Arc mRNA induction in striatal efferent neurons associated with response learning

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Abstract
The dorsal striatum is involved in motor-response learning, but the extent to which distinct populations of striatal efferent neurons are differentially involved in such learning is unknown. Activity-regulated, cytoskeleton-associated (Arc) protein is an effector immediate–early gene implicated in synaptic plasticity. We examined arc mRNA expression in striatopallidal vs. striatongiral efferent neurons in dorsomedial and dorsolateral striatum of rats engaged in reversal learning on a T-maze motor-response task. Male Sprague–Dawley rats learned to turn right or left for 3 days. Half of the rats then underwent reversal training. The remaining rats were yoked to rats undergoing reversal training, such that they ran the same number of trials but ran them as continued-acquisition trials. Brains were removed and processed using double-label fluorescent in situ hybridization for arc and preproenkephalin (PPE) mRNA. In the reversal, but not the continued-acquisition, group there was a significant relation between the overall arc mRNA signal in dorsomedial striatum and the number of trials run, with rats reaching criterion in fewer trials having higher levels of arc mRNA expression. A similar relation was seen between the numbers of PPE+ and PPE− neurons in dorsomedial striatum with cytoplasmic arc mRNA expression. Interestingly, in behaviourally activated animals significantly more PPE− neurons had cytoplasmic arc mRNA expression. These data suggest that Arc in both striatongiral and striatopallidal efferent neurons is involved in striatal synaptic plasticity mediating motor-response learning in the T-maze and that there is differential processing of arc mRNA in distinct subpopulations of striatal efferent neurons.

Introduction
The dorsal striatum is involved in response learning, with specific subregions mediating different aspects of such learning. Inactivation of dorsal striatum impairs response learning on a T-maze (Packard & McGaugh, 1996). Furthermore, manipulations of dorsolateral (DL) striatum impair acquisition learning on such a task, whereas manipulations of dorsomedial (DM) striatum impair reversal learning in the same paradigm (Ragozzino et al., 2002a; Palencia & Ragozzino, 2004, 2005). Changes in activity of striatal neurons also occur during motor-response learning on the T-maze (Jog et al., 1999; Barnes et al., 2005).

Approximately ninety-five percent of neurons in striatum are spiny efferent neurons (Kemp & Powell, 1971). Half of these efferent neurons, striatopallidal neurons, express preproenkephalin (PPE) mRNA (PPE−), which is thus used as a phenotypic marker of these neurons (Gerfen & Young, 1988; Gerfen et al., 1990; Le Moine & Bloch, 1995). The other half do not express PPE (PPE+), and thus can be phenotypically identified as striatongiral neurons (Gerfen & Young, 1988; Le Moine & Bloch, 1995). Models of basal ganglia function suggest that activation of striatongiral neurons facilitates, whereas activation of striatopallidal neurons inhibits, behaviour (Mink & Thach, 1993). However, the extent to which these efferent neuron populations are similarly or differentially engaged during striatally based learning is completely unknown.

Response learning is associated with increased phosphorylated cAMP response element-binding protein levels in dorsal striatum (Colombo et al., 2003). Furthermore, expression of activity-regulated cytoskeleton-associated protein (Arc) is elevated in striatum of rats undergoing training or pseudotraining on an operant lever-pressing task (Kelly & Deadwyler, 2002, 2003) and is correlated with lever pressing rates and total session length (Kelly & Deadwyler, 2003). However, no one has examined the relation between immediate–early gene (IEG) expression in distinct striatal subregions and efferent neuronal subpopulations and striatally based learning.

Arc is implicated in synaptic plasticity underlying learning and memory. Firstly, spatial learning in the Morris water maze increases arc mRNA expression in hippocampus, and this expression is correlated with the learning (Guzowski et al., 2001). Secondly, arc mRNA is translocated to activated dendrites in hippocampus where it is thought to play a role in synaptic modifications underlying learning and memory (Steward et al., 1998). Thirdly, inhibiting arc mRNA production in hippocampus disrupts spatial learning (Guzowski et al., 2000). Finally, initiation of arc mRNA transcription occurs within minutes of neuronal activation, and then arc mRNA is transported into the cytoplasm and out to activated dendrites (Guzowski et al., 1999).

Given the importance of Arc for synaptic plasticity and the temporal relation between arc mRNA expression and neuronal activation, cellular compartment analysis of temporal activity by fluorescent
in situ hybridization (catFISH) of arc mRNA expression provides a powerful tool for assessing activation and involvement of neuronal populations in learning paradigms (Guzowski et al., 1999). The purpose of the present study therefore was to examine the relation between learning and the expression and subcellular distribution of arc mRNA in phenotypically identified efferent neurons of dorsal striatum.

Materials and methods

Animals

Sixteen male Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA, USA) weighing 300–350 g were housed in tub cages in a room controlled for temperature and lighting (12 : 12 h). During the first 2 weeks of the habituation phase, the rats were food-deprived to 80% of their free-feeding weight. Additionally, a group of control rats (caged controls; n = 4) were housed and fed over the same period of time as the rats undergoing motor-response training; however, these rats were not exposed to any aspect of the behavioural training. All animal care and experimental procedures conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the University of Utah.

Apparatus

A four-arm plus-maze constructed of painted black wood was used throughout the behavioural training and testing phase in a room void of extra-maze cues and under normal lighting conditions. On each trial, one of the arms of the plus maze was closed off by a removable door at the centre platform to make a T-maze configuration. Each arm was 55 cm long \times 10 cm wide. The height of the arm walls was 15 cm. The maze was placed on a platform 72 cm above the floor. Removable start boxes with doors were at the end of each arm and contained a food well 2.5 cm in diameter and 1.5 cm deep.

