Mesolimbic Component of the Ascending Cholinergic Pathways: Electrophysiological-Pharmacological Study

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Brudzynski, Stefan M., Ludmila Kadishevitz, and Xiao-Wen Fu. Mesolimbic component of the ascending cholinergic pathways: electrophysiological-pharmacological study. J. Neurophysiol. 79: 1675–1686, 1998. The cholinergic input from the pontomesencephalic cholinergic neurons to the diencephalic and basal forebrain structures has been implicated in a number of limbically controlled overt behaviors. The cellular mechanism by which the cholinergic terminals initiate behavioral manifestations is not clear. The objective of this study was to investigate the effects of the ascending cholinergic projection from the laterodorsal tegmental nucleus (LDT) on neuronal firing in the anterior hypothalamic-medial preoptic region (AHMP), known to be involved in agonistic behavior. Experiments were performed on urethane-anesthetized rats. Iontophoretic application of carbachol (CCh) into the vicinity of single cells in the AHMP caused a dose-dependent decrease in the mean firing rate of 83% of units and an increase in 10% of units. The inhibitory effect of CCh, but not the excitatory effect, was reversed by iontophoretic pretreatment with scopolamine. The inhibition of the firing rate was repeatable for the same dose of CCh and dose dependent. Electrical stimulation of neurons in the LDT caused a comparable, current-dependent decrease in the mean firing rate of AHMP neurons that also was reversed by pretreatment of neurons in the AHMP with scopolamine. The antagonizing effects of scopolamine were reversible with time. The same units in the AHMP that inhibited their firing to stimulation of the LDT also responded with a similar inhibition to local iontophoretic CCh. Finally, the fluorescent carbocyanine dye, 4-(4-hexadecylamo-nostyril)-N-methylpyridinium iodide, (DiA), has been used as a retrograde axonal tracer and was injected into the recording sites immediately after the electrophysiological recordings. After 1 wk, DiA dye was found in numerous neurons in the LDT as shown by the fluorescence confocal microscopy. Results of the study suggest that LDT cholinergic neurons project and terminate in the AHMP and that their activation causes a decrease in the mean firing rate of the AHMP neurons. It is postulated that this inhibitory effect is implicated in the initiation of some of the behavioral patterns like defensive or alarm vocalization and behavioral inhibition.

INTRODUCTION

On the basis of results of immunohistochemical studies and in situ hybridization for choline acetyltransferase mRNA, two distinctive subgroups of cholinergic perikarya (CH5 and CH6) were distinguished in the pontomesencephalic region within the pedunculopontine and in the laterodorsal tegmental nuclei (LDT) (Armstrong et al. 1983; Butcher 1995; Kimura et al. 1981; Lauterborn et al. 1993; Mesulam et al. 1983, 1984, 1989; Vilaro et al. 1994). Although a substantial portion of the pedunculopontine cholinergic neurons has descending projections (Jones 1990; Woolf and Butcher 1989), the LDT has diverse and extensive ascending projections. The ascending projections innervate numerous nuclei in the thalamus, hypothalamus, basal forebrain, septum, basal ganglia and medial frontal, and olfactory cortices (Cornwall et al. 1990; Fibiger 1982; Hallanger et al. 1987; Herrero et al. 1991; Jones and Beaudet 1987; Paxinos and Butcher 1985; Satoh and Fibiger 1986; Somba et al. 1988; Vincent et al. 1986; Woolf and Butcher 1986; Woolf et al. 1990). The functional role of this ascending cholinergic system remains the key for understanding sleep-waking cycle, emotional arousal, and the initiation of a number of defensive and alarm behavioral patterns.

