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Cholinergic neurons in the pedunculopontine tegmental nucleus are involved in the mediation of prepulse inhibition of the acoustic startle response in the rat

Michael Koch, Martin Kungel, Horst Herbert

Tierphysiologie, Universität Tübingen, Auf der Morgenstelle 28, D-72076 Tübingen, Germany

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Abstract. The amplitude of the acoustic startle response (ASR) is markedly reduced when the startle eliciting pulse is preceded by a weak, non-startling stimulus at an appropriate lead time, usually about 100 ms. This phenomenon is termed prepulse inhibition (PPI) and has received considerable attention in recent years as a model of sensorimotor gating. We report here on experiments which were undertaken in order to investigate some of the neural mechanisms of PPI. We focused on the characterization of the cholinergic innervation of the pontine reticular nucleus, caudal part (PnC), an obligatory relay station in the primary startle pathway. The combination of retrograde tracing with choline acetyltransferase-immunocytochemistry revealed a cholinergic projection from the pedunculopontine tegmental nucleus (PPTg) and laterodorsal tegmental nucleus (LDTg) to the PnC. Extracellular recording from single PnC units, combined with microiontophoretic application of the acetylcholine (ACh) agonists acetyl-β-methylcholine (AMCH) and carbachol revealed that ACh inhibits the majority of acoustically responsive PnC neurons. Neurotoxic lesions of the cholinergic neurons of the PPTg significantly reduced PPI without affecting the ASR amplitude in the absence of prepulses. No effect on long-term habituation of the ASR was observed. The present data indicate that the pathway mediating PPI impinges upon the primary acoustic startle circuit through an inhibitory cholinergic projection from the PPTg to the PnC.

Key words: Acetylcholine – Acoustic startle response – Pedunculopontine tegmental nucleus – pontine reticular nucleus, caudal part – Prepulse inhibition – Schizophrenia

Introduction

The acoustic startle response (ASR) is a contraction of the facial and skeletal muscles following a sudden and loud acoustic stimulus. This simple behavior serves as a model for the study of the mechanisms underlying sensorimotor transfer in vertebrates and of the mechanisms of its extrinsic modulations (Davis and File 1984). A marked reduction of the ASR amplitude can be observed when a weak stimulus is presented 50–150 ms before the startle pulse occurs (Hoffman and Ison 1980), a phenomenon termed prepulse inhibition (PPI). PPI is thought to represent a "gating" mechanism, which can be activated by stimuli of several sensory modalities, thereby inhibiting neurons of the primary startle pathway. Since PPI of the ASR was found to be impaired in patients suffering from schizophrenia (Braff et al. 1978), there has been considerable interest in determining the neural and neurochemical substrates of this phenomenon of sensorimotor gating. Hence, a good deal of information is already available on the pharmacological mechanisms and the neuroanatomical basis of PPI, mainly due to the work of Swerdlow and co-workers: it is evident from a recent series of experiments in rats that an overactivity of the mesolimbic dopamine system, involving D2-receptors in the nucleus accumbens, leads to a disruption of PPI. Furthermore, it has been shown that a GABAergic pathway descending from the nucleus accumbens to the ventral pallidum is a substrate for the decrease in PPI induced by mesolimbic dopamine overactivity (summarized in Swerdlow et al. 1992a). How the information conveyed by this pathway ultimately influences the primary acoustic startle circuit is as yet not fully understood. It was suggested that the pedunculopontine tegmental nucleus (PPTg) could be an important part of the prepulse circuit and might act as a relay between the ventral pallidum and the pontine reticular nucleus, caudal part (PnC), an obligatory part of the primary startle pathway (Swerdlow and Geyer 1993). Acoustically evoked potentials occurring in conjunction with the ASR were recorded from the PPTg by Ebert and Ostwald (1991) who consequently suggested that the PPTg might be involved in the inhibition of the ASR. Direct evidence for an involvement of the midbrain tegmentum, including the PPTg, in mediating PPI comes from lesion studies

Correspondence to: M. Koch

(Leitner et al. 1981; Swerdlow and Geyer 1993) and from electrical stimulation experiments (Saitoh et al. 1987; Lai and Siegel 1990). Furthermore, electrophysiological data also suggest that the PnC could be the site where the prepulse circuit impinges upon the primary startle circuit (Wu et al. 1988).

Another phenomenon of sensorimotor gating is habituation, which leads to a decline of the ASR amplitude following repeated stimulation. Electrolytic lesion experiments have suggested that the PPTg might also be involved in mediating the habituation of the ASR (Capps and Stockwell 1968; Groves et al. 1974; Jordan and Leaton 1983).

The PPTg has been thoroughly characterized physiologically and neuroanatomically (Garcia-Rill 1991), but its exact nuclear boundaries are still a matter of debate. Based on cytoarchitectural, hodological and neurochemical criteria the PPTg has been defined as consisting of large cholinergic neurons which do not receive input from extrapyramidal areas, and which can therefore be differentiated from the adjacent non-cholinergic midbrain extrapyramidal area (Rye et al. 1987, 1988; Lee et al. 1988; Steininger et al. 1992). Recent papers have challenged this strict definition of the PPTg by showing that non-cholinergic and cholinergic cells are intermingled in the PPTg and by demonstrating input from extrapyramidal structures (Semba and Fibiger 1992; Spann and Grofova 1992).

A cholinergic projection from the PPTg to the pontine reticular formation has been shown in cats (Mitani et al. 1988; Shiromani et al. 1988) and rats (Semba et al. 1990). It is very likely that this cholinergic input directly influences the giant reticulospinal neurons of the PnC (Jones 1990; Grofova and Keane 1991) which are known to be of particular importance for the mediation of the ASR (Wu et al. 1988; Koch et al. 1992; Lingenhöhl and Friauf 1992). The PnC also receives a direct input from the caudal part of the basal nucleus of Meynert (Koch and Ebert 1993), which also contains cholinergic neurons (e.g. Woolf 1991), but the neurotransmitter of this descending projection is as yet not identified. A recent investigation provided neurochemical support for the action of ACh as a transmitter in the PnC (Camacho-Arroyo 1991) and its physiological effects in the pontine brainstem have already been described with respect to its role in REM sleep (Greene and Carpenter 1985; Baghdoyan et al. 1987; Gerber et al. 1991; Jones 1991). The effects of ACh on the ASR are not completely clear (Hughes 1984), although a recent paper has suggested a role for cholinergic systems in the modulation of the ASR (Wu et al. 1993).

The aim of the present study was to investigate whether the mesopontine cholinergic cell groups represent the sole source of cholinergic input to the PnC, or whether additional cholinergic pathways, e.g. from the basal nucleus of Meynert, impinge upon PnC neurons. To achieve this goal, we combined retrograde tracing of the efferents to the PnC with immunocytochemical labeling of choline acetyltransferase (ChAT). Furthermore, we used extracellular single unit recording combined with microiontophoresis in order to characterize the effects of ACh-agonists on the acoustically responsive neurons in the PnC. Finally, we performed behavioral tests to measure the effects of lesioning the cholinergic neurons of the PPTg on PPI and on long-term habituation of the ASR.

