INACTIVATION OF PREFRONTAL CORTEX ABOLISHES CORTICAL ACETYLCHOLINE RELEASE EVOKED BY SENSORY OR SENSORY PATHWAY STIMULATION IN THE RAT

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Abstract—Sensory stimulation and electrical stimulation of sensory pathways evoke an increase in acetylcholine release from the corresponding cortical areas. The pathways by which such sensory information reaches the cholinergic neurons of the basal forebrain that are responsible for this release are unclear, but have been hypothesized to pass through the prefrontal cortex (PFC). This hypothesis was tested in urethane-anesthetized rats using microdialysis to collect acetylcholine from somatosensory, visual, or auditory cortex, before and after the PFC was inactivated by local microdialysis delivery of the GABA-A receptor agonist muscimol (0.2% for 10 min at 2 μl/min). Before PFC inactivation, peripheral sensory stimulation and ventral posterolateral thalamic stimulation evoked 60 and 105% increases, respectively, in acetylcholine release from somatosensory cortex. Stimulation of the lateral geniculate nucleus evoked a 57% increase in acetylcholine release from visual cortex and stimulation of the medial geniculate nucleus evoked a 72% increase from auditory cortex. Muscimol delivery to the PFC completely abolished each of these evoked increases (overall mean change from baseline=−7%). In addition, the spontaneous level of acetylcholine release in somatosensory, visual, and auditory cortices was reduced by 15–59% following PFC inactivation, suggesting that PFC activity has a tonic facilitatory influence on the basal forebrain cholinergic neurons. These experiments demonstrate that the PFC is necessary for sensory pathway evoked cortical ACh release and strongly support the proposed sensory cortex-to-PFC-to-basal forebrain circuit for each of these modalities. © 2007 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: microdialysis, muscimol, cholinergic basal forebrain, neocortex, systems neuroscience, cortical circuitry.

Acetylcholine (ACh) is a neurotransmitter that has been implicated in the regulation of a variety of higher cortical functions, including plasticity, working memory and attention (Rasmusson, 2000, 2006; Sarter et al., 2003; Dalley et al., 2004). Almost all cortical ACh is extrinsic and released by the terminals of cholinergic neurons whose cell bodies are located in the basal forebrain (BF). The extracellular concentration of ACh within the cortex is commonly interpreted as indicating the level of activity of these cholinergic neurons. Studies measuring cortical ACh have demonstrated that sensory stimulation evokes an increase in release from sensory cortical areas and does so with a degree of regional specificity; for example, visual stimulation causes much greater ACh release in visual cortex than in nonvisual areas (Collier and Mitchell, 1966; Fournier et al., 2004; Laplante et al., 2005).

The mechanisms and pathways by which sensory stimulation causes this modality-specific increase in ACh release are unknown. Presynaptic facilitation of the cortical cholinergic terminals by the thalamocortical afferents appears unlikely (Materi and Semba, 2001). A more likely alternative is that the increased release reflects increased firing of the cholinergic BF neurons. The pathway by which sensory input reaches the cholinergic BF neurons is unclear; traditional anatomical tracing methods have not revealed any extensive projections from sensory relay nuclei to the BF (Grove, 1988; Semba et al., 1988; Záborszky et al., 1991). A recent proposal is that sensory stimulation first activates the sensory cortex, which then activates the prefrontal cortex (PFC), which in turn activates the cholinergic BF neurons (Záborszky et al., 1997), an example of "top-down" processing (Sarter et al., 2001). PFC was proposed as an important component of this circuit because it is one of the few cortical areas that projects to the BF (Gaykema et al., 1991; Záborszky et al., 1997; Vertes, 2004) and it receives corticocortical projections from primary and association sensory cortices (van Eden et al., 1992; Condé et al., 1995). This hypothesis was supported by the demonstration that neurons in distinct regions of PFC are activated by visual or somatosensory cortex stimulation, and that stimulation of these PFC regions elicits firing of BF neurons (Golmayo et al., 2003). In addition, chemical stimulation of PFC produces an increase in ACh release in the parietal cortex (Nelson et al., 2005), consistent with a functional connection from PFC onto cholinergic BF neurons.

A specific prediction of this proposed circuit is that interrupting the pathway by inactivating the PFC should abolish sensory-evoked ACh release in sensory cortices. We tested this prediction by measuring the evoked release of ACh from somatosensory, visual and auditory cortices before and after blocking neuronal activity in PFC by the local administration of a selective GABA-A receptor agonist, muscimol. ACh release from somatosensory cortex was evoked by peripheral stimulation in one group of animals. In three additional groups the specific thalamic nu-

clei for these modalities were electrically stimulated using the same stimulus parameters, to allow for more direct comparisons between the somatosensory, visual and auditory modalities. The comparison between sensory and thalamic stimulation within the somatosensory modality provided validation for this approach. The comparison of evoked ACh release before and after muscimol allowed each animal to serve as its own control, thereby removing possible confounding variables such as the effect of anesthesia and probe efficiency, which could vary between animals, or the composition of the perfusate, which was the same in both stimulation periods. Any differences in evoked ACh release between the two stimulation periods can therefore be confidently attributed to muscimol inactivation of the PFC.

EXPERIMENTAL PROCEDURES

Animals

Male Wistar rats (200–375 g; Charles River, St. Constant, Quebec, Canada) were used. All experimental procedures were approved by the University Animal Care Committee and were carried out in accord with the Canadian Council on Animal Care and National Institutes of Health guidelines on the ethical use of animals in research. All efforts were made to minimize the number of animals used and their suffering.

