The temporal effects of Arc on the alternation behavior and the object recognition memory

Thesis submitted during June 2020 to the University of Hyderabad in partial fulfillment of the award of a Ph.D. degree in COGNITIVE SCIENCE

By

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DECLARATION

I, **Vishnu Shandilya M.C.**, hereby declare that this thesis entitled "**The temporal effects of Arc on the alternation behavior and the object recognition memory**" submitted by me under the guidance and supervision of **Dr. Akash Gautam** is a bonafide research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

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CERTIFICATE

This is to certify that the thesis entitled "**The temporal effects of Arc on the alternation behavior and the object recognition memory**" submitted by Mr. **Vishnu Shandilya M. C.**, bearing registration number **15CCHL05**, in partial fulfillment of the requirements for the award of **Doctor of Philosophy in Cognitive Science** is a bonafide work carried out by him under my supervision and guidance.

The thesis has not been submitted previously in part or in full to this or any other University or Institution for the award of any degree or diploma.

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This work is dedicated to my beloved parents for their support, encouragement and patience

Acknowledgements

"In order to be a perfect member of a flock of sheep, one has to be, foremost, a sheep" ~ Albert Einstein

I thank my mentor, Dr. Akash Gautam, for his patience and support through out my Ph.D. duration, and providing me an opportunity to work in his Molecular Neurobiology lab. I also thank my doctoral committee members, Dr. Joby Joseph and Dr. Sudipta Saraswati, for their assistance and valuable suggestions for my research work. I would like to thank Dr. Arvind from CCMB for providing support in stereotaxic experiments. I would also like to thank all my friends at the CNCS, in the School of Medical- and Life-Sciences for their inputs.

INDEX

ABBREVIATIONS

AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid Receptor
ANOVA	Analysis of Variance
AO	Alternating Object
AP50	Adaptor protein
Arc	Activity Regulated Cytoskeleton-associated protein
Arg	Activity regulated gene
ART	Aligned rank transformation
ASN	Antisense Oligodeoxynucleotide
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
bw	Body weight
CaMKIIβ	Ca2+/calmodulin-dependent protein kinase II β
catFISH	Cellular compartment analysis of temporal activity by fluorescence in situ hybridization
cDNA	Complementary DNA
cm	Centimeter
Cq	Quantification cycle
CRE	cAMP-response element
dCq	delta of two Cq values
ECL	Enhanced chemiluminescence
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Glu	Glutamate receptor
GPCR	G-protein-coupled receptor
HRP	Horse radish peroxidase
ICI	Inter-choice interval
IEG	Immediate Early Genes
IgG	Immunoglobulin G
ISI	Inter-session interval
kb	Kilo base
kD	Kilo Dalton
kg	Kilo gram
KO	Knock out
LTD	Long Term Depression
LTM	Long Term Memory
LTP	Long Term Potentiation
m	Meter
MEF2	Myocyte enhancer factor-2
mg	Milli gram
min	Minute
ml	Milli liter
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NMDAR	N-methyl-D-aspartate Receptor

NORM	Novel object recognition memory
ODN	Oligodeoxynucleotide
OF	Open-field
ORM	Object recognition memory
PAGE	Polyacrylamide gel electrophoresis
PMSF	Phenylmethyl-sulfonyl fluoride
PVDF	Polyvinyl difluoride
RFA	Rewarded forced alternation
RO	Recurring Object
RT-qPCR	Reverse transcriptase – quantitative Polymerase chain reaction
s	Second
SA	Saline
SAB	Spontaneous alternation behavior
SC	Scopolamine hydrochloride
SDS	Sodium dodecyl sulfate
SEM	Standard error of mean
SOP	Sometimes Opponent Processes model
SRE	serum response element
SRN	Scrambled Oligodeoxynucleotide
TBS	Tris-buffered saline
TET	Total exploration time
TO	Novel object
TOM	Temporal order memory
µg	Micro grams
µl	Micro liter
ZLRE	Zeste-like response element

Figures

Chapter 1

INTRODUCTION

Learning and memory

Learning and memory is one of the important functions of the brain, and one of the unsolved problem in neuroscience is how do the learning and memory work. Learning is the process of acquiring new information. It can be categorized into the associative and non-associative process. Associative learning can be divided into classical conditioning, in which a conditioned stimulus is paired with unconditioned one, and instrumental conditioning, in which we associate our behavior with the reinforcing event. Non-associative learning can be either habituation – where the repeated occurrence of a stimulus leads to a decreased response, or sensitization – where the repeated occurrence of a stimulus leads to a stronger response (Vanderah and Gould, 2015).

On the other hand, memory is the process of recollecting the learned information. It can be categorized into short-term and long-term memories. Working memory is a type of short-term memory that requires maintenance of information. Long-term memory is further categorized into explicit memory (consisting of episodic and semantic memories) and implicit memory (consisting of skills and habits). Each type of memory is associated with different brain regions. Working memory, skills and habits are associated with Neocortex; Episodic and semantic memories (events occurred during particular time and location) with medial temporal lobe, and medial diencephalon; Skills and habits (ability to learn and perform a task) with Basal ganglia and Cerebellum; Emotional memory with Amygdala (Vanderah and Gould, 2015).

Some of the commonly used laboratory methods to study memory in animal models include brain lesions, pharmacological studies, genetic manipulations of memory-related genes, functional imaging, and cell recordings from different brain areas after or during behavioral tasks. For example, memory impairment in animals can be generated by NMDA-induced lesions via stereotaxic surgery (Toscano et al., 2005; Pothuizen et al., 2004); deficits in long-term memory formation occur in scopolamine-treated or Arc KO mice (Plath, 2006; Klinkenberg and Blokland, 2010); long-term potentiation can be observed at glutamatergic synapses through the recordings from CA1 region (Derkach et al., 1999); scopolamine produces a dose-dependent reduction of functional connectivity between brain regions involved in memory during the functional imaging (Shah et al., 2015).

Memory: From behavior to molecules

Memory operations involve processes occurring at various levels, from neural networks to molecules. At the network level, widespread regions of the brain cooperate with each other and form systems that maintain or contribute to memory processes. For example, the gamma oscillations couple the hippocampal CA1 and CA3 regions during the spontaneous alterations task in rats and facilitate the retrieval of hippocampus-dependent memories

(Montgomery and Buzsáki, 2007). Some of the questions asked at this level are: how does the information is encoded and decoded?, how and where is it represented, and how is it transformed from input to output? Some of the basic properties of a complex network include excitation/inhibition, feed-forward/backward, and convergence/divergence or parallel processing (Byrne et al., 2014).

Neural circuits are surprisingly plastic, as they are constantly adjusted throughout the lifetime at the synaptic level. These adjustments can be either short-term or long-term changes. Short-term changes include changes in receptor sensitivity, the kinetics of vesicle, and some forms of the signaling process. Long-term changes include the addition or deletion of receptors, enzymatic modifications of cellular components, and some forms of retrograde signaling. These synaptic changes can also lead to structural plasticity.

At the molecular level, the neural activity is determined by the movement of ions and molecules across the neural membrane. This active and passive movement of most of the ions is regulated by various membrane transport proteins, which in turn can be affected by synaptic plasticity genes (Hammond, 2014).

Synaptic plasticity genes

Genes whose expression regulates the synaptic plasticity are referred to as synaptic plasticity genes. These genes are functionally categorized into different groups, like Immediate-Early genes (IEGs) (e.g., ARC, BDNF, CREB1, EGR1, HOMER1), Late Response Genes (LRGs) (e.g., INHBA, SYNPO), Long Term Potentiation (LTP) genes (e.g., ADCY1, ADCY8, BDNF), Long Term Depression genes (LTD) (e.g., GNAI1, GRIA1, GRIA2), Cell Adhesion genes (e.g., ADAM10, CDH2, GRIN2A), Extracellular Matrix & Proteolytic Processing genes (e.g., ADAM10, MMP9, PLAT), CREB Cofactors (e.g., AKT1, CAMK2G, NMDAR1), Neuronal Receptor genes (e.g., EPHB2, GABRA5, GRIA1), Postsynaptic Density genes (e.g., ARC, GRIA1, HOMER1) (Atluri et al., 2013). One of the IEGs, Arc, is the candidate gene for our present study as it has a greater functional importance in learning and memory processes (Shepherd and Bear, 2011).

Immediate Early Genes (IEGs)

IEGs are a group of genes whose expression is regulated by synaptic plasticity within a short period of time. They get quickly transcribed and/or translated in response to neuronal activity (Gallo et al., 2018). IEGs encode effector proteins (e.g., Arc, Narp, and Homer) and transcription factors (e.g., MEF2, c-fos, and zif268) to control various cellular functions. They regulate growth factors, metabolic enzymes, and cytoskeletal proteins in the cell (Minatohara et al., 2016). Most of the IEGs are expressed in neurons, and these neuronal IEGs are involved in regulating the morphology and density of dendritic spines, implying their importance in learning, memory, and synaptic plasticity (Peebles et al., 2010; Lanahan and Worley, 1998). Some of the well-known examples of neural IEGs are given in the table 1.

Gene	**Function**
Arc	Memory consolidation, LTP, LTD, homeostatic plasticity
c-fos	Transcription factor, Memory
zif268	Transcription factor, Memory
Homer 1a	Synaptic plasticity
cox-2	Prostaglandin synthesis, Inflammation
Narp	Growth function
BDNF	Growth factor

Table 1: Some of the IEGs and their function

Activity-regulated cytoskeletal-associated protein (Arc)

Arc (Activity-regulated cytoskeletal-associated protein) or Arg3.1 is one of the neuronal IEGs that are expressed transiently and temporally after the synaptic activity. It was discovered through differential cloning method during the induction of seizures in the hippocampus of the rat brain (Link et al., 1995; Lyford et al., 1995). It is an important component in the generation of all the known forms of synaptic plasticity viz. long-term potentiation (LTP), long-term depression (LTD), and homeostatic plasticity. Homologs of human Arc have been found in mammals (eg., mice, rats), birds (chicken), and reptiles (turtle), but not in fish or invertebrates (Byrne, 2017). Compared to a human, the Arc gene has 92.9%. 92.7%, 70.6%, and 26.5% sequence similarity with that of mouse, rat, chick, and fruit fly, respectively. Arc sequence is similar to Gag protein in retroviruses (such as HIV), which form capsids around the viral material that is transferred between the cells during infections, which suggests that the evolutionary origin of Arc could be these viral proteins (Pastuzyn et al., 2018).

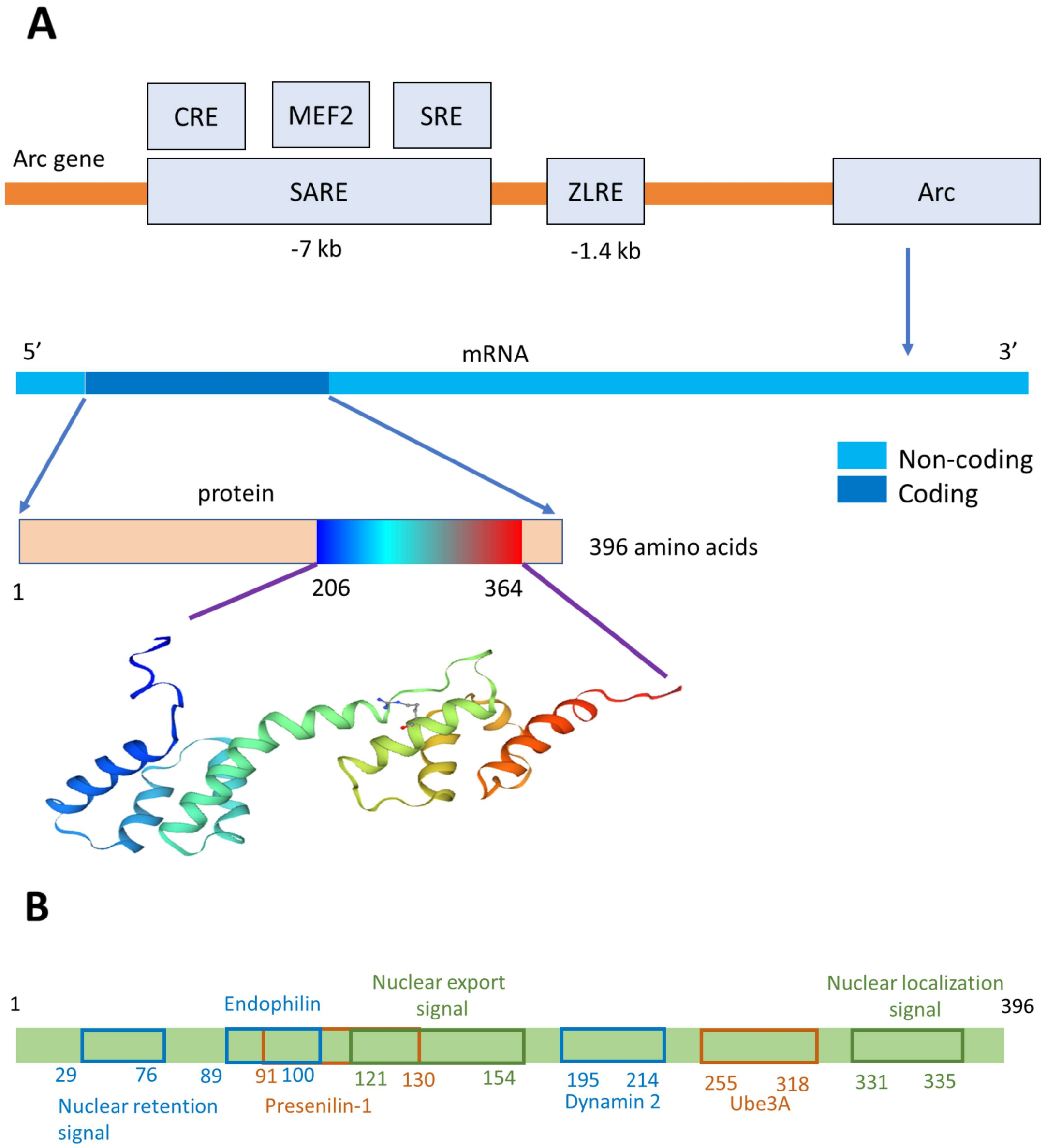

Figure 1. (A) Transcription factors regulating Arc expression, and the partial structure of human ARC protein (Bienert et al., 2016). (B) Schematic representation of human Arc and functionally relevant regions (Myrum et al., 2015).

Regulation of Arc mRNA

Arc gene is located in chromosome 8 in humans (location: 8q24.3) and in chromosome 15 (location: 34.25 cM) in the mouse. Human mRNA has one variant with 2950 base pairs length, and mouse mRNA has two variants with 3059 and 3056 base pairs long. The difference in mouse arises due to the addition of three nucleotides *CAG* from 2007th to 2009th position, which is outside the protein-coding region. Both variants have three exons and two introns and encode a same protein of 396 amino acids length. Arc is an effector molecule that is regulated by several downstream signaling pathways, including PKA, PKC, and ERK (Korb and Finkbeiner, 2011). The Arc transcription is regulated by various transcription factors, including CRE (cAMP-response element), MEF2 (myocyte enhancer factor-2), SRE (serum response element), and ZLRE (zeste-like response element) (Epstein and Finkbeiner, 2018) (Fig. 1A).

Memory storage is believed to incorporate changes in the synaptic characteristics, which are marked by neuronal activity and further facilitated by gene expression and mRNA translation (Pfeiffer and Huber, 2006). In the study by Guzowski et al., 1999, Arc mRNA expression increased steadily two min after electroconvulsive shock (100 Hz) treatment in hippocampus, peaked at 30 min, and reached a basal level at 60 min (Guzowski et al., 1999). During behavioral experiments in rats, it has been observed that the transcription of Arc is quickly induced and this transcribed mRNA enters cytoplasm within a short period of time (~5 to 15 min) (Guzowski et al., 2000; Vazdarjanova et al., 2002). Some of these cytoplasmic mRNA binds to kinesin motor complex, an active transporter, and migrates to dendrites at the speed of around 65 μm/min (Dynes and Steward, 2007; Kanai et al., 2004). Interestingly, these transporter proteins are also controlled by the processes that are dependent on α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPAR) and N-methyl-D-aspartate Receptor (NMDAR) activity (Raju, et al., 2011). The 3'UTR in Arc mediates the transport of Arc mRNA as well as its decay by binding to microRNAs.

The localization of this migrated mRNA is presumably selective and more studies are needed to elucidate whether the destination of mRNA is near the synapses that have recently experienced an activity, or near all synapses indiscriminately (Steward et al., 2015; Farris et al., 2014; Steward and Worley, 2001). However, it is interesting to note that this selectivity of localization is temporally modulated (Steward et al., 2015). It implies that all the functional effects of Arc mRNA on the plasticity of a particular synapse are dependent on both how recent was the original activity that triggered Arc transcription and whether that synapse was activated before.

Regulation of Arc protein

As the full-length Arc has not been crystallized yet, we have knowledge only about the partial structure of Arc protein (Bienert et al., 2016), as shown in figure 1A. Arc protein is translated from the first exon of Arc mRNA (199 to 1386 bp) and consists of 396 amino acids length with about 55 kD of molecular weight. Arc translation is inhibited by some of the microRNAs, including, miR-34a, miR-19a, and miR-326 in the rat hippocampal primary neurons in culture (Wibrand, et al., 2012). It is suggested that one of the reasons of Arc's ability to have diverse interactions is that it is a flexible protein with oppositely charged domains (C and N) that interact with each other, which results in biphasic (open and closed) conformational states, and its ability to undergo reversible self-oligomerization (Myrum, et al., 2015). Some of the known Arc protein-interacting regions are shown in figure 1B.

Memory consolidation can be categorized into the system– and synaptic–consolidation. System consolidation occurs over a period of weeks to months, whereas synaptic consolidation occurs within hours (Dudai, 2004). Alterations in the synaptic strength upon the glutamatergic signals that induce synaptic consolidation generally require new transcription and protein synthesis (Panja and Bramham, 2014; Bramham and Messaoudi, 2005). Arc is one of the proteins essential for glutamate-induced synaptic consolidation (Shepherd and Bear, 2011). In fact, the synaptic consolidation is occluded when Arc protein synthesis is inhibited by Arc-antisense oligodeoxynucleotides (ODNs) (Guzowski et al., 2000). Also, unlike many proteins, Arc translation is controlled by various signaling cascades that depend on the current activity level of receptors such as NMDAR and G-protein-coupled receptor (GPCR) (Korb and Finkbeiner, 2011). In principle, this creates a temporal association between the ongoing activity and Arc functionality. Arc has the ability to interplay with a diverse set of proteins at nuclear and dendritic domains to facilitate different forms of synaptic plasticity (Nikolaienko et al., 2018).

Role of Arc in synaptic plasticity

Neuroplasticity is the ability of the brain to constantly adapt to the environment. It can be categorized into structural and synaptic plasticity. Structural plasticity refers to a modification of physical structures of cellular components after learning, and synaptic plasticity refers to change in the strength of the synapse, as observed by a change in firing patterns. Moreover, an increase in synaptic strength could also affect structural plasticity. Arc affects all the known types of plasticity (Minatohara et al., 2016). Structural plasticity requires modifications in tubulin or actin cytoskeleton. Many studies have shown that the Arc mediates the changes in the structure of both tubulin and actin cytoskeleton (Fujimoto et al., 2004; Fu and Zuo, 2011; Dillon and Goda, 2005; Lyford et al., 1995). Sustained Arc synthesis is required for the regulation of local polymerization of F-actin (Messaoudi et al., 2007). Spine density (predominantly the thinner spines) increases by Arc over-expression *in vitro* and decreases for Arc KO mice *in vivo* (Peebles et al., 2010). Additionally, it activates Notch1, a receptor that regulates spine morphology and required for structural plasticity (Alberi et al., 2011).

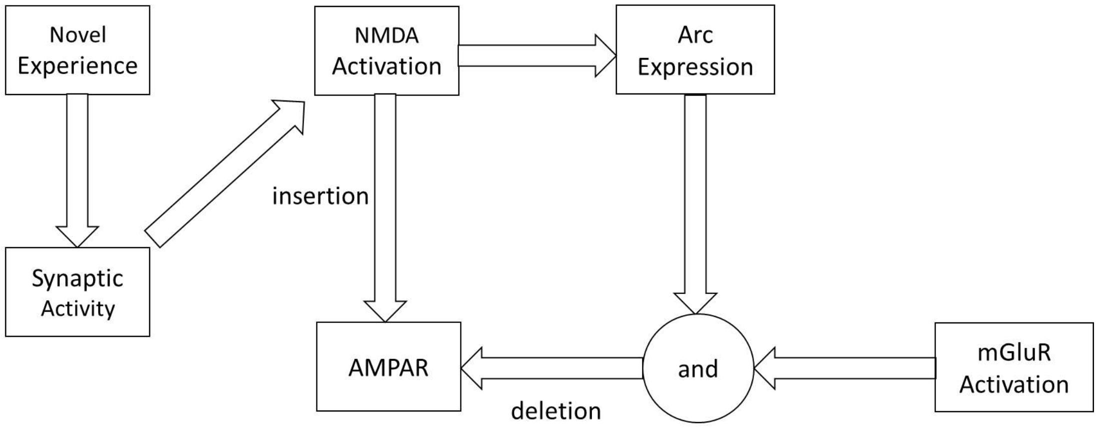

Figure 2. Schematic figure of Arc modulating surface expression of AMPA receptor. Arc expression is induced by NMDA activation upon a novel experience and synaptic activity. In the presence of mGluR activation, Arc decreases the surface AMPAR expression. In parallel, surface AMPAR expression is increased by NMDA activation.

Long-term potentiation (LTP) is a process of enhancing the strength between synapses. LTP facilitates actin polymerization and increases the number of spines (Yuste and Bonhoeffer, 2001). Arc is essential for the late-phase LTP. When Arc KO mice were subjected to high-frequency simulation, there was an increased response during the early phase, and a decreased response during the late-phase (Plath, 2006). Arc decreases early-LTP when Arc antisense ODNs are infused shortly before or after the high-frequency stimulation (Messaoudi, 2007).

On the other hand, long-term depression (LTD) is a process of decreasing the strength between synapses. Arc is essential for mGluR1s-mediated LTD in CA1 neurons (Waung et al., 2008). This LTD is achieved by Arc-mediated AMPA receptor endocytosis and thereby decreasing the synaptic efficacy (Chowdhury et al., 2006; Shepherd et al., 2006) (Fig. 2). However, this does not need new Arc transcription, but it is dependent on existing Arc mRNA previously delivered to dendrites (Waung et al., 2008). This localized translation is controlled by Ca2+/ Calmodulin-dependent eEF2K and FMRP (Park et al., 2008). The presence of SRE site on the Arc promoter is also necessary for observing LTD in cultured Purkinje neurons (Smith-Hicks et al., 2010). Arc interacts with endophilin and dynamin to facilitate AMPAR endocytosis (Verde et al., 2006).

Arc also plays a role in homeostatic scaling. Arc associates with promyelocytic leukemia (PML), a nuclear protein, in a temporal manner and this facilitates the downregulation of GluA1 mRNA and results in homeostatic scaling of AMPARs (Nikolaienko et al., 2018).

Arc and memory processes

Animal models with Arc KO or knockdown showed impairment in the consolidation of memory in various behavioral tasks, including, taste aversion, spatial learning, contextual inhibitory avoidance task, object recognition, and fear conditioning (Holloway and McIntyre, 2010; Ploski et al., 2008; Plath et al., 2006; Guzowski et al., 2000). Arc expression increases upon exposure to the novel environments, such as auditory, visual, and spatial (Ivanova et al., 2011; Daberkow et al., 2007; Vazdarjanova et al., 2002). Due to this novelty association, Arc is being studied in many learning tests like inhibitory avoidance, working memory, and long-term memory (Korb and Finkbeiner, 2011).

In cortical neurons, Arc is necessary for persistent firing in NMDA-activated synapses, and hence, it is a vital component for the retention of working memory (Ren et al., 2014). In the inhibitory avoidance (IA) task, the inhibition of Arc translation by its antisense-ODNs averts the formation of long-term memory during the behavioral tagging process (Martinez et al., 2012). Depending upon the degree of hippocampal necessity for a task, Arc can interfere with the speed of learning in rodents. Higher levels of Arc in hippocampus or striatum lead to quicker learning in spatial- and reverse-learning tasks – which are hippocampal-dependent (Daberkow et al., 2007; Guzowski et al., 2002); whereas it leads to delayed learning in a lever-pressing task – which is hippocampal independent (Kelly and Deadwyler, 2003). Arc has been associated with decreased freezing in rats after fear conditioning exercise (Meloni et al., 2019). Arc serves as a principal tool to detect the neural substrates of cognition due to its tight regulation in spatial and temporal neural processes (Sauvage et al., 2013).

In the last decade, Arc has been shown to be involved in several cognitive disorders including, fragile-X syndrome, Angelman syndrome, and Alzheimer's disease, where abnormal plasticity is one of the main causes of such disorders (Korb and Finkbeiner, 2011). In rodent models of Fragile X Syndrome, there is an abnormal Arc-mediated mGluR-LTD level (Santoro et al., 2012). In a mice model of Angelman syndrome (E3 ubiquitin ligase KO), there were higher levels of synaptic Arc expression in cultured neurons, which reduced the surface AMPAR expression and decreased the synaptic transmission (Buiting et al., 2016). In Alzheimer's disease, Amyloid β proteins contribute to the increase in Arc protein expression to abnormally high levels, where it is suggested that high levels of Arc mediated-AMPAR endocytosis could contribute to the cognitive decline (Kerrigan and Randall, 2013). The association of Arc with several disorders could also be due to Arc's ability to bind with various proteins that enable synaptic plasticity, including F-Actin (necessary for spine growth), AP50 (involved in spine shrinkage), Crebbp (involved in homeostatic scaling) and CaMKIIβ (required for inverse synaptic tagging) (Nikolaienko et al., 2018). Additionally, disruption of Arc ubiquitination and preventing its degradation has been associated with general cognitive decline and dementia in Gordon Holmes syndrome (Husain, et al., 2017).

Memory tests in rodents

Based on the type of memory to be tested, there are different methods to analyze the memory of laboratory animals. Commonly used memory tests in rodents include forced alternation, radial arm maze, novel object recognition, Y-maze spontaneous alteration, and Morris water maze.

In forced alternation test, Y- or T-maze can be used to test spatial memory of previously visited arm. Rodents are placed in the start-arm and forced to navigate only one of the side arms. After a certain interval, rodents are re-placed in start-arm, and they are allowed to choose one of the side-arm. At a shorter interval, rodents generally enter the arm that was not visited previously. We can test the short-term spatial memory at different intervals through this forced alternation test.

A radial arm maze consists of eight equal-length arms, and the rodents are initially placed at the center. By placing rewards in some of the arms during training trials, we can test both reference memory errors and working memory errors during testing trails. At the test trial, rodents entering an arm that is not associated with any reward is considered a reference memory error, and re-entering an arm after consuming a reward is considered as a working memory error.

In novel-object recognition memory, rodents are placed in an open-field along with few objects. As rodents typically prefer to explore the novel objects, we can test the object recognition memory of previously familiarized objects when presented along with a novel object after a certain inter-familiarization interval.

A higher exploration of the novel-object shows a greater memory of the familiarized object.

In Y-maze spontaneous alteration, rodents are placed in the center of the Y-maze and allowed to explore all the three arms (designated as A, B, and C) for a certain period of time. Alternations can be defined as consecutive visits in three different arms (i.e., A → B → C), divided by the number of maximum possible alternations (i.e., total arm visits minus two). Naive rodents typically tend to alternate the arms on successive opportunities. This test can be used to test spatial working memory. A higher alternation rate implies intact working memory (Wolf et al., 2016).

Morris water maze test consists of a circular pool filled with water and a hidden rescue platform. The maze is surrounded by extra maze cues to aid the rodent navigation. Rodents are placed in the maze and allowed to swim until they reach the platform. During training trials, conducted across several days, rodents learn the spatial location of the hidden platform. Escape latency, defined as the time a rodent takes to find the platform, is measured at all trails. While the animals learn the location of the platform, their escape latency decreases. During the testing trail, the platform is removed, and the rodents are tested their spatial memory retention.

Spontaneous alternation behavior

Spontaneous alternation behavior (SAB) is a phenomenon where rodents tend to alternate their choices, as assessed through Y (or T) mazes on successive opportunities (Hughes, 2004) (Fig. 3). SAB is used to test several mechanisms, including spatial working memory, habituation to novelty, and curiosity (Deacon and Rawlins, 2006; Hughes, 2004). In all the applications of SAB, the animal needs to remember the previously visited arm in order to alternate. Hence, SAB is a test for spatial memory. It is a simple test that requires no training, which is an advantage (Gerlai, 1998). Hence, it could be well suited for testing the working memory processes without any interference that otherwise arises out of training. SAB can be used for testing temporal order spatial memory, where the order of arm visits has to be remembered by rodents for successful alternations.

Two types of SAB are commonly used, two-trial SAB and continuous SAB. In two-trial SAB, a mouse is placed in the start-arm, and it chooses to enter one of the two opposite arms (sample trial). The mouse is confined in that arm for a certain period of time (intra-trial interval) before it is transferred back to its cage. After a predetermined time (inter-trial interval) mouse is re-placed in start-arm and tested which arm it enters next (test trial). An alternation is said to have occurred if the mouse enters a different arm than the sample-arm. In continuous SAB, the mouse is allowed to freely visit all the three arms for several mins (no concept of start-arm). Here, an alternation is said to have occurred if the mouse enters a different arm than the previous arm. Each of these SABs has its own advantages. Two-trial SAB is ideal for testing short-term memory because of the facilitation of inter-trial interval, whereas continuous SAB is preferred for working memory due to multiple arm visits in a single trial (Hughes, 2004).

Figure 3. Y-maze commonly used for testing spontaneous alternation behavior in rodents.

Though several brain areas are involved in SAB phenomenon, hippocampus and prefrontal cortex are of primary interest in various memory studies (Lalonde, 2002). Lesions to any of the above areas impaired SAB when the retention interval between trials or a delay in a particular arm was longer. This supports the involvement of working memory paradigm in alternation behavior (Deacon and Rawlins, 2006). Although memory factor is commonly assessed in SAB, it has to be noted that factors such as motivation, attention, sensory, and anxiety can also influence an animal's tendency to alternate (Lalonde, 2002).

Object recognition memory test

Object recognition memory (ORM) tests can be used for testing several types of information retention, including spatial ORM, temporal order memory, and novel ORM. All these types of memory tasks tests the ability of an animal to recollect previously presented information. Spatial ORM tests the location memory of previously presented object; temporal order memory tests whether the animal remembers the least recently presented object against the most recent one; and novel ORM will test whether the animal recognizes the previously presented object when presented against a novel object (Barker and Warburton, 2011; Ennaceur, 2010; Ennaceur and Delacour, 1988).

In general, animals are habituated to the testing apparatus for two to four days before the experiments start. Spatial and novel ORM tests commonly have two trials, where the objects are familiarized in the first trial, and the paradigm is tested in the second trial. Temporal order memory (TOM) test has three trials, where two different objects are presented in different trials consecutively (i.e., pair of object X in trial-1 and pair of object Y in trial-2), and both objects are presented together in trial-3 (X and Y). An animal with intact TOM will spend more time exploring object least recently presented (i.e., object X) (Hannesson et al., 2004).

The prefrontal cortex, hippocampus, and perirhinal cortex are the important brain regions that affect the ORM. All three regions interact with each other during the retrieval of memory; although hippocampus is involved to a lesser degree for the novel ORM if the animal is highly familiarized to the testing apparatus before the start of experiments (Hannesson et al., 2004, de Lima et al., 2006). Perirhinal cortex is also a crucial region due to the afferent connections from visual, olfactory, and somatosensory neurons. The prefrontal cortex plays a role in TOM but not object familiarity memory (Barker et al., 2007).

In ORM tasks, the hippocampal 5-HT7 receptor has been implicated in spatial memory (Sarkisyan and Hedlund 2009), whereas CA1 specific NMDAR subunit-1 is involved in non-spatial memory formation (Rampon et al., 2000). Additionally, exposure to the ORM testing apparatus results in an increased expression of brain-derived neurotrophic factor (BDNF) in the hippocampus, which further affects the expression of downstream molecules (Goulart et al., 2010). Pharmacologically, several drugs (including scopolamine, lidocaine, caffeine, cocaine, and methamphetamine) significantly affect ORM (Antunes and Biala, 2011).

Finally, it has to be noted that ORM tests are sensitive to an animal's age. Aged animals are generally less responsive to the novelty; for e.g., aged Sprague–Dawley rats do not discriminate novel objects against the familiar ones (Baxter, 2010). Overall, ORM tests have a wide range of applications due to its sensitivity in detecting the effects of genetic, neurological, and pharmacological processes on memory (Lueptow, 2017).

Rationale of the study

Intact spatial working memory aids in successful discrimination between familiar and novel arms in Y maze and facilitates the alternations, a measure of temporal order spatial memory, in animals (Hughes, 2004). However, alternations could also be affected by the degree of habituation to the surrounding environment (Sanderson and Bannerman, 2012). Hence, there is a possibility that alternations are affected in mice when they are subjected to the repeated exposure to an environment, and is dependent on the time interval between the exposures i.e., inter-exposure intervals. This scenario of environment re-exposure had facilitated LTD in rats in the absence of previously familiarized objects (Kemp and Manahan-Vaughan, 2004). Hence, we predict that the recognition memory of the familiarized object depends on the re-exposure interval to the environment.

Minatohara et al., 2016 has discussed an association between the Arc gene and the degree of familiarization of an environment. The expression of Arc transiently increases after the exploration of unfamiliarized or novel environments (Vazdarjanova et al., 2002). Re-exposure to an environment induces Arc in the same neural ensembles, whereas it is induced in distinct ensembles if a rodent is re-exposed to a different environment (Guzowski et al., 1999). This also causes mGluR-dependent LTD in earlier activated synapses, where Arc mRNA is necessary to prime these synapses (Jakkamsetti et al., 2013). If the environment contains objects, then after the recurrent exposures to the objects as well as their environment, Arc consolidates the memory of these objects and promotes LTP (Plath, 2006). On the other hand, if the objects are absent or present in a different location during the re-exposures of that environment, then Arc facilitates LTD (Kemp and Manahan-Vaughan, 2004). However, it is unknown how the memory of these objects is affected by the Arc level. Manago et al., 2016 has shown that the Arc is required for intact temporal-order object memory, as assessed by the temporal-order object memory task, which requires the re-exposures to the maze. However, it is unknown how the temporal-order spatial memory is regulated by Arc expression.