Material and methods

Retrograde tracing and ChAT-immunocytochemistry

Five male Wistar rats weighing 200–250 g were anesthetized with chloral hydrate (420 mg/kg injected intraperitoneally), supplemented by the topical application of lidocain, which was applied prior to craniotomy. The retrograde tracer Fluoro-Gold (FG; 2% solution in 0.1 M cacodylate buffer pH 7.5; Fluorochrome Inc.) was injected iontophoretically (+5 μA, 20 min pulsed anodal current, 5 s on/off) through glass micropipettes (30 μm tip diameter) into the PnC using stereotaxic coordinates from Paxinos and Watson (1986). After a survival period of seven days, the animals were anesthetized and perfused through the aorta with 0.01 M phosphate-buffered saline (PBS) followed by 500 ml of cold 4% paraformaldehyde (PFA) and 0.01% glutaraldehyde in 0.1 M phosphate buffer (PB), and then with 200 ml of cold 10% sucrose in 0.1 M PB. The brains were removed and placed in 20% sucrose in PB at 4° C until they sank. Coronal sections (50 μm) were taken on a freezing microtome, mounted, coverslipped with DePeX, and examined under a fluorescence microscope. Adjacent sections were collected in 0.01 M PBS and rinsed twice. They were then incubated at room temperature in 3% goat serum in PBS with 0.1% Triton X-100. After rinsing the sections three times in PBS they were incubated for 48 hours in a polyclonal rabbit anti-human choline acetyltransferase (generously donated by Dr Louis B. Hersh) which was diluted 1:500 in carrier (PBS with 3% goat serum). To visualize the immunoreactivity, sections were incubated for 1.5 h at room temperature in a solution containing rhodamine isothiocyanate conjugated goat anti-rabbit IgGs (DAKO 1:100). Sections were washed again in PBS, mounted on gelatin-coated slides, air dried, cleared in xylene, and coverslipped with DePeX. Sections were viewed and photographed under a Reichert-Jung Polyvar microscope. Peroxidase stained sections (see below) were analyzed under bright-field illumination while double-labeled material was viewed with fluorescence illumination employing a UV-filter system for Fluoro-Gold and a G1-filter system for rhodamine. Retrogradely labeled, immunofluorescent, and double-labeled neurons were counted and plotted with the aid of a microscope stage position plotter system (Minnesota Datametrics). Areas with double-labeled neurons were also subsequently photographed with the two filter systems.

ChAT-staining for the examination of the quinolinic acid lesions

The procedure was the same as described above, except that the immunoreactivity was visualized by the peroxidase-antiperoxidase (PAP) technique. After washing in PBS, the sections were incubated in swine anti-rabbit IgGs (DAKO) diluted 1:50 in carrier (3% goat serum in PBS) for 2 h at room temperature, rinsed again in several changes of PBS, placed in the rabbit PAP-complex (DAKO) diluted 1:150 in carrier and incubated for 2 h at room temperature. After rinsing in PBS the sections were reacted with 0.05% diaminobenzidine and 0.01% hydrogen peroxide for 5 min. The reaction was terminated by transfering the sections into PBS. Sections were then mounted onto gelatin-coated slides, air dried, dehydrated in a graded series of ethanol, cleared in xylene and coverslipped with Entellan. Adjacent sections were stained with thionin.

Electrophysiology and pharmacology

Sixteen rats (200–300 g) were anesthetized with urethane (1.9 g/kg i.p.) supplemented by topical application of lidocain. During the experiment the animal's electrocardiogram was continuosly monitored, its body temperature kept at 37° C, and its electrolytic balance maintained by subcutaneous injection of isotonic saline (1 ml/h). The animal's brainstem was exposed by aspirating parts of the cerebellum located underneath the parietal bone. Acoustic stimulation was performed in an open field condition using a high frequency speaker located in a sound attenuated chamber. Pure tone stimuli of 50 ms duration and 2.5 ms rise and fall times were presented at a rate of 1 Hz. Peristimulus time histograms (PSTHs) from dot displays were produced online by a PDP 11 computer. Teflon-insulated tungsten electrodes (impedance: 10 MΩ) were used for extracellular single unit recordings and multibarreled glass pipettes were used for drug application. The pipettes were pulled to a tip diameter of 3–5 µm and glued to the recording electrodes, with the tips separated by 30 µm. The barrels of the multi-barreled electrode were filled with solutions of the muscarinic/nicotinic acetylcholine agonists acetyl-β-methylcholine (AMCH; Sigma; 0.5 M) or carbachol (RBI; 0.01 M). The drugs were ejected microiontophoretically by a programmable constant-current source (custom-made at McGill University, Montreal, Department of Physiology) with positive currents up to 100 nA. Retaining currents were 5–10 nA. The electrode assemblys were lowered through the pontine reticular formation by a hydraulic motor microdrive, at a caudorostral angle of 20° and a mediolateral angle of 10°. From each neuron characteristic frequency, minimal threshold, and minimal response latency were determined. Spike rates of spontanous and tone-evoked activity were calculated from the PSTHs. Spikes occurring in a period of 50 ms before the stimulus onset were taken as the spontaneous activity. Spikes occurring during the stimulus of 50 ms were taken as the tone-evoked activity (including the spontaneous activity). PSTHs were calculated from 50 consecutive stimuli. After the recording of at least three PSTHs for control, drugs were iontophoretically applied for at least the time of one PSTH. After drug application, a varying number of PSTHs were recorded until the overall spike numbers had again reached control values (recovery). Electrode tracks were marked by electrolytic lesions (6 µA, 20 s). Upon termination of the experiments the rats were decapitated, and the brains were removed and immersion-fixed with formaldehyde. Coronal sections (50 µm) of the brain were cut on a freezing microtome, mounted and stained with thionin or neutral red. Recording sites were reconstructed from the electrolytic lesions.

Brain lesions and behavioral tests

Twenty five male Wistar rats (200–300 g) were kept in groups of five animals per cage under a continuous light-dark cycle (7.00 on, 19.00 off) with food and water freely available. Lesions of the PPTg were done according to Rugg and co-workers (1992) with slight modifications of their protocol. Rats were anesthetized with chloral hydrate (420 mg/kg i.p.) and placed in a stereotaxic apparatus. They received bilateral injections of 18 or 36 nmol (0.1 or 0.2 µl of a 0.18 M solution) of quinolinic acid (QA, Sigma) dissolved in 0.1 M PB (pH 7.4) at two different rostrocaudal levels of the PPTg (coordinates according to Paxinos and Watson 1986: −7.3 posterior to Bregma, ±1.8 mm lateral, −7.5 mm ventral; −8.2 mm posterior to Bregma, ±1.8 mm lateral, −7.0 mm ventral) through glass micropipettes (tip diameter 30 µm). Pressure injections of QA were made at a rate of 0.02 µl/10 s. The injection pipette was left in situ for five minutes in order to allow for diffusion of QA. Control animals sustained the same surgical procedures except that PBS was injected instead of QA. After completion of surgery, anesthesia was prologued by injection of one third of the initial dose of chloral hydrate in order to prevent the occurrence of convulsions.

