Postsynaptic Cell Type–Dependent Cholinergic Regulation of GABAergic Synaptic Transmission in Rat Insular Cortex

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1Department of Pharmacology, 2Department of Anesthesiology, and 3Division of Oral and Craniomaxillofacial Research, Dental Research Center, Nihon University School of Dentistry, Tokyo; and 4Functional Probe Research Laboratory, Molecular Imaging Research Program, RIKEN, Kobe, Japan

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Yamamoto K, Koyanagi Y, Koshikawa N, Kobayashi M. Postsynaptic cell type–dependent cholinergic regulation of GABAergic synaptic transmission in rat insular cortex. J Neurophysiol 104: 1933–1945, 2010. First published August 4, 2010; doi:10.1152/jn.00438.2010. The cerebral cortex consists of multiple neuron subtypes whose electrophysiological properties exhibit diverse modulation patterns in response to neurotransmitters, including noradrenaline and acetylcholine (ACh). We performed multiple whole cell patch-clamp recording from layer V GABAergic interneurons and pyramidal cells of rat insular cortex (IC) to examine whether cholinergic effects on unitary inhibitory postsynaptic currents (uIPSCs) are differentially regulated by ACh receptors, depending on their presynaptic and postsynaptic cell subtypes. In fast-spiking (FS) to pyramidal cell synapses, carbachol (10 μM) invariably decreased uIPSC amplitude by 51.0%, accompanied by increases in paired-pulse ratio (PPR) of the second to first uIPSC amplitude, coefficient of variation (CV) of the first uIPSC amplitude, and failure rate. Carbachol-induced uIPSC suppression was dose dependent and blocked by atropine, a muscarinic ACh receptor antagonist. Similar cholinergic suppression was observed in non-FS to pyramidal cell synapses. In contrast, FS to FS/non-FS cell synapses showed heterogeneous effects on uIPSC amplitude by carbachol. In roughly 40% of pairs, carbachol suppressed uIPSCs by 35.8%, whereas in a similar percentage of pairs uIPSCs were increased by 34.8%. Non-FS to FS/non-FS cell synapses also showed carbachol-induced uIPSC facilitation by 29.2% in about half of the pairs, whereas nearly 40% of pairs showed carbachol-induced suppression of uIPSCs by 40.3%. Carbachol tended to increase uIPSC amplitude in interneuron-to-interneuron synapses with higher PPR, suggesting that carbachol facilitates GABA release in interneuron synapses with lower release probability. These results suggest that carbachol-induced effects on uIPSCs are not homogeneous but prepotropic: i.e., cholinergic modulation of GABAergic synaptic transmission is differentially regulated depending on postsynaptic neuron subtypes.

INTRODUCTION

The cholinergic system arising from the basal forebrain (Eckenstein et al. 1988; Rye et al. 1984) plays crucial roles in high-order neocortical brain functions including attention (Yu and Dayan 2005), sleep–wake alternation (Jones 1993), plasticity (Rasmussen and Dykes 1988), and learning and memory (Hasselmo and Bower 1993). Conditioned taste aversion (CTA), in which the subject learns to associate a taste with delayed malaise by administration of an illness-inducing agent such as lithium chloride, is a fascinating paradigm for studying the mechanisms of learning and memory because CTA is acquired by a single trial and its memory is robust and long-lasting (Yamamoto et al. 1994). Relationship between cholinergic inputs to the insular cortex (IC), the primary gustatory cortex, and gustatory processing are well established by studies of behavioral pharmacology and physiology (reviewed by Miranda et al. 2003). Novel tastes increase the concentration of acetylcholine (ACh) in the IC, whereas familiar tastes do not (Miranda et al. 2000). Inactivation of the nucleus basalis magnocellularis, which is one cholinergic source in the basal forebrain, impairs CTA acquisition (Miranda and Bermúdez-Rattoni 1999). Pharmacological manipulation provides further evidence for the involvement of ACh in CTA: injection of the nonselective muscarinic acetylcholine receptor (mAChRs) antagonists, atropine and scopolamine, abolishes CTA acquisition (Berman et al. 2000; Ramirez-Lugo et al. 2003a), which is mimicked by selective mAChR1 (M1) antagonist, pirenzepine (Naor and Dudai 1996; Ramirez-Lugo et al. 2003). Jones et al. (1999) reported that long-term potentiation (LTP) in the IC, which is considered to be at least one neural mechanism of CTA, is modified by activation of mAChRs.

Electrophysiological studies have revealed the suppressive effects of ACh on evoked excitatory postsynaptic potentials (EPSPs) recorded from pyramidal neuron in the neocortex (Kimura and Baughman 1997; Levy et al. 2006). These suppressive effects of ACh are likely to be mediated by presynaptic mAChRs (Kimura and Baughman 1997; Murakoshi et al. 2001), although Levy et al. (2006) reported a nicotinic receptor contribution in addition to mAChRs. Similar results were obtained in the case of inhibitory postsynaptic potentials (IPSPs) or currents (IPSCs): thus ACh decreased the amplitude of evoked IPSPs/IPSCs recorded from pyramidal neurons via presynaptic mAChRs (Kimura and Baughman 1997; Xiao et al. 2009). Another important role of ACh is modulation of neuronal excitability by depolarizing or hyperpolarizing the resting membrane potential. In pyramidal cells and a subset of interneurons, low-threshold spike (LTS) cells, ACh depolarizes the membrane potential and induces spontaneous spike firing, whereas the resting membrane potential of fast-spiking (FS) and late-spiking (LS) interneurons is not affected or hyperpolarized by carbachol (Kawaguchi 1997; Xiang et al. 1998; Xiao et al. 2009). These contradictory effects of ACh among neuron subtypes suggest the possibility that neural mechanisms of cholinergic modulation are pleiotropic.