Hypothesis

Arc regulates both temporal order spatial memory and the object recognition memory, which in turn are dependent on inter-exposure interval during the re-exposure to an environment

Objectives

To address our hypothesis, we framed the following objectives:

1. To study the temporal changes in alternation behavior of mice after re-exposures to a novel or familiar environment
2. To find out the changes in object recognition memory of mice at different inter-exposure intervals after re-exposures to an environment
3. To analyze the role of Arc in alternation behavior during re-exposures to a novel environment
4. To study the involvement of Arc in object recognition memory during re-exposures to a novel environment

Chapter 2

MATERIALS AND METHODS

Chemicals

PCR Master Mix (2X) kit - *DreamTaq* (catalog# K1081) was purchased from Thermo Fisher Scientific, India. Tri-reagent (catalog# 93289), polyvinyl difluoride membrane (PVDF) (catalog# 3010040001) and Scopolamine hydrochloride (catalog# S1013) were purchased from Sigma Aldrich (Merck), Germany. 1st strand cDNA synthesis kit - *PrimeScript* (catalog#6110A) was purchased from Takara Bio Inc., India. Gene-specific primers for PCR and oligodeoxynucleotides (Arc-antisense and Scrambled) were manufactured by Cassia Siamia Tech. India. *HI-SYBR* Green Master Mix (catalog# MBT074) for Quantitative-PCR was purchased from Himedia Laboratories, India. β-Actin mouse antibody (monoclonal) (catalog# 3700S) and HRP conjugated – Anti-mouse IgG Rabbit antibody (monoclonal) (catalog# 58802S) was from Cell Signal Technology, USA. Arc mouse antibody (monoclonal) (catalog# sc-17839) was purchased from Santa Cruz Biotechnology, USA. ECL Reagent (catalog# WBLUC0100) was purchased from Millipore, USA. All other general biochemicals were purchased from SRL and Himedia Laboratories.

Animals

Swiss Albino, male inbred mice, aged 15±3 weeks, were used in this study. Mice were maintained in the animal house facility of the University of Hyderabad. The food and water were provided to them *ad libitum*. They were exposed to 12-hour dark/light schedule. Mice were euthanized through cervical dislocation.

Animal experiments were approved by the institute’s animal ethics committee (IAEC/UH/151/2017/05/AG/P11).

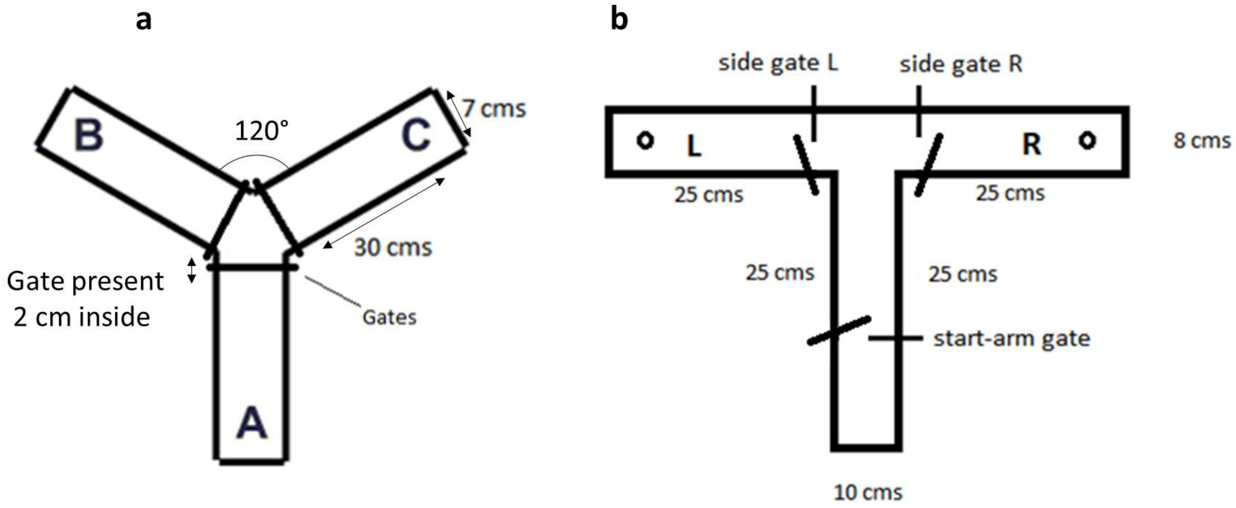

Figure 4. Dimensions of Y and T mazes. (a) Y-maze: Three similar arms placed at equal angles of 120° with the length, width, and height of 30x7x20 cm respectively, 0.5 cm thick gates placed 2 cm inside of each arm. (b) T-maze: Two arms placed at 90° with a length of 25 cm for all arms and width of 8 cm for sidearms and 10 cm for start arm.

Y- and T-maze apparatus

The Y-maze apparatus was constructed of black painted wood. It consists of three gated arms with equal angles among them (i.e., 120°). The dimensions of maze were 30x20x7cm of length, width, and height, respectively (Deacon and Rawlins, 2006). The wooden gates were vertically slidable into the grooves. The thickness of the gate is 5 mm, and the distance of the grooves from the open-end of the arm was 2 cm. The maze was bottomless, and it was kept on a grey wooden table for the mice behavior tests (Fig. 4). T-maze was made of polystyrene, with three arms, each of 25x20 cm, length, and height, respectively. Width of start and sidearms was 10 and 8 cm, respectively.

ORM apparatus

The apparatus for testing object recognition memory (ORM) consisted of an open field maze, with dimensions: 40 x 40 x 40 cm and a pair of objects, as mentioned earlier by Lueptow, 2017. In all the sessions, mice were placed in the mid-point of the adjacent quadrants, and the objects were placed in the center of two adjacent quadrants opposite to mice placement (Fig. 5). The exploration time of objects was scored manually frame-by-frame using Apple's QuickTime™ video player. The distance traveled by mice was measured by the animal tracking software (Stoelting Co's ANY-maze version 6.05).

Reverse transcription–quantitative PCR

Total RNA was isolated after cortex and hippocampus dissection and quantified as per the protocol by Gautam et al., 2013. Then, RNA samples with A260/280 ≥ 1.8 were used for reverse transcription-quantitative PCR. Initially, 0.5 µg of total mRNA was reverse transcribed into cDNA. Using this cDNA as a template and gene-specific primers for GAPDH (reference gene; 1 µM) and Arc (target gene; 4 µM), the amplicon was amplified by quantitative-PCR. The PCR sample mixture was prepared by mixing SYBR Master Mix (5 µl) (SYBR Green dye, Taq polymerase, and dNTPs), forward and reverse primers (2 µl each), and cDNA

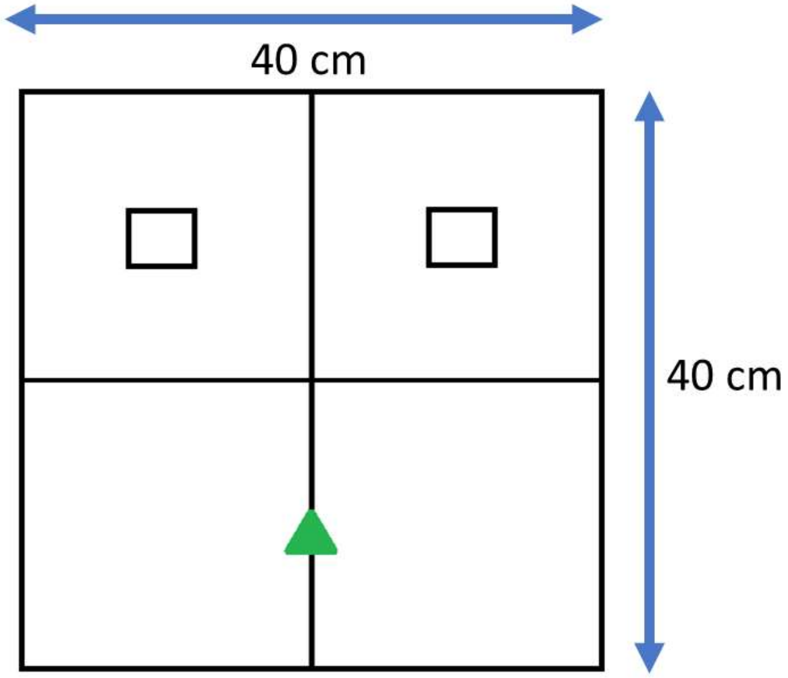

Figure 5. Open field maze showing the placement of objects (squares) and mouse (triangle) during behavioral tasks.

sample (1 µl). The sample mixture was denatured at 95° C for 120 s before the start of the PCR cycle. The PCR cycle settings were performed according to the manufacturer's recommendations (Denaturation: 95° C for 40 s, Annealing: 57° C for 40 s, Extension: 72° C for 60 s). The signal from the dye was measured at Extension (72° C) step of the cycle. The primer sequence is the following: Arc (forward: ACGATCTGGCTTCCTCATTCTGCT, reverse: AGGTTCCCTCAGCATCTCTG CTTT) amplicon was 146 bp (Barnhart et al., 2015); and GAPDH (forward: GTCTCCTGCGACTTCAG, reverse: TCATTGTCATACCAGGAAATGAGC) amplicon was 107 bp (Gautam et al., 2013). Melt-curve analysis (60° C to 95° C at 0.5° C/10 s) was performed to measure the Tm (melting temperature) of Arc and

GAPDH. Tm of Arc and GAPDH were around 85° C. To find the efficiency of PCR amplification, a three-point 10-fold dilution series for both the GAPDH and Arc was performed. PCR efficiency for Arc was 97.94% with correlation (r) = - 0.999 and slope = -3.37 and for GAPDH was 92.64% with correlation (r) = - 0.995 and slope = -3.51. No-template control for both the genes had Cq values > 30.0. We analyzed the amplified product on 1.5% Agarose gel (stained with EtBr) using DNA loading dye. Both the amplicon sizes of Arc and GAPDH were resolved correctly according to the DNA ladder, and no other band was present in their lanes. The quantification cycle (Cq) was measured using Insta Q96 (Himedia's qPCR machine). Any improper threshold and baseline assignments were corrected.

Western Blotting

RIPA lysis buffer was used to prepare 10% protein homogenate of cortex and hippocampus, and this homogenate was quantified using Bradford assay. RIPA buffer consisted of 25mM Tris-Cl, 1% Triton X–100, 1% sodium deoxycholate, 0.1% SDS and 150mM NaCl. 1mM of PMSF was added just before use. For protein quantification, Bradford standard curve was initially created by using BSA as standard protein solution. Four concentrations of BSA from 2.5 µg/ml to 10 µg/ml were prepared and 1 ml of Coomassie brilliant blue was added to each solution. Triplicate aliquots were prepared for each concentration. Then, A_{595} was measured for each aliquot. A_{595} is absorbance co-efficient when the protein solution was presented with light of wavelength 595 nm. Linear regression was calculated by Spectrophotometer as: Concentration (of unknown) = 29.8 * A_{595} (of unknown) + 2.26. The coefficient of determination (R^2) is 0.977. Later, 40 µg of protein was mixed with 6X denaturing sample buffer, and the resulting mixture was loaded in 10% SDS-PAGE. Using tank electroblotting apparatus, the resolved protein bands in the gel were trans-blotted onto the PVDF membrane. To visualize the bands on the membrane, we stained the membrane using 0.5% (w/v) Ponceau-S.

Immunoprobing

The PVDF membranes were initially blocked in 5% (w/v) fat-free milk (blocking-buffer) for one hour. Then, membranes were incubated in Arc mouse monoclonal antibody (1:400) solution for one hour at room temperature. After that, membranes were washed thrice with 0.5% Tween-TBS for 5 min each. After washing, membranes were incubated in HRP conjugated – anti-mouse IgG rabbit monoclonal antibody (1:2000) for two hours at room temperature.
Finally, membranes were washed thrice in 0.5% Tween-TBS for 10 min each and incubated in ECL (enhanced chemiluminescence) reagent for 2 min to detect the chemiluminescence signal inside the Bio-Rad's *ChemiDoc* imaging system.

After chemiluminescence detection of Arc protein, the same membranes were stripped by incubating in 0.2 M NaOH for 10 min. Then, membranes were incubated with β-Actin mouse monoclonal antibody (1:10,000) for one hour at room temperature (β-Actin was used as the loading control). After that, membranes were washed thrice with 0.5% Tween-TBS for 5 min each. After washing, membranes were incubated in HRP conjugated – anti-mouse IgG rabbit monoclonal antibody (1:2000) for two hours at room temperature. Finally, membranes were washed thrice in 0.5% Tween-TBS for 10 min each and incubated in ECL reagent for 2 min to detect the chemiluminescence signal inside Bio-Rad's *ChemiDoc* imaging system. All antibody dilutions were prepared in the blocking buffer. Protein quantification was done based on the ECL signal intensity values analyzed by the Bio-Rad's software Image Lab.

Stereotactic surgery

Several studies by inhibiting Arc via antisense oligodeoxynucleotides (ODNs) have found that Arc is necessary for LTP consolidation and modification of F-actin cytoskeleton (Minatohara et al., 2016). Hence, by the stereotactic surgery, the translation of Arc was inhibited in the dorsal hippocampus using the Arc antisense (scrambled as control) oligodeoxynucleotides (ODNs) similar to Gautam et al., 2016. The coordinates for bilateral infusion of ODNs were chosen as -2.46 mm anterior-posterior from bregma, ±1.35 mm medial-lateral and 1.35 mm dorsal-ventral (Franklin and Paxinos, 2001) (Fig. 6).

Sequences of ODNs were:

Arc-Antisense: 5–G*T*C*CAGCTCCATCTGCT*C*G*C–3 and

Scrambled: 5–C*G*T*GCACCTCTCGCAGC*T*T*C–3

where * represents phosphorothioate linkage.

For SAB-related experiments, phosphodiester linkage was used instead of phosphorothioate.

Statistical analysis

We performed the statistics using GraphPad's Prism (version 6.01) and SPSS (version 16.0). The significance level (type-I error threshold) was 5%. All the p-values which were mentioned along with the multiple-comparison tests were adjusted p-values as done by the statistical analysis software. A comparison was reported as statistically significant if its adjusted p-value was less than 0.05. Parametric tests were used if both the normality and equal variance were satisfied or corrected by the test. For data that was not normal and homoscedastic, either the non-parametric tests were directly used, or the data was aligned and ranked (if there were two independent variables). Aligned rank transformation (ART) was performed on such data using the web software available at http://depts.washington.edu/acelab/proj/art/index.html (Wobbrock *et al.*, 2011).

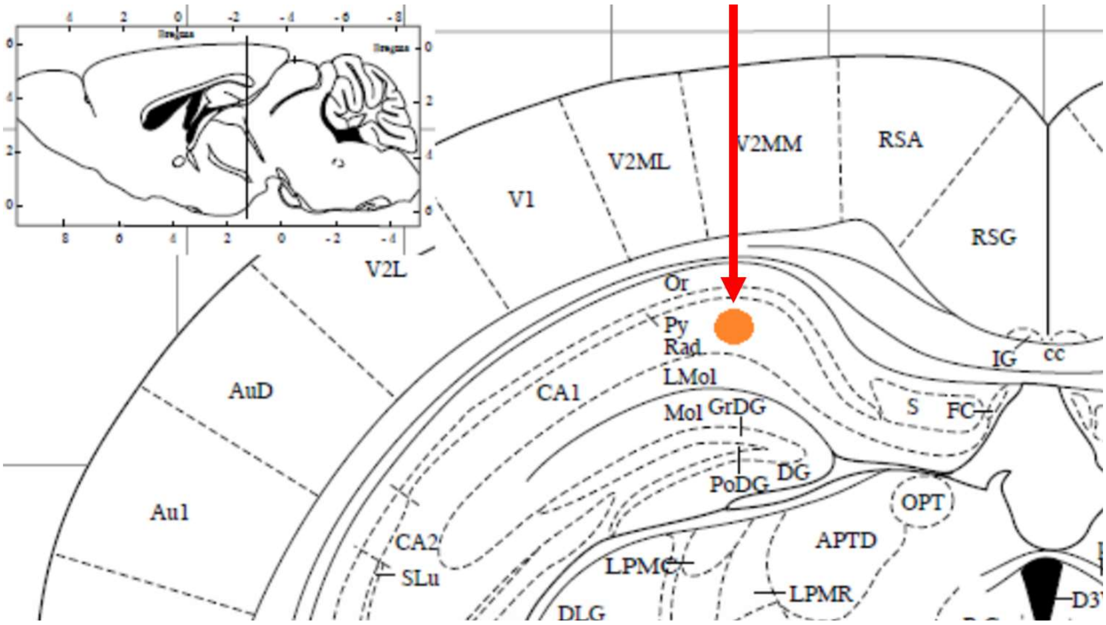

Figure 6. Stereotaxic infusion of oligonucleotides at the dorsal hippocampus as shown by the arrow in the coronal section; Inlet figure shows sagittal view (Franklin and Paxinos, 2001).

Chapter 3

The temporal changes in alternations of mice after re-exposures to an environment

INTRODUCTION

Working memory requires maintenance and manipulation of information. There is growing evidence that the process of training on the testing procedure can alter the working memory performance in the subsequent sessions in humans and non-human primates (Constantinidis and Klingberg, 2016). Spontaneous alternation behavior (SAB) is often used to evaluate the spatial component of working memory in animals (Hughes, 2004). SAB is the tendency of animals to alternate the choices (e.g., arm visits in Y-maze) when provided the opportunity in subsequent sessions. This phenomenon is commonly employed in behavioral tests, where the alternation rates are measured to gauge the working memory. Animals with intact working memory successfully discriminate between familiar and novel arms (Hughes, 2004). However, alternations could also be affected by the degree of habituation to the surrounding environment (Sanderson and Bannerman, 2012). Hence, there is a possibility that alternation behavior could be affected in mice when subjected to the repeated testing. Incorporating both repetition and novelty of the environment, we tried to study the temporal changes in the alternations of mice when tested under novel and familiar experiences.

The ‘continuous SAB’ is the commonly used procedure for testing SAB. However, the alternation rates in continuous SAB are typically low (usually less than 65%) and they are close to the chance level (i.e., 50%) (Deacon and Rawlins, 2006). Therefore to increase the alternation rates, a modified SAB procedure of

Y-maze was used in this study. To make sure that the modified procedure is exclusive for testing working memory, the scopolamine-treated mice were used as a negative control for the alternations. Scopolamine, a non-selective muscarinic-receptor antagonist, is a chemical that is commonly used for impairing the spatial working memory in rodents during SAB testing in Y-maze (Hughes, 2004; Bolden et al., 1992).

METHOD

SAB tests

Two types of SAB are generally used for testing the alternation rates: i) Two-trial SAB (through T- and Y- mazes) and, ii) Continuous SAB (through Y-maze) (Hughes, 2004). We chose Y-maze to test alternations as more number of trials can be run in this maze, and it has less experimenter interference (handling) than T-maze; however, the alternation rates are usually low (~65%) (Deacon and Rawlins, 2006). We tried to increase the alternations by introducing a delay component in continuous SAB procedure in Y-maze, referred to as 'delayed-SAB' in this study.

Effect of delay and scopolamine on alternations in T-maze

Initially, we used T-maze to test the optimal delay time as well as to validate the effect of scopolamine on alternations. The advantage of T-maze over Y-maze is that it has less inter-trial interference, and it is easier to optimize the delay time.

As reviewed by Klinkenberg and Blokland (2010), dosages between 1 to 10 mg/kgbw of scopolamine (i.p.) reduces the alternation rates in mice; therefore we used 3- and 5-mg/kgbw in our study.

To measure the alternations in T-maze, we used the rewarded forced alternation (RFA) test. Milk solution was used as the reward (12.5% v/v milk powder), and it was made available on both the side-arms. During the sample trial, the mouse was initially placed in start arm, and it was forced to enter only one side-arm (left or right), which was achieved by using a gate to block the entrance of opposite side-arm. Once the mouse enters a side-arm, it was retained there for a certain period of time (referred to as 'delay'), during which it consumed reward. After the delay, mouse was lifted and placed back in start arm (test trial), where it now chooses to enter any one of the side-arms (i.e., both side-arms were accessible). The mouse is said to have alternated if it chooses the side-arm that was blocked during the sample trial.

In our initial RFA test, we used a delay of 30s. Mice (n=4) were divided into two groups, SC and SA. SC group was injected (i.p.) with 3mg/kgbw of scopolamine, and SA group was injected (i.p.) with 0.9% w/v saline. Each mouse performed four sessions of RFA.

After this, we studied RFA with different delays of 30, 50, 90 and 105s, and tested with a dosage of 5 mg/kgbw of scopolamine. In this test, mice (n=5) were placed in side-arm until it has consumed the reward and then they were lifted and re-placed in the start-arm for the remaining period of delay time (retained using the start-arm gate) to increase the interval between the experimenter handling and the test trial; where the delay time is the sum of retention times of both side-arm and start-arm. After the delay, the gate of the start-arm was lifted and mice chose between side-arms.

Modification of SAB testing procedure

For the SAB modification procedure, we chose the delay time of 30s and scopolamine of 3 mg/kgbw based on T-maze results. Initially, mice were placed in the center of the Y-maze with all the gates open, and they were allowed to enter one of the three arms (referred to as first choice). Then the gate of that arm was closed for 30 seconds (delay time). After that delay, the gate was re-opened for the mice to make the next choice. This procedure was continued until the mouse made seven choices. The gates were closed for 30 s after the mice made every choice. Re-visiting the same arm is not considered as a different choice. Mice were removed from the maze after they made the seventh choice. A choice was made if the entire animal body (excluding tail) crosses the groove. This whole procedure constitutes one session. Every session had two parameters: the alternation rate and the inter-choice interval (ICI). A mouse was said to have alternated if it chooses a different arm than the previous one. ICI was defined as the time interval between two consecutive choices.

Delayed-SAB test

To test the effect of inter-choice delay on the number of alternations, the mice were divided into three groups: (i) No-Delay, (ii) Delay, and (iii) Scopolamine-Delay. No-Delay group was injected saline (i.p. 0.9% w/v) and underwent continuous SAB test. In continuous SAB, mice were not constrained by any delay between their choices. Delay group was injected saline (i.p. 0.9% w/v) and underwent the delayed-SAB test. Scopolamine-Delay group was injected with scopolamine hydrochloride (i.p. 3 mg/kgbw), and they underwent the delayed-SAB test. The scopolamine treated group acts as a negative control for alternations in SAB tests (Hughes, 2004). All tests were conducted two-and-half hours after the injections. All groups were allowed to make seven choices, and a delay of 30 seconds was used, except for the No-Delay group. For each mouse, we used (1) the number of alternations, and (2) average ICI (which is the mean of all ICIs) as the parameters to analyze the test result. Both parameters were measured using frame-by-frame analysis using a standard video player.

Effect of inter-session intervals on alternations

Preliminary experiments

Initially, we conducted two preliminary experiments to study the temporal changes in the alternations of the SAB test. Each experiment consisted of three SAB sessions. The time interval between session-1 and session-2 is referred to as ISI-1, and the interval between session-2 and session-3 is referred to as ISI-2.

ISI-2 was two hours (constant) for both the experiments. In the initial experiment, we checked the effect of ISI-1 on the alternation rates at session-2 and session-3. There were four groups of mice – based on different ISI-1 values: 30-, 60-, 120- and 240-min (n=5). In session-1, all groups were habituated to Y-maze for five min. Then, in both session-2 and session-3, the 7-choice delayed-SAB test was conducted for all groups. The delay period was set to 30s.

In the next experiment, we chose a fixed ISI-1 of 30 min (based on the results of first preliminary), where we compared the performance of naive mice with a negative control for alternations (i.e., scopolamine). There were two groups of mice(n=7); one group was injected 0.9% w/v saline (i.p.), and the other was injected 3mg/kgbw scopolamine (i.p.). Similar to previous preliminary experiment, all groups were habituated to Y-maze in their session-1, and the 7-choice delayed-SAB test (with 30s of delay) was conducted at both session-2 and session-3. Mice were dissected from both the groups immediately after each session to study the mRNA expression of Arc in cerebral cortex and hippocampus (n=3). As a control for this mRNA study, saline-injected mice were dissected at cage.

Main experiment

Mice performed three sessions of the 7-choice delayed-SAB test (Fig. 7). Inter-session interval-1 (ISI-1) is defined as the time interval between session-2 and session-1. We tried to study the effect of ISI-1 on both the alternation rate and the average-ICI at both session-2 and session-3. Hence, mice were divided into two groups based on the ISI-1, 30 min (n=8) and 120 min (n=8). The selection of intervals was based on our preliminary studies, where various ISI-1 intervals were tested, and their performances were similar to one of the selected intervals; however, mice in this experiment performed delayed-SAB in session-1, rather than just habituation as in the preliminary study.

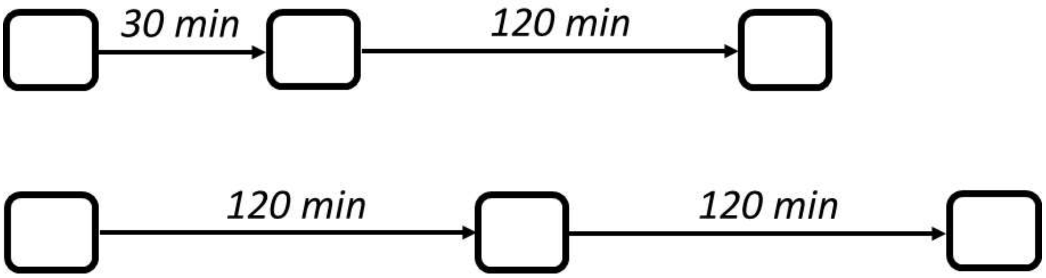

Figure 7. Schematic diagram to test the effect of inter-session intervals on the SAB. Each rectangular box represents a session of 7-choice delayed-SAB test. Inter-session interval-1 (ISI-1) was defined as an interval between session-2 and session-1. Mice are grouped based on their ISI-1 of 30 min or 120 min. Both groups underwent three sessions of testing. Each triangle represents the time when an independent set of mice were euthanized for Arc expression analysis. They were euthanized (i) 30 min after session-1, (ii) 120 min after session-1, or (iii) without any session (control).

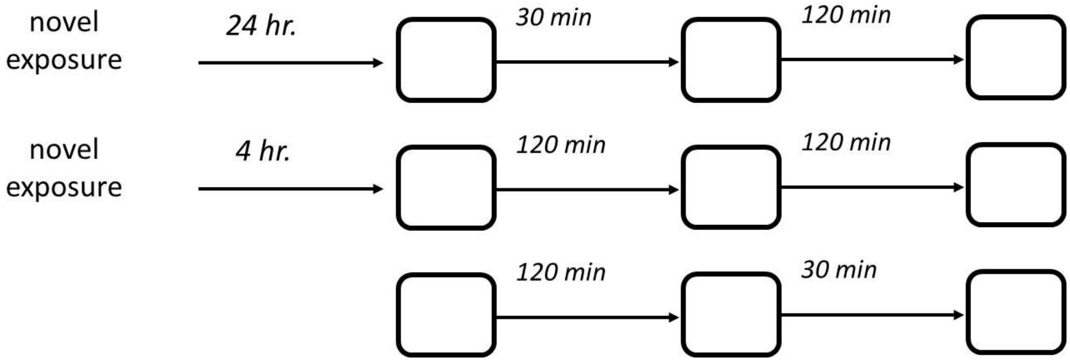

Figure 8. Schematic diagram to test the effect of familiarity of the environment on the alternations. Based on how recent they were exposed to novel environment (if any), three independent mice groups performed three sessions of delayed-SAB each.

Effect of familiarity of the environment on the alternations

To test if the observed changes in alternations across sessions were dependent of the degree of familiarity of environment (rather than ISI-1 alone), three experiments were performed based on how recently (2.5, 8, or >24 hours) mice were exposed to the environment before they perform test session (session-3) (Fig. 8).

(1) Mice (n=6) were habituated to the maze for 20 min on day-1, and they performed the delayed-SAB test on day-2. This mice group had ISI-1 of 30 min, and it will test if familiarity (at long time-scale) is a factor for the changes in alternations.

(2) Another independent group of mice (n=8) performed a delayed-SAB test every 120 min for five sessions. This mice group faced a novel Y-maze. For analysis, we only considered the last three sessions (referred to as session-1, -2, and -3) to keep statistical analysis similar to other experiment's multiple comparisons. Hence, this group performed session-3 eight hours after novel exposure. The initial two sessions will be considered as part of familiarization. This group will test if familiarity (at intermediate time-scale) is a factor for the changes in alternations.

(3) Another independent group of mice (n=6) performed session-3 150 min after novel exposure. However, this group had ISI-1 of 120 min. This group will

test if ISI-1 is a factor for the changes in alternations for a given period since novel exposure.

RESULTS

Scopolamine decreased the alternation rates in RFA tasks

For rewarded forced alternation (RFA) test with 5mg/kgbw scopolamine, the alternation rates were measured between saline (SA) and scopolamine (SC) groups. SC group had significantly lower alternation rate than SA group; average alternations for the four sessions of SC and SA groups are 2.5 and 3.5, respectively (p=0.049, unpaired t-test) (Fig. 9).

Similarly, for RFA tests with 5mg/kgbw scopolamine, SC groups had a significantly lower alternation rates than SA groups for all the delays (30s (p=0.012), 50s (p=0.008), 90s (p=0.0005), and 105s (p=0.008); unpaired-tests; s=seconds) (Fig. 10).

Inter-choice delay increases the alternations in Y-maze

We observed a significant difference in the number of alternations among No-Delay, Delay, and Scopolamine-Delay groups (p=0.005, Kruskal-Wallis test, and

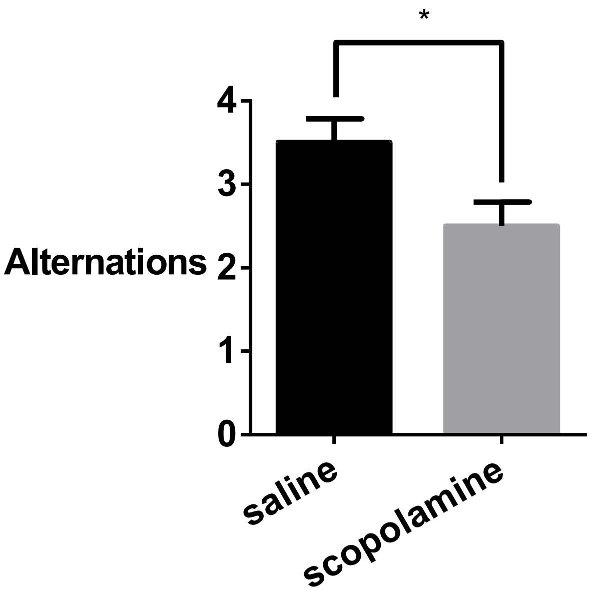

*Figure 9. Scopolamine decreased the alternations in the RFA task with 30 s of delay at the side-arm. * p<0.05. All bars represent mean ± SEM.*

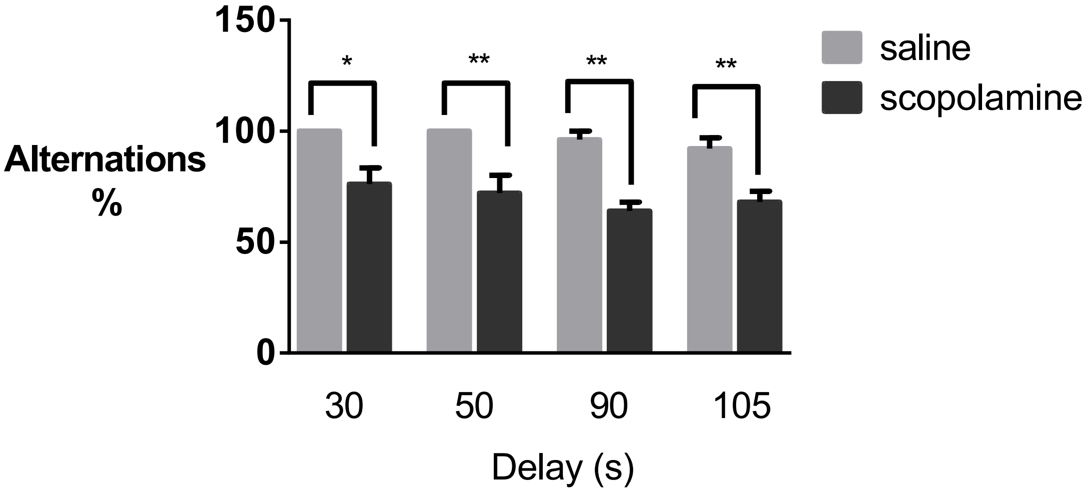

*Figure 10. Scopolamine decreased the alternation rates in RFA task with various delays at start-arm. * p<0.05, ** p<0.01. All bars represent mean ± SEM.*

Dunn's multiple comparisons). Delay group had higher alternations than both Scopolamine-Delay (p=0.011) and No-Delay (p=0.028). There was no difference between other groups (p > 0.99) (Fig. 11 a).

We observed a significant change in the average ICI among the groups (p = 0.0005, Kruskal-Wallis test and test and Dunn's multiple comparisons). No-Delay group had lower average ICI than both Delay (p = 0.001) and Scopolamine-Delay (p = 0.003). There was no difference between other groups (p > 0.99) (Fig. 11 b).

The number of alternations decreases in subsequent sessions for the inter-session interval of 30 min

Among all four groups (ISI-1 of 30-, 60- 120-, and 240-min), we found that the session-3 alternations were significantly lower than session-2 for 30 min ISI-1 group (p=0.03, paired t-test), and there was no change for the rest of the groups (Fig. 12). Additionally, we compared session-2 alternations among all the groups and found no difference among them (p=0.901, one-way ANOVA), implying that all groups performed similarly in session-2.

