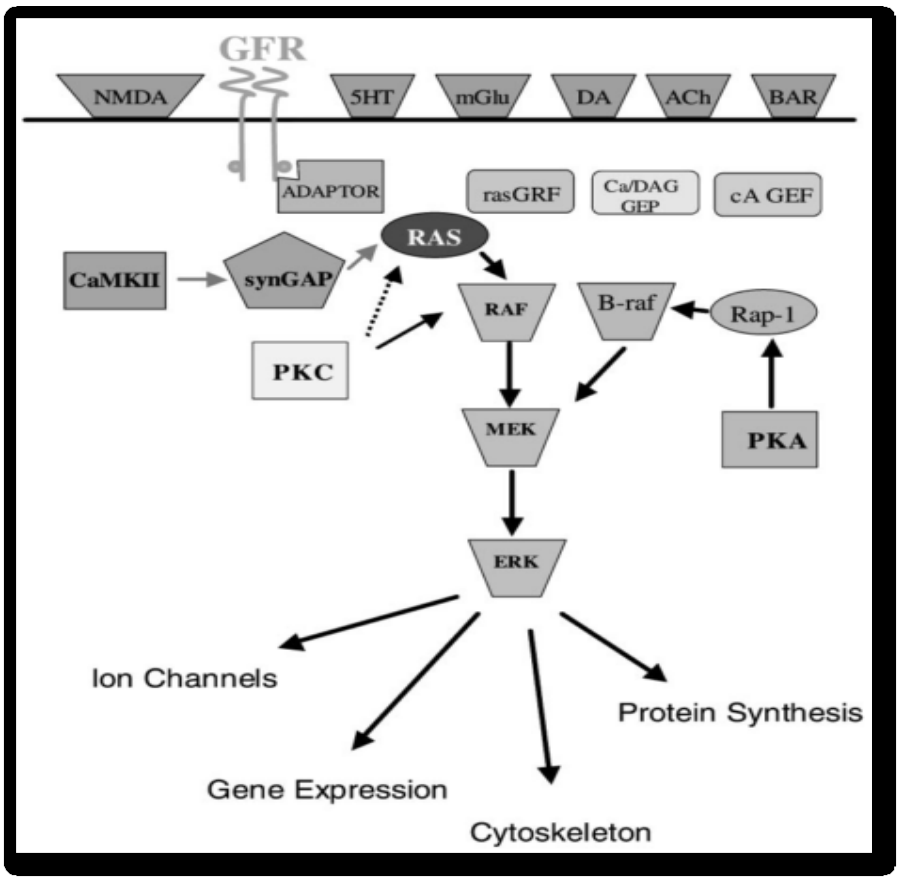


Figure 1.7.2.1.1 Activation of Raf via Ras-signalling. Activation of Raf via Ras is thought to result from an increase in Ca^{2+} via membrane receptors, NMDAR and VGCC. Ras-GTPase-activating proteins (Ras-GTPs) and Ras-guanyl nucleotide exchange factors (Ras-GEFs) are thought to regulate activity of Ras. Taken from (Thomas and Huganir 2004).

Some of the better characterised ERK-inducing pathways include:

1. Phosphorylation of tyrosine kinase receptors (RTK) following activation by growth factors, such as EGF and NGF. Adaptor proteins bind to the phosphorylated tyrosine residues of RTK and activate mSOS, a guanine nucleotide exchange protein that causes GTP exchange using Ras (Davis 1993; Seger and Krebs 1995; Downward 1996; Roux and Blenis 2004).
2. Activation of Ras resulting from increased levels of intracellular $Ca2^{+}$ (via NMDARs and VGCC), or PKC (Walker, Cullen et al. 2003; Peng, Zhang et al. 2010).
3. Ras- dependent and independent mechanisms via GPCRs (Peng, Zhang et al. 2010) (Figure 1.7.2.1.2).

Figure 1.7.2.1.2 Activation of the Raf-MEK-ERK cascade. Simplified diagrammatic representation of ERK signalling via Ras-dependent and independent pathways (Taken from Adams and Sweatt. 2002).



1.7.3 p38 MAPK signalling cascade

As characteristic of all MAPK subtypes, the p38 MAPKs are arranged in a linear fashion. Downstream kinase, p38s, exists in four isoforms that are encoded by different genes (p38 α, p38 β, p38 γ, p38 δ) and are named according to their molecular weight (38kDa) (Jahn, Schiebler et al. 1985; Kyriakis and Avruch 2001; Cuadrado and Nebreda 2010). P38 MAPKs are further characterised by the conserved, threonine-glycine- tyrosine (Thr-Gly-Tyr) motif in their activation loop thus requiring dual phosphorylation on both Thr180 and Tyr182 residues (Pearson, Robinson et al. 2001; Cuadrado and Nebreda 2010; Gong, Ming et al. 2010). ERK and p38 share approximately 48 % amino acid sequence (Fox, Coll et al. 1998).

P38α is ubiquitously expressed whereas the others are found to be tissue specific; p38 α and p38 β are highly expressed in the cerebral cortex, cerebellum, hippocampus and a few nuclei of the brain stem, but exist in different subcellular regions (Lee, Park et al. 2000). In contrast, p38 γ is expressed in skeletal muscle and is upregulated during muscle differentiation; whereas p38 δ is found in the endocrine glands, small intestines, kidneys and lungs (Lechner, Zahalka et al. 1996; Hu, Wang et al. 1999; Cuadrado and Nebreda 2010).

MEK 3/6 are the intermediate kinases in the p38 pathway and phosphorylate downstream p38s. MEK-3 has a higher affinity for p38 α and p38 β; whereas MEK-6 phosphorylates all isoforms (Enslen, Brancho et al. 2000). Crosstalk between p38 and JNK pathways exists, suggesting that perhaps the MAPK families are not as exclusively activated as first thought. MEK-3/6 can activate JNK/SAPK (Pearson, Greenwood et al.) and p38 α can also be phosphorylated by the JNK intermediate kinase, MKK4 (Brancho, Tanaka et al. 2003). The upstream kinase in the p38 cascade is not as selectively activated as its counterpart in the ERK signalling pathway. A plethora of MAPKKKs, including MEKK 1-4 and Rho family GTPases, Rap, can active MEK-3/-6 through a diverse range of stimuli (Dong, Ramachandiran et al. 2004; Roux and Blenis 2004).

1.7.3.1 Activation and roles for p38 MAPK signalling

P38 MAPKs are strongly activated in response to cellular stresses such as osmotic, oxidative, UV and DNA damage, inflammatory cytokines and growth factors (Chen, Hitomi et al. 2000; Ono and Han 2000; Kyriakis and Avruch 2001; Pearson, Robinson et al. 2001; Cuenda and Rousseau 2007; Cuadrado and Nebreda 2010). Although previously considered a stress-activated kinase, p38 is also known to be activated by G-protein coupled receptor agonists and neurotransmitters (Zhen, Uryu et al. 1998; Nebreda and Porras 2000).

Actions of p38 include inflammatory responses (such as cytokine expression), apoptosis induction, and modulation of gene expression (Lee, Laydon et al. 1994; Xia, Dickens et al. 1995; Hensley, Floyd et al. 1999; Dong, Ramachandiran et al. 2004). High p38 expression is known to induce apoptosis, whereas lower levels enhance cell survival (Dolado and Nebreda 2008).

1.7.4 Crosstalk between ERK and p38 pathways

The exact mechanisms underlying interaction between ERK and p38 are somewhat unknown. Evidence exists that the p38 cascade is negatively coupled to the ERK pathway (Chen, Hitomi et al. 2000) and results in rapid dephosphorylation of the intermediate kinase, MEK, via protein phosphatase 2 (PP2A) (Johnson and Lapadat 2002; Junttila, Li et al. 2008; Cuadrado and Nebreda 2010). However, research has also suggested that inhibition of p38 blocks ERK activation (Vincent, Sebben et al. 1998).

Despite consisting of specific, exclusive kinase cascades, activation of the ERK and p38 pathways and their target substrates are not mutually exclusive (Dong and Bode 2003). Downstream effectors of ERK and p38 include cytoskeletal proteins, signalling proteins and nuclear targets, such as transcription factors and IEGs (Treisman 1996; Hazzalin and Mahadevan 2002). This section will focus on substrates of MAPK signalling: MSKs and RSKs, voltage-gated potassium channel (Kv4.2) and transcription factors: CREB and Elk-1, which are important in mechanisms underlying synaptic

plasticity and learning and memory. Although p38 signalling is known to activate such targets in response to stress, whether or not p38 acts on these targets in mechanisms underlying synaptic plasticity is yet to be fully investigated.

1.7.4.1 Membrane ion channels and cytoplasmic targets

An important component in the regulation of neuronal excitability is the phosphorylation of voltage-gated potassium channels, in particular, Kv4.2 (Sweatt 2001; Rosenkranz, Frick et al. 2009). Kv4.2 channels are expressed in the soma and dendrites (Sheng, Tsaur et al. 1992) and regulate the magnitude of the excitatory post-synaptic potential (EPSP) following synaptic stimulation (Hoffman, Magee et al. 1997). Suppression of Kv4.2 channels increases membrane excitability, facilitating the induction of synaptic plasticity (Watanabe, Hoffman et al. 2002). ERK phosphorylates the pore forming subunit of Kv4.2 which results in increased excitability, leading to increased Ca^{2+} entry into the postsynaptic neuron via NMDA-receptors and voltage-dependent calcium channels, which in turn results in the induction of synaptic plasticity (Adams, Anderson et al. 2000; Sweatt 2001; Birnbaum, Varga et al. 2004). Whether or not p38 acts upon potassium channels during synaptic plasticity is yet to be established; however, p38s are thought to target mitochondrial K^+ATP channels (Ye, Guo et al. 2011).

1.7.4.1.1 MSK and RSK

Immediately downstream of MAPK signalling are mitogen and stress-activated protein kinases (MSKs) and 90 kDa ribosomal protein S6 kinases (RSKs), referred to as MAPKAPs. RSKs (1-4) are a family of serine/ threonine protein kinases downstream of the MAPK cascades that reside in the cytoplasm until phosphorylated (Pearson, Robinson et al. 2001; Anjum and Blenis 2008). MSK-1 and MSK-2 are required for transcriptional activation of cAMP-response element binding protein (Waskiewicz, Flynn et al. 1997; Deak, Clifton et al. 1998; Pierrat, Correia et al. 1998; Pearson, Robinson et al. 2001; Rakhit, Clark et al. 2005).

1.7.4.1.2 Transcription factors

The capacity for MAPKs to translocate between the cytoplasm and nucleus is consistent with critical roles for these kinases in regulating gene transcription (Patterson, Pittenger et al. 2001).

1.7.4.1.2.1 CREB

As previously discussed (section1.6.4.1), the transcription factor, CREB, is essential for learning and memory. It regulates late-phase gene-dependent plasticity, and numerous studies have shown that CREB transcription is downstream of MAPK signalling (Sweatt 2001). ERK is known to activate CREB, *in vivo*, (Davis, Vanhoutte et al. 2000; Zhang, Okutani et al. 2003) and *in vitro* (Treisman 1996; Impey, Obrietan et al. 1999; Roberson, English et al. 1999; Vanhoutte, Barnier et al. 1999). P38 can activate CREB directly, or via MSK and RSK (Price, Cruzalegui et al. 1996; Treisman 1996; Pierrat, Correia et al. 1998; Schiller, Bohm et al. 2006).

1.7.4.1.2.2 Elk-1

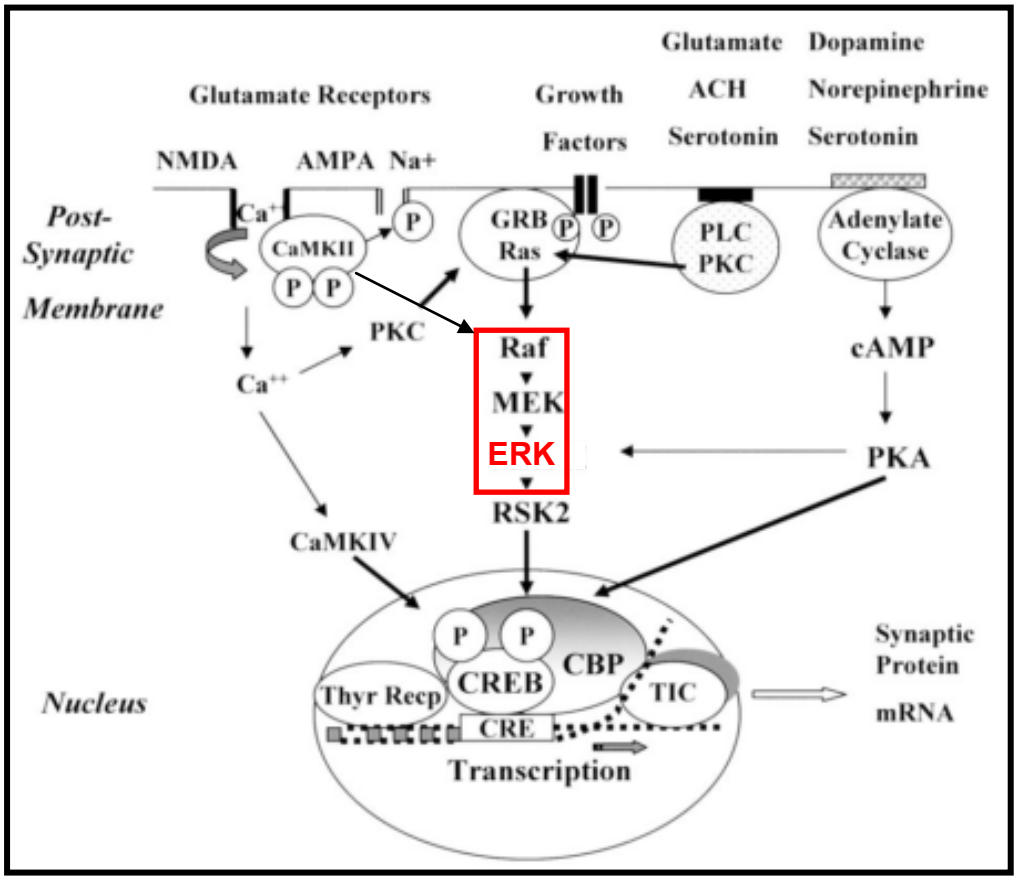
The ternary complex factor Elk-1 is a transcription factor that regulates expression of immediate early genes (IEGs) via the serum response element (SRE) site (Hill, Marais et al. 1993; Price, Cruzalegui et al. 1996; Besnard, Galan-Rodriguez et al. 2011). MAPK activates Elk-1 by phosphorylating two compulsory residues Ser 383 and Ser 389, resulting in nuclear translocation (Janknecht, Ernst et al. 1993; Gille, Kortenjann et al. 1995; Price, Cruzalegui et al. 1996; Treisman 1996; Whitmarsh, Yang et al. 1997; Besnard, Galan-Rodriguez et al. 2011). In addition to expression of IEGs such as c-*fos*, Elk- 1 promotes chromatin remodelling through histone phosphorylation in a MAPK-dependent manner (Thomson, Clayton et al. 1999; Thomson, Mahadevan et al. 1999; Chwang, O'Riordan et al. 2006; Besnard, Galan-Rodriguez et al. 2011). Post-translation, Elk-1 is translocated to the cytoplasm by a small ubiquitin-like modifier protein (SUMO) for reactivation (Besnard, Galan-Rodriguez et al. 2011). In vivo, the pattern of gene expression required for the induction of LTP is dependent on Elk-1 and CREB phosphorylation via ERK signalling (Davis, Vanhoutte 2000). Berman et al. (1998) demonstrated an

increase in levels of ERK and Elk-1 in the insular cortex, in response to novel tastes.

1.7.5 MAPK signalling as a point of convergence for intracellular kinases

Due to the linear arrangement of the MAPK cascade, an increase in abundance of MEK and ERK kinases compared to that of upstream Raf allows for amplification of intracellular signals (Huang and Ferrell 1996; English, Pearson et al. 1999).Several of the kinases identified to be involved in familiarity discrimination (reviewed in section 1.6.3) are known to influence the MAPK cascades both *in vitro* and *in vivo* (see below). Connections with the ERK pathway in mechanisms underlying learning and memory have been more widely documented than those of p38 (Figure 1.7.5.1); however, it is not understood whether these interactions occur in PRH.

1.7.5.1 MAPK signalling as a point of convergence for intracellular kinases. CaMKII, PKA and PKC can act upon the ERK cascade. Modified from Johnson and Lapadat. (2002).



- Hippocampal CaMKK inhibition blocks: ERK-dependent LTP induction (Schmitt, Guire et al. 2005), CaMKII-mediated AMPAR trafficking (Zhu, Qin et al. 2002) and increases in levels of ERK, *in vivo* (Vanhoutte, Barnier et al. 1999)

- In the hippocampus, PKA or PKC activation increases levels of p-ERK (Roberson and Sweatt 1996; English and Sweatt 1997; Banko, Hou et al. 2004; Sindreu, Scheiner et al. 2007)

- PKA and PKC regulate potassium channels via ERK signalling (Hoffman, Magee et al. 1997; Winder, Martin et al. 1999; Watabe, Zaki et al. 2000; Watanabe, Hoffman et al. 2002; Yuan, Adams et al. 2002; Morozov, Muzzio et al. 2003)

- PKA-induced CREB activation is mediated by ERK signalling in striatal neurons (Zanassi, Paolillo et al. 2001)

- BDNF-induced LTP activates p38 (Ying, Futter et al. 2002). *In vivo* inhibition of intrahippocampal BDNF blocks CREB-phosphorylation in an ERK-1/-2 dependent manner, with no effect on p38 levels (Alonso, Vianna et al. 2002).

1.7.6 Roles for MAPKs in synaptic plasticity

1.7.6.1 Roles for ERKs in synaptic plasticity

The seminal article by English & Sweatt, 1996, describing an increase in levels of phosphorylated ERK2 following LTP induction in area CA1 of the hippocampus, has spawned a plethora of evidence demonstrating additional roles for ERKs in synaptic plasticity in multiple brain regions, such as the dentate gyrus (Davis, Vanhoutte et al. 2000). ERK is critical for maintenance of NMDA-R-dependent LTP (English and Sweatt 1997; Atkins, Selcher et al. 1998; Coogan, O'Leary et al. 1999) (English and Sweatt 1997; Impey, Obrietan et al. 1999; Jin and Feig 2010) and NMDA-R-independent LTP, induced by bath application of forskolin (Wu, Lu et al. 1999; Kanterewicz, Urban et al. 2000). Additional roles for ERK have been established during early-LTP induction (Winder, Martin et al. 1999; Rosenblum, Futter et al. 2002). Following LTP induction, levels of pERK2 increase within 2 min and persists for 1 h, in area CA1 of the hippocampus (English and Sweatt 1996). Subsequent studies have demonstrated a role for this pathway in both early and late phase LTP in the hippocampus (Atkins, Selcher et al. 1998; Impey, Obrietan et al. 1999; Winder, Martin et al. 1999; Wu, Lu et al. 1999; Rosenblum, Futter et al. 2002).

In addition to a critical role in LTP, ERK activation has been associated with the mechanisms underlying LTD. ERK is activated following induction of both NMDAR-dependent (Thiels, Kanterewicz et al. 2002; Besnard, Galan-Rodriguez et al. 2011) and mGluR-dependent LTD (Kawasaki, Fujii et al. 1999; Otani, Auclair et al. 1999; Gallagher, Daly et al. 2004).

Early roles for ERK in synaptic plasticity include phosphorylation of K^+ channels, and regulation of AMPARs; whereas a critical role in gene transcription, via activation of CREB and Elk-1, is fulfilled during the late-phase (Endo and Launey 2003; Krapivinsky, Krapivinsky et al. 2003).

1.7.5.2 Roles for p38 in synaptic plasticity

The p38s have been identified to be involved in mechanisms underlying synaptic plasticity mechanisms *in vitro*. They are required for mGlu-R dependent LTD in hippocampal regions: CA1 (Bolshakov, Carboni et al. 2000; Moult, Correa et al. 2008) and dentate gyrus (Rush, Wu et al. 2002; Murray and O'Connor 2003); PFC (Zhong, Liu et al. 2008). In addition to mGluR-dependent LTD, p38 is also required for NMDAR-dependent LTD in the hippocampus (Schwarze, Ho et al. 1999; Zhu, Qin et al. 2002). In the primary visual cortex, LTD is dependent on p38 activation (Xiong, Kojic et al. 2006).

Addition roles for p38 in mechanisms underlying LTP have been established (Coogan and O'Connor 1999; Ying, Futter et al. 2002; Gisabella, Rowan et al. 2003; Toyoda, Zhao et al. 2007; Arai, Li et al. 2009). Inhibition of p38 rescues the inhibitory effect of beta amyloid peptide on LTP (Saleshando and O'Connor 2000).

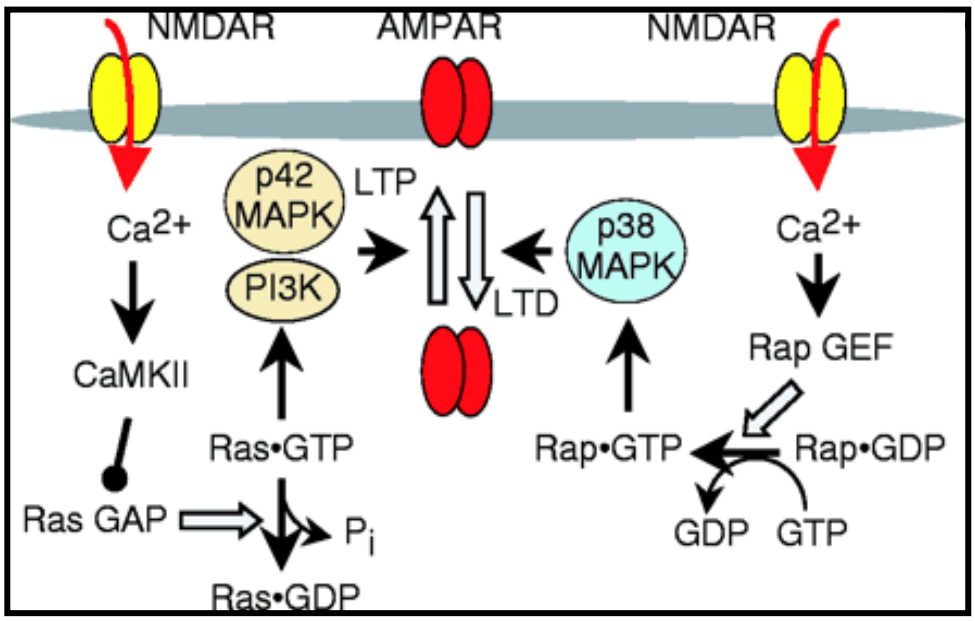
1.7.5.3 Biochemical changes and morphological changes associated with synaptic plasticity

AMPAR trafficking and remodelling of dendritic spines are cellular processes thought to underlie learning and memory (Thomas and Huganir 2004). Although the literature is somewhat inconsistent with regards to specific roles for ERK and p38 in plasticity functions, ERK is more widely associated with mechanisms underlying LTP, whereas p38 is more frequently suggested to underlie LTD. A simplified model suggests that differential roles for ERK and p38 in synaptic plasticity are dependent on upstream small GTPases, Ras and Rap, AMPA receptor trafficking, respectively (Zhu, Qin et al. 2002; Dong, Ramachandiran et al. 2004).

Ras activation leads to the insertion of AMPARs into the synapse following ERK activation; whereas Rap removes AMPARs in a p38 dependent manner (Zhu, Qin et al. 2002) (refer to Figure 1.7.5.3.1). The small G-protein Ras GTPases (Ras, Rap-1, Rap-2) that are found upstream of MAPK signalling cascades alternate between their inactive GDP-bound state and a GTP-bound

state that can elicit a downstream intracellular response, resulting in the insertion or removal of AMPARs from the synapse (Takai, Sasaki et al. 2001).

Figure 1.7.5.3.1 AMPA receptor trafficking is differentially regulated by MAPK/ERK and p38. ERK- 2 (p42) is required for insertion of AMPAR into the post synaptic membrane, associated with LTP. LTD is characterised by removal of AMPAR by p38 (Zhu, Qin et al. 2002).



The regulation of dendritic morphology is critical for neuronal development (Cohen and Greenberg 2008) . The formation of new dendritic spines is associated with LTP (Engert and Bonhoeffer 1999). Repetitive stimulation results in ERK-dependent changes in dendritic morphology and increase in the number of filopodia protrusions (Wu, Deisseroth et al. 2001).

1.7.6 MAPK identified to have critical role in learning and memory

Downstream targets of MAPKs are widely associated with mechanisms underlying learning and memory. ERKs are required for long-term memory consolidation, reconsolidation and retrieval, established in multiple brain regions (Schafe, Atkins et al. 2000; Sweatt 2001; Bozon, Kelly et al. 2003; Kelly, Laroche et al. 2003) (reviewed in section 3.1.1). Investigation into the role of p38 in memory processing is less well established, although a role in learning has been reported (Zhen, Du et al. 2001; Alonso, Bevilaqua et al. 2003; Rossato, Bevilaqua et al. 2006). Few studies have simultaneously investigated the roles of both kinases and therefore little is understood about possible interactions between these pathways during learning and memory. Dependent on the brain region and task investigated, the requirement for these kinases can differ.

1.7.7 Clinical roles for MAPK signalling

Clinical studies have demonstrated disruption to ERK signalling in cognitive disorders. Patients with low expression levels of ERK are characterised by microcephaly and deficits in neurodevelopment (Samuels, Karlo et al. 2008). Further, mutations to the ERK cascade have been associated with neurofibromatosis type 1 (Silva, Frankland et al. 1997) and rare human neuro-cardio-facial-cutaneous (NCFC) syndromes, whose symptoms include mental retardation (Rodriguez-Viciana, Tetsu et al. 2006). ERK can be activated by mutated oncogenic-Ras in which constant ERK activation results in an increase in proliferation, as seen in tumour cells, and thus inhibition of this pathway has been investigated as a potential anti-cancer treatment (Johnson and Lapadat 2002).

Increased levels of cytokine production associated with neurodegenerative diseases such as Alzheimer's disease result in an increase in p38 activation (Hensley, Floyd et al. 1999; Sheng, Jones et al. 2001). Inhibition of the p38 pathway has been suggested as a possible therapeutic target for such disorders (Munoz and Ammit 2010; Munoz, Ramsay et al. 2010; Yasuda, Sugiura et al. 2011).

1.8 Pharmacological inhibition of MAPK signalling

To elucidate the role of MAPK signalling in PRH-dependent familiarity discrimination, the use of reversible pharmacological inhibitors will be used to assess the requirement for ERK and p38 at various time points during the NOPT (Chapter 3). ERK and p38 pathways will be inhibited using MEK inhibitor, U0126, and p38 inhibitor, SB203580, respectively, to assess the role of these kinases in processes underlying recognition memory.

1.8.1 MEK inhibitor, U0126

U0126 (1,4-Diamino-2,3-dicyano-1,4-*bis*[2-aminophenylthio]butadiene) is a potent, non-competitive MEK inhibitor first discovered to block AP-1 transcriptional activity via MAPK signalling, *in vitro* (Alessi, Cuenda et al. 1995; Duncia, Santella et al. 1998; Favata, Horiuchi et al. 1998; English and Cobb 2002). Enzymatic assays have demonstrated that U0126 blocks MEK phosphorylation and subsequent activation of ERK as a result of binding to a distinct, alternative site to that of MEK substrates ERK and ATP (Favata, Horiuchi et al. 1998; English and Cobb 2002). U0126 interacts with inactive unphosphorylated MEK and prevents conformational changes required to aid phosphorylation. The half maximal inhibitory concentrations (IC_{50}) of MEK 1 and MEK 2 are 0.07 μM $\pm$ 0.02 and 0.06 μM $\pm$ 0.02, respectively. Further, *in vitro* kinase assays have established U0126 has a considerably lower IC_{50} value for MEK than other protein kinases (Table 1.8.1.1) (Davies, Reddy et al. 2000). U0126 (20 μM) has no effect on protein kinases: PKA, PKC and CaMKII (Roberson, English et al. 1999). The inactive analogue, U0124, does not inhibit MEK at concentrations up to 100 μM (Favata, Horiuchi et al. 1998).

Table 1.8.1.1 U0126 IC_{50} values. *In vitro* kinase assay IC_{50} values demonstrating U0126 inhibition of protein kinases (modified from Favata et al., 1998).

Protein Kinase	IC50 (μM)
MEK 1	0.072
MEK 2	0.058
ERK 1/ 2	> 50
MKK3 (p38)	38
MKK6 (p38)	20
JNK	> 100
PKC	> 100

1.8.2 p38 inhibitor, SB 203580

The pyridinyl imidazole, SB 203580(4-[5-(4-Fluorophenyl)-2-[4-(methylsulphonyl)phenyl]-1*H*-imidazol-4-yl]pyridine hydrochloride), has been identified as a highly specific inhibitor of p38α and p38β, downstream kinases in the p38 cascade (Cuenda, Rouse et al. 1995; Davies, Reddy et al. 2000).The use of pyridinyl imidazoles have been widely established as important treatments in inflammatory diseases (Lee, Laydon et al. 1994; Badger, Bradbeer et al. 1996; Wadsworth, Cavender et al. 1999; Lee, Kumar et al. 2000; Underwood, Osborn et al. 2000). The fluorophenyl ring of the SB 203580 inhibitor attaches to the ATP binding pocket of the p38 (ATP competitor). P38 has a relatively high K_m for ATP (binds ATP weakly) and therefore allows SB 203580 to successfully compete (Thomas and Huganir 2004). P38 isoforms p38α and p38β are sensitive to SB 203580 inhibition at submicromolar concentrations, whereas p38 δ and p38γ are not, due to a methionine-threonine substitution at position 106, within the active site (English and Cobb 2002). Further, SB 203580 (100μM) *in vitro* shows no selectivity for other MAPK subfamilies: ERK and JNK (Cuenda, Rouse et al.

1995). IC_{50} values for p38α and p38β are 50 and 500 nM, respectively (Tocris, UK). SB 203580 has been widely used to determine the role of p38 both *in vitro* (Moult, Correa et al. 2008; Peineau, Nicolas et al. 2009) and *in vivo* (Zhen, Du et al. 2001; Alonso, Bevilaqua et al. 2003; Rossato, Bevilaqua et al. 2006).

Table 1.8.2.1The effects of SB 203580 on protein kinase activity. Data listed demonstrates % activity remaining for each specified target at each SB203580 concentration. Modified from Cuenda et al, (1995).

Protein Kinase	SB 203580 Concentration (μM)	Activity (% control)
p38 α, β	1	35 ± 1
p38 α, β	10	5 ± 1
ERK 2	100	97 ± 3
MAPKK	100	93 ± 4
JNK	100	86 ± 4
cRaf	100	87 ± 2
PKA	100	97 ± 1

1.9 Thesis objectives

The MAPK signalling pathway has been widely associated with synaptic plasticity and processes underlying learning and memory. MAPK subtype, ERKs, have been widely established in mechanisms underlying learning and memory; however, the role of p38s have been studied to a lesser extent. Some findings suggest these kinases are differentially induced, whilst other studies have found they can be activated simultaneously, dependent on the task and brain region assessed. The role of both kinases in processes underlying perirhinal-dependent familiarity discrimination, a component of recognition memory, has yet to be considered. This thesis will investigate the roles of ERK and p38 signalling cascades in recognition memory using the single-trial novel-object preference task (NOPT) and associative object-in-place (OIP) task, using selective inhibitors of each pathway. Furthermore, the time course of kinase activation following the presentation of previously encountered and novel visual stimuli using the PVP will be assessed.

Chapter 2. Materials and Methods

2.1 Experimental subjects

All experiments were conducted using male pigmented rats (Dark Agouti strain; Bantin and Kingman, Hull, UK), weighing between 200–250 g at the start of the experiment. Animals were housed under a 12 h light/dark cycle (light phase: 6:00 PM to 6:00 AM), with behavioural training and testing conducted during the dark phase of the cycle.

All animal procedures used throughout this thesis were performed in accordance with the United Kingdom Animals Scientific Procedures Act (1986) and associated guidelines and had approval from the University of Bristol Ethics Committee. All efforts were made to minimize any suffering and the number of animals used during all experimental procedures.

2.2 Behavioural Tasks

All experimental details described in the behavioural methodology of this thesis are based on techniques previously published within the research group (Warburton, Koder et al. 2003; Barker, Warburton et al. 2006; Barker, Bird et al. 2007; Barker and Warburton 2008; Griffiths, Scott et al. 2008; Seoane, Massey et al. 2009; Tinsley, Narduzzo et al. 2009; Seoane, Tinsley et al. 2011; Tinsley, Narduzzo et al. 2011).

