

**The role of pontomesencephalic cholinergic neurons and their
neighboring GABAergic and putative glutamatergic neurons in
modulating cortical activity and sleep-wake states**

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TO:

- *My sweet and lovely son Charafeddine and my faithful wife, who
inspire me with the passion of love and life,*
- *My mother, the best mom in the world, who inspires me with her
eternal kindness, love and moral support,*
 - *My father and all members of my family,*
- *All my teachers, from my elementary school to my graduate studies,*
 - *All my friends,*
- *The two best countries in the world, Algeria & Canada.*

Abstract

Neurons within the brainstem pontomesencephalic tegmentum (PMT) are suggested to play a critical role in influencing cortical activity and behavior across sleep-wake states. Cholinergic neurons in the PMT form part of the ascending activating system and are thought to participate in stimulating cortical activation during both waking (W) and paradoxical sleep (PS). They are also suggested to trigger PS with muscle atonia through their descending projections into the brainstem reticular formation. Yet in the laterodorsal tegmental and pedunculo pontine tegmental nuclei (LDT and PPT), they lie intermingled with GABAergic and glutamatergic neurons, which could also modulate cortical activity and sleep-wake states.

In the present work, by immunohistochemical identification of recorded and labeled single cells in urethane-anesthetized and natural sleeping/waking rats, I described the activity profiles of LDT and PPT cholinergic neurons, in addition to GABAergic and putative glutamatergic neurons, first, under anesthesia in relation to cortical activity, and second, during natural sleep-wake states in relation to state, cortical activity and muscle tone.

In anesthetized rats, I found that all LDT/PPT cholinergic neurons increased their discharge in association with cortical activation evoked by somatic stimulation. They could thus function to stimulate this cortical activation. In contrast, LDT/PPT GABAergic and putative glutamatergic neurons were heterogeneous: they could either increase or decrease their discharge in relation to cortical activation. They could thus work differently to stimulate cortical activation or to dampen behavioral arousal.

In natural sleeping/waking rats, I found that a cholinergic neuron was active during both W and PS, as a W/PS-max active neuron. LDT/PPT Cholinergic neurons could thus function to stimulate cortical activation during W and during PS, and trigger motor inhibition and muscle atonia associated with PS. In contrast, LDT/PPT GABAergic and putative glutamatergic neurons were heterogeneous in their sleep-wake discharge profiles. Some were active during both W and PS and were considered as W/PS-max active neurons. They could thus participate in stimulating cortical activation during both W and PS. Others were maximally active during PS, as PS-max active neurons, and could thus participate in dampening behavioral arousal and muscle tone during PS. Some putative glutamatergic neurons were maximally active during W, as W-max active neurons, and could thus participate in stimulating behavioral arousal with muscle tone during wakefulness.

Together, these findings indicate that different LDT/PPT neurons are working in coordination to either mediate cortical activation during W and PS, to dampen behavioral arousal and muscle tone during PS or to stimulate behavioral arousal and muscle tone during wakefulness.

Résumé

Les neurones situés dans le tronc cérébral au niveau du pontomesencephalic tegmentum (PMT) ont été suggérés de jouer un rôle critique pour influencer l'activité corticale et comportementale durant les états de veille et de sommeil ou états, dits, de vigilance. Les neurones cholinergiques dans le PMT font partie du système d'activation ascendant qui contribue à la genèse de l'activation corticale durant l'éveil (E) et le sommeil paradoxal (SP). Ils sont aussi suggérés promouvoir l'état de SP accompagné d'atonie musculaire via leurs projections descendantes vers la formation réticulaire du tronc cérébral. Dans les noyaux laterodorsal tegmentale et pédonculopontin tegmentale (LDT et PPT), ces neurones cholinergiques sont entremêlés avec d'autres neurones GABAergiques et glutamatergiques, qui peuvent à leur tour contribuer à la modulation de l'activité corticale et donc aux états de vigilance.

Dans le présent travail, des cellules ont été enregistrées, marquées et identifiées immunohistochimiquement comme des neurones cholinergiques, GABAergiques ou présumés glutamatergiques dans le LDT et le PPT chez des rats anesthésiés à l'uréthane et chez des rats qui dorment et se réveillent naturellement. Premièrement, sous anesthésie, nous avons déterminé l'activité de ces neurones en relation avec l'activation corticale. Deuxièmement, à travers les différents états de veille et de sommeil, nous avons déterminé l'activité de ces neurones en relation avec ces états de veille et de sommeil, les activités corticales pertinentes caractérisant ces états et le tonus musculaire.

Chez les rats anesthésiés, j'ai constaté que tous les neurones cholinergiques du LDT / PPT augmentaient leur décharge en association avec l'activation corticale évoquée par une stimulation somatique. Ils pourraient donc participer à cette activation corticale.

Les neurones GABAergiques et les neurones présumés glutamatergiques, quant à eux, étaient hétérogènes. Soit, ils augmentaient ou ils diminuaient leur décharge en relation avec l'activation corticale. Ils pourraient ainsi contribuer différemment soit pour stimuler l'activation corticale ou au contraire freiner l'éveil comportemental.

Chez des rats qui dorment et se réveillent naturellement, j'ai constaté qu'un neurone cholinergique est actif au cours des deux états de vigilance l'éveil et SP, il est considéré comme étant un neurone E/SP-max. Les neurones cholinergiques de LDT/PPT pourraient ainsi stimuler l'activation corticale lors de l'éveil et du SP, comme ils pourraient également promouvoir l'inhibition motrice et l'induction de l'atonie musculaire associée au SP. En revanche, les neurones GABAergiques et les neurones présumés glutamatergiques du LDT/PPT sont hétérogènes dans leurs profils de décharge. Certains, sont actifs pendant l'éveil et le SP, comme étant des neurones E/SP-max. Comme pour les neurones cholinergiques, ils pourraient également stimuler l'activation corticale au cours de l'éveil et du SP. D'autres, sont actifs au maximum pendant le SP, comme étant des neurones SP-max. Ils pourraient éventuellement participer à freiner l'éveil comportemental ainsi que le tonus musculaire au cours du SP. Quelques neurones présumés glutamatergiques sont actifs au maximum pendant l'éveil. Ils pourraient participer à stimuler l'éveil comportemental ainsi que le tonus musculaire au cours de l'éveil.

L'ensemble de ces travaux montre que les différents neurones du LDT/PPT travaillent en coordination pour soit influencer l'activation corticale pendant l'éveil et le SP, soit freiner l'éveil comportemental et le tonus musculaire au cours du SP ou au contraire stimuler l'éveil comportemental et le tonus musculaire au cours de l'éveil.

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Note to reviewers

The present thesis is a chapter-based thesis. The work contained in this thesis is comprised of two experimental projects. The first one, "Characterization of the activity profiles of LDT/PPT neurons in anesthetized rats" has already been published as the following citation: "Boucetta S, Jones BE (2009) Activity profiles of cholinergic and intermingled GABAergic and putative glutamatergic neurons in the pontomesencephalic tegmentum of urethane-anesthetized rats. *J Neurosci* 29: 4664-4674". The second one, "Characterization of the activity profiles of LDT/PPT neurons during natural sleep-wake states" is still an ongoing project that will be published in the near future.

As a first author in the published manuscript, I obtained authorization to include part of its original text in the present thesis.

Contribution of authors

As a principal investigator of the two projects presented in this thesis, I performed all the electrophysiological experiments and analysis including the surgery, the implantation and the habituation of rats, recording and labeling of cells and fixation of the brains, as well as data and statistical analysis and the production of the figures and the tables. Experimental and analytical design was planned with the guidance of Dr. Barbara E. Jones.

Lynda Mainville, the laboratory technician, performed the immunohistochemical staining of the brain tissue with the labeled cells. I performed the microscopic examination with image acquisition and analysis for data collection and neuroanatomical mapping.

I wrote the published manuscript that was later edited by Dr. Jones to be adequate for publication.

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List of Abbreviations

ACh	Acetylcholine
CAs	Catecholamines
CG	Central gray
CNS	Central nervous system
DA	Dopamine
DMT	Dorsomedial tegmental nucleus
DpMe	Deep mesencephalic reticular nucleus
DR	Dorsal raphe nucleus
EEG	Electroencephalogram
EMG	Electromyogram
GABA	Gamma-aminobutyric acid
GAD	Glutamic acid decarboxylase
GiA	Gigantocellular reticular nucleus, pars alpha
GiRF	Gigantocellular reticular formation nucleus
GiV	Gigantocellular reticular nucleus, pars ventralis
LC	Locus coeruleus
LDT	Laterodorsal tegmental nucleus
MAAs	Monoamines
NA	Noradrenaline
Orex	Orexin

PMT	Pontomesencephalic tegmentum
PnC	Pontine reticular nucleus, caudal part
PnO	Pontine reticular nucleus, oral part
PPT	Pedunculopontine tegmental nucleus
lPPT	Lateral pedunculopontine tegmental nucleus
mPPT	Medial pedunculopontine tegmental nucleus
PS	Paradoxical sleep
RF	Reticular formation
SN	Substantia Nigra
5-HT	Serotonin
SubC	Subcoeruleus
SubLDT	Sublaterodorsal tegmental nucleus
SWS	Slow wave sleep
VAcHT	Vesicular transporter protein for acetylcholine
VGlut2	Vesicular glutamate transporter 2
VTA	Ventral tegmental area
W	Wakefulness, waking

“Allah takes the souls at the time of their death, and those that do not die [He takes] during their sleep. Then He keeps those for which He has decreed death and releases the others for a specified term. Indeed in that are signs for a people who give thought”.

(The Holy Quran, Surat Az-Zumar, quran.com/39:42)

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1. Chapter One

Introduction

1.1 Background information

1.1.1 Sleep-wake states

Since the discovery of the electroencephalogram (EEG) in the early twentieth century, neurophysiologists such as Berger and Loomis remarked that recorded EEG signals from the brain showed specific patterns across different behavioral states (Loomis et al., 1935a; Haas, 2003). Wakefulness (waking; W) was marked by low voltage and fast EEG activity, whereas sleep was marked by high voltage spindles and slow EEG activity (Loomis et al., 1935b). In the 1950s, researchers had found that sleep is, in fact, composed by two distinct states: slow-wave sleep (SWS) and paradoxical sleep (PS) (Aserinsky and Kleitman, 1953; Jouvet et al., 1959). There are thus, in most mammals, three distinctive and unique behavioral states: W, SWS and PS. W is characterized by a relatively low sensory threshold, high muscle tone and an activated EEG. SWS (also known as non-rapid eye movement (NREM) sleep) is distinguished by behavioral inactivity, higher sensory threshold, lower muscle tone and an irregular slow EEG activity. Finally, PS (also called rapid eye movement sleep, REM) is characterized by an even higher sensory threshold, rapid eye movements, a minimal muscle tone (or atonia) and an activated EEG, "paradoxically" similar to W. It is during this state that dreaming is considered to occur (Dement and Kleitman, 1957). These states alternate in an ultradian rhythm (more than one per day) to form the sleep-wake cycle. Many neuronal systems in the brain work together to orchestrate this cycle (Fig. 1.1; for review see (Jones, 2005)).

Over the years, immense research work has been realized to understand how can the brain generate these different behavioral states. Initial studies, which used lesion and

electrical stimulation techniques, made evident the importance of the brainstem for the generation of cortical activation and wakefulness (Lindsley et al., 1949; Moruzzi and Magoun, 1949; Lindsley et al., 1950). Later, with the development of techniques in neurochemistry and immunohistochemistry, researchers further explored the chemical identity of the neural systems involved in regulating sleep-wake states. Thus, different neurotransmitters and neurochemical pathways were suggested to be differentially involved in the generation of the sleep-wake states (Jones, 1989). Recording from a single cell across the sleep-wake cycle is another used technique to demonstrate that certain cells in certain brain areas exhibit specific firing patterns across the sleep-wake cycle (McGinty et al., 1974; Steriade and Hobson, 1976; Steriade et al., 1982; Steriade et al., 1990a; Datta, 1995; McCarley et al., 1995; Koyama et al., 2003; Lee et al., 2005b; Lee et al., 2005a). Furthermore, different neuronal populations are interconnected and receive input from specific neurotransmitter systems that could thus neurochemically modulate their firing characteristics across the sleep-wake cycle (Jones, 2008).

1.1.2 The reticular activating system

In the 1930s, by transections of the brainstem, Bremer showed that separation of the cerebrum (*cerveau isolé*) from the brainstem and spinal cord resulted in SWS-like patterns in the EEG, whereas separation of the encephalon (*encéphale isolé*) from the spinal cord did not affect the activity of the waking state (Steriade, 2003). Bremer suggested then that an important cortical activating mechanism might be located in the brainstem between the medulla and midbrain (Steriade, 2003). Later on, Moruzzi and Magoun showed that electrical stimulation of the brainstem reticular formation (a large network of neurons and fibers located in the brainstem), evoked diffuse and long lasting

cortical activation in sleeping and anesthetized cats (Moruzzi and Magoun, 1949). They then hypothesized that the reticular formation is the key structure to activate the cortex. Moreover, lesions of the reticular formation induced cortical slow-wave activity in association with motor immobility in cats (Lindsley et al., 1949; Lindsley et al., 1950), which supports the hypothesis of Moruzzi and Magoun that the reticular formation is critical for cortical activation.

The reticular formation influences the sleep-wake states by modulating both ascending and descending neuronal systems (Fig. 1.1).

Reticular neurons receive input from sensory systems and passing fibers in the brainstem; they send in turn both long ascending and descending fibers (Jones, 1995). They send their ascending fibers into the forebrain via two pathways, *the dorsal pathway* (also called thalamic) that terminates in the non-specific nuclei of the thalamus and *the ventral pathway* (also called extra-thalamic) that terminates in the hypothalamus, subthalamus and basal forebrain (Nauta and Kuypers, 1958; Scheibel and Scheibel, 1958; Jones and Yang, 1985). Electrical stimulation of the brainstem reticular formation evoked desynchronization of EEG and cortical activation in sleeping and anesthetized animals (Moruzzi and Magoun, 1949). Therefore, the brainstem reticular formation is suggested to be the elemental generator of cortical activation and as a potential generator of waking, which established the concept of the "ascending reticular activating system" (Jones, 1990a). Some of reticular neurons, particularly those located in the caudal pontine and the medullary reticular formations could also, via their descending projections to lower brainstem and the spinal cord (Jones and Yang, 1985), stimulate muscle tone and movement during waking (Siegel and McGinty, 1977; Siegel, 1979).

Much evidence suggested that reticular neurons were the most critical for the generation of PS and its two most prominent characteristics, the EEG characteristic of cortical activation and the behavioral characteristic of immobility with postural muscle atonia (Steriade and Hobson, 1976; Jones, 1991c). Via their ascending projections through the dorsal, thalamic as well as the ventral, extrathalamic relay systems to the cerebral cortex, rostral (oral pontine and mesencephalic) reticular neurons could stimulate cortical activation that is associated with PS (Fig. 1.1). Indeed transections rostral to the pons eliminated tonic cortical activation as well as phasic manifestations of PS but not muscle atonia (Jouvet, 1962). Via their descending projections, pontomedullary reticular neurons are considered to be critical in the generation of muscle atonia that is associated with PS (Jones, 1991c). Stimulation of the brainstem reticular formation produced a generalized motor inhibition (Magoun and Rhines, 1946; Chase et al., 1986). Transections caudal to the pons eliminated muscle atonia associated with PS (Jouvet, 1962; Webster et al., 1986), suggesting the presence of neurons in the pons, which are critical for muscle atonia. Indeed, early lesion studies in cats indicated the importance of the oral pontine reticular formation (PnO) for the generation of PS and muscle atonia (Carli and Zanchetti, 1965). Subsequent lesion studies also indicated the importance of the tegmentum lateral to the PnO and ventral to the locus coeruleus nucleus (LC), referred to by Sakai and colleagues as the peri-LC-alpha in the cat (Sakai et al., 1979) and here as the Subcoeruleus area (SubC). More recently in rats this region was termed the sublaterodorsal nucleus (Boissard et al., 2002; Lu et al., 2006), though referring to an area in the rat located caudal to the subLDT, where cholinergic neurons are located in continuity with those in the LDT. From lesion studies in both cat and rat, it appears thus that the PnO and SubC regions collectively represent a PS effector zone (Jones, 2004).

Indeed, injection of the cholinergic agonist, carbachol into this zone produces a state closely resembling PS, as marked by muscle atonia in association with cortical activation and theta activity in the hippocampus (George et al., 1964; Baxter, 1969; Mitler and Dement, 1974; Amatruda et al., 1975; Vertes and Kocsis, 1997). Moreover, neurons located in the SubC were found to discharge in relation to muscle atonia (Sakai et al., 1981). These putative glutamatergic neurons project to the ventral medullary gigantocellular reticular nucleus (GiV) (Sakai et al., 1979). Furthermore, neurons in GiV were found to discharge with muscle atonia (Kanamori et al., 1980; Siegel et al., 1991) and to project to the spinal cord (Holmes and Jones, 1994). In the same region, c-Fos studies showed that GABAergic/glycinergic neurons are active during PS (Maloney et al., 2000; Sapin et al., 2009). Therefore, it is probable that the PS-active neurons of the PS effector zone stimulate the GiV GABAergic/glycinergic neurons, which in turn provide a descending inhibitory influence to the spinal motoneurons that result in the induction of the behavioral characteristic of PS, muscle atonia (Fig. 1.1). Based upon results from lesion studies, it is also likely that some glutamatergic neurons in the pontine RF project directly to the spinal cord to excite GABA/glycinergic neurons located there (Holmes and Jones, 1994; Krenzer et al., 2011).

1.1.3 Neuronal components of the reticular activating system

Pontomesencephalic cholinergic neurons

Many studies of vigilance and behavioral state control were directed toward identifying and characterizing the neurons of the ascending reticular activating system, particularly those that should be responsible for cortical activation during both waking and PS on one hand and triggering PS on the other hand (Jones, 1990a). Although many

candidates have been suggested over the years, attention focused early upon cholinergic neurons which were later identified immunohistochemically and localized to the laterodorsal and pedunculopontine tegmental (LDT and PPT) nuclei (Steriade et al., 1990a; Jones, 1993; Steriade, 2004). Since pontomesencephalic cholinergic neurons are one of the principal candidates to be investigated in the present thesis, I will detail them in subsequent sections of this chapter.

Monoaminergic neurons

Monoaminergic neurons include noradrenergic, dopaminergic and serotonergic neurons. They are considered to be part of the arousal system (Jones, 2003). The reduction of Catecholamines (CAs; noradrenaline and dopamine) by Alpha-methyl-para-tyrosine (AMPT) or Reserpine decreases cortical activation and behavioral arousal and has a tranquilizing effect (Keane et al., 1976). Whereas, the enhancement of CAs by the dopamine precursor L-DOPA or Cocaine results in an increase of cortical activation along with behavioral arousal, insomnia and a decrease in PS (Jones, 1972; Keane et al., 1976; Hernandez-Lopez et al., 1996; Johanson et al., 1999). Electrophysiological study showed that noradrenergic neurons, which are located in the LC, discharged maximally during waking and ceased firing during PS (Aston-Jones and Bloom, 1981). Moreover, pharmacological administration of noradrenaline (NA) in the basal forebrain cholinergic cell area increased high frequency, gamma EEG activity and prolonged the waking period (Cape and Jones, 1998). The serotonergic neurons, located in the dorsal raphe nucleus (DR), were also found to be active during waking and silent during sleep (McGinty and Harper, 1976b; Trulson and Jacobs, 1979). Moreover, the release of serotonin (5-HT) was found to be high during waking compared to SWS and PS (Wilkinson et al., 1991;

Portas et al., 1998). The dopaminergic neurons, located in the ventral tegmental area (VTA) and the substantia nigra (SN), were found to discharge in bursts during waking in association with reward (Mirenowicz and Schultz, 1996). Moreover, the release of dopamine (DA) was found to be high during waking in rewarding conditions (Di Chiara and Imperato, 1988; Richardson and Gratton, 1996). Lesions of the dopaminergic cell area, the ventral mesencephalic tegmentum, resulted in the diminution of fast EEG activity and attentive behavior (Montaron et al., 1982), as well as "akinesia" and "aphagia" (Ungerstedt, 1971; Jones et al., 1973).

Monoaminergic neurons are also suggested to be "permissive" for PS. (Hobson et al., 1975). Monoaminergic neurons are suggested to block cholinergic transmission to prevent PS (Hobson et al., 1975). Indeed, the injection of the acetylcholinesterase inhibitor physostigmine (eserine) elicited a PS-like state only after depletion of MAs with reserpine (Karczmar et al., 1970). Moreover, the increase of monoamines (MAs) levels by monoamine oxidase inhibitors eliminated PS and its phasic characteristic, ponto-geniculo-occipital (PGO) spikes for long periods (Jones, 1972; Jouvet, 1972).

Hence, monoaminergic neurons appear to contribute to the mechanisms associated with behavioral arousal and cortical activation and appear to antagonize mechanisms associated with sleep induction including PS with muscle atonia (Jones, 1991b, 2003).

Reticular formation neurons

The reticular formation, which is formed by a large network of neurons and fibers, is located through the central core of the brainstem (from the medulla to the midbrain). The neurons of the reticular formation receive afferents from different sources passing through the brainstem, including ascending somatosensory, visual, auditory and vestibular

input, and cortical descending output (Jones, 2003). In turn, reticular neurons form a large contingent of the ascending reticular activating system that send ascending fibers to the forebrain (Jones, 1990a). They also send descending projections down to the spinal cord (Jones, 1995). Although the reticular formation forms a large network of neurons and fibers through the entire core of the brainstem, there is a degree of differentiation. The major population of neurons which form the ascending pathways to the forebrain and are responsible for cortical activation are concentrated in the mesencephalic and oral pontine reticular fields (Jones, 1990a). The major population of neurons which form the descending pathways to lower brainstem and spinal cord are concentrated in the caudal pontine and the medullary reticular formation (Jones and Yang, 1985). These reticulospinal neurons are suggested to stimulate muscle tone and behavioral arousal during waking (Fig. 1.1; Siegel and McGinty, 1977; Siegel, 1979) or to dampen behavioral arousal during sleep and induce muscle atonia during PS (Magoun and Rhines, 1946; Kanamori et al., 1980; Siegel et al., 1991).

As discovered recently, the majority of reticular neurons utilize glutamate as a neurotransmitter (Kaneko et al., 1989; Jones, 1995). A small portion of the reticular neurons use GABA as neurotransmitter and are mainly considered to be locally projecting neurons (Holmes et al., 1994; Ford et al., 1995; Jones, 1995). However, some of these GABAergic neurons are found to be long projecting neurons, particularly the pontomesencephalic GABAergic neurons that send long ascending projections to the forebrain (Ford et al., 1995) or medullary GABAergic neurons that send long descending projections to the spinal cord (Jones et al., 1991; Holmes et al., 1994). Thus, as locally projecting or long projecting, the GABAergic neurons could serve to inhibit glutamatergic or other neurons of the activating and arousing systems. Many anesthetic

agents work either by blocking glutamatergic transmission or by enhancing GABAergic transmission (Schulz and Macdonald, 1981; Yamamura et al., 1990; MacIver et al., 1996). Reticular glutamatergic and GABAergic systems could thus respectively work to stimulate or to dampen cortical activation and behavioral arousal.

1.1.4 Anatomy and physiology of the LDT/PPT nuclei

LDT and PPT nuclei are located at the level of the oral pons and caudal mesencephalon in the region called the pontomesencephalic tegmentum. These nuclei were found to contain the major group of brainstem cholinergic neurons which were suspected to be involved in controlling sleep-wake states (Jones, 1993). However, the cholinergic neurons are not alone in these nuclei; they lie intermingled with other GABAergic and glutamatergic neurons which are actually more numerous than cholinergic neurons (Ford et al., 1995; Wang and Morales, 2009).

1.1.4.1 LDT/PPT cholinergic neurons

Anatomy

In the early 1940s, it was suggested that acetylcholine (ACh) is synthesized within distinct neurons in the brain (MacIntosh, 1941; Feldberg and Vogt, 1948). In the 1950s, with the development of the histochemical technique to detect acetylcholinesterase (AChE), the catabolic enzyme for ACh, distinct populations of neurons were found to contain AChE and thus thought likely to be cholinergic (Koelle, 1951). A considerable number of neurons located in the brainstem reticular formation were subsequently found to contain AChE (Shute and Lewis, 1963). Surprisingly, the origin and the projections of these neurons corresponded closely to the ascending reticular activating system that had

been previously described by Moruzzi, Magoun and their colleagues (Moruzzi and Magoun, 1949; Starzl et al., 1951). Nevertheless, the presence of AChE in these neurons did not with any certainty indicate that they synthesized and released ACh (Fibiger, 1982; Butcher and Woolf, 1984). Later in the 1980s, with the development of immunohistochemistry for the synthetic enzyme of ACh, choline acetyl transferase (ChAT), the indisputable evidence for the identity and location of cholinergic neurons in the brain was brought forward (Sofroniew et al., 1985; Woolf and Butcher, 1986; Hallanger et al., 1987; Jones and Beaudet, 1987; Jones and Webster, 1988; Pare et al., 1988; Steriade et al., 1988). In the brainstem, the largest group of cholinergic neurons that project rostrally was found in the pontomesencephalic tegmentum within the LDT and PPT nuclei (Armstrong et al., 1983; Houser et al., 1983; Mesulam et al., 1983; Satoh et al., 1983; Mesulam et al., 1984; Jones and Beaudet, 1987). Recently, proof of the uptake, storage and release of specific neurotransmitters from nerve terminals has also become possible by immunohistochemical staining for specific vesicular transporter proteins, including notably that for ACh (VACHT; (Gilmor et al., 1996)). VACHT can be revealed in the soma as well as the terminals of cholinergic neurons in the LDT and PPT (Garzon and Pickel, 2000).

LDT/PPT cholinergic neurons receive input from neurons of the brainstem reticular formation and from the noradrenergic LC neurons (Jones, 1990b). In turn, cholinergic neurons send ascending and descending projections, as well as local ramifications to target some surrounding neurons particularly those of the reticular formation. For the ascending projections, cholinergic neurons project rostrally, in parallel with other reticular neurons, through two pathways, the dorsal or thalamic pathway and the ventral or extra-thalamic pathway. Via the dorsal pathway, of which they represent an

important contingent, they provide a rich innervation to the nuclei of the non-specific thalamo-cortical projection system (Sofroniew et al., 1985; Woolf and Butcher, 1986; Hallanger et al., 1987; Jones and Webster, 1988; Pare et al., 1988; Steriade et al., 1988). Via the ventral pathway, which is less dense than the dorsal pathway, they send fibers up to and through the lateral hypothalamus and into the basal forebrain (Woolf and Butcher, 1986). They would also influence other reticular neurons that send ascending projections via the dorsal, thalamic as well as the ventral, extrathalamic relay systems to the cerebral cortex (Jones and Webster, 1988; Jones, 1990b). For the descending projections, cholinergic neurons send projections to the PnO and SubC (Mitani et al., 1988; Jones, 1990b; Semba et al., 1990). Cholinergic neurons project also to reticulospinal neurons in the medulla (Jones, 1990b) as well as to motor neurons in the brainstem (Rukhadze and Kubin, 2007).

Physiology

Increasing evidence suggests the involvement of LDT/PPT cholinergic neurons in modulating sleep-wake states particularly by driving cortical activation and triggering PS.

A first functional role postulated for LDT/PPT cholinergic neurons is driving cortical activation during both waking and PS (Fig. 1.1). Moruzzi and Magoun had found that electrical stimulation of the pontomesencephalic tegmental region elicits EEG desynchronization (Moruzzi and Magoun, 1949). Given their location, the LDT/PPT cholinergic neurons were suspected to drive cortical activation and EEG desynchronization (Shute and Lewis, 1967; Jones and Beaudet, 1987; Vincent and Reiner, 1987). electrical stimulation of the LDT/PPT elicits cortical activation, while exciting thalamic neurons (Steriade et al., 1991). In vivo recordings of neurons with broad spikes

and presumed (but not identified) LDT/PPT cholinergic neurons have shown that these cells fire tonically at low to moderate rates during wakefulness and PS, as W/PS-active cells (Sakai, 1985; El Mansari et al., 1989; Steriade et al., 1990a; Kayama et al., 1992). The increased firing of these neurons was positively correlated with cortical activation on the EEG (Steriade et al., 1990a). Cholinergic neurons could depolarize thalamo-cortical relay neurons, to which they project and thus mediate the cortical activation (McCormick and Prince, 1986, 1987; Steriade et al., 1990a; Steriade et al., 1990b). Indeed, the release of ACh is high in the thalamus in positive correlation with cortical activation during waking and PS (Williams et al., 1994). Thus, the important role of ACh in thalamo-cortical processes of activation would come into play during both wakefulness and PS. Webster and Jones in 1988 showed that neurotoxic lesions of LDT/PPT cholinergic neurons had minimal effects on the maintenance of cortical activation during waking (Webster and Jones, 1988). They nonetheless did not exclude the importance of cholinergic neurons in driving cortical activation; rather, they hypothesized that other non-cholinergic neurons, which are part of the reticular activating system, are sufficient to drive this cortical activation (Webster and Jones, 1988). On the other hand, they claimed that their neurochemical lesion resulted in only 60-70 % loss of cholinergic neurons (Webster and Jones, 1988).

Although the EEG, EMG and state related discharge of LDT/PPT cholinergic neurons remains to be established, it is suggested that these cholinergic neurons could stimulate cortical activation and EEG desynchronization during active waking, which is associated with high muscle tone, as well as during PS, which is associated with complete immobility and muscle atonia (Fig. 1.1; Jones, 2005). As Barbara Jones suggests,

"cholinergic neurons can stimulate cortical activation irrespective of behavioral arousal or motor activity and muscle tone" (Jones, 2008).

A second functional role postulated for LDT/PPT cholinergic neurons is the triggering of PS. Neurotoxic lesions of LDT/PPT cholinergic neurons resulted in the immediate loss followed by a partial recovery of PS, which however, was characterized by diminished phasic activity and incomplete muscle atonia (Webster and Jones, 1988). In addition to the W/PS-active group found in LDT/PPT (see above), some single-unit recordings studies suggested the presence of another group of presumed cholinergic neurons that discharge maximally during PS, as PS-active (El Mansari *et al.*, 1989; Steriade *et al.*, 1990b; Kayama *et al.*, 1992). Furthermore, expression of c-Fos, which reflects neural activity, occurs in immunohistochemically identified LDT/PPT cholinergic neurons following PS rebound after deprivation in rats (Maloney *et al.*, 1999). But we could ask how do cholinergic neurons trigger PS and elicit many of its components? They could do so that through their influence upon the forebrain and brainstem targets.

Through ascending pathways, cholinergic neurons may participate in parallel with other neurons of the reticular activating system to elicit an activated EEG paradoxically similar to the EEG of the waking state. Indeed, as stated above, ACh release was found to be as high in the thalamus during PS as it was during waking, in association with EEG fast activity (Williams *et al.*, 1994). ACh evoked a single spike mode firing by thalamocortical neurons, which is associated with desynchronization of the thalamo-cortical system and fast EEG activity (Steriade and Deschenes, 1984; Steriade and Llinas, 1988). Furthermore, ACh release was found to be as high in the cerebral cortex during PS as it was during waking (Jasper and Tessier, 1971). The increase in the release of ACh in the cerebral cortex is particularly derived from the cholinergic neurons in the

basal forebrain that project directly to the cortex (Manns et al., 2001; Henny and Jones, 2008) and were shown to be as active during PS as during waking (Fig. 1.1; Lee et al., 2005b). The cholinergic neurons of the pontomesencephalic tegmentum could also indirectly evoke the increase of the ACh release in the cortex by stimulating basal forebrain cholinergic neurons via the ventral pathway (Fig. 1.1). Yet, in this same region, cholinergic neurons are intermingled with other non-cholinergic neurons such as GABAergic and glutamatergic neurons that are shown to influence differently the sleep-wake cycle (Fig. 1.1; Hassani et al., 2009). LDT/PPT cholinergic neurons could thus evoke cortical activation during PS by exciting the thalamo-cortical and basalo-cortical relays along their ascending systems (Fig 1.1).

