CONDITIONED *ARC* EXPRESSION IN CA1 OF THE RAT HIPPOCAMPUS SUPPORTS LONG-TERM MEMORY FOR TEMPORAL EVENTS

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Abstract

The hippocampus is important for the acquisition, storage, and retrieval of memories. It has been largely examined for its role in spatial and contextual memory, but the mechanisms of encoding temporal cues are not well understood. The present work explores the expression of an immediate early gene which contributes to synaptic changes associated with long-term plasticity, *Arc*, as a temporally-specific marker of hippocampal memory to a time-of-day cue in a novel conditioning procedure in rats. We video-recorded locomotor activity and delivered a clock time-specific, multimodal alarm signal to cage-paired rats for 14 days, and quantified *Arc* mRNA expression at conditioned and unconditioned times. We predicted that neural activity associated with a temporally-conditioned response to a time-of-day cue would include expression of *Arc* genes in the hippocampus and interactions with circadian cycles of sleep-wake behaviour.

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Arc - activity-regulated cytoskeleton-associated protein
cAMP - cyclic adenosine monophosphate
CA1 - cornu Ammon 1
CFC - contextual fear-conditioning
CR - conditioned response
CREB – cAMP-response element-binding
CRE - CREB response element
CS - conditioning stimulus
DAPI - 4′, 6-diamidino-2-phenylindole
ERK - extracellular signal-regulated kinase
IEG - immediate early gene
LTM - long-term memory
LTP - long-term potentiation
MAPK - mitogen-activated protein kinase
MTT - multiple trace theory
NREM - non-rapid eye movement
NS - neutral stimulus
REM - rapid-eye movement
ROI – region of interest
SCN - suprachiasmatic nucleus
SMC - standard model of systems consolidation
STM - short-term memory
SWS - slow-wave sleep
TCH - temporal coding hypothesis
TTFL – transcription-translation feedback loops
UCS - unconditioned stimulus
UR - unconditioned response

1. Introduction

1.1 Conditioned behaviours

Behaviours are the patterns of activity that organisms present in within their specific biological and environmental circumstances. The brain of an organism is able to maintain memory, such that new information can be integrated with learned experiences to produce behavioural responses that are more adaptive in their environment. With this flexible memory, organisms may become better suited to navigate the unique climates, resources, and threats they face, which are subject to change over periods of time. Despite the unlearned actions which guide them, individuals must navigate novel circumstances in their lifetime, and therefore importantly, behaviours must be both maintained yet flexible (Robinson & Barron, 2017).

The early work of Pavlov (1903) showed that behaviours can be modified through association. Pavlov observed that a dog would produce an unlearned physiological response in response to the presentation of food, demonstrating a relationship between a stimulus (food) and its linked biological response (production of saliva). He termed the food stimulus an example of an unconditioned stimulus (US) and the salivation as an unconditioned response (UR). Pavlov found that by pairing a neutral stimulus (NS) - the sound of a ringing bell - with the US, a new conditioning stimulus (CS) could be formed through temporal association, that is, stimuli that consistently occur at roughly the same point in time. The ringing bell could then elicit what he termed a conditioned response (CR), with behavioural and physiological outcomes that resemble the UR. Here, memory serves to temporally associate relevant stimuli, eliciting an adaptive physiological-behavioural response to an arbitrary stimulus: a readiness to begin the digestive process in preparation to consume nutrients (Lucas, 2019). Pavlovian conditioned behaviours are susceptible to further modification, as early studies showed that a conditioned salivary response

would decrease when dogs were repeatedly presented a food stimulus without being fed, a concept termed extinction (Windholz, 1989). Researchers in Pavlov's laboratory also discovered a time-dependent phenomena known as spontaneous recovery - whereby a CR would eventually recur following extinction – which could be brought on by the introduction of any external stimulus during experimentation (Windholz, 1989). These findings guided an early paradigm for flexibly conditional behaviour, which included temporal elements of memory association.

Our understanding of conditioning has developed substantially since Pavlov's time, with the procession of technology and animal research methods. Continued investigation revealed that learning had much to do with context; spatial and temporal factors that serve as cues or stimuli (Bouton & Hendrix, 2011). The discovery that fear elicits a strong neurobiological response has led to its use in many contextual conditioning studies, largely in rodents (Curzon et al., 2009). Of importance was the recognition that conditioned memory was partly a result of processes occurring at the cellular level (Athos et al., 2002). Taking advantage of the natural behaviours of animals has been key to the way that researchers have addressed the biological mechanisms of conditioning. Upon investigation in both animals and humans, types of conditioned behaviours seem to engage specific regions of the brain.

1.2 Hippocampus and long-term memory

The acquisition of memory for classical conditioning requires a system which can detect temporally predictive relationships between components of events. The hippocampus, a bilateral structure in the medial temporal lobes (MTL) of vertebrates, is key to the brain's ability to learn and recall information, although the neurobiological mechanisms are not entirely understood. It has been hypothesized that the hippocampus constructs memories by creating configural

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associations between sensory information represented throughout the neocortex (Sutherland & Rudy, 1989). When any unit of the configuration is recalled, the entire pattern of connectivity is completed and restores the memory (George & Pearce, 2012). In a conditioning paradigm, this means that the hippocampus doesn't just create associations between stimuli, but rather creates a new representation of all the combined elements of the circumstance as they relate to the stimuli (Rudy & Sutherland, 1995). Remembering associations helps to guide behaviour when faced with particular stimuli again, even if some components are novel, since having some basis for reacting appropriately may enhance reward or survival chances.

Tulving (1972) distinguished between semantic and episodic forms of explicit memory; "knowing" and "remembering" respectively, which can be attributed to differing domains and networks in the brain. Episodic memory describes a subtype of memory for events – lived experiences with autobiographical features for what happened, where, and when - and these combined features are linked together in the hippocampus. Memory for facts and information that are not recalled with specific experiences is semantic in nature (Tulving, 1972). A component-process model of memory was used to explain the need for hippocampal networks to be active when acquiring conscious, episodic-like information, and that they must also be reactivated when this type of information is recalled (Moscovitch, 1995). This posits that the hippocampus is necessary for both initially encoding episodic information, and also in the continuous representation of that type of memory.

Episodic features of memory have been the focus of many neuroscientists who study the hippocampus. Spatial elements are represented by place cells in the hippocampus - neurons which fire in specific locations when an animal explores an environment (Moser et al., 2008). Since place cells were discovered, research regarding the architecture of spatial memory in the hippocampus

has exploded, and with it, to a lesser extent insights into the organization of memory for time. A Temporal Coding Hypothesis (TCH) was described by Savastano & Miller (1998), to posit that in conditioning two stimuli can be associated through a temporal relationship with one another (1998). Later work showed that cells in the hippocampus activate in response to specific elapsed time intervals, and to time points across days (Kraus et al., 2013). Specifically, temporal sequences and the proximity of associable events such as paired stimuli in Pavlovian conditioning paradigms, are associated with neuronal activity largely in CA1 and CA3 subfields of the hippocampus (Salz et al., 2016). Eventually time cells were posited in analogy to place cells, described as individual neurons which fire at specific elapsed intervals instead of specific locations in space (Eichenbaum, 2017). However, electrophysiological studies have observed that some neurons in CA1 encode both time and space (Salz et al., 2016). Time encoding cells show changes to firing network patterns after time as modifications are made to consolidate a temporally-associated memory. Although the activity of specific neurons is changed, the hippocampus is able to maintain memory on timescales of moments to decades (Eichenbaum, 2017).

Neuroscientists who study memory have given much attention to understanding the mechanism by which initial short-term memories (STM) become sustained through processes of consolidation to be stored as long-term memories (LTM). This state change is supported by mechanisms of synaptic and systems level consolidation, which represent respective "fast/rapid" and "prolonged" processes known collectively as memory consolidation (Nadel et al., 2007). Two theories came to dominate the research regarding the mediation of LTM. The Standard Model of Systems Consolidation (SMC) posits that recollection of LTM is an independent process from the hippocampus, relying instead on the reconstruction of information stored in the neocortex (Squire & Alvarez, 1995). SMC is at odds with research findings that the hippocampus continues to

support memory storage and retrieval regardless of time passed since an initial learning experience, forming an opposing view of systems consolidation referred to as Multiple Trace Theory (MTT) (Nadel & Moscovitch, 1997). As it stands, no complete explanation exists for how memories represented in the hippocampus affect the organization of neocortical circuitry. Currently, neuroscientists continue to investigate whether activity in the hippocampus is associated with retrieving LTM (Nadel et al., 2007).

