Published in final edited form as:

Neurobiol Learn Mem. 2009 January ; 91(1): 13–22. doi:10.1016/j.nlm.2008.09.008.

Acetylcholine Activity in Selective Striatal Regions Supports Behavioral Flexibility

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Abstract

Daily living often requires individuals to flexibly respond to new circumstances. There is considerable evidence that the striatum is part of a larger neural network that supports flexible adaptations. Cholinergic interneurons are situated to strongly influence striatal output patterns which may enable flexible adaptations. The present experiments investigated whether acetylcholine actions in different striatal regions support behavioral flexibility by measuring acetylcholine efflux during place reversal learning. Acetylcholine efflux selectively increased in the dorsomedial striatum, but not dorsolateral or ventromedial striatum during place reversal learning. In order to modulate the M2-class of autoreceptors, administration of oxotremorine sesquifumurate (100 nM) into the dorsomedial striatum, concomitantly impaired reversal learning and an increase in acetylcholine output. These effects were reversed by the m₂ muscarinic receptor antagonist, AF-DX-116 (20 nM). The effects of oxotremorine sesquifumurate and AF-DX-116 on acetylcholine efflux were selective to behaviorally-induced changes as neither treatment affected acetylcholine output in a resting condition. In contrast to reversal learning, acetylcholine efflux in the dorsomedial striatum did not change during place acquisition. The results reveal an essential role for cholinergic activity and define its locus of control to the dorsomedial striatum in cognitive flexibility.

The ability to inhibit one strategy and learn a new strategy represents an essential form of adaptive behavior in daily living and often survival. Prefrontal cortex – basal ganglia circuitry plays a critical role in facilitating a shift in strategies or response patterns (Block et al., 2007; Monchi et al., 2001; Muhammad et al., 2006; Owen et al., 1993; Stefani & Moghaddam, 2006; Wise, Murray & Gerfen, 1996). There is considerable evidence in different mammalian species that the basal ganglia nuclei support cognitive flexibility (Monchi et al., 2001; Owen et al., 1993; Ragozzino et al., 2002a). More specifically, several experiments have demonstrated that the striatum, the largest component of the basal ganglia, enables learning when conditions demand a shift in choice patterns, e.g. place reversal learning, as well as a shift in strategies, e.g. switch between basing a choice on visual object information to basing a choice on egocentric response information (Block et al., 2007; Ragozzino et al., 2002a; Ragozzino & Choi, 2004).

At present, less is known about the specific circuitry and neurochemical processes in the striatum that may enable cognitive flexibility. One neurotransmitter in the striatum that may play a key role in facilitating cognitive flexibility is acetylcholine (ACh). The principle source

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of striatal ACh content originates almost entirely from interneurons (Bolam et al., 1984). The cholinergic interneurons are distinguished from the more plentiful projection neurons by their large somata, as well as extensive axonal fields (Bolam et al., 1984; Wilson et al., 1990). This anatomical feature suggests that cholinergic interneurons may be important for shaping the nature of striatal output to other brain regions also critical for cognitive flexibility. Furthermore, ACh in the striatum is critical for modulating synaptic plasticity that may underlie different forms of learning and memory (Calabresi et al., 1998).

ACh actions at muscarinic cholinergic receptors in the striatum may alter synaptic plasticity that supports certain forms of learning and memory. In particular, several experiments have demonstrated that intra-cranial infusions of muscarinic cholinergic antagonists into the dorsal striatum prior to or after training impairs memory consolidation in rats (Diaz del Guante et al., 1991; Giordano & Prado-Alcala, 1986; Solana-Figueroa & Prado-Alcala, 1990). Furthermore, cholinergic agents infused into the dorsal striatum also affect memory retrieval (Figueroa & Prado-Alcala, 1990). However, when muscarinic cholinergic receptors are blocked specifically in the dorsomedial striatum there is no effect on memory retrieval (McCool et al., 2008; Ragozzino et al., 2002b; Tvazos et al., 2004), suggesting that cholinergic actions at muscarinic cholinergic receptors outside the dorsomedial striatum may affect memory processes.

In addition to mnemonic processing, there is indirect evidence that suggests that ACh actions in the striatum play a role in learning and possibly cognitive flexibility. For example, the activity of striatal tonically active neurons is correlated with the presentation of primary rewards or stimuli associated with reward (Aosaki et al., 1984). A significant proportion of the tonically active neurons are likely cholinergic interneurons and thus may represent plastic changes in these neurons during learning (Wilson et al., 1990). These neurons also exhibit changes in the temporal relationship between stimuli or events that may be critical when conditions require a shift in learned response patterns. (Apicella, 2002). Other experiments have found changes in striatal ACh efflux during learning and strategy switching, but have not demonstrated that changes in striatal ACh output are actually critical for specific aspects of learning (Chang & Gold, 2003; Ragozzino & Choi, 2004). Limitations of these past experiments have prevented a direct link between cholinergic activity and behavioral flexibility. Moreover, cholinergic interneurons are found throughout the striatum, thus these neurons may support behavioral flexibility in multiple striatal subregions.

The present experiments address the issues raised above by investigating whether ACh efflux in different striatal regions changes during reversal learning of a place discrimination. To more directly link a change in striatal ACh efflux in underlying a shift in choice patterns, the experiments also examined the effects of modulating striatal ACh efflux during place reversal learning.