The rats were allowed to recover for one week and were then tested for their ASR amplitudes with and without prior presentation of a weak prepulse. The measurement of the ASR was accomplished after placing the rat in a wire mesh cage (19.5 × 9 × 8 cm) mounted on a digital balance (Sartorius L2200 S) inside a sound-attenuated chamber (100 × 80 × 60 cm). The deflections of the balance caused by the rat's movements were digitized and fed into a computer for further analysis. Following a 5 min acclimation period, during which time the rats received no stimuli except for a continuous white background noise of 55 dB SPL (RMS) the tests began. The test session included an initial startle-stimulus followed by four different trial types: pulse alone, prepulse followed by pulse 100 ms after prepulse-onset, prepulse alone, and no stimulus. A total of 15 presentations of each trial type were given in a pseudorandom order. Interstimulus interval was 30 s. Acoustic stimuli ("Pulse": 100 dB SPL broad band noise bursts, 20 ms duration. "Prepulse": 75 dB SPL 10 kHz tone pulse, 0.4 ms rise/fall times, 20 ms duration) were delivered through loudspeakers at a distance of 40 cm from the test cage. The intensity of the prepulse was 10 dB below the startle threshold at 10 kHz (Pilz et al. 1987). The whole-body startle amplitude was calculated from the difference between the peak-to-peak amplitudes of the output of the balance within time-windows of 80 ms after and 80 ms before the onset of the acoustic startle stimulus ("Pulse"). The mean startle amplitude was calculated from the 15 values obtained for each trial block. The response to the single startle pulse at the beginning of the test session was discarded. The amplitudes of the ASR with and without prepulses were calculated for the sham and lesion groups. Percent PPI was calculated as 100 - (startle amplitude on prepulse trials/startle amplitude on pulse alone trials) × 100 and compared between the groups. Since the evaluation of percent scores might confound the results (Davis 1988), we also analyzed the arithmetic difference between the ASR amplitudes with and without prepulses (difference scores) with respect to treatment. In order to investigate the lesions' effects on long-term habituation of the ASR the animals were further tested on three days following the prepulse tests. On those days the rats received 60 startle stimuli as described above without presentation of prepulses.

After completion of the behavioral tests the animals were perfused and their brains processed for ChAT-immunocytochemistry according to the protocol described above. Adjacent sections were stained with thionin. ChAT-immunopositive cells were counted in the PPTg bilaterally on representative sections taken at six different rostrocaudal levels. The rats' eardrums were examined upon termination of the experiments and no damage resulting from the earbars of the stereotaxic apparatus was observed. All statistical comparisons between the two experimental groups were performed with Mann-Whitney's U-test (two-tailed). Long-term habituation of the ASR was analyzed with Kruskal-Wallis' one-way analysis of variance (H-test; two-tailed).

Results

Retrograde tracing and ChAT-immunocytochemistry

A co-distribution of retrogradely labeled cells after FG injection into the PnC (Fig. 1a) and choline acetyltransferase (ChAT) immunopositive cells was found in the pedunculopontine (PPTg) and laterodorsal (LDTg) tegmental nuclei, as well as in the basal nucleus of Meynert (Fig. 1b-e). Double-labeled cells, however, were only seen in the PPTg (Fig. 2) and LDTg, but not in the basal nucleus of Meynert. We estimated that about 12% of the cholinergic cells in the PPTg on the ipsilateral side, and 7% on the contralateral side project to the PnC.

Retrogradely labeled neurons were also found in the medullary, pontine and mesencephalic reticular formation, the cochlear nucleus, the superior olivary complex (SOC), the central gray (CG), the deep mesencephalic nu-

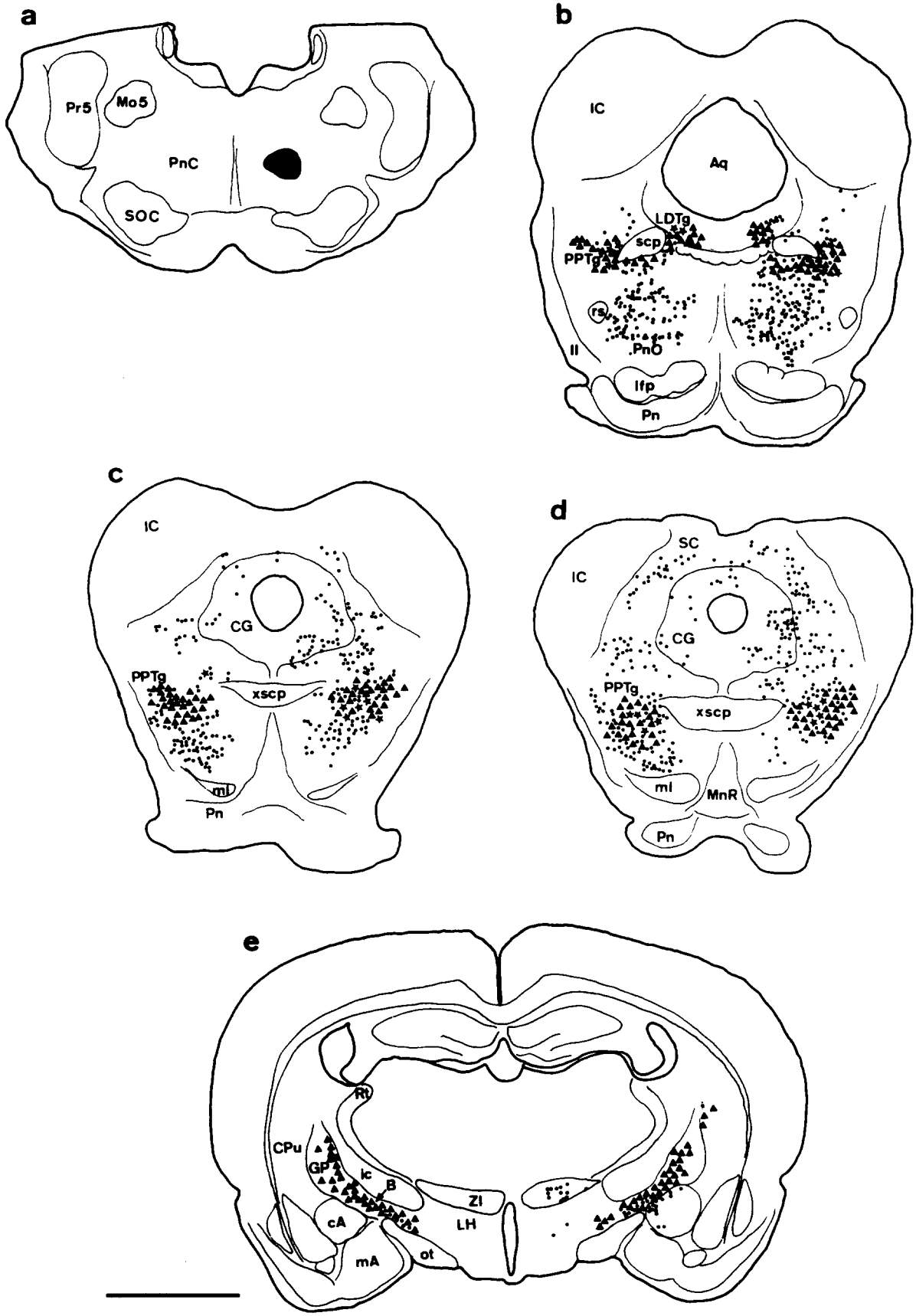


Fig. 1a–e. Line drawings of coronal sections through the brain illustrating an injection site of Fluoro-Gold in the PnC (black spot in **a**), retrogradely labeled cells (dots), ChAT-immunopositive cells (triangles), and double-labeled cells (stars) in midbrain and forebrain nuclei (**b-e**: caudal to rostral). One dot or triangle corresponds to 1–2 labeled cells. Note that double-labeled cells are only found in the PPTg and LDTg, but not in the basal nucleus of Meynert (B). Abbreviations (if not included in the text): Aq: aqueduct, CPu: caudate putamen, GP: globus pallidus, ic: internal capsule, IC: inferior colliculus, lfp: longitudinal fasciculus pons, ll: lateral lemniscus, mA: medial amygdaloid nucleus, ml: medial lemniscus, MnR: median raphe nucleus, Mo5: motor trigeminal nucleus, Pn: pontine nuclei, PnO: oral pontine reticular nucleus, Pr5: principal sensory trigeminal nucleus, rs: rubrospinal tract, Rt: reticular thalamic nucleus, ot: optic tract, xscp: decussation of the superior cerebellar peduncle. Scale bar 2.5 mm