Time as a distinct feature of episodic memory has implicated the hippocampus in a number of studies. As previously introduced, the order of temporal associations between stimuli in Pavlovian conditioning experiments rely on memory for temporal order of events (Windholz, 1989). Temporal maps have been suggested as a possible mechanism for encoding such sequential relationships, given the nature of spatial memory encoding by the hippocampus (Eichenbaum, 2017). Further exploration of the mechanisms by which the hippocampus continuously meditates temporally-associated LTMs might be addressed by investigating other types of physiological temporal activity. Temporally-associated behaviours, and hippocampal dependent memory in general, appear to be mediated largely by endogenous rhythms (Lehr et al., 2020).

1.3 Circadian rhythm

Circadian rhythms are cycles of roughly 24-hour (daily) biorhythms which dictate organic physiology and behaviours. Such biorhythms are regulated in large part by the suprachiasmatic nucleus (SCN) in mammals, and these feedback-controlled physiological functions are regulated by mechanisms at the genetic, proteomic, and metabolic level (Bolsius et al., 2021). This brain structure is primarily modulated by a cell signalled-response to light passing through the retinas of the eyes, becoming entrained, or aligned, with this external light cue, coordinating phases of expression in a specific subset of genes (Dibner et al., 2010; Pittendrigh, 1960). The transcription-

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translation feedback loops (TTFLs) of genes are the understood mechanism by which circadian rhythms can affect physiological processes, so that time-based environmental cues are integrated to the behaviours of an individual (Hurley et al., 2016).

Cyclic expression of "clock genes" in response to light-dark cycles results in more organised time-of-day -associated behaviour (Dibner et al., 2010). As introduced, clock genes - such as *Per1, Per2, Bmal1,* and *Clock* - have peak transcription and translation windows that repeat every 24-hours. Their products inter-interact, but also communicate with other proteins or genes directly to regulate cellular functions (Bolsius et al., 2021). Entrainment of SCN gene oscillations is strictly synchronised, as these oscillations mediate hormonal and metabolic signals relating to periods of activity, rest, temperature, feeding, and fasting, which require the collaboration of multiple physiological functions (Dibner et al., 2010). This is supported by evidence that feeding-cycles are one of the central Zeitgebers (literally "time-giver"); circadian entrainers of gene oscillations. However, feeding and fasting cycles result from sleep-wake rhythms, and therefore it is primarily activity level directed by light-dark cycles which underlie the circadian physiology - and therefore behaviour - of individuals (Dibner et al., 2010).

Research has uncovered that circadian rhythms produce physiological cycles which interact with memory performance (Holloway & Wansley, 1973). In fact, it has been posited that due to its importance in episodic forms of memory, the hippocampus may contain a "memory clock," which influences memory-associated endogenous oscillations, such as clock gene expression (Lehr et al., 2020). Based on evidence of disrupted hippocampal-learning in *Per1* suppressed mice, the expression of clock genes in the hippocampus is thought to play a role in mediating LTM (Kwapis et al., 2018). Hyperexcitable neurons which result in epileptic seizures are often time-of-day specific, especially those which occur in the temporal lobes (Cho, 2010).

Seizure activity was reduced in a study of *Bmal1* knockout mice, implicating clock genes as circadian drivers of neuronal activity (Gerstner et al., 2014). A parallel decline in LTM and circadian rhythm regulation in aging, and the interaction between circadian rhythms and hippocampal gene expression, call for investigation of temporal gene expression as it relates to LTM consolidation (Kondratova & Kondratov, 2012).

1.4 CREB and immediate early genes (IEGs)

The photosensitive SCN entrains all cells of the body to temporally-significant circadian cycles of gene expression (Dibner et al., 2010). Light information processed in the SCN results in a cascade of signalling pathways that influence clock gene expression, as well as immediate early gene (IEG) expression (Welsh et al., 2010). Immediate early genes (IEGs) are a class of genes which are rapidly transcribed in response to a stimulus, and their expression in the hippocampus is linked to neuronal activity associated with learning and behaviour (Minatohara, 2016). In particular, the activity-regulated cytoskeleton-associated (*Arc)* IEG is mediated by CREB expression, and is essential for hippocampal-dependant learning

CREB is crucial to the support of LTM consolidation through its activity as a transcription factor for memory genes (Eckel-Mahan et al., 2008). An SCN-modulated signalling mechanism of importance to LTM is the extracellular signal-regulated kinase (ERK) pathway. When activated, ERK causes phosphorylated cAMP-response element-binding (CREB) proteins to attach with cAMP-response elements (CRE) in the promoters of clock genes like *Per1 and Per2* (Dibner et al., 2010). CREB activation and subsequent cAMP-mediated gene transcription is a signature of hippocampal cell biology, and studies have shown that maintenance of LTM through the action of long-term potentiation (LTP) requires this genomic activity in the hippocampus. Rodents whose

hippocampal CREB expression was suppressed showed disrupted long-term LTP (Bourtchuladze et al., 1994). Further, levels of clock gene *Per1* expression were increased in rodents following training in tasks which rely on the hippocampus. The *Per1* clock gene was found to mediate hippocampal CREB phosphorylation for processing information related to time-of-day (Rawashdeh et al., 2016). CREB enhances transcription of *Per1/Per2* via the cAMP/mitogen-activated protein kinase (MAPK)-signaling cascade, ultimately supporting LTM consolidation (Bolsius et al., 2021). Clock gene protein products BMAL1 and CLOCK are components of cellular gene expression, serving as transcription and translational factors for molecules involved in synaptic plasticity (Bolsius et al., 2021).

Gene expression in the hippocampus has been strongly implicated as a mechanism for reconsolidation of LTM. In light of this, a hypothesis called *de novo* protein synthesis predicts that newly formed memory traces consistently require new proteins to be synthesized to maintain LTP processes (Rudy, 2008). LTP produces strengthened synaptic connections and utilizes the cAMP-protein kinase A (PKA) signalling pathway (Silva et al. 1998). Studies which block translation of proteins during or immediately following a learning event result in disruption to LTM, however, blocking translation of proteins hours after a learning event does not, serving evidence for early protein synthesis as an essential step to LTP (Bolsius et al., 2021). STM traces are immediately consolidated by genes, beginning the process of protein synthesis that eventually results in robust LTM, especially when those traces are reactivated in distributed learning episodes (Litman & Davachi, 2008).

Interestingly, CREB is the primary modulator of the *Arc* IEG. Neurons of the hippocampus express *Arc* in order to support consolidation of memory traces - primarily by altering dendritic synapse α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor density

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(Chawla et al., 2018; Minatohara et al., 2016). The hippocampus contains highly plastic synapses, and the CA1 region in particular displays strong *Arc* expression. Intranuclear *Arc* mRNA is detectable within a few minutes of neuronal stimulation, reaching a peak expression at roughly 8 minutes post-stimulus (Rudy, 2008). The same *Arc*-expressing neurons can undergo a new round of *Arc* expression after just 20 minutes of initiation of the previous cycle, making it an especially discrete and transient marker for neuronal activity (Guzowski et al., 2006). *Arc* has been shown to express repeatedly in the same population of CA1 neurons in relation to place field activity, when exposure to an environment was limited to once per day (Guzowski et al., 2006).

CA1 is part of a network structure in the hippocampus called the trisynaptic circuit, so called because of its connectivity with dentate gyrus and CA3 synapses (Rudy, 2008). When any of these three subfields underwent optogenetic suppression of *Arc*-expressing neurons, memory for a conditioned response to fear was impaired (Minatohara et al., 2016). Additionally, *Arc* expression in CA1 increases with stimulus intensity, although it is present at lower levels of stimulation than other subfields, such as CA3, suggesting a preferential sensitivity to stimulation than other hippocampal regions (Chawla et al., 2018). CA1 receives signals from all other trisynaptic structures, is mediated by temporally-specific CREB and IEG expression, and as a final output from the hippocampus it may serve a vital structural role in LTM for temporally-associated memory.