ACh release from sensory cortical regions was studied in 44 rats anesthetized with urethane (1.6 g/kg, i.p.; Sigma, St. Louis, MO, USA) and placed in a stereotaxic frame. Holes were drilled in the skull for implantation of two microdialysis probes (CMA/12; CMA Microdialysis AB, Solna, Sweden; 20 kDa cutoff; 0.5 mm o.d.): one for muscimol delivery into region M2 (secondary motor area) of PFC (anterior 3, lateral 2, ventral 2; mm with respect to bregma, Fig. 1A), and a second for ACh collection into primary somatosensory cortex (posterior 1.5, lateral 2.5, Fig. 1B), primary visual cortex (posterior 7.5, lateral 4.6, Fig. 1C), or primary auditory cortex (30° angle at posterior 4.0, lateral 6.5, Fig. 1D). All microdialysis probes had 2 mm of exposed membrane and were inserted so the entire membrane was within the cortex.

Perfusion with artificial cerebrospinal fluid (aCSF: 125 mM NaCl, 3 mM KCl, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 23 mM $NaHCO_3$, and 1.5 mM H_2PO_4) was begun immediately after inserting the microdialysis probes at a rate of 2 μl/min. In order to obtain sufficient basal amounts of ACh that were consistently measurable in the anesthetized preparation, the perfusate in the ACh collection probe also contained neostigmine methyl sulfate and atropine sulfate (Sigma) (10 μM for both drugs, except in one experiment in which 1 μM neostigmine and 0.2 μM atropine were used). The comparison of release before vs. after PFC inactivation in the same animals removed the possibility that high resting levels of ACh were responsible for any changes in ACh release resulting from muscimol treatment.

The probes were perfused for 60 min to allow equilibration, before collecting 15 15-min samples. These samples were analyzed for ACh content using high-performance liquid chromatography (HPLC; Waters, Mississauga, ON, Canada) with electrochemical detection. The procedures and solutions for HPLC were as described previously (Materi et al., 2000), except that in the present experiment the working electrode was a horseradish peroxidase–coated carbon electrode (BAS, Indianapolis, IN, USA) with an oxidation potential of +500 mV.

The probe in PFC was perfused with aCSF at a rate of 2 μl/min. At the beginning of collection sample 7, the solution was changed using a liquid switch (CMA) to aCSF containing 0.2% muscimol for 10 min and then switched back to aCSF alone. The

dead space in the tubing was not a concern, as the time involved (1–1.5 min) was a small fraction of the time to the second stimulation test (60–75 min). This concentration was chosen based on findings showing a very long-lasting (several hours) inhibition of neural activity over a distance of at least 2–2.5 mm (Partsalis et al., 1995; Arikan et al., 2002; Edeline et al., 2002).

Evoked release of ACh from primary somatosensory cortex was measured in response to either peripheral stimulation of the contralateral forepaw or electrical stimulation of the ipsilateral specific thalamic nucleus (ventral posterolateral nucleus, VPL) that projects to the cortical collection site. In one group ($n=7$), peripheral somatosensory stimulation was delivered throughout samples 4 (before muscimol, S1) and 13 (after muscimol, S2) via two s.c. wires inserted into the contralateral forepaw at the beginning of the experiment. Constant current pulses (2 mA, 0.1 ms duration) were delivered every 30 s by a constant-current stimulator and isolation unit (Master-8, A.M.P.I., Jerusalem, Israel). ACh release from somatosensory cortex was monitored in nine rats with the stimulating electrode implanted into ipsilateral VPL (posterior 3.4, lateral 3.4, ventral 6.4, Fig. 1E). The VPL/somatosensory cortex procedure was repeated in another group of eight animals using reduced concentrations of neostigmine (1 μM) and atropine (0.2 μM). A control group ($n=4$) received VPL stimulation without muscimol administration. ACh release from visual cortex was measured in seven rats using thalamic stimulation of the dorsal lateral geniculate nucleus (DLG) with the electrode placed at posterior 4.7, lateral 3.8, ventral 5.0 (Fig. 1F). ACh release from auditory cortex was measured in nine rats with the stimulation electrode in the ventral medial geniculate nucleus (MGV) at posterior 5.2, lateral 3.6, ventral 6.4 (Fig. 1G). Thalamic stimulation in all cases consisted of single 0.5 mA pulses (0.5 ms duration) delivered via a concentric bipolar electrode (FHC, Bowdoin, ME, USA) every 15 s throughout samples 4 (pre-muscimol, S1) and 12 (post-muscimol, S2).

For each animal the three initial baseline samples (B1) were averaged and used to normalize the release across all samples. This removed inter-animal variability due to possible differences in probe efficiency or anesthetic level. The mean of the two samples prior to S2 provided a baseline measure of spontaneous release after muscimol (B2) and was used to calculate evoked ACh release during the subsequent stimulation sample (S2/B2).

At the end of each experiment the ACh collection and muscimol delivery probes were removed from the brain and placed in a standard ACh solution; the subsequent sample was measured with HPLC to determine probe efficiency. The animal was perfused transcardially with 0.9% buffered saline followed by 10% formalin for histological confirmation of the sites of probes and stimulation electrodes. Data from animals in which the entire probes were not within 500 μm of the intended sites were excluded from analysis.

