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**Regulation of Src Family Tyrosine Kinases in the Rat Striatum by
Muscarinic Acetylcholine Receptors**

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Abstract

Acetylcholine is an important neurotransmitter in the mammalian brain. This transmitter binds to muscarinic acetylcholine receptors (mAChR) to regulate activity of a variety of intracellular signaling molecules. Fyn and Src are two members of the Src family kinase (SFK). They are highly expressed in many brain regions, including the striatum, an area in the forebrain critical for cognitive, reward, mood, and movement. Since the striatum is also among brain regions showing a high level of mAChR expression, it is intriguing to investigate whether mAChRs regulate Fyn and Src. In this study, this topic was investigated by testing the effect of pharmacological blockade of mAChRs on phosphorylation of Fyn and Src at a specific tyrosine site, tyrosine 416 (Y416), a phosphorylation event leading to activation of Fyn and Src. A widely used mAChR antagonist scopolamine was used to block mAChRs and changes in phosphorylation of SFK Y416 were examined in the two subdivisions of the striatum, i.e., the caudate putamen (CPu) and nucleus accumbens (NAc), using Western blot with a phospho- and site-specific anti-Y416 antibody. We found that a single intraperitoneal injection of scopolamine at an effective dose (5 mg/kg) induced a significant increase in Y416 phosphorylation in the CPu. A similar increase in Y416 phosphorylation was also seen in the NAc following scopolamine administration. The scopolamine-stimulated Y416 phosphorylation was time-dependent. No significant change occurred to the amount of total Fyn and Src proteins in the two regions. These results indicate that mAChRs exert an inhibitory effect on basal phosphorylation of Fyn and Src in striatal neurons under normal conditions.

1. Introduction

Acetylcholine (ACh) is a key neurotransmitter in the striatum, an area which contains more choline acetyltransferase (ChAT), ACh, muscarinic acetylcholine receptors (mAChR), and acetylcholinesterase than other tissues^{9, 13}. The striatum which consists of the caudate putamen (CPu) and nucleus accumbens (NAc) is involved in the regulation of sensorimotor, cognitive, and limbic functions and is linked to various neuropsychiatric and neurodegenerative disorders³. ACh within the striatum is mainly provided by spiny cholinergic interneurons (ChI) that are the largest locally found but are few in numbers in the striatum (only 1-2% of the total striatal cell population)^{2, 3, 7}. Despite low numbers, ChIs exert their significant influence over GABAergic projection neurons also called medium spiny neurons (MSNs) by extremely dense and branched axonal arbors^{3, 13}.

Cholinergic receptors are divided into two subclasses: ionotropic nicotinic cholinergic receptors (nAChRs) and G protein-coupled mAChRs³. mAChRs have been classified into five subtypes (M1-M5) with all transcripts expressed in the striatum^{3, 4}. These subtypes are further categorized into two groups on the basis of their distinct pharmacological properties upon activation: the G_q -coupled M1-like receptors (M1, M3, and M5) that enhance internal Ca^{2+} release through stimulation of phospholipases, and the $G_{i/o}$ -coupled M2-like receptors (M2 and M4) that inhibit adenylyl cyclase and thereby reduce cAMP formation^{3, 12}.

There is a highly heterogeneous distribution of M1-M5 receptors throughout the striatum, which contributes to the distinguishing features of the cholinergic system in the region³. The highest level of M1 receptor binding is seen in the striosomes of the caudate nucleus and the lowest expression is in the insula major of Calleja, although M1 mAChR mRNA and binding sites are heterogeneously expressed throughout each striatal region^{3, 9}.

M2 expression is moderate in the dorsal CPu and the NAc core, while the NAc shell and the insula major of Calleja express denser M2 receptor binding sites³. M3 receptor mRNA and binding site pattern of expression significantly differs from that of M1 and M2 receptors. Specifically, they are mainly enriched in the mid-ventral and ventral regions of the caudate and striatum³. M4 are the most prevalent striatal mAChRs^{3,9}. M5 mAChRs have shown minimal staining in the striatum; however, lack of sensitive M5 receptor antibodies may explain these findings³.