2.2.1 Bilateral perirhinal cannulation surgery

Animals were initially anaesthetized in a chamber with 4% isoflurane (Merial Animal Health Ltd, UK) in medical gas (95% O_2, 5% CO_2) at the rate of 1.5-2 l/ min until muscular tone had been lost, before being secured in a Kopf stereotaxic frame with the incisor bar set 3.3 mm below the interaural line. The animals' head was shaved and cleared from hair clippings before a midline incision was made to reveal skull landmarks, lambda and bregma. The height of lambda and bregma were calculated to ensure both values were

within 0.5 mm of each other. Isoflurane levels were monitored and maintained between 2-3 % throughout surgery to ensure breathing was maintained at a steady rate. Two stainless steel guide cannulae (26 gauge; Plastics One, via Bilaney, UK) targeted at perirhinal cortex were implanted through burr holes made in the skull at an angle of 20° to the vertical, with coordinates relative to bregma: AP = - 5.6 mm; L = ± 4.47 mm; V = - 6.7 mm (relative to top of the dura). Guide cannulae were anchored to the skull using two stainless steel skull screws (Plastics One, via Bilaney, UK) and fixed in place using DePuy CMW dental acrylic (Johnson & Johnson company, UK). Between experimental infusions, 33 gauge obdurators (Plastics One, via Bilaney, UK) were used to seal the cannulae to prevent infection and blockages. Throughout the surgical procedure animals were given a total of 5 ml saline fluid replacement therapy (Animalcare Ltd, UK) subcutaneously. Following midline suturing (Mersilk W501, Ethicon) 0.05 ml Vetgesic (Alstoe Animal Health, UK) was administered intramuscularly to provide post-operative analgesia. Animals were further treated with antiseptic veterinary wound powder (Hayward & Bower Ltd, UK) to prevent infections of the sutured area.

Table 2.2.1.1 Bilateral perirhinal surgery coordinates. Anterior-posterior and medial-lateral measurements were taken relative to bregma on a flat skull, and dorsal-ventral measurements were taken from the top of the dura at the angle stated.

Cannula	anterior-posterior	medial-lateral	dorsal-ventral	angle
PRH	- 5.6 mm	± 4.5 mm	- 6.7 mm	20°

2.2.2 Post-Surgery care

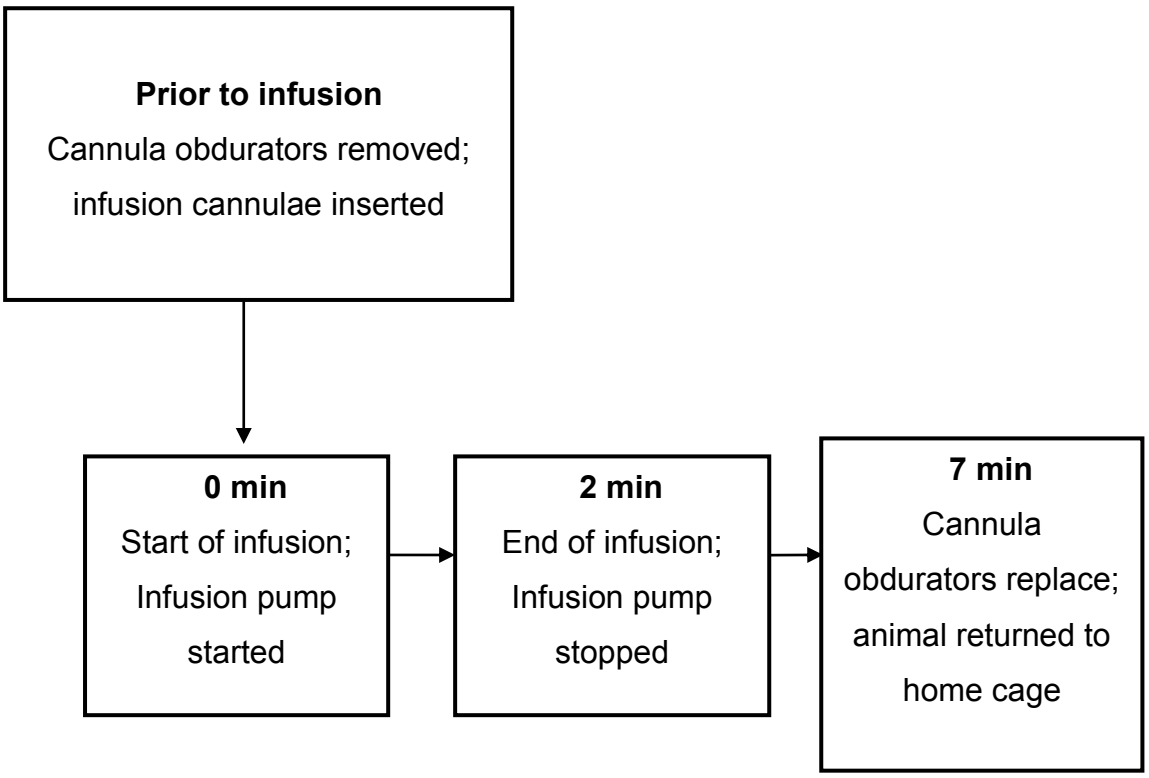
After surgery, animals were individually housed in cages adapted from terrapin tanks (40 x 25 cm, height measuring 30 cm) for one week before being housed in pairs. All animals were allowed to recover for 14 days before habituation to the testing arena began. During the recovery period, animals were monitored daily to ensure they had returned to their pre-surgery body weight.

2.2.3 Intracranial drug infusions

Guide cannulae obdurators were removed prior to drug infusions and stored in 70% alcohol until dried and replaced. Intraperirhinal infusions were administered bilaterally via guide cannulae using infusion cannulae with a 1 mm projection (Plastics One via Bilaney, UK). Drug- or vehicle- filled plastic tubing (0.5 mm internal diameter, 1.0 mm outer diameter) was attached to silicone tubing (0.5 mm bore, 0.5 mm wall), in turn attached to an infusion pump (Harvard Apparatus, US) via 25 µl glass Hamilton syringes, filled with distilled water (dH_2O). Tubing was loaded with drug solutions using disposable plastic syringes fitted with 0.6 mm gauge needles and flushed through with dH_2O following each infusion; plastic tubing was assigned to each drug and vehicle treatment to prevent contamination between solutions.

All PRH infusions were administered at a rate of 0.5 µl/min/hemisphere for 2 min. Infusion cannulae remained in place for a further 5 min to prevent backflow of the drug.

Figure 2.2.3.1 Schematic representation of typical drug infusion protocol. Following removal of cannula obdurators, infusion cannulae were inserted into the guide cannulae for the start of a 2 min infusion. Infusion cannulae remained in place for a further 5 min before obdurators were replaced and animals returned to their home cage.



2.2.4 Pharmacological inhibitors

Inhibitors U0126, and SB203580 (SB203580 hydrochloride) were used to inhibit MAPK pathways: ERK and p38, respectively. Control drug infusions were of the vehicle in which the active drug compound was diluted (Table 2.2.4.1).

Drug concentrations of U0126 and SB203580 were calculated using IC_{50} values to ensure that each compound was specifically inhibiting MEK, the intermediate kinase in the ERK cascade (Duncia, Santella et al. 1998; Favata, Horiuchi et al. 1998) or p38, the downstream kinase in the p38 cascade (Cuenda et al., 1995), respectively (1.8.1 and 1.8.2).

Table 2.2.4.1 Pharmacological inhibitors: dose and vehicle composition. Control solutions were composed of the vehicle in which the drug was diluted. All drugs were supplied by Tocris Bioscience, Bristol, UK.

Drug	Dose	Vehicle
U0126 (1,4-diamino-2,3-diacyano-1,4-bis 2-aminophenylthio]butadiene)	1 μM	0.05% DMSO/saline
SB203580 hydrochloride (4-[5-(4-Fluorophenyl)-2-[4-(methylsulphonyl)phenyl]-1H- imidazol-4-yl]pyridine hydrochloride)	50 μM	0.25% DMSO/saline

2.2.5 Spontaneous novelty preference tasks and behavioural arena

All experiments described in Chapter 3 took place in an open-top wooden arena measuring 50 x 90 x 100 cm, covered with a layer of sawdust on the floor. Objects were placed near two neighbouring corners of the arena, approximately 15 cm from each adjacent wall. The grey inner walls of the arena were surrounded with a black cloth to a height of 1.5 m to reduce the effects of room stimuli during the object recognition experiments. An overhead camera and a video recorder were used to monitor and record the animal's behaviour for subsequent re-analysis. All behaviour took place in a room dimly lit by two 40 watt lamps.

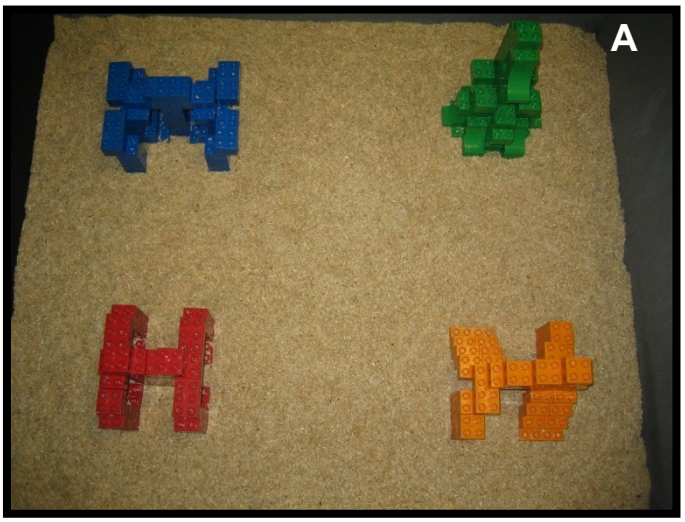
For the object-in-place task, one of the grey arena walls was replaced with a black wall and the black cloths surrounding the arena on two of the four sides were removed in order to allow extra-arena spatial cues to be seen.

2.2.6 Objects

Objects used throughout the behavioural experiments consisted of either Duplo™ blocks (Lego UK) or junk objects. Three identical copies of each object were used for the novel object preference task (A1-A3; B1-B3). Four different objects were used for the object-in-place task (A-D); the position of a pair of objects was rearranged in the test phase (Figure 2.2.8.1).

Duplo™ objects were constructed by the experimenter and designed in pairs to be of similar intrinsic interest to the rat, ensuring that animals exhibited equal exploration levels for an individual object. Duplo™ objects increased in complexity as the number of experiments on a given rat increased. Suitable junk objects, e.g. glass jars and plastic bottles that were too heavy for the animal to displace, were also used (Figure 2.2.6.1). Between each usage, objects were wiped clean with absolute alcohol to reduce olfactory cues.

Figure 2.2.6.1 Examples of objects used in the preferential exploration tasks. A - examples of Duplo™ objects built by the experimenter; B - examples of pairs of junk objects tested in the novel object preference task.



2.2.7 Habituation

During habituation to infusion procedures and the test arena, animals were handled daily by the experimenter, allowing for cannula obdurators to be removed and for any cannula blockages to be detected. This training was also used as an opportunity to familiarise the rat with the sound of the infusion pump, although no drug infusions were administered during this time. In addition to handling, animals were allowed to freely explore the empty arena for 5-10 min daily, 4 consecutive days before the start of any experimental procedures.

2.2.8 Spontaneous novelty preference exploration tasks

The spontaneous novelty preference exploration paradigms exploit animals' natural propensity to explore novelty. This paradigm consists of 3 phases: a sample phase, a delay and a test phase (Figure 2.2.8.1). Depending on the nature of the paradigm, the initial sample phase allows the animal to become familiarised with a pair of identical objects (novel object preference task) or the relative positions of a set of four objects (object-in-place task). The sample phase is followed by a retention delay in which the animal is removed from the arena, and finally a test phase in which the animal's preference for novelty is assessed.

2.2.8.1 Novel object preference Task

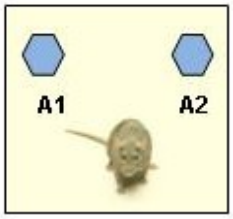
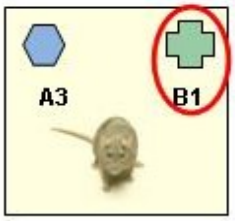
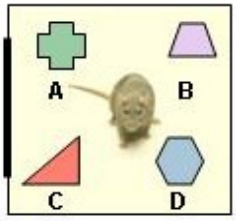
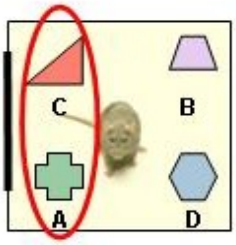
The novel object preference task (NOPT) allows visual recognition memory to be tested by measuring spontaneous behaviour in the rat (Ennaceur and Delacour 1988). During the sample phase of the NOPT, the animal was allowed to explore duplicate copies of an object (A1 and A2) which was then considered the familiar object in the test phase. The subject was placed into the arena facing the centre of a wall with its head facing away from the objects and allowed a total of either 40 s of exploration of A1 and A2, or a total of 4 min in the arena. Objects were placed approximately 15 cm away from the far corners of the arena. Following the sample phase, the animal was

returned to its home cage and later, following varying delays, reintroduced to the arena for the 3 min test phase in which a third copy of the previously seen object (A3) and a novel object (B1) were presented (Figure 2.2.8.1).

2.2.8.2 Object-in-place task

The object-in-place (OIP) task is a variation on the spontaneous NOPT in which associations between objects and the positions in which they are encountered are assessed (Dix and Aggleton 1999). As described in 2.2.5 the arena was modified for the OIP task to encourage the use of spatial cues. The OIP sample phase allowed the animal to explore four objects that were each positioned 15 cm from two walls of the arena for a total of 5 min. No maximum total sample length was imposed (Barker, Bird et al. 2007) (Barker et al., 2007). Twenty four hours after the sample phase, animals were reintroduced to the arena where the same four objects were present and the same four positions were occupied; however, two of the objects had exchanged positions (Figure 2.2.8.1).

Figure 2.2.8.1 Schematic representation of behavioural tasks: novel object preference task (NOPT) and object-in-place (OIP). Each task consists of three phases: sample, delay and test. The sample phase allows the animal to become familiarized with either: an object or arrangement of four objects in the NOPT and OIP tasks, respectively. Following a retention delay, dependent on the experiment, the animal was reintroduced to the arena for the test phase in which either: a novel object had been introduced or the positions of a pair of objects had been exchanged. The position of the rat illustrates the start position of the animal for each task. The thick black line indicates a change in colour of the arena inner wall, in order to encourage the use of spatial cues.

Task	Sample phase	Delay	Test phase
Novel object preference task (NOPT)	 4 min (40 sec exploration)	Varying delays	 3 min
Object-In-Place (OIP)	 5 min	24h	 3 min

2.2.9 Experimental design and counterbalancing

Each object was used as novel for half the animals and familiar for the other half, so that usage of the objects was counterbalanced. Experiments were further counterbalanced to ensure the position of the novel object in the arena in the test phase was varied between left and right, correcting for any possible position bias.

The novel object preference task was tested over two sessions in a cross-over design, with each subject receiving both drug and vehicle treatments over the course of the experiment. Equal numbers of animals were assigned to drug and control conditions during the first half of an experiment; in the second half of the experiment those previously administered vehicle received drug, and vice versa (Table 2.2.9.1). In the OIP task, the experiment was tested in three parts, with subjects' assigned vehicle, U0126 and SB203580 over the course of the experiment.

Table 2.2.9.1 An example of experimental counterbalancing in the novel object preference task. Equal numbers of animals were presented object A and object B in the sample phase, which would become 'familiar' in the test phase. The position of novel object was counterbalanced in the test phase to correct for any possible position bias. Assigned drug treatments were countered balanced between object presented in sample and novel position in the test phase.

Rat	Experiment part I				Experiment part II			
	Object presented in sample	Novel object in test phase	Position of novel object	Treatment (Vehicle/drug)	Object presented in sample	Novel object in test phase	Position of novel object	Treatment (Vehicle/drug)
1	*A*	*B*	*LHS*	D	*C*	*D*	*RHS*	V
2	*A*	*B*	*RHS*	D	*C*	*D*	*LHS*	V
3	*A*	*B*	*LHS*	V	*C*	*D*	*RHS*	D
4	*A*	*B*	*RHS*	V	*C*	*D*	*LHS*	D
5	*B*	*A*	*RHS*	V	*D*	*C*	*LHS*	D
6	*B*	*A*	*LHS*	D	*D*	*C*	*RHS*	V
7	*B*	*A*	*RHS*	D	*D*	*C*	*LHS*	V
8	*B*	*A*	*LHS*	V	*D*	*C*	*RHS*	D

2.2.10 Classification of exploration

Throughout the behavioural experiments, the experimenter was blind to the drug treatment assigned to each animal, in order to prevent a scoring bias during the test phase. Exploratory behaviour was classified as the time spent actively exploring the objects, with the animal's nose being < 2 cm from the object. Biting, climbing or sitting on the objects was not included as active exploration.

Exploration was recorded using the *Object Recognition Scoring Programme* created by Dr.T.J.Bussey, University of Cambridge, as used in previous

studies (Warburton, Koder et al. 2003; Warburton, Glover et al. 2005; Barker, Bashir et al. 2006; Barker, Warburton et al. 2006; Barker, Bird et al. 2007; Barker and Warburton 2008; Seoane, Massey et al. 2009; Tinsley, Narduzzo et al. 2009; Seoane, Tinsley et al. 2011; Tinsley, Narduzzo et al. 2011). One key was assigned to mark exploration for the object(s) on the left hand side of the arena, with a second key assigned as that for the right hand side. A bout of exploration was initiated by the first keystroke and a second keystroke marked the end. The total exploration for each object was calculated at the end of the session using the scoring programme.

The discrimination ratio, DR, was used as an index of the preference for novelty versus familiarity and calculated using exploration times from the test phase. A DR value of zero represents equal exploration of both novel and familiar objects in the test phase and thus no novelty preference.

Discrimination ratio, DR
= (time spent exploring novel object – time spent exploring familiar object)/ total exploration time (novel + familiar)

Sample exploration, total sample length (during the NOPT 240 s sample phase, 40 s exploration terminates the session) and test exploration times were calculated for each experiment using the scoring programme and used for subsequent statistical analysis. Total exploration times were measured to allow comparisons of motivation and motor activity between drug and control groups. Subjects failing to complete the minimum level of exploration in the sample or test phase were excluded from statistical analysis as in previously published studies (Barker, Bird et al. 2007; Barker and Warburton 2008). A minimum level of 10 s exploration in each sample and test phase was set for behavioural experiments in this thesis (Tinsley, Narduzzo et al. 2009)

2.2.11 Statistical analysis

Parametric statistical analysis was used to investigate interactions between treatment (vehicle or inhibitor) and delay (test delay or post-infusion delay). Mean DR values were analysed using two-factor (drug treatment, delay) repeated-measures analysis of variance (ANOVA). Interactions between drug treatment and delay, and effects of drug treatment, and delay on DR values were reported. Subdividing the analysis, ANOVA (treatment) was used to determine the effect of drug treatments at individual time points. Univariate analysis was used to analyse individual drug treatments, e.g. U0126 DR values across delay time points. Subsequent post-hoc Tukey's test analysis was used to make pairwise comparisons when a significant effect of delay was found. Novelty preference was established by one sample *t*-test of mean DR values versus zero (where zero represents an equal time spent exploring the novel and familiar objects, thus no novelty preference).

To determine if drug treatment or delay time points had any effect on levels of exploration, repeated-measures ANOVA (drug treatment, delay) for mean sample exploration times, total sample lengths and test exploration times were analysed as above (see Chapter 3). All statistical analyses used a significance level of 0.05.

2.2.12 Histology

Following the completion of behavioural experiments, subjects were euthanized using sodium pentobarbital (Euthatal, Merial Animal Health Ltd, Harlow, UK) administered intraperitoneally and perfused transcardially with 0.1 M phosphate buffer (PB) pH 7.4 and fixed with approximately 250 ml 4% paraformaldehyde (Adams, Anderson et al.) pH 7.4. Brains were post-fixed in PFA for a minimum of 24 h and cryoprotected in 30% sucrose in 0.2 M PB for 48 h at 4^{o}C. Coronal sections were cut at 40 µm on a cryostat (Leica CM2050S) and directly mounted onto gelatine coated slides. Once dry, sections were dehydrated using 70% ethanol before being staining for Nissl bodies using cresyl violet for approximately 15 min. Once heavily stained, sections were reduced used using 70% ethanol plus six drops acetic acid

before being placed in xylene and cover-slipped using DPX mounting medium, allowing confirmation of cannula positions using a light microscope (DM5000B, Leica, Germany) (Figure 2.2.12.1).

Figure 2.2.12.1 Cresyl violet staining protocol. Detection of nissl bodies in mounted brain sections using cresyl violet stain.

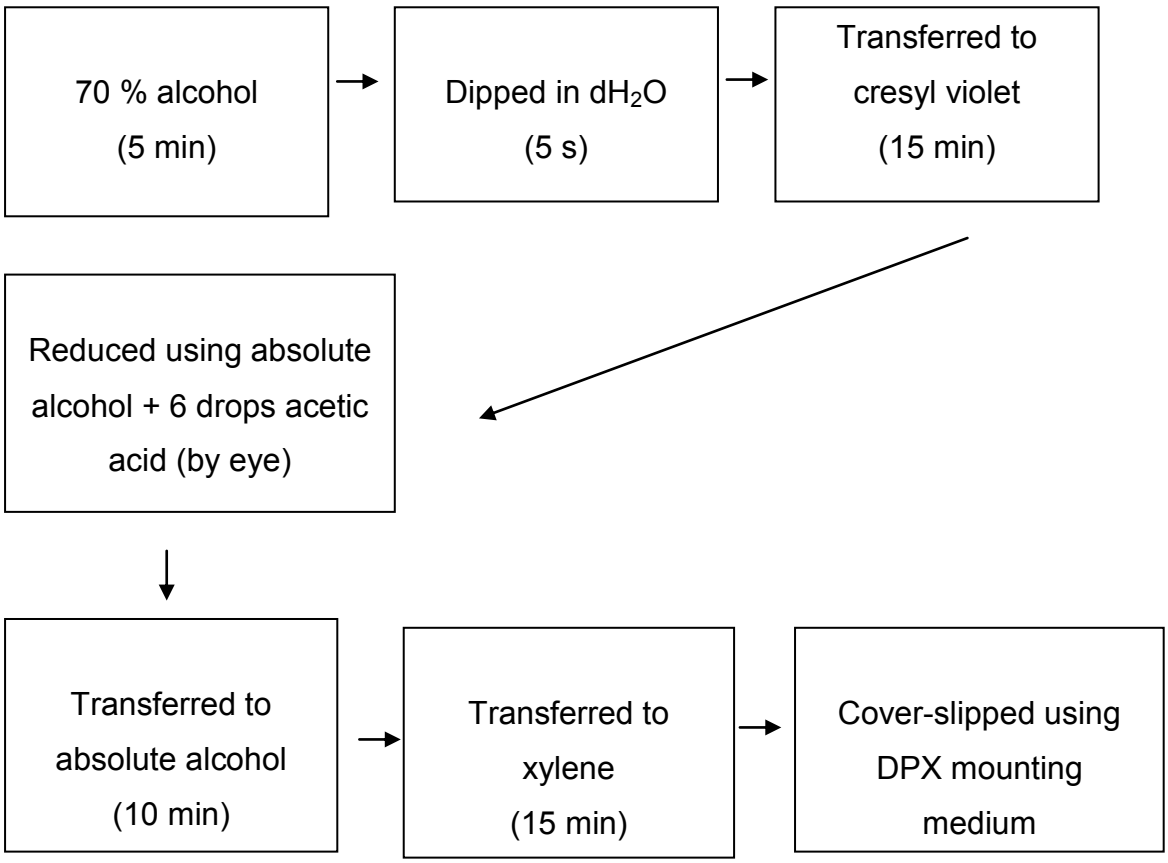
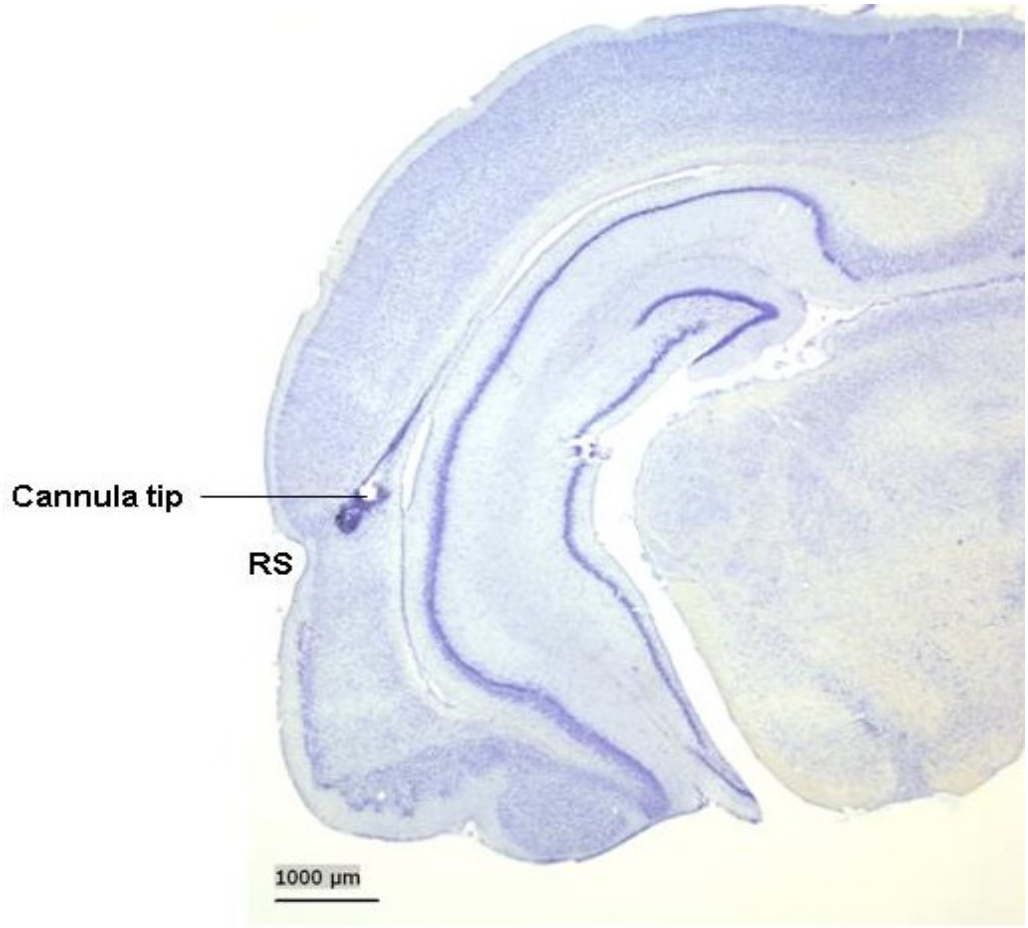


Figure 2.2.12.2 Coronal nissl-stained brain section displaying intraperirhinal cannula track. Infusion cannulae extend 1mm from the cannula tip and demonstrate site of drug infusion. Cannula position demonstrated on a section approximately -5.6 mm from bregma. (Abbreviations: RS – rhinal sulcus).



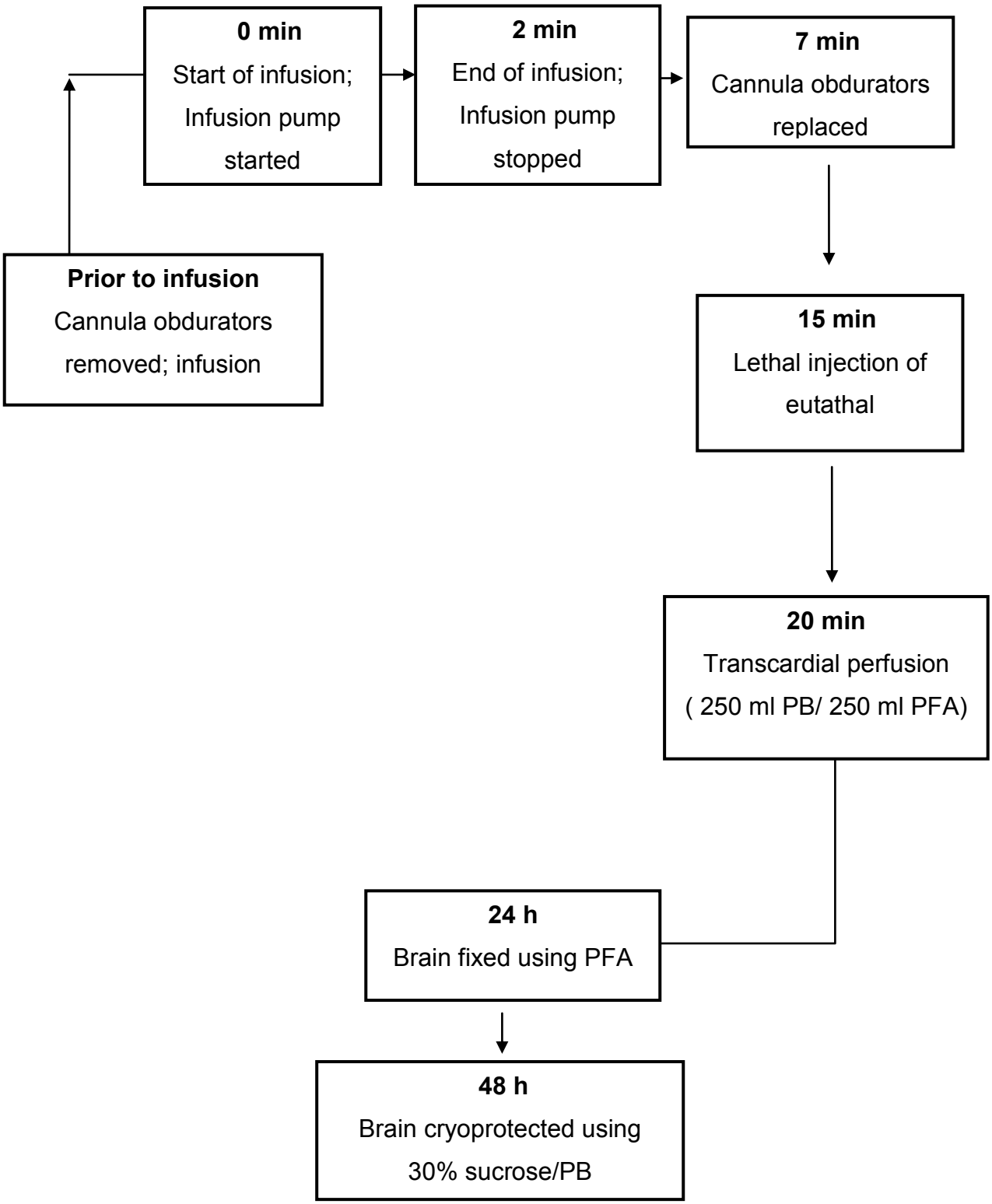
2.3 Quantification of drug spread using intraperirhinal infusions of fluorophore-conjugated muscimol

To determine the extent of drug following intraperirhinal infusions, flurophore-conjugated muscimol (FCM) (Muscimol, BODIPY® TMR-X conjugate; Invitrogen, UK) was used to visualise the localisation of spread (Allen, Narayanan et al. 2008). FCM was diluted to a concentration 0.5 mg/ ml (5 % DMSO/saline). FCM filled tubing was prepared as previously described in 2.2.3. Drug handling and infusion took place in a darkened room in order to prevent fluorescent degradation.

2.3.2 Intraperirhinal infusions and tissue perfusion

Animals were bilaterally infused with FCM for 2 min at a rate of 0.5µl/ min per hemisphere. Infusion cannulae were left in place for a further 5 min to prevent backflow of the drug. Animals were administered 1 ml of Euthatal 15 min following the start of the infusion and transcardially perfused using approximately 150 ml of both PB and PFA once breathing had become shallow and before the heart had stopped. Perfused brains were immediately removed and stored in PFA for 24 h before being transferred to 30% PB/sucrose for cryoprotection.

Figure 2.3.2.1 FCM infusion and post-perfusion timeline. Details of the time course of the FCM intraperirhinal infusion.



2.3.3 Brain sectioning and microscopic imaging

Once cryoprotected, brains were sectioned using a cryostat (Leica CM2050S) at 40 µm. Every slice was collected and mounted directly onto positively charged glass slides. In order to visualise the fluorescent dye and cellular location, brain sections were mounted with Vectashield HardSet ^{TM} Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector laboratories, UK), coverslipped and imaged using a microscope (DM5000B, Leica, Germany). Images were taken using both L5-blue filters (to detect DAPI stained cells) and A4-UV filter (image fluorescence) and merged to create an overlaid image (Chapter 3).

2.3.4 Quantification of FCM spread

Anterior-posterior (A-P) drug spread was calculated measuring the extent of FCM spread across serial sections (each section corresponded to 40 µm). Medial-lateral (M-L) and dorsal-ventral (D-V) spread was measured using imaging programme, IMAGEJ (National Institutes of Health, USA). Mean A-P, M-L and D-V values were calculated before maximal volume was determined using equation (elliptical volume = 4/3 π(radius A-P.M-L.D-V)).

