



Barker, G. R., Wong, L. F., Uney, J. B., & Warburton, E. C. (2020). CREB transcription in the medial prefrontal cortex regulates the formation of long-term associative recognition memory. *Learning and Memory*, 27(2), 45-51. <https://doi.org/10.1101/lm.050021.119>

Peer reviewed version

Link to published version (if available):
[10.1101/lm.050021.119](https://doi.org/10.1101/lm.050021.119)

[Link to publication record in Explore Bristol Research](#)
PDF-document

This is the author accepted manuscript (AAM). The final published version (version of record) is available online via Cold Spring Harbour Laboratory Press at <http://learnmem.cshlp.org/content/27/2/45>. Please refer to any applicable terms of use of the publisher.

University of Bristol - Explore Bristol Research

General rights

This document is made available in accordance with publisher policies. Please cite only the published version using the reference above. Full terms of use are available: <http://www.bristol.ac.uk/red/research-policy/pure/user-guides/ebr-terms/>

CREB transcription in the medial prefrontal cortex regulates the formation of long-term associative recognition memory.

Running title: mPFC CREB crucial for long-term recognition memory

Barker, G.R.I.¹, Wong, L-F.², Uney J.B.² and Warburton, E.C.¹

1. School of Physiology, Pharmacology and Neuroscience, University of Bristol, Bristol BS8 1TD, U.K.
2. School of Translational Health Sciences, University of Bristol, Bristol BS8 1TD, U.K.

ABSTRACT

The medial prefrontal cortex (mPFC) is known to be critical for specific forms of long-term recognition memory, however the cellular mechanisms in the mPFC that underpin memory maintenance have not been well characterised. This study examined the importance of phosphorylation of cAMP responsive element binding protein (CREB) in the mPFC for different forms of long-term recognition memory in the rat. Adenoviral transduction of the mPFC with a dominant-negative inhibitor of CREB impaired object-in-place memory following a 6h or 24h retention delay, but no impairment was observed following delays of 5min or 3h. Long-term object temporal order memory and spatial temporal order memory was also impaired. In contrast, there were no impairments in novel object recognition or object location memory. These results establish, for the first time, the importance of CREB phosphorylation within the mPFC for memory of associative and temporal information crucial to recognition.

INTRODUCTION

Neural activity within the frontal lobes is crucial for declarative memory processing. Patients with lesions in the prefrontal cortex (PFC) show impairments in both episodic memory (Rugg and Vilberg 2013) and in the recognition of stimuli associated with specific spatial and temporal information (Kopelman et al. 1997; Zhang et al. 2018) and fMRI studies have revealed activation in PFC subregions during associative recognition and temporal order memory (Dobbins et al., 2003; St Jacques et al. 2008; Park et al., 2012; Roberts et al., 2018). More selective recording and lesion studies in non-human primates and rodents, have demonstrated a role for the orbital or medial PFC (mPFC) in delayed matching to sample, object-in-place recognition memory and temporal order memory (Mitchell and Laiacona 1998; Hannesson et al. 2004ab; Browning et al. 2005; Johnston and Everling, 2006; Baxter et al. 2007; Barker et al. 2007; Barker and Warburton 2011a). Thus, across species evidence strongly points to an involvement of the mPFC in associative and recency memory.

In the rat *in vivo* electrophysiological recording studies have revealed populations of neurons in the mPFC, which display object and context-dependent modulation (Weible et al. 2012;

Hyman et al. 2012; Kim et al. 2011;2013;2018). Such firing patterns may thus represent a cellular mechanism for recognition memory involving spatio-temporal information, although these mechanisms have been reported to operate over relatively short timescales. The intracellular mechanisms that support the formation and long-term storage of such memories in the mPFC have been little investigated, perhaps in part because traditionally the prefrontal cortex has been associated with short-term memory processing (Baddeley, 1992).

For long-term memory formation, phosphorylation of the transcription factor cAMP responsive element binding protein (CREB) has been shown to be a critical step in other brain regions. Inactivation of CREB in the hippocampus has been shown to impair contextual fear and spatial memory (Silva et al. 1998; Kida et al. 2002) in the perirhinal cortex CREB inactivation impaired long-term object recognition memory (Warburton et al. 2005) and in the mPFC disruption of CREB signalling impaired object-location memory in a delay-dependent manner (Vieira and Kozus 2015). Enhancement of CREB function in the amygdala has been shown to improve fear memory (Josselyn et al. 2001) and expression of CREB binding protein has been shown to increase in the mPFC following fear learning (Siddiqui et al. 2017). Thus, CREB activation in the mPFC may be a key step in the maintenance of object recognition memory yet this hypothesis has not yet been tested.

Here we investigated the effect of blocking CREB phosphorylation in the mPFC on the formation of object-in-place, temporal order and temporal location associative recognition memory using a virus expressing a dominant-negative inhibitor of CREB (here denoted A-CREB). The A-CREB construct consists of an acidic amphipathic extension that replaces the natural basic region fused on to the N terminus of the CREB leucine domain. The acidic extension interacts with the basic region of endogenous CREB-forming a coiled-coil extension of the leucine zipper and preventing CREB from binding DNA and initiating CRE-mediated transcription. We have previously used this approach to investigate the importance of CREB phosphorylation to recognition memory formation in the perirhinal cortex region of the medial temporal lobe (Warburton et al. 2005).

