

ACETYLCHOLINE RELEASE IS ELICITED IN THE VISUAL CORTEX, BUT NOT IN THE PREFRONTAL CORTEX, BY PATTERNED VISUAL STIMULATION: A DUAL *IN VIVO* MICRODIALYSIS STUDY WITH FUNCTIONAL CORRELATES IN THE RAT BRAIN

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Abstract—By its projections to the primary visual and the prefrontal cortices, the basal forebrain cholinergic system is involved in cognitive processing of sensory stimuli. It has been suggested that visual stimulus-induced cholinergic activation of the visual cortex may exert a permissive role on thalamocortical inputs. However, it is not known if visual stimulation elicits cholinergic activation of high-order brain areas in the absence of attentional need. In the present study, we measured the effects of patterned visual stimulation (horizontal grating) on the release of acetylcholine with dual-probe *in vivo* microdialysis in the visual and the prefrontal cortices of anesthetized rats. We also used retrograde tracing to determine the anatomical relationships of cholinergic neurons with neurons of the visual system and the prefrontal cortex. Finally, we evaluated a functional correlate of this stimulation, namely *c-fos* immunolabeling. Patterned visual stimulation elicited significant increases in acetylcholine release in the visual cortex, accompanied by an increased number of *c-fos* immunoreactive neurons in this brain area. In contrast, in the prefrontal cortex, neither the level of acetylcholine release nor the number of *c-fos* immunoreactive neurons was significantly changed because of the stimulation. Cholinergic basal forebrain neurons projecting to the visual or the prefrontal cortices were both localized within the horizontal limb of the diagonal band of Broca but were not immunoreactive for *c-fos* during visual stimulation. No parts of the visual system were found to directly project to these basal forebrain neurons. These results suggest the differential involvement of cholinergic projections in the integration of sensory stimuli, depending on the level of activity of the targeted cortical area. © 2005 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: basal forebrain, *c-fos* immunostaining, cholinergic system, sensory stimuli processing, retrograde tracing, vision.

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Abbreviations: ACh, acetylcholine; ANOVA, analysis of variance; BF, basal forebrain; ChAT, choline acetyltransferase; DY, Diamino Yellow; FB, Fast Blue; HDB, horizontal limb of the diagonal band of Broca; PBS, phosphate buffer; PFC, prefrontal cortex; SI, substantia innominata; VDB, vertical limb of the diagonal band of Broca; V1, primary visual cortex; V1M, monocular primary visual cortex.

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Basal forebrain (BF) cholinergic neurons are involved in the processing of sensory information and task-relevant stimuli through their projections to the cerebral cortex. Regions of cholinergic innervation include both primary cortical sensory areas and high-order cognitive regions (association cortical areas), such as the prefrontal cortex (PFC; Mesulam et al., 1983; Luiten et al., 1987; Gaykema et al., 1990; Woolf, 1991). This implies a role for the BF in both primary as well as a high-order (i.e. PFC) cortical processing of sensory stimuli.

Cortical acetylcholine (ACh) release is enhanced in certain sensory cortical areas by a variety of sensory stimuli (Collier and Mitchell, 1966; Akaishi et al., 1990; Inglis and Fibiger, 1995; Kilgard and Merzenich, 1998). For example, ACh efflux is increased in the rat primary visual cortex (V1) in response to stroboscopic stimulation (Inglis and Fibiger, 1995) and diffuse light (Collier and Mitchell, 1966). Pharmacological and physiological studies suggest a critical role for ACh in cortical facilitation or long-term potentiation of neuronal responses to visual stimuli (Donoghue and Carroll, 1987; Greuel et al., 1988; Lamour et al., 1988; Kirkwood et al., 1999). Moreover, coupling of sensory stimuli with BF stimulation leads to long-term enhancement of neuronal excitability in the somatosensory cortex (Verdier and Dykes, 2001) and dramatic reorganization of cortical maps in the auditory cortex (Kilgard and Merzenich, 1998). It has hence been proposed that ACh released from BF projections may be involved in the processing of thalamocortical inputs in primary sensory cortices.

Likewise, release of ACh in the PFC may contribute to high-level cognitive functions by facilitating the final association of sensory stimuli within this cortical area. Lesions of the BF cholinergic system induce decreases in PFC neuronal firing rate (Gill et al., 2000) and impairments of attentional abilities (Muir et al., 1992; Voytko, 1996; Gill et al., 2000; McGaughy et al., 2000; Passetti et al., 2000; Arnold et al., 2002). Moreover, demands for sustained attention are accompanied by increases in ACh efflux in the rat PFC (Gill et al., 2000; Passetti et al., 2000; Dalley et al., 2001) and cholinergic agents modulate performance in attentional tasks (Granon et al., 1995). These data have led to the proposal that BF cholinergic neurons contribute to attentional function through their projections to the PFC.

Although these studies suggest that the BF plays an important role in cortical integration of sensory stimuli, it is not known how ongoing sensory stimuli might induce activity in BF neurons, given that thalamic sensory regions

apparently do not directly project to the BF (Zaborszky et al., 1991). BF neurons receive input from the PFC (Vertes, 2004) and are activated by electrical activation of the PFC (Golmayo et al., 2003). In relation to these findings, a new perspective is now emerging that suggests cholinergic BF neurons may be activated by PFC neurons receiving convergent sensory inputs. Following input from the PFC, BF neurons may, in turn, modulate activity in sensory areas. As such, the BF may play a role in top-down attentional control of sensory stimuli processing in response to activity in cortical areas associated with higher cognitive functions (Sarter et al., 2001; Golmayo et al., 2003).

