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Post-training disruption of Arc protein expression in the anterior cingulate cortex impairs long-term memory for inhibitory avoidance training

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ABSTRACT

The activity-regulated-cytoskeletal-associated protein (Arc) has a well established role in memory consolidation and synaptic plasticity in the hippocampus and amygdala. However the role of Arc within the anterior cingulate cortex (ACC), an area of the brain involved in processing memory for pain, has yet to be examined. Here we sought to determine if Arc protein within neurons of the rat ACC is necessary for the consolidation of a single-trial, contextual inhibitory avoidance (IA) task. Immunohistochemistry and western blotting revealed an increase in Arc protein within the ACC following IA training in a shock-specific manner, suggesting that ACC Arc expression may play a critical role in the consolidation of the aversive task. To directly test this hypothesis, male Sprague–Dawley rats were trained on the IA task and given post-training intra-ACC infusions of Arc antisense oligodeoxynucleotides (ODNs), designed to suppress Arc translation, or control scrambled ODNs that do not suppress Arc translation. Memory retention was tested 48 h after training. Arc antisense-induced disruption of Arc protein expression in the ACC impaired long-term memory for the IA task as compared to rats given intra-ACC infusions of the scrambled control ODNS, suggesting that Arc expression in the ACC is important for the consolidation of emotional memory. Results further indicate that knock down of Arc 6 h after training impairs IA memory. This is consistent with time course findings indicating elevated Arc expression at 3 and 6 h after IA training but not 12 or 48 h. Taken together, these findings support the hypothesis that Arc expression in the ACC participates in synaptic plasticity that underlies long-term memory.

1. Introduction

It is generally accepted that memories are consolidated by neuronal plasticity, the process by which connections between neurons are strengthened or weakened due to experience. Expression of the immediate early gene known as activity-regulated-cytoskeletal-associated protein (*Arc*), or Arg3.1, is often used as a marker for this neuronal plasticity (Guzowski et al., 2000; Ramirez-Amaya et al., 2005; Vazdarjanova, McNaughton, Barnes, Worley, & Guzowski, 2002; Vazdarjanova et al., 2006). *Arc* is a gene of interest in part because its mRNA is delivered to stimulated regions of dendrites (Lyford et al., 1995; Steward & Worley, 2002) where it is translated to protein (Bloomer, VanDongen, & VanDongen, 2007; Yin, Edelman, & Vanderklish, 2002) upon synaptic activity induced by long-term potentiation (LTP; Messaoudi et al., 2007) and following behavioral stimulation (Vazdarjanova et al., 2006). The local translation of proteins such as Arc may participate in synapse-specific changes in synaptic transmission and spine morphology (Steward & Schuman, 2001; Troca-Marín, Alves-Sampaio, Tejedor, & Montesinos, 2010).

Although Arc's involvement in hippocampal plasticity is well-documented, its precise role at the synapse is unknown (for review see Chowdhury et al., 2006; Fukazawa et al., 2003; Huang, Chotiner, & Steward, 2007; Lee, Liu, Wang, & Sheng, 2002; Mahanty & Sah, 1998; Messaoudi et al., 2007; Remondes & Schuman, 2002; Tanaka et al., 2008; Waung, Pfeiffer, Nosyreva, Ronesi, & Huber, 2008). Whatever its role at the synapse may be, Arc expression appears to be necessary for the consolidation of hippocampal synapses and spatial and contextual memories. For example, intra-dorsal hippocampus infusions of Arc antisense oligodeoxynucleotides that knock down Arc protein expression attenuate the maintenance of LTP, without affecting induction, and they impair long-term memory without affecting acquisition of a spatial water maze task (Guzowski et al., 2000). Likewise, intra-hippocampus infusions of Arc antisense oligodeoxynucleotides impair long-term memory, but not acquisition, of the aversive, contextual inhibitory avoidance task (McIntyre et al., 2005). Interestingly, several studies have identified an uncoupling of *Arc* mRNA and protein expression (Bloomer, VanDongen, & VanDongen, 2008; Kelly & Deadwyler, 2003; Zalfa et al., 2003). Although inhibitory avoidance training produces increases in Arc transcript and protein in the hippocampus, memory-enhancing activation of noradrenergic receptors in the basolateral complex of the amygdala (BLA) further increase

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hippocampal expression of Arc protein only (McIntyre et al., 2005). It remains to be seen whether Arc protein expression patterns are similar in other areas of the brain that participate in memory processing.

Arc is expressed in areas of the brain outside of the hippocampus. Both aversive and non-aversive tasks increase Arc mRNA in cortical and limbic areas of the brain (Kelly & Deadwyler, 2002, 2003; Ons, MartÍ, & Armario, 2004; Ploski et al., 2008). In mice, Arc mRNA is increased by a fear conditioning task in the hippocampus as well as the anterior cingulate cortex (ACC), the olfactory bulb, the piriform, parietal, sensory and motor cortices, and the amygdala (Montag-sallaz & Montag, 2003). Similarly, stress and fear conditioning increase Arc protein expression in many of the same areas of the rat brain, including the medial prefrontal cortex (mPFC) (Kelly & Deadwyler, 2003; Koya et al., 2005; Mikkelsen & Larsen, 2006; Ploski et al., 2008). The ACC is an area where increases in Arc mRNA are observed following training on an appetitive task or restraint stress task (Kelly & Deadwyler, 2003; Mikkelsen & Larsen, 2006).