The present study aimed at testing the hypothesis that inhibitory synaptic transmission mediated by γ-aminobutyric acid (GABA)ergic synapses is differentially regulated by ACh in fast-spiking and pyramidal interneurons located in layer V of rat insular cortex. In fast-spiking to pyramidal cell synapses, carbachol suppressed unitary inhibitory postsynaptic currents (uIPSCs) by 40.3%. Carbachol tended to increase uIPSC amplitude, whereas nearly 40% of pairs showed carbachol-induced suppression of uIPSCs by 35.8%, suggesting that carbachol facilitates GABA release in fast-spiking interneuron synapses with lower release probability. These results suggest that carbachol-induced effects on uIPSCs are not homogeneous but prepotropic: i.e., cholinergic modulation of GABAergic synaptic transmission is differentially regulated depending on postsynaptic neuron subtypes.
acid (GABA) is differentially regulated by cholinergic receptors, depending on their presynaptic and postsynaptic cell subtypes in the IC. We performed multipaired whole cell patch-clamp recording from GABAergic interneurons and pyramidal cells in layer V of the IC and recorded unitary IPSCs (uIPSCs). We found that carbachol suppressed uIPSC amplitude in most pairs of interneurons to pyramidal cells. On the other hand, roughly 40% of inhibitory synapses between interneurons showed uIPSC facilitation by carbachol, although a similar population of interneuron–interneuron pairs showed carbachol-induced suppression of uIPSCs.

METHODS

All experiments were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee in the Nihon University School of Dentistry. All efforts were made to minimize the number of animals used and their suffering.

Slice preparations

The techniques for preparing and maintaining rat cortical slices in vitro were similar to those described previously (Koyanagi et al. 2010). Briefly, vascular GABA transporter (VGAT)-Venus line A transgenic rats (Nagai et al. 2002; Uematsu et al. 2008) of either sex aged from postnatal day 16 (PD16) to PD44 were deeply anesthetized with sodium pentobarbital (75 mg/kg, administered intraperitoneally) and decapitated. Similar to the frontal cortex (Uematsu et al. 2008), >95% of Venus-positive cells in the IC were GABA-immunopositive (data not shown). Tissue blocks including the IC were rapidly removed and stored for 3 min in ice-cold modified artificial cerebrospinal fluid (ACSF, containing in mM): 230 sucrose, 2.5 KCl, 10 MgSO4, 1.25 NaH2PO4, 26 NaHCO3, 2.5 CaCl2, and 10 D-glucose. Coronal slices were cut at 350 μm thickness using a microslicer (Laminicslicer Pro 7; Dosaka EM, Kyoto, Japan). Slices were incubated at 32°C for 40 min in a submersion-type holding chamber that contained 50% modified ACSF and 50% normal ACSF (pH 7.35–7.40). Normal ACSF contained (in mM): 126 NaCl, 3 KCl, 2 MgSO4, 1.25 NaH2PO4, 26 NaHCO3, 2.0 CaCl2, and 10 D-glucose. Coronal slices were cut at 350 μm thickness using a microslicer (Laminicslicer Pro 7; Dosaka EM, Kyoto, Japan). Slices were incubated at 32°C for 40 min in a submersion-type holding chamber that contained 50% modified ACSF and 50% normal ACSF (pH 7.35–7.40). Normal ACSF contained (in mM): 126 NaCl, 3 KCl, 2 MgSO4, 1.25 NaH2PO4, 26 NaHCO3, 2.0 CaCl2, and 10 D-glucose. Modified and normal ACSFs were continuously aerated with a mixture of 95% O2-5% CO2. Slices were then placed in normal ACSF at 32°C for 40 min and only data obtained from electrodes with access resistance of 6–20 MΩ were used for quantification of the effects of carbachol on uIPSCs. The present study included the results obtained from pairs that satisfy the criterion of least-squares regression lines fitted to V–I curves measured at the peak voltage deflection (current pulse amplitude up to −100 pA). The amplitude of the action potential and afterhyperpolarization (AHP) were measured from the action potential threshold. By application of depolarizing step current pulses (300–1,000 ms), rheobase was identified as the minimal current that consistently elicited an action potential. Repetitive firing was evaluated by measuring the maximal firing rate and slope of least-squares regression lines in a plot of the number of spikes versus the amplitude of injected current (F–I curve; up to ~400 pA).

Amplitudes of uIPSCs were measured as the difference between the peak postsynaptic currents and the baseline currents taken from a 2- to 3-ms time window close to the onset of the uIPSCs. Average amplitude, paired-pulse ratio (PPR) of the second to first uIPSC amplitude, coefficient of variation (CV) of the first uIPSC amplitude, and failure rate were calculated from 10–30 consecutive sweeps. uIPSC amplitude in the range of synaptic noise was taken as failure. The 20–80% rise time, 80–20% decay time, and onset latency of uIPSCs were measured from average traces, which were obtained from traces aligned to the peak of presynaptic action potentials.

The dose–response curve of uIPSC amplitude in response to carbachol was fitted by logistic function (Origin 8; OriginLab, Northampton, MA)

\[
E = E_{\text{max}} + \left( E_{\text{min}} - E_{\text{max}} \right) \left\{ 1 + \left[ \frac{[\text{agonist}]}{EC_{50}} \right]^n \right\}^{-1}
\]

where \( E_{\text{max}} \) is the minimal effect, \( E_{\text{max}} \) is the maximal effect, \( EC_{50} \) is the half-maximal effective concentration, and \( n \) is the Hill coefficient.