The present study is focused on the component of the ascending projections from the pontomesencephalic cholinergic cells, which are targeted at the medial limbic structures (Consolo et al. 1990; Cornwall et al. 1990; Lewis and Shute 1967; Mesulam et al. 1989). The ascending projections form a mesolimbic component of the ascending reticular activating system (Moruzzi and Magoun 1949; Shute and Lewis 1963, 1967), which plays an important role in the regulation of limbically driven behavior. The ascending cholinergic projections are associated with the activation that accompany the states of arousal, wakefulness, and paradoxical sleep, behavioral activation, emotional arousal manifested by species-typical threatening or alarm vocalizations, and autonomic symptoms (Brudzynski 1981, 1994; Imeri et al. 1995; Jones 1993; Talwar and Kumar 1994). It has been demonstrated in the past that systemic or intraventricular cholinomimetic agents with strong muscarinic properties have potent effects on the general behavior, particularly in inducing autonomic manifestations, defensive vocalization, and agonistic behavioral responses to external stimuli (Baker et al. 1960; Funke et al. 1962; Leslie 1965; Koff and Langfitt 1966). However, all these symptoms could not be obtained when lesions of various limbic, particularly medial diencephalic structures, had been made (Gellen et al. 1972; Koff and Langfitt 1966). It has been demonstrated by using direct intracerebral injection of muscarinic cholinergic agents in cats, that these behavioral symptoms were induced from an elongated, but limited, strip of medial structures, from the periaqueductal gray, through the medial hypothalamic and preoptic regions, and intralaminar thalamic nuclei to the septal nuclei (Baxter 1967, 1968; Brudzynski and Eckersdorf 1988; Brudzynski et al. 1995; Decsi 1974; Decsi and Nagy 1977; Decsi et al. 1969; Myers 1964; Varszegi and Decsi 1967).

Similar responses to direct cholinergic stimulation, measured by changes in locomotor activity and by the production of 22-kHz ultrasonic alarm calls, also have been reported for the rat (Brudzynski and Bihari 1990; Brudzynski et al.
Functional mapping of the decrease in locomotor activity and vocalizational responses in the rat brain delineated a similar brain system to that in the cat brain (Brudzynski 1994; Brudzynski et al. 1989). Moreover, results of the mapping studies in these two species outlined a system strikingly similar to the pattern of projection of the ascending pontomesencephalic cholinergic neurons (Satoh and Fibiger 1986; Woolf et al. 1990).

It has been postulated, therefore, that cholinergically induced behavioral responses are triggered by cholinergic terminals originating from the pontomesencephalic cholinergic cells (Brudzynski and Barnabi 1996). It has been shown in recent experiments on rats, that stimulation of neurons of the LDT with L-glutamate induced comparable 22-kHz alarm calls to those induced by cholinergic stimulation of mediobasal diencephalic structures. Moreover, L-glutamate stimulation of the LDT was ineffective, or less effective, when the terminal fields in the anterior hypothalamic-medial preoptic region (AHMP) were pretreated with scopolamine, a muscarinic antagonist (Brudzynski and Barnabi 1996).

The cellular mechanism by which the ascending cholinergic inputs initiate behavioral manifestations is not clear. It has been found in the acute rat preparation that a predominantly muscarinic agent—carbachol (CCh)—caused a decrease in the firing rate of spontaneously active neurons in the AHMP (Brudzynski et al. 1991). The question arises whether or not these neuronal responses can be attributed to the effects of the ascending cholinergic projection from the pontomesencephalic cell groups.

The goal of the present study is to test the hypothesis that the ascending cholinergic fibers originating from the pontomesencephalic cells have a predominantly inhibitory influence on the firing rate of neurons in the rat mediobasal diencephalic regions from which behavioral responses to cholinergic stimulation have been obtained. The AHMP region has been chosen for the study because the prominent behavioral manifestations with the decrease in locomotor activity (Brudzynski et al. 1989) and high magnitude of alarm vocalizations (Brudzynski 1994) have been obtained from that region.

**Methods**

**Animals and surgery**

Seventy-four male, Wistar rats, weighing 230–350 g were used in the study. Animals were anesthetized with an initial urethane dose of 1.15 g/kg ip and a supplementary dose of 0.08 g/kg was added after a couple of hours if necessary. The total dose of urethane was within the range of doses that do not suppress spontaneous activity of neurons in explored areas (Cross and Dyer 1971; Dyball and McPhail 1974). Rats were placed in a Kopf stereotaxic apparatus in the Faraday enclosure and their body temperature maintained at 37.5 ± 0.3°C as measured by the rectal probe. The skull was opened, the dura reflected, and the exposed brain surface was covered with warmed 4% agar in Ringer solution after the electrode penetration. The recording of single-unit activity was performed from the AHMP between stereotaxic frontal planes 7.0 and 8.5 mm anterior from the interaural zero plane, between sagittal planes 0.1 and 1.3 mm lateral from midline, and from 7 to 9.5 mm below the surface of the cortex according to the stereotaxic atlas by Paxinos and Watson (1986). Electrodes for electrical stimulation were placed stereotaxically in the LDT and adjoining structures, between frontal planes 0.9 and −0.4 mm, between sagittal planes 0.1 and 1.5 mm from midline, and from 5.0 to 6.5 mm below the cortex surface (Paxinos and Watson 1986).

