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Muscarinic receptor/G-protein coupling is reduced in the dorsomedial striatum of cognitively impaired aged rats

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Abstract

Behavioral flexibility, the ability to modify responses due to changing task demands, is detrimentally affected by aging with a shift towards increased cognitive rigidity. The neurobiological basis of this cognitive deficit is not clear although striatal cholinergic neurotransmission has been implicated. To investigate the possible association between striatal acetylcholine signaling with age-related changes in behavioral flexibility, young, middle-aged, and aged F344 X Brown Norway F1 rats were assessed using an attentional set-shifting task that includes two tests of behavioral flexibility: reversal learning and an extra-dimensional shift. Rats were also assessed in the Morris water maze to compare potential fronto-striatal-dependent deficits with hippocampal-dependent deficits. Behaviorally characterized rats were then assessed for acetylcholine muscarinic signaling within the striatum using oxotremorine-M-stimulated [³⁵S]GTPγS binding and [³H]AFDX-384 receptor binding autoradiography. The results showed that by old age, cognitive deficits were pronounced across cognitive domains, suggesting deterioration of both hippocampal and fronto-striatal regions. A significant decline in oxotremorine-M-stimulated [³⁵S]GTPγS binding was limited to the dorsomedial striatum of aged rats when compared to young and middle-aged rats. There was no effect of age on striatal [³H]AFDX-384 receptor binding. These results suggest that a decrease in M2/M4 muscarinic receptor coupling is involved in the age-associated decline in behavioral flexibility.

Keywords

aging; striatum; muscarinic; attentional set shifting

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1. INTRODUCTION

Under the umbrella term of executive function is the flexible use of knowledge, termed here as behavioral flexibility (Ragozzino, 2007). Behavioral flexibility is impaired in aged primates (Hara et al., 2011), dogs (Tapp et al., 2003) and rodents (Schoenbaum et al., 2002). Although the neurobiological mechanism underlying the decline in behavioral flexibility in the aged brain is unknown, pharmacological and lesion studies in adult rats indicate that fronto-striatal function is critical. In adult rats, impairments in reversal learning occur after lesions or inactivation of the orbito-frontal region of the prefrontal cortex or the dorsomedial region of the striatum (Bissonette et al., 2008; Boulougouris et al., 2007; Ragozzino et al., 2002). However, based on the anatomical connections between the orbito-frontal cortex and the dorsomedial striatum (Clarke et al., 2008; Devan et al., 2011; Fuster, 1997; Ragozzino, 2007), mechanistic dysfunction in either or both of these regions have the potential to result in an age-related decline in behavioral flexibility and increase in cognitive rigidity.

In the striatum, age-related declines in striatal acetylcholine synthesis, release and degradation have been identified (Das et al., 2001; Joseph et al., 1993; Ogawa et al., 1994; Sherman and Friedman, 1990). Additionally, pharmacological antagonism of striatal muscarinic acetylcholine receptors with pirenzepine, a muscarinic receptor antagonist, impairs behavioral flexibility (reversal learning) when infused into the dorsomedial striatum of adult rats (McCool et al., 2008). However, it is not known if age-related alterations in striatal cholinergic signaling are associated with the decrease in behavioral flexibility observed in aging.

The predominate muscarinic receptor subtypes in the striatum are M1, M2 and M4 (reviewed in (Threlfell and Cragg, 2011)). M2/M4 AChRs are located on the large, aspiny striatal cholinergic interneurons, also called tonically active neurons (TANS). TANS make up about 2% of all striatal neurons and have a major modulatory affect on striatal neurotransmission, particularly in regard to dopaminergic regulation (see (Threlfell and Cragg, 2011)). Because of the major influence of the TANS on striatal neurotransmission, we focused our study on the M2/M4 muscarinic receptor subtypes.

