EFFECTS OF CONSTANT TRACK RUNNING EXERCISE ON THE EXPRESSION OF THE IMMEDIATE EARLY GENE *ARC* IN THE RAT HIPPOCAMPUS

By

SABHYA KUMAR

Molecular and Cellular Biology

THE UNIVERSITY OF ARIZONA

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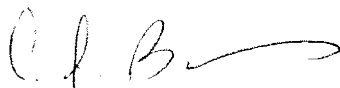
Approved by:



Carol A. Barnes, Ph. D.
Department of Psychology and Neurology

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Abstract

Arc is an IEG expressed in the CA1 sub-field of the hippocampus, following stimulus from an external environment and is expressed in the place cells involved in spatial learning. An initial experiment showed that *Arc* expression levels decreased with 25 minute rest periods between sessions. The current study aimed to see if similar results are seen with track running, if the rest periods are removed.

The rats ran on a small circular track, ensuring that a small ensemble of place cells activate on each lap. Animals were assigned to 4 groups: positive control for *Arc* induced by MECS; negative control for animals sacrificed from their home cage; a 5 minute group and a 45 minute group. The brain tissue was stained using FISH. The 45 minute group had a 4% lower *Arc* expression than the 5 minute group. A univariate ANOVA indicated a statistically significant effect in the main factor of Behavioral Group ($F = 6.955$, $p = 0.03$). Tukey tests showed that the differences between the MECS and the CC groups ($p < 0.05$) and the MECS and the 45 minute groups ($p < 0.05$) were significant. The behavioral groups showed no statistical significance meaning that massed exposure without rest does not attenuate *Arc* signal.

Background and Introduction

The hippocampus and memory

The hippocampus, a brain structure located inside the temporal lobe (Anderson et al., 2007) is known to be critical for the normal operation of learning and memory in both rats and humans (Barnes, 1991). A great amount of evidence from various studies suggests that the hippocampus plays a critical role in the formation of new memories about experienced events (Xiong, 2008) and much research done to study the hippocampus suggests that it plays a crucial role in acquisition of new information (Barnes, 1991). In the rodent, the term hippocampus refers to the subfields of CA1, CA2, CA3 and the dentate gyrus, (Martina and Clark, 2007), where the information flows from the dentate gyrus to CA3 to CA1 (Xiong, 2008). For an image of the rodent hippocamous, refer to Figure 1. These subfields not only differ in their anatomical inputs but also in their internal circuitry (Lee et al., 2004) as can be seen in the instance of CA1 and CA3 subfields, which perform different, yet complementary functions when processing spatial and contextual information (Vazdarjanova and Guzowski, 2004). In the rodent model, damage to the hippocampus results in impaired learning and spatial memory (Barnes, 1991), which supports the theory that the hipocampus is crucial in memory formation.

Place cells

Place cells are pyramidal cells in the hippocampus that fire when an animal is in a particular location in its environment (O'Keefe and Dostrovsky, 1971). When an animal is in any environment, it is known from electrophysiology that there is a collection of hippocampal place cells that are active and form a spatial map, and the membership of these neurons is the code for space changes when the environment changes. When the animal returns to the same

environment, the same neurons from previous visits fire each time, forming a code for that specific location in space; hence why these neurons are referred to as places cells (Eichenbaum, 2000). That location where a place cell fires rapidly every time the animal occupies it is referred to as that cell's place field (Anderson et al., 2007). For instance, when a rat is in its home cage, the neural activity taking place in its place cells encodes the different place fields in that cage. The results from an experiment done by Guzowski et al. (1999) demonstrated that separate populations of place cells are activated when the animal visits different environments, whereas the same populations are activated by two visits to the same environment, and these populations can be detected by the expression of genes that are induced by behaviorally specific electrophysiological activity.

Immediate Early Genes (IEGs)

Following specific extracellular signals, the first genes that neurons express are called Immediate Early Genes, or IEGs (Guzowski et al., 2005). A crucial event in the sequence of IEG expression is the phosphorylation of CREB (cAMP-responsive element-binding protein), which when phosphorylated leads to the transcription of an IEG mRNA that can be later translated into a protein (Burke and Barnes, 2006). Neurons express IEGs whenever an animal is involved in an activity that evokes associated neural activity, such as spatial learning. IEG expression is especially important in forming memories because they play a critical role in transformation of activity in neural circuits (Kubik et al., 2007) that is important in helping to stabilize those neural circuits involved in storing learning experiences as long-term memory (Guzowski et al., 2005). IEGs result in downstream expression of a varied range of proteins including structural and scaffolding proteins, signal transduction proteins, growth factors,

proteases, and enzymes (Guzowski et al., 2005). IEGs can also regulate long-term cellular responses by encoding transcription factors and cytokines (Lyford et al., 1995).

IEG expression in the hippocampus of a resting animal is extremely low as compared to that of an animal that has been subjected to a behavioral experience (Guzowski et al., 2006). In addition, since IEG expression is crucial in stabilizing recent changes in synaptic efficacy and is important for the molecular processes that are fundamental to memory consolidation (Guzowski 2005), IEGs have the potential to serve as excellent markers for neuronal activity (Guzowski et al., 2006). Using them as activity markers makes it possible to conduct studies involving various behaviors like drug addiction and withdrawal, learning and memory, sensory processing, mating, feeding, and maternal behaviors, circadian rhythm entrainment, pain, and fear and stress (Guzowski et al., 2006). In the hippocampus, IEG expression has often been used as a marker for neurons involved in spatial learning.

