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Research Report

Cholinergic modulation of local pyramid–interneuron synapses exhibiting divergent short-term dynamics in rat sensory cortex

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ABSTRACT

Acetylcholine (ACh) influences attention, short-term memory, and sleep/waking transitions, through its modulatory influence on cortical neurons. It has been proposed that behavioral state changes mediated by ACh result from its selective effects on the intrinsic membrane properties of diverse cortical inhibitory interneuron classes. ACh has been widely shown to reduce the strength of excitatory (glutamatergic) synapses. But past studies using extracellular stimulation have not been able to examine the effects of ACh on local cortical connections important for shaping sensory processing. Here, using dual intracellular recording in slices of rat somatosensory cortex, we show that reduction of local excitatory input to inhibitory neurons by ACh is coupled to differences in the underlying short-term synaptic plasticity (STP). In synapses with short-term depression, where successive evoked excitatory postsynaptic potentials (EPSPs; >5 Hz) usually diminish in strength (short-term depression), cholinergic agonist (5–10 μM carbachol (CCh)) reduced the amplitude of the first EPSP in an evoked train, but CCh's net effect on subsequent EPSPs rapidly diminished. In synapses where successive EPSPs increased in strength (facilitation), the effect of CCh on later EPSPs in an evoked train became progressively greater. The effect of CCh on both depressing and facilitating synapses was blocked by the muscarinic antagonist, 1–5 μM atropine. It is suggested that selective influence on STP contributes fundamentally to cholinergic "switching" between cortical rhythms that underlie different behavioral states.

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Abbreviations: ACh, acetylcholine; ACSF, artificial cerebrospinal fluid; AI, adaptation index; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor; AP, action potential; CCh, carbachol; DAB–HRP, 3,3-diaminobenzidine/horseradish peroxidase; D-AP5, D-2-amino-5-phosphonovaleric acid; DNQX, 6,7-dinitroquinoxaline-2,3 (1H,4H)-dione; EPSP, excitatory postsynaptic potential; FS, fast-spiking interneuron; IPSP, inhibitory postsynaptic potential; IR-DIC, infrared differential interference contrast; LTS, low-threshold-spiking interneuron; NMDA, *N*-methyl-D-aspartate; NMDAR, *N*-methyl-D-aspartate receptor; P, pyramidal neuron; PPR, paired-pulse ratio; R_{in}, input resistance; SRA, spike rate adaptation; STP, short-term plasticity; V_m, membrane potential

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1. Introduction

Acetylcholine (ACh) promotes attention and arousal by shaping the flow of excitation in sensory cortex (Descarries et al., 2004; Sarter et al., 2005). One mechanism for ACh's action may be its selective effects on diverse types of cortical inhibitory interneurons. These cells belong to many overlapping morphological, physiological and immunocytochemical classes. In practice they are typically divided into two broad groups, fast-spiking (FS) cells that display high maximum firing rates (>100 Hz), and non-fast-spiking (non-FS) cells that fire at <100 Hz and display some degree of spike rate adaptation (SRA) in response to prolonged current injection *in vitro*. FS cells are a relatively homogeneous group while non-FS cells comprise a wide variety of cell types that are difficult to classify by any one scheme (Kawaguchi and Kubota, 1997; Gupta et al., 2000; Markram et al., 2004). ACh has been reported to selectively excite several types of non-FS (relative to FS) cells, potentially shifting cortical inhibition toward a state dominated by activity of non-FS cells (Gulledge et al., 2007; Kawaguchi, 1997; Porter et al., 1999; Xiang et al., 1998).

The activity of cortical interneurons is also affected by excitatory input, most of which comes from nearby pyramidal cells. ACh weakens excitatory transmission in numerous cortical pathways (but selective enhancement of some pathways has been reported (see Aramakis and Metherate, 1998; Gil et al., 1997)); however, it has not been possible to examine modulation of local connections between specific excitatory and inhibitory neurons in defined cortical layers in past studies using extracellular stimulation of afferents. A striking feature of local pyramid–interneuron synapses is their varied dynamics: when action potentials (APs) are evoked by repetitive stimulation (>5 Hz) of a presynaptic pyramidal cell, EPSPs in some target cells are initially strong, but successively weaken (depress); EPSPs in other target cells are initially weak, but grow stronger (facilitate). Collectively, this phenomenon is known as short-term plasticity (STP). The form of STP is correlated with target cell type; EPSP depression is correlated with multipolar cell shape and fast-spiking behavior, while facilitation is correlated with bipolar/bitufted cell shape and non-FS, adapting firing patterns (Ali and Nelson, 2005; Markram et al., 1998; Reyes et al., 1998; Thomson et al., 1994; but see Angulo et al., 1999, 2003); conversely, axon terminals arising from a single presynaptic pyramidal cell may form either depressing or facilitating synapses, depending on target cell type (Reyes et al., 1998). Although many studies have addressed the functional significance of ACh-dependent changes in synaptic strength, the interrelationship between these changes and STP has rarely been considered (for an exception, see Tsodyks and Markram, 1997). Here we used dual intracellular recording in slices of rat somatosensory cortex to examine effects of cholinergic agonist on both depressing and facilitating pyramid–interneuron synapses. We found that cholinergic effects are coupled to