2.4 Quantification of ERK and p38 activation in response to visual stimuli

All experimental details described in the PVP methodology of this thesis are based on techniques previously published within the research group (Warburton, Koder et al. 2003; Wan, Warburton et al. 2004; Warburton, Glover et al. 2005; Seoane, Massey et al. 2009; Tinsley, Narduzzo et al. 2009; Tinsley, Narduzzo et al. 2011).

2.4.1 Experimental subjects

All experiments were conducted using male pigmented rats (Dark Agouti strain; Bantin and Kingman, Hull, UK) weighing 150-175 g at the start of the experiment. Animals were restricted to 2 h *ad-libitum* water per day. Animals were maintained under a 12 h light/dark cycle (light phase: 6:00 PM to 6:00 AM), with behavioural training and testing conducted during the dark phase of the cycle.

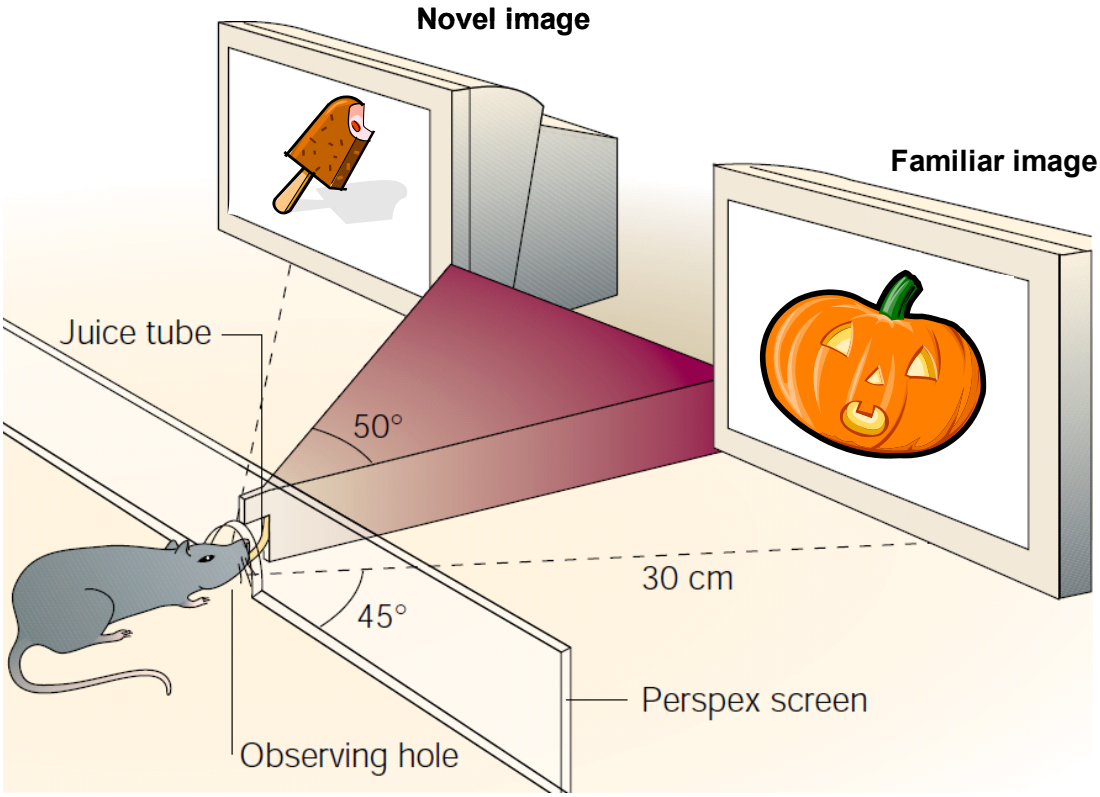
2.4.2 Paired viewing apparatus

The PVP apparatus consisted of a black-sided, open top viewing chamber (30 x 30 x 35 cm) with a Perspex screen to the front. The chamber was housed within a Faraday cage, painted black on the inside. Approximately 6 cm above floor level in the centre of the Perspex screen was an observing hole, large enough for the rat to poke its head through. Barriers (12 cm long x 9 cm high) were placed either side of the observation hole to ensure the rat's body was kept perpendicular with respect to the two computer monitors positioned approximately 30 cm in front of the observing hole. A metal divider prevented the rat's left eye from seeing the right screen and its right eye from seeing the left screen. The images were presented in the monocular field of each eye so that information was initially transmitted to the opposite hemisphere. A video camera was directed at the observing hole in order to detect the head movements and alertness of the rats throughout the procedure (Figure 2.4.2.1).

When the subject was correctly positioned in the observing hole, an infrared beam was interrupted, sending a signal to the computer (Viglen, Pentium II).

Presentation of visual stimuli on both computer monitors (Spectrum 4Vlr; AOC, UK) began when the interruption had lasted for a randomly variable 1-2 s interval. Images were presented for a total of 4.5 s during training and resulted in a juice reward (diluted blackcurrant juice) given 4 s after stimulus onset and administered by a metal juice tube positioned in front of the observing hole where it could be just reached by the rat's tongue when its head was fully engaged in the observing hole .

Figure 2.4.2.1 Schematic representation of the paired-viewing procedure (PVP) apparatus. The PVP allows simultaneous presentation of novel and familiar stimuli to different brain hemispheres within the same subject (Adapted from Brown & Aggleton 2001).



2.4.3 Paired viewing procedure

2.4.3.1 Pre-training

Animals were pre-trained daily over two consecutive days, in order to become familiar with the apparatus. During this time, animals were allowed to freely explore the chamber. Each time the rat placed its head through the observing hole, for a minimum of 1.5 s, thus breaking the LED beam, a droplet of juice was administered via the juice tube (Figure 2.4.2.1). Throughout pre-training the computer screens were blank and therefore, no visual stimuli were given. Animals were expected to achieve approximately 74 trials before being returned to their home cage.

2.4.3.2 Training protocol

After pre-training, animals were trained twice daily for six days, with each training day composed of both a morning session and an afternoon session. Sets of visual stimuli (each 15 x 12 cm in size) were compiled in order to create training lists; each set comprised 30 images: sets $A_{(1-30)}$,$B_{(1-30)}$,$C_{(1-30)}$,…..$H_{(1-30)}$. During each morning session, animals were exposed to a set of 30 images so that they might become familiar. This set (Set A) was shown to both eyes in a randomised order so that the simultaneously displayed images were different on the two screens and both eyes saw the complete set of 30 images. Once 30 pairs of images had been shown, the animal was returned to its home cage until the second session of the day. Following a delay of ≥3 h, animals were reintroduced into the viewing box for an afternoon session. During the afternoon session on the first five days, each eye saw 15 images from Set A and 15 novel (never seen previously) images. The image order was randomised but at each presentation one eye saw a novel image and the other an image from Set A; accordingly, all the images from Set A and 30 novel images were displayed, Each afternoon a different set of novel images was used so that the sets of images were as follows: Day 1: A + B; Day 2: A + C – Day 5: A+F. The 15 images of Set A shown to the left eye on Day 1 were shown to the right eye on Day 2, and alternated daily thereafter. Thus before the afternoon session of the 6th day each eye had been exposed to an equal number of novel and familiar images.

In the final afternoon session of the 6th day, the test session, one eye was shown the set (A) of familiar images and the other eye was exposed to a further set (Set H) of 30 novel images (Day 6: A+H). The novel images were shown to the left eye in one rat and the right eye in the next so as to counterbalance presentation, in order to control for any interhemispheric biases. In addition, Sets A and H were counterbalanced across rats so that the novel images in half the rats were the familiar images in the other half in order to remove biases due to the particular stimuli in the two sets.

Figure 2.4.3.2.1 Examples of visual stimuli presented during paired-viewing. Examples of 12 x 15 cm images presented during a standard morning session in which familiar objects are presented, and a test session in which one eye is presented familiar stimuli (seen in the morning session) and one eye is presented novel images.

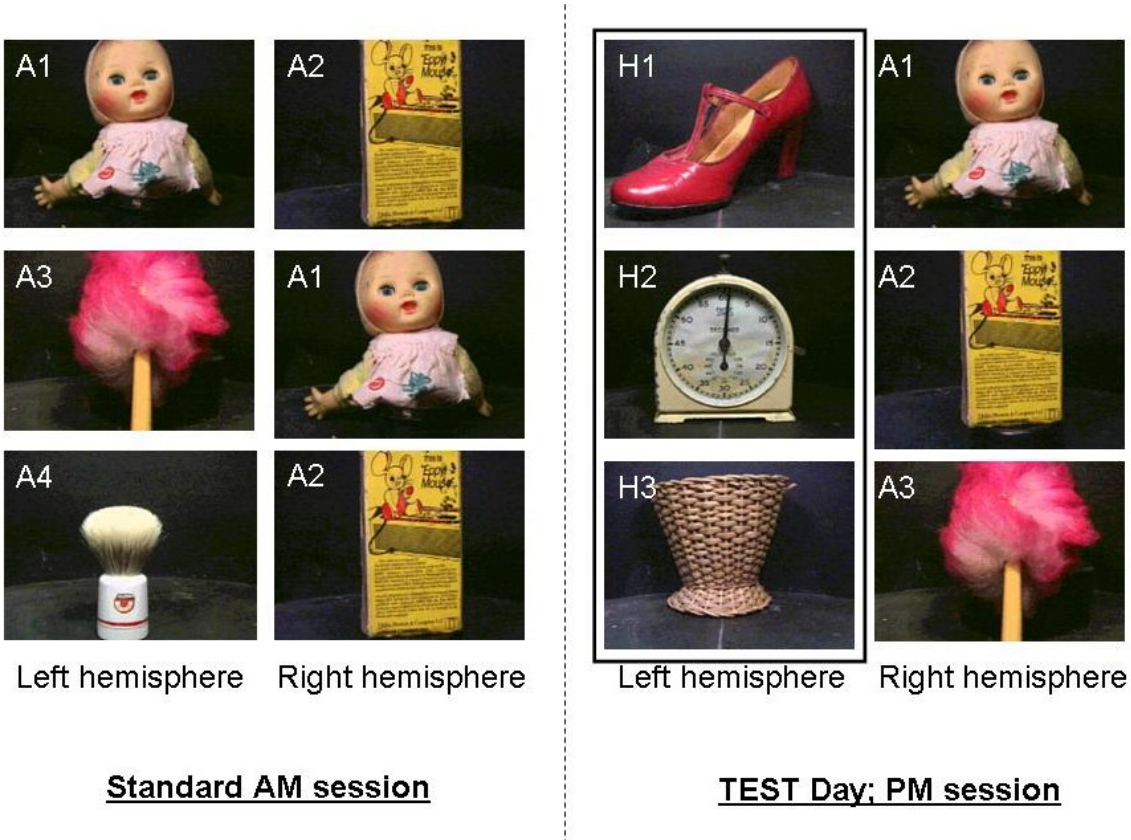


Table 2.4.3.2.1 Standard PVP protocol. Details of pre-training days (2.4.3.1) and training schedules (day 1-6) of the PVP (2.4.3.2).

Rat	Training session	Morning	Number of images/juice rewards	Afternoon	Number of images/juice rewards
1	**Pre train 1**	**Blanks**	**74 trials**	**-**	**-**
2					
3					
4					
1	**Pre train 2**	**Blanks**	**74 trials**	**-**	**-**
2					
3					
4					
1	**Day 1**	**AA**	**30 trials**	**AB**	**30L**
2		**AA**		**AB**	
3		**HH**		**HB**	
4		**HH**		**HB**	
1	**Day 2**	**AA**	**30 trials**	**AC**	**30L**
2		**AA**		**AC**	
3		**HH**		**HC**	
4		**HH**		**HC**	
1	**Day 3**	**AA**	**30 trials**	**AD**	**30L**
2		**AA**		**AD**	
3		**HH**		**HD**	
4		**HH**		**HD**	
1	**Day 4**	**AA**	**30 trials**	**AE**	**30L**
2		**AA**		**AE**	
3		**HH**		**HE**	
4		**HH**		**HE**	
1	**Day 5**	**AA**	**30 trials**	**AF**	**30L**
2		**AA**		**AF**	
3		**HH**		**HF**	
4		**HH**		**HF**	
1	**Day 6**	**AA**	**30 trials**	**AH**	**30L**
2		**AA**		**HA**	
3		**HH**		**HA**	
4		**HH**		**AH**	

2.4.3.3 Measurement of the time course of ERK and p38 kinases following PVP

The expression of dually phosphorylated ERK (pERK) or p38 (p-p38) was used as a measure of neuronal activation in response to visual stimuli. Certain perirhinal neurons respond more strongly to novel than familiar stimuli (Brown & Xiang 1998; Zhu et al 1995) and this difference has been imaged immunohistochemically using phosphorylated CaMKs, phosphorylated CREB and Fos expression (Zhu, Brown et al. 1995; Wan, Aggleton et al. 1999; Warburton, Koder et al. 2003; Warburton, Glover et al. 2005; Seoane, Massey et al. 2009; Tinsley, Narduzzo et al. 2009; Tinsley, Narduzzo et al. 2011).

Following the end of the PVP test session, different groups of animals were transcardially perfused after one of four delays: 10 min, 40 min, 70 min and 100 min. Levels of pERK and p38 were not all measured within the same brains.

2.4.4 Quantification of phosphorylated ERK 10 min following PVP using MEK inhibitor, U0126, in response to novel visual stimuli

2.4.4.1 Experimental subjects

Male DA PRH bilaterally cannulated animals were used throughout this procedure (for details of PRH cannula surgery refer to 2.1.2).

2.4.4.2 PVP design

PVP details were the same as those described for pre-training and training in 2.4.3; however, the task varied on the final test session in that novel visual stimuli were presented to both eyes. Novel lists presented to left or right eyes and the brain hemisphere of drug and control infusions were counterbalanced across rats. Fifteen minutes prior to the test session, unilateral infusions of 1 μM MEK inhibitor, U0126, and control peptide, U0124, (Table 2.4.4.2.1) were made into perirhinal cortex in opposite hemispheres, using the standard infusion protocol.

Table 2.4.4.2.1 Drug concentrations administered prior to the PVP test session. Unilateral infusions of MEK inhibitor, U0126, and control peptide, U0124, in the opposite hemisphere, were administered 15 min prior to the test session.

Drug	Dose	Vehicle
U0126 (1,4-diamino-2,3-diacyano-1,4-bis 2-aminophenylthio]butadiene)	1μM	0.05% DMSO/saline
U0124 Bis[amino(methylthio)methylene]butanedinitrile	1μM	0.05% DMSO/saline
Drugs were supplied by Tocris Bioscience, Bristol, UK.		

2.5 Phosphorylated ERK & p38 immunohistochemistry

Immunohistochemical techniques used in this thesis were based on protocols detailed in Tinsley et al. (2009).

Animals were perfused initially using 250 ml of phosphate buffer (PB)/ 5% sodium fluoride and fixed using 250 ml 4% paraformaldehyde (Adams, Anderson et al.) in PB/ 5% NaF. Following the perfusion, brains were rapidly removed and post-fixed in 4% PFA in PB/ 5% NaF for a minimum of 24 h.

Following tissue perfusion, brains were cryoprotected using 30% PB/sucrose for approximately 48 h before being coronally sectioned at a thickness of 40 µM using a cryostat (Leica CM2050S). An incision was made along the right hand side of the brain in order to distinguish between the left and right hemispheres during subsequent mounting onto slides. The brain was secured onto a metal stage using mounting medium and positioned into a ladle of isopentane, before being suspended into a dewar of liquid nitrogen until suitably frozen. Brains were allowed to acclimatise to -18^{o}C in the cryostat for approximately 30 min before sectioning commenced.

Brain sections from regions between -5.2 mm and -6.3 mm posterior to bregma were collected and free floating sections were stored in plastic wells containing PB, ready for immunohistochemical staining (Figure 2.5.2.1).

Sections were transferred into Triton Tris-buffered saline (TTBS) with 0.2% Triton X-100, 0.08 M Tris and 0.9% NaCl containing 5% bovine serum albumin (BSA) and 5 % normal goat serum (NGS) and placed onto an orbital shaker to maintain gentle agitation of the slices. All washes and solutions were made using TTBS, a solution containing the detergent Triton X-100 to improve antibody penetration. Tissue was blocked for 2 h using 5 % NGS and 1% BSA to prevent non- specific background staining. Slices were incubated with 1% BSA/1% NGS and primary polyclonal rabbit antibody for pERK (1:250) or p38 (1:500) for 48 h at 4^{o} (Table 2.5.1). On day 3 of the protocol, sections were washed and incubated for 2 h in the secondary antibody (BA

1000 polyclonal ANTI- rabbit in goat) (1:250) (Table 2.5.1), further washes followed before a 1 h incubation of with an avidin-biotin horseradish peroxidise complex (Vectastain Elite ABC kit, Vector Laboratories, UK). Sections were washed using Tris buffer pH 7.6 before being visualised using 3,3'-diaminobenzidine and finally washed in cold PB in order to stop the reaction.

Table 2.5.1 Antibody concentrations and suppliers. Details of antibody concentrations used for immunohistochemical labelling of phosphorylated ERK and phosphorylated p38.

Antibody	Concentration	Supplier
Anti-ACTIVE MAPK pAb, polyclonal, rabbit	1:250	Promega, UK Cat # V8031
Anti-ACTIVE p38 pAb, polyclonal, rabbit	1:500	Promega, UK Cat # V1121
BA 1000 ANTI-rabbit (raised in goat)	1:250	Vector Laboratories, UK
S1000 Normal Goat serum	-	Vector Laboratories, UK

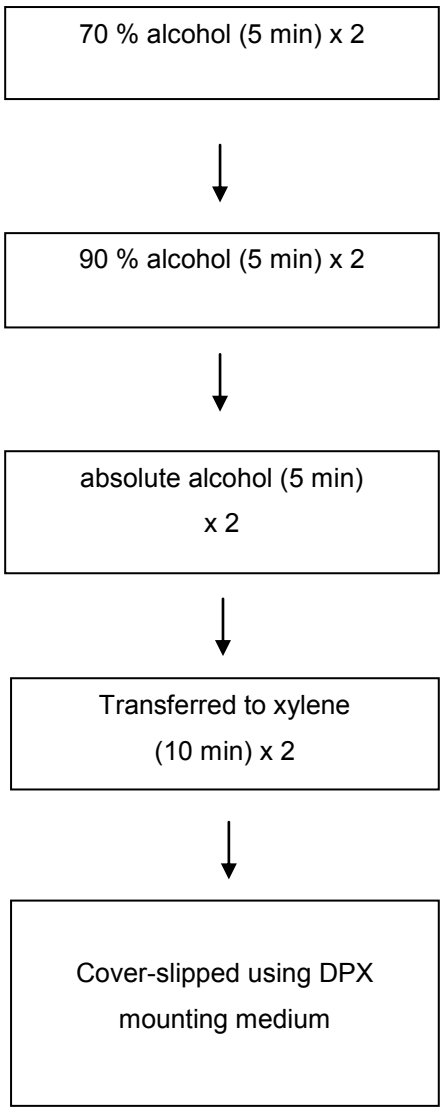
Phosphorylated ERK and phosphorylated p38 were labelled using primary polyclonal antibodies specific for dually activated ERK and p38 respectively (Table 2.5.1). Anti-active MAPK is an antibody raised against a synthetic peptide of the catalytic core of the ERK2 isoform (pThr^{183}-Glu-pTyr^{185}) and recognizes the dually activated ERK1 and ERK2 enzymes (Jarvis and Schaefer, Promega Notes, 1997) (4.2.2). Anti-active p38 antibody is raised against pThr^{180}- Gly-pTyr^{182} and detects dually phosphorylated α, γ, δ subtypes of the enzyme (Jarvis and Huang, Promega Notes, 2000) (4.4.2).

Table 2.5.2 Solutions used for immunohistochemical staining.

Solution	Composition	pH
0.1 M Phosphate buffer (PB) + Sodium fluoride	4 L dH_2O 46.008 g Na_2HPO_4 10.4g $NaH_2PO_4.2H_2O$	7.4
4 % paraformaldehyde (Adams, Anderson et al.) + Sodium fluoride (Fadiel, Eichenbaum et al.)	40 g PFA powder 1 L PB Heat ~60^o	7.4
30 % PB/ sucrose	100 ml PB 30g sucrose	7.4
Tris buffer	1.57 g Tris/HCl 200 ml d H_2O	7.6
Triton Tris-buffered saline	0.08 M Tris buffer 0.9 % NaCl 0.2 % Triton X-100	7.4

Stained sections were mounted onto positively charged slides and prepared for coverslipping by dehydrating steps using ethanol and xylene; once ready, sections were coverslipped using DPX mounting medium (Figure 2.5.1).

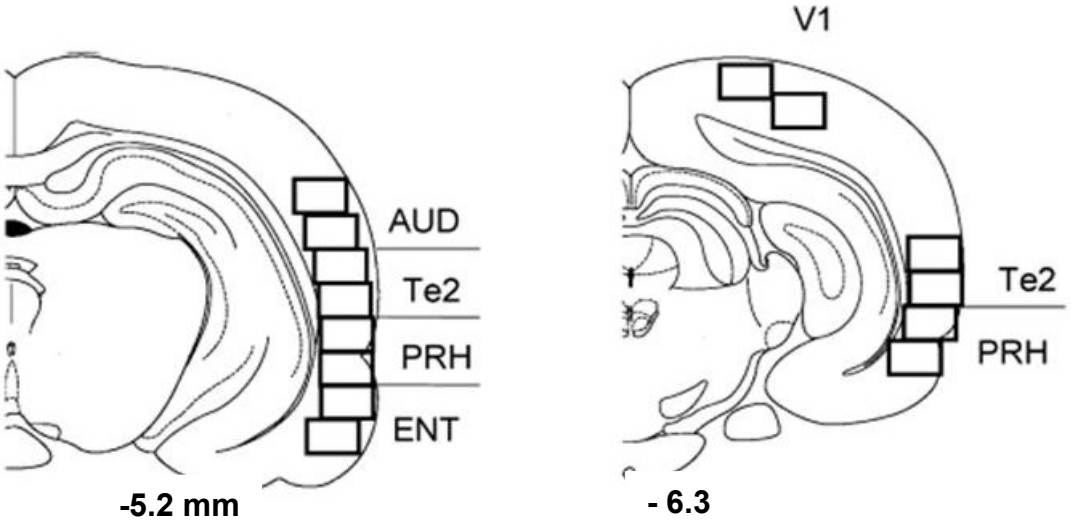
Figure 2.5.1 Section coverslipping (Protocol)



2.5.1 Brain regions of interest

Evenly stained brain sections collected at approximately -5.2 mm and -6.3 mm from bregma were imaged in order to measure phosphorylated ERK (pERK) and p-p38. The following brain regions were measured on sections at -5.2 mm from bregma: entorhinal cortex (ENT); perirhinal cortex (PRH); associative visual area (Te_2), and auditory cortex (AUD). Further sections of perirhinal cortex (PRH), associative visual area (Te2), and primary visual cortex (V1) were collected at -6.3 mm from bregma.

Figure 2.5.1.1 Brain regions of interest Brain sections of 40 µm thickness were collected and used for immunohistochemical staining of phosphorylated ERK and phosphorylated p38. Counts for both PRH and Te2 were pooled from sections taken at -5.2 mm and -6.3 mm from bregma. Counting frames were positioned to include all cortical cell layers.



2.5.2 Data analysis

All processing and counting were performed blind as to which eye had been exposed to novel or familiar stimuli or hemisphere of vehicle or drug infusion. Sections were viewed and captured using a microscope (DM5000B, Leica, Germany). A standardised rectangular counting frame (0.94 x 0.62 mm) was positioned over brain regions to include all cortical areas. Images (512 x 768 pixels) were captured at an effective 240 x magnification with 256 grey level resolution. Staining was quantified using an in-house programme in which a pre-set threshold against a smoothed background was determined in preliminary experiments so as to count clearly stained neuronal nuclei. The automated image analysis software counted oval or irregular objects measuring between 10-22 μm (CellCountv3.1, J. Leendertz). Stereological corrections were not required as only relative measures were sought.

Once counted, data was organised and analysed according to time points and brain regions. Average counts for each region in each hemisphere were calculated; PRH and Te2 counts at - 5.2 mm and - 6.3 mm from bregma were averaged between each area and hemisphere. Raw and normalised counts were tested during statistical analysis. Counts were normalised within individual rats, by dividing the mean count for a region over the total count for both hemispheres.

2.5.3 Statistical analysis

All statistical analyses used a significance level $p < 0.05$.

2.5.3.1 Statistical analysis of pERK and p-p38 counts in response to novel and familiar stimuli

Levels of phosphorylated ERK in response to novel and familiar stimuli at different delays were analysed using repeated measures three-factor ANOVA (novelty, delay, region) for raw and normalised counts. Counts were combined into control regions (AUD, ENT, V1) and regions of interest (PRH and Te2) before analysis. Subsequently, analysis was subdivided into control regions and regions of interest and analysed using two-factor ANOVA (novelty, delay). Regions in which response changes were expected (PRH and Te2) were

analysed individually. Post-hoc Tukey's tests were performed to make pairwise comparisons between novel counts across time delays and between counts at significant time points. Analysis of p-p38 counts were conducted in the same manner.

2.5.3.2 Statistical analysis of pERK levels following intraperirhinal MEK inhibition

Normalised counts were calculated to reduce variance between rats and different batches of immunohistochemical staining. Individual mean pERK counts were analysed using repeated measures ANOVA (drug treatment, area) (4.3.3). Planned comparisons analysis [ANOVA (drug treatment)] in PRH was analysed. Area Te2 and combined control areas (AUD, ENT, V1) were analysed individually [ANOVA (drug treatment)].

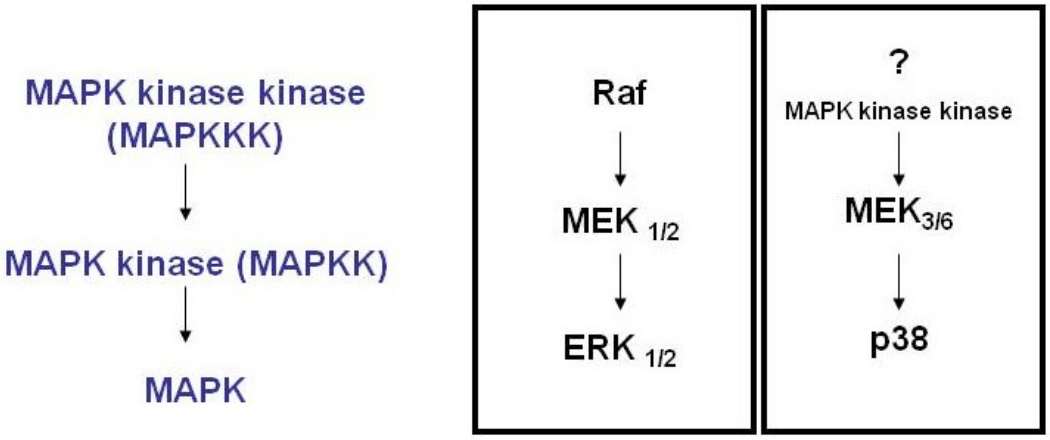
Chapter 3. The effect of intraperirhinal inhibition of ERK and p38 on spontaneous novelty preference exploration behaviour.

3.1 Introduction

Investigations into the role of signal transduction pathways in PRH have demonstrated roles for protein kinases: PKA, PKC, CAMKII and CAMKK in long-term familiarity discrimination (Tinsley, Narduzzo et al. 2009; Tinsley, Narduzzo et al. 2011). However, the role of the MAPK/ERK signalling pathway, a point of convergence for PKA, PKC and CAMKK pathways (Roberson, English et al. 1999; Dolmetsch, Pajvani et al. 2001; Thomas and Huganir 2004), had not previously been investigated.

Although the MAPK isoforms differ in some components, the MAPK family is characterised by a three-tier kinase core cascade in which the upstream kinase sequentially dually phosphorylates the downstream kinase (Chen, Gibson et al. 2001; Sweatt 2001). The nomenclature of each cascade is determined by its downstream kinase. Extracellular-signal regulated kinases (ERKs) and p38s are among the most common MAPK isoforms.

Figure 3.1.1 Simplified schematic representation of the ERK and p38 cascade. MAPKs are characterised by a three kinase cascade in which kinases are sequentially dually phosphorylated.



Initial interest in MAPK signalling cascades focused on their roles as critical regulators of cell proliferation, differentiation and involvement in neuronal apoptosis and cytokine expression (Lee, Laydon et al. 1994; Kawasaki, Morooka et al. 1997; Roberson, English et al. 1999). The discovery of their presence in post-mitotic neurons in the CNS suggested an additional role for these kinases. ERK and p38 are abundantly expressed throughout the cerebral cortex, including the hippocampus, and in the cerebellum; areas widely associated with learning and memory (Ortiz, Harris et al. 1995; Lee, Park et al. 2000; Zhen, Du et al. 2001).

The expression of MAPKs in PRH has been less extensively studied. Nonetheless, a decrease in levels of phosphorylated ERK (pERK) were inversely associated with neuronal degradation in several brain regions, including PRH in a genetic alcoholic rodent model (Crews and Braun 2003). In an additional study, levels of pERK were measured in multiple brain regions following the repeat presentation of two objects in the sample phase of a variant of the NOPT. No increase in pERK levels in PRH was found 5 min following memory encoding (Kelly, Laroche et al. 2003).

3.1.1 MAPKs and learning and memory

Much evidence now exists for the involvement of ERK in different types of learning in the rodent. Fewer studies have focused on p38 as a critical kinase involved in learning and memory. Contextual fear conditioning requires ERK activation in the hippocampus (Atkins, Selcher et al. 1998; Chwang, O'Riordan et al. 2006), amygdala (Schafe, Atkins et al. 2000), and prefrontal cortex (Runyan, Moore et al. 2004). Inhibitory avoidance (IA) learning increases levels of pERK (Cammarota, Bevilaqua et al. 2000) and p-p38 (Cammarota, Bevilaqua et al. 2000; Alonso, Bevilaqua et al. 2003) in the CA1 region of the hippocampus. Phosphorylated ERK is increased in the entorhinal and parietal cortex following IA learning (Walz, Roesler et al. 1999). ERK (Szapiro, Vianna et al. 2003) and p38 activations are critical for extinction of IA memories in the hippocampus (Rossato, Bevilaqua et al.

2006). Levels of pERK also increase in the hippocampus following retrieval in the IA learning task (Szapiro, Izquierdo et al. 2000).

Moreover, pERK levels increase in areas CA1/CA2 of the dorsal hippocampus (Blum, Moore et al. 1999; Selcher, Atkins et al. 1999) and entorhinal cortex (Hebert and Dash 2002) following performance in the Morris Water Maze spatial memory task. Phosphorylated ERK is increased in the insular cortex following exposure to novel saccharin in a conditioned taste aversion task (Berman, Hazvi et al. 1998) and in the olfactory bulb following olfactory aversion learning in rat pups (Zhang, Okutani et al. 2003). Following acquisition of conditioned eye-blink response pERK and p-p38 levels increase in the anterior cerebellar vermis, in the same animals elevated pERK levels were also found in the hippocampus (Zhen, Wei et al. 2001) (Table 3.1.1.1).

Table 3.1.1.1 Identified roles for ERK and p38 in learning and memory. Roles for ERK have been identified in multiple brain regions following tasks associated with learning and memory. The involvement of p38 in such tasks has been studied to a lesser extent. Levels of ERK and p38 have been found to be differentially activated in brain regions following such tasks. [Symbol ↑- indicates increase in level of kinase following task; **-** not investigated].

Task		**Brain Region**	**MAPK**	
			ERK	**P38**
Fear conditioning		AMYGDALA	↑	-
		HIPPOCAMPUS		↑
		PREFRONTAL CORTEX		-
Inhibitory Avoidance learning	**consolidation**	ENTORHINAL CORTEX	↑	-
		HIPPOCAMPUS	↑	↑
		PARIETAL CORTEX	↑	-
	Extinction	HIPPOCAMPUS	↑	↑
Taste aversion task		INSULAR CORTEX	↑	no change
Olfactory aversion Learning		OLFACTORY BULB	↑	-
Condition eye blink response		CEREBRAL VERMIS	↑	↑
		HIPPOCAMPUS	↑	no change
Morris Water Maze (spatial)		ENTORHINAL CORTEX	↑	-
		HIPPOCAMPUS	↑	↑

3.1.2 ERK is required for long-term recognition memory

A role for ERK in recognition memory has been identified using a variant of the NOPT. Intracerebroventricular (ICV) infusion of MEK inhibitor, U0126, 40 min prior to acquisition resulted in memory impairments when tested at 24 h delay but not when tested at 10 min delay (Bozon, Kelly et al. 2003; Kelly, Laroche et al. 2003). As this study used ICV infusions to impair long-term recognition memory, the precise location of action of the inhibitor was not established.

