

**The Role of Basal Forebrain Neurons in the Modulation of Cortical Activity:  
A Physiological and Anatomical Examination**

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**A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment  
of the requirements of the degree of  
Doctorate of Philosophy.**

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0-612-78725-7

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## **Abstract**

The basal forebrain is essential for stimulating the cortical activation associated with waking and paradoxical sleep, yet it is also important for attenuating cortical activation and promoting SWS. Heterogeneous neuronal groups of this region, which include cortically projecting cholinergic, GABAergic and other noncholinergic/nonGABAergic possibly glutamatergic neurons, may mediate these different processes. In order to understand this differential modulation, the discharge profiles of the neuronal groups must be studied in association with cortical activity.

Juxtacellular recording and labeling of neurons in the magnocellular preoptic area (MCPO) and substantia innominata (SI) of the basal forebrain with Neurobiotin (Nb) followed by immunohistochemical staining for neurotransmitter enzymes revealed the discharge properties of identified neurons during slow irregular electroencephalographic (EEG) activity and stimulation-induced cortical activation in urethane-anesthetized rats. Choline acetyltransferase immunopositive (ChAT+) neurons increased their average discharge rate with stimulation, and the majority shifted from an irregular tonic discharge during slow irregular EEG activity to a rhythmic burst discharge during activated and rhythmic EEG activity. In contrast, a minority of glutamic acid decarboxylase immunopositive (GAD+) neurons increased their average discharge rate, while the majority made up of specific subgroups decreased their average discharge rate in association with stimulation-induced cortical activation. Another group of neurons that discharged in rhythmic clusters during cortical activation was both ChAT and GAD immunonegative. The possibility that these were glutamatergic was investigated by immuno-staining for phosphate-activated-glutaminase (PAG), the synthetic enzyme for neurotransmitter glutamate. A parallel study determined that a significant proportion of cortically projecting basal forebrain neurons contained PAG. The rhythmically discharging ChAT and GAD immunonegative cells were PAG+. Their rhythmic discharge occurred at the same frequency as that of the cholinergic and GABAergic neurons and was simultaneously correlated with the activity of the olfactory bulb and that of other limbic neocortical regions.

Together these findings indicate that cholinergic and GABAergic neurons, together with glutamatergic neurons function in a parallel manner to mediate cortical

activation including rhythmic theta and high frequency gamma activity. Distinct subgroups of GABAergic neurons may be involved in dampening cortical activation and mediating the slow irregular cortical activity of slow wave sleep.

## Résumé

Le cerveau antérieur basal est essentiel pour l'activation du cortex associée à l'éveil et au sommeil paradoxal. Il est également important pour l'atténuation de l'activation du cortex nécessaire pour favoriser le sommeil à ondes lentes. Ces actions du cerveau antérieur basal semblent être régularisées par un groupe hétérogène de neurones projetant au cortex. Ces neurones incluent des neurones cholinergiques, GABAergiques et autres neurones non-cholinergiques/non-GABAergiques. Afin de mieux comprendre le rôle spécifique de chacune de ces populations neuronales dans la régulation des différents stades sommeil/éveil, nous avons entrepris la caractérisation des profils d'activité électrophysiologique au cours de différents stades d'activation du cortex. Pour ce faire, nous avons procédé à des enregistrements juxtacellulaires de neurones localisés au niveau de l'aire préoptique magnocellulaire et de la substantia innominata. Les enregistrements ont été faits chez des rats anesthésiés à l'uréthane et dont le cortex était activé suite à des stimulus physiques alors que les animaux présentaient un profil d'EEG lent et irrégulier. Les neurones enregistrés ont été injectés avec de la neurobiotine (Nb), afin de pouvoir les identifier puis ont été marqués immunohistochimiquement pour les enzymes de synthèse des neurotransmetteurs d'intérêt. Ces études ont permis de démontrer que de nombreux neurones marqués par la choline acetyltransférase (ChAT+) augmentaient leur taux de décharge suite à l'excitation corticale puisque la majorité d'entre eux passaient d'un mode de décharge tonique et irrégulier à une activité en salves rythmiques durant l'activité rythmée et activée de l'EEG. Par opposition, une sous-population de neurones marqués par l'acide glutamique décarboxylate (GAD+) avait un taux de décharge plus rapide tandis qu'une majorité de ces neurones avaient leur activité électrique diminuée suite à la stimulation corticale. Nous avons également observé un autre groupe de neurones qui déchargeaient de façon rythmique pendant l'activation corticale et qui étaient ChAT- et GAD-. Afin de déterminer si ces neurones étaient glutaminergiques, nous avons testé s'ils étaient immunoréactifs au marqueur glutaminergique spécifique de la glutaminase activée par phosphate (PAG). Nos études ont démontré que les neurones CHAT- et GAD- étaient glutaminergiques et qu'en plus ils projetaient au cortex cérébral. La décharge rythmique des neurones glutaminergiques était similaire à celle observée chez les neurones cholinergiques et GABAergiques que

nous avons identifié et, elle aussi, était corrélée avec l'activation du bulbe olfactif et de d'autres régions néocorticales limbiques. En somme, nos résultats indiquent que les neurones cholinergiques, GABAergiques et glutaminergiques sont activés en concert pour générer des ondes d'activité theta rythmiques et de haute fréquence gamma. Une sous-population de neurones GABAergiques semble plutôt modérer l'activité corticale et favoriser ainsi les ondes lentes et irrégulières caractéristiques des ondes lentes du sommeil.

## **Statement of Originality**

This series of experiments introduce many novel findings concerning both the physiology and anatomy of the basal forebrain neurons that regulate cortical activity and sleep-wake states. The studies have all been prepared in manuscript format for separate publication elsewhere, and the first three have already been published. Many of the results have also been presented orally or as posters at the 1998, 1999, 2000 Society for Neuroscience meetings, the 2001 Canadian Sleep Research Congress, and the 1999 and 2001 World Federation of Sleep Research Societies Congress.

The initial two series as well as the final series of studies were the first to combine physiological and anatomical methods to describe the *in vivo* firing properties of immunohistochemically identified cholinergic, GABAergic and glutamatergic basal forebrain neurons. I performed the experiments and analysis, while experimental and analytical design was planned with the guidance of Drs. Barbara E. Jones and Angel Alonso, and Dr. Jones' technician Lynda Mainville assisted with the immunohistochemistry.

The third series of experiments was of an exclusively anatomical nature and was the first to describe a glutamatergic neuronal component of the basal forebrain cortical projection system. Lynda Mainville and I shared in the retrograde tracer injections. Lynda Mainville did the immunohistochemistry, and I completed the anatomical data collection and subsequent analysis. These experiments and analysis were done under the guidance and supervision of Dr. Jones.

The manuscripts were written by myself before being co-edited for publication by Dr. Jones. The findings stemming from this body of work represent a unique

contribution to the understanding of how subcortical modulatory systems potentially mediate cortical function and behavioral state.

## **Acknowledgements**

Firstly, I must express my deepest gratitude to Dr. Barbara Jones. Her encouragement and unwavering enthusiasm played no small part in the success of this work. Beyond her scientific achievement, I must also commend her professionalism, attention to detail, and pedagogical acumen; it was enjoyable and enlightening to work with someone so well rounded.

I must also convey my gratitude to Dr. Angel Alonso for his insights, assistance, and advice. His experience and advice helped to shape the experimental setup and analysis of much of this thesis, and our conversations often helped to lighten my mood.

I thank Lynda Mainville teaching me immunohistochemistry, as well as for helping and advising me with so many of the experiments. It was a pleasure working with you. I must also thank Naomi Takeda for being so helpful in so many ways that they are difficult to enumerate.

Special thanks go to those who passed through Dr. Alonso's Lab during my stay: Drs. Clayton Dickson, Jacopo Magestretti, and Li Ma, and the soon to be Drs. Mark Shalinsky and Bassam Hamam. I also want to thank many others who have made my time at the Neuro all the more memorable: Drs. Ahmed Elhusseiny, Antoine Ramjaun, Thomas Stroh, Edmund Cape, Karen Maloney, Yi-ping Hou, and the soon to be Dr. Pierre Villeneuve.

Of course my parents and family must be thanked for their continual support and love. As well, thanks go to of course Natasha, who receives my profoundest appreciation. And, thanks to the many others who have helped to guide or influence me along the way, so to you – thank-you.

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## **List of Abbreviations**

AAT, aspartate aminotransferase  
ac, anterior commissure  
Acb, accumbens  
ACh, acetylcholine  
AChE, acetylcholine esterase  
BLA, basolateral amygdaloid nuclei  
BST, bed of stria terminalis  
CeA, central amygdaloid nucleus  
ChAT, choline acetyl-transferase  
CoA, cortical amygdaloid nuclei  
CPu, caudate putamen  
CT, cholera toxin  
DAB, diaminobenzidine  
DBB, diagonal band of Broca  
En, endopiriform nucleus  
EP, entopeduncular nucleus  
f, fornix  
ic, internal capsule  
GABA, gamma-aminobutyric acid  
GAD glutamic acid decarboxylase  
GP, globus pallidus  
LEA, lateral entorhinal area  
LH, lateral hypothalamus  
LOT, lateral olfactory tract  
LPOA, lateral preoptic area  
LS, lateral septum  
MCPO, magnocellular preoptic area  
MeA, medial amygdaloid nuclei  
MEA, medial entorhinal area  
MS, medial septum  
oc, optic chiasm  
OTu, olfactory tubercle  
ot, optic tract  
PAG, phosphate-activated-glutaminase  
PAP, peroxidase-antiperoxidase  
PaS, parasubiculum  
Pir, piriform cortex  
PRh, perirhinal cortex  
PrS, presubiculum  
Re, reuniens  
ret, reticular nucleus of thalamus  
S, subiculum  
SI, substantia innominata  
SIa, substantia innominata anterior  
SIp, substantia innominata posterior

## **Chapter One**

### **1. Introduction**

---

### **1.1 Sleep-wake states**

In the early twentieth century, neurophysiologists observed that electrical potentials recorded from the head, the electroencephalogram (EEG), displayed distinct patterns in association with different behavioral states (Berger, 1930; Loomis, 1935). Sleep was marked by high voltage slow irregular activity, and attentive waking was marked by relatively low voltage fast activity (Berger, 1930; Loomis, 1935). Despite variability of the EEG, sleep was generally considered to be a unitary physiological state, until in the 1950s it was found to be comprised of two distinct states. One was characterized by high voltage slow EEG activity and was thus called slow-wave sleep (SWS). The other was characterized by low voltage relatively fast EEG activity similar to that of waking and accompanied by the occurrence of rapid eye movements (REM) together with muscle atonia, and was thus called REM or paradoxical sleep (PS) (Aserinsky and Kleitman, 1953; Jouvet et al., 1959).

Since the early studies, different patterns of EEG activity have been classified by amplitude and frequency, as characterizing different stages of SWS, the state of PS and different behaviors of waking. The variations in amplitude and frequency of the EEG are a function of the degree of synchronous activity of large populations of cortical neurons (Gloor, 1985). In all mammals, waking is typically associated with low voltage fast activity in the beta range (15 - 30 Hz range) or gamma range (>30Hz), which is maximal in amplitude during attentiveness and heightened arousal. In rodents, a sinusoidal theta wave (4 - 8 Hz) is also present in hippocampus and limbic cortex during behavioral arousal and locomotion. In humans and other primates and cats, a sinusoidal alpha wave (10 - 12 Hz) is prominent during inattentive states with the eyes

closed. During slow wave sleep (SWS), the EEG is considerably different from that of waking and marked by a decrease in fast gamma activity, with however an increase in beta activity (Maloney et al., 1997). With the onset of sleep, prominent spindle waves (12 – 14 Hz) occur intermittently. Then as sleep progresses, a marked increase in the EEG amplitude occurs with a concomitant slowing of its frequency, which occurs as delta activity (1 – 4 Hz) and the slow oscillation (<1Hz). In the hippocampal formation of rodents high frequency bursts of activity, referred to as sharp waves (~200 - 300 msec duration) with high frequency ripples (>200Hz), occur during SWS. The state of paradoxical sleep, which in normal subjects is always preceded by a period of SWS, shows increased fast gamma activity and continuous theta activity in rodents.

With these physiological findings, considerable research has concentrated on localizing areas of the CNS capable of modulating the cortical EEG pattern as well as sleep-wake states and understanding the mechanisms by which they do so. The general objective of this thesis is to further characterize the neuronal substrates involved in these processes. To better define this objective a review of the work that has succeeded in identifying the major structures that are integral to the sleep-wake process will follow.

### **1.1.1 Cortical Arousal Systems**

Experiments in the 1930s by Bremer utilizing transections of the brainstem showed that separation of the cerebrum (*cerveau isolé*) from the brainstem and spinal cord resulted in SWS-like patterns in the EEG (Bremer, 1935). Bremer's studies indicated to others that an important cortical arousal mechanism might be located in the

brainstem. Subsequently in the 1940s, Moruzzi and Magoun demonstrated that electrical stimulation of the brainstem reticular formation, but not sensory pathways passing through the brainstem, produced cortical activation in sleeping cats (Moruzzi and Magoun, 1949). This major finding led them to postulate that the reticular formation could be the ascending activating system for the cortex. Supporting their hypothesis and findings were reports that lesions of the reticular formation produced behavioral immobility and cortical slow-wave activity in chronic experiments (Lindsley et al., 1949; Lindsley et al., 1950). Further neurophysiological and neuroanatomical work indicated that reticular neurons receive collaterals from sensory systems and send ascending fibres into the forebrain by a dorsal thalamic pathway and a ventral extra-thalamic pathway terminating in the hypothalamus, subthalamus, and basal forebrain (Dempsey et al., 1941; Starzl et al., 1951a; Starzl et al., 1951b; Nauta and Kuypers, 1958; Scheibel and Scheibel, 1958; Jones and Yang, 1985). This work set in place the theory of the 'ascending reticular activating system' and the concept that this system is necessary for the maintenance of cortical activation and behavioral arousal.

Further examination of the ventral extra-thalamic pathway demonstrated that lesions of the thalamus failed to eliminate cortical activation evoked by stimulation of the reticular formation (Dempsey et al., 1941; Starzl et al., 1951b). These results suggested that the ventral extra-thalamic relay through the basal forebrain area is sufficient for basic state modulation. It should be noted that in the chronic course of experiments studying separation of the brainstem from the forebrain, cortical activation did return, and the forebrain alternately displayed fast and slow activity similar to that of wake and sleep in normal animals (Batsel, 1960; Villablanca, 1962). It is also

noteworthy that cortical activation could be triggered by olfaction (Villablanca, 1965), while in experiments with permanent olfactory and visual deafferentation, an alternation between slow and fast cortical activity was also evident (Batsel, 1964; Villablanca, 1965). These studies indicated the existence of an autochthonous forebrain activating system. Anatomical studies revealed that the basal forebrain gave rise to a cortical projection system that was the primary cortical source of acetylcholine (ACh) (Shute and Lewis, 1963; Mesulam et al., 1983a; Rye et al., 1984; Luiten et al., 1987). The potential importance for ACh as a neurotransmitter responsible for general cortical arousal was becoming evident. Electrophysiological studies concerning the action of ACh showed that it caused excitatory effects on cortical neurons (Krnjevic and Phillis, 1963), while both muscarinic and nicotinic antagonists diminished cortical activation (Longo, 1966; Domino et al., 1968). ACh release studies showed it to be maximally released in association with cortical activation, notably during wakefulness and PS (Celesia and Jasper, 1966; Jasper and Tessier, 1971). Pharmacological blocking of ACh degradation at the synapse, by physostigmine, increased cortical arousal and wakefulness and shortened onset to PS, while prolonging its duration (Domino et al., 1968). Pharmacological prevention of ACh synthesis, by hemicholinium-3, resulted in a decrease in waking and an elimination of PS (Hazra, 1970). In addition, lesions of the basal forebrain with kainic or ibotenic acid caused parallel decreases in cortical ACh release and cortical activation (LoConte et al., 1982; Stewart et al., 1984; Buzsaki et al., 1988). In contrast, direct stimulation of the basal forebrain was shown to produce a seven-fold increase in cortical ACh release and cortical activation (Kanai and Szerb, 1965; Casamenti et al., 1986; Kurosawa et al., 1989). Unit recordings in freely moving

cats have shown discharge patterns of some basal forebrain neurons are elevated during waking and REM sleep, compared to non-REM sleep (Detari et al., 1984; Szymusiak and McGinty, 1986a; Detari et al., 1987; Szymusiak and McGinty, 1989). Subpopulations of these cells were found to project cortically and were found to fire faster during waking as compared to non-REM sleep and also during waking with movement as compared to quiet waking (Szymusiak and McGinty, 1989). Although unidentified, some of these cells were presumed to be cholinergic. Together these findings indicated that ACh and the cholinergic neurons of the basal forebrain have an important global effect on cortical activity.

#### **1.12 Sleep-Promoting Systems**

It had been long assumed that sleep was a passive process representing fatigue caused by the cessation of peripheral sensory input and allowing rest for the cerebral cortex. This concept was entitled the “deafferentation” theory by Purkinje in 1846 (Thorpy, 1991). During the period when the ascending reticular activating system was identified, this notion continued to be favored despite several lines of evidence suggesting this was not necessarily the case. In the early part of the century, von Economo (von Economo, 1931) noted in cases of *encephalitis lethargica*, that individuals with excessive somnolence, had lesions in the posterior hypothalamus, whereas other individuals with insomnia had lesions of the preoptic area and anterior hypothalamus. In order to verify the observations of von Economo, Nauta conducted a series of experimental lesions transactions through different levels of the hypothalamus or preoptic area in rats (Nauta, 1946). He showed that transections placed at the level of

the posterior hypothalamus resulted in persistent somnolence suggesting that the forebrain had been isolated from its activating system. Furthermore, transections placed in the region of the preoptic area and anterior hypothalamus (POAH) produced a complete insomnia, leading to lethal exhaustion. These findings indicated that the POAH contained an important somnogenic system. More importantly, these studies indicated that sleep was not simply a passive process of fatigue.

This work lent support to the earlier findings of Hess, who had demonstrated that electrical stimulation of the POAH could elicit behavioral suppression in freely moving cats (Hess, 1931; Hess, 1954). Sterman and Clemente (Sterman and Clemente, 1962a, b) expanded on this work and showed that electrical stimulation of the preoptic area and adjacent basal forebrain was able to induce sleep. Researchers have found that lesions in the basal forebrain can result in insomnia and a disruption of EEG sleep patterns (McGinty and Sterman, 1968; Szymusiak and McGinty, 1986b). The contradictory nature of these findings in relation to those studies demonstrating that the basal forebrain region was important for cortical activation appeared difficult to resolve. However, other work demonstrated that electrical stimulation to some basal forebrain sites elicited excitation of cortical neurons, while that to adjacent locations produced strong inhibition of cortical neurons (Jimenez-Capdeville et al., 1997). The explanation would thus seem to lie in the precise anatomical location of the sites of stimulation or lesion and the discrete populations of cells and fibers affected. Yet, the precise neuronal substrates for the potential hypnotic influence of the basal forebrain have been elusive. Some single unit recording studies in freely moving cats have found a minority of neurons in preoptic and other areas of the basal forebrain that display peak discharge

during SWS and comparatively reduced rates during waking and PS (Szymusiak and McGinty, 1986a; Detari et al., 1987; Detari and Vanderwolf, 1987; Koyama and Hayaishi, 1994; Osaka and Matsumura, 1994, 1995). Some cells of this type were found to project to the cortex or brainstem (Szymusiak and McGinty, 1989). In fact, midbrain reticular formation cells involved in ascending activation during waking have been found to be inhibited by stimulation of the basal forebrain (Lineberry and Siegel, 1971). These results indicated that sleep-active basal forebrain neurons might be involved in the inhibition of activity at the level of cortex and brainstem and support the idea that sleep may be an actively generated behavioral state as suggested decades earlier independently by von Economo, Nauta and Hess.

Recent findings have emphasized the importance of the somnogenic role of the preoptic area. A group of neurons concentrated in the ventrolateral preoptic area (VLPO) are activated at sleep onset as revealed by c-fos studies (Sherin et al., 1996) and single unit recordings, showing an increased discharge of these cells, preceding and during sleep (Szymusiak et al., 1998). Lesion of this discrete set of neurons reduces sleep time and results in a fragmented sleep pattern (Lu et al., 2000).

Besides the forebrain regions being important in the generation of sleep, work has also suggested that certain brainstem structures may facilitate behavioral and physiological sleep. Studies of brainstem lesions indicated that transection behind the oral pontine tegmentum induce total insomnia (Batini et al., 1959), while electrical stimulation in the dorsal medullary reticular formation and solitary tract nucleus were shown to induce SWS in awake animals (Magnes et al., 1961). The solitary tract

also sends inhibitory fibres into the reticular formation and many forebrain regions (Ricardo and Koh, 1978), thus potentially inhibiting cortical activation.

Collectively these results have indicated that the basal forebrain, the POAH, and the lower brainstem reticular formation are important in the generation of SWS, and that sleep is not simply a passive process, but one in which inhibition of the activating system is a crucial process.

In reviewing this work, it becomes clear that the regulation of sleep and wake is not strictly localized and is in fact influenced by disparate collections of neurons across the neuraxis. However, central to the maintenance of the both normal sleep and wake states is the basal forebrain. Revealing the mechanisms by which this area may regulate cortical activity and state will guide the objectives of this thesis. As such, further insights from the pre-existing literature into each particular component of the basal forebrain system and its potential functional effects will be examined.

## **1.2 Anatomy and Physiology of the Basal Forebrain**

The basal forebrain region, which contains cholinergic neurons, sits at the base of the telencephalon and consists of the following nuclei, rostrally to caudally: the medial septum (MS), vertical and horizontal diagonal bands of Broca (vDBB, hDBB), magnocellular preoptic nucleus (MCPO), substantia innominata anterior and posterior (SIa, SIp), and globus pallidus (GP) (Mesulam et al., 1983a; Schwaber et al., 1987; Gritti et al., 1993), although differing cytoarchitectonic divisions and nomenclature for these nuclei have been employed (see (Heimer and Alheid, 1991; Gritti et al., 1993)).

Meynert originally described the large neurons of the SI and GP, which was subsequently referred to as the nucleus basalis of Meynert or magnocellular basal nucleus (Heimer and Alheid, 1991). Kievit and Kuypers (Kievit and Kuypers, 1975) used retrograde transport techniques to reveal that cells of this region projected to the cortex. This projection that forms the ventral extrathalamic relay between the ascending reticular formation and the cortex was found to be composed of cholinergic and other noncholinergic neurons (Rye et al., 1984; Zaborszky et al., 1986; Fisher et al., 1988; Gritti et al., 1993; Gritti et al., 1997). The proportion of the basal forebrain cell population which is represented by the cholinergic cells, remains uncertain, however they would appear to be a minority of the neurons and only  $\sim 1/3$  of the cortically projecting cells (Zaborszky et al., 1986; Gritti et al., 1993, 1994; Gritti et al., 1997). A large group of neurons in this region contains the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) (Fisher et al., 1988; Gritti et al., 1997). GABAergic neurons comprise  $\sim 1/3$  of cortically projecting neurons of this region, in addition to other projections (Zaborszky et al., 1986; Fisher et al., 1988; Gritti et al., 1993, 1994; Gritti et al., 1997). Finally, the neurotransmitter of the noncholinergic/nonGABAergic cells, which represents another  $\sim 1/3$ , of the cortically projecting cells remains to be determined.

Neurons of the basal forebrain project to the cortex in a topographically organized manner (Saper, 1984; Luiten et al., 1987). Neurons in the MS and vDBB innervate the hippocampus, those in the hDBB and rostral MCPO target primarily the olfactory bulb, piriform and entorhinal cortices, and those in the SI and more caudal MCPO innervate the neocortex (Rye et al., 1984; Saper, 1984; Luiten et al., 1987). In

addition, the projections from the SI and MCPO are organized in a rostral to caudal topographical fashion, innervating medial to lateral neocortical sites (Bigl et al., 1982; Mesulam et al., 1983b; Rye et al., 1984; Saper, 1984; Luiten et al., 1987; Gritti et al., 1997).

There is some controversy regarding the extent of cortex that each basal forebrain cell innervates. Many studies utilizing retrograde transport have found very wide-ranging cortical target areas of individual neurons. For instance, cells of the DBB have been found to project simultaneously to the olfactory bulb and hippocampus (Okoyama et al., 1987) and cells of the MCPO to project simultaneously to the olfactory bulb, piriform cortex and/or entorhinal cortex (Paolini and McKenzie, 1997). Conversely, the work of Price and colleagues has suggested a very restricted projection profile of cortically projecting neurons with their axon collaterals encompassing one narrow cortical area of ~1mm in width (Price and Stern, 1983).

Considerable physiological research has been done towards understanding how the medial septum and DBB control hippocampal EEG activity. This thesis is directed at understanding how the MCPO and SI of the basal magnocellular nuclei control the activity of the neocortex along with behavioral state. As such the following discussion will pertain largely to them, however, for comparative purposes at many points through the thesis, the particular parallels and discrepancies between the rostral-medial (MS-DBB) septal region and the caudal-lateral (MCPO-SI) basal magnocellular region of the basal forebrain will be noted.

### **1.2.1 Basal Forebrain Afferents**

The neurons of the basal forebrain receive afferents from multiple chemically and anatomically diverse sources. This input is undoubtedly critical in modulating or determining the physiological activity of neurons in this region, and thus shaping its role in behavioral state modulation. The neurons receive particularly important input from the reticular activating system via medial forebrain bundle originating in the brainstem, and additionally from several other subcortical and cortical areas.

Monoaminergic input to the basal forebrain from brainstem cell groups has been identified from the locus coeruleus and the dorsal raphe nuclei (Jones and Cuello, 1989). For many years, monoaminergic influences on the sleep-waking cycle have been recognized. Both the noradrenergic and serotonergic neurons have been found to be most active during wakefulness (McGinty and Harper, 1976; Trulson and Jacobs, 1979; Aston-Jones and Bloom, 1981) and thus are likely to exert their maximal influence during this period. The noradrenergic locus coeruleus projects into all of the major subdivisions of the basal forebrain (Semba et al., 1988; Jones and Cuello, 1989). As well, serotonergic afferents from the raphe nuclei are distributed through the basal forebrain (Vertes, 1988; Jones and Cuello, 1989). Both of the noradrenergic and serotonergic afferents synapse onto cholinergic and non-cholinergic basal forebrain neurons (Zaborszky et al., 1991).

Glutamatergic innervation of the basal forebrain appears to arise from different sources, and is likely to be very important in shaping the activity of this region. Quantitative L-glutamate autoradiographic experiments revealed that L-glutamate

binding sites in the basal forebrain outnumber muscarinic or monoaminergic sites (Zilles et al., 1991), and glutamatergic antagonists injected into the basal forebrain result in a reduction of cortical ACh levels (Rasmusson et al., 1994).

Neurons of the mesencephalic reticular formation, a major component of the ascending reticular activating system, send a major projection to the basal forebrain (Jones and Cuello, 1989). Although this area contains the primary cholinergic nuclei of the brainstem, (Jones and Beaudet, 1987) anatomical studies suggest that other neurons in the mesencephalic reticular formation which are glutamatergic project to the basal forebrain, and only a minority of the cholinergic neurons project to the basal forebrain (Semba et al., 1988; Jones and Cuello, 1989). These glutamatergic afferents likely innervate both cholinergic and non-cholinergic neurons within the basal forebrain (Jones, 1995). There is nonetheless a cholinergic innervation of the basal forebrain, and evidence that ACh is released in the basal forebrain from fibers ascending from these nuclei (Consolo et al., 1990). The cholinergic fibers that originate from the brainstem appear to be primarily directed to non-cholinergic neurons (Zaborszky et al., 1991).

The basal forebrain also receives glutamatergic projections from the cortex (Davies et al., 1984). These primarily glutamatergic fibers originate in many cortical areas and terminate throughout the basal forebrain (Lehmann and Saper, 1985; Gaykema et al., 1991; Zaborszky et al., 1991).

Neurons from several hypothalamic nuclei innervate the basal forebrain. Anatomical studies have found that cells of the posterior and lateral hypothalamus project in a topographical manner to regions of the basal forebrain (Cullinan and Zaborszky, 1991). Cells of the posterior hypothalamus terminate on cholinergic and

non-cholinergic cells (Cullinan and Zaborszky, 1991) and are potentially the wake discharging histaminergic neurons (Reiner and McGeer, 1987; Sakai et al., 1990). The orexinergic neurons of the lateral hypothalamus, which have recently been demonstrated to be extremely important for the maintenance of waking, have also been shown to innervate the cholinergic basal forebrain neurons (Chemelli et al., 1999). The medial preoptic area and anterior hypothalamus have been shown, in contrast, to possess inhibitory neurons that innervate the basal forebrain cholinergic cells (Cullinan and Zaborszky, 1991). Inhibitory GABAergic and galaninergic neurons of the ventrolateral and medial preoptic areas have been associated with sleep promotion (Sherin et al., 1996; Szymusiak et al., 1998). Moreover, several other types of neuropeptidergic afferents from diverse sources throughout the hypothalamus have been found to innervate basal forebrain neurons, these include fibers containing neurotensin, substance-P, somatostatin, neuropeptide-Y and vasopressin (Zaborszky, 1982; Haber and Nauta, 1983; Levey et al., 1986; Szigethy and Beaudet, 1987; Chan-Palay, 1988a, b; Zaborszky and Cullinan, 1989; Zaborszky et al., 1991). The presence of these basal forebrain afferents suggests hypothalamic influences to be important to this system across the sleep-waking cycle.

It is these multiple afferents, as well as small collections from a variety of other regions, to the basal forebrain that will dictate to a large extent the activity of this region across the sleep-wake cycle and will thus participate in the regulation of cortical activation and behavioral state. These inputs are organized with temporal specificity, so that different systems will affect their basal forebrain targets differentially across

behavioral states. However, multiple afferent systems will be active during any one state and the complex interaction of several neurotransmitter and neuromodulatory inputs arriving together is likely to produce equally complicated physiological responses. The physiological action of the putative transmitters impinging upon different populations of basal forebrain neurons is considered below in the context of their specific anatomical and physiological properties.

### **1.2.2 Cholinergic neurons**

#### **1.2.2.1 Cholinergic neurons – anatomy**

Early histochemical work by Shute and Lewis (Shute and Lewis, 1963) using the antibody for acetylcholine esterase (AChE), the catabolic enzyme for ACh, suggested that there might be a cholinergic projection from the basal forebrain to the cortex. The existence of cortically projecting cholinergic basal forebrain neurons was ultimately confirmed using immunohistochemical-staining procedures for choline acetyltransferase (ChAT), the enzyme for ACh synthesis, combined with retrograde tracer techniques (Mesulam et al., 1983a; Rye et al., 1984). The magnocellular acetylcholine-containing cells, of the basal forebrain, were found to be the primary source of cholinergic innervation to the limbic system and neocortex (Shute and Lewis, 1967; Lehmann et al., 1980; Mesulam et al., 1983b; Rye et al., 1984; Luiten et al., 1987).

Cholinergic cells of the basal forebrain often occur in clusters within the many fiber-tracts that course within the basal forebrain (Gritti et al., 1993). These neurons have medium to large cell bodies, and multiple, radiating dendrites. Subpopulations of basal forebrain cholinergic cells have been shown to co-express several peptide markers

such as the neuropeptide galanin (Melandar et al., 1985) and nitric oxide synthase (Kitchener and Diamond, 1993; Sugaya and McKinney, 1994). Another marker, which has been shown to be very important in functional lesion studies, is the p75 nerve growth factor receptor that is present selectively on most cholinergic neurons of the basal forebrain (Batchelor et al., 1989).

Different subpopulations of cholinergic cells project to different cortical regions following the general pattern of projection of basal forebrain neurons described above. However, the prefrontal cortex receives the densest cholinergic innervation of all cortical regions, receiving its input primarily from the MCPO, SI and GP nuclei (Lysakowski et al., 1989; Gritti et al., 1997).

There is a projection from cholinergic neurons of the SI to the basolateral nucleus of the amygdala (Nagai et al., 1982; Woolf and Butcher, 1982). Some cholinergic neurons project to thalamic nuclei, including the reticularis nucleus (Steriade et al., 1987; Groenewegen, 1988; Asanuma and Porter, 1990; Bickford et al., 1994). A minority of cholinergic basal forebrain cells project caudally to innervate the posterior hypothalamus, caudal midbrain, pons and medulla (Semba et al., 1989; Gritti et al., 1994). These projection patterns have the potential to thus differentially modulate various regions of the cortical mantle as well as subcortical structures.

#### **1.2.2.2 Cholinergic neurons – physiology**

The cholinergic cells of the MCPO and SI have distinct electrophysiological properties. *In vitro* studies in the guinea pig determined that these cells have low threshold  $\text{Ca}^{2+}$ -spikes, transient outward rectification, and a long after-hyperpolarization (Khateb et al.,

1992). The low threshold  $\text{Ca}^{2+}$ -spike was particular to the cholinergic basal forebrain cells in the guinea pig brain slices. Depolarization from a hyperpolarized level or release from a hyperpolarizing holding potential causes these cells to rebound with a series of rhythmic spike-bursts, with the frequency of the bursts being 100-250 Hz. Blockade of the  $\text{Na}^+$  spikes with tetrodotoxin revealed a broad  $\text{Ca}^{2+}$ -spike. The emergence of the spikes following the hyperpolarization occurred with a delay generated by the presence of a transient outward  $\text{K}^+$ -current, the A-current. The spike bursts were followed by a long lasting after-hyperpolarization (~300msec) potentially due to a  $\text{Ca}^{2+}$ -dependent  $\text{K}^+$ -current. In contrast to the rhythmic-burst responses evoked from hyperpolarized levels, depolarizing current injection induced single spike firing at slow frequencies <15 Hz. Thus, the cholinergic neurons were shown *in vitro* to fire in two distinct modes of discharge: a rhythmic bursting mode and a tonic firing mode

It has been determined, *in vitro*, that cholinergic cells respond to the neurotransmitters that are released by basal forebrain afferents (see anatomy above). Noradrenaline, glutamate agonists and histamine are all capable of depolarizing these cells (Fort et al., 1995; Khateb et al., 1995b; Khateb et al., 1995a), while muscarinic cholinergic agonists and serotonin hyperpolarize them (Khateb et al., 1993; Khateb et al., 1997). Furthermore, other *in vitro* studies have indicated that neurotransmitter interactions can uniquely affect the behavior of cholinergic neurons. Administration of NMDA in the presence of the cholinergic agonist carbachol, which hyperpolarizes the membrane, induces prolonged rhythmic low threshold bursting (Khateb et al., 1997). The potential for important modulation by peptidergic influences is also present. Cholinergic cells are selectively associated with high affinity binding sites to for the

neuropeptide neurotensin (Szigethy and Beaudet, 1987; Szigethy et al., 1990). The potential physiological effects of neurotensin were thus examined in *in vitro* electrophysiological studies; the findings showed that neurotensin promoted a slow depolarization of these cells and a sustained rhythmic bursting activity (Alonso et al., 1994).

It has been proposed that basal forebrain neurons, which discharge in both wake and PS, might correspond to the cholinergic neurons (Szymusiak and McGinty, 1986a, 1989). They would be expected to be active during these states since ACh is maximally released from the cortex during both states relative to SWS (Celesia and Jasper, 1966; Jasper and Tessier, 1971). Understanding the manner in which these neurons discharge in association with cortical activity is critical in understanding their role in mediating cortical activation during these states, and can only be accomplished by the *in vivo* recording and identification of cholinergic neurons.

### **1.2.3 Noncholinergic Neurons**

#### **1.2.3.1 GABAergic Neurons – Anatomy**

Immunohistochemical techniques have been used to detect GABAergic neurons using antibodies for GABA synthesizing enzyme GAD (Mugnaini and Oertel, 1985). These GAD positive neurons in the basal forebrain outnumber the cholinergic cells by a ratio of approximately two-to-one (Gritti et al., 1993). They have been hypothesized to be implicated in the mediation of sleep-wake states (Gritti et al., 1993). However, this GABAergic population is very heterogeneous in both size and projections. Similar to the cholinergic cells, many large GABAergic cells are retrogradely labeled from the

hippocampus, olfactory bulb and cortex (Kohler et al., 1984; Zaborszky et al., 1986; Fisher et al., 1988; Gritti et al., 1997). Studies have shown that the GAD enzyme is contained for the most part within different neurons than the ChAT enzyme (Brashear et al., 1986; Kosaka et al., 1988; Fisher and Levine, 1989). The basal forebrain GABAergic projections appear to be topographically organized in the same manner as the cholinergic neurons with widespread collaterals in addition to more focused regional projections (Kohler et al., 1984; Gritti et al., 1997). There is evidence that these cortically projecting GABAergic neurons preferentially terminate on hippocampal and cortical GABAergic interneurons (Freund and Antal, 1988; Freund and Meskenaite, 1992). These findings suggested that the forebrain GABAergic cells may function in a feed-forward disinhibitory circuit with cortical pyramidal neurons.

GABAergic projections have been identified to the posterior hypothalamus (Gritti et al., 1994), as well as thalamic nuclei, like some cholinergic neurons (Asanuma and Porter, 1990; Bickford et al., 1994; Gritti et al., 1998). Basal forebrain cortical and thalamic projections seem to be somewhat overlapping in that some cells have been found to project to both the reticularis nucleus as well as the cortex (Jourdain et al., 1989). Morphologic features may partially differentiate the cells projecting to different target areas, as cortically projecting GABAergic neurons are significantly larger than the GABAergic cells that give rise to descending projections to the posterior hypothalamus (Gritti et al., 1994 1068; Gritti et al., 1997 1512). This descending projection has been suggested to serve in the inhibition of the cortical activating function that has been ascribed to the posterior hypothalamus (Nauta, 1946; McGinty, 1969; Szymusiak et al., 1989; Gritti et al., 1994). Non-cholinergic projections

descending from the basal forebrain to the midbrain, pons and medulla have also been documented (Swanson et al., 1984; Semba et al., 1989), but these have not been directly examined for GAD immunoreactivity. Many GABAergic basal forebrain cells are small ( $<15\ \mu\text{m}$ ) and are likely interneurons. There is evidence that GABAergic terminals from putatively local GABAergic neurons densely innervate the cholinergic basal forebrain cells (Khateb et al., 1998). Clearly, this large and heterogeneous GABAergic group of neurons is integral to the function of the basal forebrain system.

#### **1.2.3.2 Noncholinergic NonGABAergic Neurons – Anatomy**

Based on immunohistochemical cortical retrograde tracing studies, in the rat, a significant proportion  $\sim 1/3$  of the cells projecting to the cortex from the basal forebrain were found to be neither cholinergic nor GABAergic (Zaborszky et al., 1986; Gritti et al., 1997). It was proposed that part of this projection could be glutamatergic. Immunohistochemical staining for glutamate has been difficult, as glutamate is present as a metabolite in most cells, and in GABAergic neurons where it can be used as a precursor for GABA synthesis (Roberts, 1981; Ottersen and Storm-Mathisen, 1984). However, recent evidence suggests that the glutamate used for neurotransmission is produced via a unique synthetic pathway utilizing phosphate-activated glutaminase (PAG). Staining for this enzyme in the cortex showed its presence in pyramidal cells, known to utilize glutamate in transmission and its absence in interneurons, which utilize GABA (Bradford et al., 1978; Kaneko and Mizuno, 1988; Kaneko et al., 1992). Using an antibody for PAG will allow the testing of the hypothesis that the final component of the basalocortical projection system is glutamatergic.

### **1.2.3.3 Noncholinergic neurons – physiology**

Magnocellular neurons of the MCPO and SI, which were immunonegative for the ChAT enzyme, have been shown by *in vitro* recordings to possess electrophysiological characteristics distinct and unique from those of the cholinergic cells (Alonso et al., 1996; Fort et al., 1998). However, these studies did not positively distinguish the neurotransmitter phenotype of these noncholinergic cells. These experiments determined that noncholinergic cells do not display low threshold bursts, and they exhibit relatively fast after-hyperpolarization potentials (AHPs) and fast spiking (>20Hz) distinguishing them from the cholinergic cells. On the other hand, some neurons had a transient outward rectifying current, the A-current, similar to the cholinergic cells (Alonso et al., 1996). A majority exhibited rhythmic clustering of spikes and subthreshold membrane-potential oscillations (Alonso et al., 1996). These cells had subthreshold oscillations evoked by depolarizing current that varied from 20 to 70Hz, as did their intra-cluster spike frequency (Alonso et al., 1996). These cells tended to be large and located in the MCPO and SI regions suggesting they may be projection neurons. *In vitro* studies have also determined that the non-cholinergic cells in the basal forebrain respond non-uniformly to neurotransmitters, likely to be released by subcortical afferents (Fort et al., 1998). The majority of cells respond to noradrenaline by depolarization (69%) while a minority is hyperpolarized and inhibited (17%) (Fort et al., 1998). Of the neurons that are depolarized by NA, Type A is depolarized by muscarine (44%), and Type B is hyperpolarized by muscarine (23%). The neurons that are hyperpolarized by NA (Type C) are also hyperpolarized by

muscarine. Further pharmacological investigations found the three cell types to differ in their response to serotonin and histamine as well. Serotonin evoked no response or a small hyperpolarization in Type A and strong hyperpolarization in Type B and C. Histamine evoked a strong depolarization in Type A and B, while no response was noted in Type C. These pharmacologically distinct profiles are potentially important for correlating the *in vitro* data with extracellular recordings made *in vivo* in association with cortical activation, as well as understanding the potential activity profiles across sleep-waking states when different neurotransmitters are known to be released.

GABAergic and other non-cholinergic neurons may function in the modulation of cortical activation and behavioral state by many potential means. There is a potential disinhibitory role that basalocortical GABAergic neurons may play by innervating cortical interneurons, which suggests they may be active during cortical activation allowing the discharge of principal cells. Studies in the MS and DBB have indicated that putatively GABAergic cells fire in synchrony and would appear to be involved in the pacing of hippocampal theta activity (Lee et al., 1994; Brazhnik and Fox, 1999). On the other hand, studies recording basal forebrain neurons, including cortically projecting neurons, across the sleep-wake cycle have identified cells that increase firing during sleep (Szymusiak and McGinty, 1989). It has been suggested that these could be GABAergic or noncholinergic neurons, and may correspond to those that are inhibited by noradrenaline *in vitro*. It also been suggested that local circuit GABAergic neurons could potentially be active during sleep and thus be important in the control and modulation of the cholinergic cells, by keeping them inhibited during cortical slow wave activity (Khateb et al., 1998). All considered, the potential roles of

noncholinergic cells are varied and emphasize the need for specific correlations of neurotransmitter phenotypes with specific physiological profiles to be made.

### **1.3 Basalocortical Influence on Cortical Activity and Cognition**

The direct influence of the basal forebrain is effected on the cortex by the release of neurotransmitter by basalocortical afferents on their cortical targets. The most widely studied transmitter released by basalocortical afferents has been ACh, as many studies have explored the effects of ACh and its agonists in the cortex. These studies show that ACh has a predominantly excitatory effect (Krnjevic and Phillis, 1963; Krnjevic et al., 1971). This excitatory effect of ACh results in a depolarization of the neurons accompanied by an increase in membrane resistance resulting from the closure of  $K^+$ -channels (Krnjevic et al., 1971; Woody et al., 1978). This effect is typically a slow response that persists for several minutes after application of ACh (Krnjevic and Phillis, 1963). The response of most cortical neurons to ACh is not met with an increase in firing, but instead a facilitory affect on responses driven by glutamate or synaptic excitation. These effects are mediated by muscarinic receptors, which are present throughout all cortical layers and are denser than nicotinic receptors (Parkinson et al., 1988). However, cholinergic inputs also seem to be able to facilitate synaptic transmission via a presynaptic action on nicotinic receptors, particularly on thalamic terminals (Prusky et al., 1987). Different types of cortical interneurons have been shown to have relatively fast responses to muscarinic agonists as well as to nicotine (Xiang et al., 1998).

ACh release in the neocortex in rats is highest during waking movement (Day et al., 1991; Kurosawa et al., 1993), and it is considered to be a source for general neocortical arousal. However, a more specific role in modulating cortical activation has been shown in response to particular environmental stimuli, and additionally activation of the basal forebrain cholinergic system has been shown to produce regional enhancement of sensory processing within the cortex (Rasmusson and Dykes, 1988; Webster et al., 1991a; Webster et al., 1991b; Metherate and Ashe, 1993).

The effects of chemical stimulation of the basal forebrain on EEG activity have been studied with the aim of understanding how this area may modulate cortical activation in response to the chemically varied afferent inputs (Cape and Jones, 1998). Noradrenaline and serotonin, which were found to act in opposite ways upon cholinergic and some non-cholinergic cells *in vitro*, were found to produce opposite effects upon the EEG and sleep-wake states (Cape and Jones, 1998). The injection of noradrenaline, which excites cholinergic neurons, produced an increase in cortical gamma activity and a decrease in delta activity, associated with a behaviorally awake state. In contrast, the injection of serotonin, which inhibits cholinergic neurons, produced a decrease in gamma activity and the occurrence of delta activity, associated with either sleep or quiet waking during the period in which the rats were normally asleep. In other experiments, the injection of the glutamate agonists AMPA or NMDA evoked cortical activation indicated by high frequency gamma activity and behavioral waking during the period in which the rat is normally asleep (Cape and Jones, 2000). Finally, the effects of the injection of the neuropeptide neurotensin, whose receptors are selectively associated with the cholinergic neurons and which induces depolarization

and bursting in them, were also examined. This peptide induced an increase in both theta and gamma activity, and produced an increase in PS as well as waking (Cape et al., 2000). These results indicate that modulation of neurons in the basal forebrain by natural neurotransmitters is associated with selective changes in cortical activity and sleep-wake states.

Another focus of research on the basal forebrain has been on its potential role in learning and cognitive processes. The initial realization of this possible function came from experimental lesions of the MS that eliminated hippocampal theta activity and disrupted memory of spatial information (Winson, 1978). It had been previously realized that rhythmically discharging cells of the MS and DBB fire synchronously with the theta rhythm and are responsible for its generation in the hippocampus (Green and Arduini, 1954; Petsche et al., 1962; Gray, 1971; Andersen et al., 1979; Mitchell et al., 1982; Borst et al., 1987). Lesion of the MS cholinergic cells using the IgG-saporin lesions targeted to the p75 nerve growth factor receptor have revealed that although its amplitude is dramatically decreased, theta continues to occur and is presumably generated by extant GABAergic neurons (Lee et al., 1994). Acetylcholine is thus important in mediating the rhythmic theta activity that is present during waking and REM sleep, when it is maximally released (Jasper and Tessier, 1971), but seemingly works in parallel with other transmitters released from other basal forebrain neurons.