Due to the dramatic change in evoked release observed after muscimol administration, it was necessary to ensure that muscimol did not diffuse from the PFC to the sites at which ACh was collected. Therefore, electrophysiological experiments were carried out in three additional rats. Evoked responses were recorded from somatosensory cortex, the sensory cortical area closest to PFC, before and after muscimol was delivered to the same PFC site used in the collection experiments. Field potentials were recorded using tungsten microelectrodes (FHC), amplified (AM Systems, Carlsborg, WA, USA), filtered between 1 and 500 Hz, and collected on a microcomputer at a sampling frequency of 10 kHz using a DataWave interface and software (Englewood, CO, USA). The contralateral paw was stimulated electrically every 2 s (4 mA, 0.1 ms pulses; Master-8) and evoked potentials were saved for 30 min before and 155–225 min (average 189 min) after 10 min administration of 0.2% muscimol. The amplitude of the initial negative wave of the average of 10 potentials was measured and compared before and after muscimol inactivation of the PFC.
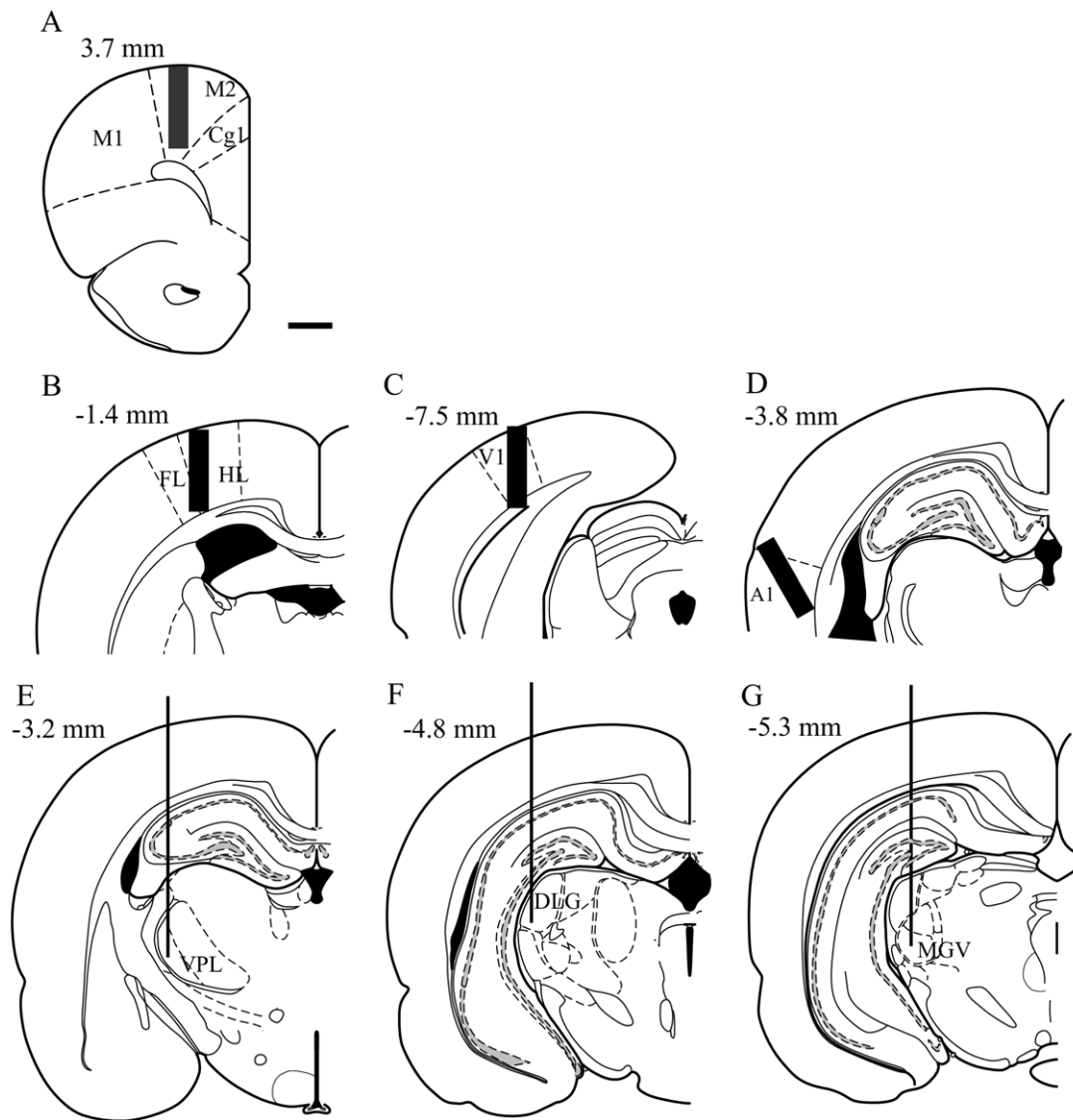
Fig. 1. Locations of microdialysis probes and thalamic stimulation electrodes. Probes in PFC (A), somatosensory cortex (B), visual cortex (C) and auditory cortex (D) are indicated by rectangles representing the dimensions of the exposed membrane of the dialysis probes (2 mm long, 0.5 mm diameter). Scale bar in A (1 mm) pertains to all figures. The locations of the thalamic stimulation electrodes are shown in: (E) the somatosensory relay nucleus, (F) the visual relay nucleus and (G) the auditory relay nucleus. Numbers indicate the distance in mm of the coronal section from bregma (Paxinos and Watson, 2005). Abbreviations: A1, primary auditory area; Cg1, cingulate area 1; FL, forelimb region of primary somatosensory cortex; HL, hindlimb region of primary somatosensory cortex; M1, primary motor area; M2, secondary motor area; V1 binocular region of primary visual cortex.

Statistics

Statistical analysis included repeated-measures analysis of variance (ANOVA) and *t*-tests using Prism software (GraphPad, San Diego, CA, USA). As a decrease was predicted, one-tailed *t*-tests were used to compare values before and after muscimol. The level of significance for type I errors was set at 5%. Data are presented as mean±S.E.M. in the text and figures.