M2/M4 receptor function and receptor density was analyzed in the striatum from behaviorally characterized young, middle-aged and aged rats. All rats were tested in an attentional set-shifting task and in the Morris water maze to assess fronto-striatal and hippocampal function, respectively. M2/M4 AChRs were assessed for both the function (receptor/G-protein coupling) and level. G-protein-coupled receptor function can be assessed by the ability of agonist to activate the binding of GTP to the alpha subunit of the G-protein complex. M2/M4 muscarinic receptors were stimulated with the agonist oxotremorine-M (Gillard et al., 1987; Miller et al., 1991). Receptor stimulation by oxotremorine-M activates the G-protein $G\alpha_{q11}$ and the bound GDP ("off state") is replaced with GTP ("on state"). The GTP is radiolabeled ($GTP\gamma S^{35}$) and non-hydrolysable, allowing for quantification using an autoradiographic method (Sim et al., 1995). To measure M2/M4 receptor levels, the muscarinic receptor antagonist [3H]AFDX-398 was used. It preferentially binds to the M2 and M4 AChRs at the concentration used in this study (2nM) (Quirion et al 1993; Miller et al 1991; Aubert 1992). Muscarinic receptor function and levels were quantified in the same brain regions of the same rats and data were analyzed relative to both age and cognitive ability.

2. MATERIALS AND METHODS

2.1 Subjects

Male Fisher 344 × Brown Norway (F344BNF1, NIA-Harlan, Chicago, IL) rats were all of the same cohort with all ages arriving in the same shipment. Rats were housed individually once they arrived at the laboratory for use as subjects. Rats were on a 12-hr light cycle and food and water were available *ad lib* unless otherwise indicated. There were three age groups: Young (N=7), middle-aged (N=7) and aged (N=7). Rats were assessed at 8, 18 and 27 months of age for spatial learning in the water maze using a standardized protocol (LaSarge and Nicolle, 2009). Three months later, at the ages of 12, 21 and 29 months of age, the same animals were assessed using an attentional set-shifting task (Birrell and Brown, 2000). For the attentional set-shifting task, rats were maintained on a restricted diet at 85% of baseline body weight. Behavioral testing was conducted during the light phase of a 12-h light/dark cycle. Rats were euthanized by decapitation within a week after the completion of the set shifting task. Brains were extracted and flash frozen in −80 °C isopentane, and stored at −80°C. Coronal brain sections (20 µm) through the striatum were cut on a cryostat at −20 °C, mounted onto gelatin-coated slides, and stored at −80°C until use for the oxotremorine-M-stimulated [³⁵S]GTPγS assay or the [³H]AFDX-398 binding assay.

2.2 Spatial Learning in the Water Maze

Spatial learning ability was assessed using a standardized protocol (LaSarge and Nicolle, 2009) and a video tracking system (Ethovision, Noldus, Leesburg, VA). The water maze is a circular tank that is 1.83m diameter and 0.58 m in height filled with water. It contains a retractable escape platform that is centered and submerged 2 cm in one of the quadrants. The water is made opaque with non-toxic white food coloring. The maze was surrounded with black curtains with white patterns to provide spatial cues. Rats were trained for three trials/day for eight days to locate a camouflaged platform that remained in a consistent location. Every sixth trial was a probe trial (described below). For training trials, rats were placed in the water at the perimeter of the pool and allowed 90 sec to locate the escape platform using randomized start locations. The rats remained on the platform for 30 sec before being placed in a holding cage for a 60 sec inter-trial interval. The probe trial assesses the development of a spatial bias for the platform location. During these trials, rats were allowed to search for 30 sec for the escape platform which had been made unavailable by retraction. A “proximity score” was generated from the proximity of the rat to the platform during probe trials 2–4.

Sensory-motor ability was tested on the last day and determined by cue training to a visible platform. Cue training consisted of one session of six trials. On each of these six trials, the platform was placed in different locations to test for sensory, motor and motivational factors that may influence spatial learning. No spatial cues were used for cue testing.

