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Two distinct populations of projection neurons in the rat lateral parafascicular thalamic nucleus and their cholinergic responsiveness

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Abstract

The lateral parafascicular nucleus (IPf) is a member of the intralaminar thalamic nuclei, a collection of nuclei that characteristically provides widespread projections to the neocortex and basal ganglia and is associated with arousal, sensory, and motor functions. Recently, IPf neurons have been shown to possess different characteristics than other cortical-projecting thalamic relay neurons. We performed whole cell recordings from IPf neurons using an *in vitro* rat slice preparation and found two distinct neuronal subtypes that were differentiated by distinct morphological and physiological characteristics: diffuse and bushy. Diffuse neurons, which had been previously described, were the predominant neuronal subtype (66%). These neurons had few, poorly-branching, extended dendrites, and rarely displayed burst-like action potential discharge, a ubiquitous feature of thalamocortical relay neurons. Interestingly, we discovered a smaller population of bushy neurons (34%) that shared similar morphological and physiological characteristics with thalamocortical relay neurons of primary sensory thalamic nuclei.

In contrast to other thalamocortical relay neurons, activation of muscarinic cholinergic receptors produced a membrane hyperpolarization via activation of M₂ receptors in most IPf neurons (60%). In a minority of IPf neurons (33%), muscarinic agonists produced a membrane depolarization via activation of predominantly M₃ receptors. The muscarinic receptor-mediated actions were independent of IPf neuronal subtype (i.e., diffuse or bushy neurons); however the cholinergic actions were correlated with IPf neurons with different efferent targets. Retrogradely-labeled IPf neurons from frontal cortical fluorescent bead injections primarily consisted of bushy type IPf neurons (78%), but more importantly, all of these neurons were depolarized by muscarinic agonists. On the other hand, IPf neurons labeled by striatal injections were predominantly hyperpolarized by muscarinic agonists (63%). Our results indicate two distinct subpopulations of IPf projection neurons, and interestingly IPf neurons respond differentially to muscarinic receptor activation based on their axonal target.

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Keywords

Thalamus; Basal Ganglia; Thalamostriatal; Burst; Acetylcholine; Muscarinic

INTRODUCTION

Intralaminar thalamic nuclei have long been hypothesized to play an important role in cortical activation and speculated to potentially serve as cellular substrates of consciousness (Morison and Dempsey, 1941; Scheibel and Scheibel, 1967; Llinas and Ribary, 1993; Llinas et al., 1998). The parafascicular nucleus is a bilateral cluster of neurons in the caudal intralaminar thalamic group split into a medial and lateral portion by the fasciculus retroflexus in the rat. The lateral parafascicular nucleus (IPf) is homologous to the centre-médian nucleus in monkeys and humans, and this nucleus has been associated with motor-related structures (Jones, 1985; Groenewegen and Berendse, 1994). The IPf is primarily innervated by the basal ganglia, neocortex, and brainstem structures (Cornwall and Phillipson, 1988; Tsumori et al., 2000; Krout and Loewy, 2000; Krout et al., 2002; Tsumori et al., 2002). The major efferent projections of IPf neurons innervate the basal ganglia (striatum, globus pallidus, and subthalamic nucleus), with fewer cortical efferents relative to other thalamocortical projecting nuclei (Jones and Leavitt, 1974; Berendse and Groenewegen, 1990; Berendse and Groenewegen, 1991; Kincaid et al., 1991; Feger et al., 1994; Deschenes et al., 1996).

While the anatomical relationship between the IPf, neocortex, and basal ganglia is well established, the understanding of the functional relationship is limited. The IPf provides excitatory glutamatergic synaptic innervation to both basal ganglia and neocortex (Wilson et al., 1983; Sugimoto and Hattori, 1983; Dube et al., 1988; Mouroux and Feger, 1993; Mouroux et al., 1995; Marini et al., 1996). The centre-médian-parafascicular complex is of particular interest considering the significant neuronal loss in this region in patients with Parkinson's disease and progressive supranuclear palsy (Henderson et al., 2000b). In light of this pathology, alterations in IPf activity may play a potentially important role in the manifestations of these motor diseases.

Anatomical studies indicate rat IPf neurons are morphologically distinct from thalamocortical neurons of primary sensory thalamic nuclei in that they have long poorly branching dendrites (Scheibel and Scheibel, 1967; Deschenes et al., 1996). Until recently only these anatomical studies have existed regarding the neurons of the rat IPf. More recently, several labs have reported that IPf neurons display electrophysiological properties distinct from thalamocortical relay neurons, most notably being their decrease in burst discharge (Smith et al., 2006; Phelan et al., 2006; Lacey et al., 2007). However, all of these studies indicate a morphologically similar population that is distinct from stereotypical thalamocortical neurons.

Ascending cholinergic projections from the pedunculopontine nucleus and the laterodorsal tegmental nucleus to various thalamic neurons are thought to be involved in regulating behavioral states such as arousal, attention, and sleep/wake states (Moruzzi and Magoun, 1949; Hallanger et al., 1987; Pare et al., 1988; Steriade and Llinas, 1988). Autoradiographic studies reveal the localization of both muscarinic and nicotinic receptors throughout the thalamus (Rotter et al., 1979; Clarke et al., 1985). The majority of studies involving cholinergic and thalamic actions are limited to primary sensory thalamic nuclei. In rat thalamocortical relay neurons of primary sensory nuclei, activation of muscarinic receptors depolarizes all relay neurons, presumably via activation of M1 and M3 (Zhu and Uhlich, 1998; Varela and Sherman, 2007). In cat and guinea pig thalamocortical neurons, activation of muscarinic receptors produces biphasic responses consisting of a brief hyperpolarization followed by a membrane depolarization, or only membrane depolarizations (McCormick and Prince, 1987). In the

present study, we obtained whole cell recordings from IPf neurons and distinguished two distinct subtypes of neurons, diffuse and bushy, based upon electrophysiological and morphological differences. Furthermore, we surprisingly found that in contrast to primary sensory thalamocortical neurons, muscarinic agonists produced an inhibitory action on the majority of IPf neurons with a minority population responding to the muscarinic agonists with an excitatory, depolarizing action. The inhibitory or excitatory response to the muscarinic agonist is correlated with the different efferent targets of IPf neurons. That is, muscarinic agonists only depolarized cortical projecting IPf neurons, whereas muscarinic agonists predominantly hyperpolarized striatal-projecting neurons. These distinct actions suggest differential function of cholinergic actions in the different neural circuits, thalamocortical versus thalamostriatal.

METHODS AND MATERIALS

All experimental procedures were carried out in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the University of Illinois Institutional Animal Care and Use Committee. Thalamic slices were prepared from young Sprague-Dawley rats (postnatal age 10–20 days). The rats were deeply anesthetized with sodium pentobarbital (50 mg/kg) and decapitated. The brains were quickly removed and placed into cold ($\sim 4^{\circ}\text{C}$), oxygenated (95% O_2 /5% CO_2) slicing medium containing (in mM): 2.5 KCl, 1.25 NaH_2PO_4 , 10.0 MgCl_2 , 0.5 CaCl_2 , 26.0 NaHCO_3 , 11.0 glucose, and 234.0 sucrose. Tissue slices (300 μm thickness) were cut in the horizontal plane using a vibrating slicer and transferred to a holding chamber containing warmed ($\sim 35^{\circ}\text{C}$), oxygenated (95% O_2 /5% CO_2) physiological solution containing (in mM): 126.0 NaCl, 2.5 KCl, 1.25 NaH_2PO_4 , 2.0 MgCl_2 , 2.0 CaCl_2 , 26.0 NaHCO_3 , and 10.0 glucose for at least one hour before recording. In experiments with lanthanum chloride, we used a physiological solution containing (in mM): 151.0 NaCl, 2.5 KCl, 1.25 MgCl_2 , 2.0 CaCl_2 , 10.0 HEPES, and 10.0 glucose, and the pH adjusted to 7.3 with NaOH and oxygenated with 100% O_2 . Individual slices were then transferred to a submersion-type recording chamber and superfused (~ 2 ml/min) with oxygenated physiological solution maintained at $\sim 30^{\circ}\text{C}$.