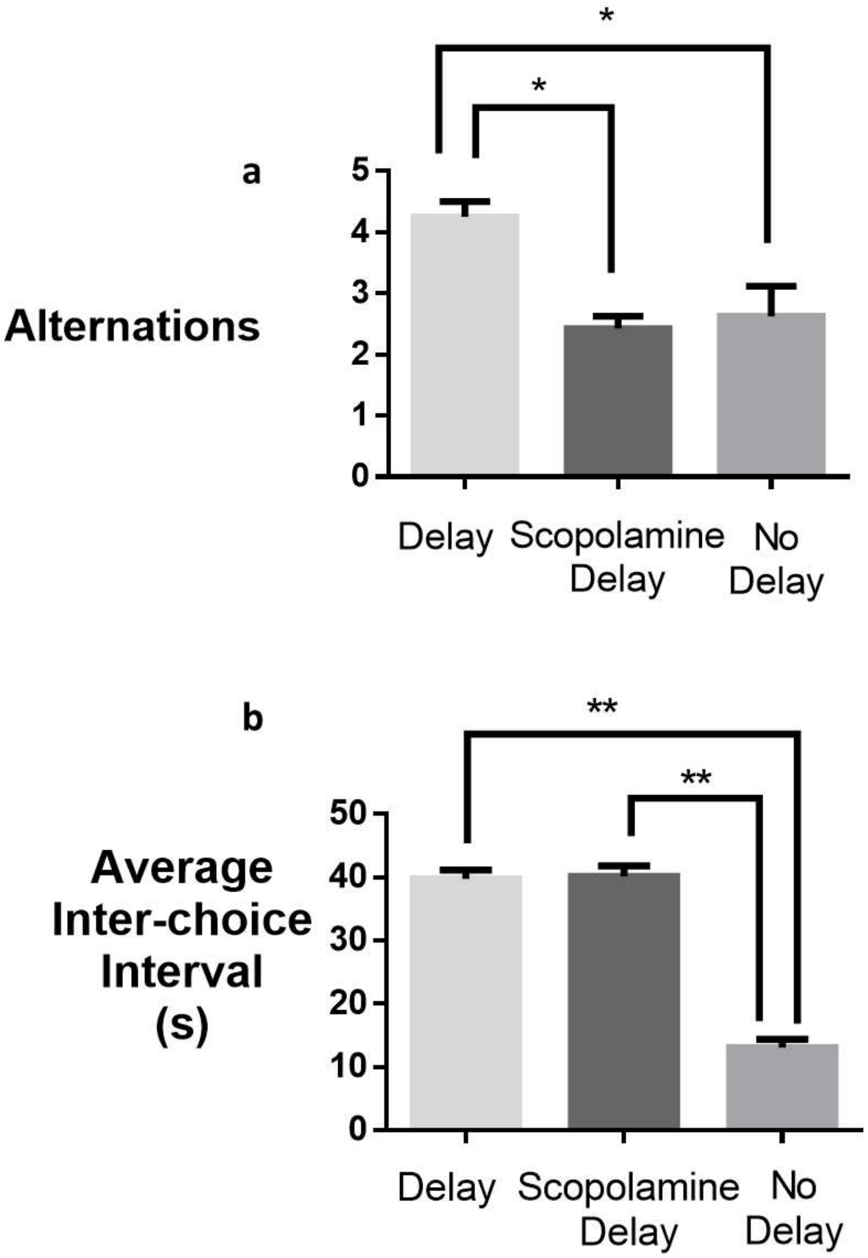

*Figure 11. (a) Alternations and (b) average ICI were higher for Delay-group with respect to No-Delay. As expected, Scopolamine-Delay (negative control) showed lower alternations, validating the test. * p<0.05, ** p<0.01. All bars represent mean ± SEM.*

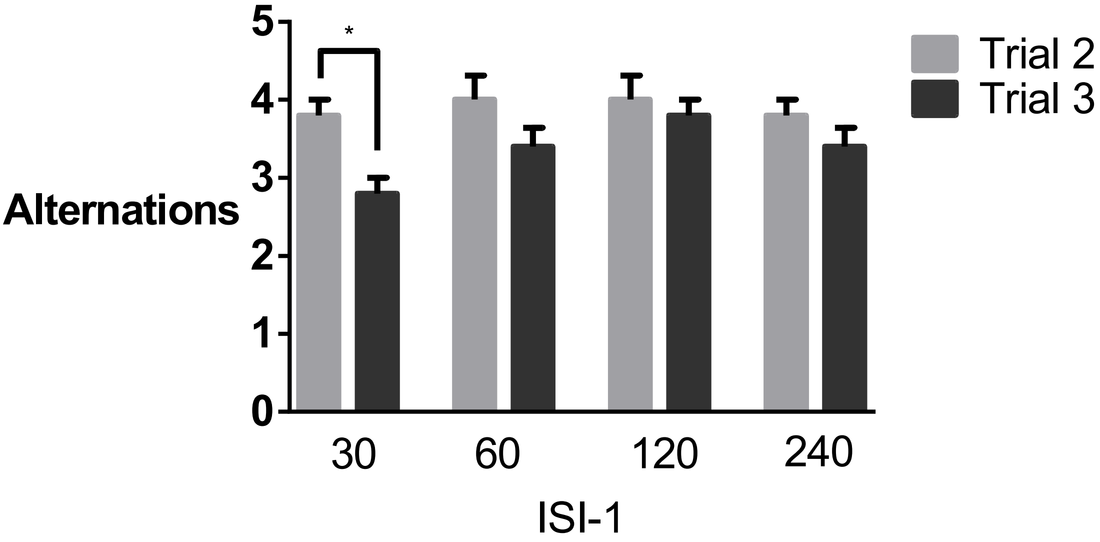

Figure 12. Alternations at various inter-session intervals-1. ISI-1 of 30 min showed a decrease in alternations in its trial-3 as compared to its trial-2. p<0.05. All bars represent mean ± SEM.*

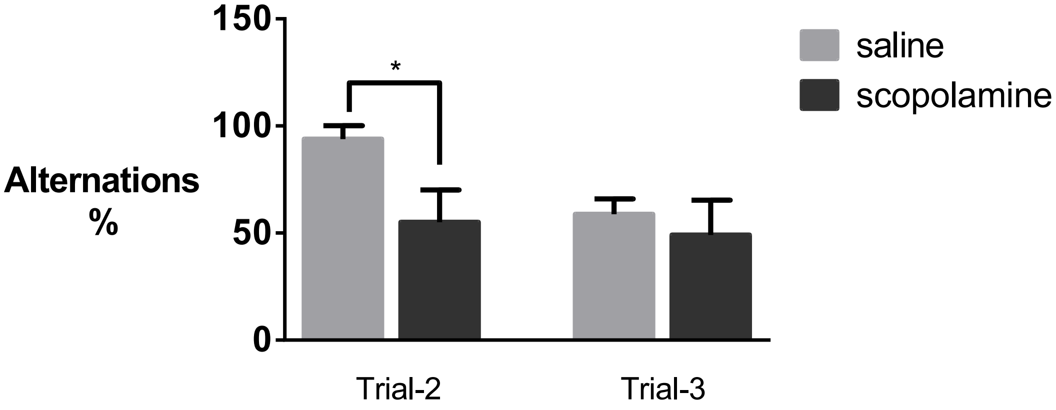

Figure 13. Effect of scopolamine on temporal changes in alternations. Scopolamine-treated mice had lower alternations in all sessions. p<0.05. All bars represent mean ± SEM.*

We then also observed, in the next preliminary experiment, that the alternations at session-3 for the saline group were similar to the scopolamine group (p=0.85, paired t-test). At session-2, the alternations for the saline group were higher than the scopolamine group (p=0.03, paired t-test) (Fig. 13).

In the main experiment, there was a significant difference in alternations among the sessions of 30 min ISI-1 group (p=0.002, Friedman test) (Fig. 14 a). However, there was no such difference for the 120 min ISI-1 group (p=0.876, Friedman test) (Fig. 14 b). Among the sessions of 30 min ISI-1 group, session-3 alternations were reduced as compared with session-1 (p=0.008, Dunn's multiple comparisons). No differences were found between other pairs of sessions. However, there was no difference in average ICI among the sessions for both groups (p=0.57 for 30 min ISI-1; p=0.54 for 120 min ISI-1; repeated-measure ANOVA) (Fig. 14 c, d).

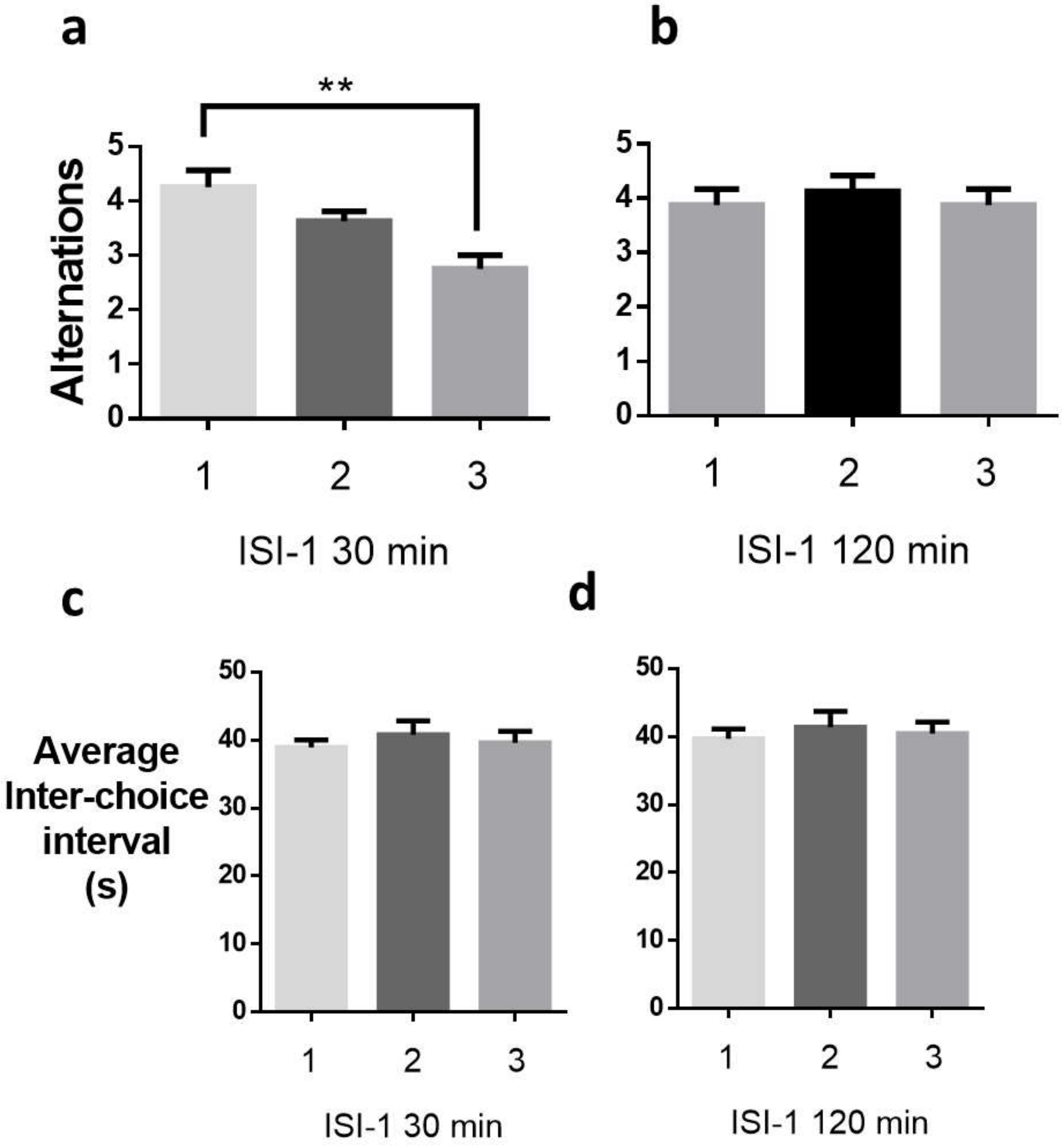

*Figure 14. Comparison of alternations (a, b) and average ICI (c, d) across sessions (at X-axis) in mice that performed the delayed-SAB test with (a, c) ISI-1 of 30 min and (b, d) ISI-1 of 120 min. ISI-1 represents the interval between session-2 and -1. There was a significant difference in the number of alternations among sessions of mice group ISI-1 30 min, but not for 120 min. There was no difference in average ICI among the sessions for both ISI-1s. Both groups were presented novel maze at session-1. ** p<0.01. All bars represent mean ± SEM.*

Effect of familiarity on alternations

Familiarization 24 hours before testing does not decrease alternations: For mice that were previously habituated to Y-maze, there was no change in the number of alternations (p=0.76, Friedman test) (Fig. 15 c) and average ICI (p=0.44, ANOVA, F (1.726, 8.63) = 0.85) among their sessions (Fig. 16 c).

Familiarization 8 hours before testing does not decrease alternations: Even though there was a significant main effect in the number of alternations among the sessions (p=0.0085, Friedman test), there was no significant difference between individual sessions through multiple comparisons (p>0.05, Dunn's multiple comparisons) (Fig. 15 b). There was no difference in average ICI among sessions (p=0.22, ANOVA, F (2.373, 16.61) = 1.614) (Fig. 16 b).

ISI-1 of 120 min does not decrease alternations: There was no difference in the number of alternations (p=0.66, Friedman test) (Fig. 15 a) and average ICI (p=0.32, F (1.974, 9.872) = 1.244, ANOVA) (Fig. 16 a) for group with ISI-1 of 120 min that faced novel maze 2.5 hours before session-3.

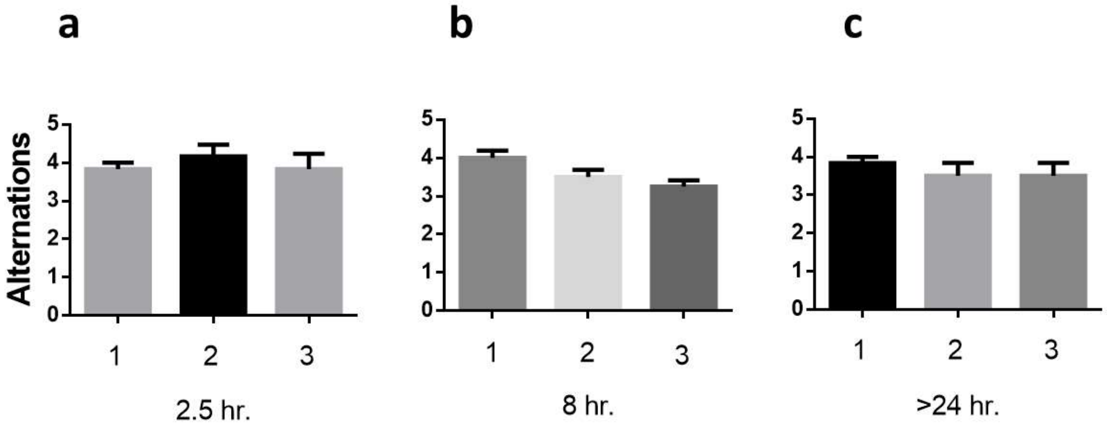

*Figure 15. Alternations in mice that performed delayed-SAB session-3 at (a) 2.5 hours, (b) 8 hours, and (c) more than 24 hours after novel exposure of maze. There was no difference in alternations among sessions for each group. * p<0.05, ** p<0.01. All bars represent mean ± SEM.*

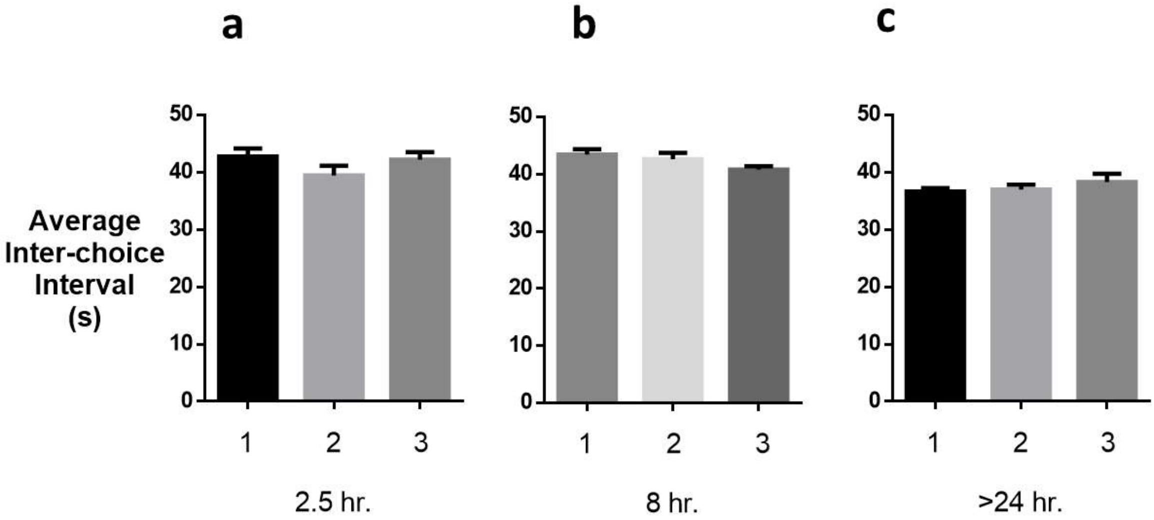

*Figure 16. Average ICI in mice that performed delayed-SAB session-3 at (a) 2.5 hours, (b) 8 hours, and (c) more than 24 hours after novel exposure of maze. There was no difference in average ICI among sessions for each group. * p<0.05, ** p<0.01. All bars represent mean ± SEM.*

DISCUSSION

By presenting a delay between choices, alternations have increased in continuous spontaneous–alternation task. We used scopolamine, a chemical known for causing attention and working memory deficits, as a negative control in our experiments to confirm the absence of ques (Cheal, 1981). We could successfully validate the test procedure as the scopolamine-treated mice performed poorly in alternations. Also, the delay had increased ICI. These results conclude that the mouse will alternate if it has been exposed to an arm for a greater amount of time. Apart from working memory, alternations also require short-term habituation of the testing environment (Sanderson and Bannerman, 2012; Wolf et al., 2016). Our above results support, at least in a smaller time scale (10 to 30 s), the necessity of short-term habituation of the testing apparatus for successful alternations. Spontaneous alternation behavior is commonly used in pharmacological experiments interested to study memory retention (Hughes, 2004). It is, therefore, important to note the possibility of a drug's effect on attention and habituation, apart from memory, while measuring the alternations.

Continuous spontaneous alternations are generally modest. However, the multiple-choice delayed task that we used had increased the alternations. The results of our multiple-choice, delayed task suggests that it has (1) minimal interference between the trial (as facilitated by delay), (2) more accurate measurement of animal's alternation rate than two-trial SAB, and (3) higher alternations even under novel exposure. On the other hand, continuous tasks without delay could be more useful to study attention enhancement instead of memory (Cheal, 1981; Hughes, 2004).

Results of the temporal study of alternations showed that there was no significant difference in ICI in all the sessions of all the groups. This showed that the amount of time the mice spent in an arm was independent of changes in the rate of alternations. Additionally, the time since novel exposure did not affect the alternations. Rather, we found that the inter-session interval had an effect, with the shorter interval resulting in a significant drop in alternations in the test trail. However, this interval did not affect the second session, and it suggests that the observed drop in alternations could be due to the physiological changes occurring in the interval between trial-3 and trial-2. We hypothesize that the observed changes could have been affected by plasticity-related proteins, which are synthesized after a novel experience. Therefore, the role of one of such proteins, Arc, has been studied further in this research work.

Additionally, we were interested to see if such decreases in alternations could be observed if the testing environment was familiar to mice. Here, when we familiarized mice to the Y-maze for 20 min the previous day, there was no decrease in the alternation. This concludes that the change in alternation occurring upon repeated exposure is dependent on the novelty of the environment.

Chapter 4

The temporal changes in object recognition memory of mice after re-exposure to an environment

INTRODUCTION

Object recognition memory (ORM) tasks will test the ability of an animal to recollect previously presented information. The degree of familiarity of the previously presented object is estimated by the amount of an animal's exploration towards it when presented against a novel one – where lower exploration rates imply higher familiarity and vice versa. The availability of hippocampal functions, up to few hours (~3 hours) after the object familiarization, can facilitate memory retention for longer periods (~24 hours) (de Lima et al., 2006). This represents the hippocampus-dependent memory consolidation time. However, the hippocampus is mainly needed for storing contextual information (i.e., storing object's information with respect to its immediate surroundings) rather than the object-specific information (Piterkin et al. 2008). Based on these findings, we test if the ORM of an initially presented object could be affected if it is replaced by a non-identical object in a similar context. The re-exposure to the environment with a different object could interfere with the consolidation process of the former one, and the efficiency of this interference could depend on the inter-familiarization interval (interval between familiarization of these objects).

We tested the above hypothesis for two cases, (1) testing the familiar objects against each other at different test sessions, referred to as temporal order memory (TOM), and (2) testing each familiar object against a novel one, referred to as novel-ORM (NORM).

METHOD

Behavioral tests

Our modified behavioral procedure consists of two familiarization phases (session-1 and session-2) and one test phase (session-3). In both the familiarization phases, a distinct pair of objects was presented temporally, and in the test phase, the familiarity of these objects was tested. During the test phase, the familiarity of these objects was assessed either against each other (TOM task) or against a novel object (NORM task). Both these procedures generally include the habituation of rodents to the testing maze for few days before the object familiarization day (Manago et al., 2016; Barker et al., 2011; Barker et al., 2007; Hannesson et al., 2004; Mitchell and Laiacona, 1998). However, in NORM tests, we presented a novel environment (OF and its surroundings) to the rodents during the familiarization phases. The mice were placed in the maze for five min in each session.

Temporal order memory test

Mice were habituated for 5 min in the OF maze without objects one day before the familiarization phases. The task consisted of three sessions with an inter-session interval of either 30, 120, or 240 min between session-1 and session-2 (referred to as ISI), and an interval of 120 min between session-2 and session-3 (Fig. 17). The mice were divided into three groups (30, 120, and 240 min) based on their ISI (n=8). In the session-1 (first familiarization phase), the mice were familiarized with two non-identical objects: RO (Recurring Object - present in all subsequent sessions) and AO (Alternating Object - present in alternating sessions). In session-2 (second familiarization phase), the mice were exposed to two identical RO objects, with one object in a familiar location and the other in the location previously occupied by object AO (of session-1). The positions of objects RO and AO were counter-balanced among all the groups. We designated the RO objects present in session-2 as either 'ROr' or 'ROa' based on their location that had been previously (as in session-1) used by objects 'RO' and 'AO' respectively. In session-3 (test phase), the mice were exposed to both the objects RO and AO, both located as in the session-1.

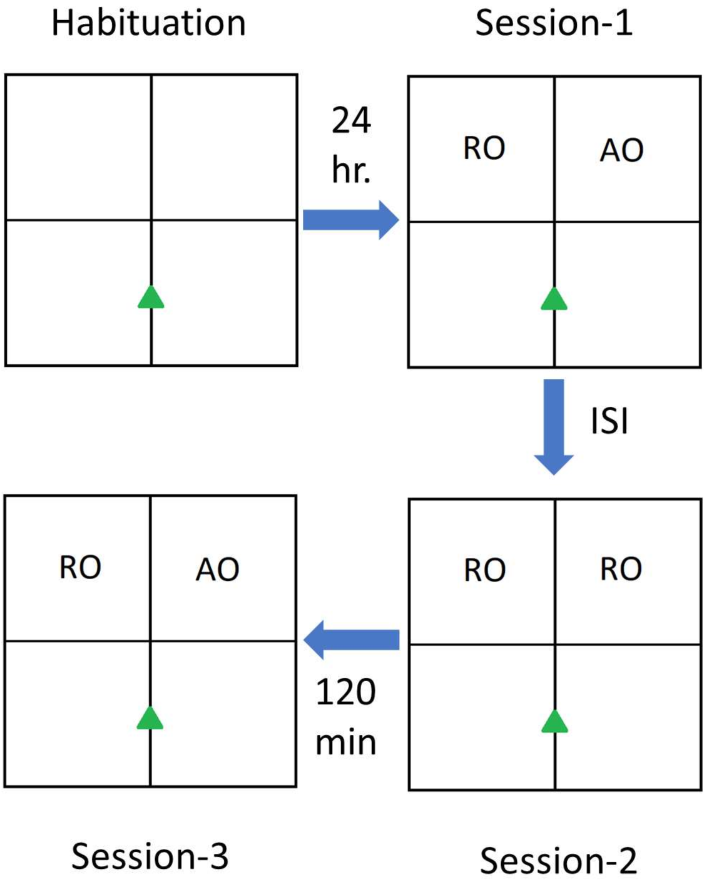

Figure 17. Schematic view of TOM test. After the habituation to the open field one day before, mice undergo three sessions of exposure. Session-1 has RO (Recurring Object – present in all sessions) and AO (Alternating Object – present in alternating sessions). Session-2 has identical objects RO (designated as ROr and ROa based on the location shared by objects RO and AO, respectively, in session-1), and it was performed either after 30-, 120- or 240 min (ISI-1) after session-1. Session-3 has objects RO and AO, and it was performed 120 min after session-2.

An object was considered explored if the animal’s nose was less than 3 cm away from the object and facing towards the object. Mice, which spent a minimum of 15s to explore both the objects in the session-1, were considered for analysis.

Test parameters

We calculated the location preference (left- or right-side) by mice and compared all group means with each other. The distance traveled by mice (head) was measured using the animal tracking software (Stoelting Co’s ANY-maze, version 6.05). The object exploration time was manually scored frame-by-frame using Apple’s QuickTime™ video player. Additionally, we calculated the total exploration time (TET) for each session, which was the sum of exploration times of both objects.

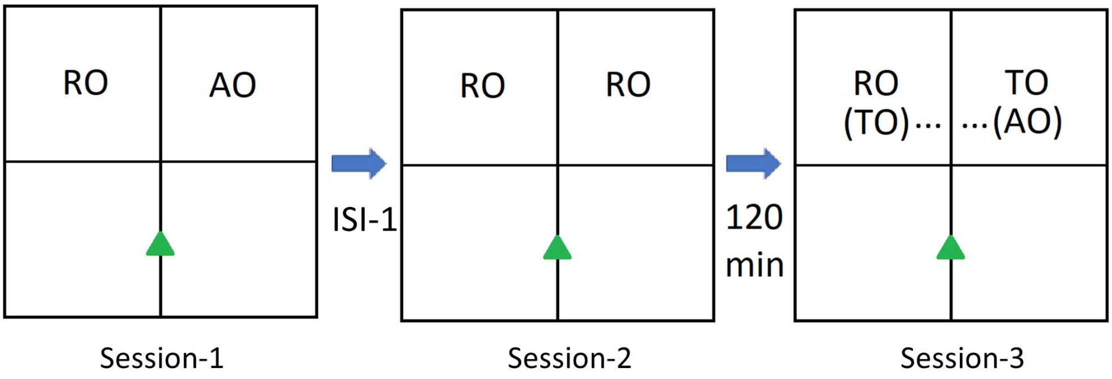

Figure 18. Schematic view of NORM experiment. Mice performed three sessions in the task. session-1 had objects RO and AO. session-2 had identical objects RO, designated as ROr or ROa based on the location shared by objects RO or AO, respectively, in session-1, and it was performed either 30- or 120-min after session-1. session-3 has objects RO and TO (a novel object) in experiment-2.1; or TO and AO in experiment-2.2 (dashed line). session-3 was performed 120 min after session-2 in both experiments.

Novel–object recognition memory tests

The experimental procedure for NORM tests was similar to that of the TOM test except that we introduced a novel object (TO) in the test session (Fig. 18). The location of AO and RO was the same as in their respective session-1. Additionally, mice were not habituated to the testing apparatus before the test. As confirmed through pilot studies, mice had no innate object preference between any pair of objects. Based on the object present at the test session, two experiments have been performed.

1. Exploration between Recurring– and Novel–object during the test phase:

Object AO was replaced by TO. Mice were divided into two groups having ISI of either 30- or 120-min (n=7 each).

2. Exploration between Alternating– and Novel–object during the test phase:

Object RO was replaced by TO. Mice were divided into two groups having ISI of either 30 min (n=5) or 120 min (n=4).

Effect of duration of habituation and the ISI on OF exploration

We tested the effect of duration of habituation and the ISI on the subsequent sessions in OF. The duration of habituation per session and the ISI values were the same as in the TOM task. Additionally, we used 30 min habituation as a positive control for inter-day habituation (Ballarini et al., 2009). We divided mice into three groups: A, B, and C (Fig. 19). Group A underwent two sessions of OF exposure, whereas groups B and C underwent three sessions. Group A was exposed to OF for 30 min in the session-1 on day-1 and five min in the session-2, which was 24 hours later. Groups B and C were exposed to OF for five min in

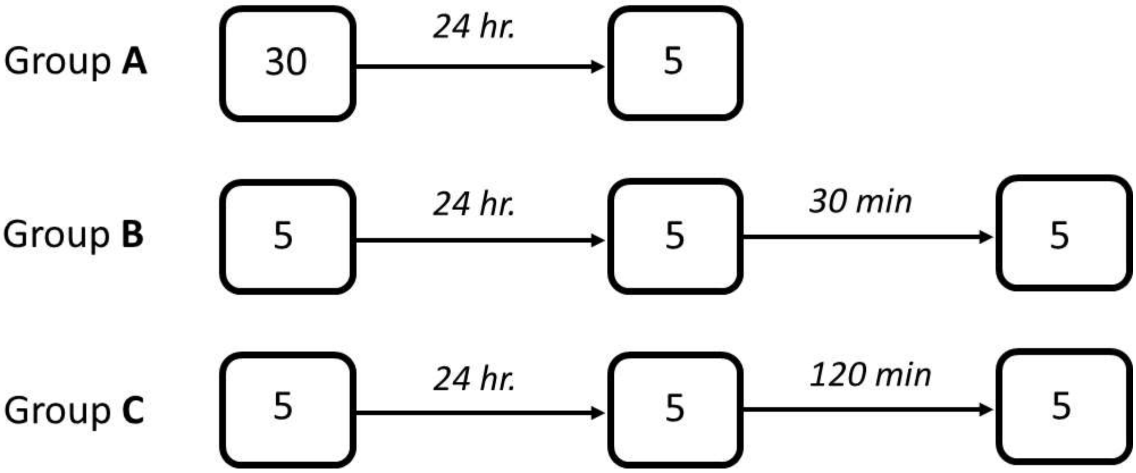

Figure 19. Schematic diagram for the series of habituation sessions to the open-field. Each session is represented by a box. Mice were divided into three groups (A, B, and C) based upon their OF exposure time per session and the inter-session intervals. Time of OF exposure per session is mentioned inside the boxes, whereas inter-trial intervals are displayed by arrows.

session-1 on day-1 and 5 min in session-2, which was 24 hours later. Additionally, both groups B and C were exposed to OF for five min in the session-3, which was performed 30 min- and 120 min- after session-2 for group B and group C, respectively. As the most common measure for assessing the habituation to the novelty (Bolivar, 2009), the distance covered by mice was measured.

To check the effect of habituation duration in session-1 on session-2 exploration, we compared the distance between those sessions. For 30 min habituation (group A), we compared distance traveled during the first five min of session-1 with its session-2. For 5 min habituation (groups B and C), we compared the distance traveled of session-1 (pooled data for both groups) with its session-2 (pooled). To check the effect of ISI on exploration, we compared the distance traveled between session-2 and session-3 for both ISI of 30 min (group B) and ISI of 120-min (group C).

RESULTS

Temporal order memory test

The location preference was not significantly different among the groups (one-way ANOVA, F (2, 21) = 0.84, p = 0.44). Aligned rank transformation (ART) was performed on the distance-traveled data. Distance traveled by mice was not significantly different among the groups (F (2, 21) = 1.25, p=0.30), but there was a difference among sessions (F (2, 42) = 48.88, p<0.0001). For all the groups, mice traveled less distance at session-3 than session-1 (Dunn's multiple comparisons test, p=0.001 (ISI of 30 min), p=0.0005 (ISI of 120 min), and p=0.008 (ISI of 240 min)). No difference exists between other inter-session comparisons (Fig. 20 a).

ART was performed on the TET data. TET was not significantly different among the groups (F (2, 21) = 0.80, p=0.45) but there was a difference among sessions (F (2, 42) = 43.48, p<0.0001). For all the groups, mice explored objects less at session-2 than session-1 (Dunn's multiple comparisons test, p=0.017 (ISI of 30 min), p=0.037 (ISI of 120 min), p=0.003 (ISI of 240 min)). Additionally, mice explored the objects less at session-3 than session-1 for ISI of 30 min (p=0.003) and 120 min (p=0.017), but not for 240 min (p=0.136). No difference exists between trail-2 and session-3 for all groups (Fig. 20 b).

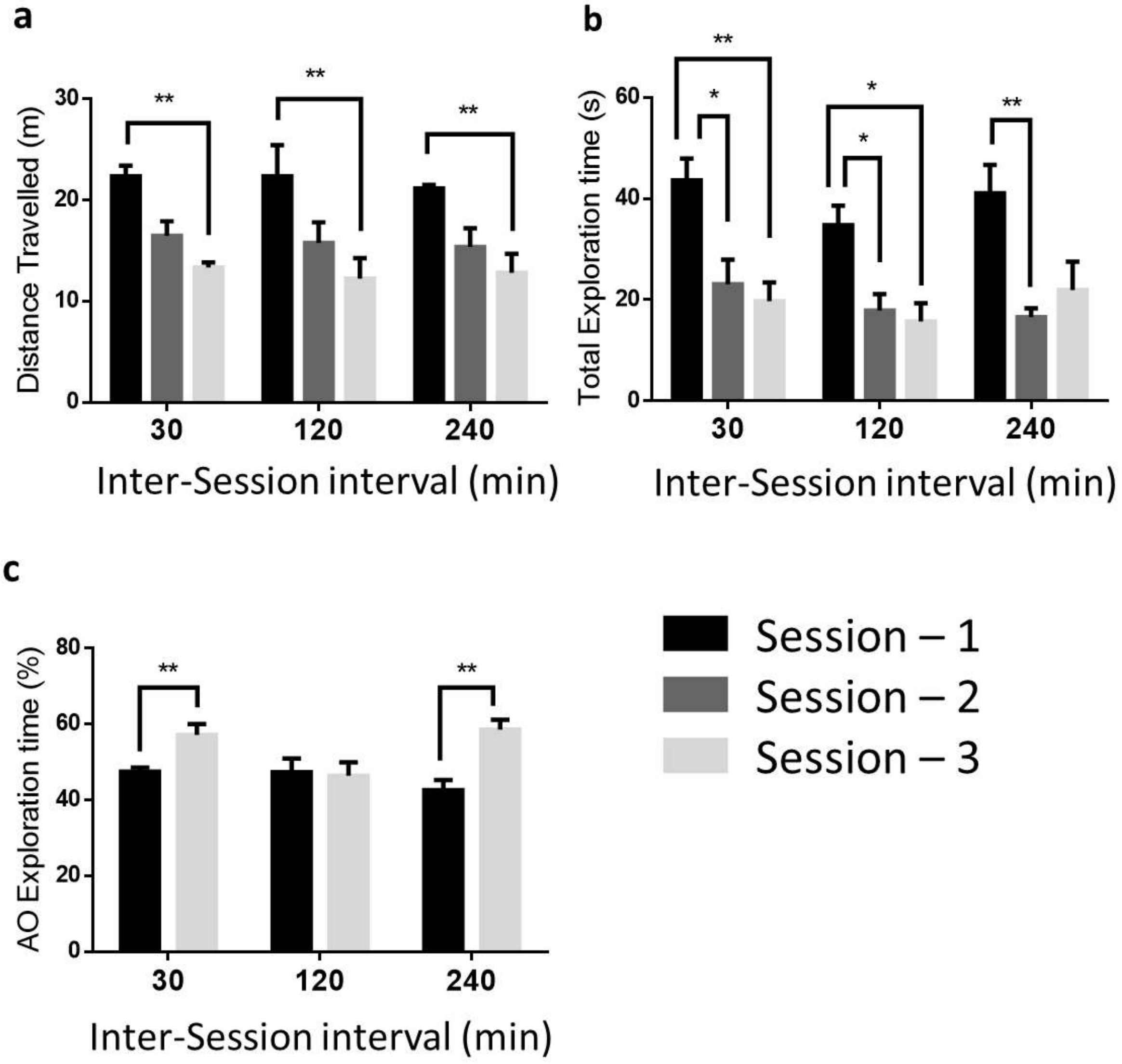

*Figure 20. Analysis of parameters of TOM task. (a) There was no difference in distance travelled among the ITI groups. However, for all three groups, mice travelled less distance in session-3 than session-1. No difference existed between other comparisons. (b) There was no difference in TET among the ITI groups. However, TET was higher in session-1 than both session-2 and session-3 (except 240 min ITI). No difference existed between other comparisons. (c) In session-3 of the test, mice with ITI of 30 min and 240 min have explored object AO significantly more than their session-1, whereas 120 min group had no change. All bars represent mean ± SEM. * p<0.05, ** p<0.01.*

There was no significant difference in the percentage of exploration time of object ROa among the groups (58.98% for 30 min ISI, 52.99% for 120 min ISI, and 51.26% for 240 min ISI; Kruskal-Wallis test, p=0.39). ART was performed on the object AO exploration (%) data. There was a significant interaction between groups and sessions (F (2, 21) = 8.571, p=0.0019). The change in object AO exploration between session-1 and session-3 was analyzed. Both ISI of 30 min and 240 min increased their exploration of the object AO, whereas the exploration remained same in 120 min ISI group (Wilcoxon test; p=0.007 (30 min ISI), p=0.945 (120 min ISI), and p=0.007 (240 min ISI)) (Fig. 20 c).