2.3 Attentional Set-Shifting

Attentional set-shifting is a digging task that requires the subjects to discriminate between relevant and irrelevant cues for a food reward (McAlonan and Brown, 2003). Small terra-cotta flower pots were used as digging bowls. The reward was 1/2 a piece of peanut butter crunch cereal (Quaker Oats Company, Illinois) placed at the bottom of the pot. The odor was masked with cereal dust sprinkled liberally in to the digging medium. Essential oils (Aura Cacia, Norway, Iowa) were used to scent the rims of the pots. Animals were tested in their home cage that was surrounded by an opaque barrier to minimize visual cues and distractions outside of the testing environment.

Habituation and shaping—After food deprivation, subjects were habituated to the test apparatus and then shaped to dig for a reward. Two pots were filled with bedding and the

cereal was placed at the very bottom of both pots to encourage the rats to dig vigorously. When the reward was found, the rat was moved to the holding area at the back of its home cage. This session lasted 10–15 minutes for 8 days. At the end of this period, rats were trained sequentially on two simple discriminations (SD) problems. One SD was between odors that served as the relevant stimulus and the other used digging media as the relevant stimulus. The odor and media pairs presented were not used again in subsequent testing.

Testing Paradigm—The testing phase consisted of five test problems: a simple discrimination (SD), compound discrimination (CD), intra-dimensional shift and a reversal (IDS and IDS-Rev), and the extra-dimensional shift (EDS). All five tests were run sequentially within one session lasting approximately 2 hours. During training, the left/right locations of the pots were randomized. The first four trials of each discrimination consisted of an exploratory period in which the rat was allowed to dig in both pots. An error was recorded if the rat first dug in the unbaited pot. Once the first four trials were completed, the rats were only allowed to dig in one of the two pots and were corrected by returning to the holding area after the selection. An error was recorded if the rat vigorously dug in the incorrect pot. Testing continued until reaching a criterion of six consecutive correct trials. Once criterion was reached the next discrimination followed.

Table 1 describes the general protocol for the set-shifting task. Table 2 shows examples of the discriminanda used in testing. The first discrimination was a simple discrimination (SD) between either two odors or two digging media, followed by a compound discrimination (CD) with the same positive stimulus as the initial SD. Importantly in the CD, the new dimension did not reliably predict the location of the reward. Once criterion was reached, an intra-dimensional shift (IDS) was presented. The IDS was a new CD with two new pairs of odors and media. The IDS was then reversed (IDS-R). In the IDS-R phase, the formerly negative stimulus became the positive stimulus (and vice versa). Lastly, an extra-dimensional shift (EDS) was presented. During this phase, the irrelevant dimension became the relevant, thus the former rewarded dimension no longer predicted the reward.

2.4 Agonist-Stimulated [³⁵S]GTPγS Autoradiography

At the time of assay, sections from all subjects were processed simultaneously. All assay conditions were at 25 °C. Sections were rinsed in TME buffer (50 mM Tris-HCl, 3 mM MgCl₂, 0.2 mM EGTA, and 100 mM NaCl, pH 7.4) for 10 min and then pre-incubated in TME with 2 mM GDP and 1 μl/100ml adenosine deaminase (“assay buffer”) for 15 min. Following the pre-incubation, sections were incubated for 120 minutes at 25 °C in assay buffer containing 0.04 nM [³⁵S]GTPγS. Basal [³⁵S]GTPγS binding was defined by binding in the absence of agonist, and M2/M4-stimulated binding was determined by the addition of (oxotremorine-M, 10 μM). Non-specific [³⁵S]GTPγS binding was defined by competition with 10 μM unlabeled GTPγS. The reaction was terminated by a quick rinse in ice-cold 50 mM Tris-HCl buffer (pH 7.4), followed by one rinse in deionized distilled H₂O. Slides were dried overnight, and then exposed to phosphor imaging screens along with [¹⁴C] standards for approximately 36 h.