LDT/PPT cholinergic neurons could also participate in the cognitive correlate of PS, dreaming (Hobson, 1992), via the ascending system through either direct stimulation of the limbic and cortical structures to which they project (Satoh and Fibiger, 1986) or indirect stimulation by exiting the VTA dopaminergic neurons (Forster and Blaha, 2000) that were found to burst during PS (Dahan et al., 2007) and to project to limbic and cortical structures (Loughlin and Fallon, 1983; Sobel and Corbett, 1984; Gasbarri et al., 1991; Narita et al., 2010). Given the implication of these terminal structures in emotions, memory, and even hallucinations (Portavella and Vargas, 2005; Behrendt, 2010), which are often associated with PS and dreaming (Paiva et al., 2011), cholinergic neurons may be implicated in the dreaming process.

Through their projections into the brainstem reticular formation, LDT/PPT cholinergic neurons would have the capacity to trigger PS (Jones, 2004). It has been shown that injection of the cholinergic agonist, carbachol into the PS effector zone (PnO/SubC) produces a state closely resembling PS, as marked by muscle atonia in

association with cortical activation and theta activity in the hippocampus (George et al., 1964; Baxter, 1969; Mitler and Dement, 1974; Amatruda et al., 1975; Vertes and Kocsis, 1997). In this same region, endogenous ACh is released in higher concentrations during natural PS than during waking and SWS (Kodama et al., 1990). The generation of the EEG components of PS, cortical activation, could be mediated by excitation of W/PS-active neurons in the PnO and SubC through muscarinic type 1 ACh receptors (M1 AChRs) (Greene and Carpenter, 1985; Greene et al., 1989). As ascending projections to the forebrain in parallel to the reticular activating system, these reticular neurons could participate in inducing cortical activation (Fig 1.1; Jones, 1990a). Yet, elicitation of the EMG component of PS, muscle atonia, in this region was found to be predominantly mediated by the muscarinic type 2 (M2) ACh receptors (M2 AChRs), which mediate hyperpolarization and inhibition (Velazquez-Moctezuma et al., 1989; Velazquez-Moctezuma et al., 1991; Imeri et al., 1994; Baghdoyan and Lydic, 1999). Indeed, a large number of presumed reticulo-spinal neurons in the PnO and caudal pontine reticular formation (PnC) were found to bear M2 AChRs (Brischoux et al., 2008), suggesting that LDT/PPT cholinergic neurons could inhibit reticulospinal neurons which normally promote behavioral arousal with muscle tone (Brischoux et al., 2008). Motor neurons in the brainstem and spinal cord could thus be indirectly disfacilitated during PS. Cholinergic neurons could also directly inhibit brainstem motor neurons (Rukhadze and Kubin, 2007).

According to what is cited above, the LDT/PPT cholinergic neurons could normally stimulate cortical activation, via their influence upon reticular and forebrain structures, during waking and during PS. They also could elicit tonic motor inhibition and

muscle atonia through influence upon brainstem and reticulo-spinal systems to promote PS (Fig. 1.1).

Electrophysiological evidence has suggested the existence of more than one functional type of cholinergic neuron in the LDT/PPT. Neurons in the LDT/PPT that had broad spikes were considered 'possibly' cholinergic and found to be active during both waking and PS (W/PS-active) or during PS (PS-active) in cat (El Mansari et al., 1989; Steriade et al., 1990a; Koyama and Sakai, 2000) and in rat (Kayama et al., 1992). Moreover, an electrophysiological study claimed also the presence of two sub-groups of putative cholinergic neurons in LDT/PPT, one was inhibited by 5-HT and considered as PS-active, and another was not affected by 5-HT and considered as W/PS-active (Thakkar et al., 1998). Furthermore, a c-Fos study demonstrated that cholinergic neurons were active, whereas monoaminergic neurons were reciprocally inactive during PS (Maloney et al., 1999). This electrophysiological evidence received support from immunohistochemical studies showing that some cholinergic neurons in LDT/PPT bear excitatory α_1 adrenergic receptors (α_1 -ARs) and were thus proposed to be W/PS-active, and others bear inhibitory α_2 adrenergic receptors (α_2 -ARs) and were thus proposed to be PS-active neurons (Hou et al., 2001). The latter group would conform to the conceptual model of reciprocal roles between cholinergic and monoaminergic systems in eliciting PS and waking respectively (Hobson et al., 1975). In a "prey-predator" like model (McCarley and Hobson, 1975), cholinergic neurons are proposed as PS-active while monoaminergic neurons are W-active (McCarley and Hobson, 1975; Maloney et al., 1999; Hou et al., 2001). Thus, these different studies and theories would allow for the possibility that cholinergic LDT/PPT neurons are W/PS-active and/or PS-active subtypes.

1.1.4.2 LDT/PPT GABAergic neurons

Anatomy

Gamma-aminobutyric acid (GABA) is derived from glutamate by the enzyme glutamic acid decarboxylase (GAD). Following the development of immunohistochemical staining for GAD, GABA was found to be mostly synthesized by local interneurons located in almost all the areas of the central nervous system (CNS) (Mugnaini and Oertel, 1985; Watanabe et al., 2002). Many nuclei in the brainstem such as LDT, PPT, DR, VTA and SN were found to include GABAergic neurons in addition to the main type of neurons that characterize these nuclei (Fonnum et al., 1978; Jones, 1991c; Jones, 1991a; Holmes et al., 1994; Ford et al., 1995; Jones, 1995; Tepper et al., 1995; Maloney et al., 2002; Wang and Morales, 2009). In the LDT and PPT nuclei, GABAergic neurons were found to be intermingled with cholinergic neurons (Ford et al., 1995; Wang and Morales, 2009). Surprisingly, these GABAergic neurons were found to be more numerous than the cholinergic neurons (Ford et al., 1995; Wang and Morales, 2009). They were small (10-15 μm) to medium (up to 20 μm) GAD-immunoreactive neurons and intermingled with medium sized ChAT⁺ cells through the LDT, SubLDT, and medial and lateral (m and l) PPT (Ford et al., 1995). Although many GABAergic neurons give rise to a dense local innervation of neighboring cells, including cholinergic cells (Mugnaini and Oertel, 1985; Ford et al., 1995), a minority of the GABAergic cells contribute to long ascending projections from the tegmentum into the region of cortically projecting neurons in the posterior lateral hypothalamus (Ford et al., 1995) and into the subthalamic nucleus (Bevan and Bolam, 1995). GAD-positive terminals are also present near and around GAD-positive cell bodies in LDT/PPT (Ford et al., 1995), suggesting

that as in many other areas of the CNS, GABA-GABA interactions may occur and underlie processes of disinhibition (Oertel et al., 1984; Mugnaini and Oertel, 1985).

Physiology

GABA, the main inhibitory neurotransmitter in the brain, has long been suggested to play a role in sleep, since its agonists provoke a sedative effects (Mendelson, 1985). It has been suggested that some GABAergic neurons, particularly those located in basal forebrain and preoptic area, are sleep promoting neurons (Gong et al., 2004; Modirrousta et al., 2004). However, GABAergic neurons are not always active in a state-selective manner throughout the brain (Steriade et al., 1986; Steriade et al., 2001; Hassani et al., 2009). Although the discharge pattern of GABAergic neurons in the LDT-PPT was not known, neurons with brief action potentials and high frequency discharge, were recorded in the LDT/PPT and proposed to be GABAergic (Sakai, 1985; El Mansari et al., 1989; Steriade et al., 1990a; Kayama et al., 1992). Some of these putative GABAergic cells were found to be PS-active neurons (Steriade et al., 1990a). Indeed, LDT/PPT GABAergic neurons have been described to be active, according to their c-Fos expression, during the rebound from PS that follows sleep deprivation in the rat (Maloney et al., 1999). Furthermore, these GABAergic neurons have been suggested to play an intermediary role between cholinergic and monoaminergic (serotonergic and noradrenergic) neurons to affect the inhibition of the neighboring monoaminergic neurons during PS (Maloney et al., 1999). Given the large numbers of relatively small GABAergic neurons through the pontomesencephalic tegmentum, it is likely that the majority of these cells provide a proportion of the rich local innervation to surrounding neurons (Ford et al., 1995). The neurons of the "ascending reticular activating system"

may accordingly be regulated by important inhibitory influences through local GABAergic neurons (Maloney et al., 1999). LDT/PPT GABAergic neurons contribute also, although in a minor proportion, to the major ascending projections from the brainstem reticular activating system (Ford et al., 1995). In such parallel projections, the GABAergic neurons could potentially oppose, support or modulate the action of the major excitatory transmitter line depending upon their target neurons (Freund and Meskenaite, 1992; Somogyi and Klausberger, 2005). Acting as local neurons and/or projection neurons, the LDT/PPT GABAergic cells would be expected to play a very important role in regulating the influence of the ascending reticular activating system upon cortical activation (Fig. 1.1).

1.1.4.3 LDT/PPT glutamatergic neurons

Anatomy

Glutamate is the main excitatory neurotransmitter in the brain and is present in neurons throughout the CNS. Some previous studies showed the presence of glutamate in LDT and PPT neurons (Clements and Grant, 1990; Jones, 1995). But since glutamate serves as a precursor for GABA as well, it could also be present in GABAergic neurons; therefore, the presence of glutamate in neurons does not serve to identify these neurons as glutamatergic. With the discovery of vesicular glutamate transporters (VGluTs), VGluT1, VGluT2 and VGluT3 as specific markers for the uptake and the release of glutamate (Bellocchio et al., 2000; Fremeau et al., 2001; Fremeau et al., 2002), it became possible to identify glutamatergic neurons in the brain (Fujiyama et al., 2001; Fremeau et al., 2004; Herzog et al., 2004; Nickerson Poulin et al., 2006). Unlike VACHT, VGluTs protein is present and visible only in terminals and not in cell bodies (Fujiyama et al.,

2001). However, using in situ hybridization technique for VGluTs mRNA, which is present in cell bodies, could confirm the glutamatergic identity of these cells (Freneau et al., 2004). Indeed, a recent study using in situ hybridization for VGluT2 mRNA, has indicated the presence of glutamatergic cells in the LDT/PPT where they are intermingled with cholinergic and GABAergic cells and represent an important contingent of these nuclei (Wang and Morales, 2009). Although there is no information about the specific projections of LDT/PPT glutamatergic neurons, evidence has suggested that they form an important contingent of long ascending projections from LDT/PPT into the forebrain in parallel with the cholinergic neurons (Pare et al., 1988; Jones and Cuello, 1989; Rasmusson et al., 1994; Ford et al., 1995) and GABAergic neurons (Ford et al., 1995). It is also the case that a major proportion of neurons in the LDT/PPT which project to the pontine and medullary reticular formation are noncholinergic and could be thus glutamatergic neurons (Jones, 1990b).

Physiology

To date, there is a complete ignorance concerning the physiological role of LDT/PPT glutamatergic neurons in sleep-wake cycle, since their activity across sleep-wake states was relatively unknown and interest was focused on the cholinergic neurons in this region. Therefore, the activity of LDT/PPT glutamatergic neurons across sleep-wake states and their relation with their neighboring cholinergic and GABAergic neurons remains to be explored.

1.2 Figure 1.1

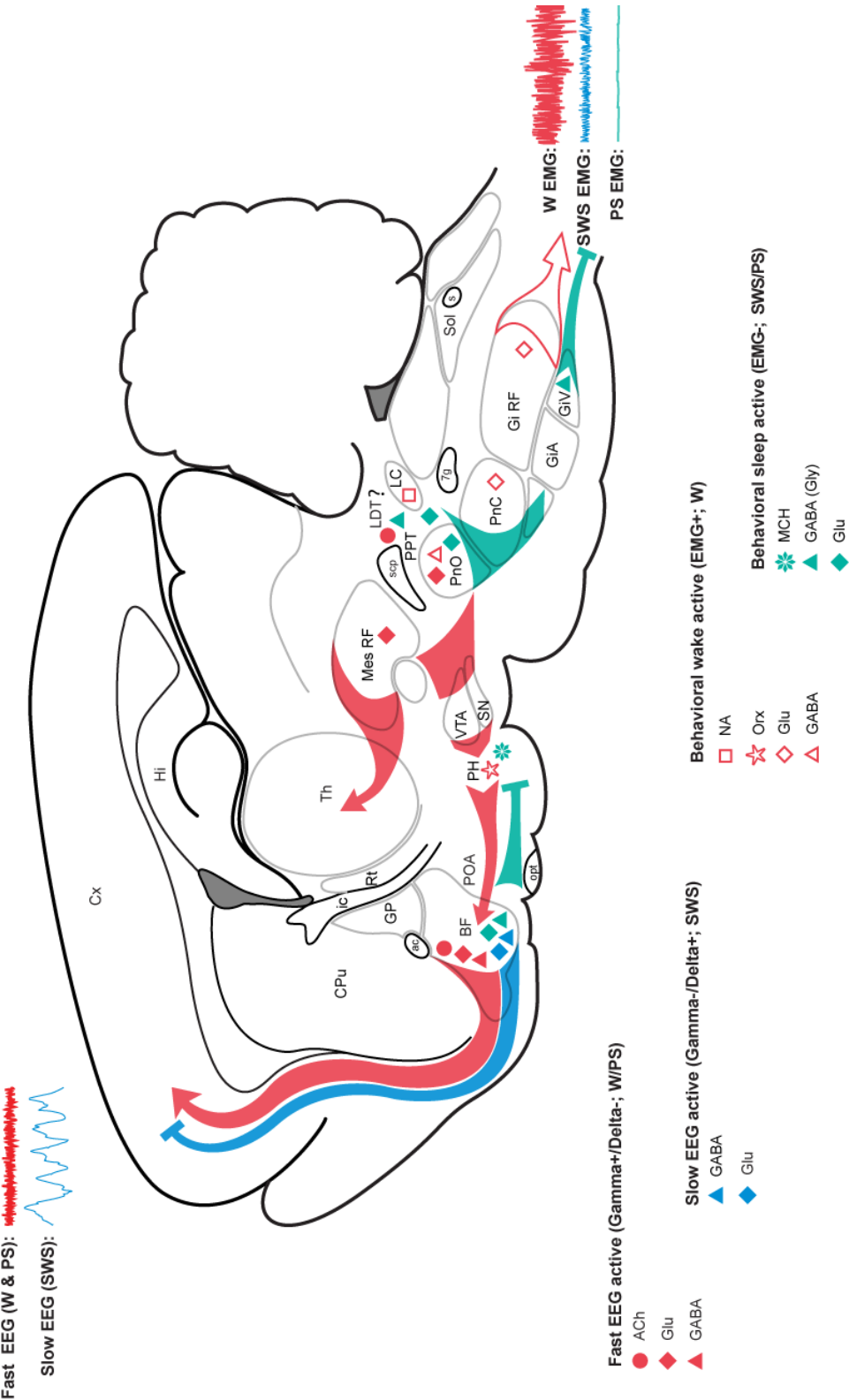


Figure 1.1. Neural systems orchestrating the sleep-wake cycle

Figure 1.1. Neural systems orchestrating the sleep-wake cycle. Sagittal schematic view of the rat brain representing different neurons classified according to their chemical neurotransmitters, pathways and discharge profiles. Wake (W) is characterized by cortical activation with fast (gamma, >40 Hz) EEG activity (upper left, red trace) and postural muscle tone with high neck EMG activity (lower right, red trace); slow wave sleep (SWS) by slow (delta, < 4 Hz) EEG activity (upper left, blue trace) and low muscle tone with low EMG activity (lower right, blue trace); and paradoxical sleep (PS) by fast EEG activity (upper left, red trace) and muscle atonia with virtually no tonic EMG activity (lower right, aqua trace). Neurons that stimulate cortical activation compose the ascending activating system and are comprised of neurons which discharge in positive association with cortical activation (gamma+) and in negative association with slow EEG activity (delta-) to thus be active during both W and PS (W/PS-active, filled red symbols). They include cholinergic (ACh), glutamatergic (Glu) and GABAergic (GABA) neurons. Neurons that oppose the cortical activating system discharge in positive association with slow EEG activity (delta+) and in negative association with fast EEG activity (gamma-) to thus be active during SWS (SWS-active neurons, blue symbols). They include cortically projecting basal forebrain neurons (GABA and Glu). Neurons that stimulate behavioral arousal with postural muscle tone ultimately influence neurons in the brainstem reticular formation and spinal cord and discharge in positive association with EMG activity (EMG+) as W-active neurons (open red symbols). Many give rise to descending or diffuse projections in the brain and include noradrenergic (NA), orexinergic (Orx), putative glutamatergic (Glu) and GABAergic neurons. Neurons that promote behavioral quiescence with decreases in muscle tone or atonia and sleep discharge in negative association with EMG activity (EMG-) to fire at progressively

higher rates during SWS and PS as SWS-PS-active neurons (aqua symbols). They include GABAergic and putative glutamatergic neurons in the forebrain and brainstem and MCH neurons in the hypothalamus. The EEG, EMG and state related discharge of the cholinergic, GABAergic and glutamatergic neurons in the pontomesencephalic tegmentum remains to be established ('?'). Abbreviations: 7g, genu 7th nerve; ac, anterior commissure; CPu, caudate putamen; Cx, cortex; EEG, electroencephalogram; EMG, electromyogram; Gi RF, gigantocellular RF; GiA, gigantocellular, alpha part RF; GiV, gigantocellular, ventral part RF; GP, globus pallidus; Hi, hippocampus; ic, internal capsule; LC, locus coeruleus nucleus; LDT, laterodorsal tegmental nucleus; MCH, melanin concentrating hormone; Mes RF, mesencephalic RF; NA, noradrenaline; opt, optic tract; Orx, orexin; PH, posterior hypothalamus; PnC, pontine, caudal part RF; PnO, pontine, oral part RF; POA, preoptic area; PPT, pedunculopontine tegmental nucleus; RF, reticular formation; Rt, reticularis nucleus of the thalamus; s, solitary tract; scp, superior cerebellar peduncle; SN, substantia nigra; Sol, solitary tract nucleus; Th, thalamus; TM, tuberomammillary nuclei; VTA, ventral tegmental area. (Modified with permission from (Jones, 2005)).

1.3 Considerations and Objectives:

In the preceding sections, I have discussed compelling data that suggests an involvement of LDT/PPT neurons in the control of cortical activation and sleep-wake states. The different types of neurons that exist in the region most probably carry out this control by means of their anatomical projections either through the ascending pathways to the forebrain, or through the descending pathways to the brainstem and spinal cord, and/or through local projections influencing these systems.

With the help of extracellular recording techniques, distinct cell types have been electrophysiologically identified *in vivo* in the area of LDT and PPT, where slow firing cells with broad spikes were presumed to be cholinergic neurons, and fast firing cells with narrow spikes were presumed to be GABAergic neurons (Sakai, 1985; El Mansari et al., 1989; Steriade et al., 1990a; Kayama et al., 1992). However, since these conventional techniques for extracellular recording *in vivo* do not allow the subsequent neurochemical identification of the recorded units, they are likely to confuse different cell populations in the LDT/PPT where three chemically distinct cell types have been identified (Wang and Morales, 2009). Thus, the behavior of neurochemically identified LDT/PPT cell groups in relation to the changes in cortical activity and thereby in relation to sleep-wake states has not yet been possible to study. It is therefore necessary to identify the chemical phenotype of recorded units to unequivocally identify different neuronal populations. Fortunately, the development of the juxtacellular technique (Pinault, 1996) allowed the labeling of single units recorded extracellularly and thereby a subsequent study of neurochemical identity, shape, location and, possibly, axonal projections. To overcome the limitations of previous electrophysiological studies, we used the juxtacellular

recording and labeling technique to record different LDT/PPT cells in anesthetized and naturally sleeping/waking rats and subsequently to identify these cells immunohistochemically.

Previous studies from our laboratory showed the benefit of using juxtacellular recording and labeling to identify chemically distinct neurons in the basal forebrain in urethane-anaesthetized animals in relation to cortical activation and slow irregular EEG activity (Manns et al., 2000a, b; Manns et al., 2003). As it was in the case of basal forebrain, the characterization of activity profiles that LDT/PPT neurons should present in relation to cortical activation in anaesthetized animals can give answers to some important questions: What is the reaction of the neurochemically distinguished cell populations in relation the changes in cortical activity evoked by sensory stimulation? Are the properties and discharge characteristics consistent within the cell populations? Are these different cell populations distinct based on their electrophysiological properties? To answer such questions, we developed in an acute preparation, in urethane-anaesthetized rats, the first study of my thesis project: "Characterization of activity profiles of LDT/PPT neurons in relation to cortical activation and slow irregular activity in urethane-anaesthetized rats".

Although the acute study could suggest the way these neurons might behave during natural sleep-wake states, many questions remain open: What are the activity profiles of cholinergic, GABAergic and glutamatergic neurons during W, SWS and PS across natural sleep-wake states? Do LDT/PPT neurons behave in the same way during cortical activation in W and PS as they do in the anaesthetized preparation? Do the LDT/PPT cholinergic neurons represent a physiologically homogeneous sleep-wake sub-group as some previous studies suggested (Domino et al., 1968; El Mansari et al., 1989; Steriade et

al., 1990b; Williams et al., 1994)? Or do they, as other studies suggested (Kayama et al., 1992; Sakai and Koyama, 1996), form different subgroups? Some could be active during both waking and PS, as "W/PS-max active cells"; others however, could be specifically active during PS, as "PS-max active cells". Do their activities relate to EEG activity, muscle tone or other related phenomena during W, SWS or PS? Are these different cell populations different in their electrophysiological properties? To answer such questions, we developed in a chronic preparation of naturally sleeping-waking head-fixed rats, the second study of my thesis project: "Characterization of the discharge profiles of LDT/PPT neurons during natural sleep-wake states".

The subsequent parts of the thesis will be divided into different chapters. In chapter 2, *Material and Methods*, I will present the appropriate experimental context that allows us to realize both projects. Next, in chapters 3 and 4, I will present the results of the acute study and the chronic study respectively. Finally, the results of both studies will be discussed in chapter 5, *Discussion*, in light of their significance for LDT/PPT neuronal function, and will be followed by a general conclusion.

2. Chapter Two

Materials and Methods

A portion of this chapter was published in Journal of Neuroscience, Vol 29(14): 4664-4674, 2009

2.1 The activity profiles of LDT/PPT neurons in anesthetized rats

2.1.1 Animals and surgery

Experiments were performed on 66 adult male Long-Evans rats (200 – 250 gm; Charles River, St. Constant, 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 gm/kg, intraperitoneally (i.p.) and supplementary doses if necessary of 0.1 – 0.15 gm/kg, i.p. 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. 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 (antero-posterior, AP -4.0 mm, lateral ± 0.5 mm relative to bregma) and in the frontal bone as a reference.

2.1.2 Unit recording and labeling

As described previously (Manns et al., 2000a), juxtacellular recording and labeling was performed using an intracellular amplifier (IR-283, Neurodata Instruments, New York, NY). Unit recordings were performed with glass microelectrodes (~ 0.5 - 1.5 μm tip and ~ 25 - 40 M Ω) filled with 0.5 M NaCl and 5.0% Neurobiotin (Nb, Vector Laboratories, Burlingame, CA). To reach the pontomesencephalic tegmentum, holes were drilled in the skull (AP -5.0 mm from bregma, L ± 0.9 mm) and after opening the dura, the electrode descended from anterior to posterior at an angle of 30° from the

vertical, so as to avoid the tentorium. Single units were then isolated as the electrode was descended through the pontomesencephalic tegmentum into the region of the LDT. Once isolated, the unit was characterized during spontaneous irregular slow EEG activity and during somatosensory stimulation. The somatic stimulation consisted of a continuous pinch of the tail applied by large, blunt forceps such as to apply pressure but not to evoke pain. The stimulation was repeated several times to confirm that the response was constant. After the recording and characterization of isolated neurons, they were labeled by applying the juxtacellular method, as originally developed and described by Pinault (Pinault, 1996). Labeling was accomplished by modulating the firing of the cell through positive current pulses (1 – 10 nA) for a period of 2 - 10 min. In this study, for a total of 149 units submitted to this juxtacellular labeling protocol in 66 rats, 118 units were successfully labeled with Nb (~80%).

Within a few hours after the juxtacellular labeling, the animals were administered an overdose of urethane and perfused transcardially with physiological saline (0.9% NaCl), followed by 500 ml of a fixative containing 3% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were removed, post-fixed overnight in the fixative solution and immersed for 2 days in 30% sucrose in phosphate buffer for cryoprotection. They were frozen at -50°C and stored at -80°C .

2.1.3 Histochemistry

Serial sections were cut at 25 μm thickness in the coronal plane on a freezing microtome and collected for histochemical processing. For revelation of Nb, sections were incubated for 2.5 h in Cy2-conjugated streptavidin (1:1000, Jackson ImmunoResearch Laboratories, West Grove, PA). Following location of an Nb-labeled

cell, the relevant section was dual-immunostained for vesicular transporter protein for acetylcholine (VACHT, with a goat, Gt, polyclonal antibody, AB1578 from Chemicon International, Temecula, CA and a Cy5-conjugated Donkey, Dky-anti-Gt antibody from Jackson) and glutamic acid decarboxylase (GAD, with a Mouse, Ms, monoclonal anti-GAD67 antibody, MAB5406 from Chemicon and a Cy3-conjugated Dky-anti-Ms antibody from Jackson) for identification of cells as cholinergic, GABAergic or noncholinergic-nonGABAergic. Sections were viewed and images acquired by epifluorescence using a Nikon Eclipse E800 (Nikon Instruments Inc., Melville, NY) equipped with a digital camera (Microfire S99808, Optronics, Goleta, CA). The labeled cells were mapped onto a computer resident atlas with the aid of Neurolucida (v7, MicroBrightField, Williston, VT). Cell size was measured by the long axis of the cell and cells classified as small ($\leq 15 \mu\text{m}$) or medium-to-large ($16 - 35 \mu\text{m}$). Of 118 Nb-labeled cells, 73 were judged unequivocally positively (by bright fluorescence in soma, "+") immunostained or negatively (by no fluorescence in soma, "-") immunostained for VACHT and GAD and were located within the confines of the LDT, subLDT or adjacent MPPT, so as to be included and reported in the Results.

2.1.4 Data analysis

Analysis of physiological data was performed on 40 sec periods corresponding to Pre-Stimulation, Stimulation and Post-Stimulation conditions. For the EEG, spectral analysis was performed to determine the dominant peak frequency and integrated power of the spectra in the slow, delta (0.5 – 4.5 Hz) and fast high beta–gamma (20 – 60 Hz) frequencies. As established previously (Manns et al., 2000a), Somatic Stimulation resulted in a change in the EEG from a predominantly slow, irregular delta-like pattern to

a faster rhythmic theta-like pattern and increased fast high beta-gamma activity, typical of a degree of cortical activation despite a lack of behavioral response in the urethane anesthetized animal (see Fig.3.7).

Spike duration was determined from all spikes averaged across the Pre-Stimulation period for each unit. The duration was measured at the first and second zero crossings and thus from the initial positive to the negative deflection and to the subsequent return to resting level (measured with an offset from resting corresponding to 10% of the positive peak amplitude so as to avoid variations in baseline zero) (see Fig.3.8A). For unit discharge, average discharge rate (ADR) was calculated as spikes/sec per condition, and instantaneous firing frequency (IFF) as the corresponding frequency of the primary mode of the interspike interval (ISI) histogram per condition (see Fig. 3.8B, C). Using the condition during which a unit discharged maximally, each unit was classified according to several characteristics of its discharge. First, it was classified according to the IFF as “fast” (>14.5 Hz), “slow” (0.25 - 14 Hz), “very slow” (< 0.25 Hz) or “silent” (0 Hz). Second, each unit was classified as “tonic” or “phasic” by comparing the IFF to the ADR, or specifically the corresponding interval of the ADR to the ISI distribution. If the ADR interval fell within 95% of the ISI distribution, the unit was classified as “tonic”, if outside 95%, as “phasic”. Among the tonically firing units, their discharge was further distinguished as “tonic regular” if their ADR interval fell within 82% of the ISI distribution or as “tonic irregular” if outside 82%. Among phasically firing units, their discharge was further distinguished according to the IFF as comprised by high-frequency spike bursts, as >80 Hz, or lower frequency spike clusters, as <80 Hz. Finally, cells were classified according to their response to Somatic Stimulation as “On”, if their ADR increased, “Off” if it decreased or “No” if it showed no change (< 1 Hz).

The discharge of units was further examined by autocorrelation histogram (ACH) to determine if it was rhythmic or not and by spike triggered averaging (STA) with EEG activity to determine if it was cross-correlated with cortical activity (see Fig. 3.9).

All analyses of raw data were done using Matlab R2007a (MathWorks, Natick, MA) and statistical analysis using Systat 11 (SPSS, Chicago, IL). Comparisons were made across cell types using Chi square, ANOVA with Bonferroni adjustment for post-hoc paired comparisons, student t tests and Kruskal-Wallis, Mann-Whitney or Wilcoxon non-parametric tests (for variables which contained zeros or were irregularly distributed). Figures were made using Adobe Photoshop CS (Adobe Systems, San Jose, CA) for photomicrographs and Adobe Illustrator Creative Suite (CS2, Adobe Systems) for electrophysiological data.

2.2 The activity profiles of LDT/PPT neurons during natural sleep-wake states

2.2.1 Surgery and habituation to head-fixation

All experiments were performed on 40 adult male Long-Evans rats (200-250 g, Charles River, St. Constant, Quebec, Canada). All procedures were approved by the McGill University Animal Care Committee and the Canadian Council on Animal Care. The Animals were hosted under a 12:12 hour light-dark schedule with lights on from 7:00 am to 7:00 pm and they had free access to food and water. The surgery was performed under deep anesthesia (ketamine, xylazine and acepromazine: 65/5/1 mg/kg in a cocktail of 2 ml/kg initial dose and 1 ml/kg booster if needed, i.p.). Anesthesia levels were assessed throughout the procedure by testing the reaction of the tail or hind limbs to

pinching. Using a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA), the rats were first implanted with EEG (epi-dural stainless screws over olfactory bulb (OB), anterior medial prefrontal (PF) and retrosplenial (RS) cortices) and two EMG (Teflon-coated silver wire loops in neck muscles) electrodes. Then, a metal U-shaped frame was attached to the head skull with acrylic dental cement, leaving a space accessible to the skull over the retrosplenial region and Lambda, which was sealed temporarily with sterile gauze. Eventually, the U-shaped frame was screwed to a carriage adapter that in turn was fixed to the main stereotaxic frame. These settings restrained the animal's head with minimum discomfort since neither ear nor tooth bars are required. Rats were allowed to recover from surgery (~2 days) and were gradually introduced to the head fixation in the carriage adapter while lying within a small Plexiglas box, which prevented twisting but not moving their bodies and limbs. Animals were habituated to head fixation by repetitive sessions of increasing time duration, until they were able to sleep and be awake comfortably for up to 6 hours. The adaptation period takes 7 to 10 days or even more in some rats.

2.2.2 Unit recording and labeling

One day previous to the experiment, rats were anesthetized again (as described above) and a craniotomy was drilled over the retrosplenial region to allow the electrode to reach the pontomesencephalic tegmentum via a rostrocaudally angled orientation. This rostrocaudally angled orientation of the electrode was made to avoid interference with the tentorium or the cerebellum. The skull was cleaned and covered with sterile gauze.

The day of the experiment, the dura mater was cut following application of one drop of lidocaine, and a glass micropipette ($\sim 1\ \mu\text{m}$ tip and $\sim 40\ \text{M}\Omega$) filled with $\sim 5\%$ Neurobiotin (Nb, Vector Laboratories, Burlingame, CA) in $0.5\ \text{M}$ NaCl solution was lowered with an angle of 30° to reach the pontomesencephalic tegmentum region by using a David Kopf micropositioner (David Kopf Instruments). Single units were recorded and labeled using an intracellular amplifier (Neurodata IR-283A, Cygnus Technology, Inc., Delaware Water Gap, PA). The unit signal was amplified ($2000\times$), digitized at sampling rate of $8\ \text{kHz}$ and filtered (bandpass-filters: $0.3 - 3\ \text{kHz}$) using a CyberAmp (380, Axon Instruments, Union City, CA) and acquired for online viewing with the Axoscope software (v10.1, Axon Instruments). The unit was simultaneously recorded with EEG (digitized at sampling rate of $250\ \text{Hz}$, amplified $5000\times$ and filtered $0.5-60\ \text{Hz}$), EMG (digitized at sampling rate of $250\ \text{Hz}$, amplified $5000\times$ and filtered $10-100\ \text{Hz}$) signals and video recordings of the animal behavior by using Harmonie software (v5.2, Stellate Co, Montreal, Canada). A single unit was isolated and recorded for a minimum of 5 minutes and during at least one full sleep-wake cycle including one episode of each of the three major states, active wake (aW), slow wave sleep (SWS) and paradoxical sleep (PS). After the recording of the isolated unit, labeling was started by applying the juxtacellular technique (Pinault, 1996; Manns et al., 2000b). In short, positive currents ($\sim 10\ \text{nA}$) were applied in $200\ \text{ms}$ pulses in order to modulate the activity of the cell, a procedure needed for the marker (Nb) to be taken by the cell.