**Electrical stimulation and extracellular single-unit recordings**

Electrical stimulation was delivered by stainless steel concentric bipolar electrodes (NE-100, Rhodes Medical Instruments, Woodland Hills, CA) with tip separation of 0.5 mm. The monophasic square-wave pulses (1-ms width, 100–800 μA, 1 Hz) were generated by a Grass S44 stimulator coupled to a Grass stimulation isolation unit (SIU-5). Six-barrel pipettes for recording and iontophoresis were pulled from F. Haer capillary glass tubing (1.0 mm OD) with a Narishige vertical puller (Model PE-2). The central barrel was filled with 2 M NaCl solution and served as a recording electrode. The electrode impedance ranged from 3 to 7 MΩ, as measured by microelectrode tester (model BL-100, Winstm Electronics, San Francisco, CA) using 135 Hz AC test current of \( \frac{5 \times 10^{-10}}{A} \). One of the side barrels was filled with 2% pontamine sky blue (Chicago sky blue, Sigma Chemical, St. Louis, MO) in 0.5 M sodium acetate for marking of the recording site. Recorded signals were connected to the probe headstage of the Axoprobe-1 DC amplifier (Axon Instruments, Forest City, CA) and then coupled to a signal processing unit for high-pass filtering and further amplification (Intronix, Rexdale, ON). The amplified signal was fed into a window discriminator (Frederick Haer, Brunswick, MA) to isolate action potentials. Standard pulses corresponding to the individual action potentials were multiplexed with stimulus triggers and event markers and fed to an AST 286 personal computer equipped with a Data-Translation DT2801A A/D conversion card. The multiplexed signal was sampled and decoded at 1 kHz by a PCIPEE program (CY Electronics, London, ON). The data were displayed continuously in real time as running time histogram or poststimulus time histogram during sampling and saved for analysis. Peristimulus time histograms were compiled from the stored data files off-line. Significant changes in activity of neurons after stimulation were quantified by comparing the height of each 1-ms poststimulus bin of the perstimulus time histogram with the average height of the bin for 100 ms before the stimulus. These data also were recalculated for the mean firing rate/s and compared for three arbitrary chosen, 100-ms bouts of time: 100 ms before the stimulus, 100 ms immediately after the stimulus, and 101–200 ms after the stimulus. Significant changes in the activity of neurons after iontophoretic ejection were quantified by comparing the mean firing rate for 20-s ejection bout with the mean firing rate/20 s immediately before and after the ejection.

**Microiontophoresis**

Side barrels of the multibarrel pipette were filled with 0.5 M centrifuged solutions of carbachol (CCh, carbamylcholine chloride), sodium glutamate, or scopolamine hydrochloride, saline and 2% pontamine sky blue (all compounds from Sigma Chemical). All pharmacological agents were dissolved in distilled water and retained with 1 nA current. The current was generated by a Dagan 640 Micro-iontophoresis current generator (Dagan, Minneapolis, MN). For the technical description of the generator, see Stone (1985). The drugs were ejected using ±120 nA current (usually 20–60 nA), positive for CCh, scopolamine, or saline and negative for glutamate, applied for 20–30 s for a single agent and ±70 s for double ejection. Periods of maintaining the retention current between ejections were controlled in time to avoid build-up of solute at the tip of the pipette and to allow a quantitative assessment of the successive ejection-induced changes in the firing rate (Bradshaw et al. 1973). The current effect was compensated automatically by an additional barrel filled with 2 M NaCl. For further details of the recording and iontophoresis, see Brudzynski et al. (1991). To antagonize the effects of CCh, scopolamine was ejected.
dialkylaminostyryl probe, DiA [4-(4-(dihexadecylamino)styryl)-N-methylpyridinium iodide, D-3883, Molecular Probes, Eugene OR], has been used as a retrograde axonal tracer. DiA is bright fluorescent green (Snider et al. 1992), diffuses more rapidly in membranes, and stains membranes more uniformly than other carbocyanine dyes. Injection solution of DiA was prepared by dissolving 2.5 mg of DiA in 0.9 ml of 100% ethanol with 0.1 ml of dimethylsulfoxide and subsequently filtering through 5-µm pore filter (Millipore, Bedford, MA). After the electrophysiological recordings were finished, 0.1 µl of DiA was injected into the recording site in the AHMP via a glass pipette (50- to 60-µm tip diam) connected with a Hamilton 1-µl syringe. Seven animals with a body weight not exceeding 300 g were injected. After 6 h survival time, the rats were anesthetized deeply with urethan and perfused transcardially with 0.9% NaCl solution followed by 10% formaldehyde in 0.1 M phosphate buffer saline (PBS). The brains were removed, stored in fixative for 48 h at 27°C and subsequently immersed in 10, 20, and 30% sucrose in PBS for 24 h in each concentration. After 1 wk of storage in a black box at room temperature, the brains were sectioned for 50-µm horizontal preparations on a cryostat microtome (Leitz, Canada), transferred into PBS solution, mounted immediately on glass slides, coverslipped using DPX mountant (BDH Chemicals, Toronto, ON) and stored at 0°C for confocal microscopy analysis.