Studies furthered the hypothesis that ACh may mediate learning and memory through theta activity. It was originally found that theta frequency stimulation was optimal for long-term potentiation in the hippocampus (Larson et al., 1986; Greenstein et al., 1988). Subsequently, it was found that ACh application induces rhythmic theta

activity in the hippocampus and during this oscillatory period, synapses are also potentiated by stimulation that would otherwise be ineffective (Huerta and Lisman, 1993). Many cortical areas exhibit theta activity; one prominent area is the entorhinal cortex, which also receives a dense projection from medial septum as well as the more lateral and caudal basal forebrain nuclei (Saper, 1984). The entorhinal cortex receives cortical information from widespread cortical regions and projects it to the hippocampus via the perforant path, then in turn receives output from the hippocampus and projects it back upon the neocortex (Van Hoesen and Pandya, 1975a; Van Hoesen et al., 1975; Van Hoesen and Pandya, 1975b; Swanson and Cowan, 1977; Van Hoesen, 1982; Swanson and Kohler, 1986; Kohler, 1988). Rats with lesions of MS showed a loss of AChE staining in the medial entorhinal cortex and showed deficits in learning a radial arm maze (Mitchell et al., 1982). During specific sniffing and exploratory behaviors theta activity is also present through the olfactory cortices, including the olfactory bulb and the piriform cortices (Macrides et al., 1982; Vanderwolf, 1992). Theta rhythms are present in other neocortical limbic areas, such as the retrosplenial cortex where it is dependent upon input from the basal forebrain (Borst et al., 1987; Leung and Borst, 1987). Thus, the basal forebrain may have the potential to drive the activity of these regions in a parallel manner to which the MS drives theta activity in the hippocampal formation, and similarly may be equally important for promoting plastic changes in cortical networks. Indeed research has shown neocortical plasticity in response to cholinergic agonists. In the somatosensory cortex, ACh administration paired with sensory stimulation was found to enhance the response to successive stimulation (Metherate et al., 1987, 1988). Stimulation of the basal forebrain paired

with auditory stimuli resulted in a progressive reorganization of the primary auditory cortex, which was blocked with cholinergic antagonists (Kilgard and Merzenich, 1998). These studies have linked the role of ACh, and basal forebrain induced theta activity with synaptic plasticity.

Cholinergic neurons of the basal forebrain have gained some notoriety as their degeneration has been noted to be one of the key early neuropathological symptoms of Alzheimer's disease (for review see (Davies and Moloney, 1976; Terry and Davies, 1980)). The effects of basal forebrain excitotoxic lesions in animals have been well documented, and indicate that this area is necessary for normal acquisition and retention of newly learned responses (Dunnett et al., 1991). The cholinergic basal forebrain neurons had long been thought to be critically implicated in global aspects of cognitive function (Dunnett et al., 1991; Wenk, 1997). However, lesions that nonselectively destroy neuronal populations of the basal forebrain using ibotenic acid have been shown to be more effective in impairing learning than lesions that selectively destroy the cholinergic neurons using IgG-saporin (Dunnett et al., 1991). These selective lesions, which produce long-lasting selective depletions in cholinergic markers throughout the forebrain (Book et al., 1992; Berger-Sweeney et al., 1994; Heckers et al., 1994; Torres et al., 1994; Wenk et al., 1994; Baxter et al., 1995), produce disruptions of specific forms of attentional processing, but not learning and memory (McGaughy et al., 1996; Stoehr et al., 1997; Turchi et al., 1997; Waite et al., 1999). The role of noncholinergic basal forebrain neurons in cognitive function remains largely unexplored, and the presence of parallel projections of GABAergic and other noncholinergic nonGABAergic neurons may aid in explaining the lack of global cognitive impairments

following selective lesions of basal forebrain cholinergic neurons. Thus, basal forebrain mediation of cognitive function may be attributable to the entire basal forebrain cell population.

#### **1.4 Considerations and Objectives**

Ample evidence of the basal forebrain's functional importance in both sleep-wake regulation and cognitive function exists, however the mechanisms by which these processes are influenced remain obscure.

Unit recording studies done in freely moving animals have only been able to hint at which cell types display which types of firing profiles. While recordings done *in vitro* have identified neurons as to their cholinergic or non-cholinergic nature, the particular role of cholinergic and non-cholinergic neurons in relation to cortical activity remains undefined. At the current level of technology, it remains difficult to positively identify neurons recorded *in vivo* as to their neurotransmitter and projections. One powerful technique for doing just this is an extracellular labeling method developed by Pinault (Pinault, 1996), called the 'juxtacellular' method according to the proximity of the electrode to the neuron. This method using dye-filled micropipettes allows the labeling and subsequent immunohistochemical identification of recorded neurons, requires a very stable recording set-up, and is thus most easily applied in anesthetized rats. Urethane anesthesia, which does not greatly affect glutamatergic nor GABAergic receptors like other anesthetics (Moroni et al., 1981; Maggi and Meli, 1986; Goodchild, 1993; Pocock and Richards, 1993), is associated with EEG activity that appears very similar to SWS in the undisturbed animal and to cortical activation in the stimulated

animal (Maggi and Meli, 1986). So, juxtacellular recording and labeling can be used in animals under urethane anesthesia to study the activity of specific neurons under conditions of both cortical slow wave activity and cortical activation. This procedure could thus be very powerful in identifying the discharge properties of neurochemically distinct groups of neurons during different levels of cortical activity. The major objective of this thesis will be to use this technique to identify the discharge properties of identified cholinergic, GABAergic and non-cholinergic/nonGABAergic basal forebrain neurons in association with different patterns of EEG activity. Such information is essential for gaining a richer understanding of the mechanisms by which the basal forebrain region mediates behavioral states.

The chemoneuroanatomical understanding of this region is also incomplete, as the neurotransmitter content of a component of the basolocortical projection has yet to be positively identified. This subset of cortically projecting neurons, which has been suggested to be glutamatergic, is surely of functional relevance for cortical activity. Thus, another major objective of this thesis will be to examine this possibility utilizing retrograde transport of tracers from the cortex coupled with immunohistochemical staining for the PAG enzyme, in an effort to identify cortically projecting glutamatergic neurons. This chemoneuroanatomical information together with the physiological will provide a clearer picture of the basal forebrain's role in the modulation of cortical activity and its importance in sleep-wake states.

## 1.5 References

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## **Chapter Two**

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### **2. Discharge Properties of Juxtacellularly Labeled and Immunohistochemically Identified Cholinergic Basal Forebrain Neurons Recorded in Association with the Electroencephalogram in Anesthetized Rats<sup>1</sup>**

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<sup>1</sup> Reprinted from The Journal of Neuroscience, 2000, Manns ID, Alonso A, Jones BE: " Discharge profiles of juxtacellularly labeled and immunohistochemically identified cholinergic basal forebrain neurons recorded in association with the electroencephalogram in anesthetized rats ", 20, 1505-1518, Copyright (2000), with permission from the Society for Neuroscience.

## 2.1 Preface

As reviewed in the Introduction, multiple lines of evidence have indicated that the cholinergic neurons of the basal forebrain are important for cortical activation. However, no studies have examined the activity of identified cholinergic neurons in relation to cortical activity. Cholinergic neurons were identified *in vitro* (Khateb et al., 1992; Alonso et al., 1996), however only the biophysical characteristics of the cells could be elucidated by these studies. Other studies done *in vivo* in both freely moving sleep-waking animals and anesthetized animals have generally been extracellular recording studies in which the recorded neurons could not be subsequently identified as to their chemical identity, that is, whether they were cholinergic or noncholinergic. A few studies had endeavored to label the cells they had recorded, but were unsuccessful in identifying cholinergic neurons (Semba et al., 1987; Paolini and McKenzie, 1997; Pang et al., 1998). As such, it had been difficult to know what the precise physiological discharge of these cells would be *in vivo* and thus the precise function they may have in modulating cortical activity. The first study was conducted to thus determine the discharge properties of identified cholinergic neurons in relation to cortical activity.

## **2.2 Abstract**

Multiple lines of evidence indicate that cholinergic basal forebrain neurons play an important role in the regulation of cortical activity and state. However, the discharge properties of cholinergic cells in relation to the electroencephalogram (EEG) are not yet known. In the present study, cells were recorded in the basal forebrain in association with cortical EEG activity in urethane-anesthetized rats and their discharge examined during EEG irregular slow activity and during stimulation-induced cortical activation, characterized by rhythmic slow (theta) and high frequency (gamma) activities. Recorded cells were labeled with Neurobiotin (Nb) using the juxtacellular technique and identified as cholinergic by immunohistochemical staining for choline-acetyltransferase (ChAT). Nb-positive/ChAT-positive neurons were distinctive and significantly different from Nb-positive/ChAT-negative neurons, which were heterogeneous in their discharge properties. All Nb<sup>+</sup>/ChAT<sup>+</sup> cells increased their discharge rate with stimulation, and most shifted from an irregular tonic discharge during EEG slow irregular activity to a rhythmic burst discharge during rhythmic slow activity. The stimulation-induced rhythmic discharge was cross-correlated with the EEG rhythmic slow activity. In some units, the rhythmic discharge matched the rhythmic slow activity of the retrosplenial cortex; in others, it matched that of the prefrontal cortex, which occurred at a slower frequency, suggesting that subsets of cholinergic neurons may rhythmically influence their cortical target areas at particular frequencies. Cholinergic basal forebrain neurons may thus evoke and enhance cortical activation through both an increase in rate and a change in pattern to rhythmic bursting that would stimulate rhythmic slow (theta-like) activity in cortical fields during active waking and paradoxical sleep states.

## **2.3 Introduction**

The basal forebrain has been shown to be critically involved in the regulation of cortical activity and behavioral state (Jones, 1993). As known since early physiological studies, it serves as the extra-thalamic relay from the reticular formation to the cerebral cortex (Dempsey et al., 1941; Moruzzi and Magoun, 1949; Starzl et al., 1951), conveying activation evident as fast activity on the EEG. The neurons that form this relay were revealed to be cholinergic (Lewis and Shute, 1967; Kievit and Kuypers, 1975; Rye et al., 1984). Blocking cholinergic receptors resulted in diminished cortical activation (Wikler, 1952; Longo, 1966) and impaired memory function (Peterson, 1977). Lesions of the basal forebrain produced decreased cortical activation in parallel with decreased acetylcholine (ACh) release (LoConte et al., 1982; Stewart et al., 1984). Moreover, maximal ACh release was found to be naturally associated with the cortical activation of active wake and paradoxical sleep states (Celesia and Jasper, 1966; Jasper and Tessier, 1971; Marrosu et al., 1995).

The activating influence of ACh in the cerebral cortex has been known to involve the depolarization and excitation of cortical neurons (Krnjevic and Phillis, 1963; McCormick and Prince, 1986), resulting in a shift of cortical activity from very slow (delta) to fast (beta-gamma) activity (Metherate et al., 1992). ACh and cholinergic neurons have also been implicated in the facilitation of rhythmic slow activity or theta which occurs during active waking and paradoxical sleep (Jouvet et al., 1960; Parmeggiani and Zanocho, 1963; Vanderwolf, 1975) in the hippocampus (Gaztelu and Buno Jr., 1982; Buzsaki et al., 1983; Alonso et al., 1987; Lee et al., 1994; Dringenberg and Vanderwolf, 1997; Brazhnik and Fox, 1999) and in cingulate, retrosplenial and

entorhinal cortex (Borst et al., 1987; Dickson and Alonso, 1997). In fact, theta-band (4-9 Hz) activity occurs in parallel with increased high frequency gamma-band (30-60 Hz) activity in the EEG across neo- in addition to allo-cortical regions (Parmeggiani and Zanocco, 1963; Stumpf, 1965; Maloney et al., 1997). Such slow rhythmic modulation could be important for the role in plasticity and memory that has been attributed to the cholinergic input in both allo- and neo-cortex (Landfield et al., 1972; Larson et al., 1986; Greenstein et al., 1988; Metherate et al., 1988; Huerta and Lisman, 1995; Kilgard and Merzenich, 1998).

The precise modulation of cortical activity by cholinergic basal forebrain neurons is not yet known since their *in vivo* discharge properties have not been characterized. *In vivo* recording studies have found many cell types with many different activity profiles in the basal forebrain, leaving uncertain which cell type might be cholinergic (Aston-Jones et al., 1984; Detari et al., 1984; Szymusiak and McGinty, 1986; Detari and Vanderwolf, 1987; Reiner et al., 1987; Buzsaki et al., 1988; Szymusiak and McGinty, 1989; Nunez, 1996). This uncertainty is not surprising since the basal forebrain cell population is made up predominantly of non-cholinergic cells (Zaborszky et al., 1986; Gritti et al., 1993; Gritti et al., 1994; Gritti et al., 1997), which have been shown to be electrophysiologically diverse (Pang et al., 1998).

Recent *in vitro* studies have characterized the electrophysiological properties of immunohistochemically identified cholinergic basal forebrain neurons (Khateb et al., 1992). These cells were found to be distinctive, having calcium conductances which endow them with the capacity to discharge rhythmically in high frequency bursts of spikes, as well as in a slow tonic mode (Khateb et al., 1992). Based upon these *in vitro*

findings, the present study sought to find cholinergic basal forebrain cells by extracellular recording *in vivo* in anesthetized rats and to determine their pattern of discharge in association with cortical activation. Units were recorded and characterized with the EEG, labeled with Neurobiotin (Nb) using the juxtacellular technique (Pinault, 1996; Manns et al., 1998) and subsequently examined by immunohistochemical staining for ChAT, the synthetic enzyme for acetylcholine (Manns et al., 1999).

## **2.4 Methods and Materials**

### **Animals and surgery**

Experiments were performed on adult Long Evans rats (200 - 250 g., Charles River, Canada) anesthetized with urethane (ethyl carbamate; Sigma, St. Louis, Mo; initial dose 1.4 g/kg, i.p.). Adequate anesthesia was confirmed by the lack of withdrawal in response to pinching of the hind limb. Additional doses of anesthetic (0.1 – 0.15 g/kg, i.p.) were given if and when this response appeared. Body temperature was kept at 37°C with a heating pad attached to a thermostatic control instrument (Yellow Springs Instruments, Yellow Springs, OH).

The animals were placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA) and held there for the duration of the experiment. Trephine windows were made in the cranium over the left and right basal forebrain for subsequent descent of microelectrodes. For recording of EEG, stainless steel screws were threaded into the skull to come into contact with the dura over the retrosplenial cortex (anterior-posterior, AP -4 mm, lateral, L  $\pm$  0.5 mm relative to bregma (Paxinos and Watson, 1986)). A reference electrode was placed in the frontal bone and a ground electrode over the right cerebellum. For the purpose of antidromic activation of basal forebrain units, a bipolar stimulating electrode (with a separation of 0.5 mm between the tips) was placed in the prefrontal cortex on each side (AP +2.0 mm, L  $\pm$  1.0 mm, and V -2.0 mm). This electrode was also used to record the field potential in the prefrontal cortex in several experiments.

## **Unit recording and labeling**

Unit recordings were performed with glass microelectrodes, which were pulled to a fine tip, broken to an external diameter of  $\sim 0.5 - 1.5 \mu\text{m}$ , and filled with 0.5 M sodium acetate and 2.5 - 5.0% Neurobiotin (Nb, Vector Laboratories, Burlingame, CA). Using a hydrostatic micromanipulator (MX510, Newport Corp., Irvine, CA), the electrode was moved into the region of the basal forebrain to a position just below the anterior commissure. At this juncture, single units were isolated as the electrode was descended through the basal forebrain. Upon isolation, the unit was characterized in association with spontaneous irregular slow wave activity and stimulation-induced rhythmic slow activity on the EEG. The stimulation consisted of a continuous pinch of the tail. Neurons were also tested for their response to antidromic stimulation from the prefrontal cortex. Antidromic criteria included the ability to follow single pulses (0.3 ms, 100 - 600  $\mu\text{A}$ ) with a constant latency and to follow high frequency stimulus trains of two or three pulses at 100 - 200 Hz. The extracellular recording of units was done using an intracellular amplifier (IR-283; Neuro Data Instruments, New York, NY). Extracellular voltage signals were amplified and bandpass filtered between 0.3 and 3 kHz. The EEG signal was filtered between 0.5 and 125 Hz. Both signals were then digitized with a sampling rate of 6.6 kHz. Spike widths were measured from positive inflection to first zero-crossing using greater than 200 averaged spikes. Antidromic latencies were measured from time of stimulation artifact.

After the recording and characterization of isolated neurons, they were labeled using the 'juxtacellular' method as developed and described by Pinault (Pinault, 1996). The labeling procedure involved moving the microelectrode as close as possible to the

cell's membrane, thus maximizing the action potential amplitude. Current was then applied, and Nb iontophoresed with the bridge circuitry of the amplifier. Currents consisted of a 50% duty cycle (200ms pulses) and initially involved high intensities of a  $\sim +10$  nA delivered from a DC current of  $\sim -5$  nA. Once it became apparent that the current pulses resulted in a robust modulation of the neuron's firing, the current intensities were lowered (usually to  $\sim +2$  nA from a DC current of  $\sim -1$  nA). Throughout the labeling procedure, it was important to monitor the neuron's response and adjust the stimulation parameters and distance of the electrode from the membrane, so as to maintain robust modulation yet, avoid damage to the cell. In preliminary studies, it was found that weak modulation of the cell resulted in no neuronal labeling, whereas, overly strong modulation of the cell could result in cell death, usually heralded by widening of the action potential and paroxysmal discharge. The labeling procedure was applied for periods of 3 to 20 minutes. Short labeling protocols or those with poor modulation tended to result in weakly labeled neurons, while longer protocols or those with robust modulation tended to result in very strong neuronal labeling. Post-labeling survival periods ranged from a few minutes to several hours and were found adequate when short, given that the duration and modulation during the labeling protocol were adequate. The animals received an overdose of urethane and were then transcardially perfused with physiological saline (0.9% NaCl) followed by 500 mL of a fixative containing 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). The brains were removed and immersed overnight in a 30% sucrose/phosphate buffered solution for cryoprotection, and then frozen at  $-50^{\circ}$  C and stored at  $-80^{\circ}$  C.

## **Histochemistry**

In preliminary experiments, brains were prepared for simple revelation of Nb. For this purpose, frontal sections were cut at 50  $\mu\text{m}$  thickness on a freezing microtome. They were then thoroughly washed in phosphate buffer before being incubated with avidin-biotin peroxidase complex (ABC; Vector Laboratories, Burlingame, CA) for at least 4 hours. The Nb was then revealed with  $\text{H}_2\text{O}_2$  and the chromogen 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Horikawa and Armstrong, 1988) using nickel intensification.

In the main study, brains were prepared for dual staining of Nb and choline acetyltransferase (ChAT) to determine if the labeled neurons were cholinergic. Coronal frozen sections were cut at 30  $\mu\text{m}$ , washed thoroughly in phosphate buffer and incubated overnight in a primary antibody for ChAT (rabbit anti-ChAT antiserum, 1:3500, Chemicon, Temecula, CA). The following day, the sections were washed and incubated with secondary antibodies for 2.5 hours. A Cy2-conjugated streptavidin (1:800, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), was used to reveal Nb. A Cy3-conjugated donkey anti-rabbit antiserum (1:1000, Jackson ImmunoResearch Laboratories), was used to reveal ChAT-immunostaining. Sections were then mounted and viewed by fluorescent microscopy using a Leitz Dialux microscope equipped with a Ploemopak 2 reflected light fluorescence illuminator with Leica filter cubes for fluorescein (I3) and rhodamine (N2.1). Cell size was measured from film transparencies, and cells classified as small ( $\leq 15 \mu\text{m}$ ) or medium-to-large (16 to 35  $\mu\text{m}$ ) according to their large diameter.

## **Data Analysis**

Analysis of physiological data was performed on stationary periods of recording from pre-stimulated and stimulated conditions. For the EEG, spectral analysis was performed to determine the dominant peak frequency. Four contiguous EEG segments (4 seconds each) were tapered through a Hanning window and converted by fast Fourier transform. Power spectra were averaged and plotted ( $\text{mV}^2/\text{Hz}$ ) for presentation. The oscillatory nature of the same EEG segments were assessed by an auto-correlation function (ACF). To assess The amplitude of the gamma frequency activity in the EEG during pre-stimulation and stimulation conditions, the the area of the amplitude spectra was measured between 30 - 58 Hz.

For all unit and unit-to-EEG analyses, calculations were done on at least 60 seconds of artifact-free data. For unit discharge, the average discharge rate was calculated as average spikes per second from the peristimulus histogram (PSH) of the pre-stimulation and stimulation periods. Using this data, units were categorized as increasing ('on') or decreasing ('off') their discharge in response to the stimulation. The calculation of the predominant instantaneous firing frequency was determined from the first-order interspike interval histogram (ISIH), using the same segments as for the PSH. Assessment of rhythmic and higher-order interspike interval tendencies was performed using an auto-correlation histogram (ACH) on the same data segments as for the other unit calculations. Determination of the dominant frequency of rhythmic ACHs was done using a fast Fourier transform to convert the ACH data to the frequency domain. Unit discharge was considered to be 'rhythmic', if the spectrum of the ACH had a peak that was at least 3 times the amplitude of the average power. Classification of units according

to their predominant pattern of discharge was effected by consulting the raw records together with the PSH and ISIH, such as to initially characterize the predominant firing pattern as tonic and/or phasic. Whether the phasic activity was comprised of burst or cluster-like discharge was assessed by visually examining the records and also by determining the percentage of high frequency interspike interval incidents (according to which the discharge was considered burst-like with >80 Hz activity representing >5% of the ISIH distribution). The spike-triggered average (STA) was used to estimate the extent of cross-correlation between spike-trains and EEG activity. The time of each individual spike in a spike-train was used as a reference to gather and average concomitant windows of EEG data (usually  $\pm 2.5$  seconds before and after the spike), thus allowing estimation of the EEG pattern, which is preferentially associated with any given spike discharge. To determine whether the actual unit-EEG STA was significantly different from random unit-EEG patterns, it was compared with an STA computed using a spike-train generated from randomly shuffled interspike intervals of the original spike train. The actual unit-EEG STA was considered significantly different from the random unit-EEG STA with a probability of  $\leq 0.05$  using the Wilcoxon test. In such cases, the unit discharge was considered to be significantly cross-correlated with the EEG activity. All analysis of raw data was done with Matlab (5, The MathWorks Inc., Natick, MA).

For statistical comparison of the properties of cholinergic and non-cholinergic cells, both non-parametric and parametric tests were used. Chi square analyses were employed to determine if the groups differed according to the classification of units on the multiple criteria detailed above. Analysis of variance (ANOVA) was employed to examine differences in unit properties between the cholinergic and non-cholinergic cell

groups. Paired and non-paired Student's t tests were employed for both *post-hoc* comparisons and also simple tests involving only two conditions or groups. All statistics were performed using Systat (7.0, SPSS Inc., Chicago, IL).

Figures were compiled using Adobe Photoshop (4.0 Adobe Systems, Inc., San Jose, CA) for photomicrographs and Origin (5.0, Microcal Software Inc., Northhampton, MA) for plotting electrophysiological data and analyses.

## **2.5 Results**

### **Labeling and characterization of basal forebrain units**

In preliminary studies aimed at establishing the juxtacellular technique and also surveying the population of basal forebrain neurons, single units were recorded in association with EEG activity and labeled with Nb for single staining using peroxidase. Applying the juxtacellular technique in the manner described by Pinault (Pinault, 1996), it was confirmed that following modulation of a recorded unit's discharge by current pulses, the soma and dendrites of a single neuron and only a single neuron were labeled with Neurobiotin (Nb+) (Fig. 1). Nb+ cells that had been electrophysiologically characterized were subsequently selected for the preliminary sample if they were located within the basal forebrain cholinergic cell area (n = 90). These Nb+ cells were distributed through the substantia innominata (SI, n = 32) and magnocellular preoptic nucleus (MCPO) or located in the immediately adjacent lateral preoptic area or olfactory tubercle (and grouped with those in the MCPO, n = 58). The cells were oval to fusiform (bipolar) or polygonal (multipolar), and commonly had long radiating dendrites (Fig. 1). Although some cells (~27%) were small ( $\leq 15 \mu\text{m}$ ), the vast majority was medium-to-large (16 to 35  $\mu\text{m}$  in long diameter). The profiles of unit discharge varied considerably in this population, as did the responses of units to stimulation of the animal and the relationships of unit discharge to EEG activity.

In urethane-anesthetized rats, the EEG recorded from the retrosplenial cortex was characterized by relatively irregular low frequency activity (Fig. 2). Continuous pressure applied to the tail of the animal resulted in a change in EEG activity, even though it did not elicit a motor response. During the stimulation, the EEG was characterized by higher

frequency, rhythmic slow (theta-like) activity and the presence of high frequency fast activity riding on the rhythmic slow activity (Fig. 2C). In the preliminary studies, approximately 45% of the cells demonstrated a only tonic type of discharge in both conditions, whereas the remaining cells also demonstrated a phasic type of discharge in one or both conditions (Manns et al., 1998). Irrespective of discharge profile, the majority of cells (~58%) increased their average discharge rate with stimulation, and a minority (~34%) decreased their average discharge rate, whereas some did not change their rate. Among those cells that increased their discharge rate, a proportion appeared to discharge rhythmically in bursts in association with rhythmic EEG activity that occurred during stimulation (Fig. 2C and D). The frequency of the spike bursts was highly variable, differing between and varying within spike trains of the same cell (Fig. 2E). Bursting cells could often be antidromically activated from the cerebral cortex (Fig. 2F). Such cells displaying a burst and/or a tonic discharge profile were considered as likely candidates for being cholinergic neurons, since high frequency burst (>100 Hz) and slow tonic (<20 Hz) modes of firing were previously described in identified basal forebrain cholinergic neurons *in vitro* (Khateb et al., 1992). A certain number of cells also displayed phasic rhythmic discharge as regular trains of spikes lacking high frequency bursts. Such cells were thought less likely to be cholinergic and to correspond possibly to some non-cholinergic cells identified *in vitro* by rhythmically occurring clusters of spikes (<80 Hz) (Alonso et al., 1996). In the total preliminary sample, cells were classified according to their distinctive pattern of discharge as: 1) cells discharging only in a tonic manner, 45.5%, 2) cells discharging in a burst-like manner (containing high frequency

bursts of >80 Hz), 32.5%, and 3) cells discharging in a cluster pattern (containing no high frequency bursts of >80 Hz), 22%.

### **Identification and comparison of cholinergic and non-cholinergic cells**

In the subsequent main study, cells were characterized and labeled with Nb for dual staining for Nb and ChAT. In this process, neurons were first recorded and, depending upon their discharge profile, subsequently selected for labeling by the juxtacellular procedure. Of the total sample of cells ( $n = 52$ ), many were selected which displayed tonic activity or which displayed a burst-like discharge in addition to tonic activity. A number of more rarely encountered cluster discharge cells were also selected for inclusion in the sample.

Of 52 Nb-labeled cells located in the basal forebrain cholinergic cell area, 12 were established as immuno-positive for ChAT (Nb+/ChAT+), and 40 were established as immuno-negative for ChAT (Nb+/ChAT-, Fig. 3, Table 1). The Nb+/ChAT+ and Nb+/ChAT- cells were located within the SI or MCPO (including a few cells in the adjacent lateral preoptic area and olfactory tubercle), the greater proportion being in the MCPO (Fig. 4, Table 1). The Nb+/ChAT+ cells were not morphologically distinct from the Nb+/ChAT- cells, since both groups included oval to fusiform (bipolar) and polygonal (multipolar) neurons (Table 1). Many cells of each type appeared to have long radiating dendrites. The total sample of cells ranged in size from small to medium or large. The Nb+/ChAT+ cells were comprised entirely of medium-to-large cells (range: 17.3 to 24.8  $\mu\text{m}$ ), whereas the Nb+/ChAT- cells were comprised by small, as well as

medium-to-large cells (range: 12.0 to 29.1  $\mu\text{m}$ ) and accordingly differed significantly from the Nb+/ChAT+ cells in this regard (Table 1).

As in the preliminary study, the retrosplenial EEG during the pre-stimulation recording was characterized by irregular low frequency activity that shifted to relatively faster rhythmic slow (theta-like) activity during stimulation, as evident in the EEG record and in the spectral analysis and ACF of those records. Across experiments, the average dominant peak frequency of the retrosplenial EEG activity increased from  $1.03 \pm 0.06$  to  $3.02 \pm 0.11$  Hz in the samples for all units ( $t = 19.03$ ,  $df = 51$ ,  $p < 0.001$ ). Across experiments, stimulation was also marked by a significant increase in average amplitude of EEG activity in the gamma frequency band (30 - 58 Hz;  $t = 3.13$ ,  $df = 25$ ,  $p < 0.01$ ). The stimulation thus evoked a degree of cortical activation evidenced by a parallel increase in the dominant low peak frequency and high frequency gamma band amplitude in the EEG of the anesthetized animals.

Of the total sample of recorded and labeled neurons, ~44% discharged only in a tonic manner, ~38.5% with high frequency bursts (>80 Hz) and ~17.5% in a cluster type of discharge (lacking high frequency activity of >80 Hz) in the pre-stimulation and/or stimulation conditions (Table 1). These proportions of cells with their different discharge patterns were similar to those obtained in the preliminary study (above). In the experimental sample, the majority of cells also increased their average rate of discharge (measured by PSH) with stimulation (83%, Table 1). Within this sample, the Nb+/ChAT+ cells differed significantly from the Nb+/ChAT- cells both in their predominant discharge pattern and response to stimulation. The majority of the Nb+/ChAT+ cells displayed a burst discharge pattern in addition to a tonic discharge

pattern (75%), with the remaining displaying a tonic type of discharge; they all increased their average rate of discharge with stimulation (Table 1). Of the Nb+/ChAT- cells, the largest proportion displayed a predominantly tonic discharge pattern (50%), a smaller proportion a bursting discharge pattern (27%), and the remaining a cluster discharge pattern (22.5%). The majority of the Nb+/ChAT- cells increased their average discharge rate with stimulation ('on', 78%), however a minority decreased their rate ('off', 22%, Table 1).

It was evident in the total sample that many phasically firing cells displayed rhythmicity in their discharge during stimulation, as assessed from the recordings, ACHs and corresponding spectra (Table 1). The activity of a substantial number of cells was also significantly cross-correlated with the EEG rhythmic slow activity during stimulation as evident from the recordings and STAs (Table 1). In this sample, both Nb+/ChAT+ and Nb+/ChAT- cells showed rhythmic activity and significant cross-correlations with the EEG during stimulation-induced rhythmic slow activity, although the proportion of Nb+/ChAT+ cells doing so was significantly larger than that of the Nb+/ChAT- cells (Table 1).

### **Distinctive properties of cholinergic cells**

Nb+/ChAT+ cells were located in the MCPO or SI (Figures 3 and 4, Table 1) and were on average medium in size. In several cases, they could be identified as cortically projecting by antidromic activation from a stimulating electrode in the prefrontal cortex (Table 2).

As shown for the Nb+/ChAT+ cell in Fig. 3A, which was located in the MCPO and antidromically activated from the prefrontal cortex, cholinergic cells always increased their rate of discharge and most commonly also changed their mode of discharge from tonic to burst discharge with stimulation of the animal (Fig. 5I.). This response to stimulation is reflected in the significant increases in the average discharge rate (from the PSH) and the mean instantaneous firing frequency (from the ISIH) for the Nb+/ChAT+ cells (Fig. 5I and II, Table 3). In most cases, the burst discharge was rhythmic (as evident in the ACH). In some cases, the frequency of the rhythmic discharge matched that of the dominant frequency of the rhythmic EEG activity and spectral peak from the retrosplenial cortex (Fig. 5IIA and B). In such cases, there was a significant cross-correlation of the unit discharge with the EEG activity (evident in the STA) at the same frequency (Fig. 5IIC).

As shown for the Nb+/ChAT+ cell in Fig. 3B, which was located in the SI, other cholinergic cells also increased their rate of discharge and discharged rhythmically in bursts during stimulation-evoked EEG rhythmic slow activity, but their discharge did not appear to be at the same frequency as the dominant EEG frequency of the retrosplenial cortex (Fig. 6I). From the ACH for the unit and the cross-correlation (STA) with the EEG, it became apparent that the unit was discharging rhythmically at a slower frequency and cross-correlated with the EEG at this slower frequency (Fig. 6II). This difference was reflected in the mean frequencies of the unit rhythmic discharge (from the ACH) and cross-correlated EEG rhythmic activity (from the STA), which were slower than the average EEG peak frequency from the retrosplenial cortex (Table 3). This slower frequency corresponded to a secondary peak in the retrosplenial EEG power spectrum

(Fig. 6II). This observation suggested that the slower unit rhythmic discharge may be correlated with the dominant EEG frequency of another cortical region.

To examine the possibility that the unit rhythmic discharge might be more closely correlated with the dominant EEG signal of another cortical area, a field potential from the prefrontal cortex stimulating electrode was simultaneously acquired with the retrosplenial EEG signal. As in recordings from the retrosplenial EEG, the prefrontal field potential was characterized during the pre-stimulation condition by irregular low frequency activity, which increased in frequency and regularity during stimulation. Across experiments, stimulation caused a significant increase in the dominant peak frequency recorded in the prefrontal field activity from  $0.83 \pm 0.04$  to  $1.63 \pm 0.10$  Hz ( $t = 7.53$ ,  $df = 26$ ,  $p < 0.001$ ), although this dominant frequency was significantly slower during stimulation in the prefrontal field recording compared to the retrosplenial EEG ( $t = -6.40$ ,  $df = 26$ ,  $p < 0.001$ ). In addition, parallel to the effect in the retrosplenial EEG signal, stimulation elicited a significant increase in average amplitude in the gamma frequency band in the prefrontal field activity (30 - 58 Hz;  $t = 4.17$ ,  $df = 25$ ,  $p < 0.001$ ).

The rhythmic discharge of several Nb+/ChAT+ neurons was found to be cross-correlated with the dominant rhythmic activity on the prefrontal cortex during stimulation-evoked cortical activation. In one such unit, which could also be antidromically activated from the prefrontal cortex, the rhythmic discharge (evident in the ACH) appeared to match the prominent rhythmic slow activity of the prefrontal cortex (evident in the ACF, Fig. 7A and B) and corresponded in frequency to the dominant spectral peak of the prefrontal cortex (evident in the power spectra, Fig. 7C). This frequency also corresponded to a secondary peak in the retrosplenial cortex (Fig. 7C),

which was often present (Fig. 5II and 6II). Following this observation, the Nb+/ChAT+ neurons were subdivided according to whether their rhythmic discharge (from the spectrum of the ACH) most closely matched the dominant spectral peak of the retrosplenial or the prefrontal cortex, in those cases in which the EEG was simultaneously recorded from both areas (n = 7, Table 4). The units' rhythmic discharge frequencies (from the ACH) matching to the retrosplenial dominant peak were of a significantly higher frequency than those matching to the prefrontal dominant peak (Table 4), indicating that subgroups of cholinergic cells may discharge rhythmically at different frequencies depending upon the cortical region to which they project.

#### **Different properties of non-cholinergic cell groups**

Nb+/ChAT- cells were differentiated according to their response to stimulation as 'on' or 'off' cells (Table 1). The 'on' and 'off' cells were both located in the MCPO and SI and in similar proportions in those nuclei as those for the total Nb+/ChAT- cell group (Table 1). The Nb+/ChAT- 'on' cells were on average significantly smaller than the Nb+/ChAT+ neurons (Table 2) and were comprised by a substantial number of small (10) in addition to medium-to-large cells (21). The Nb+/ChAT- 'off' cells did not differ significantly in size from the cholinergic cells (Table 2) and were comprised predominantly of medium-to-large cells (8/9). Cells from both groups were identified as cortically projecting by antidromic activation, and neither their average latency of activation nor their average spike width were significantly different from those of the Nb+/ChAT+ cells (Table 2).

As illustrated for the Nb+/ChAT- cell in Fig. 3C, which was located in the MCPO, most non-cholinergic cells increased their average rate of discharge with stimulation, thus being classified as 'on' cells, and most often discharged in a tonic manner (Fig. 8I, Table 1). They tended to fire sporadically during pre-stimulation and more rapidly in a repetitive tonic manner during stimulation (Fig. 8). These predominant characteristics were reflected in the increases in average discharge rate (from PSH) and predominant instantaneous firing frequency (from the ISIH, Fig. 8II, Table 3). The non-cholinergic 'on' cells differed from the cholinergic (all 'on') cells by significantly lower frequencies of firing (from both PSH and ISIH) during stimulation, reflecting the differing predominantly tonic vs. phasic bursting discharge patterns of these cell types (Table 3). Whereas the predominantly, tonically discharging non-cholinergic 'on' cells (n = 19) tended not to show low frequency rhythmicity in their discharge (Fig. 8IIB), some Nb+/ChAT- 'on' cells did discharge phasically and did display low frequency rhythmicity in their discharge during stimulation (not shown). These included Nb+/ChAT- 'on' cells that discharged in clusters (n = 7) and others which displayed a burst-like discharge (with high frequency components of >80 Hz, n = 5). Several of these Nb+/ChAT- 'on' cells showed a significant cross-correlation with the EEG during stimulation (5 cluster, 3 burst and 2 tonic). As was the case for the cholinergic cells, the mean frequency of the unit rhythmic discharge (from the ACH) and the unit-to-EEG cross-correlation (from the STA) tended to be slower than that of the mean dominant EEG spectral peak from the retrosplenial cortex for the non-cholinergic 'on' cells, and the rhythmically discharging non-cholinergic 'on' cells did not differ from the cholinergic cells in this regard (Table 3). Similarly, the rhythmic activity of some non-

cholinergic units, like those of some cholinergic units, more closely matched the dominant spectral peak from the prefrontal cortical field potential ( $n = 5$ ) than from the retrosplenial EEG ( $n = 2$ ).

Nb+/ChAT- 'off' neurons were heterogeneous in their firing patterns (not shown). However as reflected in the mean predominant instantaneous firing frequency (from the ISIH), many discharged phasically in high frequency bursts during pre-stimulation and virtually ceased firing during stimulation (Table 3). They accordingly differed from the cholinergic cells in their mean instantaneous firing frequencies (from the ISIH) in both the pre-stimulation and stimulation conditions, reflecting an almost mirror image to that of the cholinergic cells in the changes of firing frequencies and patterns with stimulation. Some Nb+/ChAT- 'off' cells also showed rhythmic discharge and significant cross-correlation with the EEG during stimulation at frequencies that did not differ significantly from those of the cholinergic cells (Table 3).

## 2.6 Discussion

To our knowledge, the present results document for the first time the discharge properties of immunohistochemically identified basal forebrain cholinergic neurons *in vivo* and in relationship to EEG activity. All Nb+/ChAT+ neurons increased their discharge rate, and the majority shifted from a tonic or irregular discharge to a robust rhythmic bursting discharge pattern in association with cortical activation. Moreover, in the majority of Nb+/ChAT+ neurons, their discharge was temporally cross-correlated with the stimulation-induced rhythmic slow activity in the EEG at frequencies which matched the prominent activity of the retrosplenial or the prefrontal cortex. These data indicate that cholinergic basal forebrain neurons have the capacity to modulate rhythmically their cortical target areas at particular frequencies during cortical activation.

Previous *in vitro* studies established that identified cholinergic neurons discharged in two intrinsic modes, a tonic mode and a rhythmic bursting mode, which is subtended by calcium conductances (Khateb et al., 1992; Alonso et al., 1996). From the *in vitro* data, it could not be ascertained whether the bursting activity would be associated with EEG activity of slow wave sleep, as is the case for the thalamocortical neurons (reviewed by (Steriade and Llinas, 1988; Steriade et al., 1994)), or that of cortical activation normally occurring during waking and paradoxical sleep (Khateb et al., 1992). Indeed, previous *in vivo* studies in anesthetized animals had reported phasic discharge in chemically unidentified basal forebrain neurons in association with irregular slow activity (Nunez, 1996; Detari et al., 1997). Here, we found in the urethane-anesthetized rat that identified cholinergic cortically projecting neurons discharged in rhythmic bursts in association with stimulation-induced cortical activation, which was evidenced by an

increase in high frequency gamma activity and the appearance of rhythmic slow (theta-like) activity on the EEG. The rhythmic discharge was cross-correlated with the rhythmic slow activity suggesting that it may be induced by the cholinergic neurons. The rhythmic slow activity on the retrosplenial cortex occurs at the same frequency as that of hippocampal theta activity in urethane-anesthetized rats; it is generated locally in the retrosplenial cortex but is dependent upon input from basal forebrain cholinergic neurons (Holsheimer, 1982; Borst et al., 1987; Leung and Borst, 1987). Here in many units, the rhythmic discharge did not correspond to the dominant spectral peak of the retrosplenial cortex, but instead to a lower frequency secondary peak of the retrosplenial cortex and prominent spectral peak of the prefrontal cortex. Although it is not possible to say what the frequency of this rhythmic slow activity in the prefrontal cortex would be in the unanesthetized animal, it could correspond to activity at the lower end of the theta-band, given the relatively slow frequency of the theta activity recorded over the retrosplenial cortex in the anesthetized animal. In any event, the current results suggest that during activation, different subsets of basal forebrain cholinergic cells with different cortical target areas may discharge rhythmically at different frequencies and modulate their cortical target areas at those particular frequencies.

Similar to the significant increase in the prominent EEG peak frequency found here for both the retrosplenial and prefrontal cortex with stimulation-induced cortical activation, an increase in peak frequency in the EEG has been documented for all cortical leads with cortical activation in freely moving rats (Maloney et al., 1997). The increased low peak frequency parallels increased gamma activity across cortical regions in association with active waking behaviors and paradoxical sleep. During active waking,

the peak frequency is significantly slower over the anterior medial frontal cortex (and parietal cortex) with a mean frequency of ~4 or 5 Hz (low theta-band activity), as compared to the retrosplenial (or posterior) cortex with a mean frequency of ~7 Hz (high theta-band activity) (Bringmann, 1995) ((Maloney et al., 1997) and unpublished results). These results indicate that during active waking, theta-band activity occurs across all cortical regions though on average at differing frequencies across those regions. However, during coordinated olfacto-motor behaviors (involving investigative sniffing), rhythmic slow activity in the olfactory bulbs can become loosely coupled to theta in the hippocampus at the same frequency (Macrides et al., 1982; Vanderwolf, 1992). During paradoxical sleep, theta activity is also evident at similarly high frequencies from all cortical leads (Parmeggiani and Zanooco, 1963; Maloney et al., 1997). Viewed together with the results of the present study, it would appear that different subsets of cholinergic basal forebrain neurons with different primary cortical projections may discharge rhythmically at particular theta-band frequencies in association with some behaviors, but also have the possibility of discharging at similar theta-band frequencies during certain behaviors or states to permit coherent phasic modulation across allo- and neo-cortical areas for coordination of sensory, motor and higher-order processes.

Although identification of units recorded *in vivo* in the medial septal-diagonal band (MS-DB) complex as cholinergic has not yet been achieved, most units therein, including possibly cholinergic units, discharge rhythmically in relation to hippocampal theta in the urethane-anesthetized rat (Brazhnik and Fox, 1999). In the present study, a minority of the cholinergic cells discharged in relation to the retrosplenial, equivalent to hippocampal, rhythmic slow activity; the majority discharged in relation to the slower,

prefrontal rhythmic slow activity. Cells in both groups were located in the magnocellular preoptic nucleus (MCPO) and substantia innominata (SI) and could be antidromically activated from prefrontal cortex, thus not being differentiated by their location or course of cortically projecting fibers. Yet, their different frequencies of rhythmic discharge suggest different subsets of cells distributed along the continuum of cholinergic MS-DB:MCPO-SI neurons, which are known to be topographically organized according to their primary cortical projections (Bigl et al., 1982; Rye et al., 1984; Saper, 1984). However, the cortically projecting cell groups are also overlapping in the basal forebrain and their axonal terminal fields overlapping in the cerebral cortex by fine collaterals extending beyond their primary cortical projection areas (Boylan et al., 1986; Luiten et al., 1987; Okoyama et al., 1987; Gritti et al., 1997), thus being disposed in a manner to provide an integrated, in addition to a particularized, influence on cortical targets.

*In vivo* experiments in freely-moving, naturally sleeping-waking rats employing microinjections of neurotransmitters or their agonists into the basal forebrain cholinergic cell area have also indicated that the bursting discharge of the cholinergic cells would be associated with theta-band in parallel with increased gamma-band EEG activity (Cape and Jones, 1998; Jones and Muhlethaler, 1999). NMDA, which *in vitro* induces rhythmic bursting by the cholinergic cells (Khateb et al., 1995; Khateb et al., 1997), produced an increase in theta- and gamma-band EEG activity across cortical regions (Cape and Jones, 1994; Jones and Muhlethaler, 1999; Cape and Jones, Submitted). Similarly, neurotensin, which generates intense and extended rhythmic bursting discharge *in vitro* by the cholinergic cells (Alonso et al., 1994), evoked cortical activation with increased theta-

band EEG activity across cortical regions and in association with increases in wake and paradoxical sleep states ((Cape et al., 1996; Cape et al., 1999) and in preparation).

Burst discharge by the cholinergic neurons likely acts to increase the probability of neurotransmitter release, particularly along collateralized axons (Hessler et al., 1993; Lisman, 1997). The burst discharge in addition to increased rate of discharge by the cholinergic neurons could underlie the documented increased ACh release during active waking and paradoxical sleep, as compared to slow wave sleep (Celesia and Jasper, 1966; Jasper and Tessier, 1971; Marrosu et al., 1995). Whether the phasic release of ACh in the cortex or hippocampus would in turn directly drive cortical activity in a phasic manner has not yet been established and would appear unlikely, since muscarinic actions are slow (McCormick and Prince, 1986). Nonetheless, a sustained muscarinic-induced depolarization can bring cortical interneurons and pyramidal cells close to firing threshold where many of them express theta-like, as well as gamma-like, subthreshold oscillations (Llinas et al., 1991; Silva et al., 1991; Metherate et al., 1992; Klink and Alonso, 1993). But, it is also possible that phasic modulation could be subtended by the relatively fast muscarinic and/or nicotinic action on cortical interneurons (McCormick and Prince, 1986; Roerig et al., 1997; Xiang et al., 1998; Porter et al., 1999). The slow rhythmic modulation might serve as an envelope for faster gamma activity facilitating the coherent discharge of spatially distant but functionally related cortical neurons (Singer, 1993).

In addition, theta activity and cholinergic actions have also both been shown to modulate synaptic plasticity and retrieval dynamics in hippocampal-cortical networks, where these processes are believed to underlie memory (Larson et al., 1986; Huerta and Lisman,

1995; Wallenstein and Hasselmo, 1997; Metherate et al., 1988; Metherate and Ashe, 1991; Kilgard and Merzenich, 1998; Klink and Alonso, 1997; Fransen et al., 1999),

### **Non-cholinergic neurons**

In contrast to the cholinergic neurons, non-cholinergic neurons were physiologically and morphologically heterogeneous, including a minority which decreased ('off'), in addition to the majority which increased ('on') their discharge rate with stimulation and including small, potentially, locally or diencephalically projecting cells (Gritti et al., 1994), in addition to medium-to-large cortically projecting cells (Gritti et al., 1997). The largest proportion of non-cholinergic 'on' cells discharged tonically and would accordingly correspond to the major cell type reported in sleep-wake recording experiments in which most chemically unidentified basal forebrain cells simply discharged at higher rates during waking or paradoxical sleep than during slow wave sleep (Detari et al., 1984; Szymusiak and McGinty, 1986; Detari and Vanderwolf, 1987; Szymusiak and McGinty, 1989). Another minor proportion of the non-cholinergic 'on' cells discharged in a rhythmic manner, particularly in a cluster discharge pattern which resembled that described for non-cholinergic neurons recorded *in vitro* (Alonso et al., 1996). Among the non-cholinergic 'off' cells, was a proportion which discharged phasically in bursts and often in association with the high amplitude irregular slow EEG activity. These could correspond to cells identified *in vitro* as showing phasic discharge patterns and being hyperpolarized by noradrenaline (Fort et al., 1998). They could also, according to their size and antidromic activation, correspond to cortically projecting slow wave sleep-active neurons recorded in naturally sleeping-waking cats (Szymusiak and McGinty, 1989).