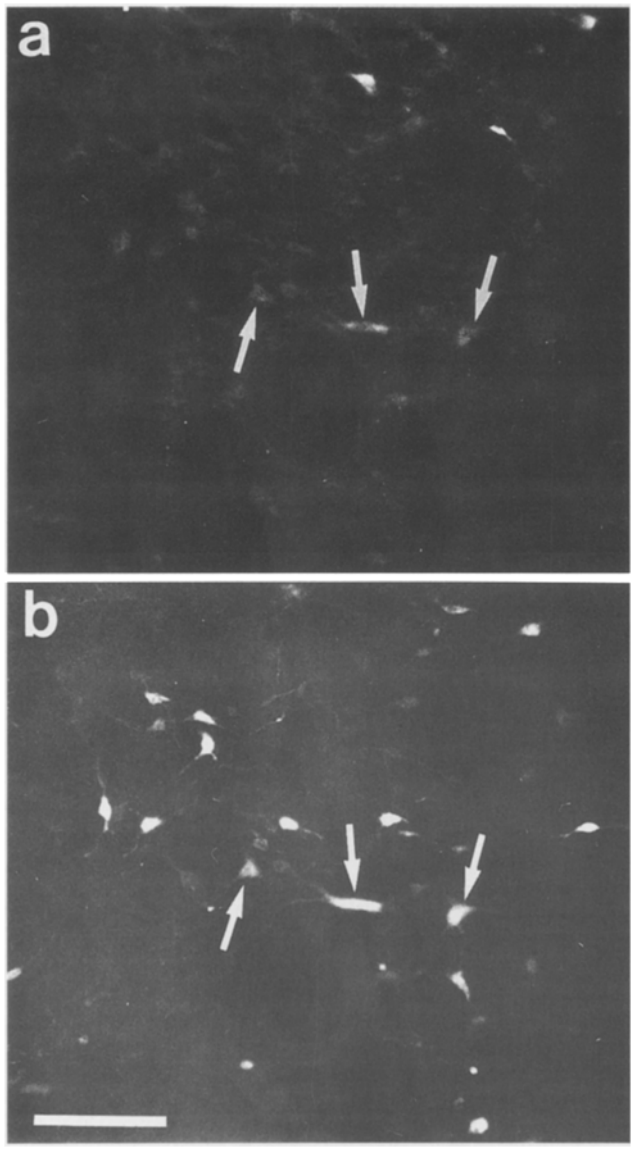


Fig. 2a,b. Fluorescence photomicrographs of a coronal section through the PPTg illustrating neurons immunoreactive to an antiserum against ChAT (**a**) and neurons retrogradely labeled after injection of FG into the PnC (**b**). The double-labeled neurons are indicated by arrows. Scale bar 100 μm

clei, the substantia nigra, the zona incerta (ZI), the lateral hypothalamus (LH), and the central nucleus of the amygdala (cA).

The overall distribution of ChAT-immunopositive cells in the brain as found in the present study closely resembles the ones described in the literature, i.e. the antibody labeled pericarya in the cholinergic cell groups Ch1-Ch6 (according to Mesulam's nomenclature; see Wainer et al. 1984), as well as in the striatum and cranial nerve motor nuclei.

Electrophysiology and pharmacology

Extracellular recordings were obtained from 57 single units in the PnC. Thirty seven units were acoustically responsive, 20 single units did not respond to acoustic stimulation but were spontaneously active. The latency of the first acoustically evoked spike ranged from 2–10 ms (mean 5.8 ms ± 0.4 ms s.e.m.), frequency tuning was generally broad, with characteristic frequencies ranging from 7–15 kHz (mean 10.5 kHz ± 0.3 kHz s.e.m.), and the minimal threshold intensity was 87 ± 1.6 dB SPL. Acetyl-β-methylcholine (AMCH) was tested in 28 acoustically responsive PnC units, 61% (17) were inhibited by 58.5% ± 8% (mean ± s.e.m) and 39% (11) were excited by 59.5% ± 12% (mean ± s.e.m). Carbachol was tested in 9 acoustically responsive PnC units, 78% (7) were inhibited by 35.9% ± 6% (mean ± s.e.m) and 22% (2) were excited by 38.2% ± 19% (mean ± s.e.m). A typical example of the inhibitory effect of AMCH on the acoustic responsiveness of a PnC neuron is given in Fig. 3. AMCH and carbachol did not lead to a complete block of the firing rate of the units, but instead dose-dependently reduced the activity until the maximal suppression of about 60% was reached. The spontaneous activity of the acoustically responsive units was inhibited to the same extent. Of the 20 non-acoustic PnC units 45% (9) were inhibited, and 45% (9) were excited by AMCH, in 10% (2) of the units AMCH application had no effect at all.

Brain lesions and behavioral tests

The number of ChAT-immunopositive cells in the PPTg was markedly reduced one week after the injection of 72 nmol of QA (two injections of 18 nmol bilaterally into the PPTg) compared to PBS-injected rats (Fig. 4a,b). This low dosage of QA did not lead to a total destruction of the PPTg, since no gliosis was observed (Fig. 4c). After injection of the higher dose of QA (144 nmol: two injections of 36 nmol bilaterally) into the PPTg the relative specificity of the neurotoxin was obviously lost, as indicated by a marked gliosis at the injection site. Rats with heavy gliosis in and around the PPTg (n = 7) and animals where the ChAT-immunocytochemistry did not work properly (n = 2) were excluded from the final analysis.

The statistically significant reduction of cholinergic cells in the PPTg (Mann-Whitney's U-test: $P = 0.003$ Fig. 5a) had no significant effect on the ASR amplitude in the absence of prepulses (Fig. 5b). Prepulses reduced the ASR amplitude by 51% in sham-lesioned rats compared to 22% in QA-lesioned rats (Fig. 5c). This statistically significant reduction of PPI following QA lesions was found for both percent scores and difference scores of PPI. A significant correlation between the number of ChAT-immunopositive cells in the PPTg and PPI was found by linear regression analysis ($r = 0.54$, $df = 12$, $P < 0.05$ for percent scores and $r = 0.59$, $df = 12$, $P < 0.03$ for difference scores), but not between the number of ChAT-immunopositive cells in the PPTg and the ASR amplitude measured in the absence of prepulses ($r = 0.50$, $df = 12$, $P = 0.07$).