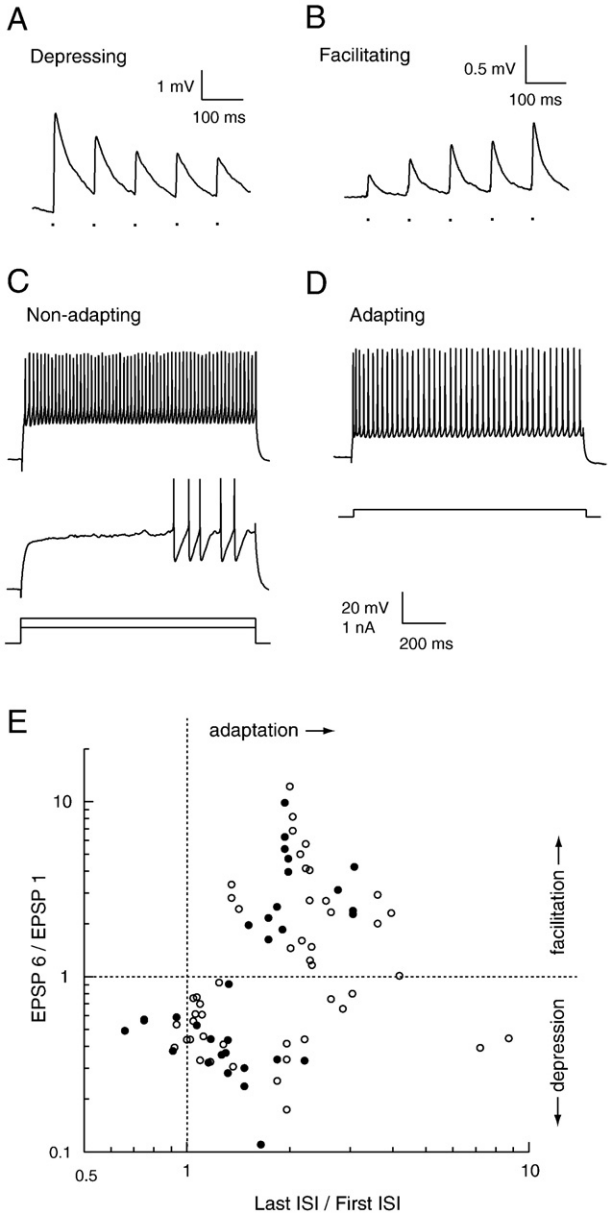

Fig. 1 – Correlation between intrinsic firing properties and synaptic dynamics. A,B, Synaptic dynamics. Repetitive evoked presynaptic APs (10 Hz) generate EPSPs (average traces, 100 trials) that successively depress (A) or facilitate (B). Dots indicate AP times. Resting V_m = −59 mV (A), −60 mV (B). C,D, Intracellular voltage traces evoked by 1 s depolarizing current steps. C, Example of a non-adapting, fast-spiking (FS) inhibitory cell; note sustained high-frequency firing (upper trace), and type II onset (lower trace). D, inhibitory cell displaying spike rate adaptation. Resting V_m = −69 mV (C), −70 mV (D). E, Correlation between firing properties and synaptic dynamics. X axis shows ratio of last to first ISI elicited by current injection (adaptation index, as defined in Experimental Procedures; c.f. panels C,D). Y axis shows the ratio of peak amplitudes of the last versus the first EPSP in a 20 Hz evoked train. Dashed lines denote X = 1 (no adaptation), Y = 1 (no short-term plasticity), respectively. Each open circle represents one cell pair from the entire data set (*n* = 104); filled circles denote pairs in the carbachol data set (Fig. 2C,D and 3A,D; *n* = 37). Three postsynaptic cells that fired initial bursts and two with irregular steady-state firing rates (stuttering cells; c.f. Gupta et al., 2000) were omitted from the analysis.