Methods

Subjects

Male Long-Evans rats (Harlan, Indianapolis, USA) weighing between 350-400 grams at the start of the experiment served as subjects. Rats were singly housed in plastic cages (26.5 cm wide × 50 cm long × 20 cm high) in a humidity (30%) and temperature (22°C) controlled room with a 12-h light/dark cycle (lights on at 0700 h). Rat received surgery approximately 5-7 days after arriving at the colony. Animal care and use was in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and was approved by the Institutional Laboratory Animal Care and Use Committee at the University of Illinois at Chicago.

Surgery

Each rat received stereotaxic surgery to bilaterally implant cannula into the striatum. Before surgery each rat received an intraperitoneal (i.p.) injection of atropine sulfate (0.2 ml of a 250 µg/ml solution) followed 10 min later by an i.p. injection of sodium pentobarbital (50 mg/kg). In Experiments 1 and 2, twelve-mm guide cannula (CMA Microdialysis Inc., North Chelmsford, MA, USA) were bilaterally implanted and aimed toward the dorsomedial striatum (see Figure 1). The cannulae were implanted at a 10° angle. The stereotaxic coordinates were 1.1 mm anterior to bregma, $\pm$ 2.8 mm lateral to the midline and 4.0 mm below dura. In Experiment 3, cannulae were bilaterally implanted aimed at either the ventromedial striatum or dorsolateral striatum. The coordinates were based on Paxinos and Watson's (1996) rat brain atlas. An omega-shaped ring was placed behind the guide cannulae. The omega ring allowed the rat to be connected to the liquid swivel/balance arm by a wire attached with a hook that extended from the liquid swivel. This setup prevented the tubing from being twisted during microdialysis collection. Four jeweler's screws were positioned in the skull surrounding the guide cannulae. Dental acrylic (Stoelting, Wood Dale, USA) was used to secure the guide cannula. In Experiment 3, guide cannulae were bilaterally implanted aimed toward the dorsolateral striatum or ventromedial striatum (see Figure 1). The stereotaxic coordinates for the dorsolateral striatum were 0.6 mm anterior to bregma, $\pm$ 3.5 mm lateral to the midline and 4.0 mm below dura. The stereotaxic coordinates for the ventromedial striatum were 1.1 mm anterior to bregma, $\pm$ 2.8 mm lateral to the midline and 6.0 mm below dura. The cannulae aimed for the ventromedial striatum were implanted at a 10° angle. After surgery, the rats received 6 ml of saline subcutaneously (s.c.) and fed ground rat chow and sugar mixed with water for 1 day. For 5-7 days after surgery the rats were allowed to recover and handled for 5 min each day. After 2 days of recovery all rats were food-restricted to maintain their weight at about 85% of their free-feed weight. Each rat had free access to water throughout the study. Behavioral training commenced 5-7 days after surgery.

Apparatus

Behavioral testing experiments were conducted in a black plastic four-arm cross maze. The height of the maze walls was 15.0 cm and each of the arms measured 55 cm long $\times$ 10 cm wide. There was a food well (3.2 cm diameter $\times$ 1.6 cm high) in each arm located 3 cm away from the end wall. The hole in the food well measures 2.3 cm in diameter and was 1.6 cm deep. The maze was elevated 72 cm above the floor in a room with extra-maze cues.

Pretraining

Except where noted, the experiments involved behavioral testing that required pretraining. On the first day of pretraining all arms of the maze were baited with a half piece of Froot Loops cereal (Kelloggs, Battle Creek, MI). Each rat was placed at the end of a maze arm and allowed to explore and consume the Froot Loop pieces. One pretraining trial was completed after a rat consumed all the cereal pieces. Between trials a rat was placed in a holding cage while the maze was rebaited with Froot Loops pieces. The pretraining session was terminated after 15 min had elapsed and the number of trials completed was recorded. If a rat did not complete 1 pretraining trial within 15 min it stayed in the maze until it consumed all cereal pieces or until 20 min had elapsed. During subsequent pretraining sessions, when a rat had consumed a piece of Froot Loops cereal from two baited arms it was picked up and placed in an unbaited arm. After eating from a third baited arm it was picked up and placed in another unbaited arm. After consuming cereal from the fourth arm, a rat was removed, the arms were rebaited and another trial was started. This procedure was used to acclimate a rat to being picked up in the maze after consuming a cereal piece. This procedure was continued until a rat was able to complete 5-7 trials in 15 min across 2 consecutive days. After reaching this criterion, a final day of pretraining occurred in which a black plastic block (9 cm wide $\times$ 13 cm high $\times$ 1 cm thick) was

placed at the entrance of one arm so that it prevented entry, giving the maze a T-shape. Therefore, there were only two arm choices available to a rat. A rat was placed at the end of a stem arm and allowed to enter either choice arm to obtain a cereal piece. After the initial choice, a rat was placed back in the stem arm. If a rat chose the same arm as the initial choice, it was returned to the stem arm until it chose the other arm. When a rat had chosen both arms it was placed in the holding cage while the two choice arms were re-baited. The session ended after a rat had completed 7 of these trials. Rats in all experiments required a total of 4-8 days of pretraining. In experiments 1 and 4, rats were pseudorandomly assigned to an experimental group such that all the groups received a similar amount of pretraining.