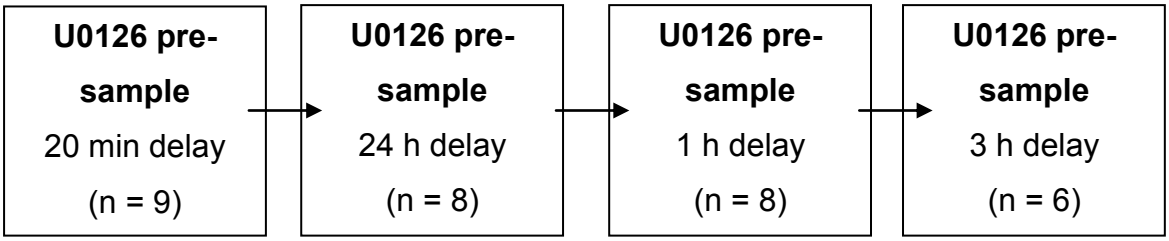
3.1.3 Chapter objectives

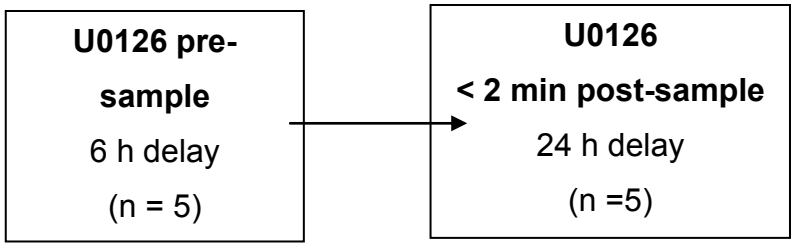
To further investigate the finding reported by Kelly et al. (2003), intraperirhinal infusions of MEK inhibitor, U0126, were made to determine if recognition memory impairment is produced as a result of disruption to the PRH. Additionally, the role of MAPK isoform p38 in recognition memory was investigated to establish if that pathway is also necessary for this type of learning. Further, the roles of ERK and p38 in OIP memory were assessed to ascertain if they are involved in associative recognition memory.

3.2 Timescale of behavioural experiments.

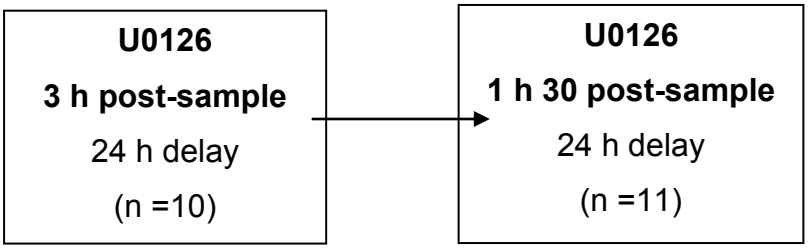
Figure 3.2.1 Timescale of behavioural experiments. Chronological order of behavioural experiments, detailing delay between sample and test phase and the number of animals (n) used for statistical analysis in each experiment.

Cohort 1 and 2- PRH cannulations. Intraperirhinal infusions prior to- and post- sample phase

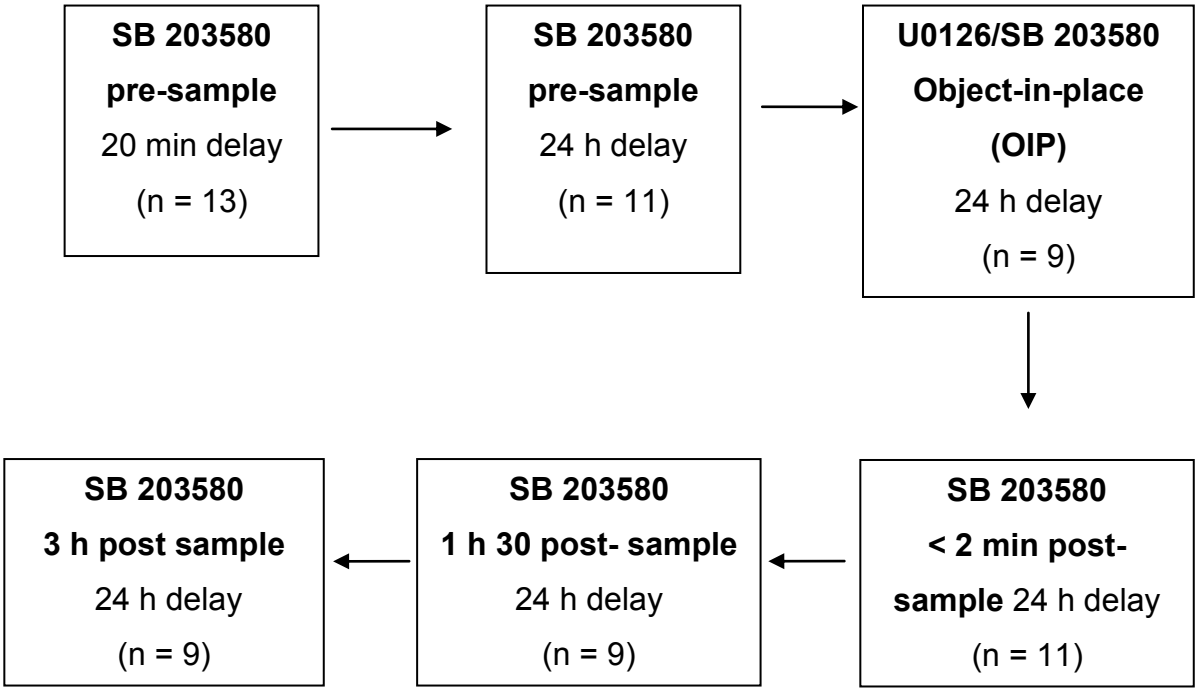




Cohort 3- PRH cannulations. Intraperirhinal infusions post- sample phase



Cohort 4- PRH cannulations. Intraperirhinal infusions prior to- and post-sample phase



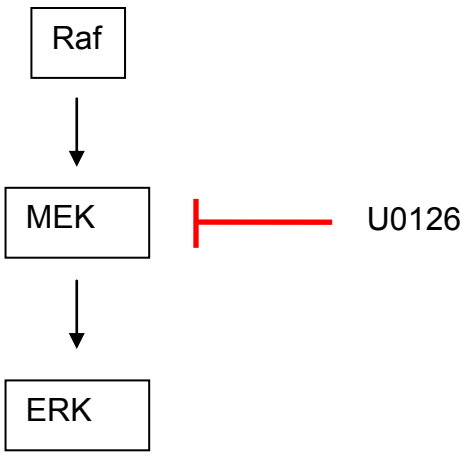
3.2.1 Spontaneous novel-object preference task: pharmacological intervention of MAPK cascades

The NOPT provides an established method of testing the effect of pharmacological inhibition on recognition memory (Dere, Huston et al. 2007). Reversible inhibitors can be administered at different stages of the task in order to determine the effect of the target on different stages of the mnemonic process (acquisition/consolidation/retrieval). This Chapter will assess the effects of MEK inhibitor, U0126, and p38 inhibitor, SB 203580, when administered prior to the sample phase (to affect the acquisition and possible consolidation of the memory) and following the sample phase (to disrupt memory consolidation).

3.2.1.1 MEK inhibitor, U0126

MEK inhibitor, U0126, is selective for the intermediate kinases - MEK-1 and MEK- 2 in the ERK cascade (Favata, Horiuchi et al. 1998). A dose of 1 µm was selected for the intracerebral infusions described in this Chapter to specifically target the intermediate MEK kinase in the ERK cascade without interfering with other kinases (Duncia, Santella et al. 1998) (General Introduction 2.8.1).

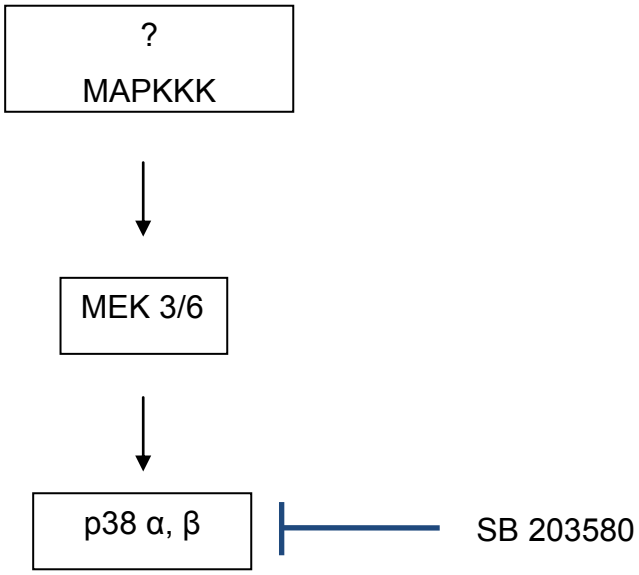
Figure 3.2.1.1.1 Targeting the ERK signalling pathway. MEK inhibitor, U0126, blocks the intermediate step in the ERK cascade. U0126 is a non-competitive inhibitor of MEK 1 and MEK 2 (Favata, Horiuchi et al. 1998).



3.2.1.2 p38 inhibitor, SB 203580

Throughout this Chapter, SB 203580 (50 μm) was used to selectively inhibit the downstream kinase in the p38 pathway. The p38 selective inhibitor, SB 203580, is specific for p38 isoforms α and β.

Figure 3.2.1.2.1 Targeting the p38 signalling pathway. p38 inhibitor, SB 203580, blocks p38 α, β, downstream kinases in the p38 cascade (Cuenda, Rouse et al. 1995). P38 is activated by MEK -3 / -6. A vast array of stimuli can stimuli MEKs (Dong, Ramachandiran et al. 2004; Roux and Blenis 2004); the upstream MAPKKK in PRH is unknown.

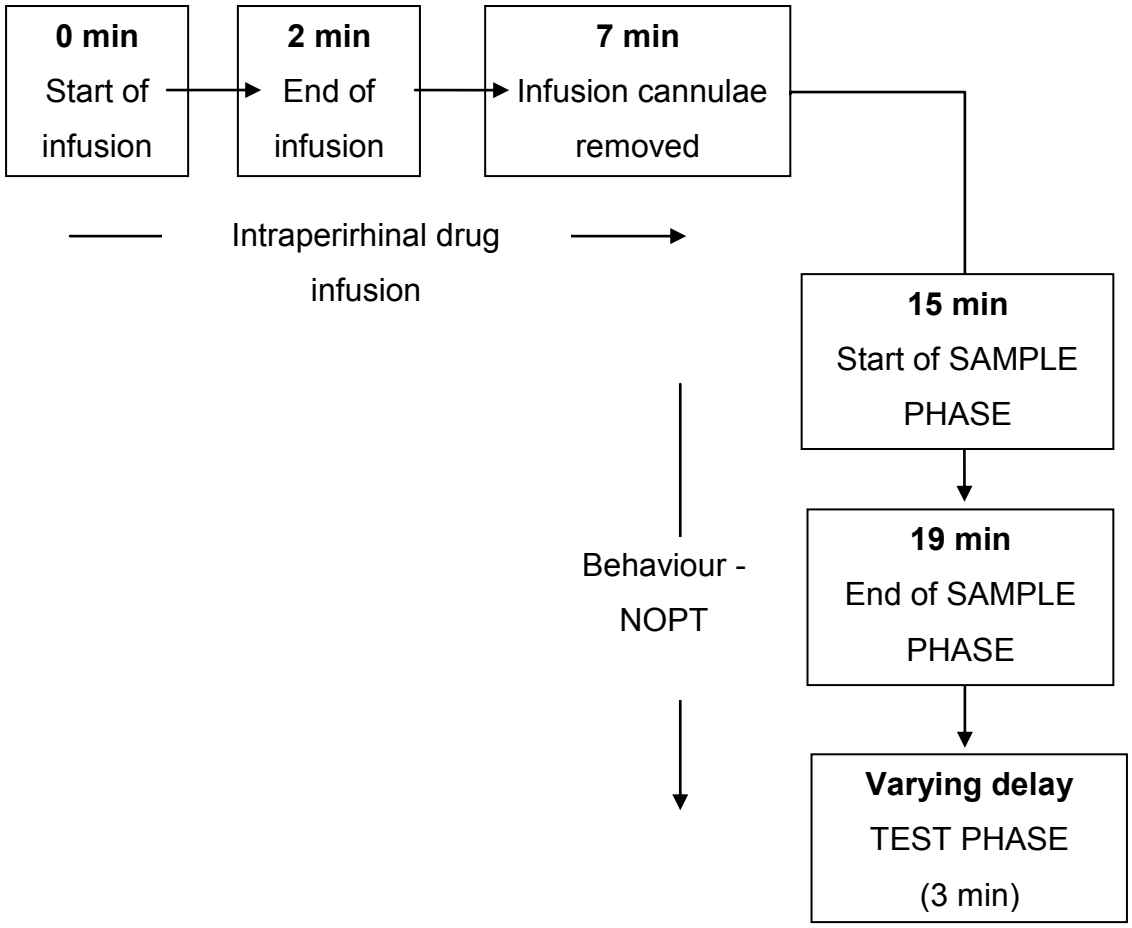


I) The effect of intraperirhinal MEK inhibition on familiarity discrimination

3.3 Spontaneous novelty preference exploration task: intraperirhinal U0126 infusions made prior to the sample phase.

U0126 infusions were administered 15 min prior to the start of the sample phase to determine the effect of MEK inhibition on the acquisition and early consolidation of familiarity discrimination memory. Experiments were carried out in the test delay order: 20 min (n = 9), 24 h (n = 8), 1 h (n = 8), 3 h (n = 6) and 6 h (n = 5). Numbers of animals used in the statistical analyses are listed (Figure 3.2.1).

Figure 3.3.1 Experimental time course. Drug infusions were made at a rate of 0.5 µl/min/hemisphere for 2 min, with infusion cannulae remaining in place for a further 5 min. The sample phase of the experiment started15 min from the start of the infusion and lasted for a maximum of 4 min or 40 s of exploration, depending on which came sooner. Animals were returned to their home cages and reintroduced to the arena at delays of 20 min, 1 h, 3 h, 6 h or 24 h for the test phase.

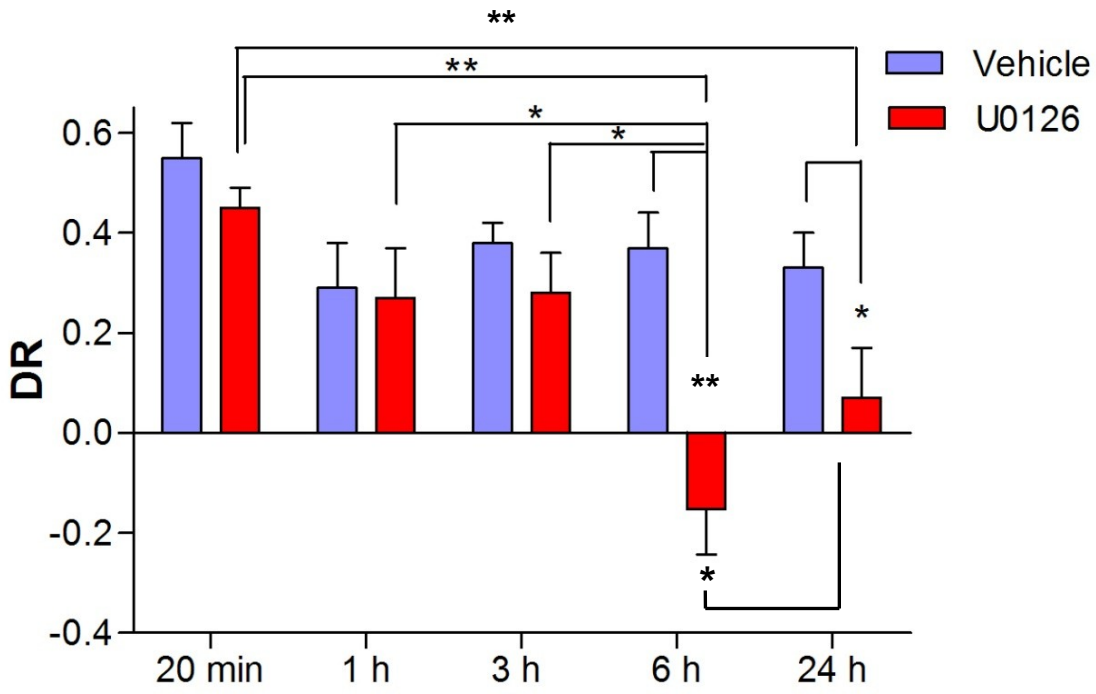


3.3.1 ERK is required for long-term familiarity discrimination

One rat was excluded from statistical analysis in the 20 min experiment as the discrimination ratio following treatment with vehicle was > 3 standard deviations from the group mean. One animal in the 3 h delay and two in the 6 h delay groups were also removed for this reason. In addition, one animal was removed from the 3 h experiment for not meeting the sample exploration minimum criteria (see section 2.2.10).

As detailed in Materials and Methods (section 2.2.10) a DR value was calculated as the proportion of total exploration time in the test phase spent exploring the novel object less that spent exploring the familiar object (all 3 min).

Figure 3.3.1.1 Inhibition of the ERK cascade using MEK inhibitor, U0126, prior to acquisition results in recognition memory impairments when tested at delays greater than 6 h. ANOVA with repeated measures (drug treatment, delay) of mean DR values revealed a significant drug by delay interaction ($p < 0.01$). Significant effects of drug treatment were found at 6 h ($p < 0.01$) and 24 h ($p < 0.05$). Post-hoc Tukey's test revealed significant differences of mean DR values for U0126 treatment between time delays: 20 min - 6 h ($p < 0.01$); 6 h - 24h min ($p < 0.05$); 6 h - 1 h ($p < 0.05$); 6 h 3 h ($p < 0.05$). Bars represent standard error of mean. Significance levels: * = $p < 0.05$; ** = $p < 0.01$.



ANOVA with repeated measures (drug treatment, delay) of mean DRs revealed a significant drug by delay interaction [$F_{(4,30)} = 4.04$, $p < 0.01$]. Significant main effects of drug [$F_{(1,30)} = 23.86$, $p < 0.001$] and delay [$F_{(4,30)} = 4.82$, $p < 0.01$] were also found. ANOVA (drug treatment) at individual delays revealed no significant effect at 20 min [$F_{(1,7)} = 1.05$, $p > 0.1$], 1 h [$F_{(1,7)} < 1$, $p > 0.1$] or 3 h [$F_{(1,5)} = 1.0$, $p > 0.1$]. An effect of treatment was significant for DR means at delays of: 6 h [$F_{(1,4)} = 66.47$, $p < 0.01$] and 24 h [$F_{(1,7)} = 10.41$, $p < 0.05$]. Analysis of U0126 treatment across the different delays revealed a

significant effect of delay [$F_{(4,30)} = 6.30$, $p < 0.01$]. Post-hoc pairwise comparisons (Tukey's test) revealed significant differences between U0126 treatments at 20 min delay - 6 h ($p < 0.01$) and 20 min - 24 h ($p < 0.05$), while the 6 h mean was significantly different from the DR mean at 1 h ($p < 0.05$) and 3 h ($p < 0.05$). No effect of vehicle treatment across delay was found [$F_{(4,30)} = 2.19$, $p > 0.05$].

One-sample *t*-tests revealed that all vehicle mean DRs were significantly different from zero, indicating greater exploration of the novel object compared to the object previously encountered. Mean DR values for U0126 treatment tested at 6 h ($p > 0.1$) and 24 h ($p > 0.1$) were not significantly different from zero, demonstrating an inability to discriminate between novel and previously encountered stimuli at these test delays (Table 3.3.1.1).

Table 3.3.1.1 DR values and t values and significance levels of novelty preferences for vehicle and U0126 treated animals at each test delay. Mean DR values for vehicle treated animals were not significantly different from zero when tested at 6 h ($p > 0.1$) and 24 h delay ($p > 0.1$), indicating an inability to discriminate between novel and familiar stimuli.

Delay	Treatment	DR mean ± SEM	T value (df) and significance
20 min	Vehicle	0.55 ± 0.07	t(7) 8.43, p < 0.001
	U0126	0.45 ± 0.04	t(7) 10.50, p < 0.001
1 h	Vehicle	0.29 ± 0.09	t(7) 3.21, p < 0.05
	U0126	0.27 ± 0.10	t(7) 2.81, p < 0.05
3 h	Vehicle	0.38 ± 0.04	t(5) 8.53, p <.001
	U0126	0.28 ± 0.08	t(5) 3.31, p < 0.05
6 h	Vehicle	0.37 ± 0.07	t(4) 5.19, p < 0.01
	U0126	-0.15 ± 0.09	**t(4) -1.63, p > 0.1**
24 h	Vehicle	0.33 ± 0.07	t(7) 4.47, p < 0.01
	U0126	0.07 ± 0.1	**t(7) 0.67 p > 0.1**

Total exploration times in the sample and test phase, and sample length (maximum exploration is 40 s, total sample length is 240 s) in the NOPT were analysed to determine if drug treatment or test delay altered exploration levels. Two-factor ANOVA (drug treatment, delay) found no significant interactions [$F_{(4,30)} < 1$, $p > 0.1$] for sample exploration times. No significant effect of treatment was found [$F_{(1,30)} < 1$, $p > 0.1$]; although a significant effect of delay [$F_{(4,30)} = 3.20$, $p < 0.5$] was reported. Subsequent post-hoc Tukey tests revealed differences between sample exploration times at 20 min and 3 h delay ($p < 0.05$). This finding is explained by increased levels of exploration for objects presented during the sample phase for the 20 min delay experiment compared to that for the 3 h delay. For sample length, no drug

treatment by delay interaction, [$F_{(4,30)} < 1$, $p > 0.1$], treatment [$F_{(1,30)} < 1$, $p > 0.1$] or delay [$F_{(4,30)} < 1$, $p > 0.1$] effect was found. Repeated measures ANOVA (drug treatment, delay) for test exploration times revealed no drug by delay interaction [$F_{(4,30)} < 1$, $p > 0.1$] or significant effect of treatment [$F_{(1,30)} = 1.40$, $p > 0.1$]; however, a significant effect of delay was found [$F_{(4,30)} = 3.18$, $p < 0.05$]. Post-hoc Tukey's test revealed differences between test total exploration times at 20 min and 1 h ($p < 0.05$) (Table 3.3.1.2). This result arises from increased levels of exploration in the test session at 20 min delay. As all animals had met the minimum exploration criteria and were statistically different from zero, there is no reason to believe that animals when tested at 1 h delay were in anyway inhibited in their ability to explore.

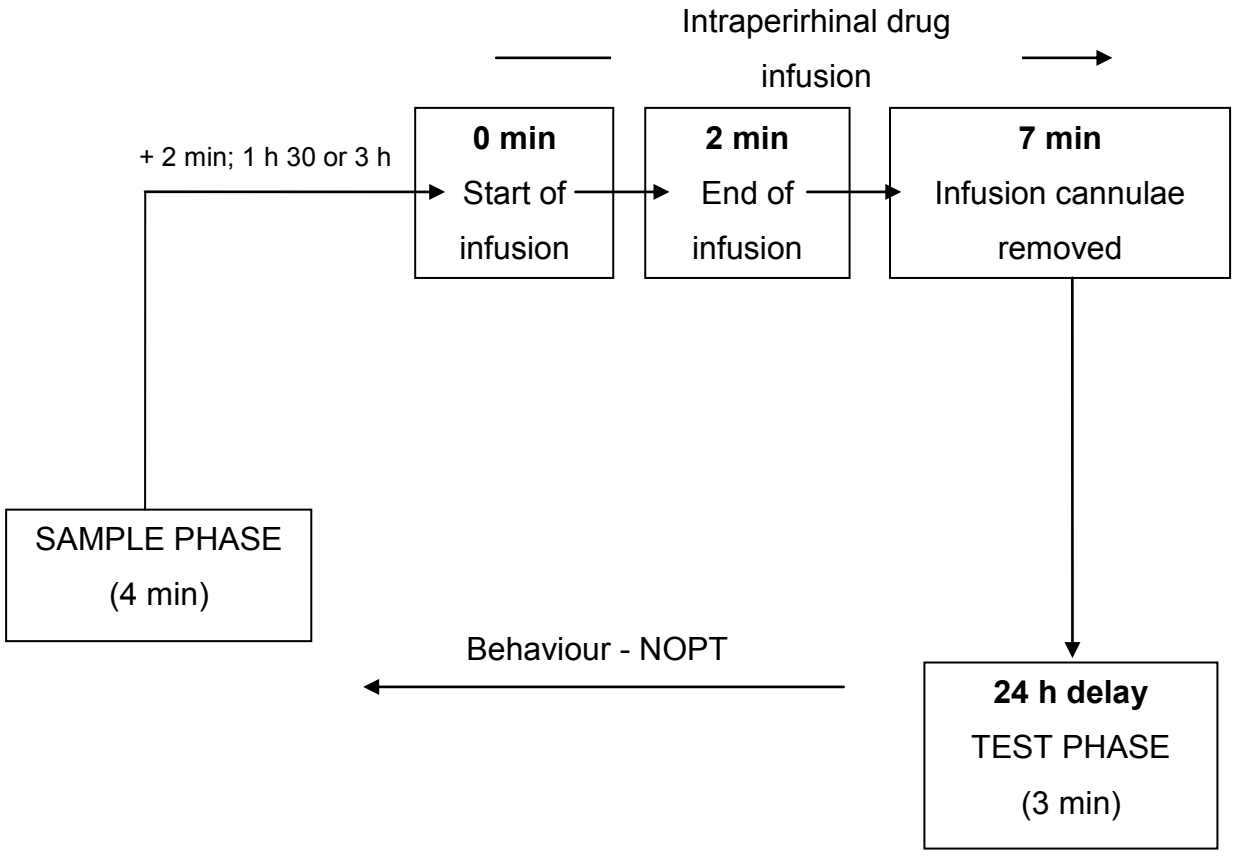
Table 3.3.1.2 Total exploration times in the sample and test phases and sample length for vehicle and U0126 infused groups at each test delay. No significant drug treatment by delay interaction was found of sample exploration times, total sample length or test exploration times ($p > 0.1$). Further, there was no effect of drug on sample exploration times, total sample length or test exploration times ($p > 0.1$). However, significant effects of delay were found for sample exploration times ($p < 0.05$) and test exploration times ($p < 0.05$). No effect of delay was found for total sample length ($p > 0.1$). Results presented are means ± SEM.

Delay	Treatment	Total sample exploration time (s)	Sample length (s)	Total test exploration time (s)
20 min	Vehicle	28.75 ± 3.46	222.63 ± 10.40	24.04 ± 1.48
	U0126	30.38 ± 3.18	227.5 ± 12.5	21.66 ± 2.29
1 h	Vehicle	26.25 ± 4.02	217.25 ± 15.57	17.59 ± 1.83
	U0126	21 ± 2.75	240	15.35 ± 1.52
3 h	Vehicle	19.5 ± 2.55	240	20.19 ± 2.67
	U0126	19 ± 1.91	240	21.89 ± 1.94
6 h	Vehicle	29 ± 4.63	233.4 ± 4.04	21.17 ± 3.47
	U0126	30.4 ± 2.75	235.8 ± 4.2	16.09 ± 1.79
24 h	Vehicle	25.13 ± 3.19	237.25 ± 2.57	18.65 ± 2.14
	U0126	22.38 ± 2.99	234.25 ± 5.75	18.37 ± 2.16

3.3.3 ERK has a critical, early role in the consolidation of long-term familiarity discrimination

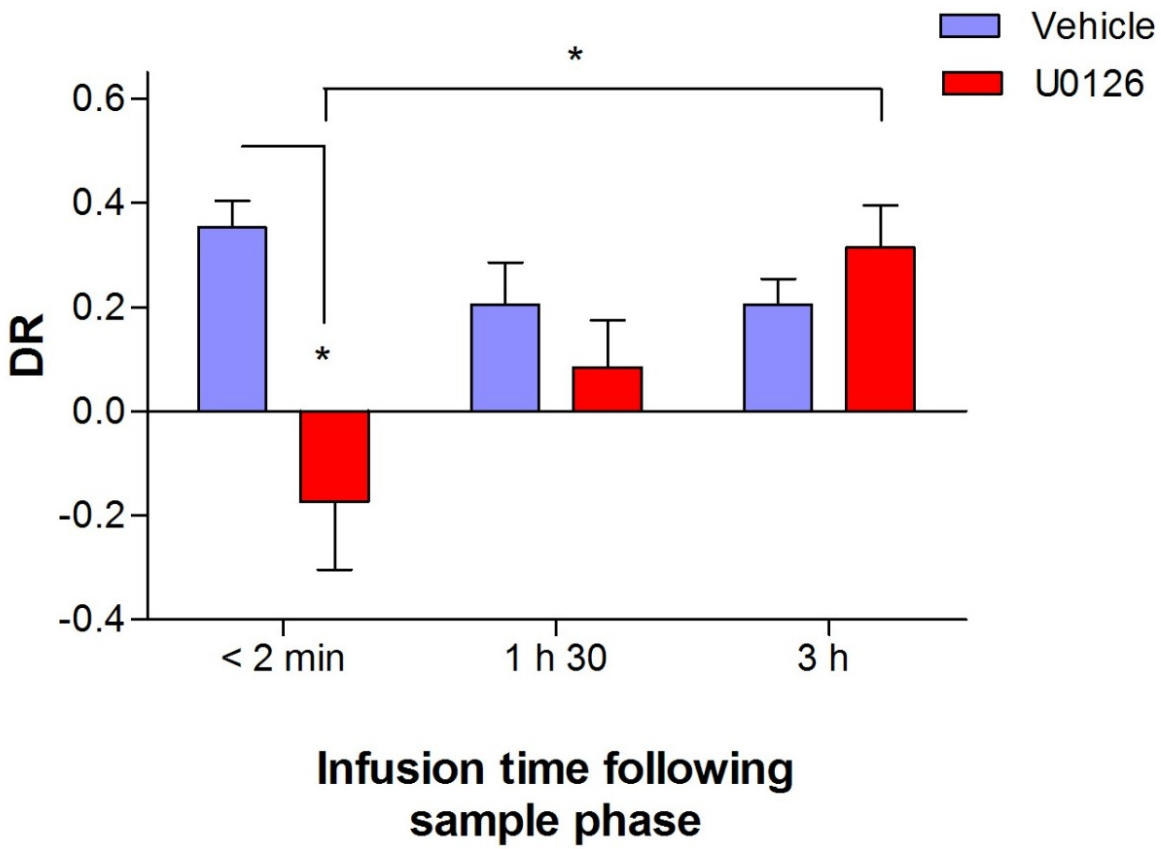
To determine if the memory impairment found at longer delays was a result of the effect of U0126 on consolidation rather than acquisition, infusions were administered at varying time points following the sample phase and memory tested at a 24 h delay (Figure 3.3.2.1). Memory was tested at 24 h as this time delay was found to result in recognition memory impairments when U0126 was infused pre-sample in 3.3.2. The infusion protocol is described in section 2.2.3.

Figure 3.3.2.1 Time course of experiment. Drug infusions were made at a rate of 0.5 µl/min/hemisphere for 2 min, at varying delays following completion of the sample phase. Animals were returned to their home cages and reintroduced to the arena 24 h following the start of the sample phase.



One rat that did not meet the exploration criterion in the test phase was removed from the experiment in which infusions were made immediately (< 2 min) following the sample phase.

Figure 2.3.2.2 Immediate inhibition of MEK following encoding results in impairments in long-term familiarity discrimination. Two-factor ANOVA (drug treatment, infusion delay) for mean DR values revealed a significant interaction ($p < 0.05$). A significant effect of drug treatment ($p < 0.05$) but not delay ($p > 0.1$) was found. ANOVAs of DRs for the two treatment conditions at individual infusion delays revealed a significant effect of drug when U0126 was administered immediately (< 2 min) following the sample phase ($p < 0.05$). No significant effect of drug treatment was found when infused 1 h 30 ($p > 0.1$) or 3 h ($p > 0.1$) following the sample phase. Bars represent standard error of mean. Significance level * = $p < 0.05$.



Repeated-measures ANOVA (drug treatment, infusion delay) for DR values revealed a significant interaction [$F_{(2,23)} = 5.33$, $p < 0.05$]. A significant effect of drug treatment [$F_{(1,23)} = 5.80$, $p < 0.05$] but not delay [$F_{(2,23)} = 1.75$, $p > 0.1$] was found. ANOVA for treatment conditions at individual infusion delays revealed a significant effect of drug when U0126 was administered immediately (< 2 min) following the sample phase [$F_{(1,4)} = 10.69$, $p < 0.05$]. No effect of treatment was found when infusions were made 1 h 30 [$F_{(1,10)} = 1.23$, $p > 0.1$] or 3 h [$F_{(1,9)} < 1$, $p > 0.1$] following acquisition. Analysis of DR values for U0126 infusions at different delays post-sample revealed a significant effect of infusion delay [$F_{(2,23)} = 5.03$, $p < 0.05$]. Post-hoc Tukey tests revealed significant differences between DR mean values when infused immediately and 3 h post-sample ($p < 0.05$). No significant effect of infusion time for vehicle DR values was found [$F_{(2,23)} < 1$, $p > 0.1$].