**Confocal microscopy**

The DiA-injected preparations were analyzed under Bio-Rad MRC-600 confocal laser scanning microscope equipped with krypton.

**Fluorescent tracer injections**

To study neuronal connections of the recording site, the group of a long-alkyl chain, carbocyanine and aminostyryl dyes have been selected (Balice-Gordon et al. 1993; Honing and Hume 1986, 1989). This group of dyes was chosen because they can be injected postmortem in fixed tissues (Godement et al. 1987; Mufson et al. 1990) in the same animals that were used for electrophysiological recordings in nonsurvival experiments. The dyes do not transfer between intact membranes and have been used as retrograde tracers (Godement et al. 1987; Honig and Hume 1989). These lipophilic dyes can bind both in vivo and in vitro to lipid components of the plasma membrane, diffuse in the plane of the membrane, resulting in uniform staining of the entire cell plasma membrane (Godement et al. 1987). After electrophysiological recording, the lipophilic
FIG. 4. Location of the recording site with ejecting pipettes for CCh and SCO in the anterior hypothalamic medial preoptic region (AHMP) and the stimulating electrode in the laterodorsal tegmental nucleus (LDT) shown on the parasagittal section of the rat brain. Scale is in mm from the frontal, interaural zero plane, according to the Paxinos and Watson (1986) stereotaxic atlas. BN, bed nucleus of stria terminalis; cc, corpus callosum; CE, cerebellum; HI, hippocampal formation; HY, medial and posterior hypothalamus; NA, nucleus accumbens; OB, olfactory bulb; PAG, periqueudal grey matter; RF, reticular formation; SE, septal nuclei; TH, thalamic nuclei.

Histological verification and statistics

Recording sites were marked by expelling pontamine sky blue by a cathodal current of 5–8 µA for 10–20 min (Lodge et al. 1974)
by a local concurrent application of scopolamine in the AHMP. Some of the neurons were tested for both, iontophoretic CCh and stimulation of the LDT and for the effects of scopolamine on both of these responses. These results would demonstrate electrophysiologically that activation of the LDT may produce comparable, muscarinic neuronal responses in the AHMP to those induced by CCh in the same structure. Finally, the last step included the injection of DiA into the recording site to demonstrate retrogradely labeled cells within the region of the LDT.

RESULTS

Effects of CCh on firing rate of AHMP neurons

A total of 100 spontaneously active neurons (100%) were studied in the AHMP. Neurons with a stable firing rate for at least 40–60 s were selected for further study. The average firing rate of units was 2.01 ± 0.22 Hz (mean ± SE). In response to iontophoretically applied CCh (20- to 60-nA ejecting current), the vast majority of these cells (83%) responded with a decrease in the mean firing rate and only 10% of cells showed increase in the mean firing rate (Fig. 1A). On average, the mean firing rate of 2.48 ± 0.15 Hz was significantly reduced to 0.9 ± 0.09 Hz during ejection and later were observed as an intense blue dot on the histological preparations. Stimulation sites were marked with an electrolytic iron deposition by passing 10 μA anodal current through the stimulating electrode for 1 min. The animal then was killed with an overdose of urethan and transcardially perfused with 0.9% NaCl solution followed by 10% formaldehyde in PBS containing 2% potassium ferricyanide. The ferricyanide reacted in the iron deposit in the tissue to form a Prussian blue spot that marked the tip of the stimulating electrode. The sites of recording and stimulation were marked on composite diagrams using a drawing attachment of the stereo-zoom microscope (SZH, Olympus Optical, Japan). Results were analyzed using Student’s t-test for paired comparisons.