Response learning paradigm

For 2 weeks the rats were handled daily, being placed on a flat table and fed the food reward (Froot Loops cereal; Kellogg, Battle Creek, MI, USA) for 10 min, or until the rat consumed 10 Froot Loops, to allow habituation to the handling involved in the behavioural testing. Rats were then exposed to the T-maze for 5 days, being allowed to freely explore the maze and consume half-pieces of cereal in the food wells for 15 min each day.

The day prior to the first learning session, the turn bias of each rat was determined. To this end, the rats were run on the T-maze from randomly selected arms. After the initial trial, the rat was moved back into the same arm. The rat continued to run trials from this stem arm until it turned in the direction (right or left) opposite to its initial choice. This was done to prevent the reinforcement of only the initial turn choice. Once the rat made both right and left turns from the initial stem arm, it was started from a different arm. That is, the door at the centre platform was moved to make a T-shape in another orientation, and the two new choice arms were baited. This procedure continued for seven trials. The direction in which the rat initially turned four or more times was recorded as the turn bias.

Two groups of rats were run on the response-learning task: reversal learners (n = 8) and yoked, ‘continued-acquisition’ controls (n = 8). All rats underwent acquisition training for three days, during which the rats were trained to turn opposite their turn bias when started from all four arms chosen in a pseudorandom order. Correct turns were rewarded with half of a Froot Loop. The intertrial interval was \sim 10 s, during which time the rat was moved to a new start arm and the new goal arm was loaded. The rat was considered to have learned the response when it made 90% correct responses over 10 trials. This criterion was chosen based on pilot work in our laboratory, indicating that rats reaching this criterion consistently turned in the reinforced direction when tested in the same apparatus on subsequent days. Once criterion was reached on the first and second days, the rat was returned to its home cage until the next day. On the third day, the rats were again run to criterion on the acquisition trials. Then, half of the rats were reversed; that is, they were now rewarded to turn in the direction opposite to that reinforced during the acquisition phase. Reversal trials were run until the rat made 90% correct responses in the new direction over 10 trials. The other rats served as yoked, continued-acquisition control rats. Each of these rats was run immediately after each reversal learner, and ran the same number of trials as the preceding reversal learner but continued to run them as acquisition trials. The continued-acquisition group was used specifically to control for general motor behaviour, number of trials run, and the time spent in the maze. Importantly, although both groups ran the same number of trials, there was no ‘new’ learning of a motor response in the continued-acquisition group, only in the reversal group. All rats were killed 5 min after reaching criterion in the reversal direction or after completing the yoked, continued-acquisition trials. All rats were killed

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by exposure to CO2 and decapitated. The brains were rapidly removed and flash-frozen in 2-methyl-butane chilled on dry ice. At the beginning and end of behavioural training, caged control rats were removed from their home cages and immediately killed in order to determine the basal level of arc mRNA expression. All brains were stored at −80 °C until they were processed for in situ hybridization.

**Behavioural activation via novelty exposure**

A separate group of rats was run on another, simpler, form of behavioural activation to assess whether there were differences in the transcriptional activation or cytoplasmic distribution of arc mRNA in the two subpopulations of striatal efferent neurons. For this experiment, a group of 15 rats was used to assess the effects of novelty exposure and exploratory behaviour, as previously described by Guzowski et al. (1999), on arc mRNA expression in the nucleus and cytoplasm of phenotypically identified striatal efferent neurons. Rats were removed from their home cage after having been isolated in the cage for the previous 24 h to allow for low levels of basal arc mRNA expression. Once removed, they were immediately placed into one of two novel environments, each consisting of a 2-ft × 2-ft Plexiglas chamber surrounded by a variety of salient stimuli (different stimuli surrounding each chamber). The rat was moved back and forth between the two environments every 15 s for a total of 5 min. One group of rats (n = 3) was killed immediately at the end of this 5-min period. Other rats were returned to their home (isolation) cages at the end of the 5 min and then killed 10 min (n = 3), 25 min (n = 3) or 55 min (n = 3) later, such that they were killed 15, 30 or 60 min after initial exposure to the novel environments. Another group of rats (n = 3) remained in the home cage and were killed immediately upon removal from the home cage, thus serving as a caged control group.

**In situ hybridization histochemistry**

Brain tissue from all groups to be directly compared was processed and hybridized in parallel. Brains were sectioned (12-μm coronal sections) in a cryostat (Cryocut 1800; Cambridge Instruments, Heidelberg, Germany). Sections were thaw-mounted onto SuperFrost (Fisher Scientific, Pittsburg, PA, USA) slides and stored at −20 °C.

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**Fig. 2.** Confocal, z-stack images showing arc mRNA (cy-3, red), PPE mRNA (cy-5, blue), and nuclear (Sytox Green) staining in the dorsomedial striata of rats. Animals were killed (A) 5 min, (B) 15 min or (C) 30 min after exposure to a novel environment. Asterisks highlight neurons of interest across each z-stack that are PPE− (blue). Plus-signs highlight PPE− neurons. In A, one or two discrete foci of arc mRNA signal appear and then disappear as one progresses through the nucleus of the highlighted cell. The red signal within the green nucleus appears pink. This cell is a neuron with intranuclear foci. In B, the plus sign highlights a PPE− neuron with arc signal (red) present prior to the level of the nucleus (green), expressed in the nucleus (pink colouring as highlighted with arrowheads), and persisting in the cytoplasm (red) around the green nucleus. This cell is a neuron with both nuclear and cytoplasmic labelling. In C, the plus sign highlights a PPE− neuron in which the arc signal (red) does not overlap with the green nuclear counterstain, representative of a cell with only cytoplasmic labelling of arc mRNA. Scale bar, 10 μm.
Fig. 3. Confocal images showing arc mRNA (cy-3, red), PPE mRNA (cy-5, blue), and nuclear (Sytox Green) staining in the DM and DL striata of (A) caged control rats, (B) rats engaged in reversal learning on the T-maze, and (C) rats engaged in continued-acquisition trials on the T-maze. Scale bar, 10 μm.
postfixed in 4% paraformaldehyde in 0.9% NaCl, acetylated in fresh 0.25% acetic anhydride in 0.9% NaCl with 0.1 M triethanolamine (pH 8.0), dehydrated in an ascending series of alcohols, delipidated in chloroform and then rehydrated in a descending series of alcohols. Slides were air-dried and stored at −20 °C.