***In vivo* Microdialysis**

On test days, subjects were placed in a round-bottom plastic bowl, and a 2-mm microdialysis probe was inserted through each guide cannula. The wire extending from the liquid swivel was hooked onto the omega ring. Artificial cerebrospinal fluid (aCSF) was perfused through the microdialysis probes at a rate of 2.0 μ l/min. The aCSF contained NaCl (0.128 M), KCl (0.004M), dibasic Na₂HPO₄ (0.002 M), monobasic Na₂HPO₄ (0.0006 M), CaCl₂ (0.001 M), MgCl₂ (0.0009 M) and glucose (0.001 M). The solution was brought to a pH of 7.4 by NaOH. To reliably detect ACh levels, the reversible acetylcholinesterase inhibitor, neostigmine bromide (100 nM) was added to the aCSF. The perfusate collected during the first 60 min was not analyzed to allow equilibration between the brain tissue and perfusion solution. For each rat, in all of the experiments, microdialysis samples were simultaneously collected from each hemisphere.

Experiment 1: Dorsomedial Striatal ACh Output during Place Reversal Learning

The purpose of Experiment 1 was to determine whether ACh output from the dorsomedial striatum is important for behavioral flexibility. This was investigated by determining whether dorsomedial striatal ACh output changes during place reversal learning. If dorsomedial striatal ACh output changes during place reversal learning, then does directly modulating ACh output in this region by infusing oxotremorine sesquifumurate (Oxo-S), which has its highest affinity for m₂ muscarinic cholinergic receptors, block the change in striatal ACh output during reversal learning? If so, can the effect of Oxo-S be reversed by the m₂ muscarinic cholinergic antagonist, AF-DX-116?

Behavioral testing involved two phases across two consecutive days: acquisition phase and reversal learning phase. Prior to each session, a rat was hooked up to the liquid swivel wire and placed in the holding chamber. No microdialysis collection was conducted during place acquisition. Therefore, only the wire was attached to the rat without probes inserted. In the acquisition phase, a rat learned to enter the same arm to receive a cereal reinforcement. The maze was arranged in a T-shape such that there was a stem arm and two choice arms. Each rat was started randomly from the “east” or “west” arm. If a rat entered the correct arm it was allowed to consume the cereal piece in the food well. If a rat made an incorrect choice, it was removed from the non-reinforced arm after reaching the food well. In the acquisition phase, rats were tested for 24 min. This length of testing was chosen because it was similar to that of a previous study that combined discrimination testing with *in vivo* microdialysis collection (Ragozzino & Choi, 2004). The trials were separated into four 6-minute blocks in which the number of correct trials and total number of trials was recorded. For each 6-min block, a percent correct score was determined by calculating the number of trials a rat chose the correct arm divided by the total number of trials for that block.

For reversal learning, rats were pseudorandomly assigned to one of the following treatments such that each group exhibited similar performance in the last block of acquisition and completed a similar number of trials throughout acquisition testing: 1) aCSF (n = 11); 2) Oxo-

S 100 nM (n = 8); 3) AF-DX 116 20 nM (n = 12); 4) Oxo-S 100 nM/AF-DX 116 20 nM (n = 13). The doses of Oxo-S and AF-DX-116 were chosen based on experiments demonstrating that higher doses affected basal ACh output in the striatum, but comparable doses as used in this experiment, did not affect basal ACh output in other brain areas (Bertorelli et al., 1991; DeBoer et al., 1990; Douglas et al., 2001; Stillman et al., 1996). In the reversal learning phase, a rat was placed in the plastic bowl and a microdialysis probe was placed in each guide cannula. The perfusate collected during the first 60 min was not analyzed, to allow equilibration between the brain tissue and perfusion solution. Subsequently, five baseline samples were collected at 6-min intervals. Following baseline collection, a rat was placed in the maze for the reversal learning test. A rat was required to turn in the opposite direction as in acquisition to receive a ½ piece of cereal. Each rat was tested for a total of 30 min. A rat's behavioral performance was separated into 6-min blocks, which corresponded with the collection of microdialysis samples. For each 6-min block, a percent correct score was determined by calculating the number of trials a rat chose the correct arm divided by the total number of trials for that block. Five microdialysis samples were collected concomitantly in 6-min intervals. At the onset of testing, the control group was perfused with aCSF through the microdialysis probes while the drug groups were perfused with either Oxo-S, AF-DX 116 or a combination of Oxo-S and AF-DX 116. All drugs were mixed in aCSF and perfused through the microdialysis probes. Because samples were collected from both hemispheres in each rat, the drugs were infused via reverse dialysis bilaterally. The drug infusion occurred throughout the 30 min of behavioral testing.

Experiment 2: Effect of Oxo-S and AF-DX 116 on Dorsomedial Striatal ACh Output in a Resting Condition

The purpose of Experiment 2 was to determine whether the doses of the Oxo-S and AF-DX 116 used during behavioral testing had any effect on basal levels of dorsomedial striatal ACh output. If in Experiment 1, Oxo-S infused into the dorsomedial striatum simultaneously blocks ACh output and reversal learning, then the effect on ACh output can be interpreted in two ways. First, Oxo-S may block an increase in dorsomedial striatal ACh output by selectively affecting the behaviorally-induced increase. Second, the dose of Oxo-S used may generally decrease dorsomedial striatal ACh output in which the lack of increase in ACh output during reversal learning is just a result of a subtraction between the decrease in a basal output effect of Oxo-S and the behaviorally-induced increase in ACh output. In a somewhat related manner, if AF-DX 116 reverses the effect of Oxo-S on dorsomedial striatal ACh output during reversal learning, then unknown is whether AF-DX 116 selectively affects behaviorally-induced changes or whether AF-DX 116 increases basal ACh levels in the dorsomedial striatum.