The IEG *Arc*

Arc, or activity regulated cytoskeleton-associated protein, is an important IEG whose expression is induced by stimuli known to increase hippocampal place cell activity (Guzowski et al., 1999). It is important for maintenance of long-term potentiation, a process thought to underlie memory formation by increasing synaptic strength that results from repeated stimulation of the hippocampal neurons (Frey and Moris, 1997) and requires synthesis of new RNA and proteins (Lyford et al., 1995). *Arc* has been shown to be expressed in the CA1 sub-field of the hippocampus, where neurons are specifically activated during the exploration of a given environment (Guzowski et al., 1999). Studies have shown that, in rats, the expression of *Arc* RNA occurs in about 40% of the CA1 neuronal cells of the dorsal hippocampus upon exploration

of a new environment, which in this case was a 60 x 60 cm square platform divided into nine 400cm² grids (Chawla et al., 2005). This proportion of cells that express *Arc* in the CA1 sub-field of the hippocampus is consistent with the values found in cells that show electrophysiologically-recorded place fields from an environment of the same size (Guzowski et al., 1999).

According to Chawla et al. (2005), when the expression of *Arc* protein was blocked by using antisense oligodoexynucleotides, it interfered with the consolidation of spatial memory and maintenance of long-term potentiation. This provided evidence that *Arc* is involved in the long-term stabilization of newly formed memories from spatial experiences (Guzowski et al., 1999). Due to its role in the synaptic processes that mediate learning and its expression in the place cells of the hippocampus, which are critical for spatial learning, *Arc* can be used reliably as a marker for activity in these place cells in an environment-specific manner after spatial exploration (Chawla et al., 2005). *Arc* would be expressed whenever the place cells reactivate and can thus be used to mark neurons during repeated explorations of the same space.

Confocal imaging suggests that *Arc* is present in dendrites and this presence corresponds to the localization of F-actin, which happens to be the protein with which *Arc* coprecipitates, suggesting that expression of *Arc* may be linked to the cytoskeleton *in vivo* (Lyford et al., 1995). *Arc* is also known to associate with the N-methyl-D-aspartate (NMDA) receptor complexes (Kelly and Deadwyler, 2003), which are required for transcriptional activation and dendritic trafficking of mRNAs and proteins (Husi et al., 2000). According to one study, blockade of the NMDA receptors in the hippocampus results in impairment of both synpatic plasticity and learning (Husi et al., 2000). In addition, *Arc* is involved in modulating AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor trafficking by regulating synaptic strength

(Rao et al., 2006). AMPA receptors allow for fast synaptic transmission in the central nervous system. Regulation of *Arc* transcription starts when synaptic activity triggers *Arc* expression, leading to an induction in AMPA receptor surface delivery. This causes a decreased NMDA/AMPA ratio, which results in excessive *Arc* transcription inhibition. To resume *Arc* transcription however, the ratio of these two receptors can be reset, allowing for subsequent *Arc* expression and synaptic plasticity (Rao et al., 2006).

The expression of *Arc* mRNA can be seen as early as within 2-3 minutes of a new activity, at which time, it is found at the genomic sites of transcription in the nucleus (Guzowski et al., 2005). Within 20-45 minutes, however, the mRNA can be found accumulated in the cytoplasm (Guzowski et al., 2005). This difference in localization is helpful in determining when a single neuron was activated prior to harvesting the brain. By identifying the subcellular location of *Arc* within positive neurons, it is possible to analyze how a certain behavior affects activity of the neuron across time.

The Rat Model

By studying the hippocampus of rats, we can learn important information about the function of this structure in humans, as the neural circuitry of the rat and human hippocampus is fundamentally the same, due to conservation in mammals throughout evolution (Barnes, 1991). Rats also serve as a good model of spatial learning because they tend to be excellent foragers, an ability that helps them learn spatial memory problems more easily than some other animals and contributing to excellent performance in tests such as the Morris water maze (Barnes, 1991).

In this study we used specifically young rats because previous studies have shown that older animals show poorer memory retention than younger animals, especially during spatial

learning tasks, something similar to what is found in humans where older humans show problems in processing spatial information (Barnes, 1991). During some non-spatial tasks, however, no difference in performance between young and old rats is seen (Barnes, 1991). But since this study is measuring the effects of running on a track in a fixed location, the task is spatial and therefore young rats were ideal.

Previous Studies

In a previous wheel running experiment, rats that ran on an exercise wheel for 5 min were compared to rats that ran for 45 min, with reward after every 16 laps in both cases, to see if the prolonged exposure to a single location within an environment led to an attenuation of the *Arc* signal in the hippocampus (Lister et al., 2008). As can be seen in Figure 2 the results of the study showed that the rats that ran for 45 minutes actually showed a higher expression of *Arc* signal than the cage control rats and the rats that ran for 5 minutes (Lister et al., 2008). Thus spending more time in the same location of an environment (the single physical spot within the exercise wheel) for a continuous period of time did not lead to attenuation of *Arc* signal in the hippocampus.

These results were contradictory to the results found in another study, where rats were exposed to a new environment for 5 min, 9 times in a single day, each exposure separated by 25 minutes (MASSED-I group). These rats showed less expression of *Arc* when compared to groups NOVEL, in which rats were exposed to a new environment once, or SPACED, where rats were exposed to a new environment for 5 minutes every day for 9 days (Guzowski et al., 2006). This experiment showed that repetitive exposure results in a loss of *Arc* expression in the hippocampal neurons (Guzowski et al., 2006), despite the fact that the neurons were still firing

during each exposure. These results show that if an animal is exposed to the same environment over and over again, but with longer inter-exposure periods, it has a higher *Arc* expression as compared to an animal that is exposed to a novel environment multiple times in the same day. Based on these results, Guzowski et al. (2006) concluded that massed training affects memory consolidation by reducing coupling between neural activity and IEG expression.