Experimental design

The study was conducted in three steps. The first step was focused on the effects of iontophoretically ejected CCh on the firing rate of spontaneously active AHMP neurons and the antagonizing effects of scopolamine on CCh-induced responses. The results would demonstrate predominance of cholinergically induced inhibitory neuronal responses in the AHMP mediated by muscarinic receptors. Second, responses of AHMP neurons to electrical stimulation of the LDT and its vicinity were recorded and antagonized by a local concurrent application of scopolamine in the AHMP. Some of the neurons were tested for both, iontophoretic CCh and stimulation of the LDT and for the effects of scopolamine on both of these responses. These results would demonstrate electrophysiologically that activation of the LDT may produce comparable, muscarinic neuronal responses in the AHMP to those induced by CCh in the same structure. Finally, the last step included the injection of DiA into the recording site to demonstrate retrogradely labeled cells within the region of the LDT.
increased their firing rate from an average of 1.53 to 2.79 Hz, or by 72% (Fig. 2, A). The increase was dose dependent (Fig. 2, B, left) and remained unchanged 45 min later (Fig. 2, B, right). The antagonist effects of scopolamine were repeatable on the same cells (Fig. 3, A). Scopolamine applied concurrently with excitation attenuated or reversed the CCh-induced inhibitory effects (Fig. 3, B). Although two types of excitatory responses could be distinguished, excitation (Fig. 5, B) and excitation followed by inhibition (Fig. 5, C), both types showed primary excitation and were counted jointly. Thus three types of responses were distinguished: inhibition, excitation, and no change in the mean firing rate. Sixty-nine units (27%) responded to LDT (Fig. 6A). The majority of the responding neurons (75%) were inhibited by LDT stimulation, i.e., three times more cells responded with inhibition than with excitation. Moreover, the latency period (from the time of the stimulus occurrence to the first significantly changed bin) was different for the excitatory and inhibitory effects. Average latency for the total inhibition of firing was 3.2 ± 0.5 ms, whereas the average latency for the significant excitatory effects was 11.8 ± 1.8 ms (see Fig. 5, A and B). Most of CCh at 20- to 40-nA level (63.7% reduction, t[82] = 5.5, P < 0.0001, Fig. 1B). Firing rate returned to the pre-ejection level almost instantly with termination of the ejecting current for each neuron (Fig. 1B). Ten neurons increased their firing rate from an average of 1.53 ± 0.41 Hz to 2.79 ± 0.57 Hz during ejection of the same doses of CCh. The increase, however, did not reach the significance level (t[12] = 2.0, n.s.). The inhibitory responses to CCh were dose dependent (Fig. 2, A and B) and repeatable for the same current (Fig. 2B, left). The same cells responded invariably with an increase in the mean firing rate to ejection of 10–20 nA of glutamate as tested for n = 65 units (Fig. 2B, right).

Effects of scopolamine on CCh-induced effects

Ejection of scopolamine (1–5 nA) before or during the application of CCh attenuated or reversed the CCh-induced inhibitory effects (Fig. 3, A and B). The antagonizing effects of scopolamine were repeatable on the same cells (Fig. 3A). As tested for eight cells, an average decrease in the mean firing rate by 72% (t[7] = 6.5, P < 0.0001) was reversed by scopolamine to a nonsignificant value of 25% below the pre-ejection control level (t[7] = 3.48, n.s., Fig. 3B). The increase in firing rate to CCh was not sensitive to preceding or concurrent ejection of scopolamine for the range of the ejecting current of 1–5 nA.