A significant decrease of the ASR amplitude across four days was observed both in the sham (Kruskal-Wallis

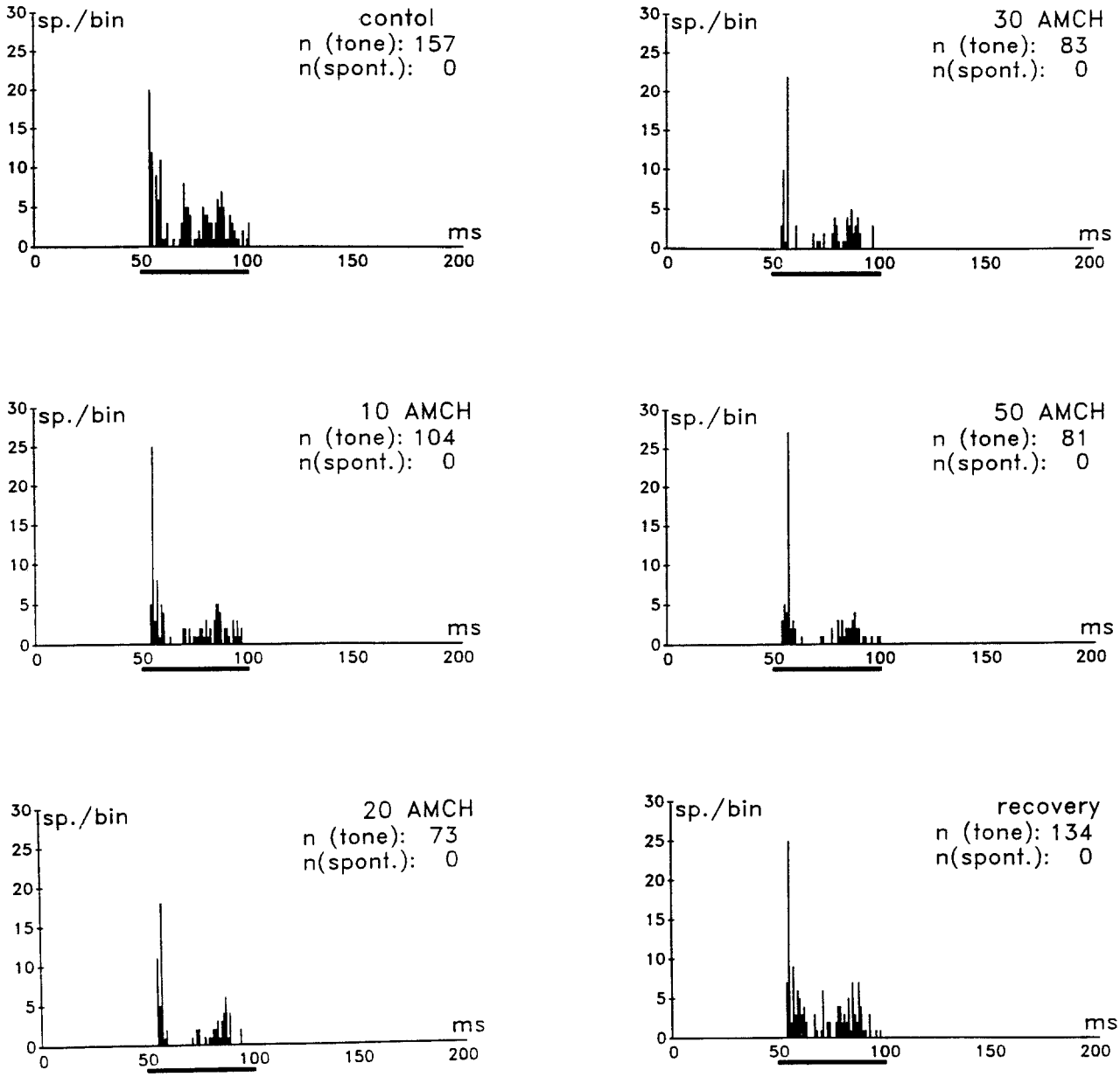


Fig. 3. Peristimulus time histograms (PSTHs) illustrating the response of a PnC unit to acoustic stimulation (8 kHz, 90 dB) and the inhibitory effect of different doses (in nA of iontophoretically applied current) of AMCH on the number of tone-evoked spikes. n's indicate the total number of tone-evoked and spontaneously occurring spikes calculated from 50 consecutive stimuli (bin width 1 ms)

H-test $P = 0.02$) and the lesion (Kruskal-Wallis H-test $P = 0.03$) groups, indicating that the destruction of cholinergic cells in the PPTg, following QA lesions, did not abolish long-term habituation of the ASR (Fig. 5d).

Discussion

Retrograde tracing and ChAT-immunocytochemistry

Our data show that the descending projections from the pedunculopontine and laterodorsal tegmental nuclei (Ch5 and Ch6 cell groups, respectively) provide the sole cholinergic input to the caudal pontine reticular nucleus. A cholinergic projection from the PPTg and LDTg to the PnC has been described in cats (Mitani et al. 1988; Shiromani et al. 1988) and in rats (Semba et al. 1990), but it was unclear whether the PnC receives additional cholinergic input from the basal nucleus of Meynert (Koch and Ebert 1993). We found that retrogradely labeled neurons were co-distributed with the ChAT-immunopositive cells in the caudal part of the basal nucleus of Meynert, but they were not double-labeled. This finding supports the data of Semba and co-workers (1989), who showed that the majority of basal forebrain neurons projecting to the lower brainstem are noncholinergic and have different physiological properties than the cholinergic neurons projecting to the cortex. Recent anatomical studies have shown that ChAT-immunoreactive varicosities terminate mainly on the cell bodies and proximal dendrites of

giant neurons in the reticular formation (Jones 1990) and that descending fibers from the PPTg form terminal-like boutons on giant PnC neurons (Grofova and Keane 1991). Thus, our anatomical findings, together with the data reported in the literature, strongly suggest a cholinergic projection from the PPTg to the giant neurons of the PnC. The PnC has long been implicated in the mediation of the ASR (Davis et al. 1982) and, more recently, the subpopulation of giant reticulospinal neurons in the PnC was suggested to function as the sensorimotor interface for the fast transmission of direct acoustic input to motor output necessary for the ASR (Wu et al. 1988; Kandler and Herbert 1991; Ebert and Koch 1992; Koch et al. 1992; Lingenhöhl and Friauf 1992). The PnC has also been described as the recipient of excitatory input from the amygdaloid complex mediating modulatory influences enhancing the ASR (Rosen et al. 1991; Koch and Ebert 1993).

The overall distribution of cholinergic cells throughout the rat brain as revealed by immunocytochemical staining with the present antibody against human ChAT is fully compatible with the various ChAT maps published by others using the same (Tago et al. 1989) or different (Armstrong et al. 1983; Wainer et al. 1984; Kimura et al. 1984; Tatehata et al. 1987; Woolf 1991; Henderson and Sherriff 1991) antisera. The results of the retrograde tracing experiment using FG as a tracer are largely consistent with those of a previous study where horseradish peroxidase was used (Shammah-Lagnado et al. 1987).