Expression of arc mRNA in identified subpopulations of striatal efferent neurons was accomplished by double-label FISH for arc and PPE mRNAs. Full-length ribonucleotide probes complementary to the mRNAs for arc (Lyford et al., 1995) and PPE (Yoshikawa et al., 1984) were synthesized from cDNAs using digoxigenin-UTP (DIG-UTP; arc) and fluorescein-UTP (FITC-UTP; PPE) with T7 (arc) and SP6 (PPE) RNA polymerases and DIG and FITC RNA labelling kits (Roche Applied Science, Indianapolis, IN, USA), respectively. Each cDNA (1 μL) was combined with 2 μL RNA labelling mix, 2 μL transcription buffer, 2 μL RNA polymerase and 13 μL dH2O. The probes were incubated for 2 h at 37 °C and then treated with DNase I for 15 min at 37 °C. EDTA (2 μL, 0.25 M, pH 8.0) was added, and the probes were purified using a G-50 spin column (Amersham Biosciences, Piscataway, NJ, USA) and stored at −20 °C.

The ribonucleotide probes were mixed with nuclease-free water and RNA mix (final concentrations: salmon sperm DNA, 100 μg/mL; yeast total RNA, 250 μg/mL; yeast transfer RNA (tRNA), 250 μg/mL). The probe, water and RNA mix were denatured at 90 °C for 5 min and then cooled on wet ice for 2 min. The DIG- and FITC-labelled probes were diluted 1:1000 in hybridization buffer consisting of (final concentrations): Tris buffer, pH 7.4, 23.8 mM; EDTA, pH 8.0, 1.2 mM; NaCl, 357 mM; dextran sulphate, 11.9% wt/vol; Denhardt’s solution, 1.2%; and formamide, 59.9% v/v.

Ninety microliters of hybridization buffer with probe was applied to each slide containing four sections and covered with a glass coverslip. Slides were hybridized overnight (12–18 h) in humid chambers at 56 °C. Once removed, slides were vigorously washed four times in 2 × SSC (0.15 M NaCl with 0.015 M sodium citrate). Slides were then washed in ribonuclease A (RNase A; 10 μg/mL; Roche Applied Science, Indianapolis, IN, USA) in 2 × SSC for 15 min at 37 °C. After incubation with RNase A, slides were washed 2 × 10 min in 2 × SSC at RT, 2 × 10 min in 0.2 × SSC at RT, 2 × 15 min in 0.2 × SSC at 56 °C, and 2 × 10 min in 0.2 × SSC at RT. Endogenous peroxidase activity was quenched with 2% H2O2 for 15 min, and slides then were washed in TNT buffer solution (tris-HCl, pH 7.5, 0.1 M; NaCl, 0.15 M; and Tween®0%, 0.05%) and incubated overnight at 4 °C with an antidigoxigenin antibody (1:1000) coupled to horseradish peroxidase (HRP; Roche Applied Science, Indianapolis, IN, USA). Probes were detected using a tyramide signal amplification (TSA) and cyanine-3 (cy-3) substrate kit (Perkin–Elmer Life Sciences, Boston, MA, USA) as per the manufacturer’s specifications. After detection of the DIG-labelled ribonucleotide probe, slides were treated again with 2% H2O2 for 15 min to quench residual HRP activity. The FITC-labelled ribonucleotide probe was then detected via incubation overnight at 4 °C with an anti-FITC antibody (1:1000 dilution) coupled to HRP (Roche Applied Science, Indianapolis, IN, USA) followed by visualization with a TSA-cyanine-5 (cy-5) substrate kit (Perkin–Elmer Life Sciences, Boston, MA, USA). Slides were washed in TNT buffer, counterstained with Sytox Green (1:35 000; Molecular Probes) and mounted in antifade agent (Molecular Probes).

To control for nonspecific labelling, additional slides were hybridized in parallel with the experimental tissue, except that each ribonucleotide probe, each antibody, or the TSA reagent were omitted from the assay. In these cases, no fluorescent signal was detected, attesting to the lack of nonspecific signal being generated from these reagents.

**Imaging and statistical analyses**

Images were collected using an Olympus FVX confocal microscopy system with an IX70 microscope, 488 nm argon laser line, and 60×, 1.2 NA oil-immersion lens (plan APO). Photomultiplier tube (PMT) assignments and pinhole size contrast values were kept constant across different confocal sessions. Areas of analysis were z-sectioned in 1-μm-thick optical sections, and a total of 10 z-sections were analysed for each field. A sample area of 0.7 × 0.7 mm was collected in the DM and DL subregions of one striatal section (1.0 mm anterior to bregma; Fig. 1A) for each rat. In addition, single fields (236 × 236 μm) were taken of the CA1, CA3 and dentate gyrus regions of one hippocampal section (3.3 mm posterior to bregma) for each rat (Fig. 1B).