1.5 Sleep and memory consolidation

LTM is supported not only by LTP through protein synthesis, but importantly, through sleep. Marked by decreased motor activity, sleep is defined by stages constituting unique brain wave features. 4-8 Hz theta oscillations in the hippocampus are detectable using

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electrophysiological recording, and are associated with rapid-eye movement (REM) sleep, as well as wakeful behaviours (Boyce et al., 2017). Activity of hippocampal neural networks during REM sleep has been shown to follow a temporal sequence reflecting that of prior waking experience (Xia & Storm, 2021). REM sleep cycles are characterized by 90 minute cycles of theta rhythm; activity which has been linked to spatial encoding and memory consolidation (Xia & Storm, 2021). An increase in the quantity of REM cycles has been reported in sleep following novel environmental exposure in rats, as well as motor and language task learning in humans (Boyce et al., 2017). Experimental optogenetic inhibition of hippocampal theta activity during REM sleep in mice disrupted memory for object recognition and contextual fear-conditioning tasks (Boyce et al., 2016).

Research has identified successive stages of sleep, which are individually defined by unique features of brainwave activity and their various influences on memory consolidation (Rasch & Born, 2013). Slower, irregular oscillations known as large irregular activity (LIA) are associated with non-REM (NREM) sleep, further divided into stages 1, 2, and 3. Like REM, NREM has been reported as increased following learning events. Synchronized firing of hippocampal neurons occurs in stage 3, or slow-wave sleep (SWS) of NREM, activity known as sharp wave ripples (SWRs) which support LTP and interrupt theta rhythm (Samsonovich & Ascoli, 2005). It was hypothesized that SWRs may coordinate the transfer of information between the neocortex and hippocampus (Sirota et al., 2003). A holistic theory suggests that a homeostatic mechanism regulating the cycles of REM and SWS sleep stages aids in memory performance, based on work in both human and animal studies. A two-process model of sleep regulation has implicated a circadian control of REM and NREM sleep periods (Borbely et al., 2016). SWS in NREM is affected by previous waking experience, a sorting process which refines memory traces and is

followed by the REM stage, a deeper state of sleep which is thought to consolidate memory for the most salient information (Giuditta, 2014). In humans, sleep containing only both states improved memory in a discrimination task (Mednick et al., 2003). Quantity of cycles was positively correlated with successful learning in a two-way active avoidance task in rats (Langella et al., 1992).

Like LTM, sleep itself is marked by the expression of specific genes in the hippocampus and neocortex (Bolsius et al., 2021, Honjoh et al., 2017). The cAMP/MAPK-CRE pathway, a circadian-regulated transcription pathway which promotes protein synthesis of CREB and some IEGs, is most active in the hippocampus during REM sleep (Boyce et al., 2017; Xia & Storm, 2021). Circadian oscillations of these factors are essential to memory persistence - a study in mice found disrupted hippocampal gene oscillations and deficits in contextual memory when exposed to inconsistent light cycles and consequently sleep-wake activity (Eckel-Mahan et al., 2008). *Arc* knockout mice displayed longer REM sleep cycles and disruption to homeostatic sleep responses after sleep deprivation (Suzuki et al., 2020). Cortical intranuclear *Arc* expression increased with sleep deprivation in wild-type mice, and a higher nucleus:cytoplasmic ratio of *Arc* was found in sleeping mice compared with awake and sleep-deprived mice (Honjoh et al., 2017).

1.6 Temporal conditioning in rats

This research aimed to investigate the nature of *Arc* IEG expression associated with long-term, time-associated episodic memory recall. Using a rat model of the hippocampus, and a novel classical conditioning procedure, our goal was to determine whether expression of hippocampal CA1 *Arc* mRNA is associated with anticipation, inferring recollection, of a temporally-significant event.

To evaluate the function of the hippocampus in representing a time-associated LTM, we can measure the expression of the *Arc* gene, which transiently responds to neuronal synaptic activity and is associated with memory reconsolidation in a rat model of the hippocampal formation (Kawashima et al., 2009). The CA1 subfield of the hippocampus has been shown to express *Arc* when place field neurons were activated, and studies using a temporal context show activity in CA1 corresponding to temporal events (Eichenbaum, 2017; Guzowski et al., 2006). In the outlined experiment, rats remained in a consistent physical environment with only distinct temporal cues delivered during their resting phase (daytime) as an experimental manipulation, in order to separately characterize environmental spatial and temporal elements. Rats were conditioned to a time-of-day once every 24-hours for 14 days, with no stimulus presented on the testing day, ensuring that the targeted conditioned *Arc* IEG response supported a LTM representation of the conditioning stimulus (Rudy, 2008). Previous experiments have determined that *Arc* mRNA reaches a peak expression in hippocampal CA1 neurons at 5-10 minutes post-activation, allowing for a specific time window associated with the prior conditioning stimulus to be targeted (Guzowski et al., 2001). Expression of *Arc* correlated to these time points provides evidence that the hippocampal formation continues to store and retrieve an event-based memory, utilizing memory-associated genes to modify an existing memory trace, an argument for long-term hippocampal reconsolidation (Nadel et al., 2007).

The role of CA1 *Arc* expression in consolidating memory for a temporally-conditioned stimulus remains unclear in the current research of temporal memory. This work will explore whether memory for a time-associated stimulus can be conditioned and observed in the rat, and whether *Arc* IEG expression would be temporally-associated with the conditioning stimulus. We predicted an association between time-of-day and *Arc* expression that would condition a change

in locomotor activity. Further, we predicted that temporally relevant experiences condition neuronal cells in CA1 to reactivate at the conditioned time-of-day, increasing *Arc* IEG expression.

2. Materials and Methods

2.1 Subjects and animal housing

This work was approved by the University of Lethbridge Animal Care and Use Committee and agreed with Canadian Council of Animal Care standards. Twelve experimentally naive, young-adult, male Long-Evans rats were used in the presently outlined experiment. They were cage-paired and acclimated to the University of Lethbridge vivarium rat colony room for seven days, then handled for 5 minutes per day for the five days preceding experimentation.

The cage-paired rats were then divided into three identical rooms, with an opaque black curtain dividing two modified, rectangular plexiglass-cages per room. The three rooms had identical 12:12 light:dark cycles; 7:30AM-7:30PM. Rats were given access to food and water ad libitum. At two points in the experiment, animals were transferred into identically setup cages to ensure sanitary conditions. Husbandry checks were performed daily by the experimenter at consistent time windows for each cage, between 6:00PM-7:00PM

The cages were labelled as Cages A, B, C, D, E, F. In room one were cages A & F, in room two were cages B & D, and in room three were cages C & E. The pairs remained in their respective room and cage throughout the duration of the 15-day experiment, with a 24hr acclimation period to the room and cage context prior to experiment start.

2.2 Video recording data analysis and;

2.3 Conditioning procedure

To gather video recordings of rat activity, each outer cage lid was fitted with a Raspberry Pi computer with an Arducam NOIR 5MP OV5647 camera module, facing downward into the cage to obtain a full view of the cage floor area. The video recordings were obtained at a 640x480

pixel resolution, at 15 frames per second, for 24 hours per-day over the course of the 15-day experiment.

Every 24 hours for 14 consecutive days, an identical combined visual, auditory, and vibrational signal was delivered using a mobile device to the six pairs of rats at a specified time-of-day. The time-of-day conditioning signal was delivered using the Reminder application on inactive (no cellular radio connectivity) Apple iPhone devices, which were velcro secured to the outer length of each cage. The Reminder signal can be set to a specific alert tone and vibration in the device settings. The selected alert tone setting for this experiment is one paired tone-vibration (5-seconds total duration), named "Chord" (3 pitches: 333Hz/506Hz/695Hz), at ~65dB volume. In order to ensure no other activity of the mobile device besides one 5-second signal every 24 hours, each device was placed on a scheduled "Do Not Disturb" setting for the remainder of the 24 hours that it was not in use (this option is activated in the Apple iPhone device Settings). Time-of-day conditioning signals were: Cage A, 9:00AM; Cage B, 10:30AM; Cage C, 12:00PM; Cage D, 1:30PM; Cage E, 3:00PM; Cage F, 4:30PM (Table 1).

Acquired video data were subdivided into 15-minute intervals for analysis of cage motion as an indicator of paired rat locomotor activity, producing 96 data points per cage, per day. A further analysis of cage motion, at 1-minute respective cage pre-stimulus time was performed. These analyses were performed using a Python script which compared adjacent frames to determine whether they are identical (1) or different (0). Each numerical output therefore equated to the inactivity of the rats, such that a higher numerical output equated to less cage motion.

2.4 Targeting Arc IEG expression

Table 1.   Summary of Experimental and Control group treatments.