The same microdialysis procedure was used as in Experiment 1. After 5 baseline samples, one group of rats received Oxo-S 100 nM (n = 6) and another group received AF-DX 116 20 nM (n = 7) via reverse dialysis for a total of 30 minutes. In this experiment, there was no behavioral testing and a rat was left in the plastic bowl throughout the collection of microdialysis samples.

Experiment 3: Dorsomedial Striatal ACh Output during Place Acquisition

The purpose of this experiment was to determine whether changes in ACh output in the dorsomedial striatum occur during any learning situation including the initial learning of a place discrimination or only when a rat has to inhibit one learned choice pattern and learn a new choice pattern, e.g. place reversal learning. This was investigated by determining whether dorsomedial striatal ACh output changes during acquisition of a place discrimination.

The behavioral testing and microdialysis procedures were similar to those as in Experiment 1. However, the microdialysis procedure was conducted during place acquisition. In the acquisition phase, rats were tested for 24 min with microdialysis samples collected at 6 minute intervals. For place acquisition, the trials were separated into four 6-minute blocks in which

the number of correct trials and total number of trials was recorded. For each 6-min block, a percent correct score was determined by calculating the number of trials a rat chose the correct arm divided by the total number of trials for that block. The sample size in this group was 10.

Experiment 4: ACh Output from the Dorsolateral Striatum and Ventromedial Striatum during Place Reversal Learning

The purpose of Experiment 4 was to determine whether changes in ACh output are observed in the other striatal regions during place reversal learning. Specifically, in one group microdialysis samples were collected from the dorsolateral striatum ($n = 7$), while in a second group microdialysis samples were collected from the ventromedial striatum ($n = 10$). The same behavioral testing and microdialysis procedures were used as in Experiment 1.

Histology

After completion of testing, each rat received a lethal dose of sodium pentobarbital. Just prior to the perfusion procedure a 2-mm microdialysis probe dipped in 2.5% Chicago Blue stain was inserted in each guide cannula to highlight the location of the probe placement. All rats were perfused intracardially with 0.9% phosphate-buffered saline, followed by a 4% formaldehyde solution. Brains were removed and stored in a 4% formaldehyde solution. The brains were frozen and cut in coronal sections (40 μm) on a cryostat. The brain sections were mounted on slides, dried, and examined to determine the spread of the stain. Subsequently, the brain sections were stained with cresyl violet to assess the location of the probes.

Statistical Analysis

The microdialysis data were analyzed by converting the raw values to percentages from each subject's baseline output. The baseline output was calculated from the mean of the first five samples for each subject. The percent values were analyzed by a repeated measures ANOVA. A repeated-measures ANOVA was used to analyze the percent correct scores across blocks for reversal learning between the groups.

Results

Figure 1 illustrates the location of the microdialysis probes in the various striatal subregions for Experiments 1-4. The dorsomedial striatal probe placements were concentrated at the level of the genu of the corpus callosum in the medial and central regions of the dorsal striatum. Dorsolateral striatal probe placements were concentrated at the level of the genu of the corpus callosum but in the lateral region of the dorsal striatum. The ventromedial striatal probe placements were concentrated in the nucleus accumbens and the most ventral portion of the dorsal striatum.

Experiment 1: ACh Output from the Dorsomedial Striatum during Reversal Learning

The dorsomedial striatal ACh output results are illustrated in Figure 2a. Prior to reversal learning and drug infusions, all groups exhibited stable basal ACh output. In control rats, there was an increase of approximately 25% above basal levels during the initial block (T1) of reversal learning which increased to 40% or greater above basal levels in all subsequent blocks (T2 – T5). Oxo-S treatment led to a negligible increase in ACh output of approximately 10% throughout the test session. In contrast, AF-DX 116 or combined treatment of AF-DX 116/Oxo-S produced a similar pattern of dorsomedial striatal ACh output as controls. An ANOVA with repeated measures revealed that there was a significant group effect, $F_{3,40} = 10.69$, $P < 0.01$, reflecting the different treatment effects on dorsomedial striatal ACh output. There was a significant effect of condition (baseline and reversal learning), $F_{1,40} = 232.75$, $P < 0.01$, indicating increases in dorsomedial striatal ACh output during the reversal learning condition