Densitometric analysis of the autoradiograms was analyzed using NIH IMAGEJ. Measurements of optical density were referenced to [¹⁴C] standards and were converted to nCi/g [³⁵S] (Sim et al., 1995). Data are reported as mean values ± SEM of three to six sections per subject and condition. Non-specific binding was subtracted from basal and stimulated sections. Net binding was obtained by subtracting stimulated binding from basal binding. A schematic illustration of the sampling procedure is shown in Figure 5.

2.5 [³H]AFDX-384 Autoradiography

Tissue sections from a subset of animals were used for receptor binding analysis (young, N = 3, MA, N = 4, Aged = 5). Brain sections stored at -80°C were placed in a -20°C freezer overnight before each experiment. On the day of the experiment, sections were allowed to come to room temperature. Sections were preincubated at room temperature in Krebs Buffer (120 mM NaCl, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 5.6 mM glucose, 25 mM NaHCO₃, 2.5 mM CaCl₂, and 4.7 mM KCl, pH 7.4) for 15 minutes followed by incubation for 1 hour at 22°C in Krebs Buffer containing 2nM [³H]AFDX-384 (Perkin Elmer, Boston, MA) with or without 1μM atropine for assessment of non-specific binding. Following incubation, the slides were placed in three sequential 4 minute washes of 50 mM Tris-HCl, pH 7.4 at 4°C. After rinsing in cold, distilled water, slides were blown dry under a stream of air and placed in a vacuum container over night. Slides were set tightly against tritium-sensitive film along with [³H] standards for 4 weeks to convert optical density to fmol/mg wet tissue. The films were manually developed. [³H] AFDX-384 binding was determined by quantitative densitometry using a computerized image analysis program (MCID Imaging Research, Cambridge, UK) and the anatomical boundaries are shown in Figure 3A.

2.6 Data Analysis

A repeated measures ANOVA was used to compare spatial learning across the acquisition blocks and attentional set shifting across test phases. Post *hoc* Fisher's PLSD tests explored significant main effects of groups or interactions. Unless otherwise described, one-way ANOVAs (age) were used for all other comparisons.

3 RESULTS

3.1 Attentional Set-Shifting

Behavioral flexibility was assessed using an attentional set-shifting discrimination paradigm that assesses both reversal learning and set shifting. Figure 1 shows the number of training trials needed to reach criterion for all phases of the attentional set-shifting task. A main effect of age (Phase, $F(2,18) = 5.07$, $p < 0.05$) indicates that the aged group required significantly more trials to reach criterion than the young and middle-aged groups and that the young and middle-aged groups did not differ from each other (Fisher PLSD; young vs aged, $p < 0.05$; middle aged vs aged, $p < 0.05$). An interaction between test phase and age ($F(4,8) = 3.79$, $p < 0.001$) and subsequent post *hoc* analysis for each test phase indicates that there was significantly poorer performance by the aged rats on only two phases—reversal learning (the IDS-reversal) and extra-dimensional set shifting (the EDS). Similar results were observed with an analysis of errors to criterion [Age: $F(2,18) = 5.35$, $p < 0.05$]. No differences between young and middle-aged rats in the trials or errors to criterion occurred in any discrimination phase. Overall, the data indicate that reversal learning and extra-dimensional set-shifting ability remain stable through middle-age and that a robust impairment in these abilities emerges by old age.

3.2 Spatial Learning

The ability of rats to acquire a spatial bias for the location of the escape platform in a reference memory water maze task was used to compare a predominately hippocampal-mediated task with the behavioral flexibility measures. Age-related deficits in spatial learning were apparent at middle-age in contrast to the preservation of behavioral flexibility at this age. Training trial data are shown in Figure 2A. The path length taken to reach the escape platform during the training trials was not significantly affected by age [age, $F(2,18) = 2.22$, $p = 0.14$]. There was, however a trend towards an interaction between path length and age [$F(3,6) = 2.07$, $p = 0.07$]. Because of the interaction trend and the historical