Src family kinases (SFK) are non-receptor tyrosine kinases and the largest known family of this type of kinases^{5,8}. They play key roles in cell differentiation, motility, proliferation, and survival¹¹. Their 52-62 kDa protein structure is composed of six distinct functional regions: (1) the Src homology (SH) 4 domain, (2) the unique region, (3) the SH3 domain, (4) the SH2 domain, (5) the catalytic domain, and (6) a short negative regulatory tail¹. The SH2 and SH3 domains play a central role in regulating SFK catalytic activity by stabilizing the inactive conformation¹¹. The inactive conformation is displaced by two main modulatory actions: (1) dephosphorylation of pY527 in the negative regulatory tail, which normally promotes intramolecular interactions with the SH2 domain keeping the kinase in a closed inactive conformation, and (2) phosphorylation of Y416 in the catalytic domain, which induces a conformational change that allows the kinase to assume an active conformation^{8,11}. Importantly, these modular domains endow SFKs with the ability to be regulated by and to communicate with a diverse group of proteins¹¹. As such, SFKs play a vital role in regulating the cellular responsiveness to extracellular stimulation. In regard to Src and Fyn specifically, they are shown to be ubiquitously expressed in the cell body; however, Src has particularly higher levels of expression (5-200 times more) in platelets, neurons, and

osteoclasts^{5,6,11}. Src is implicated in a variety of cellular processes such as proliferation, differentiation, and migration, while Fyn is also diversely implicated in such processes as cellular adhesion, T-cell signaling, and brain functions^{5,6}. Interestingly, Fyn knockout mice have shown abnormal hippocampal development, defects in learning and memory, and decreased myelination, which further enforces its potential for being integral in striatal ChI function⁵.

SFK activity has previously shown to be regulated by many different G protein-coupled receptors (GPCR), including $G_{i/o}$ and G_q protein-coupled receptors¹¹. Evidence for this is apparent as multiple GPCRs increased SFK catalytic activity (two- to threefold)¹¹. One particular intracellular signaling molecule downstream to GPCRs is cAMP which has been shown to modulate SFKs activity^{1,10}. Schmitt and Stork have demonstrated a link between cAMP and SFKs by showing that protein kinase A (PKA) phosphorylated Src at serine 17, which in turn caused autophosphorylation of Src at Y416 and led to activation of Src¹⁰. In addition, Yeo *et al.* preformed a similar experiment to determine whether PKA regulates Fyn activity and found that PKA phosphorylated Fyn at serine 21, which is a step deemed to be essential for Fyn kinase activity¹⁴.

How GPCRs induce SFK activation has not been elucidated in many tissues. This indicates an intriguing question to whether or not there is a connection between mAChRs in the striatum and modulation of SFK phosphorylation. Here we investigated the role of mAChRs in regulating SFK phosphorylation at the Y416 residue in the rat striatum *in vivo*. By understanding this potential link, further exploration can be made to better understand the role of striatal ChIs in the regulation of striatal neurons.

2. Materials and methods

2.1. *Animals*

Adult male Wistar rats (210-300 g; Charles River, New York, NY) were housed in pairs in a controlled environment at a constant temperature of 23°C and humidity of 50 ± 10% with food and water available ad libitum. The animal room was on a 12-h/12-h light/dark cycle. Rats were allowed 6-7 days of habituation to the facility. All animal use and procedures were in strict accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

2.2. *Drug administration and protein extraction*

Rats received an intraperitoneal (i.p.) injection of systemically active agents in a volume of 1 ml/kg. Doses of agents were calculated as the salt. An effective dose (5 mg/kg, i.p.) of the mAChR antagonist scopolamine for a systemic injection was determined by literature and our previous studies. After drug injection, rats were anesthetized and decapitated. Rat brains were removed and coronal slices were cut. The CPu and NAc regions were dissected from slices. Brain tissue was homogenized in a radioimmunoprecipitation assay (RIPA) buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, and 1 μg/ml leupeptin (Cell Signaling Technology, Danvers, MA). Homogenates were centrifuged at 800 g for 10 min (4°C) and supernatants were collected. Protein concentrations were determined. Samples were stored at -80°C until use.

2.3. *Western Blot*

As described previously (Van Dolah et al., 2011; Jin et al. 2013), proteins were separated on SDS NuPAGE Novex 4-12% gels (Invitrogen, Carlsbad, CA) and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA). Membranes after blocking were incubated in a solution containing a primary rabbit antibody overnight at 4°C. This was followed by incubation in a horseradish peroxidase-linked secondary antibody. Immunoblots were developed with an enhanced chemiluminescence reagent (GE Healthcare Life Sciences, Piscataway, NJ). Optical density of immunoblots was measured using NIH gel analysis software.