Pharmacological studies implicate the ACC in the consolidation of long-term memory for the IA task (Malin & McGaugh, 2006). Specifically, immediate post-training infusions of the muscarinic agonist oxotremorine directly into the rostral ACC (rACC) enhance memory for the nociceptive component of the task – the footshock (Malin & McGaugh, 2006). Likewise, the ACC plays a role in the processing of nociceptive stimuli in humans (Davis et al., 2005; Hutchison, Davis, Lozano, Tasker, & Dostrovsky, 1999; Phan, Liberzon, Welsh, Britton, & Taylor, 2003). The research described here seeks to determine if the role of Arc in hippocampal synaptic plasticity and memory can be extended to other brain regions such as the ACC. In Experiment 1, Arc expression was measured in the ACC 1 h after training. In Experiment 2, antisense or scrambled ODNs were infused into the rostral portion of the ACC immediately, 6 h, or 45 h after training, and memory retention was tested 48 h after training. In Experiment 3, a timeline of Arc expression in the ACC was examined in rats 3, 6, 12, and 48 h after training.

2. Methods

2.1. Subjects

One hundred and sixty-two male Sprague–Dawley rats (300–325 g), purchased from Charles River Laboratories (Wilmington, MA) were housed separately in a temperature controlled setting (19.5 °C), maintained on a 12 h light/dark cycle, and given food and water *ad libitum*. All procedures were in accordance with NIH guidelines and approved by the University of Texas at Dallas Institutional Animal Care and Use Committee.

2.2. Inhibitory avoidance (IA) training

All rats that received IA training were handled for 3 min every day for 5 days prior to the training session. The IA apparatus was a trough-shaped box, 90 cm in length, with a sliding guillotine style door dividing two compartments. The lighted compartment was 30 cm long, with plastic sides. A table lamp sat directly over this side, shining down into the compartment. The dark compartment was 60 cm long, with metal sides. This compartment was completely dark when the sliding door was closed. The box was placed in a dark room, and the rats were brought into the room immediately before training. Each rat was removed from its home cage and placed in the light compartment facing away from the dark compartment. Once the rat turned around (180°) and crossed over into the dark compartment, the sliding guillotine door was closed and the rat was trapped in the dark compartment. When the rat walked all the way to the end of the box and turned around again, an inescapable 0.45 mA footshock lasting 1.0 s was applied to the floor plates. The rat was left in the dark compartment for 10 s following the shock. In Experiments 2 and 3, rats were removed from the IA box and given immediate or delayed (6 or 45 h), post-training intra-rACC infusions of either antisense (AS) or scrambled (SC) ODNs.

2.3. Inhibitory avoidance training without shock

Some rats included in Experiment 1 were placed in the IA box and allowed to cross over into the dark compartment. They were blocked into the dark compartment for 30 s but no shock was administered.

2.4. Testing

Rats were returned to the IA apparatus 48 h later. They were again placed in the light compartment. Latency to cross into the dark compartment was recorded and used as a measure of memory, if the rat failed to cross into the dark compartment before 10 min they were removed and a time of 600 s was recorded.

2.5. Surgeries

For Experiments 2 and 3, rats were anesthetized with isoflurane (1% in O_2). A continuous flow of isoflurane was delivered to the rat throughout the surgery. Once anesthetized, the rats' heads were leveled in a rat stereotaxic device. Rats were given injections of atropine sulfate (0.4 mg/kg, i.p.) to keep airways clear and 0.5 ml of the local anesthetic marcaine at the incision site. Cannulae were placed directly above the rostral ACC (rACC), mounted with dental cement and secured with skull screws. The following stereotaxic coordinates (in mm) were adapted from Malin and McGaugh (2006): anteroposterior (AP), +2.7 mm from bregma; mediolateral (ML), ±0.5 mm from midline; dorsoventral (DV), −1.6 mm from the surface of the skull. Rats were given 3–5 ml of saline subcutaneously to help prevent dehydration, and moved to a warm recovery chamber until they were awake and moving. They were then allowed to recover for 1 week before training.

2.6. Infusions

Infusions were administered through 30 gauge dental needles extending 1 mm beyond the cannulae, attached to 1 μL Hamilton syringes. A volume of 0.5 μL of ODN was administered by a KD Scientific (Harvard Instruments) infusion pump, at a constant rate, for the duration of 120 s. ODNs (Midland Certified; pH 7.5) were dissolved in deionized water to a final concentration of 2 ng/μL.