Responses of AHMP neurons to stimulation of LDT

The experimental set-up is illustrated in Fig. 4. The mean firing rate of AHMP neurons was recorded before and immediately after a 1-msec electrical stimulus was delivered to the LDT. A total of 255 spontaneously active neurons (100%) were tested in the AHMP with the mean firing rate of 1.01 ± 0.09 (for n = 106, which includes all responding cells and 37 not responding cells). Responses of the units were classified according to their primary response. Examples of an inhibitory and two excitatory responses are shown in Fig. 5, A–C. Although two types of excitatory responses could be distinguished, excitation (Fig. 5B) and excitation followed by inhibition (Fig. 5C), both types showed primary excitation and were counted jointly. Thus three types of responses were distinguished: inhibition, excitation, and no change in the mean firing rate. Sixty-nine units (27%) responded to LDT (Fig. 6A). The majority of the responding neurons (75%) were inhibited by LDT stimulation, i.e., three times more cells responded with inhibition than with excitation. Moreover, the latency period (from the time of the stimulus occurrence to the first significantly changed bin) was different for the excitatory and inhibitory effects. Average latency for the total inhibition of firing was 3.2 ± 0.5 ms, whereas the average latency for the significant excitatory effects was 11.8 ± 1.8 ms (see Fig. 5, A and B). Most of CCh at 20- to 40-nA level (63.7% reduction, t[82] = 5.5, P < 0.0001, Fig. 1B). Firing rate returned to the pre-ejection level almost instantly with termination of the ejecting current for each neuron (Fig. 1B). Ten neurons increased their firing rate from an average of 1.53 ± 0.41 Hz to 2.79 ± 0.57 Hz during ejection of the same doses of CCh. The increase, however, did not reach the significance level (t[12] = 2.0, n.s.). The inhibitory responses to CCh were dose dependent (Fig. 2, A and B) and repeatable for the same current (Fig. 2B, left). The same cells responded invariably with an increase in the mean firing rate to ejection of 10–20 nA of glutamate as tested for n = 65 units (Fig. 2B, right).

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of the neurons inhibited by stimulation of the LDT had a lower firing rate than cells responding with excitation, however, there was no significant difference between baseline firing of cells showing inhibitory versus excitatory responses (*t*-test, *P* > 0.08). On average, the value of firing rate per second, calculated for the 100-ms bouts of time, significantly decreased from 0.77 ± 0.1 Hz before the stimulus to 0.36 ± 0.05 Hz immediately after the stimulus (*t* [51] = 8.14, *P* < 0.0001, Fig. 6B) and returned the a similar level in the following 100-ms bout after the stimulus. The average time of the total inhibitory period induced by stimulation of LDT was 26.1 ± 1.4 ms (maximum 57 ms). The inhibition time was dependent on the stimulating current and increased proportionally to the intensity of the stimulus in the LDT (Fig. 7, A and B).

**Effects of scopolamine on effects induced by LDT stimulation**

Iontophoretic application of scopolamine into the AHMP during the period of stimulation of the LDT (see Fig. 4) reversed the effects of stimulation. The mean firing rate per second, calculated for 100-ms bouts of time, showed a significant decrease immediately after the stimulus, as compared with 100-ms bout before the stimulus (Fig. 8A). The average value of the firing rate significantly decreased from 0.48 ± 0.2 Hz to 0.23 ± 0.09 Hz as a result of the stimulation of the LDT (*t* [5] = 2.86, *P* < 0.03) and returned to the prestimulus level in the following 100-ms bout (Fig. 8A). However, similar LDT stimulation had no effect on the firing of the same neurons when scopolamine was ejected into the vicinity of the recorded neuron (Fig. 8B). Effects of scopolamine were reversible as tested in four neurons (Fig. 9, A–C). Stimulation of the LDT caused significant inhibition of the firing rate of a AHMP neuron before the drug application (Fig. 9A). This inhibitory effect was reversed when scopolamine was ejected concurrently into the vicinity of the recorded neuron in the AHMP (Fig. 9B, +SCO). The inhibitory effects of the LDT reappeared in the same neuron 45 min after application of scopolamine (Fig. 9C). Ejection of scopolamine into the vicinity of neurons that responded with an excitation to the LDT stimulation had no effect on the response (Fig. 9, D–F).

Finally, responses of two neurons were studied both to iontophoretic CCh and to stimulation of the LDT (Fig. 10). Stimulation of LDT, which caused inhibition of the firing rate in the AHMP neurons (Fig. 10A), was ineffective when scopolamine was ejected into the vicinity of the recorded neuron. The same neuron was tested for its responses to ejection of CCh (Fig. 10B). Ejection of CCh caused a current (dose)-dependent decrease in the mean firing rate of this neuron (40–120 nA CCh). Application of scopolamine (in this case by passive diffusion from the pipette tip) partially antagonized the effects of CCh (Fig. 10B, SCO + CCh). The same neuron responded with the usual excitatory response to iontophoretic glutamate (Fig. 10B, GLU).

**Localization of recording and stimulation sites and their anatomic connection**

Localization of all neurons tested with CCh and studied in response to the stimulation of the LDT is illustrated in Fig. 11, on the right, and the left side of the brain, respectively. The majority of cells that showed inhibitory responses occupied the region of the medial AHMP and, partially, the lateral portion of the paraventricular nucleus. Neurons with excitatory responses are more scattered but show a tendency to be located in the bed nucleus of stria terminalis, perifornical region, and in the vicinity of the retrochiasmatic region.