The results from wheel running study were different from the study conducted by Guzowski et al. (2006). The goal of the wheel running study was to look at the effect of constant, uninterrupted running on the relationship between neuronal firing and *Arc* expression; that study did not find a decoupling of *Arc* expression from neuronal firing, however, there was an abnormally high proportion of neurons (~77%, Figure 1) expressing *Arc* as would be expected for an animal running in place. It is possible that the hippocampus of the rat running on the wheel was treating the running session as if the wheel was unwrapped, like a length of space in the real world, thereby activating ensembles of place cells in sequence. Since the rats were rewarded on schedule after every 16 laps, they may have been able to estimate the distance traveled before they would get a reward based on the number of laps they had learned to run and because of that their activity on the wheel was proportional to an animal running a longer distance, which in turn affected the proportion of neurons expressing *Arc.* If this was the case, then the original hypothesis was not tested properly, as the hippocampus would have been cycling through the sequences of neuronal ensembles during each 16-lap interval instead of having the same neurons firing constantly for the 45 minutes.

The objective of this study was to test the original hypothesis by using a track instead of a wheel and keeping the distance for each lap very short, so that the same neurons would be firing

as close to constantly as possible during successive laps. Additionally a random reward schedule was used to prevent the animal learning a fixed distance between rewards and constructing a longer sequence of ensemble activity compared to the actual distance traveled per lap, in the event that that was possible in the hippocampus of the wheel running paradigm. With the current design, we are trying to investigate if massed exposure with constant neuronal activation also leads to attenuation of *Arc* signal in the hippocampus, resulting in lower *Arc* expression in rats running on the track for a longer period of time.

Methods

Behavioral Handling and training.

For this study, we used twelve nine months old male F344 rats. In the beginning, the rats were handled to get them familiarized with the experimenter. This was done by wrapping the rats in a towel and holding the towel up to the expeirmenter's chest for five to ten minutes everyday for three days. This helped get the rats used to being picked up and handled. Before training, the rats were put on a food restriction diet to bring their weights to about 85%.

A circular track with a black surface and an outer diameter of 56 cm was used in the study (Figure 3). The twelve rats were divided into four groups, with three rats randomly assigned to each group: caged congtrol (CC), rats were sacrificed directly from their home cages; 5 Minute Running Group, in which the rats ran around the track for five minutes; 45 Minute Running Group, in which the rats ran on the track uniterrupted for forty-five minutes; and MECS group, in which maximal electroconclusive shock was used to induce *Arc* expression in rats straight from their home cages. The MECS and CC group served as positive and negative controls, respectively, and the other two groups were the behavioral groups.

All of the rats were trained to run on the track continuously for forty-five minutes every day for five months. Rewards were placed on the track on the same place every time but at random intervals (laps ranging from 1-4) in order to avoid expectation of reward behavior in the rats because in the previous wheel running experiment the rats were rewarded after every 16 laps, and due to that, it is possible that they were able to estimate when they would get their rewards and produce sequence activity on the wheel in hippocampal populations proportional to an animal running a longer distance. This may have affected the proportion of neurons expressing *Arc* (Lister et al., 2008) and means there may have been significant delays between the firing of each neuron in the sequence.

Brain Extraction, tissue blocking, and cutting

On the day of the experiment, according to their groups, the rats were transported in a glass jar with isofluorane vapors to anestheizse them. The CC rats were taken in directly from the rat colony room. The MECS rats were induced with an electroconclusive shock and then transferred into the jar. The behavioral groups were transferred after the track running period. It took the rats around one minute to become unconscious, at which point, they were sacrificed using a rodent guillotine. Immediately after the brains were extracted from each rat, they were flash frozen at -80°C in an isopentane bath that was set up earlier. Once the brains had frozen completely, they were hemisected and one of the hemispheres from each brain was chosen to be blocked.

Four hemispheres were chosen to be placed in one block (total three blocks were made), where one hemisphere from each group was placed in each block. OCT was used as the blocking agent and the entire procedure was done inside a cryostat at -25°C. When the blocks were frozen

and ready to be used, they were mounted on the cryostat and cut at a thickness of 20μm in the coronal plane from posterior to anterior. The slides were then collected in series, where series 1 included slides 1, 11, 21, etc., series 2-5 included slides 2, 3, 4, 5, 12, 13, 14, 15, etc., and series 6-10 included slides 6,7,8,9,10, 16,17,18,19,20 etc. Classifying the slides in this order helped keep slides in separate sets, some of which were used while the other were set aside for potential later use. Once all of the tissue was collected, the slides were stored overnight at -20°C and then moved to -80°C until further use.

Nissl staining

The main purpose of Nissl staining was to use slides from series 1 in order to identify the most anterior part of the hippocampus in each brain section, which was to be used to select sections from the same level of anterior hippocampus in series 6-10 for fluorescence *in* situ hybidization (FISH) staining. The slides were first immersed in paraformaldahyde for twenty minutes to fix the tissue and were then left in a 1:1 mixtue of chloroform and 100% ethanol for one hour. In the next four steps, the tissue was rehydrated by moving the slides through the following order: 100% EtOH for one minute, 95% EtOH for one minute, 70% EtOH for one minute, and 50% EtOH for one minute. After rehydrating the tissue, it was moved to Thionin (dye) for three minutes to five minutes, depending on how long it took for the dye to stain the tissue. Once the tissue was stained to pereference, the slides were moved to distilled for four minutes, to wash off any excess dye, after which they were treated in the following order, to dehydate the tissue: 50% EtOH for four minutes, 70% EtOH for four minutes, 95% EtOH for four minutes, and 100 % EtOH for four minutes. Lastly the tissue was cleared by soaking two

times in xylene for five minutes each. Finally, coverslips of thickness 1.5 were placed on the slides using Permount and left to dry for two days.

