Cholinergic Responses in Crossed Tecto-Reticular Neurons of Rat Superior Colliculus

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Sooksawate T, Isa K, Isa T. Cholinergic responses in crossed tecto-reticular neurons of rat superior colliculus. J Neurophysiol 100: 2702–2711, 2008. First published August 27, 2008; doi:10.1152/jn.90723.2008. Neurons in the intermediate gray layer (SGI) of mammalian superior colliculus (SC) receive cholinergic innervation from the brain stem parabrachial region, which seems to modulate the signal processing in the SC. To clarify its role particularly in orienting behaviors, we studied cholinergic effects on the major output neuron group of the SGI, crossed tecto-reticular neurons (cTRNs), identified by retrograde labeling from the contralateral brain stem gaze center in SC slices obtained from rats (PND 17–22) by whole cell patch-clamp techniques. Bath application of carbachol induced either (i) nicotinic inward (nIN) + muscarinic inward (mIN) (11/24) or (ii) nIN + mIN + muscarinic outward (mOUT) (13/24) current responses. Transient pressure application of 1 mM acetylcholine elicited nIN in all neurons tested (n = 58). In a majority of these neurons (52/58), the nIN was completely suppressed by dihydro-β-erythroidine, a specific antagonist for α4β2 nicotinic receptor subtype. The remaining 6/58 neurons exhibited not only the slower α4β2 receptor-mediated component but also a faster component that was inhibited by a specific antagonist for α7 nicotinic receptor, α-bungarotoxin. cTRNs expressing α7 nicotinic receptors tended to be smaller in size than those lacking α7 receptors. Bath application of muscarine induced two response patterns: mIN only (17/58) and mIN + mOUT (21/58). The mIN and mOUT were mediated by M3 (plus M1) and M2 muscarinic receptors, respectively. These results suggest that a major response to cholinergic inputs to cTRNs is excitatory. This would indicate the facilitatory role of the brain stem cholinergic system in the execution of orienting behaviors including saccadic eye movements.

INTRODUCTION

The mammalian superior colliculus (SC) is involved in various types of sensori-motor processing (King 2004). Among these functions, orienting behaviors are known to be controlled by a group of output neurons of its intermediate gray layer (SGI) that send descending projections to the contralateral brain stem gaze center in the medial pontine reticular formation (MPRF) (Grantyn and Berthoz 1985; Isa and Sasaki 2002). We call these output neurons crossed tecto-reticular neurons (cTRNs). In our previous study, we showed that a majority of them exhibit regular spiking properties to depolarizing current pulses and exhibit the N-methyl-D-aspartate (NMDA) receptor–dependent bursting responses to visual inputs using slice preparations of the rat SC (Sooksawate et al. 2005b).

Many neuroanatomical studies analyzed cholinergic projections to the SGI, which originate from the pedunculopontine and laterodorsal tegmental nuclei (PPTN and LDTN) in the parabrachial region of the brain stem (Beninato and Spencer 1986; Graybiel 1978a; Hall et al. 1989; Illing and Graybiel 1985; Ma et al. 1991). Several anatomical studies using immunohistochemistry, in situ hybridization, receptor autoradiography, or immunoprecipitation have shown the expression of α3, α4, α5, α6, α7, β2, β3, and β4 nicotinic receptor (nAChR) subunits in the SC (Clarke et al. 1985; Dominguez del Toro et al. 1994; Gotti et al. 2006; Perry et al. 2007; Prusky and Cynader 1988; Swanson et al. 1987; Tribollet et al. 2004; Wada et al. 1989; Whiteaker et al. 2000, 2002). α4β2- and α7-like receptors are the predominant forms in the SGI, whereas α3/α6β2 receptors are mostly expressed in the superficial gray layer (SGS) (Gotti et al. 2006; Nguyen et al. 2004; Perry et al. 2002, 2007; Tribollet et al. 2004; Whiteaker et al. 2000). In addition, previous binding studies have reported that that M1, M2, and M3 muscarinic receptor (mAChR) subtypes are all expressed in the SC (Cortes et al. 1984; Cymerman et al. 1987; Frey and Howland 1992; Hess et al. 1990; Levey 1993; Miyoshi et al. 1989; Price et al. 1986; Zubieta and Frey 1993). These cholinergic inputs seem to play an important role in modulating the signal processing in the SC in a context-dependent manner. However, until recently, the physiological roles of the cholinergic inputs was not clear. Our laboratory has been analyzing the role of cholinergic inputs to the SC, especially their relation to regulation of saccadic eye movements, with different techniques. It was found that tonic activity of a group of PPTN neurons was related to a successful performance in a visually guided saccade task in awake behaving monkeys (Kobayashi et al. 2002). Li et al. (2004) showed that activation of M1 and M3 type mAChRs caused presynaptic inhibition of GABAergic transmission in the SGI by using the whole cell patch-clamp technique. Injection of nicotine into the monkey SGI shortened the reaction times of saccades toward the movement field of the neurons at the injection site (Aizawa et al. 1999; Watanabe et al. 2005). In a previous study on rodent slices, we reported that bath application of 30 μM carbachol (CCh) induced depolarization and spike firing in all the SGI neurons tested (45/45). The CCh-induced inward currents were partially suppressed by nAChR antagonist, 1 μM DHβE. Moreover, 30 μM nicotine could also induce depolar-
ization in most of the cTRNs (Kobayashi and Isa 2002). However, details of the receptor mechanism of the cholinergic actions are still unclear.