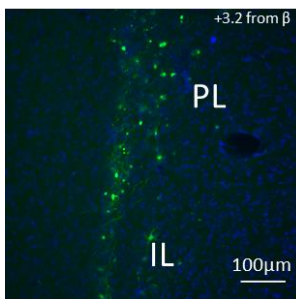
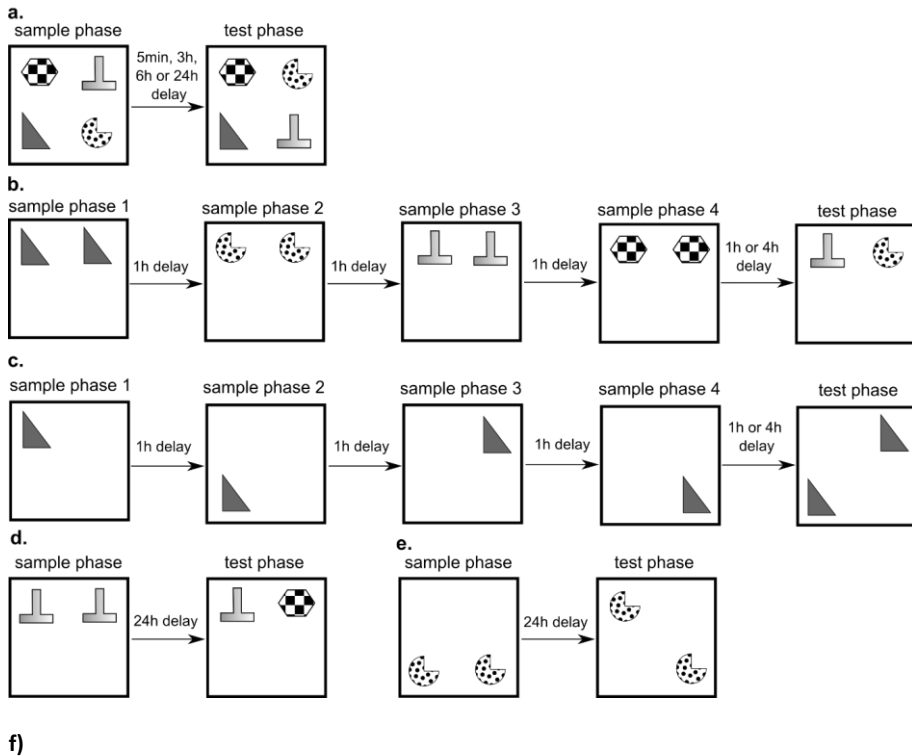


Figure 1: Diagram of the spontaneous object recognition tasks used: (a) Object-in-place task, (b) object temporal order task, (c) object temporal location task, (d) object recognition task, (e) object location task, (f) EGFP expression in the prelimbic (PL) and infralimbic (IL) region of the mPFC after infusion of adenoviral vector. Image taken at +3.2 from bregma (β).

RESULTS

Histology

Both control and A-CREB adenoviral vectors were tagged with enhanced green fluorescent protein (EGFP) for visualisation. Infusion of the vectors produced localised transgene expression, in all animals, centred in the prelimbic/infralimbic (PL/IL) region of mPFC at the injection site, +3.2mm AP from bregma (Figure 1f). Expression was observed up to 0.5mm from the injection site both in the anterior and posterior direction and throughout the dorsal-ventral extent of PL and IL, although expression was restricted to a 150µM width in the mediolateral direction. Transgene expression was observed in the anterior cingulate cortex particularly in that region adjacent to PL but only at the injection site. There was minimal tissue damage as assessed by cresyl violet staining.

Disruption of CREB phosphorylation impairs long term object-in-place memory formation

To examine the effects of CREB-disruption in the mPFC on object-in-place memory, we injected one group of rats with the virus expressing a dominant negative inhibitor of CREB (A-CREB group) while a second group of rats received a virus expressing EGFP only (EGFP group) as a control group. In the sample phase of the object-in-place the subject was placed in the arena to explore four different objects (Fig. 1a). After a delay period, the rat was replaced in the arena, which contained the four same objects, but two of the objects had exchanged position. Discrimination between two objects which had exchanged position and the two objects which were in the same position (Fig. 1a) was calculated using a discrimination ratio (DR) which controlled for individual differences in exploration levels. Object-in-place memory performance was assessed after four different retention delays (5min, 3h, 6h and 24h) in four separate runs. Consistent with our previous studies it was predicted that control animals will spend more time exploring the moved objects compared to the unmoved objects across each delay, and thus demonstrate memory for both the objects and the places in which they were previously encountered.

As overall object exploration levels could have an effect on memory encoding, we examined such exploration during different stages of the task and found no differences during the sample phase between the A-CREB and EGFP groups in any of the delay conditions [delay x virus interaction $F(3,72)=0.12$, $p=0.95$]. Similarly, there were no differences in total object exploration during the test phase in any of the delay conditions [delay x virus interaction $F(3,72)=0.29$, $p=0.83$]. The mean sample and test phase exploration for each condition at each delay is shown in Table 1.

Fig. 2a shows the mean DR for the EGFP and A-CREB groups at each retention delay and it can be seen that object-in-place memory was impaired in the A-CREB group at the longer (6h and 24h) but not at the shorter retention delays (5min, 3h) while performance in the EGFP group was unimpaired at any of the delays tested. Statistical analyses revealed a significant delay x virus interaction [$F(3,72)= 10.36$, $p=0.0001$] and post hoc analyses showed a significant difference between the memory performance of the A-CREB and EGFP rats at the longer delays (6h $p=0.0001$; 24h $p=0.0001$) but not at the shorter delays (5min $p=0.650$; 3h $p=0.702$).

Delay	Condition	Exploration in sample phase (s)	Exploration in test phase (s)
5min	EGFP	86.3±6.5	32.0±3.77
	A-CREB	100.1±4.2	33.6±3.1
3h	EGFP	73.3±7.6	35.4±2.5
	A-CREB	81.5±5.0	39.0±2.2
6h	EGFP	66.3±4.3	29.5±4.2
	A-CREB	79.7±3.8	38.5±1.9
24h	EGFP	88.7±5.9	40.0±3.2
	A-CREB	103.3±4.7	47.2±3.6

Table 1: Total object exploration times during the sample and test phases of the object in place task. Data presented as mean ± SEM (n=10).