2.4. *Antibodies and pharmacological agents*

Primary antibodies used in this study include rabbit antibodies against phospho-SFKs at Y416 (Cell Signaling Technology, Beverly, MA) which detects a phosphorylated tyrosine at position 416 on SFKs, Src (Cell Signaling), Fyn (Santa Cruz Biotechnology, Santa Cruz, CA), or actin (Cell Signaling). The pharmacological agent used is (-)-scopolamine hydrobromide, which was purchased from Sigma-Aldrich. Scopolamine was dissolved in a physiological saline solution and was freshly prepared on the day of the experiment.

2.5. *Statistics*

The results are presented as means \pm SEM. These results were statistically analyzed using a one way analysis of variance followed by a Bonferroni (Dunn) comparison of groups using least squares-adjusted means. Probability levels of <0.05 were considered statistically significant.

Figure 1. Effects of scopolamine (5 mg/kg, i.p.) on phosphorylation of Src family tyrosine kinases in the rat caudate putamen.

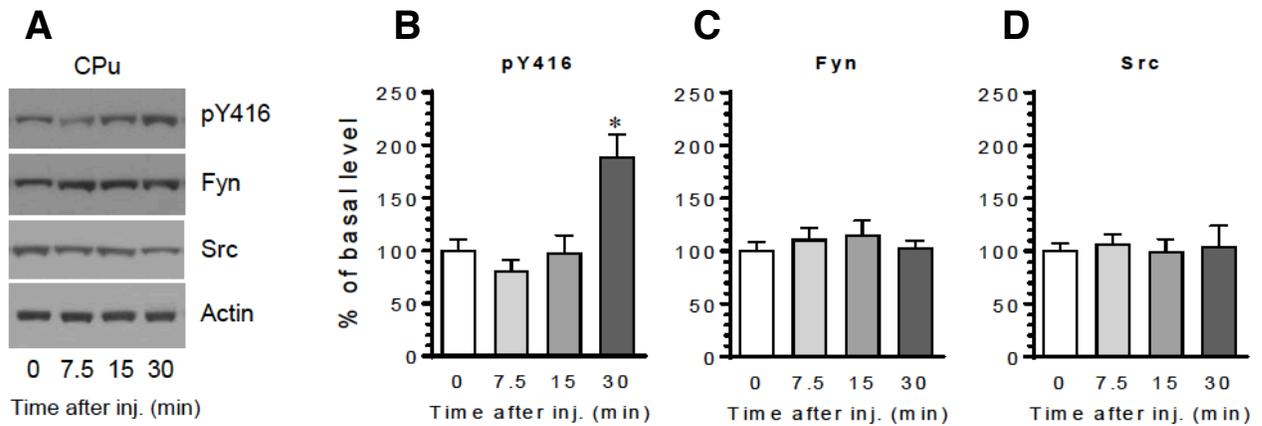


Fig. 1. (A) Representative immunoblots showing effects of scopolamine on pY416 phosphorylation, Fyn total, Src total, and Actin (control) in the caudate putamen. (B-D) Quantification of effects of scopolamine on SFKs phosphorylation at pY416 (B), Fyn total protein (C), and Src total protein (D) at sacrifice intervals of 0, 7.5, 15, 30 min. Data are presented as mean \pm SEM (n = 4 per group).

Figure 2. Effects of scopolamine (5 mg/kg, i.p.) on phosphorylation of Src family tyrosine kinases in the rat nucleus accumbens.