compared to baseline condition. The analysis also revealed that there was a significant effect of block (5 blocks each of baseline and test), $F_{4,160} = 20.30$, $P < 0.01$. Moreover, there was a significant condition by group interaction, $F_{3,40} = 7.24$, $P < 0.01$. A subsequent analysis using *F*-tests for simple effects revealed that there was not a difference in ACh output among the groups during the baseline condition (P 's > 0.05). In contrast, the Oxo-S group exhibited significantly less ACh output during the reversal learning condition compared to that of the other treatment groups (P 's < 0.05). A further analyses on the block $\times$ group interaction with a Bonferroni t-test revealed that the groups exhibited comparable ACh output during the different blocks of baseline (P 's > 0.05). During reversal learning, there was not a significant difference in ACh output among the groups for the 1st block (P 's > 0.05). However, in blocks T2-T5 ACh output was significantly less in the Oxo-S compared to that of other treatment groups (P 's < 0.05). Thus, Oxo-S treatment blocked the reversal learning-induced increase in dorsomedial striatal ACh output during blocks T2-T5. In contrast, AF-DX 116 infused alone did not alter ACh output compared to that of controls as revealed by Bonferroni t-tests (P 's > 0.05). Furthermore, AF-DX 116 infused in combination with Oxo-S, prevented the blockade of ACh output during reversal learning compared to that with Oxo-S treatment alone.

The different cholinergic drugs affected, in a similar way, both reversal learning performance and dorsomedial striatal ACh efflux during behavioral testing. In the last test block of place acquisition, rats from all groups achieved approximately 90% correct (see Figure 2b). In the reversal learning session, control rats initially dropped to chance levels as rats would initially choose the arm that was reinforced on acquisition, but begin to choose the new correct arm in the first test block. Performance steadily improved across the reversal learning session with control rats achieving approximately 90% of trials correct by the last test block. Oxo-S treatment reduced place reversal learning performance leading to 50-60% accuracy across the first four blocks with 70% accuracy on the final test block. Infusion of AF-DX 116 into the dorsomedial striatum led to a behavioral performance that was comparable to that of controls. When AF-DX 116 was combined with Oxo-S the behavioral deficit induced by Oxo-S alone was reversed. A two-way ANOVA with repeated measures indicated that there was a significant group effect $F_{3,40} = 9.37$, $P < 0.01$. To further explore the significant group effect, a subsequent analysis using *F*-tests for simple effects revealed that reversal learning performance in the Oxo-S group was significantly reduced compared to that of all other treatment groups (P 's < 0.01). The analysis also revealed a significant test effect of block $F_{4,160} = 55.12$, $P < 0.01$, indicating learning across the test blocks. Finally, there was not a significant group $\times$ test interaction, $F_{12,160} = 0.37$, $P > 0.05$.

One possibility is that the various drug effects observed during reversal learning were due to an alteration in the number of test trials completed in the different blocks. To examine this, the number of trials completed in each test block was calculated for all groups. As illustrated in Figure 3, all groups completed a similar number of trials across the five test blocks. An ANOVA with repeated measures revealed that there was not a significant group effect, $F_{3,40} = 0.19$, $P > 0.05$; nor a significant effect of block, $F_{4,160} = 0.87$, $P > 0.05$; and there was not a significant group $\times$ block interaction, $F_{12,160} = 0.63$, $P > 0.05$. Thus, the different drugs while affecting dorsomedial striatal ACh output and reversal learning did not affect the number of trials completed during reversal learning.

Experiment 2: Effect of Oxotremorine Sesquifumurate and AF-DX 116 on Dorsomedial Striatal ACh Output in a Resting Condition

The findings from Experiment 1 leave unanswered whether the effect of Oxo-S at 100 nM and/or AF-DX 116 at 20 nM directly affect behaviorally-induced changes in ACh output or also affect basal ACh output. To determine this, Experiment 2 investigated the effects of Oxo-S and AF-DX 116 infusions on dorsomedial striatal ACh output in a condition that did not involve

behavioral testing. In this experiment, rats remained in a holding chamber during the entire period in which samples were collected. As shown in Figure 4, Oxo-S and AF-DX 116 did not affect basal ACh output from the dorsomedial striatum. An ANOVA with repeated measures indicated that there was not a significant difference in ACh output between the groups, $F_{1,11} = 0.01$, $P > 0.05$. The difference in ACh output between baseline and test conditions was not significant, $F_{1,11} = 4.17$, $P > 0.05$, nor was there a significant group $\times$ condition interaction, $F_{1,11} = 0.01$, $P > 0.05$.

Experiment 3: ACh Output from the Dorsomedial Striatum during Acquisition

Experiment 1 demonstrated that ACh output increases in the dorsomedial striatum during place reversal learning and modifying the increase in ACh output concomitantly alters reversal learning performance. The goal of Experiment 3 was to determine whether changes in dorsomedial striatal ACh output are not only critical when conditions require a shift in choice patterns, but play a more general role in discrimination learning. To investigate this possibility a group of rats was tested on the initial learning of a place discrimination with simultaneous sampling of ACh output. Figure 5 illustrates that dorsomedial striatal ACh output did not change during place acquisition from basal levels. An ANOVA revealed that there was not a significant difference in ACh output during testing vs. baseline conditions, $F_{1,7} = 0.71$, $P > 0.05$.

Although ACh output from the dorsomedial striatum did not change during acquisition of a place discrimination, rats displayed learning of the place discrimination, performing slightly above chance during the 1st test block, 55.3% and achieving 83.8% correct by the final test block. In addition, examination of the trials completed revealed that rats in this experiment completed a similar number of trials across the four test blocks ranging from a mean of 8.25 ± 0.31 to 8.5 ± 0.42 .