Recording pipettes were pulled from 1.5 mm outer diameter capillary tubing and had tip resistances of 3–6 $\text{M}\Omega$ when filled with solution containing (in mM): 117.0 K-gluconate, 13.0 KCl, 1.0 MgCl_2 , 0.07 CaCl_2 , 0.1 EGTA, 10.0 HEPES, 2.0 $\text{Na}_2\text{-ATP}$, 0.4 Na-GTP, and 0.2% biocytin. The pH of this solution was adjusted to 7.3 and osmolarity was adjusted to 290 mosm. The use of this intracellular solution results in ~ 10 mV junction potential that has been corrected for in all voltage measures.

Whole-cell recordings were obtained with the visual aid of a modified microscope equipped with differential interference contrast optics (Axioskop 2FS, Carl Zeiss Instruments). A low-power objective was used to identify specific thalamic nuclei and anatomical landmarks, and a high-power water immersion objective was used to visualize individual neurons. Recordings were limited to 500 μm lateral to the fasciculus retroflexus. An Axoclamp 2B or Multiclamp 700A amplifier (Molecular Devices Corporation) was used in bridge mode for voltage recordings. An active bridge circuit was continuously adjusted to balance the drop in potential produced by passing current through the recording electrode. Voltage clamp recordings were limited to neurons that had a stable access resistance of <18 $\text{M}\Omega$. Current and voltage protocols were generated using pClamp software (Molecular Devices Corporation), and data were digitized and stored on computer for off-line analyses.

Initial input resistances were calculated from the linear slope of the voltage-current relationship obtained by applying constant current pulses ranging from -40 to $+40$ pA (1 s duration). To evoke the transient inward current, we used an inactivation protocol in which command voltage

steps were given to different hyperpolarized levels (-110 mV to -50 mV, 5 mV increments, 5 s duration) followed by a return to the holding potential (-50 mV). Peak amplitudes were measured and averaged over three consecutive trials for each neuron tested. Action potential characteristics were measured in a subset of neurons where the membrane potential was manually adjusted to -50 mV, and a series of depolarizing current pulses were injected in each neuron (range: 5 – 225 pA, 5 – 10 pA increments, 1 s duration, 0.2 Hz). The slopes of the frequency-intensity relationships were calculated from the linear plots of current versus maximum discharge rate, typically the first interspike interval. The calculated correlation coefficient of the resulting linear regression was > 0.9 in all neurons tested. Spike adaptation was calculated by the ratio of the last instantaneous frequency and initial instantaneous frequency in response to the maximum current intensity tested in each neuron. Only neurons that responded with greater than six action potentials to the current step were included in this analysis. All data are presented as mean $\pm$ standard deviation. Differences between means were considered significant when $p < 0.05$.

Concentrated stock solutions of pharmacological agents were prepared in distilled water and diluted in physiological solution to final concentration before use. Agonists were applied by injecting a bolus into the input line of the chamber for 60 – 80 seconds using a motorized syringe pump. Based on the rate of agonist injection and chamber perfusion rate, the final bath concentrations of agonists were estimated to be approximately one-fourth of the concentration introduced in the flow line (Cox et al., 1995). Concentrations listed in the text are the final bath concentrations after the four-fold dilution. During drug application, changes in input resistance were determined by membrane responses to single-intensity constant hyperpolarizing current pulses (5 – 20 pA, 250 – 500 ms, 0.2 Hz). Antagonists were diluted to a final concentration from stock solutions and bath applied. All compounds were purchased from Sigma (St. Louis, MO) or Tocris (Ellisville, MO).

Retrograde tracer injections

Rats (postnatal age 10 – 20 days) were deeply anaesthetized with an intraperitoneal injection of ketamine (40 mg/kg) and xylazine (5 mg/kg). Animals were placed in a stereotaxic frame, an incision was made in the scalp, and holes drilled in the skull to allow placement of a glass pipette (20 – 30 μ m tip diameter) in frontal cortex (anterior 1.0 mm, lateral 1.0 mm, ventral 0.5 mm, relative to bregma) or the lateral striatum (anterior 0.0 mm, lateral 3.5 mm, ventral 3.5 mm, relative to bregma; Paxinos and Watson, 1986). Pressure injections of fluorescent retrograde microspheres (100 – 300 nL, Molecular Probes) were made bilaterally in frontal cortex or the lateral striatum. Animals were allowed to recover for approximately 24 hours, and then brain slices were prepared for electrophysiological recordings as described above.

Histological procedures

Following recordings, slices were fixed overnight in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C . The slices were then reacted using an immunoperoxidase procedure (Vectastain Elite ABC; Vector Labs, CA) previously described in our laboratory (Cox et al., 1996). Brain slices were then mounted (Permount) on gelatin coated coverslips and neuronal morphology and histological measurements were determined using 2-dimensional reconstruction of the biocytin-filled neurons under a light microscope with the aid of Axiovision software (Carl Zeiss Instruments). Neurons that were >500 μ m lateral to the fasciculus retroflexus were not included in this study.

RESULTS

Intracellular recordings using the whole cell configuration were obtained from 288 rat IPf neurons using an *in vitro* slice preparation. All recordings were limited to the IPf, a region that

lies adjacent to the lateral side of the fasciculus retroflexus (Figure 1C). The dorsal-ventral aspect of the IPf was identified using the third ventricle, the fasciculus retroflexus, and the posterior commissure as visual landmarks (Paxinos and Watson, 1986; see Figure 1C). All IPf recordings were restricted to within 500 μm of the fasciculus retroflexus. Within the developing rodent, P10–P21, the IPf extends 800–1000 μm lateral to the fasciculus retroflexus, well within our criteria of 500 μm (Sherwood and Timiras, 1970).

Morphological distinction of IPf neurons

In order to correlate electrophysiological properties with morphological characteristics of IPf neurons, biocytin was included in the recording pipettes. We were able to recover 227 of the 288 biocytin-filled IPf neurons. Based on cell morphology, IPf neurons were easily differentiated into two subtypes: diffuse and bushy (Figure 1A, B). Diffuse type neurons were the more common cell type in the IPf (66%: 149/227) and were characterized by having few primary dendrites (3.0 ± 0.8 , $n=139$; Figure 1A). The dendritic arbor appears relatively simple with long, thin primary dendrites and fewer higher-order branches. Diffuse neurons had somata with an average area of $315 \pm 119 \mu\text{m}^2$ ($n=141$, Table 1). The ratio of the major and minor axis of the soma was calculated to determine if the somata shape was round or fusiform. Diffuse neurons had a major/minor axis ratio of 1.44 ± 0.27 ($n=141$). The average maximal dendritic spread of diffuse neurons averaged $396 \pm 133 \mu\text{m}$ ($n=137$). This population of neurons is similar to previous reports in this nucleus (Scheibel and Scheibel, 1967; Deschenes et al., 1996; Smith et al., 2006; Phelan et al., 2006; Lacey et al., 2007).

Bushy neurons compose the remainder of the population (34%: 78/227), and appear very similar to stereotypical thalamocortical relay neurons in primary sensory thalamic nuclei (Guillery, 1966; Jones, 1985; Govindaiah and Cox, 2004). Bushy neurons had several radially projecting primary dendrites (5.3 ± 1.3 , $n=71$) with extensive dendritic arborizations (Figure 1B). The average maximal length of a primary dendrite in bushy IPf neurons was $195 \pm 60 \mu\text{m}$ ($n=75$), significantly shorter than that of diffuse neurons ($p<0.01$, Mann-Whitney test, Table 1). Bushy neurons had an average soma area of $299 \pm 94 \mu\text{m}^2$ ($n=76$) and a major/minor axis ratio of 1.43 ± 0.31 ($n=76$), which did not significantly differ from the diffuse type neuron measurements ($p>0.1$, Mann-Whitney test, Table 1).