Data are presented as mean ± SE. The intrinsic electrophysiological properties of interneurons and kinetics of uIPSCs were analyzed using one-way ANOVA followed by post hoc Tukey’s test. In the present study, we recorded uIPSCs under application of normal ACSF for 5–10 min, then applied carbachol for 7.5–10 min, and washed for 10–15 min. To obtain the time course of the amplitude of uIPSCs before, during, and after carbachol application, uIPSC amplitude was normalized to the average of uIPSCs 0–5 min before carbachol application. Typically the last 10–20 events of each period were used for quantification of the effects of carbachol on uIPSCs. The present study included the results obtained from pairs that satisfy the criterion that the difference in uIPSC amplitude of first and last 5–10 events in control was <10%. In each cell pair, Student’s t-test was used to classify the carbachol-induced changes of uIPSC amplitude, i.e., facilitation, suppression, or no change. Comparisons of the uIPSC current-clamp and voltage-clamp recordings were ~9 mV and voltage was corrected accordingly. Thin-wall borosilicate patch electrodes (2–5 MΩ) were pulled on a Flaming-Brown micropipette puller (P-97; Sutter Instrument, Novato, CA).

Recordings were obtained at 30–31°C. Seal resistance was >5 GΩ and only data obtained from electrodes with access resistance of 6–20 MΩ and <20% change during recordings were included in this study. Series resistance was 70% compensated. Before uIPSC recordings, voltage responses of presynaptic and postsynaptic cells were recorded by application of long hyperpolarizing and depolarizing current pulse (300–1,000 ms) injections to examine basic electrophysiological properties, including input resistance, single spike kinetics, voltage–current (V–I) relationship, and repetitive firing pattern and frequency. Since a part of cell pairs had mutual or at least two connections, all cells were recorded under voltage-clamp condition (holding potential = −70 mV) during uIPSC recording. Short depolarizing voltage step pulses (2 ms, 80 mV) were applied to presynaptic cells to induce action currents. Carbachol and atropine were added directly to the perfusate. Membrane currents and potentials were low-pass filtered at 5–10 kHz and digitized at 20 kHz.

Data analysis

Clampfit (pClamp 10; Axon Instruments) was used for analyses of electrophysiological data. Input resistance was measured from slopes of least-squares regression lines fitted to V–I curves measured at the peak voltage deflection (current pulse amplitude up to −100 pA). The amplitudes of the action potential and afterhyperpolarization (AHP) were measured from the action potential threshold. By application of depolarizing step current pulses (300–1,000 ms), rheobase was identified as the minimal current that consistently elicited an action potential. Repetitive firing was evaluated by measuring the maximal firing rate and slope of least-squares regression lines in a plot of the number of spikes versus the amplitude of injected current (F–I curve; up to ~400 pA).

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The dose–response curve of uIPSC amplitude in response to carbachol was fitted by logistic function (Origin 8; OriginLab, Northampton, MA)
amplitude, PPR, and CV between control and drug application were conducted by paired \(t\)-test. Wilcoxon test was used for comparison of failure rate of uIPSCs between control and drug application. Normalized amplitude of the first to fifth uIPSCs was fitted by a single-exponential function (Origin 8; OriginLab). Relationship between carbachol-induced changes of uIPSC amplitude and PPR was analyzed using Pearson’s correlation coefficient test. The level of \(P < 0.05\) was adopted to indicate significance.

**Histology**

To visualize biocytin-labeled neurons after whole cell patch-clamp recording, slices were fixed, cryoprotected, and sectioned (60 \(\mu\)m). Sections were processed using the ABC method (Vector Laboratories, Burlingame, CA) and nickel-intensified dianinobenzidine as the chromogen. The slices were examined microscopically to verify their morphology and location. All chemicals, unless specified otherwise, were purchased from Sigma-Aldrich (St. Louis, MO).

**Results**

To examine cholinergic effects on uIPSCs from interneurons to pyramidal cells or interneurons, dual or triple whole cell patch-clamp recording was performed from Venus-positive GABAergic and Venus-negative pyramidal cells in layer V of the IC. Venus-negative cells with pyramidal somata showed repetitive firing with spike adaptation, as shown in Fig. 1C. Venus-positive interneurons were classified into FS, LTS, LS, and regular-spiking (RS) cells. FS cells were characterized by their low input resistance, short duration of action potential and AHP, and high frequency of repetitive firing (Fig. 1B; Kawaguchi and Kubota 1997; Kobayashi et al. 2008; Koyanagi et al. 2010; Xiang et al. 1998, 2002). Table 1 summarizes electrophysiological properties of IC interneurons, including the resting membrane potential, input resistance, single action potential, and repetitive firing. Cholinergic effects on uIPSCs were not significantly different among LTS, LS, and RS cells, which do not exhibit a fast-spiking firing pattern. In the present study these interneuron subtypes were categorized as “non-FS” cells. Thus uIPSC recordings were classified into six groups: uIPSCs obtained from 1) FS to pyramidal cells (FS \(\rightarrow\) Pyr), 2) FS to FS cells (FS \(\rightarrow\) FS), 3) FS to non-FS cells (FS \(\rightarrow\) non-FS), 4) non-FS to pyramidal cells (non-FS \(\rightarrow\) Pyr), 5) non-FS to FS cells (non-FS \(\rightarrow\) FS), and 6) non-FS cells to non-FS cells (non-FS \(\rightarrow\) non-FS; Table 2). To obtain averaged time course of cholinergic effects on uIPSC amplitude, FS \(\rightarrow\) FS, FS \(\rightarrow\) non-FS, non-FS \(\rightarrow\) FS, and non-FS \(\rightarrow\) non-FS synapses were analyzed as FS to interneurons (FS \(\rightarrow\) interneuron) and non-FS to interneuron (non-FS \(\rightarrow\) interneuron) synapses, respectively.