Cage	Signal Time	Perfusion Time (Experimental Group)	Perfusion Time (Control Group)
A	9:00 AM	9:00 AM	1:30 PM
B	10:30 AM	10:30 AM	3:00 PM
C	12:00 PM	12:00 PM	4:30 PM
D	1:30 PM	1:30 PM	9:00 AM
E	3:00 PM	3:00 PM	10:30 AM
F	4:30 PM	4:30 PM	12:00 PM

On the 15th and final day of the experiment, no signals were delivered to the rats. Instead, one rat from each cage was removed from the cage by an experimenter 60 seconds after their cage-signal time (Experimental Group animals, 6), and at the same time, one rat from a different cage/different room was removed to serve as a control (Control Group animals, 6). Rats were then injected intraperitoneally with 1.5-2.5ml of Euthansol (sodium pentobarbital) for immediate perfusion and tissue collection. All experimental and control pairings were perfused simultaneously, between 8-13 minutes following conditioned signal time to target a peak IEG expression window (Supplementary Table 1).

2.5 Brain acquisition and fISH tissue processing

Rats were perfused on-ice intracardially using 100 ml of 1x phosphate-buffered saline (PBS) in diethyl pyrocarbonate (DEPC) solution, followed immediately by 150 ml of 4% paraformaldehyde (PFA) in 1x PBS-DEPC solution. Brains were collected and stored in an ice-cold post-fix solution of 4% PFA for 120 minutes, then transferred to 30% sucrose PBS solution at 4 °C for at least 48 hrs before sectioning. One hemisphere of each brain was sliced coronally at 40 um thickness using a freezing sliding microtome in a ⅙ section series. Sections were mounted

on Superfrost+ (Fisher Scientific) ionized slides, and stored at -80 °C in preparation for fluorescent in-situ hybridization (fISH).

Thawed slides were incubated in 4% PFA for 4 minutes, washed with saline-sodium citrate (SSC) buffer, and treated with proteinase K buffer (p4580, Sigma-Aldrich, USA) for 30 minutes in a tris-buffered saline (TBS) chamber. Then slides were incubated in 4% PFA for 3 minutes, washed with SSC, before incubation in acetic anhydride for 10 minutes, followed by incubation in 1:1 acetone-methanol at -20 °C for 5 minutes. Slides were then washed with SSC before application of a hybridization buffer for 60 minutes in a 50% formamide humid chamber, followed by a 16-hour humid chamber incubation at 56 °C with a hybridization buffer containing IEG-targeted riboprobes for hippocampal CA1 *Arc* IEG quantification. Probes were designed with an online software program (National Centre for Biotechnology Information Primer-Blast), and contained intranuclear-specific sequences found in portions of intron 1, exon 2, and intron 2 of *Arc* mRNA molecules. After several washes with SSC, slides were quenched with 2% hydrogen peroxide in SSC, followed by two, 5 minute washes in TBS with Tween-20 (TBST), and a 5 minute wash in TBS. They were then treated with a TSA blocking buffer containing 5% sheep serum for 60 minutes in a TBS humid chamber, then anti-DIG (1:300 in blocking buffer) was added before incubation at 4 °C in TBS humid chamber for 20 hours. After several washes in TBST, biotin-tyramide dye (1:100) was added to the slides for 60 minutes, then Streptavidin-Texas Red dye (1:200 in blocking buffer) for 30 minutes, incubated in a TBS chamber for both steps. Following washes in TBST, cell nuclei were counterstained with 4′, 6′-diamidino-2-phenylindole (DAPI; 1:2000; Sigma-Aldrich), and cover slipped using Vectashield mounting medium (Vector Laboratories Inc, USA).

2.6 CA1 Arc quantification

Expression of the *Arc* IEG in unilateral sections of CA1 were quantified using StereoInvestigator's stereological optical fractionator workflow. Image z-stacks were acquired at 60x oil-immersion objective in a 50 x 50 um counting frame positioned using a 250 x 250 um grid overlaying the CA1 contour in a systematic-random sampling procedural method and were captured using Fluoview FV10-ASW software on an Olympus FV1000 confocal laser scanning microscope. Eight (512 x 512 px) images were collected at each counting frame site, at a 2 um step between image slices, producing a stereological 3D reconstruction of 16 um of tissue thickness overall. Texas Red and DAPI channel thresholds were set to account and control for noise and signal intensity from subject to subject, and depth of section within z-stacks. Only intranuclear *Arc* transcription foci within DAPI-stained cells were quantified. Each DAPI-stained cell was quantified by inclusion of its widest point in the z-stack dissector height zone. Unbiased whole CA1 *Arc* and DAPI estimates were generated. Unbiased estimates of CA1 *Arc*:DAPI generated with StereoInvestigator had coefficients of error of <0.1 for each individual rat.

2.7 Statistical Analysis

Comparisons of activity levels in Control and Experimental groups were analysed to determine whether there was a significant effect of conditioning on motor activity. Paired t-tests were performed on the conditioned activity vs. average unconditioned activity at each time-of-day, as well as for morning- and afternoon-only comparisons. Microsoft Office Excel was used to organize data and generate boxplots of interquartile ranges (IQR) of Unconditioned (Control) and Conditioned groups.

Locomotor activity was captured using video recordings of whole cage activity and processed frame-by-frame to determine if adjacent frames were different or not, with final outputs denoting the total number of "same" frames, equating to "stillness" of both rats in each cage. For comparison, the relative activity between all cages was calculated and represented on the y-axis, such that 0 denotes the lowest activity measured for Unconditioned cages at 1-minute pre-stimulus (Cage B, 9AM/Unconditioned), and 1 represents the highest possible activity (all adjacent video frames different).
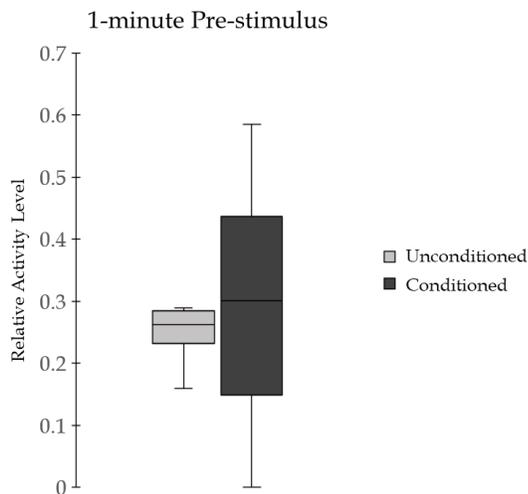
Pearson R correlation coefficients were calculated to determine *Arc* IEG expression vs. relative activity levels using JASP open-source software, and scatter plots were generated using Excel.

3. Results

3.1 Relative Activity Levels of Rats

The results of conditioning rats (n = 12) to a time-of-day–specific stimulus revealed a greater variability of activity levels in rats at one minute prior to the normal time of the unconditioned stimulus (Conditioned group, $\mu = 0.2944$, $\sigma = 0.2181$), compared to activity levels of rats at times never paired with the unconditioned stimulus (Unconditioned group, $\mu = 0.2481$, $\sigma = 0.0500$) (Figure 1A, Table 2). A paired t-test showed that the difference between the means for the relative activity levels of our compared groups was not significant ($p = 0.29$, $t = 0.5993$). Further analysis comparing each Conditioned cage with the average of all other cages (Unconditioned) at each time-of-day showed that greater Conditioned group variability was an effect of higher than average activity in the first half of the light cycle (ns., $p = 0.06$, $t = 2.62$), and significantly lower than average activity in the second half of the light cycle ($p = 0.04$, $t = 3.32$) (Figure 1B, Table 2).