Effect of ISI on RO recognition memory

The location preference between the groups: 30 min and 120 min ISI, was not significantly different (unpaired t-test, p=0.17). There was no significant difference in the distance traveled between the groups (two-way ANOVA, F (1, 12) = 1.80, p=0.20), but a difference existed among sessions (two-way ANOVA, F (2, 24) = 33.67, p<0.0001). For both groups, mice traveled less distance in later sessions than session-1 (Bonferroni's multiple comparisons test, p<0.01) (Fig.

21 a). No difference existed between session-2 and session-3 for both groups (p>0.1).

There was no significant difference in the TET between the groups (two-way ANOVA, F (1, 12) = 0.02, p=0.88), but a difference existed among sessions (two-way ANOVA, F (2, 24) = 5.79, p=0.0089). However, no difference exists among their sessions through multiple comparisons (p>0.5) (Fig. 21 b). We found no significant difference in the exploration time (%) of object ROa between the groups (57.6% for 30 min ISI and 53.6% for 120 min ISI; p=0.13, unpaired t-test).

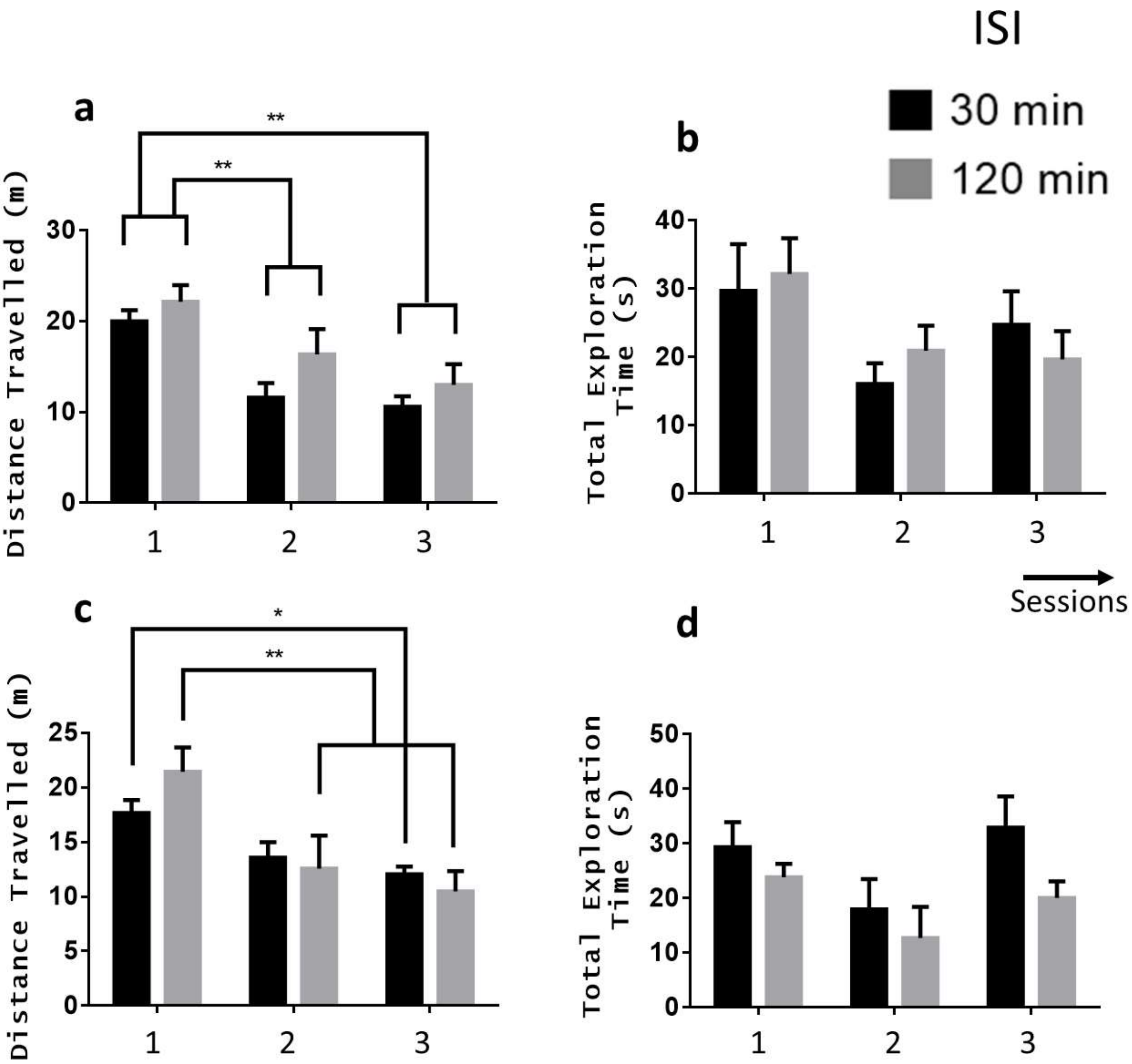

*Figure 21. Distance traveled (a, c) and TET (b, d) by mice in NORM tests having either RO (a, b) or AO (c, d) at session-3. The x-axis represents three sessions. (a) There was no difference in the distance among the groups. However, more distance was traveled in session-1 than both session-2 and session-3 for all three groups. (b) There was no difference in the TET among groups and among sessions. (c) There was no difference among the groups, but more distance was traveled in session-1 than both session-3 (for all groups), and session-2 (for 30 min ITI). (d) There was no difference in the TET among groups and among sessions. All bars represent mean ± SEM. * p<0.05, ** p<0.01.*

There was no significant difference in RO exploration between the groups (two-way ANOVA, F (1, 12) = 0.008, p=0.92) but a difference existed among sessions (two-way ANOVA, F (1, 12) = 51.23, p<0.0001). Mice with ISI of both 30 min and 120 min explored object RO less in their session-3 than in their session-1 (Bonferroni multiple comparisons, p<0.001) (Fig. 22 a).

Effect of ISI on AO recognition memory

The location preference between the groups: 30 min and 120 min ISI, was not significantly different (unpaired t-test, p=0.30). There was no significant difference in the distance traveled between the groups (two-way ANOVA, F (1, 7) = 0.05, p=0.82), but a difference existed among sessions (two-way ANOVA, F (2, 14) = 15.87, p=0.0003). More distance was traveled in session-1 than both session-3 (for both groups, p<0.01), and session-2 (for 30 min ISI, p<0.05) (Bonferroni's multiple comparisons test) (Fig. 21 c). No difference exists between session-2 and session-3 for both groups (p>0.1).

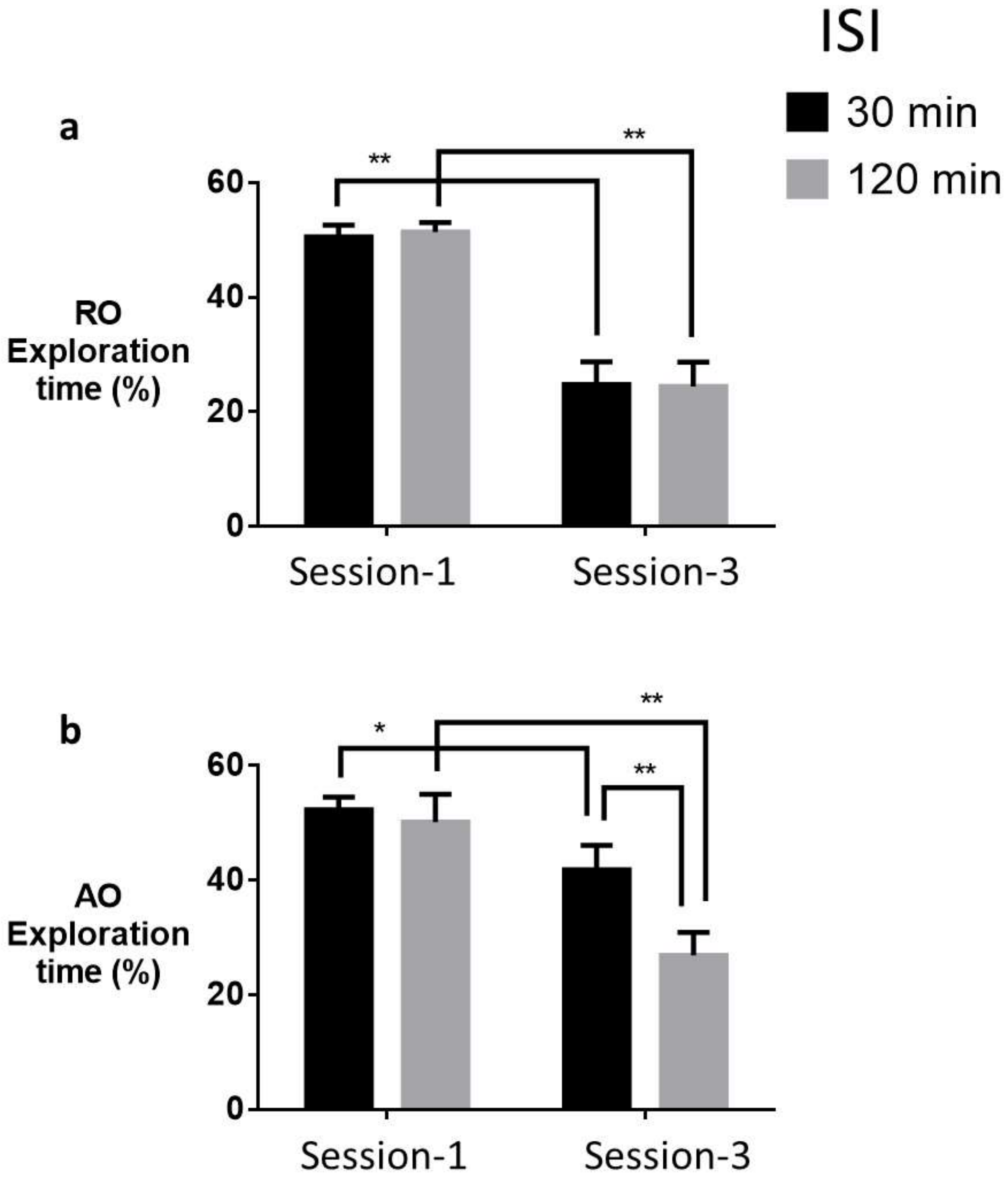

*Figure 22. Exploration time (%) of object (a) RO and (b) AO by mice at session-1 and -3 of NORM tests. (a) There was no difference in RO exploration among the groups, but all groups explored RO less in their session-3 than session-1. (b) Overall, AO exploration was less in session-3 than session-1 for both groups. However, at session-3, mice with ITI of 30 min have explored object AO more than that of 120 min. All bars represent mean ± SEM. * p<0.05, ** p<0.01.*

There was no significant difference in the TET between the groups (two-way ANOVA, F (1, 7) = 2.49, p=0.15), but a difference existed among sessions (two-way ANOVA, F (2, 14) = 4.69, p=0.02). However, no difference exists among the sessions through multiple comparisons (p>0.05) (Fig. 21 d). We found no significant difference in the exploration time (%) of object ROa between the groups (53.17% for 30 min ISI, and 49.17% for 120 min ISI; p=0.38, unpaired t-test – Welch corrected).

There was a significant interaction between groups and sessions (two-way ANOVA, F (1, 7) = 6.803, p=0.03). For both groups, object AO exploration (%) was less in the session-3 as compared to its session-1 (Bonferroni's multiple comparisons, p=0.03 (for ISI 30 min), and p=0.0008 (for ISI 120 min). Notably, in session-3, mice group with ISI of 30 min have explored object AO more than that of 120 min (Bonferroni's multiple comparisons, p<0.0001) (Fig. 22 b). There was no difference in AO exploration in session-1 between both groups (p=0.84).

In both TOM and NORM tests, 20% of the videos were re-scored by an independent experimenter who was blind to the experimental design, animal assignment, and other details. Pearson correlation was measured between the original and blind-scored results. There was a significant correlation between the original and re-scored results (r=0.879, p<0.01).

Effects of habituation duration and ISI on subsequent session's exploration

We found that the distance traveled had significantly decreased in the following day if mice were habituated to OF for 30 min (paired t-test, p=0.002) but not for 5 min (pooled data of group B and C) (paired t-test, p=0.798). Additionally, at five min habituation per session, neither 30 min (paired t-test, p=0.85) nor 120 min ISI (paired t-test, p=0.82) significantly decreased the novelty of the OF (Fig. 23).

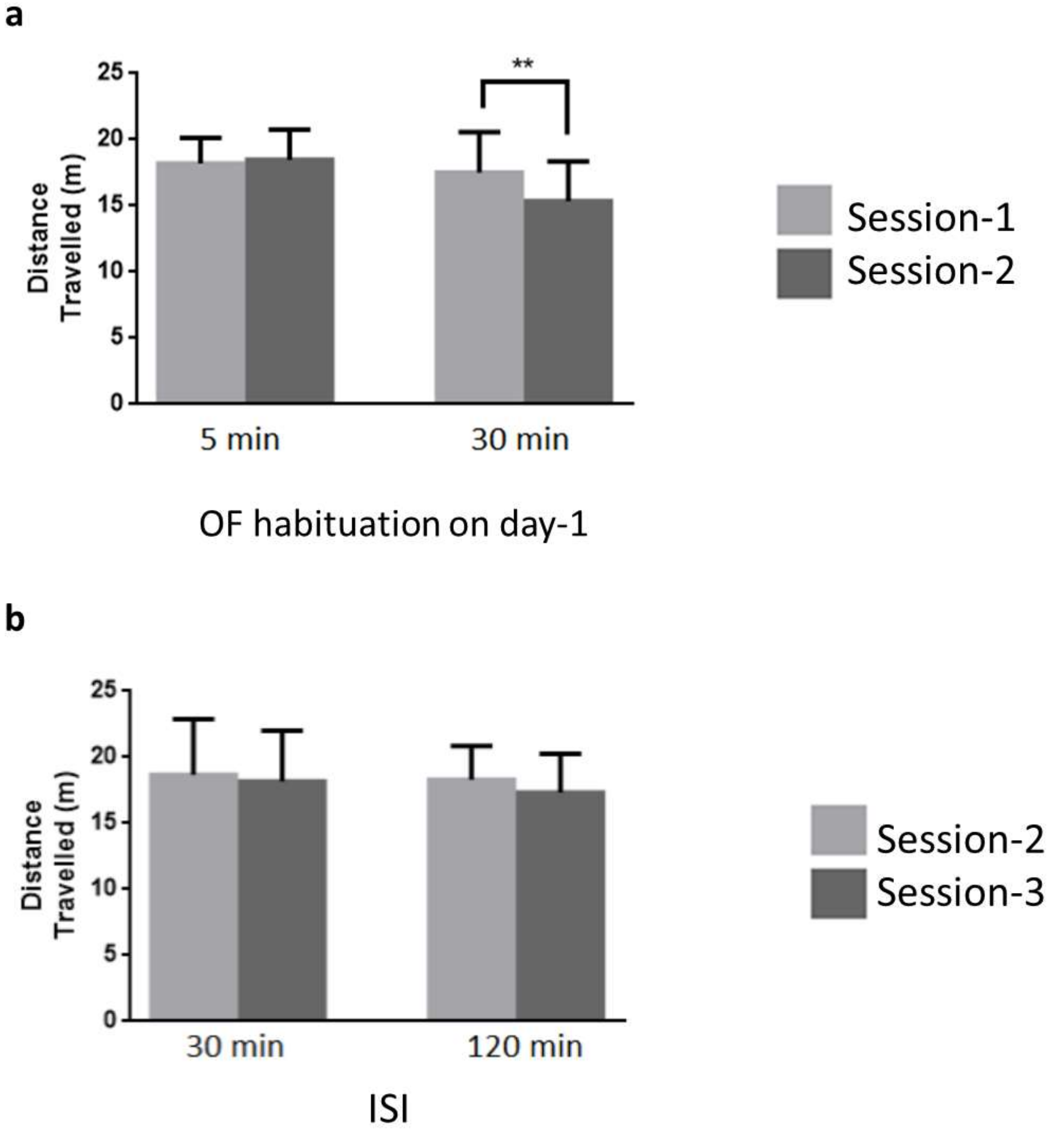

*Figure 23. (a) Distance traveled by mice in the first five minutes of session-1 and session-2 for both cases of 5-min and 30-min habituation on day-1 is shown. session-1 was performed on day-1 for either 5 or 30 min, and session-2 was performed on day-2 for 5 min. There was a significant decrease in distance traveled in session-2 with respect to session-1 for mice habituated for 30-min on day-1, but no difference for the mice habituated for 5-min (pooled of groups, B and C) on day-1 (paired t-test, ** p<0.01). (b) Distance traveled by mice in session-2 and session-3 for both ITI of 30 min (group B) and 120 min (group C). We found no change in distance traveled between session-2 and session-3 for both ITI 30–min (paired t-test, p=0.85) and 120–min (paired t-test, p=0.82). All bars represent Mean ± SEM.*

DISCUSSION

Generally, the recognition memory of an object (ORM) that has been exposed at a single instance previously is intact only for two to four hours in mice. After this period, mice cannot discriminate between familiar and novel objects (Lueptow, 2017). Initially, we studied the effect of inter-session interval (ISI) between two familiarization phases of TOM task at the test phase. As both objects were familiarized, an equal exploration between them was expected at the test phase. We found that mice explored objects equally at ISI of 120 min; however, object exploration was unequal at ISI of 30- and 240-min. A higher exploration of alternating-object (AO) for mice group with ISI of 240 min could be due to the decay of short-term memory AO, resulting from a higher interval between test and familiarization phase of six hours, which is expected from the memory paradigm that the longer the interval between the familiarization and testing phase more the memory decay of an object. However, the mice group with ISI of 30 min also had higher exploration of AO. Overall, this shows that object recognition memory is inter-session interval specific.

With a similar experimental design, we investigated the effect of ISI on the NORM task. A novel object was presented in the test phase against each of the familiarized objects. We found that ORM of alternating-object (AO), but not recurring-object (RO), was affected by ISI. Within ORM of AO, mice preferred the novel object significantly more for ISI of 120 min than 30 min, contrary to the expectation that a larger interval leads to more memory decay. For ORM of AO, mice explored novel object 58% and 73% for ISI of 30- and 120-min, respectively. This shows that ORM is intact for AO for ISI of 120 min. For ORM of RO, mice explored novel object around 75% of the time for both ISI; showing intact RO memory for both ISI. Overall, this shows that for the ORM for an object that is exposed for single-time in a repeated exposure design is ISI specific. We have ruled out the role of other parameters, including distance traveled and TET, on the observations.

Chapter 5

Involvement of Arc in working memory paradigm after re-exposure to a novel environment

INTRODUCTION

When rodents are exposed to the novel environment, Arc mRNA is expressed within a quick period (around 5 to15 min). Some of the mRNA is transported to dendrites and translated locally in the dendritic spines (Minatohara et al., 2016). In dendrites, Arc protein mediates the changes in the spine morphology (Kitanishi et al., 2009; Steward et al., 1998; Vazdarjanova et al., 2002). Both cytoplasmic- and dendritic-translated Arc activates CaMKIIβ and triggers LTD through AMPAR endocytosis (Okuno et al., 2012).

The availability of Arc mRNA and the activation of metabotropic glutamate receptors in the spines are necessary for LTD (Jakkamsetti et al., 2013; Park et al., 2008). This temporal correlation between LTD and Arc was also observed during the novel exploration by rats in the reference memory paradigms, and this LTD had facilitated the familiarization of these novel environments (Dong et al., 2012; Kemp and Manahan-Vaughan, 2004). However, it is unclear whether the temporal changes in the working memory paradigm and Arc expression are associated. Therefore, the present objective was to investigate the temporal effects of Arc expression on the working memory upon novel exploration in Y-maze.

In this objective, we analyzed the Arc mRNA expression by qPCR and its protein by Western blotting in both hippocampus and cortex (the two important brain regions involved in the spatial working memory) of these mice. We then confirmed the role of Arc in the temporal changes of the working memory in mice by inhibiting the Arc protein expression in the dorsal hippocampus using the stereotaxic apparatus.

METHOD

Effect of scopolamine on Arc expression

In this preliminary study, mice were divided into three groups of mice(n=3). The first group was injected 3mg/kgbw scopolamine (i.p.); the second group was injected saline 0.9% w/v; the third group (control) was also injected saline but did not perform any behavioral test. Similar to the preliminary behavior experiment, both behavioral groups were habituated to Y-maze in their session-1, and the 7-choice delayed-SAB test (with 30 s of delay) was conducted at both session-2 and session-3, with ISI-1 of 30 min. All mice were dissected immediately after each session to study the mRNA expression of Arc in the cerebral cortex and hippocampus.

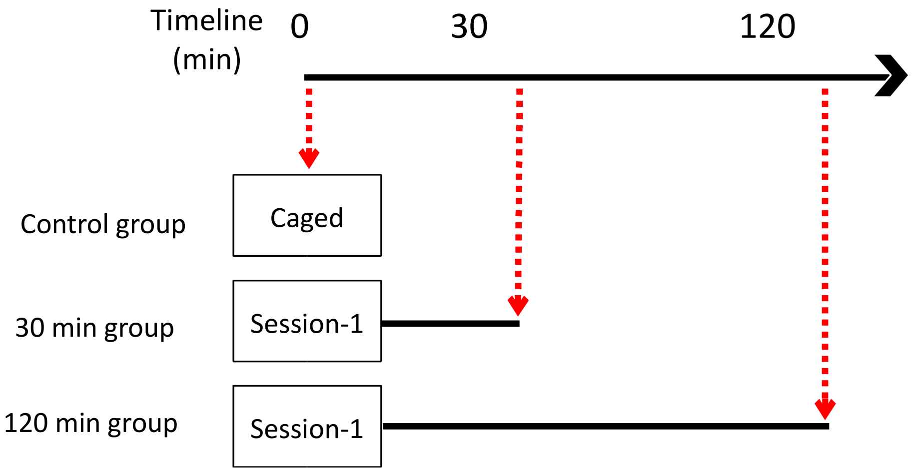

Figure 24. Schematic view of experiment design for Arc expression study for SAB tests. Groups were divided based on different dissection times, as indicated by arrows. Group 30 min and 120 min underwent delayed SAB test at t=0 min, and were euthanized at 30 and 120 min, respectively. Control was dissected from cage.

Effect of ISI-1 on Arc expression level

For Arc expression studies, mice were euthanized 30 min or 120 min after session-1 of delayed-SAB. Additionally, control mice were dissected at the cage without any behavior test. All mice were sacrificed by cervical dislocation, and their cortex and hippocampus were dissected out quickly in ice-cold saline for mRNA and protein expression studies. Dissection timings were represented by dotted arrows in figure-24. For mRNA study, dCq values are used for statistical comparison. For each sample, the dCq values were calculated as: Cq (Arc) - Cq (GAPDH). For statistical analysis of dCq values, we used one-way ANOVA followed by Tukey's multiple comparisons test. For protein study, the Arc/β-Actin ratio was calculated for all groups. The resultant ratios of each group were compared using one-way ANOVA test and Tukey's multiple comparisons for both cortex and hippocampus.

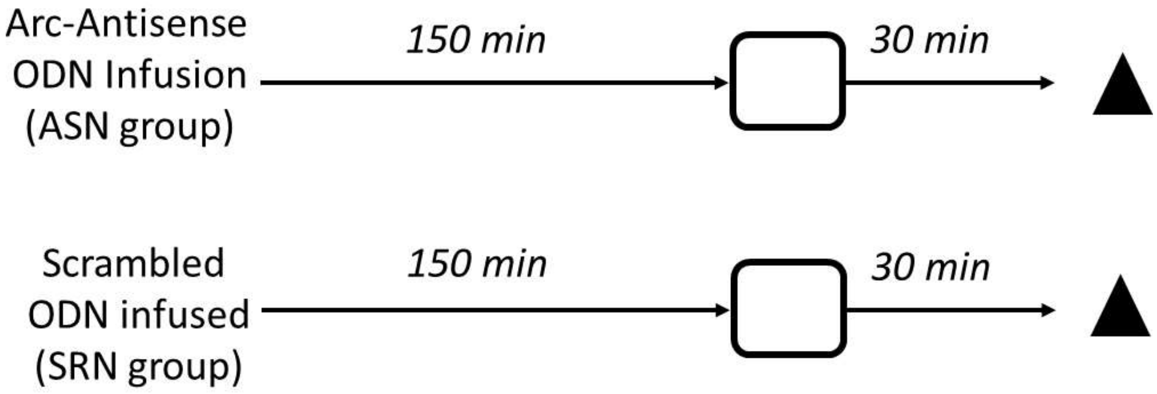

Figure 25. Schematic diagram for the stereotaxic experiment for Arc protein analysis. Each rectangular box represents a session of 7-choice delayed-SAB test. Mice were divided into two groups based on the type of ODNs were infused: (i) Arc-Antisense ODN, and (ii) Scrambled ODN. Each group underwent one session delayed-SAB test. Each triangle represents the time mice were euthanized for Arc protein expression analysis.

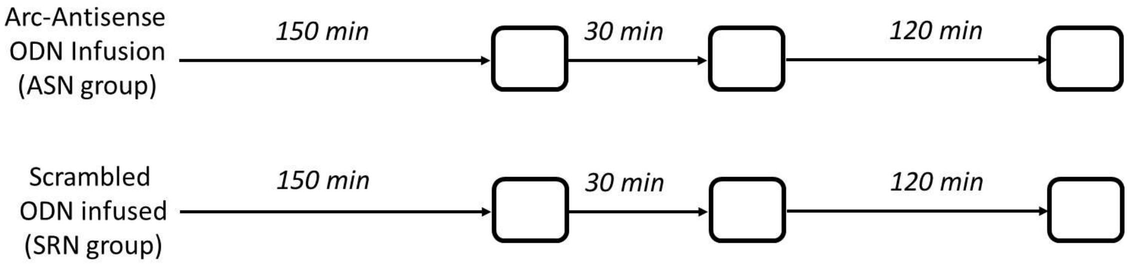

Figure 26. Schematic diagram for the stereotaxic experiment for SAB tests. Each rectangular box represents a session of 7-choice delayed-SAB test. Mice were divided into two groups based on the type of ODNs were infused: (i) Arc-Antisense ODN, and (ii) Scrambled ODN. Each group underwent three sessions with the same inter-session intervals.

Effect of Arc inhibition on spontaneous alternation behavior

Initially, to confirm the efficiency of infused ODNs, we performed Western blotting of the hippocampus to analyze the Arc protein level. An independent set of mice (n=3), grouped similarly to the above behavioral test (i.e., ASN and SRN), were euthanized by cervical dislocation 30 min after session-1 of delayed-SAB (without performing session-2) (represented by triangles in Fig. 25).

Further, based on the type of ODNs infused in the dorsal hippocampus, mice were divided into two groups (n=6) for behavior study: (1) ASN (Antisense ODN-injected) and (2) SRN (Scrambled ODN-injected) groups. 2.5 hours post ODN injection, each group performed three sessions of the delayed-SAB test, with ISI-1 of 30 min and faced a novel Y-maze (Fig. 26). As alternations were not affected for mice group with ISI-1 of 120 min, we did not choose it for further studies.

RESULTS

Scopolamine reduces the Arc expression

In the preliminary experiment, for both cortex and hippocampus, dCq values were significantly different between the groups (saline and scopolamine) in both session-1 and session-2, but not session-3 (Fig. 27). In cortex, –dCq is higher in saline as compared to scopolamine in session-1 (p=0.0013, unpaired t-test), and session-2 (p=0.015, unpaired t-test). Similarly, in hippocampus, –dCq is higher in saline as compared to scopolamine in session-1 (p=0.002, unpaired t-test), and session-2 (p=0.001, unpaired t-test).

Arc mRNA expression peaks after 30 min of the first session

dCq was significantly different among mice groups in both cortex (One-way ANOVA, F (2, 6) = 20.4, p=0.0021) and hippocampus (One-way ANOVA, F (2, 6) = 126.7, p<0.0001). In cortex, A30 group has lower dCq than both control (p=0.0029, Tukey's multiple comparisons) and B120 (p=0.0045, Tukey's multiple comparisons).

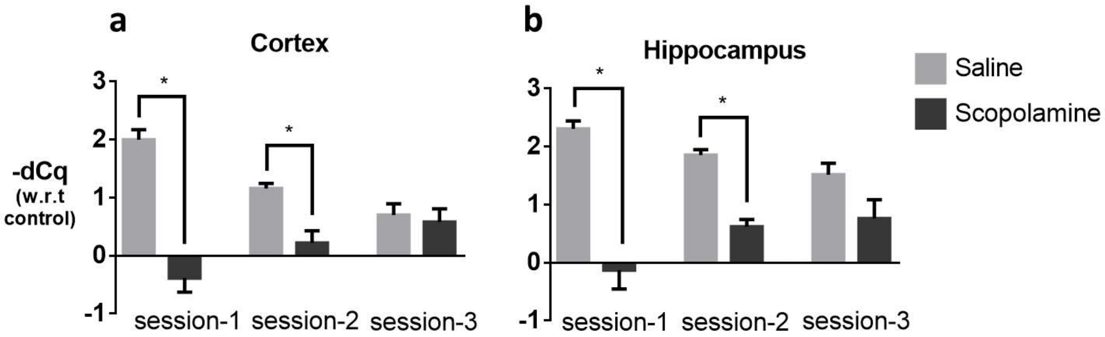

*Figure 27. Scopolamine reduces the Arc expression during SAB task. Scopolamine treated mice have lesser Arc expression at session-1 and -2. * p<0.05. All bars represent mean ± SEM.*

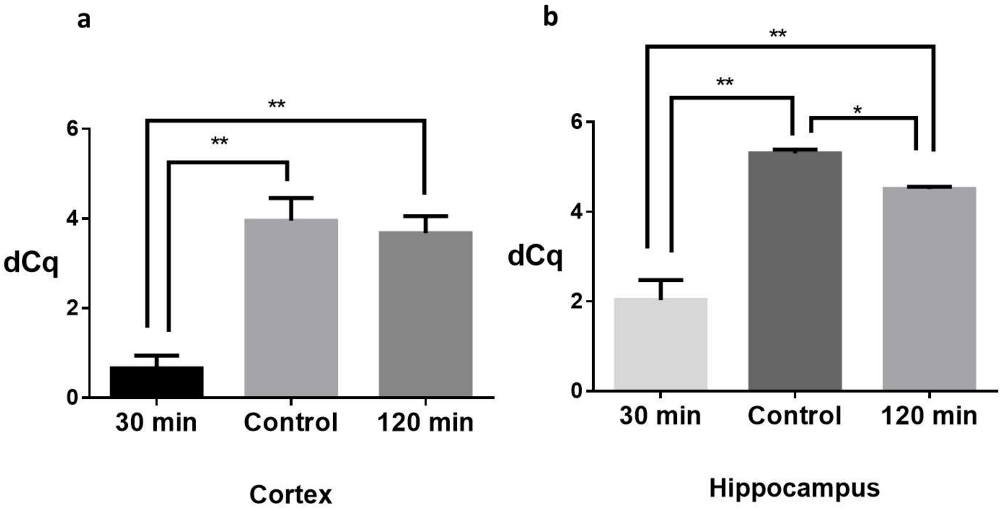

*Figure 28. Analysis of expression of Arc mRNA induced during SAB tasks. Arc mRNA expression was higher in the 30 min group than other groups in both (a) cortex and (b) hippocampus. Additionally, mRNA expression was higher in the120 min group than control group in hippocampus. *p<0.05; **p<0.01. All bars represent mean ± SEM.*

However, dCq was similar between control and B120 (Tukey's multiple comparisons, p=0.878) (Fig. 28a). Similarly, in the hippocampus, the A30 group has lower dCq than both control (p<0.0001, Tukey's multiple comparisons), and B120 (p<0.0001, Tukey's multiple comparisons). Additionally, B120 has lower dCq than control (p<0.023, Tukey's multiple comparisons) (Fig. 28b).

Arc protein expression peaks after 30 min of the first session

Arc protein expression was significantly different among mice groups in both cortex and hippocampus (One-way ANOVA, p < 0.005). In cortex, there was a 4.4-fold increase in the Arc protein expression in the A30 group as compared to the control (Tukey's multiple comparisons, p<0.0001). Additionally, there was a 2.5-fold decrease in the expression of Arc protein in the B120 group as compared to the A30 group (Tukey's multiple comparisons, p<0.005). Also, as compared to the control, there was a 1.7-fold increase in the Arc protein expression in the B120 group (Tukey's multiple comparisons, p<0.05). (Fig. 29a).

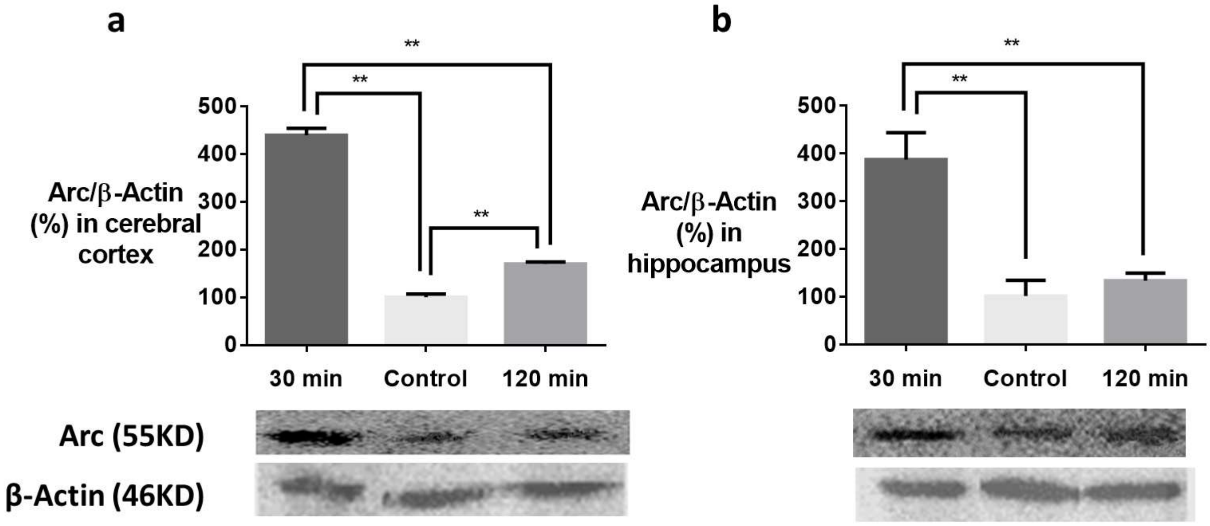

Figure 29. Analysis of expression of Arc protein induced during SAB tasks. (a) In Cortex, there was higher expression of Arc protein in 30 min group than both Control and 120 min groups. Additionally, 120 min group had higher expression than control. (b) Similarly, in Hippocampus, there was higher expression of Arc protein in 30 min group than both Control and 120 min groups. All Bars represent mean ± SEM.

