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Excitatory Actions of Orexins in Rat Paraventricular Nucleus of Thalamus

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EXCITATORY ACTIONS OF OREXINS IN RAT PARAVENTRICULAR NUCLEUS OF THALAMUS

By

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ABSTRACT

The midline thalamic paraventricular nucleus (PVT) receives a unique orexinergic innervation. To address possible function, this investigation used patch clamp recordings in rat brain slice preparations to evaluate intrinsic properties of PVT neurons and neuronal responses to bath-applied orexin peptides (A and/or B). PVT neurons displayed distinct state-dependent burst or tonic firing patterns, time dependent and time-independent inward rectification, T-type currents and low threshold spikes, action potential after-hyperpolarizations, spike frequency adaptation and spike broadening. A majority responded to both orexin peptides with slowly rising and prolonged membrane depolarizations, and inward currents that involved closure of potassium channels and/or opening of nonselective cationic channels. These data imply that endogenously released orexins likely act at both types of orexin receptors that can engage two conductances to modulate and increase neuronal excitability in PVT, a role that may be important for 'arousal' and neurotransmission within this midline thalamic nucleus.

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ABBREVIATIONS

ABC	avidin-biotin-peroxidase complex
aCSF	artificial cerebrospinal fluid
ACTH	adrenocorticotropic hormone
AHP	afterhyperpolarizations
AP	area postrema
ARC	arcuate nucleus
CART	cocaine and amphetamine related transcript
CCK	cholecystokinin
CRF	corticotrophin-releasing factor
CRF-R	corticotrophin-releasing factor receptor
DAB	diaminobenzidine
DAG	diacylglyceride
DAP	depolarizing afterpotential
DMV	dorsal motor nucleus of vagus
DRN	dorsal raphe nucleus
EPSPs	excitatory postsynaptic potentials
GAD	glutamate decarboxylase
GnRH	gonadotropin-releasing hormone
GPCR	G protein-coupled receptor
GSNs	glucose-sensitive neurons
HLA	human leukocyte antigen
HPA	hypothalamic-pituitary axis

ICV	intracerebroventricular
IP_3	inositol triphosphate
IPSCs	inhibitory postsynaptic currents
ISI	interspike interval
LC	locus coeruleus
LDT	laterodorsal tegmental nucleus
LH	luteinizing hormone
LHA	lateral hypothalamic area
LTS	low threshold spike
αMSH	alpha-melanocyte-stimulating hormone
NB	forebrain nucleus basalis
NGS	normal goat serum
NMDG	N-methyl-D-glucamine
NPO	nucleus pontis oralis
NPY	neuropeptide Y
NTS	nucleus tractus solitarius
OX_1R	orexin type one receptor
OX_2R	orexin type two receptor
PBS	phosphate buffered saline
PK_2	prokineticin
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C

POMC	pro-opiomelanocortin
PTX	pertussis toxin
PVN	paraventricular nucleus
PVT	thalamic paraventricular nucleus
REM	rapid eye movement
SCN	suprachiasmatic nucleus
SFA	spike frequency adaptation
SN	substantia nigra
SP	substance P
TMN	tuberomammillary nucleus
TRP	transient receptor potential
TTC: GFP	nontoxic C-terminal fragment of tetanus toxin and green fluorescent protein
TTX	tetrodotoxin
VLPO	ventrolateral preoptic area
VIP	vasoactive intestinal polypeptide
VTA	ventral tegmental area

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ABSTRACT

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CHAPTER 1

INTRODUCTION

1.1 Preface-overview

Levels of awareness and sleep have long been known to involve interactions between the cortex and the thalamus (reviewed in McCormick and Bal, 1997). Important in this regard are both the "specific" thalamic relay nuclei and thalamic reticular nucleus, with additional contributions from the so called "non-specific" midline and intralaminar thalamic nuclei (Groenewegen and Berendse, 1994; Jones, 2001; Steriade et al., 1993). Many in-vivo and in-vitro investigations have focused on thalamocortical neurons in the specific sensory relay nuclei and their interactions with reticular nucleus neurons. The midline and intralaminar thalamocortical neurons project to the prefrontal cortex, especially the anterior cingulate gyrus (Berendse and Groenewegen, 1991; Marini et al., 1996) to promote activity that correlates with levels of arousal (Hofle et al., 1997; Paus et al., 1998; Yamasaki et al., 2002). While the established property of thalamocortical neurons during quiet sleep is the appearance of bursting activity patterns, intralaminar neurons are unique by virtue of rhythmic activity and bursting during wakefulness (Steriade et al., 1993).

One of these midline cells groups that is relatively unexplored is the thalamic paraventricular nucleus (PVT), a cell group that receives a prominent innervation from recently discovered lateral hypothalamic "orexinergic" neurons, cells that are critical for sustaining arousal (see below). The research described in this thesis is an initial description of the membrane properties of PVT neurons recorded in a rat brain slice

preparation and how these neurons respond to exogenous applications of orexin (see Fig.1.1).

1.2 Hypothesis and Objectives

The interactions between the cortex and the thalamus are considered important determinates for levels of arousal and sleep (reviewed in McCormick and Bal, 1997). Neurotransmitters from the brainstem activating systems acting on thalamocortical neurons can induce membrane potential changes that result in state-dependent firing patterns (bursting, tonic), activity subtending both sensory transmission and cortical activation during arousal (Steriade and Llinás, 1988; McCormick and Bal, 1997; Steriade et al., 1997; Jones, 2000). It is particularly interesting to note that the newly discovered lateral hypothalamic orexinergic neurons, regarded as functionally important for maintaining arousal, do not project to specific thalamocortical relay nuclei but rather to select midline and intralaminar thalamic nuclei, the so called "nonspecific thalamocortical relay system", with a dense projection to PVT where in situ hybridization data reveal the presence of mRNA for both orexin receptors (Marcus, 2001). Considering that orexins are reported to excite neurons in other locations where there are orexin fibers and receptors, this research was undertaken to test the hypotheses that: a) orexin A and B will both excite (depolarize) PVT neurons; b) orexin-induced activity will alter intrinsic properties of PVT neurons and affect firing patterns. The objectives of this research are, first, to characterize the intrinsic electrophysiological properties of PVT neurons and, second, to assess the effects and mechanisms of activation of orexin receptors on PVT cell excitability.

1.3 The midline thalamic paraventricular nucleus (PVT)

The midline group of thalamic nuclei is relatively poorly represented in higher mammals and in man. In a considerable percentage of human brains there is no massa intermedia connecting the two halves of the dorsal thalamus (Sheps, 1945). By contrast, in subprimates and lower vertebrate brains, including rodents, a well developed system of gray matter links the dorsal thalamus. Part of this gray matter is a discrete group of neurons that form the thalamic paraventricular nucleus (PVT), a midline structure that extends the length of the thalamus along its dorsal border below the third ventricle. Rostrally, PVT follows the surface of the massa intermedia, curving ventrally to form a wedge between the anterior poles of the nucleus reuniens. At caudal levels, it ends ventral to the habenula. PVT originates from a pronuclear mass that also gives rise to the pineal and habenular nuclei; it is highly conserved through phylogeny and is a stable thalamic structure through lower mammalian evolution (Bentivoglio et al.,1991). Its anatomical connectivity across various mammals appears to be very similar (Fiset et al., 1999; Jones, 1998). Traditionally, PVT and other thalamic midline and intralaminar nuclei have been considered as components of the 'non-specific' thalamocortical relay system.

1.3.1 Cellular morphology

Relatively little morphological data exist on PVT and midline thalamic neurons when compared with the data available on cells located within the 'specific' sensory thalamocortical projection system. Earlier reports that utilized Golgi staining techniques suggested a cellular morphology that was similar to that in the more lateral thalamic cell groups. Recent studies have employed retrograde tracers to profile PVT neurons whose

axons target specific sites, notably the amygdala, the hippocampus and the accumbens. PVT neurons that project to each area appear to display qualitative and topographic differences (see efferent projections below). In the study by Su and Bentivoglio (1990), these labeled cells displayed similar shapes (spindle or ovoid) and measured 12-22 microns in their long axis. Labeled neurons in the ventral PVT were noted to be generally smaller than those located dorsally and appeared mainly round, with a bipolar dendritic arborization. In the medial PVT the long axis of labeled cells was oriented vertically, parallel to the midline. At the ultrastructural level, dendrites of the more dorsal neurons approached but did not penetrate to the ependymal surface, a feature that warrants against the notion of a specialized dendritic-CSF relationship (Balercia et al., 1992).

1.3.2 Afferent connections

Anatomical tracer studies reveal that the PVT receives many afferent connections, and these can be described both by site of origin as well as their chemical neuroanatomy.

A) Suprachiasmatic nucleus (SCN)

From the hypothalamus, fibers arise from neurons in the suprachiasmatic nucleus (SCN), the brain's biological clock, and appear to be densest in the anterior PVT (e.g. Watts and Swanson, 1987; Kawano et al., 2001). Dual tracer studies reveal that this innervation is direct to PVT neurons whose axons project to the amygdala (Peng and Bentivoglio, 2004) and express a glutamatergic phenotype (Heilbronner et al., 2004; Hur and Zaborsky, 2005; Huang et al., 2006). Possible transmitters in this SCN innervation include arginine vasopressin or AVP (Buijs, 1978), vasoactive intestinal polypeptide or

VIP (Kalamatianos et al., 2004) and prokineticin or PK2 (Cheng et al., 2002). This SCN innervation, which is reciprocal (see below), implies that PVT is a component of the "circadian system".

B) Posterolateral hypothalamus

Relevant to this thesis is a prominent PVT innervation arising from the posterolateral hypothalamus, an input that in part arises from neurons that display immunoreactivity for the recently characterized orexin (also called hypocretin) peptides (Peyron et al., 1998; Sakurai et al., 1998; Nambu et al, 1999; Baldo et al., 2003). The axonal arborization of these orexinergic neurons clearly outlines the borders of PVT (Kirouac et al., 2005; see Fig 2.2) and corresponds with the distribution of orexin receptor mRNA in the midline thalamus (Marcus et al., 2001). The orexin expressing neurons have a glutamatergic phenotype (Abrahamson et al., 2001; Rosin et al., 2003) and also co-express dynorphin (Chou et al., 2001). Additionally, PVT receives a modest innervation from a separate population of lateral hypothalamic neurons that synthesize melanin-concentrating hormone (Bittencourt et al., 1992; Saito et al., 2001). Afferents presumed to generate nitric oxide arise from non-cholinergic neurons in the lateral hypothalamus (Otake and Ruggiero, 1995).

C) Hypothalamic tuberomammillary nucleus

The hypothalamic tuberomammillary nucleus (Panula et al., 1989), cells that synthesize histamine, GABA and other possible transmitters, send axons to PVT. It is notable that PVT contains a strong histamine H3 receptor distribution (Pillot et al., 2002).