We investigated here whether the cholinergic system plays a local or more global role in cortical processing of visual stimuli. We used a dual *in vivo* microdialysis approach to evaluate whether visual stimulation elicits ACh release specifically in the V1 region or in both the V1 and PFC areas. We eliminated attentional demand using anesthetized animals and used patterned visual stimuli that specifically activate visual neurons. Retrograde tracing experiments were conducted to establish possible overlap in cholinergic BF neurons projecting to V1 and PFC. In addition, also with retrograde tracing, we asked whether visual structures (e.g. retina) project directly to BF neurons. Finally, the pattern of visual stimulus-activated V1, PFC and/or the BF neurons was examined using *c-fos* immunoreactivity. Some of these data have appeared in abstract form (Laplante et al., 2002).

EXPERIMENTAL PROCEDURES

Long-Evans rats (275–325 g) were obtained from Charles River Canada (St-Constant, Québec, Canada) and housed individually in a 12-h light/dark cycle with free food access. Distinct groups of rats were used for three different experiments: (1) *in vivo* microdialysis ($n=7$), (2) *c-fos* immunostaining ($n=8$) and (3) retrograde tracing ($n=10$). The effects of monocular visual stimulation on cortical ACh release (*in vivo* microdialysis) and neuronal activation (*c-fos* immunostaining) were investigated. Animal care and protocols conformed to the Canadian Council for Animal Care guidelines on the ethical use of animals. All efforts were made to minimize both the suffering and number of animals used. All experiments were approved by le Comité de déontologie de l'expérimentation sur les animaux de l'Université de Montréal and the McGill University Animal Care Committee.

Visual stimulation

The following monocular visual stimulation paradigm, adapted from Girman et al. (1999) and Porciatti (1999), was used for the *in vivo* microdialysis experiments (after basal level of ACh release had been established in the perfusate) or before *c-fos* immunocytochemistry. Rats were anesthetized with urethane (1.2 g/kg; Sigma Chemical Co., Oakville, Ontario, Canada) to prevent head movements, which ensured consistent ocular stimulation, stress and attention demand. Urethane is commonly used in experiments with rats, since it produces long periods of anesthesia, has a wide safety margin, and has little effect on normal blood pressure and respiration (according to the Guide to the Care and Use of Experimental Animals of the Canadian Council for Animal Care). Rats were positioned in a stereotaxic frame to ensure constant exposure to the stimulus in the desired eye. Animals were then placed into a closed black chamber. Body temperature

was maintained at 37 °C with a thermostatically controlled heating pad (FHC, Bowdoinham, ME, USA). Pupil dilation and accommodation paralysis with atropine was not used. Drying of the eye was prevented with a natural eye lubricant (Ophtapharma, Montréal, Québec, Canada). A computer monitor (30×25 cm, Titanium; Apple Computer Inc., Cupertino, CA, USA) was placed 32 cm parallel to the long axis of the rat and centered on the eye. A horizontal sinusoidal grating (contrast 90%, 0.08 c/day, 3.4 Hz) was generated by VPixx software (v 8.5; Sentinel Medical Research Corp., Québec, Canada) and displayed on the computer monitor for 30 min. We selected the orientation, temporal and spatial frequency of the grating based on published values that have been shown to induce an optimal response in most V1 neurons (Girman et al., 1999; Porciatti et al., 1999).

In vivo microdialysis

We used a dual-dialysis probe approach to simultaneously measure ACh release in the monocular V1 (V1M) and the medial PFC. The microdialysis procedure was similar to that which has been previously described (Quirion et al., 1994; Day et al., 2001; Laplante et al., 2004), with minor modifications. Surgery was performed 2 days before the *in vivo* dialysis experiment. Rats were anesthetized with a mixture of ketamine (50 mg/kg; Vetrep-harm, Belleville, Ontario, Canada), xylazine (5 mg/kg; Novopharm, Toronto, Ontario, Canada) and acepromazine (0.5 mg/kg; Ayerst, Montréal, Québec, Canada) and immobilized in a stereotaxic frame. The skull was exposed through a midline incision and the insertion of the temporal muscle displaced to expose the parietal bone. For the visual cortex, we used a transverse horizontal probe made as described previously (Day et al., 2001). The dialysis membrane (molecular weight cutoff 60 kDa, i.d.=0.22 mm, o.d.=0.31 mm; Hospal Industry, Lyon, France) was covered with epoxy glue along its whole length except for 3 mm corresponding to the area of dialysis. The transverse probe was inserted into the V1M contralateral to the stimulated eye (mm from Bregma: AP -6.7, L +2.0–5.0, V -1.8; Paxinos and Watson, 1995). The ends of the dialysis tube were connected to a stainless steel cannula with epoxy glue. For the medial PFC, a vertical intracerebral guide cannula (MAB 2.14.G; Scipro, Concord, Ontario, Canada) was lowered just over the medial PFC contralateral to the stimulated eye (mm from Bregma: AP +2.8, L +0.5, V -2.0; Paxinos and Watson, 1995). Steel cannulas were secured on the top of the skull using dental cement. The microdialysis probe (MAB 6.14.4; molecular weight cutoff 15 kDa, o.d. 0.6 mm, 4 mm; Scipro) was prepared according to the manufacturer's instructions and inserted within the vertical intracerebral guide cannula 1 h prior to the *in vivo* dialysis experiment. Each animal was dialyzed only once.

Dialysis probes were connected to a microliter-syringe pump and perfused with a cerebrospinal fluid-like solution containing (in mM) NaCl, 123; KCl, 3; CaCl₂, 1.3; MgCl₂, 1; NaHCO₃, 23 and sodium phosphate buffer (PBS), 10 (pH 7.4). Neostigmine bromide (5 μM), an acetylcholinesterase inhibitor, was added to the solution to increase recovery, as commonly used in anesthetized animals (Fournier et al., 2004; Antoniou et al., 1997; Kurosawa et al., 1989). This concentration of acetylcholinesterase inhibitor does not induce changes in sensory-evoked cortical activity as assessed by field potentials recording (Oldford and Castro-Alamancos, 2003). Moreover it has been demonstrated that varying the concentration of neostigmine in the microdialysis perfusate does not affect significantly the magnitude or the duration of the sensory-evoked ACh efflux (Himmelheber et al., 1998). Probes were perfused at a flow rate of 5 μl/min. After a 1 h wash out, 10 min dialysate fractions were collected for a 1 h period. When the basal level of ACh release stabilized, unilateral visual stimulation began. Dialysates were collected up to 2 h after end of the stimulation to establish the return to basal levels.