2.7. Oligodeoxynucleotides

Arc AS ODNs are sequenced specifically to bind to the start codon of Arc mRNA, thus disrupting the expression of Arc protein. Arc SC ODNs are sequenced to be the same atomic weight as the AS ODNs, with random base pairs so that it has no affinity to any known mRNA. Guzowski and colleagues used the AS sequence used in this experiment (GTCCAGCTCCATCTGCTCGC) and showed it to have the highest efficacy and specificity with the least toxicity (Guzowski et al., 2000).

To ensure that the effects of infusing Arc AS ODNs were due to a knock down of Arc protein expression and not the knock down of another protein important for the long-term consolidation of memory, knock down of CamKII-α was also determined using Western immunoblotting. A second ODN sequence (ATGGTCCAGCTCCATCTG), targeting the start codon of Arc mRNA, was used

in the behavioral paradigm to ensure the memory-impairing effects were due specifically to a decrease in Arc protein expression. This sequence was previously used by Guzowski et al. (2000) and shown to be effective at knocking down Arc expression. This sequence is an 18 mer reverse complement of bases 214–231 of the published Arc sequence (GenBank accession number U19866).

To quantify the reduction of Arc protein expression as a result of ODN infusions, six rats underwent the previously reported surgery protocol and IA training. These rats were then given unilateral intra-rACC infusions of AS (into one hemisphere) and the SC ODN (into the other), and therefore each rat served as its own control. Rats were sacrificed 3 and 6 h after the infusion to determine the relative expression of Arc protein in the rACC across both hemispheres.

According to a recent report, Arc AS ODNs are maximally diffused at 3 h after infusion in the lateral amygdala (Ploski et al., 2008). Accordingly, infusions given 45 h after training should knock down Arc expression in the rACC at the time of the retrieval test given 48 h after training. To verify diffusion of the ODN in the cortex one rat was infused with a biotinylated ODN (Midland Certified Reagent Company) and sacrificed 3 h later. An ABC–DAB reaction (Vector Laboratories) was used to visualize the diffusion.

2.8. Tissue preparation

Rats were deeply anesthetized using isoflurane 1, 3, 6, 12 or 48 h after training. Brains were removed within 2 min of death and flash frozen in 2-methylbutane submerged in a dry ice/ethanol bath. Brains were stored at −80 °C until use.

Only brains with correct cannulae placement were used. To determine cannula placement, 40 μm sections were taken across the rACC (+4.2–+2.10 mm from bregma), mounted on gelatin-subbed slides, and stained with thionin. Slides were analyzed under a light microscope to determine the location of the cannulae and drug infusion sites. Any brains that did not have cannulae marks in the rACC were not used for this study, a total of 34 rats were excluded due to incorrect cannulae placement. For Experiment 1, coronal 20 μm cryosections were taken from the rACC and mounted on microscope slides for immunohistochemical analysis. Sections were arranged so that each slide had a sample from each of the experimental groups. In Experiment 1, 500 μm cryosections were collected starting +4.2 mm from bregma and continuing to −3.0 mm from bregma. Tissue punches were taken, using a tissue punch tool (0.5 mm diameter), from both rostral and caudal portions of the ACC, 0.5 mm from midline to the medial edge of each hemisphere and starting from the top of the brain and continuing −2 mm. Based on the findings of Malin and colleagues (2007) indicating a specific contribution of the rostral portion of the ACC in the consolidation of IA memory, punches were refined for Experiments 2 and 3 to include only the rostral portion of the ACC. These punches were collected starting at +4.2 mm from bregma and continuing to +2.1 mm from bregma.

2.9. Immunohistochemistry

Tissue was submerged in 4% paraformaldehyde and then rinsed in TBS. Endogenous activity was quenched with a peroxide mixture and a series of TBS rinses. Arc antibody (Synaptic Systems, 1:500) in blocking agent was left on the tissue overnight. Tissue was then rinsed with a series of washes of TBS–Tween (0.02%, pH 7.5). The secondary antibody (anti-rabbit-HRP; Cell Signaling, 1:500) was applied to slides and allowed to incubate for at least 2 h. After another series of washes with TBS–Tween, a Tyramide Signal Amplification (Fisher) with CY-3 was performed. The slides were then washed again in TBS–Tween (0.02%, pH 7.5) and counter stained with DAPI (Molecular Probes, 1:500 in TBS). Images were acquired on a Zeiss Axio Imager Fluorescence microscope.

2.10. Immunoblotting

Tissue was sonicated in a 0.1 M phosphate buffer containing 10% glycerol, 20% protease inhibitor cocktail (Sigma), and 10% phosphatase inhibitor (Sigma). Protein amounts were determined using a Qubit fluorometer and protein assay kit (Invitrogen). Approximately 15 μg of protein from each sample were heated with a sample buffer and reducing agent. The samples were run on 4–12% Bis–Tris MIDI Gels (Invitrogen) using electrophoresis. Each gel contained samples from each experimental condition. Gels were transferred by electroblotting onto a nitrocellulose membrane. The membrane was then washed in Tris-buffered saline (pH 7.5) and incubated overnight at 4 °C in Anti-Arc (Synaptic Systems, 1:6000) and Anti-Actin (Sigma, 1:2000) primary antibodies diluted in 5% non-fat dry milk in TBS–Tween. The membrane was washed in TBS with Tween, and incubated at room temperature for 1 h in a secondary HRP-linked antibody (Cell Signaling, 1:6000) also diluted in 5% non-fat dry milk. Immunoreactivity was detected using chemiluminescence (ECL Western Blot Kit; Pierce). A marker (Invitrogen) was run to determine the relative mobility of immunoreactive bands. Films were scanned and converted into TIF files to be analyzed using Image-J software. The average density of each band was measured and Arc immunoreactivity levels were normalized to the Actin immunoreactivity level for each sample.