2.2.3 Histochemistry

After recording and labelling of one unit on each side of the brain, the animals were perfused under anesthesia (Euthanyl, $\sim 100\ \text{ml/kg}$, i.p.) with $\sim 400\ \text{ml}$ of 3%

paraformaldehyde fixative solution. The brains were removed and immersed in a 30% sucrose solution for 2 to 3 days or until they sank, then they were frozen at -50° C and stored at -80° C. The brains were cut in a freezing microtome and adjacent sections (25 μ m) were collected. Sections containing the pontomesencephalic tegmentum region were washed for 30 min, incubated for ~3 hours in a Cy2-conjugated streptavidin solution (SA-Cy2, 1:1000, Jackson Immunoresearch Laboratories) and mounted in non-coated sections under glycerol 70%. Visualization and localization of the Nb-labeled neuron was done under a Leica DMLB microscope with epi-fluorescence. Subsequently, the section containing the neuron was re-incubated and processed immunohistochemically for vesicular transporter protein for acetylcholine (VACHT, with a goat, Gt anti-VACHT, Chemicon, and Cy5-conjugated Donkey, Dky-anti-Gt) and glutamic acid decarboxylase (GAD with a mouse, Ms anti-GAD67, Chemicon and Cy3-conjugated Dky-anti-Ms). The location of Nb-labeled cells was determined by epi-fluorescence using a Leica DMLB microscope and photographed using a Nikon Eclipse E800 (Nikon Instruments Inc., Melville, NY, USA) equipped with a digital camera (Optronics, Microfire S99808). The labeled cells were mapped onto a computer resident atlas using Neurolucida (v9, MicroBrightField, Williston, VT). Cell size was measured by the long axis of the cell and cells classified as small (≤ 15 μ m) or medium-to-large (16 – 35 μ m). The Nb-labeled cells (Nb+) were judged unequivocally positively (by bright fluorescence in soma, “+”) immunostained or negatively (by no fluorescence in soma, “-”) immunostained for VACHT and GAD and were located within the confines of the LDT, SubLDT or adjacent MPPT, so as to be included and reported in the Results.

2.2.4 Data analysis

Units were considered for analysis only if they recorded for 5 minutes periods or longer and showed at least one episode of aW, SWS and PS. Manual scoring of different sleep-wake states was done by analyzing EEG, EMG and video recordings in 10 s epochs. As defined previously (Maloney et al., 1997; Lee et al., 2004) , six different states were scored as: active wake (aW), quiet wake (qW), transition to slow wave sleep (tSWS), slow wave sleep (SWS), transition to paradoxical sleep (tPS) or paradoxical sleep (PS). aW was identified by the presence of high frequency gamma and theta (4.5-8 Hz) activity in the EEG, a high and phasic EMG amplitude and limited movement of the animal's body; qW was identified by the presence of low voltage and fast cortical EEG activity as well a relatively low EMG; tSWS was identified by the presence of a slower and medium voltage of cortical EEG activity with the presence of occasional spindling activity (12-14 Hz) along with a low EMG; SWS was identified by a high voltage slow delta EEG activity (0.5-4 Hz) together with a low EMG; tPS was identified by the presence of a continuous spindling in EEG activity slowing down to ~10 Hz and a low EMG; finally, PS was identified by the presence of a prominent theta EEG activity together with fast and low voltage cortical activity, a minimal EMG amplitude and phasic activity of the animal's whiskers.

Electrophysiological records of EEG and EMG were scored (using Matlab program) by 10 sec epochs to determine the sleep-wake states (hypnogram). In the meantime, the unit activity was analyzed to determine various parameters. The average discharge rate (ADR) was calculated as spikes/sec per state and the instantaneous firing frequency (IFF) as the corresponding frequency of the primary mode of the interspike interval histogram per state (ISIH). The rhythmicity of discharge and its frequency was calculated by using

using the autocorrelation histogram (ACH) and cross-correlated EEG activity by using the spike triggered average (STA). To be correlated with unit spike rate, Gamma (30 – 58 Hz), delta (1 – 4.5 Hz) and theta activity (4.5 – 8 Hz) (measured as the ratio of theta/delta powers), and EMG amplitude (30 – 100 Hz) were measured per epoch. Units were further classified into sleep-wake sub-groups according to whether their discharge rate varied significantly across states ($p < 0.05$ by ANOVA) and if so, according to the state in which their maximal discharge rate occurred ($p < 0.05$ by post-hoc paired comparisons) (Lee et al., 2004). Each unit was further distinguished according to its firing properties during the maximally active state. *First*, the unit was classified according to the IFF as “fast” (>14.5 Hz) or slow firing (<14.5 Hz). *Second*, each unit was classified as “tonic” or “phasic” by comparing the IFF to the ADR, or specifically the corresponding interval of the ADR to the ISI distribution. If the ADR interval fell within 95% of the ISI distribution, the unit was classified as “tonic”, if outside 95%, as “phasic”. Among the tonically firing units, their discharge was further distinguished as “tonic regular” if their ADR interval fell within 82% of the ISI distribution or as “tonic irregular” if outside 82%. Among phasically firing units, their discharge was further distinguished according to the IFF as comprised by high-frequency spike bursts, as >80 Hz, or lower frequency spike clusters, as <80 Hz. For spike duration measurement, to avoid variations in zero on the return to baseline following the negative deflection, the measurement of the average spike duration was based upon second zero crossing but calculated with an offset from zero corresponding to 10% of the positive peak amplitude.

All analyzes of raw data were done using Matlab 5.3 (MathWorks, Natick, MA) and statistical analysis using Systat 12.0 (SPSS, Chicago, IL). Figures were made using

Adobe Photoshop CS (Adobe Systems, San Jose, CA) for photomicrographs and Adobe Illustrator CS (Adobe Systems, San Jose, CA) for plotting electrophysiological data.

3. Chapter Three

The activity profiles of LDT/PPT neurons in anesthetized rats

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3.1 Preface

In this study we applied the technique of juxtacellular labeling of recorded neurons for subsequent immunohistochemical identification of cholinergic, GABAergic and putative glutamatergic neurons in the LDT, SubLDT and medial PPT (MPPT). As previously employed for identification of the cholinergic and other neurons in the basal forebrain (Manns et al., 2000a, b; Manns et al., 2003), we labeled recorded cells with Neurobiotin (Nb) following their characterization in relation to cortical activity in urethane anesthetized rats. As possible with this anesthesia, somatic stimulation was used to evoke enhanced fast, beta-gamma along with rhythmic slow, theta-like activity, which resembles cortical activation of natural waking (W) and paradoxical sleep (PS), from a baseline of irregular slow activity, which resembles slow wave activity of natural slow wave sleep (SWS) (Maloney et al., 1997; Clement et al., 2008). Similar to comparable populations of cells in the basal forebrain, the cell groups were found to be heterogeneous in their properties and their response to stimulation with associated cortical activation.

3.2 Results

Of 149 cells, which were recorded, characterized and submitted to juxtacellular labeling, 73 were successfully labeled with Nb, could be judged unequivocally immunopositive or negative for VACHT and GAD and were located in the region of the pontomesencephalic cholinergic cell area, centered upon the LDT. The Nb-labeled cells were distributed through the caudal to rostral LDT ($n = 28$) or its ventral extension beneath the central gray in the SubLDT ($n = 25$) or in the MPPT ($n = 20$) (Fig. 3.1). Of the 73 Nb-labeled cells, 11 were established as immunopositive for VACHT (Nb+/VACHT+), 29 as immunopositive for GAD (Nb+/GAD+) and 33 as immunonegative for both VACHT and GAD (Nb+/VACHT-/GAD-) (Table 3.1). The Nb-labeled cholinergic, GABAergic and noncholinergic/nonGABAergic cells were co-distributed through the LDT, subLDT and MPPT (Fig. 3.1 and Table 3.1). Morphologically, the Nb+/VACHT+ cells were in the vast majority polygonal and multipolar, whereas the Nb+/GAD+ and the Nb+/VACHT-/GAD- cells were in the majority round, oval or fusiform, accordingly bipolar and only in the minority polygonal and multipolar, thus differing significantly according to shape (Table 3.1). All the Nb-labeled cholinergic cells were medium-to-large cells, whereas the GABAergic and noncholinergic/nonGABAergic cells were in large number small in addition to medium-to-large, thus differing significantly according to size (Table 3.1). This difference was reflected by a significant difference in the average (long axis) cell size among the cell groups, with the cholinergic cells being significantly larger on average than the GABAergic and noncholinergic/nonGABAergic cells (Table 3.2).

Physiologically, the Nb-labeled cells were heterogeneous in their discharge properties. They responded differentially to somatic stimulation and the evoked changes in EEG activity, which were characterized by a decrease in slow irregular, delta-like activity (0.5 – 4 Hz) and an increase in fast, high beta–gamma activity (20 - 60 Hz), sometimes riding upon rhythmic slow, theta-like activity (Fig. 3.7) (Table 1). The cholinergic cells had on average longer duration spikes than the GABAergic and noncholinergic/nonGABAergic cells (Fig. 3.8A) (Table 2). However, given large variability they could not be distinguished on an individual basis by this feature (Fig. 3.10A). According to their instantaneous firing frequency (calculated from the modal value of the interspike interval) during somatic stimulation, the majority of all cells were slow firing (0.25 to 14.5 Hz), irrespective of cell type (Fig. 3.8C) (Table 1). The cell groups could thus not be clearly distinguished according to average discharge rate or instantaneous firing frequency during stimulation (Fig. 3.10B, C). Nonetheless, the GABAergic and noncholinergic/nonGABAergic cells were more heterogeneous than the cholinergic cells and included a significant number of fast firing (>14.5 Hz) neurons along with very slow firing (<0.25 Hz) and silent neurons in the stimulation condition. According to their firing pattern (assessed from the interspike interval histograms) during stimulation, the vast majority of all cells were tonic and regular or irregular, irrespective of cell type (Table 3.1). In contrast to cholinergic cells, GABAergic and noncholinergic/nonGABAergic cells also included neurons which discharged in a phasic manner, characterized by clusters of spikes (<80 Hz instantaneous firing frequency within the cluster). No cells discharged in high frequency bursts (>80 Hz instantaneous firing frequency). No cholinergic and few of the noncholinergic cells showed rhythmic firing that was cross-correlated with the EEG activity (n = 3) in either pre-stimulation or

stimulation conditions (Fig. 3.9). Classified according to their response to somatic stimulation as “On” if they increased, “Off” if they decreased and “No” if they showed no change in their rate of discharge, the three cell groups differed significantly (Table 3.1). Whereas all cholinergic cells were “On”, the GABAergic and noncholinergic/nonGABAergic cells comprised “On”, “Off” and “No” cells (Table 3.1).

Given the heterogeneity of the noncholinergic cells, their further study was performed according to the functional subgroups of “On”, “Off” or “No”. The “On” and “Off” Nb+/GAD+ and Nb+/VACHT-/GAD- cell groups were further examined in detail and compared to the “On” Nb+/VACHT+ cells (Table 3.3). As will be presented below for each of these cell types, despite differing degrees of variation in discharge properties, the GABAergic and noncholinergic/nonGABAergic “On” cells behaved in a similar manner to the cholinergic cells. Moreover, the overlap in electrophysiological properties of the three “On” cell groups was such that a single cell could not be distinguished as cholinergic, GABAergic or noncholinergic/nonGABAergic according to those properties (Fig. 3.11). “Off” cells behaved in an opposite manner to the cholinergic and other “On” cells in their response to stimulation and relationship to EEG activity. As for the “On” cells, the overlap in their electrophysiological properties did not allow any single cell to be distinguished as GABAergic or nonGABAergic according to those properties (Fig. 3.12).

3.2.1 Cholinergic (Nb+/VACHT+) neurons

Nb-labeled cholinergic cells (n = 11) represented a relatively homogeneous group according to both morphological and physiological characteristics (Table 3.1). They were commonly polygonal, multipolar and medium-to-large in size (mean $\pm$ SEM: 24.32 $\pm$

2.50 μm in long diameter) (Table 3.2, Fig. 3.2A). They had on average a relatively long spike duration (see Fig. 3.8A and Fig. 3.2C) (0.55 ± 0.04 ms mean duration to first zero crossing and 1.71 ± 0.17 ms duration to second zero crossing) (Table 3.2), yet comprised cells with short as well as long duration spikes (Fig. 3.10A). In absence of stimulation, they fired minimally in association with the irregular slow EEG activity during the pre-stimulation period (Fig. 3.2B, C). They greatly increased their firing during somatic stimulation and the associated increase in fast cortical activity. Following cessation of the stimulation, the cholinergic cells slowed and often subsequently ceased firing (Fig. 3.2B, C). Across cells, the average discharge rate increased significantly during stimulation (3.99 ± 1.09 Hz) as compared to the pre-stimulation period (0.48 ± 0.29 Hz) (Table 3.3) and the post-stimulation period (0.25 ± 0.15 Hz; $p < 0.05$). During stimulation, the cholinergic cell discharge was characterized by a regular (Fig. 3.2C) or irregular tonic firing pattern typified by a slow instantaneous firing frequency (mean 3.19 ± 0.68 Hz) (Table 3.3) that was similar to the average discharge rate (Fig. 3.8B, C). Neither during stimulation or pre-stimulation was the discharge of the cells rhythmic or cross-correlated with slow EEG activity (Fig. 3.9). At the beginning of somatic stimulation, the majority of cholinergic cells ($n = 7$) increased their firing before the change in EEG activity (by ~ 180 ms for the cell shown in Fig. 3.2, up to ~ 1.6 sec across cells).

3.2.2 GABAergic (Nb+/GAD+) neurons

Based on their response to somatic stimulation, the Nb-labeled GABAergic neurons formed a heterogeneous group, the majority showing an increase, as “On” cells (~50%), some a decrease, as “Off” cells (~34%), and a small minority no change, as “No” cells (~16%) (Table 3.1). These functionally different cell types were co-distributed through the LDT, SubLDT and MPPT. They did not differ significantly according to cell size or spike duration (data not shown).

3.2.2.1 GABAergic “On” neurons

The GABAergic “On” cells ($n = 14$) were not homogeneous in their morphological or physiological properties. As for the total Nb+/GAD+ cell group (Table 3.1), the “On” subgroup varied in size and shape, being either small ($n = 7$) (Fig. 3.3A) or medium-large ($n = 7$) and either bipolar ($n = 8$) (Fig. 3.3A) or multipolar ($n = 6$). Similar to all GABAergic cells (Table 3.2), the “On” cells had a relatively short spike duration (Fig. 3.3C) (0.42 ± 0.02 ms duration to first zero crossing and 1.18 ± 0.08 ms duration to second zero crossing), yet also comprised cells with longer duration spikes (Fig. 3.11A). These GABAergic neurons discharged at low rates during spontaneous irregular slow EEG activity (pre-stimulation) and markedly increased their firing in response to somatic stimulation and the associated cortical activation (Fig. 3.3B, C). Across the GABAergic “On” cells, the average discharge rate was significantly higher during stimulation (7.31 ± 1.43 Hz) as compared to pre-stimulation (3.56 ± 1.17 Hz) (Table 3.3) and post-stimulation (3.49 ± 1.35 Hz, $p < 0.05$). During stimulation, they commonly discharged in a tonic regular to irregular manner at low to fast instantaneous firing frequencies (6.39 ± 1.34 Hz) (Table 3.3). Some GABAergic “On” cells exhibited a phasic, cluster firing

pattern during stimulation ($n = 2$, data not shown), however during neither stimulation nor pre-stimulation conditions was the unit discharge rhythmic or cross-correlated with slow EEG activity (data not shown). Among the GABAergic “On” cells which changed their activity at a discernibly different time than the EEG in response to somatic stimulation ($n = 9$), most ($n = 7$) increased their firing after the EEG activation (by ~ 1 to 5 sec) (Fig. 3.3B, C).

3.2.2.2 GABAergic “Off” neurons

The GABAergic “Off” cells ($n = 11$) were also heterogeneous in their morphological and physiological characteristics. They could be either small (Fig. 3.4A) ($n = 5$) or medium-large ($n = 6$) in size, but were predominantly bipolar, fusiform-oval-round (Fig. 3.4A) ($n = 9$) in shape. Like all GABAergic cells (Table 3.2), the “Off” cells had on average a relatively short spike duration (Fig. 3.4C) (0.41 ± 0.31 ms duration to first zero crossing and 1.25 ± 0.12 ms to second zero crossing), yet also comprised cells with longer duration spikes (Fig. 3.12A). The “Off” cells discharged at slow rates during spontaneous irregular slow EEG activity (pre-stimulation) and ceased or markedly decreased their firing in response to somatic stimulation and the associated cortical activation (Fig. 3.4B, C). They resumed discharge at rates similar to pre-stimulation following cessation of the stimulation. Across the identified GABAergic “Off” cells, the average discharge rate decreased significantly during stimulation (2.14 ± 1.18 Hz) as compared to the pre-stimulation period (4.42 ± 1.76 Hz) (Table 3.3) and post-stimulation period (4.50 ± 1.76 Hz, $p < 0.05$). During the pre-stimulation condition in association with irregular slow wave EEG activity, they commonly exhibited slow irregular tonic firing (Fig. 3.4C) (with average instantaneous firing frequency of 3.91 ± 1.59 Hz). Some

cells displayed phasic firing in clusters of spikes ($n = 4$) (data not shown), and one showed rhythmic firing which was cross-correlated with rhythmic slow theta-like activity during stimulation (data not shown). Among the GABAergic “Off” cells which changed their activity at a discernibly different time than the EEG in response to somatic stimulation ($n = 7$), most ($n = 5$) decreased their firing following the EEG activation (with a delay of ~ 1 to 8 sec) (Fig. 3.4B, C).

3.2.3 Non-cholinergic/non-GABAergic (Nb+/VACHT-/GAD-) neurons

Nb-labeled noncholinergic/nonGABAergic cells ($n = 33$) were heterogeneous in their properties and comprised “On” ($\sim 44\%$), “Off” ($\sim 42\%$) and “No” cells ($\sim 14\%$) (Table 1), which were co-distributed through the LDT, SubLDT and MPPT. These cell subgroups did not differ significantly according to cell size or spike duration (data not shown).

3.2.3.1 Non-cholinergic/non-GABAergic “On” neurons

Most noncholinergic/nonGABAergic “On” cells were medium-large (Fig. 3.5A, $n = 10$) and the remaining small ($n = 4$). For the shape, the majority was bipolar (Fig. 3.5A) ($n = 11$) and the remaining multipolar ($n = 3$). They had on average a relatively short spike duration (Fig. 3.5C) (0.42 ± 0.02 ms to first zero crossing and 1.20 ± 0.11 ms to second zero crossing), yet comprised cells with longer duration spikes (Fig. 3.11A). These neurons discharged at low rates during spontaneous irregular slow EEG activity (pre-stimulation) and markedly increased their rate in response to somatic stimulation and the associated cortical activation (Fig. 3.5B, C). They decreased and ceased firing during the post-stimulation period. Across cells, the average discharge rate increased

significantly during stimulation (6.42 ± 1.88 Hz) as compared to the pre-stimulation period (1.88 ± 0.79 Hz) (Table 3.3) and post-stimulation period (3.01 ± 1.51 Hz, $p < 0.05$). They generally exhibited a tonic firing pattern, either regular (Fig. 3.5C) or irregular (5.24 ± 1.78 Hz, average instantaneous firing frequency). Some cells exhibited a phasic cluster firing pattern during pre-stimulation or stimulation periods ($n = 3$) (data not shown), of which one was rhythmic and showed cross-correlated discharge with rhythmic slow theta-like activity during stimulation (data not shown). Of the VAcHT-/GAD- “On” cells which changed their discharge at a discernibly different time than the EEG following somatic stimulation ($n = 11$), most ($n = 8$) started to fire before EEG activation (from ~ 100 ms to 1.5 sec) (Fig. 3.5B, C).

3.2.3.2 Non-cholinergic/non-GABAergic “Off” neurons

The VAcHT-/GAD- “Off” cells ($n = 12$) varied in size. Like all such cells (Table 3.1), the “Off” cells could be either medium-large (Fig. 3.6A) ($n = 7$) or small ($n = 4$). For the shape, the majority was multipolar ($n = 8$) and the remaining bipolar (Fig. 3.6A) ($n = 3$). Like all VAcHT-/GAD- (Table 3.2), the “Off” cells had on average a medium spike duration (Fig. 3.6C) (0.44 ± 0.03 ms to first zero crossing and 1.25 ± 0.11 ms to second zero crossing) and comprised cells with short, medium or long duration spikes (Fig. 3.12A). These “Off” neurons discharged at slow rates during spontaneous irregular slow EEG activity (pre-stimulation) and ceased or significantly decreased their firing in response to somatic stimulation and the associated cortical activation (Fig. 3.6B, C). After cessation of the stimulation, they increased their firing to pre-stimulation discharge rate. The average discharge rate decreased significantly during stimulation (1.98 ± 0.86 Hz) as compared to the pre-stimulation period (4.96 ± 1.77 Hz) (Table 3.3) and post-

stimulation period (4.77 ± 1.64 Hz, $p < 0.05$). During the pre-stimulation condition in association with irregular slow wave EEG activity, they commonly exhibited slow irregular tonic firing (Fig. 3.6C) (with average instantaneous firing frequency of 3.69 ± 1.36 Hz). Some cells exhibited a phasic cluster firing pattern ($n = 3$) (data not shown), and one showed rhythmic activity during stimulation, which was cross-correlated with EEG activity (data not shown). Although only for a minority of VACHT-/GAD- “Off” cells could their decrease in firing be clearly assessed in relation to the EEG following somatic stimulation ($n = 5$), most of these ($n = 4$) decreased their firing prior to the change in EEG activity (by ~ 130 ms to 4.3 sec) (data not shown).

3.3 Tables and Figures

Table 3.1. Frequency of cholinergic, GABAergic and noncholinergic/nonGABAergic cells with different anatomical and physiological characteristics¹

<i>n</i>	All cells	Nb+/VAcHT+	Nb+/GAD+	Nb+/VAcHT-/GAD-	Statistic :
	73	11	29	33	χ^2 (df)
Anatomy					
Area					7.58 (4)
LDT	28	7	7	14	
SubLDT	25	3	10	12	
MPPT	20	1	12	7	
Shape ²					6.76 (2)*
Round-oval-fusiform	36	2	18	16	
Polygonal	33	9	10	14	
Size ²					7.03 (2)*
Small ($\leq 15\mu\text{m}$)	24	0	12	12	
Medium-large ($> 15\mu\text{m}$)	45	11	16	18	
Physiology					
Frequency (Stimulation) ³					5.35 (6)
Fast (> 14.5 Hz)	12	1	6	5	
Slow (< 14.5 Hz)	50	10	19	21	
Very slow (< 0.25 Hz)	6	0	3	3	
Silent (0 Hz)	5	0	1	4	
Firing type (Stimulation) ⁴					3.27 (4)
Tonic Regular	29	6	11	12	
Tonic Irregular	22	5	9	8	
Phasic Cluster	12	0	6	6	
Phasic Burst	0	0	0	0	
Response to stimulation					
Increase ("On")	41	11	14	16	10.14 (4)*
Decrease ("Off")	23	0	11	12	
No change ("No")	9	0	4	5	

¹Frequency (n, number of cells) for the three groups are presented and compared, using the likelihood ratio χ^2 statistic; *p < 0.05; **p < 0.01; ***p < 0.001.

²Shape and size (long axis) of whole cell bodies only (n = 69).

³According to instantaneous firing frequency calculated from interspike interval modal value during stimulation.

⁴Including cells with discharge rates > 0.25 Hz (n = 63).

Table 3.2. Morphological and physiological measures of cholinergic, GABAergic and noncholinergic/non-GABAergic cell groups¹

	Nb+/VACHT ⁺ ^a	Nb+/GAD ⁺ ^b	Nb+/VACHT-/GAD ⁻ ^c	Cell group statistic:
Morphology				
Size (μM) ²	24.32 ± 2.50 (11) ^{b,c}	16.34 ± 0.87 (28) ^a	17.42 ± 1.19 (30) ^a	4.96 (2, 65)**
Physiology				
Spike width 1(msec) ³	0.55 ± 0.04 (11) ^b	0.42 ± 0.02 (29) ^a	0.45 ± 0.02 (33)	4.91 (2, 70)**
Spike width 2(msec) ⁴	1.71 ± 0.17 (11) ^{b,c}	1.22 ± 0.06 (29) ^a	1.29 ± 0.08 (33) ^a	5.26 (2, 70)**

¹Mean ± SEM values (with number of cells) are presented and compared by one-way ANOVA (*F* (df)), with main effect of cell group indicated by ** (*p* < 0.01). *Post-hoc* Bonferroni-corrected pairwise differences in means are indicated between groups by corresponding letters.

²According to long axis of cell body measured in whole cells (*n* = 69).

³Measured at base of first zero crossing.

⁴Measured at base of second zero crossing.

Table 3.3. Average discharge rate and instantaneous firing frequency during pre-stimulation and stimulation conditions of cholinergic, GABAergic and noncholinergic/nonGABAergic cell groups¹

	Nb+/VACht+ cells ^a		Nb+/GAD+ cells ^b		Nb+/VACht-/GAD- cells ^c	
Average Discharge Rate ²	Pre-Stimulation	Stimulation	Pre-Stimulation	Stimulation	Pre-Stimulation	Stimulation
"On" Subgroup [†]	0.48 ± 0.29 (11) ^b	3.99 ± 1.09 (11)**	3.56 ± 1.17 (14) ^a	7.31 ± 1.43 (14)***	1.88 ± 0.79 (15)	6.42 ± 1.88 (15)***
"Off " Subgroup			4.42 ± 1.76 (11)	2.14 ± 1.18 (11)**	4.96 ± 1.77 (12)	1.98 ± 0.86 (12)**
Instantaneous Firing frequency ³						
"On" Subgroup	1.07 ± 0.51 (9)	3.19 ± 0.68 (11)*	2.80 ± 1.06 (14)	6.39 ± 1.34 (14)**	1.47 ± 0.47 (14)	5.24 ± 1.78 (15)**
"Off " Subgroup			3.91 ± 1.59 (11)	2.99 ± 1.22 (10)	3.69 ± 1.36 (12)	1.44 ± 0.72 (6)

¹Mean ± SEM values (with number of cells) of average discharge rate and instantaneous firing frequency are presented and compared between the Pre-Stimulation and Stimulation conditions by nonparametric Wilcoxon tests for each cell subgroup (*p < 0.05; **p < 0.01; ***p < 0.001). Rates and frequencies were compared among cell groups for the Pre-Stimulation and Stimulation conditions by nonparametric Kruskal-Wallis tests. A main effect of cell group was significant only for the average discharge rate during Pre-Stimulation among the On subgroups ([†], p < 0.05). According to *post-hoc* Mann-Whitney (p < 0.017, allowing 3 comparisons), pairwise differences in means are indicated between groups by corresponding letters.

²Average discharge rate (Hz) calculated for ~40 sec periods of each condition.

³Instantaneous firing frequency (Hz) calculated from the interspike interval modal value for ~40 sec periods having >2 spikes.

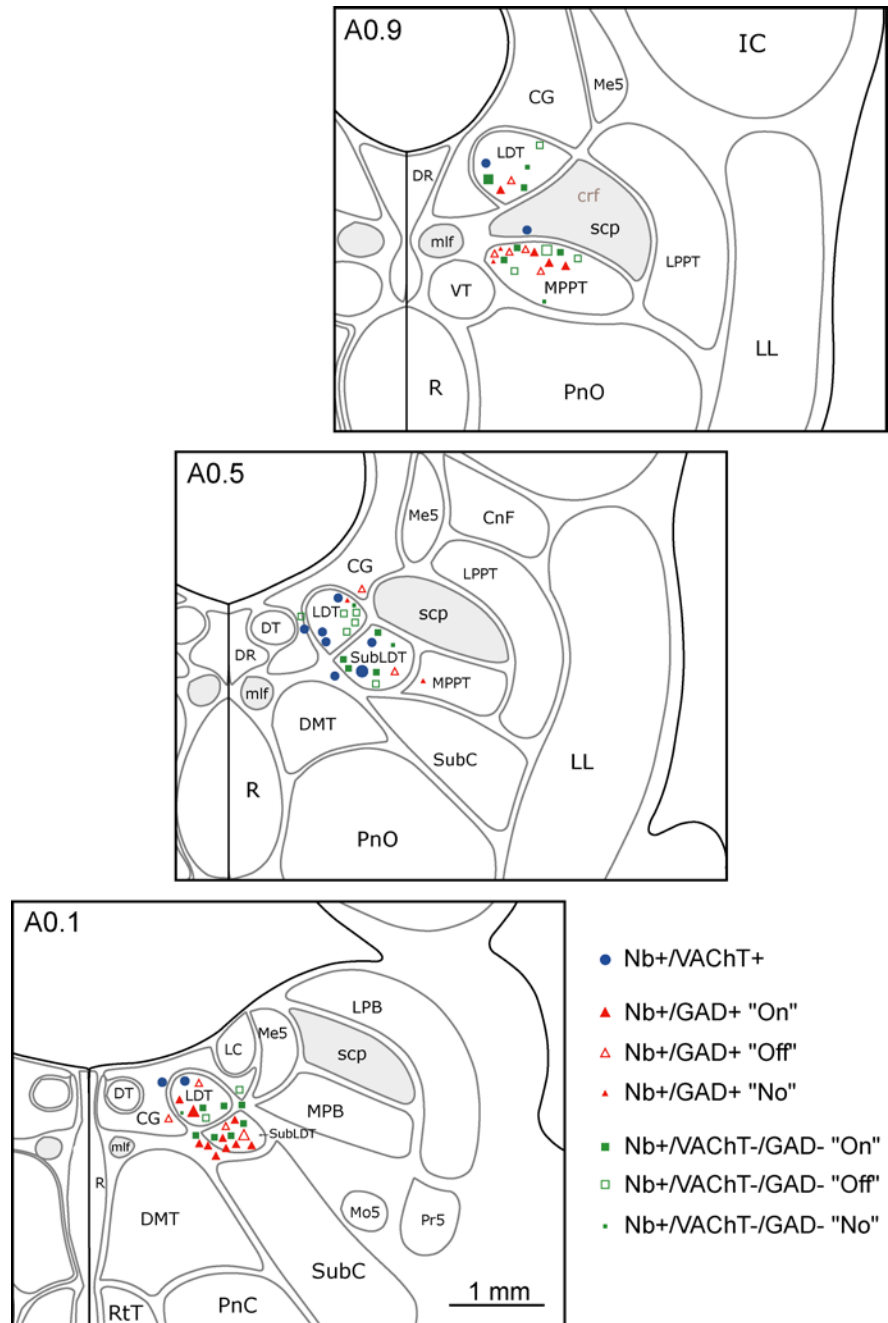
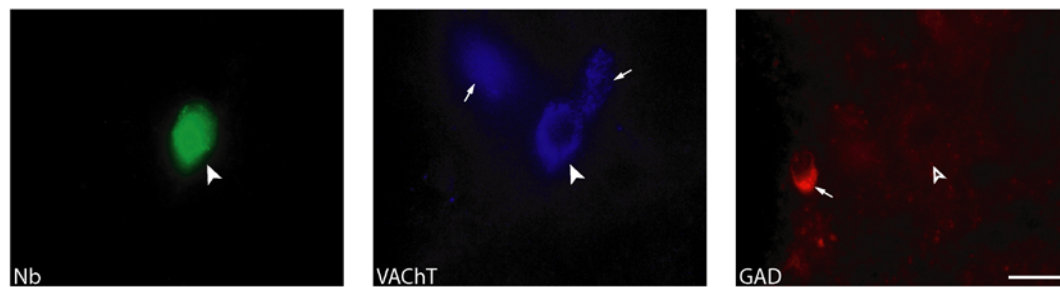


Figure 3.1. Distribution of recorded cells in the mesopontine tegmentum.