Localization of the stimulation sites in the tegmentum is shown in Fig. 12. All stimulation sites that caused inhibition of the firing rate of AHMP neurons were located in or at the border of the LDT. Locations of the stimulation electrode that yielded an increase in the firing rate or no change are scattered further around from the LDT. The excitatory ef-
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acetyltransferase (Dencev et al. 1996). These observations will be further studied and published separately.

DISCUSSION

Inhibitory responses of medial AHMP neurons to cholinergic stimulation

Our results have indicated that iontophoretic application of CCh into the vicinity of single cells in the AHMP caused a dose-dependent decrease in the mean firing rate of the majority of units. The inhibitory effect of CCh, but not the excitatory effect, was reversed by iontophoretic pretreatment with scopolamine. Electrical stimulation of the LDT neurons caused a comparable current-dependent decrease in the mean firing rate of AHMP neurons. Effects of LDT stimulation were reversed by iontophoretic pretreatment of the recorded neurons in the AHMP with scopolamine. The same AHMP neurons that were inhibited by LDT stimulation also responded to CCh with the decrease in firing rate.

Numerous studies reported both the excitatory and inhibitory effects of cholinergic agents on the firing rate of AHMP neurons (Bloom et al. 1963; Hsieh and Pan 1990; Jell 1973; Kow and Pfaff 1985; Oomura et al. 1969). It seems, however, that the most medial neurons, which are not necessarily embedded within any particular nucleus and are not projecting to the neurohypophysis, show predominantly inhibitory responses to iontophoretic ejection of CCh or acetylcholine (Brudzynski et al. 1991; Hsieh and Pan 1990; Moss et al. 1972). More caudally, neurons of the ventromedial hypothalamic nucleus showed a comparable number of excited or inhibited neurons to acetylcholine (Kow and Pfaff 1985). On the other hand, cells of the paraventricular nucleus that project to the neurohypophysis or neurons in the supraoptic-complex showed no response to CCh (Dreifuss and Kelly 1970; Gribkoff et al. 1988; Lin et al. 1993; Moss et al. 1972). Excitatory responses to acetylcholine or CCh also were obtained from the forebrain cholinergic neurons projecting to the neocortex (Lamour et al. 1986; Levine et al. 1986).

All of these results suggest that there is a subpopulation of medially located, cholinceptive neurons in AHMP that respond with a decrease in firing rate to acetylcholine. Medial localization of these neurons is consistent with results of functional mapping of behavioral responses in the rat and cat brain (Brudzynski 1994; Brudzynski et al. 1995). Further functional and anatomic evidence suggest that these inhibitory effects are caused by cholinergic input from the LDT nucleus.

Inhibitory responses of AHMP neurons to LDT stimulation

Inhibitory neuronal responses in the AHMP to stimulation of the LDT were obtained only from electrodes localized almost precisely within the LDT nucleus. Furthermore, after injection of the fluorescent lipophilic carbocyanine dye, DiA, into the recording sites immediately after an electrophysiological experiment, DiA was found in numerous neurons in the LDT, 1 wk later.

Inhibitory responses of the AHMP neurons to stimulation of the mesencephalic tegmentum have been reported. High-frequency electrical stimulation of the mesencephalic reticul-
lar formation in the central tegmental field resulted in the inhibition of a majority of the preoptic neurons (Mallick et al. 1986; Mohan-Kumar et al. 1984, 1985). Inhibition of firing in the subpopulation of the basal forebrain neurons in response to a short train stimulation of the medial pontomesencephalic region also has been reported recently (Semba et al. 1995).

Cholinergic input to the AHMP, presumably from the LDT, has been implicated in the sleep-wakefulness cycle (Imeri et al. 1995, 1996; Jones 1993; Mallik and Joseph 1997). Low-amplitude stimulation of the LDT induced significant increase in total rapid eye movement (REM) and in the duration of REM sleep (Thakkar et al. 1996). Similar stimulation of neighboring areas in the mesencephalic reticular formation during non-REM sleep evoked long-lasting suppression of discharges in sleep-related neurons in the magnocellular preoptic nucleus (Szymusiak 1995; Szymusiak and McGinty 1989). The inhibitory effects of the ascending cholinergic fibers are not unique for the mediobasal forebrain. Cholinergic inhibitory input, which modulates the thalamo-cortical relay transmission, has been well documented (Ben-Ari et al. 1976; Dingledine and Kelly 1977; Purpura et al. 1966; Steriade and Buzsaki 1990). Although inhibitory influences in the thalamus are associated with the regulation of the cortical activity, the mesolimbic component seems to be involved in the regulation of limbic activity in aversive and agonistic behavioral situations.