In conclusion, cholinergic basal forebrain neurons discharge in rhythmic bursts that may be important in mediating cortical activation associated with active waking and paradoxical sleep and in promoting the particular rhythmicity and coherent activity in cortical networks that may facilitate processes of binding and plasticity occurring during these states.

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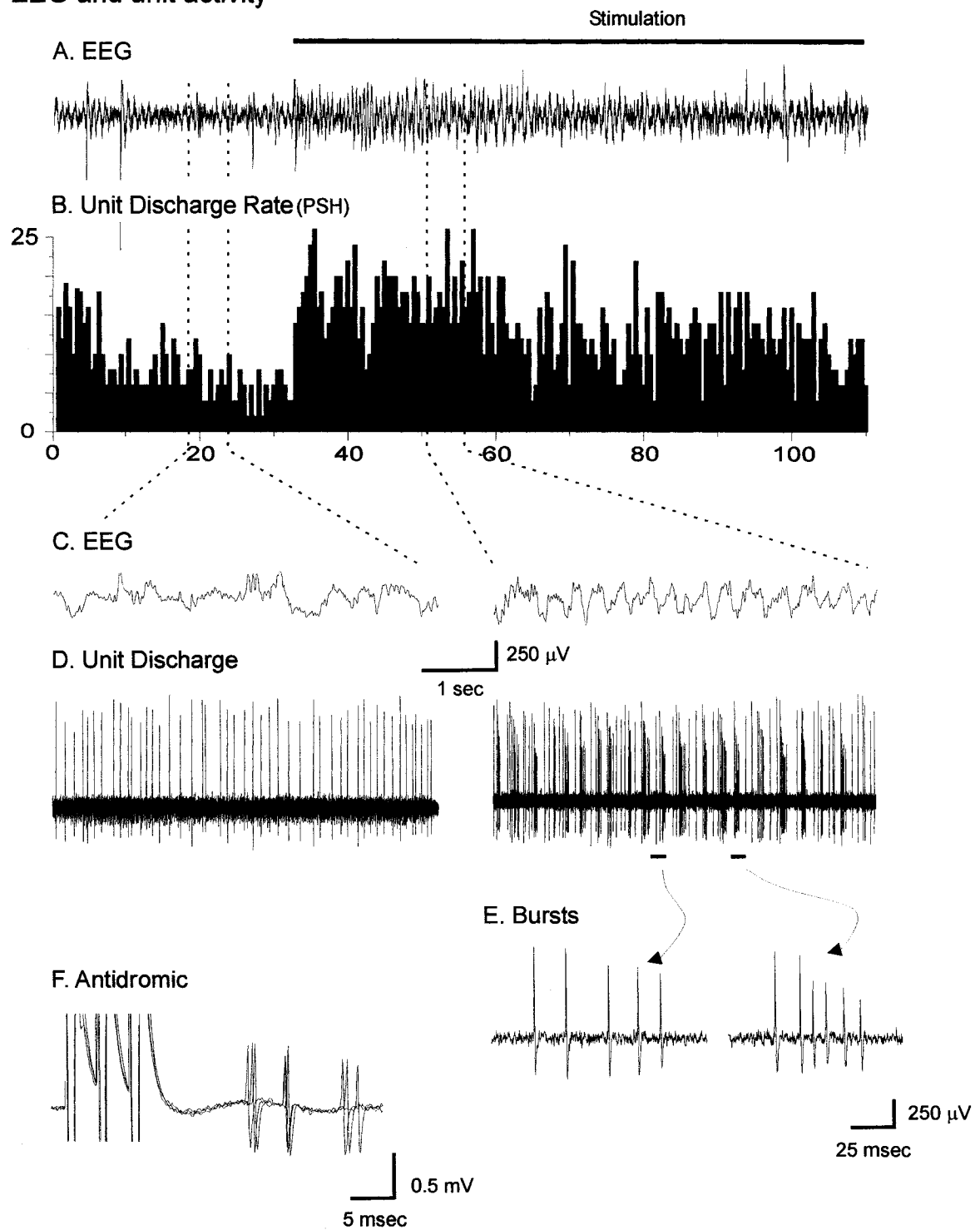
## **2.8 Figures**

Figure 1. Medium-sized multipolar neuron recorded and labeled by the juxtacellular technique with Neurobiotin (Nb, and revealed with nickel-enhanced DAB) in the MCPO region of the basal forebrain. Scale bar 50 $\mu$ m.



**Figure 2. Discharge pattern of Nb-labeled neuron in the MCPO prior to and during stimulation of the animal. (A) EEG (from retrosplenial cortex) and (B) peristimulus histogram (PSH) of the mean rate of discharge (in spikes per second) during pre-stimulation and stimulation conditions. (C) EEG and (D) unit discharge traces are expanded from the two conditions (left and right). Note increase in rate of discharge and change in pattern of discharge from tonic to bursting with stimulation of the unit in association with a change in the pattern of EEG activity. (E) Expanded traces of individual bursts showing variable firing frequencies. (F) Antidromic activation of bursting MCPO neuron from prefrontal cortex.**

MCPO cell  
EEG and unit activity



**Figure 3. Photomicrographs of recorded and juxtacellularly labeled Nb+/ChAT+ and Nb+/ChAT- neurons located in the basal forebrain cholinergic cell area. Nb was revealed with green fluorescent Cy2-conjugated streptavidin (left) and ChAT-immunostaining with red fluorescent Cy3-conjugated secondary antibodies (right). (A) Nb+/ChAT+ neuron (#98o18003/6) in MCPO lying among other ChAT+ cells. (B) Nb+/ChAT+ neuron (# 98812009/10) in SI. (C) Nb+/ChAT- neuron (#98629000) in MCPO surrounded by ChAT+ neurons. Scale bar 20µm.**

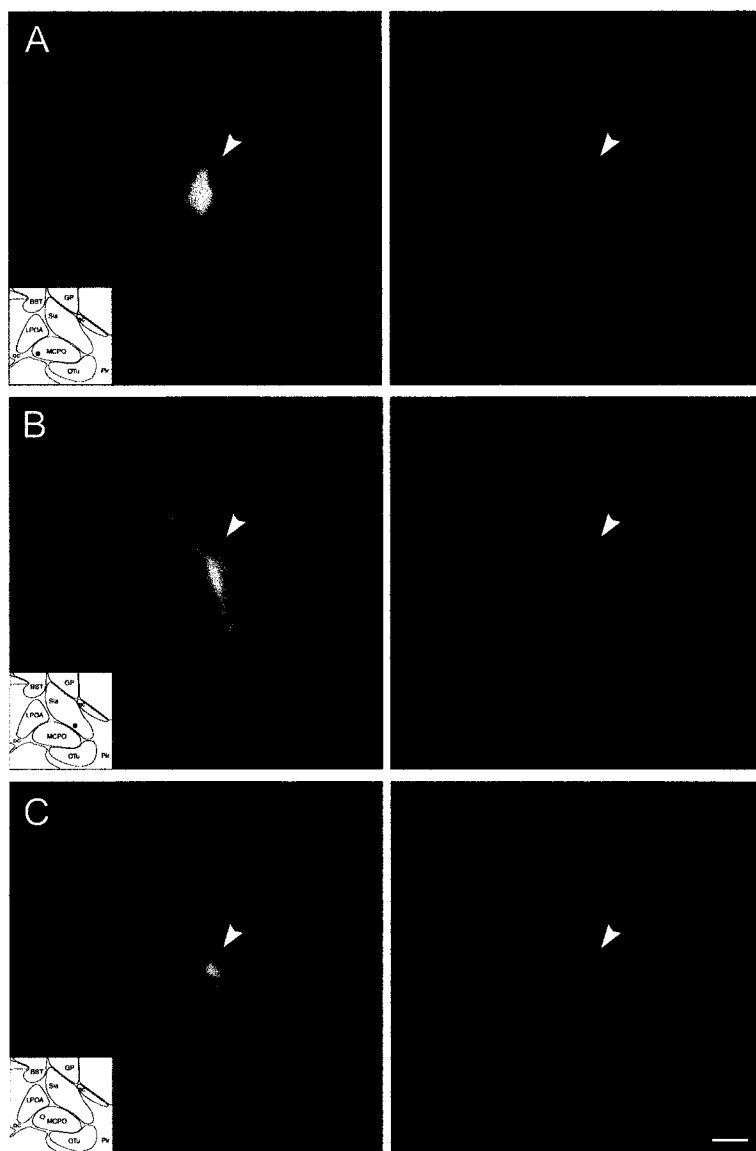


Figure 4. Distribution of recorded and characterized Nb<sup>+</sup>/ChAT<sup>+</sup> (filled circles) and Nb<sup>+</sup>/ChAT<sup>-</sup> (open circles) neurons in the basal forebrain (represented on atlas sections adapted from (Gritti et al., 1993)). Scale bar 1mm.

Abbreviations: Acb, accumbens nucleus; ac, anterior commissure; BST, bed of the stria terminalis; CPu, caudate putamen; DBB, diagonal band of Broca nucleus ; f, fornix; FStr, fundus of striatum; GP, globus pallidus; LPOA, lateral preoptic area; LS, lateral septum; MCPO, magnocellular preoptic nucleus; MS, medial septum; oc, optic chiasm; OTu., Olfactory tubercle; Pir, Piriform cortex; Ret, Reticularis nucleus; SIa, substantia innominata pars anterior; SIp, substantia innominata pars posterior; sm, stria medullaris.

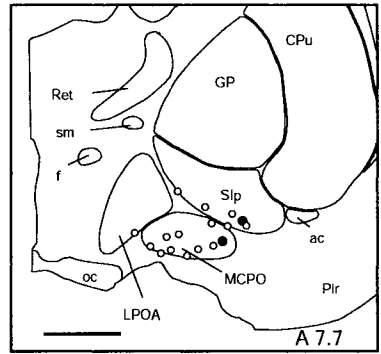
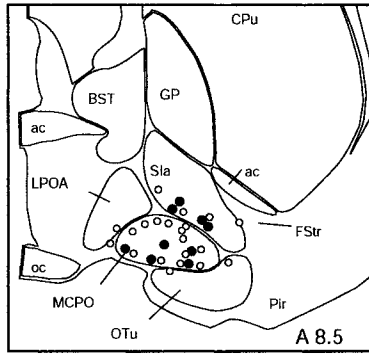
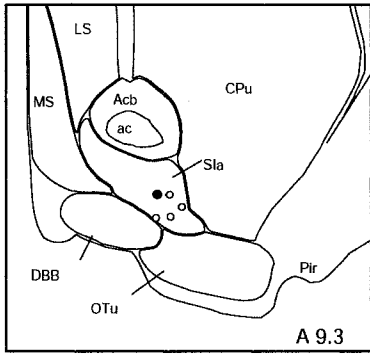
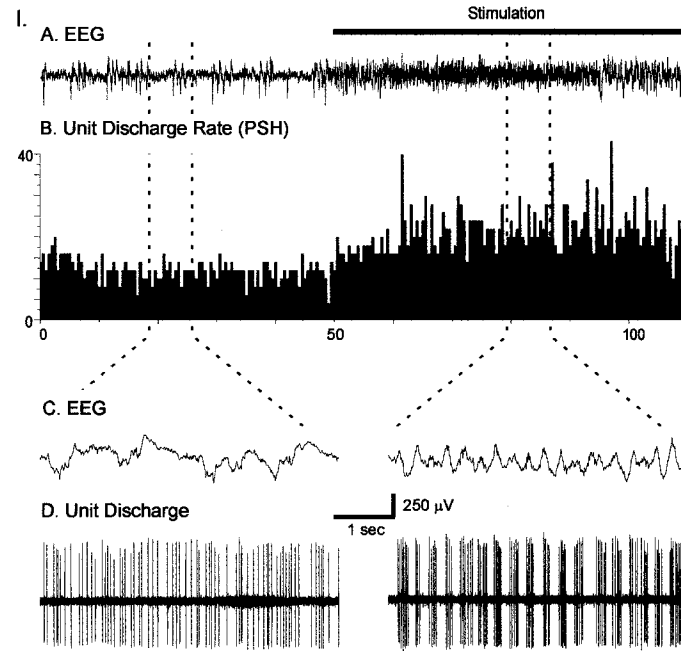


Figure 5. I. Discharge pattern of Nb+/ChAT+ neuron (#98o18003/6) in the MCPO (Fig. 3A). (A) EEG (from retrosplenial cortex) and (B) peristimulus histogram (PSH) of the mean rate of discharge (in spikes per second) prior to and during stimulation of the animal. (C) EEG and (D) unit discharge traces are expanded for both pre-stimulation and stimulation conditions (left and right). Note the change from a tonic discharge pattern to a burst-like discharge pattern in addition to the increased rate of discharge with stimulation and in association with a change in EEG activity.

II. EEG and unit analysis during pre-stimulation and stimulation conditions. (A) Autocorrelation functions (ACF, with correlation coefficients on vertical axes) of the pre- and stimulation EEG recordings and corresponding power spectra. (B) Autocorrelation histograms (ACH, with correlation coefficients on vertical axes) of pre- and stimulation period unit spike-trains and insets of corresponding interspike interval histograms (ISIH). A power spectrum is shown (inset) for the stimulation ACH in which rhythmic activity is apparent. (C) Spike-triggered averages (STA) of unit-EEG cross-correlation (with mV on vertical axes) for actual unit (black line) and randomized-spike train (gray line). A power spectrum is shown (inset) for the stimulation STA in which the actual unit-EEG function was significantly different from the randomized spike-train unit-EEG function (Wilcoxon test,  $p < 0.05$ , \*). Note with stimulation the appearance of cross-correlated EEG and unit rhythmic activity with a peak frequency of  $\sim 3.8$  Hz, which also corresponds to the prominent EEG rhythmic slow activity and spectral peak frequency.

# Nb+/ChAT+ MCPO cell



## II.

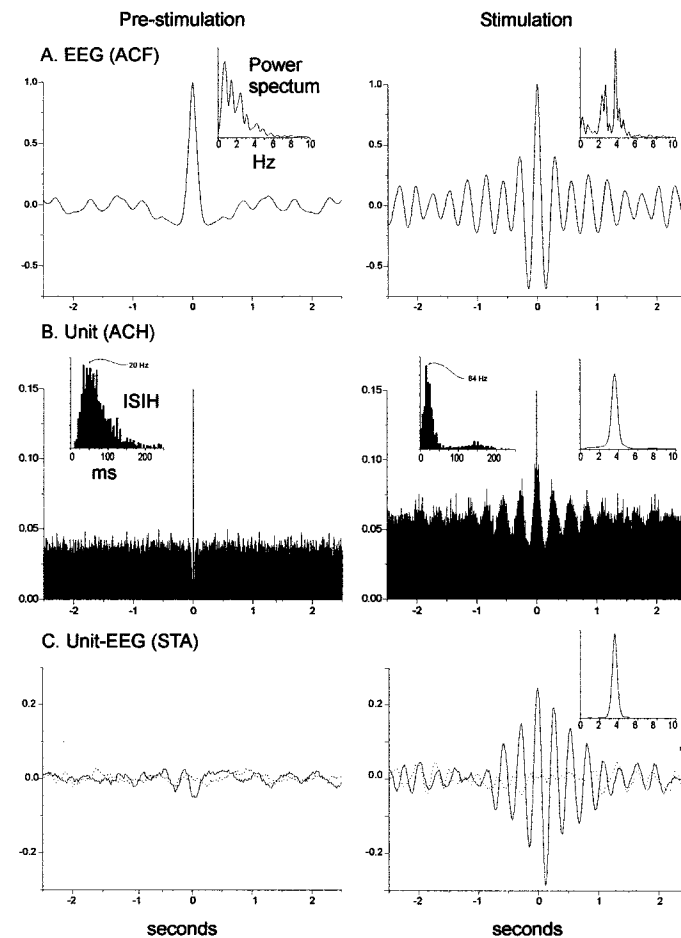
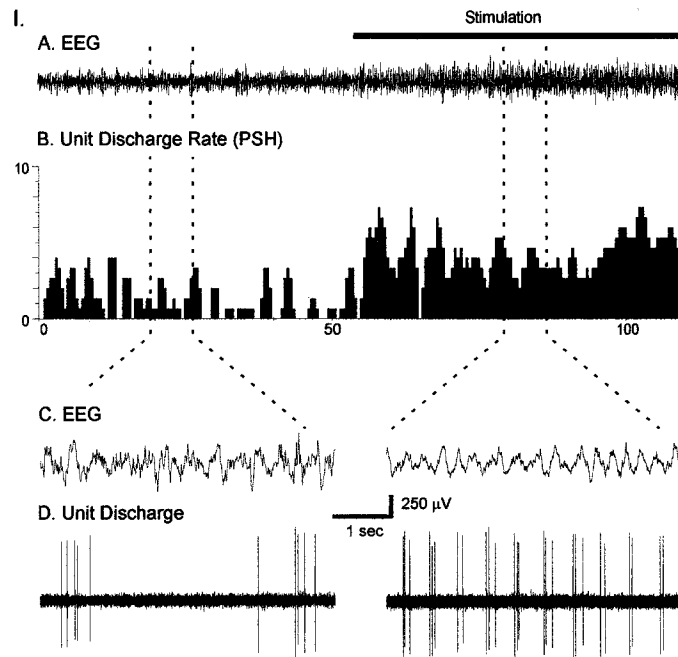


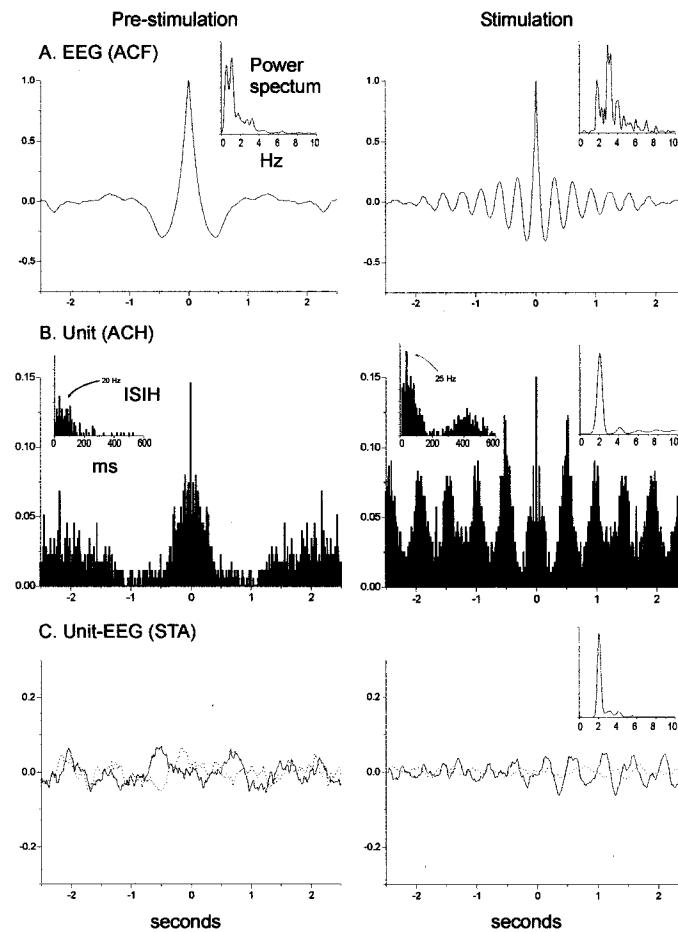
Figure 6. I. Discharge pattern of Nb+/ChAT+ neuron (#98812009/10) in the SI (Fig. 3B). (A) EEG (from retrosplenial cortex) and (B) peristimulus histogram (PSH) of the mean rate of discharge (in spikes per second) prior to and during stimulation of the animal. (C) EEG and (D) unit discharge traces are expanded for both pre-stimulation and stimulation conditions (left and right). Note the change from an irregular discharge pattern to a rhythmic burst-like discharge pattern in addition to the increased rate of discharge and in association with a change in EEG activity with stimulation.

II. EEG and unit analysis during pre-stimulation and stimulation conditions. (A) Autocorrelation functions (ACF, with correlation coefficients on vertical axes) of the pre- and stimulation EEG recordings and corresponding power spectra. (B) Autocorrelation histograms (ACH, with correlation coefficients on vertical axes) of pre- and stimulation unit spike-trains and insets of corresponding interspike interval histograms (ISIH). A power spectrum is shown (inset) for the stimulation ACH in which rhythmic activity is apparent. (C) Spike-triggered averages (STA) of unit-EEG cross-correlation (with mV on vertical axes) for actual unit (black line) and randomized-spike train (gray line). A power spectrum is shown (inset) for the stimulation STA in which the actual unit-EEG function was significantly different from the randomized spike-train unit-EEG function (Wilcoxon test,  $p < 0.05$ , \*). Note with stimulation the appearance of cross-correlated EEG and unit rhythmic activity with a peak frequency of  $\sim 2$  Hz, which did not correspond to the prominent EEG rhythmic slow activity or spectral peak but to a secondary peak in the EEG power spectrum.

# Nb+/ChAT+ SI cell

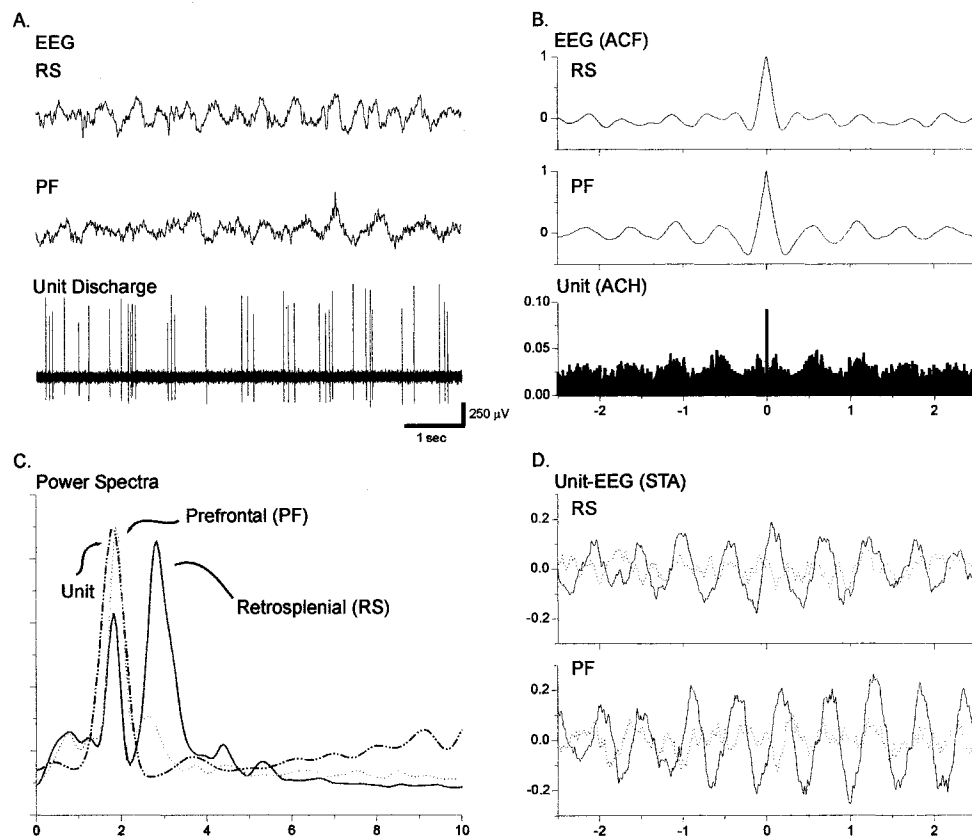


## II.



**Figure 7. EEG and unit analysis for Nb+/ChAT+ MCPO neuron (#98o070021/23) during stimulation. (A) Representative traces showing retrosplenial (RF) and prefrontal (PF) cortical leads recorded simultaneously with unit. (B) Autocorrelation functions and histogram (ACF, ACH) of respective recordings. Note that the unit rhythmic discharge most closely matches the rhythmic activity of the prefrontal cortex. (C) Power spectra of EEG leads and unit ACH indicating that the rhythmic discharge of the unit closely matches the dominant peak frequency of the prefrontal cortex, whereas it matches a secondary peak of the retrosplenial cortex. (D) Spike-triggered averages (STA) of unit-EEG cross-correlation for both retrosplenial and prefrontal cortices (with normalized units on vertical axis) for actual unit (black line) and randomized-spike train (gray line).**

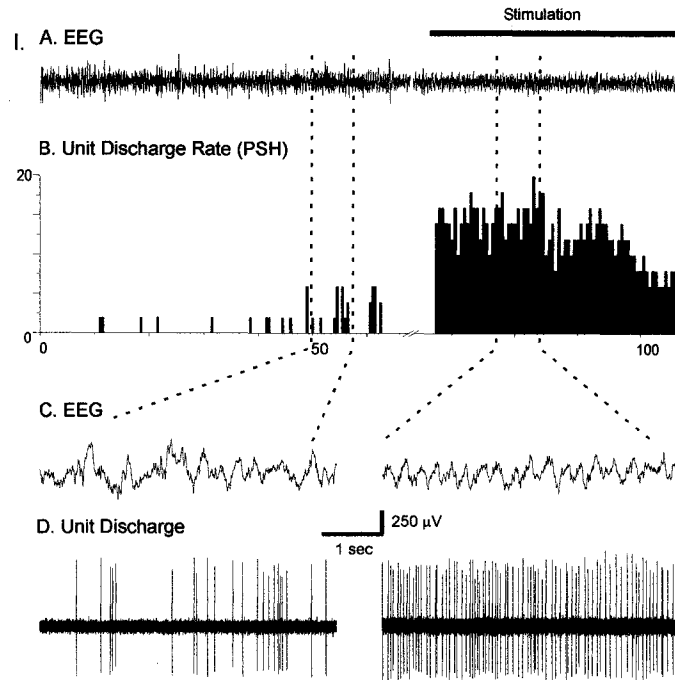
Nb+/ChAT+ MCPO cell  
EEG and unit analysis



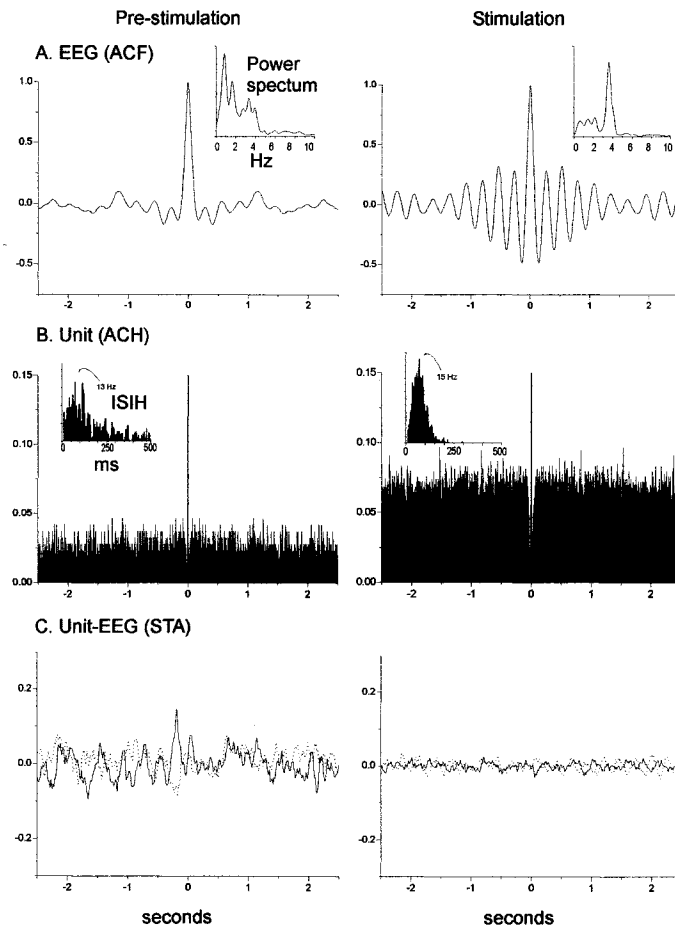
**Figure 8. I. Discharge pattern of Nb+/ChAT- neuron (#98629000) in the MCPO (Fig. 3C). (A) EEG (from retrosplenial cortex) and (B) peristimulus histogram (PSH) of the mean rate of discharge (in spikes per second) prior to and during stimulation of the animal. (C) EEG and (D) unit discharge traces are expanded for both pre-stimulation and stimulation conditions (left and right). Note the change from an irregular discharge pattern to a tonic discharge pattern and slightly increased rate of discharge in association with a change in EEG activity with stimulation.**

**II. EEG and unit analysis during pre-stimulation and stimulation conditions. (A) Autocorrelation functions (ACF, with correlation coefficients on vertical axes) of the pre- and stimulation EEG recordings and corresponding power spectra. (B) Autocorrelation histograms (ACH, with correlation coefficients on vertical axes) of pre- and stimulation unit spike-trains and insets of corresponding interspike interval histograms (ISIH). (C) Spike-triggered averages (STA) of unit-EEG cross-correlation (with mV on vertical axes) for actual unit (black line) and randomized-spike train (gray line). Note the lack of low frequency rhythmic activity in the unit discharge and the absence of cross-correlated unit-EEG activity with stimulation.**

# Nb+/ChAT- MCPO cell



## II.



## 2.9 Tables

**Table 1. Frequency of anatomical and physiological characteristics in cholinergic and non-cholinergic cell groups.<sup>1</sup>**

|                                                  | <u>All cells</u> | <u>Nb+/ChAT+ cells</u> | <u>Nb+/ChAT- cells</u> | <u>Statistic:</u><br><u><math>\chi^2</math>(df)</u> |
|--------------------------------------------------|------------------|------------------------|------------------------|-----------------------------------------------------|
| n                                                | 52               | 12                     | 40                     |                                                     |
| <b>ANATOMY</b>                                   |                  |                        |                        |                                                     |
| <b>Area</b>                                      |                  |                        |                        | 0.34(1)                                             |
| MCPO                                             | 34               | 7                      | 27                     |                                                     |
| SI                                               | 18               | 5                      | 13                     |                                                     |
| <b>Shape</b>                                     |                  |                        |                        | 0.59 (1)                                            |
| Oval-Fusiform (Bipolar)                          | 21               | 6                      | 15                     |                                                     |
| Polygonal (Multipolar)                           | 31               | 6                      | 25                     |                                                     |
| <b>Size</b>                                      |                  |                        |                        | 6.61 (1)**                                          |
| Small ( $\leq 15\mu\text{m}$ )                   | 11               | 0                      | 11                     |                                                     |
| Medium-Large ( $>15\mu\text{m}$ )                | 41               | 12                     | 29                     |                                                     |
| <b>PHYSIOLOGY</b>                                |                  |                        |                        |                                                     |
| <b>Pattern</b>                                   |                  |                        |                        | 10.84 (2)**                                         |
| Tonic                                            | 23               | 3                      | 20                     |                                                     |
| Tonic/Burst                                      | 20               | 9                      | 11                     |                                                     |
| Tonic/Cluster                                    | 9                | 0                      | 9                      |                                                     |
| <b>Response to Stimulation (PSH<sup>2</sup>)</b> |                  |                        |                        | 5.26 (1)*                                           |
| Increase ('on')                                  | 43               | 12                     | 31                     |                                                     |
| Decrease ('off')                                 | 9                | 0                      | 9                      |                                                     |
| <b>Unit Rhythmicity</b>                          |                  |                        |                        |                                                     |
| <b>During Stimulation (ACH<sup>3</sup>)</b>      |                  |                        |                        | 6.91 (1)**                                          |
| Rhythmic                                         | 22               | 9                      | 13                     |                                                     |
| Not Rhythmic                                     | 30               | 3                      | 27                     |                                                     |
| <b>Unit-EEG Cross-Correlation</b>                |                  |                        |                        |                                                     |
| <b>During Stimulation (STA<sup>4</sup>)</b>      |                  |                        |                        | 10.13 (1)**                                         |
| Correlated                                       | 23               | 10                     | 13                     |                                                     |
| Not Correlated                                   | 29               | 2                      | 27                     |                                                     |

<sup>1</sup> Frequencies (n, number of cells) for the two groups are presented and compared using the likelihood ratio chi square ( $\chi^2$ ) statistic, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

<sup>2</sup> Classification based upon peristimulus histogram (PSH) measure of average discharge rate.

<sup>3</sup> Based upon autocorrelation histogram (ACH) measure of rhythmicity.

<sup>4</sup> Based upon spike-triggered average (STA) measure of unit-to-EEG cross-correlation (retrosplenial cortex).

**Table 2. Morphological and physiological measures in cholinergic and non-cholinergic cell groups.<sup>1</sup>**

|                                | <u>Nb+/ChAT+ cells</u> | <u>Nb+/ChAT- cells</u> |                  | Cell Group<br>Statistic: F (df) |
|--------------------------------|------------------------|------------------------|------------------|---------------------------------|
|                                |                        | <u>'on'</u>            | <u>'off'</u>     |                                 |
| <b>MORPHOLOGY</b>              |                        |                        |                  |                                 |
| <b>Size (μM)</b>               | 21.34 ± 0.62 (12)      | 17.99 ± 0.77 (31)†     | 20.29 ± 1.37 (9) | 3.73 (2,49)*                    |
| <b>PHYSIOLOGY</b>              |                        |                        |                  |                                 |
| <b>Spike Width (ms)</b>        | 0.72 ± 0.05 (12)       | 0.68 ± 0.04 (31)       | 0.68 ± 0.06 (9)  | 0.13 (2,49)                     |
| <b>Antidromic Latency (ms)</b> | 13.18 ± 2.58 (4)       | 16.83 ± 2.78 (4)       | 14.90 ± 0.9 (2)  | 0.53 (2,7)                      |

<sup>1</sup> Mean  $\pm$  SEM (number of cells) are presented for Nb+/ChAT+, Nb+/ChAT- 'on' and Nb+/ChAT- 'off' cell groups. The three groups were compared by ANOVA. With a main effect of cell group indicated by \* ( $p < 0.05$ ), *post hoc* comparisons were performed using Fisher's Least-Significant-Difference test (according to which † indicates significant difference relative to the Nb+/ChAT+ cell group and § to significant differences relative to the Nb+/ChAT- 'on' cell group).

**Table 3. Frequencies (Hz) of dominant EEG activity (from retrosplenial cortex) and units' average discharge, instantaneous firing, and unit-to-EEG cross-correlated activity during pre-stimulation and stimulation conditions in cholinergic and different non-cholinergic cell groups.<sup>1</sup>**

| Frequency Measure                                    | Nb+/ChAT+ cells    |                      | Nb+/ChAT- cells   |                                    |                                                   |                                                | Cell Group Statistic: F (df) |               |
|------------------------------------------------------|--------------------|----------------------|-------------------|------------------------------------|---------------------------------------------------|------------------------------------------------|------------------------------|---------------|
|                                                      | Pre-Stimulation    | Stimulation          | Pre-Stimulation   | <u>'on'</u>                        | Pre-Stimulation                                   | <u>'off'</u>                                   |                              |               |
|                                                      |                    |                      |                   | Stimulation                        |                                                   | Stimulation                                    |                              |               |
| EEG<br>(Spectral Peak)                               | 0.91 ± 0.09 (12)   | 3.21 ± 0.13 (12)***  | 1.11 ± 0.08 (27)  | 3.30 ± 0.08 (27)***                | 1.28 ± 0.17 (6)                                   | 3.32 ± 0.09 (6)***                             | 1.83 (2,42)                  | 0.23 (2,42)   |
| Average discharge<br>(PSH) <sup>2</sup>              | 5.29 ± 1.39 (12)   | 12.49 ± 2.24 (12)*** | 3.83 ± 0.61 (31)  | 7.96 ± 0.97 (31) ***, <sup>†</sup> | 5.96 ± 2.30 (9)                                   | 3.28 ± 1.42 (9)*, <sup>††</sup> , <sup>§</sup> | 1.00 (2,49)                  | 6.42 (2,49)** |
| Instantaneous firing<br>(ISIH) <sup>3</sup>          | 34.44 ± 12.60 (12) | 68.23 ± 23.65 (12)*  | 13.04 ± 1.74 (31) | 23.02 ± 4.68 (31)*, <sup>††</sup>  | 149.82 ± 60.51 (9) <sup>††</sup> , <sup>§§§</sup> | 13.88 ± 6.98 (9) <sup>††</sup>                 | 11.20 (2,49)***              | 5.32 (2,49)** |
| Rhythmic discharge<br>(ACH) <sup>4</sup>             | 1.15 ± 0.17 (7)    | 2.25 ± 0.27 (9)**    | 0.94 ± 0.18 (6)   | 1.65 ± 0.27 (9)*                   | 1.86 ± 0.76 (4)                                   | 1.65 ± 1.08 (4)                                | 1.65 (2,14)                  | 1.72 (2,19)   |
| Unit-to-EEG cross-<br>correlation (STA) <sup>5</sup> | 1.07 ± 0.11 (6)    | 2.05 ± 0.31 (10)*    | 0.62 ± 0.12 (5)   | 1.53 ± 0.36 (10)*                  | 1.11 ± 0.25 (5)                                   | 1.55 ± 0.05 (3)                                | 2.66 (2,13)                  | 0.74 (2,20)   |

<sup>1</sup>Mean ± SEM frequencies (Hz) are presented for the pre-stimulation and stimulation conditions for Nb+/ChAT+, Nb+/ChAT- 'on' and Nb+/ChAT- 'off' cell groups. Stimulation was compared to pre-stimulation for all cells within each group by paired t-tests for the EEG, PSH, and ISIH values, or unpaired t-tests for ACH and STA values (\*p<0.05, \*\*p<0.01, \*\*\*p<0.001). The three groups were compared in the pre-stimulation and 'stimulation' conditions by ANOVA. With a main effect of cell group for each condition indicated by \*, *post hoc* comparisons were performed using Fisher's Least-Significant-Difference test (according to which † indicates significant differences relative to the Nb+/ChAT+ cell group and § to significant differences relative to the Nb+/ChAT- 'on' cell group).

<sup>2</sup> Peristimulus histogram (PSH) measurements of average discharge rate.

<sup>3</sup> Interspike interval histogram (ISIH) measurements of the instantaneous firing frequency.

<sup>4</sup> Autocorrelation histogram (ACH) measurements of rhythmic discharge.

<sup>5</sup> Spike-triggered average (STA) measurements of unit-to-EEG cross-correlation frequency.

**Table 4. Correspondence of rhythmic discharge frequency with the dominant EEG spectral peak frequency from retrosplenial (RS) or prefrontal (PF) cortical EEG during stimulation in cholinergic cells.<sup>1</sup>**

| Unit activity matches<br>dominant spectral peak |          | <u>Frequency (Hz)</u> |                         |                         |
|-------------------------------------------------|----------|-----------------------|-------------------------|-------------------------|
|                                                 | <b>n</b> | <u>Unit ACH</u>       | <u>RS Dominant Peak</u> | <u>PF Dominant Peak</u> |
| <b>RS</b>                                       | 2        | 3.53 ± 0.23           | 3.35 ± 0.45             | 2.50 ± 0.30             |
| <b>PF</b>                                       | 5        | 1.94 ± 0.22 **        | 3.31 ± 0.18             | 2.01 ± 0.22             |

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<sup>1</sup> Mean ± SEM are presented and compared between RS and PF groups by Student's t-test (\*p<0.05, \*\*p<0.01, \*\*\*p<0.001).

## **Chapter Three**

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### **3. Discharge Profiles of Juxtacellularly Labeled and Immunohistochemically Identified GABAergic Basal Forebrain Neurons Recorded in Association with the Electroencephalogram in Anesthetized Rats<sup>1</sup>**

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<sup>1</sup> Reprinted from The Journal of Neuroscience, 2000, Manns ID, Alonso A, Jones BE: " Discharge profiles of juxtacellularly labeled and immunohistochemically identified GABAergic basal forebrain neurons recorded in association with the electroencephalogram in anesthetized rats ",20 , 9252-9263, Copyright (2000), with permission from the Society for Neuroscience.

### 3.1 Preface

As reviewed in the Introduction, the anatomical identification and description of the GABAergic basal forebrain neurons revealed that they outnumber the cholinergic neurons (Gritti et al., 1993). This group of neurons is anatomically heterogeneous, as different subgroups may comprise locally projecting interneurons, caudally projecting neurons to the posterior hypothalamus (Gritti et al., 1994), and rostrally projecting neurons to the cortex in numbers approximately equal to the cholinergic neurons (Fisher et al., 1988; Gritti et al., 1997). The precise role the cortically projecting GABAergic neurons play, however, has remained obscure. One hypothesis has been that, they could underlie the sleep promoting function long attributed to the basal forebrain. Alternatively, they could promote cortical activation through projections into cortical interneurons (Freund and Meskenaite, 1992). Unlike the cholinergic neurons, GABAergic neurons were not specifically characterized physiologically by *in vitro* studies, since immunohistochemical staining for GAD was not successful in the *in vitro* brain slice. Thus, only non-cholinergic neurons have been characterized according to their biophysical properties (Alonso et al., 1996) and hypothesized to be potentially GABAergic. Again, no studies have characterized the physiological properties of these cells in association with EEG activity or behavioral state for the same reasons noted for the cholinergic neurons in the previous chapter. This study was thus aimed at understanding the potential functions of this heterogeneous group of neurons, by learning how they fire in relation to cortical EEG activity.

### **3.2 Abstract**

The basal forebrain ostensibly plays a dual role in the modulation of cortical activation and behavioral state. It is essential for stimulating cortical activation in association with waking (and paradoxical sleep), yet also important for attenuating cortical activation and promoting slow wave sleep. Using juxtacellular recording and labeling of neurons with Neurobiotin followed by immunohistochemical staining for glutamic acid decarboxylase (GAD), we studied the discharge properties of identified GABAergic basal forebrain neurons in relation to electroencephalographic (EEG) activity in urethane-anesthetized rats to determine the part (s) that they may play in this dual role.

The GABAergic neurons displayed distinct discharge profiles in relation to somatosensory stimulation-evoked cortical activation. Whereas a significant minority increased its average discharge rate, the majority decreased its average discharge rate in association with cortical activation. Moreover, subgroups displayed distinct discharge patterns related to different cortical activities, including very regular high frequency tonic spiking within a gamma EEG frequency range and rhythmic cluster spiking within a theta-like frequency range during cortical activation. During irregular slow EEG activity in absence of stimulation, one subgroup displayed spike bursts correlated with cortical slow oscillations. As relatively large in size and also antidromically activated from the cortex, many GABAergic neurons recorded were considered to be cortically projecting and thus capable of directly modulating cortical activity. Subgroups of GABAergic basal forebrain neurons would thus have the capacity to promote cortical activation by modulating gamma or theta activity and others to attenuate cortical activation by modulating irregular slow oscillations which normally occur during slow wave sleep.

### 3.3 Introduction

Although the basal forebrain has been long considered to play an important role in the modulation of cortical activity and sleep-wake states, its role has appeared to be dual and to comprise ostensibly antagonistic processes (see for review, (Jones, 2000)). As the ventral, extrathalamic relay to the cerebral cortex from the brainstem reticular activating system, it was initially implicated in the stimulation of cortical activation (Starzl et al., 1951). Yet, together with the adjacent preoptic region, it was also shown to be important for cortical slow wave activity and slow wave sleep (Serman and Clemente, 1962; McGinty and Serman, 1968). More recent studies applying neurotoxic lesions to the basal forebrain have also yielded seemingly contradictory results with some reporting deficits in cortical activation (Stewart et al., 1984; Buzsaki et al., 1988) and others reductions in slow wave sleep (Szymusiak and McGinty, 1986). Paralleling these, are recent results applying electrical stimulation to the basal forebrain showing that some sites elicit excitation, while adjacent sites produce strong inhibition of cortical neurons (Jimenez-Capdeville et al., 1997). The excitation was associated with enhanced release of acetylcholine (ACh), whereas the inhibition was not. These results confirmed the long conceived role of cholinergic basal forebrain neurons in cortical activation (Krnjevic and Phillis, 1963; Celesia and Jasper, 1966), yet also suggested a role for adjacent non-cholinergic neurons in antagonistic processes that could be important for slow wave sleep.

Unit recordings in the basal forebrain and adjacent preoptic area during the natural sleep-waking cycle have revealed cell types that behave differently in relation to cortical activity and sleep-wake state (Szymusiak and McGinty, 1986; Detari et al., 1987;

Szymusiak and McGinty, 1989). Some discharge maximally in association with cortical activation during waking called “wake-active”, and others discharge maximally in association with slow-wave sleep, called “sleep-active”. Both types could be antidromically activated from the cerebral cortex and some sleep-active cells from the brainstem. These different profiles of activity suggest that different neurons are involved in fulfilling the dual role of the basal forebrain in promoting cortical activation and sleep.

By application of juxtacellular recording-labeling and immunohistochemical identification of basal forebrain neurons, we recently determined that identified cholinergic neurons fire during cortical activation, discharging in rhythmic bursts in association with theta and gamma EEG activity (Manns et al., 2000; Cape et al., In press). Intermingled with cholinergic are non-cholinergic neurons, including a large population of GABAergic neurons, which outnumber the cholinergic and comprise cortically, in addition to caudally or locally projecting neurons (Gritti et al., 1993; Gritti et al., 1994; Gritti et al., 1997). The aim of the present study was to characterize the discharge properties of basal forebrain GABAergic neurons in relation to cortical activity and to determine if they may act in parallel with or antagonistic to the cholinergic neurons and thus potentially participate in processes of cortical activation or its attenuation. For this purpose, single units were recorded during irregular slow cortical activity and somatosensory-stimulation evoked cortical activation in urethane-anesthetized rats. Physiologically characterized neurons were labeled with Neurobiotin (Nb) by the juxtacellular technique and subsequently immunostained for glutamic acid decarboxylase (GAD).

### **3.4 Methods and Materials**

#### **Animals and Surgery**

Experiments were performed on 60 adult male Long Evans rats (200 - 250 g., Charles River, Canada). All procedures were approved by the McGill University Animal Care Committee and the Canadian Council on Animal Care. The animals were anesthetized with urethane (ethyl carbamate; Sigma, St. Louis, Mo) using an initial dose 1.4 g/kg, i.p. and supplementary doses if necessary of 0.1 – 0.15 g/kg, i.p., in order to insure an adequate level of anesthesia, as determined by the lack of response to pinching of the hind limb. Body temperature was maintained at 36 - 37°C by a thermostatically controlled heating pad. According to procedures described in detail in a previous study (Manns et al., 2000), the anesthetized animals were positioned in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA) for both the surgery and subsequent recording. For EEG recording, stainless steel screws were placed over the retrosplenial cortex (anterior-posterior, AP -4 mm, lateral, L  $\pm$  0.5 mm relative to bregma (Paxinos and Watson, 1986)) and in the frontal bone as a reference. The retrosplenial (posterior cingulate) cortex was chosen for recording EEG due to the presence of a prominent theta rhythm during states of cortical activation that was moreover shown to not depend upon hippocampal theta and input from the medial septum/diagonal band complex (Borst et al., 1987; Leung and Borst, 1987). For the purpose of antidromic activation of basal forebrain units and recording of the local field potential, a bipolar stimulating electrode was placed in the prefrontal cortex on each side (AP +2.0 mm, L  $\pm$  1.0 mm, and V -2.0 mm). This region was selected for this purpose since it is known to receive afferents from GABAergic in addition to cholinergic basal forebrain neurons (Gritti et al., 1997) as

well as to sit in the path of the major medial basalo-cortical fiber system (Saper, 1984; Luiten et al., 1987).