In our recent study, as the initial step to clarify the cholinergic action on SGI neurons, we analyzed the effect of bath application of CCh on a large population of randomly sampled SGI neurons to cover all the different response patterns. Five different patterns with various combinations of nicotinic inward (nIN), muscarinic inward (mIN), and muscarinic outward (mOUT) current responses were observed: 1) nIN only, 2) nIN + mIN, 3) nIN + mIN + mOUT, 4) nIN + mOUT, and 5) mOUT only (Sooksawate and Isa 2006). In that study, we could identify some of the recorded cells as output neurons based on the trajectories of their axons that were stained by intracellularly injected biocytin. However, they still showed four different response patterns to CCh, which made interpretation of the role of cholinergic input difficult. In this study, we focused on the cholinergic action on a defined population of SGI output neurons, cTRNs, to clarify the role of cholinergic inputs for the control of orienting behaviors. These projection neurons were identified by retrograde labeling from the contralateral MPRF (Isa and Sasaki 2002; Sooksawate et al. 2005b). We first analyzed their responses to bath-applied CCh. Second, we studied the nAChR and mAChR subtypes in these identified population of SGI neurons. Based on the results, functional implication of the cholinergic inputs to the SGI will be discussed.

METHODS

The animal experiments were conducted in accordance with the Guideline for the Use of Animals in Research (the Physiological Society of Japan) and were approved by the Animal Research Committee of the Okazaki National Institutes. All attempts were made to minimize both the suffering and number of animals used in this study. A total of 28 rat pups were used in the experiments.

Retrograde labeling of identified cTRNs

cTRNs were identified by retrograde labeling as previously reported (Sooksawate et al. 2005b). In brief, dextran-conjugated Texas red (5% in Tris-buffered saline, Molecular Probes, Eugene, OR) was unilaterally injected under xylazine/ketamine (10 mg/kg/60 mg/kg) anesthesia into the MPRF of 14- to 16-day-old Long Evan rats, 3–6 days before the acute slice experiment. After injection of the tracer, the incision was sutured, and the rat pups were allowed to be with their mother until the day of the acute slice experiment.

Slice preparations of the SC

The SC slices were obtained from 17- to 22-day-old rats that received injection of dextran-conjugated Texas red as described above. Rats were decapitated under deep ether anesthesia. Brains were quickly removed and submerged for 5 min in an ice-cold sucrose-Ringer solution containing (in mM) 234 sucrose, 2.5 KCl, 1.25 NaH2PO4, 10 MgSO4, 0.5 CaCl2, 26 NaHCO3, and 11 D-glucose, pH 7.4 (after equilibration with 95% O2-5% CO2) at room temperature (22–25°C) for >1 h before recording. After incubation, SC slices were placed in a recording chamber on an upright fluorescence microscope (Axioskop FS, Zeiss) and continuously superfused with the Ringer solution at a rate of 2–3 ml/min using two peristaltic pumps (Minipuls 3, Gilson). The first pump was used to superfuse the Ringer solution or drug solutions to the recording chamber. The second pump was used to pump the solution out from the chamber. The method for switching between Ringer solution and drug solution was through a manual valve. The brain stem caudal to the SC was incubated in a fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer for 2–4 days and cut into frontal sections to confirm the injection site of the tracer (Fig. 1A).