D) Other monoaminergic afferents

Other monoaminergic fibers that converge in PVT include dopaminergic inputs from the A8, A10, A11, A13 and A14 cell groups in the ventral tegmental area and retrorubral region (Takada et al., 1990; Otake and Ruggiero, 1995), noradrenergic inputs from the locus coeruleus, the commissural nucleus of solitary tract and caudal ventrolateral medulla (Otake and Ruggiero, 1995; Otake et al., 2002), and serotonergic inputs from the dorsal and median raphé nuclei (Otake et al., 2002).

E) Afferents displaying immunoreactivity for cholecystokinin (CCK)

Sources of afferents that display immunoreactivity for cholecystokinin (CCK) include cells in the dorsomedial hypothalamus, periaqueductal gray, dorsal raphe and parabrachial nucleus (Bhatnagar et al., 2000; Otake, 2005).

F) Afferents displaying immunoreactivity for cocaine and amphetamine related transcript (CART)

PVT displays a high density of fibers that are immunoreactive for cocaine and amphetamine related transcript (CART; Parsons et al., 2006) possibly arising from CART-synthesizing neurons in the reticular thalamic nucleus (Koylu et al., 1998).

G) Afferents displaying immunoreactivity for substance P (SP)

Sources of afferents that display substance P (SP) immunoreactivity include the Edinger-Westphal nucleus, the mesopontine tegmentum and raphe nuclei (Otake, 2005).

H) Afferents displaying immunoreactivity for corticotrophin-releasing factor (CRF)

PVT receives additional inputs from corticotrophin-releasing factor (CRF) immunoreactive cells in the central nucleus of amygdala (Otake and Nakamura, 1995), bed nucleus of the stria terminalis, the septal nuclei, the supramammillary nuclei, infralimbic and prelimbic cortices and subiculum (Vertes, 2004).

1.3.3 Efferent connections

The efferent projections of the PVT are mainly to limbic related brain regions, namely the deep layers of the prefrontal cortex, accumbens and amygdala, and are organized along anterior-posterior axes. (Mogo, 1995; Pinto et al., 2003; Parsons et al., 2006). As mentioned above, and typical of most thalamocortical projecting neurons, these PVT efferent neurons express a predominantly glutamatergic phenotype (Heilbronner et al., 2004; Hur and Zaborsky, 2005; Huang et al., 2006). The anterior PVT has a reciprocal connection with the hypothalamic SCN (Krout et al., 2002; Moga et al., 1995) suggesting an important link in the control of circadian rhythmicity. Anterior PVT neurons also project to dorsomedial and ventromedial hypothalamic nuclei, the lateral septum, bed nucleus of the stria terminalis, and have prominent projections to central and basomedial amygdaloid nuclei, anterior olfactory nucleus and olfactory tubercle, shell of the nucleus accumbens, infralimbic, piriform, and perirhinal cortices, ventral subiculum; and endopiriform nucleus. Posterior PVT neurons project to the central, basolateral and basomedial amygdaloid nuclei, the core and shell regions of nucleus accumbens, anterior olfactory nucleus and olfactory tubercle but have few projections to the lateral septum and hypothalamus. Dual tracer studies reveal a significant number of neurons throughout

the rostrocaudal PVT send axons to both the ventral striatum and prefrontal cortex (Otake and Nakamura, 1998) whereas fewer neurons innervate both prefrontal cortex and medial accumbens (Bubser and Deutch, 1998).

1.3.4 Electrophysiology

The cellular electrophysiology of PVT neurons is largely unknown and will be a topic of this research. Since this research was initiated, two papers have appeared (Ishibashi et al., 2005, in rat; Huang et al., 2006, in mouse) that refer to patch clamp data from PVT neurons. In rodents, cells are reported to have mean resting membrane potentials in the order of -55 to -60 mV and input resistances in the 350-450 MΩ range. Similar to observations in the adjacent midline centromedial and rhomboid nuclei (Bayer et al., 2002), PVT neurons are reported to display tonic firing when depolarized from resting membrane potentials, and the presence of a calcium-dependent low threshold spike (LTS) crowned by a burst of fast action potentials when depolarized from a hyperpolarized level, features characteristic of thalamocortical relay neurons (Deschenes et al., 1984; Jahnsen and Llinas, 1984; reviewed in Sherman, 2001).

1.3.5 Functional aspects

The midline and intralaminar thalamic nuclei are regarded as components of the neural representations of motivational drives and aspects of pain, need for sleep and nurturance, hunger, thirst, fear, stress and power-dominance (Sewards and Sewards, 2003). The following sections focus on selected topics in this domain.

A) Sleep and arousal

PVT and other thalamic midline nuclei have long been considered a component of the 'ascending reticular activating system" that regulate sleep, wakefulness and arousal (Moruzzi and Magoun, 1949). Indeed, many PVT afferents mentioned above arise from cell groups known to regulate arousal and vigilance i.e. histaminergic cells of the tuberomammillary nucleus, serotonergic cells of the dorsal raphe nuclei, the noradrenergic cells of the locus coeruleus, and dopaminergic cell groups in the ventral tegmentum. Activation of the immediate early gene c-Fos and expression of its Fos protein has been utilized to reveal a functional association between its expression in neurons and a specific 'stimulus' or state. A number of findings point to PVT as a site of circadian changes in neuronal gene expression. Fos expression in PVT neurons displays a daily rhythm, with greater expression during wakefulness in both nocturnal and diurnal animals (Cirelli et al., 1993; Novak and Nunez, 1998; Peng and Grassi-Zucconi, 1995).

It has also been proposed that PVT plays a pivotal role in awareness of stimuli of various sensory modalities (Smythies, 1997), such as auditory vigilance (Paus et al., 1997), visual awareness (Purpura et al., 1997), awareness of tactile and nociceptive information (Giesleret al., 1981; Peschanski and Besson,1984; Sturm et al., 1999). It has been suggested that a concerted action of the midline-intralaminar complex might bring the entire basal-ganglia and thalamocortical system to a higher level of activity, that is, to a state of readiness. In keeping with this notion is the PVT innervation by the recently discovered orexins, peptides important for the promotion and maintenance of wakefulness (Marcus, 2001; Peyron, 1998).

B) Circadian rhythms

SCN is the site of the principal biological clock in the mammalian brain, entraining behaviours (e.g. sleep-wake cycles) and physiological processes (e.g. morning peaks in glucose and cortisol) to the solar cycle (Reppert and Weaver, 2001; Buijs et al., 2003). As mentioned above, PVT is one of two thalamic targets of SCN efferents, the other being the intergeniculate leaflet (Moga and Moore, 1997). The SCN connection to PVT has been proposed to participate in the control of the sleep/wake cycle (Bujis, 1996). A projection from PVT back to SCN may provide feedback regulation of circadian timing. The observation that lesions of the anterior PVT abolish the advances induced by light during late subjective night whereas mid-posterior PVT lesions did not affect phase shifts (Salazar-Juárez et al., 2002) suggests that anterior PVT neurons in particular modulate entrainment of circadian rhythms to light.

PVT also sends projections to the VLPO (Chou et al., 2002; Van der Werf, 2002). Fos expression in PVT shows a positive correlation and a pattern that is 180° out of phase with the Fos rhythm in VLPO (Novak and Nunez, 1998). The role for PVT in circadian rhythmicity and in sleep-waking cycles appears to be closely intertwined.

C) Stress

PVT innervates a range of forebrain structures directly implicated in the generation of behavioural, endocrine and/ or autonomic responses to stress, notably medial prefrontal cortex, central and medial amygdala, bed nucleus of the stria terminalis and hypothalamic dorsomedial and paraventricular nuclei. PVT has been proposed as a relay nucleus that transfers stress-related information to the limbic forebrain (Bubser and

Deutch, 1999; Otake et al., 2002). PVT neurons express Fos protein as part of a concerted response to a wide variety of stressors that include pain, e.g. colorectal distention (Traub et al., 1996), foot shock (Bubser and Deutch, 1999; Li and Sawchenko, 1998), restraint or immobilization (Cullinan et al., 1995; Senba et al., 1993), and higher temperatures (Bachtell et al., 1993). Experiments examining adrenocorticotropic hormone (ACTH) and corticosterone responses suggest that an intact posterior PVT inhibits habituation in the hypothalamic-pituitary axis (HPA) activity under conditions of chronic stress (Bhatnagar and Dallman, 1998; Bhatnagar et al., 2002), but some investigators have differing viewpoints on this issue (see Fernanades et al., 2002). Although PVT shows increased neuronal activity with exposure to a range of acute stressors, PVT and central amygdala do not appear to contribute to the HPA axis response to acute psychological stress since PVT lesions do not block either the diurnal pattern of HPA activity or the ability of the animals to respond to the acute stimuli (Bhatnagar and Dallman, 1999; Dayas, 1999). The observation that PVT lesions did not alter acute stress-induced corticotrophin-releasing factor (CRF) cell recruitment in the parvocellular hypothalamic paraventricular nucleus (Spencer et al., 2004) suggests that different neural circuits may be involved in the mechanisms underlying responses of PVT to acute or chronic stressors. It is interesting that the density of glucocorticoid receptors in modest within the thalamus, and densest in PVT (Morimoto et al., 1996) and disappears after adrenalectomy (Visser et al., 1996). Stress affects the brain noradrenergic circuitry and it is notable that chronic psychosocial stress in male tree shrews selectively upregulates α2B adrenoceptors in PVT but not in the adjacent anteroventral thalamic nucleus (Heilbronner et al., 2004).

D) Reward and psychostimulation

The nucleus accumbens is a major site of psychostimulant drug actions (Kalivas and Stewart, 1991; Wise, 1995), and data from a variety of studies indicate that a convergence of dopaminergic and glutamatergic innervations regulate psychostimulant-induced locomotor behaviour (Churchill et al., 1995; Daunais and McGinty, 1995). Selective antipsychotic drugs (clozapine) and psychostimulants (cocaine, amphetamine and ecstasy) induce Fos expression in PVT (Deutch et al., 1995, 1998; Stephenson et al., 1999). Moreover, significant increases in brain κ-opioid receptors are noted in PVT in response to withdrawal from physical dependence on butorphanol, a synthetic opioid that is structurally similar to naloxone and whose chronic use produces physical dependence (Fan et al., 2003). Lesions of PVT block cocaine-induced locomotor sensitization, specifically disrupting the contextual conservation of the sensitization (Young and Deutch, 1998). Thus PVT appears to be a key node in extended cortico-striato-pallido-fugal circuits that subserve both the arousing properties and reinforcing aspects of psychostimulants. These data are consistent with those of Brown et al (1992), who noted earlier that PVT is one of the few sites that are activated in cocaine-induced environment-specific conditioning, suggesting that PVT is implicated in conditioned addictive behavior.

E) Feeding

Lesions of posterior PVT result in an increase in food intake mainly during the light phase, possibly due to the disruption of information of feeding regulation relayed by PVT from the circadian timing system; this may reflect a strictly circadian role with

respect to food intake (Dallman et al., 1999). Neuropeptide Y (NPY) is a potent inducer of feeding behaviour (Parrott et al., 1986). Reduced NPY receptor density is noted in PVT in dietary restricted rats, and significantly increased in dietary obese rats (Widdowson et al., 1997), perhaps reflecting an overall response in central feeding circuits to negative and positive energy balance.