### **Unit recording and labeling**

Juxtacellular recording and labeling was done with an intracellular amplifier (IR-283; Neurodata Instruments, New York, NY). Unit recordings were performed with glass microelectrodes, filled with 0.5 M sodium acetate and ~ 5.0% Neurobiotin (Nb, Vector Laboratories, Burlingame, CA). Recorded units were characterized during spontaneous EEG activity and during somatosensory stimulation. The stimulation consisted of a continuous pinch of the tail applied by large, blunt forceps. Antidromic activation was tested from the prefrontal cortex. Antidromic responses were distinguished from orthodromic responses if they displayed a constant latency to stimulation, an ability to follow high frequency discharge, and when present, collision with spontaneously occurring spikes. Orthodromic responses were frequently observed but were not analyzed in the present study. Spike widths were measured from positive inflection to first zero-crossing using greater than 128 averaged spikes.

After the recording and characterization of isolated neurons, they were labeled using the 'juxtacellular' method (Pinault, 1996). In the majority of rats (58), only one cell on one side of the brain was submitted to the labeling procedure; in some rats (2), one cell on each side of the brain was so labelled. The animals then received an overdose of urethane and were transcardially perfused with a 4% paraformaldehyde solution.

## **Histochemistry**

Coronal frozen sections were cut at 30  $\mu\text{m}$  and incubated overnight in a primary antibody for GAD (rabbit anti-GAD antiserum, 1:3000, Chemicon, Temecula, CA). They were subsequently co-incubated in a Cy2-conjugated streptavidin (1:800) to reveal Nb and a Cy3-conjugated donkey anti-rabbit antiserum (1:1000, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) to reveal GAD-immunostaining. Sections were viewed by fluorescent microscopy using a Leitz Dialux microscope equipped with a Ploemopak 2 reflected light fluorescence illuminator with Leica filter cubes for fluorescein (I3) and rhodamine (N2.1). Cell size was measured from film transparencies, and cells classified as small ( $\leq 15 \mu\text{m}$ ) or medium-to-large (16 to 35  $\mu\text{m}$ ) according to their large diameter.

Of the 62 cells submitted to the juxtacellular labeling procedure, 55 cells were recovered in sections from 53 brains which were also successfully dual-immunostained for GAD and thus included in the analysis and results of the present study. In 51 brains, one Nb<sup>+</sup> cell was present, and in 2 brains, one Nb<sup>+</sup> cell was present on each side, yielding two cells, for a total of 55 Nb<sup>+</sup> cells. Accordingly 55 of 62 cells (~90%) were successfully labeled and recovered following application of the juxtacellular procedure. The electrophysiological data from these Nb<sup>-</sup> cells were not included in the Results of the study.

## **Data Analysis**

Analysis of physiological data was performed on 40 - 80 sec periods during the spontaneous, pre-stimulation condition and the stimulation condition, as previously

described in detail (Manns et al., 2000). To include the most stationary and artifact-free periods in the pre-stimulation and stimulation conditions, the epochs at the stimulation onset (~1-2 sec) were excluded since they could be associated with transient DC shifts in the recording. For the EEG, spectral analysis was performed to determine the dominant peak frequency of the power spectra ( $\text{mV}^2/\text{Hz}$ ) in the low frequency end of the spectrum and to calculate the amplitude of beta (15 – 29 Hz) and gamma band activity (30 – 58 Hz) in the high frequency end. The same EEG segments were analyzed by an auto-correlation function (ACF). For unit discharge, average discharge rate was calculated from the peristimulus histogram (PSH), and predominant instantaneous firing frequency from the interspike interval histogram (ISIH). Assessment of rhythmic and higher-order interspike interval tendencies was performed using an auto-correlation histogram (ACH). Determination of the dominant frequency of rhythmic ACHs was done using a fast Fourier transform to convert the ACH data to the frequency domain. Unit discharge was considered “rhythmic”, if the spectrum of the ACH had a peak that was at least 3 times the amplitude of the average power. The spike-triggered average (STA) was used to estimate the extent of cross-correlation between spike-trains and EEG activity and tested for being different from random (shuffled) spike trains with the Wilcoxon test. All analyses of raw data were done using Matlab (5, The MathWorks Inc., Natick, MA) and statistical analyses using Systat (9.0, SPSS Inc., Chicago, IL). Figures were compiled using Adobe Photoshop (5.0 Adobe Systems, Inc., San Jose, CA) for photomicrographs and Origin (5.0, Microcal Software Inc., Northhampton, MA) for plotting electrophysiological data.

### **3.5 Results**

#### **Characterization of EEG and Unit Activity**

Basal forebrain neurons were recorded and characterized in association with EEG activity prior to their juxtacellular labeling. Unit discharge was examined in relation to EEG occurring spontaneously and to that occurring during somatosensory stimulation under the urethane anesthesia (Fig. 1). Although in no case did the stimulation evoke a motoric response, it consistently caused a prominent change in the EEG. Its pattern shifted from irregular slow activity to rhythmic slow activity. The spontaneous irregular slow activity contained prominent slow waves, which are similar to the slow oscillation (0 – 1 Hz) (originally described in cats, (Steriade et al., 1993)), as well as irregular delta waves. Due to variability in the level of anesthesia within and across experiments, the irregular slow activity during the pre-stimulation period varied in peak frequency from 0.3 to 2.4 Hz and in amplitude from 3.0mV to 50 $\mu$ V as reflecting deeper to lighter levels of anesthesia. Also varying as a function of the depth of anesthesia, the EEG change evoked by the somatosensory stimulation (applied as continuous pressure to the tail) could be somewhat transient (~20 sec) or long lasting (up to the full duration of the stimulation which was maintained for ~40 - 80 sec). As measured during the period of stimulation, the evoked rhythmic slow activity varied in frequency from 2.2 to 4.6 Hz and amplitude from 350 to 50  $\mu$ V. Both in its rhythmicity and frequency, this rhythmic slow activity is the same as that previously described on the retrosplenial (or posterior cingulate) cortex and shown to be correlated with rhythmic slow activity in the hippocampus, corresponding to “theta” in urethane-anesthetized rats (Holsheimer, 1982). Here, despite variability in the level of anesthesia resulting in variability in the

spontaneous and evoked frequency and amplitude of the EEG, there was a systematic increase in the average dominant low frequency spectral peak from  $1.14 \pm 0.10$  to  $2.90 \pm 0.14$  Hz ( $t = 11.36$ ,  $df = 41$ ,  $p < 0.001$ ). This increase in low peak frequency was always associated with an increase in rhythmicity as evident in the ACF for the EEG (Fig. 1, II) and was thus interpreted as a shift from irregular slow activity, indicative of the slow oscillation combined with delta activity, to rhythmic slow activity, indicative of a theta-like oscillation. In addition, there was an increase in high frequency EEG activity with somatosensory stimulation, marked by a significant increase in the average amplitude of gamma band activity (30 - 58 Hz;  $t = 3.33$ ,  $df = 38$ ,  $p < 0.01$ ) and no significant change in that of beta band activity (15 - 30 Hz) across experiments. The increase in gamma EEG activity, which has been shown to reflect cortical activation in naturally sleeping-waking rats (Maloney et al., 1997), is considered, together with the parallel appearance of theta-like activity, to reflect an increase in cortical activation evoked by somatosensory stimulation in the urethane-anesthetized rats.

Basal forebrain units were classified into two major categories according to whether they increased or decreased their mean average rate of discharge (by PSH measures) in association with the somatosensory stimulation-induced cortical activation. Although the change in firing rate was not always long-lasting (Fig. 1), the average rate during the stimulation as compared to that during pre-stimulation reflected the predominant response of the units (as verified statistically by t-tests which compared all 1 sec epochs between the two conditions for each unit and were significant for 52/55). Units were respectively referred to as “on” or “off” for simplicity, even though only a

small minority of cells discharged at frequencies of less than 1 Hz during either the pre-stimulation or stimulation conditions.

### **Identification and categorization of Nb+/GAD+ and Nb+/GAD- neurons**

Of 55 basal forebrain neurons, which were successfully labeled with Nb (Nb+) and processed for GAD immunohistochemistry, 21 were found to be GAD-positive (GAD+) and 34 GAD-negative (GAD-, Figs. 2 and 3, Table 1). The Nb+/GAD+ and Nb+/GAD- cells were distributed through the MCPO and SI nuclei of the basal forebrain with a greater number of both located in the MCPO (Table 1). The GAD+ and GAD- cell groups were indistinguishable morphologically, since both were comprised of proportions of oval-to-fusiform (bipolar) and polygonal (multipolar) neurons (Table 1). The GAD+ neurons were all medium-to-large neurons (range: 16.7 – 30.0  $\mu\text{m}$  in large diameter), whereas the GAD- neurons, were comprised of some small neurons in addition to medium-to-large ones (range: 13.0 – 31.1  $\mu\text{m}$ ).

The major proportion of the cells in the total sample were “on” cells (67%) and a minor proportion were “off” cells (33%). Between the GAD+ and GAD- cell groups, the respective proportions of “on” and “off” cells differed significantly, such that the GAD+ neurons represented the largest proportion of the “off” and the GAD- cells the largest proportion of the “on” cells in the sample (Table 1). In fact, the majority of the GAD+ neurons (60%) decreased their average discharge rate in response to stimulation while the majority of the GAD- cells (82%) increased their average discharge rate (Table 1). Across the pre-stimulation and stimulation conditions, the discharge patterns of the sampled units were highly diverse. The predominant patterns were categorized and the

cells accordingly grouped as manifesting across the two conditions: 1) a tonic mode of discharge, 2) a cluster mode of discharge in addition to tonic or irregular spiking or 3) a burst mode of discharge in addition to tonic or irregular spiking (Table 1). The burst mode was characterized as recurring high frequency spike bursts (>80Hz), and the cluster mode distinguished from bursting as recurring trains of spikes lacking in high frequency spike bursts (Manns et al., 2000). GAD+ and GAD- cell groups comprised cells of the three categories and did not differ in the proportions of cells in each (Table 1). However, they did differ in the precise relationship of the unit discharge pattern to EEG activity (below). During stimulation-evoked cortical activation, the proportion of GAD+ neurons displaying low frequency rhythmic discharge was less than that of the GAD- neurons (24% versus 44%; Table 1, though not significantly so,  $p = 0.13$ ). Similarly, the proportion of GAD+ neurons exhibiting a cross-correlated discharge with EEG activity at low frequency was also less than that of the GAD- neurons (19% versus 44%; Table 1,  $p = .051$ ). The proportion of GAD+ cells displaying high frequency regular discharge, which met the criterion of rhythmic, (33%) was higher than that of the GAD- cells (15%;  $\chi^2 = 7.31$ ,  $df = 1$ ,  $p = 0.007$ ).

### **Characteristics of Nb+/GAD+ cell subgroups**

Although the Nb+/GAD+ cells could be characterized as comprising a majority of “off” and tonically discharging cells, their precise response to stimulation according to both frequency and pattern of discharge differentiated these cells into multiple subgroups with distinctive features. Of the GAD+ “on” cells, all fired in a predominantly tonic mode and were accordingly categorized as *on (tonic)*. As a subgroup, they showed a significant

increase in their average rate of discharge with stimulation (Table 3). The GAD+ “off” cells, which collectively showed a significant decrease in their average rate of discharge with stimulation (Table 3), fired in different patterns and were also accordingly further subdivided. Some cells, displaying a decrease in average discharge rate, discharged tonically during the pre-stimulation condition and discharged in spike clusters during stimulation with cortical activation, such that they were categorized as *off (tonic/cluster)* cells (Table 3). Other cells displaying a decrease in average discharge rate, discharged in high frequency bursts during the pre-stimulation condition to virtually cease firing with stimulation and thus be categorized as *off (burst)* cells (Table 3). Lastly, other cells showing a decrease in rate discharged tonically or irregularly during both the pre-stimulation and stimulation conditions, accordingly being called *off (tonic)*. The two major subgroups of GAD+ cells (“on” and “off”) differed morphologically in terms of their average large diameter with the “off” cells being larger than the “on” cells ( $t = 2.055$ ,  $df = 18$ ,  $p = 0.055$ ; Table 2). They did not differ according to their average spike width. Not all cell types could be antidromically activated from the prefrontal cortex, but the latency of antidromic activation did not differ among the subgroups which were so activated (Table 2).

The Nb+/GAD+ *on (tonic)* cells (mapped in Fig. 3) represented the single largest subgroup of GAD+ neurons sampled (40%; Table 3). Several could be antidromically activated from the prefrontal cortex ( $n = 3$ ; Table 2). As illustrated in an exemplary cell (Fig. 4 corresponding to Fig. 2A), these *on (tonic)* cells increased discharge rate with stimulation and fired in a tonic mode at a moderately fast rate (up to 65 Hz according to PSH values), often during both pre-stimulation and stimulation conditions (Table 3). As

for the cell illustrated (Fig. 4), GAD+ *on (tonic)* cells showed very regular spiking at relatively high frequencies (~10 – 50 Hz, according to ACH values). This spiking was considered to be rhythmic (according to the established criterion detailed in Methods) for some cells (4/8) during irregular slow activity and for most cells (7/8) during the rhythmic slow activity and accompanying increased gamma on the EEG, which reflect stimulation-evoked cortical activation (Table 3).

The Nb+/GAD+ *off (tonic/cluster)* neurons (mapped in Fig. 3), were the second largest subgroup of GAD+ neurons sampled (25%; Table 3). None of these could be antidromically activated from the prefrontal cortex. As shown for an exemplary cell (Fig. 5 corresponding to the cell in Fig. 2B), their firing pattern was tonic and moderately fast (10 – 30 Hz) during pre-stimulation with irregular slow cortical activity, then shifted to a rhythmic cluster spike discharge with stimulation-induced rhythmic slow activity. Although their average rate of discharge decreased significantly with stimulation, their instantaneous firing frequency did not, and the stimulation within cluster spike frequency (15 to 55 Hz) was similar to the pre-stimulation tonic spike frequency (10 to 40 Hz; Fig. 5 and Table 3). The cluster discharge during stimulation occurred rhythmically (at 1 to 2 Hz) in all *(tonic/cluster)* cells. It was also cross-correlated with the retrosplenial EEG signal in most cells (Fig. 5 and Table 3), although typically not with the predominant spectral peak, but with a secondary peak of lower frequency. The rhythmic cluster discharge of some of these units was cross-correlated with the dominant spectral peak of the EEG signal from the prefrontal cortex (not shown). The within cluster spike discharge was often rather regular (as for the cell shown in Fig. 5), though it did not reach criterion for being classified as rhythmic.

The Nb+/GAD+ *off (burst)* cells (mapped in Fig. 3) represented 20% of the GAD+ neurons sampled (Table 3). Most of these cells were antidromically activated from the prefrontal cortex (n = 3; Table 2). As illustrated for an exemplary cell (Fig. 6 corresponding to Fig. 2C), they typically fired in high frequency bursts (up to 550 Hz from ISIH values) during irregular slow cortical activity and often ceased firing altogether during stimulation-induced cortical activation. The burst firing of these neurons was reflected in the large difference between the average discharge rates (from the PSH) and instantaneous firing frequencies (from the ISIH) during both pre-stimulation and stimulation conditions (Table 3). The burst discharge during pre-stimulation occurred at similar frequencies (<1 Hz) as the irregular slow waves on the cortex and as for the cell illustrated (Fig 6), was in some cases cross-correlated with the slow EEG activity (Table 3).

The Nb+/GAD+ *off (tonic)* cells (mapped in Fig. 3) represented the smallest proportion of the GAD+ neurons sampled (15%; Table 3). None of these cells could be antidromically activated from the prefrontal cortex (Table 2). As for the cell illustrated (Fig. 7 corresponding to Fig. 2D), GAD+ *off (tonic)* neurons tended to discharge in a slow (< 5 Hz average rate according to PSH values) and irregular tonic manner in association with irregular slow cortical activity and to cease firing with stimulation-induced cortical activation (Table 3). The spiking by these neurons was in some cases cross-correlated with the irregular slow cortical activity (Table 3).

### **Characteristics of Nb+/GAD- cell subgroups**

That the majority of the Nb+/GAD- neurons were “on” cells (Table 1) was reflected in a significant increase in their mean average discharge rate with stimulation (Table 3). In their response to stimulation, they thus differed significantly as a group from the Nb+/GAD+ cells (Table 3). Moreover, their average discharge rate during both pre-stimulation and stimulation conditions was significantly slower than that of the GAD+ “on” cells (Table 3). In further contrast, the GAD- “on” neurons were comprised of subgroups of cells with predominant tonic, cluster, and burst discharge patterns (Table 3). The GAD- “off” subgroup significantly decreased its average discharge rate with stimulation and was not different in this regard from the GAD+ “off” subgroup. However, the GAD- “off” subgroup was different in being homogeneous and composed entirely of tonically discharging cells. The GAD- cells were on average significantly smaller than GAD+ cells, and the GAD- “off” cells were also significantly smaller than the GAD+ “off” cells (Table 2). They did not differ significantly according to their spike width or latency of antidromic activation (Table 2).

The Nb+/GAD- *on (tonic)* neurons (mapped in Fig. 3) represented the largest group of GAD- neurons (35%; Table 3). Many of these cells were antidromically activated from the prefrontal cortex (Table 2). As for the cell illustrated (Fig. 8 corresponding to Fig. 2E), these cells tended to fire regularly and to increase their average rate of discharge (PSH) without markedly changing their instantaneous firing frequency (ISIH; Table 3). When compared to the Nb+/GAD+ *on (tonic)* subgroup, the average discharge rate of the GAD- *on (tonic)* cells was significantly slower across conditions (Table 3). Moreover, a lower proportion of these GAD- cells (17%) displayed rhythmic high frequency spiking during stimulation than the GAD+ *on (tonic)*

cells (88%;  $\chi^2 = 19.87$ ,  $df = 1$ ,  $p < .001$ ; Table 3). They did not differ significantly in size, spike width or latency of antidromic activation from the GAD+ *on (tonic)* cells.

As a group, the Nb+/GAD- *on (cluster)* neurons (mapped in Fig. 3) represented the second largest subgroup of GAD- neurons (29%; Table 3). None of these cells could be antidromically activated from the prefrontal cortex. Their average rate of discharge increased significantly with stimulation (Table 3) and simultaneously shifted from tonic or irregular spiking to a regular cluster discharge in association with stimulation-induced cortical activation (not shown). This cluster discharge was rhythmic in all cells (Table 3). In the majority of cases, their rhythmic discharge was also cross-correlated with a signal in the retrosplenial EEG (Table 3), though usually corresponding to a slower frequency, secondary peak here and the primary peak on the prefrontal cortex in the power spectra (not shown). Some of these cells showed very regular to rhythmic high frequency spiking within the spike clusters during stimulation (Table 3). During the stimulation-evoked cortical activation, the discharge pattern of the GAD- *on (cluster)* neurons was similar to that of the GAD+ *off (tonic/cluster)* neurons. During the pre-stimulation irregular slow activity however, they differed, (as reflected in their different response to stimulation) having a significantly lower average discharge rate than the GAD+ *off (tonic/cluster)* cells ( $t = -2.729$ ,  $df = 13$ ,  $p = 0.017$ ).

The Nb+/GAD- *on (burst)* neurons (mapped in Fig. 3) represented 18% of the GAD- cell sample (Table 3). One of these neurons was antidromically activated from prefrontal cortex (Table 2). These cells discharged in an irregular manner during pre-stimulation conditions, and shifted their discharge to high frequency bursting with stimulation (not shown). They displayed a significant increase in average rate of

discharge (PSH) and greater, although insignificant, increase in instantaneous firing frequency (ISIH; Table 3), reflecting their bursting activity. The discharge of most cells was rhythmic, and cross-correlated with the retrosplenial EEG during stimulation (Table 3), although with activity corresponding to a secondary peak on the retrosplenial EEG and a primary peak on the prefrontal cortex. These GAD- *on (burst)* neurons did not resemble any GAD+ cells in discharge characteristics across conditions.

The Nb+/GAD- “off” neurons (mapped in Fig. 3) accounted for a small proportion of the GAD- cells sampled (18%, Table 3) and were composed entirely of *off (tonic)* neurons. One of these cells was antidromically activated from prefrontal cortex (Table 2). They tended to fire in a irregular slow tonic pattern with irregular slow cortical activity and to cease firing with stimulation (not shown). They did not differ from Nb+/GAD+ *off (tonic)* neurons in their response to stimulation and average discharge rates across conditions. They were on average smaller in size than the GAD+ homologous subgroup ( $t = -2.157$   $df = 12$ ,  $p = 0.052$ , Table 2).

### 3.6 Discussion

This, to our knowledge, is the first physiological characterization of immunohistochemically identified GABA-synthesizing basal forebrain neurons. GABAergic cells behave differently from non-GABAergic cells in terms of their discharge profile in relation to cortical activity. They comprise unique and distinct subgroups of neurons, that could collectively serve in the dual role of the basal forebrain in promoting cortical activation during waking and attenuating cortical activation during slow wave sleep. Moreover, particular subgroups display phasic discharge patterns that may modulate rhythmic gamma or theta oscillations during cortical activation and others, irregular slow wave activity during cortical slow wave activity.

A minority, though significant (40%), of the GABAergic neurons increased their average discharge rate with somatosensory-evoked cortical activation, as compared to the vast majority (~80%) of non-GABAergic neurons in the current sample and the totality of cholinergic neurons in our previous sample (Manns et al., 2000). All these GAD+ “on” cells discharged in a tonic mode and were distinguished from the GAD- “on” cells, including specifically the *on (tonic)* cells, by their higher frequency discharge (up to 65 Hz). They were furthermore distinguished by the regularity of their tonic discharge, such that it met criterion for being rhythmic at high frequency in almost all cells during cortical activation. Their range of firing frequencies extended across the ranges of EEG activities corresponding to beta (15 to 30 Hz) and gamma (30 – 60 Hz) activities, and shifted on average from a beta range into a gamma range with somatosensory stimulation. In naturally sleeping-waking rats, beta activity is higher during slow wave sleep than during waking, while gamma activity is higher during waking and highest during active

or attentive waking behavior (and during paradoxical sleep), thus reflecting cortical activation in the rat (Maloney et al., 1997). Here, gamma EEG activity was significantly higher during somatosensory-stimulation than during the pre-stimulation condition. Large in size and antidromically activated from the pre-frontal cortex, these presumed cortically projecting GABAergic neurons, could contribute by their high frequency rhythmic discharge to the promotion and/or pacing of gamma EEG activity. Such action could be achieved through the cortical inhibitory interneurons, which GABAergic basal forebrain neurons innervate (Freund and Meskenaite, 1992), by the rhythmic timing of their discharge with IPSPs. Accordingly, they would act in parallel with the cholinergic basalo-cortical neurons, since the cholinergic neurons increase firing in association with increased gamma (Manns et al., 2000), and ACh promotes gamma by providing a long-lasting facilitation to cortical interneurons and pyramidal cells (Metherate et al., 1992; Buhl et al., 1998).

The majority of GAD+ neurons decreased their average discharge rate with somatosensory stimulation-evoked cortical activation and were, for simplicity, categorized as “off” cells. These “off” cells could be further subdivided into distinct subgroups. Among these, the *off (tonic/cluster)* cells were unique in their discharge profile. During pre-stimulation, they fired in a tonic spike discharge at a relatively high rate, in what would correspond to a beta range of EEG frequencies. During stimulation, though they decreased their average rate of discharge, they shifted to a rhythmic cluster spike discharge, that was cross-correlated with the stimulation-evoked theta-like EEG activity. They could thus potentially maintain target neurons in a non-rhythmic mode by their tonic discharge during irregular slow wave activity and then in a rhythmic mode by

their cluster discharge during rhythmic slow activity. They would accordingly subserve a dual role in preventing theta during slow wave sleep and then promoting theta during waking (and paradoxical sleep). Non-cholinergic neurons, that have been presumed to be, though not yet identified as, GABAergic neurons in the medial septum also discharge rhythmically and appear to be important in stimulating theta activity in the hippocampus (Lee et al., 1994; Dragoi et al., 1999). The cluster discharge of the GAD+ *off (tonic/cluster)* cells recorded here resembled the cluster discharge of the GAD- *on (cluster)* cells. Together, the cluster pattern of discharge of these two cell groups resembled that of non-cholinergic cells previously described *in vitro* in the basal forebrain (Alonso et al., 1996). Given the relationship of their cluster discharge to the EEG activity and their relatively large size, the GAD+ *off (tonic/cluster)* neurons, like the GAD- *on (cluster)* neurons, are likely to project to cortical areas (Zaborszky et al., 1986; Gritti et al., 1997), even though they could not be antidromically activated from the prefrontal cortex. As previously found for the rhythmic bursting discharge of the cholinergic cells (which would correspond to the GAD- *on (burst)* cells in the present sample), the rhythmic discharge of the cluster spiking cells tended to be cross-correlated with rhythmic slow activity that was slower than that of the primary retrosplenial theta-like activity and more similar to that of the prefrontal activity. Accordingly, the GABAergic cluster discharging neurons could serve, along with the other non-GABAergic cluster discharging cells, in parallel with the burst discharging cholinergic neurons to modulate cortical activity in a rhythmic slow manner at frequencies within the theta range but particular to a cortical region as well as behavioral state (Manns et al., 2000).

The GAD+ *off (burst)* and *off (tonic)* cells arrested their discharge in association with cortical activation. With respect to cortical activity, their profile of discharge is similar to that of neurons in the basal forebrain and adjacent preoptic area that were characterized as “sleep-active” in naturally sleeping-waking, freely moving animals (Szymusiak and McGinty, 1986; Koyama and Hayaishi, 1994). The majority of “sleep-active” cells were inhibited by stimulation of the midbrain reticular formation, locus coeruleus or by iontophoretic application of noradrenaline (NA) (Szymusiak and McGinty, 1989; Osaka and Matsumura, 1994; Osaka and Matsumura, 1995). In brain slices, a small proportion of non-cholinergic basal forebrain neurons were identified that were hyperpolarized by NA and ACh, and thus proposed to potentially represent slow wave sleep-active neurons (Fort et al., 1998). Also in brain slices more recently, a major proportion of neurons in the adjacent lateral preoptic area has been shown to be inhibited by these neurotransmitters and identified (by PCR) as potentially GABAergic (Gallopín et al., 2000). Together with the present results, it would appear that a population of GABAergic neurons distributed through the basal forebrain and in partial continuity with cells in the preoptic region, which are inhibited by ascending activating impulses transmitted in part by noradrenergic and cholinergic fibers (Jones and Cuello, 1989), may serve to dampen cortical activation and promote slow wave sleep.

The GAD+ *off (burst)* cells fired high frequency bursts associated with the irregular cortical slow waves during the pre-stimulation condition. These neurons appear similar to a type of “sleep-active” cell recorded in the preoptic area and adjacent basal forebrain of freely moving rats which discharged in a phasic manner during slow wave sleep (Koyama and Hayaishi, 1994). They may also be similar to cells in the septum that

discharge in association with hippocampal sharp waves (Dragoi et al., 1999). GAD+ *off (burst)* cells were identified by antidromic activation as cortically projecting neurons and their discharge was cross-correlated with the irregular slow activity on the cortex. They could accordingly fire in a synchronous manner with the ( $< 1$  Hz) slow oscillation (or delta waves, 1 – 4 Hz) of slow wave sleep, during which cortical neurons discharge in a highly synchronous slow manner (Steriade et al., 1993). GABAergic *off (burst)* basal forebrain cells could thus attenuate fast cortical activity while modulating irregular slow activity during slow wave sleep.

Nb+/GAD+ *off (tonic)* neurons discharged in single spikes at very low average rates ( $< 5$  Hz) in association with irregular slow cortical activity and virtually ceased firing with cortical activation ( $< 1$  Hz). These cells appear to be similar to “sleep-active neurons” that were described in the cat as firing at  $< 10$  Hz during slow wave sleep and  $< 1$  Hz during waking (Szymusiak and McGinty, 1986). Some of the latter neurons were antidromically activated from the cortex, others from the brainstem (Szymusiak and McGinty, 1989). In the present sample, none of the GAD+ *off (tonic)* cells could be antidromically activated from prefrontal cortex. However, given the relatively large size of these GAD+ cells, it is likely that they are projection neurons and could project to cortical areas (Gritti et al., 1997). On the other hand, given the full range of cell sizes among projecting cells, it is also possible that they could project to subcortical regions, including the thalamus (Asanuma and Porter, 1990; Gritti et al., 1998) and posterior hypothalamus or brainstem (Gritti et al., 1994). Accordingly, they could be involved in attenuating cortical or subcortical activity in a slow tonic manner during slow wave sleep.

In conclusion, the unique and distinct GABAergic basal forebrain cell subgroups identified here reveal GABAergic neurons with the potential to act in parallel with cholinergic and other non-cholinergic cortically projecting neurons such as to promote and/or pace gamma activity (by GAD+ *on (tonic)* cells) and theta-like activity (by GAD+ *off (tonic/cluster)* cells) during the cortical activation of waking (and/or paradoxical sleep). GABAergic cells would also have the capacity to attenuate cortical activation, including theta activity (by the GAD+ *off (tonic/cluster)* cells), and modulate cortical activity at very slow frequencies (by GAD+ *off (burst)* cells) or induce other cortical, subcortical or behavioral changes (by GAD+ *off (tonic)* cells) associated with slow wave sleep.

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### 3.8 Figures

Figure 1. Example of EEG and unit activity patterns prior to and during somatosensory stimulation.

I. EEG (A) from retrosplenial cortex and unit discharge rate (B, peristimulus histogram (PSH) of the rate of discharge in spikes per second) for periods preceding and during somatic stimulation conditions. The EEG (C) and unit discharge (D) traces are expanded for the period of transition from irregular slow cortical activity to rhythmic slow activity. Note the change in pattern of the EEG activity and concomitant increase in rate of tonic discharge by the unit.

II. EEG analysis during pre-stimulation and stimulation conditions. EEG autocorrelation functions (ACF, in A, with correlation coefficient on vertical axes) and power spectra (with low and high frequency ranges respectively placed in left and right insets) are shown for pre- and stimulation records. These indicate the shift from low frequency irregular slow activity to a higher frequency rhythmic slow activity with stimulation, in addition to the concomitant increase in gamma amplitude seen in the high frequency power spectra. (Data from neuron #9918014.)

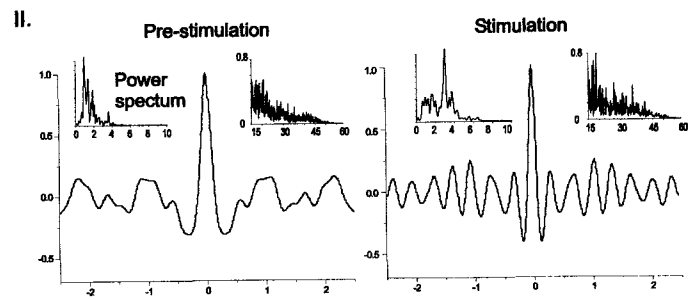
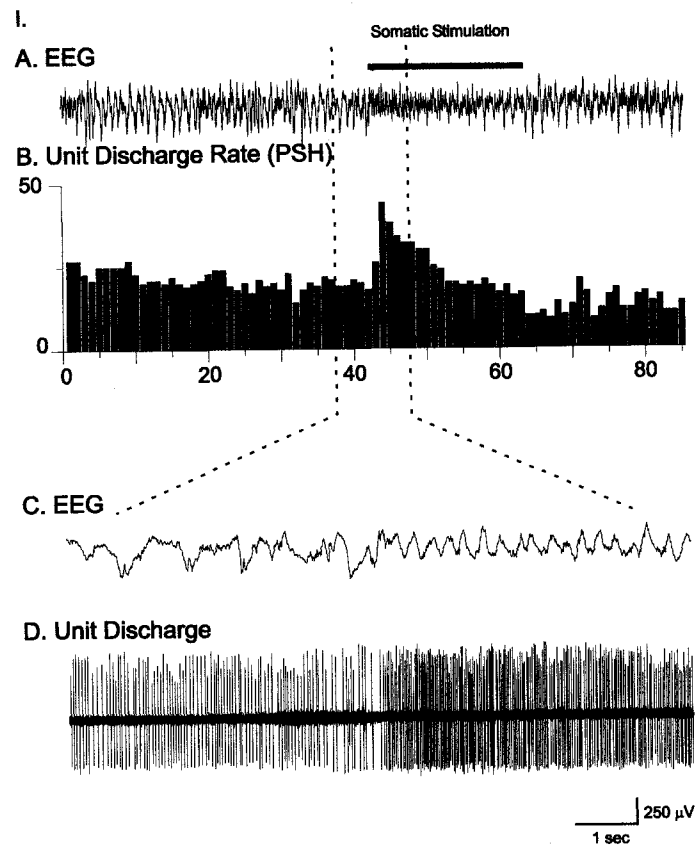
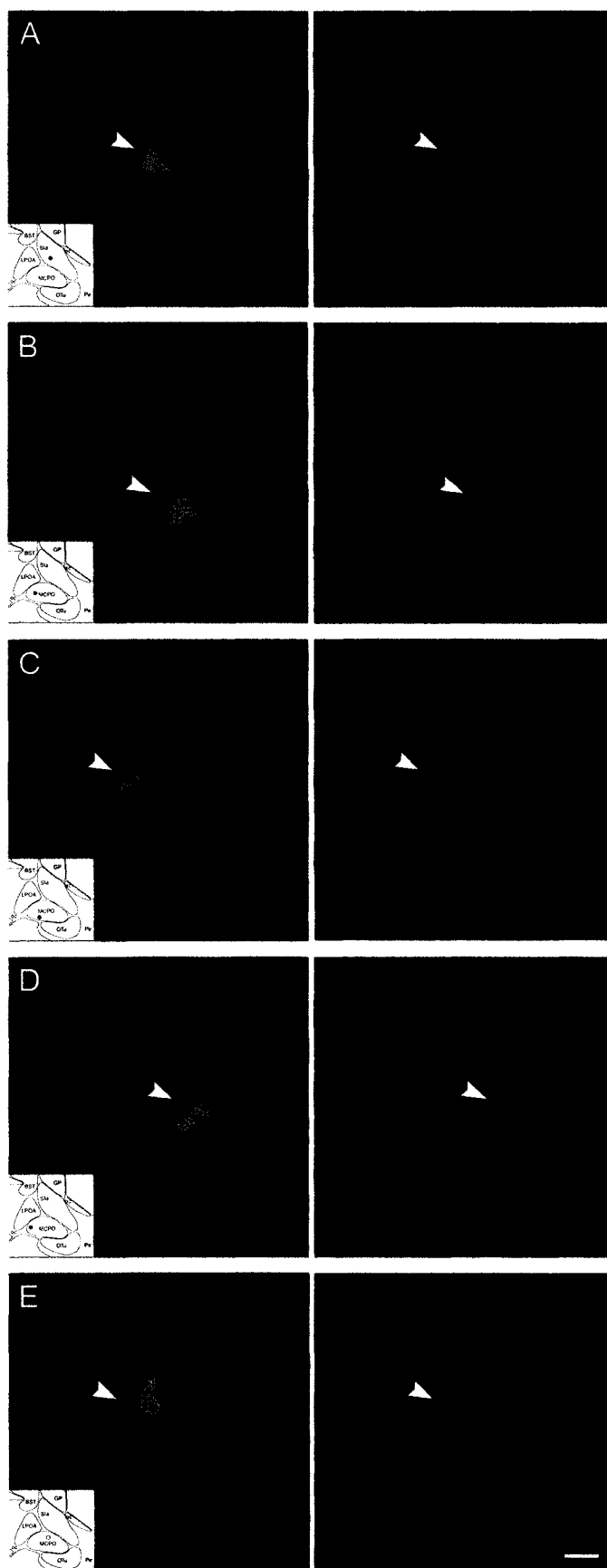


Figure 2. Photomicrographs of recorded and juxtacellularly labeled Nb+/GAD+ or Nb+/GAD- neurons located in the basal forebrain cholinergic cell area. Nb was revealed with green fluorescent Cy2-conjugated streptavidin (left) and GAD-immunostaining with red fluorescent Cy3-conjugated secondary antibodies (right). (A) Nb+/GAD+ *on (tonic)* neuron in SI. (B) Nb+/GAD+ *off (tonic/cluster)* neuron in MCPO. (C) Nb+/GAD+ *off (burst)* neuron in MCPO. (D) Nb+/GAD+ *off (tonic)* neuron in MCPO. (E) Nb+/GAD- *on (tonic)* neuron in MCPO. The location of each cell is shown in the atlas inset to the lower left of each cell, and together with other cells, as the largest symbol for its subgroup in Fig. 2; the recording of each cell (A, B, C, D, and E) is shown in order in subsequent figures (3, 4, 5, 6, and 7 respectively). Scale bar 20µm.



**Figure 3. Location of Nb+/GAD+ and Nb+/GAD- neurons in the basal forebrain**  
(represented on atlas sections adapted from (Gritti et al., 1993)). Each subgroup of GAD+ cells (triangular symbols) and GAD- cells (circular symbols) is represented by a particular symbol (as indicated in the figure), and the exemplary cell from each subgroup (illustrated in Fig. 1 A-E and Fig. 3 - 7, respectively) is represented by the largest symbol. Scale bar 1mm.

Abbreviations: Acb, accumbens nucleus; ac, anterior commissure; BST, bed of the stria terminalis; CPu, caudate putamen; DBB, diagonal band of Broca nucleus ; f, fornix; FStr, fundus of striatum; GP, globus pallidus; LPOA, lateral preoptic area; LS, lateral septum; MCPO, magnocellular preoptic nucleus; MS, medial septum; oc, optic chiasm; OTu., Olfactory tubercle; Pir, Piriform cortex; Ret, Reticularis nucleus; SIa, substantia innominata pars anterior; SIp, substantia innominata pars posterior; sm, stria medullaris.

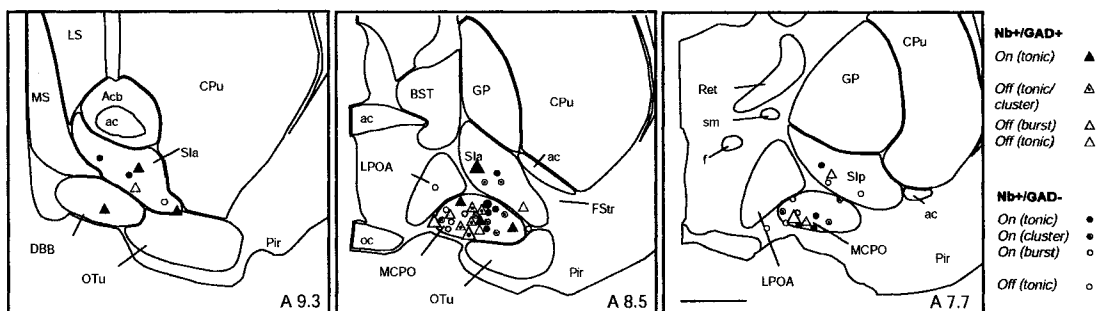


Figure 4. Nb+/GAD+ *on (tonic)* cell (#98o18009/11 shown in A of Fig. 1).

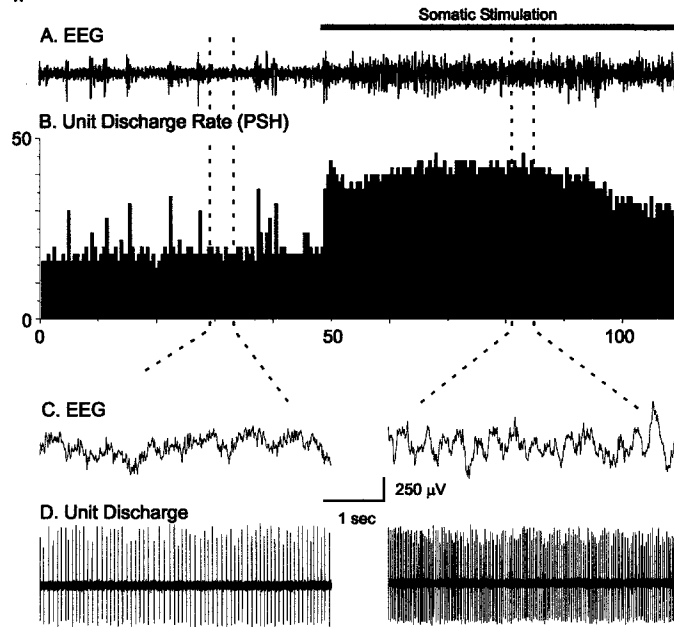
I. EEG and unit recording during pre-stimulation and stimulation conditions. EEG (A) from retrosplenial cortex and unit discharge rate (B, peristimulus histogram, PSH, plotting spikes per second) are shown for periods preceding and during somatic stimulation. The EEG (C) is expanded and shown with the unit traces (D) for each condition (below, left and right). Note that the unit discharge is tonic during both the pre-stimulation and stimulation conditions and increases in rate in association with the stimulation-evoked EEG changes from irregular slow to rhythmic slow activity, indicative of cortical activation.

II. EEG and unit analysis during pre-stimulation and stimulation conditions. EEG autocorrelation functions (ACF, in A, with correlation coefficient on vertical axes) and power spectra (with low and high frequency ranges respectively placed in left and right insets) are shown for pre- and stimulation records. These illustrate the shift from low frequency irregular slow activity to a higher frequency rhythmic slow activity with stimulation, and the concomitant increase in gamma amplitude seen in the high frequency range of the power spectra. Unit autocorrelation histograms (ACH, in B, with normalized incidence on vertical axes) and interspike interval histograms (ISIH, in insets on right) are shown for the same records. An expansion of the ACH is shown (below each) for shorter intervals along with the spectra of the ACH (as insets in upper right corners in which † indicates rhythmic activity according to established criterion). Note that the unit discharge is moderately high and relatively regular during the pre-stimulation condition with a frequency at ~ 20 Hz and higher and very regular (such as to be considered

rhythmic) with a frequency at  $\sim 40$  Hz during the stimulation-evoked increase in gamma activity and cortical activation.

(A) Nb+/GAD+ On (tonic) cell

I.



II.

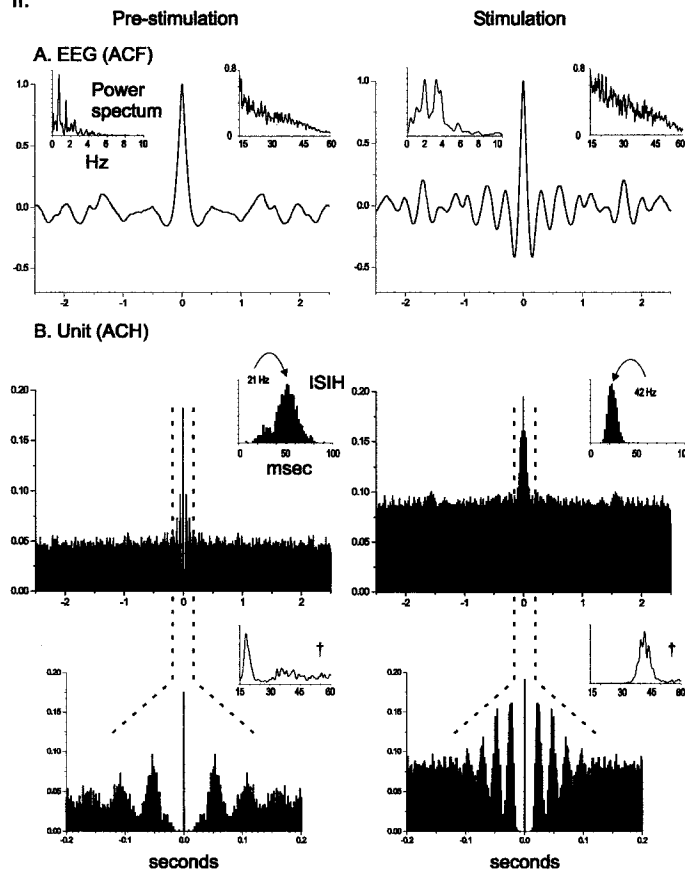


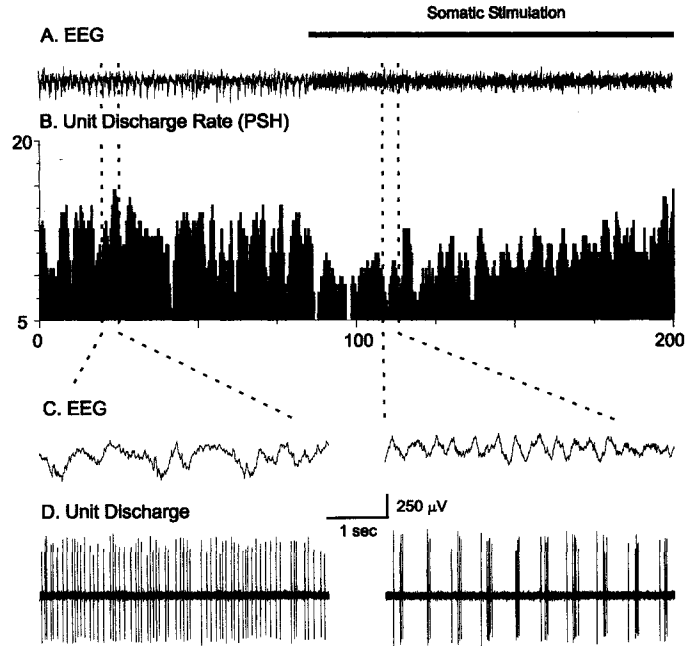
Figure 5. Nb+/GAD+ *off* (tonic/cluster) cell (#03/25/LM2 shown in B of Fig. 1).

I. Note in the PSH (shown in B), that the average rate of discharge decreases in association with cortical activation (evident in A). At the same time, the pattern of discharge changed from tonic spiking during pre-stimulation to a cluster discharge pattern (D) in association with the appearance of rhythmic slow activity that occurred on the EEG (C) during stimulation.