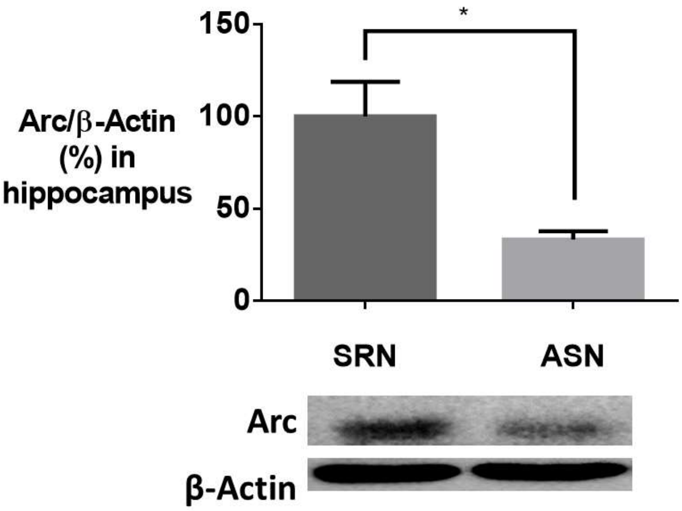

Figure 30. Analysis of expression of Arc protein induced during SAB task in mice infused with ODNs in the dorsal hippocampus. There was around 3-fold decrease in protein expression in Arc-Antisense ODN infused mice (ASN group) than Scramble ODN (SRN group). All bars represent mean ± SEM.

Similarly, in the hippocampus, there was a 3.7-fold increase in the Arc protein expression of A30 group as compared to the control (Tukey's multiple comparisons, $p<0.005$). Additionally, there was a 2.8-fold decrease in the Arc protein expression in B120 group as compared to the A30 group (Tukey's multiple comparisons on dCq values, $p<0.01$). However, we did not find any significant difference in the expression of Arc protein in hippocampus of B120 group as compared to its control (Tukey's multiple comparisons, $p>0.05$) (Fig. 29b).

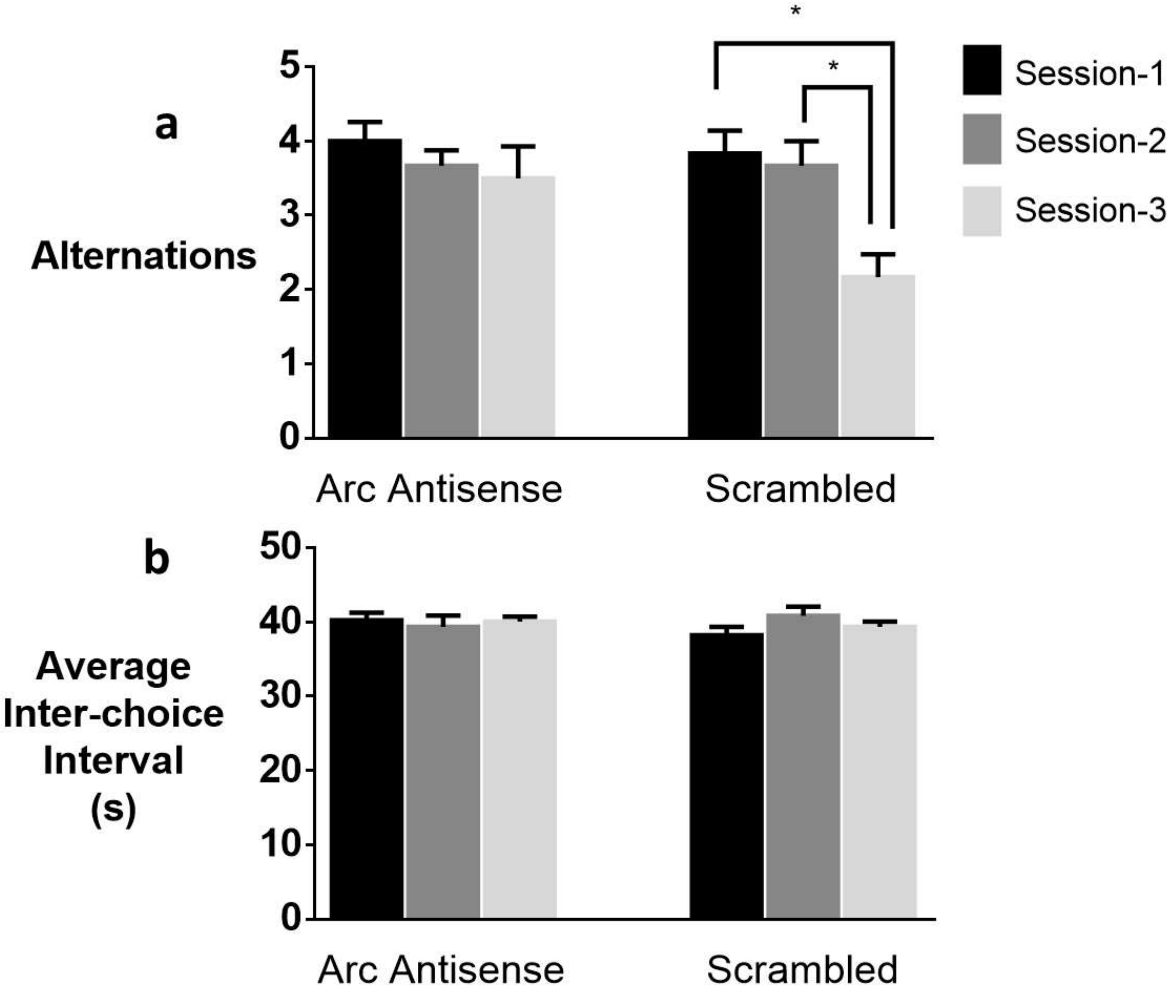

Figure 31. Alternations (a) and average-ICI (b) in the SAB test for mice infused with Arc antisense (ASN) and Scrambled (SRN) ODNs. (a) We found that session-3 alternations were decreased only for SRN but not for ASN group. (b) We found no significant differences in average ICI among the sessions of both the groups. All bars represent mean ± SEM.

Arc antisense ODNs decreases Arc protein expression

There was a three-fold decrease in hippocampal Arc protein expression in ASN group as compared to SRN (control) group ($p=0.02$, t-test) (Fig. 30).

Down-regulation of Arc in mice hippocampus does not decrease the number of alternations

After the confirmation of the decreased Arc level in the hippocampus, we checked alternations in the delayed-SAB test. The number of alternations was significantly different among the sessions of Scrambled ODN infused (SRN) group ($p=0.005$, Kruskal-Wallis test). However, no significant difference was found among the sessions of the Arc-antisense ODN (ASN) infused group ($p=0.60$, Kruskal-Wallis test) (Fig. 31a). Session-3 alternations of SRN group were lower than both its session-1 ($p=0.015$) and session-2 ($p=0.042$) alternations. Additionally, there were no significant changes in alternations between session-1 and session-2 of SRN ($p>0.999$). In contrast, there were no significant differences between any pair of sessions of ASN (all $p>0.83$) (Fig. 31a). For multiple comparisons, Dunn's test was used. On the other hand, there were no significant differences in average ICI among the sessions for both SRN (one-way ANOVA, F (2, 15) = 1.557, p=0.243) and ASN (one-way ANOVA, F (2, 15) = 0.173, p=0.842) groups (Fig. 31b).

DISCUSSION

It is expected that Arc transiently increases in rodents after a novel experience (Vazdarjanova et al., 2002). We observed such transient increases in both SAB and ORM tasks. In both tasks, mRNA expressions were at peak 30 min after novel experience, and it returned to near basal levels at 120 min. Similarly, Arc protein expression was highest at 30 min, and it reached near the basal level at 120 min in SAB task. This concludes that Arc expression is ISI-1 specific.

In cohort with SAB ISI-1 timing and its corresponding Arc expression levels, it suggests that the current level of Arc by itself does not affect the alternation rate during a session. Rather, SAB results imply that previously expressed Arc is necessary for the decrease in the number of alternations (i.e., as seen in session-3). This implies a temporal association of Arc in the working memory paradigm.

To confirm the necessity of Arc for the temporal decrease in alternations, we infused Arc antisense oligodeoxynucleotides (ODNs) using a stereotaxic apparatus into mouse dorsal hippocampus for inhibiting Arc protein expression. We confirmed the downregulation of the protein by Western blotting. We repeated the SAB experiment and observed similar results for control (scrambled ODNs), but not for Arc antisense ODN infusions. The alternations did not decrease in session-3 for Arc-inhibited mice. This confirms the involvement of Arc in the temporal decrease in alternations.

Arc has been suggested to contribute to the familiarization of a novel environment via LTD (Jakkamsetti et al., 2013). Arc decreases the surface-AMPAR expression and reduces the synaptic strength of those neurons, which induced Arc during the previous exposures (Okuno et al., 2012; Park et al., 2008). This facilitates increased relative strengths of synapses during the novel exploration (Kemp and Manahan-Vaughan, 2004; Dong et al., 2012). An earlier study by Nomoto et al., 2016 has shown that there is around 70% of overlapping Arc-positive neurons after exposures to different spatial environments. This implies that the re-exposure to the same environment might activate a greater percent of neurons that induced Arc in the earlier sessions, and thus a significant number of synapses could be weakened. On the other hand, activated synapses without substantial Arc induction could undergo enhanced early-phase LTP (Shepherd and Bear, 2011). Early-phase LTP was strongly induced in the synapses of Arc KO mice, and it can last up to three hours before reaching the baseline (Plath et al., 2006), implying the possibility of stronger expression of early LTP in Arc negative neurons in naïve mice after spatial exploration. In addition, Vazdarjanova et al., 2006 showed that only 20 to 30% of neurons express Arc in the hippocampal regions in naïve rats after spatial exploration in open-field. These studies suggest that early-phase LTP was also maintained in some of the neurons during our experiment's interval (of 2 hours) between session-2 and session-3. Hence, in the naïve mice, the habituation is affected differently due to the parallel incidence of both synaptic weakening and its lack thereof in the distinct neurons; whereas, the habituation would be affected more uniformly in the Arc-inhibited mice. This non-uniformity in naive mice increases the information entropy, and we suggest that this reduces short-term habituation ability during subsequent exposures in the working memory paradigm.

Our present findings on the temporal effects of Arc during the working memory paradigm may also be explained in the light of the Sometimes opponent process (SOP) model proposed by Wagner (Wagner, 2014; Vogel et al., 2019). SOP supposes an inverse relationship between the abilities of short-term habituation and associative memories formation. The short-term habituation results in the alternation behavior (Sanderson and Bannerman, 2012), which was also observed in our study where more habituation to the arms increased alternations rate. We also found no change in alternations with a familiarized environment, a scenario that likely requires lesser new associative memories formation. Interestingly, it has already been demonstrated that the Arc has a role in the facilitation of associative memories (Hudgins and Otto, 2019; Hashikawa et al., 2011). Overall these add to the theory that Arc reduces the short-term habituation ability.

Chapter 6

Involvement of Arc in object recognition memory after re-exposure to a novel environment

INTRODUCTION

Studies through Arc KO mice have shown the involvement of Arc in both temporal order memory and the long-term spatial object recognition memory. Upon re-exposure to the environment, it was found that the existence of Arc mRNA primes mGluR-dependent LTD in earlier activated synapses (Jakkamsetti et al., 2013). In addition, during the exposure to a novel environment, the placement of familiar objects in new locations or unfamiliar objects in any location induces LTD in rats and facilities ORM (Dong et al., 2012; Okuno et al., 2011; Kemp and Manahan-Vaughan, 2004); this suggests that the novel ORM could be affected by the expression of Arc at the re-exposure time. Therefore, in this chapter, we tried to investigate the involvement of Arc in the temporal changes of ORM upon the re-exposures in a modified novel ORM test.

Initially, we analyzed the Arc mRNA expression by RT-qPCR during the temporal scales similar to behavioral test, in both the cerebral cortex and hippocampus. These areas have a role in the recognition memory in both short (~30 to 60 min) and intermediate (~ 3 hours) retention intervals (Antunes and Biala, 2011). Finally, to test whether Arc is necessary for the temporal changes that were observed in ORM, the modified novel ORM task was performed after inhibiting the Arc protein expression in the mouse's dorsal hippocampus via stereotaxic infusion of Arc antisense ODNs. The Arc protein expression in the hippocampus was analyzed by Western blotting.

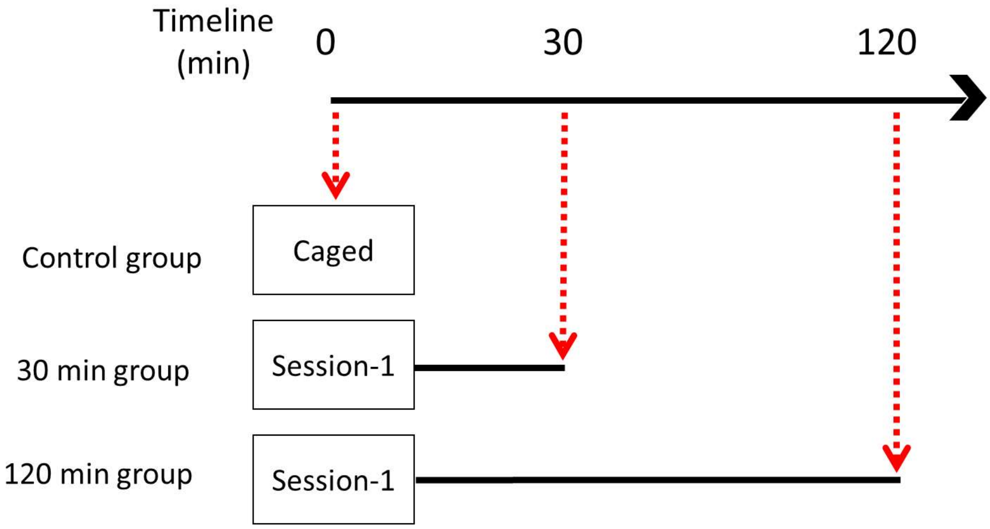

Figure 32. Schematic view of experimental design for Arc mRNA and protein expression study for NORM experiments on naïve mice. Mice were grouped as Control, 30- and 120-min, representing the dissection times after session-1, indicated by the dotted arrows. Control group was dissected at cage.

METHOD

Effect of ISI on Arc expression level

For Arc expression studies, mice were euthanized 30 min or 120 min after session-1 of NORM task. Additionally, control mice were dissected at the cage without any behavior. All mice were sacrificed by cervical dislocation, and their cortex and hippocampus were dissected out quickly in ice-cold saline for mRNA study and hippocampus for protein expression study. Dissection timings were represented by dotted arrows in figure-33. For mRNA study, dCq values are used for statistical comparison. For each sample, the dCq values were calculated as: Cq (Arc) - Cq (GAPDH). For statistical analysis of dCq values, we used one-way ANOVA followed by Tukey's multiple comparisons test. For protein study, the Arc/β-Actin ratio was calculated for all groups. The resultant ratios of each group were compared using one-way ANOVA test and Tukey's multiple comparisons.

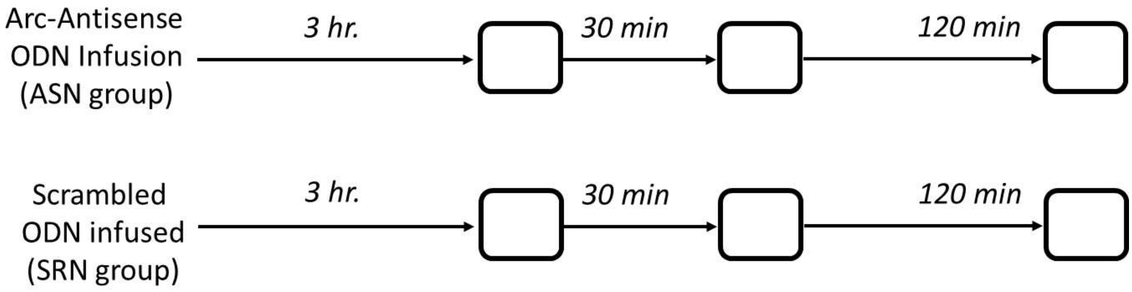

Figure 33. Schematic view of experimental design for NORM test for mice infused with ODNs. ASN (Arc-antisense) and SRN (scrambled) groups underwent NORM test as per the mentioned time intervals.

Effect of Arc inhibition on NORM of AO

Based on the type of ODNs infused in the dorsal hippocampus, mice were divided into two groups (n=5 each): (1) ASN (Arc Antisense-ODN injected) and (2) SRN (Scrambled-ODN injected) (Fig. 34). Three hours post ODN injection, both groups have performed three sessions of NORM test with an ISI of 30 min. The duration of session-1 and session-2 was eight min each, and session-3 (test session) was five min. Objects AO and TO were presented at the test session.

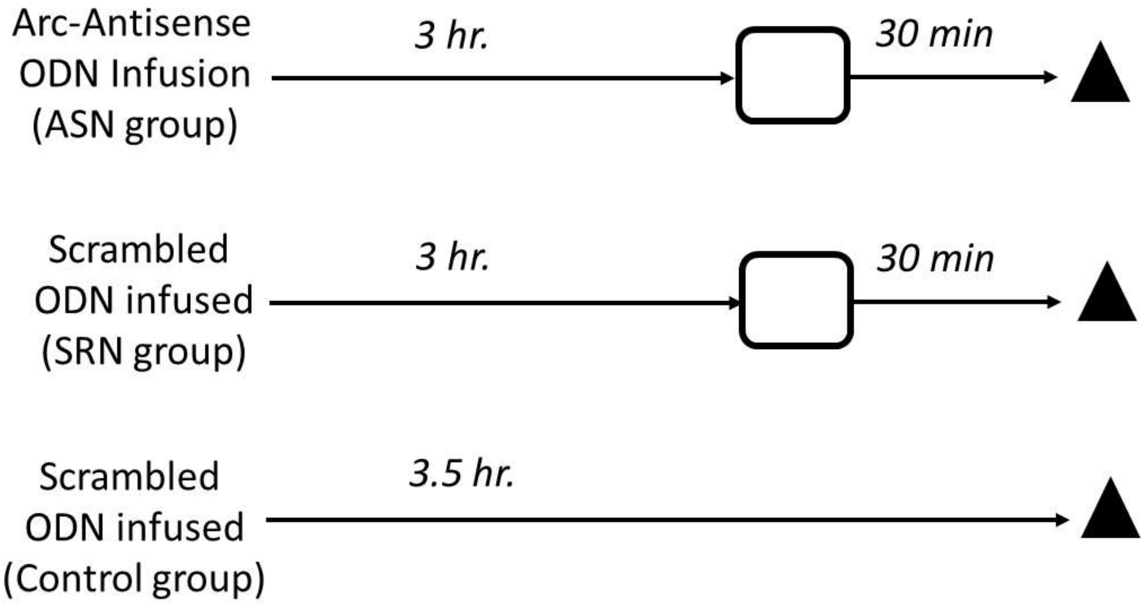

Figure 34. Schematic view of experimental design for Arc protein analysis for the mice infused with ODNs. ASN (Arc-antisense ODN), SRN (scrambled ODN), and Control (scrambled ODN – without behavior session) groups (n=3) were euthanized, at the specific times as represented by triangles, to check the Arc protein expression in the hippocampus.

Western blotting

To confirm the efficiency of infused ODNs, we performed Western blotting of the hippocampus to analyze the Arc protein level. Mice (n=3) were grouped similarly to the NORM test for the Arc inhibited mice (i.e., *ASN* and *SRN*), and they were euthanized by cervical dislocation 30 min after session-1 of NORM test (Fig. 35). A third group, Control, was infused scrambled ODNs but did not undergo any behavior session. All groups were euthanized 3.5 hours post ODN infusions.

RESULTS

Arc mRNA expression was highest at ISI of 30 min after the novel experience

-dCq values were normalized (%) with respect to control. There was a significant difference between -dCq values among mice groups (ISI of 30 min, 120 min, and control) in both cortex (F (2, 6) = 8.722, p=0.0168) and hippocampus (F (2, 6) = 8.851, p=0.0162) (one-way ANOVA, followed by Tukey's multiple comparisons). Arc expression significantly increased at 30 min

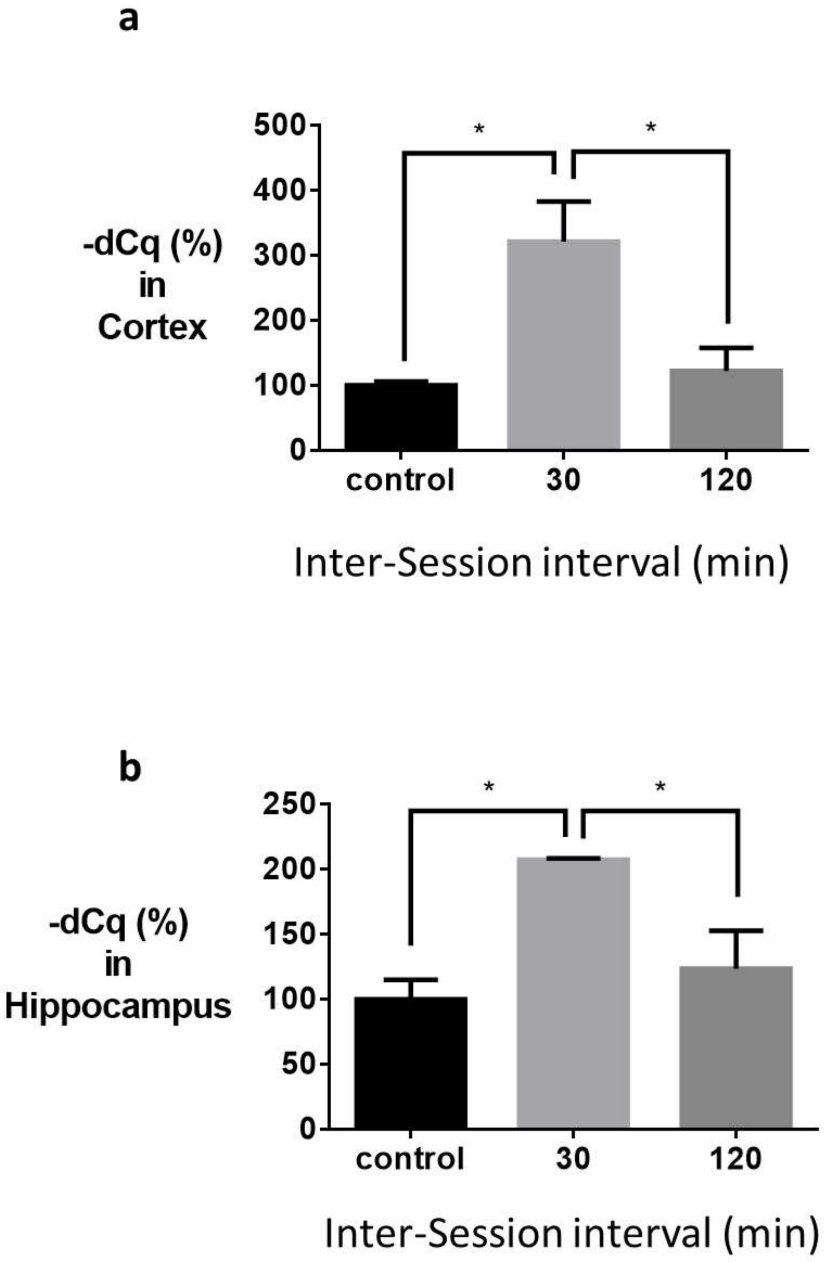

*Figure 35. Analysis of expression of Arc mRNA induced after session-1 of NORM test. In both (a) cortex and (b) hippocampus, the mRNA expression was higher in 30 min ISI group than both the control and 120 min. All bars represent mean ± SEM. *p<0.05.*

ISI, as compared to control, in both cortex (p=0.021) and hippocampus (p=0.016). Additionally, Arc expression significantly decreased at 120 min ISI as compared to 30 min in both cortex (p=0.033) and hippocampus (p=0.046). There was no difference between 120 min ISI and control for both brain regions (p>0.5) (Fig. 36).

Arc protein expression was maximum after 30 min of novel experience

In the hippocampus, Arc protein expression was significantly different among mice groups (ISI of 30 min, 120 min, and control) (one-way ANOVA, F (2, 6) = 12.27, p=0.007). A higher Arc expression was found at 30 min ISI as compared to both control (p=0.009) and 120 min ISI (p=0.017) (Tukey's multiple comparisons). There was no difference between control and 120 min ISI (p=0.84) (Fig. 37).

Infusion of Arc antisense ODNs inhibits Arc protein expression

Arc protein level was significantly different among ASN, Control, and SRN groups (one-way ANOVA, F (2, 6) = 51.58, p=0.0002) (Fig. 38). SRN group (scrambled ODN infused + session-1 of NORM task) showed higher Arc protein expression than that of the Control group (scrambled ODN infused, without ORT) (Tukey's multiple comparisons test, p=0.0003). We found over a four-fold decrease in the hippocampal Arc protein level in the ASN group as compared to the SRN group (Tukey's multiple comparisons test, p=0.0003).

SRN group explored AO significantly more than ASN group

The location preference between the groups: SRN and ASN, was not significantly different (unpaired t-test, p=0.31). There was no significant difference in the distance traveled between the groups (two-way ANOVA, F (1, 8) = 0.023, p=0.88) and among the sessions (two-way ANOVA, F (2, 16) = 0.105, p=0.90) (Fig. 39 a). There was no significant difference in the TET between the groups (two-way ANOVA, F (1, 8) = 0.00137, p=0.97) and among sessions (two-way ANOVA, F (2, 16) = 2.62, p=0.103) (Fig. 39 b). We found no significant difference in the exploration time (%) of object ROa between the groups (57.80% for SRN and 60.72% for ASN; p=0.60, unpaired t-test).

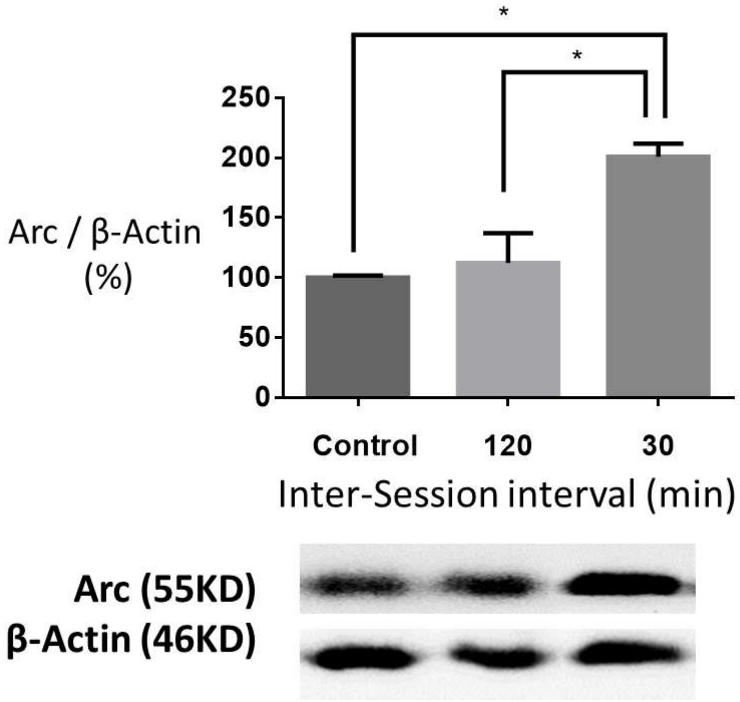

*Figure 36. Analysis of expression of hippocampal Arc protein that was induced after session-1 of NORM test. Protein expression was higher in 30 min ISI than both the control and 120 min. All bars represent mean ± SEM. *p<0.02.*

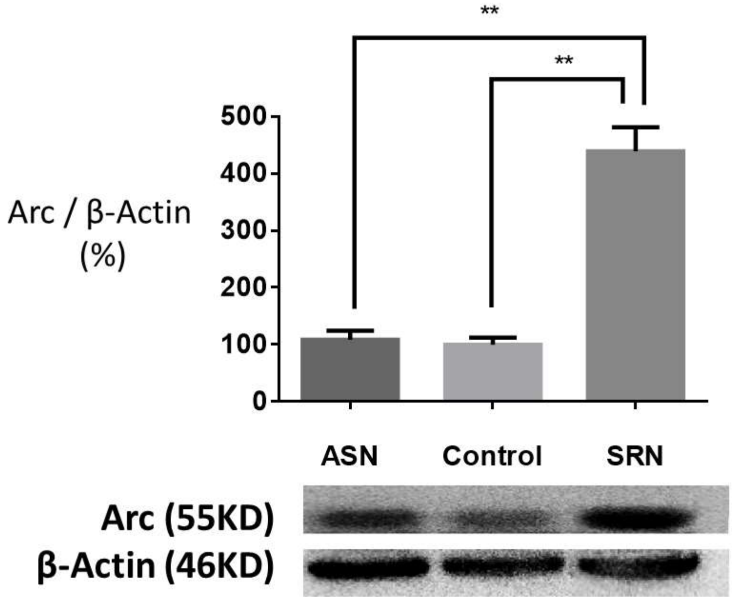

*Figure 37. Analysis of expression of hippocampal Arc protein that was induced after session-1 of NORM test in mice that were infused ODNs. SRN (scrambled ODN infused + NORM trail-1) had higher expression than both Control (scrambled ODN infused, without NORM session-1), and ASN (Arc antisense ODN infused + NORM session-1). All bars represent mean ± SEM. ** p<0.0005.*

There was a significant interaction between the groups and the sessions (two-way ANOVA, F (1, 8) = 5.751, p=0.043). For both groups, object AO exploration (%) was less in the session-3 as compared to its session-1 (Holm-Sidak multiple comparisons, p=0.042 (for SRN), and p=0.0008 (for ASN). Importantly, in session-3, SRN mice have explored the object AO more than ASN mice (Holm-Sidak multiple comparisons, p=0.013). There was no difference in AO exploration in session-1 between both the groups (p=0.98) (Fig. 39 c).

DISCUSSION

In cohort with ORM ISI timing and its corresponding Arc expression levels, it suggests that the current expression level of Arc during the repeated exposure to the environment (i.e., second exposure in our case) – but not the object – influences the memory retention ability of that object at the later phase. This is different from the single exposure memory tests that require Arc for memory consolidation (i.e., long-term storage). The results of ORM and Arc expression studies suggest that Arc is necessary for greater decay of ORM.

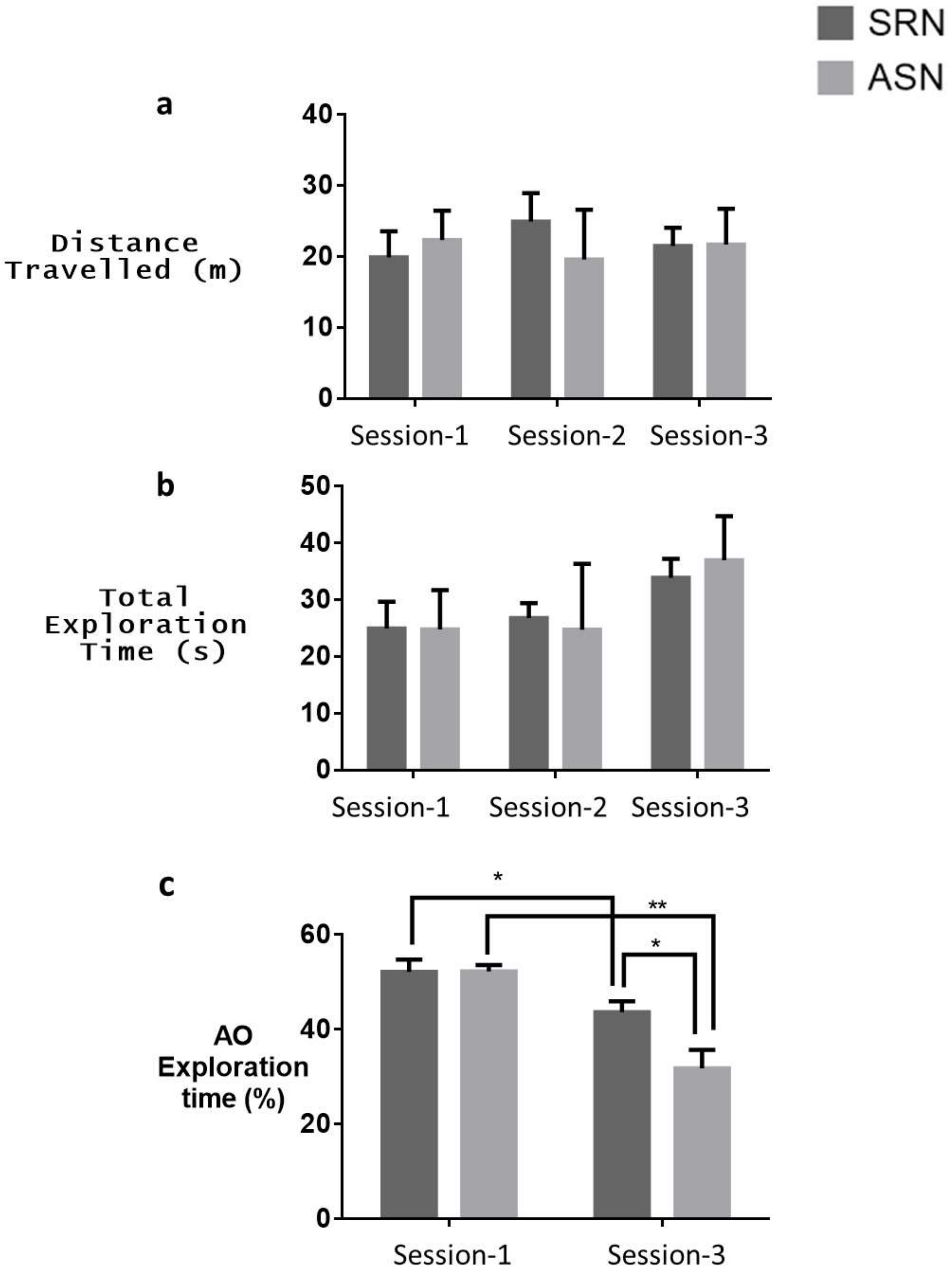

*Figure 38. Analysis of parameters of NORM test for ODN infused mice. There was no difference between the groups and among the sessions in (a) Distance travelled, and (b) TET by mice. (c) Exploration time (%) of object AO by mice at session-1 and session-3. Both the groups decreased their AO exploration at session-3, however, SRN mice have explored AO more than ASN mice. All bars represent mean ± SEM. * p<0.05, ** p=0.0008.*

To confirm the necessity of Arc in greater memory decay, we inhibited its protein expression using the stereotaxic infusion of Arc antisense ODNs in the dorsal hippocampus. Both control (scrambled ODN-infused) and Arc-inhibited mice decreased their exploration of alternating-object at test session (i.e., session-3). This shows that some memory of the alternating-object was present. However, Arc-inhibited mice explored that object significantly more than control ones. This confirms the involvement of Arc in accelerated decay of object memory occurring upon repeated exposure to the environment without the object. We have ruled out the association of other parameters, viz., distance traveled and TET, with the observations, as they are similar for control and Arc-inhibited mice.

During object familiarization, the novel environment was used in our ORM tasks to facilitate the dorsal hippocampus involvement (de Lima et al., 2006; Antunes and Biala, 2011). Moreover, we also tested mice behavior in ORM test apparatus in a similar temporal design (including ISI) but without the objects. When each session was of five min duration, the distance traveled did not decrease as sessions increased, which suggests that the decrease in distance traveled by mice that was observed during ORM tasks (same five min per session) was primarily influenced by the presence of objects rather than the surrounding environment. However, when the duration of the session was 30 min, there was a significant decrease in the distance, indicating a greater familiarization of the environment. Additionally, TET decreased with an increase in sessions in TOM tasks, but not in novel ORM tasks, the difference being the presence of a novel object in ORM tasks. Under these novel environments, we found that mice did not discriminate a familiar object that was present in a novel location. Overall, this shows that distance traveled and exploration time are predominantly decided by the familiarization of object than the environment.