Carbachol induced slight inward currents in pyramidal neurons (55 ± 14.1 to 105 ± 27.0 pA, \(n = 65\); \(P < 0.01\), paired \(t\)-test), FS (27.0 ± 10.7 to −14.3 ± 12.0 pA, \(n = 21\); \(P < 0.01\), paired \(t\)-test), and non-FS cells (8.7 ± 9.4 to −13.6 ± 10.8 pA, \(n = 29\); \(P < 0.001\), paired \(t\)-test). Input resistance was not significantly affected by carbachol in pyramidal (185.5 ± 11.0 to 189.0 ± 12.0 \(\Omega\), \(n = 54\); \(P > 0.1\), paired \(t\)-test), FS (211.9 ± 23.1 to 214.2 ± 25.5 \(\Omega\), \(n = 22\); \(P > 0.1\), paired \(t\)-test), and non-FS cells (327.2 ± 30.9 to 339.4 ± 35.2 \(\Omega\), \(n = 20\); \(P > 0.1\), paired \(t\)-test). Therefore we did not scale the pulses in the present study.

To examine the properties of short-term dynamics of uIPSCs, five train pulses (2 ms) were applied at 20 Hz to presynaptic cells. uIPSCs were almost completely diminished by bath application of bicusculine methiodide (10 \(\mu\)M), indicating that uIPSCs were mediated by GABA\(_A\) receptors as previously demonstrated (Koyanagi et al. 2010).

**Carbachol suppresses uIPSCs from FS to pyramidal cells**

Figure 1 shows a typical example of the effects of carbachol on uIPSCs. In this case, a presynaptic FS cell (Fig. 1, A and B) was connected to two postsynaptic pyramidal cells (Fig. 1, A and C). Presynaptic action current induced by depolarized voltage pulse injection induced uIPSCs in the postsynaptic pyramidal cells. Bath application of carbachol (10 \(\mu\)M) suppressed the amplitude of uIPSCs in both pyramidal cells and the suppressive effects of carbachol were reversible (Fig. 1, D and E). In 16/16 (100%) FS \(\rightarrow\) Pyr synapses, carbachol consistently suppressed uIPSC amplitude by 51.0 ± 6.2% (Fig. 2A). The dose–response curve of the effect of carbachol shows a typical sigmoid curve (Fig. 2B). By fitting the curve with the logistic function (see METHODS), the following values were obtained: the maximum effect (\(E_{\text{max}}\)), 90.7%; \(E_{\text{calc}}\), 3.7 \(\mu\)M; Hill coefficient, 0.5. Preapplication of 100 \(\mu\)M atropine, a mAChR antagonist, blocked uIPSC suppression by 30 \(\mu\)M carbachol (Fig. 2C), suggesting that carbachol-induced suppression of uIPSCs was mediated by mAChRs.

As shown in Fig. 2D, suppression of uIPSCs by carbachol was accompanied by increases in PPR (0.68 ± 0.04 to 0.82 ± 0.05, \(n = 16\); \(P < 0.01\), paired \(t\)-test) and CV (0.20 ± 0.03 to 0.45 ± 0.06, \(n = 16\); \(P < 0.001\), paired \(t\)-test). The failure rate was also increased by carbachol (0.0 ± 0.0 to 8.8 ± 4.5%, \(n = 16\); \(P < 0.05\), Wilcoxon test). In these cell pairs, the second to fifth uIPSCs were decreased by carbachol (Fig. 2E). Values of the decay time constant of the amplitude of five train uIPSCs were 46.4 ms in control and 97.0 ms in carbachol application. These results suggest that a decrease in uIPSCs by carbachol may be ascribed to presynaptic modulation via mAChRs.

**Carbachol-induced modulation of uIPSCs from FS cells to interneurons**

To obtain uIPSCs from FS cell–interneuron pairs, we performed whole cell patch-clamp recordings only from Venus-positive cells. Figure 3 shows an example of triple whole cell recording from two FS and one RS cell, in which two inhibitory synaptic connections were found. Interestingly, the presynaptic FS cell (FS1; Fig. 3A) exhibited heterogeneous carbachol-induced modulation of uIPSCs, i.e., the FS1 \(\rightarrow\) RS synapse showed carbachol-induced facilitation of uIPSCs, whereas the FS1 \(\rightarrow\) FS2 synapse showed suppression (Fig. 3, B and C). These results suggest that the effects of carbachol on uIPSCs were different depending on the postsynaptic interneuron cell type, even though the uIPSCs were generated by the same presynaptic FS cell. The facilitative or suppressive effects of carbachol on uIPSCs were partially reversible (Fig. 3B).

A summary of the effects of carbachol on uIPSC amplitude in FS \(\rightarrow\) FS/non-FS synapses is listed in Table 2. Carbachol suppressed uIPSCs in 50% of FS \(\rightarrow\) FS synapses and 22.2% of FS \(\rightarrow\) non-FS synapses. The degree of suppression of uIPSC amplitude in FS \(\rightarrow\) FS and FS \(\rightarrow\) non-FS synapses was 32.5 ± 4.7% (\(n = 10\)) and 52.3 ± 28.9% (\(n = 2\)), respectively. In contrast to the case of FS \(\rightarrow\) Pyr synapses, carbachol enhanced
uIPSC amplitude in 30.0% of FS → FS synapses and 55.6% of FS → non-FS synapses. The extent of uIPSC amplitude facilitation was comparable between FS and non-FS synapses (34.9 ± 9.7%, n = 5; P > 0.1, Student’s t-test).