The detection limit of the assay was between 20 and 100 fmol of ACh/50 μ l of perfusate. ACh content in dialysate fractions was determined using HPLC separation, post-column enzymatic reaction and electrochemical detection. ACh was separated from other molecules in the dialysate on a reverse-phase column (75 \times 2.1 mm) that was pre-treated with sodium lauryl sulfate. All column hardware and packing materials were obtained from Chrompack (Ratiran, ME, USA). Following separation, the eluate passed through an enzyme reactor (10 \times 2.1 mm) containing acetylcholinesterase (EC 3.1.1.7; type VI-S; Sigma Chemical Co.) and choline oxidase (1.1.3.17; Sigma Chemical Co.) covalently bound to glutaraldehyde-activated Lichrosorb NH₂ (10 μ m; Merck, Darmstadt, Germany). ACh reacted with the enzymes to give a stoichiometric yield of hydrogen peroxide, which was electrochemically detected at a platinum electrode at a potential of +500 mV versus an Ag/AgCl reference electrode (VT-03 flow cell; Antec, Fremont, CA, USA). The mobile phase consisted of a 0.2 M aqueous potassium PBS, pH 8.0, containing 1 mM tetramethylammonium hydroxide and was degassed online (CMA 260; Cargenie Medicin, Stockholm, Sweden) and delivered at 0.35–0.45 ml/min by a dual-piston pump (ESA 580; ESA, Chelmsford, ME, USA). Sample concentrations were calculated by comparison to known standards and expressed in fmol of ACh/ μ l of perfusate.

After each experiment, brains were removed for histological processing to confirm probe location (Fig. 1). Twenty micrometer coronal cryostat sections of frozen brains were mounted onto gelatin-coated slides and stained with Cresyl Violet. Probes location was established using a rat brain atlas (Paxinos and Watson, 1995). Results from animals with inadequately located probes were discarded.

C-fos immunocytochemistry

Rats ($n=7$) were used to evaluate the effects of monocular visual stimulation on *c-fos* immunoreactivity within PFC, horizontal limb of the diagonal band of Broca (HDB) and V1M regions (Paxinos and Watson, 1995). At the end of the visual stimulation period, rats were removed from the stereotaxic apparatus and kept in darkness for 90 min. They were then killed and brains were preserved with an intracardiac perfusion with 4% paraformaldehyde. Brains were collected and sliced into 35 μ m sections using a vibratome. Sections were pre-incubated for 1.5 h at room temperature in PBS (pH 7.4) containing 0.5% Triton X-100 and 1.5% normal donkey serum. They were then incubated overnight at room temperature with rabbit-anti-*c-fos* primary antibody (1:10,000; Oncogene Research Products, San Diego, CA, USA) in PBS-Triton-0.5% normal donkey serum. This was followed by a 2 h incubation in donkey-anti-rabbit secondary antibody (Jackson ImmunoResearch, Westgrove, PA, USA) and then for 1 h in the avidin-biotin complex (ABC Elite kit; Vector Laboratories, Burlingame, CA, USA). After each incubation step, rinses were carried out in PBS containing 0.25% Triton. A peroxidase-substrate kit Vector SG (Vector Laboratories) was used to develop the reaction product. Sections were then mounted on slides, dehydrated and cover-slipped with permount. Photographs were taken with a Leica DC 500 digital camera on three consecutive sections in the selected regions (mm from Bregma, PFC: AP from +2.70 to +2.80; HDB: AP from +0.20 to +0.30; and V1M: from -6.70 to -6.80). Full-image reconstruction of the entire region was carried out using Adobe Photoshop. Labeled cell counting was accomplished with ImageJ (public domain NIH program). Control animals were processed in the same manner, except that they did not receive visual stimulation.

Retrograde track tracing

Retrograde tracing was performed to determine the location of BF neurons sending projections to the PFC and V1M. According to the findings of this experiment, a second retrograde tracing ex-

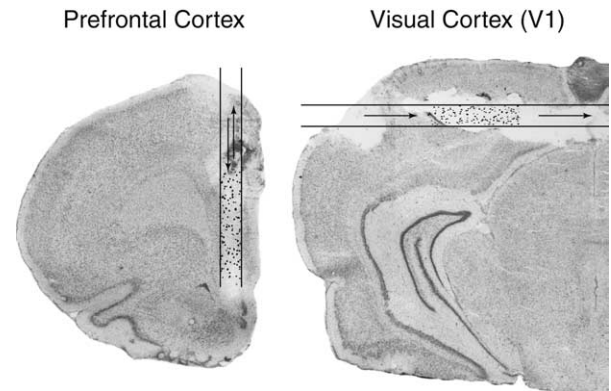


Fig. 1. Cresyl Violet-stained coronal sections of one representative rat brain at the level of microdialysis probe. The probes are represented schematically with arrows showing the direction of the perfusing solution. Dots represent the active microdialysis membrane of the probe. Left: location of the vertical probe implanted within the PFC (Bregma +2.7 mm); right: location of the horizontal probe inserted within the visual cortex contralateral to the stimulation (Bregma -6.7 mm; lateral 2–5 mm).

periment was performed to examine cerebral structures sending projections to the horizontal part of the diagonal band of Broca.