Whole cell patch-clamp recordings

Whole cell patch-clamp recordings were obtained from the SGI neurons under visual control of patch pipettes using a patch clamp...
amplifier (EPC-7, List, Darmstadt, Germany). Retrogradely labeled cTRNs were located using epifluorescence optics and visualized with Nomarski optics and a ×63, water immersion objective (Fig. 1B). Most of the recordings were obtained from neurons located in SGI, the terminal field of cholinergic input fibers (Beninato and Spencer 1986; Graybiel 1978a; Hall et al. 1989; Illing and Graybiel 1985; Ma et al. 1991). Recording electrodes were pulled from borosilicate glass capillaries (Clark Electromedical Instruments) using a horizontal electrode puller (P-97, Sutter Instruments) and filled with an intrapipette solution containing (in mM) 140 K-gluconate, 20 KCl, 0.2 EGTA, 2 MgCl2, 4 Na2ATP, 10 Na3GTP, 10 HEPES, and 0.1 spermine (pH 7.3). In the experiment to identify nAChR subtypes, the pipettes were filled with solution containing (in mM) 120 Cs-gluconate, 10 CsCl, 2 MgCl2, 4 Na2ATP, 10 EGTA, 10 HEPES, and 0.1 spermine (pH 7.3). Because the liquid junction potential between the Ringer solution and the gluconate-based intrapipette solution was estimated to be −10 mV, the actual membrane potential was corrected by this value. The osmolarity of the intrapipette solution was 280–290 mOsm/l. The resistance of the electrodes was 4–7 MΩ. The resistance of the electrodes was 4–7 MΩ. 

Ringer solution and the gluconate-based intrapipette solution was applied to the cells tested, except for the neurons in the experiment to identify nAChR subtypes, with a duration of 500 ms at 40-pA steps to −70 mV and −75 to −90 mV set by a constant current injection. Data were acquired using a pClamp system (pClamp 8.0, Axon Instruments).

Histology and morphological analysis

In all the experiments, biocytin was dissolved in the intrapipette solution at a concentration of 5 mg/ml just before recording for later morphological analysis of the recorded neurons. The recorded cells were filled with biocytin by diffusion from the pipettes. After recordings, the slices were fixed with 4% phosphate-buffered paraformaldehyde solution for 4–24 h. They were processed with the avidin-biotin peroxidase method (ABC kit, Vectorlab), visualized with diaminobenzidine tetrahydrochloride (DAB; Dojin, Kumamoto, Japan), and intensified with nickel ammonium sulfate (Isa et al. 1998; Saito and Isa 1999). Finally, the slices were counterstained with cresyl violet or neutral red. Only cells with intact somas and proximal dendrites were drawn using a camera lucida attached to a light microscope (Olympus) and analyzed using the MetaMorph software version 6.3 (Molecular Devices).

Drugs and chemicals

All the drugs and chemicals were purchased from Sigma (St. Louis, MO) with the exception of TTX and α-conotoxin MII (α-CTX MII), which were purchased from Sankyo (Tokyo, Japan) and Tocris, respectively.

Carbachol (CCh; 30 μM), dihydro-β-erythroidine (DHβE; 1 μM), and atropine (1 μM) were used in this study as a nonselective cholinergic agonist, nAChR antagonist, and mAChR antagonist, respectively. To study fast responses of cholinergic receptor activation, 1 mM ACh was applied with air pressure pulses (20–30 psi, 30–50 ms duration) through a micropipette identical to the patch pipette to induce nicotinic currents. Muscarinic response was suppressed by 1 mM atropine added into the Ringer solution. The micropipette was placed within 50 μm of the recorded neurons. α-Bungarotoxin (α-BTX; 100 nM), α-CTX MII (50 nM), and DHβE (200 nM) were used as relatively specific agonists for α7, α3/α6β2, and α4β2 subtypes of nAChRs, respectively, and applied to every neuron tested in order, from α-BTX, α-CTX MII, and DHβE. Muscarine (10 μM) was used as a specific agonist of mAChRs. Pirenzepine (1 μM), methoctramine (10 μM), and 4-diphenylacetoxy-N-methylpiperidino-methide (4-DAMP; 50 nM) were used as relatively specific antagonists of M1, M2, and M3 subtypes of mAChRs, respectively. Stock solutions of all drugs were prepared in distilled water, stored at −20°C, and diluted to their final concentrations in Ringer solution on the day of the experiment. All the drug solutions were applied to the recorded neurons via the bath superfusion system with the exception for ACh. After application of the drugs, the Ringer solution without any drugs was perfused continuously to wash out all the drugs.