STP, such that local synaptic excitation of cells receiving facilitating input may be selectively turned down by ACh during periods of high activity.

2. Results

2.1. *Correlation of synaptic properties, firing properties, and morphology*

Unitary EPSPs evoked by stimulation of a presynaptic pyramidal cell were abolished by bath application of 10 μM DNQX (AMPAR antagonist) in conjunction with 50 μM D-AP5 (NMDAR antagonist), indicating that the connections were glutamatergic. In about half of all identified pairs, the mean amplitude of the largest EPSP in an evoked train (at least 5 successive EPSPs at 20 Hz) was <0.2 mV. These pairs were not analyzed because of poor signal:noise ratio. In the remaining connected pairs, mean amplitude of the first EPSP in an evoked train was 0.79+/−0.98 mV (n=104 pairs). The subsequent EPSPs in an evoked train (20 Hz) depressed in 58 pairs; the remainder facilitated (Fig. 1A–B,E).

Interneurons showed varying degrees of spike rate adaptation. This was quantified as adaptation index (AI; ratio of first to last ISI in a train of spikes; Fig. 1C,D; c.f. Experimental procedures, Table 1). AI was positively correlated with maximum firing rate (r=0.70; p<0.0001, n=82; not shown). Cells that received facilitating synapses displayed significantly more spike rate adaptation than those that received depressing synapses (Fig. 1E; Table 1). The degree of depression or facilitation was not significantly correlated with the age of the slice over the range of ages tested (11–26 days; not shown) although a significant age related shift toward facilitation in depressing synapses has been documented elsewhere (Reyes and Sakmann, 1999) and pyramid–FS synapses from 28–52 day-old rats showed a mix of depression and facilitation (Angulo et al., 1999).

A random subset of synaptically connected pairs was filled with biocytin to allow later classification of cells by light microscopy after slice fixation (see Experimental procedures). Bipolar/bitufted cells displayed more spike rate adaptation (AI=2.67+/−1.67; n=18, including 3 Martinotti cells) than multipolar cells (AI=1.60+/−0.95; n=16, including basket and one chandelier cell). This difference was significant (p<0.05, two-tailed unpaired t-test; two cells that fired initial bursts, one bipolar/bitufted and one multipolar, were excluded). Cells that received depressing synapses were usually multipolar

Table 1 – Summary of results

		Depressing synapses:	Facilitating synapses:
Cell shape:	Multipolar:	13/22 [1]	4/14
	Bipolar/bitufted:	9/22 [2]	10/14 [3]
	Adaptation index (control):	1.59+/−1.33 [4] (58)*	2.22+/−0.65 [4] (46)*
Synaptic properties (control):	EPSP1, mV:	1.49+/−1.22 (22)	0.25+/−0.22 (15)
	EPSP2, mV:	0.82+/−0.63	0.41+/−0.26
	EPSP3, mV:	0.66+/−0.51	0.51+/−0.36
	EPSP4, mV:	0.61+/−0.49	0.66+/−0.44
	EPSP5, mV:	0.61+/−0.48	0.78+/−0.55
	EPSP6, mV:	0.56+/−0.45	0.90+/−0.59
	PPR (EPSP2/EPSP1):	0.62+/−0.19	2.03+/−0.85
Synaptic properties (carbachol):	EPSP1, mV:	0.97+/−0.78*	0.20+/−0.24
	EPSP2, mV:	0.57+/−0.42*	0.23+/−0.26*
	EPSP3, mV:	0.52+/−0.36*	0.31+/−0.32*
	EPSP4, mV:	0.46+/−0.32*	0.37+/−0.42*
	EPSP5, mV:	0.49+/−0.36	0.46+/−0.49*
	EPSP6, mV:	0.47+/−0.33	0.53+/−0.49*
	PPR (EPSP2/EPSP1):	0.64+/−0.16	1.27+/−0.89*
Intrinsic properties (carbachol):	R_{in}, normalized to control:	0.98+/−0.09 (5)	1.15+/−1.12 [5] (6)*
	V_m, mV, relative to control:	1.57+/−2.88 (17)	3.82+/−2.60 [5] (8)*

Data are classified according to synaptic dynamics (depressing vs. facilitating) under control conditions. Values are given as mean+/−S.D., with number of cells or pairs in parentheses.
* Indicates significance (p<0.05) relative to control value, except as noted.
[1] i.e., 13/22 morphologically characterized cells receiving depressing synapses were multipolar. Includes 21 basket cells and one chandelier cell.
[2] Includes one Martinotti cell.
[3] Includes two Martinotti cells.
[4] Depressing significantly different from facilitating.
[5] Excludes cells where carbachol produced >0.5 Hz non-evoked firing (~one third of all cells).