Retrograde tracers were infused in two adjacent sites in order to label a reasonable portion of the structure to match probes position in the microdialysis experiments and the location of areas selected for the analysis of *c-fos* immunostaining. A dual-injection of 2% Diamino Yellow (DY; Sigma Chemical Co.) within the PFC (mm from Bregma: AP +2.7, L +0.5, V -4.5 and AP +3.7, L +0.5, V -4.5) and 2% Fast Blue (FB; Sigma Chemical Co.) within the V1M (AP -6.3, L +3, V -1 and AP -7.0, L +3, V -1) was performed for each rat. Two percent FB was also injected within the HDB (AP +0.3, L +0.8, V -7.8, AP -0.3, L +2, V -8.4) of another group of rats. Dyes were diluted in a sterile saline solution and injected with a glass micropipet (60 μ m tip diameter) by intracerebral pressure injection (Micropump; Harvard Apparatus, St-Laurent, Québec, Canada) at a rate of 0.1 μ l/min for each site (0.4 μ l/site). Micropipets were left in place for 5 min after injection in order to reduce diffusion along the insertion track. After 10 days, rats were transcardially perfused with 4% paraformaldehyde and the entire brain was sectioned (30 μ m thick sections) using a vibratome (VT 1000s; Leica Microsystems, Richmond Hill, Ontario, Canada). Sections of the BF region were further immunostained for choline acetyltransferase (ChAT). Other sections were mounted in Vectamount (Vector Laboratories) and observed with a fluorescent microscope (DMR HC; Leica Microsystems). The locations of the injection sites were determined using a rat brain atlas (Paxinos and Watson, 1995). For HDB-injection experiments, retrogradely labeled neurons located in the visual system, including the dorsal geniculate body, the superior colliculus, the pretectal nuclei and the retina, were examined.

ChAT immunocytochemistry

In order to verify whether BF neurons projecting to PFC or V1M (labeled with DY or FB, respectively) were cholinergic, we performed ChAT immunostaining (Chemicon, Temecula, CA, USA). After 1 h incubation in a blocking solution (PBS containing 0.5% Triton, 1.5% normal goat serum, pH 7.4), free floating sections were incubated for 48 h in goat-anti-ChAT antibody (1:200) at 4 °C on a rotating platform, followed by a biotinylated, rabbit-anti-goat, secondary antibody (Vector Laboratories). Sections were then incubated in a solution of TRITC-conjugated streptavidin (Jackson ImmunoResearch Laboratories). After each incubation step, rinses were carried out in PBS containing 0.5% Triton.

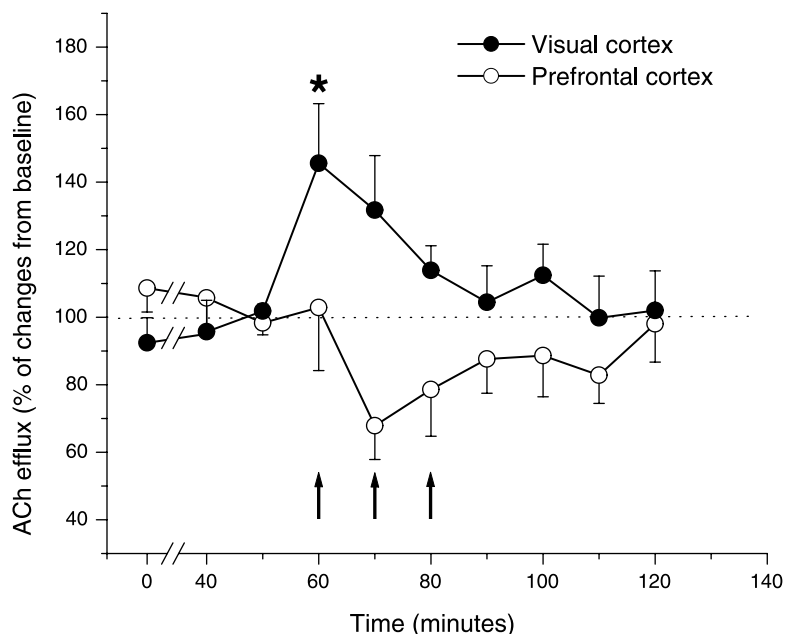


Fig. 2. Graphic representation of the mean values of ACh release expressed in percentage of change from baseline as a function of time (in minutes). Visual stimulation (vertical arrows) was applied for 30 min, starting after the stabilization of the basal level of ACh release. In the V1 (black circles), ACh release was significantly increased in the first dialysate fraction during the visual stimulation (* $P < 0.05$). In the PFC (open circles) the visual stimulation did not induce consistent changes.

The proportion of cholinergic neurons (red) among retrogradely labeled neurons (yellow or blue) was determined in the vertical limb of the diagonal band of Broca (VDB) and HDB, and substantia innominata (SI) by dividing the number of double labeled neurons by the total number of DY or FB labeled neurons in each region.

Statistical analysis

For *in vivo* microdialysis experiments, four samples prior to the visual stimulation were averaged and taken as 100%. A two-way analysis of variance (ANOVA) was performed on the percentage of baseline values with both factors, time (before and during the stimulation) and brain areas, as repeated measures. Significant interactions were decomposed using simple main effects tests. Post hoc pairwise contrasts between means were conducted using Tukey tests, with $P < 0.05$ being considered significant.

For *c-fos* immunoreactivity, a paired Student's *t*-test was used to analyze the significant differences between the number of *c-fos* positive neurons in cortices contralateral to the stimulated eye (which receive 90% of the input from the stimulated eye) versus ipsilateral side.