2.11. Statistical analysis

Two-sample *t*-tests were used to analyze inhibitory avoidance retention latencies. Pair-wise comparisons were made between the AS and SC ODN-treated rats. Western blot densitometry results are expressed as a ratio of Arc to Actin and are normalized to a cage control to account for film variation and expressed as a percentage of the control vehicle group. The ratios were then compared using a Student's *t*-test to make pair-wise comparisons between the groups. A probability level of $p < 0.05$ was considered significant. Data are presented as means ± SEM.

3. Results

3.1. Experiment 1

Immunohistochemistry was used to initially identify brain regions expressing Arc. Results indicated prominent expression of Arc in the ACC of trained rats (Fig. 1). Western immunoblotting showed an increase in Arc expression in the ACC of rats that were trained with footshock vs. rats that were exposed to the training apparatus in the absence of footshock ($p < 0.01$; Fig. 2). Arc expression in the ACC of the no-shock rats appeared to be elevated, but was not significantly different from that of naive cage controls. Therefore, Arc protein expression is elevated in the ACC in a shock-specific manner that is consistent with findings suggesting that the ACC is important specifically for the consolidation of memory for the painful stimulus (Malin & McGaugh, 2006).

3.2. Experiment 2

Rats ($n = 28$) were trained on the aversive, single-trail inhibitory avoidance (IA) task, and were given bilateral infusions of either Antisense (AS) or Scrambled (SC) ODN, into the rostral portion of the ACC, immediately after training. Latencies to cross into the shock compartment during the retention test were lower in Arc

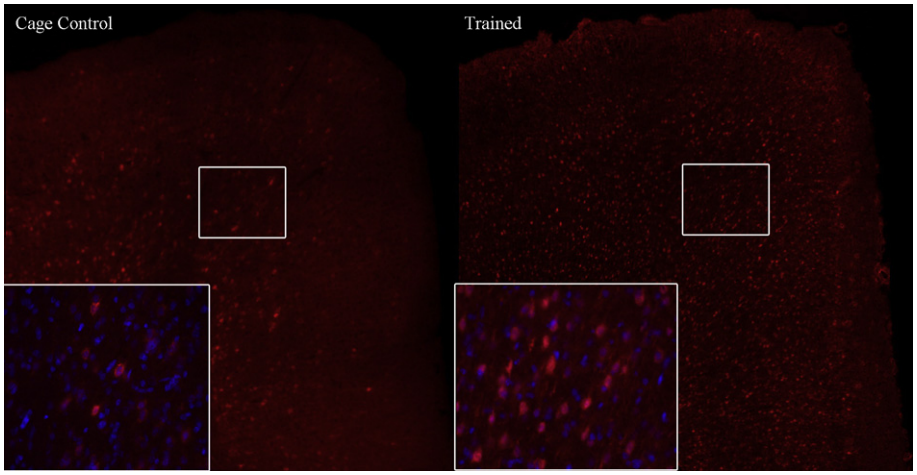

Fig. 1. Immunohistochemistry was used to visualize Arc protein expression after training on a one trial IA task. Representative photomicrographs show Arc protein expression in the rACC of a naive cage control (left) and a rat trained on IA with footshock (right). Arc expression appears to be elevated in the rACC of the trained rat.

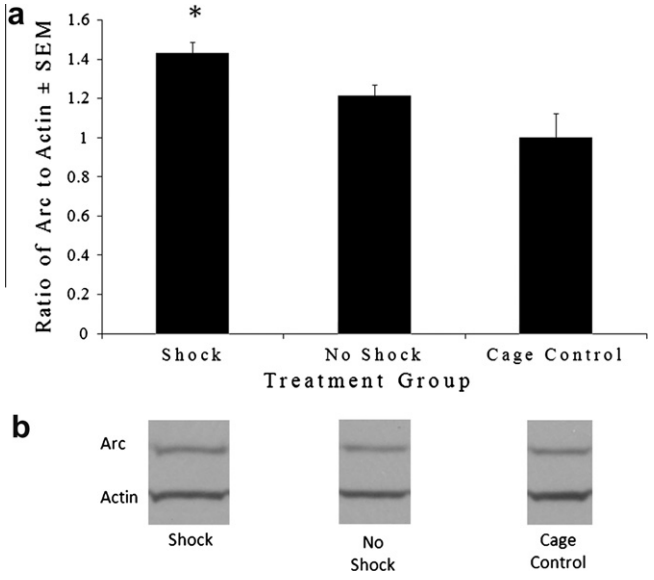

Fig. 2. Western immunoblotting was used to quantify the increase in Arc protein expression seen in the ACC after IA training. (a) Arc values were significantly greater in rats trained with footshock ($m = 1.4 \pm 0.05$) than in rats trained with no shock ($m = 1.21 \pm 0.05$). Arc is normalized to Actin and by calculating the ratio of band density of Arc to that of Actin and then expressed as a percentage of normalized cage control values. $^{*}p < 0.01$. (b) Representative western immunoblot showing Arc protein expression in the ACC of rats trained with and without a shock and a naïve cage control.