A)

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B)

1-minute Pre-stimulus

(Column chart: Relative activity level (y-axis, -0.1 to 0.6) vs time-of-day (x-axis: 9:00 AM, 10:30 AM, 12:00 PM, 1:30 PM, 3:00 PM, 4:30 PM). Legend: Unconditioned, Conditioned.)
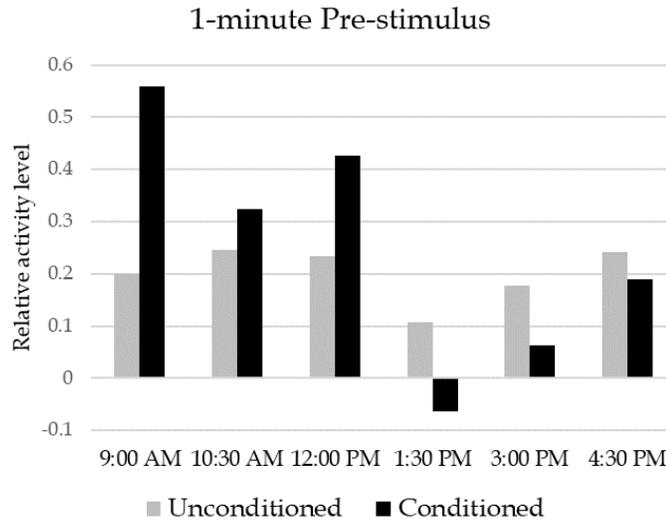
Figure 1. A) Boxplot showing a comparison of cage activity levels in the 1-minute prior to a conditioned signal time (Conditioned) with the cage activity of all other cages at those times (Unconditioned). B) Column chart displaying the comparison of average relative cage activity for all Unconditioned cages at 1-minute prior to each timepoint, with the cage activity of the cage signal-conditioned to that time-of-day (Conditioned).

Table 2. Relative activity level of cages at 1-minute pre-stimulus for each time-of-day. Data points are normalized so that the lowest average recorded activity level at an unconditioned time-of-day for all cages at 1-minute pre-stimulus (Cage B, 9:00AM) represents minimum locomotor activity (0), and all frames contain motion is the maximum locomotor activity (1).

	Cage A	Cage B	Cage C	Cage D	Cage E	Cage F	Timepoint Average (Unconditioned)
9:00 AM	**0.55933**	0.00009	0.18297	0.16249	0.20156	0.45382	0.20019
10:30 AM	0.32968	**0.32507**	0.12636	0.35115	0.21495	0.20258	0.24494
12:00 PM	0.24708	0.28109	**0.42631**	0.10481	0.45596	0.07606	0.23300
1:30 PM	0.11430	0.12750	0.00922	**-0.06256**	0.15819	0.12411	0.10666
3:00 PM	0.26167	0.13653	0.04392	0.34963	**0.06360**	0.10045	0.17844
4:30 PM	0.25716	0.09187	0.34862	0.22164	0.29437	**0.18985**	0.24273
Cage Average (All timepoints)	0.29487	0.16036	0.18957	0.18786	0.23144	0.19114	
(Unconditioned timepoints)	0.24198	0.12741	0.14222	0.23794	0.26501	0.19140	

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Analysis of relative activity levels for each cage across the experimental period showed an increase in activity levels at one minute prior to conditioning time for cages A, B, D, E, and F, and an overall decrease in activity levels in cage C (Table 3, Supplementary Figure 2). Most cages showed a significant change across 14 conditioning days, while two showed a trending direction of change (Cage A: $p = 0.000001$, $t = 8.08$; Cage B: ns., $p = 0.134$, $t = 1.16$; Cage C: $p = 0.001$, $t = 3.85$; Cage D: ns., $p = 0.164$, $t = 1.02$; Cage E: $p = 0.046$, $t = 1.82$; Cage F: $p = 0.002$, $t = 3.49$).

Table 3.   Relative activity level at 1-minute pre-stimulus of cages on days 1, 7, and 14 of conditioning. Data points are normalized so that the lowest recorded activity level at an unconditioned time-of-day across all conditioning days (Cage C, 9:00AM, Conditioning Day 10) represents the minimum (0), and all frames contain motion is the maximum (1).

Conditioning Day	Cage A	Cage B	Cage C	Cage D	Cage E	Cage F	Average All
1	-0.26198	0.17320	0.90142	-0.11601	-0.11492	-0.19553	0.06436
7	0.02996	0.74020	0.80392	-0.01797	-0.18192	-0.06481	0.21823
14	0.98257	0.65686	-0.20153	-0.11329	0.16939	1.00000	0.41567

3.2 Arc IEG Activation in Control and Experimental Groups

Results of CA1 *Arc* IEG quantification (Figure 2) reveal a trend of an average higher *Arc*:DAPI in the Experimental (Table 1) group ($\mu = 0.1273$, $\sigma = 0.043$) compared with the Control group ($\mu = 0.1130$, $\sigma = 0.0307$) (ns., $p = 0.28$, $t = 0.62$) (Supplementary Figure 3). Figure 3A and 3B show that much like relative activity levels, there is a bimodal *Arc:*DAPI relationship related roughly to the midpoint of the light cycle. Experimental group rats (those perfused at their conditioned time) have on average a higher *Arc*:DAPI than their cage-matched controls until 1:30PM, where the average *Arc*:DAPI observed is lower than Control group rats. Differences between Control and Experimental groups were stronger in the Morning condition (Figure 3A), where the Experimental rats also had the highest average CA1 *Arc* activation.
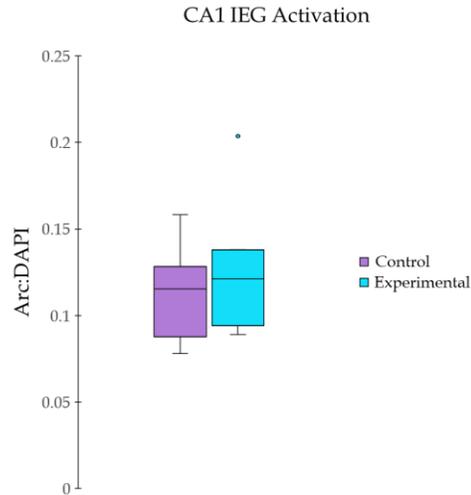
Figure 2. Stereologic *Arc* IEG and DAPI quantification results, showing estimates of *Arc*:DAPI in CA1 of both Experimental (perfused at conditioned time, n = 6) and matched Control (perfused at an unconditioned time, n = 6) groups.

A) **B)**
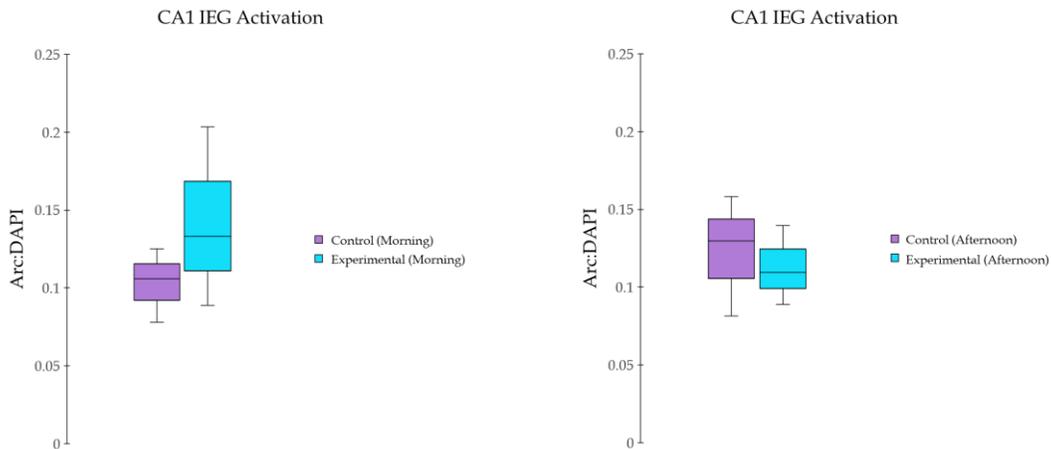
Figure 3. Stereologic *Arc* IEG and DAPI quantification results, showing estimates of *Arc*:DAPI in CA1 of both Control (perfused at an unconditioned time, n = 6) and Experimental (perfused at conditioned time, n = 6) groups, comparing (A) only subjects who were perfused at all timepoints before midday (1:30PM), ie. Morning (ns., $p = 0.21$, $t = 1.007$), and (B) only subjects who were perfused after midday, ie. Afternoon (ns., $p = 0.32$, $t = 0.55$).

Arc IEG expression in CA1 of individual rats is presented in Table 4, showing that Control (Table 1) group rats perfused in the morning at unconditioned signal times have on average lower levels of *Arc* ($\mu = 0.10302$, $\sigma = 0.02362$) than Experimental group rats, perfused at their conditioned time-of-day ($\mu = 0.14189$, $\sigma = 0.05778$), though the difference between groups was not significant ($p = 0.21$). The Experimental rat in Cage A was the only animal to show a lower *Arc* expression than its cage-matched control, a rat from Cage D perfused at 9:00AM (Table 4). Afternoon Control group rats, who were all conditioned to signals in the morning, showed on average higher *Arc* expression ($\mu = 0.12301$, $\sigma = 0.03876$) than Afternoon-conditioned (Experimental) rats ($\mu = 0.11265$, $\sigma = 0.02542$), although again the difference between these groups was not significant ($p = 0.32$).