ImageJ software (http://rsb.info.nih.gov/ij/) was used to determine the total arc mRNA signal in each sampled area. Briefly, the red (cy-3) channel for each z-stack image was selected, and the 10 z-stacks collapsed to produce a single image. This image was thresholded such that the lower limit of the look-up table was set to 25. The minimum particle size was set to 3 pixels. A particle analysis was then performed on the thresholded images yielding the area, in pixels, containing cy-3 signal above threshold. This value was then corrected to yield a dependent measure of arc-positive pixels as a percentage of the total field size. This measure of arc mRNA expression is hereafter referred to as ‘particle analysis’.

Cells positive for arc mRNA in each field were also counted by an experimenter blinded to the treatment groups of the animals to determine the cellular distribution of the arc signal in phenotypically identified neurons. Overall, all Sytox Green-stained cellular profiles in each field were examined through the 10 z-planes. Based on the catFISH labelling technique, each cell identified as being positive for arc mRNA expression was designated as having intranuclear foci, nuclear plus cytoplasmic signal, or only cytoplasmic signal (Fig. 2; Guzowski et al., 1999). These determinations were based on analysis of individual z-sections through each labelled cell to determine the presence of the cy-3 arc signal in the Sytox Green-stained nucleus. A cell was considered to have intranuclear foci if it contained one or two intense areas of cy-3 signal that appeared coincident with the Sytox Green nuclear counterstain (Fig. 2A). A cell was considered to have both nuclear and cytoplasmic staining if it contained arc mRNA signal both within and outside of the Sytox-stained nucleus (Fig. 2B). Cells were considered to have only cytoplasmic staining if they contained perinuclear or cytoplasmic labelling of arc mRNA that did not overlap with the Sytox-stained nucleus over the multiple optical z-sections (Fig. 2C; Guzowski et al., 1999). During this cellular analysis, cells containing the blue cy-5 PPE mRNA signal in and around the nucleus of the cell were classified as PPE+. Arc-positive cells that did not also contain blue cy-5 staining were classified as PPE−. As a matter of reference, on average, 1034 ± 19.7, 1042 ± 13.8

**FIG. 4.** Results of particle analysis of arc mRNA signal (cy-3, red staining) in the DM and DL striata. Data in (A) are the mean ± SEM (n = 4 for caged controls and n = 8 each for reversal and continued-acquisition groups) arc mRNA signal (number of cy-3-positive pixels) expressed as a percentage of the total field size, as determined by the particle analysis function of the ImageJ program. Data in (B) and (C) show the correlation between the degree of arc mRNA signal in DM and DL striatum and the number of trials required to reach criterion (9/10 correct turns) during (B) reversal training or (C) the same number of trials run as continued-acquisition trials. *P < 0.05 vs. caged control group; †P < 0.05 vs. reversal group.
and 1037 ± 14.6 Sytox Green-stained cellular profiles were counted in the DM subregion of striatum per animal in the caged control, continued-acquisition, and reversal groups, respectively (means ± SEM). The percentages of these Sytox Green cellular profiles that were positive for arc mRNA expression in the cytoplasm (nuclear plus cytoplasmic or cytoplasmic only) were 1.0 ± 0.04, 8.1 ± 1.1 and 6.9 ± 1.3%, respectively, in the DM striata of caged control, continued-acquisition, and reversal rats.

For this catFISH analysis, values are reported as the total number of arc-PPE+ and arc-PPE− neurons with nuclear, nuclear plus cytoplasmic, and cytoplasmic-only staining in each striatal region. Importantly, there was a high degree of correlation between the particle analysis of mRNA expression and the total number of arc-positive cells counted in each animal (PPE+ and PPE− combined) in both the DM (r² = 0.94, P = 0.0001) and DL (r² = 0.879, P = 0.0001) striatum, suggesting that the overall amount of arc signal detected was highly related to the total number of cells expressing arc mRNA above detectable levels.

Differences in each of these dependent measures in each brain region between the caged control, continued-acquisition, and reversal groups were analysed with a two-way ANOVA (treatment × cell type) followed by Student–Newman–Keuls or post hoc t-tests to determine significant differences. Likewise, analysis of tissue from rats exposed to a novel environment was completed with a two-way ANOVA (time × cell type) followed by similar post hoc analyses. For analysis of total arc mRNA signal in the hippocampus, a one-way ANOVA (treatment) was used for each region; this was followed by post hoc analysis with the Student–Newman–Keuls analysis. Statistical significance was set at P ≤ 0.05.

Results

Arc mRNA expression in striatum was increased in animals engaged in motor response learning on a T-maze

Particle analysis of the overall arc mRNA signal revealed that arc mRNA was increased over caged controls in the DM striatum of animals engaged in either reversal or continued-acquisition training (F²,17 = 5.58, P = 0.014; Figs 3 and 4A). In the DL striatum, the arc mRNA signal was significantly elevated (F²,17 = 9.72, P = 0.002) in the continued-acquisition group relative to both the caged controls and the reversal group. In the DL striatum, the overall amount of arc mRNA signal in the reversal group was not significantly different from that of the caged controls.