Electrophysiology and pharmacology

Our study reports on the effects of acetylcholine agonists on the single unit activity in the PnC, with special emphasis on the inhibitory effects of muscarinic ACh agonists on the acoustically responsive PnC neurons. The response characteristics of the acoustically driven PnC neurons (short latency, high minimal threshold intensity, broad frequency tuning with characteristic frequency around 10 kHz) found in the present study are very similar to those found in previous extracellular (Ebert and Koch 1992) and intracellular (Lingenhöhl and Friauf 1992) recordings from identified giant reticulospinal PnC neurons. The muscarinic/nicotinic ACh agonists tested in the present study both increased and reduced the spontaneous and the tone-evoked activity in the PnC. This finding is largely consistent with the results of Greene and co-workers' recordings in cats (Greene and Carpenter 1985) and brain slices of the rat (Greene et. al 1989) which show that the PnC includes a mixed population of cholinoceptive neurons. However a larger number of the *acoustic* units were inhibited by ACh agonists, whereas equal numbers of the *non-acoustic* neurons were excited and inhibited. The fact that we did not observe inhibition in *all* acoustically driven PnC units could be explained by the fact that the PnC receives also cholinergic input from the LDTg, a projection which might subserve a different function than the one from the PPTg. Since neither AMCH nor carbachol is a pure muscarinic agonist, the occurrence of excitatory effects might be mediated also by activation of the nicotinic receptors. This is unlikely, however, since it has been shown that the excitatory effects of carbachol on neurons in the medial PnC are mediated by the muscarinic receptor (Greene et al. 1989). The fact that maximal suppression of single unit activity was about 60% is suggestive of a *modulatory* inhibition. Gerber and co-workers found that muscarinic agonists hyperpolarize a subpopulation of PnC neurons by activation of an inward rectifying potassium conductance and they speculated that predominantly the *reticulospinal* PnC neurons are inhibited by ACh (Gerber et al. 1991). Since almost all acoustically responsive PnC neurons project to the spinal cord (Lingenhöhl and Friauf 1992), our present data, showing inhibition of tone-evoked activity of a major subpopulation of PnC neurons, support the assumption of Gerber and co-workers. Stimulation of the PPTg at an appropriate rate inhibits muscle activity, but can also activate muscles leading to stepping-like activity in the animals if the stimulation rate is changed (Kelland and Asdourian 1989; Lai and Siegel 1990). Thus, the neurons producing muscle tone suppression are likely to be intermingled with those increasing the activity of muscles. In this context it is interesting to note that PPTg neurons projecting to the spinal cord are *not* cholinergic (Goldsmith and Van der Kooy 1988) and that glutamatergic neurons have been found in the PPTg and LDTg (Clements and Grant 1990). It is therefore conceivable that the activation of muscles observed after PPTg-stimulation is based on the glutamatergic pedunculospinal pathway, whereas atonia is produced via the cholinergic pedunculoreticular circuit.

Brain lesions and behavioral tests

The results of our lesion experiment show that the PPTg is involved in PPI, but not in long-term habituation of the ASR. After submission of this article, Swerdlow and Geyer (1993) published a paper showing that electrolytic lesions of the PPTg decreased PPI. Their results are fully supported by our data. Our findings are based on selective excitotoxic lesions that are not confounded by the possibility of damage to fibers-of-passage, which must be taken into account for the interpretation of electrolytic and also some excitotoxic lesions. The low dose of QA (18 nmol per injection) used here has been shown to leave the fibers in the pontine reticular formation intact (Koch et al. 1992). We followed the protocol of Rugg and co-workers to produce relatively selective lesions of cholinergic cells in the PPTg, a technique that is based on the differential vulnerability of neurochemically distinct neurons to QA. These authors have shown that the cholinergic cells in the PPTg are vulnerable to the neurotoxic effects of QA and that the relative selectivity for destroying cholinergic cells in the brainstem is highest for QA compared to other neurotoxins. They reported that QA produced a 40% reduction of ChAT-immunopositive cells with little damage to non-cholinergic cells (Rugg et al. 1992), which is about the same degree of destruction of cholinergic cells as we found (Fig. 5a). Large electrolytic