Electrophysiological distinction of IPf neurons

Current clamp recordings were used to examine the intrinsic properties of IPf neurons. We compared various intrinsic properties of the diffuse and bushy subtype neurons (Table 1). Diffuse type neurons had an average resting membrane potential of $-62 \pm 7 \text{ mV}$ ($n=149$) and an average input resistance of $1003 \pm 529 \text{ M}\Omega$ ($n=142$). In contrast, the average resting membrane potential of bushy neurons was significantly hyperpolarized compared to diffuse neurons ($-73 \pm 7 \text{ mV}$, $n=78$; $p<0.01$, Mann-Whitney test). The apparent input resistance of bushy neurons averaged $492 \pm 234 \text{ M}\Omega$ ($n=76$), significantly lower than that of diffuse neurons ($p<0.01$, Mann-Whitney test). It is important to note that the ages of the animals from which slices were taken overlapped, and did not differ significantly (Bushy: 14 ± 2 postnatal days; Diffuse: 14 ± 2 postnatal days, $p>0.1$, Mann-Whitney test) eliminating the possibility that these differences between cell types were due to developmental differences.

A ubiquitous feature of thalamic neurons is the ability to produce action potential discharge in two distinct modes: burst and tonic (Jahnsen and Llinas, 1984; Steriade and Llinas, 1988). To evoke burst discharge, the membrane potential was held at a relatively hyperpolarized membrane potential ($\sim -90 \text{ mV}$) and a subsequent step depolarizing current pulses produced the transient high frequency, action potential discharge. Under these conditions, the depolarizing current step produces a transient depolarization (75–200 ms duration) crowned by a high frequency discharge of multiple sodium-dependent action potentials (burst mode;

e.g. Figure 2Aii lower trace). At depolarized membrane potentials, the same current step produced action potential discharge in tonic mode (Figure 2Aii top trace). In contrast to the transient burst discharge in which the discharge rate is independent of stimulus intensity, the discharge frequency of tonic mode is linearly related to the degree of membrane depolarization (Zhan et al., 1999).

We next examined whether bushy and diffuse types of neurons could be distinguished by their discharge properties. Nearly all bushy neurons (96%, 75/78) produced transient burst discharge at resting membrane potential levels or when the membrane potential was further hyperpolarized (Figure 2Aii). At depolarized membrane potentials, these neurons produced action potentials in tonic mode (e.g., Figure 2Aii). Burst discharge in bushy neurons is indistinguishable from that in relay neurons from other primary sensory thalamic nuclei (Govindaiah and Cox, 2004). In contrast to bushy neurons, the majority of diffuse neurons (84%, 125/149) did not produce burst discharge, even when given a depolarizing step from hyperpolarized membrane potentials (~ -90 mV; Figure 2Bii). It is important to note that the number of action potentials per burst (3 ± 1 action potentials/burst, $n=24$) in diffuse neurons that did produce burst-like discharge were significantly less than that observed in bushy neurons (5 ± 2 action potentials/burst, $n=75$, $p<0.01$, Mann-Whitney test).

We also compared the characteristics of tonic action potential discharge in diffuse and bushy neurons. Action potential amplitudes did not differ significantly between the two neuronal subtypes (diffuse: 68 ± 6 mV, $n=13$; bushy: 65 ± 5 mV, $n=10$; $p>0.10$, Mann-Whitney test; Table 1). However, the half-amplitude duration of the action potentials from diffuse neurons averaged 1.63 ± 0.32 ms ($n=13$) which was significantly different from that of the bushy neurons ($p<0.03$, Mann-Whitney; 1.92 ± 0.25 ms, $n=10$; Table 1). The maximum firing frequency over the range of current intensity tested (10–225 pA) differed between the bushy and diffuse neurons (Table 1, $p<0.05$, t-test); however the slope of the stimulus intensity-spike discharge rate did not vary between the two neuronal subtypes (Table 1, $p>0.4$, t-test). Both bushy and diffuse neurons showed a significant spike frequency adaptation when comparing the initial versus final discharge frequency (Bushy: 31.2 ± 8.9 Hz, 8.9 ± 4.0 Hz; Diffuse: 23.3 ± 7.3 Hz, 11.6 ± 5.5 Hz, $p<0.001$, paired t-tests). Furthermore, the spike adaptation ratio for bushy neurons was significantly less than that in the diffuse neurons indicating a greater degree of spike frequency adaptation in the bushy neurons (Table 1, $p<0.01$, t-test).

Burst discharge requires the activation of the low threshold, transient calcium current, I_T . Activation of I_T produces the low threshold calcium spike (LTS), a transient depolarization on top of which multiple sodium-dependent action potentials can occur at a high frequency. In order to isolate the LTS, action potentials were blocked with tetrodotoxin (TTX). In bushy neurons, a depolarizing current step in the presence of TTX (0.5 μ M) produced a stereotypical LTS with an average amplitude of 34 ± 12 mV ($n=13$, Figure 3A). In a minority of diffuse neurons (10%, 4/41), a depolarizing current step produced burst discharge (Figure 3Bi). In TTX, the LTS of these diffuse neurons had an average amplitude of 21 ± 2 mV ($n=4$, Figure 3Bi). In another subpopulation of diffuse neurons (66%: 27/41), a single action potential could be produced but not multiple action potentials (Figure 3Bii). In these neurons, a small transient, depolarizing potential (13 ± 5 mV, $n=27$) was evoked in the presence of TTX (Figure 3Bii TTX). In the remaining 10 neurons (24%), the step depolarization produced single action potentials, and following the addition of TTX, there was no apparent transient depolarization evoked in these cells (Figure 3Biii). The LTS amplitude significantly differed across the bushy and diffuse subpopulations of neurons ($F_{3,50}=49.6$, $p<0.01$, one-way ANOVA). The LTS amplitude of the bushy neurons was significantly greater than that in all groups of the diffuse type neurons (Figure 3; $p<0.05$, Tukey-Kramer multiple comparisons).

We next tested whether the small transient depolarizations evoked in a subpopulation of diffuse neurons were indeed calcium-dependent depolarizations. Voltage clamp recordings were performed in order to evoke the inward current (see Experimental Procedures). In bushy neurons, a transient inward current was evoked with an average maximum amplitude of 565 ± 151 pA ($n=5$, Figure 4Ai). This inward current was significantly attenuated by the calcium channel blocker lanthanum chloride (200 μ M; Figure 4A, D; $n=5$; $p<0.01$ paired t-test). In diffuse neurons, the average maximum amplitude of the inward current was 110 ± 33 pA ($n=7$, Figure 4Bi), which was significantly less than that produced in bushy neurons. This smaller current was also blocked by lanthanum chloride (200 μ M; Figure 4B, D; $n=7$; $p<0.01$ paired t-test). In current clamp mode, prior to lanthanum chloride, these seven diffuse neurons produced a transient depolarization with an average peak amplitude of 10 ± 3 mV. In three different diffuse neurons, no inward current was evoked suggesting that these neurons have no I_T (Figure 4C). Although the diffuse neurons displayed little or no I_T from our somatic measures, we cannot discount the possibility of I_T at electrotonically distal dendrites that could not be activated due to space clamp error.