<u>Riboprobe Synthesis</u>

2µL of DNA template, 12µL of DEPC water at 37°C, 2µL of 10X transcription buffer at 37°C, 2µL of DIG RNA labeling mix at -20°C, and 2µL of RNA polymerase at -20°C was added to a 1.5mL microfuge tube in the written order. The resulting 20µL solution was mixed well with a pipet tip, spun briefly on a microfuge, and incubated at 37°C for two hours. The reaction was then treated with 1µL of DNAse for fifteen minutes at 37°C, after which the reaction was stopped by adding 2µL of RNase free 0.5 EDTA, followed by addition of 37µL of DEPC water, to bring the total volume of the solution to 60µL.

For the next step, RNA was purified using Roche's miniquick columns. The matrix was resuspended, the column was placed in a clean microfuge tube with a cut lid, and was spun for one minute at 1000xg. After that, 200µL of DEPC water was added and the column was spun for another 2 minutes at 1000xg. The 60µL sample was added to the column and spun for an additional four minutes at 1000xg.

The probe yield and integrity was evaluated on a 1% agarose ge containing ethidium bromide of 0.5µg. 2µL of probe was added to 8µL of RNA loading buffer. The riboprobe mixture was then denatured at 90°C for seven minutes and the probes were placed on ice until they were loaded on the gel. For the final step, the gel was run at 200mV in 1X TBE for 20 minutes.

<u>Fluorescence *In Situ* Hybridization (FISH) staining</u>

Slides suitable for FISH staining were picked out using the Nissl stained series 1 slides.

Since there were four brains on each slide, not all of them were level within the block and therefore multiple slides were required to image the most anterior hippocampus of all of the brain sections comprising each different block. The FISH staining was a three day process. The goal of day 1 was to hybridize *Arc* riboprobes to the endogenous *Arc* mRNA in the brain tissue. On the first day all of the containers were thoroughly washed with RNAse Away and rinsed with DEPC water to ensure that the RNA probes will not be destroyed by RNAses. On the previous day, the slides were removed from -80°C and stored at -20°C overnight, from where they were removed on the first day of staining and were thawed for three hours. Moving the tissue from -80°C to -20°C first before bringing them to room temperature avoided immediate thawing of the tissue, which can result in damage.

Once the tissue and containers were ready to be used, the tissue slides were immersed in 4% paraformaldehyde for five minutes at 4°C. The tissue was then washed in the following order: 2X SSC for two minutes at room temperature (RT); acetic anhydride for ten minutes at RT; briefly dipped in DEPC water at RT; 1:1 acetone and methanol for five minutes at -20°C; 2X SSC for five minutes at RT. The tissue was then treated with 120µL of 1X Sigma Prehybridization buffer, coverslipped, and left in a humid chamber of 50% formamide solution for thirty minutes at RT. During this time, a mixture of 30-100ng riboprobe per 100µL Amersham buffer was made and heated at 90°C for seven minutes and then cooled on ice for three minutes. Each tissue slide was treated with 120 µL of riboprobe, coverslipped, and incubated in a hybridization oven at 56°C for eighteen hours. 50% formamide solution humid chamber was used and was wrapped in saran wrap when placed in the hybridization chamber.

The goal of the second day of staining was to tag the riboprobe-mRNA complexes with an antibody for visualization. At the beginning of second day, the humid chamber was removed from the hybridization oven to let it cool at RT for fifteen minutes. Different containers than Day 1 were used to avoid risk of RNAse contamination. Once the slides were at RT, coverslips were removed by dipping the slides in 2X SSC. Tissue was then washed in 2X SSC for five minutes and again for ten min on a shaker table at RT to wash away any excess unbound riboprobe. Next step was to wash in 2X SSC with RNAse Away (concentration of 25mg/mL) for fifteen minutes at 37°C, in order to further destroy any unbound riboprobe that was not already washed away. The tissue was then washed on a shaker table to remove excess RNAse Away in 2X SSC for ten minutes at RT, 0.5X SSX for ten minutes at RT, 0.5X SSC for thirty minutes at 55°C (in an oven), 0.5X SSC for five minutes at RT. After all of the washes, the slides were placed in 1%

quench in 1X SSC for 15 minutes on a shaker table for 15 minutes at RT, followed by a wash in tris-buffered saline with tween-20 (TBS-T) for 5 minutes and tris-buffered saline (TBS) for 5 minutes on a shaker table at RT. The slides were blocked with 120 µL TSA blocking buffer with 5% normal sheep serum, coverslipped, and left in a TBS humid chamber for 30 minutes at RT. As the final step for the second day, the tissue was treated with 120 µL of anti-DG1:400 in 0.5% TSA blocking buffer, coverslipped, and stored at 4°C for 18 hours in a saran wrapped humid chamber containing TBS-damp blotting paper.