II. Note in the analysis, that upon stimulation, the EEG shifted from irregular slow activity to a faster rhythmic slow activity, accompanied by an increase in gamma activity. During stimulation the unit discharge is rhythmic (in the ACH shown in B) at  $\sim 2$  Hz (as evident in the power spectrum of the ACH shown in inset on upper left). The expanded ACH for the stimulation condition (drop down) indicates that the high frequency activity within the spike clusters is relatively regular at  $\sim 40$  Hz (but does not meet criterion for being rhythmic). Spike-triggered averages (STA, shown in C) of the unit-to-EEG cross-correlation (with mV on vertical axes) indicate that the correlation for the unit (black line) is significantly different from that for the randomized-spike train (gray line, Wilcoxon test,  $p < 0.05$ , \*). The power spectrum for the unit-to-EEG STA is shown (in inset). Note that the cross-correlated unit-to-EEG activity occurs at a frequency of  $\sim 2$  Hz, which did not correspond to the prominent EEG rhythmic slow activity or spectral peak but to a secondary peak in the power spectrum (shown in A with inset on upper left). See Fig. 3 for further explanation of measures.

(B) Nb+/GAD+ Off (tonic/cluster) cell

I.



II.

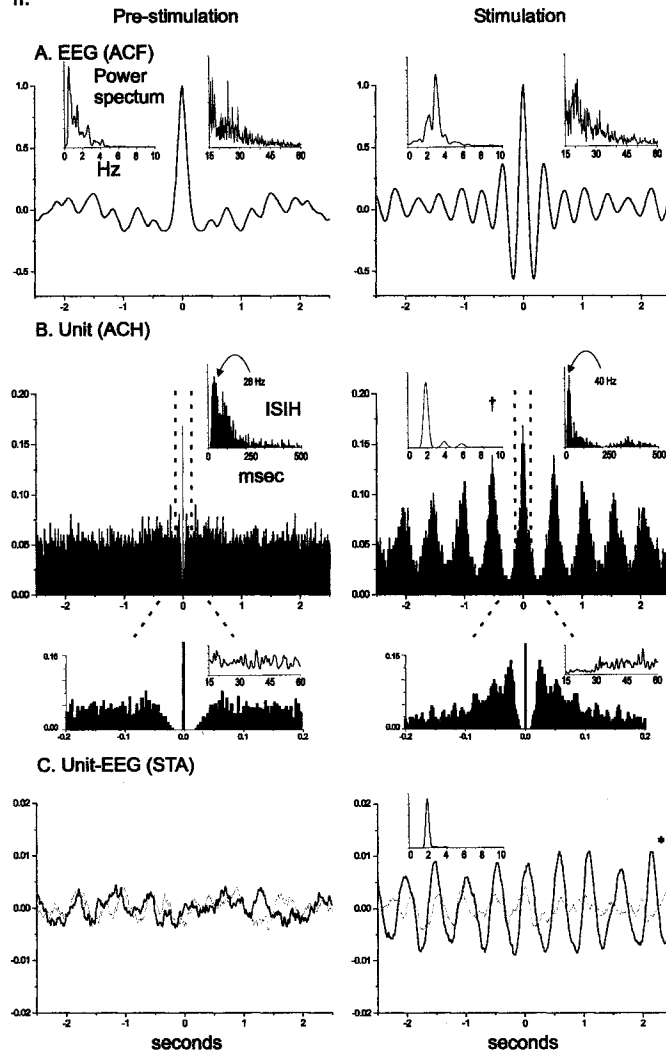


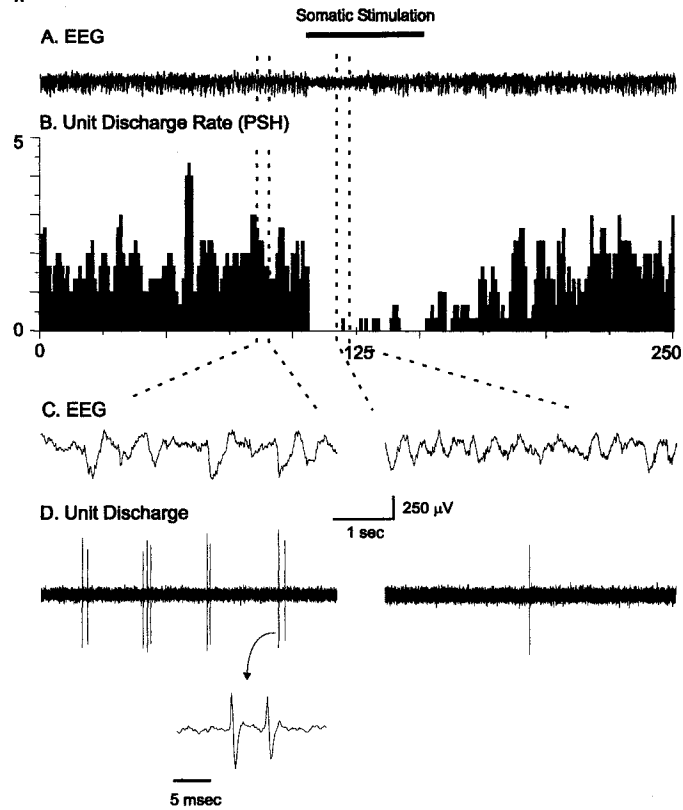
Figure 6. Nb+/GAD+ *off* (*burst*) neuron (#98n030016/19 shown in D of Fig. 1).

I. Note the virtual cessation of discharge (in the PSH in B) with somatic stimulation that evokes cortical activation in the EEG (A). In association with the irregular slow EEG activity during pre-stimulation (C), high frequency bursting occurs (D, including inset with blow up below on left).

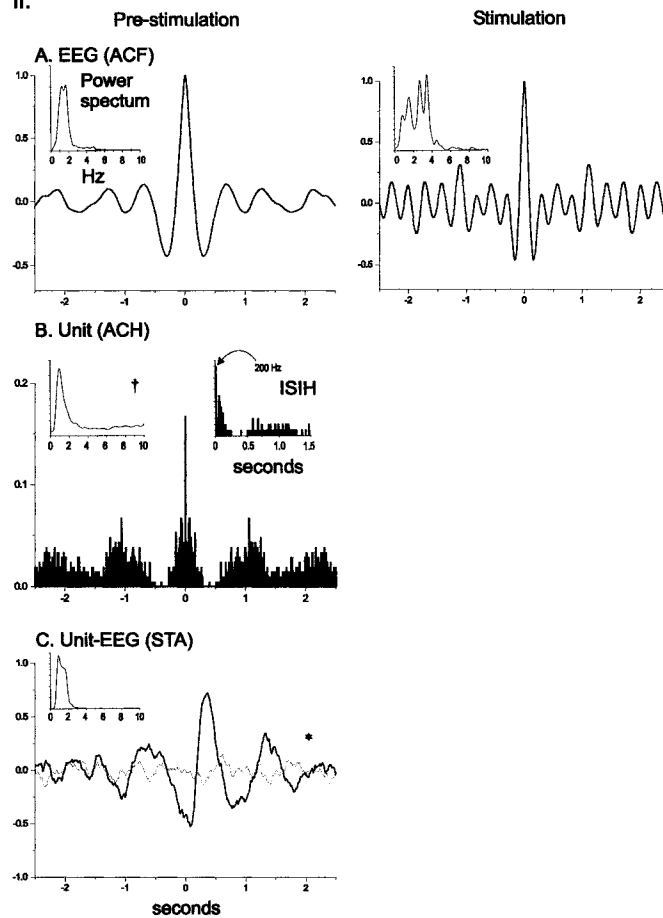
II. Note in the analysis of the EEG a shift from irregular slow to faster rhythmic slow activity with stimulation. The unit analysis for pre-stimulation indicates the high frequency spike mode (at ~ 200 Hz in the ISIH in B) reflecting the bursting and the slower frequency activity reflecting the recurrence of the bursting at a similar frequency as the EEG activity (at ~ 1.2 Hz, evident in the ISIH, ACH and spectrum of the ACH for the unit in B and in the spectrum of the ACF for the EEG in A). The unit discharge was significantly cross-correlated with the EEG (as shown in the STA in C) at this same frequency (evident in power spectrum in inset). See Figs. 3 and 4 for further explanation of measures.

(C) Nb+/GAD+ Off (burst) cell

I.



II.



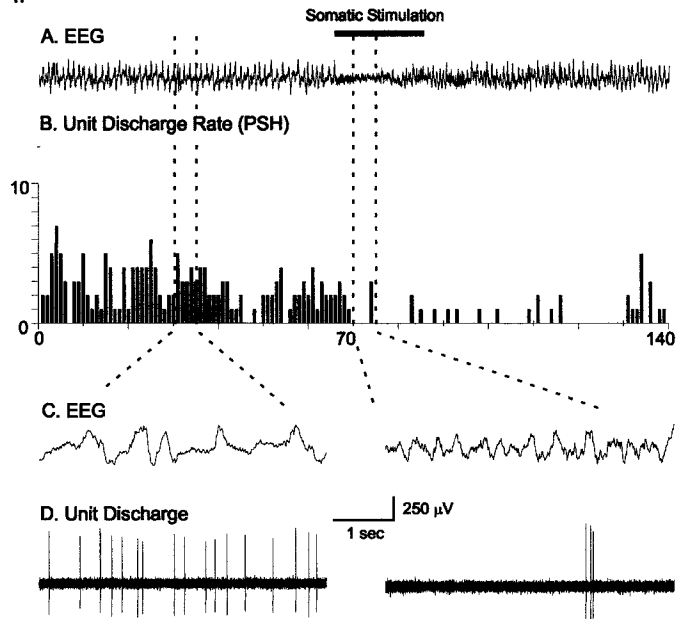
**Figure 7. I. Nb+/GAD+ *off (tonic)* cell (#98o16010 shown in C of Fig 1).**

**I. Note in the PSH (B), the marked decrease in discharge, and in the recording (C), the relatively tonic slow discharge of the unit in association with the irregular slow activity of the EEG (C).**

**II. Note in the analysis, the shift in EEG activity from the irregular slow activity to a higher frequency rhythmic slow activity. Also note, the slow (8.5 Hz mode in ISIH) and relatively irregular discharge of the unit (B) and the lack of relationship with the EEG (B and C). See Figs. 3 and 4 for further explanation of measures.**

(D) Nb+/GAD+ Off (tonic) cell

I.



II.

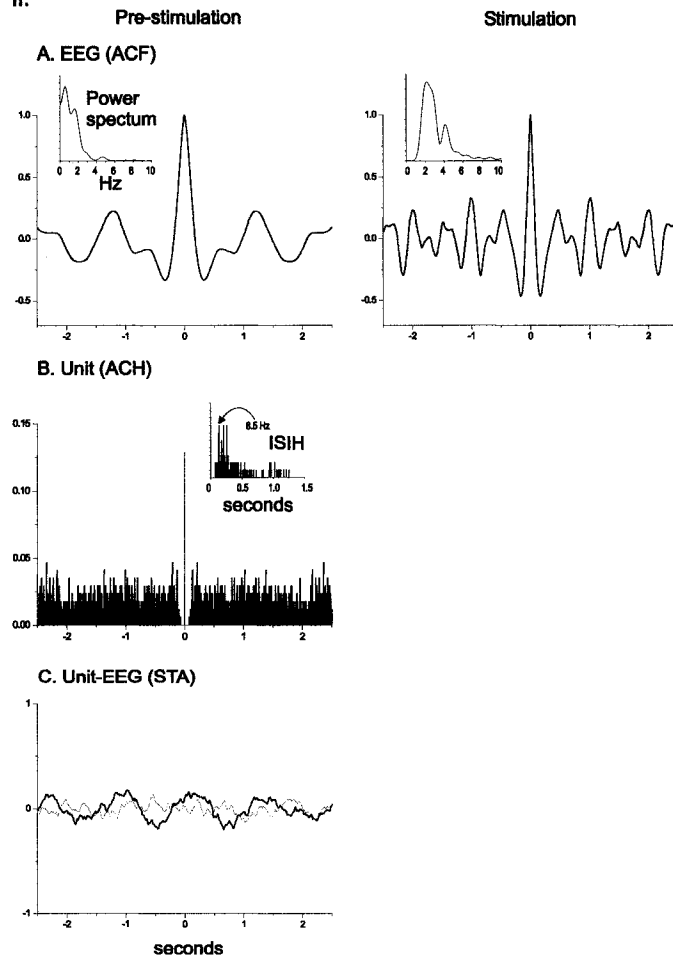


Figure 8. Nb+/GAD- *on (tonic)* neuron (#03/23/R2 as shown in E of Fig. 1).

I. Note the increase in average discharge rate (B) and regularity of discharge which becomes clearly tonic (D) in association with cortical activation (A and C) during stimulation.

II. Note in the analysis that with stimulation the EEG shifts from irregular slow activity to a faster rhythmic slow activity, concomitant with an increase in gamma activity. The unit's tonic discharge shows no sign of rhythmicity in the high frequency range (B with drop down) in contrast to the Nb+/GAD+ *on (tonic)* cell (shown in Fig. 3). See Fig. 3 for further explanation of measures.



### 3.9 Tables

**Table 1. Frequency of anatomical and physiological characteristics in GABAergic and non-GABAergic cell groups.<sup>a</sup>**

|                                                                           | <u>All cells</u> | <u>Nb+/GAD+ cells</u> | <u>Nb+/GAD- cells</u> | <u>Statistic:</u><br><u><math>\chi^2</math>(df)</u> |
|---------------------------------------------------------------------------|------------------|-----------------------|-----------------------|-----------------------------------------------------|
| n                                                                         | 55               | 21                    | 34                    |                                                     |
| <b>ANATOMY</b>                                                            |                  |                       |                       |                                                     |
| <b>Area</b>                                                               |                  |                       |                       | 0.004 (1)                                           |
| SI                                                                        | 16               | 6                     | 10                    |                                                     |
| MCPO                                                                      | 39               | 15                    | 24                    |                                                     |
| <b>Shape</b>                                                              |                  |                       |                       | 0.064 (1)                                           |
| Oval-Fusiform (Bipolar)                                                   | 30               | 11                    | 19                    |                                                     |
| Polygonal (Multipolar)                                                    | 25               | 10                    | 15                    |                                                     |
| <b>Size</b>                                                               |                  |                       |                       | 2.99 (1)                                            |
| Small ( $\leq 15\mu\text{m}$ )                                            | 3                | 0                     | 3                     |                                                     |
| Medium-Large ( $>15\mu\text{m}$ )                                         | 52               | 21                    | 31                    |                                                     |
| <b>PHYSIOLOGY</b>                                                         |                  |                       |                       |                                                     |
| <b>Response to Stimulation (PSH<sup>b</sup>)</b>                          |                  |                       |                       | 10.14 (1)***                                        |
| Increase ("on")                                                           | 36               | 8                     | 28                    |                                                     |
| Decrease ("off")                                                          | 18               | 12                    | 6                     |                                                     |
| <b>Discharge Pattern</b>                                                  |                  |                       |                       | 0.47 (2)                                            |
| Tonic                                                                     | 29               | 12                    | 17                    |                                                     |
| Tonic and/or Cluster                                                      | 16               | 5                     | 11                    |                                                     |
| Tonic and/or Burst                                                        | 10               | 4                     | 6                     |                                                     |
| <b>Low Frequency Rhythmicity during Stimulation (ACH<sup>c</sup>)</b>     |                  |                       |                       | 2.31 (1)                                            |
| Rhythmic                                                                  | 20               | 5                     | 15                    |                                                     |
| Not Rhythmic                                                              | 35               | 16                    | 19                    |                                                     |
| <b>Unit-to-EEG Cross-Correlation during Stimulation (STA<sup>d</sup>)</b> |                  |                       |                       | 3.79 (1)*                                           |
| Correlated                                                                | 19               | 4                     | 15                    |                                                     |
| Not Correlated                                                            | 36               | 17                    | 19                    |                                                     |

<sup>a</sup> Frequencies (n, number of cells) for the two groups are presented and compared using the likelihood ratio chi square ( $\chi^2$ ) statistic,

\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

<sup>b</sup> Classification based upon peristimulus histogram (PSH) measure of average discharge rate.

<sup>c</sup> Based upon autocorrelation histogram (ACH) values.

<sup>d</sup> Based upon unit-to-EEG spike-triggered averages (STA) with EEG from retrosplenial cortex.

**Table 2. Morphological and physiological measures of GABAergic and non-GABAergic cell groups.<sup>a</sup>**

|                                           | <u>Cell Size (<math>\mu\text{m}</math>)<sup>b</sup></u>     | <u>Spike Width (msec)<sup>c</sup></u>      | <u>Antidromic Latency (msec)<sup>d</sup></u> |
|-------------------------------------------|-------------------------------------------------------------|--------------------------------------------|----------------------------------------------|
| <b>Nb+/GAD+ Cells<br/>Average †</b>       | <b>23.14 <math>\pm</math> 0.99 (21)</b>                     | <b>0.56 <math>\pm</math> 0.03 (21)</b>     | <b>12.05 <math>\pm</math> 2.24 (6)</b>       |
| <b>“On” Average</b>                       | <b>20.85 <math>\pm</math> 0.70 (8)</b>                      | <b>0.57 <math>\pm</math> 0.06 (8)</b>      | <b>11.80 <math>\pm</math> 3.94 (3)</b>       |
| <i>On (tonic)</i>                         |                                                             |                                            |                                              |
| <b>“Off” Average <math>\otimes</math></b> | <b>24.80 <math>\pm</math> 1.48 (12)</b>                     | <b>0.56 <math>\pm</math> 0.03 (12)</b>     | <b>12.30 <math>\pm</math> 3.10 (3)</b>       |
| <i>Off (tonic/cluster)</i>                | 24.68 $\pm$ 3.33 (5)                                        | 0.48 $\pm$ 0.03 (5)                        | .                                            |
| <i>Off (burst)</i>                        | 23.85 $\pm$ 1.38 (4)                                        | 0.60 $\pm$ 0.04 (4)                        | 12.30 $\pm$ 3.06 (3)                         |
| <i>Off (tonic)</i>                        | 26.20 $\pm$ 2.45 (3)                                        | 0.65 $\pm$ 0.06 (3)                        | .                                            |
| <br><b>Nb+/GAD- Cells<br/>Average</b>     | <br><b>20.53 <math>\pm</math> 0.78 (34) †</b>               | <br><b>0.61 <math>\pm</math> 0.02 (34)</b> | <br><b>11.09 <math>\pm</math> 1.59 (7)</b>   |
| <b>“On” Average</b>                       | <b>21.09 <math>\pm</math> 0.88 (28)</b>                     | <b>0.61 <math>\pm</math> 0.02 (28)</b>     | <b>10.55 <math>\pm</math> 1.75 (6)</b>       |
| <i>On (tonic)</i>                         | 20.32 $\pm$ 1.00 (12)                                       | 0.63 $\pm$ 0.03 (12)                       | 11.30 $\pm$ 1.90 (5)                         |
| <i>On (cluster)</i>                       | 21.44 $\pm$ 1.76 (10)                                       | 0.56 $\pm$ 0.04 (10)                       | .                                            |
| <i>On (burst)</i>                         | 22.06 $\pm$ 2.30 (6)                                        | 0.64 $\pm$ 0.06 (6)                        | 6.50 (1)                                     |
| <b>“Off” Average</b>                      | <b>17.91 <math>\pm</math> 1.29 (6) <math>\otimes</math></b> | <b>0.58 <math>\pm</math> 0.05 (6)</b>      | <b>14.30 (1)</b>                             |
| <i>Off (tonic)</i>                        |                                                             |                                            |                                              |

<sup>a</sup>Mean  $\pm$  SEM values (within in parentheses) for Nb+/GAD+ and Nb+/GAD- cell groups and subgroups. GAD- groups or subgroups were compared with homologous GAD+ cell groups or subgroups by ANOVA, and a significant difference indicated by the specific symbol of the GAD+ group (shown in the first column) with which the comparison was made (with one symbol =  $p < 0.05$ ).

<sup>b</sup>Large diameter of cell soma.

<sup>c</sup>Spike width measured from initial positive inflection to first zero-crossing.

<sup>d</sup>Latency to antidromically activated spikes from prefrontal cortex.

**Table 3. GABAergic and non-GABAergic cells' mean average discharge rate (PSH), instantaneous firing frequency (ISIH), rhythmic discharge rate (ACH), unit-to-EEG cross-correlation frequency (STA), and associated dominant EEG spectral peak during pre-stimulation and stimulation conditions.<sup>1</sup>**

|                              | Group Percent | Unit Rate (PSH, Hz)  |                           | Unit frequency (ISIH, Hz) |                     | Unit Rhythmic Frequency (ACH, Hz)          |                                            | Unit-to-EEG Frequency (STA, Hz) |                    | EEG spectral peak (Hz) |                      |
|------------------------------|---------------|----------------------|---------------------------|---------------------------|---------------------|--------------------------------------------|--------------------------------------------|---------------------------------|--------------------|------------------------|----------------------|
|                              |               | Pre-Stimulation      | Stimulation               | Pre-Stimulation           | Stimulation         | Pre-Stimulation                            | Stimulation                                | Pre-Stimulation                 | Stimulation        | Pre-Stimulation        | Stimulation          |
| Nb+/GAD+ Cells Average †     | 100%          | 17.91 ± 2.78 (20)    | 17.31 ± 3.84 (20)         | 60.52 ± 27.69 (20)        | 60.62 ± 26.99 (20)  | 1.42 ± 0.39 (4)<br>high: 29.34 ± 3.05 (7)  | 1.52 ± 0.14 (5)<br>high: 31.81 ± 3.74 (7)  | 1.08 ± 0.21 (9)                 | 1.69 ± 0.14 (3)    | 0.97 ± 0.13 (16)       | 3.20 ± 0.21 (16) *** |
| "On" Average §<br>On (tonic) | 40%           | 23.15 ± 5.22 (8)     | 33.01 ± 5.61 (8) **       | 28.49 ± 6.32 (8)          | 49.29 ± 14.63 (8)   | 0.86 (1)<br>high: 30.88 ± 5.31 (4)         | high: 31.81 ± 3.74 (7)                     | 0.68 ± 0.01 (3)                 | .                  | 1.16 ± 0.21 (8)        | 3.21 ± 0.25 (8) ***  |
| "Off" Average                | 60%           | 14.4 ± 2.82 (12)     | 6.85 ± 2.83 (12) ***      | 81.88 ± 45.67 (12)        | 68.17 ± 44.64 (12)  | 1.61 ± 0.49 (3)<br>high: 27.30 ± 2.22 (3)  | 1.52 ± 0.14 (5)                            | 1.28 ± 0.29 (6)                 | 1.69 ± 0.14 (3)    | 0.77 ± 0.12 (8)        | 3.19 ± 0.36 (8) ***  |
| Off (tonic-cluster)          | 25%           | 18.90 ± 3.00 (5)     | 9.18 ± 3.07 (5) **        | 26.32 ± 4.26 (5)          | 37.78 ± 6.22 (5)    | 1.5 (1)<br>high: 27.30 ± 2.22 (3)          | 1.52 ± 0.14 (5)                            | 1.49 ± 0.01 (2)                 | 1.69 ± 0.14 (3)    | 0.55 ± 0.05 (2)        | 3.66 ± 0.74 (2)      |
| Off (burst)                  | 20%           | 16.88 ± 5.73 (4)     | 8.3 ± 4.23 (4)            | 208.6 ± 121.73 (4)        | 155.00 ± 133.77 (4) | 0.82 (1)                                   | .                                          | 0.82 ± 0.01 (2)                 | .                  | 1.11 ± 0.13 (3)        | 3.26 ± 0.52 (3)      |
| Off (tonic)                  | 15%           | 3.62 ± 0.75 (3)      | 0.99 ± 0.51 (3)           | 5.53 ± 1.60 (3)           | 3.02 ± 0.68 (3)     | 2.5 (1)                                    | .                                          | 1.53 ± 0.98 (2)                 | .                  | 0.61 ± 0.16 (3)        | 2.80 ± 0.79 (3)      |
| Nb+/GAD- Cells Average       | 100%          | 8.95 ± 1.51 (34) ††  | 12.93 ± 1.84 (34) ***††   | 25.17 ± 5.12 (34)         | 31.27 ± 8.38 (34)   | 1.40 ± 0.28 (10)<br>high: 23.82 ± 3.57 (6) | 2.02 ± 0.26 (15)<br>high: 33.28 ± 4.92 (5) | 1.01 ± 0.30 (12)                | 2.34 ± 0.31 (15) * | 1.24 ± 0.13 (26)       | 2.71 ± 0.18 (26) *** |
| "On" Average                 | 82%           | 8.98 ± 1.58 (28) §§§ | 14.76 ± 1.96 (28) ***§§§§ | 26.09 ± 6.00 (28)         | 35.85 ± 9.93 (28)   | 1.40 ± 0.28 (10)<br>high: 23.82 ± 3.57 (6) | 2.06 ± 0.28 (14)<br>high: 33.28 ± 4.92 (5) | 0.712 ± 0.80 (10)               | 2.17 ± 0.28 (14) * | 1.14 ± 0.09 (21)       | 2.63 ± 0.20 (21) *** |
| On (tonic)                   | 35%           | 12.1 ± 2.77 (12)     | 20.72 ± 3.19 (12) ***§§   | 26.51 ± 5.34 (12)         | 29.90 ± 4.08 (12)   | 0.84 ± 0.14 (3)<br>high: 23.70 ± 0.70 (3)  | high: 24.70 ± 5.03 (2)                     | 0.79 ± 0.12 (4)                 | 2.10 ± 1.42 (2)    | 1.30 ± 0.17 (10)       | 2.72 ± 0.31 (10) *** |
| On (cluster)                 | 29%           | 7.57 ± 2.50 (10)     | 10.28 ± 2.45 (10) **      | 19.32 ± 4.25 (10)         | 21.67 ± 4.02 (10)   | 1.41 ± 0.30 (4)<br>high: 23.93 ± 7.95 (3)  | 1.62 ± 0.13 (10)<br>high: 39.00 ± 5.51 (3) | 0.78 ± 0.14 (3)                 | 1.57 ± 0.12 (7)    | 1.14 ± 0.15 (5)        | 2.60 ± 0.27 (5) *    |
| On (burst)                   | 18%           | 5.06 ± 1.61 (6)      | 10.3 ± 2.74 (6) *         | 40.33 ± 7.67 (5)          | 71.37 ± 45.06 (6)   | 1.95 ± 0.83 (3)                            | 3.15 ± 0.71 (4)                            | 0.55 ± 0.16 (3)                 | 3.02 ± 0.44 (5) *  | 0.91 ± 0.06 (6)        | 2.86 ± 0.39 (6) **   |
| "Off" Average<br>Off (tonic) | 18%           | 8.81 ± 4.78 (6)      | 4.39 ± 3.50 (6) *         | 17.10 ± 7.68 (6)          | 9.92 ± 5.51 (6) *   | .                                          | 1.55 (1)                                   | 2.5 ± 1.71 (2)                  | 4.73 (1)           | 1.61 ± 0.60 (5)        | 3.01 ± 0.46 (5) *    |

<sup>1</sup>Mean ± SEM (with n in parentheses) values are presented for the pre-stimulation and stimulation conditions for Nb+/GAD+ and Nb+/GAD- cell groups and subgroups. Within each group or subgroup, measures were compared across the pre-stimulation and stimulation conditions by paired t-tests for the unit discharge PSH and ISIH values and for the EEG spectral peak, or by unpaired t-tests for ACH and STA values (\*p<0.05, \*\*p<0.01, \*\*\*p<0.001). GAD- groups or subgroups were compared with homologous GAD+ cell groups or subgroups by ANOVA, and a significant difference indicated by the specific symbol of the GAD+ group (shown in the first column) with which the comparison was made (with one symbol = p<0.05, two symbols = p<0.01, or three symbols = p<0.001). Symbols in parentheses indicate that the GAD- cell group differed from the GAD+ cell group according to a two-way ANOVA with group (GAD- or GAD+) as a between factor and condition (pre-stimulation and stimulation) as repeated measure within each group. Symbols out of parentheses indicate that they differed according to a one-way ANOVA between groups in the pre-stimulation or stimulation condition.

## **Chapter Four**

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### **4. Evidence for Glutamate, in Addition to Acetylcholine and GABA, Neurotransmitter Synthesis in Basal Forebrain Neurons Projecting to the Entorhinal Cortex<sup>1</sup>**

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<sup>1</sup> Reprinted from Neuroscience, 2001, Manns ID, Mainville L, Jones BE: "Evidence for glutamate, in addition to acetylcholine and GABA, neurotransmitter synthesis in basal forebrain neurons projecting to the entorhinal cortex", 107, 249-263, Copyright (2001), with permission from Elsevier Science.

## **4.1 Preface**

As reviewed in the Introduction, the basal forebrain is important in the promotion of cortical activation. Cortically projecting cholinergic and potentially subgroups of GABAergic neurons as elucidated in the previous two chapters subserve this role.

However, cholinergic and GABAergic groups do not account for the totality of the basal forebrain cortical projection and thus cannot entirely account for its influence on cortical activity (Zaborszky et al., 1986; Fisher et al., 1988; Gritti et al., 1997). It has been proposed that cortically projecting noncholinergic/nonGABAergic basalocortical neurons may be glutamatergic, and would thus be important in exciting cortical neurons. To test this hypothesis, the following study used an antibody for an enzyme (PAG), which has been shown to be responsible for synthesis of neurotransmitter glutamate (Kaneko and Mizuno, 1988; Akiyama et al., 1990; Kaneko et al., 1992; Kaneko and Mizuno, 1994), and examined whether basal forebrain neurons projecting to the entorhinal cortex contained this enzyme.

## 4.2 Abstract

Basal forebrain neurons play important parts in processes of cortical activation and memory that have been attributed to the cortically projecting, cholinergic neurons. Yet, non-cholinergic neurons also project to the cerebral cortex and also appear to participate in processes of cortical modulation and plasticity. GABAergic neurons compose a portion of the cortically projecting cell group, but do not fully account for the non-cholinergic cell contingent. In the present study in the rat, we investigated whether the non-cholinergic, non-GABAergic cell component might be composed of glutamatergic neurons. We examined afferents to the entorhinal cortex, which is known to be modulated by basal forebrain neurons and to be critically involved in memory. Dual-immunofluorescent staining was performed for cholera toxin, as retrograde tracer, and phosphate-activated-glutaminase, the synthetic enzyme for the neurotransmitter pool of glutamate. The retrogradely labeled cells were distributed across the basal forebrain through the medial septum, diagonal band, magnocellular preoptic area and substantia innominata. The major proportion (~80%) of the retrogradely labeled cells was found to be immunopositive for phosphate-activated-glutaminase. Equal minor proportions (~40%) were immunopositive for choline acetyl-transferase and glutamic acid decarboxylase. In other material dual-immunostained for neurotransmitter enzymes, ~95% of choline acetyl-transferase- and ~60% of glutamic acid decarboxylase-immunopositive neurons contained phosphate-activated-glutaminase. It thus appeared that a significant proportion of these cell groups, including their cortically projecting contingents, could synthesize glutamate together with acetylcholine or GABA as neurotransmitters and another proportion of cells could synthesize glutamate alone.

Accordingly, as either co-transmitter or primary transmitter within basalocortical afferents, glutamate could have the capacity to modulate the entorhinal cortex and promote its role in memory.

### **4.3 Introduction**

The basal forebrain has long been considered to play an important role in cortical activation and memory and to do so through projections to the cerebral cortex by cholinergic neurons (Metherate et al., 1988; Smith, 1988; Metherate et al., 1992; Huerta and Lisman, 1993; Jones, 1993; Muir et al., 1994; Kilgard and Merzenich, 1998; Cape and Jones, 2000; Cape et al., 2000). More recently, it has become apparent by more selective lesions in the basal forebrain that non-cholinergic neurons must also play an important role in these processes (Dunnett et al., 1991; Lee et al., 1994; Voytko et al., 1994; Baxter et al., 1995). As first discovered in the projection to the hippocampus from the medial septum (MS) and diagonal band of Broca (DBB) (Kohler et al., 1984; Freund and Antal, 1988) and subsequently in that to the neocortex from the magnocellular preoptic area (MCPO), substantia innominata (SI) and globus pallidus (GP) (Fisher et al., 1988; Freund and Meskenaite, 1992; Gritti et al., 1997), GABAergic neurons were found to contribute significantly to the basalocortical projection. In addition, however, another proportion of cells projecting to both the hippocampus and neocortex could not be accounted for as being either cholinergic or GABAergic (Gritti et al., 1997). It thus appeared that a third contingent of non-cholinergic, non-GABAergic cells contributed to the basalocortical projection. It was thought possible that this contingent might utilize glutamate as a neurotransmitter.

The immunohistochemical identification of glutamatergic neurons has been problematic since glutamate serves as a metabolic precursor for the synthesis of GABA (Roberts, 1981; Ottersen and Storm-Mathisen, 1984). However, substantial evidence indicates that the metabolic pool of glutamate in GABAergic neurons is derived from a

different synthetic pathway than the neurotransmitter pool of glutamate in glutamatergic neurons. In GABAergic neurons, glutamate is primarily derived from  $\alpha$ -ketoglutarate transaminated by aspartate aminotransferase (AAT), the soluble form of which is found in GABA-immunoreactive interneurons and not in pyramidal neurons of the cerebral cortex (Kaneko and Mizuno, 1994). As evidenced by glutamate released from cortical synaptosomes, neurotransmitter glutamate is principally (>75%) derived from glutamine (Bradford et al., 1978). Glutamine is supplied by the glia, which take up the glutamate released from the nerve terminal, convert it to glutamine and shuttle glutamine back to the neuron for new synthesis of glutamate. The conversion of glutamine to glutamate in neurons occurs by the mitochondrial enzyme glutaminase, which is stimulated by phosphate and thus called phosphate-activated glutaminase (PAG). In the cerebral cortex, this enzyme is negatively regulated by glutamate and not by GABA, indicating product inhibition, which is characteristic of other neurotransmitter synthetic pathways (Bradford et al., 1978). In isolated cerebellar, known to be glutamatergic, granule cell cultures, PAG is selectively activated by depolarization (Alavez et al., 1996), suggesting activity-dependent positive regulation, which is also characteristic of neurotransmitter synthetic pathways. In immunohistochemical studies, Donoghue et al. (Donoghue et al., 1985) and Kaneko and his colleagues (Kaneko and Mizuno, 1988; Akiyama et al., 1990; Kaneko et al., 1992; Kaneko and Mizuno, 1994) found that PAG was present in the pyramidal cells of the cerebral cortex and absent in non-pyramidal, presumed interneurons and identified GABAergic cells. Following from the biochemical evidence that this enzyme is regulated according to utilization of glutamate as a neurotransmitter and the immunohistochemical evidence that it is selectively present in known

glutamatergic neurons of the cerebral cortex, we considered that PAG immunohistochemistry could be effectively employed in the basal forebrain to examine whether non-cholinergic and non-GABAergic neurons might be glutamatergic.

While projecting to all regions of the cortical mantle, the basal forebrain cell population provides a particularly dense innervation to the entorhinal cortex (Rye et al., 1984; Saper, 1984; Luiten et al., 1987; Lysakowski et al., 1989). As part of the hippocampal region, the entorhinal cortex plays a particularly important role in memory (Zola-Morgan et al., 1989). As in the hippocampus, the cholinergic input to the entorhinal cortex has the capacity to stimulate rhythmic network oscillatory activity (Mitchell et al., 1982; Konopacki et al., 1992; Dickson and Alonso, 1997) that has been shown to underlie cortical activation and synaptic plasticity (Lopes da Silva et al., 1990; Huerta and Lisman, 1993). It has been postulated that non-cholinergic, presumed GABAergic input, may also play an important role in mediating by pacing such rhythmic activity (Lee et al., 1994). It is also well known that glutamate has the capacity through activation of metabotropic and ionotropic receptors to promote both rhythmic network activity and synaptic plasticity in limbic cortex (Bortolotto et al., 1994; Taylor et al., 1995; Whittington et al., 1995; Malenka and Nicoll, 1999; Cobb et al., 2000). It would thus be of great functional significance if the basal forebrain neurons projecting to the entorhinal cortex were found to synthesize and thus potentially release glutamate as a neurotransmitter. By examining in the rat retrograde transport of cholera toxin (CT) with dual immunohistochemical staining for ChAT, GAD or PAG, we thus investigated the possibility that a proportion of basal forebrain neurons projecting to the entorhinal cortex synthesize, in addition to acetylcholine (ACh) or GABA, glutamate as a neurotransmitter.

#### **4.4 Methods and Materials**

Twenty-four male Wistar rats (Charles River Canada, St. Constant, Quebec) weighing between 200 and 250 grams were employed in these studies. All procedures were approved by the McGill University Animal Care Committee and the Canadian Council on Animal Care. Surgery was performed under barbiturate anesthesia (Somnotol, 50-65 mg/kg, i.p.). In 17 rats, injections of cholera toxin (CT) followed by colchicine treatment were performed, and in 5 rats colchicine treatment alone was used. In the first operation, 100 nl 1% CT subunit b (choleragenoid, LIST Biological Laboratories, Campbell, CA) in Tris phosphate buffer was injected (over ~1 minute) into the brain using an oil-filled micropipette (tip diameter ~50µm) attached to a 1µl Hamilton syringe (Gritti et al., 1994). The injection was placed under stereotaxic guidance into the right lateral entorhinal cortex according to coordinates relative to ear bar zero at anterior-posterior (AP) +1.8 mm, lateral (L) -5.4 mm and vertical (V) +2.2mm (Paxinos and Watson, 1986). In a second operation ~24 hours after the CT injection, 50µg of colchicine (Sigma, St. Louis, MO) dissolved in 25µl saline was injected into the left lateral ventricle in order to enhance levels of GAD in the cell soma as previously employed (Gritti et al., 1997). Approximately 48 hours after the CT injection and 24 hours after the colchicine treatment, animals were killed under anesthesia (65 mg/kg, i.p., Somnotol) by intra-aortic perfusion with a fixative. Having no CT injections and with (n = 5) or without (n = 2) colchicine pretreatment, 7 rats were similarly killed by perfusion.

For fixation of the brain, we adopted with slight modification the procedure developed by Kaneko for PAG immunohistochemistry, since it was designed to fix with minimal shrinkage the tissue and mitochondria where the PAG enzyme is located

(Kaneko et al., 1992; Kaneko and Mizuno, 1994). Following a brief rinse with phosphate-buffered saline, the animals were perfused through the aorta with 500 ml of a modified Zamboni's solution, containing 0.3% paraformaldehyde and 75% saturated picric acid in 0.1 M sodium phosphate buffer (pH 7.0). The brains were post-fixed overnight at 4°C in a solution of 3% paraformaldehyde with 75% saturated picric acid. They were subsequently immersed in a solution of 30% sucrose at 4°C for 24 - 48 hours for cryoprotection. Brains were then frozen at -50°C in isopentane and kept at -80°C.

Brains were cut at 20 µm thickness on a freezing microtome, and twenty adjacent series of sections were collected at 400 µm intervals. One series was processed for single labeling of cholera toxin using peroxidase-antiperoxidase (PAP) immunohistochemistry with Nickel (Ni) enhanced 3,3'-diaminobenzidine tetrahydrochloride (DAB), as described previously (Gritti et al., 1994). Sections were incubated overnight with a goat anti-cholera toxin antiserum (1:40,000, List Biological Laboratories), followed by rabbit anti-goat antiserum (Jackson ImmunoResearch Laboratories, West Grove, PA), and finally a goat PAP (Jackson ImmunoResearch Laboratories) revealed with 0.05% 3,3'- (DAB, Sigma), in the presence of 0.1% glucose oxidase and 0.04% nickel ammonium sulfate in 0.1 M Tris-water (pH 7.4).

Due to the light granular staining of PAG, it was found necessary to employ immunofluorescence for dual-immunostaining of PAG with the other antigens, including CT. Accordingly, dual-immunostaining for CT and PAG, ChAT, or GAD was performed on adjacent series of sections using a goat anti-cholera toxin antiserum (at 1:40,000, LIST Biological Laboratories), a rabbit anti-PAG antiserum (1:6000, developed and kindly supplied by T. Kaneko, Kyoto, Japan (Akiyama et al., 1990)), a rabbit anti-ChAT

antiserum and a rabbit anti-GAD antiserum (both used at 1:3000, Chemicon, Temecula, CA). Sections were incubated overnight in the presence of antibodies to CT and one of the enzyme antisera. Control sections were run in the absence of one or both primary antibodies and replaced with normal serum of the species the primary antiserum was raised in. Following thorough rinsing, the sections were incubated with a biotin-conjugated anti-goat antiserum (1:1000, Jackson ImmunoResearch Laboratories), and after another thorough rinsing, the sections were subsequently incubated with Cy2-conjugated streptavidin (1:1000, Jackson ImmunoResearch Laboratories) for revelation of the CT and a Cy3-conjugated anti-rabbit antiserum (1:1000, Jackson ImmunoResearch Laboratories) for revelation of the transmitter enzyme. The sections were subsequently rinsed, mounted, dehydrated and cover slipped using PermOUNT (Fisher, Fairlawn, NJ).

Series were also processed to evaluate the co-localization of PAG with ChAT and PAG with GAD. For the PAG-ChAT series, sections were incubated overnight with the rabbit anti-PAG antiserum (1:6000, supplied by T. Kaneko) and rat anti-ChAT antiserum (1:3, Boehringer Mannheim, Germany). Control sections were run in the absence of one or both primary antibodies and replaced with normal serum of the species the primary antiserum was raised in. Following rinsing, the sections were incubated with Cy3-conjugated anti-rabbit antiserum (1:1000, Jackson ImmunoResearch Laboratories) for the revelation of PAG and AMCA-conjugated anti-rat antiserum (1:50, Jackson ImmunoResearch Laboratories) for the revelation of ChAT. Similarly, for the PAG-GAD series, sections were incubated overnight in the rabbit anti-PAG antiserum (1:6000) and a sheep anti-GAD antiserum (1:3000, supplied by E. Mugnaini, Chicago, IL (Oertel et al., 1982)). Following rinsing, sections were subsequently incubated with Cy3-conjugated

donkey anti-rabbit antiserum (1:1000, Jackson Immunoresearch Laboratories) for revelation of PAG and Cy2-conjugated donkey anti-sheep antiserum (1:50, Jackson Immunoresearch Laboratories) for revelation of GAD. These series were directly mounted with Aqua Poly/Mount (Polysciences, Inc., Warrington, PA).

The injection sites were evaluated in the PAP-immunostained material. Of 17 brains injected with CT, 12 were used as pilot material for perfection of the injection site and/or immunohistochemistry or were considered imperfect for inclusion in the quantitative analysis of the results. In 5 brains in which the injection sites and immunohistochemistry were considered optimal, the injection sites were drawn and the retrogradely labeled cells mapped through the basal forebrain at 800 $\mu$ m intervals from the DAB-Ni-stained material. The mapping was performed on a Leitz Othoplan microscope equipped with an x/y-sensitive stage and video camera that were connected to a computerized image-analysis system (Biocom, Les Ulis, France) equipped with a resident atlas, as described previously (Gritti et al., 1994). From these same brains, the dual-immunofluorescent stained sections were examined under fluorescent illumination using a Leitz Dialux 20 microscope equipped with a Ploemopak 2 reflected light fluorescence illuminator and Leica filter cubes for fluorescein (I3), rhodamine (N2.1) and ultraviolet (A). Single CT<sup>+</sup> and double CT<sup>+</sup>/PAG<sup>+</sup>, CT<sup>+</sup>/ChAT<sup>+</sup> and CT<sup>+</sup>/GAD<sup>+</sup> cells were examined and tabulated across basal forebrain nuclei in three adjacent series of sections from 5 animals. Profiles of cell bodies, which were judged as whole cell bodies containing nuclei, were counted within and through the entire area of each of the basal forebrain nuclei in the 20  $\mu$ m thick sections. Cell counts were performed in six sections at 800 $\mu$ m intervals starting most rostrally at ~A10.9 and moving caudally to ~A6.9.

Accordingly, counts were performed across appropriate levels for each nucleus, corresponding to at least 2 sections per nucleus (MS, 3; DBB, 2; MCPO, 2; SIa, 3; SIp, 2). Because the fluorescent images could not be captured by video camera for computer-aided image analysis, the labeled cell body profiles were mapped and counted manually on appropriate atlas sections. Morphological measurements were taken from randomly selected CT+/PAG+, CT+/ChAT+ and CT+/GAD+ cells in each of the basal forebrain nuclei (1 – 4 cells per nucleus) in 3 animals by measuring their largest diameter or length in film transparencies.

Of 7 processed brains not injected with CT, 3 were selected for optimal dual-immunostaining of PAG with ChAT (including 2 without colchicine pretreatment) and 5 of PAG with GAD (all having colchicine pretreatment). The percentage of ChAT+ cells, which was PAG+, and that of GAD+ cells, which was PAG+, were estimated by counting a sample of cell body profiles in 12, 20  $\mu$ m thick sections at 400  $\mu$ m intervals across the basal forebrain nuclei of each brain. These samples consisted of ~40 –100 cell profiles in each brain, specifically: for the ChAT+/PAG+  $76.7 \pm 12.8$  profiles (in 3 rats) and for GAD+/PAG+  $81.4 \pm 8.1$  profiles (in 5 rats).

Cell numbers and measurements were compared across cell types by ANOVA. Statistics were performed using Systat software (version 9.0, SPSS, Chicago, IL). Figures were prepared using Adobe Photoshop (version 5.0, Adobe Systems, San Jose, CA).

## **4.5 Results**

### **PAG immunostaining in basal forebrain neurons**

Viewed in immunofluorescent stained material, many cells in the basal forebrain appeared to be immunoreactive for PAG (Fig. 1A). The PAG immunostaining was granular and present within the soma and proximal dendrites of neurons. Punctate immunostaining was also apparent in the neuropil where it was presumably contained within nerve varicosities or terminals. The staining appeared to vary in intensity over neuronal cell bodies. As evident at high magnification, unambiguously PAG-immunostained cells contained numerous fluorescent granules distributed through their cytoplasm and were accordingly qualitatively deemed PAG-immunopositive (Fig. 1B). PAG<sup>+</sup> cells were present in all basal forebrain nuclei, including the medial septum (MS), nucleus of the diagonal band of Broca (DBB), magnocellular preoptic nucleus (MCPO, Fig 1A and B), substantia innominata (SI, in its anterior and posterior parts, SIa and SIp), and the globus pallidus (GP). Through these nuclei, PAG<sup>+</sup> cells ranged in shape from fusiform or oval to polygonal and in size from small (10 – 15  $\mu\text{m}$ ), to medium (15 – 25  $\mu\text{m}$ ) or large (> 25  $\mu\text{m}$ ).

### **Retrograde transport of cholera toxin from entorhinal cortex**

Neurons projecting to the entorhinal cortex were identified by retrograde transport of CT. The injection site and full distribution of retrogradely labeled cells were examined by light microscopy in PAP (DAB-Ni) immunostained material. For full analysis, brains were selected in which the injections were centered in and covered a significant proportion of the lateral entorhinal cortex (n = 5). In these brains, the

injection site extended from layer I through layer VI to the deep white matter and covered an area extending approximately 1.5 mm in the medial-lateral and rostral-caudal axes (Fig. 2A). Diffuse labeling for CT also occurred along the track of the micropipette resulting in some labeling in the subiculum in a few cases (3/5) and in CA1 in one case (1/5).