Multiple cholinergic responses in IPf neurons

The intralaminar nuclei are innervated by cholinergic neurons arising from brainstem nuclei. Considering the putative role of cholinergic brainstem nuclei as well as intralaminar nuclei in attention- and arousal-related behaviors, we next tested the action of cholinergic agonists on IPf neuronal excitability. Bath application (60–80 s) of the general cholinergic agonist, carbachol (25 μ M), altered the membrane potential of most IPf neurons tested (17/21, Figure 5A). Carbachol produced a membrane hyperpolarization in 53% of neurons (9/17) and a membrane depolarization in 41% (7/17) of neurons tested (Figure 5A). A biphasic response (hyperpolarization followed by a depolarization) was observed in the remaining neuron. In a subset of neurons ($n=10$ cells), tetrodotoxin (TTX, 0.05 μ M) was added to the bath to block sodium-dependent action potentials and the carbachol-mediated alteration in membrane potential persisted suggesting carbachol was acting via postsynaptic cholinergic receptors. Considering carbachol can activate both nicotinic and muscarinic receptors, we next tested the actions of selective cholinergic agonists.

The nicotinic agonist 1,1-dimethyl-4-phenyl-piperazinium iodide (DMPP, 25 μ M) depolarized nearly all IPf neurons tested (29/30, Figure 5B). The muscarinic agonist acetyl- β -methylcholine (MCh) also altered the membrane potential in most IPf neurons (86%, 105/122 cells, Figure 5C). In contrast to DMPP, MCh produced a membrane hyperpolarization in a majority of neurons (60%, 63/105) while a membrane depolarization was seen in a smaller population of neurons (33%, 35/105). In a minority population of cells (7%, 7/105), MCh produced a biphasic response consisting of an initial hyperpolarization followed by a longer-lasting depolarization (Figure 5C, bottom trace). In a subpopulation of neurons ($n=25$ cells), in which DMPP produced a depolarization, MCh was found to produce either a hyperpolarization or a depolarization.

Considering the two distinct subtypes of IPf neurons we described, we next determined if the different cholinergic agonist-mediated actions (i.e., hyperpolarization and depolarization) were correlated with the different IPf neuron subtypes (Table 2). In diffuse neurons, carbachol (25 μ M), produced both depolarizations (2/7, 29%) and hyperpolarizations (4/7, 57%). Similarly, carbachol (25 μ M) produced both types of responses, depolarizations (2/4, 50%) and hyperpolarizations (2/4, 50%), in bushy neurons. The nicotinic receptor agonist, DMPP (25 μ M) produced depolarizations in 19 of 20 (95%) diffuse neurons and all 4 (100%) bushy neurons tested. The muscarinic agonist, MCh (1–125 μ M), produced all three described effects on diffuse neurons. MCh predominantly produced hyperpolarization in the majority of diffuse neurons (37/61, 61%); however, we did observe depolarizations (14/61, 23%) and biphasic

responses (2/61, 3%). Similar to diffuse neurons, MCh (1–125 μ M) produced hyperpolarizations in the majority of bushy neurons (16/31, 52%), depolarizations in a smaller population (12/31, 39%) as well as biphasic responses (2/31, 6%). Despite the clear morphological and intrinsic electrophysiological differences between bushy and diffuse IPf neurons, the responses to cholinergic agonists were very similar for both populations and thus the cell morphology was not a reliable predictor of the cholinergic response.

In view of previous studies in which muscarinic receptor activation produces only depolarizations in rodent sensory thalamocortical relay neurons (Zhu and Uhlich, 1998; Varela and Sherman, 2007), the prevalence of hyperpolarizations in response to muscarinic agonists was surprising. We next tested the concentration-dependence of the MCh-mediated hyperpolarizations using various concentrations ranging from 1–125 μ M (Figure 6A). The highest MCh concentration tested (125 μ M) produced hyperpolarizations with average peak amplitude of 8 ± 6 mV (Figure 6Aiii, $n=52$). Decreasing the MCh concentration produced smaller hyperpolarizations (Figure 6A). There was a statistically significant difference in the magnitude of hyperpolarization amplitudes across concentrations tested ($p<0.005$, $F_{4/135}=5.4$, ANOVA). The concentration dependence of the MCh-mediated depolarizations was also tested across the same concentrations (1, 5, 15, 25, and 125 μ M; Figure 6B). Despite the apparent concentration dependent trend, there was not a statistically significant effect of MCh concentration on the magnitude of the membrane depolarization ($p>0.05$, $F_{4/65}=1.5$, ANOVA).

We next assessed alterations in input resistance produced by MCh. During the peak MCh-mediated effect, the membrane potential was manually adjusted to pre-drug levels for comparisons. The MCh-mediated hyperpolarizations (25, 125 μ M) were associated with a significant decrease in input resistance (Figures 5C, 6A; 24.3 ± 19.0 %, $n=13$, $p<0.01$, paired t-test). The MCh-mediated depolarizations were associated with an apparent increase in input resistance that was not statistically significant (Figures 5C, 6B; 29.8 ± 44.1 %, $n=9$, $p>0.05$, paired t-test).

Selective muscarinic receptor antagonists were used to determine if the MCh-mediated depolarizations and hyperpolarizations were mediated by distinct receptor subtypes. The M_2 selective antagonist, methoctramine was tested on 19 neurons. Short application of MCh (125 μ M) produced hyperpolarizations that averaged 9 ± 4 mV ($n=8$). In the presence of methoctramine (25 μ M), the MCh-mediated hyperpolarization was completely blocked in all neurons tested (Figure 7B right graph, paired t-test, $p<0.01$). After 30 minutes of washout, there was a partial recovery of the MCh-mediated hyperpolarization in only one cell. Considering the irreversibility of the antagonists in the previous experiments, we next tested lower concentrations of methoctramine and MCh. MCh (25 μ M) produce membrane hyperpolarizations that averaged 8 ± 5 mV ($n=11$). In the presence of methoctramine (1, 10 μ M), the MCh-mediated hyperpolarization was attenuated by 98 ± 5 % (Figure 7B left graph, $n=11$, $p<0.01$, paired t-test). After 30 minutes of washout, partial recovery (63 ± 27 % of control) was observed in five neurons (Figure 7B).

To determine if the depolarizing actions of MCh on IPf neurons was due to a different muscarinic receptor subtype, we tested the selective M_3 antagonist 1,1-Dimethyl-4-diphenylacetoxypiperidinium iodide (4-DAMP) on the MCh-mediated response. In the presence of 4-DAMP (10 nM), the MCh-mediated depolarization was attenuated by 86 ± 8 % ($n=5$), and partially recovered (50 ± 20 %) following 30 minutes washout of 4-DAMP (Figure 7C, D). In 5 additional cells, a higher 4-DAMP concentration (500 nM, $n=4$; 100 nM, $n=1$) was tested, and in all cells, the MCh-mediated depolarization (15 mM) was completely blocked in an irreversible manner. We also tested the relatively selective $M1$ antagonist telenzepine on the MCh-mediated depolarization. At relatively high concentrations (100, 500, and 1000 nM), telenzepine attenuated the MCh-mediated depolarizations by 10% ($n=3$), 46% ($n=4$) and 73%

(n=11), respectively. Taken together, our data strongly support that the MCh-mediated depolarization occurs primarily via activation of M₃ muscarinic receptors but could involve M₁ receptors to a lesser extent, whereas the MCh-mediated membrane hyperpolarization is mediated by M₂ receptors.