behavioral profile showing significant impairment in the aged group compared to the young (reviewed in (LaSarge and Nicolle, 2009) and (Zhang et al., 2007)), the interaction was further explored using one-way ANOVA for each block to determine if there was a replication of data from previous studies demonstrating impaired performance of the aged group compared to the young group. Indeed, this was the case. Blocks 2 and 4 demonstrated a significant effect of age (Block 2; $F(2,18) = 3.59$, $p < 0.05$; Block 4; $F(2,18) = 3.79$, $p < 0.05$). In Block 2, the young group has significantly shorter path length compared to the aged group ($p < 0.05$) and in Block 4 the young group had a significantly shorter path length compared to the middle-aged group ($p < 0.05$) and a trend towards shorter path length was apparent between the young and aged ($p = 0.09$). There were no significant differences between the middle-aged and aged groups in any of the blocks.

A more definitive measure of spatial learning than the training trial path length data is the proximity to the escape platform during the probe trial. The probe trial data indicates the proximity of the animal's search to the escape location for a 30 sec period during which the platform is retracted and unavailable for escape. This measure reflects search accuracy and has been shown to be the most statistically sensitive measure of spatial learning in the water maze (Gallagher et al., 1993; Maei et al., 2009). In this study, the proximity of the four probe trials was analyzed using a repeated measure ANOVA. A main effect of Age [$F(2,18) = 8.11$, $p < 0.005$] and no interactions indicates that the young group has a more accurate search (lower proximity) compared to both the middle-aged and aged groups ($p < 0.01$). Figure 2B shows the individual probe trial data and the "proximity score" which is the average proximity of probe trials 1–4. A one-way ANOVA indicates that the mean proximity score is significantly lower in the young group compared to the middle-aged and aged groups and there is no difference between the middle-aged and aged groups [Age, $F(2,18) = 8.10$, $p < 0.005$, Fisher's PLSD, young vs middle-aged or aged, $p < 0.05$). These data indicate that spatial learning becomes impaired by 18 mo of age in F344xBNFI rats.

3.3 Visible Platform

The latency of the subjects to reach the visible platform during cue training was not affected by age, $F(2,18) = 0.39$, NS. Similarly, the swim speed was not significantly different across groups, $F(2,18) = 1.45$, NS. Overall, the cue training data indicate that there is comparable sensory and motor ability across the age groups.

3.5 Oxotremorine-M-stimulated [35 S]GTP γ S binding autoradiography

Figure 3B shows autoradiograms of basal and oxotremorine-M-stimulated [35 S]GTP γ S binding in the striatum. Oxotremorine-M stimulated [35 S]GTP γ S binding was significantly decreased in the dorsal striatal regions indicated a significant effect of age with the aged group having significantly less binding than the middle-aged and young groups (main effect: $F=5.12$, $p < 0.05$; Fishers PLSD; young vs aged and middle-aged vs aged, $p < 0.05$). Because our *a priori* hypothesis was that alterations in the dorsomedial striatum might contribute to reversal learning changes with aging, we examined the medial and lateral subdivisions using separate ANOVAs, even though there was no significant interaction ($p=0.12$) between age and region (medial/lateral), $p = 0.12$. One-way ANOVA indicated that the age-related decrease in striatal oxotremorine-M-stimulated [35 S]GTP γ S binding is attributable to a decreased in binding limited to the dorsomedial striatal region [medial: $F(2,18) = 11.45$, $p < 0.001$; Fisher's PLSD, young vs middle-aged and middle-aged vs aged, $p < 0.05$; Figure 3C). In contrast, there was no effect of age in the dorsolateral striatum [lateral: $F(2,18) = 1.58$, $p = 0.23$; Figure 3D]. There were no changes in basal [35 S]GTP γ S binding between the age groups (Table 4).