RESULTS

Effect of PFC inactivation on ACh release from somatosensory cortex

ACh release was expressed as pmol/sample and then normalized for each animal to its baseline, the mean of the first three samples. The baseline release (B1 in Fig. 2) in the animals that received peripheral stimulation was 1.25±0.42 (mean±S.E.M.) pmol/sample. This mean is relatively high compared with the other groups described below, due to two animals with particularly high resting values (>2 pmol/sample) that were not observed in any other groups; removal of these two animals did not alter any of the following conclusions about the effect of muscimol. Repeated measures ANOVA over time revealed a significant change across samples ($F_{14,84}=5.05$, $P<0.001$). The first period of electrical stimulation of the forepaw evoked a 60% increase in ACh release (S1, Fig. 2), which was significantly greater than B1 (paired $t_6=4.54$,
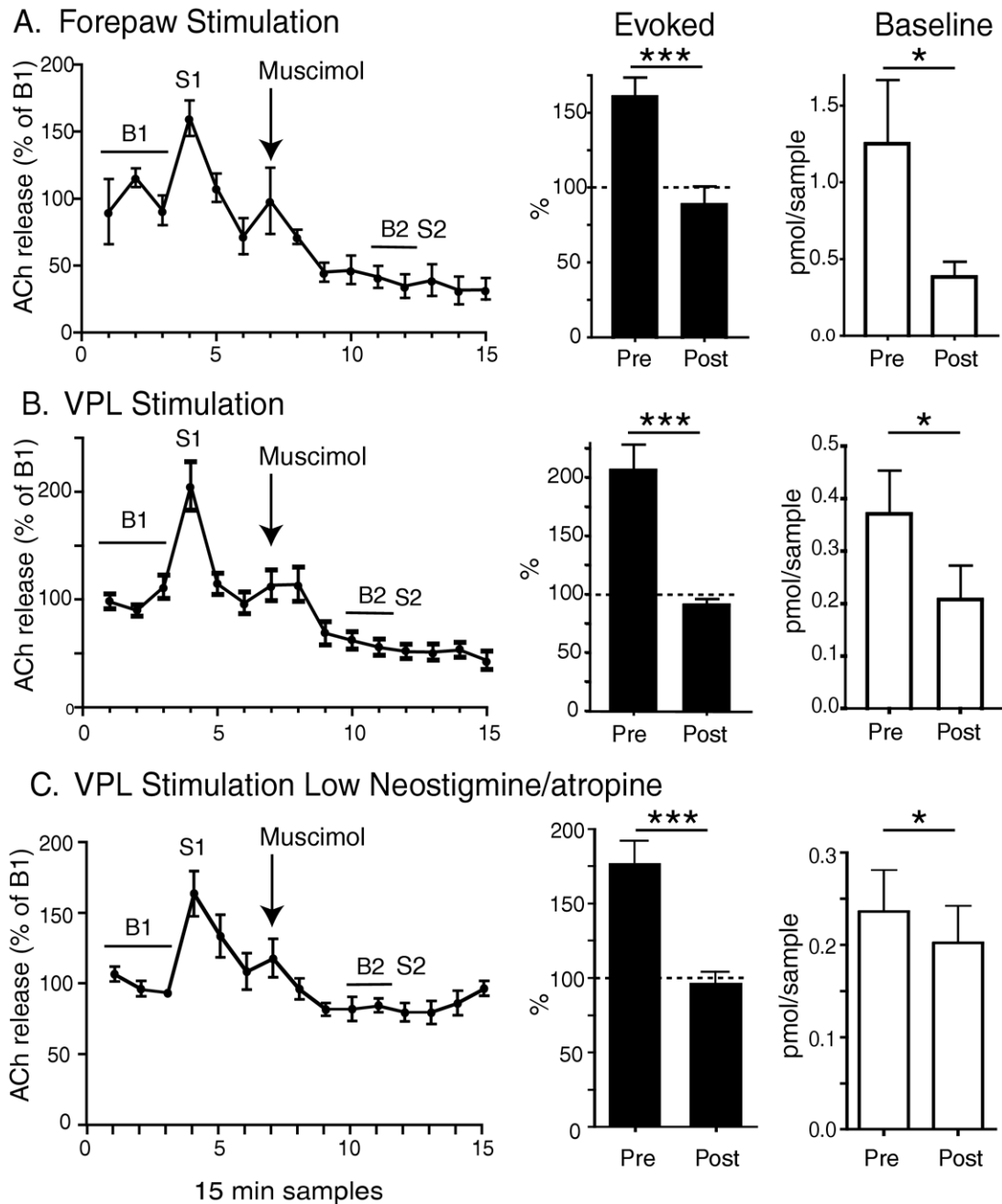
Fig. 2. Effect of PFC inactivation on ACh release from somatosensory cortex. (A) ACh release evoked by contralateral forepaw stimulation during samples 4 (S1) and 13 (S2). Left, normalized ACh release across 15-minute samples; mean±S.E.M. Middle, solid bars: evoked release pre- and post-muscimol during the stimulation periods (S1 and S2) expressed as percent of preceding baseline. Right, open bars: mean ACh release (pmol/sample) during the baseline periods, B1 and B2, pre- and post-muscimol. (B) ACh release in the group that received VPL thalamic stimulation during samples 4 (S1) and 12 (S2). (C) ACh release using reduced concentrations of neostigmine (1 μM as opposed to 10 μM) and atropine (0.2 compared with 10 μM). VPL was stimulated during samples 4 and 12. In each experiment, evoked and baseline release was reduced post-muscimol: *** $P<0.001$; * $P<0.05$.

$P=0.004$). Following administration of muscimol to PFC during the first 10 min of sample 7, there was a decrease in spontaneous release that stabilized within 45 min of muscimol onset, i.e. by sample 9. ACh release during samples 11 and 12 was averaged and used as a new baseline (B2 in Fig. 2A) for determination of evoked release by the second stimulation period. The mean baseline