Muscarinic agonists alter K⁺ conductance

To further investigate the currents altered by MCh in IPf neurons, voltage clamp recordings were obtained and slow voltage command ramps (−50 mV to −110 mV, 4 s duration, 0.1 Hz) were applied to determine voltage characteristics of the MCh-mediated conductance changes (Figures 8). Prior to MCh application, the current response to the ramped voltage command was nonlinear. To quantify MCh-mediated changes in conductance, the linear current response from a holding potential of −50 mV to −70 mV was used to calculate the “resting” conductance prior to MCh application and during the peak MCh response. At a holding potential of −50 mV, in a subpopulation of IPf neurons, MCh produced an outward current with an average peak amplitude of 17 ± 19 pA (n=13; Figure 8Ai). Prior to MCh application the “resting” conductance of the cells averaged 3.41 ± 3.04 nS and during the peak outward current the conductance significantly increased to 3.62 ± 3.30 nS (n=13, p<0.05, paired t-test). The difference in the current responses before and after MCh application represents the MCh-sensitive conductance (I_{diff} , Figure 8Aiii), which was usually linear over the voltage range of −50 mV to −70 mV and corresponded to a conductance averaging 0.21 ± 0.34 nS (n=13, p<0.05 paired t-test). Overall, the MCh-sensitive conductance was linear and reversed near the calculated potassium reversal potential (-90 ± 20 mV, n=13; Figure 8Aiii), suggesting that MCh enhances a linear K⁺ current, characteristic of K_{leak}.

In a smaller population of cells, MCh produced an inward current with an average peak amplitude of 32 ± 13 pA (n=6; Figure 8Bi). Prior to MCh application the initial conductance of these cells averaged 5.24 ± 2.15 nS and during the peak of the MCh-mediated inward current the conductance was significantly reduced to 4.35 ± 2.08 nS (p<0.05, paired t-test, n=6). The MCh-sensitive inward current was relatively linear and had an average reversal potential of -101 ± 29 mV (Figure 8Biii, n=6), which is near the K⁺ equilibrium potential and indicative of K_{leak}.

Efferent targets of IPf neurons

Considering IPf neurons project to the neocortex and/or the basal ganglia (Jones and Leavitt, 1974; Berendse and Groenewegen, 1990; Berendse and Groenewegen, 1991; Kincaid et al., 1991; Feger et al., 1994; Deschenes et al., 1996), we next investigated whether bushy and diffuse neuronal subtypes project to distinct brain regions. Fluorescent microspheres were injected into the dorsal striatum or the frontal neocortex (see Experimental Procedures). Striatal injections were primarily restricted to the dorsal lateral striatum with minor leakage in the cortex along the pipette tract (Figure 9B, Striatal injection). The neocortical injections were placed within the frontal cortical regions, and did not extend to underlying striatum (Figure 9B, Cortical injection).

Striatal injections resulted in neuronal labeling within the IPf and 58 labeled IPf neurons were recorded from 27 animals. Our recordings and subsequent histological analyses revealed that the majority of these neurons were diffuse type neurons (62%, 36/58, Figure 9Ci, 9Cii) with a smaller percentage of bushy neurons (38%, 22/58). It is possible that a proportion of these neurons may have been labeled due to tracer leakage as the pipette traversed the cortex. In animals with neocortical injections, we recorded from 37 labeled IPf neurons in 14 animals. In contrast to the striatal injections, these labeled IPf neurons were predominantly bushy type neurons (78%, 29/37, Figure 9Di, 9Dii) with fewer diffuse neurons (22%, 8/37). These diffuse and bushy neurons had similar distinguishing intrinsic properties (resting membrane potential,

input resistance, and burst incidence) as described for non-labeled IPf neurons. The proportion of bushy and diffuse neurons recorded in the striatal injections was strikingly similar to the unbiased distribution of bushy/diffuse neurons we obtained in unlabeled tissue. More interesting was the fact that following cortical injections the proportion of labeled bushy and diffuse neurons flipped with the bushy subtype being the majority population.

We also examined the responsiveness of the different retrograde labeled IPf neurons to the muscarinic agonist MCh. Our earlier results indicated that bushy and diffuse neuron subtypes did not correlate with the MCh response (hyperpolarization versus depolarization, Table 2). Application of MCh (15 μ M) to cortically labeled bushy neurons altered the membrane potential in all neurons tested (n=13, Figure 10). Interestingly, all 13 bushy neurons responded to MCh with a membrane depolarization that averaged 8 ± 3 mV (n=13). The diffuse neurons labeled from cortical injections showed a variety of responses to MCh: two neurons hyperpolarized, one neuron depolarized, and the remaining two neurons were unresponsive to MCh.

The majority of neurons labeled following striatal injections were diffuse type cells (60%, 21/35, Figure 10). MCh produced a hyperpolarization in 71% (15/21) of these diffuse neurons with average amplitude of 11 ± 7 mV. MCh produced a depolarization in two diffuse neurons and had no effect on the remaining four neurons. Bushy neurons labeled from striatum injections predominately hyperpolarized in response to MCh (50%, 7/14). Three bushy neurons showed membrane depolarizations and the remaining four neurons did not respond to MCh. Although the bushy and diffuse neurons had similar response profiles to muscarinic receptor activation, the retrograde labeling experiment indicated differential responses of IPf neurons to muscarinic receptor activation when distinguished by their efferent projections.

DISCUSSION

In this study, we found two distinct subtypes of projection neurons within the rat IPf. The two distinct subtypes, diffuse and bushy, could be easily differentiated based on their dendritic architecture. Diffuse type neurons have a simple dendritic architecture consisting of a couple, relatively long, minimally branching dendrites, and these cells are the prevalent subtype in the IPf nucleus accounting for nearly two-thirds of the population. Bushy type neurons appear similar to thalamocortical relay neurons of primary sensory thalamic nuclei with a radially branching, complex dendritic architecture, and these cells make up the remaining one-third of the overall population. Furthermore, diffuse and bushy neurons could also be distinguished based on different intrinsic properties (Table 1). The most notable difference is in the neurons' ability to produce burst discharge. Although considered a ubiquitous feature of thalamic neurons, the majority of diffuse type IPf neurons lack burst discharge. In contrast, bushy neurons produce stereotypical burst discharge. In addition, bushy neurons principally project to the frontal cortex.

We have shown that activation of cholinergic receptors on rat IPf neurons can lead to complex postsynaptic responses. Activation of nicotinic receptors strongly depolarized all IPf neurons. On the other hand, activation of muscarinic receptors leads to a membrane hyperpolarization in most neurons, a depolarization in some neurons, and a biphasic response in a few neurons. As shown above, the rat IPf is comprised of two distinct neuronal subtypes and as a whole both subtypes responded similarly to MCh application. However, when we consider the projection location of the neuronal subtypes, we find that cortical projecting bushy neurons depolarize with MCh application while striatal projecting diffuse neurons predominantly hyperpolarize in the presence of MCh. The MCh-mediated membrane hyperpolarization is due to activation of M_2 receptors, which causes an increased linear K^+ conductance. While, the membrane

depolarization seen with MCh application is due to M₃ receptor activation and appears to be mediated by a decreased resting K⁺ conductance.

Our knowledge regarding the specifics of IPf neurons is rather limited. In a limited number of individually labeled rat IPf neurons, Deschenes *et al.* (1996) only identified neurons that appear similar to diffuse neurons described here. They found that individual diffuse neurons could project to both the striatum and neocortex via a multiple branching axon (Deschenes *et al.*, 1996). Anatomical work in the cat centre-médian nucleus, which is analogous to the rat IPf, revealed both diffuse-like and bushy-like neurons based on morphological criteria; however, this is the only report of such heterogeneity (Tseng and Royce, 1986). Our results confirm the presence of multiple neuronal subtypes in the rat IPf as in the cat centre-médian nucleus. Although the morphology of diffuse neurons is strikingly similar to interneurons of other thalamic nuclei (e.g. dorsal lateral geniculate nucleus), the axons clearly project beyond the boundaries of the IPf and do not appear to have local collaterals. This clearly indicates that diffuse neurons are indeed projection neurons and not local interneurons. In addition, an axonal tracing study has observed similar axons projecting out of the IPf in the rat (Deschenes *et al.*, 1996). Furthermore, GABAergic staining has revealed very few GABA positive neurons are found in intralaminar thalamic nuclei of rodents (Arcelli *et al.*, 1997).