F) Memory

By virtue of its central position in the brain, the thalamus can regulate the flow of information from brainstem, sensory organs and visceroceptors to other areas of cortex and subcortical regions. Damage to the intralaminar and midline nuclei disrupts memory processes (Bailey and Mair, 2005; Newman et al., 2005; Van der Werf et al., 2003) and a role in classical conditional learning has been suggested (Beck and Fibiger, 1995; Brown et al., 1992; Dallman, 1999). The contribution of PVT neurons still remains largely unknown. Perhaps the answer resides in the 'plasticity' in HPA function that is a consistent finding in animals exposed to chronic stress, whether continuous or intermittent; these animals exhibit normal of enhanced HPA responses to novel, acute stressors despite the negative feedback effects of elevated circulating glucocorticoids released by chronic stress (e.g. Young et al., 1990). To account for this difference between effects of stress and glucocorticoids, it has been proposed that prior stress leaves a central facilitatory 'trace' that, upon exposure to a novel stressor, balances or overcomes the negative feedback effects of circulating glucocorticoids. Bhatnagar and Dallman (1998) propose that this facilitatory role resides as a 'memory' within PVT (also see Dallman et al., 2003).

1.4 Orexins–Hypocretins

1.4.1 Discovery

Much has been learned about brain functions by the analysis of deficits arising as a consequence of damage to specific brain regions. For example, in the early 1900s, Von Economo reported that patients afflicted with "encephalitis lethargica" were hypersomnolent and aphagic, and at autopsy many showed cell loss in the posterior lateral hypothalamus (Von Economo, 1931). Subsequent studies in various animal species indicated similar changes in behaviour could be induced by destruction of the posterolateral hypothalamic area (e.g. Anand and Brobeck, 1946). Thus began the notion that lateral hypothalamic neurons were important in regulating feeding and arousal. A major advance in our understanding not only of feeding behaviours but (curiously) also the cause of a sleep disorder known as narcolepsy resulted from simultaneous discoveries in 1998 by two independent laboratories of a family of neuropeptides and their receptors known as the hypocretins or orexins. Importantly, these peptides were found to be synthesized solely by neurons located in the posterolateral hypothalamus. In the Sutcliff lab, a search for the most abundant mRNAs in the rat hypothalamus using subtractive cDNA cloning techniques led to the isolation and identification of a novel mRNA exclusively expressed in the lateral, posterior and perifornical hypothalamus (de Lecea et al., 1998). Based on the structure of this mRNA, the structure of its pre-pro-peptide was determined and shown to cleave into two peptide products that they termed hypocretin1 (hct1) and hypocretin 2 (hct2), named from the combination of hypothalamic and incretin (based on a weak homology with secretin, which belongs to the incretin superfamily of

peptides). In the same year, a group led by Yanagisawa was seeking to identify endogenous ligands for orphan G protein-coupled receptors (GPCRs). They independently purified the same two peptides which they called *orexin A* and *orexin B*, derived from the Greek word for appetite (Sakurai et al., 1998). They also described a new orphan G protein coupled receptor (GPCR) which turned out to be the orexin B receptor, or OX_2R. While both names have remained in common use, the orexin terminology will be applied here.

1.4.2 Molecular and cellular biology

The orexin system consists of 3 genes, the precursor pre-pro-orexin gene that encodes two closely related neuropeptides: orexin A and orexin B, and two separate genes for the receptors, OX_1R and OX_2R (Sakurai et al., 1998)

A) Orexin peptides

Mammalian orexin A is a 33 amino acid peptide of 3562 Daltons that possesses an N-terminal pyroglutamyl residue, a C-terminal amidation, and two intra-molecular disulfide bridges, Cys6–Cys12 and Cys7–Cys14. The primary structure of orexin A is remarkably well preserved among human, bovine, rat, mouse, and pig. Mammalian orexin B is a 28 amino acid peptide of 2937 Daltons that possesses a C-terminal amidation, and has 46% (13/28) amino acid identity to orexin A. Orexin B from rat and mouse is identical, and only one and two amino acid residues are changed in the porcine and human counterparts, respectively. Orexins have also been described in the frog Xenopus laevis and have a high similarity to the mammalian peptides (Shibahara et al.,

1999). The human orexin gene (chromosomal localization: 17q21–22) consists of two exons and one intron, and encodes a 131 amino acid precursor peptide, pre-pro-orexin. This precursor possesses an N-terminal 33 residue secretory signal peptide, and is cleaved at sites of basic amino acid residue pairs by prohormone convertases, yielding orexins A and B (Sakurai et al., 1999). The amino acid identity between human and rat pre-pro-orexin is 83%, with most substitutions occurring near the C-terminus.

B) Orexin receptors

The orexin receptors (OX_1R and OX_2R) have a 64% amino acid identity with each other. The amino acid identity between human and rat receptors is 94% for OX_1R and 95% for OX_2R. The OX_1R has a 10-fold higher affinity (expressed as EC_{50}, the concentration of ligand needed to elicit half-maximum receptor response) for orexin A (EC_{50} = 20nM) than orexin B (EC_{50} = 250nM), whereas OX_2R binds the two peptides with equal affinity (EC_{50} = 20nM). In general terms, OX_1R is thought to activate the G protein $G_{q/11}$, causing an influx of extracellular Ca^{2+} while OX_2R is thought to couple either to $G_{q/11}$ or $G_{i/o}$, the latter enhancing potassium ion efflux (Beuckmann and Yanagisawa, 2002). Orexin receptors are highly specific for orexin neuropeptides; NPY, secretin, melanocortin, and other neuropeptides do not interfere with orexin binding to its receptors.

1.4.3 Orexin receptor distribution

Following an initial study by Trivedi and colleagues (1998), Marcus et al (2001) reported a more detailed account of the orexin receptor system in the rat brain. They

reported that OX_1R mRNA is localized mainly in prefrontal and infralimbic cortex, hippocampus, paraventricular thalamic nucleus, ventromedial hypothalamic nucleus, dorsal raphe nucleus and locus coeruleus. OX_2R mRNA was observed in cerebral cortex, basal forebrain cholinergic nuclei, hippocampus, midline and intralaminar thalamus, raphe nuclei, and hypothalamic tuberomammillary, dorsomedial, paraventricular and ventral premammillary nuclei. From this study it would appear that the distribution patterns of OX_1R and OX_2R are distinct and complementary, although partially overlapping, suggesting different physiological roles for each receptor subtype.

1.4.4 Orexin immunocytochemistry

The total number of orexin neurons in the brain has been estimated at 1,100 in rats (De Lecea et al., 1998) and 70,000 in humans (Thannickal et al., 2000). These neurons are located exclusively in the lateral hypothalamus, overlapping with a distinctly different population of melanin-concentrating hormone expressing neurons (Elias et al., 1998). Immunocytochemical studies suggest that orexin neurons utilize glutamate as a rapid co-existing transmitter (Abrahamson et al., 2001; Rosin et al., 2003), and also co-express the peptide dynorphin (Chou et al., 2001), neuronal activity-regulated pentraxin (Narp, Reti et al., 2002) and display prolactin-like immunoreactivity (Risold et al., 1999). In the rat, orexin-like immunoreactivity is detectable on embryonic day 19 (van den Pol et al., 2001), and pre-pro-orexin mRNA has been reported at birth (Yamamoto et al., 2000). Few reports describe the existence of orexin outside the central nervous system, notably in the gut and pancreas (Kirchgessner and Liu, 1999). Pre-pro-orexin mRNA has also been reported in testis but not in ovaries (Jöhren et al., 2001).

A) Efferent projections

By contrast with the restricted lateral hypothalamic location of the somata of orexinergic neurons is the wide, but also selective distribution of orexinergic axonal projections throughout the CNS and spinal cord (Kukkonen et al., 2002; Lu et al., 2000; Nambu et al., 1999; Peyron et al., 1998; van den Pol, 1999). Orexin-immunoreactive fibers can be traced to: a) hypothalamic regions implicated in the regulation of feeding behaviour (i.e dorsomedial, paraventricular and arcuate nuclei); b) selective sites in thalamus, notably the midline paraventricular (PVT) and centromedial nuclei, but avoiding the specific ventrobasal relay nuclei; c) circumventricular organs (subfornical organ and area postrema); d) elements of the limbic system (hippocampus and amygdala); e) brain stem areas regulating sleep and arousal (locus coeruleus, tubermammillary and dorsal raphe nuclei); f) sites engaged in circulatory and autonomic control (e.g. dorsomedial and ventrolateral medulla, spinal intermediolateral cell column); g) spinal cord sensorimotor areas (lamina 1 and 10). This distribution generally coincides with the distribution of orexin receptors.

B) Afferent projections

Various studies have reported on cell groups that project axons to the lateral hypothalamus, but afferents to the orexin neurons are difficult to decipher because these cells are dispersed and intermingled with many other cell types. However this has now become possible in transgenic mice that express a fusion protein exclusively in orexin neurons (Sakurai et al., 2005; Maskos et al., 2002). In addition to a small number of local

interneurons, Sakurai et al (2005) reported large numbers of retrogradely labeled neurons in different amygdaloid nuclei, shell region of the nucleus accumbens, bed nucleus of the stria terminalis, medial and lateral septum, medial and lateral preoptic areas, including the ventrolateral preoptic area or VLPO (site of sleep-promoting neurons; Gallopin et al., 2000), medial habenula, hypothalamic arcuate, ventromedial, dorsomedial and paraventricular nuclei, posterior hypothalamus, midbrain tegmentum and raphe nuclei. Double labeling and electrophysiological studies confirmed a cholinergic basal forebrain input, preoptic GABAergic input and serotonergic median / paramedian raphe nuclei input. It appears that monoamine nuclei that receive innervation from orexin neurons are not reciprocally connected with them, different from cholinergic neurons in the basal forebrain which do have reciprocal connections; this feature might be important for consolidating wakefulness. A recent study using anterograde and retrograde tracers (Yoshida et al., 2006) provides additional information on the connectivity of orexin neurons and supports the notion that these neurons may integrate a variety of interoceptive and homeostatic signals to increase behavioural arousal in response to hunger, stress, circadian signals and autonomic challenges.

1.4.5 Functional considerations

A) Feeding and appetite

Because of the exclusive location of orexin neurons and the concept of the lateral hypothalamus as a designated feeding centre, the function of the orexins was originally believed to be primarily in the regulation of appetite and food intake (reviewed in Elmquist et al., 1999, Follwell and Ferguson, 2002). Consistent with this notion are

physiological data indicating that injections of orexin into the brain increase food intake (Edwards et al., 1999; Haynes et al., 1999; Sakurai et al., 1998) and injections of orexin antiserum into the hypothalamus reduce feeding (Yamada et al., 2000). Compared with other orexigenic peptides, orexin-stimulated food intake is significantly less than NPY, but similar to that of melanin concentrating hormone. In addition, fasted animals show an increase in orexin mRNA levels (Cai et al., 1999) and orexin knock-out mice are hypophagic (Hara et al., 2001). Furthermore, isolated orexin neurons are regulated by metabolic cues with reduced activity in response to glucose and leptin, and increased activity in response to ghrelin, an orexigenic peptide derived from the stomach. Orexin neurons are also directly inhibited by NPY (Fu et al., 2004). These results suggest both direct and indirect regulation of orexin neurons by energy states.