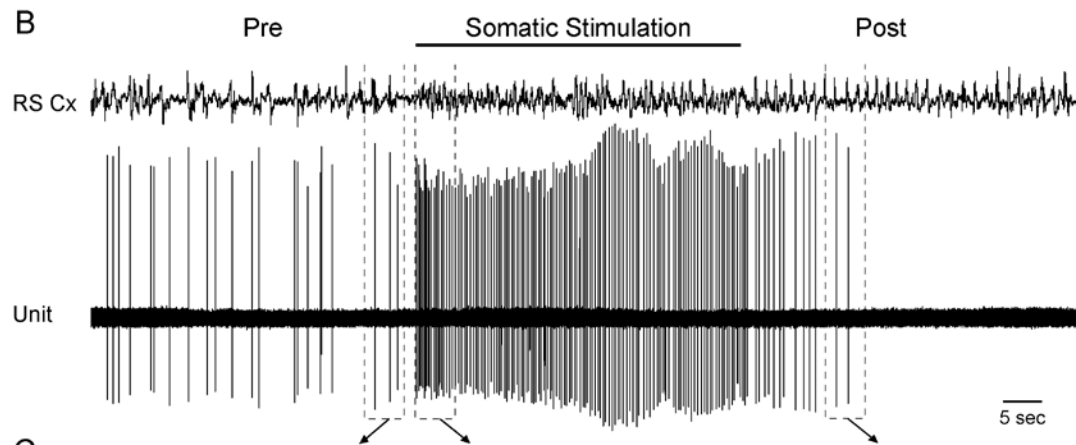
Figure 3.1. Distribution of recorded cells in the mesopontine tegmentum. The recorded, Nb-labeled cells were immunohistochemically identified as cholinergic, using VACht (Nb+/VACht+, blue circles), GABAergic, using GAD (Nb+/GAD+, red triangles) or as noncholinergic/nonGABAergic (Nb+/VACht-/GAD-, green squares). GABAergic and noncholinergic/nonGABAergic cells were further distinguished according to their response as “On” (filled symbols), “Off” (open symbols) or “No” (small filled symbols) response to somatic stimulation. Cells are mapped onto appropriate levels (Anterior, A0.9, A0.5 or A0.1 mm to interaural zero) through the LDT/SubLDT/MPPT cholinergic cell area. Recordings and images are presented for representative cells (largest symbols) of the cholinergic (Fig. 2), GABAergic “On” and “Off” (Figs. 3 and 4) and noncholinergic/nonGABAergic “On” and “Off” cells (Figs. 5 and 6). Abbreviations: CG, central grey; CnF, cuneiform nucleus; crf, central reticular fasciculus; DMT, dorsomedial tegmental area; DR, dorsal raphe nucleus; DT, dorsal tegmental nucleus; IC, inferior colliculus; LC, locus coeruleus; LDT, laterodorsal tegmental nucleus; LL, lateral lemniscus; LPB, lateral parabrachial nucleus; LPPT, lateral pedoculopontine tegmental nucleus; Me5, mesencephalic trigeminal nucleus; mlf, medial longitudinal fasciculus; Mo5, motor trigeminal nucleus; MPB, medial parabrachial nucleus; MPPT, medial pedoculopontine tegmental nucleus; PnC, pontine reticular nucleus, caudal part; PnO, pontine reticular nucleus, oral part; Pr5, principal sensory trigeminal nucleus; R, raphe nuclei; RtT, reticulotegmental nucleus of the thalamus; scp, superior cerebellar peduncle; SubC, subcoeruleus. SubLDT, sublaterodorsal tegmental nucleus; VT, ventral tegmental nucleus.

● Nb+/VACHT+ cell

A



B



C

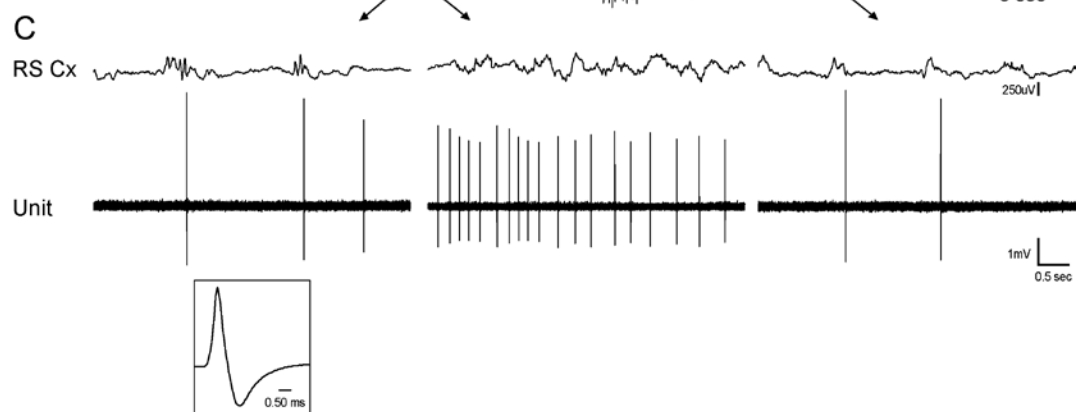


Figure 3.2. Nb+/VACHT+ cell.

Figure 3.2. Nb+/VAcHT+ cell. **A**, The Nb-labeled cell (filled arrowhead, #ABS80a) was positively immunostained for VAcHT (filled arrowhead) and negatively for GAD (open arrowhead), while near other VAcHT+ and GAD+ cells in the region (small arrows). Scale bar, 20 μ m. The cell was located in the SubLDT (largest blue circle, Fig. 3.1, A0.5). **B** and **C**, The unit discharged at an average low rate (0.70 Hz) in association with spontaneous irregular slow wave activity on the EEG of the retrosplenial cortex (RS Cx) in the period preceding stimulation (Pre). It increased its rate markedly (to 3.10 Hz) and fired tonically during somatic stimulation in association with faster activity on the EEG (See also Figs. 3.8 and 3.9). Note that the increase in unit discharge preceded the change in EEG activity (by $\sim$ 180 ms). After stimulation, the unit initially decreased then ceased firing as the EEG returned to irregular slow wave activity. Traces in B (enclosed by dashed lines) are expanded in C (arrows). The unit had a relatively long duration spike (shown in C, 0.76 and 2.55 ms at first and second zero crossings) (Fig. 3.8).

▲ Nb+/GAD+ "On" cell

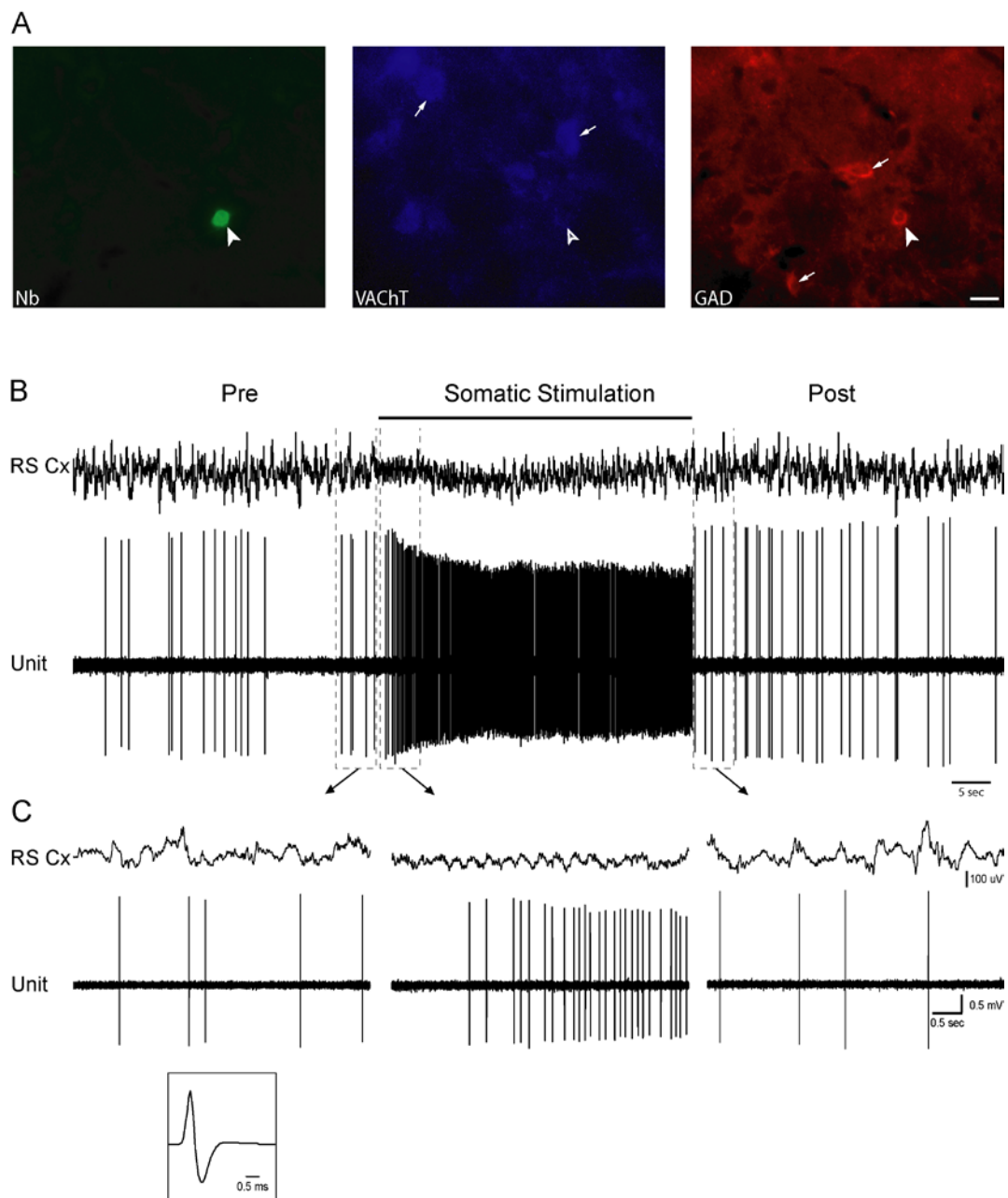


Figure 3.3. Nb+/GAD+ "On" cell.

Figure 3.3. Nb+/GAD+ “On” cell. **A**, Nb-labeled cell (filled arrowhead, #ABS78b) was negatively immunostained for VACHT (open arrowhead) and positively so for GAD (filled arrowhead), while located near other VACHT+ and GAD+ cells in the region (small arrows). Scale bar, 20 μ m. The cell was situated in the LDT (largest red filled triangle, Fig. 3.1, A0.1). **B** and **C**, The unit discharged at a low average rate (0.46 Hz) in association with irregular slow wave activity on the EEG of the retrosplenial cortex (RS Cx) prior to stimulation (Pre). It increased its firing markedly (to 11.49 Hz) and fired tonically in association with faster cortical activity riding upon rhythmic slow (theta-like) activity during somatic Stimulation. Note that the increase in unit discharge followed the change in EEG activity (by $\sim$ 1.22 sec). After stimulation, the unit decreased its rate of firing to return to pre-stimulation levels in similar association with irregular slow wave activity. Traces in B (enclosed by dashed lines) are expanded in C (arrows). The unit had a relatively short duration spike (shown in C, 0.44 and 1.21 ms at first and second zero crossings).

△ Nb+/GAD+ "Off" cell

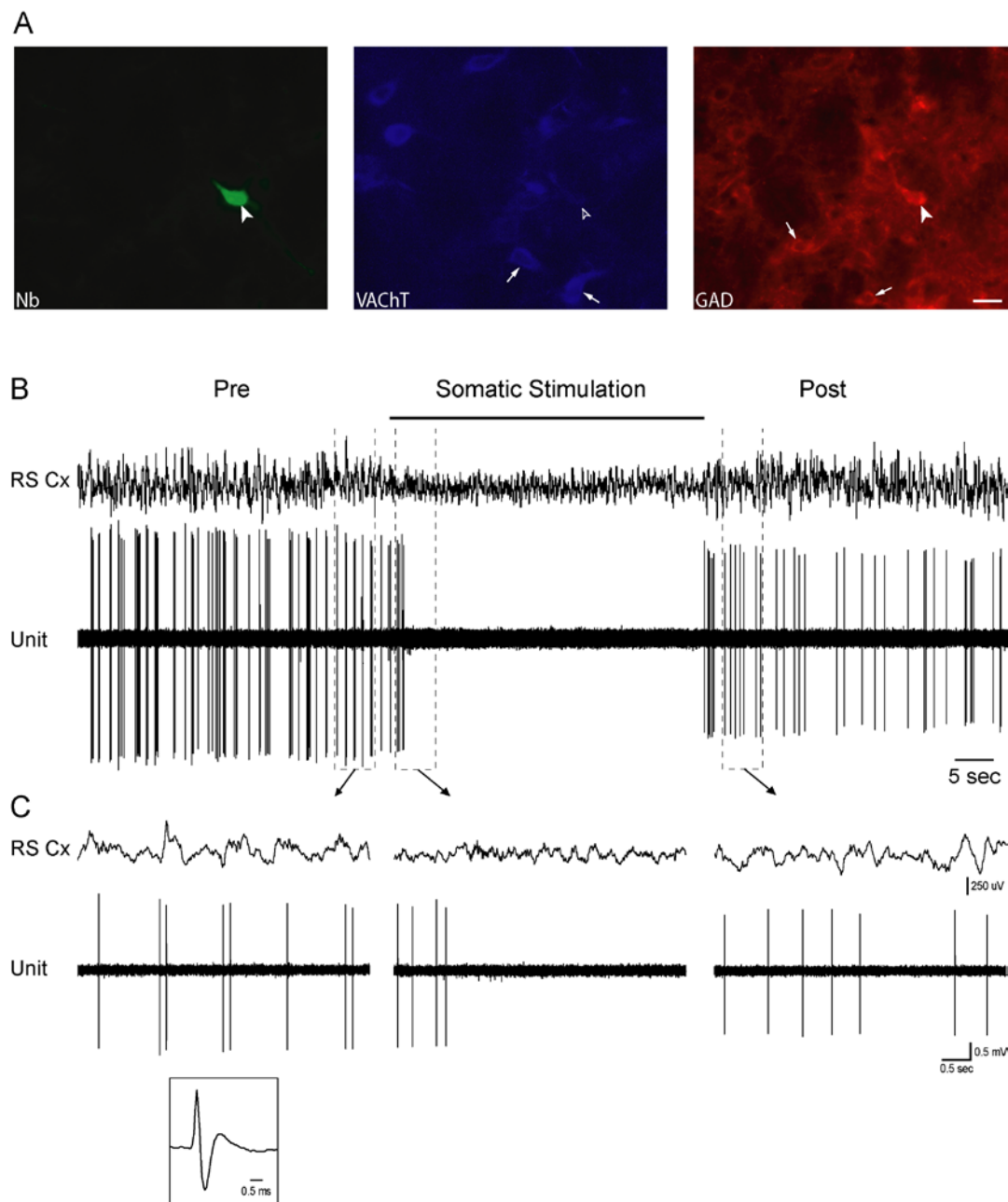


Figure 3.4. Nb+/GAD+ "Off" cell.

Figure 3.4. Nb+/GAD+ “Off” cell. **A**, Nb-labeled cell (filled arrowhead, #ABS102a) was negatively immunostained for VAcHT (open arrowhead) and positively so for GAD (filled arrowhead), while located near other VAcHT⁺ and GAD⁺ cells in the region (small arrows). Scale bar, 20 μ m. The cell was situated in the SubLDT (largest red open triangle, Fig. 3.1, A0.1). **B** and **C**, The unit discharged phasically at a moderate average rate (2.07 Hz) in association with irregular slow wave activity on the EEG of the retrosplenial cortex (RS Cx) prior to stimulation (Pre). It ceased firing in association with faster cortical activity riding upon rhythmic slow (theta-like) activity during somatic stimulation. Note that the unit ceased firing following the change in EEG activity (by ~1.31 sec). After stimulation (Veening et al.), the unit recovered its baseline rate of firing in association with irregular slow wave activity. Traces in B (enclosed by dashed lines) are expanded in C (arrows). The unit had a relatively short duration spike (shown in C, 0.35 and 0.93 ms at first and second zero crossings).

■ Nb+/VAcHT-/GAD- "On" cell

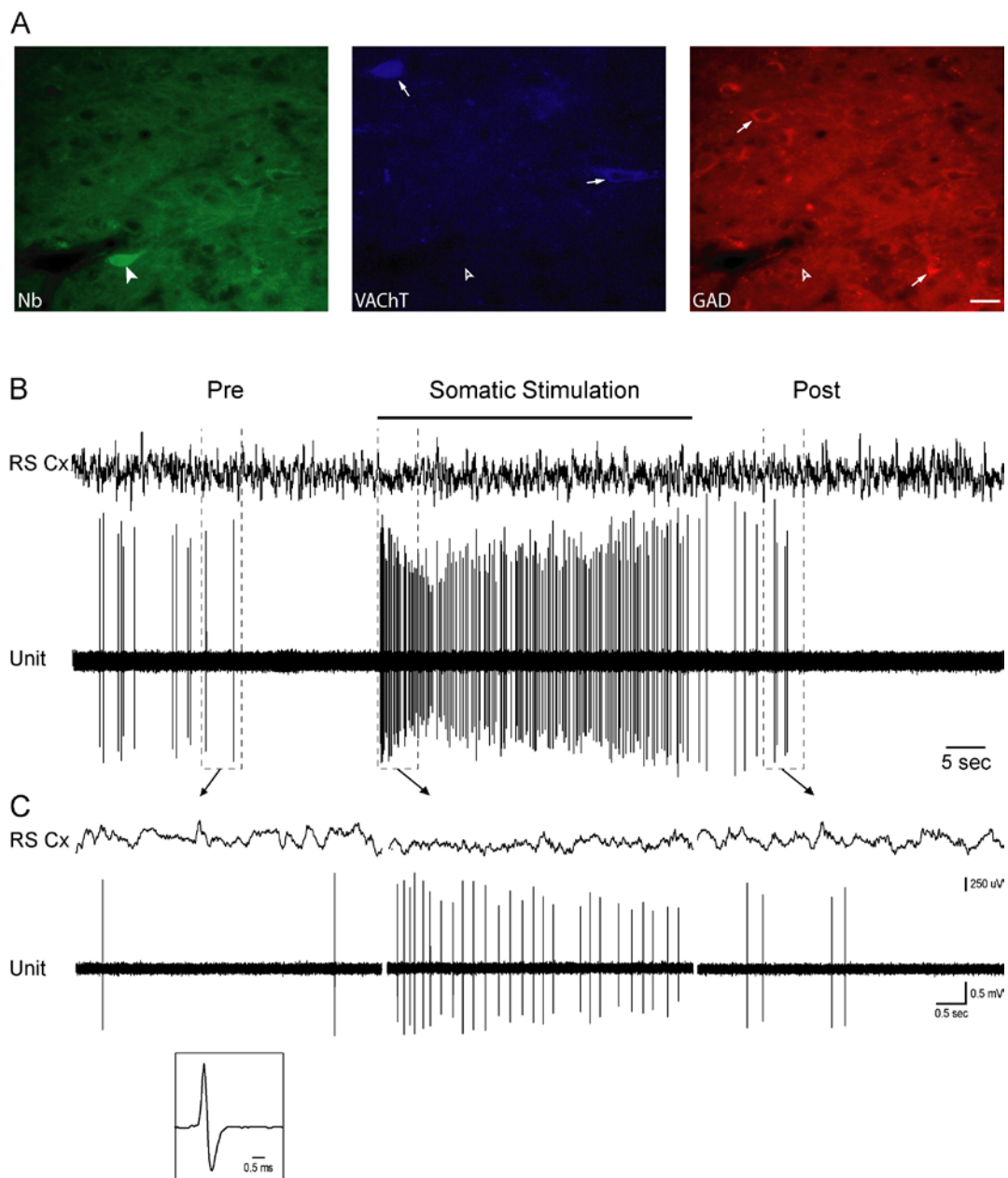


Figure 3.5. Nb+/VAcHT-/GAD- "On" cell.

Figure 3.5. Nb+/VACHT-/GAD- “On” cell. **A**, Nb-labeled cell (filled arrowhead, #ABS87c) was negatively immunostained for VACHT (open arrowhead) and for GAD (open arrowhead), while located near other VACHT+ and GAD+ cells in the region (small arrows). Scale bar, 20 μ m. The cell was situated in the LDT (largest green filled square, Fig. 3.1, A0.9). **B** and **C**, The unit discharged irregularly at a low average rate (0.385 Hz) in association with irregular slow wave activity on the EEG of the retrosplenial cortex (RS Cx) prior to stimulation (Pre). It increased its firing markedly (to 3.59 Hz) and fired tonically in association with faster cortical activity during somatic stimulation. Note that the increase in unit discharge preceded the change in EEG activity (by ~210 ms). After stimulation, the unit decreased its rate back to baseline levels in association with irregular slow wave activity. Traces in B (enclosed by dashed lines) are expanded in C (arrows). The unit had a relatively short duration spike (shown in C, 0.36 and 0.98 ms at first and second zero crossings).

□ Nb+/VAcHT-/GAD- "Off" cell

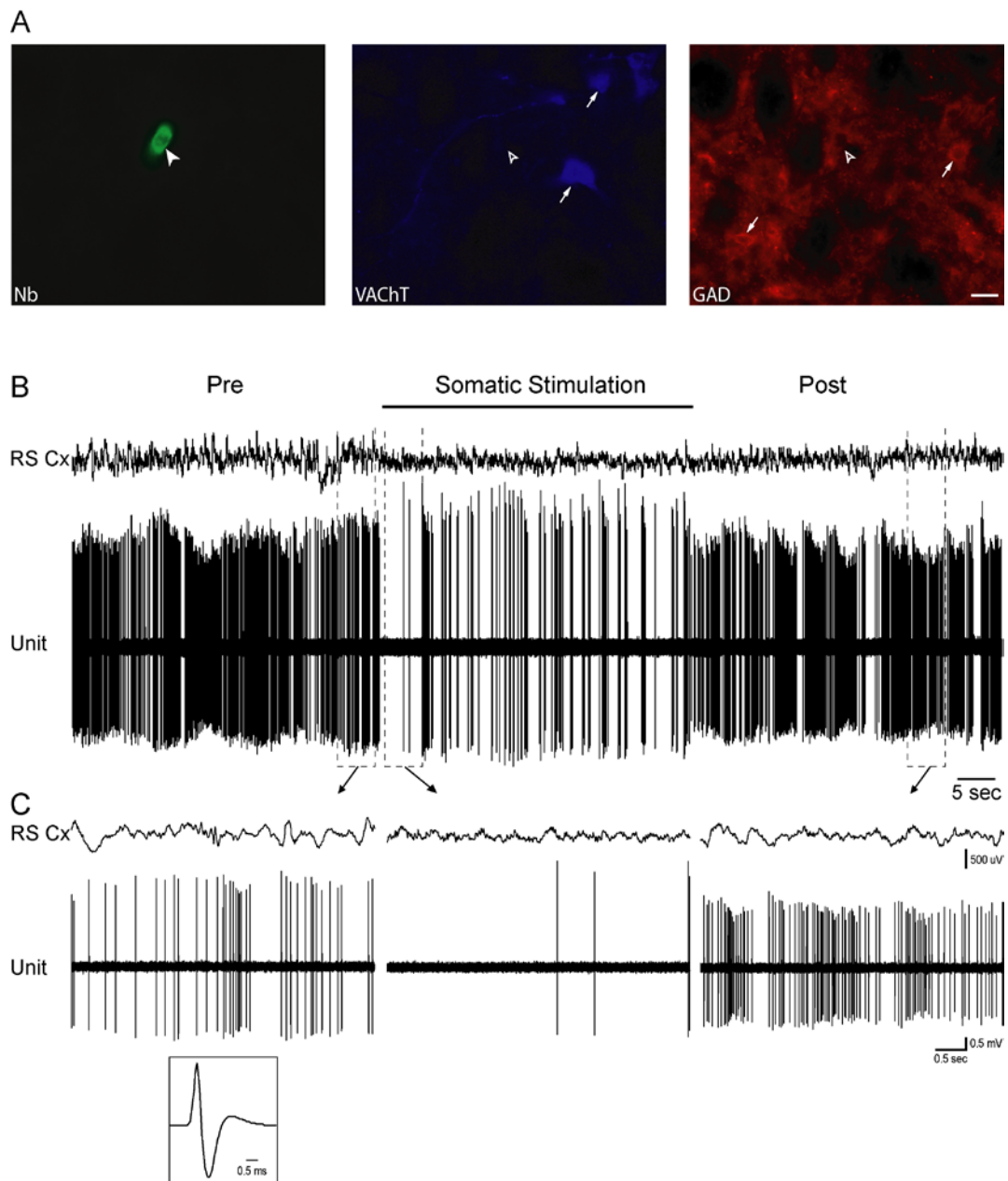


Figure 3.6. Nb+/VAcHT-/GAD- "Off" cell.

Figure 3.6. Nb+/VACHT-/GAD- “Off” cell. **A**, Nb-labeled cell (filled arrowhead, #ABS54) was negatively immunostained for VACHT (open arrowhead) and for GAD (open arrowhead), while located near other VACHT+ and GAD+ cells in the region (small arrows). Scale bar, 20 μ m. The cell was situated in the MPPT (largest green open square, Fig. 3.1, A0.9). **B** and **C**, The unit discharged at a fast average rate (13.28 Hz) in association with irregular slow wave activity on the EEG of the retrosplenial cortex (RS Cx) prior to stimulation (Pre). It decreased its average discharge rate (to 4.20 Hz) in association with faster cortical activity during somatic stimulation. After stimulation, the unit recovered its baseline rate in association with irregular slow wave activity. Traces in **B** (enclosed by dashed lines) are expanded in **C** (arrows). The unit had a medium duration spike (shown in **C**, 0.49 and 1.34 ms at first and second zero crossings).

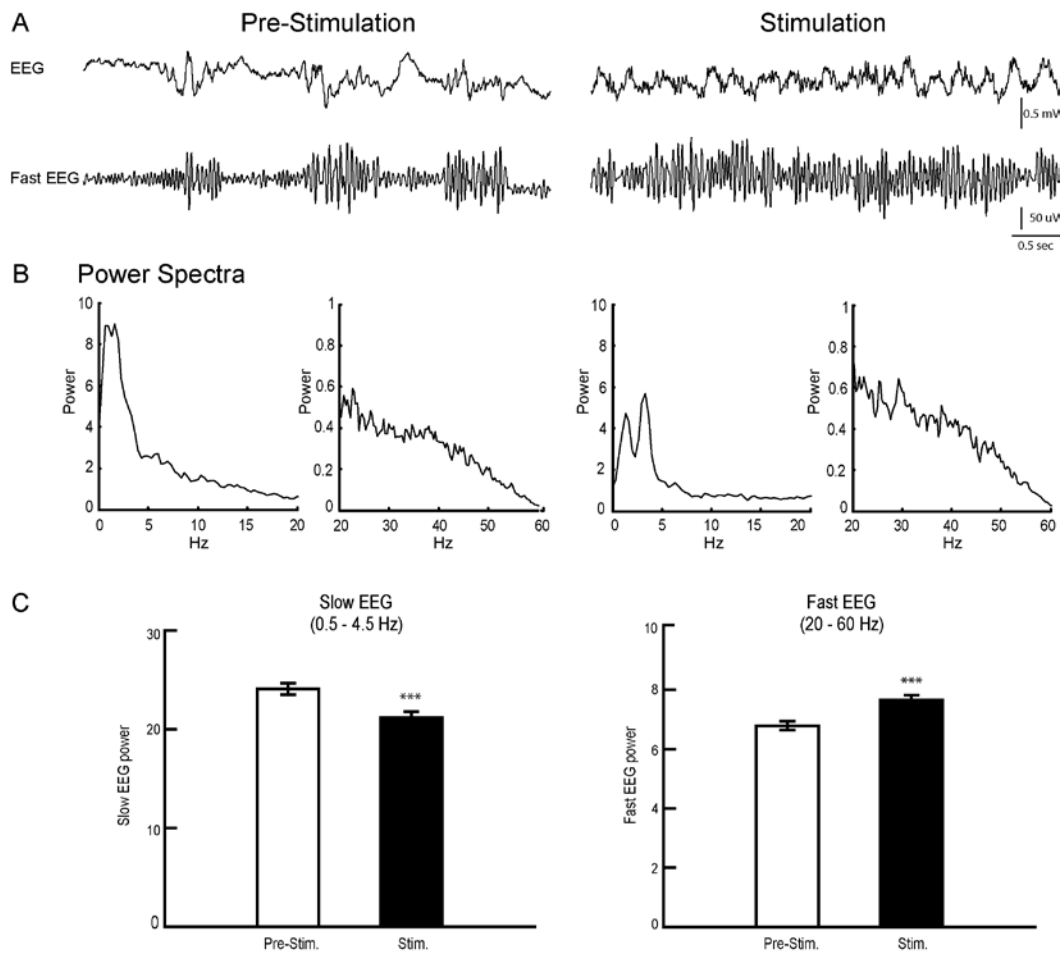


Figure 3.7. EEG activity during pre-stimulation and stimulation conditions in urethane anesthetized rat.

Figure 3.7. EEG activity during pre-stimulation and stimulation conditions in urethane anesthetized rat. **A**, EEG (0.5 - 100 Hz) and fast filtered EEG (20 - 60 Hz) during 4 sec periods of each condition in one animal (#ABS100a). Note the change from irregular slow activity (~1.5 Hz) to relatively rhythmic slow activity (~3 Hz) accompanied by an increase in fast activity during stimulation compared to pre-stimulation. **B**, Power spectra ($\mu\text{V}^2/\text{Hz}$) of the EEG activity during 40 sec periods of Pre-stimulation (left) and stimulation from the same animal as in A, for low (0 - 20 Hz) and high (20 - 60 Hz) EEG frequencies. Note the decrease in power and upward shift in peak frequency (from 1.7 to 3.3 Hz) in the slow activity reflecting a shift from irregular slow (delta-like) activity to rhythmic slow (theta-like) activity during stimulation. This change in slow activity is accompanied by an increase in the power of high beta-gamma activity. **C**, Average integrated power of slow EEG and fast EEG during pre-stimulation and stimulation conditions across all animals ($n = 73$). Note the significant decrease in slow EEG ($t = 9.05$, $df = 77$) and the significant increase in fast, high beta-gamma EEG power during stimulation period ($t = -9.19$, $df = 77$) ($***p < 0.001$, according to paired t tests). Whereas the EEG during spontaneous pre-stimulation conditions resembles that of slow wave sleep, the EEG during stimulation reflects partial cortical activation in response to somatosensory stimulation, despite the lack of any behavioral response, under urethane anesthesia.