In our study, approximately one-third of the rat IPf is comprised of bushy neurons with similar morphology and intrinsic properties as thalamocortical relay neurons from primary sensory thalamic nuclei such as the lateral geniculate nucleus and the ventrobasal nucleus. Intrinsic properties of bushy neurons are very similar to thalamocortical relay neurons of primary sensory thalamic nuclei. Both neurons possess the ability to produce action potential discharge in two distinct modes: tonic and burst. The burst discharge is due to activation of a voltage sensitive, transient low threshold calcium current, which is thought to be ubiquitous in all thalamic relay neurons. Functionally, bushy IPf neurons may possess a similar role in transferring information to the neocortex as other primary sensory relay neurons since they project to layers IV, V, and VI of primary motor, secondary motor, and primary somatosensory cortices (Berendse and Groenewegen, 1991; Marini *et al.*, 1996). In addition, these neurons may also send axonal projections to superficial layers of neocortex (layer I), and thus the burst discharge may be a useful mode to increase the efficacy of thalamocortical transmission (Berendse and Groenewegen, 1991; Lisman, 1997).

The majority of neurons we encountered in the IPf were diffuse type neurons. These cells had distinguishing characteristics (relatively depolarized resting membrane potentials, high input resistances, lack of burst discharge) that clearly set them apart from bushy neurons. The relatively depolarized resting membrane potentials and relatively higher input resistances of diffuse neurons may make these cells more responsive to afferent synaptic activity thereby potentially increase their sensitivity. As a result, these unique intrinsic characteristics would make them probable to respond in a suprathreshold manner to afferent inputs. In addition, their cell morphology was very distinguishable with far-reaching, very simple appearing dendrites with little branching. This is in stark contrast to the bushy neurons with their radially projecting, complex branching dendritic arbors. Despite some diffuse neurons having apparent low threshold calcium currents, these currents are relatively small and not sufficient to support burst firing in these neurons. Our study clearly indicates the lack of bursting in most diffuse cells which has been suggested by extracellular recordings *in vivo* (Lacey *et al.*, 2007) as well as a limited number of intracellular recordings in the slice preparation (Smith *et al.*, 2006). Therefore, the majority of diffuse neurons can only operate in the tonic discharge mode, while bushy neurons can operate in both tonic and burst discharge modes.

Considering that studies to date indicate that primary thalamic projection neurons can produce both burst and tonic output, the lack of burst output from the diffuse neurons may be a clue in

their functional role in thalamic circuit activity. Our retrograde labeling and subsequent recordings revealed that with striatal injections the distribution of labeled bushy and diffuse neurons roughly approximated that of neuronal distribution obtained in unlabeled slices. This could suggest that a larger population of diffuse neurons project to the striatum or this could correspond to the population of IPf neurons described by Deschenes *et al.* (1996). These authors reported that all of their labeled neurons, which appear similar to the diffuse neurons we report here, send axons that give rise to branches within both the striatum and neocortex. Perhaps, more surprising was that cortical injections produced significantly greater labeling of bushy neurons indicating that these neurons may primarily project to the neocortex. Our understanding of the functional role of IPf neurons is still rather limited, but perhaps the different intrinsic properties may provide insight into their function regarding information transfer to neocortex and their role in the basal ganglia circuit.

Acetylcholine application in the cat medial geniculate nucleus (MGN) *in vivo* results in both increases and decreases in spike discharge (Tebecis, 1972), whereas acetylcholine application in the cat dorsal lateral geniculate nucleus (dLGN) elicits only excitation (Phillis, 1971; Krnjevic, 1974). McCormick and Prince (McCormick and Prince, 1987) reported the nicotinic agonist DMPP produced depolarizations in all cat MGN and dLGN neurons tested in the *in vitro* slice. Our results with DMPP were very similar, producing depolarizations in a high percentage of rat IPf neurons. In addition to nicotinic activation, McCormick and Prince (McCormick and Prince, 1987) also tested muscarinic activation *in vitro*, in cat and guinea pig MGN and dLGN. They found muscarinic induced hyperpolarizations in a majority of neurons tested in the cat and guinea pig MGN and the guinea pig dLGN with a smaller percentage depolarizing with MCh application, but the majority of the neurons tested in the cat dLGN produced depolarizations with a smaller percentage producing hyperpolarizations (McCormick and Prince, 1987). In the rat dLGN, muscarinic agonists produce only depolarizations in relay neurons (Zhu and Uhlrich, 1998). Our data provide the first evidence that MCh can produce inhibitory actions in apparent projection neurons of the rat IPf nucleus. Our muscarinic results are actually similar to those in guinea pig MGN and dLGN or cat MGN in that muscarinic receptor-mediated hyperpolarizations are the predominant effect.

The cellular mechanisms of muscarinic receptor mediated hyperpolarizations and depolarizations have been previously studied in the thalamus (McCormick and Prince, 1987; McCormick, 1992; Zhu and Uhlrich, 1998). However, these studies examined the cellular mechanisms of muscarinic modulation on primary sensory thalamic nuclei and not intralaminar thalamic nuclei. The muscarinic hyperpolarizations seen in cat and guinea pig thalamic relay neurons are due to an increased K^+ conductance, whereas the depolarization in guinea pig dLGN relay neurons appears to be mediated via a reduction of a K^+ conductance (McCormick and Prince, 1987). In rat dLGN relay neurons, the muscarinic depolarization consists of two components (Zhu and Uhlrich, 1998). The early component is mediated by an increased mixed cation conductance, I_h , and the later component is mediated by a decreased K^+ conductance. The late depolarizing component was mimicked by the M_1 receptor agonist McN-A-343, suggesting that the late, K^+ -dependent component is mediated via M_1 receptor activation. Zhu and Uhlrich (1998) speculate that the early component is mediated via M_3 receptors, however pharmacological evidence for this is lacking. Our results suggest that the MCh depolarization of rat IPf neurons is primarily mediated via the M_3 muscarinic receptor activation and is associated with a decreased K^+ conductance. The hyperpolarization is mediated via activation of M_2 receptors and is associated with an increased K^+ conductance.

The functional consequences of these cholinergic actions in the IPf could be similar to the proposed significance of cholinergic modulation in primary sensory thalamus. Cholinergic input to the thalamus arises from mesencephalic brain regions that constitute the ascending reticular formation (Mesulam *et al.*, 1983), where it is believed to modulate sensory information

transfer from the thalamus to the cortex (Sillito et al., 1983; Francesconi et al., 1988; Uhlich et al., 1995) by altering the action potential firing mode of thalamic neurons (Steriade and Llinas, 1988). Thalamic relay neurons are known to produce action potentials in two distinct modes: burst firing and tonic firing. Burst discharge is dependent on activation of the low threshold, transient Ca^{2+} current, I_T , which produces a low threshold calcium spike on top of which multiple sodium-dependent action potentials can occur at a high frequency (Jahnsen and Llinas, 1984). Tonic firing occurs at depolarized membrane potentials when I_T is inactivated and the frequency of action potential discharge is linearly related to the magnitude of membrane depolarization. The majority of IPf neurons that project to the cortex are of the bushy subtype and these neurons are capable of firing in tonic and burst modes, just like primary sensory thalamic relay neurons. In addition, this study has shown that these bushy neurons only depolarize in response to muscarinic receptor activation. Similarly, rat and cat primary sensory thalamocortical relay neurons also depolarize in response to muscarinic receptor activation (McCormick and Prince, 1987; Zhu and Uhlich, 1998). This depolarization would shift the bushy neurons from burst mode to tonic mode where these neurons may transfer their signal to cortex more reliably. The role of the diffuse neuronal subtype's projection to the neocortex is still unclear since the population appears minor and the muscarinic activation results in several membrane effects.