For the last day of staining, the riboprobe-mRNA complexes tagged with antibody were further visualized with cyanine 3 (Cy3) and the cellular nuclei were counterstained with To-Pro. To start with, the coverslips were removed by dipping the slides in TBS-T at RT, followed by washing the slides in TBS-T three times, each for ten minutes on a shaker table at RT, to remove

unbound antibody and permeabilize the cellular membranes for better penetration of dye into the nucleus. Tissue was then stained with 120µL Cy3 mixed with 1:50 in amplification diluent, coverslipped, and left in an aluminum foil wrapped, TBS humid chamber for thirty minutes at RT. The coverslips were removed in TBS-T and the slides were washed three times in TBS-T for ten minutes and for five minutes in TBS at RT on a shaker table (the container was covered in foil during these washes to avoid light exposure to the tissues). This allowed for the removal of excess Cy3 solution. After these washes, the slides were stained with To-Pro 1:1000 in TBS, coverslipped, and stored in an aluminum foil covered humid chamber with TBS damp blotting paper for thirty minutes at RT. The coverslips were removed by dipping slides in TBS and the tissue was then washed in TBS for ten minutes on a shaker tabe at RT, after which final coverslips (1.5 thickness) were placed using Vecatshield. The slides were placed in an aluminum covered box and stored at 4°C for two days. Excess Vecatshield was then removed with Kim Wipes and the edges of the slides and coverslips were sealed off with nail polish.

Quantification of *Arc* postive neurons

Once the slides were stained using FISH, they were viewed under a confocal microsocpe and imaged. Three sections were selected per brain and for each slide three images were taken from proximal, middle, and distal regions of the CA1 sub-field of the dorsal hippocampus. Each image was then used to count the total number of *Arc* positive neurons. From the confocal images, a median plane, which consists of the middle 20% of the optical planes in an image, was selected and neurons expressing *Arc* were counted. This eliminated the bias of counting cells at the cut edge of tissue, as cutting can damage those cells and remove the stain, thereby favoring the negatively stained cells. The blue stain represents the cellular nuclei and the red stain

indicates positive *Arc* expression (Figure 4). At first, the total number of neurons were counted through the median plane, with the *Arc* expression turned off to eliminate bias of counting a nucleus because it has *Arc* expression, when otherwise it might not have been counted. The total number of nuclei represents the SUM of neurons in the image. Next step was to turn on the *Arc* expression channel and classify the marked nuclei as *Arc* positive or *Arc* negative. The *Arc* expression stain was red and if it was associated with a nucleus, it was classified as *Arc* positive. This number was then subtracted from the SUM to find out the total number of *Arc* negative neurons. Using these numbers, the total percentage of *Arc* positive neurons was calculated.

<u>Stastical Analysis</u>

Some of the data could not be collected due to damage inflicted during the processing steps. A univariate Analysis of Variance (ANOVA) with Tukey HSD post hoc tests was performed on the remaining data collected at each location in the CA1 subfields of caged control (N=1), 5 min runners (N=2), 45 min runners (N=2), and MECS (N=2) to analyze the significance of the data for the total number of *Arc* positive neurons. Each region (proximal, middle, and distal) within a subject was treated as a separate data point in order to be able to run statistical analysis.

Results

In the past study by Guzowski et al. (2006), rats that were exposed to an environment nine times a day, MASSED, showed a lower expression of *Arc* signal when compared to the rats that were exposed to a new environment once each day for nine days (SPACED) or those who were only exposed to a new environment once (NOVEL). Similar results were found in this experiment, where the rats that contnuously ran for 45 minutes showed lower mean proportions

of cells with *Arc* expression (25.6%) than the rats that ran for only 5 minutes (29.2%). The mean values for the data for *Arc* expression can be seen in Table 1.

The univariate ANOVA found a significant main effect of the between subjects factor of behavioral group ($F=6.955$, $p < 0.005$). In addition, Tukey HSD post hoc tests were performed to determine which groups were different from each other. The results from the Tukey tests showed that the source for the significant differences was between the MECS group and the CC group ($p=0.003$) and the MECS group and the 45 minute group ($p= 0.022$). There was also a trend towards significance noted between the MECS group and the 5 minute group, with $p=0.057$. Since this number is fairly close to the alpha significance level of $p=0.05$, it is possible that with more data that value for significance between these groups would go down towards 0.05. There was no statistical difference seen between the CC and behavioral groups and between the two behavioral groups, even though the 5 min and 45 min groups showed a higher expression of *Arc* than the CC, and the 45 minute groups showed a lower expression of *Arc* than the 5 minute group (Figure 5). Once again, low statistical power may be responsible for the lack of significance and with more data collection differences may be detected.

Discussion

The limited results from this experiment were similar to those found by Guzowski et al. (2006) in that the rats which ran on a track for a longer period of time showed about a 4% lower expression of *Arc* positive neurons in their hippocampus compared to the 5 minute running group (Table 1). If real, this difference may be due to the phenomenon known as electrotranscriptional decoupling. The Guzowski (2006) study showed that when a rat is exposed to the same environment repeatedly with short intervening rest periods, the same neurons fire each time but

after the final exposure a lower amount of *Arc* expression occurs. The direction of this change in expression is what we saw happen in the rats from the 45 minute behavioral group compared to the 5 minute group. However, if upon replication, the statistical analyses (Tukey HSD post hoc tests) suggest no differences between the *Arc* expression in rats from the 5 minute group and the 45 minute group, the results essentially mean that our massed exposure did not lead to attenuation of *Arc* signal and therefore electrotranscriptional decoupling did not occur, which is the same as what Lister et al. (2008) found in the wheel running experiment. If this turns out to be the case, the results are different from what Guzowski et al. (2006) found in their experiment.

In addition, when examining the data for the percentage of *Arc* expression, Figure 4, we see that both the behavioral groups showed a higher percentage of *Arc* positive neurons than the CC group; however, the Tukey HSD post hoc tests revealed that those differences were not statistically significant. The most likely explanation for this lack of significance is the small amount of data collected from the CC group, that is only one animal was counted. Until more data from the control group is sampled, these results cannot be definitively determined.