after muscimol (B2) was 0.38±0.10 pmol/sample, 59% less than the original baseline (Fig. 2A, right, open bars). Paired t-test on the absolute values showed that this decrease in spontaneous release from B1 to B2 was statistically significant ($t_6=2.73$, $P=0.017$). After muscimol administration, there was no significant change in ACh release during skin stimulation compared with B2 (-12%,
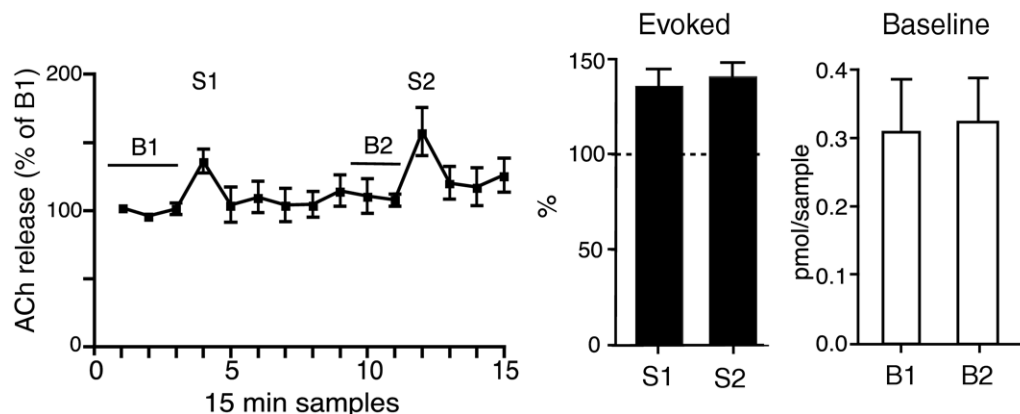
Fig. 3. Evoked ACh release is repeatable in the absence of muscimol administration. Left, normalized ACh release across 15-min samples. Middle: the amount of evoked release was similar during both stimulation periods. Right, the level of spontaneous release was also unchanged during the two baseline periods (B1 and B2).

$t=0.94$, $P=0.38$). A comparison of the percentage change in evoked release during the two stimulation periods was statistically significant (Fig. 2A, middle, solid bars; $t=5.20$, $P=0.001$).

ACh was also collected from somatosensory cortex in another group ($n=9$) with stimulation of the somatosensory thalamic nucleus (VPL) (Fig. 2B). Basal ACh release before muscimol was 0.37 ± 0.07 pmol/sample in this group. There was a significant change in release over samples ($F_{14,112}=3.67$, $P<0.001$). During the first period of VPL stimulation there was a $105\pm22\%$ increase in ACh release and this increase was completely blocked after muscimol administration (Fig. 2B, solid bars; paired $t_8=6.07$, $P<0.001$). Spontaneous ACh release also showed a significant decrease of $41\pm8\%$ following muscimol administration (Fig. 2B, open bars; paired $t_8=2.06$, $P=0.037$), as was the case in the peripheral stimulation group.

These two groups were compared using a two-factor ANOVA to determine if peripheral and thalamic stimulation had different effects. The difference in evoked release between pre- and post-muscimol periods was significant ($F_{1,14}=50.87$, $P<0.0001$). The difference between peripheral and thalamic groups was not significant ($F_{1,14}=1.64$, $P=0.22$) nor was the interaction between muscimol effect and type of stimulation significant ($F_{1,14}=5.05$, $P=0.09$). Thus, the two stimulation methods produced similar increases in ACh release before muscimol and no increase after muscimol.

The possibility that these results were due to the high basal levels of extracellular ACh was examined in another group of animals ($n=8$) receiving VPL stimulation while perfusing the probe with aCSF containing 1 μM neostigmine and 0.2 μM atropine. The results presented in Fig. 2C were similar to those obtained with the higher drug concentrations. As expected, the basal release was less than that seen with higher drug concentrations (0.24 vs. 0.37 pmol/sample). Nevertheless, the relative increase in ACh release during the initial VPL stimulation period was similar ($77\pm14\%$ vs. 105%) and this increase was completely blocked after muscimol inactivation of PFC ($-3\pm7\%$). This difference in evoked release was statistically significant

(paired $t_7=5.59$, $P<0.001$). Basal release was slightly, but significantly decreased, from 0.236 to 0.202 pmol/sample, ($t_7=2.25$, $P=0.03$). The smaller relative decrease (15%) possibly indicates a floor effect given the lower ACh values.

To control for the possibility that the block of evoked ACh release during the second stimulation period was due to an uncontrolled time-dependent decrease in release unrelated to muscimol administration, we examined four animals that received VPL stimulation during samples 4 and 12, but without muscimol in the PFC probe (Fig. 3). The second stimulation sample evoked the same increase in ACh release from somatosensory cortex as the first period ($P=0.20$). The ratio of the two stimulation periods in the four animals ranged from 0.94 to 1.09 (mean=1.04). This result is consistent with previous observations using repeated stimulation samples at similar intervals (Rasmusson et al., 1994; Szerb et al., 1994). There was also no change in basal release ($P=0.18$, Fig. 3, right panel). These results confirm that the block of ACh release seen in the muscimol treated groups was not due to a depletion of ACh stores during the first stimulation period.