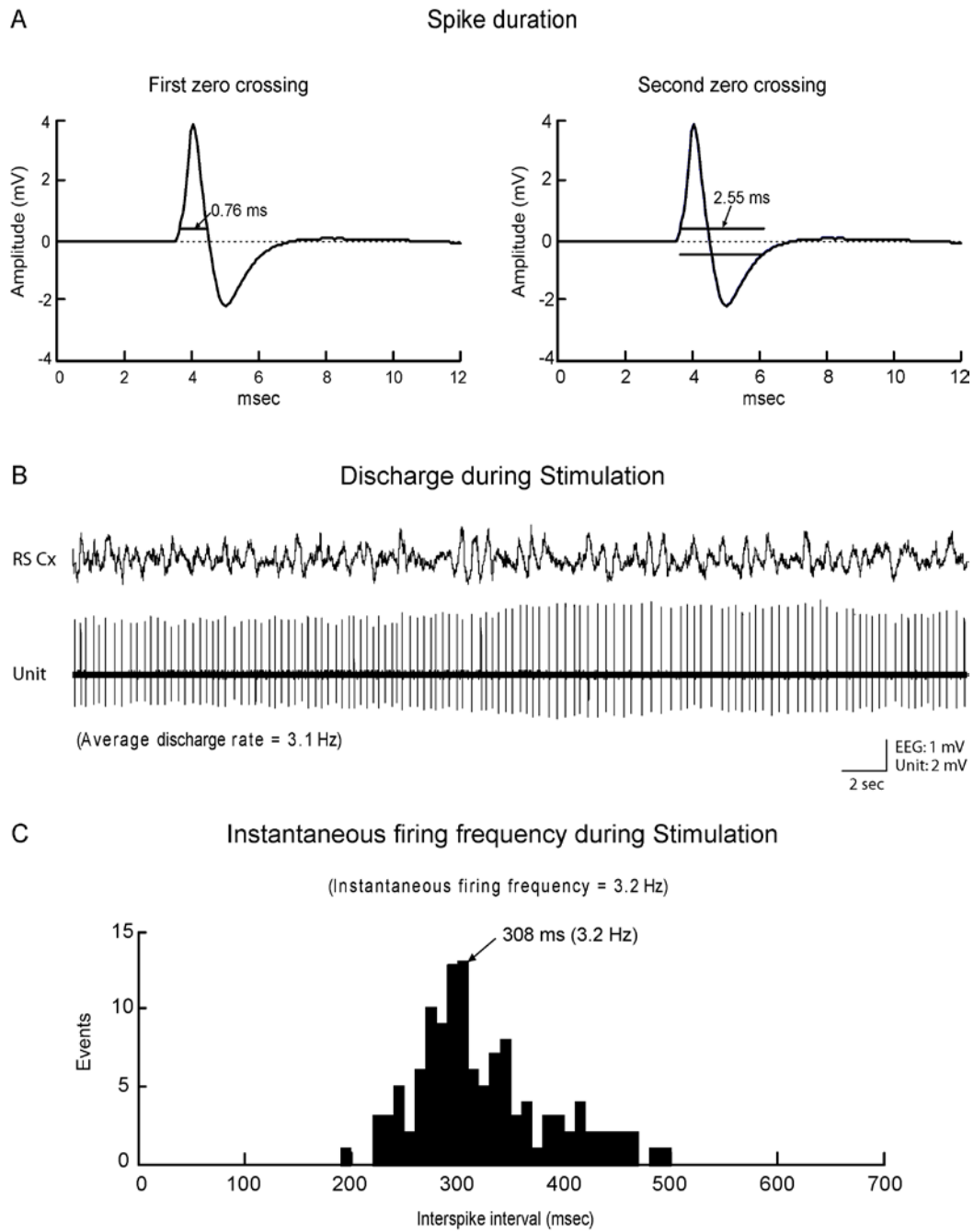


Figure 3.8. Electrophysiological properties of neurons.

Figure 3.8. Electrophysiological properties of neurons. Shown for a VACHT+ unit (#ABS80a, represented in Fig. 3.2): **A**, Average spike duration based upon first and second zero crossings (but calculated with an offset from zero corresponding to 10% of the positive peak amplitude to avoid variations in zero on the return to resting following the negative deflection). Note that the VACHT+ cell has a relatively long spike duration. **B**, EEG from retrosplenial cortex (RS Cx) and unit activity during 20 sec of the stimulation period with average discharge rate (# spikes per second) calculated from the full 40 sec period. Note that the VACHT+ cell has a regular tonic discharge of ~3 Hz in association with stimulated cortical activation. **C**, The instantaneous firing frequency during the full 40 sec stimulation period (as calculated from the mode of the interspike interval histogram, as 1000 ms/modal value). Note that for the VACHT+ cell, the instantaneous firing frequency (3.2 Hz) does not differ from the average discharge rate (3.1 Hz), reflecting the tonic, regular discharge of the cell (see also Fig. 3.9).

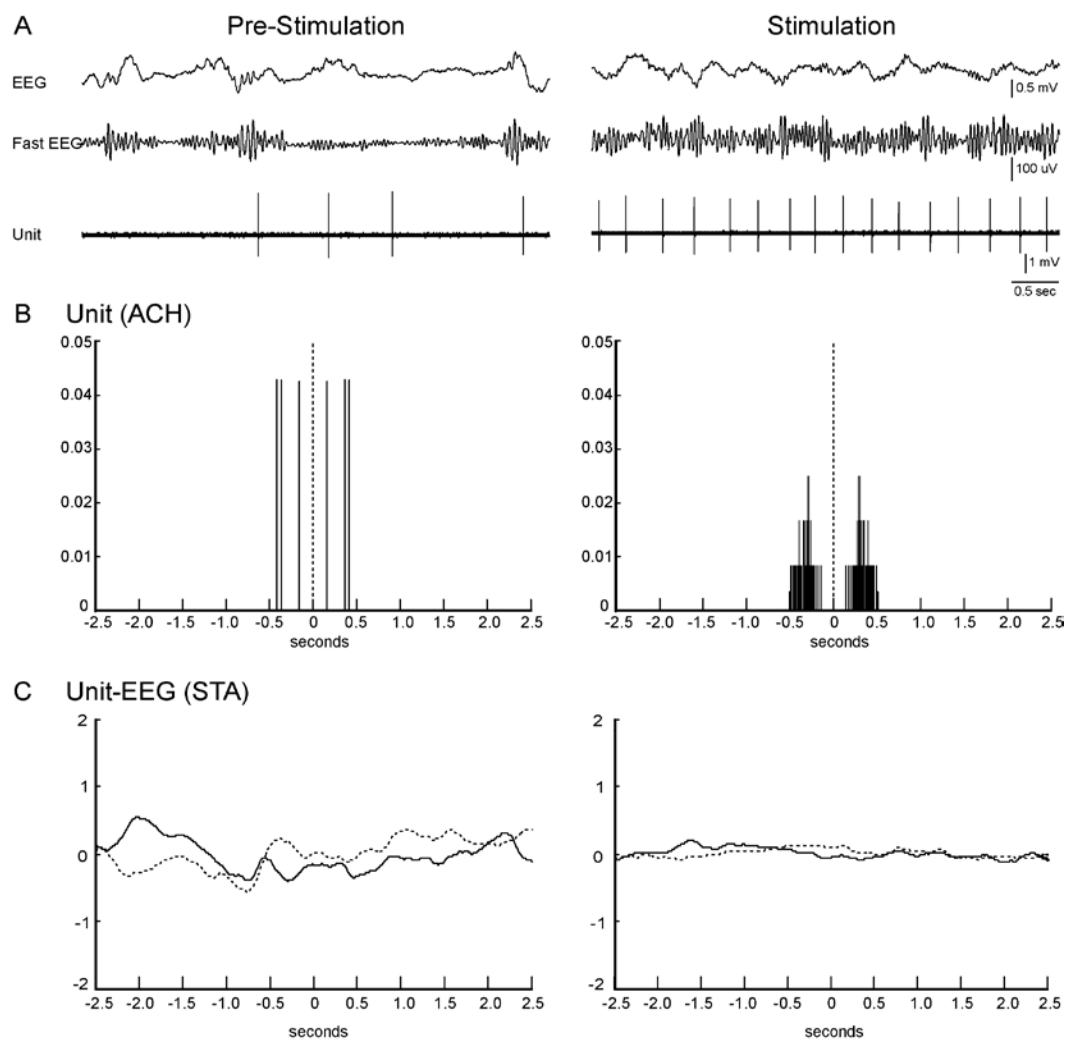


Figure 3.9. Auto-correlation and cross-correlation with EEG of unit discharge.

Figure 3.9. Auto-correlation and cross-correlation with EEG of unit discharge. As shown for a VACHT+ unit (#ABS80a, shown in Fig. 3.2 and Fig. 3.8): **A**, EEG (0 – 100 Hz) and Fast filtered EEG (20 – 60 Hz) together with unit activity during 10 sec periods of pre-stimulation and stimulation conditions. Note for this VACHT+ cell, the unit discharges minimally and irregularly in single spikes during pre-stimulation, and it discharges moderately and regularly (~3 Hz) in single spikes during stimulation. In neither condition is there any apparent relationship to EEG oscillations. **B**, Autocorrelation histograms (ACH) of unit activity (with arbitrary voltage units for spikes on vertical axes) for the full 40 sec recording periods of pre-stimulation (left) and stimulation (Cannon et al.). Note for the VACHT+ cell, there is minimal activity with no evidence of rhythmicity in spiking during the pre-stimulation period, whereas there is moderate activity with evidence of regularity in the tonic spiking (around 300 ms intervals as also seen in the ISI, Figure 3.8C) during stimulation. **C**, Spike-triggered average (STA) of unit-to-EEG (with mV EEG on vertical axes) for the 40 sec periods of pre-stimulation (left) and stimulation conditions for the actual unit spike train (*solid black line*) and randomized spike train (*dotted gray line*). Note for this VACHT+ cell, there was no cross-correlation between the unit discharge and EEG activity (as reflected by the relatively flat lines for the unit spike train and lack of difference from the shuffled spike train) in either pre-stimulation or stimulation period.

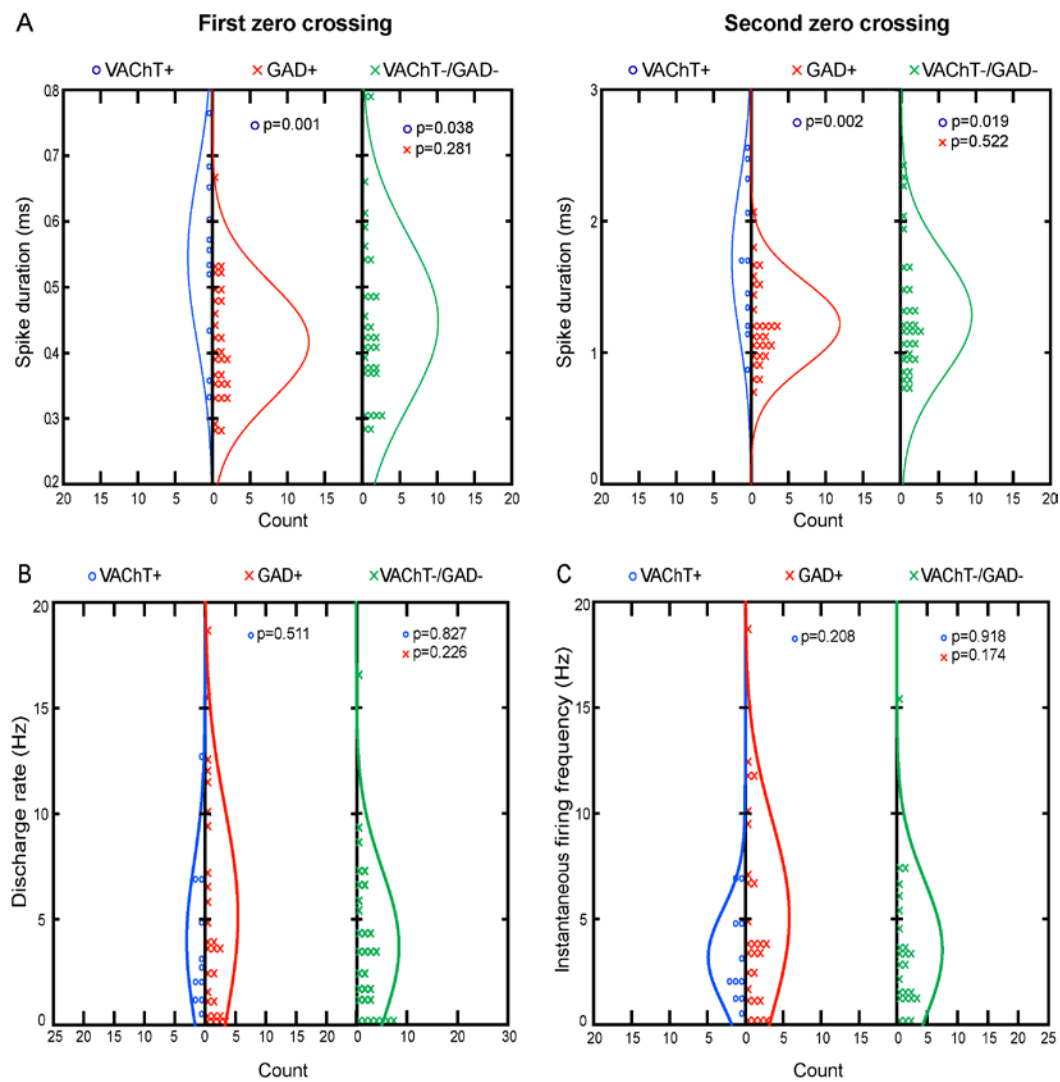


Figure 3.10. Comparison of electrophysiological variables during somatic stimulation among all Nb+/VACHT+, GAD+ and VACHT-/GAD- neurons.

Figure 3.10. Comparison of electrophysiological variables during somatic stimulation among all Nb+/VACHT+, GAD+ and VACHT-/GAD- neurons. *A, Left graph,* Average spike duration based upon first zero crossing for VACHT+ (0.55 ± 0.04 ms, mean $\pm$ SEM, n = 11; median = 0.55 ms), GAD+ (0.42 ± 0.02 ms, n= 29; median = 0.40 ms) and VACHT-/GAD- cells (0.45 ± 0.02 ms, n = 33; median = 0.41 ms). *Right graph,* Average spike duration based upon second zero crossing for VACHT+ (1.80 ± 0.19 ms, mean $\pm$ SEM, n = 11; median = 1.69 ms), GAD+ (1.22 ± 0.05 ms, n= 29; median = 1.14 ms) and VACHT-/GAD- cells (1.28 ± 0.08 ms, n = 33; median = 1.18 ms). Note that despite a significant difference in means across the three cell groups and between the cholinergic and noncholinergic cell groups in spike duration (see Table 2 and figure), the values for each cell group are highly variable and overlap extensively. *B,* Average discharge rate (Hz) during stimulation for VACHT+ (3.99 ± 1.09 Hz, n = 11), GAD+ (6.25 ± 1.47 Hz, n = 29) and VACHT-/GAD- cells (4.48 ± 0.97 Hz, n = 33). Note that there was no significant difference in mean average spike rates across cell types and extensive overlap in values across the three groups (see Table 3.3 and figure). *C,* Average instantaneous firing frequency for VACHT+ (3.19 ± 0.68 Hz, n = 11), GAD+ (6.25 ± 1.46 Hz, n = 28) and VACHT-/GAD- cells (4.27 ± 1.04 Hz, n = 27). Note that there was no significant difference in mean firing frequencies across the three groups and extensive overlap (see Table 3.3 and figure).

In attempting to select units according to the range of values of any one or all three of the electrophysiological variables, the probability of selecting a cholinergic cell from all recorded cells was only minimally increased from 15% to 26% and that of selecting a GABAergic cell from all cells was increased from 40% to 48%. Only a small

subset of GABAergic and noncholinergic/nonGABAergic cells could be distinguished as noncholinergic cells according to their high average spike rate (>13 Hz) and firing frequency (>7 Hz). On the other hand, GABAergic cells could not be distinguished from noncholinergic/nonGABAergic cells.

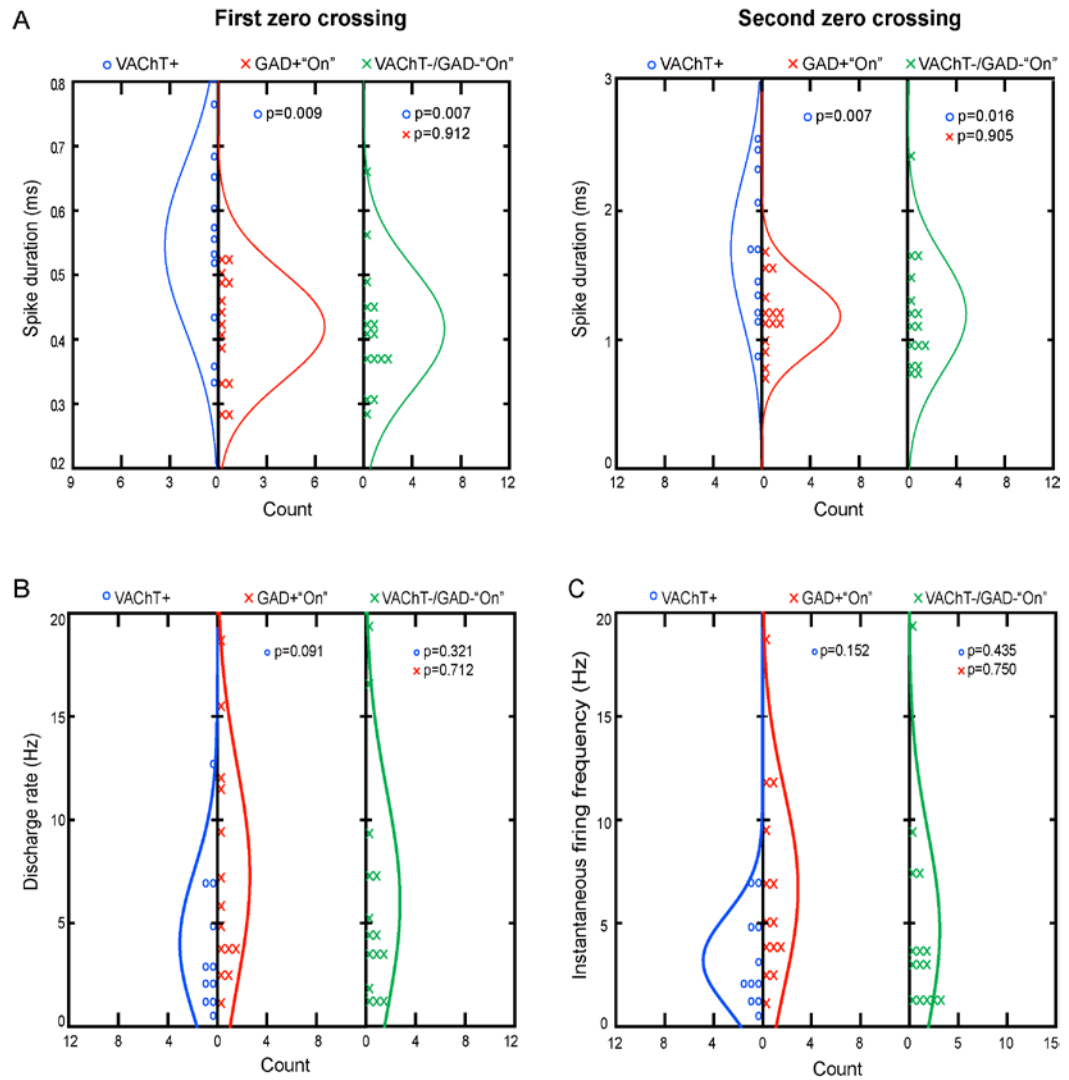


Figure 3.11. Comparison of electrophysiological variables during somatic stimulation among Nb+/VACHT+, GAD+ "On" and VACHT-/GAD- "On" cells which commonly increased their discharge during stimulation.

Figure 3.11. Comparison of electrophysiological variables during somatic stimulation among Nb+/VAcHT+, GAD+ “On” and VAcHT-/GAD- “On” cells which commonly increased their discharge during stimulation. A, Left graph, Average spike duration. based upon first zero crossing for VAcHT+ (0.55 ± 0.04 ms, mean $\pm$ SEM, n = 11; median = 0.55 ms), GAD+ “On” (0.42 ± 0.02 ms, n = 14; median = 0.43 ms) and VAcHT-/GAD- “On” cells (0.42 ± 0.02 ms, n = 16; median = 0.41 ms). Note that despite a significant difference in means across the three groups ($F = 6.08$, $df = 2, 38$, $p < 0.01$), the values for each cell group are highly variable and overlap extensively. **Right graph,** based upon second zero crossing for VAcHT+ (1.71 ± 0.17 ms, mean $\pm$ SEM, n = 11; median = 1.69 ms), GAD+ “On” (1.18 ± 0.08 ms, n = 14; median = 1.19 ms) and VAcHT-/GAD- “On” cells (1.20 ± 0.11 ms, n = 16; median = 1.14 ms). Note that despite a significant difference in means across the three groups ($F = 5.57$, $df = 2, 38$, $p < 0.01$), the values for each cell group are highly variable and overlap extensively. **B,** Average discharge rate during stimulation for VAcHT+ (3.99 ± 1.09 Hz, n = 11), GAD+ “On” (7.31 ± 1.43 Hz, n = 14) and VAcHT-/GAD- “On” cells (6.42 ± 1.88 Hz, n = 15). Note that there was no significant difference in mean average spike rates across cell types ($F = 1.06$, $df = 2, 38$, $p > 0.05$) and extensive overlap in values across the three groups. **C,** Average instantaneous firing frequency for VAcHT+ (3.19 ± 0.68 Hz, n = 11), GAD+ (6.39 ± 1.34 Hz, n = 14) and VAcHT-/GAD- cells (5.24 ± 1.78 Hz, n = 15). Note that there was no significant difference in mean firing frequencies across the three groups ($F = 1.16$, $df = 2, 38$, $p > 0.05$) and extensive overlap.

In attempting to select units according to the range of values of any one or all three of the electrophysiological variables, the probability of selecting a cholinergic

cell from all “On” cells was only minimally increased from 28% to 41% and that of selecting a GABAergic cell from all “On” cells was increased from 34% to 47%. Approximately half the cholinergic cells could be distinguished from other “On” cells by broad spikes (>1.8 ms). A small subset of GABAergic and noncholinergic/nonGABAergic “On” cells could be distinguished as noncholinergic cells according to their high average spike rate (>13 Hz) and firing frequency (>7 Hz). GABAergic cells could not be distinguished from noncholinergic/nonGABAergic cells.

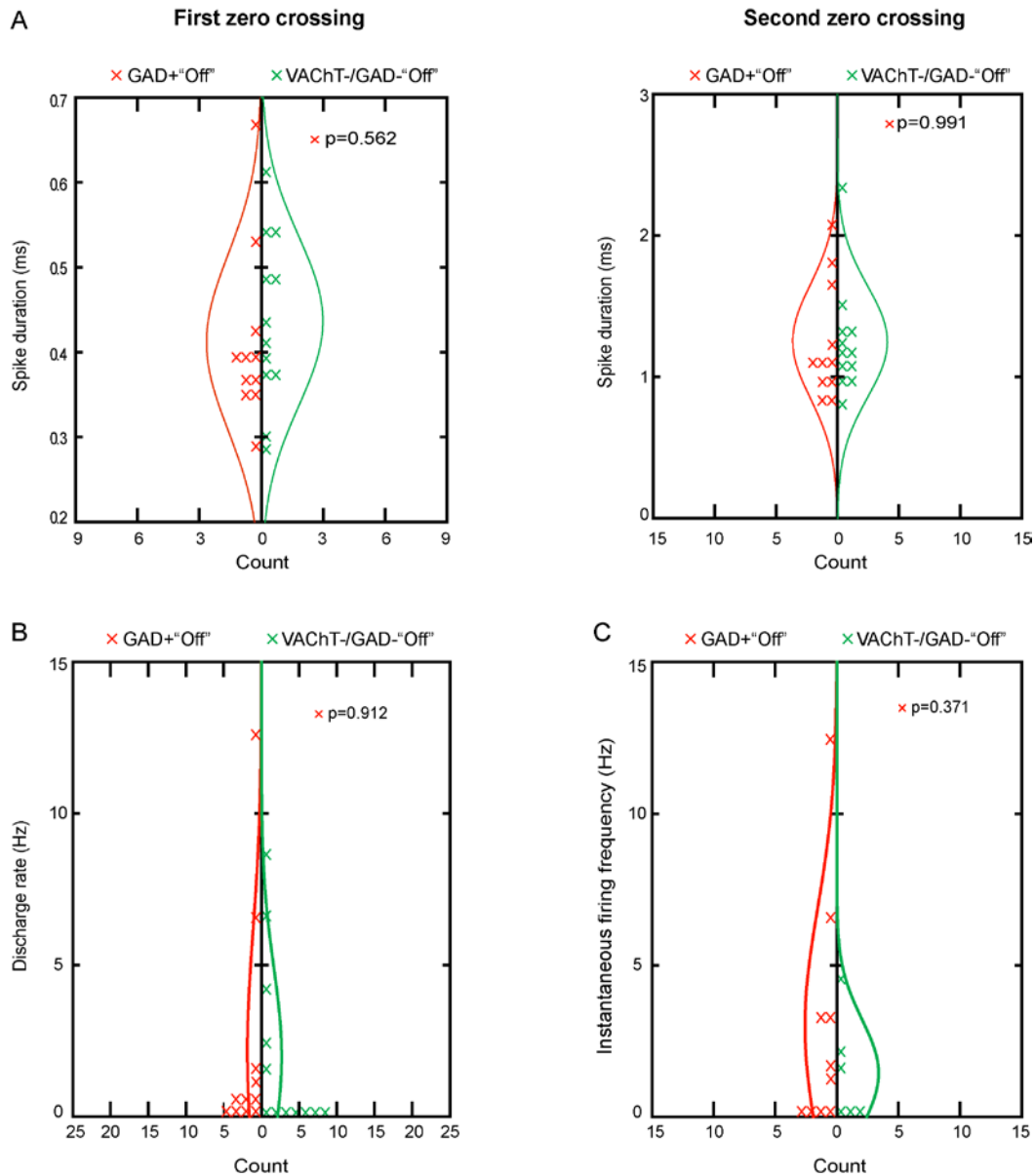


Figure 3.12 Comparison of electrophysiological variables during somatic stimulation between GAD+ "Off" and VACHT-/GAD- "Off" cells, which commonly decreased or ceased firing in response to stimulation.

Figure 3.12 Comparison of electrophysiological variables during somatic stimulation between GAD+ “Off” and VACHT-/GAD- “Off” cells, which commonly decreased or ceased firing in response to stimulation. *A, Left graph*, Average spike duration based upon first zero crossing for GAD+ “Off” (0.41 ± 0.03 ms, mean $\pm$ SEM, $n = 11$; median = 0.39 ms) and VACHT-/GAD- “Off” cells (0.44 ± 0.03 ms, $n = 12$; median = 0.42 ms). Note that there was no significant difference between the means of the cell groups ($t = -0.59$, $df = 21$, $p > 0.05$), and the values for the two groups overlap extensively. *Right graph*, average spike duration based upon second zero crossing for GAD+ “Off” (1.25 ± 0.12 ms, mean $\pm$ SEM, $n = 11$; median = 1.09 ms) and VACHT-/GAD- “Off” cells (1.25 ± 0.11 ms, $n = 12$; median = 1.18 ms). Note that there was no significant difference between the means of the cell groups ($t = -0.46$, $df = 21$, $p > 0.05$), and the values for the two groups overlap extensively. *B*, Average discharge rate (Hz) during stimulation for GAD+ “Off” (2.14 ± 1.18 Hz, $n = 11$) and VACHT-/GAD- “Off” cells (1.98 ± 0.86 Hz, $n = 12$). Note that there was no significant difference in mean average spike rate between cell types ($t = 0.11$, $df = 21$, $p > 0.05$) and extensive overlap in values between the groups. *C*, Average instantaneous firing frequency for GAD+ “Off” (2.99 ± 1.22 , Hz, $n = 10$) and VACHT-/GAD- “Off” cells (1.44 ± 0.72 Hz, $n = 6$). Note that there was no significant difference in mean firing frequencies between the groups ($t = 0.92$, $df = 14$, $p > 0.05$) and extensive overlap.

Given a virtually complete overlap in the distributions of the three variables for the GABAergic and noncholinergic/nonGABAergic “Off” cells, no selection criteria could be employed to distinguish individual cells.

4. Chapter Four

The activity profiles of LDT/PPT neurons during natural sleep-wake states

4.1 Preface

In the last chapter the activity profiles of LDT and PPT cholinergic neurons, in addition to GABAergic and putative glutamatergic neurons under anesthesia in relation to cortical activity were described. All LDT/PPT cholinergic neurons were found to increase their discharge in association with cortical activation evoked by somatic stimulation, whereas GABAergic and putative glutamatergic neurons were heterogeneous: they increased or decreased their discharge in relation to cortical activation.

Although this acute study could suggest the way these neurons might behave during natural sleep-wake states, where evoked cortical activation could resemble cortical activation of natural waking (W) and paradoxical sleep (PS) and irregular slow activity could resemble slow wave activity of natural slow wave sleep (SWS), the activity profiles of LDT and PPT cholinergic, GABAergic and putative glutamatergic neurons across natural sleep-wake states remain unknown.

In the present study, by immunohistochemical identification of recorded and labeled single cells in natural sleeping/waking rats, the activity profiles of LDT and PPT cholinergic, GABAergic and putative glutamatergic neurons across natural sleep-wake states in terms of state dependency and the relationship with cortical and behavioral activities relevant to sleep-wake states are described.

4.2 Results

4.2.1 Identification, localization and classification of cell groups

A total of 70 units were recorded in the pontomesencephalic tegmentum across a full sleep-waking cycle including the three major states, active wake (aW), SWS and PS and three transitional states, quite wake (qW), transition to SWS (tSWS) and transition to PS (tPS). According to their sleep-wake discharge profile, neurons comprised different functional sub-groups: (1) those that discharge maximally during both W and PS as W/PS-max active cells ($n = 32$), (2) those that discharge minimally during waking and progressively raised their discharge during sleep to reach their maximal discharge during PS as PS-max active cells ($n = 21$), (3) those that discharged maximally during waking as W-max active cells ($n = 13$), (4) those that did not show significant variation in their discharge across sleep-wake states as wsp-equivalent cells ($n = 3$), and (5) one that discharged maximally during SWS as SWS-max active cell ($n = 1$). Of all units recorded, 60 could be submitted to juxtacellular labelling protocol at the end of the sleep-wake cycle, and 47 of these (~78%) were successfully labelled with Nb (Nb+). Of these 47 Nb+ cells, 32 Nb+ cells were located within the pontomesencephalic cholinergic cell area, whereas 15 were located in the surrounding central gray (CG, $n = 6$), pontine reticular nucleus, oral part (PnO, $n = 3$), deep mesencephalic reticular nucleus (DpMe, $n = 2$), dorsomedial tegmental nucleus (DMT, $n = 1$) or subcoeruleus (SubC, $n = 1$). Within the cholinergic cell area, the Nb+ cells were distributed across the rostrocaudal extent of the LDT ($n = 17$), its ventral extension beneath the central grey (SubLDT) ($n = 11$) or the medial part of the PPT (mPPT) ($n = 4$) (Fig 4.1). Of the 32 Nb+ cells, one was immuno-positive for VACHT (Nb+/VACHT+), 7 were immuno-positive for GAD

(Nb+/GAD+) and 24 were immuno-negative for both VACht and GAD (Nb+/VACht-/GAD-) (Table 4.1). Morphologically, the majority of cells were medium-to-large in size, including the Nb+/VACht+ cell, but both Nb+/GAD+ and Nb+/VACht-/GAD- cells included a minority of small sized cells (Table 4.1). Physiologically, according to instantaneous firing frequency, the majority of cells were fast spiking (>14.5 Hz), yet a minority, including the Nb+/VACht+ cell was slow (<14.5 Hz) (Table 4.1). The cells were very heterogeneous in their firing pattern including tonic and phasic patterns. The Nb+/VACht+ cell and many Nb+/VACht-/GAD- cells fired in a tonic regular manner, whereas the Nb+/GAD+ cells fired tonically in an irregular manner or phasically. Phasic firing occurred in either a cluster (<80 Hz) or burst (>80 Hz) mode among these cells (Table 4.1). According to their sleep-wake discharge profile, half of the Nb+ cells in the LDT/PPT were W/PS-max active cells ($n = 16$), 37.5% PS-max active cells ($n = 12$) and 12.5% W-max active cells ($n = 4$) (Table 4.1). Neither wsp-equivalent nor SWS-max active cells were found labelled in the LDT/PPT area. Based on their anatomical and physiological features, the Nb+/VACht+, Nb+/GAD+ and Nb+/VACht-/GAD- cells could not be clearly distinguished (Table 4.1).

4.2.2 W/PS-max active neurons

The W/PS-max active neurons represent the largest sub-group found in the LDT/PPT area ($n = 16$). They discharged maximally during aW and PS than during SWS. This discharge profile was the most common profile for all three, Nb+/VACht+, Nb+/GAD+ and Nb+/VACht-/GAD- cell groups.