Conclusion

In the first objective, we studied the temporal changes in the alternation behavior of mice after re-exposures to a novel or familiar environment. Initially, we found that the alternation rates increase with the duration of exposure to the novel environment in a short time-scale (< min). It suggests that short-term habituation to an environment increases the alternation rates within it. Later, we showed that the alternation rates decrease after the short inter-session interval after repeated exposure in a novel environment, but not in the familiar one. In the second objective, we studied the temporal changes in object recognition memory (ORM) of mice at different inter-exposure intervals after re-exposures to an environment. We showed that the object recognition memory, during re-exposure to the environment without that object, depends on the inter-exposure interval, with shorter interval showing decreased object recognition memory. In the third objective, we studied the involvement of Arc on the temporal changes in alternation behavior. Initially, we showed that the behavior task increases both Arc's mRNA and protein expression in both cortex and hippocampus. We found that the expression level of Arc during the re-exposure session is in cohort with the alternation rates at the subsequent session; a higher Arc expression during re-exposure resulted in the drop of alternations at the next session. After inhibiting Arc's protein expression using Arc's antisense oligodeoxynucleotides, we showed that Arc decreases the spontaneous alternations in the subsequent sessions. We also suggest the Arc has reduced the ability of short-term habituation. In the final objective, we studied the involvement of Arc on the ORM after re-exposures to an environment. Initially, we showed that the behavior task increases both Arc's mRNA and protein expression. We found that the expression level of Arc during the re-exposure session is in cohort with ORM; a higher Arc expression during re-exposure resulted in a decrease in ORM. After inhibiting Arc's protein expression using Arc's antisense oligodeoxynucleotides, we showed that Arc decreased the ORM of an object when mice were re-exposed to the environment without that object.

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Publications from thesis

Brain Research Bulletin 158 (2020) 51–58

Contents lists available at ScienceDirect

Brain Research Bulletin

journal homepage: www.elsevier.com/locate/brainresbull

Research report

The temporal effect of hippocampal Arc in the working memory paradigm during novelty exploration

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ARTICLE INFO

Keywords:
Working memory
Spontaneous alternations
Novel experience
Arc
Temporal effects

ABSTRACT

Arc (activity-regulated cytoskeleton-associated protein) is one of the neuronal Immediate Early Genes (IEG), which is involved in the consolidation of memory and is an essential factor in the induction of Long-term Potentiation (LTP), Long-term Depression (LTD) and homeostatic synaptic plasticity. It has also been implicated in the increased familiarization of novel environments during reference memory paradigms. However, the Arc associated temporal effects in a working memory paradigm during novelty exploration are not well studied. Therefore, in the present study, we used spontaneous alternation behavior (SAB) test along with the expression analysis of Arc to study its temporal effects on the working memory paradigms. Using a modified SAB test, we found that the increase in the duration of exposure to a novel environment in the short time-scale (< min) increases the alternations showing that short-term habituation increases the alternation rate. Additionally, during repeated exposure to a novel environment, the alternation rates decrease after shorter inter-session interval. Parallelly, we observed the upregulation of Arc mRNA and protein level 30 min after the SAB test in the cortex and hippocampus of mice, which returns to near-basal level after two hours. The novel experience, associated with the enhanced expression of Arc, helps in the decrease of alternations in subsequent sessions. This change in alternations was absent if the environment was familiar. Further, the role of Arc during these SAB test was confirmed by the inhibition of hippocampal Arc protein through the stereotaxic infusion of Arc antisense oligodeoxynucleotides. We observed that the Arc is involved in the temporal decrease of spontaneous alternations during a series of exposures to a novel environment. Finally, the significance of these results has been discussed in the light of Wagner's Sometimes Opponent Processes model, where we suggest that Arc reduces the ability for short-term habituation during repeated exposures in the working memory paradigm, and the loss of this ability is more prominent when subjected to a novel environment.

1. Introduction

Immediate Early Genes (IEGs) encode transcription factors and effector proteins to regulate different cellular functions by targeting various molecules, including the growth factors, metabolic enzymes, cytoskeletal proteins, etc. (Minatohara et al., 2016). Those IEGs which are exclusively or predominately expressed in the brain (neuronal IEGs) play an important role in maintaining the dendritic spine density, regulation of spine morphology and the induction of LTP/LTD; making these IEGs an important factor for learning and memory studies (Lanahan and Worley, 1998; Peebles et al., 2010). Activity-regulated cytoskeleton-associated (Arc) gene is one of the neuronal IEGs that is rapidly upregulated after neuronal activation (Link et al., 1995; Lyford et al., 1995) and it is involved in synaptic (LTP and LTD) and homeostatic plasticity (Shepherd et al., 2006; Bramham et al., 2010).

The consolidation of long-term spatial memory (LTM) is also found to be impaired in Arc KO mice (Plath et al., 2006; Peebles et al., 2010; Yamada et al., 2011). Arc is essential for both the induction and consolidation of LTP and is required for maintaining the late-phase LTP (Messaoudi et al., 2007). Arc interacts with endophilin and dynamin to form a protein complex, which is involved in the AMPAR (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor) endocytosis. This process results in a decrease of surface-AMPAR expression and thereby induces LTD (Plath et al., 2006; Chowdhury et al., 2006; Park et al., 2008). Shepherd et al. also observed the absence of neuron-wide homeostatic synaptic scaling of AMPAR that contains GluR1 (Glutamate receptor-1) in Arc KO neurons (Shepherd et al., 2006). Due to its tight regulation in a temporal and region-specific manner, the detection of Arc product is a powerful tool to identify neural substrates of cognitive processes (Sauvage et al., 2013). It is also one of the plasticity-related

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https://doi.org/10.1016/j.brainresbull.2020.02.013
Received 18 December 2019; Received in revised form 30 January 2020; Accepted 25 February 2020

proteins (PRP), whose expression is required for the plasticity of synaptic connections (Rao et al., 2006). Arc is needed for persistent firing in NMDAR (N-methyl-D-aspartate receptor) -activated cortical neurons, and therefore, it is an essential substrate for general working memory retention (Ren et al., 2014).

After experiencing a novel environment, the Arc mRNA is transcribed in the nucleus within a short period (about 5–15 min). Some of these mRNA gets translated into Arc protein in the dendritic spines, which contributes to modification in the spine morphology (Steward et al., 1998; Vazdarjanova et al., 2002; Guzowski et al., 2006; Kitanishi et al., 2009; Minatohara et al., 2016). If metabotropic glutamate receptors are active in these spines, then Arc protein induces the mGluR-dependent LTD via AMPAR endocytosis (Park et al., 2008; Jakkamsetti et al., 2013). Apart from the dendritic spine's Arc, the cytoplasmic-translated Arc binds to inactive CaMKIIβ (Ca2 + /calmodulin-dependent protein kinase II-β) in the spines to induce LTD via AMPAR endocytosis (Okuno et al., 2012). Such kind of correlation between Arc and LTD was observed temporally during novel exploration of reference memory paradigms in rats and facilitated in the familiarization of those novel environments (Kemp and Manahan-Vaughan, 2004; Dong et al., 2012). However, there is a complete lack of studies to show the association between temporal effects in working memory paradigm and Arc expression. Therefore, in the present study, we aimed to study the temporal effects of Arc on the working memory.

To test the temporal effects on working memory, we used spontaneous alternation behavior (SAB) phenomenon using Y-maze. SAB is a widely used behavioral test to assess the spatial working memory in rodents (Hughes, 2004). SAB is a disposition for mice to alternate their successively presented routes (choices). Mice have to maintain the information of the previously visited route transiently, use it while making the next choice, and update the memory to the recent choice during this SAB phenomenon. For precise assessment of time-dependent effects on working memory and corresponding Arc level, we used a modified SAB test-procedure. Scopolamine is a widely used neuropharmacological drug, which acts as a nonselective muscarinic receptor antagonist for inducing the working memory impairment (Bolden et al., 1992). Therefore, we used a scopolamine-treated mice group on the modified Y–maze as a negative control for alternations. In this study, we analyzed the expression of Arc mRNA by qPCR and Arc protein by Western blotting in both cortex and hippocampus (the two major brain regions involved in spatial working memory) of these mice. Furthermore, we confirmed the involvement of Arc in the temporal changes of working memory by suppressing the Arc protein expression in the mice hippocampus by infusing Arc antisense oligonucleotides via stereotaxic apparatus.

2. Methods

2.1. Materials

Scopolamine hydrochloride (catalog# S1013), Tri-reagent (catalog#93289), and polyvinyl difluoride membrane (PVDF) (catalog# 3010040001) were purchased from Merck, Germany. DreamTaq Green PCR Master Mix (2X) kit (catalog# K1081) was purchased from Thermo Fisher Scientific, India. PrimeScript 1 st strand cDNA synthesis kit (catalog#6110A) was purchased from Takara Bio Inc., India. PCR gene-specific primers were manufactured locally by Cassia Siamia Tech. HI-SYBR Green Master Mix (catalog# MBT074) for Quantitative-PCR was purchased from Himedia Laboratories, India. Arc mouse monoclonal antibody (catalog# sc-17839) was purchased from Santa Cruz Biotechnology, USA; β-Actin mouse monoclonal antibody (catalog# 3700S) and HRP conjugated – Anti-mouse IgG Rabbit monoclonal antibody (catalog# 58802S) was from Cell Signal Technology, USA. ECL Reagent (catalog#WBLUC0100) was bought from Millipore, USA. Arc antisense and scrambled oligodeoxynucleotides for the stereotaxic experiment were purchased from GCC-Biotech, India.

2.2. Animals

Swiss Albino, male, inbreed mice, aged 15 ± 3 weeks, were used in this study. Mice were maintained in the animal house of the University of Hyderabad. Mice were exposed to a 12 -h dark/light schedule, and the food and water were provided to them ad libitum. Mice euthanasia was carried out through cervical dislocation. Approval for animal experiments was taken from the institute's animal ethics committee (IAEC/UH/151/2017/05/AG/P11).

2.3. Working memory

2.3.1. Apparatus

Spontaneous alternation behavior (SAB) was used to test working memory performance. The Y-maze apparatus was constructed from black painted wood and consisted of three gated arms with equal angles among them. The dimensions of the maze (30 × 20 x 7 cm of length, width, and height, respectively) were chosen as per Deacon and Rawlins (2006). The wooden gates of 5 mm thickness were vertically slidable into the grooves. The grooves were present inside all the three arms around 2 cm from the open end of the arm through which the mouse enters (Supplementary Fig. 1). The maze was bottomless and kept on a grey wooden table for the behavior-testing of mice. There were three white-light bulbs, each 36 W, located two meters above the maze to provide illumination.

2.3.2. Modification of SAB testing procedure

Two types of SAB are generally used for testing the alternation rates: i) Two-trial SAB (through T- and Y- mazes) and, ii) Continuous SAB (through Y-maze) (Hughes, 2004). However, their alternation rates are usually low (~ 65%) (Deacon and Rawlins, 2006). We tried to increase the alternations by introducing a delay component in continuous SAB procedure, referred to as 'delayed-SAB' in this study.

Initially, mice were placed in the center of the Y-maze with all the gates open, and they were allowed to enter one of the three arms (referred to as first choice). Then the entrance of that arm was closed for 30 s (delay time) using a gate. After that delay, the gate was re-opened for the mice to make the next choice. This procedure was continued until the mouse made seven choices. The gates were closed for 30 s after the mice made every choice. Re-visiting the same arm is not considered as a different choice. Mice were removed from the maze after they made the seventh choice. A choice was made if the entire animal body (excluding tail) crosses the groove. This whole procedure constitutes one session. Every session had two parameters: the alternation rate and the inter-choice interval (ICI). A mouse was said to have alternated if it chooses a different arm than the previous one. ICI was defined as the time interval between two consecutive choices.

2.3.3. Delayed-SAB test

To test the effect of inter-choice delay on the number of alternations, the mice were divided into three groups: (i) *No-Delay*, (ii) *Delay*, and (iii) *Scopolamine-Delay*. *No-Delay* group was injected saline (i.p. 0.9% w/v) and underwent continuous SAB test. In continuous SAB, mice were not constrained by any delay between their choices. *Delay* group was injected saline (i.p. 0.9% w/v) and underwent the delayed-SAB test. *Scopolamine-Delay* group was injected with scopolamine hydrochloride (i.p. 3 mg/kgbw), and they underwent the delayed-SAB test. The scopolamine treated group acts as a negative control for alternations in SAB tests (Hughes, 2004). All tests were conducted two-and-half hours after the injections. All groups were allowed to make seven choices, and a delay of 30 s was used, except for the No-Delay group. For each mouse, we used (1) the number of alternations, and (2) average ICI (which is the mean of all ICIs) as the parameters to analyze the test result. Both parameters were measured using frame-by-frame analysis using a standard video player. For statistical analysis, we used the Kruskal-Wallis test followed by Dunn's multiple comparisons (posthoc test).

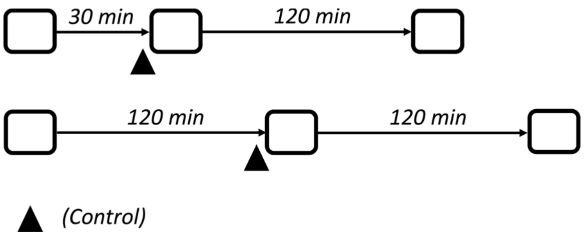

Fig. 1. Each rectangular box represents a session of a 7-choice delayed-SAB test. Inter-session interval-1 (ISI-1) was defined as an interval between session-2 and session-1. Mice are grouped based on their ISI-1 30 min or 120 min. Both groups underwent three sessions of testing. Each triangle represents the time when an independent set of mice were euthanized for Arc expression analysis. They were euthanized (i) 30 min after session-1, (ii) 120 min after session-1, or (iii) without any session (control).

2.4. Assessment of the temporal changes in alternations and corresponding Arc expression under a novel environment

2.4.1. Behavior

Mice performed three sessions of the 7-choice delayed-SAB test. Inter-session interval-1 (ISI-1) is defined as the time interval between session-2 and session-1. We tried to study the effect of ISI-1 on both the alternation rate and the average-ICI at both session-2 and session-3. Hence, mice were divided into two groups based on the ISI-1, 30 min (n = 8) and 120 min (n = 8), as shown in Fig. 1. The selection of intervals was based on our preliminary studies, where various ITI-1 intervals were tested, and their performances were similar to one of the selected intervals.

2.4.2. Arc expression

For Arc expression studies, mice were euthanized 30 min or 120 min after session-1 of delayed-SAB. Control mice were dissected at the cage without any behavior test. All mice were sacrificed by cervical dislocation, and their cortex and hippocampus were dissected out quickly in ice-cold saline for mRNA and protein expression studies. Dissection timings were represented by triangles in Fig. 1.

2.4.2.1. Reverse transcription-quantitative PCR. Total RNA was isolated from the cerebral cortex and hippocampus and further quantified similarly to the protocol mentioned earlier by Gautam et al., 2013. RNA samples with A260/280 ≥ 1.8 were used further for qPCR. Initially, 0.5 μg of mRNA was reverse transcribed into total cDNA. Using this cDNA as a template and gene-specific primers for Arc and GAPDH (reference gene), the amplicon was amplified by q-PCR. The PCR sample mixture was prepared by mixing cDNA sample (1 μl), forward and reverse primers (2 μl each of 1 μM), and SYBR Master Mix (SYBR Green dye, Taq polymerase, and dNTPs) (5 μl). PCR cycle was standardized and performed according to the manufacturer's recommendations (Denaturation: 95o C for 40 s, Annealing: 57o C for 40 s, Extension: 72o C for 60 s and 40 cycles). The primer sequence was Arc-forward (ACGATCTGGCTTCCTCAT TCTGCT), Arc-reverse (AGGTTCCCTCAGCATCTCTGCTTT), GAPDH-forward (GT CTCCTGCGA CTTCAG), and GAPDH-reverse (TCATTGTCATACCAGGAAATGAGC) (Barnhart et al., 2015; Gautam et al., 2013). Arc and GAPDH had a single peak at melt-curve analysis, and the amplified product was analyzed on 1.5% Agarose gel to confirm the sizes of amplicons. The quantification cycle (Cq) was measured using qPCR machine (Himedia's Insta Q96) automatically. Any improper baseline and threshold assignments were corrected. A three-point 10-fold dilution series for both the genes was performed to find out the efficiency of PCR amplification. PCR efficiency for GAPDH was 92.64% with correlation (r) = - 0.995 and slope = -3.51. PCR efficiency for Arc was 97.94% with correlation (r) = - 0.999 and slope = -3.37. We added no-template control for both the genes and their Cq values > 30.0. The Cq of No-Template control is more than 3.2 cycles from the highest Cq of the highly diluted samples. The dCq values were calculated as Cq (Arc) - Cq (GAPDH) for each sample and were used further for statistical analysis. A higher dCq value implies lower Arc expression and vice versa.

2.4.2.2. Western blotting. 10% protein homogenate of cortex and hippocampus in RIPA lysis buffer was prepared and quantified similarly to the protocol mentioned by Gautam et al., 2013. 40 μg of protein mixed with 6X denaturing sample buffer, and it was loaded in 10% SDS-PAGE. The resolved protein bands in the gel were trans-blotted onto the PVDF membrane using tank electroblotting apparatus. The membranes were initially blocked in 5% (w/v) fat-free milk (blocking-buffer) for one hour. Then, they were incubated with Arc mouse monoclonal antibody (1:400) or β-Actin mouse monoclonal antibody (1:10,000) for one hour at room temperature. The specificity of antibodies was confirmed earlier. Membranes were washed thrice with 0.5% Tween-TBS for 5 min each. Membranes were incubated in HRP conjugated – anti-mouse IgG rabbit monoclonal antibody (1:2000) for two hours at room temperature. Finally, membranes were washed thrice in 0.5% Tween-TBS for 10 min each and incubated in ECL reagent for 2 min to detect the chemiluminescence signal using the Bio-Rad's ChemiDoc™ imaging system. Membranes were stripped by incubating in 0.2 M NaOH for 10 min. The Arc/β-Actin ratio was calculated for all groups.

2.5. Effect of familiarity of the environment on the alternations

To test if the changes in alternations as observed in the previous experiment (section: 2.4.1) were due to ISI-1 only and not affected by the novelty of environment, three experiments were performed based on how recently (2.5, 8, or > 24 h) mice were exposed to the environment before they performed test session (session-3) (Fig. 2).

(1) Mice (n = 6) were habituated to the maze for 20 min on day-1, and they performed the delayed-SAB test on day-2. This mice group had ISI-1 of 30 min, and it will test if familiarity (at long time-scale) is a factor for the changes in alternations. (2) Another independent group of mice (n = 8) performed repeated delayed-SAB test every 120 min for five sessions. This mice group faced a novel Y-maze. For analysis, we only considered the last three sessions (referred to as session-1, -2, and -3) to keep statistical analysis similar to other experiment's multiple comparisons. Hence, this group performed session-3 eight hours after novel exposure. The initial two sessions will be considered as part of familiarization. This group will test if familiarity (at intermediate time-scale) is a factor for the changes in alternations. (3) Another independent group of mice (n = 6) performed session-3 150 min after novel exposure. However, this group had ISI-1 of 120 min. This group will test if ISI-1 is a factor for the changes in alternations for a given period since novel exposure (Fig. 2).

2.6. Effect of Arc inhibition on alternations

2.6.1. Stereotactic surgery

The translation of Arc was inhibited in the dorsal hippocampus by the stereotactic surgery using the Arc antisense or scrambled

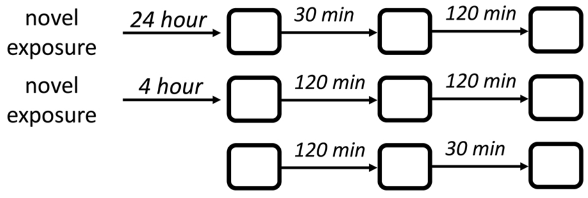

Fig. 2. Based on how recent they were exposed to a novel environment (if any), three independent mice groups performed three sessions of delayed-SAB each.

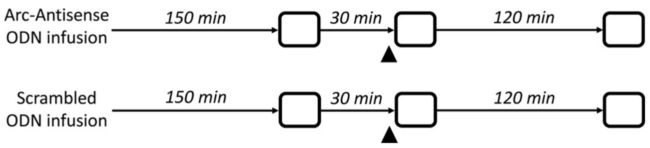

Fig. 3. Each rectangular box represents a session of a 7-choice delayed-SAB test. Mice were divided into two groups based on the type of ODNs were infused: (i) Arc-Antisense ODN, and (ii) Scrambled ODN. Each group underwent three sessions with the same inter-session intervals. Each triangle represents the time when an independent set of mice were euthanized for Arc protein expression analysis; these mice did not undergo session-2.

oligodeoxynucleotides (ODNs) similar to our previous study (Gautam et al., 2016). However, the coordinates for bilateral infusion of ODNs were chosen as -2.46 mm anterior-posterior from bregma, ± 1.35 mm medial-lateral, and 1.35 mm dorsal-ventral (Franklin and Paxinos, 2001). Sequences of ODNs were:

Arc-Antisense: 5–GTCCAGCTCCATCTGCTCGC–3 and
Scrambled: 5–CGTGCACCTCTCGCAGCTTC–3

2.6.2. *Spontaneous alternation behavior*

Based on the type of ODNs infused, mice were divided into two groups ($n = 6$): (1) ASN (Antisense ODN-injected) and (2) SRN (Scrambled ODN-injected) groups. 2.5 -h post ODN injection, each group performed three sessions of the delayed-SAB test, with ISI-1 of 30 min and faced a novel Y-maze (Fig. 3). As alternations were not affected for mice group with ISI-1 of 120 min, we did not choose it for further studies.

2.6.3. *Western blotting*

To confirm the efficiency of infused ODNs, we performed Western blotting of the hippocampus to analyze the Arc protein level. An independent set of mice ($n = 3$), grouped similarly to the above behavioral test (i.e., ASN and SRN), were euthanized by cervical dislocation 30 min after session-1 of delayed-SAB (without performing session-2) (Fig. 3).

2.7. *Statistical analysis*

The statistics were performed using GraphPad's Prism (ver. 6.01). The significance was set at 5%. All the p-values mentioned, along with multiple comparison tests, were adjusted p-values as performed by the software. For statistical significance, the adjusted p-value was set at 0.05.

3. Results

3.1. *Inter-choice delay increases the alternations*

We examined the effect of inter-choice delay on the alternations through the delayed-SAB test. We observed a significant difference in the number of alternations among No-Delay, Delay, and Scopolamine-Delay groups ($p = 0.005$, Kruskal-Wallis test, and Dunn's multiple comparisons). Delay group had higher alternations than both Scopolamine-Delay ($p = 0.011$) and No-Delay ($p = 0.028$). There was no difference between other groups ($p > 0.99$) (Fig. 4a). We observed a significant change in the average ICI among the groups ($p = 0.0005$, Kruskal-Wallis test and test and Dunn's multiple comparisons). No-Delay group had lower average ICI than both Delay ($p = 0.001$) and Scopolamine-Delay ($p = 0.003$). There was no difference between other groups ($p > 0.99$) (Fig. 4b).

3.2. *ISI-1 of 30 min affected session-3 alternations*

We assessed the effect of ISI-1 (of 30 and 120 min) on both the alternation rate and the average-ICI at session-2 and 3. There was a significant difference in alternations among sessions of 30 min ISI-1 group ($p = 0.002$, Friedman test) (Fig. 5a). However, there was no such difference for the 120 min ISI-1 group ($p = 0.876$, Friedman test) (Fig. 5b). Among the sessions of 30 min ISI-1 group, session-3 alternations were reduced as compared with session-1 ($p = 0.008$, Dunn's multiple comparisons). No differences were found between other pairs of sessions (Fig. 5a). There was no difference in average ICI among the sessions for both groups ($p = 0.57$ for 30 min ISI-1; $p = 0.54$ for 120 min ISI-1; repeated-measure ANOVA) (Fig. 5c and d).

3.3. *Arc mRNA shows maximum expression after 30 min of the first session*

The expression level of Arc mRNA was measured in the cortex and hippocampus during the temporal changes in alternations under the novel environment. dCq values were significantly different among control, 30 min, and 120 min groups in both cortex (ANOVA, $F(2,6) = 20.4$, $p = 0.0021$) and hippocampus (ANOVA, $F(2,6) = 126.7$, $p < 0.0001$). In cortex, 30 min group has lower dCq than both control ($p = 0.0029$), and 120 min ($p = 0.0045$). However, dCq was similar between control and 120 min ($p = 0.878$) (Fig. 6a). Similarly, in the hippocampus, 30 min group has lower dCq than both control ($p < 0.0001$) and 120 min ($p < 0.0001$). Additionally, 120 min has lower dCq than control ($p < 0.023$) (Fig. 6b). Tukey's multiple comparisons test was used.

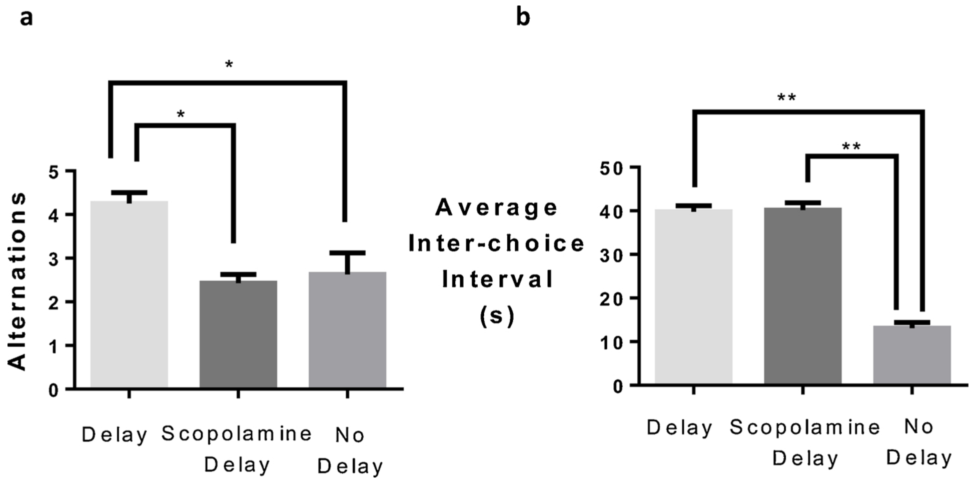

Fig. 4. (a) Alternations and **(b)** average ICI were higher for Delay-group with respect to No-Delay. As expected, Scopolamine-Delay (negative control) showed lower alternations, validating the test. All bars represent mean ± SEM.

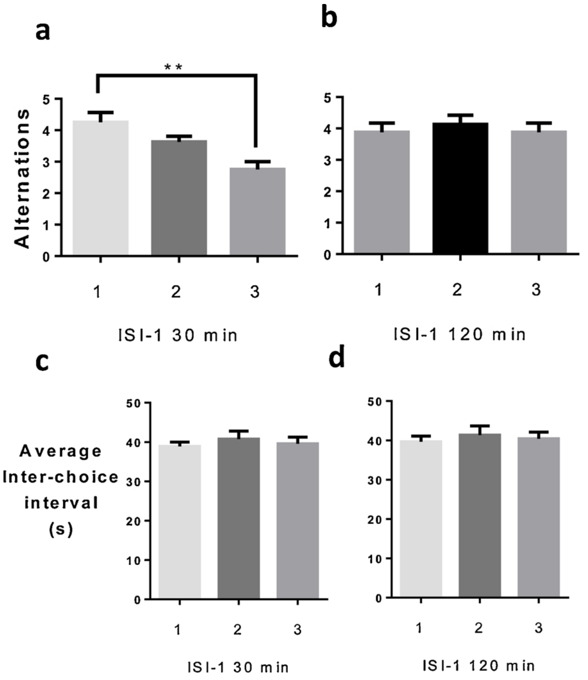

Fig. 5. Comparison of alternations **(a, b)** and average ICI **(c, d)** across sessions (at X-axis) in mice that performed the delayed-SAB test with **(a, c)** ISI-1 of 30 min and **(b, d)** ISI-1 of 120 min. ISI-1 represents the interval between session-2 and -1. There was a significant difference in the number of alternations among sessions of mice group ISI-1 30 min, but not for 120 min. There was no difference in average ICI among the sessions for both ISI-1 s. Both groups were presented novel maze at session-1. ** $p < 0.01$. All bars represent mean ± SEM.

3.4. Arc protein shows maximum expression after 30 min of session-1

In parallel, the expression level of Arc protein was also checked in the cortex and hippocampus during the temporal changes in alternations under the novel environment. Arc protein expressions were significantly different among mice groups in both cortex and hippocampus ($p < 0.005$, one-way ANOVA). As compared to Control, there was a 4.4-fold and 1.7-fold increase in protein expression in the 30 min group ($p < 0.0001$) and 120 min group ($p < 0.05$), respectively. There was a 2.5-fold decrease in 120 min group than 30 min group ($p < 0.005$) (Fig. 7a). Similarly, in the hippocampus, as compared to Control, there was a 3.7-fold increase in protein expression in the 30 min group ($p < 0.005$). There was a 2.8-fold decrease in 120 min group than 30 min group ($p < 0.01$). There was no difference between Control and 120 min groups (Fig. 7b). Tukey’s multiple comparisons test was used.

3.5. Effect of familiarity on alternations

As mentioned in Section 2.5, the following experiments were performed and analyzed to test the effect of familiarity of the environment on alternations.

3.5.1. Familiarization 24 h before does not decrease alternations

For mice that were previously habituated to Y-maze, there was no change in the number of alternations ($p = 0.76$, Friedman test) (Fig. 8a) and average ICI ($p = 0.44$, ANOVA, F (1.726, 8.63) = 0.85) among their sessions (Supplementary Fig. 2a).

3.5.2. Familiarization 8 h before does not decrease alternations

Even though there was a significant main effect in the number of alternations among the sessions ($p = 0.0085$, Friedman test), there was no significant difference between individual sessions through multiple comparisons ($p > 0.05$, Dunn's multiple comparisons) (Fig. 8b). There was no difference in average ICI among sessions ($p = 0.22$, ANOVA, F (2.373, 16.61) = 1.614) (Supplementary Fig. 2b).

3.5.3. ISI-1 of 120 min does not decrease alternations

There was no difference in number of alternations ($p = 0.66$, Friedman test) (Fig. 8c) and average ICI ($p = 0.32$, F (1.974, 9.872) = 1.244, ANOVA) (Supplementary Fig. 2c) for group with ISI-1 of 120 min that faced novel maze 2.5 h before session-3.

3.6. Effect of Arc inhibition on alternations

To confirm the role of Arc to the changes in alternations, the synthesis of Arc protein was inhibited by Arc antisense ODNs in the hippocampus, and thereafter the changes in alternations were measured.

3.6.1. Arc-antisense ODNs decreased Arc protein expression

First, we confirmed the downregulation of Arc protein by stereotaxically infused Arc antisense ODNs through Western blotting. We found around a three-fold decrease in hippocampal Arc protein

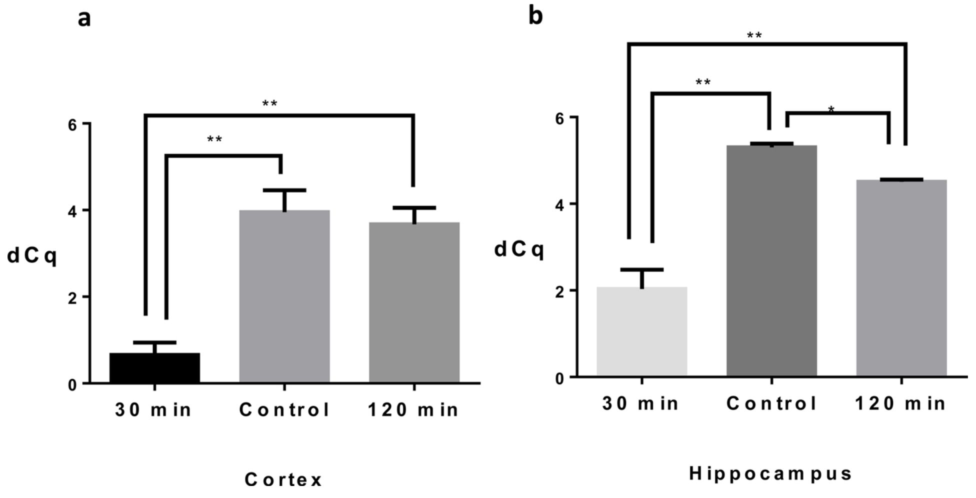

Fig. 6. Analysis of Arc mRNA level by qPCR. Arc mRNA expression was higher in the 30 min group than other groups in both **(a)** cortex and **(b)** hippocampus. Additionally, mRNA expression was higher in the 120 min group than the control group in the hippocampus. *$p < 0.05$; **$p < 0.01$. All bars represent mean ± SEM.

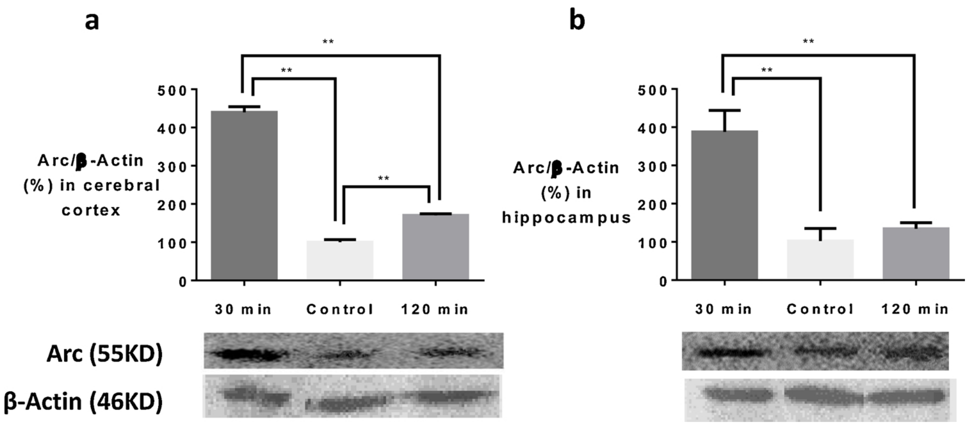

Fig. 7. Arc protein analysis by Western Blotting. **(a)** In Cortex, there was a higher expression of Arc protein in the 30 min group than both the Control and the 120 min groups. Additionally, 120 min group had higher expression than control. **(b)** Similarly, in Hippocampus, there was a higher expression of Arc protein in the 30 min group than both Control and 120 min groups. All Bars represent mean ± SEM.