The cholinergic receptor agonist (CCh or muscarine) was applied to the recorded neurons for 40–60 s. The interval between CCh and muscarine applications was 15 min to avoid desensitization and rundown of the receptors. As shown in RESULTS, the effects of DHβE and atropine did not recover within this interval; therefore they were separately applied in combination with CCh. To identify subtypes of the cholinergic receptors, the neuron was pretreated with α-BTX or α-CTX MII for >10 min, DHβE for >4 min, and pirenzepine, 4-DAMP, or methoctramine for >5 min before the additional application of the agonists. During these pretreatment times, the antagonists were applied at the appropriate concentrations in the solution containing the agonist. To prevent the effects of drugs on presynaptic elements, 0.5 μM TTX was applied after the response properties to current pulses were determined in all the experiments. We also used 10 μM bicuculline methobromide (Bic), 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNOX), and 50 μM d-2-amino-5-phosphonovaleric acid (APV) for suppression of GABA(A) receptors, AMPA/kainate receptors, and NMDA receptors, respectively, to exclude the possible contamination of current responses caused by these receptors. Because GABA(C) receptors were expressed exclusively in the superficial layers of SC (Boller and Schmidt 2003; Boue-Grabot et al. 1998), GABA(C) receptor antagonist was not added into the Ringer solution.

Data analysis

The input impedance was calculated from the steady-state voltage change in response to a −40-pA hyperpolarizing current pulse. The membrane capacitance in the whole cell configuration was measured by reading in the pClamp system after adjustment of the capacitive component. Data are expressed as mean ± SE. Significance was tested by Student’s t-test, one-way ANOVA, and Dunnett’s test where applicable, and P < 0.05 was considered significant.

RESULTS

Whole cell patch-clamp recordings were performed from a total of 132 cTRNs under video-assisted Normarski microscopy as reported previously (Saito and Isa 1999, 2000; Sooksawate and Isa 2006; Sooksawate et al. 2005b). Effects of bath application of CCh, a nonselective agonist of both nAChRs and mAChRs, were tested in 28 cTRNs. Effects of transient pressure application of ACh were tested in 66 cTRNs. Effects of bath application of muscarine, a selective agonist of mAChRs, were tested in 38 cTRNs.

Patterns of cholinergic responses in cTRNs

We studied the cholinergic responses in a major output cell group of the SGI, the cTRNs, which were identified by retrograde labeling from the contralateral brain stem gaze center. To test the desensitization and rundown of cholinergic receptors, we examined the effect of repeated application of CCh on the peak amplitudes of the inward currents. When CCh was applied at 15-min intervals, the peak amplitudes of inward currents was slightly reduced to 94.11 ± 0.81 and 91.44 ± 0.51% after two and three repeated applications, respectively (P < 0.01, ANOVA and Dunnett’s test, n = 4; Fig. 2Ab) This result indicates that our procedure of CCh application exhibited slight desensitization and rundown. Thus we chose the appli-
declining phase was completely suppressed by 1 μM atropine (Fig. 2A1a). Thus these cTRNs exhibited relatively fast inward currents mediated by nAChRs and slow inward currents by mAChRs [nicotinic inward (nIN) + muscarinic inward (mIN) current response]. In the remaining 13 cTRNs (54%), CCh induced an inward current with a relatively fast onset and a slow declining phase. In the presence of 1 μM DHβE, CCh induced a mixture of slow inward and slow outward currents in which the inward current predominated (Fig. 2A2a) or the slow outward current appeared to override the smaller and slower inward current (Fig. 2A2b). Both of the remaining slow inward and outward currents completely disappeared when the neuron was pretreated with 1 μM atropine. Therefore these cTRNs exhibited relatively fast inward currents mediated by nAChRs and slow inward and outward currents mediated by mAChRs [nicotinic inward (nIN) + muscarinic inward (mIN) + muscarinic outward (mOUT) current response]. Typical examples of the morphology of these cTRNs that exhibited the nIN + mIN current responses and nIN + mIN + mOUT current responses are shown in Fig. 2B. Both of these types of cells possessed relatively large soma and multipolar dendrites. In some cells, the axons were found to leave the SC and traveled along the outer border of the periaqueductal gray matter, a typical axonal course of cTRNs (Fig. 2Bd). Regular spiking was a major firing pattern in both cTRNs exhibited nIN + mIN (9/11) and nIN + mIN + mOUT (9/13) current responses. When we compared the neurons with the above two types of the cholinergic responses, there were no significant differences in somatic area [281.9 ± 32.94 (neurons with nIN + mIN, n = 11) vs. 265.8 ± 17.55 μm² (neurons with nIN + mIN + mOUT, n = 11), P = 0.67, Student’s t-test], membrane capacitance [37.45 ± 3.24 (neurons with nIN + mIN, n = 11) vs. 43.14 ± 2.87 pF (neurons with nIN + mIN + mOUT, n = 13), P = 0.20], and input impedance [460.7 ± 73.93 (neurons with nIN + mIN, n = 11) vs. 418.5 ± 59.98 MΩ (neurons with nIN + mIN + mOUT, n = 13), P = 0.66].