4.2.2.1 The Nb+/VAcHT+ neuron

The Nb+/VAcHT+ neuron ($n = 1$), represented ~6 % of the W/PS-max sub-group ($n = 16$). As typical of this sub-group, the cell discharged maximally during aW and PS than during SWS and it reached its highest rate during PS. Across sleep-wake states, its discharge was positively correlated with gamma EEG amplitude ($r = 0.37$, $p < 0.05$, $n = 1$) (Fig. 4.3A) and with theta activity ($r = 0.92$, $p < 0.005$, $n = 1$). Across sleep-wake states, its firing rate was strongly changed. As shown for CBS28u03 in Fig. 4.4, the unit discharged at slow rates during aW (1.90 Hz), progressively decreased rates in qW to discharge minimally during the tSWS and to be virtually silent during SWS (0.06 Hz), increased back significantly its firing during the tPS to reach maximal rates during PS (9.70 Hz). The cholinergic cell was a slow firing neuron with a typical tonic regular pattern since its instantaneous firing frequency (10.1 Hz) was almost equivalent to its mean discharge rate (9.7 Hz) (Table 4.2). It had a relatively long duration spike (1.9 ms).

4.2.2.2 Nb+/GAD+ neurons

Nb+/GAD+ neurons ($n = 4$) represented 25% of the W/PS-max sub-group ($n = 16$). They discharged at higher rates during aW and PS compared to SWS. The majority ($n = 3$) reached their maximal discharge during PS (as was the case for the cell CBS37u02 shown in Fig. 4.5). One, however, discharged during aW. They showed a strong modulation of their discharge rate across sleep-wake states. Their average discharge rate was relatively high during aW (4.67 ± 3.16 Hz); it markedly decreased to be minimal during tSWS and SWS (0.22 ± 0.17 Hz) and then increased progressively during tPS to be maximal during PS (9.93 ± 5.18 Hz) (Fig. 4.3A). Across sleep-wake states, their average discharge rate was positively correlated with gamma EEG activity in 3/4 cells (r

= 0.75 ± 0.07 , $p < 0.005$) (Fig. 4.3A) and with theta activity in 3/4 cells ($r = 0.74 \pm 0.11$, $p < 0.005$, $n = 3$). Most of them were fast firing neurons ($n = 3$). Their mean instantaneous firing frequency (52.93 ± 21.21 Hz) was much higher than the average discharge rate (12.99 ± 4.46 Hz) (Table 4.2), which reflected a tonic irregular firing pattern ($n = 1$) or phasic firing pattern which could be either a cluster pattern ($n = 1$) or a bursting pattern ($n = 2$). They had, on average, a relatively narrow spike duration (1.03 ± 0.08 ms) which significantly differed from the Nb+/VAcHT-/GAD- W/PS-max neurons (1.51 ± 0.11 ms) (Table 4.2). However, spike duration measurements were found to overlap extensive among these two sub-groups (Fig. 4.9B).

4.2.2.3 Nb+/VAcHT-/GAD- neurons

Nb+/VAcHT-/GAD- neurons ($n = 11$) represented 69% of the W/PS-max sub-group ($n = 16$). They discharged at their highest rates during aW and PS in association with cortical activation. Most cells ($n = 7$) reached their maximal discharge during PS (as was the case for the cell CBS22u01 shown in Fig. 3.6). Some others ($n = 4$), however, reached their maximal discharge during aW. They showed a strong modulation of their discharge rate across sleep-wake states. Their mean average discharge rate was relatively high during aW (4.99 ± 1.33 Hz) and progressively decreased during tSWS to be minimum during SWS (0.34 ± 0.17 Hz), and then progressively increased during tPS to be maximal during PS (8.77 ± 2.13 Hz) (Fig. 4.3A). Across sleep-wake states, their average discharge rate was significantly and positively correlated with gamma EEG activity in all cells ($r = 0.40 \pm 0.03$, $p < 0.05$, $n = 11$) (Fig. 3.3A) and with theta activity in 9/11 cells ($r = 0.69 \pm 0.07$, $p < 0.005$, $n = 9$). Some VAcHT-/GAD- W/PS- max cells were fast firing ($n = 6$), but others were slow firing ($n = 5$). They included cells with

different firing patterns, from tonic regular ($n = 5$) to tonic irregular ($n = 2$) to phasic cluster ($n = 4$). Their mean instantaneous firing frequency was moderately high (25.47 ± 6.66 Hz) and higher than the average discharge rate (9.87 ± 2.11 Hz) (Table 4.2). They had, on average, a relatively medium spike duration (1.51 ± 0.11 ms), which significantly differed from the Nb+/GAD+ W/PS-max sub-group (1.03 ± 0.08 ms) (Table 4.2).

4.2.3 PS-max active neurons

The PS-max active neurons represent the second most numerous sub-group found in the LDT/PPT area ($n = 12$). They discharged at their highest rates during PS in association with muscle atonia. They included Nb+/GAD+ and Nb+/VACHT-/GAD- cell groups.

4.2.3.1 Nb+/GAD+ neurons

Nb+/GAD+ neurons ($n = 3$) represented 25% of the PS-max sub-group ($n = 12$). Across the sleep-wake cycle, they discharged at their lowest rate during aW (3.94 ± 0.08 Hz), they increased slightly their discharge during SWS (4.70 ± 0.79 Hz) and then further increased their discharge during tPS to reach their maximal rates during PS (18.99 ± 1.08 Hz) (Fig. 4.3B). Their average discharge rate was negatively correlated with EMG amplitude across sleep-wake states ($r = -0.54 \pm 0.12$, $p < 0.01$, $n = 3$) (Fig. 4.3B). They were all fast firing cells, and their mean instantaneous firing frequency (46.50 ± 15.21 Hz) was largely greater than their average discharge rate (18.99 ± 1.08 Hz) (Table 4.2), reflecting a tonic irregular firing pattern ($n = 2$) or a phasic cluster firing pattern ($n = 1$, cell CBS28u04 shown in Fig. 4.7C with rhythmic firing in association with theta activity during PS). They had, on average, a relatively narrow spike width (0.92 ± 0.11 ms),

which did not differ significantly from the other PS-max sub-group (1.23 ± 0.11 ms) (Table 4.2, Fig. 4.9C).

4.2.3.2 Nb+/VAcHT-/GAD- neurons

Nb+/VAcHT-/GAD- neurons ($n = 9$) represented 75% of the PS-max sub-group ($n = 12$). As typical of PS-max cells (as shown for the cell CBS27u01 in Fig. 4.8), they discharged at their lowest rate during aW (2.42 ± 0.84 Hz), progressively increased their discharge during qW and tSWS to be higher during SWS (4.98 ± 1.18 Hz) and then increased further their discharge during tPS to reach their maximal rates during PS (17.03 ± 4.13 Hz) (Fig. 4.3B). Their average discharge rate was significantly, negatively correlated with EMG amplitude across sleep-wake states in 6/9 cells ($r = -0.49 \pm 0.05$, $p < 0.01$, $n = 6$) (Fig. 4.3B). They were all fast firing cells and included cells with different firing patterns, from tonic regular ($n = 2$) to phasic cluster ($n = 4$) to phasic burst ($n = 3$). Their mean instantaneous firing frequency was faster (77.69 ± 20.53 Hz) than the average discharge rate (17.08 ± 4.13 Hz) (Table 4.2). They had, on average, a relatively medium spike duration (1.23 ± 0.11 ms), which did not differ significantly from the other PS-max sub-group (0.92 ± 0.11 ms) (Table 4.2, Fig. 4.9C).

4.2.4 W-max active neurons

W-max active neurons represented the smallest sub-group in the LDT/PPT area ($n = 4$). This sub-group comprised only VAcHT-/GAD- cells. They discharged at their highest rates during aW (1.27 ± 0.68 Hz), then decreased their discharge rate progressively through SWS (0.22 ± 0.04 Hz) to reach minimum rates during PS (0.17 ± 0.14 Hz) (Fig. 4.3C). Their average discharge rate was significantly, positively

correlated with EMG amplitude across sleep-wake states in 2/4 cells ($r = 0.45 \pm 0.12$, $p < 0.01$, $n = 2$) (Fig. 4.3C). They were all slow firing cells. They included cells with either tonic regular ($n = 1$) or phasic cluster ($n = 2$) firing pattern (representative cell not showed). Their mean instantaneous firing frequency (7.01 ± 3.36 Hz) and their average discharge rate (1.27 ± 0.68 Hz) were slow (Table 4.2). They had, on average, a relatively large spike duration (1.78 ± 0.15 ms) (Table 4.2).

4.3 Tables and Figures

Table 4.1. Frequency of anatomical and physiological characteristics among cholinergic, GABAergic and noncholinergic/nonGABAergic cell groups

<i>n</i>	All cells	Nb+/VAcHT+	Nb+/GAD+	Nb+/VAcHT-/GAD-
Anatomy	32	1	7	24
Area				
LDT	17	1	3	13
SubLDT	11	0	3	8
mPPT	4	0	1	3
Size ^a				
Small (≤15μm)	9	0	3	6
Medium-large (>15μm)	19	1	4	14
Physiology				
Firing frequency ^b				
Slow (<14.5 Hz)	11	1	1	9
Fast (>14.5 Hz)	21	0	6	15
Firing pattern ^c				
Tonic regular	9	1	0	8
Tonic irregular	5	0	3	2
Phasic cluster	13	0	2	11
Phasic burst	5	0	2	3
Discharge profile ^d				
WP-max	16	1	4	11
P-max	12	0	3	9
W-max	4	0	0	4

Numbers (n) of Nb-labeled cells identified as VAcHT+, GAD+ or VAcHT-/GAD-. Whereas the three groups could not be compared due to the n of 1 for VAcHT+ cells, between the GAD+ and VAcHT-/GAD- no significant differences in frequencies were found on any parameter according to χ^2 tests of association.

^aAccording to long axis of whole cell bodies (n=28).

^bAccording to instantaneous firing frequency calculated from the peak of the primary mode of the interspike interval histogram (ISI_H) during the state of maximal discharge.

^cFiring pattern determined by comparison of the instantaneous firing frequency and average discharge rate during the state of maximal discharge.

^dAccording to classification of units based on statistical analysis (by ANOVA) of significant differences in discharge rate across and between the three principle states: aW (W), SWS (S) and PS (PS), indicating the state (s) during which the highest rate occurred (-max).

Table 4.2. Electrophysiological properties of cholinergic, GABAergic and noncholinergic/nonGABAergic cell groups

Cell type and subgroup	<i>n</i>	Spike duration (ms)	Instantaneous firing frequency (Hz)	Average discharge rate (Hz)
VChT+ ^a				
WP-max	1	1.90	10.10	9.70
GAD+ ^b				
WP-max	4	1.03 ± 0.08 ^c	52.93 ± 21.21	12.99 ± 4.46
P-max	3	0.92 ± 0.11	46.50 ± 15.21	18.99 ± 1.08
Average	7	0.98 ± 0.06^c	50.17 ± 12.78	15.56 ± 2.71
VChT-/GAD- ^c				
WP-max	11	1.51 ± 0.11 ^b	25.47 ± 6.66	9.87 ± 2.11
P-max	9	1.23 ± 0.11	77.69 ± 20.53	17.08 ± 4.13
W-max	4	1.78 ± 0.15	7.01 ± 3.36	1.27 ± 0.68
Average	24	1.45 ± 0.08^b	41.98 ± 9.96	11.14 ± 2.11

Means ± SEM per cell subgroup are presented along with statistical results. Whereas comparisons could not be made with the VChT+^a cell (n=1), significant differences (by t test) are indicated relative to GAD+^b or VChT-/GAD-^c cell groups. Whereas the spike width derives from the average across all states, the firing frequency and discharge rate are those during the state of maximal discharge for each sleep-wake subgroup.

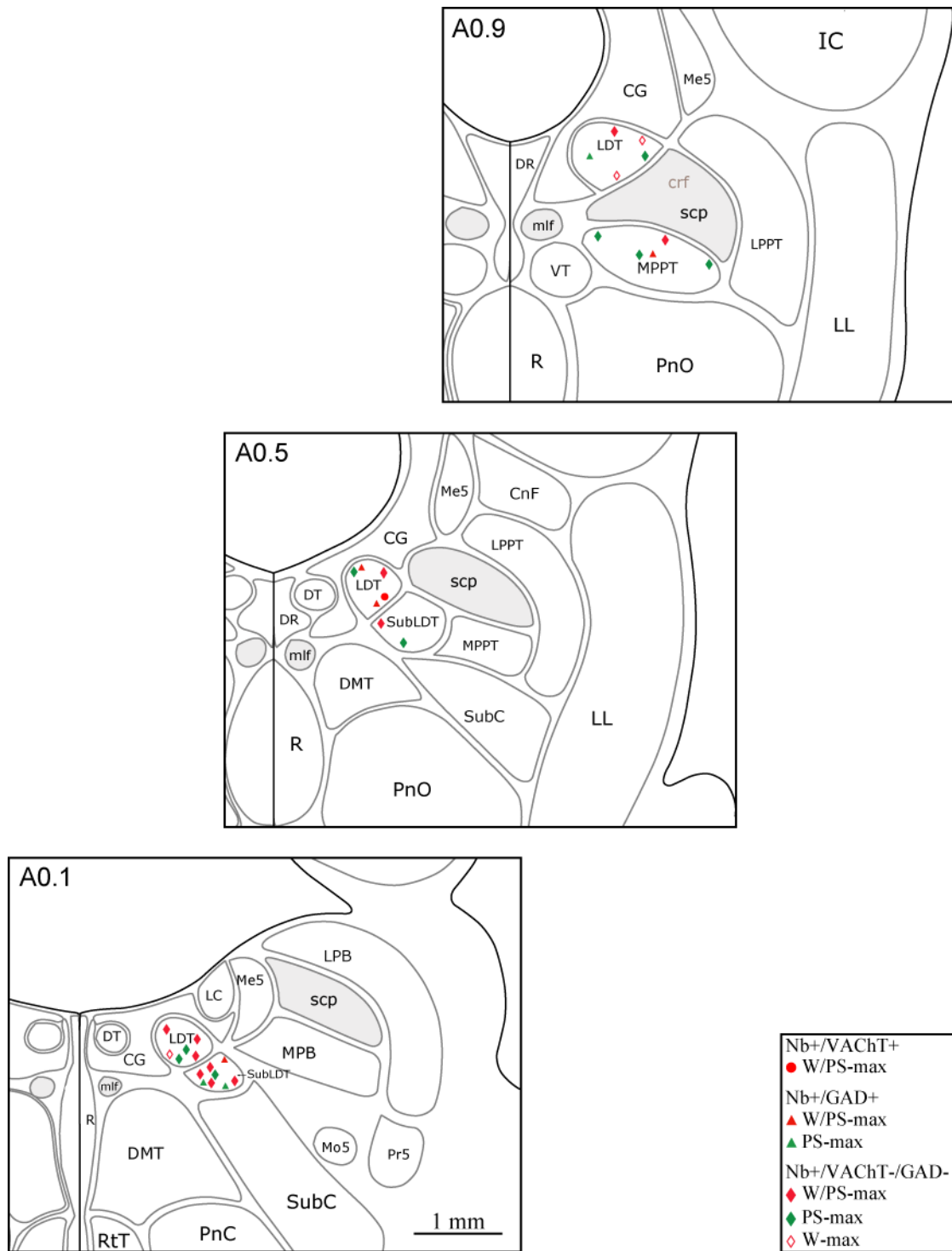


Figure 4.1. Distribution of recorded cells in the mesopontine tegmentum.

Figure 4.1. Distribution of recorded cells in the mesopontine tegmentum. The recorded, Nb-labeled cells (n=32) were immunohistochemically identified as cholinergic, using VACht (Nb+/VACht+, circles), as GABAergic, using GAD (Nb+/GAD+, triangles), or as noncholinergic/non-GABAergic (Nb+/VACht-/GAD-, diamonds). They were further distinguished according to their maximally discharging state(s): aW and PS as “W/PS-max” (filled red), PS as “PS-max” (filled green), aW as “W-max” (open red). Cells are mapped onto appropriate levels (anterior, A0.9, A0.5, or A0.1 mm to interaural zero) through the LDT/SubLDT/MPPT cholinergic cell area. CG, Central gray; CnF, cuneiform nucleus; crf, central reticular fasciculus; DMT, dorsomedial tegmental area; DR, dorsal raphe nucleus; DT, dorsal tegmental nucleus; IC, inferior colliculus; LC, locus coeruleus; LL, lateral lemniscus; LPB, lateral parabrachial nucleus; LPPT, lateral pedunclopontine tegmental nucleus; Me5, mesencephalic trigeminal nucleus; mlf, medial longitudinal fasciculus; Mo5, motor trigeminal nucleus; MPB, medial parabrachial nucleus; MPPT, medial pedunclopontine tegmental nucleus; PnC, pontine reticular nucleus, caudal part; PnO, pontine reticular nucleus, oral part; Pr5, principal sensory trigeminal nucleus; R, raphe nuclei; RtT, reticulotegmental nucleus of the thalamus; scp, superior cerebellar peduncle; SubC, subceruleus; VT, ventral tegmental nucleus.

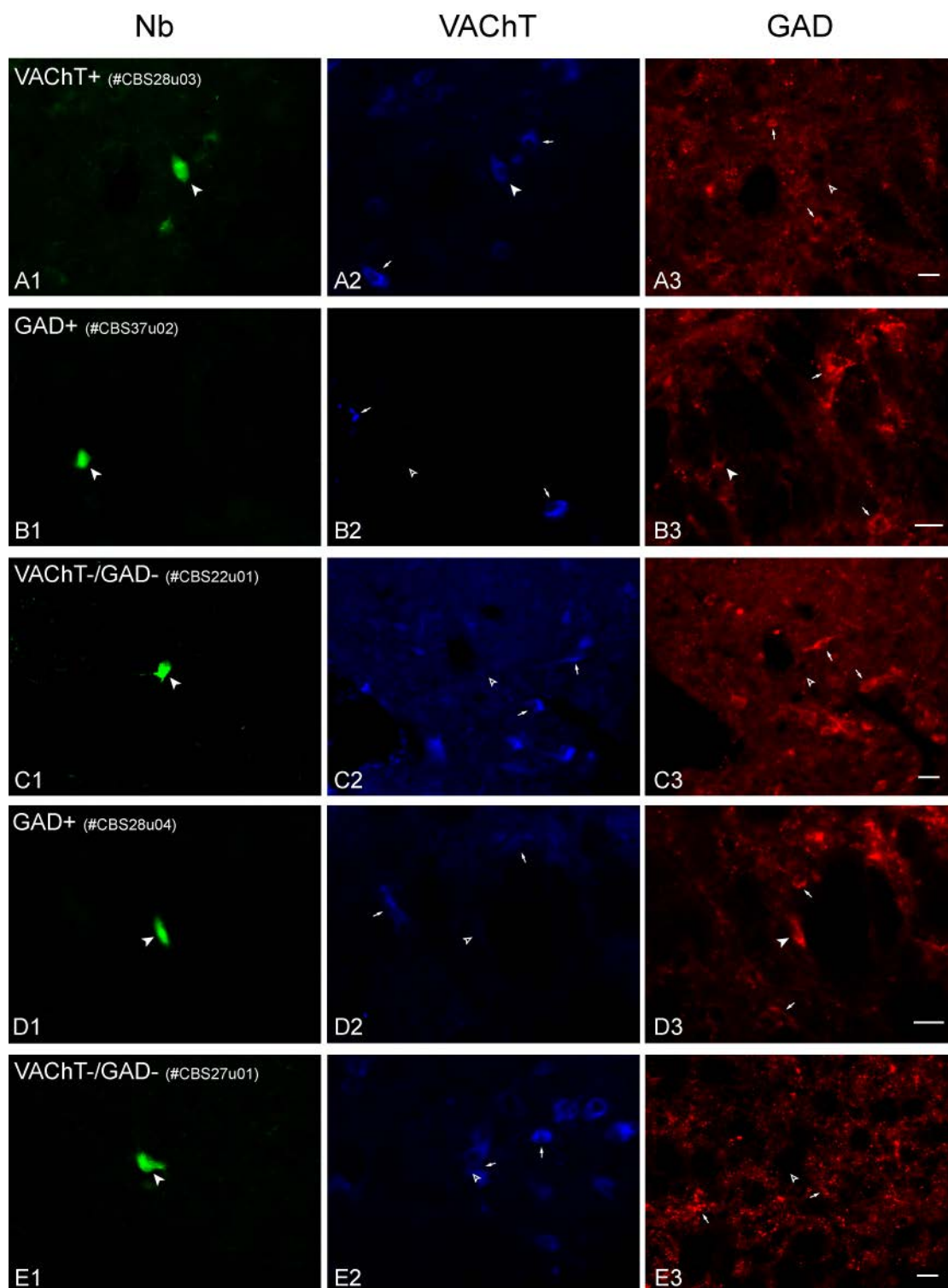


Figure 4.2. Immunostaining of recorded and Nb-labeled neurons

Figure 4.2. Immunostaining of recorded and Nb-labeled neurons. Five Nb⁺ cells (green, filled arrowheads are shown A-E in plates A1 to E1 and correspond to recorded units representative of the five major subgroups and presented in Figs. 4.4 – 4.8, respectively. **A1** Unit (#CBS28u03) classified as “W/PS-max” was immunopositive for VACHT (A2, blue, filled arrowhead), immunonegative for GAD (A3, red, open arrowhead), and located among other VACHT⁺ and GAD⁺ cells (small arrows) in the LDT. **B1** Unit (#CBS37u02), “W/PS-max”, was negative for VACHT (B2, blue, open arrowhead), positive for GAD (B3, red, filled arrowhead), and located among other VACHT⁺ and GAD⁺ cells (small arrows) in the SubLDT. **C1** Unit (#CBS22u01), “W/PS-max”, was negative for VACHT (C2, blue, open arrowhead) and GAD (C3, red, open arrowhead) and located among other VACHT⁺ and GAD⁺ cells (small arrows) in the LDT. **D1** Unit (#CBS28u04), “PS-max”, was negative for VACHT (D2, blue, open arrowhead), positive for GAD (D3, red, filled arrowhead) and located near other VACHT⁺ and GAD⁺ cells (small arrows) in the SubLDT. **E1** Unit (#CBS27u01), “PS-max”, was negative for VACHT (E2, blue, open arrowhead) and GAD (E3, red, open arrowhead) and located near other VACHT⁺ and GAD⁺ cells (small arrows) in the LDT. Scale bars, 20 μ m.

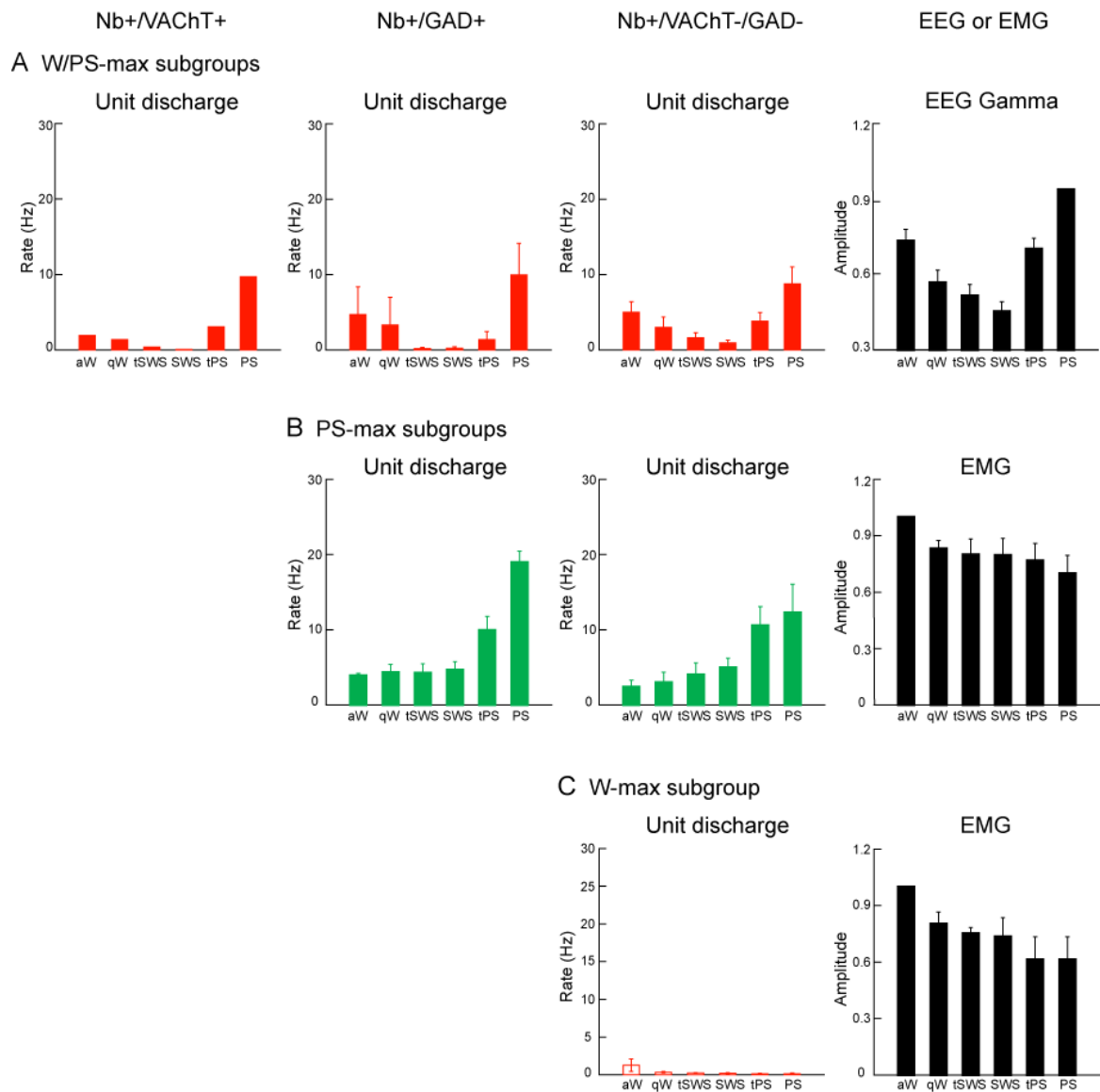


Figure 4.3. Mean discharge rates of different cell types and subgroups across sleep-wake stages in association with EEG and EMG activity.

Figure 4.3. Mean discharge rates of different cell types and subgroups across sleep-wake stages in association with EEG and EMG activity. **A**, Discharge rates of cholinergic (Nb+/VACHT+) (n=1), GABAergic (Nb+/GAD+) (n=4) and putative glutamatergic (Nb+/VACHT-/GAD-) (n=11) neurons (mean of average rates per stage per cell) that discharged maximally during aW and PS in positive correlation with gamma EEG activity (normalized average amplitude from 9 W/PS-max units) across sleep-wake stages. **B**, Discharge rates of GABAergic (n=3) and putative glutamatergic (n=9) neurons that discharged maximally during PS in negative correlation with EMG amplitude (normalized average amplitudes from 7 PS-max units) across sleep-wake stages. **C**, Discharge rates of putative glutamatergic neurons (n=4) that discharged maximally during aW in positive correlation with EMG amplitude (normalized average amplitudes from 3 W/PS-max units) across sleep-wake stages.

W/PS-max: Nb+/VACHT+ neuron (#CBS28u03)

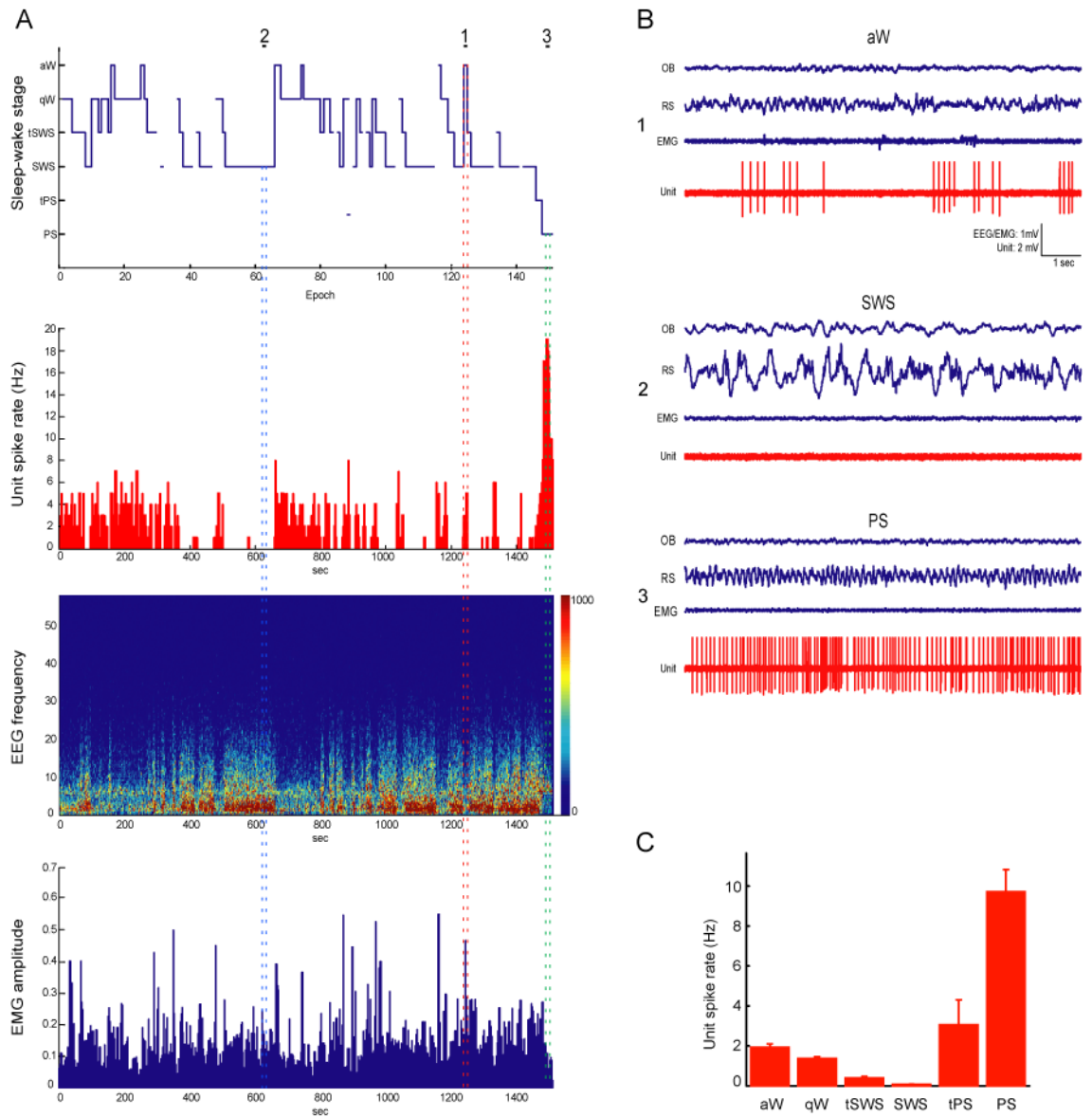


Figure 4.4. Discharge of cholinergic W/PS-max active unit across sleep-wake states.

Figure 4.4. Discharge of cholinergic W/PS-max active unit across sleep-wake states.

Data from Nb-labeled cell (#CBS28u03) that was immunopositive for VACHT (Fig. 4.2A). **A**, Sleep-wake recording, scored (per 10 sec epoch) for sleep-wake stages, is shown with simultaneous unit spike rate (Hz), EEG frequency and amplitude ($\mu\text{V}/\text{Hz}$ with frequency on y axis and amplitude scaled according to color from blue to red) and EMG amplitude (arbitrary units) over the recording session. Representative epochs indicated by dashed vertical lines of aW (red), SWS and PS (green) are shown in **B**. **B**, Polygraphic records from 10 sec epochs (indicated by horizontal lines in **A**) of the unit together with EEG and EMG activity during aW (1), SWS (2) and PS (3). **C**, Bar graph showing mean spike rate (Hz) of the unit across sleep-wake stages. Note that during aW, the unit discharged tonically at moderate rate with prominence of fast EEG activity, ceased firing during SWS in association with slow EEG activity ($\sim 1 - 4$ Hz), and discharged maximally and tonically to reach its highest rates during PS in association with prominent theta along with fast EEG activity. Abbreviations: OB, olfactory bulb; PF, prefrontal cortex; RS, retrosplenial cortex; aW, active wake; qW, quiet wake; tSWS, transition to slow wave sleep; SWS, slow wave sleep; tPS, transition to paradoxical sleep; PS, paradoxical sleep.