The hypothalamic arcuate nucleus has emerged as a key region for appetite regulation. Its neurons synthesize molecules with defined roles in feeding, namely orexigenic neuropeptide Y (NPY), pro-opiomelanocortin (POMC, the precursor for the appetite-suppressing neuropeptide α-MSH), and GABAergic neurons, some of which co-express NPY. Orexin neurons receive innervation from arcuate NPY neurons (Broberger et al., 1998; Elias et al., 1998) and orexin-immunoreactive terminals in turn synapse on arcuate NPY neurons (Horvath et al., 1999a; Jain et al., 2000). Pretreatment of animals with the NPY Y_1 or Y_5 antagonists blocks the orexigenic effect of exogenously administered orexins (Ida et al., 2000; Yamanaka et al., 2000). The observation that Fos expression in NPY neurons is stimulated by orexin suggests that orexin may exert its effects on food intake by activating arcuate NPY neurons. Several reviews summarize the main neuroanatomical and electrophysiological information currently available in feeding

behaviour (Jobst et al., 2004; Leibowitz and Wortley, 2004; Stanley et al., 2005).

B) Regulation of sleep-waking states

A major function emerging from research on orexins is their role in regulating sleep and wakefulness. Orexin neurons project to regions classically involved in sleep regulation and vigilance, including the monoaminergic systems (locus coeruleus; dorsal raphe; tuberomammillary nuclei), cholinergic neurons in the brainstem (Burlet et al., 2002) and basal forebrain (Eggermann et al., 2003) and GABA/galaninergic neurons in the VLPO. As mentioned below, exogenous application of orexin is excitatory, depolarizing cells and increasing the firing rates of neurons in the locus coeruleus, raphe , tuberomammillary nuclei and cholinergic neurons in the pons and basal forebrain, but apparently is without effect on GABAergic neurons in the VLPO. Central administration of orexin in rodents reduces REM sleep and increases wakefulness (Hagan et al., 1999).

Genetic studies provide persuasive evidence for a role of orexin in regulating sleep/wake states. Briefly, animals with orexin gene knockouts and/or OX_2R gene mutations have features that resemble narcolepsy in humans. Narcolepsy, regarded as a sleep disorder for more than 100 years, is characterized by the tetrad of excessive daytime sleepiness, cataplexy (sudden, bilateral loss of muscle tone without loss of consciousness, often brought on by sudden action or emotion), hypnagogic hallucinations, and sleep paralysis (reviewed in Scammell, 2003). Known to be genetically transmitted in canines as an autosomal recessive gene called canarc-1, the mechanism of this disease in humans has remained unclear until the discovery of orexins (see Siegel, 1999). Some cases of canine narcolepsy (Doberman, Labrador, and

Dachshund) were shown to involve an OX_2R mutation, and pre-pro-orexin knock-out mice display similar manifestations. Indeed, various conditions that interfere with the integrity of the neurons, the peptides they secrete, or their receptors are associated with a deficiency in wakefulness and the development of narcolepsy (Chemelli et al., 1999; Hara et al., 2001; Lin et al., 1999; Nishino et al., 2000; Peyron et al., 2000; Thannickal et al., 2000). Several other mouse models of deficiency in orexin signaling have been developed, among which are mice lacking either the *orexin* gene (pre-pro-orexin knockout mice) or orexin neurons (orexin/ataxin-3 transgenic mice), as well as mice with null mutations in the both orexin receptor genes; all have remarkably similar phenotypes to those of human narcolepsy (Chemelli et al., 1999; Hara et al., 2001; Lin et al., 1999; Willie et al., 2003). In humans, where narcolepsy occurs in approximately 1 in 2,000 individuals, most cases are sporadic, and narcolepsy seems to be a polygenic and/or an environmentally influenced disorder. Interestingly, a link between narcolepsy and Human Leukocyte Antigen HLA-DQBI0602 has lead to the suggestion that narcolepsy might be an autoimmune disease, causing selective destruction of the orexin-synthesizing neurons in the LHA. Indeed, a majority of HLA-DQBI0602 positive patients with narcolepsy-cataplexy have undetectable orexins in their cerebrospinal fluid (Scammell, 2003). This mechanism would be consistent with the hypothesis that narcolepsy is caused by environmental factors that act on a susceptible genetic background.

C) Neuroendocrine regulation

Orexin neurons innervate medial hypothalamic neurons that are known to regulate anterior pituitary functions, and orexins have a major role in governing the hypothalamic-

pituitary adrenal axis (HPA; reviewed by Spinazzi et al., 2006). Intracerebroventricular administration of orexin increases secretion of thyrotropin releasing hormone (Mitsuma et al.,1999), enhances corticotropin (ACTH) and corticosterone release, a component of which may be mediated via the release of NPY (Russell et al., 2001). These effects may also reflect a direct action on parvocellular paraventricular nucleus releasing factor neurons which in turn are also responsive to exogenous orexin when studies in brain slice preparations (Follwell and Ferguson, 2002). Effects on the HPA axis are markedly reduced in pregnancy (Brunton and Russell, 2003). Intracerebroventricular administration of orexin also influences LH secretion, inhibitory during diestrous or low estrogen states and excitatory during surge conditions (Pu et al., 1998). Gonadotropic releasing hormone (GnRH) neurons co-express OX_1R and are directly contacted by orexin fibers (Campbell et al., 2003). The observation that orexin neurons are themselves contacted by CRF-immunoreactive terminals, express CRF-R1 receptors and are depolarized by exogenous CRF (Winsky-Sommerer et al., 2004) implies a dynamic interplay between the HPA and orexin systems. Intracerebroventricular administration of orexin also stimulates drinking behaviour, almost as potently as angiotensin (Kunii et al., 1999), possibly reflecting the excitatory action of orexins on neurons in the subfornical organ and/or area postrema (Yang and Ferguson, 2002).

D) Autonomic functions

Orexin neurons project to CNS sites involved in autonomic regulation, notably the paraventricular nucleus in the hypothalamus, the nucleus of the solitary tract and the rostral ventrolateral area in the medulla oblongata, dorsal motor nucleus of the vagus, the

caudal region of the sacral spinal cord and the intermediolateral column (see Zheng et al., 2005; van den Pol et al., 1999). Viral transneuronal labeling has demonstrated that orexin neurons are linked to different sympathetic outflow systems (Geerling et al., 2003). Collectively these observations support a role in regulation of both the sympathetic and parasympathetic parts of the autonomic nervous system. For example, data support the conclusion that orexin neurons are activated by systemic hypoglycemia, releasing orexin in the dorsal motor nucleus of the vagus and thereby stimulating pancreatic efferent firing (Wu et al., 2004). Intracerebroventricular administration of orexin administration induces a dose related increase in mean arterial pressure, heart rate and renal sympathetic nerve activity (reviewed in Shirasaka et al., 2003), responses that are similar to those observed during stress (Samson et al., 1999; Shirasaka et al., 1999). Kayaba et al (2003) find reduced baseline blood pressure and attenuated defense responses in orexin knockout mice. Potential sites of direct orexin actions include spinal preganglionic neurons (Antunes et al., 2001; van den Trop et al., 2003) and the nucleus of the solitary tract cells (Yang et al., 2003). Injection of orexin into the diagonal band of Broca has been reported to initiate thermogenesis through a sequential stimulation of the sympathetic nervous system (Monda et al., 2004). The orexin innervation to the locus coeruleus may also participate in enhancing vigilance, attention and cognitive aspects of any stress related cardiovascular response.

1.4.6 Cellular electrophysiology and receptor-mediated actions

The objective of the research described here is to use electrophysiology to examine the actions of orexins on PVT neurons. This section of the thesis provides an overview of the literature already available on the electrophysiology of orexins.

A) Spinal cord motoneurons

The presence in the spinal ventral horn of orexin fibers and OX_1Rs prompted a study (Yamuy et al., 2004) that may be the only report to date of an action mediated by endogenously released orexin. They reported that in the intact anesthetized cat, electrical stimulation in the lateral hypothalamus induced a depolarizing response in motoneurons that was selectively reduced by application of SB-334867, an OX_1R antagonist. Local orexin application induced depolarization and firing without apparent change in membrane resistance and, in a few neurons, an enhancement of synaptic activity (increased membrane noise), suggesting (but clearly not proving) the possibility of both pre- and postsynaptic sites of action.

B) Spinal superficial dorsal horn neurons

In rat slice preparations, patch clamp studies reveal that orexin B induced a tetrodotoxin (TTX)-resistant inward current and a near parallel shift in I/V plots, possibly reflecting involvement of a number of conductances, and increase in baseline noise (Grudt et al., 2002). The fact that these were blockable with suramin indicates involvement of a P2x purinergic receptor agonist, perhaps ATP, in the orexin response. Note of an increase in the frequency of spontaneous inhibitory postsynaptic currents

(IPSCs), blockable with tetrodotoxin (TTX) and strychnine, may reflect an action on presynaptic glycinergic interneurons.

C) Nucleus pontis oralis (NPO)

The NPO, which plays a key role in REM sleep, receives projections from the lateral hypothalamic orexin neurons (Peyron et al., 1998). Microinjections of orexins into NPO induced a similar behavioural state (REM sleep), and juxtacellular application of orexin A resulted in cell depolarization, increased firing, an increase in the amplitude of locally-evoked excitatory postsynaptic potentials (EPSPs) as well as frequency and amplitude of spontaneous EPSPs; collectively these data suggest orexin modulates excitatory inputs to NPO neurons (Xi et al., 2002, 2003).

D) Area postrema (AP)

AP is a midline circumventricular organ whose neurons participate in autonomic functions, body fluid balance and feeding control. In slice preparations, orexin A was reported to depolarize the majority of cells tested, effects that were considered to be mediated via non selective cationic channels since currents reverse around –43 mV (Yang and Ferguson, 2002).

E) Nucleus tractus solitarius (NTS)

In brain slices, application of orexin A is reported to depolarize ~90% of NTS neurons by activating a nonselective cationic conductance and inhibit a sustained potassium conductance, actions that are mediated through activation of phospholipase C

and protein kinase C (Yang et al., 2003a, b). In slices from caudal NTS, bath applied orexin B is reported to have a selective enhancing action on evoked, spontaneous and miniature excitatory postsynaptic currents (EPSCs), suggestive of a presynaptic site of action (Smith et al., 2002).