RESULTS

In vivo microdialysis

Average basal levels of ACh release in V1M and PFC were 6.2 ± 2.8 fmol/ μ l and 12.9 ± 3.8 fmol/ μ l, respectively. Patterned visual stimulation increased ACh release in the contralateral visual cortex, which then returned progressively to the basal level (Fig. 2). On the other hand, visual stimulation did not enhance ACh release in PFC (Fig. 2). Two-way ANOVA revealed a non-significant main effect of time ($F_{(3,55)} = 2.15$; $P = 0.12$), a significant main effect of brain area ($F_{(1,55)} = 16.54$; $P = 0.007$) and a significant in-

teraction between these factors ($F_{(3,55)} = 3.68$; $P = 0.03$). Simple main effect tests revealed a significant effect of time in the visual cortex ($F_{(3,36)} = 3.19$; $P = 0.04$), but not in the PFC ($F_{(3,36)} = 2.28$; $P = 0.10$). Tukey's post hoc tests revealed a significant difference in ACh release in the visual cortex between time 0 and 10 min, $P < 0.05$.

Retrograde track tracing

Fig. 3 shows two representative injection sites of DY or FB within the PFC and V1M cortex of the same animal,

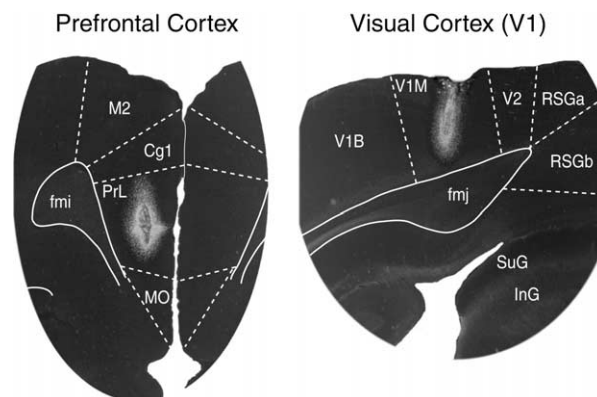


Fig. 3. Microphotographs showing the injection sites of the retrograde tracers within the PFC (left, DY; Bregma +2.7 mm) or the V1 (right, FB; Bregma -6.3 mm). For clarity, the different brain regions are indicated according to the rat brain atlas from Paxinos and Watson (1995). Cg1, cingulate cortex; fmi, fmj, fornix median; M2, motor cortex; MO, medial orbital cortex; PrL, prelimbic cortex (PFC); RSGa,b, retrosplenial cortex; SuG, InG, superior colliculus; V1B, V1, binocular area; V2, secondary visual cortex.

respectively. The volume of each injection site was approximately $2300 \mu\text{m}^3$ (500–600 μm diameter). All cortical layers were infiltrated by the dye. The general distribution pattern of neurons projecting to V1M or PFC matched that which has been shown in previous studies, with neurons being particularly abundant in the frontal cortex, claustrum, endopyriform nucleus and visual cortex, ipsilateral to the injection site.

Regarding BF neurons, those stained with FB (projecting to V1) were mainly located within the HDB and ventral part of the SI (Fig. 4), ipsilateral to the injection site. A large proportion (73%) of these neurons (34–50 FB neurons counted per animal) was double-stained with ChAT. Occasionally, neurons from the VDB were also immunolabeled for FB, 93% of them being cholinergic. Retrogradely DY-labeled BF neurons projecting to the PFC were mainly located within the VDB and HDB (Fig. 4). Most (74%) of the DY positive neurons located within the HDB/SI (20–50 DY neurons counted per animal) and 57% of DY positive neurons located within the VDB were labeled for ChAT. Cholinergic BF neurons projecting to PFC (DY-labeled) or V1M (FB-labeled) were closely apposed with intermingled neurites (Fig. 5). Occasionally, neurons showed a yellow nucleus with faint blue fluorescence in the cytoplasm, suggesting the colocalization of FB and DY. However, this blue fluorescence could also have been due to the diffusion of the DY dye from the nucleus to the cytoplasm, as reported by Keizer and collaborators (1983).

To assess whether cholinergic neurons projecting to V1M were activated during visual stimulation, we injected the retrograde dye in the HDB/SI region (data not shown). Retrogradely labeled cells were found within the PFC, entorhinal cortex, perirhinal cortex, amygdala, raphe, ventral tegmental area, parabrachial nucleus and the V1 and secondary visual cortex. In term of the hippocampus, ipsilateral to the injection site, the pyramidal cell layer of the CA1 field of the posterior hippocampus and the CA2 region of the dorsal hippocampus were labeled. These projection findings are consistent with previous studies (Zaborszky et al., 1991). Specific care was taken in the analysis of brain structures pertinent for vision, such as the dorsolateral geniculate nucleus, the superior colliculus, pretectal nuclei and the retina. These regions, however, did not contain any FB positive neurons projecting to the HDB/SI region.

C-fos immunocytochemistry

Our patterned visual stimulation induced a significant increase in the number of neurons expressing *c-fos* (16.2%, $P=0.025$, paired Student's *t*-test) in layer IV of V1M, contralateral to the stimulated eye (receiving the input from the stimulated eye) compared with the ipsilateral cortex (Table 1; Fig. 6). The number of *c-fos*-labeled neurons was not different in the PFC (contra versus ipsilateral, $P=0.698$, paired Student's *t*-test) and *c-fos*-immunostained nuclei were absent in the HDB. There was no change in the number of *c-fos* immunoreactive neurons in the visual cortex of control rats kept in darkness (data not shown).