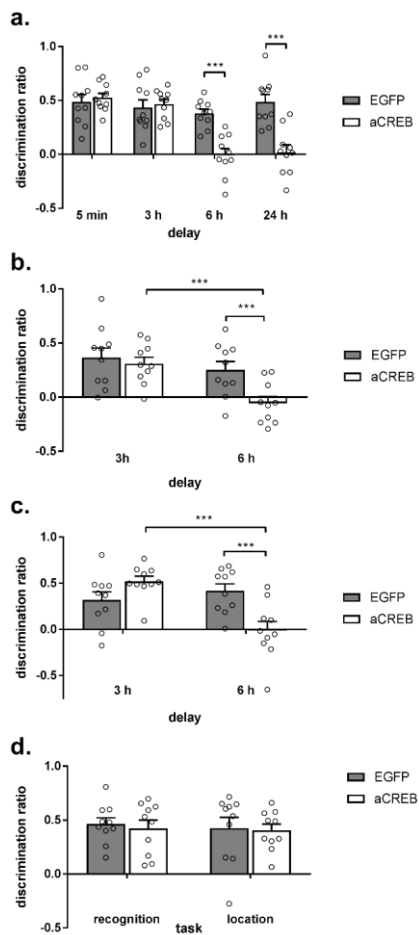


Figure 2 Blockade of CREB function in the medial prefrontal cortex impaired long-term but not short-term associative recognition memory formation and did not alter long-term non-associative recognition memory formation. (a) Blockade of CREB function in the medial prefrontal cortex impaired long-term, but not short-term object-in-place memory. T-tests comparing the discrimination ratios of each group against chance performance revealed that at the 5min and 3h delays, both the EGFP and A-CREB groups showed significant object-in-place discrimination [5min EGFP $t_{(9)} = 7.08$, $p = 0.0001$, A-CREB $t_{(9)} = 12.95$, $p = 0.0001$; 3h EGFP $t_{(9)} = 6.11$, $p = 0.0001$, A-CREB $t_{(9)} = 10.87$, $p = 0.0001$]. In contrast at the 6h and 24h delay the EGFP group showed significant discrimination [6h $t_{(9)} = 9.50$, $p = 0.0001$; 24h $t_{(9)} = 7.131$, $p = 0.0001$] whereas the A-CREB group did not [6h $t_{(9)} = -0.13$, $p = 0.899$; 24h $t_{(9)} = 0.30$, $p = 0.77$]. (b) Blockade of CREB function in the medial prefrontal cortex impaired long-term, but not short-term temporal order memory. T-tests comparing the discrimination ratios of each

group against chance performance revealed that at the 3h delay both the EGFP and A-CREB groups showed significant discrimination between the object presented in S2 and S3 [EGFP $t_{(9)} = 4.09$, $p=0.003$; A-CREB $t_{(9)} = 5.25$, $p=0.001$]. At the 6h delay the EGFP group showed significant discrimination [$t_{(9)} = 3.27$, $p=0.010$] while the A-CREB did not show significant discrimination [$t_{(9)} = -0.91$, $p=0.385$]. (c) Blockade of CREB function in the medial prefrontal cortex impaired long-term, but not short-term temporal location memory. T-tests comparing the discrimination ratios of each group against chance performance revealed that at the 3h delay both the EGFP and A-CREB groups showed significant discrimination between the object presented in S2 and S3 [EGFP $t_{(9)} = 3.54$, $p=0.006$; A-CREB $t_{(9)} = 9.24$, $p=0.000$]. At the 6h delay the EGFP group showed significant discrimination [$t_{(9)} = 5.62$, $p=0.000$] while the A-CREB did not show significant discrimination [$t_{(9)} = -0.12$, $p=0.91$]. (d) Blockade of CREB function in the medial prefrontal cortex had no effect on novel object recognition or object location memory. T-tests comparing the discrimination ratios against chance performance revealed that all groups showed significant discrimination between the novel and familiar objects in the object recognition task [EGFP $t_{(9)}=8.060$, $p=0.000$; A-CREB $t_{(9)}= 5.61$, $p=0.000$]. Both groups also showed significant discrimination between the moved and unmoved objects in the object location task [EGFP $t_{(9)}=4.23$, $p=0.002$; A-CREB $t_{(9)}= 7.00$, $p=0.000$]. Data presented as mean \pm sem. *** $p<0.001$.

Formatted: Not Highlight

Disruption of CREB phosphorylation impairs long term temporal order memory formation

We next examined performance in a temporal order memory task in which the subjects were presented with objects in a sequence of four sample phases (S1-S4). In the test phase, the objects from S2 and S3, were presented and exploration of these objects measured. Memory for order was expressed by the preferential exploration of the object which had occurred earlier in the series i.e. object from S2 (Figure 1b). Memory performance was calculated as a DR.

The A-CREB and EGFP groups showed the same levels of object exploration during each of the four sample phases [sample phase x virus x delay interaction $F(3,108) = 1.20$, $p=0.31$ n.s.] and in the test phase [delay by virus interaction $F(1,36)= 0.87$, $p=0.36$ n.s.]. The mean sample and test phase exploration at each delay is shown in Table 2.

Fig. 2b shows the mean DR for the EGFP and A-CREB groups following either 3h or 6h retention delay. Discrimination was significantly lower in the A-CREB compared to the EGFP group at the longer delay only [delay x virus interaction $F(1,36)=4.33$, $p=0.045$] confirmed by post hoc analyses showing a significant difference between the EGFP and A-CREB groups at the 6h delay ($p=0.006$) but not at the 3h delay ($p=0.92$).