F) Dorsal motor nucleus of vagus (DMV)

Orexins acting at postsynaptic receptors induced inward currents and depolarized neurons that project to the gastric fundus and corpus, effects mediated in large part by reduction in a potassium conductance but possibly also involving a non-selective cation conductance (Hwang et al., 2001). By contrast with NTS, these neurons also displayed an orexin-induced selective enhancement of inhibitory synaptic and miniature IPSCs, consistent with an action at presynaptic receptors (Grabaukas and Moises, 2003; Davis et al., 2003).

G) Locus coeruleus (LC)

Several papers have report orexin-induced excitation, inward currents and depolarization in LC neurons, mediated by reduction in a sustained potassium conductance and/or nonselective cationic conductance; also reported is action potential broadening and reduction in afterhyperpolarizations (Bourgin et al., 2000; Hagan et al., 1999; Horvath et al., 1996; Ivanov and Aston-Jones, 2000; Murai and Akaike, 2005; van den Pol et al., 2002). Excitatory actions were antagonized by SB-334867-A, an antagonist for the OX_1R (Soffin et al., 2002).

H) Dorsal Raphe Nucleus (DRN)

Orexin neurons densely innervate major aminergic cell groups including DRN (e.g. Chemelli et al., 1999). Several studies have reported excitatory actions of orexins in rat DRN cells. In a brief report, Brown et al (2001) reported a TTX resistant and prolonged orexin-A-induced dose-dependent membrane depolarization; an associated variable decrease in a leak potassium conductance was proposed as a mechanism. A subsequent and more detailed analysis (Brown et al., 2002) revealed actions by both orexin A and B inducing inward currents with increase in membrane noise; since effects were strongly reduced by replacement of external sodium by the impermeant ion N-methyl-D-glucamine (NMDG), they concluded that orexin was activating a mixed cation channel (possibly the phospholipase C coupled transient receptor potential channels) alone or in combination with reduction of a leak potassium conductance. Of note is evidence of the probable convergent actions of orexins, histamine and noradrenaline on the same channels in DRN neurons. By contrast, a study by Liu et al (2002) reported orexin-induce inward currents in DR neurons that reversed near –18mV, attributed to actions mediated by a postsynaptic sodium-dependent nonselective cationic channel; also noted was a TTX-sensitive increase in GABAergic synaptic activity, implying activity-dependent effects on GABAergic neurons that project to the recorded neurons. The increases in excitability induced by orexin receptor agonists in DRN appears to reflect activation of the OX_2R subtype rather than the OX_1R subtype (Soffin et al., 2004).

In an in vivo study, Takahashi et al (2005) reported that orexin's action on DR neurons was most pronounced during rapid eye movement (REM) sleep when the cells

are usually silent; this suggests that the orexinergic system may suppress REM sleep through activation of serotonergic DR neurons.

A recent report by Haj-Dahmane and Shen (2005) presents a novel mechanism that implicates orexins in endocannabinoid release arising through a postsynaptic OX_2R - mediated action that depends on activation of the PLC/diacylglyceride (DAG) pathway but without raising postsynaptic intracellular calcium; released endocannabinoids act retrogradely to induce depression of glutamatergic currents in DRN.

I) Tuberomammillary nucleus (TMN)

Since both orexin A and B depolarized TMN neurons, their effects were considered to be mediated via OX_2Rs (Bayer et al., 2001; Yamanaka et al., 2002). In a study by Eriksson et al (2001), orexin-induced depolarization was reported to be TTX resistant (i.e. postsynaptic) and accompanied by a slight reduction in input resistance (92.2 ± 1.8%). By contrast with reports in other neurons, the orexin responses they observed were largely unaffected by manipulation of the external potassium concentration, membrane conductance was increased rather than decreased, and nickel abolished the depolarization, none of which would be consistent with involvement of a potassium channel. However, the fact that the orexin responses could be largely abolished by KB-R7943, a selective blocker of the electrogenic sodium-calcium exchanger (Iwamoto et al., 2001), lead Ericsson et al to conclude that orexins mediated their actions in TMN neurons mainly via activation of sodium-calcium exchanger, but also a calcium current. Recent studies by Hoang et al (2003) led them to suggest that GIRK channels in tuberomammillary and locus coeruleus neurons are suppressed by orexins.

J) Hypothalamus

Studies on cultured hypothalamic neurons suggest that orexins can increase the frequency of both GABAergic and glutamatergic spontaneous postsynaptic currents, and that a subpopulation of cells display a rise in intracellular calcium that presumably reflects calcium influx from outside the cell (Van den Pol et al., 1998). Additionally, these authors reported that microdrop applications of orexin increased IPSCs evoked in putative neuroendocrine neurons in the arcuate nucleus, and increased the frequency but not amplitude of miniature IPSCs and EPSCs, features that would imply orexin receptors are located presynaptically. By and large however, most studies report an action mediated by postsynaptic receptors.

Arcuate nucleus (ARC). The ARC, a key centre for energy homeostasis and feeding behaviour, receives a dense orexinergic innervation (Peyron et al., 1998). ARC cells express markers for both OX_1Rs and OX_2Rs. However, receptor localization studies disagree as to receptor types: Marcus et al (2001) reported only OX_2Rs while Guan et al (2002) reported OX_1Rs; on the other hand, Burdakov et al (2003) reported mRNA for both receptors. Orexin injections into the ARC can stimulate feeding (Willie et al., 2001). Extracellular recordings suggest that orexin increases firing of a subset of ARC neurons (Rauch et al., 2000). In a patch clamp analysis in mouse ARC neurons, Burdakov et al (2003) noted that a subset of glutamate decarboxylase (GAD) expressing neurons are selectively and directly depolarized by orexin-A acting at OX_2Rs. In the latter analysis, orexin was seen to induce a current that reversed around -35mV, is reversed by KB-R7943, with a reversal potential that shifts in a negative direction in low Na^+ solutions

and in a positive direction in low Ca^{2+} solutions, leading them to conclude that orexin was activating the sodium-calcium exchanger and mobilizing calcium from intracellular stores rather than from outside sources. More recently van den Trop et al (2004) reported that orexins induced burst firing and pacemaker activity selectively in the NPY/Agouti-related peptide ARC neurons, leading them to comment that this induced activity might be key to the release of these peptides in central energy circuits.

Paraventricular nucleus (PVN). OX_2Rs predominate in PVN (Marcus et al., 2001; Trivedi et al., 1998), indicating that neurons here are likely to respond to both orexin A and B. Observations and conclusions differ between studies. In vivo, no PVN neurons were reported to respond to micoiontophoretic application of orexin A (Shiraishi et al., 2000). However, data obtained in vitro in slice preparations by Shirasaki et al (2001) indicated that orexin B applications could induce TTX-resistant depolarization without significant changed in membrane resistance, responses seen in both magnocellular (type 1) and parvocellular (type 2) PVN neurons, implying that both cell types possessed postsynaptic OX_2Rs. By contrast, Follwell and Ferguson (2002) reported that a majority (67%) of the magnocellular PVN neurons respond to bath applied orexin A with membrane depolarization and increase in both frequency and amplitude of spontaneous EPSCs. In the presence of TTX, they observe that these depolarizations were no longer observed, an indication of an indirect action. They also noted that orexin enhanced the inward current evoked by exogenous glutamate, suggesting that the peptide could modulate a postsynaptic glutamate receptor. By contrast, the response in a majority of parvocellular PVN neurons was a TTX-resistant membrane depolarization and inward

current that reversed near – 45 mV, suggesting involvement of a non-selective cationic conductance.

Lateral hypothalamic area (LHA). LHA contains glucose-sensitive neurons (GSNs), cells that are inhibited by rising glucose levels and excited by the converse, thus contributing to body energy homeostasis (Levin et al., 1999). Lowering glucose increases cytosolic calcium in orexin neurons, demonstrating their glucose sensitivity (Muroya et al., 2001). Two studies suggest that orexins preferentially excite GSNs, identified by an increase in firing when glucose concentration falls (see Liu et al., 2001). In vivo, Shiraishi et al (2000) reported an increased firing in 66% of glucose sensitive (also 40 % of non-glucose sensitive) neurons by microiontophoresed orexin. Interestingly, these authors also reported that orexin decreased cell firing in glucose-responsive and non-responsive neurons in the hypothalamic ventromedial nucleus (VMH), a putative satiety centre. Note that this is the only report to date where orexin appears to *decrease* cell excitability. In LHA slice preparations, Liu et al (2001) confirmed a direct (TTX-resistant) postsynaptic depolarization by orexin A that was selective for GSNs.

K) Ventral tegmental area (VTA) and laterodorsal tegmental nucleus (LDT)

Both dopaminergic and GABAergic neurons in the VTA are reported to be directly excited by orexin A and B, and a subpopulation of dopaminergic neurons to respond with bursting activity patterns (Korotkova et al., 2003). Cholinergic neurons in the LDT and adjacent pedunculopontine tegmental nucleus provide the major cholinergic input to the thalamus and medial pontine reticular formation, and their activity is linked

to REM sleep and arousal (Steriade and McCarley, 1990). In cell attached recordings, mouse LDT cells responded to orexin-A with prolonged firing, responses that were reduced in subsequent applications, possibly indicative of receptor desensitization (Burlet et al., 2002). In addition, data from whole cell recordings from both cholinergic and non-cholinergic LDT cells revealed a complexity of postsynaptic and presynaptic actions: a) in the presence of low calcium media to inhibit synaptic transmission, orexin A induced long lasting inward currents (~ –35 pA) with an associated increase in membrane conductance (105~178% of control) and membrane noise, considered due to activation of a postsynaptic cationic conductance; b) an increase in evoked EPSCs, and sEPSCs due to an action on presynaptic neuronal transmission, blockable in TTX. In a subsequent analysis, Kohlmeier et al (2004) reported that orexins elevated intracellular calcium in LDT and dorsal raphe neurons by enhancing calcium influx, in part mediated via L-type calcium channels and activation of protein kinase C (PKC). Note that these data differ from results obtained in OXR-transfected cells (Sakurai et al., 1998) where orexin was observed to increase calcium release from intracellular stores via a protein kinase A (PKA)-related mechanism.

L) Substantia nigra (SN)

SN contains both GABAergic and dopaminergic neurons, cells that can be distinguished in extracellular recordings by their fast (GABA) or slower (dopaminergic) firing frequency. In slice preparations, it appears that orexins selectively excite dopaminergic but not GABAergic neurons, and that bath applied thapsigargin significantly reduces the orexin effects, an indication that calcium release from

intracellular stores is an important component of the response. These effects are not blocked by application of a specific PKC inhibitor but are blockable with a PKA inhibitor (Korotkova et al., 2002).

M) Thalamus

In thalamus, orexin fibers project selectively to midline and intralaminar neurons, components of the so-called "non-specific" thalamocortical system (Peyron et al., 1998). In a study by Bayer et al (2002), all neurons tested with orexins in the centromedial and rhomboid nuclei demonstrated TTX-resistant depolarization, probably mediated via OX_2Rs, and involving decrease in a potassium conductance. None of the cells tested in the ventral posterolateral or lateral geniculate nuclei displayed responsiveness to orexins.