One sample *t*-tests revealed vehicle DR means at all delays were significantly different from zero, indicating a preference for novelty. DR values for U0126 infusions immediately ($p > 0.1$) and 1 h 30 ($p > 0.1$) following sample were not significantly different from zero. This indicates an equivocal result at 1 h 30 as, although no effect of drug treatment was found at this infusion time point, when treated with U0126, animals did not discriminate significantly between the novel and familiar object (Table 3.3.2.1). Hence at this time point the inhibition would appear to have been partial.

Table 3.3.2.1 DR values, t values and significance levels of novelty preference for vehicle and U0126 treated animals at each infusion delay. Mean DR values for U0126 treated animals were not significantly different from zero when infusions were made immediately- ($p > 0.1$) or 1 h 30 min ($p > 0.1$) post-sample.

Infusion time post- sample	Treatment	DR mean ± SEM	Significant difference from zero
< 2 min	Vehicle	0.35 ± 0.05	$t(4) = 7.34$, $p \leq 0.01$
	U0126	-0.17 ± 0.13	**$t(4) = -1.35$, $p > 0.1$**
1 h 30	Vehicle	0.21 ± 0.08	$t(10) = 2.32$, $p < 0.05$
	U0126	0.08 ± 0.09	**$t(10) = .906$, $p > 0.1$**
3 h	Vehicle	0.20 ± 0.05	$t(9) = 4.18$, $p < 0.01$
	U0126	0.31± 0.08	$t(9) = 3.87$, $p < 0.01$

ANOVA with repeated measures (drug treatment, delay) of exploration times found no significant interaction between treatment and delay for total sample exploration times [$F_{(2,23)} = 1.48$, $p > 0.1$], sample length [$F_{(2,23)} < 1$, $p > 0.1$] or total test exploration times [$F_{(2,23)} < 1$, $p > 0.1$]. No significant effect of treatment was found for sample exploration times [$F_{(1,23)} = 2.62$, $p > 0.1$], sample length [$F_{(1,23)} < 1$, $p > 0.1$] or total test exploration times [$F_{(1,23)} = 2.62$, $p > 0.1$]. Significant delay effects were found for sample exploration times [$F_{(2,23)} = 4.30$, $p < 0.0$], test exploration times [$F_{(2,23)} = 6.31$, $p < 0.01$] but not for sample session length [$F_{(2,23)} = 1.27$, $p > 0.1$]. Post-hoc Tukey tests revealed total sample exploration times at infusion delay 1 h 30 were significantly different to 3 h ($p < 0.05$) and total test exploration times for infusion delay 1 h 30 were significantly different from infusion delay 0 min ($p < 0.05$) and 3 h ($p < 0.05$).

Table 3.3.2.2 Total exploration times in the sample and test phases and sample length for vehicle and U0126 infused groups at each infusion delay. No significant interaction between treatment and delay was found for sample exploration times, sample length or total test exploration times ($p > 0.1$). No effect of treatment was found for any exploration times. Significant delay effects were found for sample exploration times ($p < 0.01$) and test exploration times ($p < 0.01$) but not for sample length ($p > 0.1$). Post-hoc Tukey tests revealed total sample exploration times at infusion delay 1 h 30 were significantly different to 3 h ($p < 0.05$) and total test exploration times for infusion delay 1 h 30 were significantly different from infusion delay 0 min ($p < 0.05$) and 3 h ($p < 0.05$).Results presented are mean ± SEM.

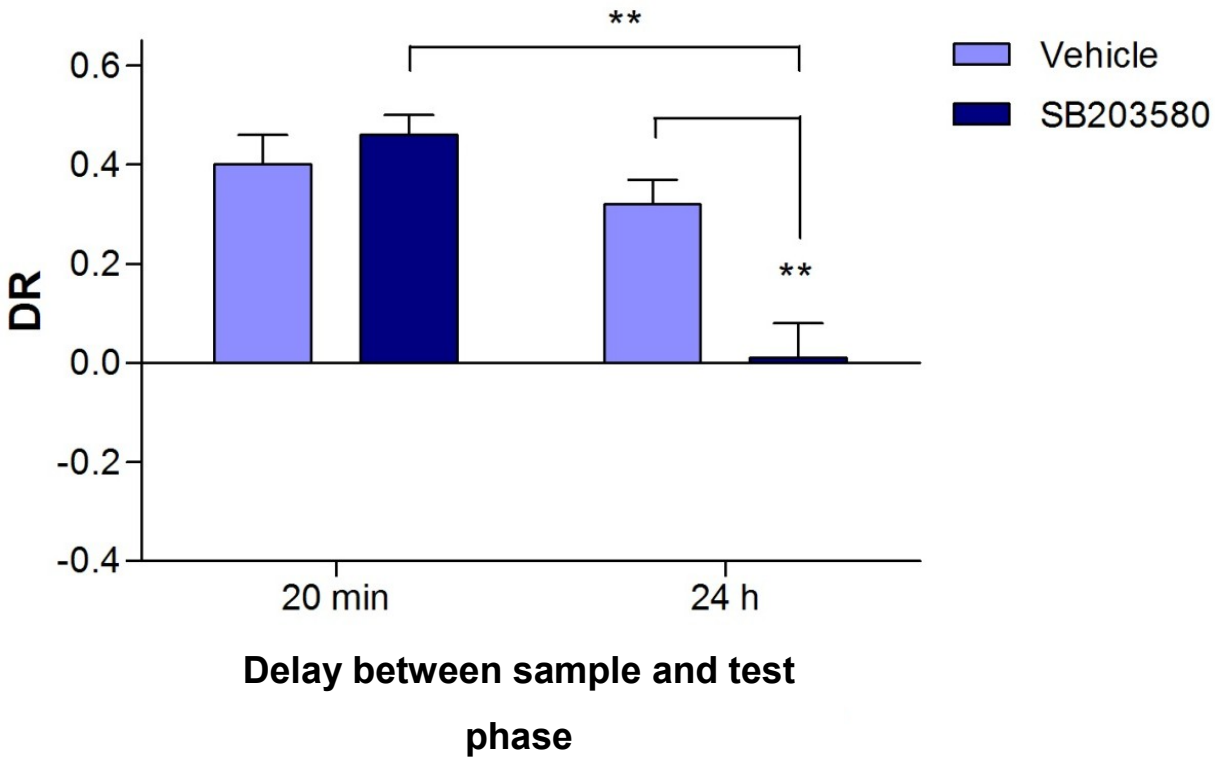
Delay	Treatment	Total sample exploration time (s)	Sample length (s)	Total test exploration time (s)
< 2 min	Vehicle	24.20 ± 3.31	240 ± 0	17.35 ± 1.84
	U0126	26.20 ± 1.2	240 ± 0	21.37 ± 2.63
1 h 30	Vehicle	25.87 ± 2.48	239.42 ± 0.54	27.23 ± 2.23
	U0126	33.91 ± 1.87	232.53 ± 5.86	24.88 ± 2.51
3 h	Vehicle	24.10 ± 2.63	240 ± 0	18.39 ± 2.14
	U0126	24.60 ± 1.61	240 ± 0	20.63 ± 2.32

II) The effect of intraperirhinal p38 inhibition on familiarity discrimination

3.3.3 p38 is required for long-term familiarity discrimination

To determine if inhibition of the p38 pathway results in the same memory impairments as those found as a result of ERK inhibition, intraperirhinal SB 203580 (50 μM) infusions were made 15 min prior to the sample phase of the NOPT and memory tested at delays of 20 min and 24 h, as described in 2.2.3.

Figure 3.3.3.1 Intraperirhinal inhibition of p38 leads to an impairment in long-term recognition memory. A significant treatment by delay interaction ($p < 0.01$) was revealed. Further, significant main effects of delay ($p < 0.001$) and treatment ($p < 0.05$) were found. ANOVA of DRs at individual time-points revealed a significant effect of treatment at 24 h ($p < 0.01$) but not at 20 min ($p > 0.1$). Bars represent mean ± SEM. Significance level ** $p < 0.01$.



Repeated measures ANOVA (drug treatment, delay) of DR values revealed a significant treatment by delay interaction [$F_{(1,22)}$ = 12.9, $p < 0.01$]. Further, significant effects of delay [$F_{(1,22)}$ = 18.87, $p < 0.001$] and treatment [$F_{(1,22)}$ = 5.48, $p < 0.05$] were found. ANOVA of DRs at individual time-points revealed a significant effect of treatment (vehicle vs. SB 203580) at 24 h [$F_{(1,10)}$ = 20.96, $p < 0.01$] and not at 20 min [$F_{(1,12)} < 1$, $p > 0.1$]. Subsequent analysis revealed no effect of delay for vehicle infused animals [$F_{(1,22)}$ = 1.01, $p > 0.1$]; conversely, for SB 203580 infusions the effect delay of was significant ($p < 0.001$). The mean DR for SB 203580-treated animals when tested at 24 h, was not significantly different from zero [$F_{(1,22)}$ = 29.61, $p < 0.001$] (Table 3.3.3.1). Vehicle treated animals at delays of 20 min and 24 h and SB 203580 treatment at 20 min were significantly different from zero ($p < 0.001$).

Table 3.3.3.1 DR values, t values and significance levels of novelty preference for vehicle and SB 203580 treated animals at different test delays (mean ± SEM). Vehicle DR values at 20 min and 24 h were significantly different from zero, demonstrating a novelty preference. At 20 min SB 203580 treated animals were also able to discriminate; however, when tested at 24 h this group were impaired (DR > 0, $p > 0.1$).

Delay	Treatment	DR mean ± SEM	Significance from zero
20 min	Vehicle	0.40 ± 0.06	t(12) 6.72, $p < 0.001$
	SB 203580	0.46 ± 0.04	t (12) 10.82, $p < 0.001$
24h	Vehicle	0.32 ± 0.05	t(10) 6.97, $p < 0.001$
	SB 203580	0.01 ± 0.07	**t(10) < 1, p > 0.1**

Table 3.3.3.2 Total exploration times in the sample and test phases and sample length for vehicle and SB 203580 infused groups at each test delay. ANOVA with repeated measures (drug treatment, delay) revealed no significant interaction or main effects for total sample exploration times, sample lengths or test exploration times. Values represent mean ± SEM.

Delay	Treatment	Total sample exploration time (s)	Total sample length (s)	Total test exploration time (s)
20 min	Vehicle	29.84 ± 2.01	227.52 ± 12.48	17.49 ± 1.08
	SB 203580	24.45 ± 2.14	234.64 ± 5.36	16.96 ± 1.17
24 h	Vehicle	29.94 ± 2.05	239.1 ± 0.9	15.81 ± 1.22
	SB 203580	28.72 ± 3.06	227.03 ± 9.24	16.61 ± 1.62

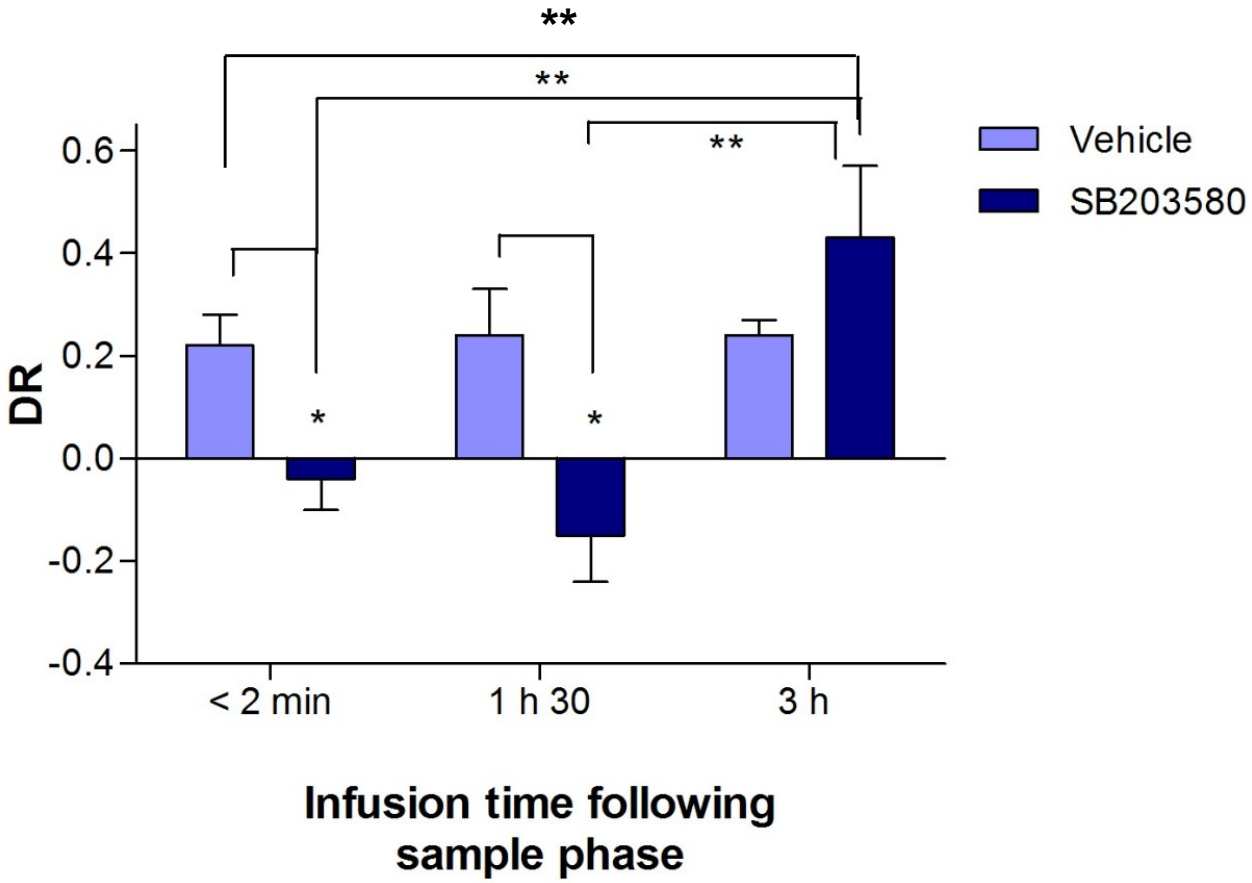
ANOVA (drug treatment by delay) revealed no significant interaction for total sample exploration time [$F_{(1,22)} < 1$, $p > 0.1$], total sample length [$F_{(1,22)} = 1.2$, $p > 0.1$] or test exploration time [$F_{(1,22)} < 1$, $p > 0.1$]. No effects of treatment on sample exploration time [$F_{(1,22)} = 2.25$, $p > 0.1$], total sample length [$F_{(1,22)} < 1$, $p > 0.1$] or total test exploration time [$F_{(1,22)} = < 1$, $p > 0.1$] were found. Further, no effect of delay was seen on sample exploration time [$F_{(1,22)} < 1$, $p > 0.1$], total sample length [$F_{(1,22)} < 1$, $p > 0.1$] or total test exploration time, [$F_{(1,22)} < 1$, $p > 0.1$].

3.3.4 Inhibition of p38 blocks long-term familiarity discrimination when infused either immediately or 1 h 30 after encoding

To determine if the inhibitory effect of p38 on familiarity discrimination results from impaired memory consolidation (as opposed to acquisition), SB 203580 (50 μM) infusions were made at varying time points following the end of the sample phase, and tested 24 h after the start of the experiment (as detailed in 3.3.2.1). A delay of 24 h was chosen as there was a memory deficit at this delay in 3.3.1.

Any DR value greater than 3 standard deviations from the mean, was removed from analysis. At each time point one animal was excluded for this reason (2.2.10).

Figure 3.3.4.1. Inhibition of p38 blocks long-term familiarity discrimination when infused either immediately or 1 h 30 after encoding. A significant drug treatment by infusion delay interaction was reported ($p < 0.01$). Significant main effects of drug treatment ($p < 0.05$) and infusion delay ($p < 0.01$) were also reported. A significant treatment effect was found when infusions were made immediately ($p < 0.05$), or 1 h 30 following the sample phase ($p < 0.05$). A significant effect of delay was found for SB 203580 infusions ($p < 0.01$), post-hoc Tukey tests revealed differences between mean DR values between SB 203580 treatments for infusions immediately - 3 h following the sample ($p < 0.01$) and 1 h 30 - 3 h ($p< 0.01$).



Repeated-measures ANOVA (treatment, infusion delay) of DR values revealed a significant treatment by infusion delay interaction [$F_{(2,26)} = 5.99$, $p < 0.01$]. Significant main effects of treatment [$F_{(1,26)} = 4.88$, $p < 0.05$] and infusion delay [$F_{(2,26)} = 7.40$, $p < 0.01$] were also reported. ANOVA at individual infusion delays revealed a significant effect of treatment when infused immediately [$F_{(1,10)} = 8.78$, $p < 0.05$], or 1 h 30 following the sample phase [$F_{(1,8)} = 9.83$, $p < 0.05$]. Analysis of mean DR values for SB 203580 infusions revealed a significant effect of delay [$F_{(2,26)} = 9.43$, $p < 0.01$]. Post-hoc Tukey's test revealed significant differences in mean DR values between SB 203580 treatments for infusions immediately following the sample compared to infusions made 3 h post-sample ($p < 0.01$). Further, mean DR values for SB 203580 infusions made 1 h 30 post-sample were significantly different from those found when infusions were made 3 h post-sample ($p < 0.01$). Analysis of DRs for the vehicle treatment revealed no significant effect of delay [$F_{(2,26)} < 1$, $p > 0.1$].

One sample *t*-tests of DR values revealed that vehicle-treated animals were able to discriminate between novel and familiar objects at each delay (DR significantly greater than zero). In contrast, when treated with SB 203580, animals were impaired when infusions were made immediately- and 1 h 30 following the sample phase ($p > 0.1$). Animals infused with SB 203580 3 h post-sample were significantly different from zero and able to discriminate novelty ($p < 0.05$) (Table 3.3.4.1).

Table 3.3.4.1 DR values, t values and significance levels of novelty preference for vehicle and SB 203580 treated animals at different infusion delays, when tested at a 24 h delay between sample and test. Vehicle-treated animals were able to discriminate between novel and familiar at each infusion delay. Following SB 203580 infusions, animals were unable to discriminate when the infusion was administered immediately ($p > 0.1$) or 1 h 30 following the sample phase ($p > 0.1$). The mean DR for animals administered SB 203580 3 h following the sample phase was significantly greater than zero.

Delay	Treatment	DR mean ± SEM	Significantly different from zero
0 min	Vehicle	0.22 + 0.06	t(10) 3.73, $p < 0.01$
	SB 203580	-0.04 + 0.06	**t(10) -0.71, p > 0.1**
1 h 30	Vehicle	0.24 ± 0.09	t(8)2.70, $p < 0.05$
	SB 203580	-0.15 ± 0.09	**t(8)-1.62, p > 0.1**
3 h	Vehicle	0.24 ± 0.03	t(8)8.43, $p < 0.001$
	SB 203580	0.43 ± 0.14	t(8)3.04, $p < 0.05$

Table 3.3.4.2 Total sample exploration times, sample length and total test exploration times for vehicle and SB 203580 treated animals infused following the memory encoding. No significant treatment by infusion delay interaction was found for total sample exploration time ($p > 0.1$), total sample length ($p > 0.1$) or total test exploration time ($p > 0.1$). No effect of treatment, or effect of infusion delay was found for sample exploration times ($p > 0.1$), total sample length ($p > 0.1$), or test exploration times ($p > 0.1$). All values are stated as mean ± SEM.

Delay	Treatment	Sample exploration time (s)	Sample length (s)	Test exploration time (s)
0 min	Vehicle	30.8 ± 2.24	233 ± 7	17.88 ± 1.55
	SB 203580	31.09 ± 2.64	223.1 ± 11.36	17.02 ± 1.23
1 h 30	Vehicle	30 ± 3.08	233.89 ± 6.11	18.75 ± 1.09
	SB 203580	32.78 ± 2.35	222.99 + 14.13	18.97 ± 1.61
3 h	Vehicle	32.18 ± 2.38	237.58 ± 2.35	20.32 ± 1.51
	SB 203580	27.13 ± 2.88	236.53 ± 3.47	20.51 ± 1.91

Repeated-measures two-factor ANOVA (treatment by infusion delay) of sample exploration times [$F_{(2,26)} = 1.35$, $p > 0.1$], total sample length [$F_{(2,26)} < 1$, $p > 0.1$] and total test exploration times [$F_{(2,26)} < 1$, $p > 0.1$] revealed no significant interaction. No effect of treatment was found on sample exploration time [$F_{(1,26)} < 1$, $p > 0.1$], total sample length [$F_{(1,26)} = 1.6$, $p > 0.1$] or test exploration time [$F_{(1,26)} < 1$, $p > 0.1$]. No effect of infusion delay was found on total sample exploration times [$F_{(2,26)} < 1$, $p > 0.1$], total sample length [$F_{(2,26)} < 1$, $p > 0.1$] or total exploration times recorded in the test session [$F_{(2,26)} = 1.70$, $p > 1$].

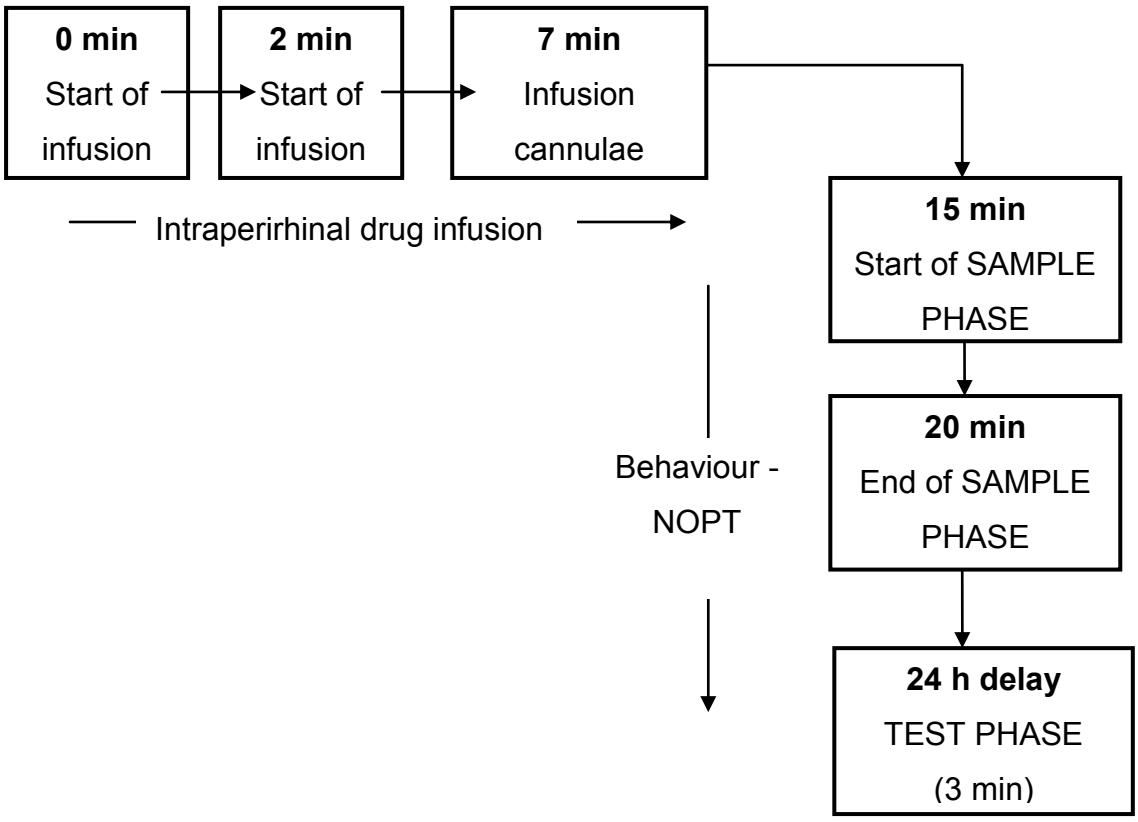
III) The effect of ERK and p38 inhibition on associative object-in-place (OIP) memory

3.5 Inhibition of ERK and p38 impairs long-term OIP memory.

To determine if ERK and p38 have a critical role in another form of recognition memory, the effect of inhibition of each pathway was tested upon associative OIP memory (Materials & Methods, section 2.2.8.2).

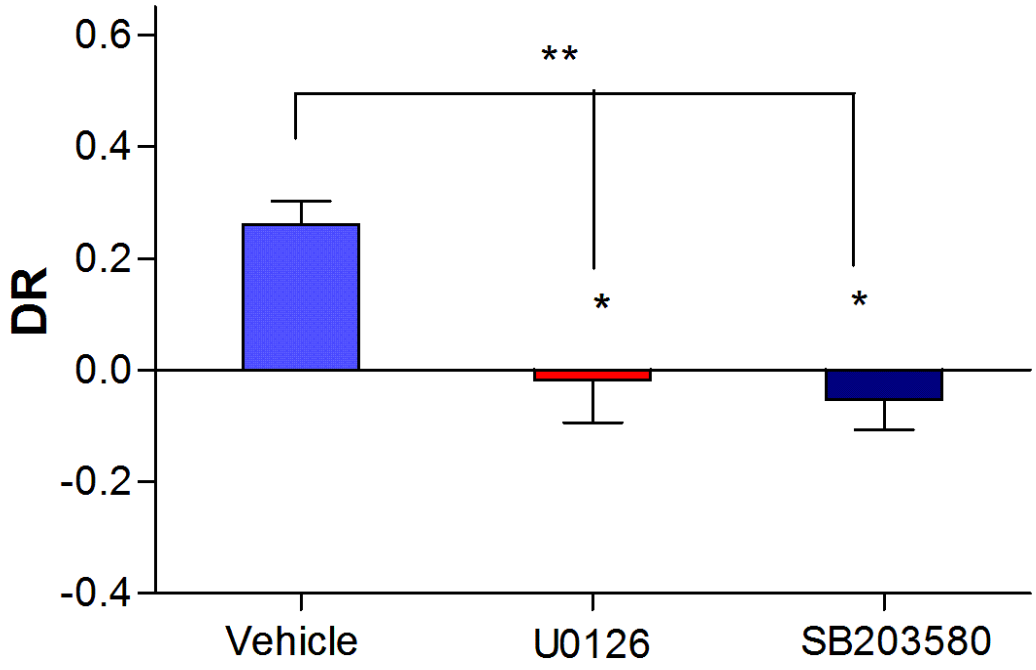
The role of ERK and p38 in the OIP task was tested using a crossover design with three sessions (vehicle, U0126, SB 203580- treatments). Inhibitors, U0126 (1 μM) or SB 203580 (50 μM), were administered bilaterally into the PRH 15 min prior to the sample phase, in which animals were allowed to explore for 300 s. Animals were tested at a 24 h delay (Materials and Methods 2.2.8.2). DR values were calculated using exploration times for the objects that had remained in the same position (familiar) versus the pair that had switched position (novel) in all 3 min of the test phase.

Figure 3.5.1 Object-in-place experimental time course. Drug infusions were made at a rate of 0.5μl /min/ hemisphere for 2 min, with infusion cannulae remaining in place for a further 5 min. The sample phase of the test started 15 min following the start of the infusion and lasted for 5 min. Animals were returned to their home cages and later reintroduced to the arena for the test phase (24 h delay).



One control animal that was more than three standard deviations from the mean DR value for the vehicle group was removed from statistical analysis (2.2.10).

Figure 3.5.1.1 Intraperirhinal infusions of ERK and p38 block long-term OIP memory formation. Infusions were made 15 min prior to the start of the sample phase and tested at 24 h delay. ANOVA of DRs revealed a significant effect of drug conditions in the OIP task ($p < 0.01$). Post-hoc Tukey tests revealed significant differences between mean DR values for vehicle and drug treatments: U0126 ($p < 0.05$) and SB 203580 ($p < 0.05$). Bars represent mean ± SEM. Significance levels: * = $p < 0.05$; ** = $p < 0.01$.



Repeated measures ANOVA (drug treatment: vehicle, U0126, SB 203580) of mean DRs in the OIP task revealed a significant difference between drug treatments [$F_{(2,16)}$ = 8.67, < 0.01]. Post-hoc analysis with Tukey's test revealed significant differences between the vehicle mean DR and mean DR value following treatment with U0126 ($p < 0.05$) or SB 203580 ($p < 0.05$). One sample *t*-tests revealed the mean DR value for the vehicle group to be significantly greater than zero, indicating a preference for the objects that had changed position in the test phase. U0126- and SB 203580- treated animals were not significant from zero (*t*-test) (Table 3.5.1).

Table 3.5.1 T-test analysis comparing the DR mean for each drug condition versus zero. The mean DR value for the vehicle condition was significantly greater than zero, indicating a preference for the objects that had changed position compared to those that had remain in the same position. Mean DR values for U0126- and SB 203580- treated animals were not significantly different from zero during the test phase of the OIP task ($p > 0.1$).

Treatment	Mean ± SEM	Significantly different from zero
Vehicle	0.26 ± 0.04	t(8) 6.35, p < 0.001
U0126	-0.02 ± 0.08	**t(8) < 1, p > 0.1**
SB 203580	-0.05 ± 0.05	**t(8) < 1, p > 0.1**

No significant differences in the sample exploration time [ANOVA: $F_{(2,16)}$ = 1.67, $p > 0.1$] or test exploration time [ANOVA: $F_{(2,16)} < 1$, $p > 0.1$] for vehicle, U0126 or SB 203580 treatments during the OIP task were found. No analysis of total sample length was performed as animals were allowed to remain in the arena for 300 s, no limit on maximum exploration was defined (2.2.8.2) (Table 3.5.2).

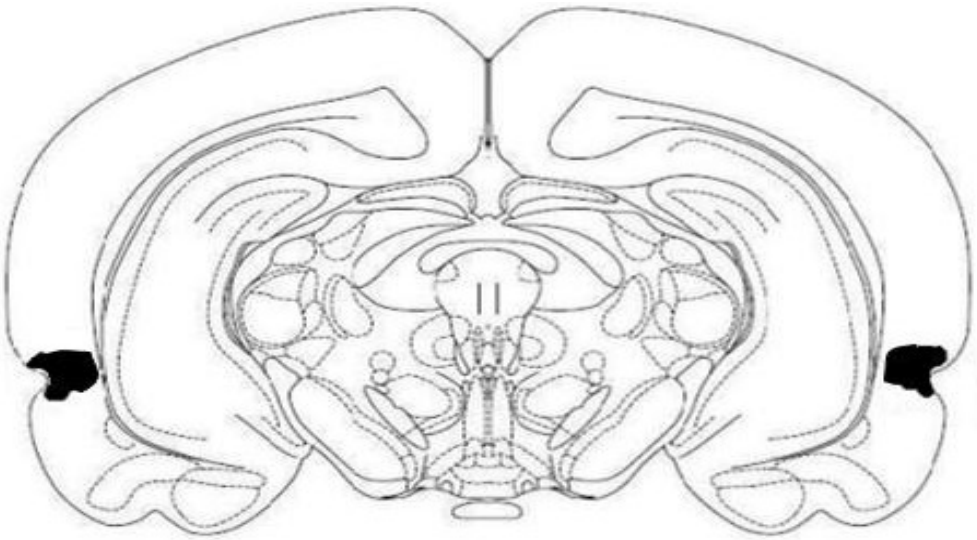
Table 3.5.2 Sample and test exploration times for vehicle and drug treatments. No significant differences between sample and test exploration times were observed during the OIP task between drug conditions ($p > 0.1$). All values are quoted as mean ± SEM.

Treatment	Total sample exploration time (s)	Total test exploration time (s)
Vehicle	44.64 ± 2.45	28.92 ± 1.45
U0126	44.37 ± 3.38	27.58 ± 1.81
SB 203580	50.76 ± 3.58	26.83 ± 1.37

3.6 Histology

Following the completion of behavioural experiments, animals were transcardially perfused before brains were removed and prepared for sectioning (Materials and Methods 2.2.12). Brain slices were stained using cresyl violet and the positions of intracerebral cannulae were determined using a stereotaxic atlas (Paxinos & Watson,1998) (Figure 3.6.1).

Figure 3.6.1 Intraperirhinal cannulae positions. PRH cannulae positions used in this Chapter were confirmed using cresyl violet stained slices and determined using a stereotaxic atlas. Cannula tips were found to be positioned between -5.2 mm and -5.8 mm posterior to bregma with all infusion sites within area 35 or 36. Approximate cannulae positions are illustrated on a section -5.6 mm posterior to bregma.