The combined results obtained from FS → FS/non-FS synapses are shown in Fig. 4. The group with carbachol-induced uIPSC facilitation showed an increase in uIPSC amplitude by 34.8 ± 5.4% (n = 11; Fig. 4A), which was accompanied with decreases in PPR (1.10 ± 0.16 to 0.67 ± 0.08, n = 11; P < 0.01, paired t-test), CV (0.78 ± 0.08 to 0.51 ± 0.05, n = 11; P < 0.01, paired t-test; Fig. 4C), and failure rate (26.4 ± 5.9 to 12.4 ± 3.7%, n = 11; P < 0.01, Wilcoxon test). In the facilitation group, the second to fifth uIPSCs were less affected by carbachol (Fig. 4E). The kinetics of normalized amplitude of the first to fifth uIPSCs could not be fitted by single-exponential function. In contrast, the group with carbachol-induced uIPSC suppression showed a decrease in uIPSC amplitude by 35.8 ± 6.6% (n = 12; Fig. 4B), with increases in PPR (0.62 ± 0.05 to 0.79 ± 0.08, n = 12; P < 0.05, paired t-test), CV (0.33 ± 0.04 to 0.58 ± 0.07, n = 12; P < 0.05, paired t-test; Fig. 4D), and failure rate (3.4 ± 1.9 to 16.0 ± 6.3%, n = 12; P < 0.05, Wilcoxon test). In these cell pairs, the second to fifth uIPSCs were less affected by carbachol (Fig. 4F). Values of the decay time constant of the amplitude of five train uIPSCs were 42.4 ms in control and 76.3 ms in carbachol application. These results suggest that increases and decreases in uIPSC amplitudes by carbachol obtained from FS → interneuron synapses are likely attributable to presynaptic modulation.

Carbachol suppresses uIPSCs from non-FS to pyramidal cells

Similar to the case of FS → Pyr synapses, the suppressive effect of carbachol (10 μM) was dominant in non-FS → Pyr synapses: 10/13 (76.9%) pairs showed carbachol-induced suppression, whereas only 1/13 (7.7%) pairs showed uIPSC facilitation (Table 2). The mean amplitude of uIPSC suppression by carbachol was 52.4 ± 5.5% (n = 10; Fig. 5A), which was almost the same degree of suppression as the case of FS-pyramidal cell pairs. The suppressive effect of carbachol could be partially washed out. As shown in Fig. 5B, suppression of uIPSC amplitude by carbachol was accompanied by increases in PPR (0.76 ± 0.09 to 1.05 ± 0.14, n = 10; P < 0.05, paired t-test), CV (0.38 ± 0.05 to 0.69 ± 0.13, n = 10; P < 0.01, paired t-test; Fig. 5B), and failure rate (5.9 ± 2.1 to 22.0 ± 7.3%, n = 10; P < 0.05, Wilcoxon test). In these cell pairs, the second to fifth uIPSCs were also decreased by carbachol (Fig. 5C). The values of decay time constant of the amplitude of five train uIPSCs were 43.8 ms in control. The kinetics of normalized amplitude of the first to fifth uIPSCs in carbachol application could not be fitted by a single-exponential function. These results suggest that a decrease in uIPSCs in non-FS → Pyr synapses is attributable, at least in part, to presynaptic mechanisms.

Carbachol-induced modulation of uIPSCs from non-FS cells to interneurons

Figure 6 shows an example of dual whole cell recording of an RS → FS synapse. Carbachol (10 μM) reversibly enhanced
the amplitude of uIPSCs (Fig. 6, D and E). In non-FS → FS synapses, 6/11 (54.5%) pairs showed carbachol-induced suppression, whereas the other 5/11 (45.5%) synapses showed facilitation (Table 2). The extent of facilitation and suppression of uIPSC amplitudes in non-FS → FS synapses were 28.0 ± 8.0 and 43.6 ± 8.1%, respectively. Some non-FS → non-FS synapses also showed carbachol-induced uIPSC facilitation: 4/6 (66.7%) and 1/6 (16.7%) pairs showed no change and suppression of uIPSCs by carbachol, respectively (Table 2).

The extents of both facilitation and suppression of uIPSC amplitude in non-FS → non-FS synapses were 30.6 ± 9.1 and 20.4% (n = 1), respectively.

Table 2. Effects of carbachol (10 μM) on unitary IPSCs obtained from FS/non-FS cell to pyramidal cells/interneurons

<table>
<thead>
<tr>
<th>Presynaptic Neuron</th>
<th>Postsynaptic Neuron</th>
<th>Suppression</th>
<th>No Change</th>
<th>Facilitation</th>
</tr>
</thead>
<tbody>
<tr>
<td>FS</td>
<td>Pyramidal</td>
<td>16/16 (100.0)</td>
<td>0/16 (0.0)</td>
<td>0/16 (0.0)</td>
</tr>
<tr>
<td>FS</td>
<td>FS</td>
<td>10/20 (50.0)</td>
<td>4/20 (20.0)</td>
<td>6/20 (30.0)</td>
</tr>
<tr>
<td>FS</td>
<td>Non-FS</td>
<td>2/9 (22.2)</td>
<td>2/9 (22.2)</td>
<td>5/9 (55.6)</td>
</tr>
<tr>
<td>Non-FS</td>
<td>Pyramidal</td>
<td>10/13 (76.9)</td>
<td>2/13 (15.4)</td>
<td>1/13 (7.7)</td>
</tr>
<tr>
<td>Non-FS</td>
<td>FS</td>
<td>6/11 (54.5)</td>
<td>0/11 (0.0)</td>
<td>5/11 (45.5)</td>
</tr>
<tr>
<td>Non-FS</td>
<td>Non-FS</td>
<td>1/6 (16.7)</td>
<td>1/6 (16.7)</td>
<td>4/6 (66.7)</td>
</tr>
</tbody>
</table>