As mentioned earlier, the thalamic paraventricular nucleus (PVT) receives a dense orexinergic innervation. The first published paper on PVT in rat brain (Ishibashi et al. 2005) reported that both orexin A and B evoked a dose-dependent excitation (10^{-8} to 10^{-6} M) of 80% of cells tested during extracellular recordings, membrane depolarization and an associated increase in membrane resistance due to closure of potassium channels. Orexin B also induced a rise in intracellular calcium that was abolished in calcium free solutions. In the mouse, a recent publication (Huang et al., 2006) reported a similar depolarizing action of orexin on PVT neurons whose axons project to the prefrontal cortex, mediated mainly via OX_2Rs that induced either closure of potassium channels or opening of nonselective cation channels.

N) Forebrain nucleus basalis (NB) cholinergic neurons

OX_1Rs and OX_2Rs are present in NB, a component of the forebrain arousal system. Basal forebrain medial-preoptic neurons, but not adjacent VLPO neurons, are reported to be excited directly by orexins acting at OX_2Rs (Eggermann et al., 2001). In cultured NB neurons, orexin A applications are reported to decrease whole cell conductances mediated by a pertussis toxin (PTX)-insensitive G-protein and that leads to activation of two families of inwardly rectifying potassium channels: GIRK and KirNB channels (Hoang et al., 2003, 2004).

O) Cerebral cortex

Postsynaptic OX_2Rs are reported to have a selective distribution in sublayer 6b in several regions of the cerebral cortices (somatosensory, primary visual, primary motor, cingulated) where their activation induces a TTX-resistant membrane depolarization via reduction in a K conductance (Bayer et al., 2004). In slice preparations of prefrontal cortex, data obtained from layer V pyramidal neurons indicated a concentration dependent (1nM-300μM) orexin-1 and orexin-2 peptide induction of TTX-sensitive spontaneous EPSPs that were due to excitation of thalamocortical boutons innervating spines on apical dendrites, causing transient calcium increases congruent with multiple spiking events in their glutamatergic afferents (Lambe and Aghajanian, 2003). This would represent a first demonstration of postsynaptic calcium transients resulting from neurotransmitter-evoked, TTX-sensitive depolarization of a population of axon terminals. The finding that orexins can excite the final synapse in the ascending arousal pathway suggests a mechanism for coordinating aspects of attention and arousal at a cortical level.

P) Endocrine and non-excitable cells

Studies in mouse peritoneal macrophages (Ichinose et al., 1998) indicate that orexin-B can activate charybdotoxin-insensitive calcium-dependent potassium currents. In primary cultured ovine somatotropes, orexin A and orexin B increased L-type calcium currents and growth hormone secretion in a dose-dependent manner, effects that involve PKC-mediated signaling pathways (Xu et al., 2002, 2003).

Q) Orexins and calcium currents

Initial data from heterologous expression systems and CHO cells transfected with OX1Rs indicate that orexins elevate intracellular calcium by a mechanism involving activation of PLC and the inositol triphosphate (IP_3)-mediated release of Ca^{2+} from SERCA pump-dependent intracellular stores, responses that are inhibited after thapsigargin-mediated depletion of internal stores (Sakurai et al., 1998; Smart et al., 1999; Lund et al., 2000). However, this appears to differ from mechanisms in native orexin receptors in neurons. For example, in laterodorsal tegmental and dorsal raphe neurons, activation of orexin receptors is noted to engage a Ca^{2+} influx pathway that is separate from store-dependent mechanisms and lacks sensitivity to thapsigargin and ryanodine (see Kohlmeier et al., 2004) . These data are consistent with observations of thapsigargin-insensitive orexin-induced Ca^{2+} transients in cultured hypothalamic neurons (van den Pol, 1999), cultured cortical neurons (Xia et al., 2005), and orexin-induced Ca^{2+} transients in isolated ventral tegmentum neurons where responses were sensitive to extracellular $[Ca^{2+}]_o$ (Uramura et al., 2001). The data from Kohlmeier et al (2004)

indicate that activation of orexin receptors increases calcium influx in part via L-type calcium channels and activation of PKC pathways. Data from recent studies in Chinese Hamster Ovary (CHO) cells indicate that OX_1Rs couple to at least three different signal pathways to regulate adenylyl cyclase activity, implying the possible importance of cAMP in orexin signaling (Holmqvist et al., 2005). Another study suggests that activation of OX_1Rs can lead to opening of Ca^{2+} permeable channels, events that seem to imply involvement of the transient receptor potential (TRP) channels and PKC (Larsson et al., 2005).

1.5 Purpose of this research

As mentioned earlier and above, the available evidence indicates that orexins appear to exert an excitatory influence on neuronal excitability. The strong innervation of PVT by orexin-immunoreactive fibers and prevalence of orexin receptors in PVT supports the notion that orexins will also depolarize PVT neurons. Such an action of orexins may be relevant to 'orchestrating' the activity of PVT neurons during arousal and motivated behaviours, functions generally assigned to this thalamic nucleus. However, since relatively little is known about the morphology and intrinsic properties of PVT neurons, this research first examines their properties, then proceeds to evaluate how PVT neurons respond to exogenous applications of orexin A and orexin B peptides, and lastly to provide an intial evaluation of the conductances that may be affected by activation of orexin receptors on PVT neurons.

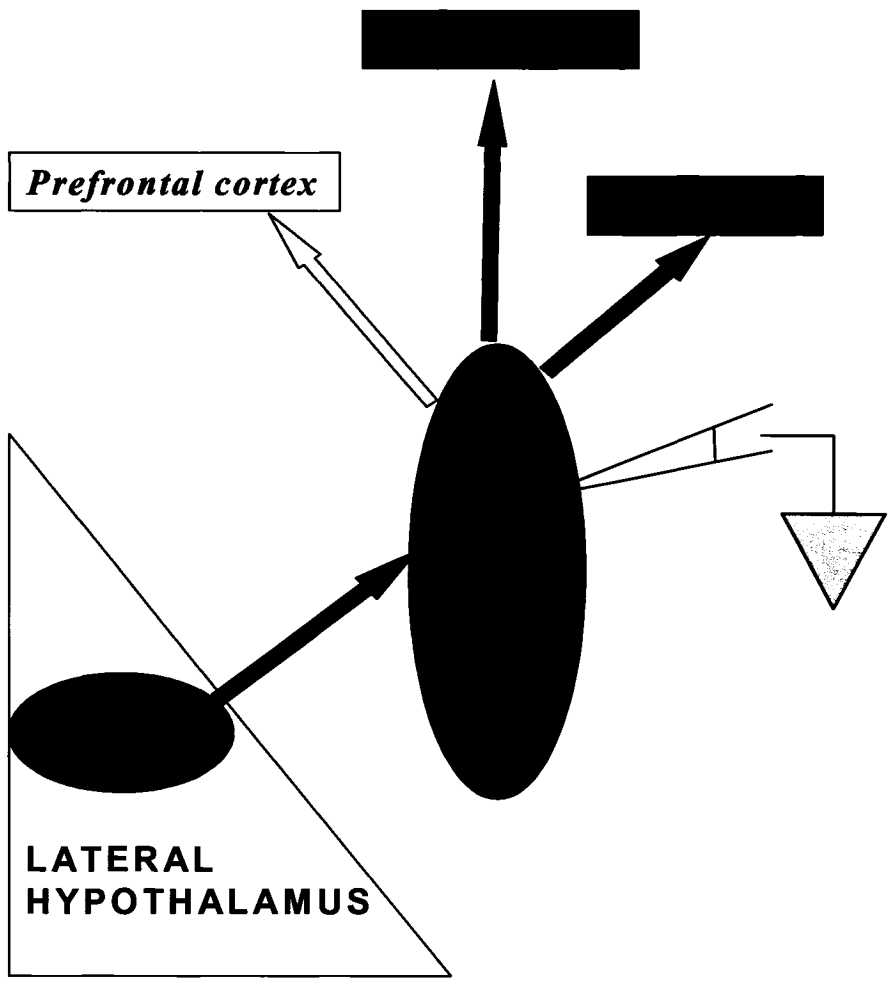

Figure 1.1. Orexin neuron-PVT connections. Schematic diagram depicts the possible linkages from the lateral hypothalamic orexinergic (also likely glutamatergic) neurons to PVT neurons that are known to project their axons to prefrontal cortex, nucleus accumbens and amygdala.

CHAPTER 2

METHODOLOGY

Experiments used Wistar rats (14~21 days) of either sex. All experiments conformed to Canadian Council for Animal Care and Ottawa Health Research Institute guidelines on the ethical use of animals in research.

2.1 Slice preparation

The animals were decapitated with a guillotine, the brain was quickly removed and immersed in oxygenated (95%O_2-5%CO_2) cooled (4~6 °C) artificial cerebrospinal fluid (ACSF) for 2-3 minutes. ACSF contained (in mM): 127 NaCl, 3.1 KCl, 1.3 $MgCl_2$, 2.4 $CaCl_2$, 26 $NaHCO_3$, 10 glucose, pH 7.3, 300 - 310 mOsm. A block of brain tissue containing the thalamic paraventricular region was dissected, glued to a small stage (LOCTITE 404 cyanoacrylate glue) and covered with ACSF. Coronal slices 400 microns thick containing anterior through to posterior PVT were made with a vibratome (Series 1000) and preincubated in oxygenated ACSF at room temperature (22~26 °C) for at least 1 hour, then transferred to a submerged chamber and superfused (2~3 ml/min) with oxygenated ACSF at room temperature.

2.2 Electrophysiological recordings

Using the cell-attached or whole-cell patch-clamp technique, and blind recording technique (Fig. 2.1), recordings were obtained from PVT neurons with borosilicate thin-walled micropipettes pulled on a Flaming-Brown puller (P87). For cell attached

recordings, patch pipettes were filled with ACSF. For whole-cell recordings, pipettes were filled with the following solution (in mM): 135 Kgluconate, 5 KCl, 5 NaCl, 1 $MgCl_2$, 10 HEPES, 1 EGTA, 3 Na_2ATP, 0.2 GTP, pH 7.3 with KOH, 290-310 mOsmol. Pipettes had resistances of 3~7 MΩ. Access resistance <15 MΩ was considered acceptable. Lucifer Yellow (0.2%) or biocytin (0.2%) was included in the pipette solution to label recorded neurons. Usually, only one neuron per slice was recorded. Whole-cell current-clamp and voltage-clamp recordings were obtained with an Axopatch 1D or Axopatch 200B (Axon Instruments, Foster City, CA). Data were filtered at 2 kHz. Data acquisition and storage for subsequent off-line analysis were achieved with Digidata 1200 interface and clampex (pClamp9) and permanent records were made on a chart recorder (Gould, Brush 220).

2.3 Application of agents

Agents were made up as concentrated stocks in distilled water and were subsequently diluted to the required concentration in ACSF prior to application. Agents included barium chloride and tetrodotoxin (TTX) from Sigma, and orexin A and B from Bachem. All peptides and channel blockers were applied by bath perfusion.