Pharmacological properties of nAChR subtypes

The pharmacological properties of nAChR-mediated current responses in cTRNs were examined in the second series of experiments. The experiments were performed using Cs gluconate–based intrapipette solution to block the current mediated by K+ channels. Brief pressure applications of 1 mM ACh (20–30 psi, 30- to 50-ms duration) in the presence of 1 μM atropine induced inward currents in all the tested cTRNs (58/58) at the holding potential of −70 mV (Fig. 3). There were two types of current responses: a slow inward current only and a fast and slow inward current. First, we examined the effect of repeated application of ACh on the peak amplitudes of the inward currents. The peak amplitude of the slow inward currents induced by ACh applied at 1-min intervals was slightly reduced to 98.14 ± 0.53, 96.14 ± 1.32, and 93.62 ± 0.99% after 10, 20, and 25 min of repeated applications, respectively (ANOVA and Dunnett’s test, n = 5; Fig. 3, A and B). The fast component of the inward currents induced by ACh, applied at 1-min intervals, was also reduced to 88.03 ± 1.86, 80.63 ± 1.39, and 70.07 ± 4.62% after 10, 20, and 25 min of repeated applications, respectively (ANOVA and Dunnett’s test, n = 3; Fig. 3, E and F). These results indicate that our procedure of ACh application exhibited slight desensitization.
and rundown. Thus we chose the application of ACh at 1-min intervals in the following experiments. In the majority of the tested cTRNs (90%, 52/58), the nIN was only slightly suppressed by 100 nM α-BTX, a specific antagonist for the α7 receptor subtype, and 50 nM α-CTX MII, a specific antagonist for the α3/α6β2 receptor subtype, although 200 nM DHβE, a specific antagonist for α4β2 subtype of nAChRs, almost completely inhibited these currents after 4 min of application (Fig. 3, C and D). The mean peak amplitude was reduced to 2.25 ± 0.25% of the control ACh response (P < 0.001, ANOVA and Dunnett’s test).
M1 receptors also contribute to the inward currents by co-
mediated mainly by M3 subtype of the mAChRs. However,
respectively. These results indicate that the mIN in cTRNs is
reduced to 60.83 (Fig. 4, C). The averaged peak amplitude of these inward currents was reduced to 5.18 (neurons with DH
and E-sensitive component only, n = 17) by applying muscarine along
with the above two types of pharmacological profiles, it was
found that neurons with a DHβE-sensitive component only had
higher membrane capacitance than α-BTX- plus DHβE-sensitive
eurons [38.75 ± 1.42 (neurons with DHβE-sensitive component only; n = 52) vs. 29.47 ± 2.35 pF (neurons
with α-BTX- plus DHβE-sensitive components, n = 6), P < 0.05,
Student’s t-test] and larger somatic area [267.1 ± 9.24 (neu-
rons with DHβE-sensitive component only, n = 44) vs. 162.0 ± 11.9 μm² (neurons with α-BTX- plus DHβE-sensi-
tive components, n = 6), P < 0.01]. These results indicate that
the mIN current response in cTRNs was mediated mainly by the
α4β2 nAChRs and partly by α7 nAChRs. Moreover, the
cTRNs that expressed mainly α4β2 nAChR possessed larger
soma size than those expressing both α7 and α4β2 nAChRs.