Suppression: a group showing uIPSC decrease in amplitude. No change: a group showing no significant change of uIPSCs. Facilitation: a group showing uIPSC increase (Student’s t-test). FS, fast-spiking cell; non-FS, non-fast-spiking cell.

Figure 7 shows the combined results obtained from non-FS → FS and non-FS → non-FS synapses. The group with carbachol-induced uIPSC facilitation showed an increase in uIPSC amplitude by 29.2 ± 6.0% (n = 9; Fig. 7A), which was accompanied with decreases in PPR (0.85 ± 0.10 to 0.64 ± 0.08, n = 9; P < 0.01, paired t-test), CV (0.55 ± 0.09 to 0.35 ± 0.07, n = 9; P < 0.01, paired t-test).
FIG. 3. Carbachol-induced pleiotropic effects on uIPSC amplitude obtained from 2 interneuron pairs. A: triple whole cell patch-clamp recording from 2 FS (FS1 and FS2) and a regular-spiking (RS) neuron in layer V IC. Repetitive firing responses induced by depolarizing/hyperpolarizing current pulse injection (300 ms) are shown. RS cell exhibited regular spike firing with adaptation in response to depolarizing current pulses and rebound action potential responding to hyperpolarizing current pulses. FS1 and FS2 cells showed repetitive firing with less adaptation to depolarizing pulses. B: time course of carbachol (10 μM)-induced uIPSC increase in RS cell (top) and decrease in FS2 cell (bottom). Lines indicate local averages of 6 adjacent points. C: uIPSC recordings from RS and FS2 responding to injection of 5 train pulses to FS1 cell (20 Hz; top trace) before (Control), during (Carbachol), and after (Wash) carbachol application. All interneurons were recorded under voltage-clamp condition (holding potential: −70 mV). Ten consecutive traces (gray) and their average traces (black) are shown. Note that RS and FS2 cells showed different effects of carbachol on uIPSCs.
of normalized amplitude of the first to fifth uIPSCs could be fitted by a single-exponential function and decay time constants were 67.9 and 34.1 ms in control and carbachol application, respectively. *P < 0.05, **P < 0.01, paired t-test. 

0.38 ± 0.05, n = 9; P < 0.05, paired t-test; Fig. 7C), and failure rate (25.8 ± 10.1 to 8.9 ± 4.3%, n = 9; P < 0.05, Wilcoxon test). In the facilitation group, the second to fifth uIPSCs were less affected by carbachol (Fig. 7E). The kinetics of normalized amplitude of the first to fifth uIPSCs could be fitted by a single-exponential function and decay time constants were 67.9 and 34.1 ms in control and carbachol application, respectively. *P < 0.05, **P < 0.01, paired t-test. 

Carbachol-induced uIPSC suppression showed a decrease in uIPSC amplitude by 40.3 ± 7.6% (n = 7; Fig. 7B). In non-FS →
interneuron synapses with carbachol-induced uIPSC suppression, PPR was significantly increased from 0.52 ± 0.11 to 0.85 ± 0.08 (n = 7; P < 0.05, paired t-test; Fig. 7D). CV and failure rate were also significantly increased from 0.36 ± 0.07 to 0.52 ± 0.08 (n = 7; P < 0.01, paired t-test; Fig. 7D) and 12.9 ± 7.5 to 30.0 ± 13.7%, n = 7; P < 0.05, Wilcoxon test), respectively. In these synapses, the second to fifth uIPSCs were less affected by carbachol (Fig. 7F). Values of the decay time constant of the amplitude of five train uIPSCs were 30.4 ms in control and 77.4 ms in carbachol application. These results suggest that both increases and decreases in uIPSCs by carbachol obtained from non-FS → interneuron synapses are likely attributable to presynaptic modulation.

**FIG. 6.** Carbachol-induced pleiotropic effects on uIPSC amplitude obtained from non-FS → FS synapses. A: dual whole cell patch-clamp recording from RS and FS neurons in layer V IC. B: repetitive firing responses induced by depolarizing/hyperpolarizing current pulse injection (300 ms) are shown. The RS cell exhibited regular spike firing to depolarizing current pulses (a) and sag and rebound action potential responding to hyperpolarizing current pulses (b). C: the FS cell showed repetitive firing without adaptation to depolarizing current pulses. D: time course of carbachol (10 μM)-induced uIPSC increase in the FS cell. The line indicates local averages of 6 adjacent points. E: uIPSC recordings from the FS cell responding to injection of 5 train pulses to the RS cell (20 Hz; top trace) before (Control), during (Carbachol), and after (Wash) carbachol application. Both presynaptic RS and postsynaptic FS cells were recorded under voltage-clamp condition (holding potential: −70 mV). Ten consecutive traces (gray) and their average traces (black) are shown.
As previously reported, the frequency of the spontaneous IPSCs (sIPSCs) was increased by application of carbachol (Kawaguchi 1997; Kondo and Kawaguchi 2001; Xiao et al. 2009). Consistent with these reports, an increase in the frequency of sIPSCs was observed in both pyramidal cells (Fig. 7A) and interneurons (Fig. 6E), although we did not perform quantitative analysis in the present study.