This experiment failed to show significant differences between the levels of *Arc* expression between a short and a prolonged behavior, thus suggesting that the rest periods used in the massed exposure paradigm were critical for whichever mechanism underlies attenuation of *Arc* expression. It is possible however that the results from this experiment are not significant because the statistical power was very small. Initially we used three animals per group (N=3) and were able to stain tissues from all twelve animals. But, after the experiment, due to loss of tissue due to damage, we did not have the full data to use for results. Therefore, there was too little data

to reasonably detect all but the most dramatic statistically significant differences between the two behaviors and the control groups tested.

While not statistically significant, we did find that the 45 minute running group had a lower percentage of neurons expressing *Arc* compared to the 5 minute group; given the size of the standard errors of the mean for the data collected, if more data were analyzed and a significant difference were found between those groups, it is likely to not be particularly large. The difference reported here would estimate a reduction from 28% in the 5 minute runners to 24% of neurons in the 45 minute group, whereas Guzowski and colleagues reported a reduction from 40% of neurons to 25% in the MASSED group. The magnitude of change in the Guzowski study is thus substantially larger than what we could estimate based on the results from our track running paradigm.

Rat Group	SUM	Arc +	Arc -	% Arc Positive
5 min	106.5	30.0	76.5	29.2
45 min	63.7	15.3	48.3	25.6
CC*	107.3	12.3	285	11.5
MECS	103.8	47.0	56.8	49.7

Table 1: Mean Percentage of *Arc* positive neurons. For each group, the expression of *Arc* was measured from two animals (except for CC, in which 1 animal was analyzed) and the percentage value for the amount of neurons expressing *Arc* is given. SUM is the total number of nuclei present in the proximal, middle, and distal sections of the CA1 subfield of the rat hippocampus.

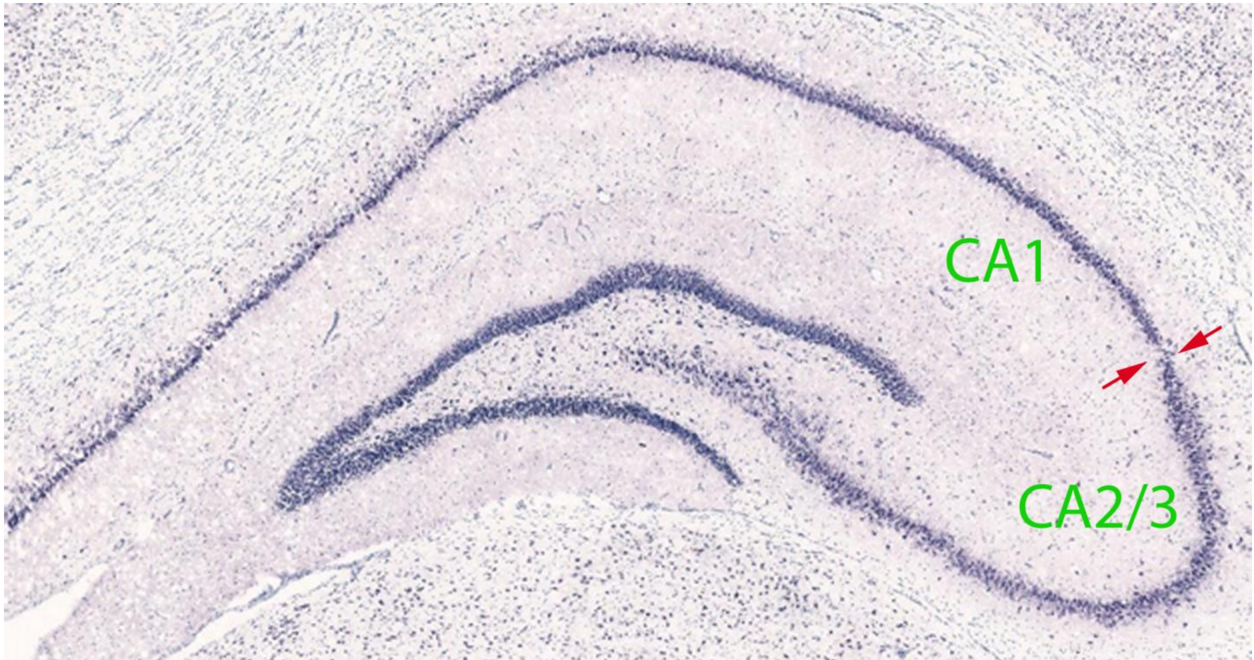

Figure 1: Coronal Section from the Dorsal Hippocampus. The red arrows indicate the narrowing thickness of the stained cellular layer that represents the border between the larger neurons of the CA2/3 subfields and the smaller neurons of the CA1 subfield, the hippocampal subfield where the images for *Arc* expression are taken.

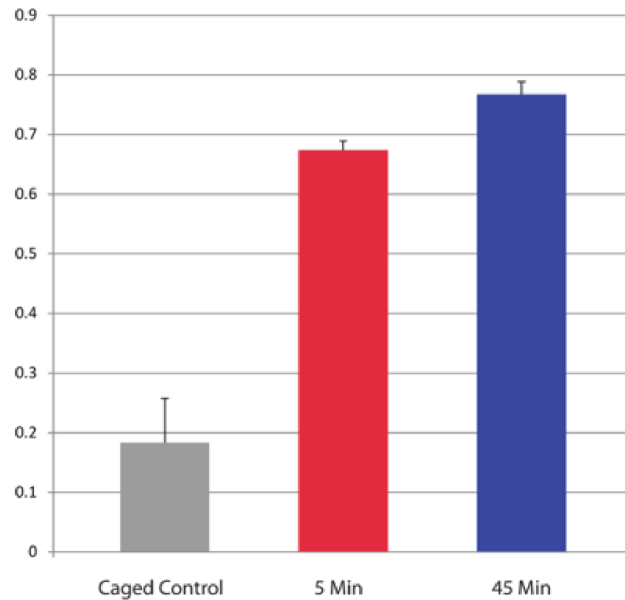

Figure 2: Percentage of *Arc* positive neurons from a previous wheel running experiment. The rats that ran for 45 minutes showed a higher proportion of *Arc* signal in the hippocampus when comapred to the rats that ran for 5 minutes (Lister et al., 2008).