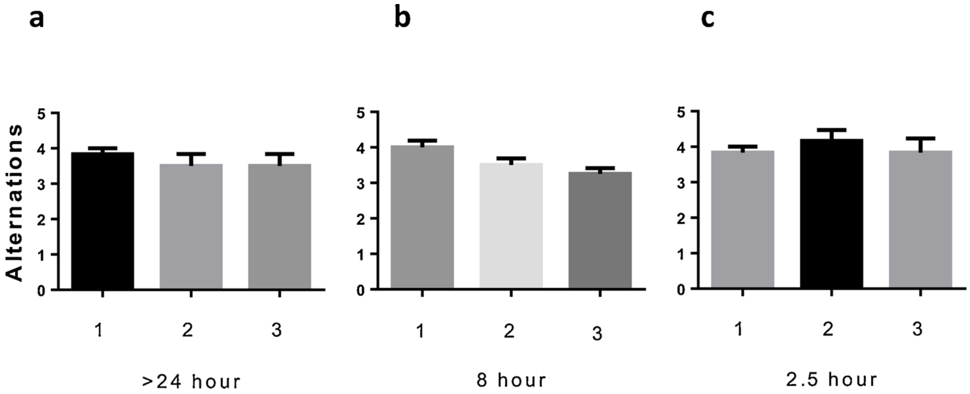

Fig. 8. Alternations in mice that performed delayed-SAB session-3 at **(a)** 2.5 h, **(b)** 8 h, and **(c)** more than 24 h after novel exposure of maze. There was no difference in alternations among sessions for each group. * $p < 0.05$, ** $p < 0.01$. All bars represent mean ± SEM.

expression in the Arc antisense group as compared to the scrambled group (t-test, $p = 0.026$) (Fig. 9).

3.6.2. Downregulation of hippocampal Arc prevents the decrease in alternations

After the confirmation of the decreased Arc level in the hippocampus, we checked alternations in the delayed-SAB test. The number of alternations was significantly different among the sessions of Scrambled ODN infused (SRN) group ($p = 0.005$, Kruskal-Wallis test). However, no significant difference was found among the sessions of the Arc-antisense ODN (ASN) infused group ($p = 0.60$, Kruskal-Wallis test) (Fig. 10a). Session-3 alternations of SRN group were lower than both its session-1 ($p = 0.015$) and session-2 ($p = 0.042$) alternations. Additionally, there were no significant changes in alternations between session-1 and session-2 of SRN ($p > 0.999$). In contrast, there were no significant differences between any pair of sessions of ASN (all $p > 0.83$) (Fig. 10a). For multiple comparisons, Dunn's test was used.

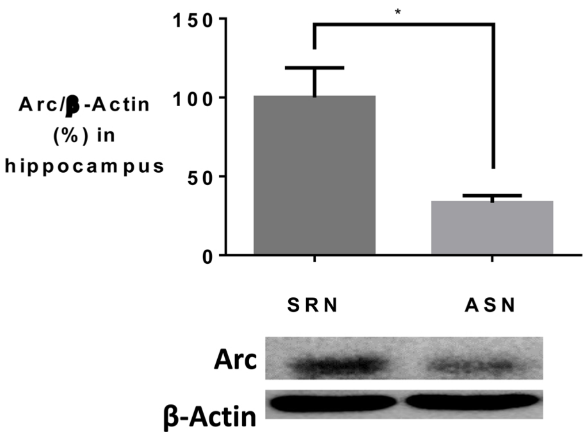

Fig. 9. Arc protein expression analysis in the dorsal hippocampus of ODN infused mice by Western blotting. There was around a 3-fold decrease in protein expression in Arc-Antisense ODN infused mice (ASN group) than Scramble ODN (SRN group). All bars represent mean ± SEM.

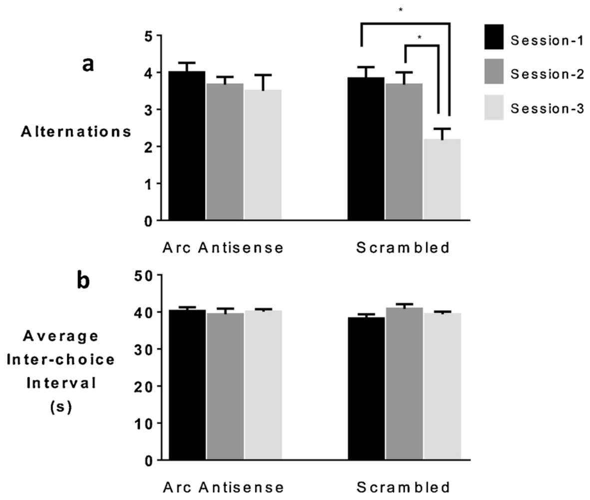

Fig. 10. Alternations **(a)** and average-ICI **(b)** were shown for Arc antisense (ASN) and Scrambled (SRN) ODN infused mice groups. **(a)** We found that session-3 alternations were decreased only for the SRN but not for the ASN group. **(b)** We found no significant differences in average ICI among the sessions of both the groups. All bars represent mean ± SEM.

On the other hand, there were no significant differences in average ICI among the sessions for both SRN (one-way ANOVA, F (2,15) = 1.557, p = 0.243) and ASN (one-way ANOVA, F (2,15) = 0.173, p = 0.842) groups (Fig. 10b).

4. Discussion

As continuous SAB has low alternations, we have used the multi-choice delayed-SAB in Y-maze due to its increased sensitivity to detect any change in the alternations. Our findings suggest that the multi-choice delayed-SAB (1) has less inter-trial interference than the continuous SAB, (2) has more accurate representation of animal's ability to alternate than 2-trial SAB as it has more measurements per animal within finite period of time, and (3) is more suitable for novel experience-concerned experimental designs.

Y-maze spontaneous alternations have been used for testing the working memory retention in pharmacological studies (Hughes, 2004). In this study, we observed that the introduction of an inter-choice delay in continuous SAB procedure has led to increased alternations. This implies that the mice spending more time in an arm (which has resulted from forced delays) have higher rates of alternation in the short time scale (~min). Alternations can result from either intact working memory or successful short-term habituation (Sanderson and Bannerman, 2012; Wolf et al., 2016). However, our present study could not support the working memory concept because it is expected that the lesser retention intervals (such as no-delay) should correlate with better working memory performances – which was not the case in this study. On the contrary, the short-term habituation concept suggests that when the inter-choice interval increases, it makes an animal attend an arm for more period of time, and this enables greater short-term habituation to that arm. Hence, the animals alternate better due to an enhanced discrimination ability between relatively novel and relatively familiar arms. We suggest that continuous SAB might be more useful to test the enhancement of attention rather than the working memory.

As all mice groups had similar inter-choice intervals, the observed temporal changes in the alternations are independent of the time spent in arms. Only the short inter-session interval (~30 min), led to a reduction in alternations in the later session, provided the presented environment was novel. Either changing the degree of novelty or ISI-1 had prevented the reduction. This showed that both novelty and shorter ISI-1 are necessary for the decrease in alternations.

We hypothesized that these results could be due to the synthesis of plasticity-related proteins (PRP) and their presence during subsequent exposure to the environment. Hence, we checked the expression level of one of such PRP, i.e., Arc in this study. We observed a high expression of Arc protein and mRNA in both cortex and hippocampus 30 min after the delayed-SAB test in Y-maze (i.e., a novel experience), which reverted to their basal level after 2 h. Similar levels were observed by Vazdarjanova et al., 2002 in these brain regions of rats of the spatial exploration of a novel environment. As seen from the 7-choice delayed-SAB results, this 30 min of ISI-1 did not affect the alternation performance at session-2, suggesting that the presence of Arc alone during a session does not affect the alternations of the same session. However, alternations were decreased only in the third session of shorter ISI-1. This suggests that the higher Arc expression in the second session results in such a decrease in the subsequent session. This result implies that Arc is associated with the temporal decrease of alternation rates.

To check if Arc is vital for the decrease in alternations, we stereotaxically infused Arc antisense ODNs at the dorsal hippocampus along with scrambled ODNs in the control group. We observed that due to the downregulation of hippocampal Arc protein by the antisense ODNs, as confirmed by Western blotting, the alternation rates did not significantly change during the subsequent Y-maze exposures. This confirms the involvement of hippocampal Arc for a temporal decrease in alternation rates. However, we were unable to perform the bilateral stereotaxic surgery in the cerebral cortex (pre-limbic cortex) due to the closeness of these regions in different hemispheres and related experimental constraints.

Arc has been suggested to contribute to the familiarization of a novel environment via LTD (Jakkamsetti et al., 2013). Arc decreases the surface-AMPAR expression and reduces the synaptic strength of those neurons, which induced Arc during the previous exposures (Okuno et al., 2012; Park et al., 2008). This facilitates increased relative strengths of synapses during the novel exploration (Kemp and Manahan-Vaughan, 2004; Dong et al., 2012). An earlier study by Nomoto et al. (2016) has shown that there is around 70% of overlapping Arc-positive neurons after exposures to different spatial environments. This implies that the re-exposure to the same environment might activate a greater percent of neurons that induced Arc in the earlier sessions, and thus a significant number of synapses could be weakened. On the other hand, activated synapses without substantial Arc induction could undergo enhanced early-phase LTP (Shepherd and Bear, 2011). Early-phase LTP was strongly induced in the synapses of Arc KO mice, and it can last up to three hours before reaching the baseline (Plath et al., 2006), implying the possibility of stronger expression of early LTP in Arc negative neurons in naïve mice after spatial exploration. In addition, Vazdarjanova et al., 2006 showed that only 20–30% of neurons express Arc in the hippocampal regions in naïve rats after spatial exploration in open-field. These studies suggest that early-phase LTP was also maintained in some of the neurons during our experiment's interval (of 2 h) between session-2 and session-3. Hence, in the naïve mice, the habituation is affected differently due to the parallel incidence of both synaptic weakening and its lack thereof in the distinct neurons; whereas, the habituation would be affected more uniformly in the Arc-inhibited mice. This nonuniformity in naive mice increases the information entropy, and we suggest that this reduces short-term habituation ability during subsequent exposures in the working memory paradigm.

Our present findings on the temporal effects of Arc during the working memory paradigm may also be explained in the light of the Sometimes opponent process (SOP) model proposed by Wagner (Wagner, 2014; Vogel et al., 2019). SOP supposes an inverse relationship between the abilities of short-term habituation and associative memories formation. The short-term habituation results in the alternation behavior (Sanderson and Bannerman, 2012), which was also observed in our study where more habituation to the arms increased alternations rate. We also found no change in alternations with a familiarized environment, a scenario that likely requires lesser new associative memories formation. Interestingly, it has already been demonstrated that the Arc has a role in the facilitation of associative memories (Hudgins and Otto, 2019; Hashikawa et al., 2011). Overall these add to the theory that Arc reduces the short-term habituation ability.

In conclusion, we observed that (1) alternation rates increase with the duration of exposure to the novel environment in short time-scale (< min), showing that short-term habituation increases the alternation rate, (2) during repeated exposure in a novel environment the alternation rates decrease after shorter inter-session interval, and (3) Arc decreases the spontaneous alternations in the subsequent sessions after the novel exposure, and we suggest that Arc reduces the ability of short-term habituation in the subsequent sessions in the working memory paradigm.

CRediT authorship contribution statement

M.C. Vishnu Shandilya: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft. **Akash Gautam:** Validation, Data curation, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

None.

Acknowledgments

The authors are thankful to the Department of Science & Technology (India) for both PURSE-II grant to the University of Hyderabad (Sanction No. SR/PURSE/Phase 2/4(C), SERB grant to AG (Grant No. EEQ/2017/000513), and International Society for Neurochemistry (Grant No. ISN/21/2101/2017/01112) which supported this study. We thank Dr. Arvind Kumar, Centre for Cellular & Molecular Biology, Hyderabad, India, for providing support in stereotaxic experiments.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.brainresbull.2020.02.013.

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NEUROSCIENCE
RESEARCH ARTICLE

M. C. V. Shandilya, A. Gautam / Neuroscience 431 (2020) 193–204

Hippocampal Arc Induces Decay of Object Recognition Memory in Male Mice

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Abstract—Activity-regulated cytoskeleton-associated (Arc) gene is one of the effector neuronal immediate early genes (IEG) that is rapidly upregulated after neuronal activation and is involved in synaptic long-term potentiation and depression. In recent years, it has been implicated in several cognitive disorders, viz. Angelman syndrome, Alzheimer's disease, fragile-X syndrome, etc. It undergoes quick transcription and highly regulated translation after exposure to a novel environment. Previous studies have shown that the presence of Arc mRNA primes mGluR-dependent long-term depression (LTD) in previously activated synapses upon re-exposure to the same environment. These studies suggest that the memory could be affected by the availability of Arc at the re-exposure time. Therefore, to confirm this, we investigated the changes in the temporal order memory and object recognition memory after the re-exposure to an environment in male mice. We studied the involvement of Arc in these changes by inhibiting Arc protein expression via stereotaxic infusions of Arc antisense oligodeoxynucleotides in the hippocampus of mice. We found that both temporal order and object recognition memories are dependent on the inter-familiarization phase interval. Strikingly, we also found that Arc accelerated the memory decay of an object when mice were re-exposed to the environment without that object. © 2020 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: object recognition, novel experience, temporal order, arc, hippocampus, memory.

INTRODUCTION

Activity-regulated cytoskeleton-associated (Arc) gene, also known as arg3.1, is one of the neuronal immediate-early genes which encode a 396 amino acid long effector protein in activated neurons. Various studies since its discovery in 1995 have shown its involvement in all the known forms of synaptic plasticity i.e., long-term potentiation (LTP), long-term depression (LTD) and homeostatic plasticity (Bramham et al., 2010; Korb and Finkbeiner, 2011; Minatohara et al., 2016). Arc KO studies have proved its importance in the spatial long-term memory (LTM) and maintenance of the late-phase LTP but no role in short-term memory (Plath et al., 2006; Messaoudi et al., 2007; Peebles et al., 2010; Yamada et al., 2011). Studies by Martínez et al. (2012) have shown that the inhibition of Arc translation averts the LTM formation during behavioral tagging in the inhibitory avoidance (IA) task. Arc mRNA transcribes quickly within five min of exploration of a novel environment. Some of these mRNA migrates to dendrites and gets translated if the mGluR is activated, which in turn induces mGluR-dependent LTD (Vazdarjanova et al., 2002; Park et al., 2008; Popkirov and Manahan-Vaughan, 2010; Jakkamsetti et al., 2013). Moreover, Arc protein selectively binds to inactive synapses and induces LTD in these synapses (Okuno et al., 2012). Arc is involved in various cognitive tasks, viz., IA, taste aversion, fear conditioning, and object recognition (Korb and Finkbeiner, 2011). Several studies by inhibiting Arc via Arc antisense oligodeoxynucleotides (ODNs) have found that Arc is necessary for LTP consolidation and modification of F-actin cytoskeleton (Minatohara et al., 2016).

During the exploration of a novel environment, the presence of unfamiliar objects in any location or familiar objects in new locations facilitates LTD in rats (Kemp and Manahan-Vaughan, 2004; Okuno, 2011; Dong et al., 2012). The object recognition memory (ORM), a type of visual-spatial memory, is commonly assessed in rodents using the ORM tests that test the animal's ability to recollect the past experiences involving the objects and the surrounding environment (Lueptow, 2017). Hence, ORM can be used for spatial- and novel-ORM. The ORM is analyzed by comparing the object exploration time between both objects. Another type of memory that can be analyzed by using the objects and OF is

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Abbreviations: ART, aligned rank transformation; IA, inhibitory avoidance; LTD, long-term depression; LTP, long-term potentiation; ODNs, oligodeoxynucleotides; ORM, object recognition memory; TOM, temporal-order memory.

https://doi.org/10.1016/j.neuroscience.2020.02.012

temporal-order memory (TOM). TOM task consists of two familiarization phases and a test phase, where an identical pair of distinct objects are presented in each familiarization phase. In the test phase, one object from each of the familiarization phase is placed at the same time to test if they can discriminate the least recent object.

Earlier catFISH studies confirmed that the percentage of ''overlapping Arc-positive neurons'' (i.e., those neurons which have expressed Arc during both the exposures of behavioral tagging) increases after ORT (Nomoto et al., 2016). Moreover, studies through KO mice have shown the involvement of Arc in both the spatial ORM and temporal order memory. However, Manago and colleagues used inter-familiarization interval of one hour (interval commonly used for TOM tasks) to conclude that the Arc is essential for TOM (Mitchell and Laiacona, 1998; Hannesson et al., 2004; Barker et al., 2007; Barker and Warburton, 2011; Manago et al., 2016). The presence of Arc mRNA primes mGluR-dependent LTD in previously activated synapses upon re-exposures to the same environment (Jakkamsetti et al., 2013); this suggests that the temporal order memory and, possibly, ORM could be affected by the availability of Arc at the re-exposure time. Therefore, we tried to study the temporal changes of temporal order memory and ORM after the re-exposures in a modified behavioral procedure and the association of Arc in this process.

To demonstrate the involvement of Arc during these temporal changes, we analyzed the expression level of Arc mRNA and protein by RT-qPCR and Western blotting, respectively, in the cortex and hippocampus. These two brain areas are involved in the recognition memory in short ($\sim$30 min to 1 h) as well as intermediate ($\sim$3 h) retention time-scales (Antunes and Biala, 2011). Several cortical areas are implicated in various memories, including object recognition and temporal order recognition memories (Barker et al., 2007). Other structures, including parahippocampal, perirhinal, and entorhinal cortices, are also involved in recognition memory. Perirhinal area is more limited to short periods, and it processes information from other areas involved in recognition memory, including somatosensory, visual, and olfactory centers. Though the hippocampus may not be directly involved in discriminating various object characteristics, it is involved in TOM and essential for comparing the temporally presented information (Clarke et al., 2010; Barker and Warburton, 2011). Moreover, in NORM, the degree of hippocampal involvement on both the recognition and temporal order memory is higher if the duration of habituation to the surrounding environment (including the OF) is less (de Lima et al., 2006; Antunes and Biala, 2011). Hence, a novel OF was presented in our behavioral tasks. Additionally, we tested the effect of duration of habituation on exploration in subsequent-trials in the OF. Finally, to confirm the role of Arc in the temporal changes of recognition memory, we also performed modified behavioral test after suppressing the Arc protein expression in the mice hippocampus using the stereotaxic injection of Arc antisense ODNs. Intracerebral administration of synthetic molecules (ODNs) bind to the sequence of the mRNA and prevents the translation of its specific protein.

EXPERIMENTAL PROCEDURES

Materials

Tri-reagent (catalog# 93289) was purchased from Merck, Germany. DreamTaq Green PCR Master Mix (2×) kit (catalog# K1081) and HI-SYBR Green Master Mix (catalog# MBT074) were procured from Thermo Fisher Scientific, India and Himedia Laboratories, India respectively. PrimeScript 1st strand cDNA synthesis kit (catalog# 6110A) was purchased from Takara Bio Inc., India. PCR gene-specific primers were synthesized by Cassia Siamia Tech, India. Arc mouse monoclonal antibody (catalog# sc-17839) was purchased from Santa Cruz Biotechnology, USA; β-Actin mouse monoclonal antibody (catalog# 3700S) and HRP conjugated – Anti-mouse IgG Rabbit monoclonal antibody (catalog# 58802S) was from Cell Signal Technology, USA. ECL Reagent (catalog#WBLUC0100) was bought from Millipore, USA. Arc antisense and scrambled ODNs for the stereotaxic experiment were purchased from GCC-Biotech, India. All other general biochemicals were purchased from SRL and Himedia Laboratories.

Animals

Swiss Albino, inbred male mice, aged 15 ± 3 weeks, were used in this study. Mice were maintained in the animal house of the University of Hyderabad. Mice were exposed to a 12-h dark/light schedule, and the food and water were provided to them *ad libitum*. A total of 92 animals were used in this study. Mice euthanasia was carried out through cervical dislocation. Approval for animal experiments was taken from the institute's animal ethics committee (IAEC/UH/151/2017/05/AG/P11).

Behavioral tests

Our modified behavioral procedure consists of two familiarization phases (trial-1 and trial-2) and one test phase (trial-3), similar to the TOM study by Barker et al. (2007). In both the familiarization phases, a distinct pair of objects was presented temporally, and in the test phase, the familiarity of these objects was tested. During the test phase, the familiarity of these objects was assessed either against each other (similar to the test phase of TOM) or against a novel object (similar to the test phase of NORM). TOM and NORM procedures generally include the habituation of rodents to the testing maze for few days before the object familiarization day (Mitchell and Laiacona, 1998; Hannesson et al., 2004; Barker et al., 2007; Barker and Warburton, 2011; Manago et al., 2016). However, in NORM tests, we presented a novel environment (OF and its surroundings) to the rodents during the familiarization phases. The testing apparatus consisted of an OF maze of size 40 × 40 × 40 cm (Lueptow, 2017). The objects were kept in the center of each of the two adjacent quadrants, and mice were placed in the mid-section of the opposite quadrants (Antunes and Biala, 2011) (Fig. 1a). The mice were placed in the maze for five min in each trial.

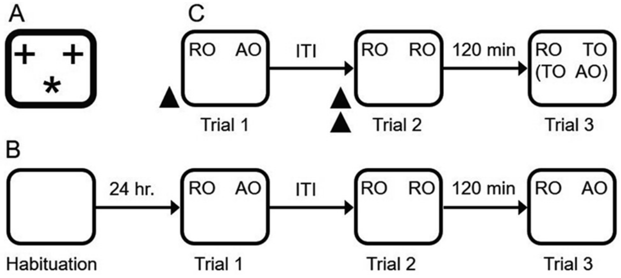

Fig. 1. **(A)** Open-field maze showing the placement of objects (+) and mouse (*) during behavioral tasks. **(B)** Schematic view of the TOM test. After the habituation to the open field one day before, mice performed the TOM task consisting of three trials. Each trial has a pair of objects, as shown. TOM test had three mice groups based on their ITI values: 30, 120, and 240 min. RO: Recurring Object; AO: Alternating Object. **(C)** Schematic view of NORM tests, consisting of three trails each. There were two NORM tests which only differ in trial-3 objects presentation (either AO or RO) against a novel object, TO. Each test has two groups of mice based on ITI values: 30, or 120 min. For Arc expression studies, an independent set of mice represented by triangles in figure were euthanized either before trial-1 at cage (control), or ITI time after trial-1 (30 or 120 min), without facing trial-2.

TOM test

We studied the TOM with different intervals between two familiarization phases. Mice were habituated for 5 min in the OF maze without objects one day before the familiarization phases. The temporal order task consisted of three trials with an inter-trial interval of either 30, 120, or 240 min between trial-1 and trial-2 (referred to as ITI), and an interval of 120 min between trial-2 and trial-3 (Fig. 1b). The mice were divided into three groups (30, 120, and 240 min) based on their ITI ($n = 8$). In the trial-1 (first familiarization phase), the mice were familiarized with two non-identical objects: *RO* (Recurring Object – present in all subsequent trials) and *AO* (Alternating Object – present in alternating trials). In trial-2 (second familiarization phase), the mice were exposed to two identical RO objects, with one object in a familiar location and the other in the location previously occupied by object AO (of trial-1). The positions of objects RO and AO were counter-balanced among all the groups. We designated the RO objects present in trial-2 as either 'ROr' or 'ROa' based on their location that had been previously (as in trial-1) used by objects 'RO' and 'AO' respectively. In trial-3 (test phase), the mice were exposed to both the objects RO and AO, both located as in the trial-1. In general, identical objects are used in TOM's first familiarization phase (Barker and Warburton, 2011). However, as recognition memory of rodents is affected by the length of habituation sessions, we used non-identical objects in first familiarization phase to increase discrimination ability of mice in the test phase (Oliveira et al., 2010).

An object was considered explored if the animal's nose was less than 3 cm away from the object and facing towards the object. Mice, which spent a minimum of 15 s to explore both the objects in the trial-1, were considered for analysis.

Test parameters. We calculated the *location preference* (left- or right-side) by mice and compared all group means with each other. The *distance traveled* by mice (head) was measured using the animal tracking software (Stoelting Co's ANY-maze, version 6.05). The object *exploration time* was manually scored frame-by-frame using Apple's QuickTime™ video player. Additionally, we calculated the *total exploration time* (TET) for each trial, which was the sum of exploration times of both objects.

Novel–ORM tests

The experimental procedure for NORM tests was similar to that of the TOM test except that we introduced a novel object (TO) in the test trial. The location of AO and RO was the same as in their respective trial-1. Additionally, mice were not habituated to the testing apparatus before the test. As confirmed through pilot studies, mice had no innate object preference between any pair of objects.

Exploration between recurring- and novel-object during the test phase. Object *AO* was replaced by *TO*. Mice were divided into two groups having ITI of either 30- or 120-min ($n = 7$ each) (Fig. 1c).

Exploration between alternating- and novel-object during the test phase. Object *RO* was replaced by *TO*. Mice were divided into two groups having ITI of either 30 min ($n = 5$) or 120 min ($n = 4$) (Fig. 1c).

In both TOM and NORM tests, 20% of the videos were re-scored by an independent experimenter who was blind to the experimental design, animal assignment, and other details. Pearson correlation was measured between the original and blind-scored results. There was a significant correlation between the original and re-scored results ($r = 0.879$, $p < 0.01$).

Effect of duration of habituation and the ITI on OF exploration

We tested the effect of duration of habituation and the ITI on the subsequent trials in OF. The duration of habituation and the ITI values were the same as the TOM test. Additionally, we used 30 min habituation as a positive control for inter-day habituation (Ballarini et al., 2009). We divided mice into three groups: A, B, and C. Group A underwent two trials of OF exposure, whereas groups B and C underwent three trials. Group A was exposed to OF for 30 min in trial-1 (on day-1) and 5 min in trial-2 (24 h later). Groups B and C were exposed to OF for 5 min in all trials. Trial-2 for groups B and C was also performed 24 h after trial-1. Additionally, trial-3 was performed 30 min- and 120 min- after trial-2 for group B and group C, respectively. As the most common measure for assessing the habituation to the novelty (Bolivar, 2009), the distance covered by mice was measured.

To check the effect of habituation duration in trial-1 on trial-2 exploration, we compared the distance of trial-1 with its trial-2. For 30 min habituation (group A), we compared the first five minutes distance of trial-1 with trial-2 (paired *t*-test). For 5 min habituation (groups B and C), we compared the distance of trial-1 (pooled data for both groups) with trial-2 (pooled) (paired *t*-test). To check the effect of ITI on exploration, we compared the distance of trial-2 vs. trial-3 for both ITI of 30 min (group B) and ITI of 120-min (group C). A paired *t*-test was used for comparison.

Reverse transcription-quantitative PCR

We checked Arc mRNA expression at the timings of trial-1 and trial-2 of the behavioral experiments. Mice were divided into three groups as Control, 30 min, and 120 min ($n = 3$) based on the interval between trial-1 and brain dissection (Fig. 1c). Groups 30 min and 120 min mice underwent the trial-1 of NORM at $t = 0$ and sacrificed at $t = 30$ and $t = 120$ min, respectively, without undergoing trial-2. The control group was dissected at cage without any trial of NORM. All mice were sacrificed by cervical dislocation, and their cerebral cortex and hippocampus were dissected quickly on ice for mRNA expression study.

Total RNA was isolated and quantified as per the protocol by Gautam et al. (2013). Further, RNA samples with A260/280 $\geq$1.8 were used for reverse transcription-quantitative PCR. Initially, 0.5 μg of mRNA was reverse transcribed into total cDNA. Using this cDNA as a template and gene-specific primers for GAPDH (reference gene; 1 μM) and Arc (4 μM), the amplicon was amplified by quantitative-PCR, where the PCR sample mixture was prepared by mixing SYBR Master Mix (SYBR Green dye, Taq polymerase, and dNTPs) (5 μl), forward and reverse primers (2 μl each), and cDNA sample (1 μl). Before the start of the PCR cycle, the sample mixture was denatured at 95 °C for 120 s. PCR cycle settings were performed according to the manufacturer's recommendations (Denaturation: 95 °C for 40 s, Annealing: 57 °C for 40 s, Extension: 72 °C for 60 s). The signal from the dye was measured at Extension (72 °C) step of the cycle. The primer details are Arc (forward: ACGATCTGG CTTCCTCATTCTGCT; reverse: AGGTTCCCTCAGCAT CTCTGCTTT) with amplicon size of 146 bp (Barnhart et al., 2015); and GAPDH (forward: GTCTCCTGCG ACTTCAG, reverse: TCATTGTCATACCAGGAAAT-GAGC) with amplicon size of 107 bp (Gautam et al., 2013). Melt-curve analysis (60 °C to 95 °C at 0.5 °C/10 s) was performed to measure the Tm (melting temperature) of Arc and GAPDH. From pilot experiments, Tm was found to be 84.6 °C and 84.9 °C for Arc and GAPDH, respectively. Using DNA loading dye, the amplified product was analyzed on 1.5% Agarose gel (stained with EtBr). Both the amplicon sizes of Arc and GAPDH were resolved correctly according to the DNA ladder, and no other band was present in their lanes. The quantification cycle (Cq) was measured using Insta Q96 (Himedia's qPCR machine). Any improper threshold and baseline assignments were corrected. To find out the efficiency of PCR amplification, a three-point 10-fold dilution series for both the genes was performed. PCR efficiency for Arc was 97.94% with correlation $(r) = -0.999$ and slope $= -3.37$ and for GAPDH was 92.64% with correlation $(r) = -0.995$ and slope $= -3.51$. No-template control for both the genes had Cq values > 30.0.

For each sample, the -dCq values were calculated as Cq (GAPDH) – Cq (Arc). A higher dCq value implies lower Arc expression and vice versa. -dCq values were normalized (%) with respect to the control group, and were used for statistical comparison across groups, using one-way ANOVA followed by Tukey's multiple comparisons test.

Stereotactic surgery

The translation of Arc was inhibited in the dorsal hippocampus by the stereotactic surgery using the Arc antisense or scrambled ODNs similar to our previous study (Gautam et al., 2016). However, we were unable to perform the bilateral stereotaxic surgery in the cerebral cortex due to experimental constraints. The coordinates for bilateral infusion of ODNs were chosen as −2.46 mm anterior-posterior from bregma, ±1.35 mm medial-lateral, and 1.35 mm dorsal-ventral (Franklin and Paxinos, 2001). Sequences of ODNs were ('*' denotes a phosphorothioate linkage):

Arc-Antisense: 5′-G*T*C*CAGCTCCATCTGCT*C*G*C-3′
Scrambled: 5′-C*G*T*GCACCTCTCGCAGC*T*T*C-3′

Novel-ORM test against the alternating object in Arc inhibited mice

Based on the type of ODNs infused, mice were divided into two groups ($n = 5$ each): (1) ASN (Arc Antisense ODN injected) and (2) SRN (Scrambled ODN injected) (Fig. 2). Three hours post ODN injection, both groups have performed three trials of NORM test with ITI of 30 min. The duration of trial-1 and trial-2 was 8 min each, and trial-3 was 5 min. Objects AO and TO were presented at the test trial.

Western blotting

Based on the ITI timings of NORM tests, we performed Western blotting of the hippocampal Arc (Fig. 1c). An independent set of mice ($n = 3$) were euthanized by cervical dislocation either 30 min or 120 min after trial-1 of the NORM test, along with control mice, which were euthanized at cage. We also performed Western blotting

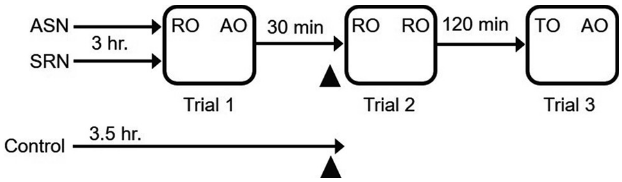

Fig. 2. Schematic view of experimental design for the NORM test and Western blotting. Arrows represent the time of ODN infusions in the dorsal hippocampus. ASN (Arc-antisense) and SRN (scrambled) groups underwent the NORM test as per the mentioned time intervals. Another independent set of mice ($n = 3$) of ASN, SRN, and control groups were euthanized at the specific times as represented by triangles to check the Arc protein expression in the hippocampus by Western blotting.

of the hippocampal Arc after stereotaxic surgery to confirm the efficiency of infused ODNs. An independent set of mice (n = 3), grouped similarly to the NORM test for Arc inhibited mice (i.e., *ASN* and *SRN*), were euthanized by cervical dislocation 30 min after trial-1 of NORM test (Fig. 2). A third group, Control, was infused scrambled ODNs but did not undergo any behavior trials. All groups were euthanized 3.5 h post ODN infusions.

The Western blotting procedure was as follows. 10% protein homogenate of the hippocampus was prepared using RIPA (lysis buffer) and quantified similarly to the protocol mentioned by Gautam et al. (2016). Forty micrograms of protein was mixed with a 6× denaturing sample buffer. The resultant mixture was loaded in 10% SDS-PAGE. The resolved protein bands in the gel were trans-blotted onto the PVDF membrane using tank electroblotting apparatus. The PVDF membranes were initially blocked in 5% (w/v) fat-free milk (blocking-buffer) for one hour. Then, membranes were incubated with Arc mouse monoclonal antibody (1:100) and β-Actin (loading control) mouse monoclonal antibody (1:10,000) for one hour at room temperature. Each antibody's specificity to our target proteins was previously confirmed. After that, membranes were washed thrice with 0.5% Tween-TBS for 5 min each. After washing, membranes were incubated in HRP conjugated – anti-mouse IgG rabbit monoclonal antibody (1:2000) for two hours at room temperature. Then, membranes were washed thrice in 0.5% Tween-TBS for 10 min each and incubated in ECL reagent for 2 min to detect the chemiluminescence signal inside the *Bio-Rad's* ChemiDoc™ imaging system.

Protein band quantification was done based on the ECL signal intensity values analyzed by the *Bio-Rad's Image Lab* software. Then, for normalization, Arc/β-Actin ratio was calculated for all groups. The resultant ratios of each group were compared using a one-way ANOVA test and Tukey's multiple comparisons.

Statistics

All the statistics were performed using GraphPad's Prism (version 6.01) and SPSS (version 16.0). The significance level (type-I error threshold) was set at 5%. All the p-values which were mentioned alongside a multiple-comparison test were adjusted p-values as done by the statistical analysis software. A comparison was reported as statistically significant if its adjusted p-value was less than 0.05. Parametric tests were used if both the normality and equal variance were satisfied or corrected by the test. For data that was not normal and homoscedastic, either the non-parametric tests were directly used, or the data was aligned and ranked (if there were two independent variables). Aligned rank transformation (ART) was performed on such data using the web software available at http://depts.washington.edu/acelab/proj/art/index.html (Wobbrock et al., 2011).