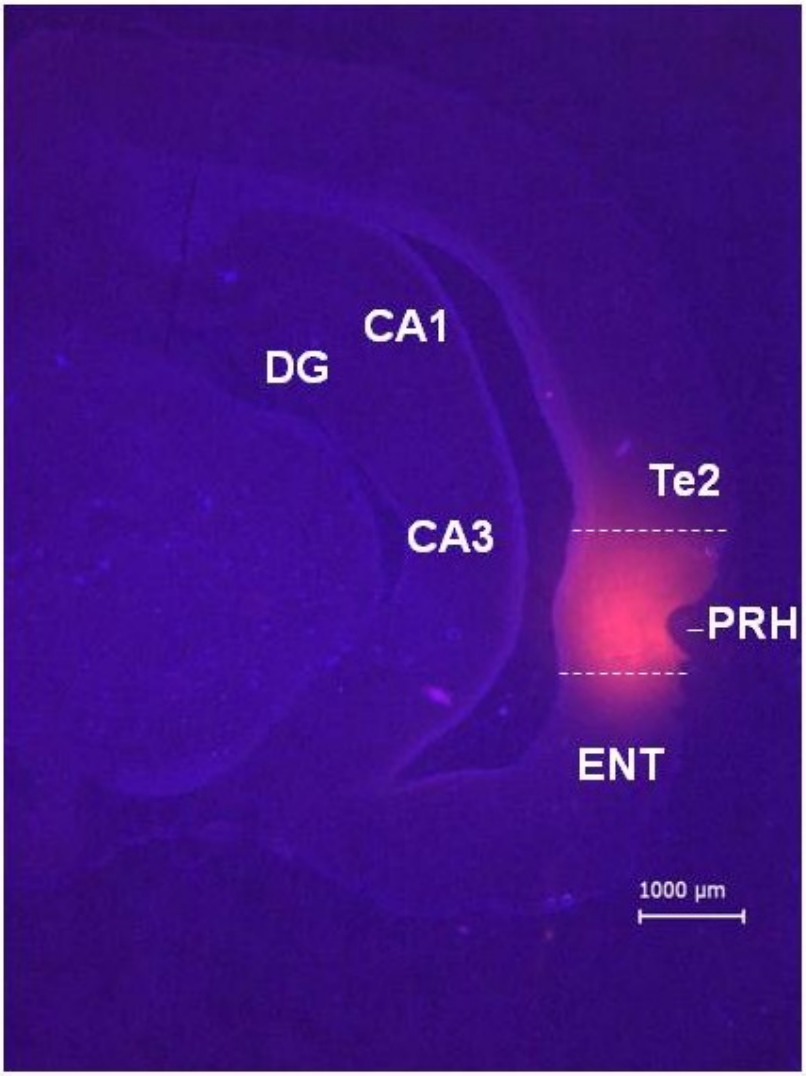


3.7 Intraperirhinal infusions of fluorophore-conjugated muscimol to visualise drug spread

Following intraperirhinal infusions, drug spread has been estimated to extend approximately 0.5 - 1 mm from the cannula tip (Martin 1991; Izquierdo, Barros et al. 2000; Attwell, Rahman et al. 2001; Seoane, Tinsley et al. 2011). Drug spread is thought to largely encompass PRH, with minimal spread extending to adjacent entorhinal cortex and area Te2 (Jo and Lee 2010; Winters, Bartko et al. 2010).

In order to visualise drug spread following PRH infusions, infusions were made using fluorophore-conjugated muscimol (FCM) (Winters, Bartko et al. 2010) (Materials and Methods 2.3). When tested behaviourally, FCM can be directly compared to the effects of $GABA_A$ receptor agonist, muscimol (Allen, Narayanan et al. 2008). Analysis of drug spread was performed on four brain hemispheres.

Figure 3.7.1 Spread of FCM following intraperirhinal infusion. Overlaid coronal image (1.25 x magnification), demonstrating maximal perirhinal drug spread. Drug spread is mainly contained within PRH, with some detection of fluorescence in adjacent ENT and area Te2. [Abbreviations: CA1/CA3- hippocampal subfields; DG- dentate gyrus ENT- entorhinal cortex; Te2 – area Te2].



Consistent with previous findings, intraperirhinal infusions resulted in drug spread chiefly restricted to PRH. The anterior-posterior spread extended 2.2 ± 0.12 mm (mean ± SEM). Traces of FCM were observed in an area extending from -3.8 mm to -6.8 mm posterior to bregma (Paxinos & Watson, 1998). In the rat, PRH is considered to begin approximately -4 mm from bregma and extend 2.5 mm caudally (Shi and Cassell 1999). FCM staining was strongest closest to the cannula tip and extended 1.7 ± 0.2 mm along the dorsal-ventral axis and 1.2 ± 0.1 mm medio-laterally. Cannula tips were positioned between - 5.2 mm and - 5.6 mm posterior to bregma (Paxinos & Watson, 1998). Maximal volume of drug penetration was calculated (2.3.4) to be 0.99 mm^3 (0.99 µl). Limited, diffuse spread to adjacent areas Te2, ENT and HPC was seen (Figure 3.7.1). Drug spread to the HPC was confined to the superficial layers.

3.8 Discussion

Inhibition of PRH ERK or p38 blocked long-term (≥ 6 h) familiarity discrimination when administered prior to- or < 3 h post-acquisition. Furthermore, intraperirhinal inhibition of either cascade resulted in OIP associative memory impairments when tested at a 24 h delay.

3.8.1 Levels of exploration in the NOPT

Levels of exploration in the sample and test phases, as well as total sample time (a maximum level of 40 s of exploration was allowed before the sample phase was terminated) were analysed to determine whether behavioural impairments result from a lack of exploration or gross lack of motivation or mobility impairments following drug treatment. No significant differences in levels of exploration in the sample or test phases were reported as a result of drug treatments. In addition, no changes in spontaneous behaviour across control groups were found.

In experiments, 3.3.1 (20 min delay), and 3.3.2 (1 h 30 post-sample infusion), significant effects of delay (test delay or infusion delay, respectively) on sample and test exploration levels were found. No difference in exploration levels of the vehicle- or U0126- treated animal were found in these experiments. Higher levels of exploration in these experiments compared to that seen at other delays result from increased interest in the objects being tested. Although the mean levels of exploration were significantly higher in the reported experiments, in each experiment, animals were expected to complete a minimum level of exploration in the sample phase to ensure they were able to discriminate in the test phase. Animals were also expected to explore to a minimum level in the test phase. Those that did not meet the exploration criteria were excluded from statistical analysis (Materials and Methods, 2.2.10).

Is the behavioural effect identified mnemonic or perceptual?

Drug-treated animals infused prior to encoding were able to discriminate novelty at shorter delays (20 min) and were therefore able to perceive the objects presented in the sample phase. The ability to discriminate at 20 min supports the hypothesis that the behavioural impairments found at longer delays result from mnemonic and not perceptual deficits. All objects tested in this Chapter were considered to have low feature ambiguity (share few overlapping features). PRH-lesioned animals are most severely impaired in visual discrimination tasks in which feature ambiguity is highest, i.e. visual stimuli presented share overlapping features (Eacott, Machin et al. 2001; Bussey, Saksida et al. 2003; Bartko, Winters et al. 2007; Bartko, Winters et al. 2007; Murray, Bussey et al. 2007).

During the NOPT, it is possible that re-exposure to the previously encountered object in the test phase may render the consolidated memory labile when reactivated (Nader, Schafe et al. 2000; Debiec, LeDoux et al. 2002; Milekic and Alberini 2002; Duvarci and Nader 2004; Duvarci, Nader et al. 2005; Nader and Einarsson 2010). In order to maintain the memory an additional process: reconsolidation is required (Kelly, Laroche et al. 2003). It is still not fully understood to what extent the mechanisms underlying reconsolidation are similar to those that underlie consolidation, although both processes have been identified to share similar substrates (Duvarci and Nader 2004; Duvarci, Nader et al. 2005; Inda, Delgado-Garcia et al. 2005; Cestari, Costanzi et al. 2006). However, the NOPT tested in this Chapter did not tax mechanisms underlying reconsolidation.

3.8.2 Immediate ERK activation is required for consolidation of long-term familiarity discrimination

MEK inhibitor, U0126, infused directly into PRH prior to the sample phase of the NOPT and tested at varying delays, resulted in memory impairments at delays ≥ 6 h. When tested at shorter delays of 20 min 1 h and 3 h, U0126- and vehicle-treated animals did not differ in showing preference for novel over visual stimuli previously encountered. These findings suggest that the ERK pathway is required for longer-term recognition memory. As infusions of U0126 following memory encoding (in the sample phase) also resulted in memory impairments at a 24 h delay, the effect of the inhibitor is explicable by interference with consolidation rather than acquisition. To further understand the time course of ERK activation in memory consolidation, U0126 was infused at varying delays following memory encoding. Inhibition of MEK immediately (< 2 min) following the sample phase and tested at 24 h delay resulted in memory impairment. No significant effect on memory consolidation was found when U0126 was infused at the 3 h delay, suggesting that the effect is on early rather than late consolidation processes. No significant difference in DR values was found between the vehicle and drug group when U0126 was infused 1 h 30 min following encoding; however, U0126-treated animals were unable to discriminate between the novel and familiar objects. This equivocal result suggests that by 1.5 - 2 h after acquisition activity of the drug could no longer fully inhibit consolidation. Infusions of (1 μM) MEK 1/ 2 inhibitor, U0126, (General Introduction 1.8.1), selectively inhibit the dual phosphorylation of downstreams kinases, ERK 1 and 2. A total volume of 1 μl U0126 would be considered to diffuse approximately 1 mm from the site of infusion (3.8.6) and selectively target ERK, with no effect on other PRH protein kinases. The effect of MEK inhibition in PRH is considered large enough to prevent function.

A wide body of experimental evidence supports a critical role for ERK in long-term memory (Atkins, Selcher et al. 1998; Blum, Moore et al. 1999; Selcher, Atkins et al. 1999); but not shorter-term memory formation (Berman et al., 1998; (Schafe, Atkins et al. 2000; Kelly, Laroche et al. 2003). Moreover,

memory impairments associated with ERK inhibition at delays $\geq$ 6 h is consistent with findings by Schafe et al. (2000). No role for ERK was found when U0126 was infused prior to acquisition and tested at delays of 20 min, 1 h or 3 h.

Further, an early role for ERK in long-term recognition memory consolidation is consistent with studies which demonstrate that MEK inhibition either prior to- or immediately following memory encoding blocks memory formation when tested at 24 h (Atkins, Selcher et al. 1998; Walz, Roesler et al. 1999) or 48 h (Berman, Hazvi et al. 1998; Blum, Moore et al. 1999; Hebert and Dash 2002; Zhang, Okutani et al. 2003) (Table 3.8.2.1).

Table 3.8.2.1 Examples of ERK and p38 activation following learning, and use of inhibitors to block memory

Task	Brain region	MAPK	
		ERK	P38
Fear conditioned learning	Amygdala	pERK increased 1 h MEK inhibition 30 min prior training blocked contextual learning ≥ 6 h	-
	Hippocampus	MEK inhibition 1 h prior or immediately post-training blocked one trial cued/contextual learning (24 h)	-
Inhibitory avoidance learning	Entorhinal cortex	MEK inhibition immediately post-training blocked LTM (24 h)	-
	Parietal cortex		-
	Hippocampus	pERK increased 2 h post-training	p-p38 increased immediately Inhibition immediately post-training blocked STM/LTM
IA extinction	Hippocampus	MEK inhibition blocked extinction when administered prior to- or immediately post retrieval	Inhibition immediately post-retrieval impaired IA extinction
Morris Water Maze (spatial)	Entorhinal cortex	MEK inhibition immediately post-training blocked LTM (48 h)	-
	Hippocampus	pERK increased 5-30 min MEK inhibition blocked LTM (48 h)	-
Conditioned eye-blink response	Cerebellar vermis	pERK increased immediately following learning MEK inhibitor (ICV) pre-conditioning blocked learning	p-p38 increased immediately following learning p38 inhibitor (ICV) blocked learning
	Hippocampus		-
Taste aversion task	Insular cortex	pERK increased within 10 min; peak = 30 min MEK inhibitor blocked LTM (72 and 120 h)	-

3.8.2.1 ERK and recognition memory

Kelly et al. (2003) demonstrated a critical role for ERK in long-term recognition memory by infusing U0126 into the lateral ventricle 40 min prior to a NOPT and testing at a 24 h delay. Intraperirhinal infusion in the present study demonstrates a specific requirement for this brain region in familiarity discrimination.

The study by Kelly et al. (2003) used a different variant of the NOPT in which the sample phase of this task allowed the animal to explore two different objects (A-B), over three five min sessions. Following a retention delay (10 min or 24 h), animals were presented with a previously investigated object and a novel object (A-C). This task differs from the NOPT variant described in this Chapter as two identical objects (A-A) were initially presented in the sample phase of the present study and explored for a maximum of 40 s or 4 min, which ever occurred sooner (refer to section 2.2.8.1). The A-B variant, in contrast to the A-A variant of the NOPT requires the functional integrity of the hippocampus for successful novelty discrimination; NMDA antagonist, AP5 infused into the hippocampus prior to encoding of two different objects impaired object recognition memory (Unpublished Barker, 2011). Animals infused with intrahippocampal AP5 were not impaired using the A-A variant of the NOPT at 24 h (Unpublished Barker, 2011).

Extensive evidence exists to indicate a critical role of PRH in identifying the prior occurrence of individual items (familiarity discrimination) (reviewed in Brown 2010). It is widely established that HPC lesioned animals are unimpaired in the version of the NOPT used in the present study (A-A variant) (Mumby 2001; Gaskin, Tremblay et al. 2003; Forwood, Winters et al. 2005; Mumby, Tremblay et al. 2005); further, fornix lesioned animals are able to perform the task (Ennaceur, Neave et al. 1996; Ennaceur and Aggleton 1997; Warburton and Aggleton 1999). It is however possible to devise conditions in which the hippocampus is also required to perform the task. Several studies have demonstrated that variants of the NOPT can be vulnerable to disruption of the hippocampus (Mansuy, Mayford et al. 1998; Pittenger, Huang et al.

2002; Oh, Han et al. 2010). The A-B variant of the NOPT appears to tax spatial memory possibly by introducing contextual cues or object association in addition to testing novel preference (Barker and Warburton 2011). By altering the NOPT to test for novelty and additional spatial components it may be possible to explain impairments associated following inhibition of additional brain regions such as PFC or HPC. Furthermore, repeat or increased familiarisation (extended or multiple sample phases) can result in PRH-independent mechanisms supporting object-recognition memory (Mumby, Piterkin et al. 2007; Albasser, Davies et al. 2009). It is therefore paramount to fully understand the precise conditions used in the task before interpreting the results.

It has been reported that ERK inhibition in the hippocampus and prefrontal cortex results in long-term recognition memory deficits using an A-B variant of the NOPT in the mouse (Kamei, Nagai et al. 2006; Nagai, Takuma et al. 2007). In addition, over-expression of type-1 adenylyl cyclase, important for coupling Ca^{2+} to cAMP (upstream of ERK signalling), in the forebrain of a mutant mouse improved object recognition memory tested using the A-B variant at 24 h compared to wild-type (WT) controls. Enhanced performance was considered to result from increased levels of pERK. Further, increased sample exploration lengths allowed mutant mice to discriminate when tested 5 days following initial training (Wang, Ferguson et al. 2004).

3.8.3 Consolidation of long-term familiarity discrimination requires p38 activation between 0 min and 3 h.

Inhibition of p38 was found to result in long-term familiarity discrimination impairments when infused into PRH 15 min prior to the sample phase and tested at a 24 h delay. Post-sample infusions of SB 203580 at 0 min and 1 h 30 also resulted in memory impairments at 24 h. No memory impairment was found when SB 203580 was infused 3 h post-sample, indicating that the time window for the effectiveness of inhibition of p38 is somewhere between 0 min and 3 h following memory encoding. SB 203580 is known to selectively inhibit p38 isoforms p38α and p38β and at the 50 μM dose administered during this

chapter, be selectively for these kinases exclusively (General Introduction 1.8.2).

Studies investigating the role of p38 in inhibitory avoidance learning found a requirement for the kinase in both short and long-term memory; SB 203580 infused directly into CA1 immediately following training resulted in an impairment when tested at 24 h. No memory impairments at 24 h were found when the inhibitor was infused 30 min or 2 h post-training (Alonso et al., 2003). Levels of both p38 and ERK phosphorylation are elevated in the anterior cerebellar vermis between 2 min and 180 min following conditioned eye-blink response in rabbits (Zhen et al., 2001). Further, intrahippocampal infusions of p38 inhibitor, SB 203580, immediately after reinforced retrieval inhibited inhibitory memory extinction (Rossato, Bevilaqua et al. 2006). An immediate requirement for p38 activation in memory consolidation and extinction, as described above, is consistent with p38 inhibition impairing recognition memory consolidation between 0 min and < 3 h following memory encoding (refer to Table 3.8.2.1).

3.8.4 ERK and p38 activation are required for the associative-object-in-place task.

Rats are sensitive to the rearrangement of familiar items and will spend more time exploring an object that has changed location compared to one that is unchanged (Aggleton & Dix 1999). Inhibition of PRH ERK or p38 cascades resulted in memory impairments in long-term OIP memory. When tested at 24 h both inhibitors impaired the ability of animals to discriminate between the pair of objects that had changed position and the pair of objects that had remained unmoved. OIP memory requires associations to be made between a group of objects and the position in which they are encountered (Dix and Aggleton 1999). This type of memory is dependent upon interaction between the PRH , HPC and PFC neural circuitry (Barker and Warburton 2008). The PRH is thought to provide object related information, whereas the HPC and prefrontal cortex are thought to provide spatial information and detect

rearrangement of the objects, respectively (Barker, Bird et al. 2007; Warburton and Brown 2010; Barker and Warburton 2011).

3.8.4.1 Roles for both ERK and p38 in recognition memory.

Differential roles of ERK and p38 have been previously established in certain previous studies. An increase in ERK activation and not p38 was found immediately following training in the Morris Water Maze in the CA1/CA2 region of the hippocampus (Blum, Moore et al. 1999). Further, exposure to novel tastes resulted in an increase in ERK phosphorylation between < 30 min – 1 h in the insular cortex; whereas no changes in phosphorylated p38 were found (Berman et al., 1998). Both phosphorylation of ERK and p38 increased in the cerebellar vermis following associative learning; however, only ERK levels increased in the hippocampus (Zhen, Du et al. 2001). The use of intraperirhinal infusions of MEK inhibitor, U0126, and p38 inhibitor, SB 203580, as tested in this Chapter demonstrates roles for both ERK and p38 in recognition memory: inhibition of either resulted in a deficit. Note similar involvement in early consolidation (similar time courses). However, it is still not understood whether the pathways are independent, or whether crosstalk exists.

3.8.5 Roles for protein kinases in recognition memory

Inhibition of protein kinases, PKA, PKC, CaMKII and CaMKK result in recognition memory impairments when tested at 24 h (Tinsley, Narduzzo et al. 2009; Tinsley, Narduzzo et al. 2011) Nadine Fontaine-Palmer, unpublished PhD thesis 2008). The ERK signalling cascade is a point of convergence for several protein kinases (Roberson, English et al. 1999). Whether or not these kinases interact with p38 is yet to be established. Further, the interaction between ERK and p38 signalling and such pathways previously identified to underlie recognition memory in PRH have yet to be established. CaMKII is widely reported to activate ERK via upstream kinases Ras and Raf-1 (Illario, Cavallo et al. 2003; Oh, Manzerra et al. 2004); however, this study has found an earlier requirement for ERK than CaMKII in long-term recognition memory consolidation. Inhibitor studies have demonstrated that CaMKII activity is

essential for consolidation of long-term object recognition memory between ~ 20 min and 100 min post-sample (Tinsley, Narduzzo et al. 2009). CaMKK activation is critical up to 20 min following encoding (Tinsley, Narduzzo et al. 2011). ERK and p38 activation is required between 0 and 3 h following memory encoding. However, even though ERK and p38 are activated earlier than both kinases described, it remains possible that there is interaction between these pathways once activated. Moreover, ERK and p38 activation is transient and, consistent with CaMKK and CaMKII, their activation is not required throughout the duration of the memory (24 h). Storage of long-term memories (memory consolidation) requires mRNA transcription and protein synthesis, whereas short-term memory is insensitive to protein synthesis inhibitors (Davis and Squire 1984; McGaugh and Izquierdo 2000). A role for ERK in transcriptional regulation in neurons is well established (Treisman 1996). Downstream targets of ERK and p38 include transcription factors CREB and ELK-1 (Atkins, Selcher et al. 1998; Blum, Moore et al. 1999; Cammarota, Bevilaqua et al. 2000; Bozon, Kelly et al. 2003; Zhang, Okutani et al. 2003; Schiller, Bohm et al. 2006). A requirement for CREB activation in PRH has been established for long-term recognition memory and not memory tested at short (15 min) delays (Warburton, Glover et al. 2005). However, it has not been established that CREB or ELK-1 are targets downstream of MAPK signalling in PRH.

3.8.6 Intraperirhinal drug infusions and limitations.

The use of intraperirhinal FCM drug infusions allowed the visualisation of drug spread within PRH. Consistent with previous findings drug spread was mainly contained within PRH and thus behavioural impairments associated with drug infusions can be attributed to inhibition within PRH. An anterior-posterior spread measuring 2.2 mm, is in accordance with previous studies in which the spread is estimated to extend ~ 1 mm (section 3.7) Maximum medial-lateral and dorsal-ventral spread was < 1 mm (Allen et al., 2001). As measurements along the x (medial-lateral), y (dorsal-ventral), z (anterior-posterior) axes were

different, FCM drug spread was considered elliptical. Maximum FCM volume calculated was 0.99 ± 0.02 mm^3.

Few fluorophore-conjugated molecules are available. Although fluorophore-conjugated muscimol serves as a useful tool in visualising drug spread, it is not possible to make direct comparisons between the spread of muscimol and the inhibitors tested in this thesis. Ordinarily, different inhibitors have varying pharmokinetic properties and thus the rate of drug spread would be expected to differ.

3.8.7 Limitations of the NOPT

When interpreting results from the NOPT, it is imperative to minimise spatial or associational factors related to the task, preventing the use of brain regions other than PRH. The use of somatosensory information, such as texture and shape, can be detected by rodents' vibrissae and can aid discrimination originally considered visual (Brown et al, 2012).

Furthermore, the use of localised infusions prevents impairment associated with global effects; reversible inhibitors should have an effect in the target region within 15 min and last up to 2 h (Day et al, 2003).The duration of the individual inhibitors' efficacy used in this Chapter was not investigated and therefore this window of activity is estimated. Care should therefore be exercised when interpreting findings in which the result is null or equivocal as drug concentrations may be too low to maintain inhibition, as opposed to a lack of kinase dependency.

3.8.8 Chapter conclusions and further experiments

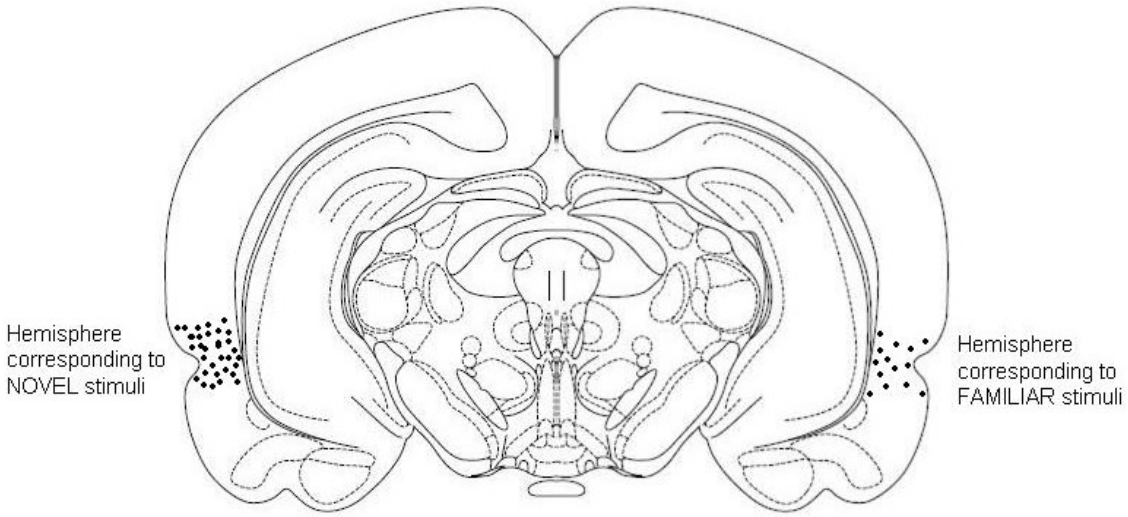
ERK has been widely established as critical for processes underlying learning and memory, although a role for this kinase within PRH recognition memory had not been unequivocally established. Fewer studies have focused on the role of MAPK subtype p38. This Chapter demonstrated that intraperirhinal inhibition of ERK or p38 blocked consolidation of long-term familiarity discrimination memory. Further, a critical role for both ERK and p38 in PRH for associative object-in-place recognition memory was found. Investigation into the interaction of ERK and p38 pathways in PRH, and further, the interaction of MAPK signalling with nuclear targets (CREB, Fos) and other kinases identified to underlie recognition memory should be explored.

Chapter 4. Measurement of neuronal activation using phosphorylated ERK and p38 in response to novel and familiar visual stimuli

4.1 Introduction

The activity of populations of neurons in PRH and adjacent temporal association cortex, Te2, change in response to the relative familiarity of visual stimuli (Brown, Wilson et al. 1987; Zhu and Brown 1995; Zhu, Brown et al. 1995; Zhu, Brown et al. 1995; Brown and Xiang 1998; Xiang and Brown 1998; Brown and Aggleton 2001; Brown and Bashir 2002). This change in neuronal activation is a putative neural substrate of familiarity discrimination (Brown and Xiang 1998; Brown and Aggleton 2001). Using the paired-viewing procedure (PVP) it is possible to present novel visual stimuli to one eye and familiar stimuli to the opposite eye and, using immunohistochemical techniques, to quantify changes in the activity of populations of neurons in the hemisphere of the brain contralateral to the presented stimuli (Materials and Methods, section 2.4). By this means, the activity generated by novel stimuli can be compared to that generated by familiar stimuli under closely controlled conditions. Fos, the protein product of immediate early gene c-*fos*, has been studied as a marker of neuronal activity following the PVP, with peak activation measured 90 min following the presentation of visual stimuli (Zhu, Brown et al. 1995; Zhu, McCabe et al. 1996; Wan, Aggleton et al. 1999; Warburton, Koder et al. 2003; Seoane, Massey et al. 2009). Fos protein expression has been established as a marker of LTD in the cerebellum (Nakazawa, Karachot et al. 1993) and hippocampus (Lindecke, Korte et al. 2006) (General Introduction, section 1.6.4.2).

Figure 4.1.1 Diagrammatic representation of neuronal activation in response to both novel and familiar stimuli presented to individual eyes and detected using Fos stained nuclei. Studies have demonstrated a decrease in neuronal activation in response to viewing previously encountered stimuli compared to novel stimuli (Brown, Wilson et al. 1987; Zhu and Brown 1995; Zhu, Brown et al. 1995; Zhu, Brown et al. 1995; Zhu, McCabe et al. 1997; Wan, Aggleton et al. 1999; Brown and Aggleton 2001).



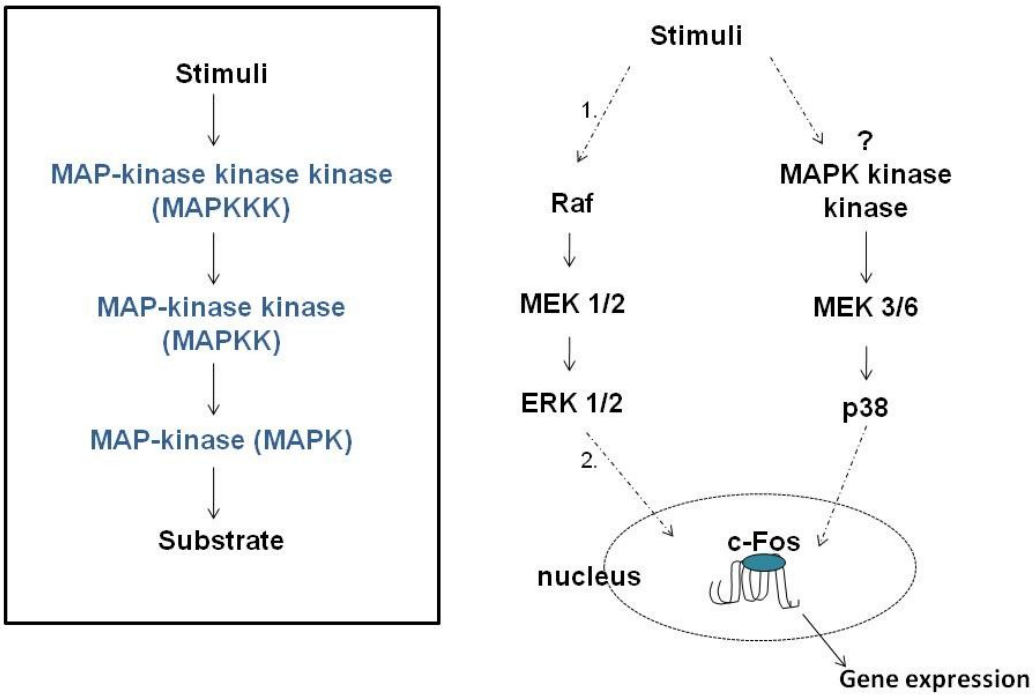
The novelty-induced increase in neuronal activation in PRH (as measured by Fos expression) can be disrupted by systemic administration of the muscarinic cholinergic antagonist scopolamine (Warburton, Koder et al. 2003), the benzodiazepine lorazepam (Wan, Warburton et al. 2004), PKC antagonist NPC15437 (Nadine Fontaine-Palmer, unpublished PhD thesis 2008), and L-type VDCC antagonist verapamil (Seoane, Massey et al. 2009), or by adenoviral transduction of PRH and area Te2 with a dominant-negative construct (A-CREB) (Warburton, Koder et al. 2003; Wan, Warburton et al. 2004; Warburton, Glover et al. 2005; Seoane, Massey et al. 2009).

In addition to Fos expression as a marker of neuronal activation in response to visual stimuli, transcription factor CREB (Warburton, Glover et al. 2005) and intracellular protein kinases, CaMKI (Tinsley, Narduzzo et al. 2011) and CaMKII (Tinsley, Narduzzo et al. 2009) have been shown to be differentially phosphorylated in response to novel and familiar stimuli using the PVP between 10 - 40 min and 70 min, respectively. Furthermore, localised inhibition of CaMKII activation by infusing AIP into PRH 20 min following presentation of novel images or of CaMKK (upstream of CaMKI) activation by STO-609 infused 15 min prior to the PVP test session reduce levels of phosphorylated CaMKII or CaMKK, respectively in the drug-infused hemisphere (Tinsley, Narduzzo et al. 2009; Tinsley, Narduzzo et al. 2011) .

ERK and p38 are downstream effectors of separate intracellular MAPK cascades. Their substrates include transcription factors, which in turn are responsible for gene expression (Leevers, Paterson et al. 1994; Treisman 1996). Both subfamilies of the MAPK signalling cascade are upstream of Fos protein (Price, Cruzalegui et al. 1996; Price, Hill et al. 1996; Schiller, Bohm et al. 2006). Therefore it would be expected that activation of ERK and p38 (if they are recruited following stimulus presentations in the PVP) would precede transcription of the Fos nuclear protein. Thus animals were sacrificed at delays of 10, 40 and 70 min and levels of pERK and p-p38 were measured in response to novel and familiar stimuli. A further delay of 100 min was introduced in order to include the time at which Fos activation is maximal.

Further, to determine if inhibition of MEK (the intermediate in the ERK cascade) could abolish the novelty-induced increase in pERK as found at 10 min following the PVP, unilateral intraperirhinal infusions of U0126 were administered prior to the test session of the PVP (in which randomised novel stimuli were presented to both eyes).

Figure 4.1.2 Simplified schematic representation of the possible interaction of ERK and p38 cascades with immediate early gene, c-*fos* in PRH. ERK and p38 are upstream kinases of immediate early gene, c-*fos* (Schiller, Bohm et al. 2006). 1). Dotted arrows represent missing steps that result in phosphorylation of ERK and p38 in PRH. 2) Dotted arrows indicate downstream targets of MAPK signalling that phosphorylate nuclear substrates e.g. c-*fos*, and result in Fos protein production.



4.1.1 Chapter objectives

This Chapter seeks to elucidate, using the PVP, the time course of activation of MAPK isoforms, ERK and p38, in response to novel and familiar visual stimuli by varying the time of perfusion following the test viewing session.

Furthermore, the effect of intraperirhinal inhibition of MEK (upstream of ERK) prior to the test session will be used to assess whether memory impairments as seen in the spontaneous preferential exploration task (Chapter 3) are paralleled by changes in levels of phosphorylated ERK (pERK) in PRH following PVP.