Task	Delay	Condition	Exploration in sample phase (s)				Exploration in test phase (s)
			S1	S2	S3	S4	
Object temporal order	3h	EGFP	50.4±4.0	55.1±5.3	61.6±5.4	35.5±4.4	37.0±3.3
		A-CREB	54.4±4.7	57.5±4.8	45.3±4.4	48.0±5.3	38.0±3.7
	6h	EGFP	55.0±5.8	51.1±6.4	40.1±4.3	46.1±6.5	34.9±5.1
		A-CREB	56.8±3.8	41.4±6.0	34.5±5.8	49.3±5.4	28.4±2.6
Temporal location	3h	EGFP	43.2±5.1	43.3±3.5	46.5±6.5	43.4±3.4	24.2±2.4
		A-CREB	41.1±5.4	52.9±2.8	49.9±5.6	42.9±5.7	26.8±4.5
	6h	EGFP	23.4±2.6	29.8±3.3	38.0±4.7	34.4±2.2	27.3±2.6
		A-CREB	29.2±2.0	26.8±6.7	39.0±8.6	32.5±3.4	27.4±2.5

Table 2: Total object exploration completed in the sample phases and the test phases of the object temporal order and the temporal location tasks. Data presented as mean ± SEM (n=10).

Disruption of CREB phosphorylation impairs object temporal location

Activity within the mPFC is required for object associative and object temporal memory performance, thus we next examined the requirement for CREB activity in the mPFC for object temporal location memory. Here, the animal was presented with the same object but in a sequence of four different locations across four sample phases (Fig. 1c). In the test phase, the animal was presented with two objects, one in location from S2, one in location S3. Intact memory for the order of locations was expressed by preferential exploration of the object in the location that appeared earlier in the list (i.e. S2). Again, memory performance was assessed using the DR.

Both the A-CREB and EGFP groups completed the same amount of exploration in each of the sample phases and between the sample phases [sample phase x virus x delay interaction $F(3,108) = 1.12, p=0.35$]. There was also no difference in object exploration between the groups in the test phase [delay x virus interaction $F(1,36) = 0.15, p=0.70$]. The mean sample and test phase exploration at each delay is shown in Table 2.

Fig.2c shows the mean DR for the EGFP and A-CREB groups following either the 3h or 6h delay. Statistical analysis revealed a significant delay by virus interaction [$F(1,36)=15.14, p=0.000$] and post hoc analyses showed that memory performance in the A-CREB group was significantly reduced compared to control at the 6h retention delay ($p=0.003$) but there was no difference between the groups at the 3h delay ($p=0.071$).

Disruption of CREB phosphorylation in the mPFC has no effect on novel object recognition or object location memory

To establish the selectivity of the mnemonic effects of CREB disruption in the mPFC, we tested the A-CREB and EGFP groups in a novel object recognition task (Fig. 1d), and an object location task (Fig. 1e), both tasks previously shown to be unaffected by mPFC ablation (Barker et al., 2007). In novel object recognition task, the animals are presented with two identical objects and in the test phase, one of the objects is replaced with a novel object. In the object location task, the animals explore two identical objects in the sample phase, and in the test phase one of the objects is moved to a new location. Intact memory is expressed by preferential exploration of the novel or moved object.

The A-CREB and EGFP groups completed the same amount of object exploration during the sample phase [$F(1,18) = 3.396, p=0.082$] and test phase [$F(1,18)= 0.80, p=0.38$] of the novel object recognition test. Statistical analysis of memory performance revealed a non-significant main effect of virus [$F(1,18)=0.174, p=0.68$] thus there was no effect of blocking CREB function in the PFC on novel object recognition (Fig. 2d). In the object location task there was a difference in the amount of object exploration in the sample phase [$F(1,18) = 6.038, p=0.024$] and inspection of the group means revealed that this difference was a result of greater sample phase exploration in the A-CREB group compared to the EGFP group. There was no difference in object exploration in the test phase [$F(1,18)=2.659, p=0.12$] and no difference in memory performance [$F(1,18)= 0.34, p=0.86$] (Fig.2d), hence there was no effect of blocking CREB function in the mPFC on object location memory. The sample and test phase exploration during each task is shown in Table 3.

Task	Condition	Time taken to complete sample phase (s).	Exploration in sample phase (s).	Exploration in test phase (s).
Object recognition	EGFP	162.8±23.1	36.5±1.5	29.8±2.6
	A-CREB	153.4±19.6	39.4±0.3	32.8±1.6
Object location	EGFP	n/a	45.1±2.9	30.1±1.9
	A-CREB	n/a	54.6±2.2	35.0±2.2

Table 3: Total object exploration completed in the sample and test phases of the object recognition and object location tasks. Data presented as mean ± SEM (n=10).

DISCUSSION

CREB activity is a key regulator in the formation of long-term memory and here we show that selective disruption of CREB phosphorylation in the mPFC, impaired long-term object-in-place associative recognition memory, object temporal order memory, and spatial temporal order memory. In contrast, there were no impairments in novel object recognition or object location memory, further there was no effect on performance at the shortest retention delays tested (i.e. 5 min or 3h). Finally, there were no changes in the animals' overall object exploration behaviour during either the sample or test phases. Together these results show that the deficits in recognition performance were not a result of a general attentional or motor deficits, but rather that disruption of CREB function in the mPFC specifically impaired long-term associative memory performance.

We have previously established the cellular and behavioural functionality of the adenoviral mediated dominant-negative construct A-CREB used in this study. Thus Warburton et al., (2005) reported that expression of A-CREB selectively in the perirhinal cortex produced a 40% reduction in overall pCREB levels, and impaired long-term (24h delay), but not short-term (15min) single item novel object recognition memory. Here histological analysis revealed transgene expression restricted along the dorsoventral extent of the prelimbic and infralimbic cortex of the mPFC, with only minimal expression in the anterior cingulate cortex.