3.6 Receptor Binding

To determine whether a loss of receptors was the cause of decreased oxotremorine-M-stimulated GTP binding, [^3H]AFDX-384 ligand binding was used to quantify M2/M4 receptor levels in the striatum. Identical anatomical boundaries were used for quantification of oxotremorine-M-stimulated [^{35}S]GTP γ S binding (shown in Figure 3). [^3H]AFDX-384 binding was completely blocked with atropine. The resulting specific binding was not altered by age and did not differ by striatal subregion (DMS, DLS) (Figure 3E,F), indicating a preservation of M2/M4 receptors with advanced age and in the presence of deficits in flexible learning.

4. DISCUSSION

The experiments described here assessed the integrity of the cholinergic system of the striatum in rats at 6, 18, and 24 months of age relative to cognitive performance on an attentional set-shifting and spatial learning task. The results of the cognitive assessment showed that spatial learning ability was impaired by middle-age whereas cognitive flexibility remained intact until very old age. Additionally, the pattern of results for oxotremorine-M-stimulated [^{35}S]GTP γ S binding were similar to the behavioral phenotype observed: there was a preservation of receptor coupling at middle-age with measurable changes not occurring until old age. Specifically, the aged rats impaired in reversal learning and set-shifting ability had reduced oxotremorine-M-mediated [^{35}S]GTP γ S binding limited to the dorsomedial subregion of the striatum but there was no overt loss of M2/M4 receptors. The results of this study suggest an association between increased cognitive rigidity and reduced muscarinic receptor function in the DMS of aged rats.

Although this report has focused its neurobiological analysis in the striatum, it should be noted that the striatum is highly interconnected with the prefrontal cortex (reviewed in (Kehagia et al., 2010)). Our observed blunting of muscarinic receptor coupling in the striatum of aged rats probably co-exists with other neurochemical alterations in fronto-striatal circuitry. For example, ionotropic glutamate receptor binding is altered in both the prefrontal cortex and the striatum of aged Long Evans rats in a pattern that is congruent with a loss of cortical input to the striatum. Specifically, in aged rats with impairment in attentional set shifting, there is a loss of both pre- and post-synaptic glutamate receptors (kainate and NMDA) in the striatum while the solely postsynaptic receptors remain unchanged (AMPA) (Nicolle and Baxter, 2003). In the prefrontal cortex of those same rats, there was also a loss of kainate and NMDA receptors. Unfortunately, the prefrontal tissue sections from the rats used in this study of muscarinic receptors were not available for analysis. Nevertheless, it is clear that the decrement in muscarinic signaling within the striatum is only one of several neurobiological changes that affect this system in aging (e.g., (Bäckman et al., 2010; Gould et al., 1996)).

In addition to fronto-striatal circuitry, there is evidence that other brain regions are important for behavioral flexibility. Lesion studies of the basal forebrain demonstrate its involvement in behavioral flexibility (Cabrera et al., 2006; Tait and Brown, 2008). Non-selective ibotenic acid lesions in the basal forebrain impair reversal learning but leaves set shifting intact (Tait and Brown, 2008). In contrast to the non-selective ibotenic acid lesion, the selective lesion of the basal forebrain cholinergics neurons with 192 IgG saporin does not impair reversal learning (Tait and Brown, 2008). These data suggest that a non-cholinergic component of the basal forebrain system is important for intact reversal learning and could be a factor in our observed deficits. Deficits in set shifting but NOT reversal learning occurs after neurotoxic lesions to the posterior parietal cortex (Fox et al., 2003). Indeed, age-related changes in neurochemical markers in the parietal cortex have been reported (Cassel et al., 2005). Further studies are needed to examine the relationship between the integrity of the

parietal cortex of aged rats and attentional set shifting. Nevertheless, multiple brain systems, both neurochemically defined and anatomically defined, are probably playing a compounding role in the observed age-related deficits in behavioral flexibility.