Pharmacological properties of mAChR subtype involved
in the muscarinic inward and outward current responses

In the third series of experiments, we examined the pharma-
cological properties of mAChR-mediated current responses in
cTRNs. The experiments were performed using K gluconate–
based intrapipette solution to allow K⁺ currents through po-
tassium channels that might be induced by activation of
mAChRs. Bath application of 10 μM muscarine induced in-
ward or inward + outward currents in all the cTRNs tested
(38/38) at the holding potential of −70 mV. Similar to the
muscarinic currents induced by bath application of CCh, mus-
carine induced only two response patterns: mIN current only
(45%, 17/38) and mIN + mOUT currents (55%, 21/38).

First, we studied the subtypes of mAChRs in neurons with
mIN only responses (n = 17) by applying muscarine along
with specific mAChR antagonists. Then, mIN could be almost
completely suppressed by 50 nM DAMP, a specific M3 recep-
tor antagonist, in a majority of this neuron group (65%, 11/17;
Fig. 4, A and B). The averaged peak amplitude of inward
currents induced by muscarine was decreased to 5.18 ± 1.49%
of the control muscarine response (P < 0.001, ANOVA and
Dunnett’s test). The effects of 10 μM methoctramine, a spe-
cific M2 receptor antagonist, and 1 μM pirenzepine, a specific
M1 receptor antagonist, were very small, if any (Fig. 4, A and
B). In the remaining 6/17 neurons (35%), muscarine-induced
inward current was partly suppressed by both 1 μM pirenz-
epine and 50 nM DAMP but not by 10 μM methoctramine
(Fig. 4, C and D). The mean peak amplitude of these inward
currents was reduced to 60.83 ± 7.13 and 6.83 ± 0.79% of the
control muscarine response after pretreating the neurons for
>5 min with pirenzepine (P < 0.001) and DAMP (P < 0.001),
respectively. These results indicate that the mIN in cTRNs is
mediated mainly by M3 subtype of the mAChRs. However,
M1 receptors also contribute to the inward currents by co-
activation with the M3 receptors in a subpopulation of these
neurons.

We studied the subtypes of mAChRs mediating the mIN +
mOUT responses. According to the above results, we isolated
the mOUT from mIN using DAMP (50 nM) to suppress the
inward current and DAMP was added with other mAChR
antagonists. In all four neurons tested, mOUT, which was
observed in the presence of DAMP, could be almost com-
pletely suppressed by 10 μM methoctramine but not by 1 μM
pirenzepine (Fig. 5, A and B). The averaged peak amplitude of
outward currents was decreased to 1.25 ± 0.75% of the control
response induced by muscarine and DAMP (P < 0.001). Based
on this result, the experiment to identify the mAChR subtypes mediating the mIN was designed as follows. We suppressed the outward currents using methoctramine (10 μM) to unmask the mIN. Then other mAChR antagonists were added with methoctramine. As shown in cTRNs that exhibited mIN only, the inward currents were almost completely inhibited by 50 nM DAMP in a majority of this group of neurons (76%, 13/17; Fig. 5, C and D). The mean peak amplitude of the inward currents induced by muscarine was decreased to 3.77 ± 1.11% of the control response induced by muscarine and methoctramine (P < 0.001). These inward currents were only slightly suppressed by 1 μM pirenzepine. In the remaining 4/17 neurons (24%), mIN was partly suppressed by each of 1 μM pirenzepine or 50 nM DAMP (Fig. 5, E and F). The mean peak amplitude of the inward currents was reduced to 69.50 ± 3.48 and 8.25 ± 1.97% of the control responses induced by muscarine and methoctramine after pretreatment with pirenzepine (P < 0.001) and DAMP (P < 0.001), respectively. There was no clear relationship between the firing patterns, the membrane capacitance, and the somatic area of these cTRNs to the muscarinic responses. These results indicate that, in this group of cTRNs, the mOUT is mediated mainly by the M2 subtype of mAChRs and mIN is mediated mainly by M3 subtype of the mAChRs and also co-activation of M1 receptors in a subpopulation of the neurons.