Effect of PFC inactivation on ACh release in visual and auditory cortex

In the experiments on visual and auditory cortices only thalamic stimulation was used to evoke increased ACh release, using the same stimulus parameters as with VPL-evoked ACh release in somatosensory cortex. In both cases the repeated ANOVA over samples was statistically significant ($F_{14,112}=16.2$, $P<0.0001$ for visual and $F_{14,84}=6.61$, $P<0.0001$ for auditory modalities). Thalamic stimulation of the visual thalamic nucleus (DLG) produced a significant increase ($57\pm4\%$) in ACh release from primary visual cortex (Fig. 4A; $t=13.3$, $P<0.001$) before muscimol. This evoked release was completely blocked after PFC inactivation with muscimol ($-7\pm6\%$; not statistically different from B2; $t=1.2$, $P=0.27$; Fig. 4A, solid bars). Spontaneous ACh release in visual cortex also decreased, by 25%, after muscimol administration, from 0.211 to 0.158 pmol/sample ($t=3.22$, $P=0.009$).
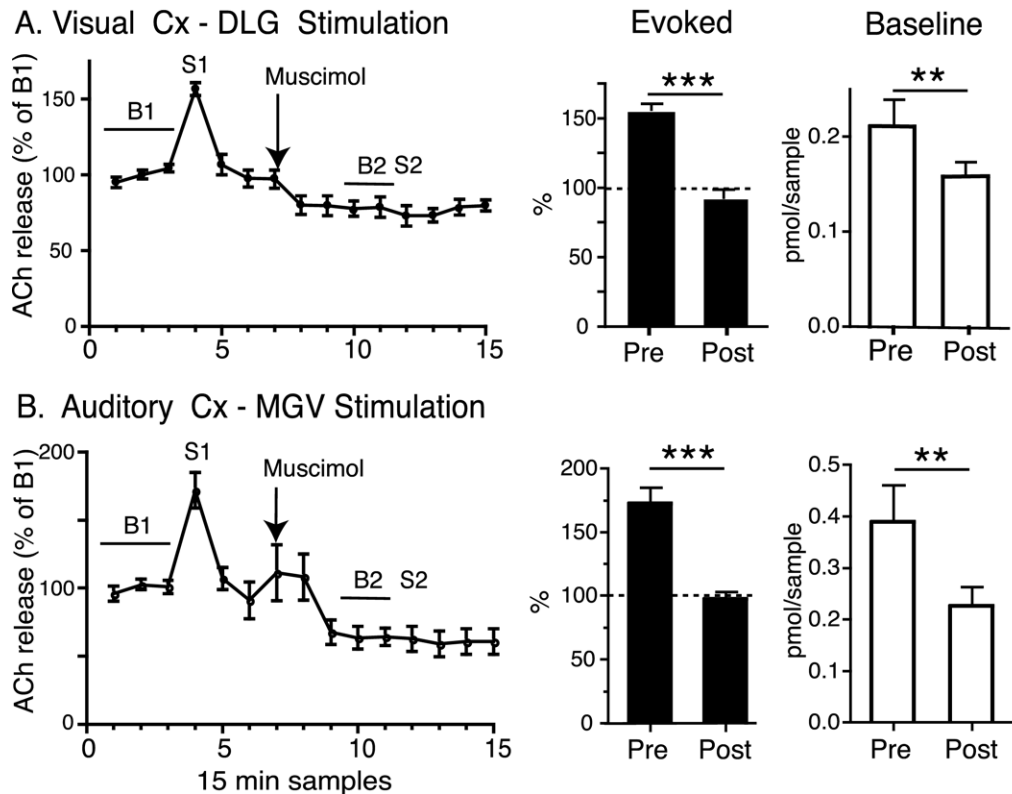
Fig. 4. Effect of PFC inactivation on ACh release in (A) visual cortex and (B) auditory cortex evoked by stimulation of DLG and MGV thalamic nuclei, respectively. Conventions as in Fig. 2. Significant differences between pre and post-muscimol periods were seen in both evoked and baseline release: *** $P<0.001$; ** $P<0.01$.

Experiments on the auditory pathway produced similar results (Fig. 4B). Auditory thalamus (MGV) stimulation evoked a 72% increase in ACh release from auditory cortex before muscimol, but no significant change (-3%) after muscimol delivery. Paired comparison of evoked changes before and after muscimol was statistically significant ($t=4.61$, $P<0.001$). A 42% decrease in spontaneous ACh release was seen in auditory cortex after muscimol (from 0.388 to 0.226 pmol/sample), which was also statistically significant ($t=3.26$, $P=0.006$).

Statistical comparison of the evoked ACh release across the three groups with thalamic stimulation of different modalities revealed a clear muscimol effect ($F_{1,22}=55.58$, $P<0.0001$), but no significant difference between the three modalities ($F_{2,22}=2.70$, $P=0.25$) and no significant interaction ($F_{2,22}=3.94$, $P=0.07$). To illustrate the consistency of the effect of PFC inactivation, the evoked release values (ratio of stimulation period over the preceding baseline) for all individual animals are shown in Fig. 5. The clear separation between the values before muscimol and after muscimol and the convergence of values after muscimol around one illustrate the effectiveness of PFC inactivation in blocking evoked ACh release.

Electrophysiological assessment of muscimol diffusion

Previous data indicate that muscimol can produce functional inactivation at least 2 mm from the site of delivery (Arikan et al., 2002; Edeline et al., 2002), but did not reveal

the maximal effective spread of muscimol. Consequently, it was important to determine that muscimol did not diffuse from the PFC to the cortical ACh collection sites in the rat model. This was tested in three rats using contralateral paw stimulation while recording evoked potentials in somatosensory cortex. The evoked potentials were recorded for 2.5–4 h after muscimol administration to the same PFC site as used for the ACh experiments. The distance between the recording site and the PFC probe in these rats ranged from 4.6 to 5.5 mm (mean 5.0 mm). In none of the animals was there any sign of a decrease in the amplitude of the evoked potential. In fact, the mean amplitude of the evoked potentials increased slightly from 158 ± 67 μV before muscimol to 195 ± 51 μV at the end of recording, perhaps due to gradual lightening of the anesthesia. These electrophysiological experiments indicate that 0.2% muscimol, delivered to the cortex via reverse microdialysis, has an effective radius less than 5 mm and therefore muscimol did not directly affect the neurons at the collection site.