Retrograde labeling of neurons with CT was evident by the presence of black immunostained granules in the soma and proximal dendrites of neurons, as described previously (Gritti et al., 1997). Retrogradely labeled neurons were evident in the vicinity of the injection site and in distal regions through the forebrain. The largest number was distributed in the hippocampal region (Fig. 2A). Near the injection site, many cells were located in the lateral entorhinal cortex (LEA) and fewer in the medial entorhinal (MEA) cortex on the ipsilateral side and some in these nuclei on the contralateral side. Ipsilaterally, large numbers of retrogradely labeled cells were distributed through the CA1 region of the hippocampus, subiculum (S), parasubiculum (PaS) and presubiculum (PrS). Laterally, cells were also present in the perirhinal cortex (PRh) at the level of the injection and more rostrally in the temporal cortex. Further rostrally (Fig. 2B), many retrogradely labeled neurons were located in the endopiriform nucleus (En), piriform cortex (Pir) and amygdaloid nuclei (particularly the cortical (CoA) and medial (MeA) nuclei). In the thalamus, retrogradely labeled cells were distributed in midline nuclei, particularly within the paraventricular (PV) and reuniens (Re) nuclei (Fig. 2B, as well as the centromedial, interanteromedial, and rhomboid nuclei). Ventrally, cells were scattered in the lateral hypothalamus (LH), and more caudally in the poster

hypothalamus). Further rostrally, cells were present in the claustrum and insular cortex, laterally and within the infralimbic region of the prefrontal cortex, medially.

Retrogradely labeled cells were located in the ipsilateral basal forebrain within the cholinergic cell nuclei (Fig. 3). A few scattered cells were also present on the contralateral side (not shown). Across the basal forebrain nuclei, the CT-immunopositive (CT+) cells were estimated to number ~7100 (according to an average total of ~350 cells counted in 20  $\mu\text{m}$  thick sections at 800  $\mu\text{m}$  intervals, multiplied by the number of sections per interval and corrected by the Abercrombie factor (Abercrombie, 1946) for an average cell length of 19  $\mu\text{m}$ ,  $n = 5$ ). They extended across the ventral expanse of the basal forebrain and were distributed in decreasing density from the medial septum to the posterior substantia innominata (in proportions of the total as MS: 35%, DBB: 19%, MCPO: 20%, SIa: 16%, SIp: 10%). Although some neurons were distributed along the edge of the GP, no neurons were present within the GP.

### **PAG, ChAT and GAD in basal forebrain cortically projecting neurons**

The dual-immunostaining of retrogradely labeled neurons for PAG, ChAT or GAD was examined by fluorescence microscopy in immunofluorescent material (Fig. 1C-H). The retrogradely transported CT was evident as punctate or granular fluorescent immunostaining within the perikarya and proximal dendrites of neurons. In this material, the CT+ cells were distributed through the basal forebrain cholinergic cell nuclei in a manner similar to although fewer (by ~2/3) in number than those in the PAP (DAB-Ni) immunostained material due to the lower sensitivity of the immunofluorescence technique (Fig. 3 and Table 1,  $n = 5$ ). In sections dual-immunostained for CT and PAG,

many CT+ cells were clearly immunopositive for PAG, while others were clearly immunonegative for PAG (Fig. 1C and D). The CT+/PAG+ cells were distributed through the basal forebrain nuclei in proportions similar to the CT+ cells (Fig. 4; Table 1). Across these nuclei, the major proportion of CT+ cells was PAG+ and on average, represented >80%. In sections dual-immunostained for CT-ChAT or CT-GAD, many CT+ cells were ChAT-immunopositive (Fig. 1E and F) and many GAD-immunopositive (Fig. 1G and H). Though differing slightly among nuclei, the proportion of CT+ cells, which was ChAT+ or GAD+, was 40% for each on average across nuclei. The CT+/PAG+ cells were co-distributed with CT+/ChAT+ and CT+/GAD+ cells through the basal forebrain nuclei. The proportion of CT+ cells double-labeled for PAG was greater than that of CT+ cells double-labeled for ChAT or GAD, whereas the proportion for ChAT did not differ significantly from that for GAD across nuclei (Table 1).

Retrogradely labeled PAG+ cells varied in shape from fusiform or oval to polygonal (Fig. 1C and D) and were on average medium in size (average  $19.5 \pm 0.48 \mu\text{m}$  in length). Retrogradely labeled CT+/ChAT+ cells were also variable in shape (Fig. 1E and F) and on average medium in size (average  $19.4 \pm 0.67 \mu\text{m}$  in length), such as to not differ from the CT+/PAG+ cells (Table 2). Similarly, the retrogradely labeled CT+/GAD+ cells were also variable in shape (Fig. 1G and H) and on average medium in size (average  $19.0 \pm 0.65 \mu\text{m}$  in length), such as to not differ significantly on average from the CT+/PAG+ or CT+/ChAT+ cells, although some CT+/GAD+ cells in some nuclei were smaller (Table 2).

#### **PAG co-localization with ChAT and GAD**

The CT+ neurons that were immunopositive for PAG represented more than 80% of the retrogradely labeled, cortically projecting neurons in the basal forebrain. Since this percentage combined with those of CT+/ChAT+ and CT+/GAD+ cells accounted for more than 100% of all the neurons projecting to the entorhinal cortex, it appeared that ChAT or GAD must be co-localized with PAG in a proportion of those cells (Table 1). This possibility was also supported by the similarity in cell shape and size of the CT+/ChAT+ and CT+/GAD+ cells with those of the CT+/PAG+ cells (Table 2). Co-localization was thus examined in material dual-immunostained by immunofluorescence for the neurotransmitter enzymes. The co-localization of GAD with ChAT was not examined, since it had been established in previous studies within the basal forebrain that this co-localization is non-existent (Gritti et al., 1993) or rare (in <2% of ChAT+ neurons, (Brashear et al., 1986; Kosaka et al., 1988; Fisher and Levine, 1989). The co-localization of PAG with ChAT or GAD was examined and assessed quantitatively by counting the number of ChAT+ or GAD+ cells that were PAG+ across nuclei (sampling ~40 – 100 cells per brain). The ChAT-PAG dual-immunostaining revealed that approximately 95% of ChAT-immunopositive neurons were unambiguously immunopositive for PAG (n = 3), while a small proportion of ChAT+ cells were clearly immunonegative for PAG (Fig. 1I and J). The GAD-PAG dual-immunostaining indicated that approximately 60% of GAD-immunopositive neurons were clearly PAG-immunopositive (n = 5), while a smaller proportion were clearly immunonegative for PAG (Fig. 1K and L).

#### **Proportions of cortically projecting cells potentially containing ChAT, GAD and/or PAG**

According to the proportions of ChAT<sup>+</sup> and GAD<sup>+</sup> cells that were also PAG<sup>+</sup> in the groups sampled, proportions were estimated of the CT<sup>+</sup> cells that could be ChAT<sup>+</sup>/PAG<sup>+</sup>, ChAT<sup>+</sup>, GAD<sup>+</sup>/PAG<sup>+</sup>, GAD<sup>+</sup> or PAG<sup>+</sup>, assuming that proportions among the cortically projecting cells could be similar to those among the total ChAT<sup>+</sup> and GAD<sup>+</sup> cell groups. Given that  $94.5 \pm 3.9\%$  of the ChAT<sup>+</sup> cells were also PAG<sup>+</sup>, it was estimated that ~38% of the CT<sup>+</sup> cells could be ChAT<sup>+</sup>/PAG<sup>+</sup> and only ~2% uniquely ChAT<sup>+</sup>. Similarly given that  $60.0 \pm 7.5\%$  of the GAD<sup>+</sup> cells were PAG<sup>+</sup>, it was estimated that ~24% of CT<sup>+</sup> cells could be GAD<sup>+</sup>/PAG<sup>+</sup> and only 16% uniquely GAD<sup>+</sup>. Finally, it was estimated that ~20% of CT<sup>+</sup> cells could be uniquely PAG<sup>+</sup>.

## **4.6 Discussion**

The present results reveal that a major proportion of basal forebrain neurons projecting to the entorhinal cortex contain the enzyme PAG that is responsible for the synthesis of the transmitter pool of glutamate. The cortically projecting neurons containing PAG were co-distributed with those containing ChAT or GAD across the cholinergic cell nuclei of the basal forebrain. In other material, a vast majority of ChAT-immunopositive and a significant proportion of GAD-immunopositive neurons were found to be dual-immunostained for PAG, indicating that glutamate may also be synthesized along with ACh or GABA in particular subsets of cortically projecting neurons. Containing only PAG, another set of neurons could synthesize and potentially release glutamate as its primary neurotransmitter. Accordingly, glutamate could play an important role in the basolocortical input to the entorhinal cortex, possibly conjointly released with ACh or GABA from some afferents and independently released from other afferents.

### **Synthesis of glutamate in basal forebrain neurons**

In the basal forebrain, many neurons displayed immunostaining for PAG, showing through their cytoplasm, multiple immunopositive granules that are indicative of staining for the enzyme that is located in the mitochondria (Kaneko and Mizuno, 1988; Laake et al., 1999). Many PAG-immunopositive cells appeared as intensely immunostained as the pyramidal cells of the cerebral cortex (unpublished observations) (Kaneko and Mizuno, 1988), which are known from electrophysiological studies to be glutamatergic. Other basal forebrain cells viewed in dual-immunostained material for PAG-CT and for PAG-ChAT or PAG-GAD, were immunonegative for PAG, similar to

the non-pyramidal cells and cells that have been identified as GABAergic in the cerebral cortex (Kaneko and Mizuno, 1988; Kaneko et al., 1992; Kaneko and Mizuno, 1994). On the other hand, significant proportions of both ChAT+ and GAD+ cells were also found to be PAG+ here in the basal forebrain. In the following discussion, we consider the functional significance of these results suggesting that different subsets of cortically projecting basal forebrain neurons may have the capacity to synthesize and thus utilize glutamate as a neurotransmitter.

### **Neurons projecting to the entorhinal cortex**

Cholera toxin injections were centered in the lateral entorhinal cortex, as confirmed by the location of the retrogradely labeled cell population, in addition to that of the injection site. The majority of the retrogradely labeled cells were located within the hippocampal-parahippocampal region, including the CA1 and subicular regions and the perirhinal cortex, as had been reported in previous studies (Van Hoesen and Pandya, 1975a; Van Hoesen et al., 1975; Van Hoesen and Pandya, 1975b; Swanson and Cowan, 1977; Van Hoesen, 1982; Kohler, 1988). Afferent neurons were also found in the temporal, piriform, insular and prefrontal cortex, endopiriform nucleus, claustrum, amygdala, midline thalamic nuclei and lateral hypothalamus, as has been previously reported (Krettek and Price, 1977; Insausti et al., 1987a, b; Dolleman-Van Der Weel and Witter, 1996; Insausti et al., 1997). It thus appeared that the injections were well centered in the lateral entorhinal cortex.

Retrogradely labeled CT+ cells were distributed through the nuclei of the basal forebrain with the highest number of cells in the MS, also in accord with previous studies

(Alonso and Kohler, 1984; Kohler et al., 1984; Saper, 1984; Luiten et al., 1987). The neurons were distributed from the MS and DBB through the ventral tier of the basal forebrain nuclei including the MCPO and SI and did not extend into the GP, which includes a portion of the nucleus Basalis. This distribution resembles that described in previous studies although in those, some retrogradely labeled cells had also been indicated in the GP (Saper, 1984), perhaps because of larger or differently placed injections of the retrograde tracer. The results of the present study, together with those of previous studies, indicate that widely distributed basal forebrain neurons project to the entorhinal cortex and provide a dense innervation to that region which is known to both project to and receive afferent information from wide areas of the cerebral cortex (Van Hoesen, 1982; Swanson and Kohler, 1986).

**PAG, as well as ChAT or GAD, in basal forebrain neurons projecting to the entorhinal cortex**

The majority (>80%) of the retrogradely labeled basal forebrain neurons showed unambiguous immunostaining for PAG in their cell bodies. Given that known glutamatergic mossy fiber afferents to cerebellar cortex were shown to contain similarly high concentrations of PAG in their terminals as in their cell bodies (Laake et al., 1999), the basolocortical afferent neurons immunostained here may also contain PAG in their terminals and thus have the capacity to synthesize and release glutamate from their terminals in the entorhinal cortex. Electrophysiological evidence for the possibility that basolocortical afferents are glutamatergic cannot be obtained from *in vivo* studies but can be assessed from *in vitro* studies employing co-cultures of septo-hippocampal slices

(Gahwiler and Brown, 1985) or basal forebrain-hippocampal dissociated cells (Lai and Brown, 1992). In these studies, stimulation of the septum or basal forebrain cells, respectively, produced fast excitatory postsynaptic currents that represented the predominant response in the postsynaptic hippocampal cells (~80% of cell pairs). These currents were not mediated by ACh but instead, by glutamate or another excitatory amino acid, since they could not be blocked by cholinergic antagonists but could be by excitatory amino acid antagonists, thus providing evidence for glutamatergic transmission in basolateral afferents. As mentioned above, such glutamatergic input from the basal forebrain could by phasic activity and/or tonic action upon postsynaptic ionotropic or metabotropic glutamate receptors, promote rhythmic network activity and synaptic plasticity in limbic cortex (Taylor et al., 1995).

ChAT+ and GAD+ neurons composed equivalent proportions of the neurons projecting to the entorhinal cortex (40% on average). These proportions are similar to though slightly higher than those documented for basal forebrain neurons projecting to the prefrontal and parietal cortex (Gritti et al., 1997). Slight differences may be due to differing cortical regions with different afferent inputs or differing immunohistochemical techniques with different sensitivities. In other studies, the projection to the olfactory bulb from the basal forebrain was estimated to comprise 10-20% cholinergic (Rye et al., 1984) and 30% GABAergic neurons (Zaborszky et al., 1986). The projection to the hippocampus and hippocampal region from the MS-DBB was estimated to comprise 25 - 75% cholinergic cells (Rye et al., 1984; Amaral and Kurz, 1985) and ~30% GABAergic cells (Kohler et al., 1984). Our findings indicate that equivalent proportions of GABAergic and cholinergic basal forebrain neurons innervate the entorhinal cortex. In

septo-hippocampal slice co-cultures (as mentioned above), although no fast cholinergic currents could be demonstrated after single shocks to the septum, a slow muscarinic postsynaptic current was revealed by prolonged stimulation (Gahwiler and Brown, 1985). In the basal forebrain-hippocampal co-cultured cell pairs (as mentioned above), fast outward postsynaptic currents were evident, which were attributed to GABA acting at GABA<sub>A</sub> receptors, in a minor proportion of the cell pairs (Laiwand and Brown, 1992). Accordingly, the *in vitro* co-culture studies provide evidence for slow, muscarinic, cholinergic transmission and fast, GABAergic transmission in the septo-hippocampal pathway. In the entorhinal cortex, ACh has been shown to promote rhythmic network activity through muscarinic receptors (Dickson and Alonso, 1997). GABA would have the potential to inhibit such activity or alternatively, to pace it by fast inhibitory postsynaptic potentials.

#### **Co-synthesis of glutamate with ACh or GABA in cholinergic and GABAergic neurons**

Given that the percentages of retrogradely labeled PAG<sup>+</sup>, ChAT<sup>+</sup> and GAD<sup>+</sup> cells represented more than 100% and that the three cell types were similarly diverse in morphology and large in size, it appeared that these enzymes could be co-localized in the same cells. Previous studies had shown that ChAT and GAD are co-localized in <2% of basal forebrain neurons of which a portion were cortically projecting (Brashear et al., 1986; Kosaka et al., 1988; Fisher and Levine, 1989; Gritti et al., 1993). Here, examining dual-immunostaining for PAG-ChAT or PAG-GAD, it was revealed that ~95% of ChAT<sup>+</sup> and ~60% of GAD<sup>+</sup> neurons contained the PAG enzyme. These results suggest

that significant proportions of presumed cholinergic and GABAergic basalocortical projection neurons might also synthesize glutamate as a neurotransmitter and thus release glutamate in addition to ACh or GABA.

Evidence for glutamate being contained in cholinergic neurons was previously reported for the cholinergic cells in the pontomesencephalic tegmentum (Kaneko et al., 1989; Clements et al., 1991). The possibility that glutamate might be released from cholinergic terminals in the cortex was previously documented in affinity-purified synaptosomes from the cerebral cortex showing that glutamate was released in addition to ACh from isolated (ChAT affinity-purified) cholinergic synaptosomes (Docherty et al., 1987). As from cholinergic synaptosomes of the cortex, glutamate is also released in addition to ACh from the prototypical cholinergic terminals of the Torpedo electric organ (Vyas and Bradford, 1987). In this preparation, glutamate or ACh release occurs differentially depending upon calcium concentrations (Israel et al., 1993). Accordingly, their differential release could depend upon firing frequency or presynaptic modulation that can each determine the degree of calcium entry into the terminal. It is perhaps for this reason that previous *in vitro* studies using co-cultures of septo-hippocampal slices found that the predominant postsynaptic response to single shocks to the septum was a non-cholinergic, fast inward current (as mentioned above) and that only with prolonged trains of stimulation did a slow muscarinic, cholinergic depolarizing current emerge (Gahwiler and Brown, 1985). Co-release of glutamate and ACh could be physiologically significant in the hippocampal region since although each is capable of stimulating rhythmic network activity (Taylor et al., 1995; Whittington et al., 1995; Cobb et al., 2000), coincident activation of metabotropic glutamate receptors and muscarinic ACh

receptors can be additive in stimulating the rhythmic activity (Cobb et al., 2000). It is also possible that glutamate released from cholinergic terminals could act upon presynaptic glutamate receptors that are known to modulate the release of ACh (Vizi and Kiss, 1998; Takumi et al., 1999; Cartmell and Schoepp, 2000). Reciprocally, presynaptic cholinergic receptors can modulate glutamate release (Vizi and Kiss, 1998), either attenuating it through muscarinic receptors (Valentino and Dingledine, 1981) or enhancing it through nicotinic receptors (Radcliffe et al., 1999). Differential receptor activation on presynaptic terminals has also been found to be frequency dependent (Takumi et al., 1999; Cartmell and Schoepp, 2000). Thus in a potentially activity-dependent manner, glutamate and ACh could be released from terminals and act upon post or presynaptic receptors of a major proportion of ChAT+ basalocortical neurons.

Co-localization of glutamate and GABA had previously been thought to indicate that glutamate was present in those neurons as the metabolic precursor for the neurotransmitter, GABA (Ottersen and Storm-Mathisen, 1984). However, mossy fibers of the hippocampus, which are well known from electrophysiological studies to function by release of glutamate and thus be 'glutamatergic' were shown to contain significant concentrations of both glutamate and GABA in their terminals, suggesting that both were present as neurotransmitters (Sandler and Smith, 1991). Their parent, granule cell bodies in the dentate gyrus have also been shown to contain both PAG (Kaneko and Mizuno, 1988) and GAD (Sloviter et al., 1996). Most recently, in the presence of glutamate receptor blockers, a GABA<sub>A</sub>-mediated postsynaptic current was unmasked in the CA3 pyramidal cells that was attributed to release of GABA from the mossy fiber terminals (Ruiz et al., 2000). That glutamate could be released from the same terminals as GABA

in the cerebral cortex was documented in isolated (GAD affinity-purified) GABAergic synaptosomes (Docherty et al., 1987). In the basal forebrain-hippocampal dissociated cell co-cultures (as mentioned above), the fast postsynaptic currents were reported as either inward or outward and thus characterized as essentially excitatory or inhibitory, with the latter representing only a small proportion (<20%) (Laiwand and Brown, 1992). Here in the basal forebrain, a larger proportion of cortically projecting cells was estimated as potentially containing both PAG and GAD than that estimated as potentially containing GAD alone (<20%). It is possible that glutamate and GABA, where co-localized, could be released in association with different frequencies of neuronal discharge and/or act upon different receptors at different post and/or presynaptic sites. Glutamate can suppress the release of GABA by acting upon presynaptic glutamate receptors (Mitchell and Silver, 2000). Reciprocally, GABA has been shown to produce presynaptic inhibition of glutamate release from mossy fiber terminals in the hippocampus (Vizi and Kiss, 1998; Vogt and Nicoll, 1999). The frequency dependence of both pre and postsynaptic receptor mediated effects (Bortolotto et al., 1994; Malenka and Nicoll, 1999; Yamada et al., 1999; Mitchell and Silver, 2000) may indicate that differential release of neurotransmitters and activation of receptors at terminals where glutamate and GABA are co-localized could occur through different firing frequencies by those neurons.

#### **Distinct roles of cortically projecting neurons synthesizing ACh, GABA and/or glutamate**

Electrophysiological recordings of basal forebrain neurons *in vivo* have revealed that cortically projecting neurons are very heterogeneous in both physiological properties and state related discharge (Aston-Jones et al., 1984; Detari and Vanderwolf, 1987; Reiner et al., 1987; Szymusiak and McGinty, 1989). From the results of the present study, it appears that basolocortical projection neurons are differentiated into several subgroups according to their neurotransmitter enzymes. Recently, *in vivo* recording studies employing juxtacellular recording and neurobiotin labeling of basal forebrain neurons have established that the neurotransmitter specific cell groups also have distinctive physiological properties (Duque et al., 2000; Manns et al., 2000a, b). ChAT-immunopositive neurons discharge in a slow irregular manner during cortical slow wave activity and increase their firing, with the majority of cells discharging in high frequency bursts in a rhythmic manner, in association with theta-like EEG activity during cortical activation (Manns et al., 2000a). From the present results, it would appear that the majority of ChAT<sup>+</sup> cells might release glutamate in addition to ACh, perhaps in an activity dependent manner. GAD-immunopositive neurons are heterogeneous and composed of distinct subgroups, which discharge in different ways in association with cortical activity, including a majority that fire during cortical activation and one that discharges rhythmically in association with theta-like activity (Manns et al., 2000b). Two minor subgroups virtually cease firing during cortical activation. From the present results, it would appear possible that these different subgroups might be distinguished by their capacity to synthesize and release glutamate in addition to GABA. Most recently, a group of ChAT-immunonegative and GAD-immunonegative basal forebrain neurons were identified as PAG-immunopositive and found to fire rhythmically in association

with rhythmic theta-like activity during cortical activation (Manns et al., 2000c).

Together with the designated cholinergic and GABAergic subgroups, these putative glutamatergic, rhythmically discharging basalocortical neurons could play an important role in stimulating rhythmic activity and with it, cortical activation and plasticity in their cortical target neurons. Thus glutamate, synthesized together with ACh and GABA but also independently in a unique set of basalocortical neurons, would have the capacity to potentially influence activity in the entorhinal cortex that is importantly involved in memory (Jarrard, 1993; Zola-Morgan and Squire, 1993).

The importance of non-cholinergic basalocortical neurons has recently been highlighted by studies showing that non-selective lesions of the entire basalocortical projection produce more devastating cognitive deficits than selective lesions of the cholinergic cells (Dunnett et al., 1991; Voytko et al., 1994; Baxter et al., 1995). And although cholinergic neurons play an important role in cortical activation, non-cholinergic neurons also appear to participate (Cape and Jones, 2000; Cape et al., 2000). Theta activity, which is eliminated by non-selective lesions, was found to be attenuated but not eliminated by selective lesions of the cholinergic neurons (Lee et al., 1994). It was proposed that the GABAergic neurons of the MS/DBB-hippocampal projection are the critical element. The present results would indicate that in addition to previously considered cholinergic and GABAergic neurons, those having the capacity to synthesize glutamate as a neurotransmitter and thus possibly being glutamatergic, represent a very significant, heretofore unidentified, component of the basalocortical projection system.

#### **4.7 References**

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## 4.8 Figures

Fig. 1. Photomicrographs of sections from the basal forebrain (MCPO) showing single (A-B) or dual (C-L) -immunostaining by immunofluorescence (using Cy3- and Cy2-conjugated secondary antibodies or avidin). PAG immunostaining (Cy3, red) was evident as granular staining within the soma and proximal dendrites of neurons and within the neuropil (A and B). CT immunostaining (Cy2, green) was also evident as granular staining in the soma and dendrites of retrogradely labeled neurons (C). In sections dual-immunostained for CT-PAG (C-D), many CT<sup>+</sup> neurons (solid white arrowheads in C) were PAG-immunopositive (solid white arrowhead in D), and others were PAG immunonegative (open white arrowhead in D). In sections dual-immunostained for CT-ChAT (E-F) or CT-GAD (G-H), CT<sup>+</sup> neurons (solid white arrowheads in E and G) were also revealed to be ChAT<sup>+</sup> or GAD<sup>+</sup> (solid white arrowheads in F and H). In sections dual-immunostained for ChAT-PAG (I-J), many ChAT<sup>+</sup> neurons were immunopositive for PAG (solid white arrowheads in J), whereas others were immunonegative for PAG (open white arrowheads in J). In sections dual-immunostained for GAD-PAG (K-L), some GAD<sup>+</sup> cells (solid white arrowheads in K) were immunopositive for PAG (solid white arrowheads in L), and others were immunonegative for PAG (open white arrowhead in L). Scale bar = 20 $\mu$ m.

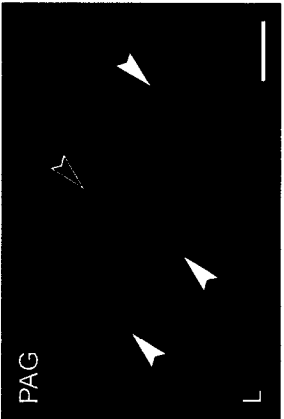
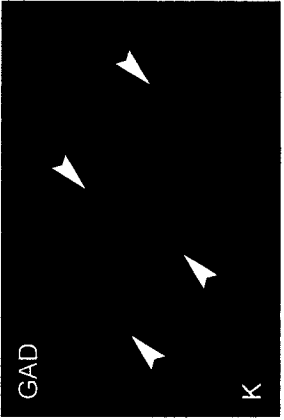
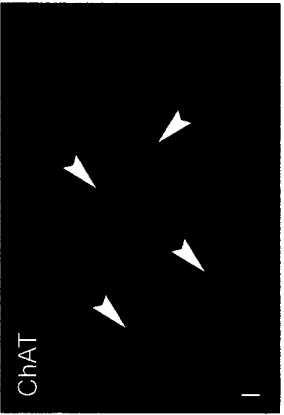
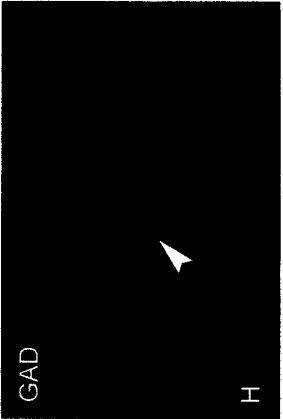
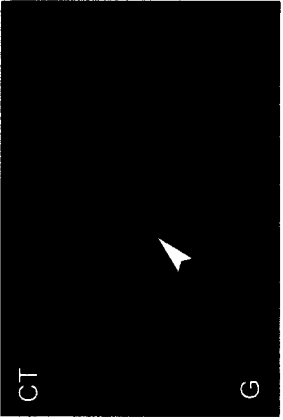
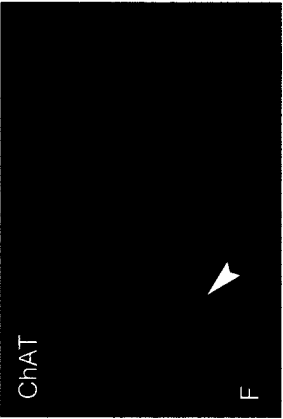
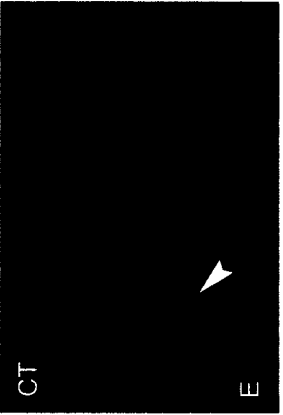
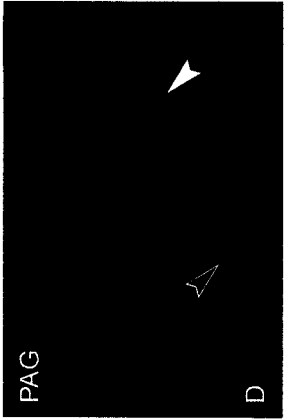
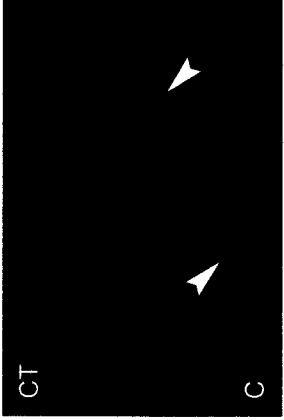
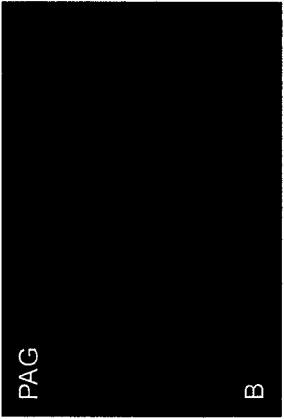
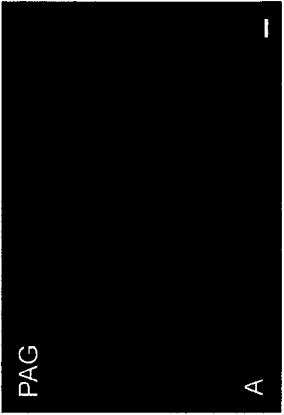


Fig. 2. Drawings of coronal sections through the forebrain showing the CT injection site in the entorhinal cortex and CT retrogradely labeled cells in the hippocampal region and diencephalon, mapped from peroxidase DAB-Ni stained material (A, ~A 2.0 and B, ~A 7.0, (Paxinos and Watson, 1986)). Each symbol indicates one retrogradely labeled cell. Abbreviations: BLA, basolateral amygdaloid nuclei; CeA, central amygdaloid nucleus; CoA, cortical amygdaloid nuclei; CPu, caudate putamen; En, endopiriform nucleus; EP, endopeduncular nucleus; GP, globus pallidus; LEA, lateral entorhinal area; LH, lateral hypothalamus; MeA, medial amygdaloid nuclei; MEA, medial entorhinal area; PaS, parasubiculum; Pir, piriform cortex; PRh, perirhinal cortex; PrS, presubiculum; PV, paraventricular nucleus; Re, reuniens; S, subiculum.

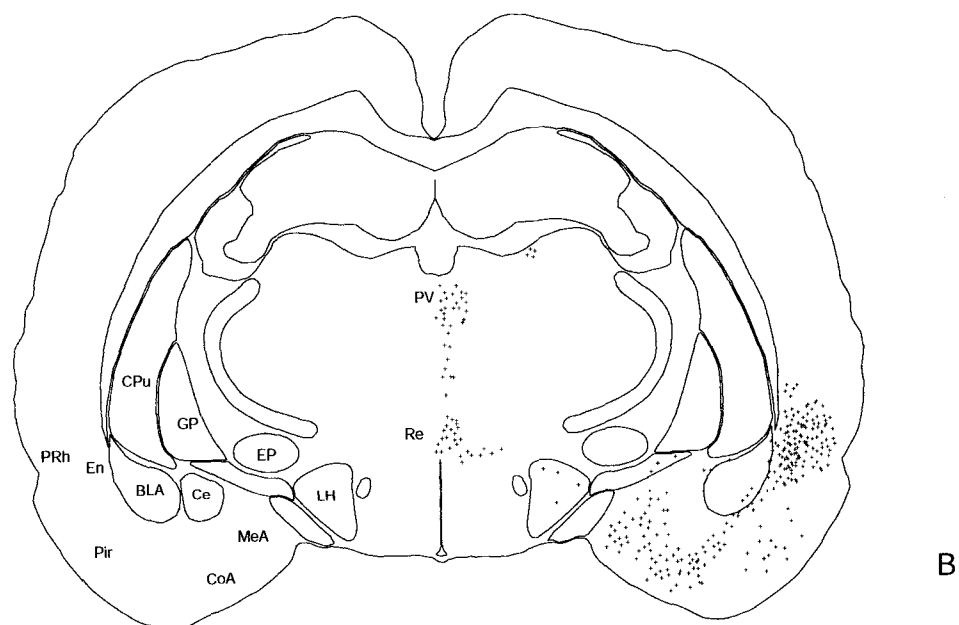
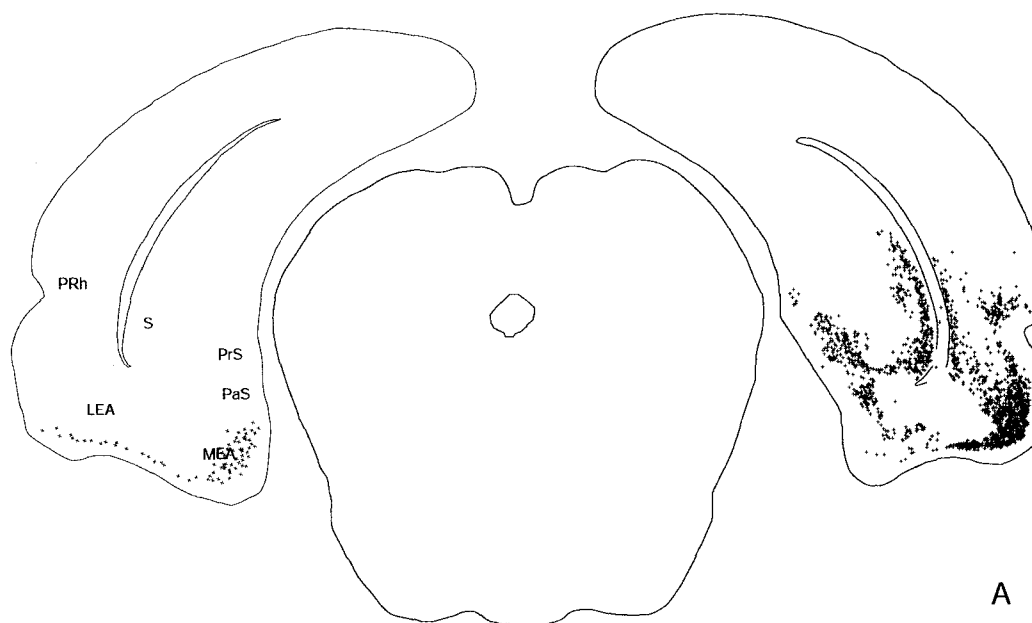
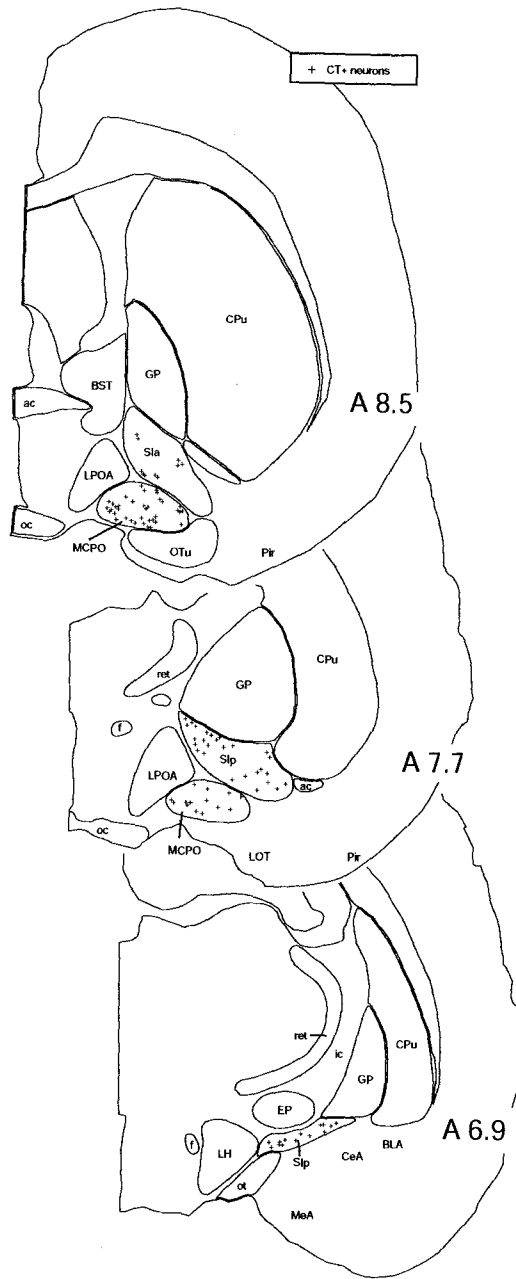
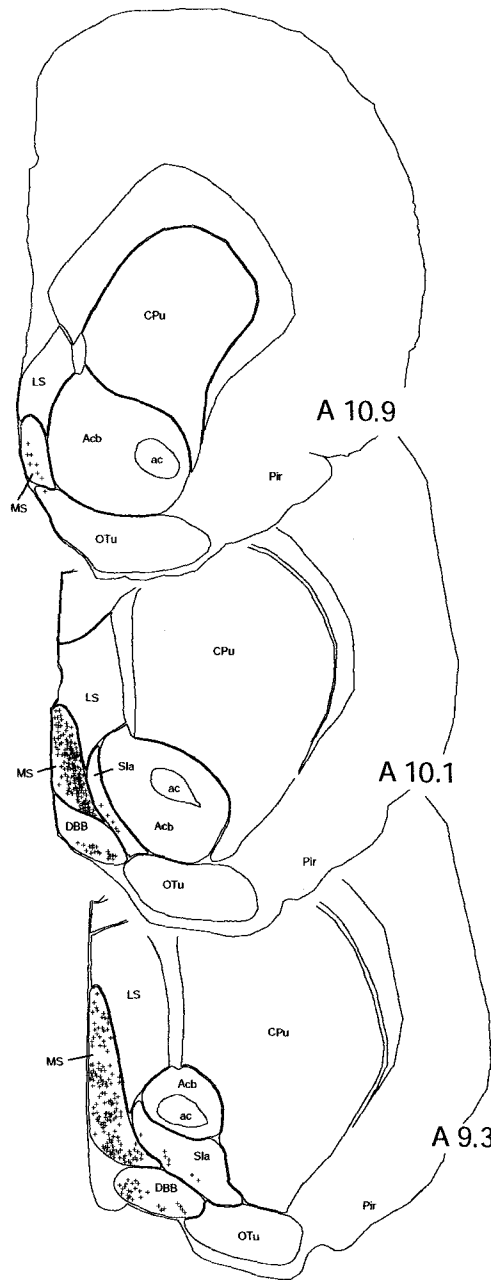
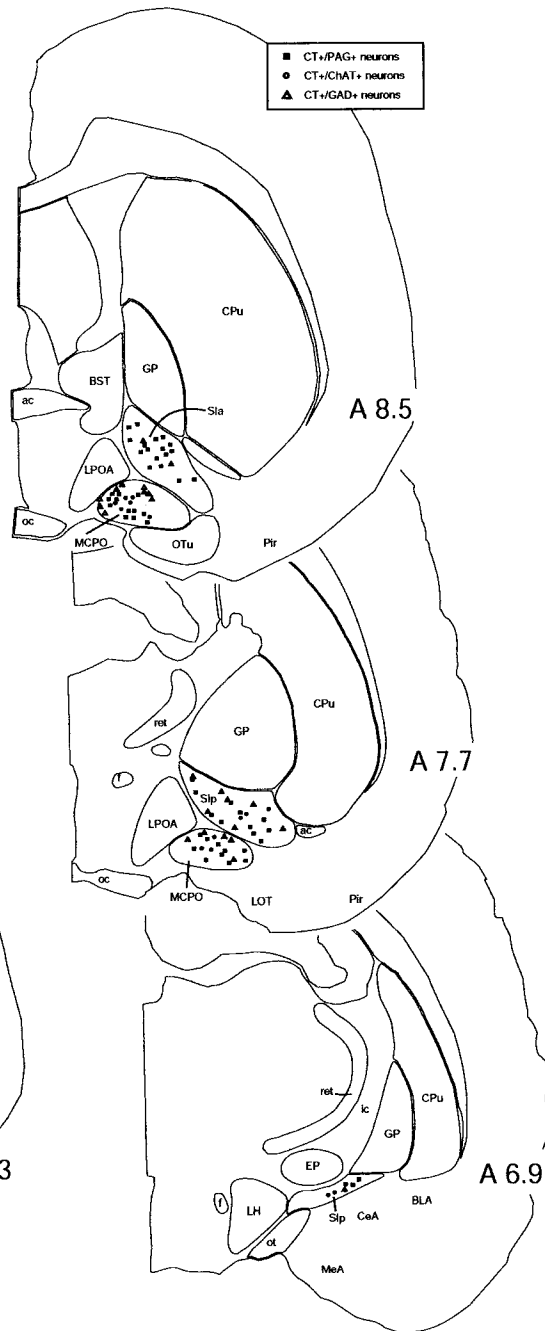
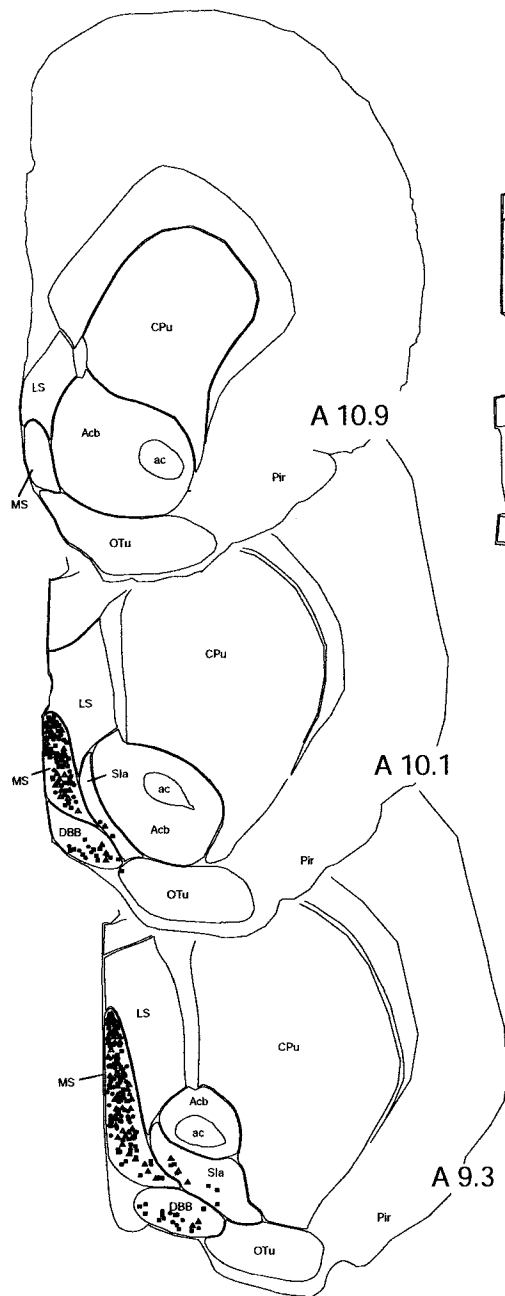


Fig. 3. Atlas figures (Gritti et al., 1993) showing neurons retrogradely labeled with CT from the entorhinal cortex, mapped from peroxidase DAB-Ni stained material. Each symbol indicates one CT+ cell. Abbreviations: ac, anterior commissure; Acb, accumbens; BLA, basolateral amygdaloid nuclei; BST, bed of stria terminalis; CeA, central amygdaloid nucleus; CPu, caudate putamen; DBB, diagonal band of Broca; EP, entopeduncular nucleus; f, fornix; GP, globus pallidus; ic, internal capsule; LH, lateral hypothalamus; LOT, lateral olfactory tract; LPOA, lateral preoptic area; LS, lateral septum; MCPO, magnocellular preoptic area; MeA, medial amygdaloid nuclei; MS, medial septum; oc, optic chiasm; OTu, olfactory tubercle; ot, optic tract; Pir, piriform cortex; ret, reticular nucleus of thalamus; SIa, anterior substantia innominata; SIp, posterior substantia innominata.



**Fig. 4. Atlas figures through the basal forebrain cholinergic cell nuclei showing retrogradely labeled (CT+) neurons dual-immunostained for PAG, ChAT or GAD, these were mapped in from immunofluorescent material. CT+/PAG+ neurons (squares), CT+/ChAT+ neurons (circles) and CT+/GAD+ neurons (triangles) are co-distributed through the nuclei. Each symbol indicates one labeled cell. For Abbreviations see Fig. 3.**



#### 4.9 Tables

Table 1. Total number of CT+ neurons counted in basal forebrain and number and percentage, which were PAG+, ChAT+, or GAD+ in fluorescent dual-immunostained material.

| Nucleus          | Number of cells counted per series <sup>a</sup> |            |            |            |            |            | Percentage double-labeled cells per series <sup>b</sup> |                |                   |
|------------------|-------------------------------------------------|------------|------------|------------|------------|------------|---------------------------------------------------------|----------------|-------------------|
|                  | CT-PAG                                          |            | CT-ChAT    |            | CT-GAD     |            |                                                         |                |                   |
|                  | CT+                                             | CT+/PAG+   | CT+        | CT+/ChAT+  | CT+        | CT+/GAD+   | CT+/PAG+                                                | CT+/ChAT+      | CT+/GAD+          |
| MS               | 68.6 ± 1.1                                      | 55.0 ± 1.1 | 61.6 ± 1.6 | 23.6 ± 1.2 | 70.6 ± 1.3 | 35.2 ± 1.2 | 80.0 ± 0.03                                             | 37.5 ± 0.02††† | 47.4 ± 0.04†††    |
| DBB              | 19.2 ± 1.0                                      | 16.6 ± 1.0 | 22.4 ± 1.1 | 10.8 ± 0.5 | 28.8 ± 1.0 | 7.2 ± 0.7  | 87.1 ± 0.05                                             | 53.7 ± 0.06††† | 23.8 ± 0.04†††,§§ |
| MCPO             | 21.8 ± 0.9                                      | 18.0 ± 1.1 | 19.6 ± 0.7 | 9.0 ± 0.6  | 22.8 ± 1.1 | 7.0 ± 0.7  | 78.7 ± 0.06                                             | 47.8 ± 0.07††  | 32.1 ± 0.05†††    |
| Sla              | 16.0 ± 0.8                                      | 13.0 ± 0.7 | 10.6 ± 0.8 | 2.6 ± 0.9  | 11.4 ± 0.5 | 4.2 ± 0.4  | 81.8 ± 0.03                                             | 22.0 ± 0.08††† | 36.4 ± 0.05†††    |
| Slp              | 15.4 ± 0.8                                      | 13.8 ± 0.7 | 12.8 ± 0.8 | 3.8 ± 0.6  | 16.6 ± 0.6 | 7.0 ± 0.3  | 91.4 ± 0.04                                             | 33.7 ± 0.08††† | 43.4 ± 0.04†††    |
|                  |                                                 |            |            |            |            |            |                                                         |                |                   |
| <b>Total/Avg</b> | 141 ± 1.3                                       | 116 ± 1.3  | 127 ± 1.7  | 49.8 ± 0.9 | 150 ± 1.7  | 60.6 ± 1.1 | 82.2 ± 0.02                                             | 40.3 ± 0.02††† | 40.2 ± 0.02†††    |
|                  |                                                 |            |            |            |            |            |                                                         |                |                   |

<sup>a</sup> CT+ and double-labeled cells were plotted (Fig. 4) and counted in fluorescent dual-immunostained material in 3 adjacent series (for CT-PAG, CT-ChAT and CT-GAD) of 20µm thick sections at 800µm intervals on the (right) side ipsilateral to the CT injection. Total numbers were tabulated for each nucleus in five animals (CT70, CT74, CT75, CT76, CT77) and presented as the mean (± SEM).