**DISCUSSION**

**Cholinergic response patterns in cTRNs**

Previous studies showed that there are several subsystems in the tectofugal pathways. For instance, Dean et al. (1989) showed that the contralateral tecto-bulbar pathway is involved in the control of orienting behaviors, whereas the ipsilateral tecto-reticular pathway is involved in avoidance behaviors. In addition, some tectofugal neurons have ascending projections to the thalamus and meso-diencephalic junctional regions (Grantyn and Grantyn 1982; Huerta and Harting 1982). More recent studies showed tectal projections to the dopaminergic neurons in the substantia nigra pars compacta (Comoli et al. 2003; McHaffie et al. 2006), which were found to be collaterals of the ascending projections to the thalamus (Coizet et al. 2007). Moreover, some of the tectofugal neurons were found to be GABAergic neurons (May et al. 2005; Sooksawate et al. 2005a).

In this study, we focused on the action of cholinergic inputs to one defined subclass of neurons in the SGI of the rat SC projecting to the brain stem gaze center: cTRNs. They send the descending command for orienting behaviors (Dean et al. 1989; Grantyn and Berthoz 1985). We identified these neurons using retrograde labeling of fluorescent tracer, dextran-conjugated Texas red, injected into the contralateral MPRF. As shown in Fig. 2A, the cholinergic responses to bath application
sensitive to 200 nM DH
Sooksawate and Isa 2006). In this study, we found that nIN was
antagonist of
/H9251
faster current component that was sensitive to
additionally, that a subset of cTRNs (10%, 6/58) exhibited a
were not. Therefore it is still unclear whether
of CCh in this group of neurons had only two patterns:
) nIN + mIN and
) nIN + mIN + mOUT. This result suggests that a major response to cholinergic input to cTRNs
would be excitatory. As we reported in our recent study (Sooksawate and Isa 2006), we found five cholinergic response patterns in randomly sampled SGI neurons: 1) nIN only, 2) nIN + mIN, 3) nIN + mIN + mOUT, 4) nIN + mOUT, and 5) mOUT only. In that study, some of the recorded neurons were identified as projection neurons because they were found to issue at least one of their axon collaterals to outside the SC with biocytin staining. In these populations of projection neurons, we found the above 1), 2), 3), and 4) response patterns. The results from this study and the former one suggest that there are at least more than two groups of projection neurons in the SGI that may differently respond to cholinergic inputs.

Functional nAChR subtypes involved in the current responses

Brief pressure application of ACh, in the presence of 1 μM atropine, was used to clarify the nAChR subtypes that mediated the nIN in cTRNs. This rapid application was fast enough to induce the fast desensitizing current component caused by α7 nAChR subtype (Albuquerque et al. 1997; Endo et al. 2005; Sooksawate and Isa 2006). In this study, we found that nIN was sensitive to 200 nM DHβE, a selective antagonist to α4β2 receptors, in all the cTRNs tested (100%, 58/58; Fig. 3), and additionally, that a subset of cTRNs (10%, 6/58) exhibited a faster current component that was sensitive to α-CTX, an antagonist of α7 nAChRs (Fig. 3, G and H). In this study, we did not find a neuron whose nicotinic current was sensitive to α-CTX MII, a selective antagonist to α3/α6β2 receptors. When these results are compared with those obtained from randomly sampled SGI neurons, it is noticeable that the ratio of SGI neurons that co-expressed both α4β2 and α7 receptors was higher (18%; 8/45) among the randomly sampled populations. In addition 1/45 neuron was sensitive to α-CTX MII in those populations (Sooksawate and Isa 2006), which suggests that nonprojection neurons of the SGI express α7 or α3/α6β2 receptors with a higher ratio than the cTRNs. The role of the nAChRs in modulation of the network activity requires more argument. This study showed that α4β2 receptors were activated by the bath application of CCh, whereas α7 receptors were not. Therefore it is still unclear whether α7 receptors can have any role in modulation of the network activity. The answer depends on how ACh is released from the presynaptic terminals innervating the SGI and reaches the receptors, that is, whether ACh is released transiently in fast synaptic action or released with slow kinetics and acts on synapses with volume transmission, which is unclear at this moment.

Functional mAChR subtypes involved in the current responses

Bath application of muscarine induced only two response patterns, inward current only (45%, 17/38) and inward + outward currents (55%, 21/38), in cTRNs in a similar manner to the muscarinic currents induced by bath application of CCh. It was clarified that, in cTRNs of mIN only pattern, M3 receptors mediate the inward currents in 11/17 cTRNs, whereas M1 receptors also seemed to be involved in mediating the mIN in the remaining 6 cTRNs (35%). Also in the case of the cTRNs expressing both mIN and mOUT, 50 nM DAMP was highly sensitive to inhibit these inward currents in the majority of cTRNs tested (14/17; Fig. 5, C and D). In the remaining 3/17 neurons, the inward currents seemed to be mediated both by M1 and M3 mAChRs (Fig. 5, E and F). These results confirmed our previous paper studied in randomly sampled SGI neurons (Sooksawate and Isa 2006).