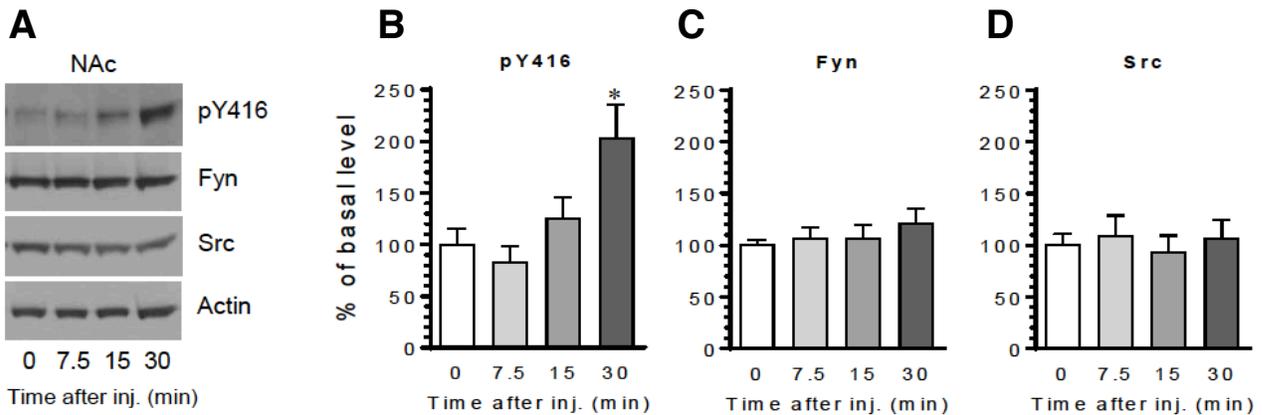


Fig. 2. (A) Representative immunoblots showing effects of scopolamine on pY416 phosphorylation, Fyn total, Src total, and Actin (control) in the nucleus accumbens. (B-D) Quantification of effects of scopolamine on SFKs phosphorylation at pY416 (B), Fyn total protein (C), and Src total protein (D) at sacrifice intervals of 0, 7.5, 15, 30 min. Data are presented as mean \pm SEM (n = 4 per group).

3. Results

We first investigated the role of mAChR antagonism in changing the phosphorylation of SFKs at a specific tyrosine site, Y416. To this end, we randomly divided rats into four different groups (n = 4 per group). This size of group was determined by a power analysis and our previous experiments of this kind. We subjected three groups of rats to a single dose of the mAChR antagonist scopolamine (5 mg/kg, i.p.). These rats were sacrificed at different time points (7.5, 15, and 30 min after drug injection) to detect changes in SFK phosphorylation at Y416 in the striatum using immunoblots. One group of rats received a saline injection and were sacrificed immediately (0 min after injection) to serve as a control. We found that a single injection of scopolamine consistently increased levels of Y416 phosphorylation in the CPu at a later time point (30 min), while scopolamine did not alter Y416 phosphorylation at two early time points (7.5 and 15 min) (**Fig. 1A** and **1B**). Similar results were seen in the NAc of scopolamine-treated rats compared to saline-treated rats (**Fig. 2A** and **2B**). These data reveal a time-dependent response of Y416 phosphorylation to scopolamine. Blockade of mAChRs with scopolamine is able to induce a relatively delayed increase in Y416 phosphorylation in both the CPu and NAc.

We next analyzed the same samples from four groups of rats to determine if scopolamine alters protein expression of the two prototypic members of SFKs, Fyn and Src, in the CPu and NAc. At all time points surveyed, Fyn and Src protein levels remained unchanged in the CPu following scopolamine administration (**Fig. 1C** and **1D**). Similarly in the NAc, we observed the insensitivity of Fyn and Src protein expression in response to scopolamine as the two proteins showed a minimal change in scopolamine-treated rats relative to saline-treated rats (**Fig. 2C** and **2D**). These data demonstrate that mAChR

blockade has no significant impact on total Fyn and Src protein expression in the CPu and NAc.