Motor response learning increased the number of PPE− and PPE+ striatal neurons with cytoplasmic arc mRNA expression

CatFISH analysis of arc mRNA expression in phenotypically identified striatal neurons revealed that there was no effect of the behavioural manipulations on the number of PPE− or PPE+ cells with intranuclear foci of arc mRNA expression (Fig. 5A; DM, F²,17 = 0.08, P = 0.9; DL, F²,17 = 0.02, P = 0.98). In all groups, there was a relatively low number of such cells in each field. Both reversal learning and continued-acquisition trials resulted in significant increases in the numbers of PPE− and PPE+ cells with combined nuclear and cytoplasmic staining in the DM striatum (Fig. 5B; F²,17 = 4.13, P = 0.03). These increases were not different in the PPE+ and PPE− neurons. In the DL striatum, the number of cells with combined nuclear and cytoplasmic labelling was significantly increased relative to the caged controls only in the rats undergoing continued-acquisition training (F²,17 = 7.21, P = 0.005), although there was also a trend for the number of cells to be increased in the DL striatum of rats undergoing reversal training relative to the caged controls (difference, 14.56; critical difference, 16.04). There was also a trend for the number of cells with combined nuclear plus cytoplasmic labelling in the continued-acquisition group to be greater than the number of cells in the DL striatum of rats in the reversal group (difference, 15.25; critical difference, 16.04). Interestingly, in the DL striatum there also was a significant overall main effect of phenotype (F²,17 = 8.49, P = 0.01), with a greater number of PPE− neurons across all treatment groups showing combined nuclear plus cytoplasmic labelling. Finally, there was a significant main effect of behavioural treatment for the number of cells with only cytoplasmic labelling in the DM striatum (Fig. 5C; F²,17 = 10.35, P = 0.001) and a strong trend for such an effect in the DL striatum (F²,17 = 3.27, P = 0.06). The number of cells showing only cytoplasmic arc mRNA staining was increased similarly in both the reversal and continued-acquisition groups relative to the caged controls. Intriguingly, there also were overall main effects for phenotype, with the number of PPE− cells with only cytoplasmic staining being greater overall than the number of PPE+ cells with only cytoplasmic staining, in both the DM (F²,17 = 21.8, P < 0.0002) and the DL (F²,17 = 21.4, P < 0.0002) striatum. This difference was most apparent in the DM striatum, where there was a significant interaction (Fig. 5C; F²,17 = 3.95, P = 0.04) between the behavioural treatment and the cellular phenotype. In this brain region, the numbers of PPE− cells with cytoplasmic-only labelling was significantly greater than the number of PPE+ cells with this labelling in both the reversal (t = 4.15, P < 0.004) and continued-acquisition (t = 4.2, P < 0.004) groups, but not in the caged controls (t = 1.7, P = 0.18).

Arc mRNA expression in dorsomedial striatum was correlated with reversal learning

Previous work has shown that the relation between arc mRNA expression and behaviour, rather than the total amount of arc mRNA expression, appears to be a better indicator of the role of arc and a particular brain region in the behavioural task (Guzowski et al., 2001). Therefore, we examined the correlation between the arc mRNA signal and new learning as reflected in the number of trials required for rats to reach criterion during reversal learning. In the DM striatum there was a significant negative correlation between the total arc mRNA signal as determined by ImageJ-based particle analysis of thresholded images and the number of trials to reach criterion in the reversal group (Figs 4B and 6A; r² = 0.55, P = 0.04), with animals who reached criterion more quickly showing greater arc mRNA signal (Figs 4B and 6A; r² = 0.55, P = 0.04). In all groups, there was a trend for the number of combined nuclear plus cytoplasmic staining in the continued-acquisition group to be greater than the number of cells in the DL striatum of rats in the reversal group (difference, 15.25; critical difference, 16.04). There was also a trend for the number of cells with combined nuclear plus cytoplasmic labelling in the continued-acquisition group to be greater than the number of cells in the DL striatum of rats in the reversal group (difference, 15.25; critical difference, 16.04). Interestingly, in the DL striatum there also was a significant overall main effect of phenotype (F²,17 = 8.49, P = 0.01), with a greater number of PPE− neurons across all treatment groups showing combined nuclear plus cytoplasmic labelling. Finally, there was a significant main effect of behavioural treatment for the number of cells with only cytoplasmic labelling in the DM striatum (Fig. 5C; F²,17 = 10.35, P = 0.001) and a strong trend for such an effect in the DL striatum (F²,17 = 3.27, P = 0.06). The number of cells showing only cytoplasmic arc mRNA staining was increased similarly in both the reversal and continued-acquisition groups relative to the caged controls. Intriguingly, there also were overall main effects for phenotype, with the number of PPE− cells with only cytoplasmic staining being greater overall than the number of PPE+ cells with only cytoplasmic staining, in both the DM (F²,17 = 21.8, P < 0.0002) and the DL (F²,17 = 21.4, P < 0.0002) striatum. This difference was most apparent in the DM striatum, where there was a significant interaction (Fig. 5C; F²,17 = 3.95, P = 0.04) between the behavioural treatment and the cellular phenotype. In this brain region, the numbers of PPE− cells with cytoplasmic-only labelling was significantly greater than the number of PPE+ cells with this labelling in both the reversal (t = 4.15, P < 0.004) and continued-acquisition (t = 4.2, P < 0.004) groups, but not in the caged controls (t = 1.7, P = 0.18).

FIG. 5. PPE− and PPE+ neurons in the DM and DL striatum with (A) intranuclear foci of arc mRNA expression, (B) nuclear plus cytoplasmic (nuclear plus cyto.) arc mRNA expression, and (C) cytoplasmic-only arc mRNA expression. Values are the mean ± SEM number of cells per 0.7 × 0.7 mm field (n = 4 for caged controls and n = 8 each for reversal and continued-acquisition groups, counted by an experimenter blinded to the treatment group of the animals). *P < 0.05 vs. respective phenotype in caged controls; †P < 0.05 vs. PPE− cells from the same behavioural group.
run by the continued-acquisition group (Fig. 4C; DM, $r^2 = 0.26$, $P = 0.20$; DL, $r^2 = 0.26$, $P = 0.19$) or between the arc mRNA signal in the DM striatum and the ‘density’ of reward (number of rewarded trials divided by the total number of trials to criterion) in the reversal group ($r^2 = 0.0006$, $P = 0.95$).