AS ODN-infused rats than SC ODN-infused rats, suggesting that the post-training disruption of Arc expression in the rACC impaired long-term memory for the IA task ($p < 0.01$; Fig. 3a).

A second ODN sequence, also targeting the start codon of Arc mRNA, was administered to ensure that behavioral effects were due specifically to the disruption of Arc expression. Rats ($n = 9$) underwent the same IA training but the second ODN sequence was infused into the rACC immediately after training. Post-training infusions of the second ODN sequence also impaired memory of IA (Fig. 3b). AS ODN-treated rats demonstrated lower retention latencies than did rats treated with SC ODN infusions ($p < 0.05$). Because the first AS sequence produced greater memory impairment than the second sequence, the first ODN sequence was used for the rest of the experiments.

In order to determine whether the impairment observed in Experiment 2 was in fact due to a requirement of Arc expression in memory consolidation, delayed infusions of Arc AS or SC ODNs were given to rats ($n = 18$) 6 h after training (Fig. 4). Infusions of AS ODNs 6 h after training impaired performance on the 48 h retention test when compared with the group treated with SC ODNs ($p < 0.01$). This finding raises several possible explanations: (1) Arc protein in the rACC is involved in memory consolidation for up to 6 h following training; (2) Treatment with Arc AS ODNs may affect the remote memory stored in the rACC and thereby interfere with expression of the memory 48 h later; or (3) Arc AS ODNs may have toxic effects on the rACC that would interfere with retention performance 48 h later. To determine whether Arc protein expression in the rACC is involved in consolidation of long-term memories or the treatment affects retrieval performance independent of a consolidation effect, the ODNs were infused into the rACC of rats ($n = 26$) 3 h before the 48 h retention test (45 h after training; Fig. 5). According to western blot results, Arc is no longer elevated in the rACC at 48 h. Our data are consistent with other findings indicating that these ODNs are effective in reducing Arc protein expression 3 h after infusion (Ploski et al., 2008). Cross-over latencies for rats receiving infusions of antisense ODNs 48 h after training were not significantly different from rats receiving infusions of scrambled ODNs (ns). These results suggest that Arc protein expression in the rACC is necessary for the consolidation, but not the retrieval of long-term memories. Although there is evidence that the ACC is required for retrieval of remote memories, there is no evidence implicating Arc protein expression in the retrieval of memories.

3.3. Experiment 3

In order to establish a timecourse for Arc expression in the rACC, rats were trained on the IA task, and sacrificed 3, 6, 12, and 48 h after training. Immunoblotting was performed to determine the amount of Arc present at these time points. Fig. 6 shows the ratio of Arc to Actin in the rACC at all four time points. Arc protein was elevated in the rACC at 3 and 6 h relative to controls ($p < 0.01$), but returned to control levels at 12 h and remained at control levels 48 h after training. Along with our initial findings of Arc elevation 1 h after IA training, here we show that Arc is elevated up to 6 h after aversive training in the rACC.

3.4. Knock down effects

Fourteen rats underwent IA training and were then given unilateral infusions of the AS ODN into the rACC of one hemisphere

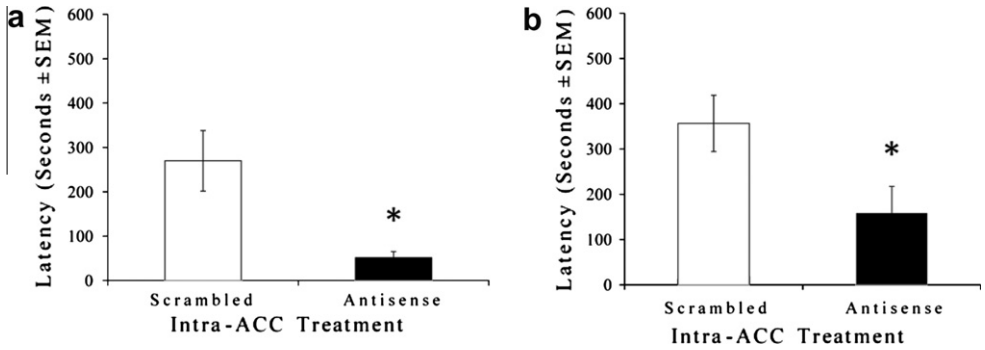

Fig. 3. (a) Latency times (s) for rats given post-training intra-rACC infusions of either antisense or scrambled ODNs. Mean latency to enter the shock compartment on the 48 h retention test was significantly shorter for Arc antisense ODN-treated rats ($m = 51.75 \pm 13.45$, $n = 8$) than for scrambled ODN-treated rats ($m = 269.85 \pm 68.11$, $n = 7$), suggesting that post-training blockade of Arc expression impaired consolidation of memory for some aspect of the event. $^{*}p < 0.01$. (b) The second ODN sequence infused into the rACC immediately after training produced a comparable memory-impairing effect. Mean latency to enter the shock compartment for antisense ODN-treated rats ($n = 4$) was significantly shorter (158.28 ± 58.99), than that for scrambled ODN-treated rats ($n = 5$; 356.48 ± 62.20; $p < 0.05$).