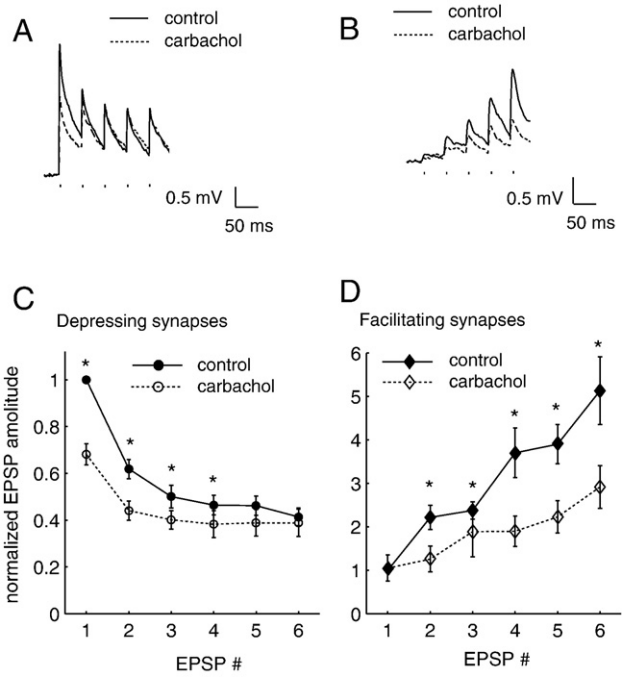

Fig. 2 – Effect of cholinergic agonist on depressing (A,C) and facilitating (B,D) synapses. A,B, examples of average postsynaptic responses evoked by 20 Hz presynaptic APs in control conditions (solid traces) and in the presence of bath-applied carbachol (5 μM, 5 min, dashed traces). Resting V_m=−66 mV (A), −64 mV (B). C,D, pooled data for all pairs. Mean and S.E.M. are shown for control (filled symbols, solid lines) and carbachol (open symbols, dashed lines) for 6 successive EPSPs (10 Hz), normalized to the first control EPSP (EPSP1) in each train. For values, see Table 1. Asterisks indicate significance (p<05) for carbachol vs. control. n=22 pairs, (C), 15 pairs (D).

while those that received facilitating synapses were usually bipolar/bitufted (Table 1; Reyes et al., 1998; c.f. Gupta et al., 2000). Over 30 presynaptic excitatory cells were recovered; all were pyramidal.

2.2. *Synaptic effects of cholinergic agonist*

EPSPs were evoked in the presence of carbachol (CCh), a non-hydrolyzable ACh analogue that acts on both nicotinic and muscarinic receptors (Brown and Taylor, 1996) but has produced mainly muscarinic effects in many *in vitro* reports (e.g. Auerbach and Segal, 1996; Fernandez de Sevilla et al., 2002; Levy et al., 2006). Depressing and facilitating synapses were analyzed separately. CCh (5–10 μM bath-applied for 3–5 min while holding resting V_m constant) reduced average peak amplitude of both depressing and facilitating EPSPs (Fig. 2A,B, compare dashed to solid traces). However, the effect on successive EPSPs in an evoked train differed between the depressing and facilitating groups. In depressing synapses, the first EPSP in the train (EPSP1) was most affected (Fig. 2A,C). As successive EPSPs depressed toward a short-term steady state, the CCh EPSP amplitudes converged with control EPSP amplitudes. In facilitating synapses, the last EPSP in the train was reduced the most by carbachol (Fig. 2B,D).

Reduction of the largest-amplitude EPSPs by CCh was significant for both depressing and facilitating pairs (Fig. 2C,D and Fig. 3A,D). The ratio of the second to the first EPSP in an evoked train (EPSP 2 / EPSP 1; a.k.a. paired-pulse ratio; PPR) was not significantly affected by CCh in depressing synapses, but was significantly reduced in facilitating synapses (Table 1). The effect of CCh on EPSP amplitude was not significantly correlated with the age (in postnatal days) of the slice.