Experiment 4: ACh Output from the Dorsolateral Striatum and Ventromedial Striatum during Place Reversal Learning

Cholinergic actions in other striatal regions, besides the dorsomedial striatum, may also enhance reversal learning. To better understand this, Experiment 4 investigated whether changes in ACh output are observed in the dorsolateral or ventromedial striatum during place reversal learning. Figure 6 shows that ACh output from the dorsolateral striatum or ventromedial striatum did not appreciably change from baseline levels throughout reversal learning. An analysis of ACh output revealed there was not a significant difference in ACh output between the groups, $F_{1,12} = 0.12$, $P > 0.05$; between baseline and test conditions, $F_{1,12} = 0.93$, $P > 0.05$ or the different blocks, $F_{4,48} = 2.10$, $P > 0.05$. The analysis also revealed that there was not a significant group $\times$ condition $\times$ block interaction, $F_{4,48} = 2.19$, $P > 0.05$.

Comparable to the dorsomedial striatal control group, both the dorsolateral and ventromedial striatum groups were able to reverse their choice patterns achieving approximately 90% correct by the end of the reversal learning session (see Figure 6B). An analysis of the percent correct scores indicated that there was not a significant group effect, $F_{1,12} = 0.12$, $P > 0.05$. There was a significant effect of block, $F_{4,48} = 26.33$, $P < 0.01$, revealing learning across the test blocks. However, there was not a significant group $\times$ block interaction, $F_{4,48} = 0.77$, $P > 0.05$.

An examination of the number of trials completed in the different blocks indicated that both groups averaged between eight and nine trials in each block as observed in the previous experiments. An ANOVA with repeated measures indicated that there was not a significant group effect, $F_{1,12} = 0.03$, $P > 0.05$; there was not a significant effect of block, $F_{4,48} = 1.03$, $P > 0.05$; and there was not a significant group $\times$ block interaction, $F_{4,48} = 0.03$, $P > 0.05$.

Discussion

The striatum is essential for flexible behavioral responses. Which subregions of the striatum and which neurochemical elements within those subregions might be responsible for behavioral flexibility has not been resolved. Here, we demonstrate that ACh output from the dorsomedial striatum selectively increases when rats must flexibly shift response patterns. This ACh output increase contributes to behavioral flexibility because an Oxo-S infusion directly into the dorsomedial striatum concomitantly reduced dorsomedial striatal ACh output and impaired place reversal learning. Oxo-S produced its effects predominantly through m_2 muscarinic cholinergic receptors because the relatively selective m_2 muscarinic cholinergic antagonist AF-DX 116 infused into the dorsomedial striatum protected against the decrease in striatal ACh output and place reversal learning deficit. Importantly, the dose of Oxo-S that blocked a reversal learning-induced increase in dorsomedial striatal ACh output had no effect on basal ACh output. This was also the case observed with AF-DX 116. These results are consistent with findings indicating that striatal cholinergic autoreceptor responses change with varying levels of extracellular ACh levels (deBoer et al., 1990). Thus, Oxo-S blocking a behaviorally-induced increase in dorsomedial ACh output can not simply be viewed as a subtraction between a behaviorally-induced increase in ACh output and Oxo-S decreasing basal ACh output. Instead, the findings indicate that the dose of Oxo-S specifically modified the behaviorally-induced increase in dorsomedial striatal ACh output. Taken together, the results demonstrate a more direct link between increases in ACh activity in a specific striatal region that supports learning when conditions require behavioral flexibility. More specifically, the findings suggest that activation of cholinergic interneurons in the dorsomedial striatum support behavioral flexibility.

The present findings indicate that modulating ACh output in the dorsomedial striatum can simultaneously modify performance in a reversal learning task. These effects were observed with Oxo-S, which preferentially binds to m_2 muscarinic cholinergic receptors (Bräuner-Osborne & Brann, 1996) and the relatively selective m_2 muscarinic cholinergic receptor antagonist, AF-DX 116. A parsimonious explanation of the neurochemical and behavioral findings is that these drugs principally modified m_2 muscarinic cholinergic receptors located on cholinergic interneurons (Alcantara et al., 2001; Hersch et al., 1994). However, m_2 muscarinic cholinergic receptors in the striatum are also located on noncholinergic cells, e.g. the terminals of excitatory synapses (Hersch et al., 1994). Therefore, one possibility is that the effects of Oxo-S and AF-DX 116 on ACh output and reversal learning are due, at least in part, to muscarinic cholinergic receptor activity directly affecting noncholinergic neurons. Thus, these drugs may have affected other neurotransmitter systems besides ACh that contributed to the behavioral effects in reversal learning. Moreover, there is some evidence suggesting that some portion of m_4 muscarinic cholinergic receptors in the striatum also serve as autoreceptors on cholinergic interneurons (Yan & Surmeier, 1996; Zang et al., 2002). Of all of the muscarinic receptor subtypes, Oxo-S does show the next highest affinity for m_4 muscarinic cholinergic receptors. Thus, another possibility is that the drug effects on dorsomedial striatal ACh efflux and reversal learning can be explained by actions at the M2-class (m_2 and m_4) of muscarinic cholinergic receptors found on cholinergic interneurons. However, because AF-DX 116 shows a high selectivity for the m_2 muscarinic receptor subtype compared to the m_4 muscarinic receptor subtype (Doods et al., 1987; Russo et al., 1993) the present findings suggest that the modulation of the behaviorally-induced changes in striatal ACh output was principally due to the m_2 muscarinic cholinergic receptor subtype.