2.4 Quantification of membrane properties

Off-line analysis was performed using Clampfit version 9 (Axon Instruments). Resting membrane potential (Vrest) was the low-pass readout of the electrode amplifier and was corrected for liquid junction potential (~12 mV) after terminating the recording. Conductance was determined from the linear slope (between -50 mV to -70 mV) of the

current-voltage (I-V; Vhold = -50mV) relationships. The spike amplitude was quantified as the difference between the Vrest (the abrupt increase in slope depolarization) and the peak voltage. The time to peak of AHP was estimated as the time from the peak of the action potential to the peak of AHP. The amplitude of AHP was estimated as the difference between the threshold and the most negative voltage reached during the AHP (defined as the peak of AHP). These spike parameters refer to the properties of spontaneously generated spikes or, in the case of silent cells, of spikes elicited by injection of threshold current. The time-dependent inward rectification (I_h) was measured as the difference between steady-state current and transient current during a voltage step from -50 to -110mV (see Fig. 3.2). The time-independent inward rectification (I_R) was measured from I-V plots by the ratio of the slope of the line fit in the -90 ~ -110 mV range to that of the line fit in the -50 ~ -70 mV range (see Fig. 3.3). The properties of firing patterns and hyperpolarizing responses were analyzed from voltage responses to injected current pulses. Instantaneous firing frequency was calculated as the reciprocal of the interspike interval (ISI). Frequency adaptation was quantified by measuring successive spike firing frequency in a train evoked by current pulses. The spike firing frequencies were plotted against the interval number since train onset. The spike adaptation ratio was defined as the ratio of the frequency of the first two spikes to the frequency of the last two spikes in the train elicited by current injections from resting membrane potential. The spike width was measured at ½ of the total spike amplitude (measured from the threshold level). All values presented are the means ± S.E.M. Statistical comparisons between control and experimental values ($p<0.05$ and better) were determined using both the paired or unpaired Student t-test and ANOVA.

2.5 Histology and Immunohistochemistry

After electrophysiological recordings, slices containing labeled thalamic neurons were fixed in a cold solution containing 4% paraformaldehyde for at least 4 hours. Lucifer Yellow-filled neurons were subsequently visualized using dimethylsulfoxide (DMSO; 1%). In this condition, the limits of the thalamic nuclei could be determined visually under the microscope by differential background staining with respect to surrounding structures. Morphology pictures for Lucifer Yellow labeled cells were taken using confocal laser scanning microscopy (LSM 510, Zeiss). Biocytin-filled neurons were incubated overnight in 1% stock Tris buffered saline containing 1% Triton solution (TBS-T), and washed two times for 10 min each in 1% TBS-T. This and all subsequent incubations were carried out in a single well of a standard well plate, gently agitated on an orbital shaker at room temperature. Endogenous peroxidase activity was suppressed by incubating slices in 250 µl of 30% H_2O_2 in 15 ml methanol for 45 min. After rinsing again in 1% TBS-T, biocytin filled cells were stained using the ABC peroxide kit. The A and B reagents were diluted in 1% TBS-T, and the tissue slices incubated overnight at room temperature. The following day, slices were equilibrated twice for 10 min each in 1% Tris buffered saline and once for 10 min in acetate buffer (pH=6) at room temperature, essential for reducing non-specific background staining. The slices were then developed using diaminobenzidine (DAB) (in 20 ml acetate buffer containing 500 mg nickel ammonium sulfate, 40 mg β–D glucose, 8 mg ammonium chloride and 5 mg DAB) to visualize peroxidase bound to biocytin. To react, glucose oxidase (14 µl/2 ml DAB solution) was added for 5~10 min and the reaction stopped by washing the slices in

acetate buffer (10 min) and TBS (twice, 10 min each). Slices were equilibrated with increasing concentrations of alcohols (50, 75, 90, 95 and 100%) for 2 min, cleared in 100% xylene (twice, 2 min each) and mounted on glass slides. Cell morphology was drawn using photomicrographs of PVT cells labeled with intracellular biocytin and camera lucida.

2.6 Orexin A Immunohistochemistry

Animals were deeply anesthetized with sodium pentobarbital (70 mg/kg, i.p.) and perfused transcardially with 0.9% saline, followed immediately by 500 ml of 4% paraformaldehyde (Sigma) in 0.01 M phosphate buffer (pH=7.4). The brains were post-fixed in the paraformaldehyde solution overnight and subsequently taken through sucrose solutions (20% sucrose in 0.01 M phosphate buffered saline [PBS], pH 7.3) at 4°C until the brains sank in the 20% sucrose solution (24 hours). The brains were then frozen and 40-μm coronal sections were taken by using a cryostat microtome. Sections were placed into an individual well containing 0.1 M PBS (pH 7.4) and stored at 5°C for at least 24 hours. For immunohistochemical processing, sections were washed twice (10 minutes each time) in Tris buffer containing 1% triton x-100 (TBS-T) and blocked for 1hour with TBS-T containing 10% normal goat serum (NGS) at room temperature. Sections were then washed again and incubated overnight at 4 °C with primary orexin A antibody (1:2,000; Sigma) diluted in TBS-T containing 2% NGS. After incubation, tissue was rinsed three times with TBS-T and then exposed to a goat anti-rabbit biotinylated secondary antibody (1:400; Vector Lab, Burlington, ON, Canada) for 1 hour. Tissue was exposed to avidin-biotin-peroxidase complex (ABC complex, 1:800; Vector Lab,

Burlington, ON, Canada) for 1 hour and stained with diaminobenzidine (DAB, Vector Lab, Burlington, ON, Canada) containing nickel ammonium sulfate to yield a blue / black reaction product. After the DAB reaction, the sections were mounted on gelatin-coated glass slides and air-dried. The sections were then taken through graded alcohols (50~100%), cleared in xylene for 10 minutes, and cover-slipped with Permount (Fisher Scientific) mounting medium. Sections were viewed and photographed using a Zeiss microscope and digital camera.

Several investigators using immunocytochemistry have clearly demonstrated that the somata of orexin-synthesizing neurons are restricted to the lateral hypothalamus while their axons have a broad central distribution (e.g. Peyron et al., 1998; Van den Pol et al., 1999). In confirmation of these observations, one animal was anesthetized, perfused, and the brain prepared for orexin immunocytochemistry (see Methods). Orexin-immunoreactive neurons were clearly visible in the lateral hypothalamic perifornical region (Fig. 2.2A and B). Relevant to the present study was the presence of a dense plexus of orexin-immunoreactive fibers through the rostro-caudal extent of PVT. Fig. 2.2A and C illustrates a section through the anterior PVT. The presence of numerous varicosities suggests that these orexin fibers form synapses on PVT neurons.

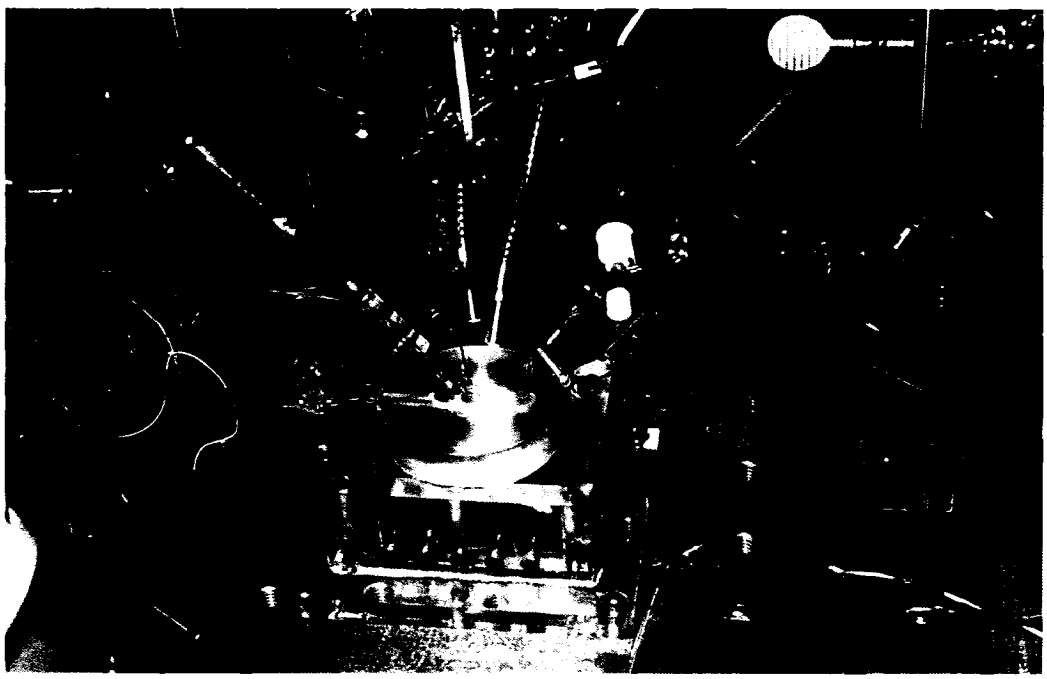

Figure 2.1. The setup for electrophysiological recordings.

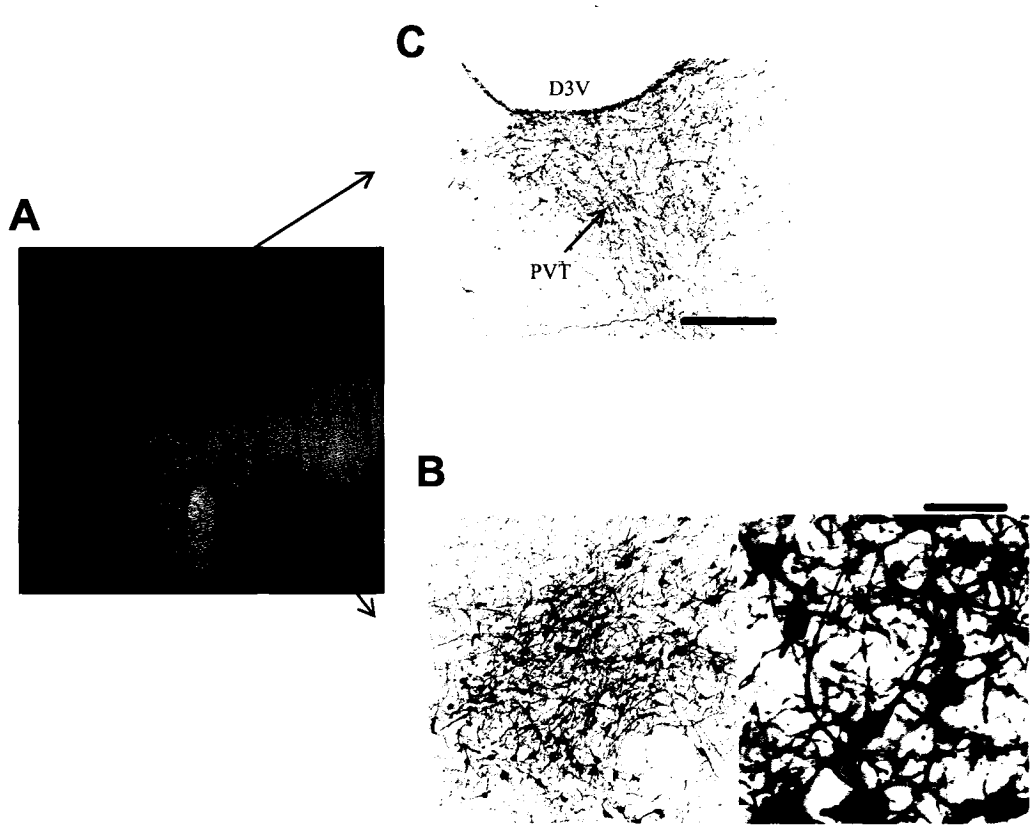

Figure 2.2. Immunohistochemical data reveal a dense distribution of Orexin fibers in the paraventricular nucleus of thalamus (PVT; primary antibody Orexin-A; 1/2000). A: Low power image of an immunostained section illustrates orexin neurons in the preifornical area (box B) and orexin axon terminal distribution in PVT (box C). **B:** Enlargements reveal details of the lateral hypothalamic orexin neuronal field with densly labeled cell bodies and fibers. **C:** Enlargement of the orexin terminal field in PVT shows how the innervation clearly outlines the contour of this section through anterior PVT, just below the dorsal third ventricle (D3V). Scale bars are 1 mm in A, 50 μm in B and 200 μm in C.