DISCUSSION

The data presented here indicate that the PFC is a necessary part of the pathway linking the somatosensory, visual and auditory pathways to cholinergic neurons of the BF. In each experiment, significant increases in ACh release were evoked during the first stimulation period, but each of these increases was completely blocked by mus-
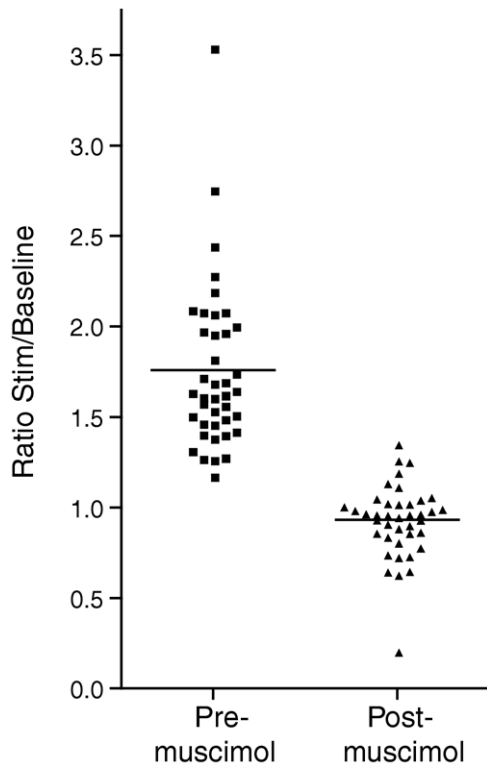
Fig. 5. Changes in ACh release evoked by stimulation for all muscimol treated animals (n=40, based on the data shown in Figs. 2 and 4). The ratio of ACh release during each stimulation period to the preceding baseline is shown for each animal during the pre-muscimol test and the post-muscimol test. The horizontal line represents the mean of each distribution (pre-muscimol=1.76±0.07, post-muscimol=0.93±0.03).

cimol inactivation of the PFC. Control animals, without muscimol administration, showed the same increase in ACh release during similarly spaced stimulation periods. PFC inactivation also produced a significant decrease in basal ACh release in each group, ranging from 15% to 59%, demonstrating that activity in the PFC provides tonic excitation to the cholinergic BF neurons.

Sensory inputs to cholinergic neurons

The observation that cortical release of ACh can be evoked by peripheral and thalamic stimulation supports early results using the cortical cup technique and more recent microdialysis studies. For example, Collier and Mitchell (1966) found that both diffuse light and lateral geniculate nucleus stimulation led to an increase in ACh release from visual cortex of anesthetized rabbits. Similar increases in cortical ACh release were seen with MGV stimulation in rabbits (Hemsworth and Mitchell, 1969) and with cutaneous nerve stimulation in cats and rats (Mullin and Phillis, 1975; Kurosawa et al., 1992). Microdialysis experiments in rats have confirmed the modality and regional specificity of sensory evoked ACh release (Fournier et al., 2004; Laplante et al., 2005). The evoked increases observed in the present experiment (57–105%) were similar in magnitude to those seen in these previous studies.

The question that arises from these experiments is how sensory information reaches the BF, since the traditional sensory pathways have few, if any, projections to the BF (Grove, 1988; Semba et al., 1988). Although the BF receives projections from the brainstem reticular formation (Jones and Beaudet, 1987; Semba et al., 1988), the nonspecificity of these reticular neurons makes it unlikely that this pathway is responsible for the modality-specific cortical ACh release. Increases in cortical ACh release with thalamic stimulation are particularly difficult to explain, as these nuclei have only ascending projections to the cortex and none to the BF (Jones, 1985). One possibility is that if cholinergic neurons sent branches to both the cortex and thalamus, electrical stimulation of the thalamus would antidromically activate the axons and thus release ACh from the cortical terminals. Although a small number of cholinergic BF neurons project to both the cortex and the reticular thalamic nucleus (Jourdain et al., 1989), cholinergic BF neurons are not known to project to the specific sensory thalamic nuclei (Semba, 2000). Similarly, the cholinergic neurons in the mesopontine tegmentum that innervate the thalamus and cortex (Hallanger et al., 1987) project only to the medial PFC and not to sensory cortical areas (Semba and Fibiger, 1989).

Anatomical evidence also indicates that cortical projections to the BF do not arise from the sensory regions, but only from PFC and perirhinal cortical areas (Grove, 1988; Gaykema et al., 1991). These anatomical factors led to the proposal of Záborszky et al. (1997) that the PFC might provide a relay in the circuit from sensory cortices to BF. Golmayo et al. (2003) tested some aspects of this hypothesis using electrophysiological techniques. They found that neurons in two regions of PFC, the secondary motor area (M2) and the cingulate area (Cg1), responded to stimulation of somatosensory and visual cortex, respectively. In addition, they found that stimulation of these two PFC areas caused an enhancement of sensory-evoked responses in the appropriate sensory cortical area that was mediated by muscarinic ACh receptors. These findings are consistent with the hypothesis that PFC activates the appropriate cholinergic neurons within BF that project to the sensory cortices.

The major finding of the present experiment is that PFC inactivation by muscimol completely abolished sensory or sensory-pathway evoked ACh release. This strongly supports the hypothesis of Záborszky et al. (1999) that the PFC is a critical part of the circuitry by which somatosensory and visual pathways can activate BF cholinergic neurons and thus produce ACh release in the cortex. In the present experiment, the PFC was inactivated via a probe placed in area M2, where Golmayo et al. (2003) found neurons that respond to somatosensory cortex stimulation (cf. Fig. 1A). The M2 and Cg1 (visually-responsive) regions of PFC extend approximately 0.5 mm anterior and 1.5 mm posterior to the level shown in Fig. 1A (Paxinos and Watson, 2005). Electrophysiological studies indicate that muscimol produces virtually complete inactivation within 1 h and over a radius of 2.5 mm of the probe (Partsalis et al., 1995; Martin and Ghez, 1999; Arikan et al., 2002; Edeline et al.,

2002). Thus we are confident that the entire PFC region shown by Golmayo et al. (2003) to be responsive to somatosensory and visual cortical stimulation was inactivated at the time of the second stimulation period. Due to the wide spread of muscimol, it was not possible to test another component of Záborszky et al. (1999) model, namely that the different modalities are relayed to the BF via distinct regions within PFC. This will require more restricted inactivation of PFC, for which muscimol is poorly suited.