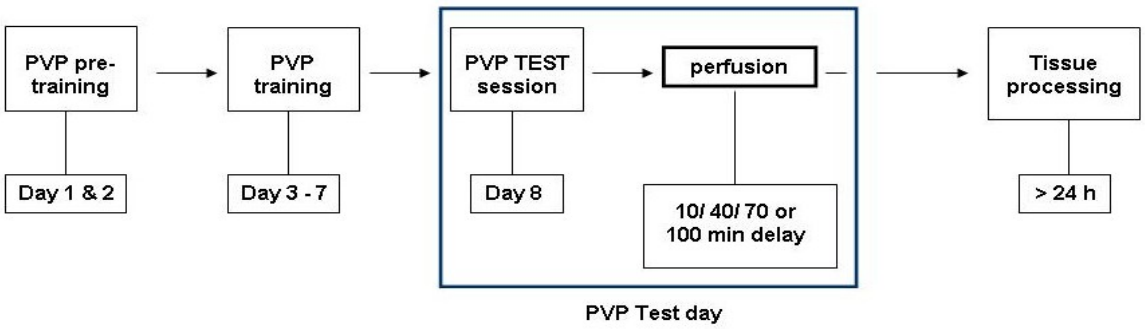
I) Measurement of pERK in response to novel and familiar visual stimuli

4.2 Measurement of phosphorylated ERK in response to novel and familiar visual stimuli

4.2.1 Method

As described in Materials & Methods, section 2.4.3.2, male DA rats were trained using the PVP procedure and perfused at varying delays (10, 40, 70 and 100 min) following the final test session. Images of brain regions - auditory cortex (AUD), entorhinal cortex (ENT), perirhinal cortex (PRH), associative visual cortex, (Te2) and visual cortex (V1) - were taken from 40 rats (10 at each delay).

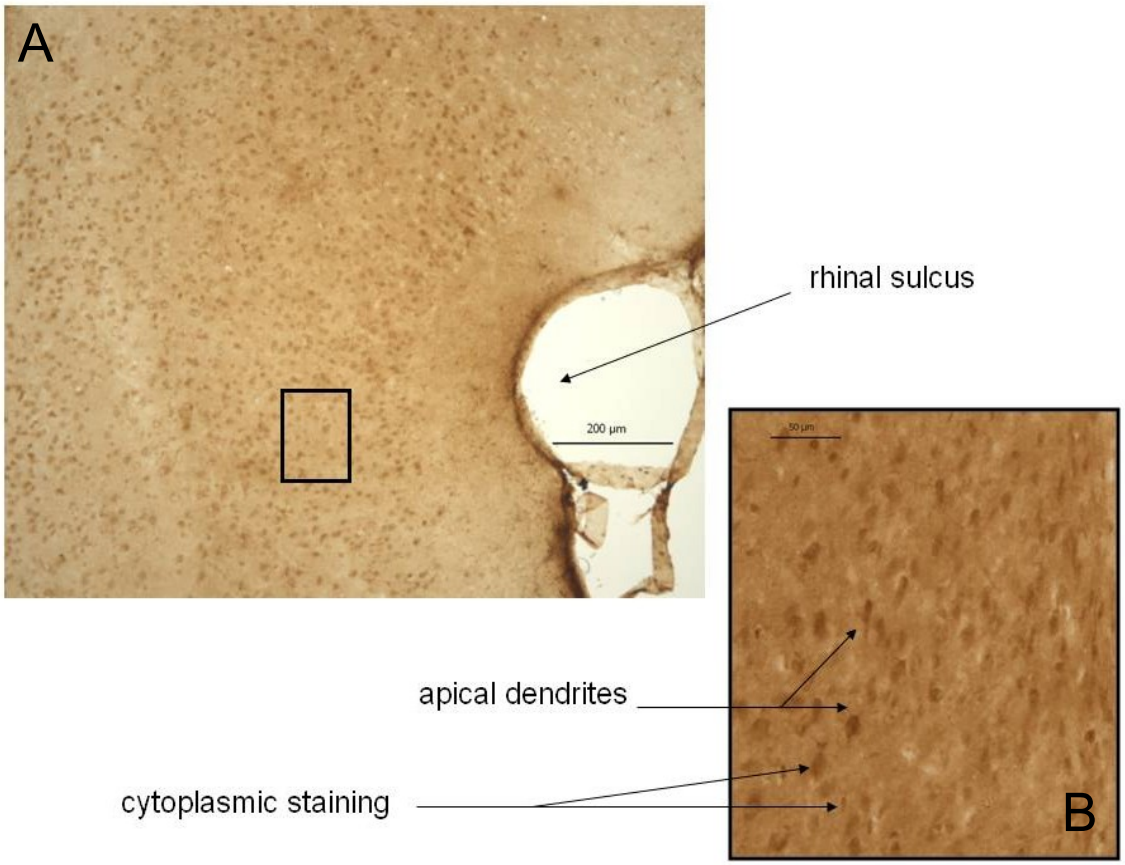
Figure 4.2.1.1 Schematic representation of the PVP procedure.
The PVP procedure comprises two days of pre-training and five days of training in which visual stimuli are presented in conjunction with a juice reward. Training days comprise a morning and afternoon session. Morning sessions present the same set of images in a randomised order to both eyes; whereas the afternoon sessions are counterbalanced so each eye sees equal numbers of novel and familiar (as seen each morning) images. The test day of the experiment is composed of a standard morning session and an afternoon session in which one eye is shown novel images and the other eye is presented with the familiar images. Primarily contralateral processing of the visual stimuli allows the neuronal responses to novel and familiar stimuli to be quantified and compared between the two brain hemispheres using immunohistochemical techniques (Zhu, McCabe et al. 1996).



4.2.2 Detection and quantification of phosphorylated ERK.

Phosphorylated ERK was detected using an anti-active MAPK antibody raised against a dually phosphorylated peptide sequence of the catalytic core of active ERK (Promega, UK). Anti-active MAPK/ERK (1:250) was used to detect dually phosphorylated ERK 1 and ERK 2 on residues Thr^{183} and Tyr^{185} (Crow, Xue-Bian et al. 1998; Schmid, Pruitt et al. 2000; Liu, Austin et al. 2002; Cottom, Salvador et al. 2003) (Material & Methods, 2.5).

Figure 4.2.2.1 Example of typical phosphorylated ERK staining
A. pERK staining in perirhinal cortex. **B.** Higher magnification of image (of area in rectangle shown in A) showing the subcellular location of pERK to be predominantly cytoplasmic, although dendritic staining was noted.

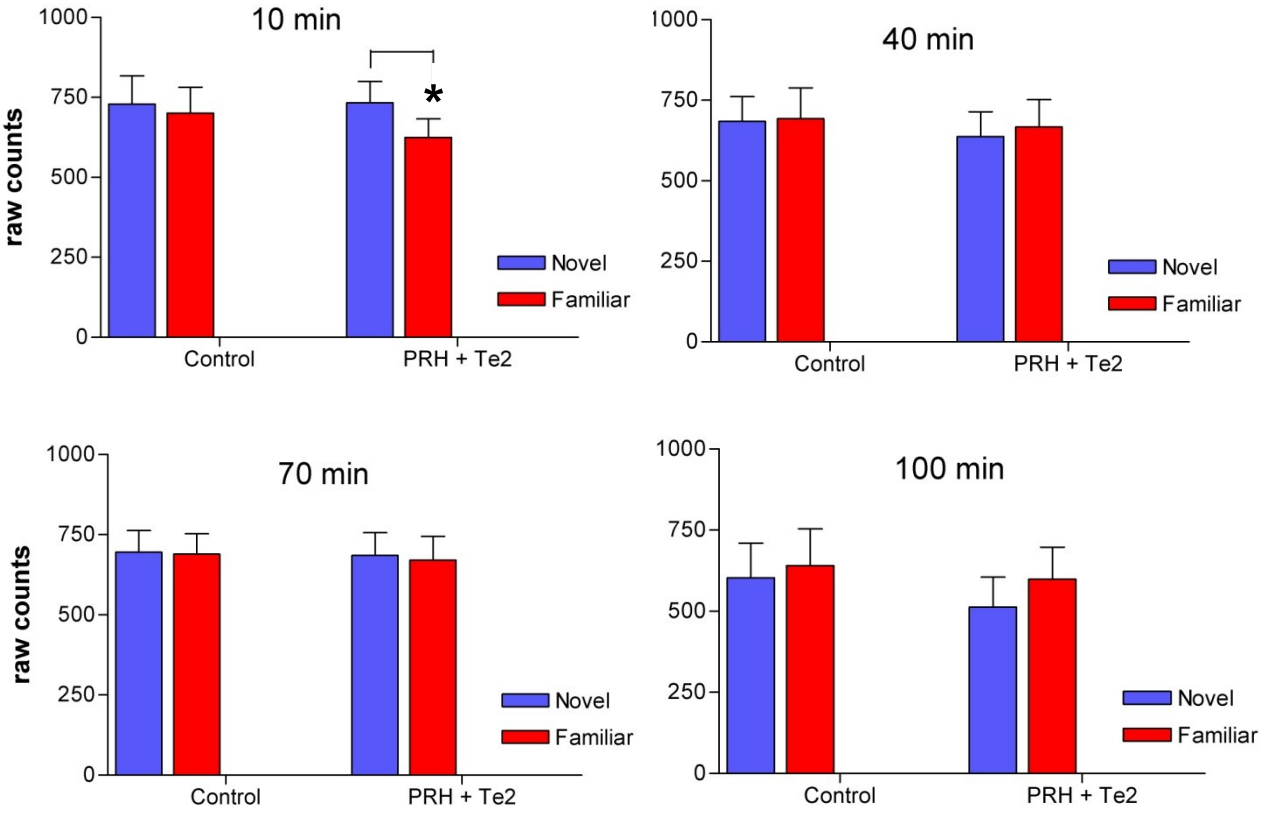


pERK labelled cells were counted using automated in-house software (CellCountMainv3.1, J. Leendertz) (Tinsley, Narduzzo et al. 2009; Tinsley, Narduzzo et al. 2011) (Materials & Methods 2.5.2). Thresholds were set in order to ensure clearly stained neurons were counted. Automatic counting was monitored by the experimenter to ensure that cells were correctly counted by the predetermined threshold; all counting and subsequent analysis was performed blind (Materials and Methods, 2.5.2).

4.2.3 Statistical analysis

Single neuronal activity changes in responses to the relative familiarity of visual stimuli are initially found in PRH and adjacent visual association area, Te2, but not surrounding regions (Zhu and Brown 1995; Zhu, Brown et al. 1995; Zhu, McCabe et al. 1996; Warburton, Koder et al. 2003; Warburton, Glover et al. 2005; Tinsley, Narduzzo et al. 2009). Therefore, the initial statistical analysis was made of combined counts in regions of interest (PRH and Te2) and combined control regions (AUD, ENT and V1), areas in which no significant neuronal changes were expected. Analysis was performed using raw counts in order to determine if any differences across time delays were present. Further, counts were normalised, to reduce variance across batches of immunohistochemical batches of processed tissue and between rats, and further analysis was performed.

Figure 4.2.3.1 Time course of ERK activation (pERK) in response to novel and familiar visual stimuli. Raw phosphorylated ERK (pERK) counts for combined control areas (AUD, ENT, V1) and regions of interest (PRH, Te2) following presentation of novel and familiar visual stimuli, across time delays. Bars represent mean ± SEM. Significance level (* $p < 0.05$).



Repeated-measures three-factor ANOVA (factors: novelty, delay, and region) of raw combined control and combined PRH and Te2 pERK counts revealed a significant three-way novelty by region by delay interaction [$F_{(3,36)}= 3.10$, $p < 0.05$]. A significant difference between regions was found [$F_{(1,36)} = 4.12$, $p < 0.05$] as a result of higher counts in combined control areas compared to PRH and Te2. No other main effect reached significance. Analysis of normalised counts did not reveal any further significant effects.

Subdividing the analysis by region, ANOVA of the counts within the combined regions of interest (combined PRH and Te2) revealed a significant novelty by delay interaction [$F_{(3,36)}$ 3.41, $p < 0.05$]. No significant difference in novelty

[$F_{(1,36)} < 1$, $p > 0.1$] or delay was found [$F_{(3,36)} < 1$, $p > 0.1$]. For the combined control regions (AUD, ENT and V1) there was no significant novelty by delay interaction [$F_{(3,36)} < 1$, $p > 0.1$] nor was there any significant novelty [$F_{(1,36)} < 1$, $p > 0.1$] or delay [$F_{(3,36)} < 1$, $p > 0.1$] main effect found. Analysis with post-hoc Tukey tests of combined PRH and Te2 counts in response to novel stimuli across delays revealed significant differences between mean counts at 10 min and 100 min ($p < 0.05$). Further post-hoc analysis of novel-familiar differences at individual 10 min and 100 min time points found a significant effect of novelty at 10 min ($p < 0.05$).

Planned comparisons with post-hoc Tukey's tests of mean pERK counts in PRH alone across time delays revealed counts at the 10 min time point were higher in the hemisphere processing novel stimuli compared to counts in the hemisphere processing previously encountered stimuli ($p < 0.05$). Further, post-hoc analysis of pERK counts in area Te2 at individual time points found an increase in response to novel stimuli at 10 min ($p <0.05$), as seen in PRH (Figure 4.2.3.2).

Figure 4.2.3.2 Phosphorylated ERK counts in response to novel and familiar stimuli across varying time delay in A.) PRH and B.) area Te2.

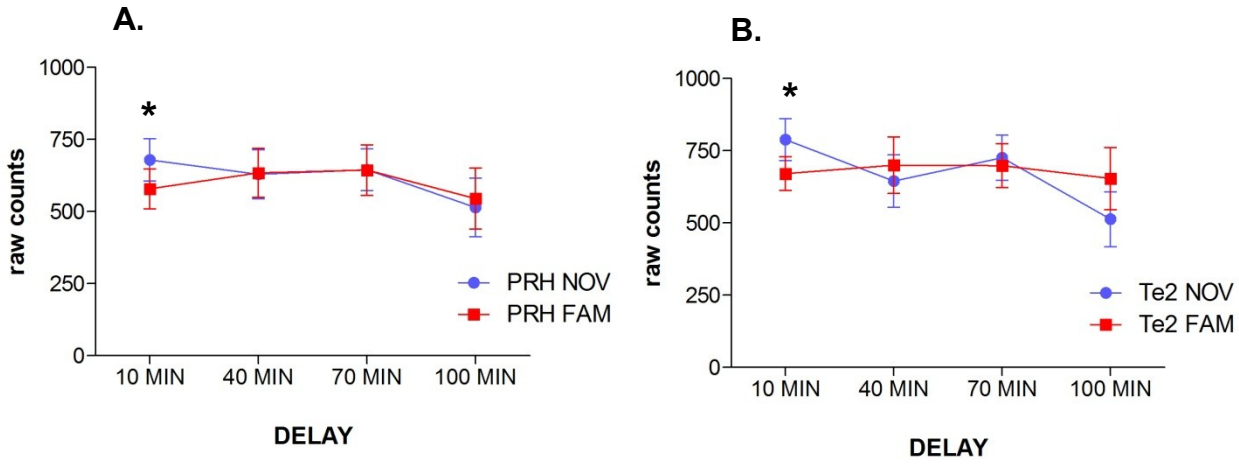


Table 4.2.3.1 pERK raw counts for combined control areas and combined regions of interest (PRH and Te2) at varying time delays. All values are stated as mean ± SEM. Significance level (* $p < 0.05$).

Brain region/ Familiarity (raw counts)	Delay			
	10 min	**40 min**	**70 min**	**100 min**
CONTROLS NOVEL	729 ± 93	684 ± 81	695 ± 71	603 ± 113
FAMILIAR	700 ± 86	693 ± 100	689 ± 67	641 ± 119
PRH + Te2 NOVEL	733 ± 71*	637 ± 81	685 ± 74	513 ± 97
FAMILIAR	625 ± 61	667 ± 89	671 ± 79	599 ± 105
PRH NOVEL	679 ± 73*	629 ± 85	645 ± 73	514 ± 102
FAMILIAR	578 ± 69	634 ± 85	644 ± 88	545 ± 105

4.3. Quantification of the effect of MEK inhibitor, U0126, on the level of pERK measured 10 min following PVP

4.3.1 Methods

Non-competitive MEK inhibitor, U0126 (1μM), (Figure 4.3.1.1) and control peptide, U0124 (1μM), were administered using the standard infusion protocol 15 min prior to the start of the PVP test session. To permit comparison of the effects of the inhibitor and its control peptide during this test session, randomised novel images were presented to both eyes. Infusions were of U0126 in PRH of one hemisphere and of U0124 in PRH of the other. Animals were transcardially perfused (Materials and Methods, 2.4.3) 10 min following the end of the session, the delay at which novelty-associated changes in pERK counts had been found in PRH.

Figure 4.3.1.1 MEK inhibitor, U0126. U0126 blocks MEK the intermediate kinase in the ERK intracellular cascade and prevents the phosphorylation of downstream ERK kinases (Duncia, Santella et al. 1998; Favata, Horiuchi et al. 1998).

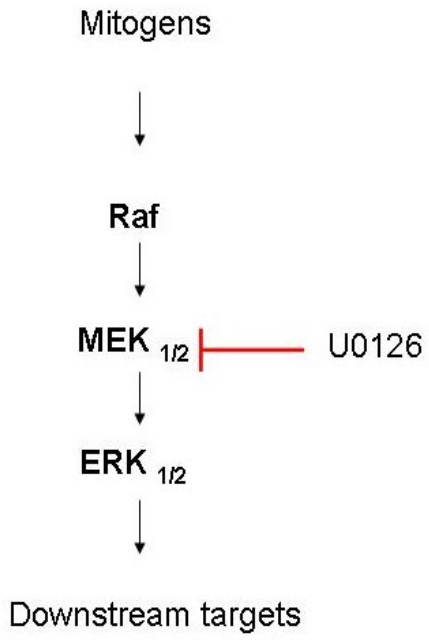
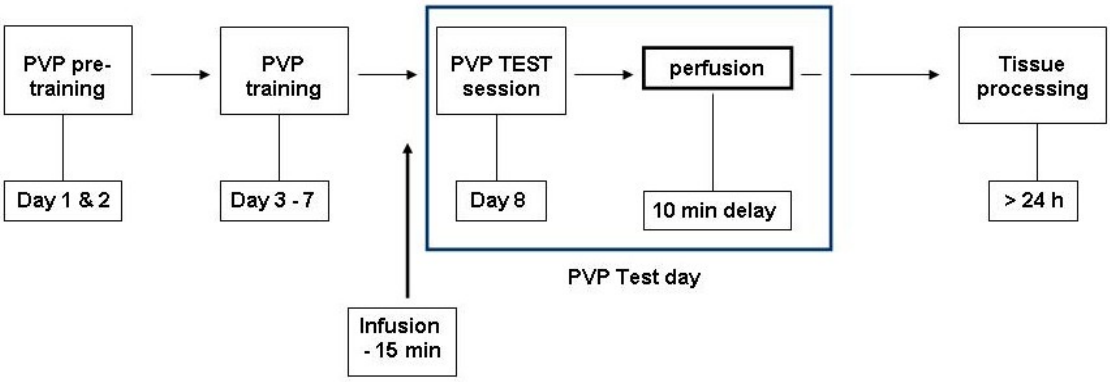


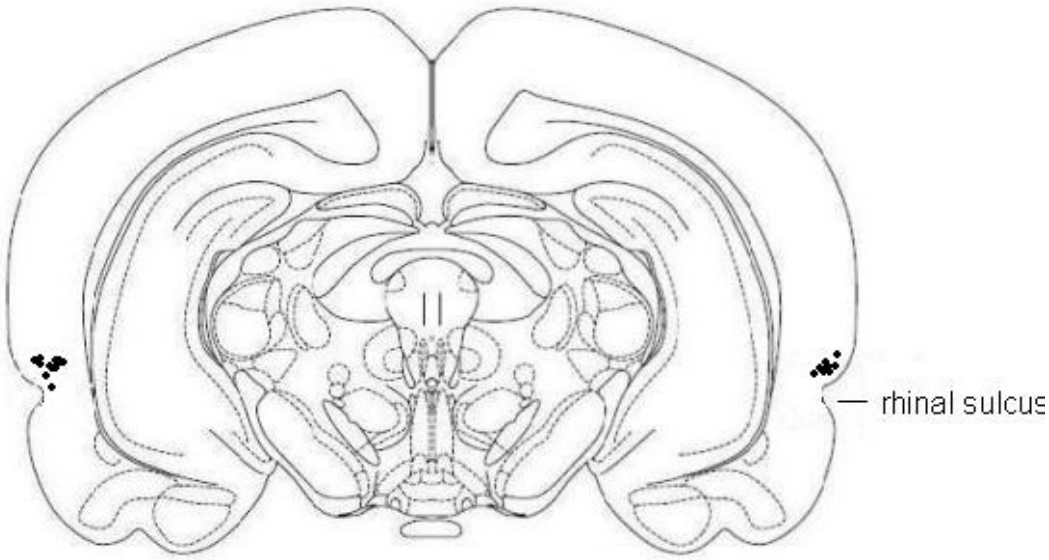
Figure 4.3.1.2 Schematic representation of the PVP procedure with unilateral U0126 infusions administered 15 min prior to the test session. Animals were trained using lists as described in 2.4.3.2. The test day of the experiment comprised a standard morning session and an afternoon session in which both eyes were shown randomised novel images. One brain hemisphere was infused with MEK inhibitor, U0126, and the other was infused with the control compound, U0124, 15 min prior to the test phase.



4.3.2 Detection of pERK

Brain sections from nine PRH cannulated rats were used for analysis in this experiment. Levels of pERK were labelled using anti-active pMAPK/ERK antibody as described in Materials and Methods, section 4.2.2.

Figure 4.2.3.1 PRH infusion sites. Intraperirhinal infusion site from nine animals plotted on a section at -5.6 mm from bregma (Paxinos & Watson, 1998). All cannula tips were within PRH between -4.5 mm and -6 mm from bregma.



4.3.3 Statistical analysis

Counts were normalised to reduce variance across rats and between different immunohistochemical batches of processed tissue. ANOVA with repeated measures revealed no significant effect of drug treatment between area [$F_{(4,32)}$, 1, $p > 0.1$]. Planned comparisons analysis of pERK counts in PRH following local infusions of MEK inhibitor, U0126, and control peptide, U0124, showed no significant interaction [ANOVA $F_{(1,8)} = 1.75$, $p > 0.1$].

No significant effect of drug treatment was found between counts in adjacent visual association area, Te2 (ANOVA $F_{(1,8)} < 1$, $p > 0.1$) or control areas; AUD, ENT and V1 (ANOVA $F_{(1,8)} < 1$, $p > 0.1$). Subsequent analysis of raw counts did not revealed any further significant effects (Table 4.3.2.1).

Figure 4.3.2.2 Normalised pERK counts in hemisphere treated with intraperirhinal U0126 or U0124 infusions. Normalised counts from auditory cortex (AUD), entorhinal cortex (ENT), perirhinal cortex (PRH), area visual association area (Te2) and primary visual cortex (V1) in the hemisphere treated with intraperirhinal infusions of U0126 or control peptide, U0124, 10 min following exposure to novel visual stimuli. Bars represent mean ± SEM.

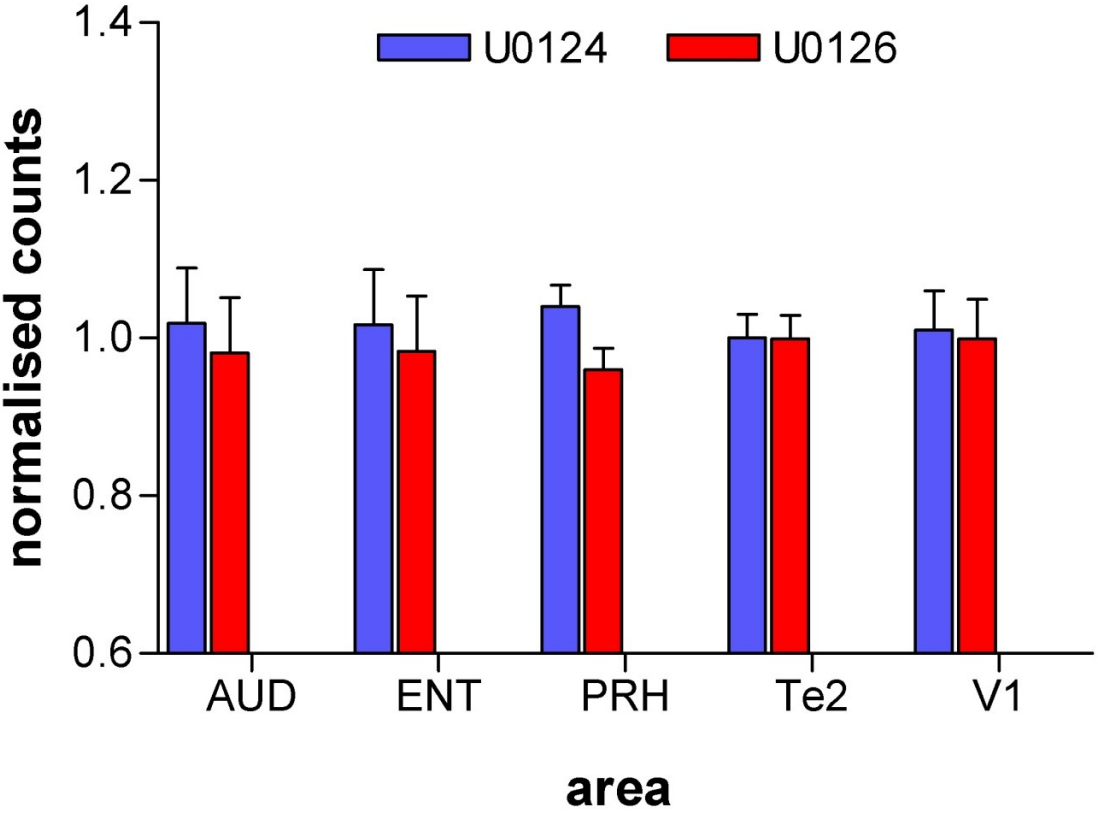


Table 4.3.2.1. Normalised and raw counts of pERK-stained neurons in hemisphere treated with U0124 or U0126. Raw counts are stated below the normalised counts**.** Values are expressed as mean ± SEM.

Intraperirhinal infusion	Area				
	AUD	**ENT**	**PRH**	**Te2**	**V1**
U0124 (control peptide)	**1.02 ± 0.07** 1261 ± 144	**1.02 ± 0.07** 1019 ± 124	**1.04 ± 0.03** 1200 ± 131	**1.00 ± 0.03** 1300 ± 122	**1.01 ± 0.05** 1338± 64
U0126 (MEK inhibitor)	**0.98 ± 0.07** 1199 ± 141	**0.98 ± 0.07** 1005 ± 149	**0.96 ± 0.03** 1085 ± 101	**1.00 ± 0.03** 1279 ± 97	**0.99 ± 0.05** 1389 ± 158

II) Measurement of p38 activation in response to novel and familiar visual stimuli

4.4 Measurement of p38 activation in response to novel and familiar visual stimuli

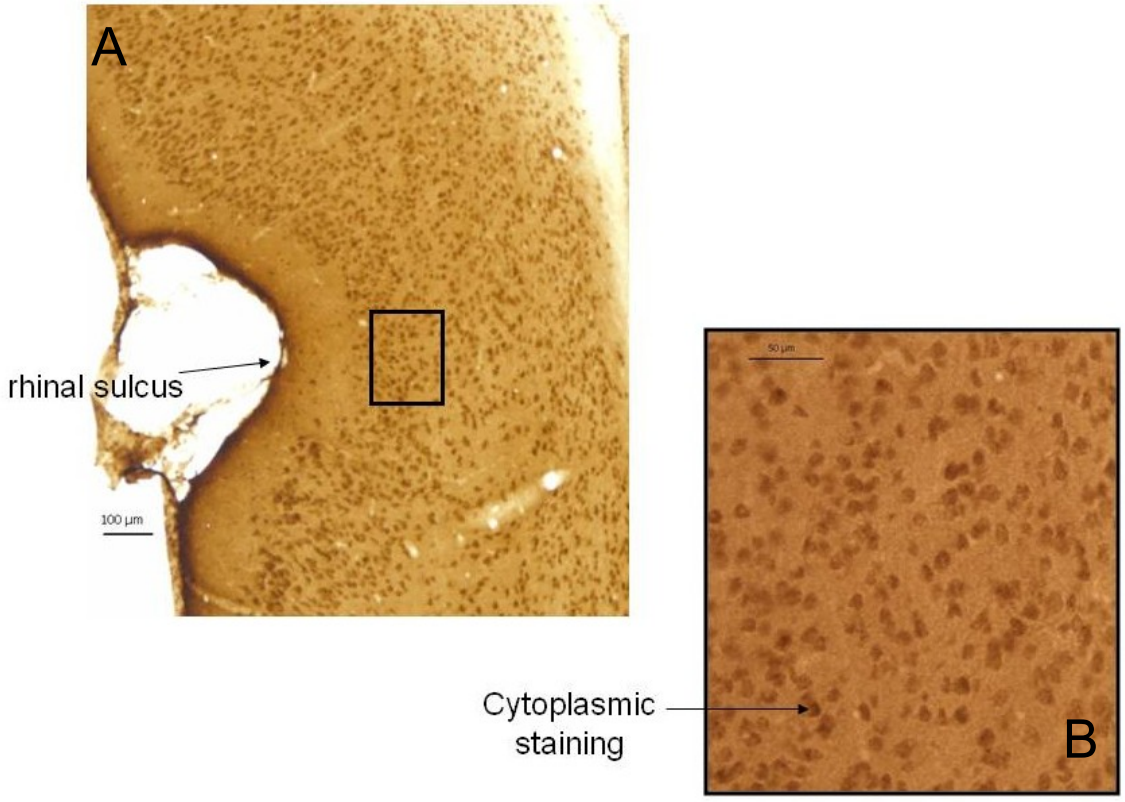
4.4.1 Methods

As described in 4.2.1, and following the experimental design used for determining pERK activation, additional male DA rats were trained using the PVP procedure and perfused at varying delays (10, 40, 70 and 100 min) following the final test session (Materials & Methods) (Figure 4.2.1.1). Brain sections were imaged from 32 rats (10 min delay: n= 8; 40 min: n=8; 70 min: n= 8; 100 min: n = 8) and used for statistical analysis.

4.4.2 Detection and quantification of phosphorylated p38.

Phosphorylated-p38 was detected using an anti-active p38 antibody raised against the dually phosphorylated amino acid residues Thr^{180} and Tyr^{182} (Promega, USA). Anti-active p38 antibody (1:500) was used to detect p38 subtypes α, γ, δ (Ferrer, Blanco et al. 2002; Keifer, Zheng et al. 2007) (Material & Methods). The subcellular localisation of p-p38 staining was found to be predominantly in the cytoplasm, with some nuclear staining identified as described in Lee et al. (2000).

Figure 4.4.2.2 Examples of phosphorylated p38 staining



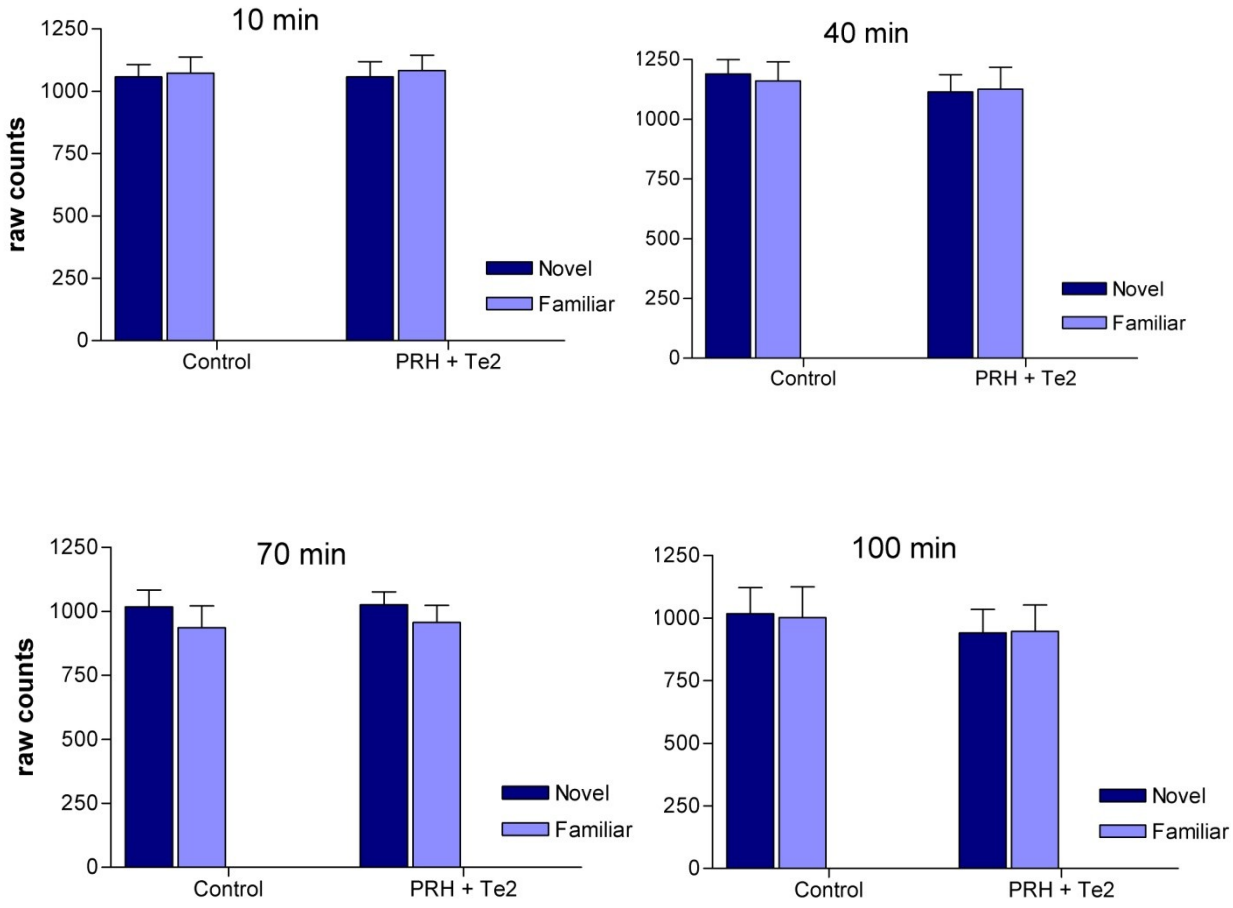
As described in 4.2.2, a threshold was set to include clearly stained neurons throughout the counting procedure.