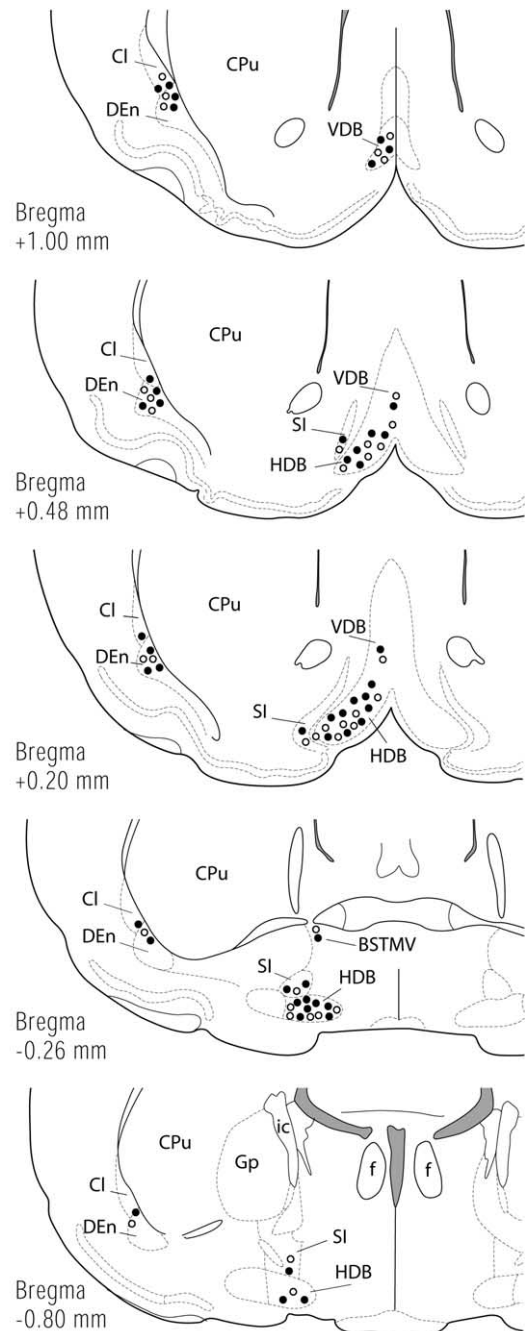


Fig. 4. Schematic representation illustrating the localization of BF neurons labeled with DY (open circles, projecting to the PFC) or FB (dark circles, projecting to the V1M) on coronal sections. Dots represent the relative proportion of each type of neuron, but not the exact number of cells. Diagrams are taken from the rat brain atlas, anterior from Bregma +1.0 to –0.80 mm. Note that these neurons are predominantly concentrated within the HDB and SI. Some neurons are contained within the VDB. Neurons located within the claustrum (Cl) and endopyriform nucleus (Den) were also represented, since these nuclei show a high proportion of both types of retrogradely labeled neurons. BSTMV, bed nucleus stria terminalis, medial division, posterior part; f, fornix; Gp, globus pallidus; ic, internal capsule; Cpu, caudate nucleus.

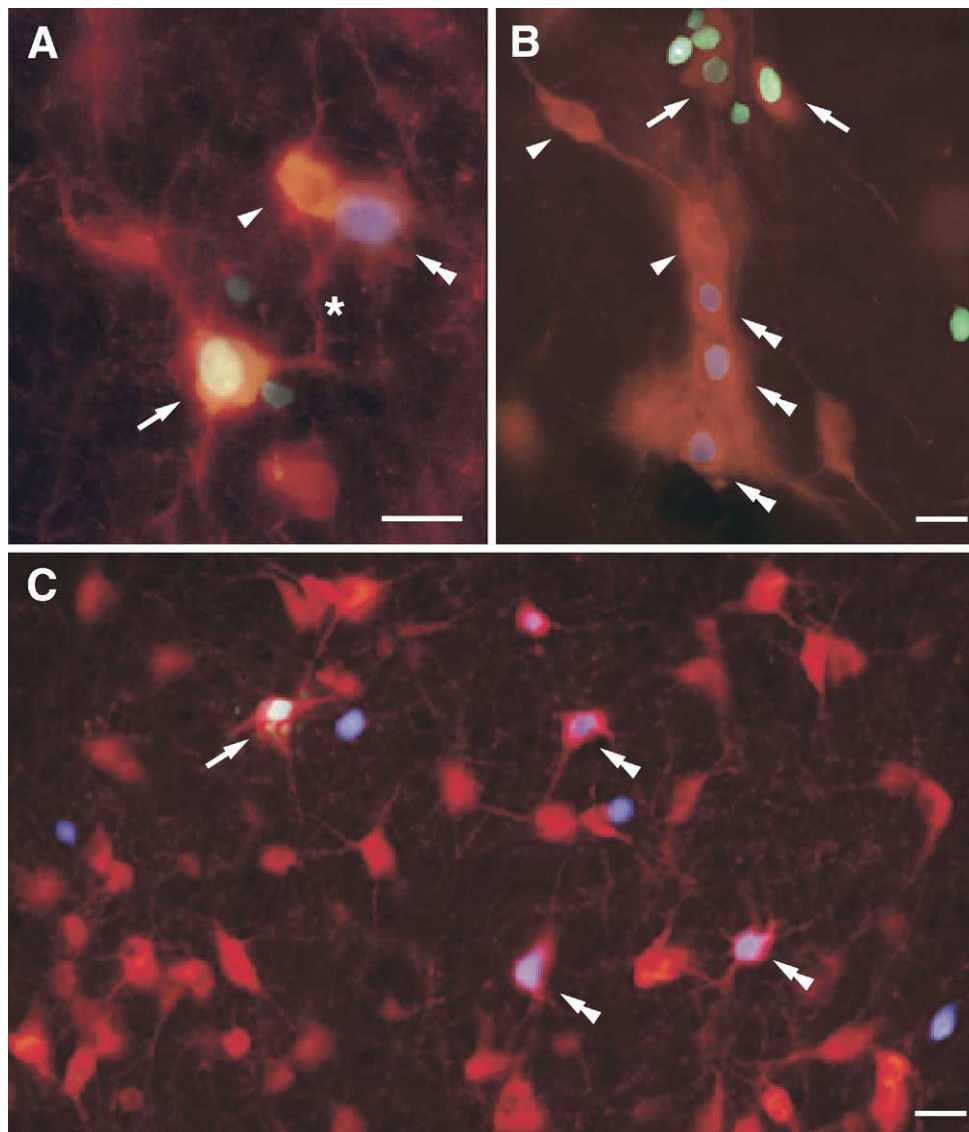


Fig. 5. Fluorescence microphotographs from neurons labeled with DY (yellow), FB (blue) and ChAT (red) located in the HDB. (A) Superposition of fluorescent photographs showing a cholinergic neuron not retrogradely labeled (arrowhead) with a cholinergic neuron projecting to the PFC (arrow) and another projecting to the visual cortex (double arrowheads). Note that neurites are intermingled (star). (B) Superposition of fluorescent photographs showing cholinergic neurons projecting to the visual (double arrowheads), prefrontal (arrow) cortices or not stained with any retrograde tracer (arrowhead). These neurons are in close contact. (C) Low magnification of neurons from the HDB showing retrogradely labeled cholinergic neurons sending projections to the visual (stained with FB, double arrowheads) or prefrontal (stained with DY, arrow) cortices. Scale bars = 10 μm in A, B; 15 μm in C.