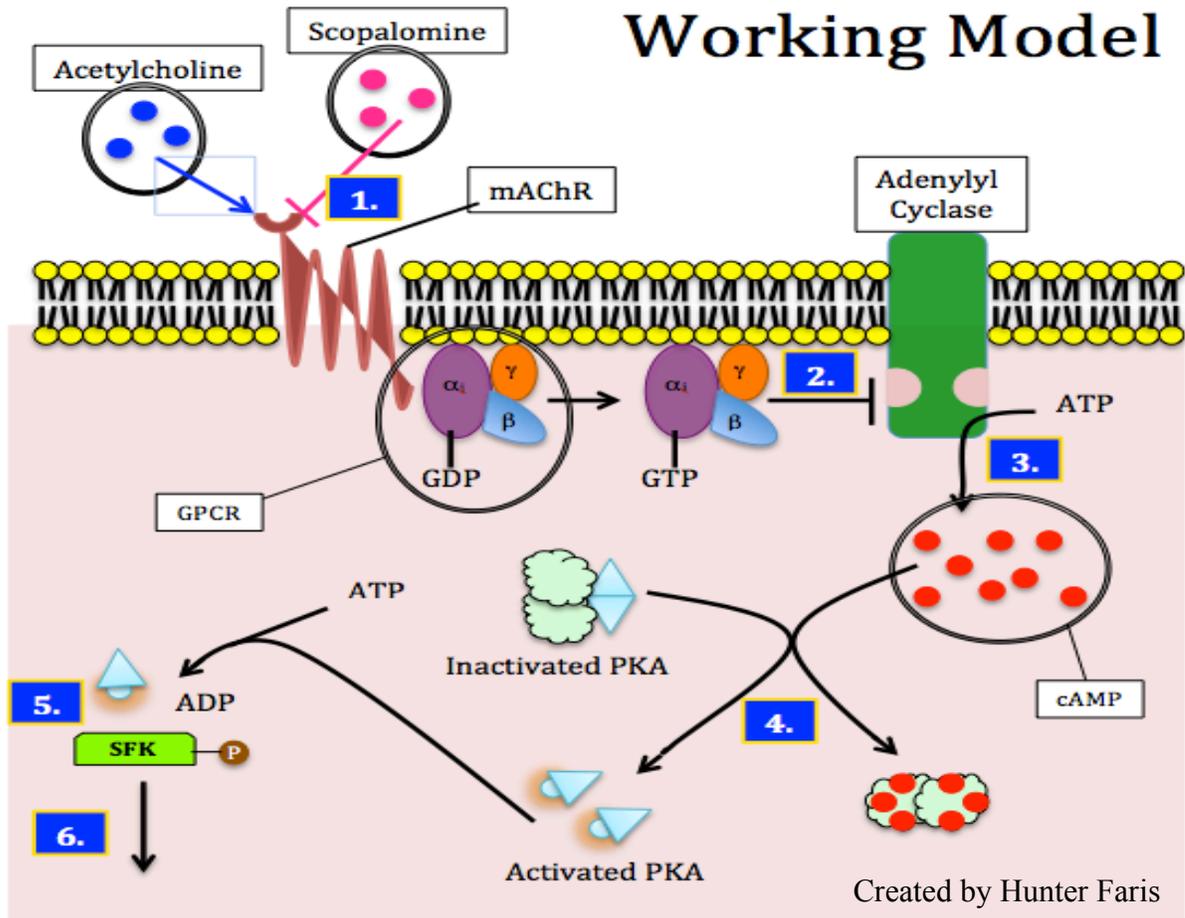


Fig. 3. Diagram depicting the signaling pathways involved in SFKs phosphorylation in the striatum in response to a single scopolamine injection (see discussion for details). **(1.)** Scopolamine blocks mAChR. **(2.)** mAChR can't activate G α_i which would inhibit adenylyl cyclase. **(3.)** Adenylyl cyclase stays active & makes cAMP. **(4.)** cAMP activates PKA. **(5.)** PKA phosphorylates SFK. **(6.)** Activated SFK causes downstream effects through molecular mechanisms that are currently poorly understood. mAChR, muscarinic acetylcholine receptor; GPCR, G-protein coupled receptor (α_i); GDP, guanosine diphosphate; GTP, guanosine triphosphate; cAMP, cyclic adenosine monophosphate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; PKA, protein kinase A; SFK, Src Family Kinase; P, phosphate.

4. Discussion

In this study, the possible role of mAChRs in the regulation of SFKs was investigated in the adult rat striatum *in vivo*. Important findings from this study include that 1) pharmacological blockade of mAChRs with a widely-used antagonist scopolamine increased phosphorylation of SFKs in the two subdivisions of the striatum, i.e., the CPu and NAc, and 2) the mAChR antagonist did not change expression of total Src and Fyn proteins within the striatum. These results for the first time provide evidence supporting a significant role of mAChRs in the regulation of SFKs. Since the mAChR antagonist elevated SFK phosphorylation, mAChRs are believed to exert an inhibitory influence on SFK activity in striatal neurons under normal conditions.

While our data demonstrate that mAChRs regulate SFKs, how mAChRs regulate SFKs is unclear. Early studies have shown that PKA is among regulators that modulate SFK activity (see Introduction). In details, activation of PKA leads to an increase in Y416 phosphorylation of SFKs^{10, 14}. Among five mAChR subtypes, M2 and M4 receptors are known to be coupled to $G_{i/o}$ proteins. As a result, activation of M2 and M4 receptors inhibits adenylyl cyclase, leading to reduction of cAMP formation and reduction of PKA activity. Thus, it is likely that scopolamine may mainly target M2 and/or M4 receptors. By blocking these receptors, scopolamine removes the M2/M4-mediated inhibition of PKA activity. This results in a higher level of PKA activity, which thereby enhances SFK phosphorylation (**Fig. 3**)^{1, 10, 14}.

Between M2 and M4 receptors, the M4 receptor is more intriguing because the M4 receptor is the most prevalent striatal mAChRs seen in all subregions of the striatum and is thus likely to exert the most profound effect on striatal signaling and activity^{3, 9}. However,

one limitation of this work is the non-subtype selective nature of scopolamine. Using this antagonist is not able to reveal information on the subtype of mAChRs responsible for the regulation of SFKs. Future studies will use an M4 receptor selective antagonist, if available, to define the selective role of M4 receptors in the regulation of SFKs.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

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