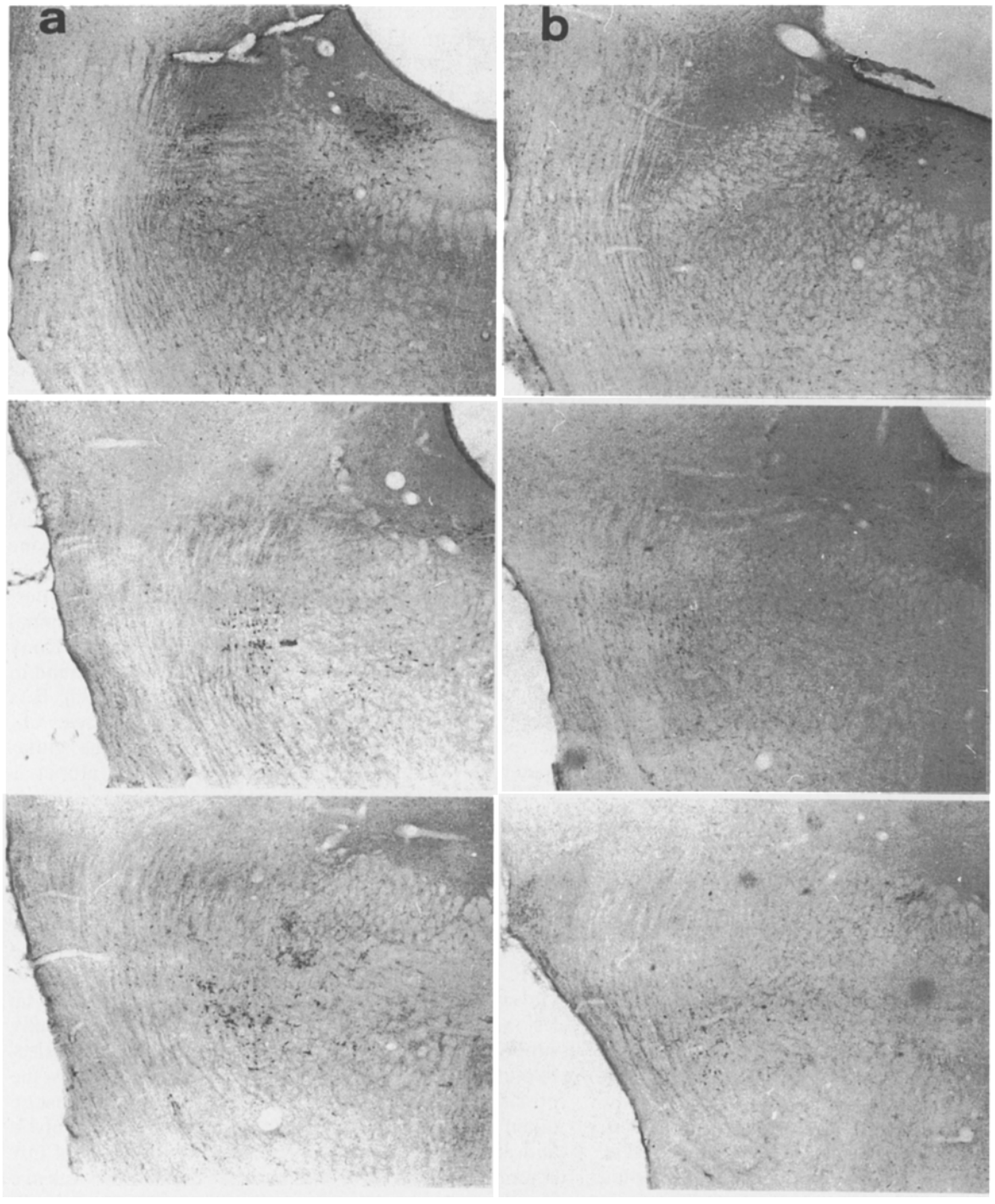
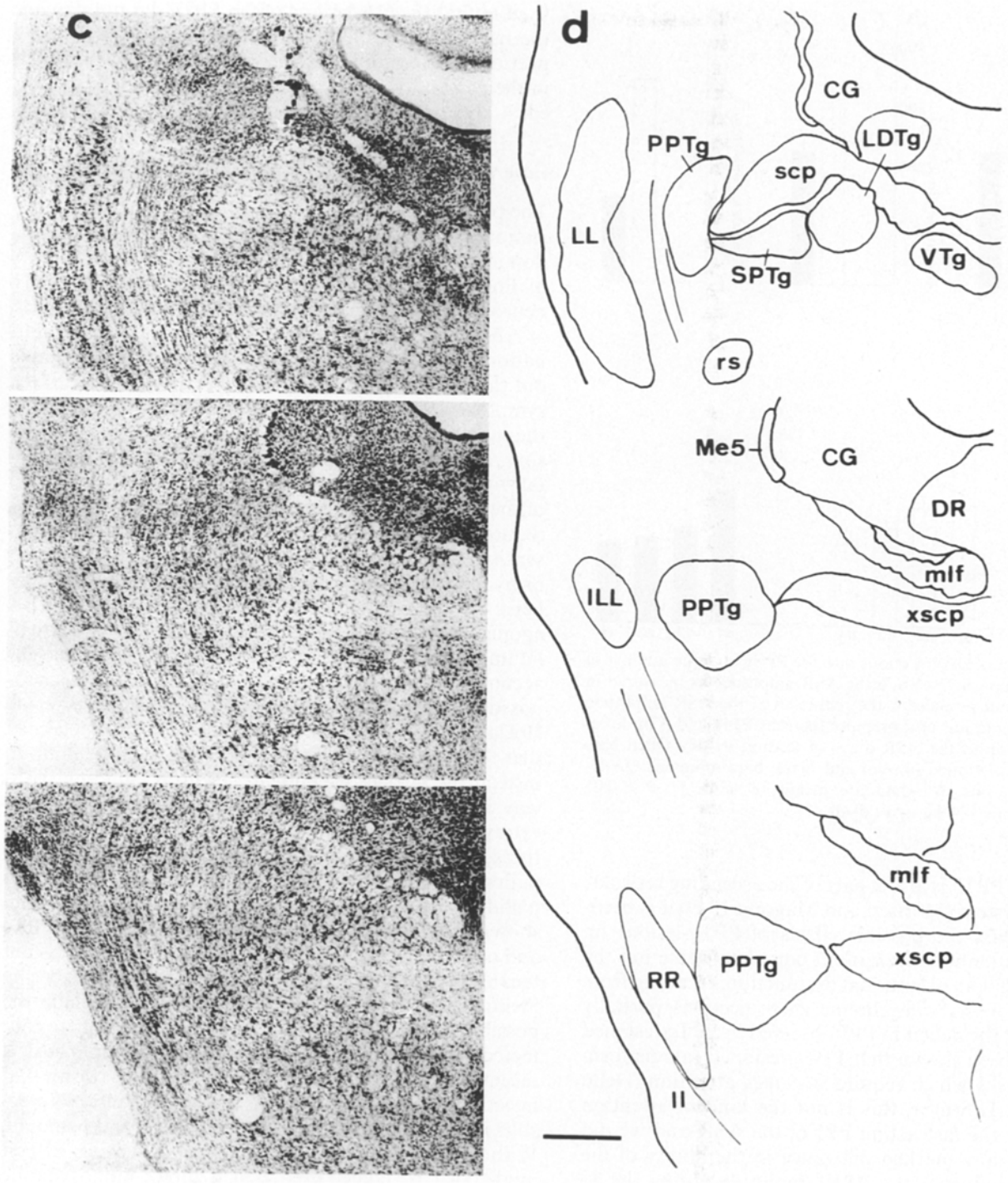


Fig. 4a–d. Representative coronal sections through the midbrain illustrating ChAT-immunopositive cells in the PPTg in sham-lesioned (**a**) and QA-lesioned (**b**) rats. Note the reduced number of ChAT-immunopositive cells in the rat lesioned with a total of 72 nmol of QA. Adjacent Nissl-stained sections of the brain shown in column **b** demonstrate that no gross tissue damage (i.e. gliosis) can be observed (**c**). Drawings of key structures of these sections from caudal to rostral (top to bottom) are depicted in **d**. Abbreviations (if not included in the text; see also legend to Fig. 1): DR: dorsal raphe nucleus, ILL: intermediate nucleus of the lateral lemniscus, LL: nuclei of the lateral lemniscus, Me5: mesencephalic trigeminal nucleus, RR: retrorubral nucleus, scp: superior cerebellar peduncle, SPTg: subpeduncular tegmental nucleus, VTg: ventral tegmental nucleus. Scale bar 500 μm

lesions of the lateral tegmentum including the PPTg reduced PPI (Leitner et al. 1981) to the same extent as we found in the present study after excitotoxic PPTg lesions, thus indicating that the PPTg is in fact the most important nucleus for PPI in the brainstem. Interestingly, however, we did not observe that PPTg lesions impaired long-term habituation of the ASR, an effect that has been observed following large electrolytic lesions of the tegmental region including the PPTg (Groves et al. 1974; Jordan and Leaton 1983). Reconciling our data with



these earlier reports, we conclude that at least the cholinergic part of the PPTg cannot be regarded as relevant for long-term habituation of the ASR. This suggestion is in line with the failure to influence the habituation of the ASR by the application of cholinergic drugs (Hughes 1984). Our data are further supported by the finding that rats treated with a choline-free diet, leading to a reduction of ACh in the brain, had impaired PPI (Wu et al. 1993). The two recently published papers reporting on the effects of PPTg lesions (Swerdlow and Geyer 1993), and cholinergic deficiency (Wu et al. 1993) on PPI have shown that a reduction of PPI is accompanied by an increase of the startle amplitude in the pulse alone trials. This is in contrast to our present data showing a slight, yet non-significant, decrease of the startle amplitude following PPTg lesions. There is no obvious explanation for this discrepancy, except for the different experimental techniques used (electrolytic lesions or choline-free diet vs. QA lesions) and for a few procedural and parametric differences, like startle pulse intensity and duration, numbers of test trials, background noise intensity and rat strain.