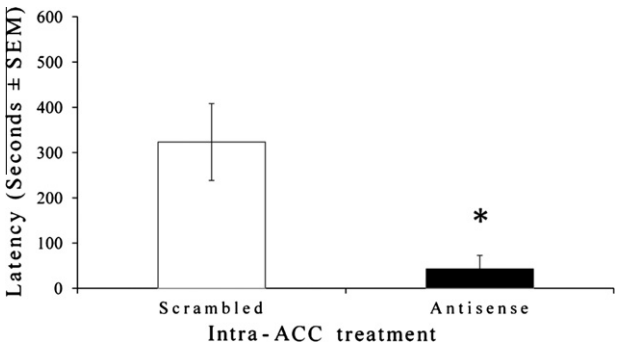

Fig. 4. Latency times (s) for rats given intra-rACC infusions of either antisense ODNs or scrambled ODNs 6 h after training. Mean latency to enter the shock compartment was significantly shorter for antisense ODN-infused rats ($m = 42.98 \pm 29.8$, $n = 6$) than for rats given scrambled ODN infusions ($m = 323.23 \pm 84.82$, $n = 5$, $p < 0.01$) suggesting that the reduction of Arc expression 6 h after training impaired consolidation of memory for some aspect of the event. $^{*}p < 0.01$.

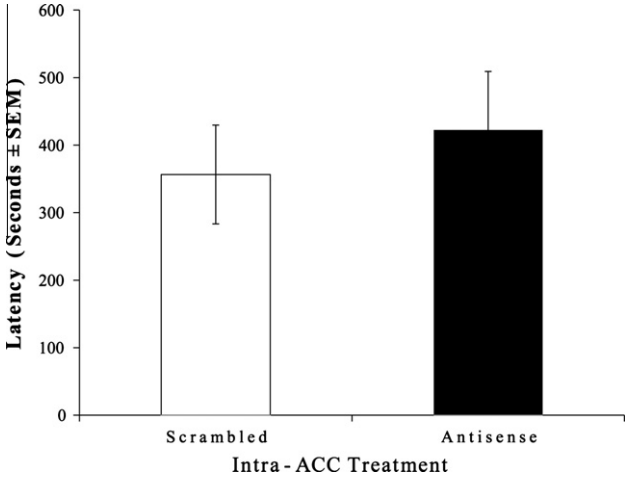

Fig. 5. Latency times (s) for rats given intra-ACC infusions of either antisense ODNs or scrambled ODNs 3 h before retention testing. Mean latency for the antisense group ($n = 7$) was 422.48 ± 86.61 s, mean latency for the scrambled group ($n = 8$) was 356.46 ± 7.16 s. There was not a significant difference between the two groups suggesting that Arc expression in the rACC does not play a critical role in memory retention performance.

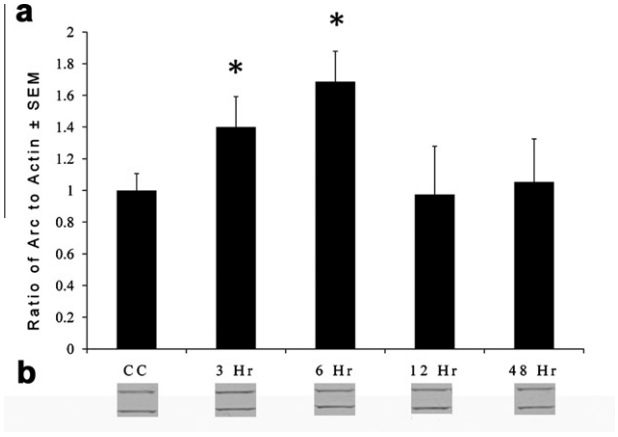

Fig. 6. (a) Mean ratio of Arc to Actin in the ACC of cage controls and rats sacrificed 3 h (1.40 ±.19, $n = 5$), 6 h (1.68 ± 0.30, $n = 3$), 12 h (0.97 ± 0.27, $n = 4$) and 48 h (1.05 ± 0.26, $n = 3$) after training. Immunoblotting revealed a significant increase in the ratio of Arc to Actin ± SEM in the ACC of rats 3 h and 6 h after inhibitory avoidance training, but not after 12 or 48 h. Values are expressed as percent to normalized cage control ratios. $^{*}p < 0.01$. (b) Representative western immunoblot showing Arc protein in the ACC of a cage control rat, and rats sacrificed at 3, 6, 12 and 48 h after training.