A.

B.

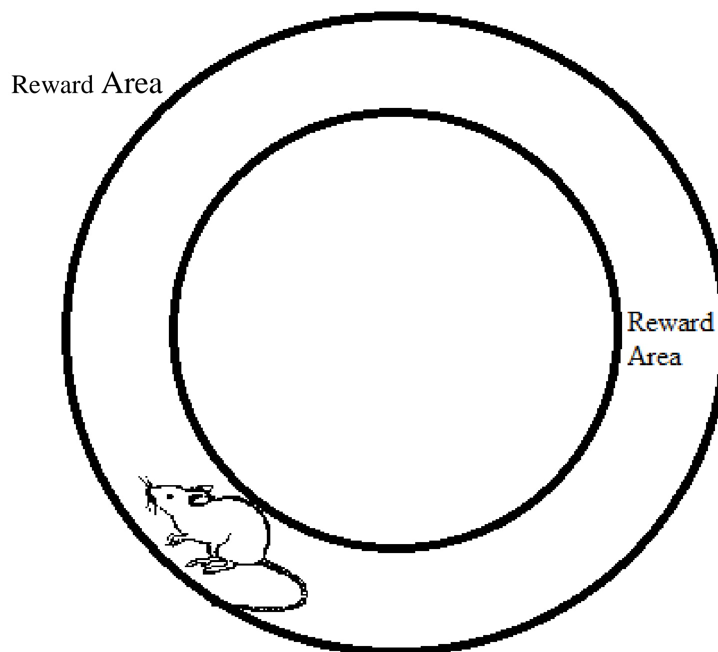

Figure 3: Pictures depicting the track used. Figure 3A shoes the actual track that was used for the 5 min and 45 min behavioral group to measure the expression of *Arc* from these behaviors. Figure 3B shows a respresntation of the track and illustrates where the rats were rewarded each time. Reward was administered using a random interval of 1-4 laps.