EPSP reduction was reversible, on average, for both depressing and facilitating synapses (Fig. 3A,D), and did not occur if vehicle (ACSF) without CCh was applied over the same time course (Fig. 3B,E). CCh also was applied in the presence of

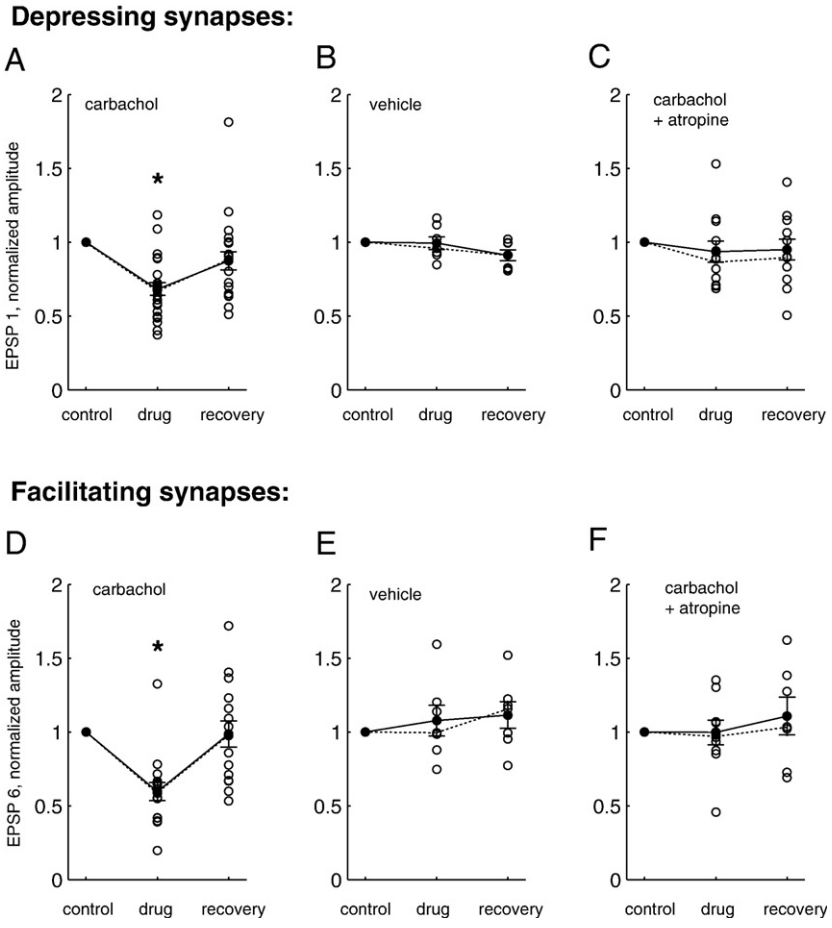

Fig. 3 – Cholinergic effect is reversible and atropine-sensitive. Each panel shows normalized EPSP amplitude before (control), during agonist application (drug), and after washout (recovery). Data are shown for the EPSP of largest average amplitude for each synaptic type: EPSP 1 for depressing (A–C), and EPSP 6 for facilitating synapses (D–F). Open circles denote average values for individual cell pairs; solid lines and filled circles with error bars denote mean and S.E.M.; dashed lines represent median values (in A and D mean and median are nearly superimposed). A,D, effect of 5 μM carbachol alone. Values are: A, drug, 0.68 +/− 0.05 (n = 22), recovery, 0.87 +/− 0.06 (n = 21); D, drug, 0.60 +/− 0.06 (n = 15), recovery, 0.99 +/− 0.09 (n = 15). B,E, effect of vehicle (ACSF) applied without any agonist. Values are B, drug, 0.99 +/− 0.04 (n = 7), recovery, 0.91 +/− 0.04 (n = 7). C,F, effect of 5 μM carbachol applied in the presence of atropine (1–5 μM); data are normalized to control value for atropine alone. Values are: C, drug, 0.94 +/− 0.07 (n = 12), recovery, 0.95 +/− 0.07(n = 12); F, drug, 1.00 +/− 0.08 (n = 10); recovery, 1.11 +/− 0.13 (n = 7). Asterisks indicate significance relative to control (p < 0.0001, A and D).

the muscarinic receptor antagonist, atropine (1 or 5 μM; results did not differ significantly among concentrations and were analyzed together). Atropine prevented CCh's reduction of both depressing and facilitating EPSPs by CCh (Fig. 3C,F).