DISCUSSION

Our results indicate that cortical ACh release is increased with regional specificity in response to a finely tuned sensory stimulus in urethane-anesthetized animals. The release in ACh in V1 is accompanied by an increase in the number of *c-fos* immunoreactive neurons in this cortical area. In contrast, no effects were seen in the PFC. Moreover, the majority of neurons projecting either to PFC or V1M are located in the HDB, although they were segregated. Approximately 75% of the retrogradely labeled neurons were cholinergic, in both cortical areas. However, HDB neurons did not show *c-fos* immunolabeling during

visual activation, nor did they receive afferents from visual structures (except, of course, from the visual cortex).

Table 1. Number of *c-fos* immunoreactive neurons in ipsilateral and contralateral cortices to the visually stimulated eye

Number of <i>c-fos</i> neurons	Ipsilateral cortex	Contralateral cortex
V1M, $n=8$	1839 \pm 572	2138 \pm 652*
PFC, $n=5$	655 \pm 94	666 \pm 93

Values are expressed in mean \pm S.E.M. of neurons/ mm^2 .

* $P < 0.03$, paired Student *t*-test.

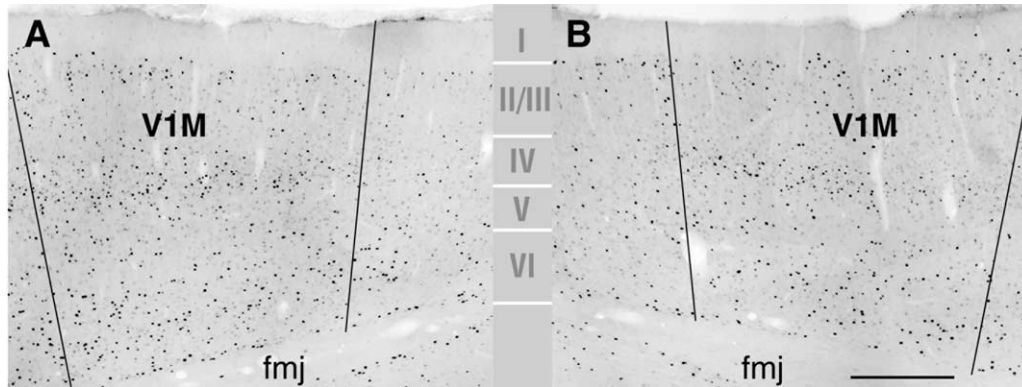


Fig. 6. Microphotographs of neurons immunolabeled for the early gene *c-fos* within the V1M (Bregma: -6.70 mm). Note that the number of neurons in layer IV of the contralateral cortex to the stimulated eye (A) is increased compared with the ipsilateral side (B). fmj, fornix median. Scale bars=50 μ m.

Regional specificity of cortical ACh release during visual stimulation

The main objective of this study was to determine whether a specific visual stimulus could induce release of ACh simultaneously in different cortical areas, or whether it would selectively activate only certain cortical regions. Considering that both attention (Gill et al., 2000; Himmelheber et al., 2001) and stress (Mark et al., 1996; Thiel et al., 1998) have previously been shown to evoke cortical ACh release in our experimental paradigm, we eliminated these possibilities by using urethane-anesthetized animals. Moreover, the chosen visual stimulus is physiologically relevant for induction of specific cortical activation. Indeed, sinusoidal horizontal grating has been shown to induce optimal selective activation of the visual cortex neurons in rats, as demonstrated by single cell or extracellular electrophysiological recordings (Girman et al., 1999; Porciatti et al., 1999). Our study is the first to report that ACh release in the V1 is enhanced by this stimulation paradigm. These results are in agreement with previous studies showing increases in ACh release in the visual cortex induced by diffuse light (Collier and Mitchell, 1966), spots of light (Arnold et al., 2002), flashes (Jimenez-Capdeville et al., 1997) or a checkerboard pattern (Fournier et al., 2004). Moreover, our results revealed the attenuation of the stimulation of ACh release at the end of the 30 min visual stimulation period. Increased extracellular ACh concentrations may have led to the stimulation of presynaptic inhibitory muscarinic autoreceptors resulting in hampered ACh release. This phasic effect suggests the adaptation of the cholinergic fibers to long-lasting visual stimulus as cortical ACh release is usually enhanced by novel or conditioned stimuli (Acquas et al., 1996). Alternatively, habituation of cortical visual neurons to repetitive stimulation could also occur.