Table 4. CA1 *Arc*:DAPI of Control and Experimental group rats.

Perfusion Time	Control Arc:DAPI		Experimental Arc:DAPI	
9:00AM	0.12500	*Morning*	0.08902	*Morning*
10:30AM	0.10600	μ=0.10302	0.20357	μ=0.14189
12:00PM	0.07804	σ=0.02362	0.13309	σ=0.05778
1:30PM	0.12950	*Afternoon*	0.13947	*Afternoon*
3:00PM	0.15810	μ=0.12301	0.10958	μ=0.11265
4:30PM	0.08141	σ=0.03876	0.08891	σ=0.02542

3.3 Arc IEG Expression Correlated to Cage Activity Levels

Our final sets of analyses combine locomotor analysis and *Arc* IEG expression results, and utilize Pearson R correlation plots in order to determine if relative activity within each cage at one minute pre-stimulus correlates with CA1 *Arc*:DAPI. Figure 4 reveals an opposite correlation between both measures for Experimental and Control group rats (ns., $p = 0.17$, $t = 0.98$), with a

positive correlative trend for Control rats and a negative correlative trend for Experimental rats. Paired t-test results showed no significant effect of activity on *Arc*:DAPI when comparing Experimental ($p = 0.13$, $t = 1.27$) nor Control groups ($p = 0.10$, $t = 1.48$).
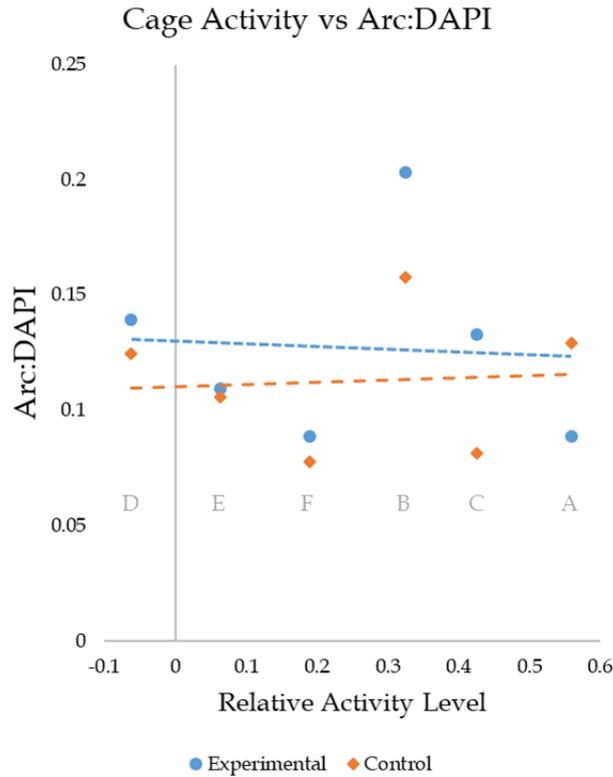
Figure 4.   Scatterplot revealing correlations between CA1 *Arc*:DAPI and relative cage activity level in both Experimental (perfused at conditioned time, n = 6) and same-cage Control (perfused at unconditioned time, n = 6) animals. Experimental animals showed a small negative correlation (r = -0.06596) between CA1 *Arc* IEG expression and relative cage activity level, while Controls showed a small positive correlation (r = 0.07241).

We observed a strong negative correlation between relative activity levels and CA1 *Arc* IEG expression in both Morning-conditioned (Figure 5A) and Afternoon-conditioned (Figure 5B)

Experimental animals. Paired t-tests revealed no significant difference between same-cage Control and Experimental animal *Arc*:DAPI for Morning ($p = 0.295$, $t = 0.64$) circumstances (Figure 5A), however there was a significant difference between same-cage Control and Experimental animal *Arc*:DAPI for Afternoon ($p = 0.0475$, $t = 3.0085$) conditions (Figure 5B).

A)

Morning Cage Activity vs Arc:DAPI

B)

Afternoon Cage Activity vs Arc:DAPI
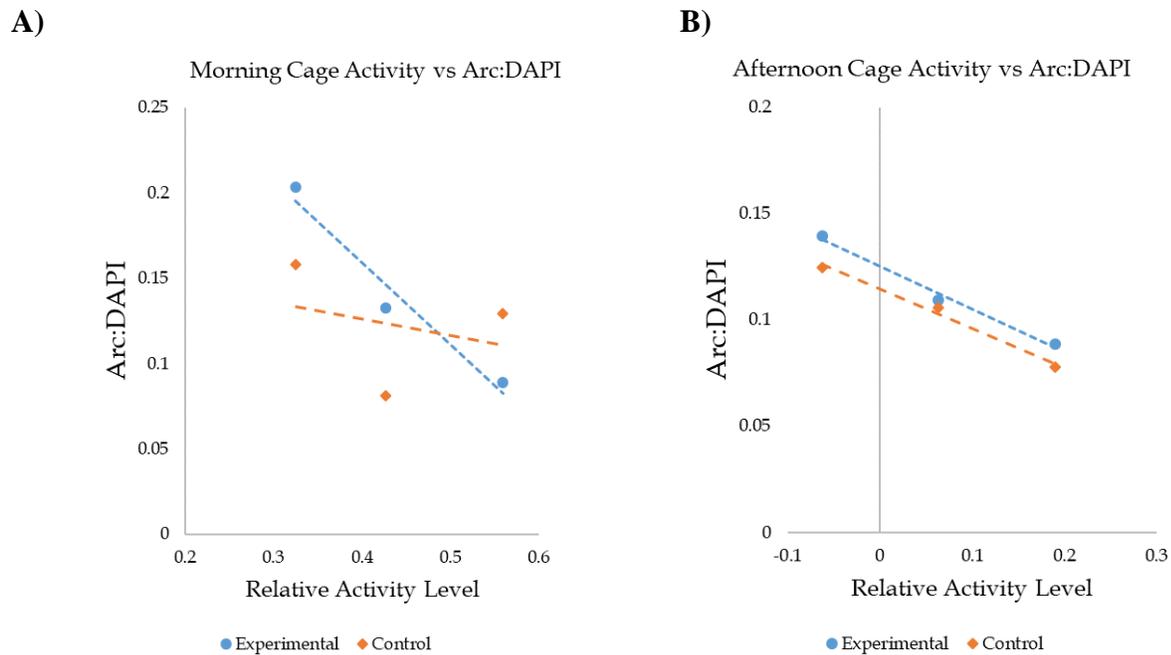
Figure 5. Scatterplots showing correlations between *Arc*:DAPI and relative cage activity level for both animals in (A) Morning signal-conditioned cages (A, B, C; n = 6) and (B) Afternoon signal-conditioned cages (D, E, F; n = 6). Morning Control animals (Figure 5A; n = 3) showed a slight negative correlation (r = -0.2953), while Morning Experimental animals (n = 3) showed a strong negative correlation (r = -0.97793) between CA1 IEG expression and relative cage activity level. Afternoon Control animals (Figure 5B; n = 3) showed a strong negative correlation (r = -0.994), and Afternoon Experimental animals (n = 3), both showed a strong negative correlation between CA1 *Arc* IEG expression and relative cage activity level (r = -0.99448).

4. Discussion

4.1 Behavioural conditioning analysis

We have shown that rat activity was conditioned to time-of-day cues as hypothesized, through analysis of locomotor behaviour (Figure 1A) one minute prior to onset of the UCS. Relative activity levels of all rats, and the effects of conditioning, were also largely impacted by circadian sleep-wake activity. Rats conditioned earlier in the light phase of their 12:12 light-dark cycle showed increased activity in response to their CS, while rats conditioned later showed a decreased activity in response to their conditioning time, compared with the average activity of all other rats at those times. These opposing trends were reflected in *Arc* IEG expression, whereby *Arc* levels were higher than controls in Morning-conditioned rats, and lower than controls in Afternoon-conditioned rats. Contrary to our hypothesis that *Arc* mediates LTM for temporal events through activation in CA1 neurons, we found that *Arc* levels were lower in Afternoon-conditioned rats perfused in both the morning (Control) and the afternoon (Experimental), compared with Morning-conditioned Experimental and Control rats.