An infusion of Oxo-S into the dorsomedial striatum reduced ACh efflux throughout the entire reversal learning session, however, rats that received Oxo-S did exhibit some learning across the test session. These results suggest that ACh actions in the dorsomedial striatum contribute to a shift in choices patterns, but are not essential for the ultimate shift in choice patterns. An

alternative possibility is that there was an incomplete functional blockade of cholinergic receptors that led to a slowed reversal learning as opposed to a complete blockade of reversal learning. However, the present findings are comparable to a recent study demonstrating that infusion of the m₁ muscarinic cholinergic antagonist, MT-7 into the dorsomedial striatum impaired place reversal learning but did not prevent reversal learning (McCool et al., 2008). Taken together, the results are consistent with the proposal that cholinergic interneurons facilitate the coordination between different striatal input-output modules, but are not necessary for the expression of a choice pattern (Graybiel, 1996).

The pattern of results also indicates that dorsomedial striatal ACh efflux remained elevated throughout the reversal learning session even at the end when rats' performance approached near 90% correct. If ACh output in the dorsomedial striatum is important specifically for inhibition of the previously relevant choice while learning the presently relevant choice, then one prediction is that when reversal learning was achieved dorsomedial striatal ACh output would no longer remain elevated. In a previous experiment, we did find that with more extensive place reversal learning dorsomedial striatal ACh output did return to basal levels (Ragozzino & Choi, 2004). Thus, one possibility is that cholinergic interneurons in this area remain activated for some period even when a "significant" level of learning has occurred during a task switch to ensure a continual accurate adaptive response, but when the choice pattern becomes a "habit" with more extensive training there is no longer enhanced cholinergic activity in this striatal subregion.

Although dorsomedial striatal ACh output increased during reversal learning there was no significant increase observed during initial learning of a place discrimination. These findings are comparable to previous results indicating that blockade of muscarinic cholinergic receptors in the dorsomedial striatum does not impair initial learning of a place or response discrimination (Ragozzino et al., 2002b; McCool et al., 2008). Thus, ACh actions in the dorsomedial striatum appear to support cognitive flexibility, but do not play a more general role in discrimination learning. The lack of change in ACh output during acquisition also suggests that the reversal learning-induced ACh efflux increase is unlikely due to simply navigating through the maze and/or consumption of cereal pieces.

In contrast to the dorsomedial striatum, a change in ACh output was not observed during reversal learning in the dorsolateral striatum or ventromedial striatum. The results suggest that cholinergic activity in the dorsomedial striatum selectively contributes to learning when conditions require cognitive flexibility. There are other conditions that have led to changes in ACh output from these striatal subregions which suggest that cholinergic interneurons in the dorsolateral and ventromedial striatum play an important role in behavioral plasticity. More specifically, dorsolateral striatal ACh output was found to gradually increase with the development of a response habit (Chang & Gold, 2003). These results are consistent with the idea that the rat dorsolateral striatum is critical for the formation and expression of a learned habit or stimulus-response learning (McDonald & White, 1993; Devan & White, 1999). In the nucleus accumbens, ACh actions may support the learning of a response with a reinforcer (Crespo et al., 2006; Pratt & Kelley, 2004). Thus, cholinergic interneurons in the striatum may play a diverse and complex role in learning with cholinergic interneurons in different striatal subregions contributing to different functions. The present experiments demonstrate that cholinergic actions in the dorsomedial striatum support learning when conditions change requiring inhibition of a previously relevant choice pattern and learning a new choice pattern.

A broader issue is understanding how the dorsomedial striatum interacts with other brain areas to alter response selection under changed environmental contingencies. In the present experiment, changes in dorsomedial striatal ACh output were observed during a spatial reversal learning task. Numerous experiments have indicated that the hippocampus and surrounding

cortical areas are critical for the learning and memory of cognitive/spatial information (Devan & White, 1999; Devan et al., 1999; Kesner et al., 1993; Morris et al., 1982; Packard & McGaugh, 1996). The dorsomedial striatum receives input from the hippocampus indirectly via other cortical areas such as the posterior cingulate, entorhinal and prefrontal cortices (McGeorge & Faull, 1989). Thus, this input to the dorsomedial striatum may be critical for the selection of appropriate responses in learning a spatial discrimination.