W/PS-max: Nb+/GAD+ neuron (#CBS37u02)

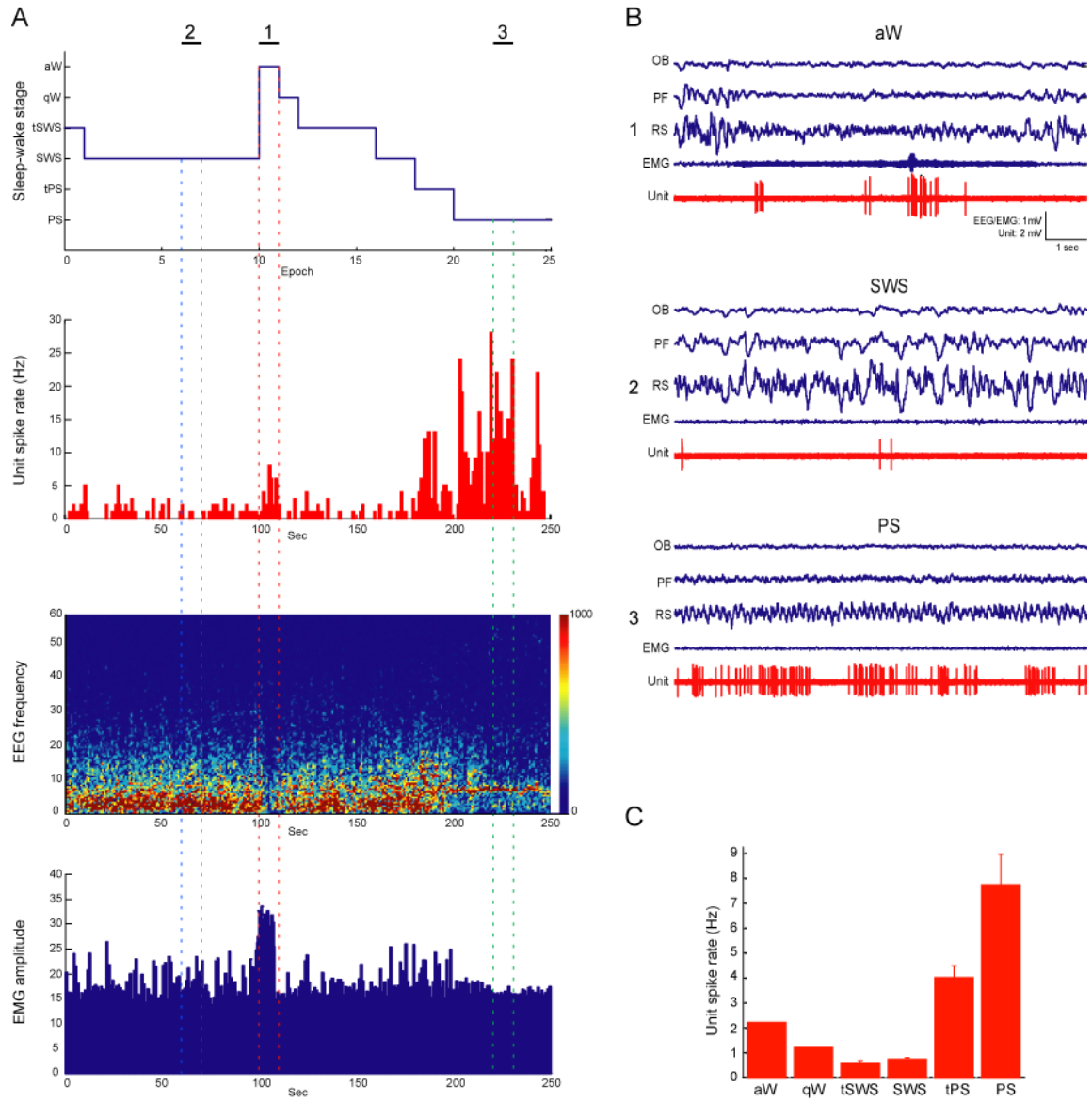


Figure 4.5. Discharge of GABAergic W/PS-max active unit across sleep-wake states.

Figure 4.5. Discharge of GABAergic W/PS-max active unit across sleep-wake states.

Data from Nb-labeled cell (#CBS37u02) that was immunoreactive for GAD (Fig. 4.2B). Note that the unit discharged moderately and phasically during aW, minimally during SWS and maximally during PS in an irregular phasic manner of clustered spikes when the EEG showed theta activity ($\sim 4.5 - 8$ Hz) along with fast activity. For details and abbreviations, see Fig. 4.4.

W/PS-max: Nb+/VAcHT-/GAD- neuron (#CBS22u01)

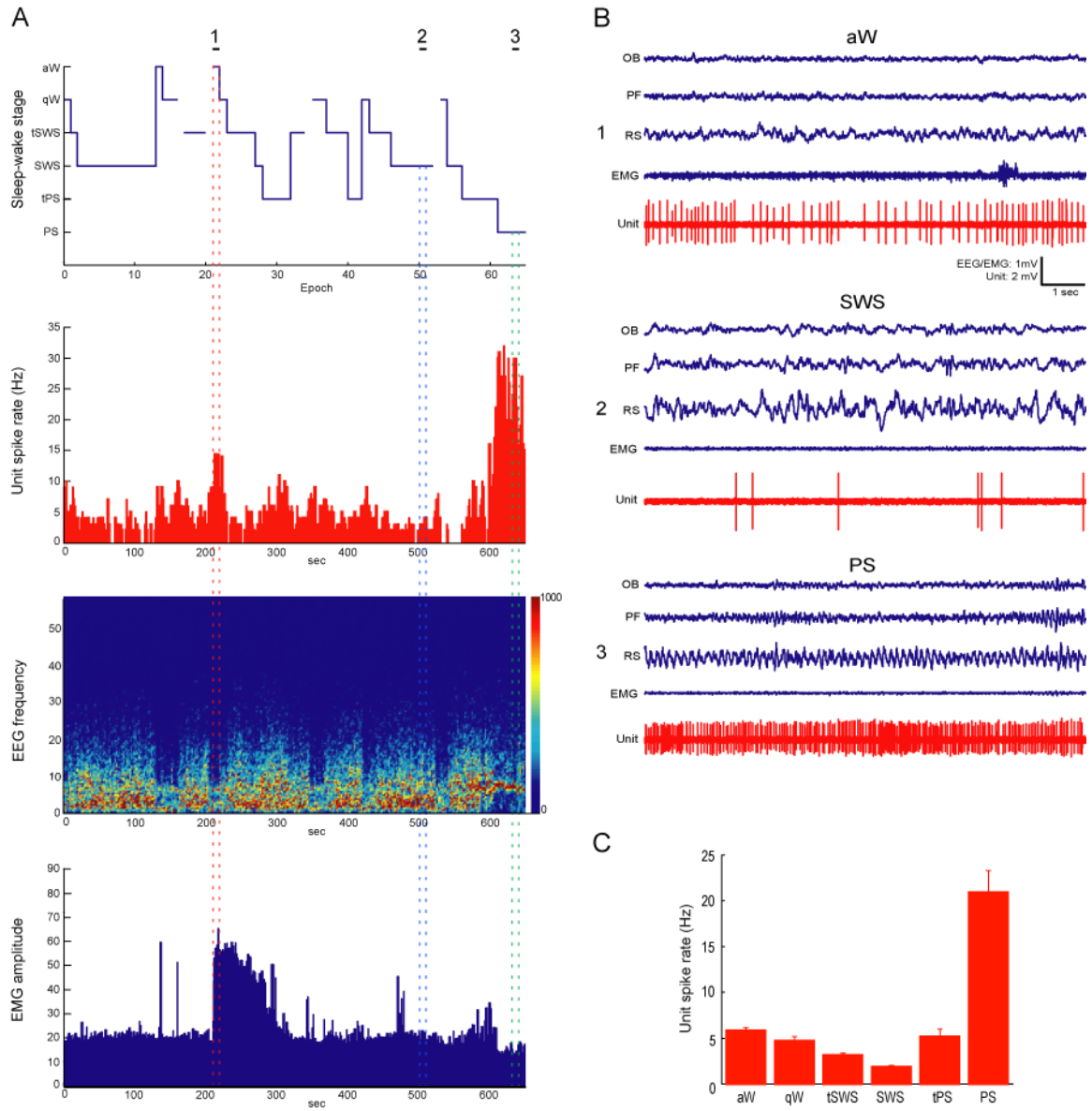


Figure 4.6. Discharge of putative glutamatergic W/PS-max active unit across sleep-wake states.

Figure 4.6. Discharge of putative glutamatergic W/PS-max active unit across sleep-wake states. Data from Nb-labeled cell (#CBS22u01) that was immunonegative for both VACht and GAD (Fig. 4.2C). Note that the unit discharged moderately during epochs of aW when the EEG showed theta (~4.5 - 8 Hz) along with fast activity, was relatively quiescent during SWS associated with slow EEG activity and discharged maximally and tonically to reach its highest rates during PS in association with prominent theta along with fast EEG activity. For details and abbreviations, see Fig. 4.4.

PS-max: Nb+/GAD+ neuron (#CBS28u04)

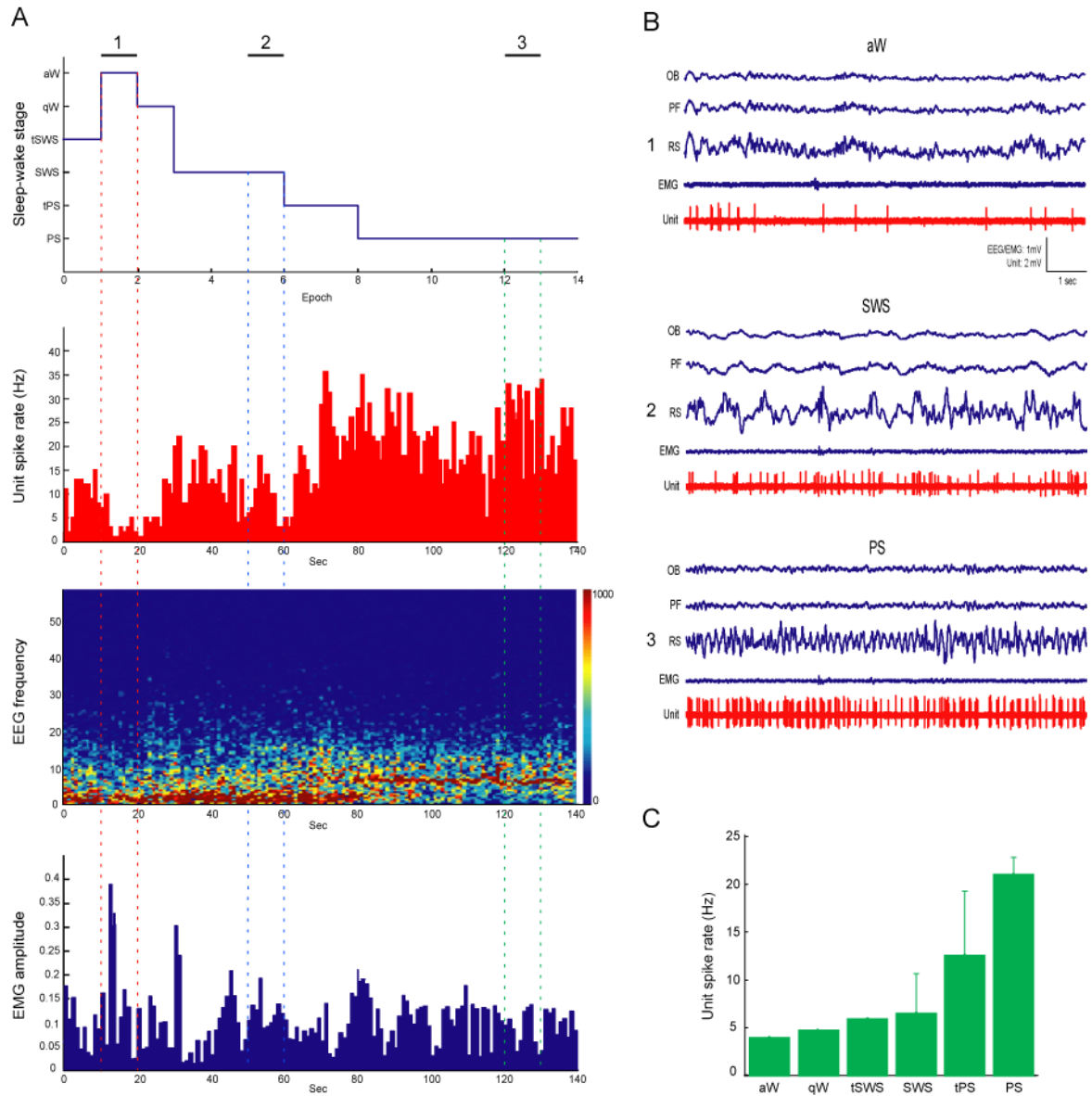


Figure 4.7. Discharge of GABAergic PS-max active unit across sleep-wake states.

Figure 4.7. Discharge of GABAergic PS-max active unit across sleep-wake states.

Data from Nb-labeled cell (#CBS28u04) that was immunostained for GAD (Fig. 4.2D). Note that the unit discharged at relatively low rates during aW with fast EEG activity and high EMG amplitude, increased firing during SWS in association with slow delta EEG activity and low muscle EMG and discharged maximally during PS in a phasic cluster pattern when theta and fast EEG activity was accompanied by muscle atonia. For details and abbreviations, see Fig. 4.4.

PS-max: Nb+/VACHT-/GAD- neuron (#CBS27u01)

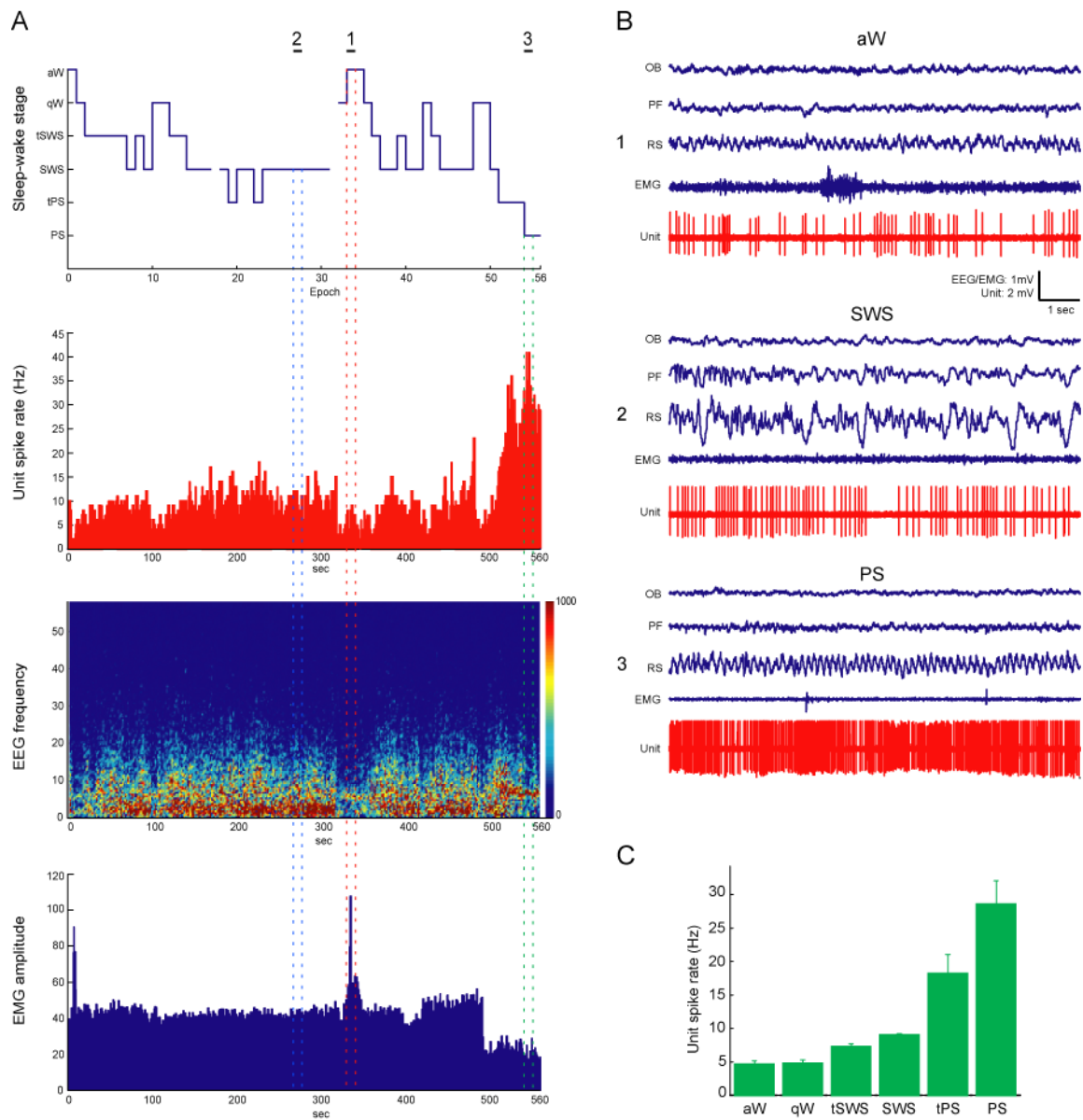


Figure 4.8 Discharge of putative glutamatergic PS-max active unit across sleep-wake states.

Figure 4.8 Discharge of putative glutamatergic PS-max active unit across sleep-wake states. Data from Nb-labeled cell (#CBS27u01) that was immunonegative for VACHT and GAD (Fig. 4.2E). Note that the unit discharged at its lowest rates during during aW with fast EEG activity and high neck muscle tone, increased its firing during SWS in association with slow EEG activity and low muscle tone and discharged maximally and tonically to reach its highest rate during PS in association with theta EEG activity and muscle. For details and abbreviations, see Fig. 4.4.

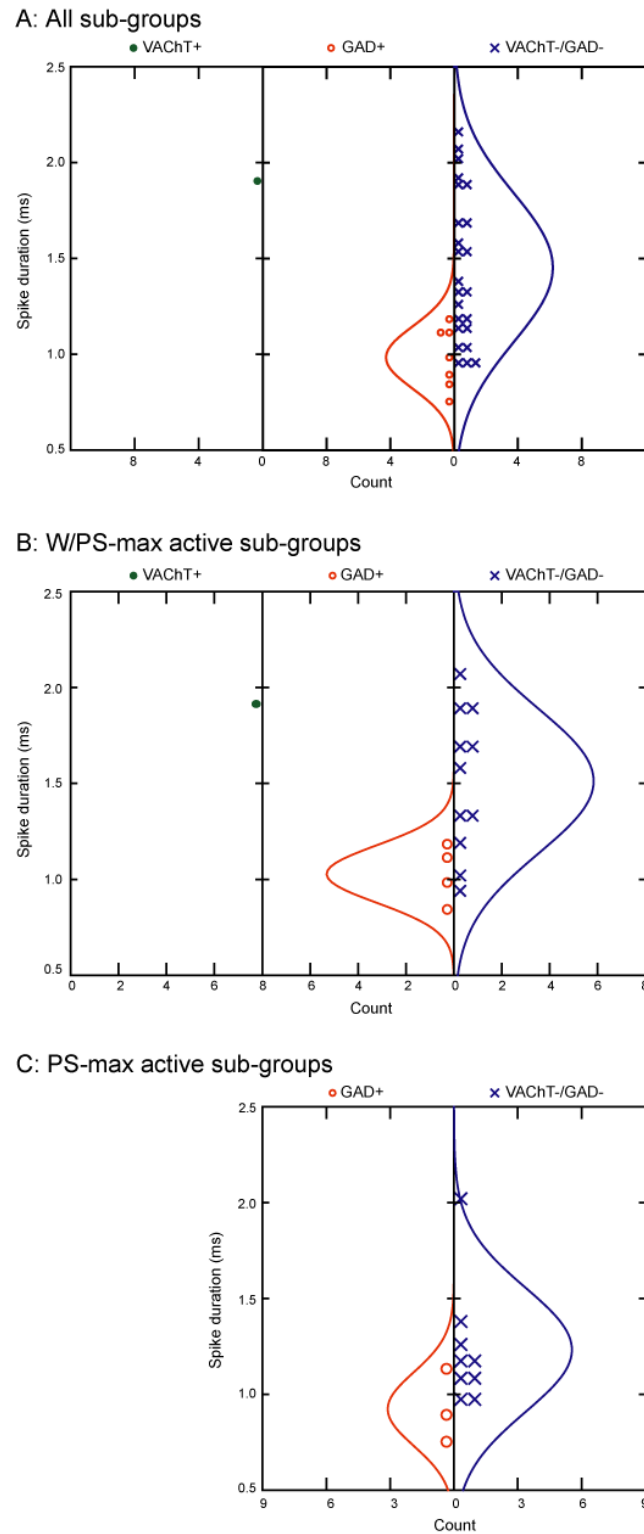


Figure 4.9. Comparison of spike duration among Nb+/VACHT+, Nb+/GAD+ and Nb+/ VACHT-/GAD- neurons.

Figure 4.9. Comparison of spike duration among Nb+/VACHT+, Nb+/GAD+ and Nb+/ VACHT-/GAD- neurons. A, Average spike duration (for second zero crossing, see Methods) among different cell groups with their distribution: VACHT+ cells (1.90 ms, n = 1), GAD+ cells (mean $\pm$ SD, 0.98 ± 0.16 ms, n = 7) and VACHT-/GAD- cells (1.45 ± 0.39 ms, n = 24). Note that despite a significant difference in means between GAD+ and VACHT-/GAD- cell groups (*t test*, $p < 0.05$, df = 29), the values for each cell group are highly variable and overlap. Note also that the one VACHT+ cell has a relatively long spike duration. B, Average spike duration of the W/PS-max active sub-groups: VACHT+ cells (1.90 ms, n = 1), GAD+ cells (1.03 ± 0.15 ms, n = 4) and VACHT-/GAD- cells (1.51 ± 0.38 ms, n = 11). Note that despite a significant difference in means between GAD+ and VACHT-/GAD- cell groups (*t test*, $p < 0.05$, df = 13), the values for each cell group overlap. C, Average spike duration of the PS-max active sub-groups: GAD+ cells (0.92 ± 0.19 ms, n = 3) and VACHT-/GAD- cells (1.23 ± 0.32 ms, n = 9). The means were not significantly different (*t test*, $p > 0.05$, df = 10).

5. Chapter Five

Discussion

A portion of this chapter was published in Journal of Neuroscience, Vol 29(14): 4664-4674, 2009

5.1 The activity profiles of LDT/PPT neurons in anesthetized rats

This study shows that in rats under urethane anesthesia, identified cholinergic pontomesencephalic neurons discharge in association with evoked cortical activation, whereas intermingled GABAergic and noncholinergic/nonGABAergic cells discharge maximally either in association with cortical activation in a parallel manner or with cortical slow wave activity in a reciprocal manner to the cholinergic cells. Thus while revealing the heterogeneity of co-distributed cholinergic, GABAergic and putative glutamatergic pontomesencephalic neurons, these results provide a basis for understanding the way in which these different cell groups might act in a coordinated manner to stimulate cortical activation and modulate sleep-wake states.

The electrophysiological properties of the three cell groups overlapped extensively and as such confirmed the importance of juxtacellular labeling and immunohistochemical staining of recorded cells for their unequivocal identification according to neurotransmitter phenotype. Based upon evidence in one study in anesthetized rats that neurons with long duration spikes (0.95 ± 0.14 , mean $\pm$ SD, for first zero crossing measured at zero line) were stained for nicotinamide adenine dinucleotide phosphate-diaphorase (Koyama et al., 1998), such cells were selected in studies of unanesthetized rats and cats as ‘presumptive’ or ‘possibly’ cholinergic cells (Kayama and Ogawa, 1987; El Mansari et al., 1989; Kayama et al., 1992). Yet, only in the present study have cells in the LDT/PPT been randomly sampled and identified as ChAT⁺, GAD⁺ or ChAT⁻/GAD⁻ to examine the full range of spike durations for each cell type. The measurements were found to overlap extensively among cell groups here, as also previously found with intracellular recording and labeling (Takakusaki et al., 1997), such that spike duration

could not be used to distinguish an individual cell as cholinergic or noncholinergic. Given a comparable overlap in average discharge rate and instantaneous firing frequency, these characteristics could also not be used alone or together with spike duration to identify individual cells as ‘presumptive’ cholinergic, GABAergic or noncholinergic/nonGABAergic.

Out of 73 Nb-labeled cells in the LDT/SubLDT/MPPT area, 15% were cholinergic, 40% were GABAergic and 45% were noncholinergic/nonGABAergic, proportions which correspond approximately to the relative frequency of these cell types in the LDT/PPT region (Ford et al., 1995; Wang and Morales, 2009). Based upon *in situ* hybridization for the vesicular glutamate transporter, VGluT2, the noncholinergic/nonGABAergic neurons would appear to be glutamatergic (Wang and Morales, 2009). Immunohistochemical staining for VGluT2 has also proven a glutamatergic identity of noncholinergic/nonGABAergic neurons in the basal forebrain (Henny and Jones, 2008). We thus propose that the VAcHt-/GAD- Nb-labeled neurons recorded here in the LDT correspond to putative glutamatergic neurons.

5.1.1 Cholinergic neurons

The cholinergic LDT/SubLDT/MPPT neurons formed a homogeneous group that discharged minimally in an irregular manner (~0.5 Hz) during irregular slow EEG activity and then in a tonic sustained manner at a moderate rate (~4 Hz) in response to somatic stimulation in association with increased high beta-gamma and theta-like EEG activity. In contrast to a recent study reporting phasic modulation of cholinergic PPT neurons in association with nested gamma oscillations during slow oscillations in urethane/xylazine/ketamine anesthetized rats (Mena-Segovia et al., 2008), the present

study did not find any phasic modulation or cross correlation with slow EEG activity of the cholinergic cell discharge during spontaneous, pre-stimulation conditions in the urethane anesthetized rats. The presence of such phasic activity in association with gamma during slow oscillations could well be due to xylazine/ketamine anesthesia which is associated with highly synchronous slow wave activity that often develops into spike-wave seizure activity in the cortex and transmission through corticofugal projections to thalamic and subcortical networks (Timofeev et al., 1998). Moreover, ketamine is associated with anomalous high amplitude and coherent gamma activity in the cortex (Pinault, 2008), whereas urethane is associated with decreased gamma activity, similarly to natural SWS (Maloney et al., 1997; Clement et al., 2008), which can moreover be augmented as in the present study by somatic sensory stimulation. Indeed, as for the basal forebrain cholinergic neurons which have been studied both under urethane anesthesia and during natural sleep (Manns et al., 2000a; Lee et al., 2005b), the LDT/PPT cholinergic neurons appear to be relatively silent in association with slow wave activity. In contrast to the former, however, the LDT/PPT cholinergic neurons did not show any phasic, rhythmic discharge in association with theta-like EEG activity. Rhythmic bursting by basal forebrain cholinergic neurons had been shown *in vitro* to be driven by low threshold calcium spikes (Khateb et al., 1992), which were also found in LDT cholinergic neurons, though rarely associated with bursts (Leonard and Llinas, 1990; Kamondi et al., 1992). Here *in vivo*, no spike bursts were observed in cholinergic LDT/SubLDT or MPPT cells. They would thus appear to provide a tonic slow input to their target neurons. According to the postsynaptic actions of Acetylcholine (ACh) in the thalamus, their tonic discharge could change the mode of firing of thalamic cells from bursting, as occurs with spindling and slow wave oscillations during SWS, to tonic, as

occurs with fast cortical activity during waking (McCormick, 1992; Steriade, 1993). In reaction to somatic stimulation, the vast majority of cholinergic neurons started to increase their firing rate before the increase in fast cortical activity, suggesting that they are among cells of the ascending activating system that are responsible for eliciting cortical activation through excitation of thalamic, hypothalamic and basal forebrain relays.

5.1.2 GABAergic neurons

The GABAergic LDT/SubLDT/MPPT neurons were heterogeneous in both their physiological and morphological properties. They comprised “On” (~48%) and “Off” (~38%) cells, which respectively increased or decreased their discharge in response to somatic stimulation. “On” and “Off” GABAergic subgroups each included small, presumed locally projecting neurons and a contingent of medium-large, presumed long projecting neurons, which send their axons into the forebrain (Ford et al., 1995). The GABAergic “On” neurons discharged and behaved in a similar manner to the cholinergic cells by increasing their rate of discharge and firing tonically during somatic stimulation. They could thus act in parallel with the cholinergic neurons either as locally projecting neurons, inhibiting other “Off” cells in the LDT region, or as projection neurons to other “Off” cells in distant regions. The GABAergic “Off” cells behaved in a reciprocal manner to the cholinergic cells. These GABAergic “Off” cells could thus normally play a role in dampening cortical activation by exerting an inhibitory influence on other neurons of the activating system. Yet, in contrast to the cholinergic neurons, most GABAergic neurons changed their rate of firing after the change in EEG evoked by somatic stimulation. This time course suggests that the GABAergic cells may be secondarily

modulated by neighboring cells, including the cholinergic via release of ACh and action upon the muscarinic type 1 (M1) ACh receptors (M1 AChRs) for excitation of “On” cells, or the muscarinic type 2 (M2) ACh receptors (M2 AChRs) for inhibition of “Off” cells. Different responses to ACh have been found among identified GABAergic neurons recorded in the pontine tegmentum *in vitro* (Brown et al., 2008). And with immunohistochemical staining, M2 AChRs have been visualized upon GABAergic neurons in subLDT and adjacent pontine tegmentum (Brischoux et al., 2008). The GABAergic neurons could also be influenced by the local glutamatergic neurons (below) or by other distant neurons involving feedback from the thalamus, hypothalamus and/or basal forebrain.

5.1.3 Putative glutamatergic neurons

Like the GABAergic neurons, the putative glutamatergic neurons were also heterogeneous in their properties and response to somatic stimulation, thus also comprising “On” (~49%) and “Off” (~35%) cell groups, which similarly varied in size and shape. In this case, however, the response of the putative glutamatergic neurons generally preceded the changes in cortical activation, as it did for the cholinergic cells. Indeed, the glutamatergic “On” cells could act in parallel to the cholinergic cells and form an important contingent of long ascending projections from the LDT/PPT into the forebrain (Pare et al., 1988; Jones and Cuello, 1989; Rasmusson et al., 1994; Ford et al., 1995). The putative glutamatergic “On” and “Off” cells could also control the local GABAergic “On” and “Off” cells (above).

Given the impossibility of identifying recorded cells as cholinergic, GABAergic or putative glutamatergic according to discharge properties here in anesthetized animals,

it is also not possible to postulate that they correspond to cells previously recorded in unanesthetized animals and characterized as discharging in particular sleep-wake states. Nonetheless, given the homogeneous response profile of cholinergic LDT/SubLDT/MPPT neurons found here, it can be stated that these cells discharge in association with cortical activation and thus could do so during both waking (W) and paradoxical sleep (PS), as was established to be the case for identified cholinergic neurons in the basal forebrain (Manns et al., 2000a; Lee et al., 2005b). Indeed, ACh release in the thalamus, which would arise largely from the brainstem cholinergic neurons, is low during slow-wave sleep (SWS) and high during both W and PS in association with cortical activation (Williams et al., 1994). In the LDT/PPT region of both cat and rat, W-PS active cells were previously postulated to be ‘presumptive’ or ‘possibly’ cholinergic neurons (El Mansari et al., 1989; Steriade et al., 1990a; Kayama et al., 1992). However, our results would indicate that such W-PS cells likely also include noncholinergic cells. ‘Possibly’ cholinergic neurons were also proposed to be most active during PS and relatively silent during waking (Kayama et al., 1992). Yet, such PS-active cells also increased their discharge during SWS relative to W (Steriade et al., 1990a; Kayama et al., 1992). They would thus more likely correspond to noncholinergic cells which were found here to have a higher rate of discharge during spontaneous irregular slow activity and thus to GABAergic or putative glutamatergic “Off” neurons. Such “Off” cells could thus gradually increase their rate of firing during sleep to fire maximally during PS in association with decreasing muscle tone in the natural sleep cycle. GABAergic “Off” cells could progressively inhibit other surrounding neurons during sleep, such as the serotonergic or noradrenergic cells which discharge during W and turn off during sleep to be silent during PS (McGinty and Harper, 1976a; Aston-Jones and

Bloom, 1981; Maloney et al., 1999). Discharging in parallel but also in the lead, glutamatergic “Off” cells may normally excite these GABAergic “Off”, postulated sleep-active cells in the region. Cells classified as W-active, which discharge during W and not during SWS or PS (Kayama et al., 1992), might include some of the putative glutamatergic and GABAergic “On” cells recorded here. Clearly, future studies applying recording with juxtacellular labeling in naturally sleeping-waking rats will be necessary to elucidate the precise roles of these cell groups in sleep-wake states. The present results indicate that cholinergic, GABAergic and putative glutamatergic neurons can function in parallel or reciprocal manners to modulate cortical activity and behavioral state across the sleep-waking cycle.