and the SC ODN into the other rACC. The hemisphere receiving AS or SC ODN was counterbalanced. The amount of Arc protein present in each hemisphere was then quantified using western immunoblotting and a percent knock down was determined for both 3 h and 6 h after training. At 3 h, an average reduction of 21% was measured in Arc protein expression in the rACC, and at 6 h after training a 60% reduction was observed (Fig. 7). These values are consistent with other findings of Arc protein reduction via ODNs in the hippocampus and the lateral amygdala (Guzowski et al., 2000; Ploski et al., 2008). A second ODN sequence was used to confirm that reduced Arc expression, and not an unknown effect of the specific ODN, was responsible for memory impairment. The same procedures were performed to determine the decrease in Arc protein in the rACC 6 h after infusing the second ODN sequence and a mean reduction of 37% was measured. To test the specificity of the AS ODNs, the same blots were probed for CamKII-α. ODN sequence #1 produced an average reduction in CamKII-α of 9.81% at 3 h and 13.6% at 6 h. Therefore the ODN sequence effectively disrupted the expression of Arc protein and was fairly specific to Arc.

3.5. Diffusion of ODN into the ACC

To determine the spread of the intra-ACC ODN infusions, biotinylated AS ODN was infused into the rACC of two rats immediately after training. The rats were sacrificed 3 h later. An ABC–DAB

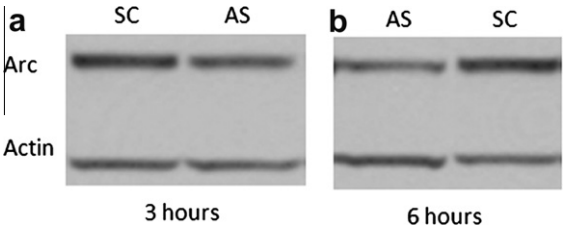

Fig. 7. Arc protein is decreased by antisense ODN. (a) Western immunoblot showing a reduction in Arc protein in the ACC of the hemisphere receiving antisense ODN infusions compared to the hemisphere receiving infusions of the scrambled ODN at (a) 3 h and (b) at 6 h.

reaction showed that the ODN did not diffuse to areas outside of the rACC (Fig. 8). Biotin labeling was identified in slices as far as 960 μm apart from rostral to caudal. This anterior–posterior spread is within the boundaries of the rACC according to the Paxinos and Watson Rat Brain Atlas (2005) suggesting that the effect of the Arc antisense ODN infusions was due specifically to blockade of Arc in the rACC and not in an adjacent area.

4. Discussion

Because Arc protein expression in the hippocampus plays a necessary role in the consolidation of IA, this research sought to determine whether Arc in the rACC is essential for the consolidation of IA memory (Guzowski et al., 2000; McIntyre et al., 2005). According to evidence reported by Malin and McGaugh (2006), stimulation of cholinergic receptors in the ACC enhances memory for IA. Additionally, the hippocampus is important for the contextual component of IA and the rACC is involved in the aversive component of IA (Malin & McGaugh, 2006). These findings are consistent with research suggesting that the ACC is important for processing the emotional saliency of pain (Johanson & Fields, 2001). We showed that Arc protein expression in the rACC is increased 6 h after IA training and this increase in Arc is specifically observed in rats given footshock during training. Our findings are consistent with those reported by Malin and McGaugh (2006), and they support the notion that the rACC is involved in consolidation of the memory of the aversive stimulus.

As observed in studies of the hippocampus, Arc expression in the rACC appears to be involved in the consolidation of memory for IA. Antisense knock down of Arc expression in the hippocampus impairs memory for the IA task (McIntyre et al., 2005). Similarly, post-training infusions of these ODNs into the rACC also impaired memory for the IA task. Antisense ODN-treated rats exhibited significantly lower cross-over latencies than rats that received Scrambled ODN infusions; suggesting an impaired memory for the aversive task. Two different antisense ODN sequences were used to ensure that the behavioral effects were not due to a non-specific effect of the ODN. Both sequences significantly impaired memory. Although a marginal decrease of 9–13% was observed in CamK-II-α following treatment with sequence #1, the ODN sequence predominately knocked down Arc (21–60%) protein expression in the ACC, further suggesting the effect is due to the decrease in Arc and not a non-specific effect of the ODN. Arc is elevated in the rACC up to 6 h after IA training. This could be analogous to the second wave of hippocampal activation observed by Ramirez-Amaya and colleagues (2005) 8 h after novel context exposure. However, the current findings do not provide any clues suggesting a drop in Arc protein expression between 1 and 6 h. Arc expression in the rACC can be further contrasted with hippocampal Arc. For example, Guzowski and colleagues (2000) infused