Interestingly, the dual-probe approach used in the present study indicated that visually induced increases in the ACh release in the V1 area was not accompanied by significant changes in PFC ACh release. This finding complements the observations of a pioneering study by Collier and Mitchell (1966), who used the cortical cup to show that

light illumination or electrical stimulation of the lateral geniculate nucleus produced a greater overflow of ACh in the visual cortex versus the motor or sensorimotor cortices in rabbit. A recent study also showed a region-specific cortical release of ACh elicited by different sensory modalities (Fournier et al., 2004). Thus, our results support a localized cortical effect of cholinergic projections. This is in keeping with the findings that electrical stimulation of different sites within the BF induces localized cortical ACh release (Jimenez-Capdeville et al., 1997). However, another study reported similar increases in the amount of ACh released in PFC and somatosensory cortex during somatosensory stimulation (Himmelheber et al., 1998), suggesting that the BF has a role in global cortical arousal. This apparent discrepancy may be due to the use of non-anesthetized animals, in which PFC may also be activated and possibly reciprocally activate additional BF neurons. This could engage BF cholinergic neurons in additional functions. The discrepancy may also result from activation of other BF neurons projecting to both cortical areas examined, or to differential stimuli intensities.

Anatomical basis for differential cortical ACh release following visual stimulation

We determined next the anatomical correlates that may support the observed differential cortical ACh release. A triple immunofluorescent method (double-retrograde tracing study and ChAT immunoreactivity) was used to delineate any overlap and/or collateralization of BF neurons that project to V1 and PFC, as well as their phenotype.

Data obtained in the present study are consistent with previous findings (Lamour et al., 1982; Carey and Rieck, 1987; Luiten et al., 1987; Dinopoulos et al., 1989; Gaykema et al., 1990; Calarco and Robertson, 1995; Woolf, 1991) demonstrating the localization of BF neurons within the HDB and ventral part of the SI that project to V1M or PFC. However, novel anatomical findings include: (1) the segregation of BF neurons projecting to V1M or PFC, such that these neurons never appear to send collaterals to both cortical areas; and (2) that the majority (75%) of retrogradely labeled neurons are positive for ChAT immunocy-

tochemistry, revealing their cholinergic nature. Moreover, these data suggest that HDB neurons projecting to the visual cortex likely interact with HDB neurons projecting to the PFC, since cholinergic fibers of FB and DY positive neurons were observed in a given region on the same section. Such interconnections between BF neurons have been demonstrated at the ultrastructural level (Zaborszky and Duque, 2000), although projection was not established. A certain proportion (25%) of FB- and DY-labeled HDB neurons did not stain for ChAT. This demonstrated that non-cholinergic HDB neurons, possibly GABAergic neurons, also project to V1M or PFC (Freund and Gulyas, 1991; Gritti et al., 1993; Sarter and Bruno, 2002).

Our findings rule out the possibility that visual subcortical structures simultaneously activate V1M and HDB neurons. Indeed, no visual non-cortical structures, including the retina, superior colliculus, dorsolateral geniculate body or pretectal nuclei, contain retrogradely labeled neurons when FB tracer was injected within the HDB. Considering that one study reported that mink retinal projections pass through the HDB on their way to the suprachiasmatic nucleus (Martinet et al., 1992), we paid particular attention to the possibility that retinal ganglion cells could be labeled. However, no retrograde tracer was observed in these cells, suggesting that there were no retinal terminals within the HDB in the rat.

Functional correlates for differential cortical ACh release

The above results suggest that ACh release is specifically evoked in the V1M area by patterned visual stimulation. This may be due to the activation of a subpopulation of HDB neurons by indirect mechanisms. The *c-fos* immunoreactivity study, however, showed that this marker was not induced within HDB neurons. As the expression of early genes is indicative of neuronal activation, this suggests that BF neurons were not activated. The apparent absence of BF neuron activation is consistent with a recent single-unit recording study showing that stroboscopic visual stimulation did not induce electrical responses in BF neurons in urethane-anesthetized rats (Golmayo et al., 2003). Activation of BF neurons was, however, seen when direct afferents were stimulated from the PFC (Golmayo et al., 2003) and in awake monkeys (Santos-Benitez et al., 1995).

The number of *c-fos* immunoreactive neurons was significantly increased by patterned visual stimulation in the V1M. This finding was expected and is in accordance with a previous study (Montero and Jian, 1995). Moreover, the number of *c-fos* immunoreactive neuron was not affected in the PFC, suggesting that neuronal activity in V1M neurons was not relayed to the PFC. This might be related to the anesthetic effect. Similarly, in anesthetized animals, visual stimulation was not accompanied by increased spike activity in PFC (Golmayo et al., 2003). Taken together, these results suggest that in absence of consciousness, visual stimulation can still elicit neuronal activation in V1M, but not in PFC, within which activity is associated with visual awareness and visual attention (Rees et al., 2002).

These functional results reveal a direct link between the cortical area exhibiting neuronal activation during visual stimulation and increased ACh release. This suggests that the differential cortical ACh release observed here is elicited by an effect on local terminals, rather than specific activation of HDB cholinergic neurons (either directly or through the activation of ascending reticular formation afferents). The increased ACh release may thus be induced by local factors released from activated visual neurons or glial cells, such as potassium, glutamate, neurotrophins, etc. Alternatively, intracortical cholinergic neurons could also contribute to cortical ACh release, but it is well known that these neurons represent only a very small fraction of the population of total cortical cholinergic fibers (Rye et al., 1984; Freund and Gulyas, 1991; Vaucher and Hamel, 1995).

CONCLUSION

Taken together, this study suggests that sensory-evoked ACh release within a given cortical area is due to intrinsic level of neuronal activity, rather than to an anticipatory role of the BF. The increased ACh release in V1M most likely reflects the role of ACh in regulating the strength of the thalamo-cortical sensory inputs. This effect may be mediated through nicotinic receptors, since nicotine but not muscarine was shown to induce sensory-evoked responses *in vivo* (Oldford and Castro-Alamancos, 2003) or by long term cholinergic enhancement through muscarinic depression of GABAergic inputs. This facilitating effect then likely leads to the selection and/or discrimination of the visual stimuli. It would be of interest to test whether further activation of the PFC would result in sustained ACh release within the V1M by a feedback mechanism that potentially might extend the contribution of the cholinergic system to cortical integration of sensory information (Sarter et al., 2001; Golmayo et al., 2003).

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