<sup>b</sup> Percentages of CT+ cells, which were double-labeled, are presented as the mean (± SEM) for each series. A two-way ANOVA was performed with 'percentage' as dependent variable, 'label' and 'nucleus' as independent variables and 'rat' as covariate. There was a significant main effect of 'label' (F = 130.6, df = 2, df<sub>error</sub> = 59, p < 0.001). In post-hoc paired comparisons (using Fisher's least significant difference test), there was a significant difference between double-labeled cells indicated by † with comparison to CT+/PAG+ and § with comparison to CT+/ChAT+ (with one symbol = p < 0.05, two symbols = p < 0.01, or three symbols = p < 0.001). There was also a significant interaction of 'label' with 'nucleus' (F = 4.2, df = 8, df<sub>error</sub> = 59, p = 0.001). A one-way ANOVA and post-hoc paired comparison was thus performed for each nucleus and significant difference indicated as above.

**Table 2. Size of double-labeled CT+/PAG+, CT+/ChAT+, and CT+/GAD+ cells in the basal forebrain<sup>a</sup>.**

| <b>Nucleus</b> | <b>PAG+/CT+</b> | <b>ChAT+/CT+</b> | <b>GAD+/CT+</b> |
|----------------|-----------------|------------------|-----------------|
| <b>MS</b>      | 18.1 ± 1.1 (9)  | 16.4 ± 1.6 (9)   | 19.6 ± 0.7 (9)  |
| <b>DBB</b>     | 20.1 ± 0.7 (9)  | 20.1 ± 0.5 (7)   | 12.7 ± 2.2 (3)  |
| <b>MCPO</b>    | 20.5 ± 0.6 (10) | 22.6 ± 1.0 (9)   | 18.1 ± 1.1 (9)  |
| <b>SIa</b>     | 18.4 ± 1.5 (9)  | 18.7 ± 2.0 (3)   | 20.3 ± 1.7 (10) |
| <b>SIp</b>     | 20.1 ± 1.2 (9)  | 18.2 ± 0.9 (5)   | 20.1 ± 1.2 (9)  |
| <b>Average</b> | 19.5 ± 0.5 (46) | 19.4 ± 0.7 (33)  | 19.0 ± 0.7 (40) |

<sup>a</sup> For each nucleus, the mean ± SEM large diameter and number of cells measured (in parentheses) in three animals (CT74, CT75, CT76) are indicated and taken from fluorescent immunostained material. A two-way ANOVA was performed with 'size' as the dependent variable, 'label' and 'nucleus' as independent variables and 'rat' as covariate. There was no significant main effect of 'label' on cell size ( $F = 1.394$ ,  $df = 2$ ,  $df_{\text{error}} = 103$ ;  $p = .253$ ). There was a significant interaction of 'label' and 'nucleus' ( $F = 3.12$ ,  $df = 8$ ,  $df_{\text{error}} = 103$ ;  $p = 0.003$ ) and in post-hoc comparisons, a significant difference in the size of CT+/GAD+ cells compared to those of the CT+/PAG+ and CT+/ChAT+ cells in the MS and DBB ( $p < .01$ , not indicated).

## **Chapter Five**

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### **5. Rhythmic Discharge of Cholinergic, GABAergic and Glutamatergic Basal Forebrain Neurons in Relation to Cortical Activity<sup>1</sup>**

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<sup>1</sup> Prepared for publication under the authorship of: Manns ID, Alonso A, Jones BE.

## 5.1 Preface

Having established in neuroanatomical studies that cortically projecting basal forebrain neurons contain the enzyme PAG, and thus that they may be glutamatergic (Chapter 4), we sought to examine whether distinct physiological cell groups could be glutamatergic. One physiological distinct group of neurons that had been noted to be ChAT and GAD immunonegative in our previous studies (Chapter 2 and 3) had a distinctive rhythmic discharge pattern, which was uncannily similar to that reported for rhythmic noncholinergic neurons *in vitro* (Alonso et al., 1996). The obvious question was whether these cells may represent cortically projecting glutamatergic neurons. The aim of this study was to test this hypothesis and then compare the rhythmicity of these cells with that of the rhythmic cholinergic and GABAergic neurons in relation to one another and EEG activity.

## **5.2 Abstract**

Cortical activity is influenced by multiple subcortical neuromodulatory inputs. Having important influence are afferents from the basal forebrain, comprising cholinergic, GABAergic and noncholinergic/nonGABAergic cortically projecting neurons. The cholinergic influence at the level of the cortex is considerable; however, its selective elimination by lesions has not been associated with major deficits in cortical function or cognitive performance, suggesting that noncholinergic inputs are equally important. As previously established, cholinergic neurons fire rhythmically in association with cortical activation and rhythmic slow theta-like activity recorded from the prefrontal cortex. In addition, some GABAergic neurons fire rhythmically with cortical activation. However, a large number of neurons that discharge rhythmically appear to be neither cholinergic nor GABAergic. Here, such cells were established as noncholinergic/nonGABAergic and found to contain phosphate-activated glutaminase (PAG), the synthetic enzyme for transmitter glutamate. Like the cholinergic and GABAergic neurons, these putative glutamatergic neurons discharged rhythmically in association with rhythmic cortical activity occurring during stimulation-induced cortical activation. Their rhythmic activity was correlated with that of multiple cortical areas, including the olfactory bulb, piriform, entorhinal and prefrontal cortex. Through coordinated rhythmic discharge of glutamatergic, GABAergic and cholinergic cortically projecting neurons, the basal forebrain may modulate cortical activity in a rhythmic manner to promote coherent activity across cortical areas during cortical activation and to facilitate synaptic plasticity within the functionally linked circuits of those areas.

### 5.3 Introduction

The basal forebrain is a cortically projecting neuromodulatory system capable of stimulating and maintaining cortical activation. Its cortical projections are distributed in a topographical manner and are well organized to co-activate multiple cortical regions in parallel during cortical activation (Saper, 1984; Luiten et al., 1987). The basalocortical system has been recognized to be involved in cortical activation and memory largely via its cholinergic projections (Rye et al., 1984; Saper, 1984; Luiten et al., 1987; Metherate et al., 1988; Smith, 1988; Metherate et al., 1992; Huerta and Lisman, 1993; Jones, 1993; Muir et al., 1994; Kilgard and Merzenich, 1998; Cape and Jones, 2000; Cape et al., 2000). The cholinergic basal forebrain neurons provide the major source of acetylcholine (ACh) to the cerebral cortex (Saper, 1984; Luiten et al., 1987). Blocking cholinergic receptors results in diminished cortical activation (Wikler, 1952; Longo, 1966) and impaired memory function (Peterson, 1977). Recent work determined that the majority of cholinergic basal forebrain neurons fire rhythmically during cortical activation and rhythmic slow theta-like activity (Manns et al., 2000a). Their rhythmic bursting discharge recorded *in vivo* substantiated *in vitro* results that found the cholinergic neurons to have intrinsic membrane properties endowing them with the potential for rhythmic bursting discharge (Khateb et al., 1992). These findings agree well with studies indicating that acetylcholine (ACh) and cholinergic neurons facilitate rhythmic slow activity, or theta, which occurs during active waking and paradoxical sleep in the hippocampus and limbic cortex (Jouvet et al., 1960; Parmeggiani and Zanocco, 1963; Vanderwolf, 1975; Gaztelu and Buno Jr., 1982; Buzsaki et al., 1983; Borst et al., 1987; Lee et al., 1994; Brazhnik and Fox, 1999). However, selective lesions of cholinergic

neurons in the medial septum-diagonal band of Broca (MS-DBB) area did not eliminate theta activity in the hippocampus suggesting that other putative GABAergic neurons participate in generating theta (Lee et al., 1994). Moreover, selective lesions of cholinergic neurons in the basal forebrain have not resulted in the same behavioral and cognitive deficits as nonselective lesions, indicating that noncholinergic neurons also play an integral role in processes of cortical activation and memory (Dunnett et al., 1991; Voytko et al., 1994; Baxter et al., 1995b). These studies clearly reveal the importance of the noncholinergic basalocortical neurons. *In vitro* studies showed that noncholinergic neurons also display robust intrinsic rhythmic activity (Alonso et al., 1996). It was thought that these rhythmically discharging neurons might correspond to the GABAergic basalocortical neurons, which project to the cortex in equal numbers to cholinergic neurons (Gritti et al., 1997). Our studies employing juxtacellular recording and labeling of GABAergic neurons found a small subgroup of GABAergic neurons that discharged in a rhythmic fashion (Manns et al., 2000b). The GABAergic neurons are believed to play an important role together with the cholinergic neurons regulating rhythmic slow activity in the cortex. However, we found in our studies that an even larger number of rhythmically discharging cells did not appear to be cholinergic or GABAergic (Manns et al., 2000a; Manns et al., 2000b). These results suggested that another important group of rhythmically discharging cells with potential influence in modulating the cortex in a rhythmic manner remained to be identified.

Noncholinergic/nonGABAergic basal forebrain neurons were also known to represent an important contingent of cortically projecting neurons (Gritti et al., 1997). In a recent study employing immunohistochemical staining for phosphate-activated

glutaminase (PAG), the enzyme responsible for synthesis of transmitter glutamate (Bradford et al., 1978), we found that cortically projecting neurons may be glutamatergic (Manns et al., 2001). In the current study, we examined rhythmically discharging basal forebrain cell groups to characterize and compare their different properties in relation to cortical activity and to determine whether noncholinergic/nonGABAergic rhythmically discharging neurons may be glutamatergic.

## **5.4 Methods and Materials**

### **Animals and Surgery**

Experiments were performed on adult urethane-anesthetized Long Evans rats ( $n = 120$ , 200 - 250g, Charles River, St. Constant, Quebec, Canada). According to procedures described in detail in a previous study (Manns et al., 2000a), the anesthetized animals were positioned in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA) for both the surgery and subsequent recording. For the purpose of antidromic activation of basal forebrain units and recording of local field potentials, bipolar electrodes were placed in the hippocampus (AP  $-3.4\text{mm}$ , L  $+2.1\text{mm}$ , and V  $-2.0\text{mm}$ ), prefrontal cortex (AP  $+2.0\text{ mm}$ , L  $+1.0\text{ mm}$ , and V  $-2.0\text{ mm}$ ), olfactory bulb (AP  $+6.4\text{mm}$ , L  $+1.0\text{mm}$ , and V  $-2.5\text{mm}$ ), entorhinal cortex (AP  $-7.4\text{mm}$ , L  $5.3\text{mm}$ , and V  $-7.8\text{mm}$ ), and piriform cortex (AP  $-2.2\text{mm}$ , L  $5.5\text{mm}$ , and V  $-8.5\text{mm}$ ). In a series of experiments, EMG activity was recorded with stainless steel wires from the intercostal muscles to monitor respiration.

### **Unit recording and labeling**

Juxtacellular recording and labeling was done with an intracellular amplifier (IR-283; Neurodata Instruments, New York, NY). Unit recordings were performed with glass microelectrodes, filled with 0.5 M sodium acetate and  $\sim 5.0\%$  Neurobiotin (Nb, Vector Laboratories, Burlingame, CA). Recorded units were characterized in association with spontaneous irregular slow activity and somatosensory stimulation-induced rhythmic slow activity along with higher frequency activity on the EEG. The stimulation consisted of a continuous pinch of the tail. Antidromic activation was tested from the prefrontal

cortex, olfactory bulb, piriform and entorhinal cortex. Spike widths were measured from positive inflection to first zero-crossing using greater than 128 averaged spikes.

After the recording and characterization of isolated neurons, they were labeled using the 'juxtacellular' method (Pinault, 1996). The animals then received an overdose of urethane and were transcardially perfused with a 4% paraformaldehyde solution or with a modified Zamboni's solution, containing 0.3% paraformaldehyde and 75% saturated picric acid in 0.1 M sodium phosphate buffer (pH 7.0) used for immunostaining of PAG (Manns et al., 2001).

### **Histochemistry**

Coronal frozen sections were cut at 30  $\mu$ m and incubated overnight in a primary antibody for ChAT (rat anti-ChAT monoclonal antibody, 1:2, Boehringer-Mannheim, Germany) and/or GAD (rabbit anti-GAD antiserum, 1:3000, Chemicon, Temecula, CA) or a rabbit anti-PAG antiserum (1:6000, supplied by T. Kaneko (Akiyama et al., 1990)). They were subsequently co-incubated in a Cy2-conjugated streptavidin (1:800, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) to reveal Nb and either an AMCA-conjugated donkey anti-rat antiserum (1:50, Jackson ImmunoResearch Laboratories) to reveal ChAT-immunostaining and/or Cy3-conjugated donkey anti-rabbit antiserum (1:1000, Jackson ImmunoResearch Laboratories) to reveal GAD-immunostaining or PAG-immunostaining. Sections were viewed by fluorescent microscopy using a Leitz Dialux microscope equipped with a Ploemopak-2 reflected light fluorescence illuminator with Leica filter cubes for ultraviolet (A), fluorescein (I3) and rhodamine (N2.1).

## **Data Analysis**

Analysis of physiological data was performed on stationary periods of recording from pre-stimulated and stimulated conditions, as previously described in detail (Manns et al., 2000a). For the EEG, spectral analysis was performed to determine the dominant peak frequency. Gamma band activity was computed as the area of the amplitude spectra between 30 - 58 Hz. For unit discharge, analysis was done on stationary periods of at least 45 seconds in duration. The average discharge rate was calculated as average spikes per second from the peristimulus histogram (PSH). The calculation of the predominant instantaneous firing frequency was determined from the first-order interspike interval histogram (ISIH). Assessment of rhythmic and higher-order interspike interval tendencies was performed using an auto-correlation histogram (ACH) on the same data segments as for the other unit calculations. The ACHs were fit with a modified Gabor function to examine oscillatory activity (Konig, 1994). For determination of the degree of rhythmicity, an oscillatory index was calculated. The ratio was computed of the oscillatory amplitude (above the offset of the random activity) of the first satellite peak to that of central peak (at time zero, above the offset of the random activity). In the pre-stimulation as well as the stimulation condition, unit activity with an oscillatory index above zero was counted as rhythmic and included in statistics for calculation of average frequency. The spike-triggered average (STA) was used to estimate the extent of cross-correlation between spike-trains and EEG activity. To determine whether the actual unit-to-EEG STA was significantly different from random unit-to-EEG patterns, it was compared with an STA computed using a spike-train generated from randomly shuffled interspike intervals of the original spike train. The actual unit-to-EEG STA was

considered significantly different from the random unit-to-EEG STA with a probability of  $\leq 0.05$  using the Wilcoxon test. The phase relationship between the unit activity and the field events was calculated by creating a histogram of spikes as they occurred in relation to the normalized field cycle. A sine wave was then fit to the phase histogram to calculate the preferential phase of the unit discharge. All analysis of raw data was done with Matlab (5, The MathWorks Inc., Natick, MA).

For statistical comparison of the properties of ChAT+, GAD+, ChAT-/GAD-, and PAG+ cells, both non-parametric and parametric tests were done using Systat (9.0, SPSS Inc., Chicago, IL). Figures were compiled using Adobe Photoshop (5.0 Adobe Systems, Inc., San Jose, CA) for photomicrographs and Origin (5.0, Microcal Software Inc., Northhampton, MA) for plotting electrophysiological data and analyses.

## **5.5 Results**

Combining previously published data concerning ChAT+, ChAT-negative, GAD+ or GAD-negative cells (n = 94, (Manns et al., 2000a; Manns et al., 2000b)) with newly obtained data for the present study of these and other cells that discharge rhythmically, 121 neurons have been recorded and juxtacellularly labeled within the magnocellular preoptic (MCPO) and substantia innominata (SI) nuclei of the basal forebrain in association with cortical activity. Of this total number of cells, ~45% discharged rhythmically during cortical activation. In the present report, complete analysis is presented for 42 rhythmically discharging neurons (of which 27 were newly obtained for this study) that were immunohistochemically identified as ChAT+, GAD+, ChAT-negative and GAD-negative, or PAG+.

### **Pattern of Unit Discharge**

As previously described (Manns et al., 2000a), the majority of cholinergic neurons discharged in a rhythmic bursting mode in association with cortical activation. The rhythmically discharging Nb+/ChAT+ cells (Fig. 1A) included here (n=11) were distributed through the MCPO and SI (Fig. 2) and were on average medium-large in size ( $23.2 \pm 1.2 \mu\text{m}$ ). The firing of the ChAT+ neuron shown in Figure 1A is depicted in Figure 3AI together with EEG activity from the hippocampus and prefrontal cortex preceding and during somatic stimulation that induced cortical activation. In association with the slow irregular cortical activity that was characteristic of the pre-stimulation condition, the firing of the ChAT+ neuron was slow and irregular. In association with the more regular slow (theta-like), along with more high frequency (gamma), cortical activity

that was characteristic of the stimulation condition, the ChAT+ neuron displayed prominent rhythmic firing, often in bursts. ChAT+ neurons always increased their average discharge rate (calculated from PSH) and their instantaneous firing frequency (from ISIH), reflecting high frequency bursting, despite considerable variability in the average firing frequency (Table 1). During stimulation, a significant proportion of the firing frequencies were above 80 Hz ( $17.6 \pm 3.9 \%$ ,  $n = 11$ ) as evidence of this discharge pattern. The degree of rhythmicity, which was assessed quantitatively by an oscillatory index (Table 1), also increased significantly with stimulation along with the frequency of the oscillatory discharge. The rhythmic unit discharge was significantly cross-correlated with the EEG activity of the prefrontal cortex (evident in the unit-to-EEG STA, Fig. 3AII).

As previously described (Manns et al., 2000b), a small subgroup of GABAergic basal forebrain neurons discharges in rhythmic clusters of spikes in association with cortical activation. The rhythmically discharging Nb+/GAD+ cells (Fig. 1B) included here ( $n = 8$ ) were distributed through the MCPO and SI (Fig. 2) and were on average medium-large ( $24.7 \pm 2.0 \mu\text{m}$ ) and not different in size from the rhythmic ChAT+ neurons. They were distinguished from the ChAT+ cells by their average spike width that was narrower (Table 1). The firing of the GAD+ neuron shown in Figure 1B is depicted in Figure 3BI in association with EEG activity. During slow irregular cortical activity, the firing of the GAD+ neuron was relatively fast and regular. During stimulation, the GAD+ neuron decreased its average discharge rate while shifting to an oscillatory discharge pattern characterized by regularly recurring clusters of spikes. In contrast to the ChAT+ neurons, the GAD+ neurons always decreased their average

discharge rate (calculated from PSH) during stimulation, and displayed a high rate of tonic discharge during slow irregular activity (Table 1). During stimulation, they discharged in clusters of spikes at an average instantaneous firing frequency of ~40 Hz (from ISIH, Table 1). Though much lower than that of the ChAT+ cells, the instantaneous firing frequency of the GAD+ cells was not significantly different from that of the ChAT+ cells, due to the high variability in frequencies of the ChAT+ cells. Only a small proportion of the instantaneous firing frequencies of the GAD+ cells included high frequency activity (>80 Hz:  $5.3 \pm 4.5\%$ ), which was significantly less than that of the ChAT+ cells ( $F = 4.27$ ,  $df = 1, 38$ ,  $p = 0.046$ ). Similar to ChAT+ cells, however, the GAD+ cells did fire in an oscillatory manner during stimulation and with a high degree of rhythmicity, as evident by the oscillatory index, which increased significantly with stimulation (Table 1). The rhythmic discharge of the GAD+ cells was significantly cross-correlated with the EEG activity of the hippocampus and prefrontal cortex during stimulation (Fig. 3BII).

In our previous studies of ChAT+ and GAD+ neurons (Manns et al., 2000a; Manns et al., 2000b), it became apparent that a number of neurons that discharged in a prominently rhythmic manner with clusters of spikes, most similarly to the GAD+ cells, were neither GAD+ nor ChAT+. Using triple staining for the present study, we have labeled a number of these rhythmically discharging cells with Nb and unequivocally identified them as ChAT-negative and GAD-negative (Fig. 1C,  $n = 10$ ). Not differing in size or distribution from the Nb+/ChAT+ and Nb+/GAD+ cells, the Nb+/ChAT-/GAD- neurons were medium-large sized neurons ( $22.6 \pm 1.6 \mu\text{m}$ ) and distributed through the MCPO and SI. On the other hand, the spike width of the ChAT-/GAD- cells was

significantly narrower than that of the ChAT+ cells (Table 1). The discharge pattern of the ChAT-/GAD- neuron shown in Figure 1C is displayed in Figure 3CI in association with EEG activity. This neuron fired in a slow and irregular manner during slow irregular cortical activity and shifted to a prominent rhythmic discharge comprised of recurring clusters of spikes with stimulation. Their average discharge rate was significantly lower than that of the GAD+ neurons during the pre-stimulation period and increased slightly with stimulation (Table 1). The instantaneous firing frequency of the ChAT-/GAD- cells did not change with stimulation, was much lower than that of the ChAT+ neurons (though not significantly so,  $F = 4.29$ ,  $df = 1$ ,  $p = 0.06$ ), and significantly lower than that of the GAD+ neurons during stimulation (Table 1). They had virtually no high frequency spiking in their discharge ( $>80$  Hz:  $0.8 \pm 4\%$  of the ISIH) as distinguished from the cholinergic cells ( $F = 8.99$ ,  $df = 1$ ,  $38$ ,  $p = 0.005$ ). The degree of rhythmicity displayed by these neurons increased significantly with stimulation and was as high as that of the ChAT+ and GAD+ cells, as evident in the oscillatory index (Table 1). During the stimulation-induced cortical activation, the STA unit-to-EEG cross-correlation was significant for the prefrontal cortex activity (Fig. 3CII).

Having found this large number of rhythmically discharging cells that was neither cholinergic nor GABAergic, we sought to determine whether they could utilize glutamate as a neurotransmitter and thus contain the enzyme PAG, identified as the synthetic enzyme for transmitter glutamate. Nb-labeled neurons with rhythmic discharge properties similar to the above-described ChAT-/GAD- cells were found to be PAG immuno-positive (Fig. 1D,  $n = 13$ ). These neurons were also confirmed by triple staining (in 11 cells) for Nb, PAG, ChAT to be both PAG+ and ChAT-negative. Not different in

size or distribution from the Nb/ChAT+, Nb+/GAD+ or Nb+/ChAT-/GAD- cells, the Nb+/PAG+ cells were medium-to-large sized neurons ( $25.5 \pm 2.4 \mu\text{m}$ ) and distributed through the MCPO and SI. They had a significantly wider spike width than the GAD+ neurons. The firing of the PAG+ neuron shown in Figure 1D is displayed in Figure 3DI. This neuron, similar to the ChAT-/GAD- neuron illustrated in Figure 3C, fired slowly and irregularly with slow irregular cortical activity and shifted to an oscillatory discharge pattern characterized by regularly recurring clusters of spikes with stimulation-induced cortical activation. Like the ChAT-/GAD- cells, the PAG+ neurons had a lower average discharge rate during slow irregular cortical activity than the GAD+ cells. Their instantaneous firing frequency did not change with stimulation, was much lower than that of the ChAT+ neurons (though not significantly so,  $F = 4.61$ ,  $df = 1$ ,  $p = 0.057$ ) and significantly lower than that of the GAD+ neurons (Table 1). The proportion of their instantaneous firing frequency that was in a high frequency range was negligible ( $>80$  Hz:  $0.4 \pm 3.6\%$  in the ISIH) as for the ChAT-/GAD- cells and significantly different from that of the ChAT+ cells ( $F = 10.69$ ,  $df = 1$ ,  $38$ ,  $p = 0.002$ ; with all statistics based upon post-hoc hypothesis testing following an overall ANOVA across groups,  $F = 4.38$ ,  $df = 3$ ,  $38$ ;  $p = 0.010$ ). As evidenced in the records and by the oscillatory indices, the rhythmic activity of these neurons increased with stimulation-induced cortical activation and was similar in degree to that of the other cell groups (Table 1). During the cortical activation, the unit-to-EEG cross-correlation was significant for the hippocampus and prefrontal cortex (Fig. 3DII).

Across the ChAT+, GAD+, ChAT-/GAD- and PAG+ cell groups, the rhythmic unit activity was similar in frequency (according to ACH, Table 1). In all groups, the

oscillatory frequency was significantly slower than the frequency of the hippocampal theta activity ( $t = 13.9$ ,  $df = 29$ ,  $p < 0.001$ ) but not than that of the prefrontal cortex activity. The proportion of rhythmic units whose activity cross-correlated with hippocampal activity was relatively low (25% of cells), whereas the proportion whose activity cross-correlated with prefrontal cortex activity was high (73% of cells) and significantly greater than the former ( $\chi^2 = 16.1$ ,  $df = 1.0$ ,  $p < 0.001$ ). However, the proportion of cells that could be antidromically activated from the prefrontal cortex was small (~10%,  $n = 4$ ), suggesting that they could have other primary cortical targets.

#### **Relation of Unit Discharge to EEG of Different Cortical Areas**

Many cholinergic, GABAergic, and noncholinergic/nonGABAergic MCPO neurons project to the olfactory bulb (Zaborszky et al., 1986). The olfactory bulb is known to have rhythmic EEG activity coupled to respiration (Sobel and Tank, 1993), which becomes locked with whisking and theta activity during certain behavioral states (Komisaruk, 1970; Macrides et al., 1982; Vanderwolf, 1992). To examine the possibility that rhythmic basal forebrain neurons may project to the olfactory bulb and discharge in correlation with olfactory bulb rhythmic activity, units were tested for antidromic activation from the olfactory bulb and recorded simultaneously with activity from the olfactory bulb and intercostal muscles, as an indicator of respiration. An example of a PAG+ neuron is shown in Figure 4I firing in association with both the olfactory bulb and intercostal activity. This unit, which was antidromically activated from the olfactory bulb, discharged rhythmically in association with stimulation and at the same frequency as the olfactory bulb and intercostal activity, as evident in the STA cross-correlation

analysis (Fig. 4II). The olfactory bulb and the intercostal activity was >90% coherent, indicating that the olfactory bulb activity reflected the respiratory activity, as previously established (Macrides et al., 1982; Sobel and Tank, 1993). During the somatosensory stimulation, the frequency of the olfactory bulb activity increased significantly from  $1.25 \pm 0.05$  to  $1.46 \pm 0.07$  ( $t = 2.5$ ,  $df = 18$ ,  $p = 0.024$ ), as did the rhythmic frequency of the units (Table 1). However, the oscillatory index of the olfactory bulb did not change with stimulation, being equally high during the pre-stimulation period ( $0.89 \pm 0.04$ ) as during the stimulation period ( $0.89 \pm 0.02$ ), in contrast to that of the units, which was much lower (average 0.43) during the pre-stimulation period than during stimulation period (average 0.84, Table 1). All rhythmic basal forebrain neurons recorded in association with olfactory bulb activity were significantly cross-correlated with its activity ( $n = 23$ ). Additionally, a relatively high proportion of these cells were antidromically activated from the olfactory bulb (~48% of cells tested,  $n = 10$ ).

Some basal forebrain neurons may project to multiple cortical regions (Saper, 1984; Luiten et al., 1987; Okoyama et al., 1987; Paolini and McKenzie, 1997); the possibility that the recorded cells did so and that their activity cross-correlated with other cortical regions was examined. Shown in Figure 5I is the discharge of a PAG+ neuron that was antidromically activated from the entorhinal cortex and discharged rhythmically during cortical activation in a cross-correlated manner with rhythmic EEG activity across multiple cortical areas, including the entorhinal cortex, prefrontal cortex, piriform cortex, and olfactory bulb (Fig. 5II). In the power spectra of those activities, the peak frequency of the unit corresponded to that of all cortical leads during slow irregular activity; however, during stimulation, it corresponded to the dominant peak frequency of all leads

excepting that of the hippocampal and entorhinal cortex, which was higher (Fig. 5III). The unit activity did correspond to a secondary peak frequency in the entorhinal cortex accounting for the significant STA cross-correlation between the two (Fig. 5II and III). The unit activity was often cross-correlated with that of the entorhinal and piriform cortex during stimulation (in 8/12 and 3/3 incidents, respectively). Additionally, instances of antidromic activation of rhythmic units were found from each of these cortical areas ( $n = 2$ ;  $n = 1$ , respectively).

### **Phase of Unit Discharge**

To understand how these groups of rhythmic basal forebrain neurons may collectively affect their cortical targets, their firing in relation to one another was examined. Since all rhythmic basal forebrain units were correlated with the activity of the olfactory bulb, the olfactory wave was used as the reference to trigger and analyze the firing of the unit across its cycle. Several recorded units (2-5) per brain ( $n = 5$ ) that were classified according to their distinctive firing properties as putatively cholinergic, GABAergic or glutamatergic were analyzed in relation to the olfactory rhythm in each brain. In Figure 6A, B, C, the firing distribution as a function of the olfactory rhythm of a putatively cholinergic, a putatively GABAergic, and a putatively glutamatergic neuron from the same brain is shown and illustrates that they may fire preferentially at different phases. Across brains, there was a consistent preferential firing phase only for the GABAergic neurons, between 135 and 180 degrees (Fig. 6D), and a significantly different phase distribution between the three cell groups (Fig. 6D; chi-square = 27.5,  $df = 10$ ,  $p = 0.002$ ). The cholinergic neurons' firing distribution was significantly different from that of the

GABAergic and glutamatergic neurons (Mann-Whitney U-test = 0, chi-square = 12.7, df = 1, p = 0.000; and, Mann-Whitney U-test = 33, chi-square = 5.6, df = 1, p = 0.018).

## **5.6 Discussion**

This is the first time that the physiology has been reported for positively identified basal forebrain cortically projecting glutamatergic neurons. The activity of the rhythmic basal forebrain units, which made up approximately one-half of the cells recorded and labeled in the basal forebrain, was slower than that of the hippocampal theta activity and preferentially cross-correlated with the that of the prefrontal cortex. It was also always correlated with olfactory bulb and often piriform and entorhinal cortical activities, all at a similar slower than hippocampal theta, frequency. Finally, examination of the phase relationship of the firing of these cells to the olfactory rhythm suggested that the GABAergic neurons had a preferred-firing phase, while no definite relationship was established for cholinergic or glutamatergic cells.

### **Physiology of Different Cell Types**

The extensive overlap in the discharge properties of the rhythmic ChAT-/GAD- with those of PAG+ cells strongly suggests that they are the same group of neurons, and as such, will be referred to collectively as the glutamatergic cell group. The glutamatergic neurons had properties that were different from those of both the cholinergic and GABAergic cell groups, which also had properties that differed between them. During slow irregular cortical activity, cholinergic and glutamatergic cells had lower average discharge rates than the relatively fast firing GABAergic cells. Additionally, during cortical activation, the instantaneous firing frequency of the glutamatergic cells (~20Hz) was significantly lower than that of the GABAergic cells (~40Hz) and much lower than that of the cholinergic cells (~70Hz) during the same period. Basal forebrain cholinergic

and non-cholinergic neurons have been identified *in vitro* as possessing intrinsic membrane properties that though differing, confer upon them both the potential for robust spontaneous rhythmic activity (Khateb et al., 1992□; Alonso et al., 1996). As compared to the burst and cluster discharge of the cholinergic and noncholinergic cells respectively identified *in vitro*, the discharge patterns of the cholinergic and noncholinergic cells described *in vivo* here and in our previous reports (Manns et al., 2000a; Manns et al., 2000b) do show remarkable similarities. From the present results, it appears that in addition to cholinergic cells that discharge in rhythmic bursts of spikes, both GABAergic and glutamatergic cells discharge in rhythmic clusters of spikes. Basal forebrain neurons sit in the pathway of the medial forebrain bundle and are innervated by many neuronal groups comprising the ascending activating system, including noradrenergic, serotonergic, and glutamatergic neurons (Jones and Cuello, 1989). Work done *in vitro* has shown the cholinergic neurons' intrinsic rhythmic properties to be intimately regulated by potential transmitters: they are depolarized by glutamate, noradrenaline and histamine agonists and are hyperpolarized by serotonin and muscarinic agonists (Khateb et al., 1993; Khateb et al., 1995b; Khateb et al., 1995a; Khateb et al., 1997; Khateb et al., 1998). Other *in vitro* work indicates that rhythmic noncholinergic neurons are comprised of pharmacologically distinct groups, the majority of which are depolarized by noradrenaline and the minority of which are hyperpolarized by noradrenaline (Fort et al., 1998). The noradrenaline-depolarized majority may correspond to the glutamatergic neurons, which made up the majority of the rhythmic cells here. The rhythmic GABAergic cells that decrease their discharge during cortical activation may correspond to the noncholinergic group identified *in vitro* that was hyperpolarized by noradrenaline,

as noradrenergic neurons of the brainstem are maximally active during the cortical activation of waking (McCarley and Hobson, 1975).

### **Relationship of Unit Discharge to Cortical Activity**

In this study, as in an earlier study (Manns et al., 2000a), the rhythmic discharge of cholinergic basal forebrain neurons had a frequency lower than that of limbic or hippocampal theta. Similarly, in a subsequent report, we noted that the small subgroup of GABAergic neurons that fired rhythmically also did so at a frequency lower than that of limbic theta (Manns et al., 2000b). The results of the present study clearly illustrate that similar to the cholinergic neurons this slower than theta activity of the GABAergic and the newly described glutamatergic neurons is also preferentially associated with the activity of the prefrontal cortex. The activity of the rhythmic cells was always correlated with olfactory activity. Neurons in the MCPO are known to project to the main olfactory bulb (Luskin and Price, 1982; Zaborszky et al., 1986) and were found here in the majority to be antidromically activated from the olfactory bulb. The activity of the olfactory bulb is linked to respiratory drive (Freeman, 1975, 1976; Freeman and Schneider, 1982; Kay et al., 1996; Kay and Freeman, 1998), and olfactory bulb unit activity discharge is synchronized with respiration due to activation of olfactory receptors (Walsh, 1956; Macrides and Chorover, 1972; Ravel et al., 1987). However, the relationship of these rhythmic basal forebrain neurons to the respiratory-olfactory cycle is not straightforward. These cells express this robust rhythmic activity maximally during states of heightened cortical arousal suggesting that afferents from the reticular formation, excited during tail-pinch stimulation, synapse on basal forebrain neurons and modulate their intrinsic

membrane properties to generate the rhythmic activity that is correlated and locked with the olfactory rhythm. These findings together with early work indicating that the inspiration-induced olfactory bulb waves are modulated by reticular stimulation (Pagano, 1966) suggest that rhythmic basal forebrain activity influences rhythmic olfactory bulb activity during arousal. However, the degree to which rhythmic olfactory activity is modulated by centrifugal inputs remains unresolved. There are reports that respiratory rhythmic olfactory bulb activity disappears immediately when nasal airflow is interrupted (Macrides and Chorover, 1972; Onoda and Mori, 1980), yet it has also been reported that respiratory patterning of olfactory bulb activity persists in tracheotomized rats (Ravel et al., 1987; Ravel and Pager, 1990) and disappears in most cases after olfactory peduncle and centrifugal fiber lesions (Chaput, 1983). Shipley (Shipley et al., 1987) found that stimulation of the basal forebrain caused very large evoked potentials in the olfactory bulb that could not theoretically be accounted for by cholinergic or GABAergic basal forebrain afferents. Thus, glutamatergic afferents may have been responsible in coordination with cholinergic and GABAergic neurons for the large field potentials observed by Shipley (Shipley et al., 1987), and moreover the activity of these three groups together could undoubtedly effect the rhythmic activity of this region.

The basal forebrain also projects to piriform and the entorhinal cortex (Alonso and Kohler, 1984; Saper, 1984), and recent work in our lab indicates that a major component of this basal forebrain projection is glutamatergic (Manns et al., 2001). Piriform and entorhinal cortex areas are olfactory cortical areas in that they receive inputs from the olfactory bulb (Haberly and Price, 1978). The entorhinal cortex also receives inputs from other association cortical areas and the hippocampus, and projects massively

into the hippocampus via the perforant path (Steward, 1976; Steward and Scoville, 1976). Our results found cross-correlations of the basal forebrain unit activity with the piriform and entorhinal cortex activity, as well as instances of neurons antidromically activated from these areas. The olfacto-hippocampal axis has been studied and shown to dynamically correlate with high levels of coherence during specific instances of olfactory behavior. This is the case in the theta (Macrides et al., 1982; Kay and Freeman, 1998) the beta and the gamma frequency bands (Boeijinga and Lopes da Silva, 1988; Kay and Freeman, 1998). The activity of the olfactory bulb and olfactory cortical areas become locked with theta of the hippocampus during exploratory or sniffing and whisking behaviors (Macrides et al., 1982; Vanderwolf, 1992). Macrides (Macrides, 1975) observed strong correlations between sniffing, olfactory EEG activity and individual cycles of hippocampal theta. Coherence and correlation analyses have shown that within and between olfactory bulb, piriform and entorhinal cortices there is significant activity that moves from the periphery inward and again back outward (Bressler, 1987a, b; Boeijinga and Lopes da Silva, 1988; Boeijinga and Lopes da Silva, 1989; Vanderwolf, 1992). The neurons of the basal forebrain may not only be involved in the slow theta activity but also the beta and gamma activity which dynamically links these distributed regions, as coincidentally their frequencies correspond to the instantaneous frequencies of the glutamatergic, ~20Hz, and rhythmic GABAergic, ~40Hz, neurons during cortical activation, respectively. Thus the caudal and lateral basal forebrain regions may influence the olfactory bulb, olfactory cortices, and other cortical regions in a dynamic way shifting with behavioral state, and potentially synchronize these areas with the hippocampal activity primarily driven by the MS/DBB nucleus. The resulting temporal

relationship may provide a neural basis for modulating olfactory input to the hippocampal formation when an animal is evaluating the behavioral relevance of odors (Macrides et al., 1982).

### **Phase of Unit Discharge in Relation to Cortical Activity**

Lesions of the MS/DBB region of the basal forebrain eliminate hippocampal theta activity (Green and Arduini, 1954) and extracellular recordings show cross-correlated discharge of MS-DBB units in phase with hippocampal theta (Petsche et al., 1962). It has been demonstrated that different populations of septo-hippocampal cells presumed to be cholinergic and GABAergic fire with different phase preferences across the theta cycle, with the relationship remaining constant in urethane-anesthetized and freely moving rats (Brazhnik and Fox, 1999). The cholinergic cells fired at the positive phase of the local dentate gyrus field, while the GABAergic cells fired at the negative phase. This relationship may be comparable to that which we observed for the basal forebrain GABAergic cells, which have a specific phase preference, at the positive phase of the olfactory bulb activity. Both the GABAergic septohippocampal cells and the GABAergic basolocortical neurons have been shown to preferentially innervate interneurons of the hippocampus and cortex respectively (Freund and Antal, 1988; Freund and Meskenaite, 1992), thus, these specific unit-to-EEG relationships may have significant implications on the resultant hippocampal and cortical activity.

## **Cognitive Implications**

Damage to the basal forebrain can result in global cognitive impairments associated with amnesia and impairments in executive function (Damasio et al., 1985b; Damasio et al., 1985a; Diamond et al., 1997; Abe et al., 1998). Cognitive deficits in both normal aging and age-related pathological conditions have also been associated with basal forebrain dysfunction. The severity of cognitive impairment observed in Alzheimer's disease is correlated with the extent of deterioration of cholinergic neurons in the basal forebrain (Perry et al., 1978; Bierer et al., 1995). Originally, the cholinergic basal forebrain system was thought to be critically implicated in global aspects of cognitive function (Dunnett et al., 1991; Wenk, 1997), but these ideas have been gradually revised as more selective lesion methods have become available. The development of a specific toxin for the basal forebrain cholinergic neurons, 192-IgG-saporin (Wiley et al., 1991) has permitted direct examination of the participation of the cholinergic neurons in cognition. It is clear that these selective lesions produce disruptions of specific forms of attention (McGaughy et al., 1996; Stoehr et al., 1997; Turchi et al., 1997; Waite et al., 1999), and that removal of cortical cholinergic input has a dramatic impact on the regulation of sensory information processing and cortical reorganization (Baskerville et al., 1997; Kilgard and Merzenich, 1998). Yet, such lesions do not impair learning and memory as originally proposed (Baxter et al., 1995a; Wenk, 1997). The role of noncholinergic basal forebrain neurons in cognitive function remains largely unexplored. The potential redundancy in the anatomical projections and rhythmic discharge of the cholinergic and the GABAergic and the glutamatergic neurons may aid in explaining the lack of global cognitive impairments following selective lesions of basal forebrain cholinergic neurons. These different groups

conceivably act in a cooperative manner to regulate cortical function, potentially regulating different aspects of cortical information processing. This may be done through their rhythmic discharge that promotes rhythmic activity in local pools of cortical neurons and through their global projections allow synchrony among spatially distributed cortical networks extending across the olfactory-hippocampal axis as well as to other cortical regions. The facilitation of cortical information processing by these basal forebrain neurons would thus occur by promoting a carrier rhythm for the coupling of these distributed populations of cells.

## 5.5 References

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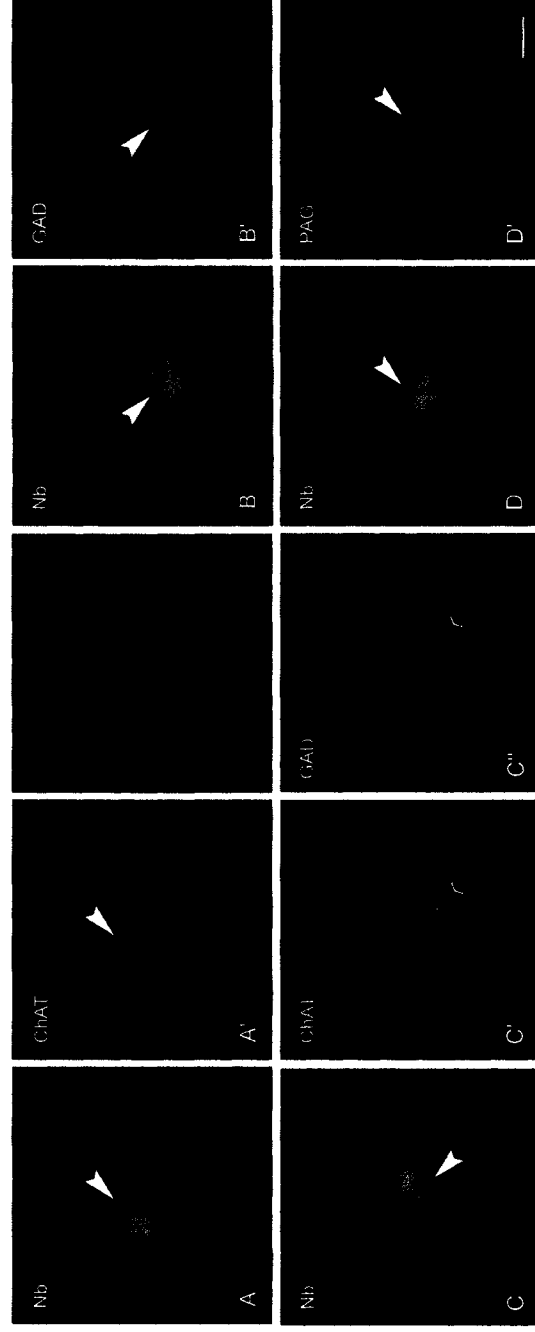
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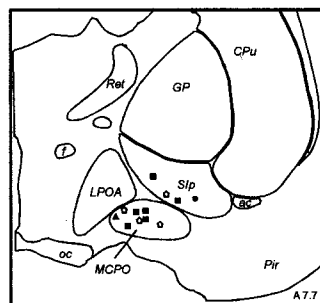
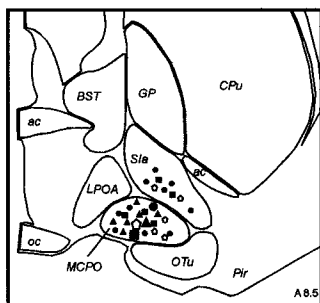
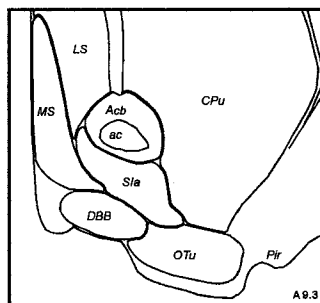
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## 5.6 Figure Legends

Figure 1. Rhythmic basal forebrain neurons juxtacellularly labeled and stained by fluorescence for Nb and neurotransmitter synthesizing enzymes. Nb was revealed with green fluorescent Cy2-conjugated streptavidin, ChAT-immunostaining with blue fluorescent AMCA-conjugated secondary antibodies, and GAD- or PAG-immunostaining with red fluorescent Cy3-conjugated secondary antibodies. (A, A') Nb+/ChAT + Cell (00011419); (B, B') Nb+/ GAD+ Cell (99032239); (C, C', C'') Nb+/ChAT-/GAD- cell (99032917); (D, D') Nb+/PAG+ cell (00010549), all in MCPO.



**Figure 2. Location of recorded and juxtacellularly labeled rhythmic ChAT+, GAD+, ChAT-/GAD- and PAG+ neurons through the basal forebrain (represented on atlas sections adapted from (Gritti et al., 1993)). Abbreviations: Acb, accumbens nucleus; ac, anterior commissure; BST, bed of the stria terminalis; CPu, caudate putamen; DBB, diagonal band of Broca nucleus; f, fornix; FStr, fundus of striatum; GP, globus pallidus; LPOA, lateral preoptic area; LS, lateral septum; MCPO, magnocellular preoptic nucleus; MS, medial septum; oc, optic chiasm; OTu., Olfactory tubercle; Pir, Piriform cortex; Ret, Reticularis nucleus; SIa, substantia innominata pars anterior; SIp, substantia innominata pars posterior; sm, stria medullaris.**



- ChAT+
- ▲ GAD+
- ChAT-/GAD-
- PAG+

Figure 3. I. Unit discharge of basal forebrain neurons in association with hippocampal (Hipp) and prefrontal cortex (PF) EEG activity during spontaneous slow irregular activity (Pre-stim.) and somatic stimulation (Stim.) -induced cortical activation and rhythmic activity (A – D). (A) ChAT<sup>+</sup> neuron shown in Fig. 1A, shifts from slow irregular to rhythmic discharge with cortical activation. (B) GAD<sup>+</sup> neuron, shown in Fig 1B, decreases its rate of discharge and changes pattern from fast tonic to rhythmic with stimulation. Both (C) ChAT<sup>-</sup>/GAD<sup>-</sup> and (D) PAG<sup>+</sup> neurons, shown in Fig. 1C and D, respectively, change from irregular to rhythmic discharge during cortical activation. II. Unit-to-EEG spike-triggered average (STA) cross-correlations for spike train, black line, and randomized-spike train, gray line (\*  $p < 0.05$ , Wilcoxon test). In all cases, rhythmic discharge is evident in the autocorrelation histograms (ACH) and significantly cross-correlated with the PF activity during the stimulation.