Activity in the Conditioned group, representing all rats which were perfused at their conditioned signal time, was higher on average than those of the Unconditioned group (Figure 1A), represented by the cage-matched cross controls (Table 1), perfused at a non-conditioned time-of-day. However, this effect was not significant. Of note, there was a greater variability for the average relative activity of rats at one minute prior to their conditioned time than when looking at Unconditioned rats. We believe this finding supports our hypothesis that time-of-day conditioning impacted locomotor activity in anticipation for the temporal stimulus, but surprisingly, in a bi-directional pattern.

Effects of time-of-day conditioning on relative activity levels become more pronounced when we examine cages throughout the day. Figure 1B displays the bi-directional Conditioned activity more clearly, showing that animals conditioned to signals at 9AM, 10:30AM, and 12:00PM, referred to here as Morning-conditioned, had greater relative activity levels compared to the average of all other (Unconditioned) cages for each of those timepoints. We see that for all respective afternoon timepoints (1:30PM, 3:00PM and 4:30PM) – Afternoon-conditioned - relative cage activity levels were significantly lower than the average for all other cages at those times. Our initial prediction would have expected a similar relationship across all timepoints, in that all animals would show the same directional activity response in anticipation of their respective conditioning stimulus timepoint. However, we instead see that the relative activity levels of the Conditioned cages reverses after 1:30PM, the midpoint of the 12:12 light-dark cycle, from a higher level of activity, to a lower level of activity compared with the Unconditioned cages. These data are summarized in Table 2.

Using a Pavlovian conditioning model to assess our findings, our UCS (alarm conditioning signal) was consistently paired with a NS (time-of-day) to form a new CS which produced a CR (activity at a time-of-day). In our experiment, rats were given conditioning signals at various times-of-day relative to their light-dark cycle, meaning that signals interacted uniquely with their circadian rhythms (Pittendrigh, 1960). Rats are nocturnal, and in a 12:12 light-dark cycle, begin to decrease in activity and increase both in SWS and REM sleep shortly after the light phase begins. Light modulates these circadian sleep rhythms, and research which tracked circadian sleep activity in rats showed that REM cycles increased with duration in the light phase (Szalontai et al., 2021). While all cages in our experiment showed the same high to low direction of activity across the light phase, Morning-conditioned cages had more pronounced increased relative activity at one

minute pre-stimulus, and Afternoon-conditioned cages showed an exaggerated decrease at one minute pre-stimulus (Figure 1B). Given that all cages received an identical stimulus, only differing with delivery across the light phase, we can infer that circadian-specific physiological states resulted in an opposing CR to time-of-day CS.

In the case of Morning-conditioned cages, we might attribute this to REM sleep rebound, whereby interrupted sleep earlier in the light phase is followed by increased REM recovery sleep (Ambrosini et al., 1992). Afternoon-conditioned cages received signals at a time when we would expect increased REM cycles, and theoretically may not experience as much REM rebound sleep before the end of the light phase. Inhibition of REM sleep in the hours following a temporally-significant event would interfere drastically with *de novo* protein synthesis by interruption of the CREB-dependent PKA signalling pathway, a requirement for consolidation of LTM (Bolsius et al., 2021). Our findings here align with research showing that circadian disruption to memory is time-of-day specific, as levels of melatonin, a sleep-related hormone, as well as synaptic plasticity are impacted by circadian cycles (Gerstner & Yin, 2010). Further, exploration of CA1 activity in rats throughout the light-dark cycle show a difference across the light-phase, from lower to higher excitatory post-synaptic potential (EPSP) (Barnes et al., 1977). These circadian-conditioning interactions might explain the oppositional effect observed in temporally-conditioned locomotor activity.

Cage A shows the highest relative activity level at conditioning time (Table 2) compared to all other cages, and while it may be that we should expect higher levels of activity earlier in the light phase when rats are less likely to be in deep NREM/SWS and REM stages of sleep, activity was more than doubled in the Cage A compared to all Unconditioned cages at 9:00AM. Further, Cage A showed a marked increase in activity (Table 3) at one minute pre-stimulus across

conditioning days (S. Figure 2). If we can interpret the increased activity as an indication that REM sleep was unlikely at conditioning time for Cage A, then it may be that behavioural conditioning was stronger due to its emergence in a more wakeful state. This would leave Cage A rats with potentially the most uninterrupted REM sleep phases, likely to be occurring later in the light cycle (Giuditta, 2014; Xia & Storm, 2021).

While we have observed that all cages showed an activity-related conditioned response to their time-of-day CS, not all cages showed the same magnitude or direction of response (Table 3, Supplementary Figure 2). Like in Cage A, a greater relative activity level at Conditioned time was observed in Cages B and C, however the direction of within-cage activity decreased across conditioning days, significantly so in Cage C ($p = 0.0011$). These cages were the only two to show an average decrease in activity in anticipation of the time-of-day stimulus across conditioning days (S. Figure 2). Boyce et al. showed that REM sleep quantity increased following learning episodes (2017). In our interpretation of cage inactivity as a likelihood of REM sleep, it is possible that across conditioning days Cages B and C rats experienced increased NREM/SWS-REM sleep sequences as a result of learning (Ambrosini et al., 1992).

The lowest activity at one minute pre-stimulus was observed in Cage D, whose conditioning time lies at the exact midpoint of the light-dark cycle. This would indicate a higher likelihood of deeper, REM sleep and conditioning-signal interference, and given that Cage D showed the least change in activity across conditioning days (S. Figure 2, Table 3), perhaps delivery of a stimulus during REM sleep interfered with memory consolidation processes (Xia & Storm, 2021). All cages exhibited reduced activity in the afternoon compared with morning, with Cage D showing the least difference about the midpoint (Table 1). However, reversing the activity effect seen in Morning-conditioned cages, the cages conditioned after midday showed a lower

relative activity on average at their respective conditioning times compared to all other cages on average at those times (Figure 1B, Table 1). Cages E and F also showed a significant increase in 1-minute pre-stimulus activity across conditioning days (Table 3). Again, the effects of conditioning seem evident in Afternoon-conditioned cages, however the direction of response is reversed after midday such that the same CS results in opposing CRs depending on the point of delivery during the light phase and correspondingly, time-specific activity levels.

4.2 CA1 IEG Activation

As discussed, rats begin to rest during the light phase, and similar to humans, enter increasingly deeper stages of sleep as rest continues, characterized by more frequent REM sleep cycles and theta wave activity in the hippocampus (Boyce et al., 2016). The pattern we observed of increased CA1 *Arc*:DAPI ratio in rats conditioned to a time-of-day early in their light phase, may result from receiving conditioning at a time when NREM sleep is more common (Figure 3A). The reversed effect observed on average in rats conditioned to times-of-day later in their light phase, may be a result of REM sleep interfering with hippocampal memory consolidation, when the hippocampus is in a more inactive state (Figure 3B) (Boyce et al., 2016; Buszaki, 2002). A study in mice found that circadian-controlled CA1 oscillations resulted in peak cAMP-MAPK signalling pathway activity at 12:00PM, and that memory persistence could be impaired if those oscillations were impeded even after LTM consolidation processes were underway (Eckel-Mahan et al., 2008). If *de novo* protein synthesis supports LTM consolidation, and initial gene expression cascade products are recruited in later REM sleep, we would expect the observed stronger *Arc* mRNA expression in Morning-conditioned Experimental group rats (Luo et al., 2013).

The reversal of Morning-conditioned *Arc*:DAPI patterns in Afternoon-conditioned Control

and Experimental rats (Figure 3B) reflects the reversal observed in relative activity levels (Figure 1B). Afternoon Control rats are Morning-conditioned, and show higher *Arc* expression than Afternoon-conditioned Experimental rats (Figure 3B). Given that these Afternoon Controls would normally experience uninterrupted sleep in the afternoon when REM sleep is more likely in rats, and that REM sleep supports LTP, we could suppose that increased *Arc* here is an indication of LTP processes taking place during sleep (Giuditta, 2014). However, our experiment also introduced a novel factor to those Afternoon control rats, whose cage-pairs were removed from the cage at their morning conditioning times on the perfusion day. Perhaps the strong novel event and potential stress of those circumstances, resulted in sleep disturbance in the Morning-conditioned, Afternoon-control rats. Suzuki et al. (2020) found that *Arc* expression was increased following a sleep deprivation episode in mice, and *Arc* expression is also markedly increased following stressful events in rodents, which may explain our finding here (Bolsius et al., 2021).