Oxotremorine-M is a non-selective muscarinic receptor agonist and as a result, the oxotremorine-M-stimulated [35 S]GTP γ S binding is a combination of the activity of distinct muscarinic receptors present in the regions analyzed (Freedman et al., 1988a; Freedman et al., 1988b; Freedman et al., 1988c; Gillard et al., 1987). However, the combination of evidence from *in situ* hybridization, neurotoxin and immunohistochemistry studies indicates that the predominant muscarinic receptor expressed in the striatum is the M4 subtype (Hersch et al., 1994; Levey et al., 1991; Purkerson and Potter, 1998; Vilaró et al., 1991). Additionally, the autoradiographic profiles obtained from the current experiments parallel the muscarinic receptor radioligand profiles of M2/M4 receptor types obtained by other studies (Aubert et al., 1995; Pilar-Cuellar et al., 2005). The current findings support the conclusion that M2/M4 muscarinic receptor activity in the DMS is necessary for behavioral flexibility. In support of the importance of the DMS in behavioral flexibility, acetylcholine efflux, measured by *in vivo* microdialysis during a place reversal test, increased exclusively in the DMS, but not in the DLS or ventral striatum (Ragozzino et al., 2009). Blocking muscarinic receptors in the DMS by infusion of oxotremorine sesquifumurate also impaired reversal learning (Ragozzino et al., 2009). These studies suggest that muscarinic cholinergic tone in the DMS is a critical regulator of behavioral flexibility, and therefore, the reduction in muscarinic G-protein signaling in aged animals is likely associated with the observed impairments in behavioral flexibility.

The loss of striatal oxotremorine-M-stimulated [35 S]GTP γ S binding is consistent with previous evidence indicating that a loss of cholinergic tone in the striatum occurs with increased age. Several aging studies have reported disrupted striatal muscarinic receptor signaling at the level of the receptor/G-protein interface, including a decrement in muscarinic receptor-mediated low-Km GTPase activity and phosphoinositide turnover (Denisova et al., 1998; Kelly et al., 1995a; Kelly et al., 1995b; Ohnuki and Nomura, 1991; Yamagami et al., 1992). In contrast to the relative agreement of signaling studies in aged striatum, the muscarinic receptor binding studies exhibit a mixed outcome. Aubert et al 1995 examined [3 H]AFDX 384 binding in 6- and 18-month-old Long-Evans rats and, in agreement with the results from this study, they observed stability of [3 H]AFDX-384 binding in the striatum of the aged group (Aubert et al., 1995). However, Tayebati et al (2004) (Tayebati et al., 2004) quantified muscarinic receptor subtypes using a completely different method. They examined 6, 18 and 22 month old Sprague Dawley rats using radioligand binding assays designed to take advantage of the differing kinetics of the muscarinic receptor subtypes (Flynn et al., 1997). Under their very different assay conditions, an age-associated loss (measured by calculation of B_{MAX}) of M4 but not M2 binding sites were reported in the dorsal striatum of 18 and 22-month-old Fisher 344 rats when compared to the 6 mo old group (Tayebati et al., 2004). These results are difficult to reconcile because substantial differences in the assays, nonetheless, the two studies using the same assay support the conclusion that there is no loss of M2/M4 muscarinic receptors in the aged striatum.

The precise mechanism underlying the age-related impairment in oxotremorine-M-mediated [35 S]GTP γ S binding is not known. Previous data have shown that the membrane molecular structure can be altered during the normal aging process (Sunshine and McNamee, 1994). It is possible that an altered membrane composition could influence receptor/G-protein coupling. For example, a strong correlation between striatal cholesterol/phospholipids molar ratio and carbachol stimulated low-Km GTPase activity was found after feeding rats with a diet high in cholesterol or low in total fat (Kelly et al., 1995a). Moreover, decreased

carbachol-stimulated low *K_m* GTPase activity in the striatum of aged F344 rats was associated with reduced membrane bilayer width (Kelly et al., 1995b). These data indicate that the impaired G-protein coupling/uncoupling in aged rats may be related to a decrease in membrane fluidity providing a plausible explanation for the blunting of muscarinic receptor-mediated GTP binding in our study.