Inhibition of firing rate of AHMP neurons in dangerous or agonistic behavioral situations

The results suggest that activity of the LDT neurons would cause a release of acetylcholine in the medial structures, including AHMP, and would cause cellular inhibition. This should be accompanied by a change in behavior or initiation of behavior, particularly associated with a dangerous or agonistic situation, as indicated by pharmacological-behavioral studies (Brudzynski et al. 1993).

Indeed, a number of studies provided evidence for a behavior-dependent decrease in the firing rate of AHMP or preoptic neurons (Adams 1968; Mink et al. 1983; Naka and Kido 1967). Adams (1968) reported that about one-third of single cells, recorded from the hypothalamus and midline thalamus of freely behaving cats, inhibited their firing rate during various manipulations. The decrease in firing rate was not specific to any particular stimulus; however, certain manipulations such as an affective defense in response to a second attacking cat, lifting and dropping the cat, or opening the cage were more effective than other stimuli. Interestingly, such an inhibition of the firing rate was not recorded from the midbrain where none of the cells were exclusively inhibited in different behavioral situations (Adams 1968). Similar results have been reported in other studies on cats. A certain class of hypothalamic neurons (‘‘medium units’’) stopped firing as soon as the cat was presented with a mouse or during a defensive response (‘‘rage’’) with accompanying growling vocalization (Naka and Kido 1967).

Significance of the cholinergic mesolimbic projection

Inhibition of neuronal firing by the ascending pontomesencephalic cholinergic projection may mean that the pontomesencephalic system can attenuate or block input from other limbic and extralimbic structures to the medial diencephalic and forebrain regions. This inhibition in the medial forebrain and diencephalon can stop the ongoing behavior or facilitate defensive responses associated with behavioral inhibition. There are experimental data to support this conclusion. High-frequency stimulation of the medial mesencephalic tegmentum, including LDT, caused inhibition of the firing rate of the ventromedial hypothalamic neurons, which were driven by amygdaloid stimulation (Tsubokawa and
of all the neurons in the zona incerta, which were excited by stimulation of the ventromedial hypothalamic nucleus, were inhibited by acetylcholine (Eaton and Moss 1989).

In summary, the results of the present study suggest that the LDT cholinergic neurons project and terminate on neurons in the mediobasal regions of the forebrain with their activation causing a decrease in the mean firing rate of these neurons. This projection can be attributed to the cholinergic component of the ascending reticular activating system (Satoh and Fibiger 1986; Shute and Lewis 1963, 1967; Vincent et al. 1986; Woolf et al. 1990). On the basis of the present results and previous behavioral studies, it is postulated that the inhibitory influence of the ascending cholinergic fibers in the mediobasal forebrain and diencephalon may play a role in the initiation of some of the behavioral patterns. Thus the inhibition of neuronal firing represent an electrophysiological correlate accompanying some agonistic behavioral manifestations, defensive or alarm vocalization and behavioral inhibition manifested by a decrease in locomotor activity.

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REFERENCES


BAXTER, B. L. Elucidation of emotional behavior by electrical or chemical stimulation applied at the same brain loci. Exp. Neurol. 19: 412–432, 1967.

BAXTER, B. L. Elicitation of emotional behavior by electrical or chemical stimulation applied at the same loci in the cat mesencephalon. Exp. Neurol. 21: 1–10, 1968.


BRUDZYNSKI, S. M. AND BHARI, F. Ultrasonic vocalization in rats produced...

BRUDZYNSKI, S. M. and ECKERSDORF, B. Vocalization accompanying emo-


BRUDZYNSKI, S. M., ECKERSDORF, B., and GOLEBIIEWSKI, H. Regional speci-


BRUDZYNSKI, S. M., MCCALCHLAN, R. S., BHIARI, F., and GIRVIN, J. P. Re-

cponse of neurons of the rat anterior hypothalamic-preoptic area to intra-


CONSOLI, S., BORRELLI, R., FORLONI, G. L., and BUTCHER, L. L. Cholin-