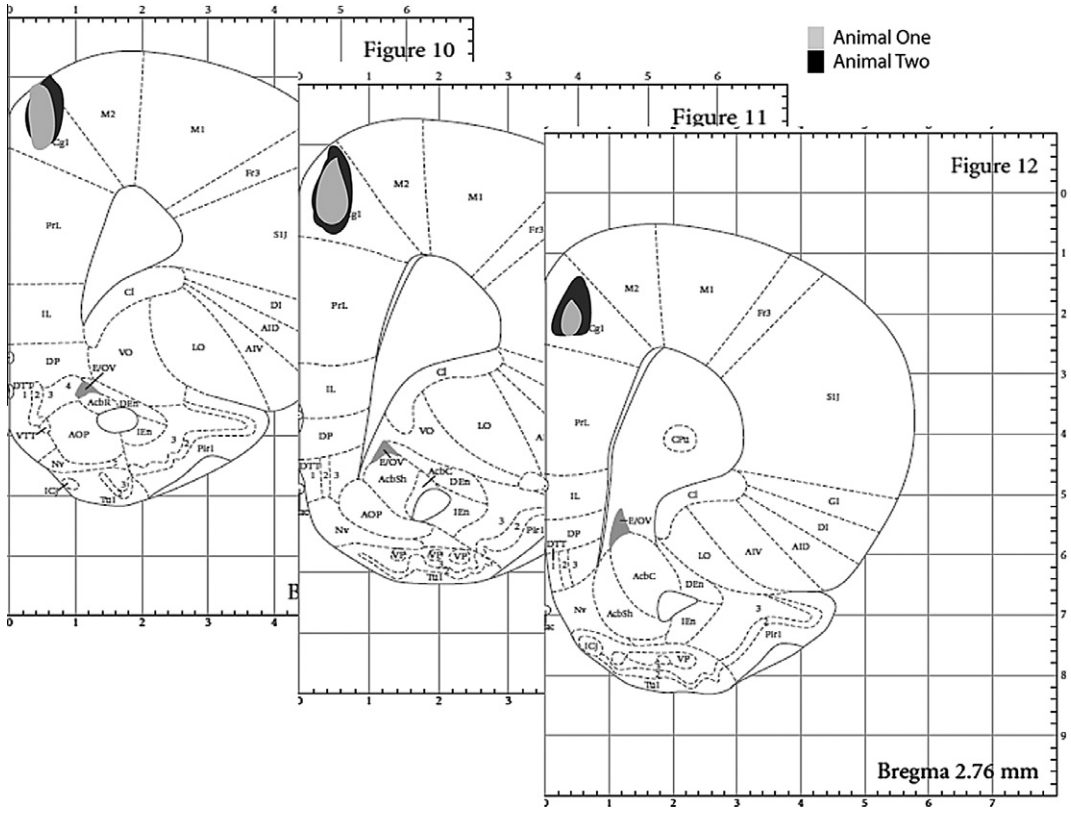

Fig. 8. Diffusion of ODN infused into the ACC. Merged representative diffusion in two rats infused with biotinylated Arc ODN and sacrificed 3 h later. Note that the ODN diffusion is restricted to the ACC and spares the adjacent motor cortex and the medial prefrontal cortex.

ODNs into the hippocampus 8 h after a training experience and did not see an effect on memory. In contrast, this experiment demonstrated that delayed intra-ACC ODN infusions (6 h after training) impaired memory for the event. Unlike the activation of the hippocampus at 8 h, the increase in Arc at 6 h in the rACC is important for long-term consolidation of the memory of the event.

It is well accepted that consolidation of memoires occurs in the minutes to hours after a training experience. This assumption is based on research examining the role of the basolateral complex of the amygdala (BLA) in consolidation of emotional memories. (Boccia, Blake, Baratti, & McGaugh, 2009; Campolongo et al., 2009; Roozendaal, Quirarte, & McGaugh, 2002). Other areas of the brain, specifically cortical areas, show a different pattern of activation following aversive training (Izquierdo et al., 1995; Ramirez-Amaya et al., 2005). Research using lesions made up to 1 month after training indicate a critical role for the ACC for the recall of remote memories for taste aversion (Ding, Teixeira, & Frankland, 2008), spatial tasks (Teixeira, Pomedli, Maei, Kee, & Frankland, 2006), and contextual fear memory (Frankland, Bontempi, Talton, Kaczmarek, & Silva, 2004). Here, we examined the involvement of the rACC in the consolidation of contextual fear memory. Although we did not observe an effect of Arc AS treatment on retrieval performance, independent of the consolidation effect, these findings do not exclude the possibility that the rACC is involved in recall. Rather, they implicate rACC expression of Arc protein in the consolidation, but not recall of memory.

This research sought to examine whether the observed role of Arc in consolidation of long-term memory is conserved across brain regions. Consistent with reports identifying a role for Arc in several cortical regions and the amygdala in fear conditioning, IA training increased Arc protein expression in the rACC (Koya et al., 2005; Mikkelsen & Larsen, 2006; Ploski et al., 2008). As seen in the hippocampus and the amygdala, post-training disruption of Arc expression in the rACC impaired long-term memory. Taken together, these findings indicate that the synaptic actions that contribute to consolidation of long-term memories may be independent of brain region. These results illuminate general mechanisms of synaptic plasticity that can be extended to multiple brain regions and raise questions about the timecourse of regional involvement in memory consolidation.

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