5.2 The activity profiles of LDT/PPT neurons during natural sleep-wake states

In the present study, we characterized for the first time the discharge profiles across natural sleep-wake states of three types of chemically identified neurons within the LDT/PPT: cholinergic, GABAergic and non-cholinergic/non-GABAergic neurons, which are referred to as "putative glutamatergic" neurons. These chemically identified neurons were functionally classified in three distinct sub-groups: (1) W/PS-max active sub-group, which had a discharge profile that correlated positively with fast EEG activity and included cholinergic, GABAergic and putative glutamatergic neurons, (2) PS-max active sub-group, which had a discharge profile that correlated negatively with EMG activity and included GABAergic and putative glutamatergic neurons, and (3) W-max active sub-group, which had a discharge profile that correlated positively with EMG activity and included only putative glutamatergic neurons. The discharge profiles of these different sub-groups were related to either fast EEG activity (W/PS-max active sub-group) or to EMG activity in a reciprocal manner (W-max active and PS-max active sub-groups), suggesting the potential role of the intermingled LDT/PPT neurons in modulating cortical or behavioral changes across sleep-wake states. Additionally, no SWS-max active cells (those that discharge maximally during SWS) were found in the LDT/PPT area, suggesting the particularity of this region in controlling W and PS states rather than SWS (Steriade et al., 1990a; Jones, 2005, 2008). Of the total cells recorded and identified in LDT/PPT, all cells of the PS-max active sub-group and many of the W/PS-max active sub-group discharged at their maximal rates during PS. Interestingly, most of these cells started to increase their rate of discharge prior to PS during the transition (tPS),

suggesting the potential role of different LDT/PPT cell groups in triggering the PS state and/or in controlling different activities related to PS (Webster and Jones, 1988; Vanni-Mercier et al., 1989; Sakai et al., 1990).

The cells of the W/PS-max active sub-group that discharged in positive correlation with fast EEG activity could potentially influence cortical activation via ascending pathways to the forebrain, as reflected in fast EEG activity during both W and PS (Satoh and Fibiger, 1986; Steriade et al., 1990a). These cells could correspond to the "On-cells" found in our acute study in anaesthetized rats, whose discharge was positively associated with evoked cortical activation. The cells of the PS-max active sub-group that discharged in negative correlation with EMG activity could potentially participate in dampening muscle tone and behavioral arousal, as reflected in minimal EMG amplitude during sleep, particularly during PS (Velazquez-Moctezuma et al., 1989; Baghdoyan and Lydic, 1999; Brischoux et al., 2008). These cells could possibly correspond to the "Off-cells" found in our acute study. The Off-cells were negatively associated with evoked cortical activation and were active during irregular slow-wave activity, possibly acting to dampen muscle tone and behavioral arousal.

Out of the 32 Nb⁺ cells identified in the LDT/PPT area, 3% were cholinergic, 22% were GABAergic and 75% were putative glutamatergic cells. This sample of identified cells does not conform to the proportions found in this area in our acute study (see above) or in histochemical studies (Ford et al., 1995; Wang and Morales, 2009). The relatively low numbers obtained in the present study, particularly of cholinergic neurons and also to a lesser degree of GABAergic neurons, could be due mainly to the challenging recording conditions in the pontomesencephalic tegmentum, which necessitated an angled approach of the recording electrode, resulting in greater recording instability, particularly

during aW. Some cells were difficult to hold through the full sleep-waking cycle and particularly during the transition from PS to aW, often associated with sudden movement. Thus, cells were often lost at the end of PS upon waking and possibly cholinergic cells were among those that are more vulnerable to such physical trauma. The particular paucity of cholinergic neurons in our findings could also be due to the difficulty of capturing them while active, particularly since recording is done mostly during SWS, when cholinergic neurons appear to be silent in the pontomesencephalic, as they are in the basal forebrain (Lee et al., 2005b). Since only one neuron per side per animal could be labeled, only a small number of cells could be submitted to the juxtacellular labelling procedure and of those less than 80% were found to be labelled. Moreover, since for each rat a minimum time of three weeks was necessary for recovery from surgery and habituation to the head-fixation before recording began, only a limited number of animals could be recorded over one year. Thus, the present approach and project requires considerable time to obtain adequate samples for the different cell populations.

Based on their anatomical and physiological features, LDT/PPT Nb+/VACHT+, Nb+/GAD+ and Nb+/VACHT-/GAD- cells could not be clearly distinguished. Moreover, except for the spike duration measurements between two W/PS-max active sub-groups, Nb+/VACHT+, Nb+/GAD+ and Nb+/VACHT-/GAD- cells did not show significant differences in their electrophysiological properties. This lack of clear differentiation was due to an extensive overlap in their respective properties. The overlap in electrophysiological properties was also found between chemically different LDT/PPT neurons in our acute study (see above) as well as between congener basal forebrain neurons (Hassani et al., 2009). Chemically different neurons could thus have similar or equivalent electrophysiological properties and discharge profiles across sleep-wake states.

It is thus not possible to distinguish between individual cells based only upon their electrophysiological characteristics. Therefore by identifying the neurotransmitter phenotypes of the recorded cells with immunohistochemical staining, the present study overcame the fundamental limitations of previous electrophysiological studies (El Mansari et al., 1989; Steriade et al., 1990b; Kayama et al., 1992) that attempted to identify cell populations based upon their electrophysiological properties.

5.2.1 Cholinergic neurons

The cholinergic neuron found in the present study was a W/PS-max active cell. It discharged maximally during both waking and PS with higher discharge during PS than waking. This neuron was silent during SWS. Supporting evidence would suggest that this single neuron could be representative of the population of LDT/PPT cholinergic neurons as W/PS-active cells: (1) In our acute study, one homogenous group of cholinergic neurons was found that increased firing in association with evoked cortical activation, mimicking fast EEG activity during both waking and PS states; (2) ACh is released in high quantities during both waking and PS in the thalamus (Williams et al., 1994), where the cholinergic LDT/PPT neurons project (Sofroniew et al., 1985; Woolf and Butcher, 1986; Hallanger et al., 1987; Jones and Webster, 1988; Pare et al., 1988; Steriade et al., 1988); (3) Basal forebrain cholinergic neurons form one homogenous group found to be W/PS-max active neurons (Lee et al., 2005b). Previous recording studies of unidentified neurons in LDT/PPT found two major groups (El Mansari *et al.*, 1989; Steriade *et al.*, 1990b; Kayama *et al.*, 1992) that were proposed to be ‘possibly’ cholinergic, one as W/PS-active and another as PS-active. The W/PS-active group could correspond to the cholinergic neuron identified here, whereas the PS-active group could

correspond to the GABAergic and/or glutamatergic PS-max active neurons identified here. It is thus possible to suggest from this evidence that LDT/PPT cholinergic neurons are W/PS-max active neurons.

However, in addition to the preceding suggestions, there is another hypothesis that proposes reciprocal roles between cholinergic and monoaminergic systems in eliciting PS and waking respectively, in which cholinergic neurons are proposed to be PS-active while monoaminergic neurons are W-active (McCarley and Hobson, 1975). Electrophysiological studies had indicated the presence of two sub-groups of possible cholinergic neurons in LDT/PPT, one W/PS-active and the other PS-active (El Mansari et al., 1989; Steriade et al., 1990a; Kayama et al., 1992; Thakkar et al., 1998). Some of these studies suggested that cholinergic PS-active neurons, particularly located in the lateral PPT (lPPT) are related to the phasic, ponto-geniculo-occipital (PGO) waves that characterize PS (El Mansari et al., 1989; Steriade et al., 1990a; Koyama and Sakai, 2000). Thus, the possible existence of another sub-group of cholinergic neurons, perhaps located in the lPPT, that are exclusively PS-max active neurons, in addition to the W/PS- max active subgroup found here in the LDT and in the medial PPT (mPPT), cannot be excluded.

As mentioned above, the present results showed that the LDT/PPT cholinergic neuron discharged at its highest rate during PS. Like the majority of the recorded cells, it started discharging prior to PS during tPS and increased its rate to maintain a high level during the entire period of PS. Thus, during PS, LDT/PPT cholinergic neurons could drive cortical activation through their two ascending pathways, the dorsal and the ventral (Fig. 5.1; Jones, 2004). They could also participate in the cognitive correlate of PS, dreaming (Hobson, 1992), by either direct stimulation of the limbic and cortical structures

to which they project (Satoh and Fibiger, 1986) or indirectly by exciting the ventral tegmental area (VTA) dopaminergic neurons (Forster and Blaha, 2000) that were found to burst during PS (Dahan et al., 2007). The VTA dopaminergic neuron could in turn release dopamine in their target areas, such as limbic and cortical structures to which these neurons project (Fallon and Moore, 1978). The activation of these terminal structures could be responsible for the cognitive correlate of PS (Portavella and Vargas, 2005; Behrendt, 2010).

Via their innervation of the PS effector zone in the pons, which includes the oral pontine reticular formation (PnO) and the subcoeruleus (SubC) regions of the medial and lateral pontine tegmentum (Jones, 2004), LDT/PPT cholinergic neurons could excite the ascending projecting glutamatergic W/PS-max neurons probably through M1 AChRs (Greene and Carpenter, 1985; Greene et al., 1989). These reticular neurons would participate in generating the EEG components of PS, cortical activation, reflected in high gamma with theta activity (Fig. 5.1; Jones, 2004). Yet, the action of LDT/PPT cholinergic neurons on the PS effector neurons could also induce the EMG components of PS, muscle atonia (Jones, 2004). Such muscle atonia could be elicited in part by inhibition, through the M2 AChRs, of the putative glutamatergic W-active reticulospinal neurons concentrated in the PnC (Brischoux et al., 2008) resulting in disfacilitation of motor neurons in the brainstem and spinal cord (Fig 5.1). Cholinergic neurons could also inhibit GABAergic W-active neurons that are located in the PnO through their M2 AChRs (Brischoux et al., 2008) and are suggested to inhibit their neighboring putative glutamatergic PS-active neurons (Maloney et al., 2000). They could also stimulate, via M1 AChRs, putative glutamatergic PS-active neurons (Greene and Carpenter, 1985; Greene et al., 1989). These PS-active neurons could in turn stimulate

GABAergic/glycinergic neurons located in the ventral medullary gigantocellular reticular formation (GiV) that could inhibit motoneurons in brainstem and spinal cord, resulting in muscle atonia during PS (Fig. 5.1).

The present results also showed that the LDT/PPT cholinergic neuron was active during wakefulness, although at lower frequency than during PS. Indeed, other evidence has suggested that the LDT/PPT cholinergic neurons could be active during wakefulness: (1) In vivo recordings of possible LDT/PPT cholinergic neurons found that they fire tonically at low to moderate rates during wakefulness (Sakai, 1985; El Mansari et al., 1989; Steriade et al., 1990a; Kayama et al., 1992); (2) High levels of ACh were reported to be released in the thalamus during waking (Williams et al., 1994); (3) Stimulation of cholinergic neurons by injection of a high dose of the excitatory amino-acid, L-glutamate into the PPT induced a waking state (Datta and Siwek, 1997). Thus, during waking, cholinergic neurons could stimulate thalamo-cortical neurons and block their rhythmic activity resulting in EEG activation and desynchronization (Steriade, 1993).

Since cholinergic neurons are active during wakefulness as well as during PS, we can then ask why cholinergic neurons do not elicit PS features, particularly muscle atonia during wakefulness? In fact, it was shown that the injection of the acetylcholinesterase inhibitor physostigmine (eserine) elicited a PS-like state with muscle atonia, yet only after the depletion of monoamines with reserpine (Karczmar et al., 1970). It is suggested therefore that monoaminergic neurons, which are active during wakefulness (McGinty and Harper, 1976b; Trulson and Jacobs, 1979; Aston-Jones and Bloom, 1981) and exert an inhibitory influence on cholinergic neurons (Luebke et al., 1992), may play a protective role to prevent cholinergic neurons from eliciting PS features during wakefulness (McCarley and Hobson, 1975). By considering that all cholinergic neurons

are W/PS-active, it is possible that monoaminergic neurons act to dampen the activity of cholinergic neurons during arousal but not to block their firing completely. Yet, the action of monoaminergic neurons in preventing cholinergic neurons from triggering PS during waking could also occur through postsynaptic actions on the cholinceptive neurons of the PS effector zone, where many monoaminergic in addition to cholinergic neurons project (Semba, 1993). Accordingly, reticulospinal and other neurons in the pontine reticular formation could be excited by noradrenaline or serotonin and inhibited by ACh through M2 AChRs (Greene et al., 1989). It is thus probable that monoaminergic and cholinergic neurons act on the same pontine neurons respectively to prevent and to elicit PS with muscle atonia (Fig. 5.1). The same counterbalancing role could be taken by orexin (also called hypocretin) neurons with cholinergic neurons, since orexin neurons, which are active only during waking (Lee et al., 2005a), project to the brainstem and can excite cholinceptive reticular neurons which are inhibited by ACh through M2 AChRs (Brischoux et al., 2008). Indeed, the deficiency of the orexinergic system in the hypothalamus results in narcolepsy with cataplexy (Chemelli et al., 1999; Lin et al., 1999; Peyron et al., 2000; Thannickal et al., 2000). This cataplexy could be induced by cholinergic neurons in the absence of orexin neurons, which degenerate in patients having narcolepsy with cataplexy (Thannickal et al., 2000).

It is thus likely that cholinergic neurons could promote cortical activation during waking when they are prevented from inducing muscle atonia either through dampening of their activity by monoaminergic neurons (McCarley and Hobson, 1975) and/or by the counterbalancing action of monoaminergic and orexin neurons on reticular, including reticulospinal, neurons (Stevens et al., 1992; Brischoux et al., 2008).

LDT/PPT cholinergic neurons could thus stimulate cortical activation, during waking and during PS, when they also could promote tonic motor inhibition and muscle atonia (Fig. 5.1).

5.2.2 GABAergic neurons

In the present study, two different sleep-wake sub-groups of LDT/PPT GABAergic neurons were found, W/PS-max active sub-group (57%) and PS-max active sub-group (43%).

5.2.2.1 GABAergic W/PS-max neurons

The GABAergic neurons of the W/PS-max active sub-group, which are predominantly fast firing, discharged in positive correlation with fast gamma EEG activity. These neurons likely correspond to the "GABAergic-On" neurons found in our acute study. It was previously shown that some GABAergic neurons in the LDT/PPT give rise to ascending projections to the forebrain (Ford et al., 1995). Thus, as projecting neurons in parallel with cholinergic neurons (Steriade et al., 1988; Ford et al., 1995), these GABAergic neurons could participate in modulating fast cortical activity by either fast pacing (Somogyi and Klausberger, 2005) or the disinhibition (Freund and Meskenaite, 1992) of their targeted neurons. They could also inhibit SWS active neurons found in the mesencephalic reticular formation (Huttenlocher, 1961; Steriade et al., 1982) which could be GABAergic and inhibit in turn glutamatergic W/PS-active neurons of that region (Fig. 5.1; Jones, 2010).

5.2.2.2 GABAergic PS-max neurons

The GABAergic cells of the PS-max active sub-group, which are all fast firing neurons, increased their discharge rate progressively across sleep stages to discharge at their maximum rate during PS. They could correspond to some neurons with brief action potentials and high frequency discharge during PS that were previously recorded but not identified in the LDT/PPT (Steriade et al., 1990a). Yet the latter could also correspond in part to non-GABAergic, putative glutamatergic neurons with the same characteristics recorded in the present study (see below). Indeed, according to a c-Fos expression study, GABAergic neurons in LDT/PPT have been demonstrated to be active during PS (Maloney et al., 1999). The GABAergic PS-max active neurons could thus participate in dampening behavioral arousal during sleep, particularly during PS. This decrease in behavioral arousal might be due to an inhibition of monoaminergic neurons in the region. Indeed, a greater release of GABA was found in both the locus coeruleus (LC) and dorsal raphe (DR) during PS compared to SWS and waking (Nitz and Siegel, 1997a; Nitz and Siegel, 1997b). Microinjections of GABA agonists induced a decrease in the activity of serotonergic neurons (Gallager and Aghajanian, 1976) and an increase of PS (Lancel et al., 1996; Nitz and Siegel, 1997a). In contrast, microinjection of the GABA_A antagonist, bicuculline, induced an increase in the activity of serotonergic neurons (Levine and Jacobs, 1992). Bicuculline had the same effect in the LC, where its microinjection resulted in a reversal of the suppression of noradrenergic neuronal activity (Gervasoni et al., 1998). This microinjection also resulted in a decrease in PS (Kaur et al., 1997). Taken together, this evidence suggests that LDT/PPT GABAergic neurons could inhibit monoaminergic neurons during PS to prevent behavioral arousal (Fig. 5.1).

There is evidence that GABA blocks reticular PS-On neurons in the PnO/SubC region, the PS effector zone, to induce PS. Indeed, injections of the GABA_A antagonist, bicuculline, into PnO region elicited state of PS (Xi et al., 1999; Sanford et al., 2003), whereas injections of the GABA_A agonist, muscimol, into the same region suppressed PS (Sanford et al., 2003). Moreover in c-Fos studies, it appeared that GABAergic neurons in the PnO become inactive during PS (Maloney et al., 2000). It is thus possible that LDT/PPT GABAergic PS-max active neurons inhibit these GABAergic W-active neurons to disinhibit PS effector neurons (Fig. 5.1).

As locally projecting interneurons, GABAergic PS-max active neurons could also inhibit their codistributed putative glutamatergic, W-max active neurons during PS (Fig. 5.1).

5.2.3 Putative glutamatergic neurons

In the present study, Nb+/VAcHT-/GAD-, putative glutamatergic neurons were found to form the largest cell group, 75% of LDT/PPT recorded and identified neurons. The glutamatergic neurons have been found to represent a slightly larger proportion (40%) than the cholinergic or GABAergic neurons in the LDT/PPT (Wang and Morales, 2009). Yet, the large proportion of the sample represented by these neurons here is probably due to the fact that these neurons are easy to target during the recording compared to cholinergic and GABAergic neurons, since they generally continue to discharge during SWS, although at a lower rate than during W or PS. LDT/PPT putative glutamatergic neurons comprise three different sleep-wake sub-groups: W/PS-max active sub-group (46%), PS-max active sub-group (37%) and W-max active sub-group (17%).

5.2.3.1 Putative glutamatergic W/PS-max neurons

The putative glutamatergic cells of the W/PS-max active sub-group would correspond to the putative "glutamatergic-On" cells found in our acute study that could be involved in inducing cortical activation during both W and PS. Surprisingly, putative glutamatergic W/PS-max active neurons were found to have a similar profile to the cholinergic (W/PS-max active) neuron. Since these putative glutamatergic neurons form, in parallel to the cholinergic neurons, an important contingent of long ascending projections from LDT/PPT into the forebrain (Pare et al., 1988; Jones and Cuello, 1989; Rasmusson et al., 1994; Ford et al., 1995), they could thus share the role of cholinergic neurons in stimulating cortical activation during both waking and PS (Fig. 5.1). Indeed, blockage of glutamatergic transmission in the basal forebrain cholinergic cell area decreased cortical ACh release and EEG activation during electrical stimulation of the PPT region (Rasmusson et al., 1994). They could also stimulate the ascending projecting glutamatergic W/PS-max neurons in the PnO (Fig. 5.1). LDT/PPT putative glutamatergic W/PS-max neurons could thus share the role of cholinergic neurons in stimulating cortical activation during both waking and PS (Fig. 5.1).

5.2.3.2 Putative glutamatergic PS-max neurons

The putative glutamatergic cells of the PS-max active sub-group, which were all fast firing neurons and fired maximally during PS in negative correlation with EMG activity, could correspond to the putative "glutamatergic-Off" cells found in our acute study. They could accordingly be involved in dampening behavioural arousal or response to stimulation and decreasing muscle tone during SWS/PS.

A c-Fos study showed that a population of glutamatergic neurons in the region of the SubC, a PS effector zone, was active during PS (Lu et al., 2006). Moreover, application of kainate, agonist for ionotropic glutamatergic receptors, into the SubC region increased PS (Onoe and Sakai, 1995). It is possible that LDT/PPT glutamatergic, PS-max active neurons stimulate the PS effector neurons in the SubC and PnO and could thus indirectly participate in inducing muscle atonia, which is suggested to be driven by these PS effector neurons (Fig.5.1, Kodama et al., 1998; Boissard et al., 2002).

As locally projecting, they could also excite, via local collaterals, other PS-max active neurons such as congener GABAergic neurons in the LDT/PPT.

5.2.3.3 Putative glutamatergic W-max neurons

As a singular profile, putative glutamatergic cells of the W-max active sub-group fired maximally during waking in positive correlation with EMG activity. Although previous electrophysiological studies showed that some neurons recorded within the pontomesencephalic tegmentum were W-active/PS-inactive neurons, they suggested that they are monoaminergic neurons (El Mansari et al., 1989; Kayama et al., 1992). Those neurons could, in fact, correspond to the VAcHT-/GAD- W-max neurons found in the present study. What are otherwise considered glutamatergic W-max neurons could participate, in response to sensory stimulation, in stimulating behavioral arousal during wakefulness, possibly by stimulating pontine reticulospinal and/or medullary reticulospinal neurons involved in driving behavioral arousal and muscle tone (Fig.5.1, Siegel and McGinty, 1977).

Finally, the present study showed that with their large number and their heterogeneous discharge profiles, LDT/PPT putative glutamatergic neurons seem to play

a role as important as other LDT/PPT cell types, notably the cholinergic cells, may play in modulating sleep-wake states. As W/PS-active neurons, they could work, in parallel with cholinergic neurons, to stimulate cortical activation during both waking and PS; as PS-active neurons, they could actively participate in inducing muscle atonia; and finally as W-active neurons, they could participate in stimulating behavioral arousal and muscle tone during wakefulness. These putative glutamatergic neurons need to be unequivocally identified as glutamatergic neurons in further investigations.

5.3 Figure 5.1

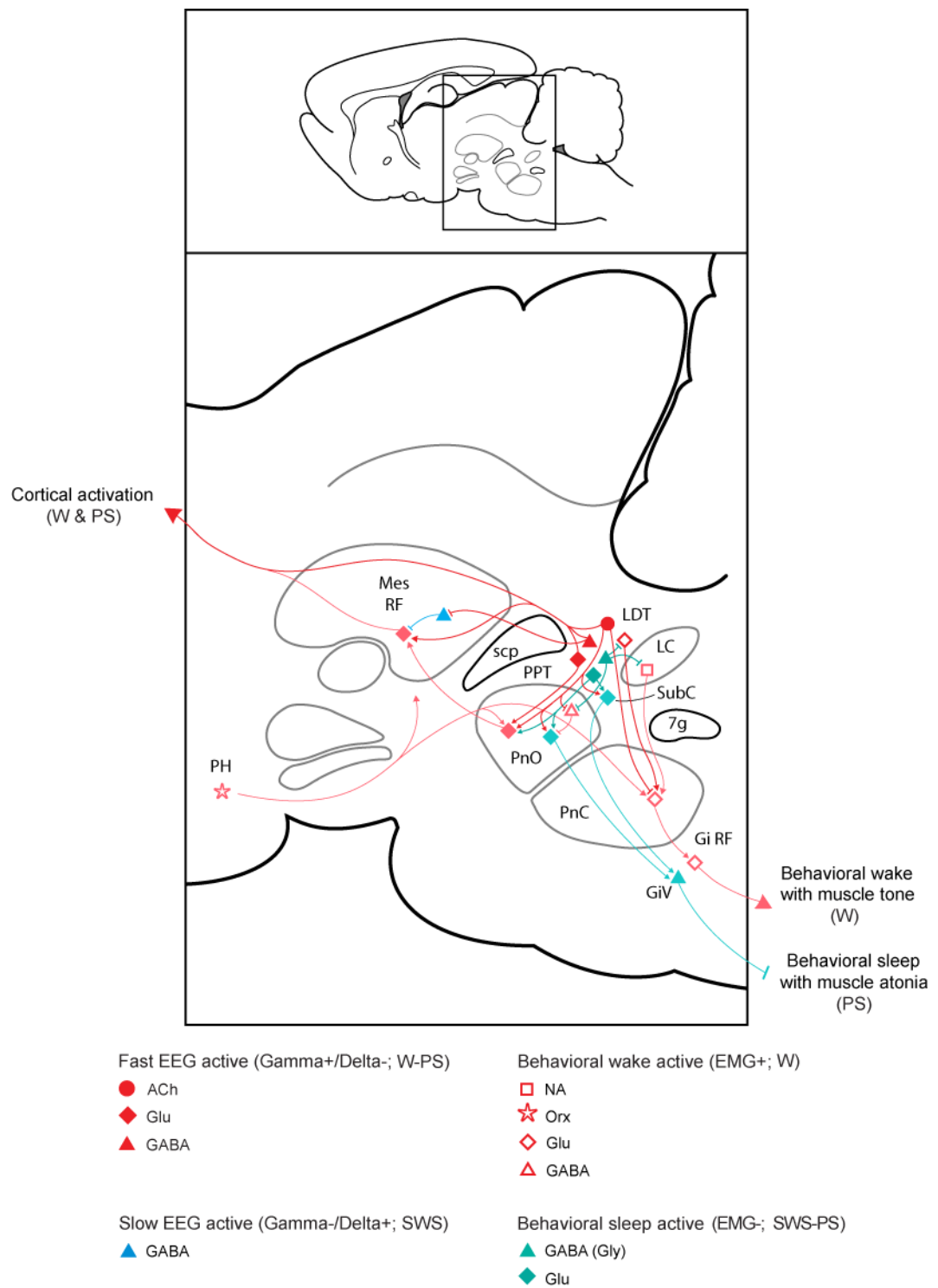


Figure 5.1. Regulation of sleep-wake states by neurons of the pontomesencephalic tegmentum.

Figure 5.1. Regulation of sleep-wake states by neurons of the pontomesencephalic tegmentum. Sagittal schematic view depicting the recorded LDT/PPT cholinergic, GABAergic and putative glutamatergic neurons and how they might influence cortical activity and behavior through actions upon other neurons in the brain. The LDT/PPT neurons that are maximally active during both waking (W) and paradoxical sleep (PS) and discharge in positive association with fast EEG activity (W/PS-active, filled red symbols) could stimulate cortical activation during both W and PS by excitation of neurons within the reticular activating system, presumed to be glutamatergic, and by excitation of neurons primarily in the thalamus and also hypothalamus and basal forebrain. They include cholinergic (ACh) and putative glutamatergic (Glu) neurons that would exert direct excitatory actions and also GABAergic (GABA) neurons that could inhibit slow wave sleep (SWS)-active neurons in part located in the mesencephalic reticular formation. Cholinergic neurons could also stimulate the PS-active neurons in the PnO and subcoeruleus (SubC) whereas they would inhibit the W-active neurons through M2 ACh Rs during PS. The inhibitory actions of ACh upon these neurons would be antagonized during W by orexin (Orx) and noradrenalinergic (NA) neurons. LDT/PPT neurons that are maximally active during PS (PS-active, aqua symbols) in negative association with EMG activity could stimulate behavioral sleep with muscle atonia during PS. They include GABAergic and putative glutamatergic neurons. The GABAergic neurons could inhibit their neighboring W-active putative glutamatergic neurons and the W-active NA LC neurons. They could also inhibit W-active GABAergic neurons in the PnO region, resulting in the disinhibition of PS-active neurons of the PnO and SubC. The putative glutamatergic neurons could stimulate PS-active neurons in the PnO and SubC, which in turn stimulate GABAergic (or glycinergic) neurons located in

the gigantocellular field of the medullary reticular formation, ventral part (GiV). These GABAergic (or glycinergic) neurons in turn inhibit motoneurons in the brainstem and spinal cord, resulting in muscle atonia. LDT/PPT neurons that are maximally active during W (W-active, open red symbols) in positive association with EMG activity could stimulate behavioral arousal with muscle tone during W. They included only putative glutamatergic neurons that could stimulate, together with Orx and NA neurons, the PnC W-active neurons, which in turn stimulate glutamatergic neurons located in the gigantocellular region of the medullary reticular formation (Gi RF). These glutamatergic neurons stimulate in turn motoneurons resulting in behavioral arousal with high muscle tone. Note that LDT/PPT neurons and their proposed projections are shown in dark colors, whereas their target neurons are shown in lighter colors. Abbreviations: 7g, genu 7th nerve; Gi RF, gigantocellular reticular formation; GiV, gigantocellular, ventral part RF; LC, locus coeruleus nucleus; LDT, laterodorsal tegmental nucleus; Mes RF, mesencephalic reticular formation; PH, posterior hypothalamus; PnC, pontine, caudal part RF; PnO, pontine, oral part RF; PPT, pedunculopontine tegmental nucleus; RF, reticular formation; scp, superior cerebellar peduncle; SubC, subcoeruleus.

5.4 General Conclusion

The studies of the present thesis illuminate for the first time the discharge profiles of chemically identified cholinergic, GABAergic and putative glutamatergic LDT/PPT neurons in relation to evoked cortical activation in anaesthetized rats and in relation to cortical activity and muscular tone across the sleep-waking cycle in naturally sleeping and waking rats. The findings advance several points that should be considered as important discoveries of the present thesis. (1) There is a similarity in the discharge profiles of chemically different cell populations (cholinergic, GABAergic and putative glutamatergic neurons) within the same functional sleep-wake sub-group on the one hand and reciprocity in their discharge profiles with other sleep-wake sub-groups on the other hand. The similarity in the discharge profiles of cholinergic, GABAergic and putative glutamatergic neurons of the W/PS-max active sub-group could suggest that these cells are working in a parallel manner to drive cortical activation during both waking and PS. Additionally, the similarity in the discharge profiles of GABAergic and putative glutamatergic neurons of the PS-max active sub-group could suggest that these cells are working in parallel manner to dampen behavioral arousal and muscle tone during PS. They could also be partially redundant in their roles. The reciprocity between the discharge profiles of putative glutamatergic neurons of the W-max active sub-group on the one hand and GABAergic and putative glutamatergic neurons of the PS-max active sub-group on the other hand could suggest that these neurons are working in reciprocal manner to stimulate and to dampen behavioral arousal and muscle tone respectively. Therefore, LDT/PPT cholinergic, GABAergic and glutamatergic neurons may be playing an important role in modulating sleep-wake states either by interacting locally or upon

their target cells. This phenomenon is not exclusive to the pontomesencephalic tegmentum region but was found also within the basal forebrain cholinergic cell area (Hassani et al., 2009), and indicates that no cell type is acting individually, but rather in parallel and/or in complement with other cell types to orchestrate the sleep-wake cycle.

(2) There is considerable heterogeneity in chemical or neurotransmitter cell phenotypes and functional sleep-wake discharge profiles even in a small area like the LDT/PPT in the pontomesencephalic tegmentum. This heterogeneity was also found in other brain areas such as the basal forebrain (Hassani et al., 2009) and lateral hypothalamus (Hassani et al., 2010). Such heterogeneity reflects the diversity of cell populations in different brain areas, which may form local networks and through interconnections, larger networks that orchestrate the sleep-wake cycle rather than isolated centers in the brain, one responsible for waking, another for SWS and another for PS as some have proposed (Saper et al., 2010).

Finally, the results of the present thesis reveal the important role that different cell types in LDT/PPT may play in controlling the sleep-wake cycle. The W/PS-max active neurons could participate in stimulating cortical activation during both W and PS; the PS-max active neurons could participate in dampening behavioral arousal and muscle tone during PS, whereas the W-max active neurons could participate in stimulating behavioral arousal and muscle tone during wakefulness. These new results should add missing pieces to the puzzle that our laboratory and others are working to complete in order to understand how the sleep-wake cycle is orchestrated.

6. References

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