Cholinergic agonists affect intrinsic membrane properties as well as synaptic properties. In layer 5 of rat visual cortex, locally applied ACh was reported to hyperpolarize FS cells via muscarinic receptor activation and to depolarize low-threshold spiking cells (LTS; the main non-FS subclass) via nicotinic receptor activation (Xiang et al., 1998). Other studies have reported that FS cells are relatively insensitive to ACh (Gulledge et al., 2007; Kawaguchi, 1997; Porter et al., 1999). ACh can also increase cell input resistance (R_{in}) via muscarinic receptor-mediated shutdown of several classes of ion channels (McCormick and Prince, 1986). In the present study, CCh produced a significant depolarization of cells in both the depressing and the facilitating groups, but the magnitude of the change was larger for the facilitating group (Table 1). The effect was reversible on washout in both groups (not shown). In cells receiving facilitating synapses, R_{in} was increased significantly by carbachol (Table 1); the change was reversed upon washout (not shown). Data in Table 1 do not include those cells where CCh elicited spontaneous firing (i.e., those cells that were most affected; this group comprised about 1/3 of those cells receiving facilitating synapses and none of the depressing type), so the average effect on intrinsic properties in the facilitating type is probably underestimated.

Bath-application of 10 μM nicotine, either in standard conditions (1 mM Mg^{+2}) or in nominally Mg^{+2}-free medium to unmask NMDA receptor activity (c.f. Levy et al., 2006) did not affect EPSPs appreciably (not shown).

3. Discussion

The main finding of the present study is that effects of cholinergic agonist are coupled to the underlying synaptic dynamics; the effect of CCh decreased for successive evoked EPSPs in a depressing train, but increased for successive facilitating EPSPs. Reduction of synaptic amplitude by CCh was reversible upon washout. However, cholinergic agonist has been shown to be permissive and in some cases sufficient to induce long-term changes in synaptic efficacy in cortex (Kirkwood et al., 1999) and hippocampus (Auerbach and Segal 1996; Maggi et al., 2004). The data presented here excluded cell pairs where EPSPs did not recover toward the control value after CCh washout (see Experimental procedures). Whether non-recovery represents long-term cholinergic effects, or conversely non-cholinergic effects such as use-dependent weakening or strengthening of the synapses, remains to be tested.

Effects of CCh were blocked by atropine and therefore are attributed to muscarinic receptor activation, although nicotinic effects might have been obscured, for instance, by receptor desensitization resulting from bath application. Muscarinic and nicotinic receptors have been identified both pre- and postsynaptically at excitatory cortical synapses (Erisir et al., 2001; Levy and Aoki, 2002; Mrzljak et al., 1993), but ACh's most prevalent synaptic effects have been attributed to reduction of presynaptic glutamate release probability (reviewed in Krnjevic, 2004). Presynaptic changes can be identified in several ways, e.g. via analysis of the coefficient of variation (CV) of synaptic amplitude (Faber and Korn, 1991), via a change in transmission failure rate, or via a change in EPSP 2/EPSP 1 (PPR). In the present study, CCh caused an increase in spontaneous background activity that was large enough to complicate the analysis of CV and failure rates. PPR was not affected in cells receiving depressing synapses but was significantly reduced in the facilitating group. This in itself is not proof of a presynaptic change, because PPR can be influenced by postsynaptic changes such as short-term receptor desensitization. Additionally, interpretation of the change in PPR for facilitating synapses is different from the depressing case, because with facilitation release probability is presumed to increase for successive EPSPs in an evoked train. The reduction of PPR via carbachol for facilitating synapses is consistent with the idea that release probability is reduced more by carbachol in later EPSPs in a train than in the initial EPSP.

It is widely agreed that depressing and facilitating synapses differ in their underlying molecular architecture (reviewed in Zucker and Regehr, 2002). However, we stress that our data do not imply (and also do not rule out) that ACh acts differently, in a mechanistic sense, at the two respective types of synapses. The simplest interpretation of the present data, in view of past work, is that ACh reduces release probability at both depressing and facilitating synapses.