Analysis of the correlation between behavioural performance and the numbers of PPE$^+$ and PPE$^-$ neurons positive for arc mRNA expression revealed a significant negative correlation between the number of PPE$^-$ cells containing nuclear plus cytoplasmic arc mRNA signal in the DM striatum and the number of trials to criterion in rats engaged in reversal learning (Fig. 7A, $r^2 = 0.67$, $P = 0.01$). This correlation was not significant for PPE$^+$ cells ($r^2 = 0.32$, $P = 0.14$). For cells with only cytoplasmic labelling of arc mRNA, there was a significant negative correlation between the number of PPE$^-$ (Fig. 7B; $r^2 = 0.58$, $P = 0.03$) and PPE$^+$ ($r^2 = 0.55$, $P = 0.04$) neurons in the DM striatum containing only cytoplasmic arc mRNA staining and the number of trials required to reach criterion on the reversal training. There were no significant correlations between the number of PPE$^-$ and PPE$^+$ cells with nuclear plus cytoplasmic staining (Fig. 7C) or with only cytoplasmic staining (Fig. 7D) and the number of trials run.

**Fig. 6.** Confocal images showing arc mRNA (cy-3, red), PPE mRNA (cy-5, blue), and nuclear (Sytox green) staining in the (A) DM and (B) DL aspects of the striatum of a rat that required few trials to reach criterion on the reversal training ('fast reverser') and in the striatum of a rat that required more trials to reach criterion ('slow reverser'). Scale bar, 10 μm.
Fig. 7. Correlations between the numbers of PPE– and PPE+ cells in the dorsomedial striatum containing (A and C) nuclear plus cytoplasmic (nuclear plus cyto.) arc mRNA label and (B and D) only cytoplasmic arc mRNA label, and the number of trials required to reach criterion (9/10 correct turns) during (A and B) the reversal training or (C and D) the same number of trials run as continued-acquisition trials. * Statistically significant correlation.
by the continued-acquisition animals. There were also no significant correlations between the number of PPE− or PPE+ cells in the DM striatum with intranuclear foci and the number of trials completed in either the reversal learning or continued-acquisition groups (data not shown). For the DL striatum, there were no significant correlations between the numbers of PPE− and PPE+ cells with arc mRNA expression in any cellular compartment and the number of trials completed (data not shown). Finally, there were no significant correlations between the numbers of PPE− and PPE+ cells in the DM striatum with nuclear plus cytoplasmic or cytoplasmic-only arc mRNA expression and ‘reward density’ in the reversal group (data not shown).

Arc mRNA expression in hippocampus was not correlated with reversal learning

ImageJ-based particle analysis of the hippocampus revealed that in the CA1 subregion arc mRNA was significantly increased over caged controls in both the reversal and continued-acquisition groups (F$_{2,17}$ = 3.95, P = 0.04; caged control, 0.4 ± 0.1 (mean ± SEM); reversal group, 4.8 ± 0.7; continued-acquisition group, 4.1 ± 1.3). Arc mRNA levels also were elevated in the CA3 subregion and the dentate gyrus; however, these increases were not statistically significant (CA3, F$_{2,17}$ = 1.97, P = 0.17; caged control, 0.2 ± 0.1 (mean ± SEM); reversal group, 1.5 ± 0.3; continued-acquisition group, 1.7 ± 0.6; DG, F$_{2,17}$ = 2.66, P = 0.10; caged control, 0.2 ± 0.2 (mean ± SEM); reversal group, 1.2 ± 0.3; continued-acquisition group, 1.0 ± 0.3). There were no significant correlations between arc mRNA expression in any of the hippocampal subregions and behavioural measures of learning, although there was a trend towards a negative correlation between the arc expression in CA1 and the number of continued-acquisition trials (r$^2$ = 0.44, P = 0.07; data not shown).

Behavioural activation was associated with greater numbers of PPE− neurons with cytoplasmic, but not intranuclear, arc mRNA localization

Because of the greater number of PPE− cells expressing arc mRNA in the cytoplasm in both the reversal and continued-acquisition groups, we wished to determine whether this difference reflected differential activation of arc mRNA transcription in PPE− neurons by exposure to a behavioural task. We therefore determined the time-course of arc mRNA induction and trafficking to the cytoplasm in PPE− and PPE+ neurons in the DM striata of rats exposed to a novel environment for 5 min and killed immediately or at different time points (10, 25, 55 min) thereafter. The number of cells with intranuclear foci of arc mRNA signal was significantly elevated (F$_{4,10}$ = 19.1, P = 0.0001) only in the striata of rats killed immediately after the 5-min exposure to the novel environment relative to caged controls (Fig. 8A). There was no difference in the number of PPE− vs. PPE+ cells showing this induction of arc mRNA expression in the nucleus (F$_{1,10}$ = 0.21, P = 0.66). In rats killed 15 min after the initial exposure to the novel environment, there was a significant increase in the number of cells with nuclear plus cytoplasmic arc mRNA signal (Fig. 8B), with a significant main effect of time point (F$_{4,10}$ = 3.87, P = 0.04) and phenotype (F$_{1,10}$ = 16.66, P = 0.002), as well as a significant interaction between time point and phenotype (F$_{4,10}$ = 4.92, P = 0.02). Post hoc analysis revealed significant increases in the numbers of neurons expressing arc mRNA in both the nucleus and cytoplasm at the 15-min time point. Post hoc analysis further revealed that the number of PPE− cells with arc mRNA signal in the nucleus
and cytoplasm was significantly greater than the number of PPE+ cells with the same type of arc distribution (t = 4.32, P = 0.05). Significant increases in the number of neurons expressing arc mRNA only in the cytoplasm were also seen in the 15- and 30-min groups, as demonstrated by an overall main effect for treatment (F_{14,10} = 6.1, P < 0.01) and significantly more cells with cytoplasmic labelling at the 15- and 30-min time points on post hoc analysis (Fig. 8C). There was also a significant main effect of cellular phenotype (F_{13,10} = 5.1, P = 0.05), with more PPE- cells having cytoplasmic arc label than PPE+ cells overall (Fig. 8C).