While Golmayo et al. (2003) did not look for auditory-responsive neurons in PFC, there is anatomical evidence for projections from primary and association auditory areas to PFC in the rat (Reep et al., 1990; van Eden et al., 1992; Condé et al., 1995). Our data suggest that the hypothesis that the PFC is a necessary component in the circuit leading to enhanced cholinergic activity in sensory cortices can be generalized to auditory as well as somatosensory and visual modalities. These data also argue against the idea that sensory-evoked ACh release might be due to presynaptic facilitation of cholinergic terminals by the thalamocortical afferents (also discussed in Materi and Semba, 2001), as these afferents would not be blocked by PFC inactivation.

The other consistent finding of these experiments was that PFC inactivation produced a significant decrease in tonic ACh levels in all three sensory cortices. This suggests that the output from the PFC provides a tonic facilitatory effect on the cholinergic BF neurons. Whether this is also the case in unanesthetized conditions remains to be determined.

While the functional data are highly consistent with the concept that PFC output leads to excitation of BF cholinergic neurons, the anatomical details of how it does so are unclear. It was initially shown that terminals of PFC neurons end in close proximity to cholinergic BF neurons (Gaykema et al., 1991). However, detailed electron microscopic studies have confirmed direct synaptic connections only onto parvalbumin-containing, presumptive GABAergic cells (Záborszky et al., 1997). These neurons are known to have local axon collaterals that synapse with cholinergic neurons; however, these synapses are likely inhibitory, which is not consistent with the present results. It is possible that the excitation of cholinergic neurons results from PFC projections to other subcortical regions that in turn innervate the BF, leading ultimately to excitation of the cholinergic neurons. Further experiments are necessary to uncover such relays, but any multisynaptic circuit must maintain the sensory-modality specificity seen in this and other ACh release studies (Collier and Mitchell, 1966; Hemsworth and Mitchell, 1969; Fournier et al., 2004; Laplante et al., 2005). Sensory evoked ACh release is unlikely to involve reciprocal cortico-cortical connections from PFC to the sensory cortices as such projections are likely glutamatergic and local application of glutamate leads to a net decrease in ACh release rather than an increase (Giorgetti et al., 2000; Materi and Semba, 2001).

Methodological issues

Inactivation of PFC was carried out by administration of the GABA-A receptor agonist muscimol, which has been used in numerous studies for its long-lasting inhibitory effects. Initially, our concern was whether the administration of muscimol via reverse dialysis would inactivate a large enough part of PFC to interrupt the proposed circuit completely. In fact, the results showing a complete block of evoked ACh release indicate that PFC inactivation was very effective. This raised the opposite concern that muscimol was diffusing from the PFC to the collection sites and/or to the BF. Therefore it was necessary to examine the electrical responsiveness of neurons at the cortical collection site using the same arrangement of PFC inactivation. We found that evoked responses in somatosensory cortex were not decreased by muscimol administration in the PFC. This result indicates that the effective diffusion of muscimol was less than 5 mm, the distance between recording and muscimol sites. As visual and auditory cortices and the regions of BF that project to the neocortex are more distant from PFC than somatosensory cortex, it is extremely unlikely that the present results are due to a direct effect of muscimol on cholinergic terminals in the cortex or on the cholinergic cell bodies in the BF.

The present study was conducted with neostigmine and atropine in the perfusate. While ACh can be measured in awake animals without using a cholinesterase inhibitor (e.g. Herzog et al., 2003; Jamal et al., 2005; Kozak et al., 2006), ACh release is greatly decreased during anesthesia (Celesia and Jasper, 1966; Collier and Mitchell, 1967; Beani et al., 1968), and therefore addition of a cholinesterase inhibitor is usually necessary in studies on anesthetized animals. Neostigmine prevents the rapid hydrolysis of ACh by the endogenous acetylcholinesterase so that detectable amounts of ACh can be picked up in the dialysate. Experiments comparing hippocampal ACh release in the presence or absence of cholinesterase inhibition have suggested that the extremely high efficiency of acetylcholinesterase may preclude the ability to measure changes in ACh levels by microdialysis in situations where cholinergic neurons are definitely activated (Chang et al., 2006). Paradoxically, the increased extracellular ACh levels produced by an anticholinesterase will decrease the amount of ACh that is actually released by the terminals, due to presynaptic inhibition via muscarinic autoreceptors (Bertels-Meeuws and Polak, 1968; Szerb and Somogyi, 1973). The use of a muscarinic antagonist such as atropine reverses this depression and increases the amount of ACh collected in the dialysate (MacIntosh and Oborin, 1953; Szerb, 1964). Given the complex interactions of these variables, as well as the continual removal of ACh in the dialysate, microdialysis provides only a rough estimate of the exact levels of ACh within the synaptic cleft. Consequently, changes in ACh levels are more easily interpretable than are absolute levels. The present study was specifically designed to examine changes produced by PFC inactivation using the same stimulation procedure delivered before and after muscimol administration. In each experiment neostigmine

and atropine were present in the dialysate throughout the experiment and thus their inclusion cannot account for the absence of evoked ACh release after PFC inactivation compared with the robust release seen initially. In addition, the fact that stimulation initially produced an approximate doubling of ACh levels in our animals indicates that the cholinergic synapses were still within a functional range. It is also worth noting that high basal release levels in the presence of neostigmine and atropine may have aided our observation of a decrease in basal release following muscimol. The ability to see a decrease in ACh release may be limited by a floor effect if the ACh levels were much lower, as suggested by the smaller decrease in the group with lower neostigmine and atropine concentrations.

CONCLUSIONS

The present study demonstrates that inactivation of PFC blocks the sensory evoked release of ACh from three sensory cortical regions (somatosensory, auditory and visual) when this release is evoked by peripheral or thalamic stimulation in the corresponding modality. These data provide strong support for the hypothesis that sensory or sensory pathway stimulation activates cholinergic BF neurons via a corticofugal circuit in which PFC is a necessary component.

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