3.1. *Potential age-dependence of STP*

In the present study no correlation was found between the age of the slice and the degree of short-term plasticity (not shown). However, layer 5 pyramid (P)–FS synapses have previously been reported to convert, on average, from uniform depression at three postnatal weeks to a mix of depression and facilitation at five weeks (Angulo et al., 1999). A similar change has been reported between two and four postnatal weeks in layer 2/3 and layer 5 P–P synapses (Reyes and Sakmann, 1999). Therefore, the distinction between depressing and facilitating synapses may be less clear in adult than young animals, and the STP-dependent difference in ACh effects may be concomitantly smaller. However, it should be noted that (1) the very pronounced facilitation seen in P–non-FS synapses appears to be qualitatively different from the relatively mild facilitation reported in P–FS and P–P synapses from older animals, and (2) the switch from depression and facilitation is slow enough to leave a long developmental window >3 weeks from birth, encompassing the critical period for sensory map refinement, during which ACh would act on cells receiving depressing versus facilitating synapses differently.

3.2. *Functional aspects*

In agreement with past studies (see the Introduction), we found that interneurons receiving depressing synapses tend to display high maximum firing rates and relatively little spike rate adaptation, and were most likely to me multipolar in shape; those receiving facilitating synapses were significantly more adapting (Fig. 1E) and were more likely to be bipolar/bitufted (Table 1). FS and LTS (a main category of non-FS) cells have been shown to form separate synaptically and electrically connected networks whose firing patterns may compete to entrain the activity of pyramidal (excitatory) cells that

generate cortical output (Beierlein et al., 2000, 2003; Gibson et al., 1999; Traub et al., 1996). The different dynamics of P–FS versus P–non-FS synapses, and consequent differences in the pattern of synaptic amplitude reduction, imply that cholinergic influence on excitation of FS versus non-FS cells is activity-dependent. With low activity (low-frequency excitatory input), EPSPs in FS cells should be strongly suppressed by ACh, but there should be little effect on non-FS cell excitation (compare CCh vs. control for EPSP 1 in Fig. 2C vs. D). With repetitive, high-frequency input, P–FS synapses are depressed and ACh should have little additional effect, but P–non-FS synapses are facilitated and should be strongly suppressed by ACh (EPSP 6 in Fig. 2C vs. D). In a hypothetical recurrently connected network where FS and non-FS sub-networks compete for entrainment of P cells, selective reduction synaptic excitation of non-FS cells might allow the FS cell network to dominate. Such an effect could correspond to the emergence of gamma-frequency oscillations seen experimentally with application of ACh in slice preparations (Buhl et al., 1998; Fisahn et al., 1998) or during periods of arousal *in vivo*. However, reduced synaptic excitation of non-FS cells via ACh would be counterbalanced by increased intrinsic excitability (Gulledge et al., 2007; Kawaguchi, 1997; Porter et al., 1999; Xiang et al., 1998), and it is difficult to predict which effect would dominate. But we propose that reduced synaptic strength could be significant during periods of high activity when both FS and non-FS cells are getting a lot of local excitatory input. *In vitro* and modeling studies assessing the combined effect of ACh on synaptic dynamics and intrinsic membrane properties will eventually be needed for a more complete picture of the functional consequences.

4. Experimental procedures

4.1. *Slice preparation and recording*

Details of the slice preparation and recording were as described previously (Reyes and Sakmann 1999; Chance et al., 2002; Levy et al., 2006), and conformed to protocols approved by the New York University Animal Welfare Committee. Wistar rats (postnatal day 11–26) were decapitated after anaesthesia via halothane inhalation, and 300 μM parasagittal slices containing somatosensory cortex were made using a vibrating microtome (Leica Instruments GmbH, Nussloch, Germany). Slices were placed in the recording chamber and perfused with oxygenated artificial cerebrospinal fluid (ACSF: 125 mM NaCl, 25 mM $NaHCO_3$, 25 mM glucose, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 2 mM $CaCl_2$, 1 mM $MgCl_2$, all purchased from Sigma-Aldrich, St Louis, MO) at 32–34 °C (flow rate 1–2 mL/min).