Relationship between effects of carbachol on uIPSC amplitude and paired-pulse ratio

To explore the mechanism of variation of carbachol-induced uIPSC modulation from FS/non-FS cells to interneurons, the normalized amplitude of uIPSCs during carbachol application was plotted against PPR under control conditions before carbachol application. Both in uIPSCs obtained from FS → interneuron and non-FS → interneuron synapses, there were significant correlations between normalized amplitude of uIPSCs during carbachol application and PPR in control conditions \( r = 0.37 \) in FS → interneuron synapses, \( P < 0.05 \), and \( r = 0.64 \) in non-FS → interneuron synapses, \( P < 0.01 \), Pearson’s correlation coefficient test; Fig. 8). These results suggest that carbachol tends to potentiate uIPSCs in inhibitory synapses with lower release probability, whereas uIPSCs with higher release probability tend to be suppressed by carbachol.

Since \( \beta \)-adrenocceptor modulation of uIPSC amplitude is age dependent in the IC (Koyanagi et al. 2010), there is a possibility that the effects of carbachol were age dependent. To explore this possibility, correlation between the normalized amplitude of uIPSCs during carbachol application and age was examined. Correlation coefficients in FS → Pyr, non-FS → Pyr, FS → interneuron, and non-FS → interneuron synapses were 0.27, 0.12, 0.18, and 0.26, respectively (Supplemental Fig. S1; \( P > 0.1 \), Pearson’s correlation coefficient test), indicating that there were no significant correlations between the normalized amplitude of uIPSCs and age.1

**DISCUSSION**

In the present study, we performed whole cell patch-clamp recordings to examine the effects of carbachol on uIPSCs in the IC. Carbachol suppressed uIPSC amplitude in most FS/non-FS → Pyr synapses, and 41.3% of FS/non-FS → interneuron synapses also showed carbachol-induced suppression of uIPSCs. In contrast, 43.5% of inhibitory synapses between interneurons showed uIPSC facilitation by carbachol and the PPR of these synapses tended to be higher than those showing carbachol-induced suppression of uIPSCs. Triple whole cell recordings showed that

1 The online version of this article contains supplemental data.
carbachol did not necessarily induce similar effects on uIPSCs obtained from two postsynaptic interneurons, even though these uIPSCs were induced by activation of the same presynaptic interneuron. Thus the present study suggests that carbachol modulates uIPSCs depending on postsynaptic cell subtypes and that presynaptic interneurons identify their targeted postsynaptic neurons and express presynaptic molecules specific to postsynaptic cell subtypes.

**Cholinergic modulation of inhibitory synapses from FS/non-FS to pyramidal cells**

Cholinergic effects on the glutamatergic excitatory synaptic transmission are considered to be predominantly suppressive. Cholinergic agonists including ACh and carbachol effectively suppress evoked EPSPs/EPSCs recorded from pyramidal cells in the cerebral cortex (Kimura and Baughman 1997; Levy et al. 2009; Murakoshi et al. 2001). These studies have provided the evidence that a decrease in EPSP/EPSCs is accompanied by an increase in PPR and are blocked by atropine, suggesting that cholinergic suppression of EPSP/EPSCs is mediated by presynaptic mAChRs. Concerning the cholinergic modulation of inhibitory synaptic transmission to pyramidal cells, previous studies have reported that the amplitude of evoked IPSPs/IPSCs is suppressed by ACh/carbachol via mAChRs (Kimura and Baughman 1997; Xiao et al. 2009). With cholinergic IPSP/IPSC suppression, presynaptic mechanisms may be involved because the suppression is accompanied by a proportional decrease in $1/CV^2$ and a decrease in miniature IPSC frequency. Our results corroborate these previous reports and extended them by demonstrating that the cholinergic suppressive effects on uIPSCs were consistent regardless of presynaptic interneuron subtypes, FS or non-FS cells. GABAergic presynaptic terminals on pyramidal cells may share a common mechanism of cholinergic modulation with glutamatergic terminals. Although it is less likely, we cannot rule out the possibility that postsynaptic mechanisms could be partially involved in carbachol-induced uIPSC suppression, since a minor part of synapses showed uIPSC suppression with decreases in PPR (Figs. 2D, 4D, 5B, and 7D); e.g., an increase in spontaneous EPSC conductance via activation of ionotropic glutamate receptors by carbachol possibly decreases input resistance, which in turn would decrease the amplitude of uIPSCs.

**Cholinergic modulation of inhibitory synapses among interneurons**

In contrast to the consistent cholinergic suppression of glutamatergic synaptic transmission, the present study revealed that cholinergic effects on uIPSCs recorded from interneurons were pleiotropic. The presumed function of inhibition of inhibitory interneurons might result in a net facilitatory effect on excitability of cortical local circuits, which may coordinate with cholinergic suppression of uIPSCs in pyramidal cells. On the other hand, reduced uIPSC amplitudes in interneurons might increase their activity and thereby suppress excitability of cortical local circuits, in contrast to uIPSC suppression in pyramidal cells. Thus the overall physiological significance of these reciprocal cholinergic effects on uIPSCs recorded from interneurons is difficult to predict. It is noteworthy that facilitation of uIPSCs tended to occur in synapses with higher PPR values, which functionally means lower release probability (Fig. 8). In other words, inhibitory local circuits with less impact on excitability of local circuits could elicit excitation via a disinhibiting mechanism of interneurons by ACh.