The connections among the hippocampus, prefrontal cortex and dorsomedial striatum may be particularly important in integrating mnemonic information for the generation and reliable execution of a strategy. In particular, there is accumulating evidence that connections between the dorsomedial striatum and either the medial prefrontal cortex or orbitofrontal cortex facilitate a shift in strategies or choice patterns (Block et al., 2007; Ragozzino, 2007; Stefani & Moghaddam, 2006; Wise et al., 1996). In particular, inactivation or lesions of the orbitofrontal cortex impair various reversal learning tests (Ghods-Sharifi et al., 2008; Kim & Ragozzino, 2005; McAlonan & Brown, 2005; Schoenbaum et al., 2002), while lesions or inactivation of the medial prefrontal cortex impair set-shifting (Birrell & Brown, 2000; Block et al., 2007; Ragozzino et al., 1999; Stefani & Moghaddam, 2006). The dorsomedial striatum, which receives direct input from both the orbitofrontal and medial prefrontal cortex (Groenewegen et al., 1990), when inactivated impairs both reversal learning and set-shifting (Ragozzino et al., 2002a; Ragozzino & Choi, 2004). However, the error patterns observed following inactivation of a prefrontal cortex subregion and the dorsomedial striatum are different. More specifically, orbitofrontal cortex or medial prefrontal cortex inactivation impairs reversal learning and set-shifting, respectively, by increasing perseveration to the previously reinforced choice pattern (Kim & Ragozzino, 2005; Ragozzino et al., 1999). This is shown by a significant increase in errors to the previously reinforced choice pattern during the initial trials of the reversal or set-shift (Kim & Ragozzino, 2005; Ragozzino et al., 1999). In contrast, dorsomedial striatal inactivation does not increase errors during the initial trials of a shift, but significantly increases errors to the previously reinforced choice after a subject has chosen the new correct choice pattern. That is rats with dorsomedial striatal inactivation inhibit using the previously reinforced choice pattern as quickly as control rats, but more often revert back to using the previously correct strategy. This pattern of results is comparable to that observed in Parkinson's disease patients in which subjects are impaired in maintaining rather initiating a new strategy (Downes et al., 1989; Flowers & Robertson, 1985). Taken together, the results are consistent with a idea proposed by Wise and colleagues (1996) that the striatum facilitates the execution of effective strategies for a particular behavioral context by reinforcing the correct response pattern when generated. The present findings suggest at the level of the dorsomedial striatum, the cholinergic interneurons, because of their wide dendritic branching and extensive axonal fields, play a critical role in facilitating a shift in response patterns with a change in environmental contingencies.

Overall, the findings from manipulations of the prefrontal cortex and dorsomedial striatum suggest that these areas are part of a larger neural system that facilitates inhibition of a previously relevant strategy and learning of a new strategy with a change in environmental contingencies. Thus, these brain areas support distinct, but complementary functions in allowing an effective switch in strategies by which the prefrontal cortex is important for the initial inhibition of a previously relevant strategy and/or generation of a strategy, while the dorsomedial striatum plays a complementary role by reliably executing a new strategy once selected.

Acknowledgments

This work was supported by grant NIH Grant NS043283.

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Figure 1.

Location of microdialysis probes in the dorsomedial, dorsolateral and ventromedial striatum of rats included in the analyses for Experiments 1-4. The length of the microdialysis probe was 2 mm. The number of probes does not match the total number of probe placements for rats included in the analyses because some probe placements overlapped to such a large extent that a single line represents more than one probe placement. The sections range from 0.3-to 1.7 mm in the anterior-posterior plane relative to bregma. The rat brain sections were modified from the atlas of Paxinos and Watson (1996).

Figure 2.

Dorsomedial striatal ACh output and behavioral performance during place reversal learning. Each rat was tested for 30 minutes across five 6-min test blocks (T1-T5). (a) ACh output from the dorsomedial striatum during place reversal learning. Controls (aCSF) displayed an increase in ACh efflux of ~ 50%. Oxotremorine sesquifumurate 100 nM infused into the dorsomedial striatum significantly reduced ACh compared to that of controls output throughout the reversal learning period. AF-DX-116 20 nM infused into the dorsomedial striatum led to a change in ACh output comparable to that of aCSF controls. AF-DX-116 20 nM infused in combination with oxotremorine sesquifumurate 100 nM elevated ACh efflux to level of aCSF controls. (b) Mean percent correct during place reversal learning. All groups performed at ~ 85-90% correct on the final block of place acquisition (ACQ). Subsequently, all groups dropped to 40-50% on

the first reversal learning block (T1). Oxotremorine sesquifumurate significantly reduced performance on blocks T2-T5 compared to that of all other groups. AF-DX-116 combined with oxotremorine sesquifumurate led to reversal learning performance that was not significantly different from that of controls.

Figure 3.

Number of trials completed per block during reversal learning. Controls, oxotremorine sesquifumurate 100 nM, AF-DX-116 20 nM and the combination of oxotremorine sesquifumurate and AF-DX-116 infused into the dorsomedial striatum led to a comparable number of trials completed across all five blocks of reversal learning.

Figure 4.

Effect of oxotremorine sesquifumurate 100 nM and AF-DX-116 20 nM infused into the dorsomedial striatum on ACh efflux in a resting condition. Samples were collected at 6-min intervals. Infusion of either drug did not affect basal ACh output.

Figure 5.

Effect of place acquisition testing on dorsomedial striatal ACh output. Each rat was tested for four 6-min blocks (a) ACh output did not change compared to baseline levels during behavioral testing. (b) Mean percent correct during place acquisition testing. Rats performed slightly above chance levels (chance = 50%) at the first test block and improved to ~ 85% by the last test block.

Figure 6.

Effect of place reversal learning on dorsolateral and ventromedial striatal ACh output. Each rat was tested for five 6-min blocks during reversal learning. (a) ACh output did not change compared to baseline levels in the dorsolateral or ventromedial striatum during place reversal learning. (b) Mean percent correct during place reversal learning. Both groups had ~ 90% accuracy on the last block of place acquisition and subsequently dropped to chance levels during the first block of reversal learning and achieved ~ 90% accuracy by the last test block of reversal learning.