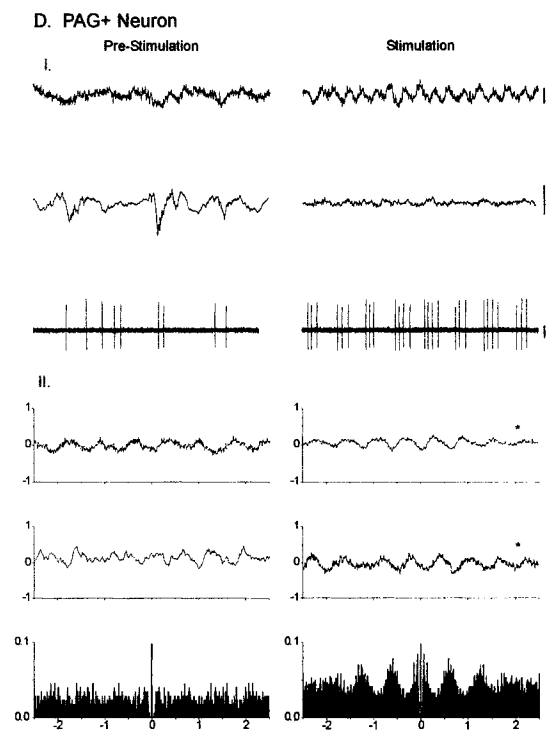
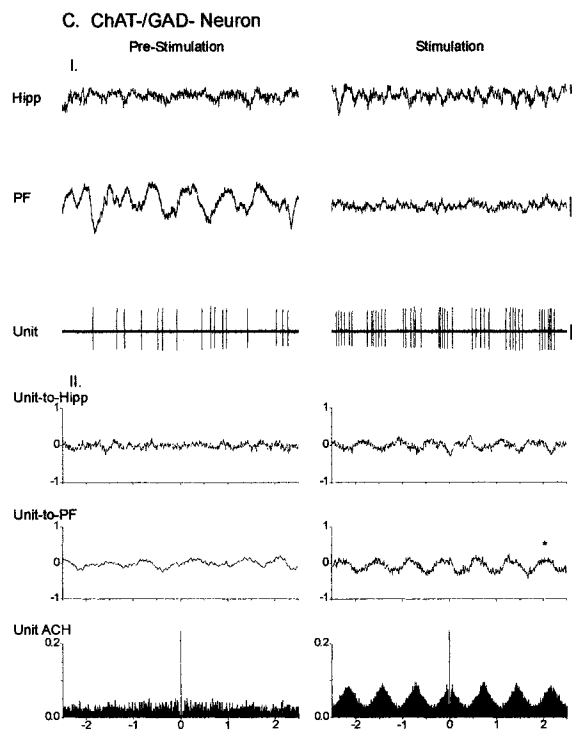
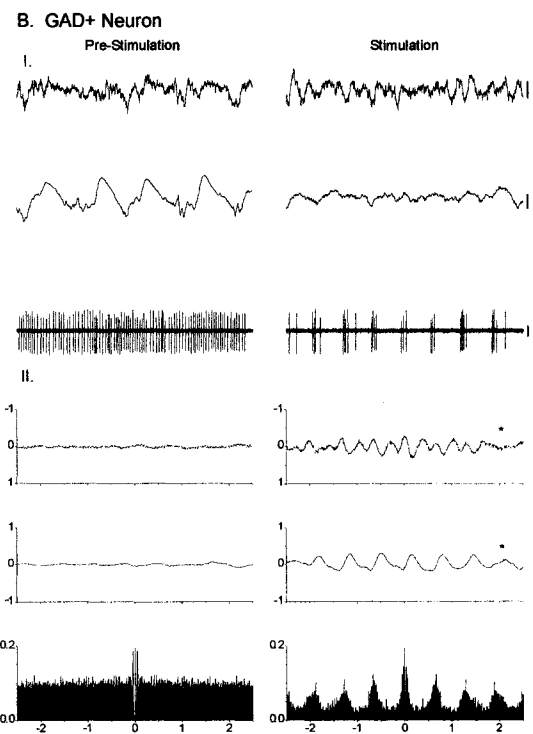
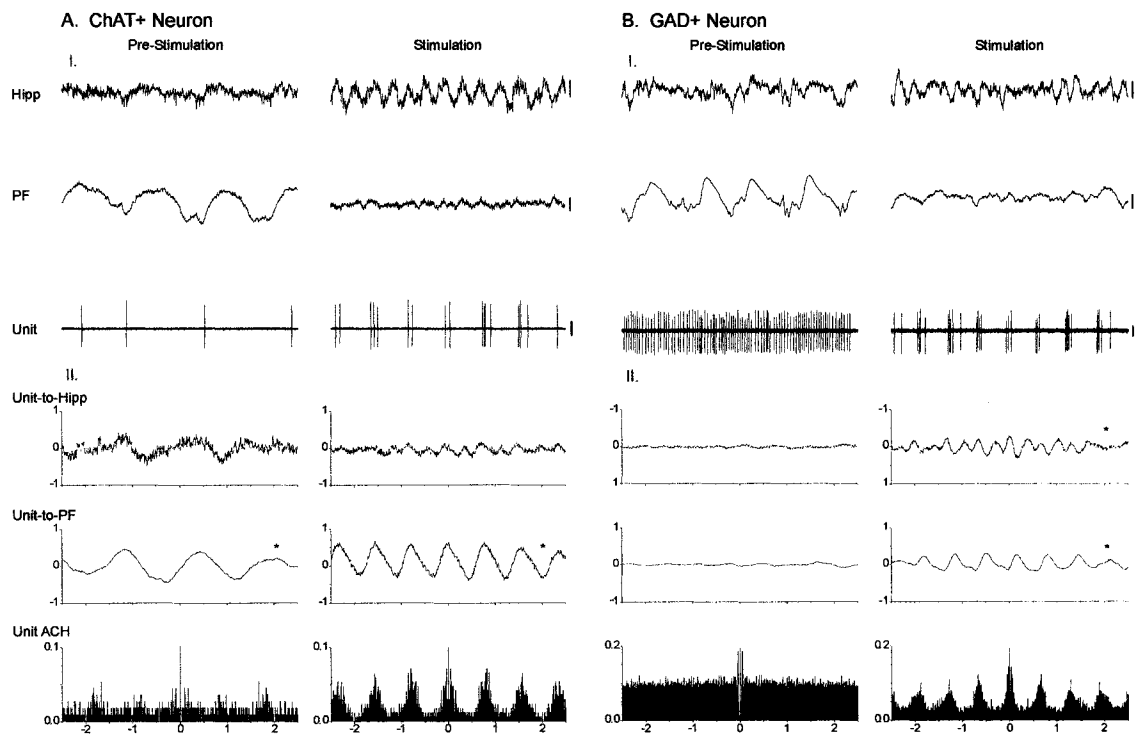


Figure 4. I. Unit discharge of rhythmic PAG<sup>+</sup>, putative glutamatergic basal forebrain neuron (00010550) in association with olfactory bulb EEG and respiratory activity (from intercostal EMG) during spontaneous pre- and stimulation conditions. II. The unit activity is significantly cross-correlated (in the STAs) with the olfactory bulb (OB) and intercostal activity during both the pre- and stimulation conditions; however, the rhythmic discharge of the unit is only evident in the ACH during the stimulation period.

# PAG+ Neuron

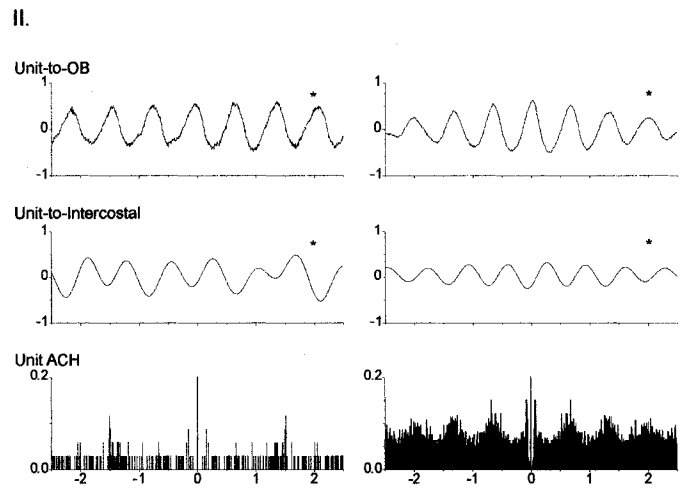
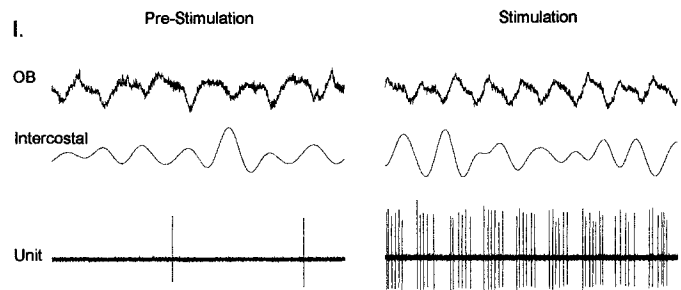
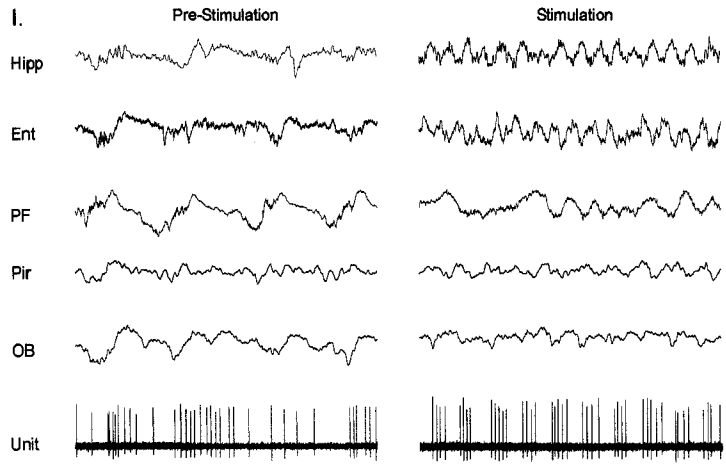
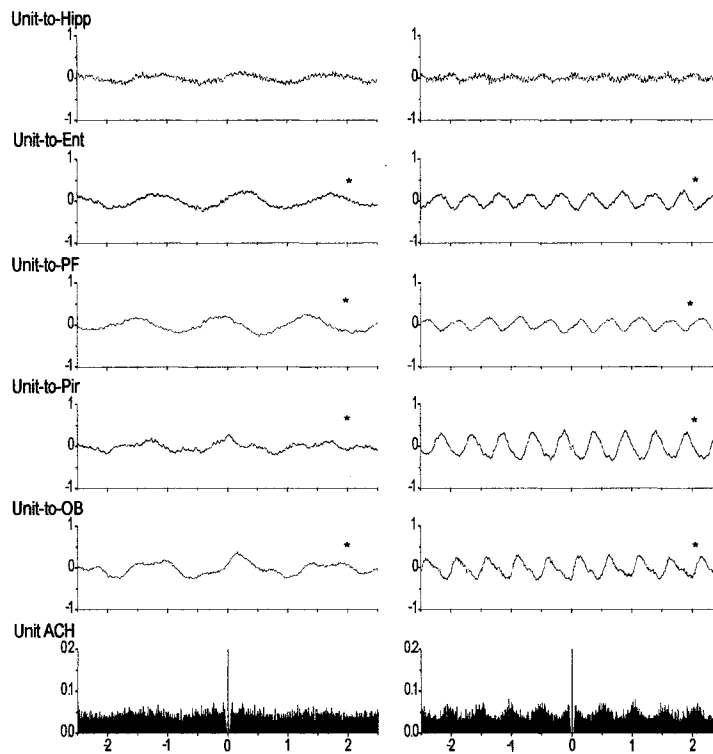


Figure 5. I. Unit discharge of a rhythmic PAG+ basal forebrain neuron (00051114) recorded in parallel with multiple cortical sites: hippocampus (Hipp), entorhinal cortex (Ent), prefrontal cortex (PF), piriform cortex (Pir) and olfactory bulb (OB). II. As evident in the STAs, the unit activity is cross-correlated with all the cortical signals except the hippocampal one. Evident in the ACHs, there is a high degree of rhythmicity during the stimulation. III. Power spectra of the EEG signals and unit ACHs indicate that the unit has the same dominant frequency as all cortical leads during the pre-stimulation period. During the stimulation period, the unit has the same dominant frequency as all leads except the hippocampal and entorhinal (Ent 1°), which however has a secondary peak (Ent 2°) at the same frequency as that of the unit.

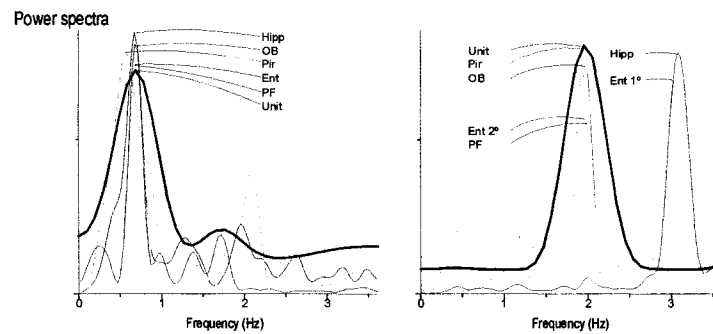
# PAG+ Neuron



## II.

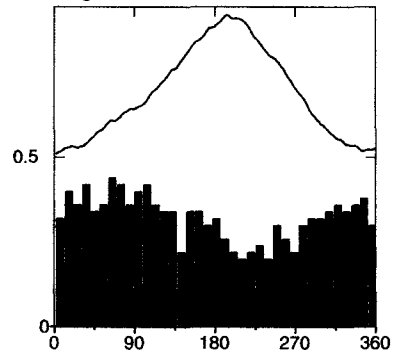


## III.

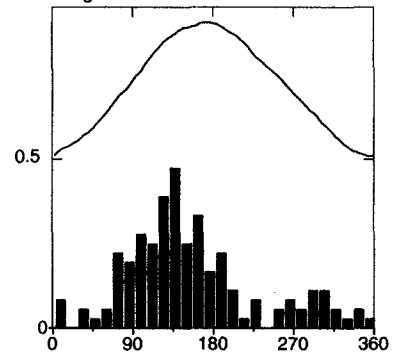


**Figure 6. Rhythmic discharge of basal forebrain units in relation to the olfactory rhythm. Phase profiles referenced to the olfactory rhythm (averaged wave in upper half of each panel) for (A) presumed cholinergic (00031590) (B) presumed GABAergic (00031508) and (C) presumed glutamatergic (00031513) neurons recorded in the same brain, illustrate the different phase preferences for each cell. (D) Frequency plot of preferential phase profiles with the olfactory rhythm (averaged wave from all neurons for the three cell groups).**

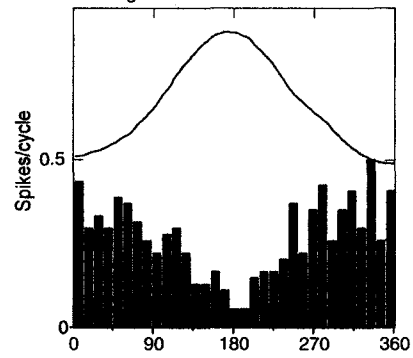
A. Cholinergic



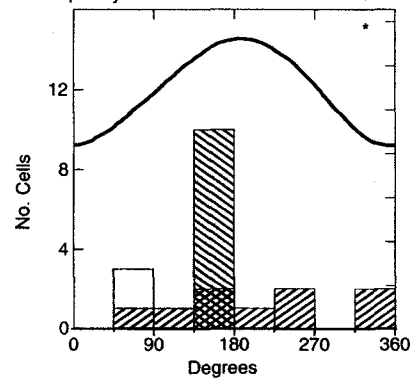
B. GABAergic



C. Glutamatergic



D. Frequency Distribution



Cell Type:

- Cholinergic
- ▨ GABAergic
- ▩ Glutamatergic

## 5.9 Tables

Table 1. Firing characteristics of ChAT+, GAD+, ChAT-/GAD-, and PAG+ rhythmically discharging basal forebrain neurons.<sup>1</sup>

| Cell Group               | Spike width (msec)           | Rate PSH (Hz) <sup>2</sup>    |                            | Frequency ISIH (Hz) <sup>3</sup> |                             | Oscillatory Index <sup>4</sup> |                 | Frequency ACH (Hz) <sup>4</sup> |                     |
|--------------------------|------------------------------|-------------------------------|----------------------------|----------------------------------|-----------------------------|--------------------------------|-----------------|---------------------------------|---------------------|
|                          |                              | Pre-Stimulation               | Stimulation                | Pre-Stimulation                  | Stimulation                 | Pre-Stimulation                | Stimulation     | Pre-Stimulation                 | Stimulation         |
| <u>ChAT+</u> (n=11)      | 0.70 ± 0.06 <sup>G,N,P</sup> | 5.73 ± 1.42 <sup>G</sup>      | 13.18 ± 2.28 <sup>G*</sup> | 38.65 ± 13.11                    | 74.15 ± 25.11 *             | 0.47 ± 0.14                    | 0.84 ± 0.04 *   | 1.09 ± 0.16 (6)                 | 2.09 ± 0.25 (11) *  |
| <u>GAD+</u> (n=8)        | 0.50 ± 0.02 <sup>C,P</sup>   | 16.78 ± 2.10 <sup>C,N,P</sup> | 7.68 ± 2.00 <sup>C*</sup>  | 29.09 ± 5.88                     | 41.18 ± 7.09 <sup>N,P</sup> | 0.26 ± 0.13                    | 0.83 ± 0.03 *   | 1.52 ± 0.02 (3)                 | 1.56 ± 0.10 (8)     |
| <u>ChAT-/GAD-</u> (n=10) | 0.58 ± 0.03 <sup>C</sup>     | 6.12 ± 1.45 <sup>G</sup>      | 8.24 ± 1.35 *              | 16.54 ± 2.69                     | 21.62 ± 3.61 <sup>G</sup>   | 0.38 ± 0.13                    | 0.85 ± 0.02 *   | 1.32 ± 0.20 (5)                 | 1.70 ± 0.21 (10)    |
| <u>PAG+</u> (n=13)       | 0.62 ± 0.03 <sup>G</sup>     | 9.26 ± 1.52 <sup>G</sup>      | 9.52 ± 1.27                | 22.46 ± 4.24                     | 19.97 ± 2.37 <sup>G</sup>   | 0.55 ± 0.11                    | 0.85 ± 0.02 *   | 1.18 ± 0.14 (9)                 | 1.45 ± 0.09(13)     |
| <b>Average (n=42)</b>    | 0.61 ± 0.02                  | 9.02 ± 0.99                   | 9.83 ± 0.90                | 26.55 ± 3.97                     | 38.59 ± 7.45                | 0.43 ± 0.06                    | 0.84 ± 0.01 *** | 1.24 ± 0.08 (23)                | 1.70 ± 0.09 (42) ** |
| <b>ANOVA<sup>5</sup></b> |                              |                               |                            |                                  |                             |                                |                 |                                 |                     |
| Stimulation: *           |                              | F=0.02 df=1 p=0.88            |                            | F=2.47 df=1 p=0.12               |                             | F=40.58 df=1 p=0.000           |                 | F= 8.58 df=1 p=0.005            |                     |
| Cell Group: C,G,N,P      | F=4.05 df=3,37 p=0.01        | F=2.58 df=3 p=0.06            |                            | F=5.04 df=3 p=0.003              |                             | F=1.28 df=3 p=0.29             |                 | F= 1.03 df=3 p=0.385            |                     |
| Interaction (StimxGroup) |                              | F=7.35 df=3,76 p=0.000        |                            | F=1.19 df=3,76 p=0.32            |                             | F=1.03 df=3,75 p=0.39          |                 | F=2.15 df=3,57 p=0.104          |                     |
| Post-hoc Comparisons     | C,G,N,P <0.05                | C,G,N,P, * <0.05              |                            | G,N,P, * <0.05                   |                             | * <0.05                        |                 | * <0.05                         |                     |

<sup>1</sup> Mean ± SEM values are presented for different physiological measures in the different cell groups.

<sup>2</sup> Peristimulus histogram (PSH) measurements of average discharge rate.

<sup>3</sup> Interspike interval histogram (ISIH) measurements of the instantaneous firing frequency.

<sup>4</sup> Frequency of rhythmic activity during pre- and stimulation periods calculated from the autocorrelation histogram (ACH, fit with a Gabor function) including all units for stimulation and only those discharging rhythmically during pre-stimulation for that condition.

<sup>5</sup> For spike width, a one-way ANOVA between cell groups was performed. For all other measures, a two-way ANOVA with stimulation and cell group as factors was performed and followed by post-hoc tests (with separate variance) for condition per cell group and/or cell group per condition if significant main or interaction effects were found. F values along with df for the main effects and interaction along with error are presented, and significant differences of post-hoc tests indicated by symbols (C, G, N, and P referring to comparisons between ChAT+, GAD+, ChAT-/GAD-negative, and PAG+ cell groups, respectively and \* between pre- and stimulation conditions).

## **Chapter Six**

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### **6. Discussion**

The basal forebrain has been known to be important in mediating cortical activation associated with waking and paradoxical sleep (PS), as well as with the promotion of cortical deactivation and slow-wave sleep (SWS). There were two primary aims of the present work: The first aim was to reveal the firing patterns of different neurochemically distinct basal forebrain neuronal groups in association with cortical activity, and more particularly to characterize how the cholinergic and GABAergic neurons may participate in the promotion of cortical activation and/or deactivation. The second aim was to determine whether the basalocortical projection system's noncholinergic/nonGABAergic component could be glutamatergic and would thus participate in an excitatory manner in the mediation of cortical activation.

Consistent with a role for the basal forebrain in mediating cortical activation, these studies found that cholinergic and particular physiological subgroups of GABAergic neurons have distinct patterns of discharge that may give them functions in the mediation of cortical activation during waking or PS. The anatomical experiments revealed that the noncholinergic/nonGABAergic basalocortical projection neurons may be uniquely glutamatergic, while physiological experiments revealed a unique rhythmic group of neurons to be glutamatergic which may modulate cortical activity in parallel with cholinergic and GABAergic neurons. Finally, consistent with a role for the basal forebrain in mediating cortical deactivation, these studies found that other subgroups of GABAergic neurons have distinct patterns of discharge that may implicate them in the mediation of slow irregular cortical activity and SWS.

## **6.1 Neurons Mediating Cortical Activation**

Since the early studies elucidating the pathways of the ascending reticular activating system, it has been suggested that the basal forebrain region was important in the mediation of cortical activation and associated sleep-wake states (Dempsey et al., 1941; Starzl et al., 1951). Other more recent work indicated that destruction of neurons in this region results in a loss of cortical activation (LoConte et al., 1982; Stewart et al., 1984; Buzsaki et al., 1988). The cortically projecting cholinergic neurons have been thought to be crucial for this mediation of cortical activation, however other groups of cortically projecting neurons had been found, including GABAergic as well noncholinergic/nonGABAergic neurons (Gritti et al., 1997). This series of experiments has found that cholinergic neurons, as well as subgroups of GABAergic and noncholinergic/nonGABAergic glutamatergic neurons display discharge patterns consistent with roles in the mediation of cortical activation. With respect to cortical activity, the firing profiles of cholinergic and the tonic-firing subtype of GABAergic neurons is similar to that of neurons in the basal forebrain that were characterized as “wake-active” in naturally sleeping-waking, freely moving animals (Detari et al., 1984; Szymusiak and McGinty, 1986; Detari et al., 1987; Szymusiak and McGinty, 1989). The other type of GABAergic neurons and the glutamatergic neurons are not obviously related to cortical activation in terms of their change in rate of discharge; however, they may participate in promoting rhythmic theta-like activity associated with cortical activation via their rhythmic pattern of discharge, which is similar to that of the cholinergic neurons. It would appear thus that several groups of neurons in the basal

forebrain including cholinergic, GABAergic, and glutamatergic could serve to enhance the cortical activation of waking and PS.

These findings were the first to unambiguously link an increase in firing of cholinergic neurons with cortical activation, which was so long ago noted to be accompanied by an increase in cortical ACh release during waking and PS (Celesia and Jasper, 1966; Jasper and Tessier, 1971). The finding that the majority of cholinergic neurons changed their discharge from a tonic irregular firing to rhythmic discharge often including high frequency bursting activity with cortical activation had been foreshadowed by the findings of Khateb and colleagues (Khateb et al., 1992), which indicated cholinergic neurons were capable of either firing in a tonic or a rhythmic burst discharge subtended by low threshold calcium spikes *in vitro*. The initial correlations of the rhythmic activity of the cholinergic neurons were done with the EEG activity of the retrosplenial cortex, which occurs at the same frequency as that of hippocampal theta activity in urethane-anesthetized rats (Holsheimer, 1982; Borst et al., 1987; Leung and Borst, 1987). The rhythmic discharge of most cholinergic units did not correspond to the dominant spectral peak of the retrosplenial cortex, but instead to a lower frequency secondary peak. Later comparisons with the theta activity of the hippocampus or entorhinal cortex also revealed correspondence to a lower frequency activity. However, when cross-correlations were done of the cholinergic neuronal activity with other cortical regions, the correlations were with their dominant frequency; this was initially found with the prefrontal cortex, and in later experiments with the activities of the olfactory bulb and piriform cortex. These results suggest that rhythmically bursting cholinergic neurons

modulate cortical activity in a theta-like manner, with potentially different frequencies depending on the cortical target area of different neurons. However, with the possibility that these neurons have dynamically changing frequencies that can converge across the neuronal population, so that during some behaviors coordinated discharge at similar frequencies may occur. As a consequence of this convergence, theta activity would be of similar frequencies in widespread cortical areas, as is the case during PS (Parmeggiani and Zanicco, 1963; Maloney et al., 1997).

The functional significance of the bursting of these neurons could be that it increases the probability of neurotransmitter release from long and widespread axon collaterals (Lisman, 1997), as are characteristic of the cortically projecting neurons of this region (Aston-Jones et al., 1985; Paolini and McKenzie, 1997). The bursting may also increase the backpropagation of action potentials into the dendrites, potentially stimulating dendritic release of ACh, which could account for the large release of ACh in this region that is not easily accounted for by brainstem afferents. Although, non-rhythmic cholinergic neurons were a minority in this study, their existence is consistent with *in vitro* reports of cholinergic neurons in the MS and DBB that do not generate rhythmic bursts, although they do similarly possess low threshold calcium spikes (Markram and Segal, 1990). Another more recent juxtacellular study found, three cholinergic basal forebrain neurons that did not fire rhythmically during cortical activation (Duque et al., 2000). It is possible that within the cholinergic cell group there are rhythmic and non-rhythmic subgroups. Regardless, without exception cholinergic neurons increase their firing rate with cortical activation.

ACh acting in the cortex on muscarinic receptors can promote rhythmic cortical theta-like activity (Konopacki et al., 1987; Dickson and Alonso, 1997). ACh has also been implicated in faster rhythmic activity as an important promoter of gamma activity (Metherate et al., 1992; Buhl et al., 1998). Many reports have noted that muscarinic-induced depolarization brings cortical pyramidal and interneurons near firing threshold where many of them exhibit slow rhythmic theta or fast gamma-like subthreshold oscillations (McCormick and Prince, 1986; Llinas et al., 1991; Lopes da Silva et al., 1991; Metherate et al., 1992; Klink and Alonso, 1993). The findings of this thesis suggest that rhythmic release of ACh by rhythmically firing cholinergic cells may phasically modulate the relatively fast muscarinic and/or nicotinic action on cortical interneurons (Roerig et al., 1997; Xiang et al., 1998; Porter et al., 1999), and thus participate in the pacing of the cortical rhythmic activity.

The rhythmic release of ACh may have consequences for plasticity related mechanisms. The importance of ACh and ACh-mechanisms in synaptic plasticity and long term potentiation (LTP) has been shown in the hippocampus by both electrical theta-frequency stimulation and by cholinergic-induced theta activity: More recently cholinergic input has also been shown to facilitate synaptic plasticity in the neocortex (Larson et al., 1986; Metherate et al., 1988; Metherate and Ashe, 1991; Huerta and Lisman, 1995; Wallenstein and Hasselmo, 1997; Kilgard and Merzenich, 1998). Accordingly, the slow theta-like rhythmic burst discharge by basal forebrain cholinergic neurons may participate in mediating plastic changes in cortical synaptic organization. This rhythmic modulation could occur during active and attentive waking behaviors or

during PS, when certain types of memory consolidation have been proposed to occur (Louie and Wilson, 2001).

Recently studies from our lab utilizing pharmacological injections into the basal forebrain have indicated the importance of the cholinergic neurons in mediating changes in cortical activity together with sleep-wake states. Glutamate is a transmitter released by the brainstem activating system in association with cortical activation. One of its agonists NMDA, which *in vitro* causes cholinergic neurons to generate rhythmic bursts (Khateb et al., 1995; Khateb et al., 1995), when injected into the basal forebrain produced decreases in delta- and increases in theta- and gamma-band EEG activity associated with decreases in SWS and PS and increases EMG activity with the appearance of active waking (Cape and Jones, 2000). Despite the indication that the cholinergic neurons may have been responsible for the increased theta and gamma activity in association with waking, NMDA could stimulate other basal forebrain cell types (Cape and Jones, 2000). A more selective activation of cholinergic neurons was found with the neuropeptide neurotensin (NT). Injection of NT into the basal forebrain specifically affected the cholinergic neurons, as fluorescently tagged NT was shown to be selectively internalized by ChAT+ neurons (Cape et al., 2000), reflecting its high-affinity binding to the ChAT cells that are selectively associated with NT receptors (Szigethy and Beaudet, 1987; Faure et al., 1995). Moreover, juxtacellularly recorded and labeled cholinergic neurons were found to increase their rate of firing and to discharge in bursts after the injection of NT into the basal forebrain of anesthetized rats (Cape et al., 2000), similar to the way in which the binding and internalization of NT *in vitro* caused depolarization associated with periods of prominent rhythmic bursting in the cholinergic neurons (Alonso et al.,

1994). The NT-induced bursting was similar to that shown in this thesis during stimulation-induced cortical activation. The NT action on cholinergic neurons was also shown to promote cortical activation, in that slow-wave delta activity decreased concomitant with an increase in gamma activity during the period when rats are normally asleep (Cape et al., 2000). Additionally, there was an increase in theta potentially caused by the stimulation of rhythmic bursting of cholinergic neurons and thus rhythmic ACh release in the cortex. This study also noted profound changes in the sleep-wake states: SWS was diminished, while quiet wake increased and was associated with low EMG, in contrast to the high EMG activity produced by NMDA, but with similarly high theta activity. The selective NT-induced activation of cholinergic neurons and their target neurons was proposed to be similar to processes that occur during natural PS with atonia. The injections of NT also lead to an increase in PS again potentially induced by the rhythmic bursting discharge of cholinergic neurons without activation of other noncholinergic neurons that are normally active during waking. Together the work in this thesis and the complimentary NT study emphasize the potent capacity cholinergic neurons have to modulate both cortical activity and sleep-wake states, during which they may rhythmically modulate plasticity while promoting synchronization and coherence at theta and gamma frequencies across cortical networks during active waking and PS states.

The finding that almost all cholinergic neurons contain the PAG enzyme leads to the speculation that these neurons may corelease glutamate. The possibility of glutamate-ACh corelease from basalocortical fibres was previously suggested by the finding that

cortical cholinergic synaptosomes in addition to releasing ACh, released glutamate (Docherty et al., 1987). It is worth noting, particularly in light of the two distinct firing modes of the majority of cholinergic neurons documented in this thesis that glutamate-ACh release could be differentially affected. Studies in the electric organ of Torpedo, where ACh and glutamate are also coreleased, indicate that the release occurs differentially depending upon calcium concentrations (Israel et al., 1993). In the context of the basal forebrain cholinergic neurons, potential differential release could depend upon firing frequency and mode of discharge as bursting subtended by calcium-spikes would result in greater calcium entry. This corelease could be of physiological significance. For instance, in the hippocampal region both ACh and glutamate are capable of stimulating rhythmic activity, and coincident activation of metabotropic glutamate receptors and muscarinic ACh receptors is additive in stimulating the rhythmic network activity (Taylor et al., 1995; Whittington et al., 1995; Cobb et al., 2000). The potential mechanisms for cholinergic basal forebrain neurons in mediating synaptic plasticity could then be occur both cholinergic and classical glutamatergic NMDA-dependent mechanisms. However, further studies still need to resolve the existence of both ACh and glutamate in neurotransmitter vesicles, or both the ACh and glutamate vesicular-transporters, in the synaptic terminals of cortically projecting cholinergic neurons.

In contrast to the obvious role of the cholinergic basalocortical system in the regulation of cortical activation, less obvious has been the importance of the GABAergic basalocortical system. Again, in contrast to the cholinergic neurons, these cells had not

been specifically identified and characterized *in vitro*, so no concrete clues existed as to their potential firing patterns. The findings reported here reveal that a proportion of the heterogeneous GABAergic basal forebrain neurons may function in parallel with the cholinergic neurons in mediating cortical activation.

The single largest subgroup of GABAergic neurons was composed of fast firing neurons that fired in a tonic manner during both slow irregular cortical activity and during stimulation induced cortical activation. Several cells were identified as cortically projecting. Concomitant with an increase in average discharge rate in these neurons, the instantaneous frequency shifted from the beta range (~20Hz) to the gamma range (~40Hz) with cortical activation. Studies done in the MS-DBB complex have revealed fast spiking parvalbumin-containing cells which are most likely GABAergic neurons (Morris et al., 1999; Knapp et al., 2000). These may be similar to the GABAergic neurons reported here in the MCPO and SI basal forebrain areas. The shift in firing frequency from the beta- to gamma-range perhaps reflects the significant increase in cortical gamma activity that is evoked with somatosensory stimulation in urethane-anesthetized rats. This change in activity pattern mirrors that of the EEG seen in naturally sleeping-waking rats, beta activity being higher during slow wave sleep than during waking, while gamma activity is higher during waking and highest during active or attentive waking and PS, thus reflecting the shift in cortical activation (Maloney et al., 1997). Basalocortical GABAergic neurons are known to preferentially innervate cortical interneurons (Freund and Antal, 1988; Freund and Meskenaite, 1992), which may be responsible for cortical gamma activity as mediated through inhibitory mechanisms (Fisahn et al., 1998). Accordingly, these GABAergic basalocortical neurons would act in

parallel with the cholinergic basalocortical neurons and ACh, which is also important in mediation of gamma activity (Fisahn et al., 1998).

The second largest subgroup of GABAergic neurons had a peculiar discharge profile: they fired in a relatively fast tonic manner during slow irregular cortical activity and then slowed their average discharge with cortical activation, while concomitantly beginning to fire in rhythmic clusters of spikes. As for the cholinergic neurons, the rhythmic activity of these cells was initially noted to be slower than that of limbic theta activity, and to be correlated preferentially with the prefrontal cortical activity. Subsequent experiments revealed that their rhythmic discharge correlated with activity of the olfactory bulb and cortex, to which they also projected. The slow rhythmic frequency of these GABAergic neurons was not different from that of the cholinergic neurons. However, the GABAergic neurons' discharge was different from the cholinergic neurons' discharge because it contained significantly lower proportion of high frequency bursts (>80Hz). In this manner, their discharge was similar to rhythmically recorded neurons shown to be ChAT or GAD immunonegative. The discharge of the GABAergic cells nonetheless remained distinct from these noncholinergic and nonGABAergic cells as their average rate of discharge with cortical activation did not decrease. These GABAergic neurons may in part reflect rhythmic noncholinergic neurons that were identified *in vitro* (Alonso et al., 1996). A subgroup of such noncholinergic neurons was found to be inhibited by noradrenaline *in vitro* (Fort et al., 1998), perhaps corresponding to the cells documented here *in vivo*, which would then be correspondingly inhibited by noradrenaline normally released during waking.

The unique activity of these neurons and their heightened average tonic firing during slow irregular cortical activity may act to efface rhythmic activity via tonic postsynaptic hyperpolarizations, promoting slow irregular activity and cortical deactivation during SWS. However, during cortical activation these cells may be quite important for the pacing and temporal locking of cortical rhythms as putative GABAergic neurons with this distinctive physiological profile fire with a specific phase preference across the olfactory rhythm. This pacemaking activity would be similar to the control MS GABAergic neurons purportedly have on the hippocampal theta activity. Neurons presumed to be, though not yet identified as, GABAergic, in the medial septum also discharge rhythmically with phase preference in pacing theta activity in the hippocampus (Lee et al., 1994; Brazhnik and Fox, 1999; Dragoi et al., 1999). Additionally, the possibility exists that the intracuster instantaneous gamma-range frequency ~40Hz of these cells may contribute to the modulation of fast activity in cortical inhibitory networks that rides on the rhythmic theta oscillations (Freund and Antal, 1988; Freund and Meskenaite, 1992; Fisahn et al., 1998). As such these physiologically complicated neurons would participate in mediating slow irregular cortical activity and rhythmic cortical activity in both the theta range, which they may help to pace, and in the gamma frequency range across the sleep-wake cycle.

The possibility exists that both of these subgroups of GABAergic neurons (and those discussed below) may corelease glutamate, considering that double labeling indicated that ~60% of GAD immunopositive basal forebrain neurons also were positive for the PAG enzyme indicating that they have the capacity to synthesize glutamate for

neurotransmitter purposes. However, further studies need to resolve the presence of both GABA and glutamate in neurotransmitter vesicles, or both the GABA and glutamate vesicular-transporters, in the synaptic terminals of cortically projecting GABAergic neurons.

One hypothesis remaining untested was that glutamate could potentially be released from basalcortical neurons and thus contribute to the stimulation of cortical activation. Using retrograde tracing techniques from the entorhinal cortex combined with PAG immunohistochemistry, it was confirmed that approximately 20% of this projection was neither cholinergic nor GABAergic and could be uniquely glutamatergic. Studies involving other cortical sites have found varying proportions of the basalcortical projection that were neither cholinergic nor GABAergic, indicating that this potentially glutamatergic projection could be variable in proportion. For instance, to the prefrontal cortex and olfactory bulb cholinergic and GABAergic projections together only accounted for  $\sim 2/3$  and  $\sim 1/2$  of the projection, respectively (Zaborszky et al., 1986; Gritti et al., 1993). Recent studies in our lab of the calcium binding proteins, dependable markers for different subtypes of inhibitory neurons in the hippocampus and cortex (Kawaguchi and Hama, 1988; Kawaguchi and Kubota, 1993; Freund and Buzsaki, 1996), indicated that only a minority of calbindin-containing basal forebrain cells, which do not contain ChAT, are GABAergic (Gritti et al., Submitted). This same study indicated that calbindin-containing cells contribute significantly to the basal forebrain cortical projection and that approximately half of the calbindin-containing cells are immunopositive for PAG (Gritti et al., Submitted). The findings of this study support the

thesis work indicating that there is a glutamatergic component of the basalocortical projection system that may be independent of the cholinergic and GABAergic components.

From the juxtacellular experiments aimed at revealing the discharge properties of the cholinergic and GABAergic neurons, many neurons that were ChAT or GAD negative were found that displayed unique rhythmic discharge properties reminiscent of those found *in vitro* by Alonso and colleagues (Alonso et al., 1996). Following the anatomical description of the glutamatergic cortically projecting neurons, a basis was established for testing the hypothesis that these unique rhythmic neurons could be glutamatergic. Experiments also revealed that these rhythmic neurons were doubly immunonegative for ChAT and GAD before cells of this physiological type were ultimately revealed to be immunopositive for PAG and negative for ChAT. These cells, which comprised the majority of rhythmic basal forebrain neurons, projected to the olfactory bulbs, piriform and entorhinal cortices and correlated with the activity of these regions. Together these findings are the first to indicate that glutamatergic basal forebrain neurons play a role in cortical activation.

The precise firing characteristics of these glutamatergic cells were different from the rhythmic GABAergic neurons in terms of average and instantaneous firing frequency and from the cholinergic neurons in that they never exhibited high frequency bursting activity. These neurons only marginally increased their average rate of discharge during cortical activation, and their instantaneous firing rate maintained ~20Hz during both conditions. However, the rhythmic firing characteristics of these cells were indistinguishable from those of the rhythmic cholinergic and GABAergic neurons

indicating that these three types of neurons may function cooperatively in mediating the slow rhythmic activity associated with cortical activation.

The rhythmic discharges of cholinergic, GABAergic, and glutamatergic neuronal groups were correlated with the EEG activity of multiple regions: in all instances with that of the olfactory bulbs and in the majority of instances with the piriform, entorhinal and prefrontal cortices. Basal forebrain neurons through their widespread projection patterns, which can span several of these regions (Paolini and McKenzie, 1997), may effectively bind the rhythmic activity of these multiple regions simultaneously. Indeed, recordings from freely moving rats have shown that activity occurring across the olfacto-hippocampal axis is coherent during exploratory sniffing and whisking behaviors in the theta (Macrides et al., 1982; Kay and Freeman, 1998), beta and gamma frequency bands (Macrides et al., 1982; Boeijinga and Lopes da Silva, 1988; Vanderwolf, 1992; Kay and Freeman, 1998). Such coherent activity may also be the case during PS when theta activity is present across many cortical areas where gamma activity increases (Parmeggiani and Zanocco, 1963; Maloney et al., 1997). These basal forebrain neurons may not only facilitate this slow rhythmic theta activity, but also the faster beta and gamma activities that dynamically interact through the olfacto-hippocampal network (Boeijinga and Lopes da Silva, 1988; Kay and Freeman, 1998), since the instantaneous frequencies of the glutamatergic and GABAergic neurons correspond to beta (~20Hz) and gamma (~40Hz) band frequencies, respectively. The rhythmic activity of basal forebrain neurons giving rise to widespread projections to the cortex may promote rhythmic activity in local cortical networks and through their global projections allow

synchrony among distant cortical networks across the olfactory-hippocampal axis and ultimately the entire cortical mantle. This activity may facilitate cortical information processing by promoting carrier rhythms for the coupling of these distributed populations of cells.

The physiological findings reported here may provide some insight into the manner in which the basal forebrain regulates cognitive function. Scores of studies in both animals and humans have noted that damage or dysfunction of this region due to aging or Alzheimer's disease results in global cognitive impairments associated with amnesia and impairments in executive function (Perry et al., 1978; Damasio et al., 1985; Damasio et al., 1985; Bierer et al., 1995; Diamond et al., 1997; Abe et al., 1998). Although, cholinergic neuronal degeneration was originally thought to underlie the dysfunction, the underlying pathology seems to be more extensive (Dunnett et al., 1991). Cholinergic and noncholinergic basal forebrain neurons are necessary for normal cognitive functioning (Dunnett et al., 1991; McGaughy et al., 1996; Stoehr et al., 1997; Turchi et al., 1997; Wenk, 1997; Waite et al., 1999). The potential role for the GABAergic and glutamatergic together with the cholinergic basal forebrain neurons in cognitive function would appear to be profound, and the findings here provide novel physiological evidence suggesting why they are collectively important.

## **6.2 Neurons Mediating Cortical Deactivation**

Since the early discoveries of von Economo, Hess and Nauta it has been suggested that the basal forebrain and preoptic and anterior hypothalamic regions were important in

actively inducing sleep (Hess, 1931; von Economo, 1931; Nauta, 1946; Hess, 1954). It has been proposed more recently that different subgroups of basal forebrain GABAergic neurons acting through either an ascending pathway to the cortex or a descending pathway to the posterior hypothalamus may be involved in promoting cortical deactivation (Gritti et al., 1994; Gritti et al., 1997). The discharge pattern of the other subgroups of GABAergic basal forebrain cells was consistent with a role in the mediation of cortical deactivation, since they arrested their discharge in association with cortical activation. With respect to cortical activity, their profile of discharge is similar to that of neurons in the basal forebrain and adjacent preoptic area that were characterized as “sleep-active” in naturally sleeping-waking, freely moving animals (Szymusiak and McGinty, 1986; Koyama and Hayaishi, 1994). In these studies, the majority of “sleep-active” cells were inhibited by stimulation of the midbrain reticular formation, locus coeruleus or by iontophoretic application of noradrenaline (Szymusiak and McGinty, 1989; Osaka and Matsumura, 1994; Osaka and Matsumura, 1995). In brain slices, a small proportion of non-cholinergic basal forebrain neurons were identified that were hyperpolarized by noradrenaline, and thus proposed to potentially represent SWS-active neurons (Fort et al., 1998). Recent juxtacellular findings in our lab have lent credence to the idea that noradrenaline may inhibit neurons, which are preferentially active during slow irregular cortical activity, during periods of cortical activation and particularly waking. Labeled neurons that were found to be GABAergic neurons and decreased their firing during cortical activation were also found, via triple immunofluorescence, to be immunopositive for the adrenergic alpha-2A receptor (Manns et al., 2001), which is known from *in vitro* studies to cause hyperpolarization in preoptic neurons (Bai and

Renaud, 1998). Thus, it would appear that a population of GABAergic neurons distributed through the basal forebrain and in partial continuity with cells in the preoptic region, which are inhibited by ascending activating impulses transmitted in part by noradrenergic fibers (Jones and Cuello, 1989; Gallopin et al., 2000), may serve to dampen cortical activation and promote SWS.

One subgroup of GABAergic cells fired high frequency bursts often associated with phasic cortical activity, which was similar to the findings of sleep-active cells that were described as firing in “grouped discharges” recorded in lateral preoptic and basal forebrain areas (Koyama and Hayaishi, 1994). The activity of these neurons, some of which were demonstrated as projecting to the cortex, was synchronous with the slow irregular activity under urethane anesthesia, and may similarly be synchronous with the slow oscillation of SWS, during which there is a very high degree of coherent firing of cortical neurons (Steriade et al., 1993; Steriade et al., 1993). The other type of GABAergic cells seemingly similar to sleep-active neurons fired at very low average rates of discharge ( $<4$  Hz) in association with slow irregular cortical activity and almost ceased firing with cortical activation ( $<1$  Hz). These firing rates are very similar to sleep-active neurons of the basal forebrain described by Szymusiak and McGinty which fire on average lower than 10 Hz during sleep and lower than 1 Hz during waking (Szymusiak and McGinty, 1986; Szymusiak and McGinty, 1989), some of which projected to the cortex (Szymusiak and McGinty, 1989). Although the majority ( $\sim 60\%$ ) of the GABAergic neurons fired preferentially in association with slow irregular cortical activity, so did a minority ( $\sim 20\%$ ) of nonGABAergic neurons. It is possible that these, as

yet, unidentified neurons participate with these two subgroups of GABAergic neurons in shaping and promoting cortical deactivation and SWS.

### **6.3 Conclusion**

Not only had early findings indicated the dual importance for the basal forebrain region in the mediation of both cortical activation and deactivation associated with wake and sleep, but other findings had showed that when the forebrain is isolated from the brainstem alternating fast and slow cortical activity reappeared similar to that of wake and sleep in normal animals (Batsel, 1960; Villablanca, 1962). Although other forebrain regions are associated with mediating, either wake or sleep processes; one forebrain area, the basal forebrain may be capable of mediating both these functions. The findings of this thesis provide unique insights into the manner in which the basal forebrain, and its several distinct neurochemical cell groups, may function in the dual roles of mediating cortical activation during the states of waking and/or PS, and promoting cortical deactivation during SWS.

## 6.4 References

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## Appendix

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