Previous studies have shown that the mPFC is not required for familiarity discrimination or simple spatial tasks (Hannesson et al. 2004b; Barker et al. 2007, but see Vieira and Korzus, 2015) likewise here disruption of CREB activity in the mPFC did not affect novel object recognition or object location. Significantly the present study found deficits in object-in-place memory following both a 6h and 24h retention delay, but not at 5min or 3h. A retention delay of 6h is much shorter than had been investigated in previous studies (Bourtchuladze et al. 1994; Guzowski and McGaugh 1997; Peters et al. 2009), and the deficit at this timepoint indicates that CREB phosphorylation is an important step in a cellular pathway for the stabilization of memory information between 3h and 6h following learning. From our previous studies initiation of this cellular pathway appears to depend on the activation of specific receptor subtypes in the mPFC such as the NMDA and D1/D5 receptor and blockade of these receptors impairs both object-in-place (Barker and Warburton 2008, 2011b; Savalli et al. 2015), and temporal order memory (Hotte et al. 2006) and interestingly activation of the NMDA and D1/D5 receptors has been shown to be crucial for CREB phosphorylation (Pittenger et al. 2002; Hotte et al. 2006; Olanas et al. 2012; Kirschmann et al. 2014; Nygard et al. 2017). CREB expression is regulated at transcriptional level by epigenetic mechanisms such as DNA methylation (Chahrour et al. 2008) and we have shown that disruption of DNA methylation impairs long-term object-in-place memory (Chahrour et al. 2008; Scott et al. 2017). Hence there are direct links between receptor activation, CREB-mediated cellular processes and gene expression in the mPFC, that leads to the long-term associative and temporal order recognition memory formation.

Previous studies have demonstrated that the PFC plays an important role in sequence behaviours specifically within the context of short-term memory, where delay-period activity in the PFC has been reported to represent temporal order, as well as a number of other

Formatted: Not Highlight

variables (Funahashi, 2017; Naya et al., 2017). Here temporal order memory for objects or locations was demonstrated by preferential exploration of the object in the location encountered earlier in the sequence. There are at least two possible mechanisms that may drive discrimination; either recency/ memory strength i.e. animals have weaker memory for the 'older' information i.e. that encoded during sample phase 2 compared to sample phase 3, or the animals remember the order of locations encountered. While some have argued that memory strength or trace decay drives the behaviour (Staddon and Higa, 1999) recent data from our lab shows that manipulations of the length of the inter-sample interval, which should disproportionately affect memory strength compared to memory for order, have little effect on performance (Barker et al. 2019). Hence, we would suggest that animals are making order judgements, at least in part, independent of memory strength, although the latter cannot be completely excluded. It should also be considered that while neural activity in the mPFC is crucial for maintenance of information to guide behaviour (for a recent review see Miller et al., 2018) processing of multiple stimuli as is required for temporal order memory, will be vulnerable to interference due to its limited capacity. Here four objects or positions were used and order memory for the middle items examined to avoid primary and recency effects associated with the first and last items, thus we did not explore the impact of manipulation of PFC processing on different order effects in the present study. Alternatively, the mPFC may play a more general role in integration and discrimination of information encoded within overlapping representations, possibly via an interaction with the hippocampus (DeVito et al. 2010; Schlichting and Preston, 2015; Morton et al. 2017). Indeed, successful object-in-place and object and spatial temporal order memory require prefrontal-hippocampal interactions (Barker and Warburton 2011; Barker et al. 2017).

In conclusion selective disruption of CREB phosphorylation within the mPFC impaired object-in-place, temporal order and temporal location memory, following delays greater than 6h. This finding suggests the existence of a common intracellular CREB-dependent mechanism for long-term recognition memory formation of associative and temporal information. The cellular processes of memory stabilization in the mPFC parallel those in other regions of the cortex i.e. the perirhinal cortex that have been shown to be required for single item object recognition memory. However, the precise molecular pathway has yet to be established. Further work is now required to define the precise interactions that comprise the molecular networks within PFC neurons, and the nature of the memory information consolidated within the mPFC.

METHODS

Subjects

All experiments were conducted in male Lister hooded rats (Harlan Laboratories, UK,) weighing 300-350g at the start of the experiments. The animals were group housed, under a 12-h/12-h light/dark cycle (light phase 18:00 – 6:00 h). Behavioural training and testing were conducted during the dark phase of the cycle. Food and water were available *ad libitum* throughout the experiment. All animal procedures were performed in accordance with United

Kingdom Animals Scientific Procedures Act (1986) and associated guidelines. All efforts were made to minimize any suffering and the number of animals used.

Adenoviral preparation

Recombinant E1-deleted Ad constructs were produced according to standard techniques (Harding et al., 1998). The cDNA for the reporter contrast enhanced green-fluorescent protein (EGFP), was excised from pEGFP1 (Clontech, Cambridge, UK) using *HindIII* and *XbaI* and inserted into the corresponding site in the multiple cloning site of the plasmid pXCXCMV (Harding et al., 1998). The cDNAs for CREB and the dominant-negative A-CREB were expressed bicistronically with EGFP by cloning each individually onto the same Ad transfer plasmid. Recombinant virus was generated by homologous recombination in human embryonic kidney 293 cells (Microbix Biosystems, Toronto, Canada), grown to a high titer and purified by CsCl density gradient centrifugation. Viral titer was determined by plaque assay: Ad-CMV-EGFP was 1×10^{10} pfu/ml, and Ad-CMV-A-CREB was 1×10^{10} pfu/ml.

Surgery

Each rat was anaesthetised with isoflurane (induction 4%, maintenance 2-3%) and secured in a stereotaxic frame with the incisor bar set at -3.5mm below the interaural line, in order to obtain flat skull. The scalp was then cut and retracted to expose the skull. Craniotomies were then made directly above the target regions, and the dura cut to expose the cortex.

Viral particles were delivered bilaterally into the medial prefrontal cortex (AP+3.2mm; ML±0.5mm, DV -4.3mm) in 2.0µl per hemisphere at a rate of 200nL/min. Animals were allowed to recover for three weeks before behavioural testing commenced.