Discussion

The dorsal striatum is involved in several types of learning. Thus, neuroplastic changes in this structure are likely to be critically involved in the adaptive processes that allow for such learning. The present results show that the cytoplasmic expression of arc mRNA, an effector IEG heavily implicated in synaptic plasticity (Guzowski et al., 2000; Guzowski et al., 2001; Steward & Worley, 2001), is increased in both PPE- (presumed striatonigral) and PPE+ (striatopallidal) neurons of the dorsal striatum of rats engaged in motor response learning and that the expression in both subpopulations of striatal efferent neurons is correlated with the behavioural measure of learning in animals engaged in reversal learning. Furthermore, the present results demonstrate that this relation between cytoplasmic arc mRNA expression and reversal learning is observed only in DM striatum, providing additional support for a critical role of this region in this aspect of motor response learning. Finally, although arc mRNA expression in the cytoplasm of both subpopulations of striatal efferent neurons is increased and correlates with learning, the current data demonstrate that the cytoplasmic distribution of arc mRNA is not uniform across distinct populations of striatal efferent neurons. Rather, greater numbers of striatonigral (PPE-) neurons have arc mRNA in the cytoplasm, despite apparently similar degrees of initiation of arc mRNA transcription in the nucleus. These data therefore provide novel evidence for a potential role of Arc in the neuroplasticity underlying basal ganglia-dependent learning processes and for cell-specific differences in the subcellular processing of arc mRNA.

Arc and learning

Although previous work has shown a relation between arc mRNA expression in hippocampus and spatial learning (Guzowski et al., 2001), the present results are the first to show a relation between arc mRNA expression in striatum and learning on a task known to be dependent on basal ganglia function. Significant correlations were seen for both dependent measures employed; that is, particle analysis of the overall amount of arc mRNA signal, as well as the number of cells positive for arc mRNA signal in the cytoplasm. Previous studies have shown that Arc is involved in learning and memory function in the hippocampus, as arc mRNA is translated to specific activated synapses in the hippocampus (Steward et al., 1998), in an N-methyl-D-aspartate (NMDA) receptor-dependent manner (Steward & Worley, 2001). Furthermore, although the expression of a number of IEGs, including arc, is increased in the hippocampi of rats engaged in different learning paradigms, it is only the expression of arc mRNA in the hippocampi of rats engaged in spatial learning, as opposed to nonspatial tasks, that is correlated with behavioural performance (Guzowski et al., 2001). The present results confirm these previous findings and extend them in three important ways. First, the data show that cytoplasmic arc mRNA expression is significantly elevated in both DM and DL striata, as well as subregions of the hippocampi, of all rats engaged in response training on the T-maze relative to caged controls, supporting the conclusion that the overall degree of gene expression may not be a valid index of the necessity of a particular brain area for the particular learning being examined. Second, the correlation between cytoplasmic arc mRNA expression in DM striatum and reversal learning (learning a ‘new’ motor response) on the T-maze, as well as the lack of correlation between cytoplasmic arc mRNA expression in either region of striatum and continued-acquisition training (no ‘new’ learning of a motor response), demonstrates that the relation between the arc mRNA expression and learning holds for response learning mediated by the striatum as well as spatial learning mediated by the hippocampus (Guzowski et al., 2001). These findings thus suggest that this relation may generally exist across brain areas mediating different types of learning. Finally, the lack of correlation between arc mRNA expression in DM and DL striatum and the number of trials completed by the continued-acquisition group in which there was no ‘new’ learning of a motor response establishes that the relation observed between arc mRNA expression and trials to criterion on a motor-response learning task is not simply a consequence of the number of trials run or the amount of time spent on the maze. The lack of correlation between the ‘density’ of reward and the arc mRNA expression further suggests that the relation between learning and arc mRNA expression in DM striatum of the reversal group is not simply due to the amount of reward per se.

The mechanisms by which the changes in arc mRNA expression in the cytoplasm contribute to striatally based learning remain to be determined. As noted above, arc mRNA is trafficked through the cytoplasm to activated dendritic spines (Steward et al., 1998), where it is locally translated into protein. The most recent evidence available suggests that Arc protein in these activated dendrites contributes to the trafficking of alpha-amino-3-hydroxy-5-methyl-4-isoxazolopropionic acid (AMPA)-type glutamate receptors at the synapse (Chowdhury et al., 2006; Rial Verde et al., 2006; Shepherd et al., 2006). The present studies do not directly address the mechanisms by which Arc alters striatal synaptic plasticity, and thus learning, in this paradigm. However, the results are the first to demonstrate that it is the expression of arc mRNA in the cytoplasm that is inversely correlated with learning, suggesting that greater distribution of arc mRNA out into the cytoplasm and sites of synaptic activity where it can contribute to synaptic reorganization may be a critical factor in determining the rapidity of learning. Clearly additional studies examining the regulation of arc mRNA trafficking, as well as the relation between Arc protein translation at synapses and behaviour will be necessary to fully test this hypothesis.