Slices were viewed under infrared differential interference contrast (IR-DIC) microscopy (Stuart et al., 1993). Layer 2/3 was located deep to layer 1 (where cell bodies are sparse) and superficial to the layer 4 barrels, which can be seen grossly at low power (4× objective; c.f. (Petersen and Sakmann, 2000, 2001)). Cells were identified provisionally as pyramidal (P; with triangular somata and prominent apical dendrites), bipolar/bitufted cells (B; with narrow, elongated somata with prominent apical and basal processes, or multipolar cells (M; with rounded somata and radially extending processes). The somata of the recorded cells were always less than 350 μM apart, and usually less than 100 μM apart. The somata of the presynaptic pyramidal cells as well as the postsynaptic interneurons were located within layer 2/3. Whole-cell recordings were made using pipettes with 8–12 MΩ resistance when filled with 100 mM K-gluconate, 20 mM KCl, 4 mM ATP-Mg, 10 mM phosphocreatine, 0.3 mM GTP, and 10 mM HEPES, pH 7.3. Voltage responses were recorded using BVC-700A amplifiers (Dagan corp., Minneapolis, MN), digitized at 10 kHz using an ITC-18 interface (Instrutech corp., Port Washington, NY), and stored on a computer using Igor software (Wavemetrics, Lake Oswego, OR). Voltages were corrected for liquid junction potential using the offset control on the amplifier. Input resistance was measured on initial break-in by measuring the voltage response to 1 s incremental current steps. Adaptation index (AI) was measured as the ratio of the last to the first interspike interval (ISI) in a train of spikes elicited by 1 s current injection (Fig. 1C,D) at a value producing one-half the cell's maximum steady-state firing rate. Maximum firing rate was measured as the inverse of the last ISI in the train at a saturating level of current injection.

In synaptically connected cells, suprathreshold stimulation evoked unitary EPSPs in the target cell(s). Connections were monosynaptic as evidenced by short average latency, monophasic rising component, and small average amplitude (see Results). Presynaptic cells were stimulated with 1–10 pulses of 5 ms each at 20 Hz (6 pulses were used in most experiments). During data collection, stimulus trains were separated by 3–6 s. The interval between stimulus trains was held constant throughout each period of data collection. After data collection, whole-cell recording was re-established with pipettes containing 0.5% biocytin for >20 min; slices were fixed immediately and the filled neurons were later visualized using avidin–biotin and 3,3′-diaminobenzidine/horseradish peroxidase histochemistry (DAB–HRP; Horikawa and Armstrong, 1988) for confirmation of cell type and location.

4.2. *Drug application*

The following compounds were used: D-2-amino-5-phosphonovaleric acid (D-AP5), atropine sulfate, carbamylcholine chloride (carbachol), 6,7-dinitroquinoxaline-2,3(1H,4H)-dione (DNQX), (–)-nicotine hydrogen tartrate. For bath application, drugs were prepared as concentrated stock solutions in 0.1 M NaOH (DNQX), 0.1 M HCl (atropine), or water (all others) and diluted in oxygenated ACSF immediately before use. Bath-applied antagonists were superfused for at least 20 min (atropine) or for at least 10 min (DNQX and D-AP5), before starting to collect data. For synaptic responses, initial experiments with continuous data collection (6–10 trials/min for >1 h) revealed slow use-dependent synaptic depression that confounded the agonist data. Therefore, data were collected discontinuously as follows: control synaptic responses were evoked; after a 20–25 min pause, carbachol was applied and allowed to reach effective concentration in the bath (3 min); synaptic responses were evoked again; after another 20–25 min pause, washout data were collected. Control, agonist and washout data each comprised 100 trials collected over ~10 min. During agonist application, resting V_m was kept at the control value by applying holding current. Cholinergic agonist was only applied once per slice to prevent possible AChR-dependent long-term synaptic changes (c.f. Maggi et al., 2004) from interfering with subsequent tests. For intrinsic

properties, data were collected either discontinuously or continuously, and agonist was sometimes applied more than once with reproducible results.

4.3. *Data analysis*

Individual and average EPSP traces were analyzed off-line using macros written with Igor. Experiments where the resting membrane potential (V_m) of either a pre- or postsynaptic cell exceeded −50 mV (where persistent spontaneous firing was observed), or where the access resistance increased substantially (>30%) during the course of the experiment, except for transient effects of agonist application, were excluded. Over a given set of trials, resting membrane potential generally did not fluctuate by more than +/−2 mV. Determination of peak EPSP amplitude and onset latency followed Feldmeyer et al. (1999). EPSP traces that contained spontaneous action potentials or large non-evoked EPSPs (>10× mean evoked EPSP amplitude) were excluded. These typically constituted less than 1% of the data set. Pairs where average EPSP amplitude showed large monotonic increases or decreases (>2× control) over the course of the experiment were also excluded. For average EPSP traces, individual traces were aligned by triggering to stimulus onset. Triggering to action potential onset (in a subset of the data) yielded results that did not differ appreciably from stimulus-triggered averages. Unless otherwise noted, mean values are presented +/−standard deviation, and *p*-values for statistical significance were obtained from two-tailed, paired *t*-tests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.brainres.2008.03.067.

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