Histology

Following completion of the experiments, each rat was anaesthetised with Euthetal (Rhône Mérieux) and perfused transcardially with phosphate-buffered saline followed by 4% paraformaldehyde. After removal the brain was post-fixed in paraformaldehyde for 24h before being transferred to 30% sucrose in 0.2M phosphate buffer for 48h. Coronal sections were cut at 40µm on a cryostat. Alternate sections were Nissl stained with cresyl violet or mounted directly onto slide using Vectorshield (Vector Laboratories, Burlingame, CA) coverslipped and examined using a fluorescent microscope (Leica DM6 B);

Behavioural Protocols (Figs 1a-e)

Object exploration occurred in a wooden open topped arena 90x100cm with walls 50cm high. Object exploration was video recorded for subsequent analysis. The stimuli presented were objects composed of Duplo blocks (Lego UK Ltd, Slough, UK) that varied in shape, colour and size (9 x 8 x 7 cm to 25 x 15 x 10 cm) and were too heavy for the animal to displace.

Habituation was conducted over four days prior to the commencement of behavioural testing. On days one and two animals were habituated to the arena for 15min with their cage mates, on days three and four, each animal was placed individually in the arena for 5mins.

Object-in-place memory. This task comprised a sample and test phase, separated by delays of 5min, 3h, 6h or 24h. Performance at each retention delay was examined in different experiments each with a sample and test phase using different quartets of objects. In each sample phase (5min duration) the rats explored four different objects. In the test phase (3min duration), two of the objects exchanged positions, see Fig. 1a. The rats were tested at the different delay conditions in an interleaved manner with an interval of 3-7 days between experiments.

Object temporal order memory. This task comprised 4 sample phases (S1-S4) separated by a one-hour inter-sample interval (ISI) in which different objects were presented. During the sample phases (4min duration each) the rats were presented with two copies of the object for 4min. The test phase occurred either 1h or 4h following S4, to give a retention delay between S2 and test phase of either 3h or 6h. In the test phase (3min duration) the rats were presented with objects from S2 and S3, see Fig. 1b.

Object temporal location memory. This task involved 4 sample phases (S1-S4) separated by a 1h ISI. In each 4min sample phase, the rats encountered one object, the position of which changed. The test phase (3min duration) occurred either 1h or 4h following the end of S4, as in the temporal order task. In the test phase the rats were presented with two objects, one in the S2 location and the other in the S3 location, see Fig. 1c.

Novel object recognition memory. In the sample phase (4min duration) the rat explored 2 identical objects. In the test phase (3min duration), 24h later, rats were placed back in the arena, which now contained the sample phase object and a novel object, see Fig. 1d.

Object location memory. In the sample phase (4min duration) the rat explored two identical objects. In the test phase (3min duration) conducted 24h later the rat was placed back in the arena which contained the object from the sample phase in the same position as in the sample phase and an identical object was in a novel location, see Fig. 1e.

Assessment of object exploration and recognition memory performance

The amount of time the animal spent exploring each object during the sample and test phases, during each task, was scored by the experimenter, blind to the animals' condition. Exploration was strictly defined as the animal directing its nose towards the object at a distance of < 2 cm. From the time spent exploring the objects during the test phase, a discrimination ratio (DR) was calculated to assess recognition memory. In the *object-in-place memory task* the DR was calculated from the difference in the time spent exploring the objects that had changed position compared to the objects that had remained in the same position. In the *object temporal order memory* and *temporal location memory tasks* the DR was calculated from a comparison of the time spent exploring the objects from S2 and S3. In the *novel object recognition memory task*, the DR was calculated from the difference in time spent exploring the novel compared to the familiar object(s) divided by the total time spent exploring all objects during the test phase. In the *object location memory task*, the DR was calculated from the difference in time spent exploring the moved and unmoved objects.

Statistical analysis

The sample size for each experiment was determined by previous studies conducted in both our and other laboratories. Power calculations on previously reported data (Barker et al. 2006; Barker and Warburton, 2011a) collected in our laboratory suggest that to achieve a power of 0.8, a group size of eight is required. Larger sample sizes were used to allow for maintenance of power should animals be excluded following histological analysis.

Memory performance between groups was compared using ANOVA analyses using SPSS (IBM). Statistical analyses were designed using an assumption of normal distribution and similar variance, but this was not formally tested. Performance in all the tasks used was compared using a two-way mixed design ANOVA with virus (EGFP or A-CREB) as the between subject factor and delay or sample phase (in the object temporal order or temporal location) as the within-subjects factor. Post-hoc tests used a Bonferroni correction for multiple comparisons. In addition to test whether each group of animals could significantly discriminate between objects or pairs of objects within each task, the discrimination ratios of each condition was compared to zero (chance performance) using a one-sample t-test (two-tailed).

ACKNOWLEDGEMENTS

This work was supported by a project grant from the Biotechnology and Biological Sciences Research Council (BB/100310X/1). We thank Jane Robbins for assistance with histology.

REFERENCES

- Baddeley A. 1992. Working memory. *Science* **255**: 556-559.
- Barker GR, Banks, PJ, Scott, H, Ralph, GS, Mitrophanous KA, Wong LF, Bashir ZI, Uney JB, Warburton, EC. 2017. Separate elements of episodic memory subserved by distinct hippocampal-prefrontal connections. *Nat Neurosci* **20**: 242-250.
- Barker GRI, Bird F, Alexander V, Warburton EC. 2007. Memory for objects, place and temporal order: A disconnection analysis of the role of the medial prefrontal cortex and perirhinal cortex. *J Neurosci* **27**: 2948-2957.
- Barker GRI, Warburton EC. 2008. NMDA receptor plasticity in the perirhinal and prefrontal cortices is crucial for the acquisition of long-term object-in-place associative memory. *J Neurosci* **28**: 2837 – 2844.
- Barker GRI, Warburton EC. 2011a. When is the hippocampus involved in recognition memory. *J Neurosci* **31**: 10721-10731.
- Barker GRI, Warburton EC. 2011b. Evaluating the neural basis of temporal order memory for visual stimuli in the rat. *Eur J Neurosci* **33**: 705-716.
- Barker GRI, Evuarherhe O, Warburton EC. 2019. Remembering the order of serially

presented objects: A matter of time? *Brain Neuroscience Advances (in press)*

Baxter MG, Gaffan D, Kyriazis DA, Mitchell, AS . 2007. Orbital prefrontal cortex is required for object-in-place scene memory but not performance of a strategy implementation task *J Neurosci* **27**: 11327-11333.