A major projection from the rat IPf is to the lateral striatum, the major input nucleus of the basal ganglia (Berendse and Groenewegen, 1990). Stimulation of the rat IPf has been shown to produce excitatory synaptic responses in striatal neurons (Wilson et al., 1983). While both bushy and diffuse neuronal subtypes project to the striatum, the majority of the neurons are diffuse. In this study we found that the predominant effect of muscarinic receptor activation on both subtypes that projected to the striatum was membrane hyperpolarizations. Most diffuse IPf neurons lack the ability to fire in burst mode. Therefore, a hyperpolarization from muscarinic receptor activation may serve to decrease the neurons firing rate, thereby dampening the glutamatergic excitatory drive onto the striatum. Hyperpolarization of the bushy neurons that project to the striatum could lead to increased burst discharge from these neurons, which may have a role in basal ganglia oscillations.

Functional Implications

The IPf is in a position to influence both basal ganglia as well as neocortical activity, which is quite unique relative to many other primary thalamic nuclei. In Parkinson's disease there is a marked degeneration of neurons within the centre-médian nucleus (Henderson et al., 2000a), the apparent analogous structure to the IPf in the rat. We would predict a selective degeneration of diffuse type neurons associated with Parkinson's disease; however the functional significance of such changes remains unexplored. One of the major symptoms of Parkinson's disease is a resting motor tremor. Oscillations of neuronal activity occur in the internal segment of globus pallidus and motor thalamus of Parkinson's disease patients at 3–6 Hz (Lenz et al., 1988; Pare et al., 1990; Lemstra et al., 1999). These oscillations have a similar frequency as intrathalamic oscillations that occur in certain sleep states and absence epilepsy (Williams, 1953; Domich et al., 1986; Steriade and Llinas, 1988; Steriade et al., 1993). An intriguing question still unanswered is: Can the IPf have a role in rhythmic oscillations?

Intrathalamic rhythmic activities in primary sensory thalamic nuclei arises from the reciprocal synaptic connectivity between thalamic relay nuclei and the adjacent thalamic reticular nucleus (Steriade et al., 1993; von Krosigk et al., 1993; Huguenard and Prince, 1994; Cox et al., 1997). The sustained activity also requires burst firing of thalamic neurons (von Krosigk et al., 1993). The IPf has been shown to project to the thalamic reticular nucleus, and in turn, receive thalamic reticular nucleus inputs (Deschenes et al., 1996; Kolmac and Mitrofanis, 1997; Tsumori et al., 2002). Whether similar rhythmic activities can occur within the IPf remains

unknown, but maybe of functional significance considering the majority of LPf neurons project to the striatum and could potentially influence rhythmic activity within the basal ganglia circuit.

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Figure 1.

Morphological characteristics of IPf neurons. **A.** Photomicrographs of two different diffuse type IPf neurons. **Ai.** This diffuse neuron has long, poorly branching dendrites and the axon clearly exits the IPf. The black dashed lines delineate the approximate border of IPf and arrows indicate the axon. **Aii.** Photomicrograph of a different diffuse neuron. As above, note the long, simple dendrites. **B.** Photomicrographs of two different bushy neurons. **Bi.** This bushy neuron also sends its axon out of the IPf (arrows) indicative of a thalamic projection neuron. **Bii.** In a different bushy neuron, note the radially projecting, complex-branching dendritic architecture. Calibration, 100 μm. **C.** Recording locations of 50 bushy (●) and 50 diffuse (x) type IPf neurons in horizontal sections. The numbers (bottom right) indicate location of section relative to

interaural zero (Paxinos and Watson, 1986). *Abbreviations:* 3V, third ventricle; MD, mediodorsal thalamic nuclei; CL=centrolateral thalamic nucleus, Po=posterior thalamic nuclear group, PVP=paraventricular thalamic nucleus, fr=fasciculus retroflexus, PC=posterior commissure, PrC= precommissural nucleus. Section diagrams were adapted from Paxinos and Watson.

Figure 2.

Distinct action potential output characteristics of LPf neuron subtypes. **Ai.** Photomicrograph of a biocytin-filled bushy type neuron. **Aii.** Voltage dependence of action potential output in the same neuron. At a depolarized membrane potential, a depolarizing current step evokes tonic action potential discharge (*top trace*). At a relatively hyperpolarized membrane potential, the same current step evokes a transient, high frequency burst of action potentials (*bottom trace*). **Bi.** Photomicrograph of a diffuse type LPf neuron. **Bii.** At a depolarized membrane potential, this neuron produces tonic action potential discharge (*top trace*). At a hyperpolarized membrane potential, this cell does not produce burst discharge (*bottom trace*). Calibration in Ai, Bi, 200 μ m.

Figure 3.

The presence of LTSs in bushy and diffuse IPf neurons. **A. Control:** In a bushy neuron, a depolarizing current pulse from a hyperpolarized membrane potential produces strong burst discharge. *Inset:* Illustrates the multiple action potentials in the burst discharge, calibration 20 mV, 20 ms. **TTX:** In TTX (0.5 μ M), the same current step produces a stereotypical transient LTS consisting of a transient, large amplitude depolarization. **Bi. Control:** In a few diffuse neurons, a burst discharge of 2 spikes is evoked by a depolarizing current step. *Inset:* Illustration of multiple action potentials in the burst discharge, calibration 20 mV, 20 ms. **TTX:** In TTX, there is an LTS with a smaller amplitude and shorter duration than that evoked in bushy neurons. **Bii.** In a different subpopulation of diffuse neurons (12/41 cells), the depolarizing current step evokes single spike discharge from hyperpolarized membrane potentials (*Control*). **TTX:** In TTX, there is a small amplitude, transient depolarization in response to the current step. **Biii. Control:** In another subpopulation of diffuse neurons, a single action potential is evoked by the depolarizing current step. **TTX:** Following the addition of TTX, there is no apparent transient depolarization in response to the depolarizing current step.

Figure 4.

Calcium-dependent transient inward currents in LPf neurons. **Ai.** Voltage clamp recording from a bushy LPf neuron. In TTX (0.5 μ M), this protocol evokes a graded transient inward current (**Aii**). Using an inactivation protocol (initial command voltage: -50 to -110 mV, 5 mV increments, 5 s duration return to holding command voltage (-50 mV)), the transient inward currents are strongly attenuated by lanthanum chloride (200 μ M). **Bi.** Voltage clamp recording from a diffuse neuron that evoked small amplitude, transient depolarization in current clamp recording (e.g., Figure 3Bii). In TTX, the inactivation protocol evokes a small amplitude transient inward current (**Bii**). This small transient current is blocked by lanthanum chloride (200 μ M). **C.** Current responses from a diffuse neuron that did not produce a low threshold calcium spike in current clamp mode (e.g., Figure 3Biii). **D.** Population data illustrating the average peak amplitude of calcium-dependent transient currents in bushy ($n=5$) and diffuse ($n=7$) neurons. * $p<0.01$, paired t-test.

Figure 5.

Cholinergic agonists alter excitability of IPf neurons. **A.** Whole cell recordings from two different IPf neurons reveal that the general cholinergic agonist carbachol (25 μ M, solid line) can produce either membrane depolarizations (top trace, bushy) or membrane hyperpolarizations (bottom trace, diffuse). **B.** The nicotinic agonist DMPP (25 μ M) produced only membrane depolarizations (bushy). **C.** The muscarinic agonist MCh (125 μ M) could produce membrane depolarizations (top trace, bushy), membrane hyperpolarizations (middle trace, diffuse), or a biphasic response (bottom trace, bushy) consisting of a membrane hyperpolarization followed by membrane depolarizations. Each trace is from a different IPf neuron.

Figure 6.