Comparison with cholinergic inputs to the superficial layers: functional implication

It has been shown that the superficial layers (sSC) of the SC receive cholinergic inputs from the nucleus parabigeminalis (Graybiel 1978b; Hall et al. 1989; Sherk 1979; Watanabe and Kawana 1979). The cholinergic input from this nucleus may stimulate presynaptic terminals to potentiate the release of glutamate onto the SGS inhibitory neurons (Binns and Salt 2000). The previous report from our laboratory also tested the function of nAChRs by a transient pressure application of ACh in the superficial layers (sSC) of the mouse SC (Endo et al. 2005). In that study, ACh application induced transient inward current response followed by a burst of synaptic currents mediated by GABA_A receptors in most of the non-GABAergic neurons. Because the GABAergic synaptic currents could be activated under the presence of TEA, they were supposed to be induced by facilitation of GABA release by activation of nAChRs in the presynaptic GABAergic neurons. The GABAergic neurons in the sSC were found to express α-CTX MII-sensitive nAChRs, α3/α6β2 subtype nAChRs, and partly α-BTX-sensitive, α7 subtype nAChRs. Thus the major action of nACh activation was supposed to be facilitation of GABAergic transmission that might be involved in enhancement of contrast sensitivity of sSC neuron.

On the other hand, preliminary previous report by Kobayashi and Isa (2002) showed that CCh and nicotine could induce depolarization and spike firing in SGI neurons and cTRNs, respectively. These results also suggested that a possible role of cholinergic input to cTRNs is facilitation of orienting behaviors including saccadic eye movements. A previous report by Weldon et al. (1983) showed that injection of CCh into the SC induced rotational behavior contralateral to the injection site and suggested the facilitatory effect of the cholinergic inputs to the SC. Our previous study also showed that injection of nicotine into the SGI facilitated the initiation of saccades (Aizawa et al. 1999; Watanabe et al. 2005). Our laboratory previously reported the results of single unit recording from the monkey PPTN during visually guided saccade task (Kobayashi et al. 2002). In that study, we showed that, when the tonic firing of a population of PPTN neurons during fixation period of the task was high, monkeys correctly performed the task, and when the tonic firing was low, they often failed in performing this task. This may suggest the facilitatory action of cholinergic inputs from the PPTN to the SGI in the control of visually guided saccades.
and mAChRs was still the inward current response in these neurons. Further study is needed to examine the functional role of this M2 receptor–mediated outward current in cTRNs. In addition to the postsynaptic facilitatory effect of cholinergic inputs to the projection neurons, a previous study from our group showed that activation of M1 and M3 receptors induced presynaptic inhibition of GABAergic synapses in the SGI (Li et al. 2004). Several reports have shown that substantia nigra pars reticulata (Hikosaka and Wurtz 1985) and fixation zone in the SC rostral pole (Meredith and Ramoa 1998; Munoz and Wurtz 1992) sent a tonic GABAergic inhibition to the presac- cadic burst neurons including projection neurons in the SGI. In this regard, therefore postsynaptic depolarization and presyn- aptic inhibition of tonic GABAergic inhibition would bring the membrane potential of cTRNs close to the threshold for the NMDA receptor (NMDAR)-dependent burst firing (Saito and Isa 2003, 2004; Sooksawate et al. 2005b). This would facilitate the animals to respond rapidly to extrinsic stimuli. On the other hand, if the cholinergic input to SGI is weak, tonic excitation of the cTRNs and the decrease of tonic GABAergic inhibition may not be sufficient to facilitate the NMDAR-dependent burst firing. This will make animals difficult to respond to the extrinsic stimulus.

As we showed above, different subclasses of SGI neurons may differently respond to cholinergic inputs. To further elucidate the mechanisms by which cholinergic inputs influence the local circuit activity in the SC, we need to examine the effects of cholinergic agonists and antagonists in other subclasses of SGI neurons, including GABAergic neurons (Lee et al. 2007; Sooksawate et al. 2005a). These results will help us to understand more how the cholinergic inputs to SGI from the parabrachial region of the brain stem modulate the various aspects of SC functions.

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