Bourtchuladze R, Frenguelli B, Blendy J, Cioffi D, Schutz G, Silva AJ. 1994. Deficient long-term memory in mice with a targeted mutation of the cAMP-responsive element-binding protein. *Cell* **7**, 59–68.

Brightwell JJ, Smith CA, Countryman, RA, Neve RL, Colombo, PJ. 2005. Hippocampal overexpression of mutant creb blocks long-term, but not short-term memory for a socially transmitted food preference. *Learn & Mem* **12**: 12-17.

Browning PG, Easton A, Buckley MJ, Gaffan D. 2005. The role of prefrontal cortex in object-in-place learning in monkeys. *Eur J Neurosci* **22**:3281–3291.

Chahrour M, Jung, SY, Shaw C, Zhou X, Wong ST, Qin J, Zoghbi HY. 2008. MeCP2, a key contributor to neurological disease, activates and represses transcription. *Science* **320**: 1224-1229.

Churchwell JC, Morris AM, Musso ND, Kesner RP. 2010. Prefrontal and hippocampal contributions to encoding and retrieval of spatial memory. *Neurobiol Learn Mem* **93**: 415–421.

DeVito LM, Lykken C, Kanter BR, Eichenbaum H. 2010. Prefrontal cortex: role in acquisition of overlapping associations and transitive inference. *Learn & Mem* **17**:161-167.

Dobbins IG, Rice HJ, Wagner AD, Schacter DL. 2003. Memory orientation and success: Separable neurocognitive components underlying episodic recognition. *Neuropsychologia* **41**:318– 333.

Frankland PW, Josselyn A, Anagnostaras SG, Kogan JH, Takahashi E, Silva, AJ. 2004. Consolidation of CS and US representations in associative fear conditioning. *Hippocampus* **14**: 557-569.

Funahashi S. 2017. Working memory in the prefrontal cortex. *Brain Sci* **7**: 49; doi:10.3390/brainsci7050049

Guzowski JF, McGaugh JL 1997. Antisense oligodeoxynucleotide-mediated disruption of hippocampal cAMP response element binding protein levels impairs consolidation of memory for water maze training. *Proc Nat Acad Sci* **94**: 2693-2698.

Haghighparast A, Taslimi Z, Ramin M, Azizi, P, Khodaghali F, Hassanpour-Ezatti M. 2011. Changes in phosphorylation of CREB, ERK, and c-fos induction in rat ventral tegmental area, hippocampus and prefrontal cortex after conditioned place preference induced by chemical stimulation of lateral hypothalamus. *Behav Brain Res* **220**: 112-118.

Hannesson DK, Howland JG, Philips AG. 2004a. Interaction between perirhinal and medial prefrontal cortex is required for temporal order but not recognition memory for object in rats. *J. Neuroscience* **24**:4596-4604.

Hannesson DK, Vacca G, Howland JG, Philips AG. 2004b. Medial prefrontal cortex is involved in spatial temporal order memory but not spatial recognition memory in tests relying on spontaneous exploration in rats. *Behav Brain Res* **153**:273-285.

Hotte M, Thuault S, Lachaise F, Dineley KT, Hemmings, HC, Nairn AC, Jay TM. 2006. D-1 receptor modulation of memory retrieval performance is associated with changes in pCREB and pDARPP-32 in rat prefrontal cortex. *Behav Brain Res* **171**: 127-133.

Hyman JM, Ma L, Balaguer-Ballester E, Durstewitz D, Seamans JK. 2012. Contextual encoding by ensembles of medial prefrontal cortex neurons. *Proc Natl Acad Sci USA* **109**: 5086-5091.

Johnston, K, Everling, S. (2006) Neural activity in monkey prefrontal cortex is modulated by task context and behavioral instruction during delayed-match-to-sample and conditional prosaccade-antisaccade tasks. *J Cog Neurosci* **18**: 749-765.

Josselyn SA, Shi CJ, Carlezon WA, Neve RL, Nestler EJ, Davis M. 2001. Long-term memory is facilitated by cAMP response element-binding protein overexpression in the amygdala. *J Neurosci* **21**: 2404-2412

Josselyn SA, Kida S, Silva, AJ. 2004. Inducible repression of CREB function disrupts amygdala-dependent memory. *Neurobiol Learn & Mem* **82**: 159-163.

Kida S, Josselyn SA, Pena de Ortiz S, Kogan JH, Chevere I, Masushige S, Silva AJ. 2002. CREB required for the stability of new and reactivated fear memories. *Nat Neurosci* **5**: 348-355.

Kim JJ, Delcasso S, Lee I. 2011. Neural correlates of object-in-place learning in hippocampus and prefrontal cortex. *J. Neurosci* **47**: 16991-17006.

Kim J, Ghim J-W, Lee JH, Jung MW. 2013. Neural correlates of interval timing in rodent prefrontal cortex. *J. Neurosci* **33**: 13834-13847.

Kim J, Kim D, Jung MW. 2018. Distinct dynamics of striatal and prefrontal neural activity during temporal discrimination. *Front Integr Neurosci* **12**: 34.

Kirschmann EK Z, Mauna JC, Willis CM, Foster RL, Chipman AM, Thiels E. 2014. Appetitive cue-evoked ERK signaling in the nucleus accumbens requires NMDA and D1 dopamine receptor activation and regulates CREB phosphorylation. *Learn & Mem* **21**: 606-615.