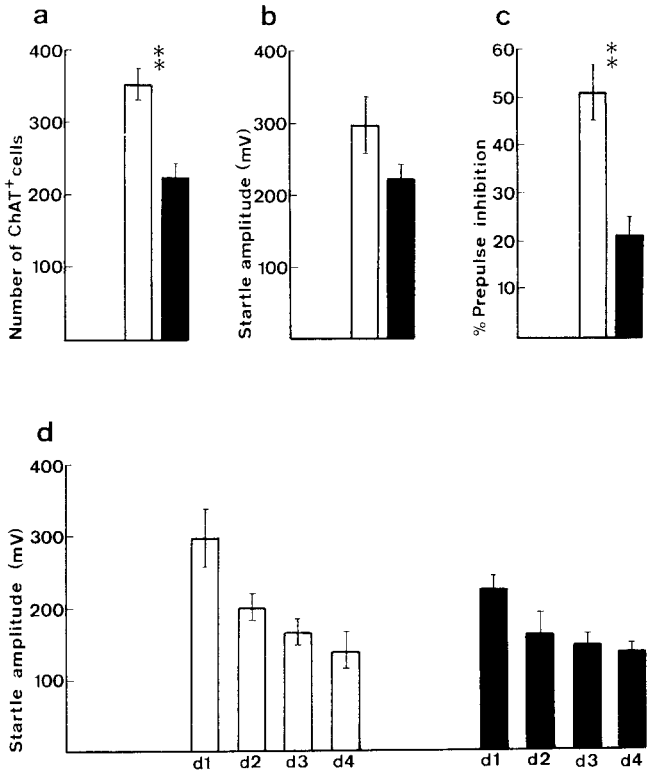


Fig. 5a–d. Effect of QA injections into the PPTg on **a** the number of ChAT-immunopositive cells, **b** the ASR amplitude as measured in the trials without prepulse, **c** the reduction of the ASR amplitude following presentation of a prepulse (percent PPI), and **d** on long-term habituation of the ASR (days of testing: d1-d4). Open bars represent sham-lesioned ($n = 6$) and black bars represent QA-lesioned ($n = 8$) rats. All data are means ± s.e.m. ** $P < 0.01$ (Mann-Whitney's U-test, two-tailed)

Since the PPTg is also a part of the ascending reticular activating system (Moruzzi and Magoun 1949) it is pertinent to discuss the possible effects of PPTg-lesions on arousal or attention. We cannot completely rule out the possibility that an attentional dysfunction, resulting from destruction of *ascending* cholinergic projections, partially accounts for the deficit in PPI observed in PPTg-lesioned rats. It has been shown that PPTg-lesioned rats perform poorly in tasks which require *sustained* attention (Dellu et al. 1991). However, this is not the kind of attention required for the fast acting PPI of the ASR and we did not observe any marked difference in the ability of the prepulse to influence the ASR amplitude during the 30 min-course of testing. It should be noted that the prepulse intensity of 75 dB SPL lies about 20 dB above the masked threshold at 10 kHz. This makes it unlikely that a deficit in the detectability of the prepulse accounts for reduced PPI in our PPTg lesioned rats, given the fact that marked PPI can be observed even when the prepulse intensity is only 5 dB above the background noise intensity (Swerdlow and Geyer 1993). Further evidence against a mere attentional deficit in PPTg-lesioned rats comes from the fact that orienting responses to the prepulses were observed in the prepulse-alone trials in our study and that the ASR amplitude in the pulse-alone trials is not significantly reduced in the present study. It is important to note that the QA lesions of the PPTg did not damage the nearby nuclei of the lateral lemniscus, which are a part of the ascending auditory system, so that no deficit in the perception of the acoustic prepulse can be expected.

General discussion

The present paper characterizes a direct cholinergic projection from the PPTg to the PnC, which exerts a predominantly inhibitory effect on the tone-evoked activity of PnC neurons. Destruction of the cholinergic PPTg neurons leads to a phasic disinhibition of PnC neurons, as reflected in the reduction of PPI while long-term habituation of the ASR was not affected. It has been pointed out that reduced PPI of the startle response is one of the symptoms of schizophrenia (Braff et al. 1978) reflecting the inability of schizophrenics to suppress intrusive sensory, motor or cognitive information. Much experimental work has therefore been dedicated to the investigation of the circuitry underlying PPI. It was found that PPI is reduced by low doses of apomorphine in rats with supersensitive dopamine receptors in the nucleus accumbens or by local infusion of dopamine into the nucleus accumbens. These effects were reversed by infusion of a GABA agonist into the ventral pallidum. Since the ventral pallidum, in turn, possibly projects to the PPTg, a nucleus accumbens - ventral pallidum - PPTg circuit was proposed to mediate PPI (summarized in Swerdlow et al. 1992a). It should be noted here that it has been claimed that the cholinergic PPTg does *not* receive pallidal input, instead the nucleus accumbens - ventral pallidum pathway would contact the non-cholinergic mesencephalic extrapyramidal area (Steininger et al. 1992). We do not think, however, that there is sufficient evidence against a pathway linking the nucleus accumbens via the ventral pallidum with the cholinergic PPTg. First, it has been shown that a clear separation of the cholinergic PPTg and non-cholinergic midbrain extrapyramidal area is not tenable (Spann and Grofova 1992). Second, the paper by Steininger and co-workers (1992) does *not* exclude the possibility that dendrites of the cholinergic PPTg are contacted by efferents from the ventral pallidum. Third, a recent paper by Semba and Fibiger (1992) has shown that injections of WGA-HRP into the cholinergic PPTg results in retrogradely labeled cells in the ventral pallidum. With regard to a possible pathway mediating PPI, it might also be conjectured that a direct monosynaptic projection from the nucleus accumbens to the cholinergic PPTg (Groenewegen and Russchen 1984; see also Fig. 6B in Steininger et al. 1992) influences PPI. Moreover, the ascending projections of the PPTg might also be considered to participate in PPI, since it has been shown that cholinergic neurons of the PPTg increase the dopamine release in the striatum through stimulation of dopaminergic cells in the substantia nigra (Blaha and Winn 1993). This latter finding has important bearings on the possible involvement of the PPTg in startle gating deficits observed in schizophrenics, since it has been found that the number of cholinergic cells in the PPTg is increased in schizophrenic patients (Karson et al. 1991). Unfortunate-

ly, dopamine efflux has only been measured in the dorsal striatum, where dopamine infusion does *not* influence PPI (Swerdlow et al. 1992b), so that presently it cannot be estimated whether the ascending cholinergic projection from the PPTg to the substantia nigra is involved in PPI. Taken together, it can be assumed that the PPTg is a relay station between the nucleus accumbens - ventral pallidum circuit and the PnC, an obligatory part of the primary startle pathway, and we suggest that ACh is the transmitter mediating the inhibitory effects of the prepulse on the ASR. The PnC has been described as a sensorimotor relay nucleus mediating the ASR (Davis et al. 1982; Wu et al. 1988; Koch et al. 1992; Lingenhöhl and Friauf 1992; Krase et al. 1993) and processing the excitatory modulation of the ASR (Rosen et al. 1991; Koch and Ebert 1993). In a parallel study we have shown that substance P excites PnC neurons and that this neuropeptide can be found immunocytochemically in neurons of the LDTg and, to a minor extent, of the PPTg (Kungel et al. submitted). We presently test the effects of substance P on the ASR. The present study shows that the PnC apparently also receives inhibitory input modulating the ASR amplitude suggesting that the PnC is a central integrator of startle-relevant information. A recent intracellular recording study from our laboratory shows PPI-like phenomena on giant PnC neurons (Lingenhöhl and Friauf 1994).

In recent years very strong emphasis has been put on the role of the PPTg in the control of several aspects of REM sleep (Garcia-Rill 1990; Jones 1991). We show here that the PPTg is also involved in modulating the ASR. Several studies have suggested a coupling between startle and the phasic events (e.g. muscle twitches) of REM sleep (Glenn 1985; Wu et al. 1989). We speculate that the release of an excitatory transmitter from PPTg neurons leads to muscle twitches, whereas the release of ACh from PPTg terminals inhibits reticulospinal PnC neurons, thereby reducing startle in the behaving animal and contributing to muscle atonia in REM sleep.

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