5. Conclusions

In this study, normal, healthy aged rats demonstrated an age-related increase in cognitive rigidity, a measure of executive function. Cognitive rigidity was associated with impaired muscarinic receptor signaling in the dorsomedial striatum. These results are consistent with lesion and pharmacological studies in adult rats that demonstrate impaired reversal learning as a consequence of dorsomedial striatal damage or cholinergic antagonism. Our data indicates that neurobiological changes in the cholinergic system of the dorsomedial striatum may underlie age-related increases in cognitive rigidity.

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Research Highlights

- Cognitive flexibility becomes impaired by old age
- Muscarinic receptor stimulation of GTP binding, a measure of receptor coupling, is blunted in the dorsal striatum of aged rats
- Muscarinic receptor levels remain stable in the dorsal striatum of aged rats
- Data indicate that muscarinic receptor function but not level contributes to increased cognitive rigidity in old age

Figure 1. Effects of aging on spatial learning in the water maze in young, middle-aged and aged rats

Aged and middle-aged rats performed poorly when compared to young on the acquisition of the platform location during training trials (A) and have a less accurate search for the platform during probe trials (B). Sensorimotor ability testing demonstrated that aging did not affect visual acuity, (C) but impaired swim speed. Old rats swam significantly slower than middle-age and young rats (D). * Aged vs. young, $p < .05$.

Figure 2. Effects of aging on reversal learning and set shifting ability

Aged rats performed poorly when compared to young and middle-aged rats in the reversal learning (IDS-R) and set shifting phases of the attentional-set shifting tasks *, ** Aged vs. young and Aged vs. MA, $p < .05$.

Figure 3. Effect of aging on muscarinic receptor signaling and receptor level in the striatum
 A) Schematic illustration of the anatomical areas sampled for optical density (shaded gray).
 B) Typical oxotremorine-M-stimulated $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ and $[^3\text{H}]\text{AFDX-384}$ autoradiograms C) and D) show the densitometric analysis of oxotremorine-M-stimulated $[^{35}\text{S}]\text{GTP}\gamma\text{S}$. E) and F) show the densitometric analysis of $[^3\text{H}]\text{AFDX-384}$ binding. *, $p < .05$.

Table 1

Possible combinations of stimulus pairs in the attentional set-shifting task. The correct exemplar for each discrimination is shown in bold.

Discrimination	Dimensions		Exemplar Combinations	
	Relevant	Irrelevant	S +	S –
SD	Medium		M1	M2
CD	Medium	Odor	M1/O1	M2/O2
			M1/O2	M2/O1
IDS	Medium	Odor	M3/O3	M4/O4
			M3/O4	M4/O3
IDS-Reversal	Medium	Odor	M4/O3	M3/O4
			M4/O4	M3/O3
EDS	Odor	Medium	O5/M5	O6/M6
			O5/M6	O6/M5

Table 2

Stimulus pairs in the attentional set-shifting task.

Odor pairs	Medium pairs
Cinnamon vs. Lavender	Shredded paper vs. Easter grass
Lemon vs. Rose	Raffia vs. Cut blue pads
Sweet orange vs. Ylang Ylang	Eco bedding vs. Cut sponge

Table 3

Cue training parameters

	Latency (sec) $\pm$ SEM	Swim Speed (cm/sec) $\pm$ SEM
Young	7.62 $\pm$ 1.01	23.87 $\pm$ 1.16
Middle-Aged	9.34 $\pm$ 1.53	22.75 $\pm$ 1.22
Aged	8.92 $\pm$ 1.69	21.13 $\pm$ 1.03

Table 4Basal [³⁵S]GTPγS binding nCi/g tissue ± SEM

	Dorsomedial Striatum	Dorsolateral Striatum
Young	218.00 ± 8.54	202.93 ± 9.37
Middle-Aged	215.43 ± 5.43	204.14 ± 6.46
Aged	219.00 ± 4.01	218.57 ± 20.89