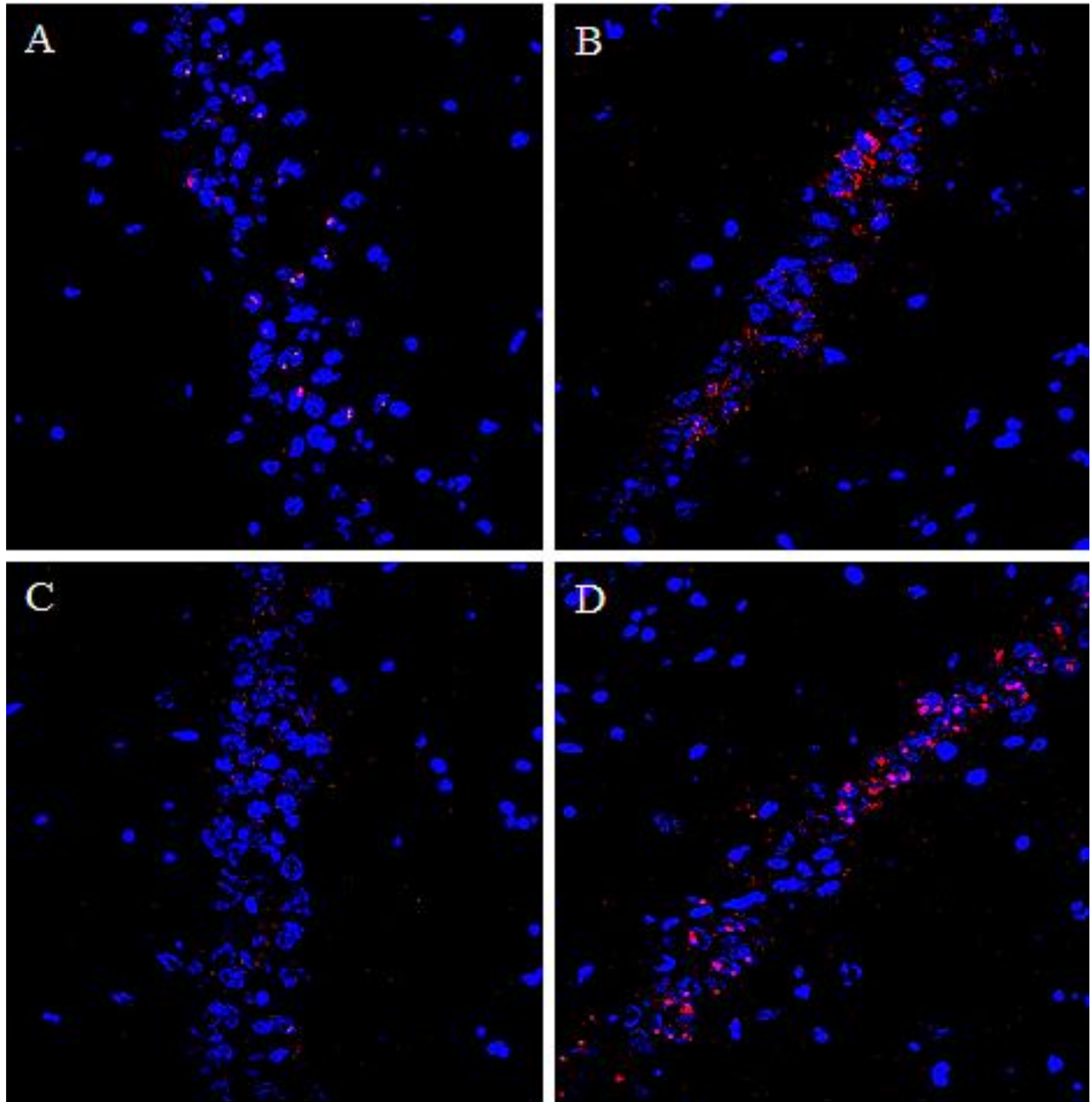

Figure 4: Image sections displaying the expression of *Arc* in the four groups. These images show the FISH staining for *Arc* expression in the groups A) 5 minutes, B) 45 minutes, C) Caged Control (CC), and D) MECS. Blue stain is for the nuclei and the red staining shows *Arc* expression.

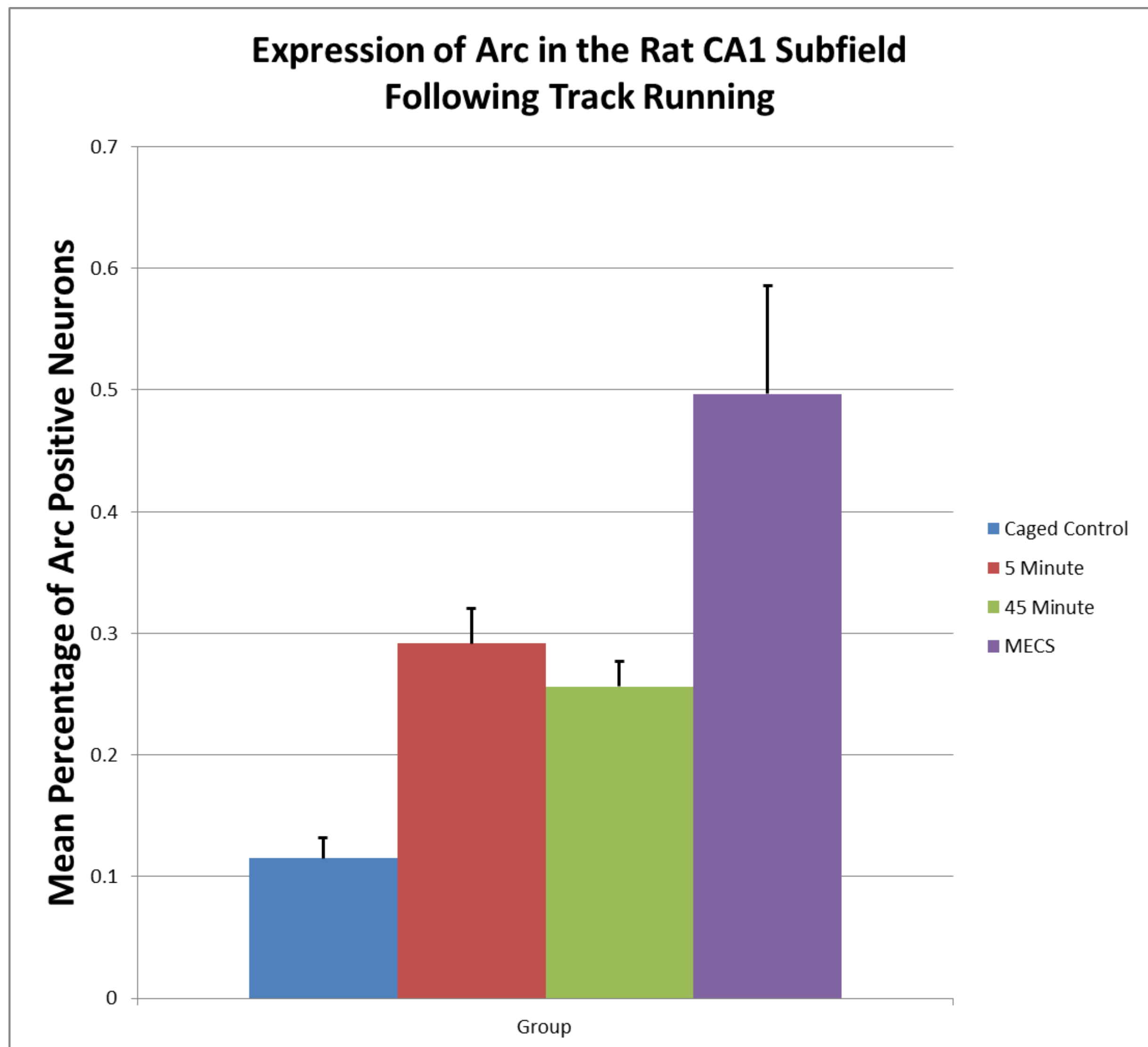

Figure 5: Percentage of *Arc* positive neurons in caged control, 5 minutes, 45 minutes, and MECS. A univariate Analysis of Variance (ANOVA) was performed on the data collected at each location in the CA1 subfields of caged control (N=1), 5min runners (N=2), 45min runners (N=2), and MECS (N=2) resulting in a significant effect of Behavioral Group ($p<0.005$). Tukey HSD post hoc tests showed statistically significant differences between MECS and CC ($p<0.05$), and MECS and 45min ($p<0.05$); and MECS and 5 min showed a trend towards significance (with $p=0.057$).

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