Kopelman MD, Stanhope N, Kingsley D. 1997. Temporal and spatial context memory in patients with focal frontal, temporal lobe, and diencephalic lesions. *Neuropsychologia* **35**: 1533-1545

Liu FC, Graybiel AM. 1996. Spatiotemporal dynamics of CREB phosphorylation: transient versus sustained phosphorylation in the developing striatum. *Neuron* **17**: 1133-1144

Miller, EK, Lundqvist, M, Bastos, AM. 2018. Working Memory 2.0. *Neuron* **100**: 463-475.

- Milner B, Petrides M, Smith ML. 1985. Frontal lobes and the temporal organization of memory. *Hum Neurobiol* **4**: 137-142.
- Mitchell JB, Laiacona J. 1998. The medial frontal cortex and temporal memory: tests using spontaneous exploratory behaviour in the rat. *Behav Brain Res* **97**:107-113.
- Morton NW, Sherrill KR, Preston AR. 2017. Memory integration constructs maps of space time and concepts. *Curr Opin Behav Sci* **17**: 161-168.
- Nygaard SK, Klambatsen A, Balouch B, Quinones-Jenab, V, Jenab S. 2017. NMDAR dependent intracellular responses associated with cocaine conditioned place preference behaviour. *Behav Brain Res* **317**: 218-225.
- Olianas MC, Dedoni S, Onali P. 2012. Potentiation of dopamine D1-like receptor signaling by concomitant activation of delta -and mu-opioid receptors in mouse medial prefrontal cortex. *Neurochem Int* **61**: 1404-1416.
- Park, H, Shannon, V, Biggan, J, Spann, C 2012. Neural activity supporting the formation of associative memory versus source memory. *Brain Res* **1471**: 81-92.
- Parkin AJ, Yeomans J, Bindschaedler C. 1994. Further characterisation of the executive memory impairment following frontal lobe lesions. *Brain and Cognition* **26**:23-46.
- Peters M, Bletsch M, Catapano R, Zhang X, Tully Y, Bourtchouladze R. 2009. RNA interference in hippocampus demonstrates opposing roles for CREB and PP1 α in contextual and temporal long-term memory. *Genes Brain Behav* **8**: 320–329.
- Pittenger C, Huang YY, Paletzki RF, Bourtchouladze R, Scanlin H, Vronskaya S, Kandel ER. 2002. Reversible inhibition of CREB/ATF transcription factors in region CA1 of the dorsal hippocampus disrupts hippocampus-dependent spatial memory. *Neuron* **34**: 447-462.
- Pozzi L, Sacchetti G, Agnoli L, Mainolfi P, Invernizzi RW, Carli M. 2011. Distinct changes in CREB phosphorylation in frontal cortex and striatum during contingent and non-contingent performance of a visual attention task. *Front Behav Neurosci* **5**: 65.
- Roberts, BM, Libby, LA, Inhoff, MC, Ranganath, C. (2018) Brain activity related to working memory for temporal order and object information. *Beh Brain Res* **354**: 55-63
- Rugg MD, Vilberg KL. 2013. Brain networks underlying episodic memory retrieval. *Curr. Opin. Neurobiol* **23**: 255-260.
- Savalli G, Bashir ZI, Warburton EC. 2015. Regionally-selective requirement for D1/D5 dopaminergic neurotransmission in the medial prefrontal cortex in object-place associative recognition memory. *Learn & Mem* **22**: 69-73.
- Schlichting ML, Preston AR. 2015. Memory integration: neural mechanisms and implications for behaviour. *Curr Opin Behav Sci* **1**:1-8.

Scott H, Smith AE, Barker GR, Uney JB, Warburton, EC. 2017. Contrasting roles for DNA methyltransferases and histone deacetylases in single-item and associative recognition memory. *Neuroepigenetics* **9**:1-9.

Sekeres MJ, Neve RL, Frankland PW, Josselyn SA. 2010. Dorsal hippocampal CREB is both necessary and sufficient for spatial memory. *Learn & Mem* **17**: 280-283.

Shimamura AP, Janowsky JS, Squire LR. 1990. Memory for the temporal order of events in patients with frontal lobe lesions and amnesic patients. *Neuropsychologia* **28**: 803-813.

Siddiqui SA, Singh S, Ranjan V, Ugale R, Saha S, Prakash A. 2017. Enhanced histone acetylation in the infralimbic prefrontal cortex is associated with fear extinction. *Cell Mol Neurobiol* **37**: 1287-1301.

Silva AJ, Kogan JH, Frankland PW, Kida S. 1998. CREB and memory. *Ann Rev Neurosci* **21**: 127-148.

St Jacques P, Rubin DC, Labar KS, Cabeza R. 2008. The short and long of it: Neural correlates of temporal-order memory for autobiographical events. *J Cog Neurosci* **20**: 1327-1341.

Tanimizu T, Kono K, Kida S. 2018. Brain networks activated to form object recognition memory. *Brain Res Bull* **141**: 27-34.

Vieira PA, Korzus E. 2015. CBP-dependent memory consolidation in the prefrontal cortex supports object-location learning. *Hippocampus* **25**: 1532-1540.

Warburton EC, Glover CPJ, Massey PV, Wan, H, Johnson B, Bienemann A, Deuschle U, Kew JNC, Aggleton JP, Bashir ZI, Uney J, Brown MW. 2005. CREB phosphorylation is necessary for perirhinal LTP and recognition memory. *J Neurosci* **25**: 6296-6303.

Weible AP, Rowland, DC, Monaghan CK, Wolfgang NT, Kentrol CG. 2012. Neural correlates of long-term object memory in the mouse anterior cingulate cortex. *J Neurosci* **32**: 5598-5608.

Zhang W, van Ast VA, Klumpers F, Roelofs K, Hermans EJ. 2018. Memory contextualization: The role of prefrontal cortex in functional integration across item and context representational regions. *J Cog Neurosci* **30**: 579-593.