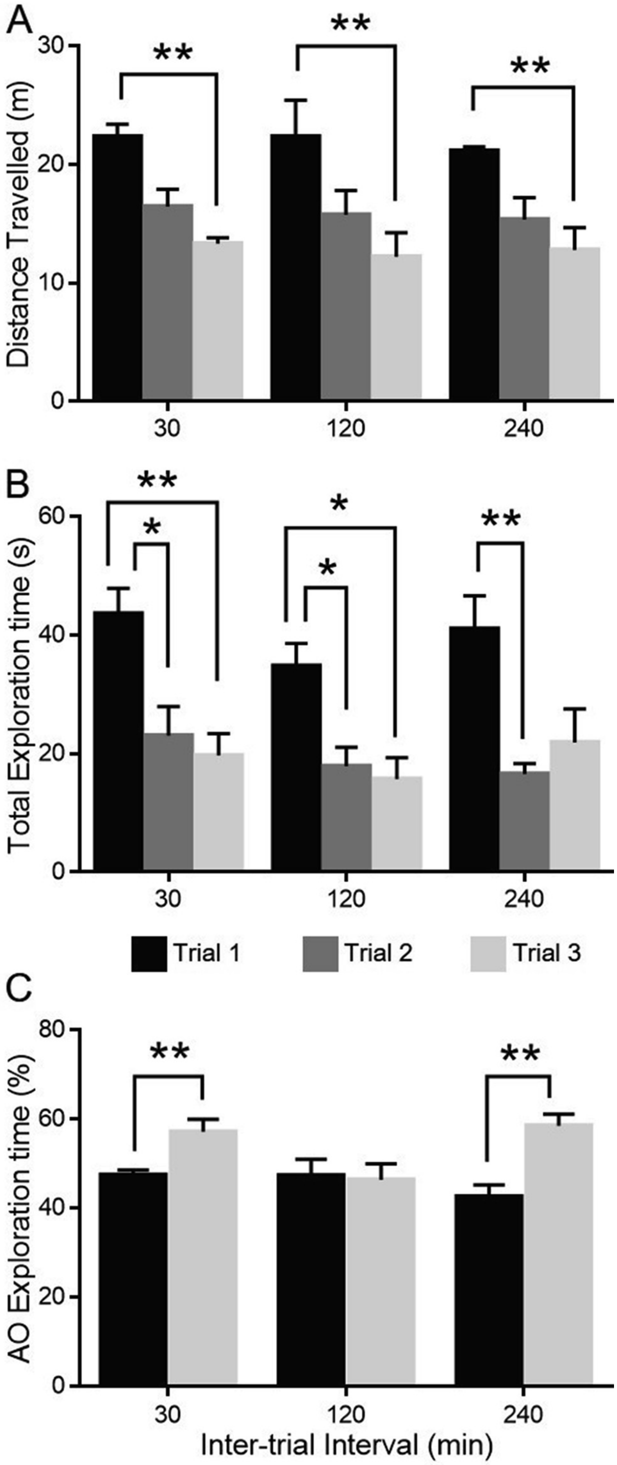

Fig. 3. Parameters of TOM task. **(A)** There was no difference in distance traveled among the ITI groups. However, for all three groups, mice traveled less distance in trial-3 than trial-1. No difference existed between other comparisons. **(B)** There was no difference in TET among the ITI groups. However, TET was higher in trial-1 than both trial-2 and trial-3 (except 240 min ITI). No difference existed between other comparisons. **(C)** In trial-3 of the TOM test, mice with ITI of 30 min and 240 min have explored object AO significantly more than their trial-1, whereas 120 min group had no change. All bars represent mean ± SEM. *p < 0.05, **p < 0.01.

RESULTS

TOM test

The location preference was not significantly different among the groups (one-way ANOVA, F (2, 21) = 0.84, $p = 0.44$). ART was performed on the distance-traveled data. Distance traveled by mice was not significantly different among the groups (F (2, 21) = 1.25, $p = 0.30$), but there was a difference among trials (F (2, 42) = 48.88, $p < 0.0001$). For all the groups, mice traveled less distance at trial-3 than trial-1 (Dunn's multiple comparisons test, $p = 0.001$ (ITI of 30 min), $p = 0.0005$ (ITI of 120 min), and $p = 0.008$ (ITI of 240 min)). No difference exists between other inter-trial comparisons (Fig. 3a).

ART was performed on the TET data. TET was not significantly different among the groups (F (2, 21) = 0.80, $p = 0.45$) but there was a difference among trials (F (2, 42) = 43.48, $p < 0.0001$). For all the groups, mice explored objects less at trial-2 than trial-1 (Dunn's multiple comparisons test, $p = 0.017$ (ITI of 30 min), $p = 0.037$ (ITI of 120 min), $p = 0.003$ (ITI of 240 min)). Additionally, mice explored the objects less at trial-3 than trial-1 for ITI of 30 min ($p = 0.003$) and 120 min ($p = 0.017$), but not for 240 min ($p = 0.136$). No difference exists between trail-2 and trial-3 for all groups (Fig. 3b).

There was no significant difference in the percentage of exploration time of object ROa among the groups (58.98% for 30 min ITI, 52.99% for 120 min ITI, and 51.26% for 240 min ITI; Kruskal-Wallis test, $p = 0.39$). ART was performed on the object AO exploration (%) data. There was a significant interaction between groups and trials (F (2, 21) = 8.571, $p = 0.0019$). The change in object AO exploration between trial-1 and trial-3 was analyzed. Both ITI of 30 min and 240 min increased their exploration of the object AO, whereas the exploration remained same in 120 min ITI group (Wilcoxon test; $p = 0.007$ (30 min ITI), $p = 0.945$ (120 min ITI), and $p = 0.007$ (240 min ITI)) (Fig. 3c).

Effect of ITI on RO recognition memory

The location preference between the groups: 30 min and 120 min ITI, was not significantly different (unpaired t-test, $p = 0.17$). There was no significant difference in the distance traveled between the groups (two-way ANOVA, F (1, 12) = 1.80, $p = 0.20$), but a difference existed among trials (two-way ANOVA, F (2, 24) = 33.67, $p < 0.0001$). For both groups, mice traveled less distance in later trials than trial-1 (Bonferroni's multiple comparisons test, $p < 0.01$) (Fig. 4a). No difference existed between trial-2 and trial-3 for both groups ($p > 0.1$).

There was no significant difference in the TET between the groups (two-way ANOVA, F (1, 12) = 0.02, $p = 0.88$), but a difference existed among trials (two-way ANOVA, F (2, 24) = 5.79, $p = 0.0089$). However, no difference exists among their trials through multiple comparisons ($p > 0.5$) (Fig. 4b). We found no significant difference in the exploration time (%) of

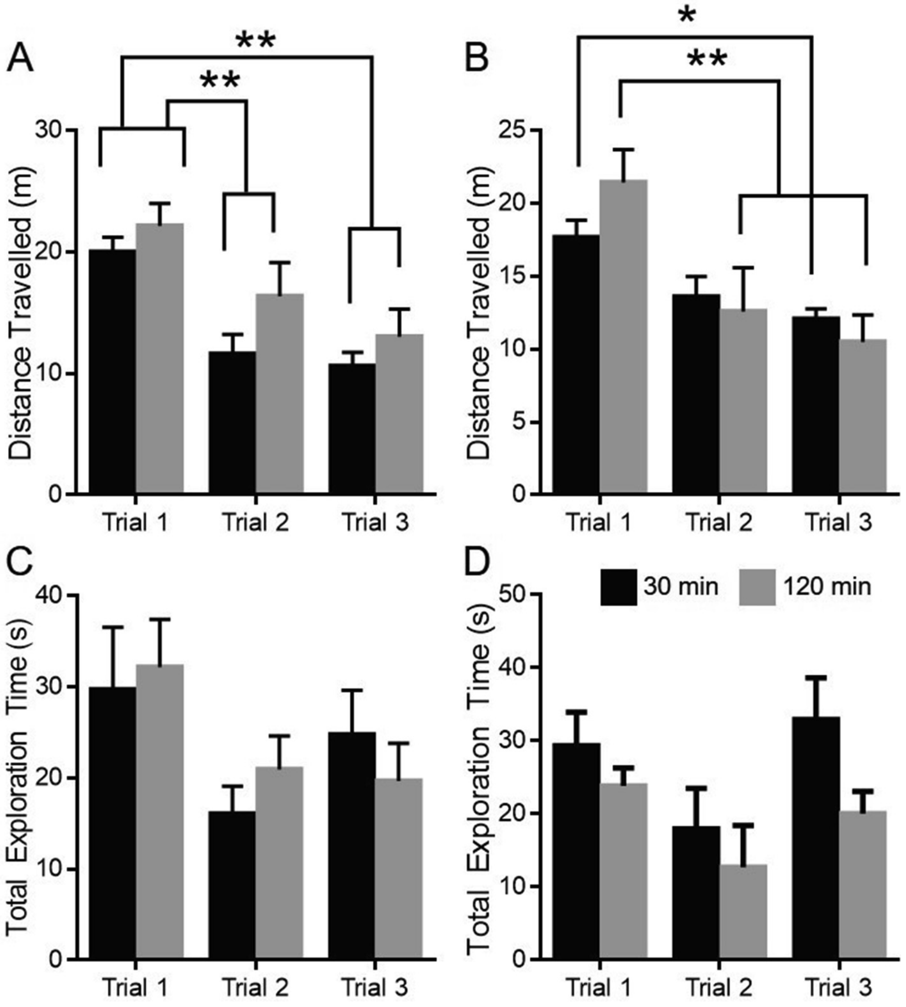

Fig. 4. Distance traveled **(A, C)** and TET **(B, D)** by mice in NORM tests having either RO **(A, B)** or AO **(C, D)** at trial-3. **(A)** There was no difference in the distance among the groups. However, more distance was traveled in trial-1 than both trial-2 and trial-3 for all three groups. **(B)** There was no difference in the TET among both groups and trials. **(C)** There was no difference among the groups, but more distance was traveled in trial-1 than both trial-3 (for all groups), and trial-2 (for 30 min ITI). **(D)** There was no difference in the TET among both groups and trials. All bars represent mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$.

object ROa between the groups (57.6% for 30 min ITI and 53.6% for 120 min ITI; $p = 0.13$, unpaired *t*-test).

There was no significant difference in RO exploration between the groups (two-way ANOVA, $F(1, 12) = 0.008$, $p = 0.92$) but a difference existed among trials (two-way ANOVA, $F(1, 12) = 51.23$, $p < 0.0001$). Mice with ITI of both 30 min and 120 min explored object RO less in their trial-3 than in their trial-1 (Bonferroni multiple comparisons, $p < 0.001$) (Fig. 5a).

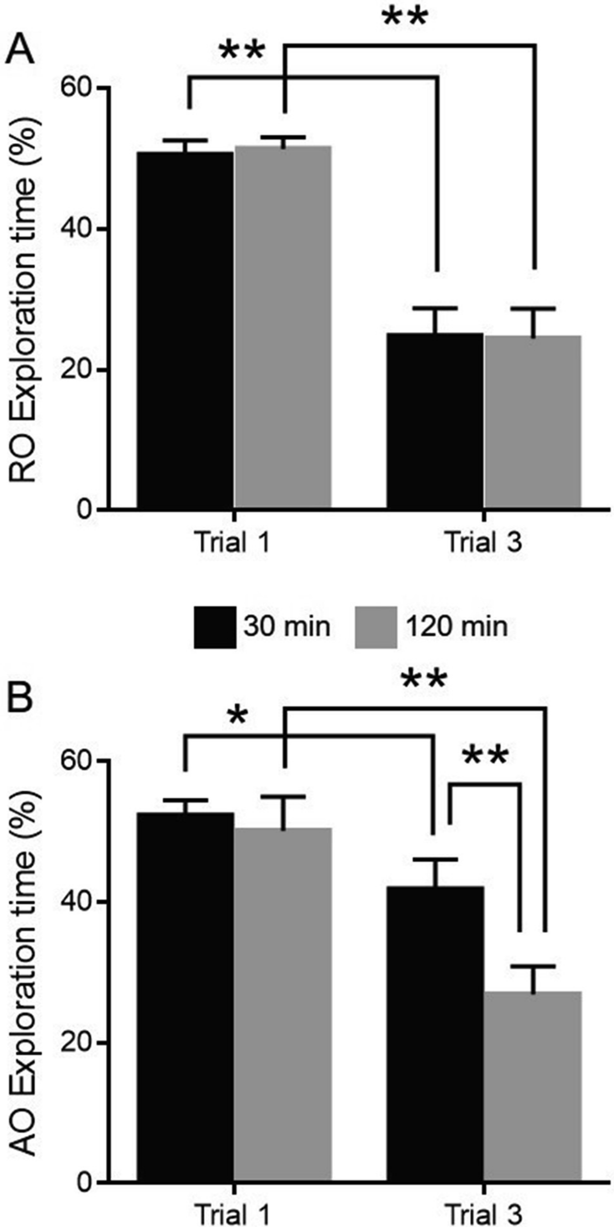

Fig. 5. Exploration time (%) of object **(A)** RO and **(B)** AO by mice at trial-1 and −3 of NORM tests. **(A)** There was no difference in RO exploration among the groups, but all groups explored RO less in their trial-3 than trial-1. **(B)** Overall, AO exploration was less in trial-3 than trial-1 for both groups. However, at trial-3, mice with ITI of 30 min have explored object AO more than that of 120 min. All bars represent mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$.

Effect of ITI on AO recognition memory

The location preference between the groups: 30 min and 120 min ITI, was not significantly different (unpaired *t*-test, $p = 0.30$). There was no significant difference in the distance traveled between the groups (two-way ANOVA, $F(1, 7) = 0.05$, $p = 0.82$), but a difference existed among trials (two-way ANOVA, $F(2, 14) = 15.87$, $p = 0.0003$). More distance was traveled in trial-1 than both trial-3 (for both groups, $p < 0.01$), and trial-2 (for 30 min ITI, $p < 0.05$) (Bonferroni's multiple comparisons test) (Fig. 4c). No difference exists between trial-2 and trial-3 for both groups ($p > 0.1$).

There was no significant difference in the TET between the groups (two-way ANOVA, $F(1, 7) = 2.49$, $p = 0.15$), but a difference existed among trials (two-way ANOVA, $F(2, 14) = 4.69$, $p = 0.02$). However, no difference exists among the trials through multiple comparisons ($p > 0.05$) (Fig. 4d). We found no significant difference in the exploration time (%) of object ROa between the groups (53.17% for 30 min ITI, and 49.17% for 120 min ITI; $p = 0.38$, unpaired *t*-test – Welch corrected).

There was a significant interaction between groups and trials (two-way ANOVA, $F(1, 7) = 6.803$, $p = 0.03$). For both groups, object AO exploration (%) was less in the trial-3 as compared to its trial-1 (Bonferroni's multiple comparisons, $p = 0.03$ (for ITI 30 min), and $p = 0.0008$ (for ITI 120 min). Notably, in trial-3, mice group with ITI of 30 min have explored object AO more than that of 120 min (Bonferroni's multiple comparisons, $p < 0.0001$) (Fig. 5b). There was no difference in AO exploration in trial-1 between both groups ($p = 0.84$).

Arc mRNA expression was maximum after 30 min of novel experience

-dCq values were normalized (%) with respect to control. There was a significant difference between -dCq values among mice groups (ITI of 30 min, 120 min, and control) in both cortex ($F(2, 6) = 8.722$, $p = 0.0168$) and hippocampus ($F(2, 6) = 8.851$, $p = 0.0162$) (one-way ANOVA, followed by Tukey's multiple comparisons). Arc expression significantly increased at 30 min ITI as compared to control, in both cortex ($p = 0.021$) and hippocampus ($p = 0.016$). Additionally, Arc expression significantly decreased at 120 min ITI as compared to 30 min in both cortex ($p = 0.033$) and hippocampus ($p = 0.046$). There was no difference between 120 min ITI and control for both brain regions ($p > 0.5$) (Fig. 6).

Temporal effects of habituation

We found that the distance traveled had significantly decreased in the following day if mice were habituated to OF for 30 min (paired *t*-test, $p = 0.002$) but not for 5 min (pooled data of group B and C) (paired *t*-test, $p = 0.798$). Additionally, at five min habituation per trial, neither 30 min (paired *t*-test, $p = 0.85$) nor 120 min ITI (paired *t*-test, $p = 0.82$) significantly decreased the novelty of the OF (Fig. 7).

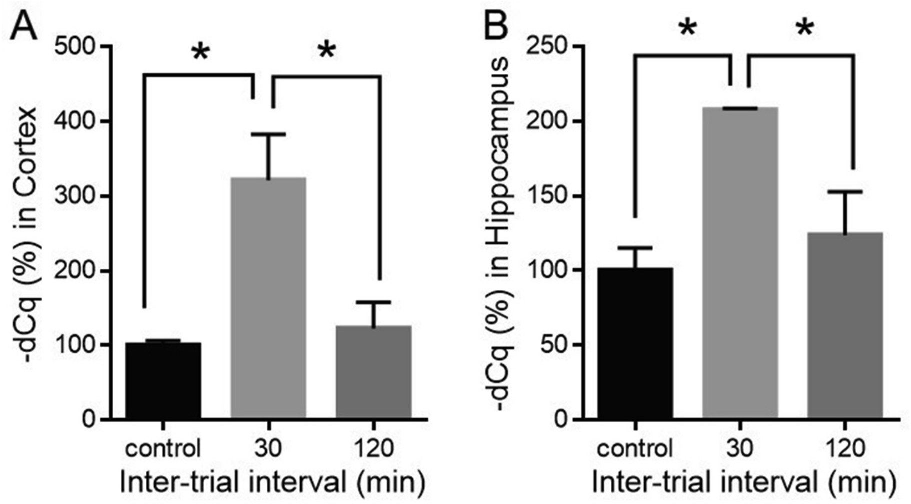

Fig. 6. Analysis of Arc mRNA level by qPCR. In both **(A)** cortex and **(B)** hippocampus, the mRNA expression was higher in the 30 min ITI group than both the control and 120 min. All bars represent mean ± SEM. *$p < 0.05$.

Arc protein expression was maximum after 30 min of novel experience

In the hippocampus, Arc protein expression was significantly different among mice groups (ITI of 30 min, 120 min, and control) (one-way ANOVA, F (2, 6) = 12.27, $p = 0.007$). A higher Arc expression was found at 30 min ITI as compared to both control ($p = 0.009$) and 120 min ITI ($p = 0.017$) (Tukey's multiple comparisons). There was no difference between control and 120 min ITI ($p = 0.84$) (Fig. 8).

Infusion of Arc antisense ODNs inhibits Arc protein expression

Arc protein level was significantly different among ASN, Control, and SRN groups (one-way ANOVA, F (2, 6) = 51.58, $p = 0.0002$) (Fig. 9). SRN group (scrambled ODN infused + ORT) showed higher Arc protein expression than that of the Control group (scrambled ODN infused, without ORT) (Tukey's multiple comparisons test, $p = 0.0003$). We found over a fourfold decrease in the hippocampal Arc protein level in the ASN group as compared to the SRN group (Tukey's multiple comparisons test, $p = 0.0003$).

SRN group explored AO significantly more than ASN group

The location preference between the groups: SRN and ASN, was not significantly different (unpaired t-test, $p = 0.31$). There was no significant difference in the distance traveled between the groups (two-way ANOVA, F (1, 8) = 0.023, $p = 0.88$) and among the trials (two-way ANOVA, F (2, 16) = 0.105, $p = 0.90$) (Fig. 10a). There was no significant difference in the TET between the groups (two-way ANOVA, F (1, 8) = 0.00137, $p = 0.97$) and among trials (two-way ANOVA, F (2, 16) = 2.62, $p = 0.103$) (Fig. 10b). We found no significant difference in the exploration time (%) of object ROa between the groups (57.80% for SRN and 60.72% for ASN; $p = 0.60$, unpaired t-test).

There was a significant interaction between the groups and the trials (two-way ANOVA, F (1, 8) = 5.751, $p = 0.043$). For both groups, object AO exploration (%) was less in the trial-3 as compared to its trial-1 (Holm-Sidak multiple comparisons, $p = 0.042$ (for SRN), and $p = 0.0008$ (for ASN). Importantly, in trial-3, SRN mice have explored the object AO more than ASN mice (Holm-Sidak multiple comparisons, $p = 0.013$). There was no difference in AO exploration in trial-1 between both the groups ($p = 0.98$) (Fig. 10c).

DISCUSSION

Arc gene plays a vital role in the regulation of synaptic plasticity, and in recent years, it has been proved to be involved in several cognitive disorders like Angelman syndrome, Alzheimer's disease, fragile-X syndrome, etc. (Nikolaienko et al., 2018). It has been shown that Arc induces mGluR-dependent LTD, and it facilitates the consolidation of recognition memory after the exploration of the novel environment (Minatohara et al., 2016). To add more to these studies, we investigated the temporal effects on both ORM and temporal order memory after the re-exposure to the environment, and the probable role of Arc in such effects. We found out that (1) both temporal order and ORM depends on the inter-trial interval of familiarization phases; and (2) Arc augments the decrease in the object familiarity upon re-exposure to the same environment without that object.

To test Arc expression at different ITI, we analyzed mRNA and protein expression in male mice using RT-qPCR and Western blotting. We found an increased mRNA and protein level at ITI of 30 min in the hippocampus and decreased levels at 120 min ITI (Figs. 6, 8). These results are similar to the earlier results obtained by Vazdarjanova et al., 2002, where they found an increased Arc mRNA expression in cortex and hippocampus within 5 to 10 min after the spatial exploration of a novel environment. From these behavioral and molecular experiments, we found that Arc expression at second re-exposure was associated in cohort with changes in ORM.

To test whether Arc is necessary for the observed decrease in object familiarity, we inhibited Arc protein expression in the dorsal hippocampus using Arc antisense ODN (Fig. 9). While both scrambled ODN (SRN) and Arc antisense ODN (ASN) infused mice displayed the presence of AO memory, they displayed a difference in the strength of memory. Results showed that Arc enhanced the (object's) memory decay when the object is absent during the re-exposure (Fig. 10c).

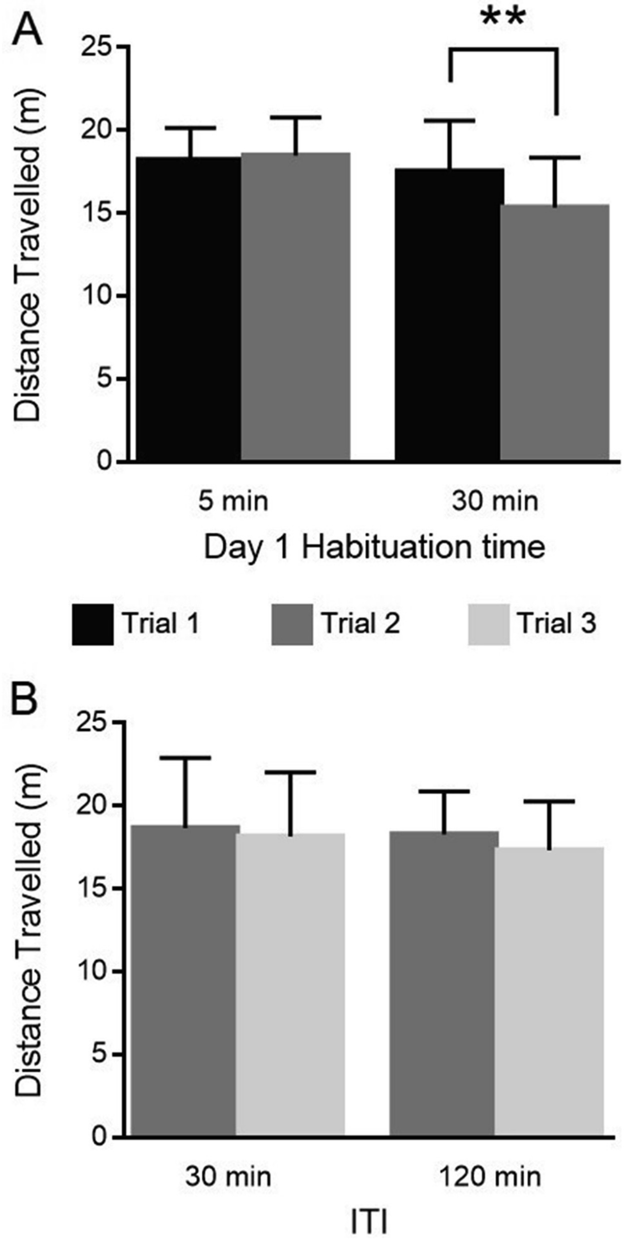

Fig. 7. **(A)** Distance traveled by mice in the first five minutes of trial-1 and trial-2 for both cases of 5-min and 30-min habituation on day-1 is shown. Trial-1 was performed on day-1 for either 5 or 30 min, and trial-2 was performed on day-2 for 5 min. There was a significant decrease in distance traveled in trial-2 with respect to trial-1 for mice habituated for 30-min on day-1, but no difference for the mice habituated for 5-min (pooled of groups, B and C) on day-1 (paired *t*-test, $^{**}p < 0.01$). **(B)** Distance traveled by mice in trial-2 and trial-3 for both ITI of 30 min (group B) and 120 min (group C). We found no change in distance traveled between trial-2 and trial-3 for both ITI 30–min (paired *t*-test, $p = 0.85$) and 120-min (paired *t*-test, $p = 0.82$). All bars represent Mean ± SEM.

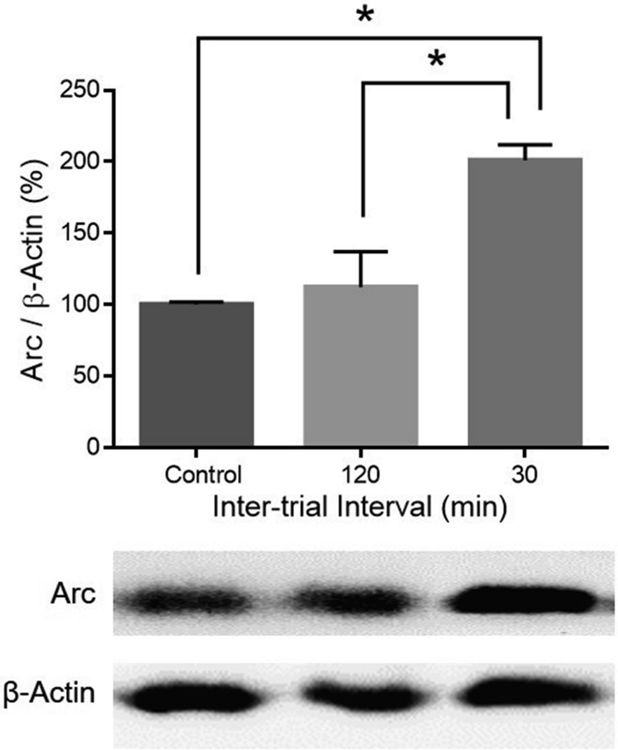

Fig. 8. Arc protein expression by Western blotting in the hippocampus of naïve mice during the NORM test. Protein expression was higher in 30 min ITI than both the control and 120 min. All bars represent mean ± SEM. $^{*}p < 0.02$.

The temporal changes in the distance traveled, and TET was found to be independent of the ODN infused (Fig. 10a, b).

Some of the Arc mRNA is translationally suppressed until it reaches spines, where it is translated if mGluR is activated and induces mGluR-dependent LTD (referred to as LTD-Priming) (Jakkamsetti et al., 2013; Steward et al. 2015; Minatohara et al., 2016). Repeated exposures to the same environment primes mGluR-dependent LTD in previously activated synapses (Jakkamsetti et al., 2013). In our context, this suggests that the synapses activated by the object which was presented during the first familiarization phase (i.e., AO) could have been weakened.

An earlier study by Lueptow (2017) showed that the ORM in mice remains intact only for a few hours (~4h), and in general, after this duration, they fail to discriminate the novel object with the familiarized one (Lueptow, 2017). Moreover, the study by Barker et al., (2007) has proved that the temporal order memories are generally weaker than the object recognition memories. In our study, we initially tried to investigate the effect of ITI on temporal order memory and found out that the memory at the test phase is ITI-specific (Fig. 3c). This result shows that the mice had forgotten the object, which was presented six hours ago, consistent with the ORT paradigm in which the higher retention interval leads to lower object recognition (Lueptow, 2017). However, mice group with shorter ITI also displayed a higher exploration (%) towards AO. This showed that the temporal order memory depends on the inter-trial interval between the familiarization phases.

We investigated the effect of ITI on ORM by introducing a novel object (TO) in the test phase to test whether either RO or AO caused the temporal changes in memory. We observed that ITI did not affect RO

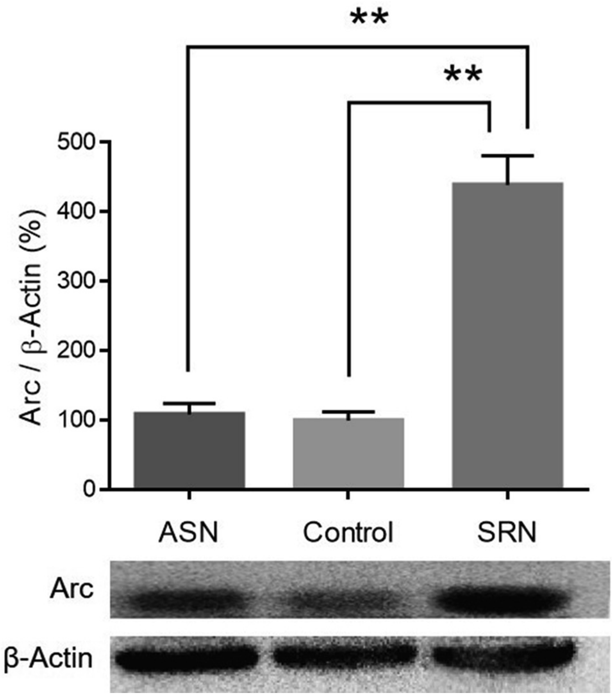

Fig. 9. Arc protein expression by Western blotting in the hippocampus of mice that underwent stereotaxic surgery. SRN (scrambled ODN infused + NORM trail-1) had higher expression than both Control (scrambled ODN infused, without NORM trial-1), and ASN (Arc antisense ODN infused + NORM trial-1). All bars represent mean ± SEM. $^{**}p < 0.0005$.

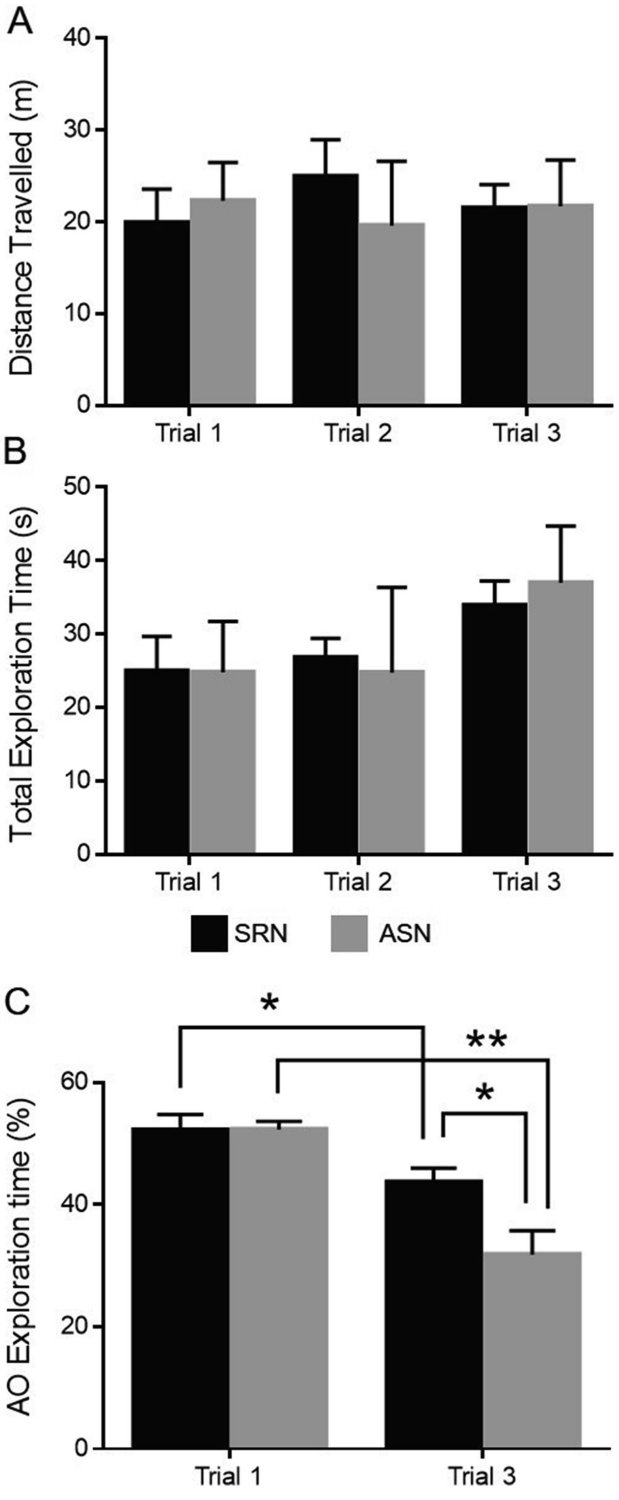

Fig. 10. Parameters of NORM test for ODN infused mice. There was no difference between the groups and among the trials in **(A)** Distance traveled, and **(B)** TET by mice. **(C)** Exploration time (%) of object AO by mice at trial-1 and trial-3. Both the groups decreased their AO exploration at trial-3. However, SRN mice have explored AO more than ASN mice. All bars represent mean ± SEM. $^{*}p < 0.05$, $^{**}p = 0.0008$.

familiarity (Fig. 5a), but it affected familiarity of AO (Fig. 5b). Mice explored TO more than RO implying an intact memory of RO in both ITIs. However, against AO, mice with shorter ITI explored TO only modestly (~58%), implying a greater decay of AO memory. In comparison, mice group with ITI of 120 min explored TO more, displaying intact AO memory. These results show that the ORM of AO during the test phase is dependent on the inter-trial interval between the familiarization phases. The temporal changes in the distance traveled and TET were found to be independent of ITI in both TOM and NORM tests (Fig. 4).

We used the novel environment in our experiments to facilitate the involvement of dorsal hippocampus in modified behavioral tests, as this brain region is involved to a greater extent when the object familiarization takes place in a novel environment rather than a familiar one (de Lima et al., 2006; Antunes and Biala, 2011). In addition, we tested how the mice would behave in similar temporal design if there were no objects. We found only 30 min habituation to OF displayed significant familiarization to the OF. Additionally, no change in distance traveled was observed between two OF exposures of ITI of 30- and 120-min, implying that the habituation is independent of the inter-trial intervals (Fig. 7). However, when objects were present in the OF, we found a decreasing trend of distance traveled as the trials increased. This suggests that the gradual decrease in exploration in TOM and NORM experiments was mainly due to the presence of objects rather than the surrounding environment. In contrast, the TET decreased with an increase in trials in TOM but not in NORM. The difference between these two tasks is the absence of OF habituation and the presence of a novel object in the test phase for NORM. As the first two trials in both tasks have the same objects, the decrease in TET in trial-2 of TOM could be due to previous-day habituation. Overall, this showed that habituation to the surrounding environment decreases both the distance traveled and object exploration time. This could explain why there was no increase in the exploration of ROa object in all the above experiments – where a familiar object was placed in a new location in the subsequent trial. Standard TOM and NORM procedures include the habituation of rodents to the testing maze (i.e., OF) for around four days before the object familiarization day (Mitchell and Laiacona, 1998; Hannesson et al., 2004; Barker et al., 2007; Barker and Warburton, 2011; Manago et al., 2016). The lack of such habituations could have made our modified TOM/NORM process a difficult memory tasks; however, this difficulty could increase the sensitivity of memory decay observations. The results from this study can be applied for understanding the molecular mechanisms of different neurodegenerative disorders like Alzheimer's disease, Parkinson's disease, etc. which involves memory decay and progress with time. However, further investigation on the association of Arc and behavioral experience in changing the memory strength is warranted.

ACKNOWLEDGMENTS

The authors are thankful to the Department of Science & Technology (India) for both PURSE-II grant to the University of Hyderabad (Sanction No. SR/PURSE/Phase 2/4(C) and SERB grant to AG (Grant No. EEQ/2017/000513), which supported this study. We thank Dr. Arvind Kumar, Centre for Cellular & Molecular Biology, Hyderabad, India, for providing support in stereotaxic experiments.

DECLARATIONS OF INTEREST

None.

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(Received 5 October 2019, Accepted 10 February 2020)
(Available online 19 February 2020)

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