4.4.3 Time course of p38 activation in response to novel and familiar visual stimuli.

Repeat-measures ANOVA of raw phosphorylated p38 counts in combined control areas AUD, ENT and V1 and combined regions of interest PRH and Te2, revealed no significant interactions between area, novelty and delay [$F_{(3,28)} < 1$, $p > 0.1$]. No significant interaction between area and novelty [$F_{(1,28)} < 1$, $p > 0.1$]; novelty and delay [$F_{(3,28)} < 1$, $p > 0.1$] or area and delay [$F_{(3,28)} < 1$, $p > 0.1$] was found. Further, no main effects were found to be significant: area [$F_{(1,28)} = 3.28$, $p < 0.1 > 0.05$] or novelty [$F_{(1,28)} < 1$, $p > 0.1$] or delay [$F_{(3,28)} = 1$, $p > 0.1$].

Counts in PRH in response to novel and familiar stimuli were analysed as a designed comparison; there was no significant novelty by delay interaction nor significant main effect of novelty or delay [ANOVA $F_{(3,28)} = 1.12$, $p > 0.1$]. To determine whether a change in response was found in area Te2, similar analysis was performed for this area in which changes are expected. ANOVA of counts in Te2 revealed no significant effects. To determine if PRH P38 counts at 10 min are the same as those measured by pERK, post-hoc Tukey's tests revealed no significant difference ($p > 0.1$). Analysis of normalised counts revealed the same pattern of results.

Figure 4.4.3.1 Time course of phosphorylated-p38 levels in control (AUD, ENT, V1) and regions of interest (PRH and Te2) in response to novel and familiar stimuli. P38 activation is not differentially activated in response to novel and familiar visual stimuli at any delay time point. Bars represent mean ± SEM.



4.4.3.1 Raw counts for phosphorylated p38 in combined control areas, combined regions of interest (PRH + Te2) and individual PRH counts following presentation of novel and familiar visual stimuli at varying time delays. All values are stated as mean ± SEM.

Brain region/ Familiarity (raw counts)	Delay			
	10 min	**40 min**	**70 min**	**100 min**
CONTROLS NOVEL	1058 ± 53	1190 ± 64	1017 ± 71	1017 ± 112
FAMILIAR	1073 ± 68	1161 ± 84	936 ± 92	1002 ± 132
PRH + Te2 NOVEL	1058 ± 64	1114 ± 78	1026 ± 53	941 ± 100
FAMILIAR	1083 ± 65	1126 ± 98	957 ± 72	947 ± 112
PRH NOVEL	968 ± 65	1005 ± 77	963 ± 55	845 ± 93
FAMILIAR	1001 ± 67	1070 ± 83	921 ± 66	857 ± 109

As no novel-familiar difference was found for phosphorylated p38 levels at any delay time point, no inhibitor experiment was performed.

4.5 Discussion

Levels of pERK are differentially expressed in response to novel and previously encountered stimuli 10 min following the PVP. No changes in p-p38 levels were found in response to visual stimuli following any delay time point. Levels of pERK and p-p38 were measured using selective polyclonal antibodies raised against the dual phosphorylated peptide catalytic core of the corresponding active enzyme. To ensure specific and satisfactory levels of immunohistochemical staining, a pilot study in which varying concentrations of primary (anti-active MAPK pAb and anti-active p38 pAb) and secondary antibody (BA 1000 Anti-rabbit, raised in goat) (2.5) should have been performed prior to the undertaking of this study. Concentrations of primary antibodies were based on previously published data (4.2.2 and 4.4.2); whereas, the concentration of BA 1000 was determined by Tinsley et al. (2009).

4.5.1 Phosphorylated ERK is differentially expressed following presentation of novel and familiar visual stimuli and activated earlier than transcription factor, CREB and immediate early gene, Fos.

Populations of neurons in PRH and area Te2, differentially express activated ERK (pERK) in response to novel and familiar visual stimuli at 10 min. Ten minutes following presentation of visual stimuli, an increase in the number of neurons expressing pERK was found in the hemisphere primarily processing novel stimuli compared to that for stimuli previously encountered. An increased activation in response to novelty compared to previously encountered stimuli has been demonstrated using Fos protein (Zhu et al., 1995; Wan et al., 1999; Zhu et al., 1997; Warburton et al., 2003;2005; Albasser et al., 2010); phosphorylated CREB (Warburton, Glover et al. 2005) and phosphorylated CaMKII expression (Tinsley, Narduzzo et al. 2009).

Transcription factor, CREB and IEG, c-*fos*, are downstream of ERK and p38; however, it is uncertain as to whether these pathway exists in PRH (Impey, Obrietan et al. 1998; Schiller, Bohm et al. 2006; Brami-Cherrier, Lavaur et al. 2007; Kidambi, Yarmush et al. 2010). Maximal activation of ERK or p38

following presentation of visual stimuli would be expected to occur earlier than that of maximal CREB and Fos activation (90 min). The increase in ERK at 10 min is consistent with this hypothesis; however, without directly inhibiting ERK signalling and measuring Fos levels, it is not possible to confirm that ERK is upstream of IEG, c-*fos*, in PRH. Differential activation of CaMKI (10-40 min) and CaMKII (70 min) following the PVP are found later than that of ERK (10 min) (Tinsley, Narduzzo et al. 2009; Seoane, Tinsley et al. 2011; Tinsley, Narduzzo et al. 2011). It has previously been reported that activation of CaMKI (Yano, Tokumitsu et al. 1998; Soderling 1999) and CaMKII (Illario, Cavallo et al. 2003; Oh, Manzerra et al. 2004) may activate the ERK pathway; however, as activation of CaMKI and CaMKII occurs later than that of ERK, this interaction may not occur in PRH. However, that is not to say that following activation, these pathways do not interact.

4.5.2 p38 α is not differentially activated in response to changes in the familiarity of visual stimuli

No change in the activation of p38 isoforms: p38 α, p38 γ and p38 δ in response to the relative familiarity of visual stimuli was found following the PVP at any time point studied. The antibody used to detect activated p38 does not detect the beta isoform and therefore behavioural changes as seen following p38 α and β inhibitor, SB 203580, may result from a direct inhibitory effect on this subtype. To confirm this, use of a selective anti-active p38 β antibody would be necessary. P38 α and p38 β are encoded by different genes and share approximately 60% amino acid sequence (Cuadrado and Nebreda 2010). As described in 1.7.3, p38 isoforms have different patterns of expression; p38 α is ubiquitous whereas p38 β is brain specific (Cuadrado and Nebreda 2010).

Critical roles for p38 activation have been identified in very few mnemonic processes, as reviewed in Chapter 3 (Zhen, Du et al. 2001; Alonso, Bevilaqua et al. 2003; Rossato, Bevilaqua et al. 2006). In contrast, ERK phosphorylation is widely involved in processes underlying long-term memory consolidation (Atkins, Selcher et al. 1998; Berman, Hazvi et al. 1998; Selcher, Atkins et al.

1999; Hebert and Dash 2002; Quevedo, Vianna et al. 2004). Results from Chapter 3 suggest that the activation of both kinases is critical for the consolidation (< 3 h following acquisition) of long-term recognition memory (Figure 3.3.2.1 and 3.3.4.1). However, p38 inhibition has been shown to block NMDA-R dependent activation of ERK (Vincent, Sebben et al. 1998); therefore, the memory impairment seen in Chapter 3 could result from inhibition of ERK signalling. Investigation into the interaction of these pathways in PRH requires further study.

An early requirement for ERK in memory consolidation has been established. ERK phosphorylation is increased in the olfactory bulb within 10 min of odour-shock training (Zhang, Okutani et al. 2003). ERK is activated within 2 min in the cerebellar vermis and hippocampus following conditioned eye blink response; whereas, increased levels of p-p38 are only found in the cerebellar vermis (Zhen, Du et al. 2001). Conditioned taste aversion learning induces an increase in levels of phosphorylated ERK within 10 min in the insular cortex in response to novel tastes; whereas levels of p38 phosphorylation remain unchanged (Berman, Hazvi et al. 1998). Levels of pERK are increased in the hippocampus < 30 min following repeat training in the Morris Water Maze; MEK inhibition prior to this training disrupted learning and abolished the associated increase in ERK. Inhibition of p38 had no effect on spatial learning (Blum, Moore et al. 1999). Such studies demonstrate differential roles for ERK and p38 in learning and memory.

4.5.3 Is the memory impairment associated with MEK inhibition consistent with changes in levels of pERK in PRH?

To further examine if the memory impairment associated with MEK inhibition as reported in Chapter 3 is consistent with a change in pERK levels in the PRH, U0126 and control peptide, U0124, were infused unilaterally directly into PRH 15 min prior to the final test session of the PVP. Subjects were perfused 10 min following the end of the test session in which randomised novel stimuli were presented to both eyes. This delay was selected because a significant difference in novel-familiar pERK counts was found at this time point (Figure

4.2.3.1). No significant change in pERK expression in PRH ($p = 0.19$) was found between hemispheres treated with U0126 and control U0124. This finding is inconsistent with PVP (Figure 4.2.3.1) and behavioural findings in Chapter 3.

Animals in the present study had received multiple infusions and had been previously trained in behavioural tasks. Histological analysis following tissue processing revealed an increase in scarring and tissue damage in PRH in brains in which multiple infusions had been administered; such scarring may also have impeded drug spread within PRH. In PVP studies by Tinsley et al. (2009; 2011) in which CAMK inhibitors were administered directly into the PRH, the animals had not previously been infused. For PVP studies, counts need to be made on sections as close as possible to the inhibitor infusion site. In the current experiments this was made difficult by the presence of scar tissue. Due to time and resource limitations, the replication of the original experiment with new, not previously infused animals was not possible.

4.5.4 PVP – *a test of reconsolidation*?

Recently, Tinsley et al. (2011) demonstrated that levels of pCaMKI were higher in PRH, in response to familiar stimuli compared to visual stimuli presented for the first time, 40 min following the PVP. Following this find, unilateral intraperirhinal inhibition of CaMKK inhibitor (upstream of CaMKI), STO-609, was infused prior to:

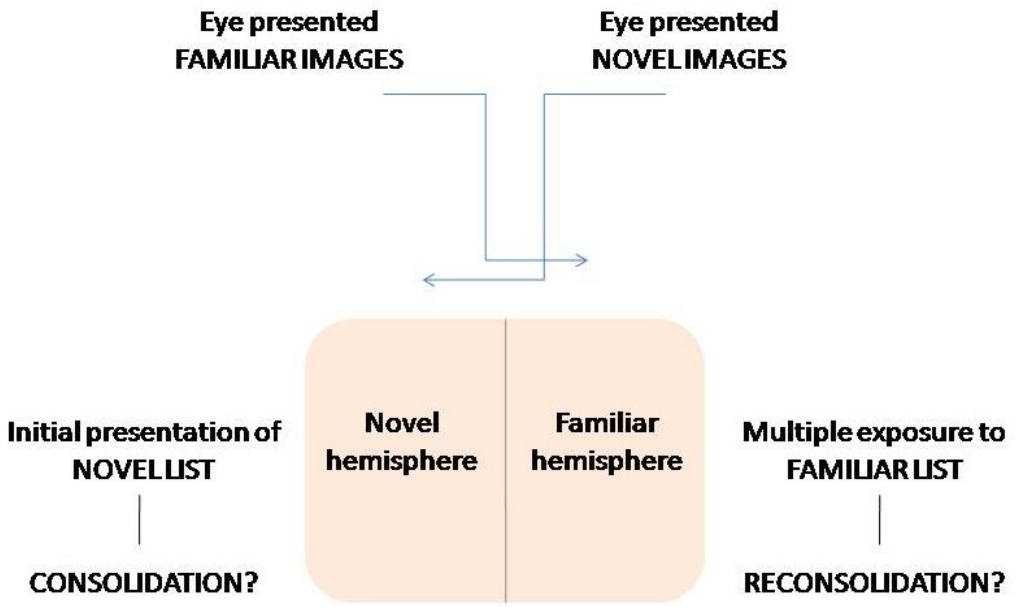
1.) Presentation of randomised novel visual stimuli to both eyes in test session
2.) Presentation of randomised familiar visual stimuli to both eyes in test session

Following both experiments, levels of pCaMKI were decreased in the hemisphere administered STO-609, compared to the vehicle-treated hemisphere, 40 min following the PVP. These findings suggest a possible requirement for kinase CAMKK in mechanisms underlying both consolidation

(the long-term storage of new information i.e. the novel images presented in the test session) and reconsolidation (consolidation mechanisms activated by the re-exposure of the familiar images presented in the test session) (Figure 4.5.4.1). When tested in the NOPT, STO-609 blocked consolidation of long-term recognition memory (Tinsley, Narduzzo et al. 2011).
To determine whether reconsolidation mechanisms in the PRH are dependent on ERK activation, it would be necessary to test whether presentation of familiar stimuli to both eyes (in final test session) following unilateral MEK inhibition, alters levels of pERK. If re-exposure to familiar stimuli in the test session does result in reconsolidation, an increase in pERK levels in the hemisphere infused with U0124 (control peptide) compared to the hemisphere treated with U0126 would be expected.

A role for ERK in reconsolidation has been established in mechanisms underlying recognition memory and fear conditioning, respectively (Kelly, Laroche et al. 2003; Duvarci, Nader et al. 2005; Valjent, Corbille et al. 2006). It is widely believed that there is a mechanistic overlap between consolidation and reconsolidation (Duvarci and Nader 2004; Tronson and Taylor 2007; Nader and Einarsson 2010); however, there are studies that suggest there is at least some distinction between these processes (Kelly, Laroche et al. 2003; Lee, Everitt et al. 2004). Using the A-B variant of the NOPT Kelly et al. (2003) found a significant increase in levels of pERK1 in the dentate gyrus when animals were sacrificed 5 min post encoding (after three 5 min sample sessions). Conversely, when sacrificed 10 min following a single re-exposure session to objects (24 h later) levels of pERK1 were found to increase in the CA1 regions of the hippocampus and adjacent, entorhinal cortex. The mechanisms underlying reconsolidation in the PRH remain to be studied.

4.5.4.1 Possible mechanisms underlying processing of novel and familiar stimuli in the PVP. On the standard test day of the PVP novel and familiar visual stimuli are presented to opposing eyes; relevant information is primarily processed in the contralateral hemispheres. It is possible that mechanisms underlying viewing familiar images may result in reconsolidation mechanisms. (Nader, Schafe et al. 2000; Duvarci and Nader 2004; Nader and Einarsson 2010).



Activation of signalling pathways may result from factors other than those designed to be tested. For example, p38 is a stress-activated MAPK (Han, Lee et al. 1994; Lee, Laydon et al. 1994; Gong, Ming et al. 2010). Throughout the PVP care was taken to minimise stress levels. Animals were habituated to the apparatus and returned to their home cages in a darkened room between morning and afternoon trials and prior to perfusion on the test day. No behavioural measurement of recognition memory is assessed during the PVP (Zhu, McCabe et al. 1996). It is therefore paramount that the animals' performance is recorded throughout each trial to determine whether the animal maintains the correct position within the apparatus, thus ensuring visual stimuli are presented to the correct monocular visual field. No animals were removed from this study based on incorrect positioning.

4.5.5 Limitations of the PVP study

As previously discussed, the PVP does not include any behavioural measure and therefore, it is assumed that animals correctly positioned within the apparatus are successfully viewing and processing the visual stimuli presented (Aggleton and Brown 2005). Each animal serves as its own control and therefore, the same level of attention should be given to both the novel and familiar visual stimuli. Albasser et al. (2010) have devised the 'bowtie maze' which allows for simultaneous measurement of novelty preference and measurement of IEG expression and determines whether the animal is able to discriminate between visual stimuli presented as would be expected in the PVP. A further limitation of the PVP study is that information is initially processed in contra lateral brain hemisphere; however, there is likely to be crossover of information, especially further down the visual processing pathway and may explain the failure of differential neuronal activation even in areas in which such changes were expected (Aggleton and Brown 2005).

An automated in-house count programme was used to quantify levels of p-ERK and p-p38. Count thresholds for each immunohistochemical stain were predetermined to correctly identify selective neuronal staining and used throughout the study; care was taken between groups to ensure that differences between batch staining were minimised. Shape thresholds were set to include typically stained neurons; however, lightly stained and clusters of cells may have resulted in their exclusion from counts. To further determine whether selective neuronal staining was being measured, double labelling with neuronal marker, NeuN, should have been carry out.

4.5.6 Further experiments

Double labelling of phosphorylated ERK and p38 would allow the co-localisation of the kinases to be assessed and would further enhance understanding of the interaction between the two pathways. Further, investigation into the sub-cellular distribution of ERK and p38 using confocal microscopy and nuclear-cytosolic fractionation (Wright et al., 2008; Du et al., 2010) 10 min after visual stimulation would be beneficial for understanding the

mechanisms by which enzymatic phosphorylation results in translocation of each kinase. In quiescent cells, ERK is present in the cytoplasm and translocates to the nucleus following phosphorylation (Karlsson, Mandl et al. 2006).

Less is understood about the localization and mechanisms underlying by p38 activation (Lee et al., 2000). Inactive p38 and upstream kinases MKK-3/6 are found to be present in both the cytoplasm and nucleus (Raingeaud, Gupta et al. 1995; Ben-Levy, Hooper et al. 1998). It is believed that p38 translocates to the nucleus upon activation and returns to the cytosol following de-phosphorylation (Wood, Thornton et al. 2009; Gong, Ming et al. 2010). However, phosphorylated (active) p38 has also been found in the cytoplasm (Roux and Blenis 2004). Furthermore, p38 substrate, MAPKAP-kinase 2 is thought to be required for translocation of activated p38 from the nucleus to the cytoplasm, suggesting a role in both nuclear and cytosolic signalling (Ben-Levy, Hooper et al. 1998).

4.5.7 Chapter conclusions

ERK is differentially activated in response to novel and familiar stimuli in PRH and adjacent area Te2, 10 min following presentation of visual stimuli. Levels of pERK were found to be higher in response to novel stimuli, suggesting a possible role for ERK in mechanisms underlying the consolidation of new visual stimuli. A role for ERK in early consolidation is consistent with behavioural findings in Chapter 3. p38 is not differentially activated in response to novel and familiar visual stimuli in the PVP; the possible reasons for this discrepancy are discussed.

Chapter 5. General Discussion

5.1. ERK and p38 are required for long-term recognition memory

PRH is critical for the familiarity discrimination component of recognition memory for objects (Brown and Aggleton 2001; Brown, Warburton et al. 2010). This thesis investigated the role of MAPK pathways: ERK and p38 in familiarity discrimination, using behavioural tasks designed to test spontaneous preferential exploration (Ennaceur and Delacour 1988; Dix and Aggleton 1999). The time course of ERK and p38 activation following presentation of novel and familiar stimuli was measured using the PVP (Zhu, Brown et al. 1995; Zhu, McCabe et al. 1996).

This study demonstrates for the first time that inhibition of ERK or p38 pathways in PRH disrupts the consolidation of long-term recognition memory. Intraperirhinal infusions of MEK inhibitor, U0126, and p38 inhibitor, SB203580, administered prior to memory encoding or following acquisition (< 3 h) impaired long-term memory formation. Furthermore, a critical role for PRH ERK and p38 was established for associative object-in-place memory tested after a delay of 24 h.

Significantly different levels of pERK were found following presentation of novel versus previously encountered stimuli using the PVP. Counts in PRH and adjacent area Te2 were higher following exposure to novel stimuli when measured after a 10 min delay. This suggests a role for ERK in early mechanisms underlying the consolidation of new visual information. In contrast, no changes in p38 activation in response to novel or familiar stimuli were found following the viewing of pictures in the PVP at any time point. P38 isoforms α and β are widely detected in the brain and are found to be differentially localised (Jiang, Chen et al. 1996; Li, Jiang et al. 1996; Hu, Wang et al. 1999; Lee, Park et al. 2000). Whereas the p38 inhibitor, SB203580, that produced behavioural impairment targeted both α and β isoforms, the anti-phosphorylated antibody used to detected p-p38 in Chapter 4 (4.4.2), did not detect the β isoform of the enzyme. As a result, the

behavioural impairment in Chapter 3, following p38 inhibition, may be due to an effect on this isoform. To date, there is no p38 β selective inhibitor available to confirm this; however, use of a selective anti-active p38 β antibody would determine if levels of p-p38 β change in response to novel and familiar stimuli, tested using the PVP.

An increase in neuronal activity in response to novelty is consistent with PVP studies using CaMKI, CaMKII, CREB and Fos as markers of neuronal activation (Zhu, McCabe et al. 1997; Warburton, Glover et al. 2005; Tinsley, Narduzzo et al. 2009; Tinsley, Narduzzo et al. 2011). ERK and p38 signalling are upstream of the transcription factor CREB and the immediate early gene protein product, Fos (Price, Cruzalegui et al. 1996; Schiller, Bohm et al. 2006). The time course of activation of ERK and p38 would therefore be expected to be earlier than 90 min, the time point at which levels of Fos expression peak, as was found.

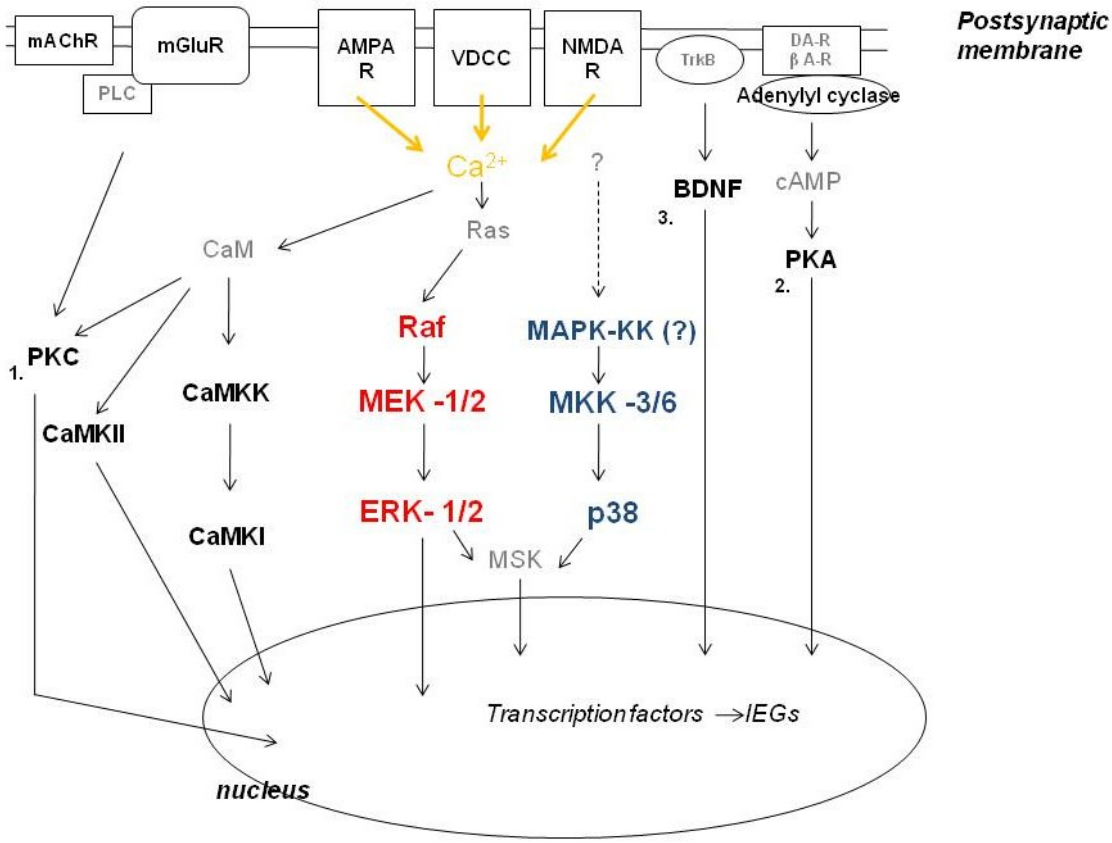
5.2. Overview of intracellular kinases underlying familiarity discrimination in PRH

Storage of long-term memories is dependent on the relay of synaptic signals to the nucleus via a network of intracellular proteins (Kelleher, Govindarajan et al. 2004). Inhibition of PKA, PKC (Nadine Fontaine-Palmer, unpublished PhD thesis 2008), CaMKK, CaMKII, or BDNF impair long-term recognition memory; (Tinsley, Narduzzo et al. 2009; Seoane, Tinsley et al. 2011; Tinsley, Narduzzo et al. 2011). This thesis has further identified critical roles for ERK and p38 in familiarity discrimination. Inhibition of either individual kinase results in long-term memory impairments, demonstrating that other pathways cannot compensate in the presence of such inhibition. However, the exact mechanisms underlying intracellular signalling in PRH necessary for recognition memory are still far from fully understood.

Recent PVP studies and findings from this thesis indicate that differential ERK activation in response to novel stimuli occurs earlier (at 10 min) than that of CaMKI (10 - 40 min) and CaMKII (70 min). CaMKII is reported to activate Ras (Oh, Manzerra et al. 2004) and Raf-1 (Illario, Cavallo et al. 2003), upstream kinases in the ERK signalling pathway. Further, CaMKI inhibition blocks ERK dependent LTP induction (Schmitt, Guire et al. 2005); and upstream CaMKK activates ERK (Soderling 1999). If ERK activation were downstream of CaMKI and CaMKII in PRH, then the time course of activation would be expected to be later than that of the upstream kinases. However, in PRH these pathways may be functionally independent (Figure 5.2.1).

It is now understood that intracellular proteins BDNF, CaMKK, CaMKII, PKA, PKC and MAPK subtypes ERK and p38 are critical for PRH recognition memory processes; however, future studies will be required to elucidate the totality of the pathways between the activation of glutamate receptors and calcium influx leading to MAPK activation and the eventual removal of glutamate receptors from the membrane (Griffiths, Scott et al. 2008).

Figure 5.2.1. Simplified schematic representation of intracellular pathways in the PRH underlying long-term familiarity discrimination. Post-synaptic membrane depolarisation results in an influx of Ca^{2+} which leads to activation of multiple intracellular signalling kinases. Inhibition of mGluR, AMPAR, NMDAR or VDCC in PRH results in long-term memory impairments. The time courses of CaMKK, CaMKII and ERK activation following PVP suggest that ERK is not activated by CaMK pathways in PRH in response to novel visual stimuli. However, possible interactions with ERK as demonstrated in alternative brain regions may exist between other pathways in PRH. 1.) ERK and p38 are activated in a PKC-dependent manner in the hippocampus (Galeotti and Ghelardini 2011); PKC inhibition blocks ERK activation in the hippocampus (Roberson, English et al. 1999); 2.) Activation of cAMP dependent PKA increases ERK activation in the hippocampus (Martin, Michael et al. 1997; Roberson, English et al. 1999; Adams, Roberson et al. 2000; Lewis, Kerr et al. 2008); 3.) ERK activation is coupled to BDNF induced synaptic plasticity (Gottschalk, Jiang et al. 1999) and learning in the hippocampus (Alonso, Vianna et al. 2002; Tyler, Alonso et al. 2002; McGauran, Moore et al. 2008). However, the interaction of ERK, p38 and these intracellular kinases in PRH is yet to be investigated. (Abbreviations: TrkB- TrkB tyrosine kinase ; DA-R- dopamine receptor; βAR – beta adrenergic receptor; PLC- phospholipase C; cAMP- cyclic adenosine monophosphate; mAChR – muscarinic acetylcholine receptor).



5.3. Further investigations

Investigations into the role of MAPKs in learning and memory have largely focused on the ERK subfamily. Extensive evidence also exists to demonstrate a role for this kinase in synaptic plasticity (Qian, Gilbert et al. 1994; English and Sweatt 1996; English and Sweatt 1997; Winder, Martin et al. 1999; Kanterewicz, Urban et al. 2000; Adams and Sweatt 2002; Peng, Zhang et al. 2010). A role for p38s in synaptic plasticity has also been established (Liang, Huang et al. 2008; Moult, Correa et al. 2008); however, there is little behavioural evidence to demonstrate a role in learning and memory (Zhen, Du et al. 2001; Alonso, Bevilaqua et al. 2003; Rossato, Bevilaqua et al. 2006). This thesis establishes a critical involvement of p38 in long-term recognition memory, although whether or not the influence of p38 results from an interaction with the ERK cascade was not investigated. ERK and p38 are known to share downstream targets such as MSK (Deak, Clifton et al. 1998; Pierrat, Correia et al. 1998; Brami-Cherrier, Lavaur et al. 2007) and transcription factors, CREB and Elk-1 (Whitmarsh, Yang et al. 1997; Hazzalin and Mahadevan 2002; Guan, Kim et al. 2003); however, the interaction between ERK and p38 pathways remains unclear. As described in Chapter 4, measurement of pERK levels 10 min following PVP demonstrates differential activation following presentation of novel versus familiar stimuli. If the effect of p38 inhibition results from direct inhibition of the ERK cascade, it would be expected that intraperirhinal p38 inhibition prior to the test session of the PVP would abolish the novelty-induced increase in ERK phosphorylation.

Downstream targets of MAPK include CREB and Fos (Cammarota, Bevilaqua et al. 2000; Zhang, Wang et al. 2004; Schiller, Bohm et al. 2006; Brami-Cherrier, Lavaur et al. 2007; Kidambi, Yarmush et al. 2010).The transcription factor, CREB, is critical for the consolidation of long-term memories (Silva, Kogan et al. 1998; Impey, Obrietan et al. 1999) and more specifically, recognition memory (Warburton, Glover et al. 2005). Oligonucleotide antagonism of Fos also produces a deficit in recognition memory (Seoane and Brown, BNA Abstract 2007). Immunohistochemical analysis of the activation of both targets following ERK inhibition prior to the PVP would determine if they are downstream of this pathway in PRH. Additionally, the role of

transcription factor, Elk-1 a downstream target of ERK and p38 is yet to be studied in the PRH. Elk-1 expression is dependent on multiple receptors known to modulate long-term recognition memory (Berman 2003).

The time courses of BDNF, PKA and PKC activation following the PVP should be further studied to understand the mechanisms underlying memory consolidation in PRH. In addition, the use of specific kinase inhibitors administered prior to the PVP and subsequent measurement of multiple kinases at various delays following the task would allow the identification of downstream targets to be confirmed. For example, the use of a PKC inhibitor administered systemically before PVP training abolished the Fos increase found in response to novel visual stimuli (Nadine Fontaine-Palmer, unpublished PhD thesis 2008), suggesting that PKC is upstream of Fos.

A role for MAPK subfamily, JNK, in synaptic plasticity has been established. Inhibition of the JNK pathway disrupts mechanisms underlying both LTD (Li, Li et al. 2007; Sherrin, Blank et al. 2011; Yang, Courtney et al. 2011) and LTP (Toyoda, Zhao et al. 2007; Liu, Wang et al. 2011) . Critical roles for this pathway in learning and memory also exist (Berman, Hazvi et al. 1998; Bevilaqua, Kerr et al. 2003; Sherrin, Blank et al. 2011). Investigations into the role of JNK, and the interaction of the MAPK signalling pathways underlying familiarity discrimination should be considered to fully understand the role of these kinases within PRH.

Chapter 6. References

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