Voltage-gated calcium channels (VGCCs) are attractive candidates for mediating cholinergic modulation of uIPSCs in the cerebral cortex. Although the glutamatergic presynaptic terminals of pyramidal cells express both N- and P/Q-type calcium channels, which trigger glutamate release (Ladera et al. 2009; Millán and Sánchez-Prieto 2002), it is controversial which types of VGCCs exist in the synaptic terminals of each interneuron subtype. Zaitsev et al. (2007) reported that GABAergic terminals of FS cells express only P/Q-type calcium channels in the cerebral cortex, similar to those in the hippocampus (Hefft and Jonas 2005), whereas there is a report that N-type
calcium channels play a major role in releasing GABA in fast-spiking GABAergic interneurons (Ali and Nelson 2006). Although G protein-mediated inhibition of N-type calcium channels is more potent than that of P/Q-type calcium channels (Currie and Fox 1997), Salgado et al. (2007) reported that activation of presynaptic mAChRs suppresses evoked IPSCs by reducing calcium influx via both N- and P/Q-type VGCCs of pyramidal cells in the auditory cortex. Consistent suppression of uIPSCs obtained from FS/non-FS Pyr synapses in the present study is in line with their study. However, the mechanism of facilitatory effect on uIPSCs by carbachol remains an open issue. It is also mysterious how interneuron terminals discriminate postsynaptic cells. It may be reasonable to speculate that the cholinergic system interacts with retrograde messengers such as nitric oxide, cannabinoids, and arachidonic acid (Regehr et al. 2009). These retrograde messengers may contribute to facilitation of GABA release from presynaptic terminals. If postsynaptic cells release these retrograde messengers depending on their cell subtypes, presynaptic interneurons may discriminate the type of postsynaptic neurons.

The increase in amplitude of uIPSCs by carbachol was followed by a slight inhibition during the washout of carbachol. Even though we excluded the results of uIPSCs that were accompanied by rundown during control recordings (Bennett et al. 1998), there is a possibility that rundown in a later recording period might prevent a recovery from the suppressive effect of carbachol. Another possibility is the biphasic effects of carbachol on uIPSCs, since in chick lateral spiriform neurons, activation of nicotinic ACh receptors leads to the biphasic effects on spike firing rate (Liu et al. 2007).

Functional implications

During carbachol application, the spontaneous synaptic inputs frequently disrupted uIPSC recording from pyramidal cells (Fig. 1E). The increase in spontaneous IPSC frequency was also observed in postsynaptic interneurons (Fig. 6E). These findings are consistent with previous studies in the cerebral cortex (Kawaguchi 1997; Kondo and Kawaguchi 2001; Xiang et al. 1998; Xiao et al. 2009). A mechanism of cholinergic increase in sIPSC frequency is considered to be due to depolarization of the resting membrane potential of a subset of interneurons, often causing spontaneous spike firing (Kawaguchi 1997; Xiang et al. 1998). Taking into account the finding that ACh suppresses AHP (Gulledge et al. 2009), cholinergic inputs could play a role in shaping and refinement of spatial and temporal patterns of excitation/inhibition in the cortical local circuits (Kimura et al. 1999) and switch the mode of excitation and inhibition in the IC. Kondo and Kawaguchi (2001) reported that application of muscarine induces periodical slow synchronized inhibition in about a quarter of neurons of the frontal cortex, whereas the other three quarters of neurons show facilitation of tonic inhibition. Functionally, ACh controls the sleep–wake cycle and sleep pattern alteration (Steriade 2004). The frequency of electroencephalography changes dramatically between slow oscillations during sleep stages and high-frequency oscillations during awake stages, including attention (Yu and Dayan 2005). Thus cholinergic inputs may regulate switching that contributes to physiological function such as encoding information converging on the IC.

The IC plays a critical role in taste aversion learning, a classical conditioning in which a single pairing of a novel taste and subsequent internal malaise prevents animals from repeated ingestion of the taste (Bermúdez-Rattoni 2004; Yamamoto 1994). Previous anatomical studies have demonstrated that the disgranular IC receives gustatory afferents from the parvicellular part of the postero medial ventral thalamic nucleus and the adjacent area of the disgranular IC, the granular IC receives visceral inputs including gastrointestinal mechanoreceptor information from the ventroposterolateral parvicellular thalamic nucleus (Allen et al. 1991; Nakashima et al. 2000). In addition, limbic afferents from the amygdala and cingulate cortex project to the IC (Allen et al. 1991; Krettek and Price 1977). Therefore visceral sensation, gestation, and limbic information are likely to be integrated in the IC during acquisition of taste aversion learning. The present study provides basic mechanisms of cholinergic effects on inhibitory synaptic transmission in the IC and we believe that our findings contribute to elucidation of mechanisms of taste aversion learning for the following reasons. First, cholinergic modulation is included in the relevant neural circuits (Miranda et al. 2003) because blockade of mAChRs by atropine, scopolamine, or pirenzepine also disrupts taste aversion learning (Berman et al. 2000; Naor and Dudai 1996; Ramirez-Lugo et al. 2003). Second, mAChRs modulate induction of LTP in the IC (Jones et al. 1999), which is considered to be a key physiological phenomenon in learning and memory. Finally, GABA-mediated inhibition of neural excitability has been considered to play a crucial role in defining the sensitive period of ocular dominance plasticity in the primary visual cortex (Hensch 2005) and manipulation of the GABAergic system restores neural plasticity in adult cerebral cortex (Sale et al. 2007).

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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