Dorsomedial striatum and learning

Specific striatal subregions, in particular DM vs. DL striatum, have been implicated in mediating different aspects of learning and behavioural flexibility (Yin & Knowlton, 2006). In general, DL striatum has been implicated in mediating relatively inflexible, stimulus-response learning or habit formation, whereas DM striatum has been implicated in mediating more behaviourally flexible, action-outcome learning. In the present work, reversal learning on the T-maze requires a shift in behavioural flexibility from a more inflexible, habitual response acquired and overtrained over three days to a new response in which action is again conceivably driven by outcome expectation. Indeed, work by Ragozzino and colleagues has previously shown that chemical inactivation (Ragozzino et al., 2002b) or blockade of muscarinic cholinergic (Ragozzino et al., 2002a; Tzavos...
et al., 2004) or NMDA glutamate receptors (Palencia & Ragozzino, 2004) in DM striatum impairs reversal learning, but not initial acquisition, on such a T-maze task. The present finding of a significant correlation between reversal learning and arc mRNA expression in DM striatum, but not DL striatum or hippocampus, further supports a critical role of DM striatum in such learning. The data also suggest that Arc-mediated synaptic modifications in both striatonigral and striatopallidal efferent neurons of the DM striatum may be critical for reversal learning on a response-based task. Also of potential interest in the present study was the observation of a trend toward greater overall arc mRNA expression in DL striatum in rats engaged in continued-acquisition training, as well as a trend toward a correlation between the number of trials run by the continued-acquisition group and arc mRNA expression in the CA1 region of hippocampus. These findings suggest that further examination of arc mRNA expression in diverse brain regions in the context of initial response learning, as well as at various points during overtraining on a task, may shed light on the systems in which plasticity occurs during different phases of the learning process and, perhaps, as organisms transition from the use of one behavioural strategy to another.

**Subcellular compartmentalization of arc mRNA**

While the present findings suggest that arc mRNA expression in both PPE + and PPE - neurons of the striatum is related to learning, the data do show differential expression of arc mRNA in the cytoplasm of these distinct neuronal populations in striatum independent of the learning task. That is, in both the reversal and continued-acquisition groups, more PPE + than PPE - neurons showed cytoplasmic arc mRNA expression. To examine the potential basis for this difference, we used a novelty exposure paradigm to specifically examine the transcriptional activation and subsequent distribution of arc mRNA in these two striatal neuron populations. The results from animals exposed to a novel environment for 5 min revealed that arc mRNA expression is induced (as demonstrated by the number of cells with intranuclear foci of arc mRNA expression) in the same number of PPE + and PPE - neurons. These data suggest that arc mRNA transcription is not differentially activated in these two populations of striatal efferent neurons. However, in the rats exposed to a novel environment, as observed in the rats engaged in continued-acquisition trials or response-reversal learning, a greater number of PPE - neurons had cytoplasmic arc mRNA expression. These findings suggest that the mechanisms underlying arc mRNA trafficking to or stability in the cytoplasm are different in these distinct striatal neuron populations. Neurons expressing PPE are known to be striatopallidal (indirect pathway) neurons (Gerfen & Young, 1988; Gerfen et al., 1990; Le Moine & Bloch, 1995). It is likely that the PPE - neurons are striatonigral (direct pathway) neurons for two reasons. First, striatal efferent neurons comprise ~95% of the neurons in the striatum. Thus, the majority, although not all, of the PPE - neurons will be striatonigral neurons (Gerfen & Young, 1988; Le Moine & Bloch, 1995). Second, Vazdarjanova and colleagues have shown that all striatal neurons expressing arc mRNA in the context of spatial navigation also express glutamic acid decarboxylase-67 (GAD67; Vazdarjanova et al., 2006). GAD67 is found in striatal efferent neurons (Mercugliano et al., 1992) and also in a small subpopulation of striatal interneurons that comprises only ~1% of the total striatal neuron population (Kawaguchi et al., 1995). Thus, overall the present data suggest that the regulation of arc mRNA trafficking and stability is different in striatonigral vs. striatopallidal efferent neurons of striatum. At this time, little is known about the specific cellular mechanisms involved in the regulation of arc mRNA trafficking, other than that the process is dependent on NMDA receptor activity (Steward & Worley, 2001), possibly involves microtubule- and actin-based mechanisms of transport (Dynes & Steward, 2006), and may be associated with or regulated similarly to z-CaMKII (Donai et al., 2003; Vazdarjanova et al., 2006). Additional studies are clearly needed to examine the processes underlying the difference in cytoplasmic arc mRNA expression we observed in striatal neurons.

The present results implicate Arc in both striatonigral and striatopallidal neurons of the DM striatum as a potential mediator of the role of this striatal subregion in striatial based learning and memory processes driven by outcome expectation. Furthermore, the data show that the subcellular distribution of arc mRNA is differentially regulated in striatopallidal and striatonigral efferent neurons of striatum, raising the possibility that the nature or degree of synaptic plasticity in these striatal efferent neuron subpopulations may differ and revealing that the processing or trafficking of arc mRNA in the cytoplasm can be differentially regulated in distinct neuronal populations within a given brain region.

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**Abbreviations**

AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; Arc, activity-regulated cytoskeletal-associated protein; catFISH, cellular compartment analysis of temporal activity by fluorescent in situ hybridization; cy-3, cyanine-3; cy-5, cyanine-5; DL, dorsolateral; DM, dorsomedial; IEG, immediate-early gene; NMDA, N-methyl-D-aspartate; PPE, preproenkephalin.

**References**


Response learning and striatal arc expression