4.3 Arc:DAPI as a Measure for Conditioned Response

The ultimate goal of this study was to determine if temporally-conditioned behaviour is associated with expression of the *Arc* IEG in CA1 of the hippocampus. Results of a Pearson R correlation scatterplot (Figure 4) for all relative cage activity and *Arc*:DAPI, indicate differences in the direction of correlation in same-cage Control and Experimental groups of animals (ns., $p = 0.17$). As relative activity increased, *Arc*:DAPI increased slightly in Control rats, and decreased slightly in Experimental rats. This is at odds with one of our hypotheses, where we would have expected a positive correlation in the Experimental animals indicating that *Arc* levels were associated with conditioning strength. However, as we have seen, activity levels and *Arc* expression were oppositely impacted by conditioning in the Morning vs. the Afternoon.

As Afternoon-conditioned cages showed lower relative activity levels in anticipation of their conditioned time-of-day (Figure 1B), we might expect that *Arc*:DAPI should be increased with decreasing activity levels, if the behaviour is associated with *Arc* expression, and this is observed in Figure 5B. However, the Afternoon-conditioned Controls show the same relationship, and were not perfused at their conditioned time-of-day. These Afternoon Controls may show increased *Arc* as a result of increased REM sleep episodes, which are essential for consolidation of a memory, though against our prediction that *Arc* would temporally signify the conditioned activity response (Ambrosini et al., 1992). We see that Cages E and F, conditioned late in the light cycle, showed a very small *Arc*:DAPI (Figure 4). Cages conditioned later would have less time to undergo REM-NREM sleep cycles before the end of the light cycle (7:30PM), and as suggested by Langella and colleagues (1992), this reduced sleep cycle quantity may have impacted the consolidation of memory at the genomic level.

Memory consolidation in sleep is a studied topic - many studies report increased performance in tasks following a sleep period (Ruch & Henke, 2020). However, information acquired during sleep is often lost. Although attention to sensory information is not lost in sleep, it might be that memory consolidation processes occurring during sleep prevent information acquired during sleep to be integrated to LTM. According to Ruch & Henke (2020), learning during sleep may only cause implicit changes to behaviour, such as activity levels seen in our experiment. They showed that explicit memory, such as hippocampus-dependent episodic memory, is not responsible for such sleep-learned behavioural outcomes. Given that *Arc* expression is associated with LTP in CA1 neurons for episodic spatio-temporal tasks, and that our results show negative correlations between *Arc* and activity levels based on time-of-day, it may be the reason that rats are wakeful during learning in order for *Arc* to impact explicit memory for the

temporally-significant event. Further, it may be that memory for a time-of-day cue recall (episodic-like memory) and for reconsolidation through LTP are distinct neurophysiological processes. In a study of circadian rhythms in memory, mice showed a greater recall in the light phase regardless of contextual fear conditioning during the light or dark phase (Chaudhury & Colwell, 2002). Therefore while *Arc* is associated with LTP, this IEG may not serve as a temporally-specific marker for the process of recalling the conditioned time-of-day memory.

The results of this work merit future investigation of the impact of hippocampal CA1 *Arc* gene expression on LTM for temporally-significant events. Investigation of IEG expression in other trisynaptic circuit structures to determine if levels of expression reflect the bidirectional pattern seen here might be of particular interest to future researchers (Rudy, 2008). Further exploration of circadian effects may also be included in future experiments, such as active (dark) cycle conditioning periods to determine if *Arc* expression is conditioned to a time stimulus more readily when delivered during wakeful behaviour. The inclusion of cage controls who received no conditioning at all would be useful in determining baseline *Arc* expression throughout the circadian cycle of the rat. The advantage of space for individually-roomed cages would prevent any bleed over of conditioning stimuli between same room cages (Supplementary Figure 1), which may have reduced the saliency of the time-of-day CS. Likewise, an increase in number of subjects might have affirmed the consistency of the trends we observed. Inclusion of female rats to determine if the difference of sex results in similar or varied responses to circadian-related conditioning would contribute to the research indicating sex differences play a role in episodic memory (Dib et al., 2021). Advances in behavioural monitoring technology allow for the tracking of individual animals, however this experiment did not utilize these techniques. Investigation of the individual activity of each rat would have allowed us to further separate the impact of *Arc* expression on

anticipatory behaviour as a measure of temporally-conditioned memory in Experimental and Control group rats.

Expanding this work with human subjects may offer another useful method for looking at time-associated LTM, using functional magnetic resonance imaging (fMRI) in temporal-conditioning experimental designs. This approach would allow for frequent monitoring of hippocampal activity, so that the impact of conditioning could be measured continuously across weeks, months, and potentially years. In humans, the strength of circadian rhythm entrainment and LTM weakens with age, and it would be particularly interesting to determine whether CA1 activity and the impact of temporal conditioning mirrors this paralleled decrease (Kondratova & Kondratov, 2012). Research investigating temporal-conditioning in phase-shifted circadian rhythms in humans, such as shift workers, or those with sleep disorders like insomnia, might reveal correlations with hippocampal activation when using fMRI clinical studies of temporal-conditioning in humans (Potter et al., 2016).

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6. Appendices

S. Table 1.   Perfusion times of Experimental and Control rats.

Time of Day	Rat Perfused (Experimental Group)	Rat Perfused (Control Group)	Perfusion Time (minutes:seconds) post- Time-of-Day
9:00 AM	Cage A_x	Cage D_c	12:32
10:30 AM	Cage B_x	Cage E_c	12:20
12:00 PM	Cage C_x	Cage F_c	10:05
1:30 PM	Cage D_x	Cage A_c	9:25
3:00 PM	Cage E_x	Cage B_c	8:55
4:30 PM	Cage F_x	Cage C_c	9:55

S. Figure 1.   Comparison of relative cage activity in conditioned, same room, and different room cages at each conditioning time.
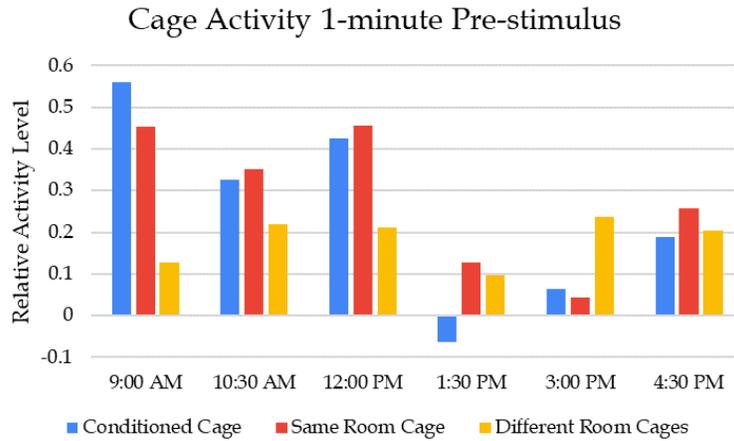
S. Figure 2.   Activity trends across conditioning days for each cage.

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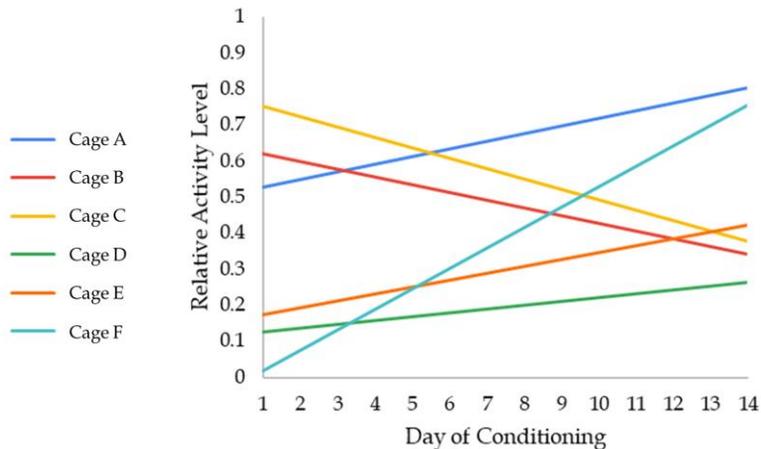
S. Figure 3.   *Arc* and DAPI-stained CA1 cells as imaged by Fluoview FV10-ASW software on an Olympus FV1000 confocal laser scanning microscope at 60x oil objective.