Concentration dependence of the MCh-mediated changes in membrane potential. **Ai-iii.** Whole cell recordings from IPf neurons (diffuse) in the presence of TTX (0.5 mM) illustrating that higher MCh concentrations (5, 25, 125 μ M) produced increasingly larger membrane hyperpolarizations. **Aiv.** Summary dose response graph showing MCh produces dose dependant hyperpolarizations in IPf neurons. Numbers in parenthesis indicates sample size, and values are presented as mean $\pm$ SEM. **Bi-iii.** Whole cell recordings from IPf neurons (bushy) illustrating the increasing concentrations of MCh produced larger membrane depolarizations. **Biv.** Population graph illustrating the concentration dependence of the MCh effect.

Figure 7.

Excitatory and inhibitory actions of MCh are mediated via different receptor subtypes. **A. TTX:** In TTX (0.5 μ M), MCh (25 μ M) produces a hyperpolarization that returns to baseline levels within 3 minutes (diffuse). **+Methoctramine:** In the presence of the selective M_2 receptor antagonist methoctramine (1 μ M), the MCh-mediated hyperpolarization is completely blocked. **Wash (TTX):** Recovery of the MCh-mediated membrane hyperpolarization following 30 minutes of wash. **B.** Summary graph of methoctramine effect on MCh-mediated hyperpolarizations. Left panel is summary of MCh (25 μ M)-mediated depolarizations pre-methoctramine (control, $n=11$), in the presence of methoctramine (Methoc., 1 or 10 μ M, $n=11$), and following washout of methoctramine (wash, $n=5$). The right panel is the summary using higher MCh (125 μ M) and methoctramine (25 μ M) concentrations ($n=8$ cells). Partial recovery was observed in only one cell with these higher concentrations. **C. TTX:** In TTX (0.5 mM), MCh (15 μ M) depolarizes an IPf neuron (bushy). **+4-DAMP:** In the selective M_3 receptor antagonist 4-DAMP (10 nM), the subsequent application of MCh results in a significantly

attenuated depolarization. **Wash (TTX):** The MCh-mediated membrane depolarization recovers following 30 minutes wash. **D.** Summary of the 4-DAMP effect on MCh-mediated depolarizations. At 500 nM, 4-DAMP completely blocked the MCh-mediated depolarization and was irreversible (n=4 cells). At 10 nM, 4-DAMP attenuated the MCh-mediated depolarization and it was partially reversible (n= 5 cells).

Figure 8.

MCh alters K^+ conductances. **Ai.** In voltage clamp recording from an IPf neuron (diffuse), slow ramped voltage commands (-50 mV to -110 mV, 4 s duration, 0.1 Hz) are used to measure conductance before and during peak MCh outward currents. Short application of MCh (25 μ M) produces an outward current. **Aii.** Expanded traces of the membrane responses to ramped voltage commands reveal an outward current and an increase in conductance by MCh (gray line). The control trace (black line) is an average of 10 consecutive responses prior to MCh application, and the MCh trace (gray line) is the average of 5 consecutive responses during the peak of the MCh-mediated outward current. **Aiii.** The difference between the control and MCh responses (Aii) is indicative of the MCh-sensitive current (I_{diff}). A linear fit (gray line) of the current response indicates that the MCh mediated current has a reversal potential of -85 mV. **Bi.** In voltage clamp recordings from a different IPf neuron (bushy), MCh (25 μ M) produces an inward current. **Bii.** Expanded traces of the membrane responses to the ramped voltage commands reveal an inward current and a decrease in conductance by MCh. These traces are averages similar to that in Aii. **Biii.** The difference between the control and MCh responses (Bii) is indicative of the MCh-sensitive current (I_{diff}). The linear fit (gray line) of the current response indicates that the MCh mediated current has a reversal potential of -86 mV.

Figure 9.

Preferential projection of IPf neuron subtypes. **A. Left panel:** Fluorescent image showing several labeled neurons in the IPf following fluorescent label injection in the dorsal striatum. **Right panel:** A differential interference contrast image illustrating the labeled neuron in the left panel (*). **B. Striatal injection:** Photomicrograph illustrating the injection site of the retrograde fluorescent latex microspheres placed in the striatum. Image is an overlap of the fluorescent image and bright-field image of slice. **Cortical injection:** Localization of the cortical injection site. Scale bar: 0.5 mm. **Ci.** An example of a biocytin-filled diffuse neuron recorded in the IPf following striatal injection of retrograde tracer. This is the same neuron illustrated in A. **Cii.** Voltage responses to hyperpolarizing and depolarizing current steps of the labeled diffuse neuron shown in Ci. Note the lack of burst discharge from this diffuse neuron. **Di.** Example of bushy neuron that was labeled by a neocortical injection and subsequently recorded from using whole cell techniques. **Dii.** The characteristic responses to

hyperpolarizing and depolarizing current steps of the labeled bushy neuron shown in Di. fr, fasciculus retroflexus. Scale bars represent 100 μm .

Figure 10.

Summary of MCh-mediated responses in distinct subtypes of IPf neurons labeled. Distributions of the MCh-mediated depolarizations and hyperpolarization amongst diffuse and bushy neurons. All neurons included in these analyses were fluorescent-positive cells that were prelabeled following either striatal or neocortical injections.

Table 1

Intrinsic properties and morphological measurements of bushy and diffuse type IPf neurons. The numbers in parentheses indicate the number of neurons tested. The asterisk (*) represents a statistically significant difference using a Mann-Whitney test.

	Bushy	Diffuse	P
Resting Membrane Potential (mV)	-73 ± 7 (78)	-62 ± 7 (149)	* <0.01
R_{input} (MΩ)	492 ± 234 (76)	1003 ± 529 (142)	* <0.01
Action Potential $\frac{1}{2}$ Width (ms)	1.92 ± 0.25 (10)	1.63 ± 0.32 (13)	* <0.03
Action Potential Amplitude (mV)	65 ± 5 (10)	68 ± 6 (13)	n.s.
Maximum AP discharge (Hz)	31.2 ± 8.9 (10)	23.3 ± 7.3 (13)	* <0.05
Slope Frequency/Intensity (Hz/pA)	0.40 ± 0.17 (10)	0.32 ± 0.20 (13)	n.s.
Spike Adaptation (1/final ISI)/(1/initial ISI)	0.30 ± 0.12 (10)	0.53 ± 0.23 (13)	* <0.01
Soma Area (mm²)	299 ± 94 (76)	315 ± 119 (141)	n.s.
Major/Minor Axis Ratio	1.43 ± 0.31 (76)	1.44 ± 0.27 (141)	n.s.
Major Dendrite Length (mm)	195 ± 60 (75)	396 ± 133 (137)	* <0.01

Table 2

The percentage of parafascicular neurons with a particular action to a given cholinergic agonist. In general, both bushy and diffuse cell types responded similar to the cholinergic agonists.

		All LPf neurons	Bushy	Diffuse
Agonist	Response	% effected	% effected	% effected
Carbachol	Depolarization	33% (7/21)	50% (2/4)	29% (2/7)
	Hyperpolarization	43% (9/21)	50% (2/4)	57% (4/7)
	Biphasic	5% (1/21)	0%	0%
	No effect	19% (4/21)	0%	14% (1/7)
DMPP	Depolarization	97% (29/30)	100% (4/4)	95% (19/20)
	Hyperpolarization	0%	0%	0%
	Biphasic	0%	0%	0%
	No effect	3% (1/30)	0%	5% (1/20)
MCh	Depolarization	28% (35/122)	39% (12/31)	23% (14/61)
	Hyperpolarization	52% (63/122)	52% (16/31)	61% (37/61)
	Biphasic	6% (7/122)	6% (2/31)	3% (2/61)
	No effect	14% (17/122)	3% (1/31)	13% (8/61)