CHAPTER 3

PROPERTIES OF THALAMIC PARAVENTRICULAR NEURONS (PVT)

3.1 Introduction

The anatomical and functional data reviewed in Chapter 1 support the notion that the thalamic paraventricular nucleus (PVT) is a relay nucleus that conveys information from the hypothalamus and other brainstem sites to telencephalic areas known for their role in motivational aspects of behavior (Groenewegen and Berendse, 1994; Berendse and Groenewegen, 1990, 1991; Su and Bentivoglio, 1990; Moga et al., 1995; Bubser and Deutch, 1998; Otake and Nakamura, 1998). Although much information exists on the cellular electrophysiology of neurons located within so-called 'specific' thalamocortical relay nuclei, there is relatively little known about the properties of midline thalamic neurons, in particular PVT. Knowledge of intrinsic properties of neurons is important for understanding how they regulate their excitability, integrate afferent information and formulate appropriate output firing patterns. This chapter provides an initial description of the intrinsic passive and active properties of a sample (n=77) of neurons located within the boundaries of the PVT as featured in the rat atlas of Paxinos and Watson (2000) and outlined by the distribution of orexin immunoreactive fibers at the beginning of Chapter 4. Resting membrane potentials were measured within 1 min after establishing the whole cell configuration. Cells with resting membrane potentials more positive than –50 mV after correction of liquid junction potential were excluded in this analysis.

3.2 Results

3.2.1 Morphology of PVT neurons

A sample of PVT neurons were labeled with intracellular Lucifer Yellow (n=12) or biocytin (n=15). Neurons displayed morphological diversity with respect to soma shape and dendritic arborizations. As illustrated in Fig. 3.1A-C, somata were usually ovoid or triangular in shape, with cross-sectional diameters ranging between 7 and 20 microns. Dendritic arborizations extending for more than 300 microns varied in their profile. Cells with 3 or more primary dendrites that extended in one general direction (e.g. Fig. 3.1A) were usually located in the dorsal part of PVT with their dendrites extending towards the ependymal lining of the 3rd ventricle, reminiscent of the cells described by Balercia et al (1992). A majority of neurons displayed 2-5 primary dendrites that extended from opposite poles of the cell (e.g. Fig. 3.1B) or in all directions (e.g. Fig. 3.1C). For reasons that are unclear, axons of the PVT neurons studied here were difficult to visualize in these preparations and could only be traced for a few tens of microns.

3.2.2 Survey of membrane properties

Basic membrane properties of 77 PVT neurons are summarized in Table 3.1. Mean resting membrane potential was -60.2 ± 0.6 mV (range -51 to -76 mV) and mean input conductance was 1.6 ± 0.1 nS (range 0.6 to 4.0 nS). Their frequency distribution is shown in Fig. 3.1D, E.

3.2.3 Time dependent inward rectification (I_h)

Many CNS neurons, including thalamocortical neurons, display an inwardly rectifying current termed I_h, carried by both sodium and potassium ions and slowly activated by membrane hyperpolarization (Pape, 1996; Munsch and Pape, 1999). I_h is commonly referred to as the pacemaker current because of its role in the generation of spontaneous pacemaker activity in the heart (DiFrancesco, 1993) and the nervous system (Pape, 1996).

During current clamp recordings in PVT, I_h can be recognized as a depolarizing sag in the membrane potential response to a hyperpolarizing current pulse (see Fig. 3.2A). To assess the value of this current, PVT neurons were voltage clamped at a potential of −50 mV, and I_h was activated by hyperpolarizing voltage steps from −40 to −110mV as illustrated in Fig. 3.2B. Net I_h current, obtained by subtracting the current at the end of a 600 msec pulse from that at the onset of the pulse at -110mV, in a sample of 58 PVT neurons was variable, ranging from 0.5 pA to 74.5 pA (Fig. 3.2D). The reversal potential for I_h, measured as the point of intersection of I-V plots measured at the beginning and at the end of the voltage pulses (Fig. 3.2C), was -40 ± 2 mV (n=4), a value consistent with a cationic type of conductance.

3.2.4 Time independent inward rectification (I_R)

Inward rectifiers are a class of potassium channels that can conduct much larger inward currents at membrane potentials negative to the K^+ equilibrium potential (E_K) than outward currents at voltages positive to it, even with equal K^+ concentrations (Katz, 1949; Lu, 2004). I_R currents are rapidly activated by hyperpolarization and exhibit a

characteristic 'inward rectification' that produces a prominent bend in the I-V relationship around E_K (Hagiwara et al., 1978; Constanti and Galvan, 1983). I_R currents are sensitive to blockade by micromolar concentrations of barium (Hagiwara et al., 1978; Constanti and Galvan, 1983).

Current clamp recordings in PVT neurons (Fig. 3.3A) featured a time independent inward rectification (I_R, arrow). In voltage clamp, a linear I-V relationship at membrane potentials between -50 and -70mv displayed a variable downward bend at more negative potentials (Fig. 3.3B, C). Values for the magnitude of I_R were determined by the ratio of the line fit in the -90 to -110mV range / that in the -50 to -70mV range. Fig. 3.3D illustrates the frequency distribution of I_R values for 58 PVT neurons.

Bath application of barium, a non-selective potassium channel blocker, induced a membrane potential depolarization and an increase in neuronal input resistance at negative membrane potentials (n= 3 cells; not illustrated), indicating that barium sensitive K^+ channels underlie I_R in these cells. The reversal potential of the barium associated increase in neuronal input resistance was around -90 mV approximating the reversal potential for potassium ions under these recording conditions (E_K = - 96 mV).

3.2.5 Membrane potential-dependent firing properties

Observations from earlier studies reveal that thalamocortical neurons can display state-dependent patterns of activity, with tonic firing at depolarizing membrane potentials and burst firing patterns at hyperpolarized membrane potentials (reviewed in McCormick and Bal, 1997). Data from PVT neurons display similar properties. At rest, a majority of PVT neurons that were recorded in these slice preparations were quiescent. A

subpopulation of cells had relatively depolarized membrane potentials and displayed tonic firing. A small subpopulation had relatively hyperpolarized membrane potentials and displayed burst firing (Fig. 3.4).

To evaluate the influence of membrane potentials on patterns of firing, cells were presented with brief (1 second) depolarizing current pulses (50 pA) while held with continuous current injections at depolarized or hyperpolarized membrane potentials. At resting or depolarized membrane potentials (e.g. ~ -55 mV), neurons responded to current injection with tonic firing (Fig. 3.5A) while the same stimulus presented to the same neurons held at more hyperpolarized membrane potentials (e.g. ~ -75 mV) elicited a burst firing pattern that was composed of a low threshold spike (LTS) and burst of action potentials (see Fig. 3.5B). When examined in more detail, the first action potential from these two firing patterns (n= 34) revealed no significant difference for their amplitudes (57.9 ± 1.7 mV for tonic firing; 59.1 ± 1.8 for burst firing; Fig. 3.5C), but did display a significant difference in their thresholds such that the threshold of the first spike in burst firing occurred at a more negative value (-43.5 ± 0.5 mV) than that for tonic firing (-36.9 ± 0.6 mV; n=34; $P<0.05$; Fig. 3.5D). Significant differences were also noted in the action potential widths (Fig. 3.5E): mean half-width measurements were larger for the spike in tonic firing (2.2 ± 0.1 ms) compared with that in burst firing (1.6 ± 0.1 ms). Also, measurements of the interval between the initial two action potentials were significantly different, corresponding to a firing frequency of 20.1 ± 1.5 Hz for tonic firing versus 83.0 ± 7.3 Hz for the burst induced pattern (Fig. 3.5F).

3.2.6 Low threshold spike (LTS) characteristics

The production of a low threshold T-current driven burst of action potentials upon release from a hyperpolarized membrane potential and the production of one or more single spikes following stimulation from depolarized potentials is a well established property of thalamocortical neurons in the specific relay nuclei (McCormick and Bal, 1997). However, to date, little information exists about the properties of the LTS in PVT neurons. In current clamp recordings, one approach tested for the voltage dependency of the LTS by applying three different levels of hyperpolarizing current (30, 40, 80 pA) for 600 msec, evoked from resting membrane potentials near -60 mV. As illustrated in the sample in Fig. 3.6A, the larger the hyperpolarizing pulse, the larger was the evoked LTS and the greater the number of action potentials per burst. This is interpreted to mean that with progressively larger hyperpolarizing pulses, a larger number of T channels become de-inactivated and are therefore able to contribute to the LTS. By contrast, when cells were held at a hyperpolarized membrane potential sufficient to de-inactivate all the T channels (e.g. -75 mV), the LTS evoked by increasing supra-threshold current injections of 20, 40 and 60 pA showed little change in amplitude (Fig. 3.6B). In voltage clamp, data from 24 PVT neurons illustrate the voltage dependency of the T current and indicate that most of the T channels are likely to be de-inactivated at membrane potentials more negative than -80 mV (Fig. 3.6 C, D)

In current clamp recordings obtained at resting membrane potentials, the LTS triggered by return to resting potentials from a 600 ms hyperpolarizing current pulse

(-80pA) was followed by variable patterns of activity. Sample traces in Fig. 3.7A-D serve to illustrate this variability. In 17 PVT neurons, the LTS was followed by a short depolarizing afterpotential (DAP) with a subsequent small afterhyperpolarization (Fig. 3.7A). In 25 PVT neurons, the LTS was followed by a DAP that lasted for more than a second (Fig. 3.7B). In 9 neurons, an intermediate duration DAP gave rise to one or more single spikes followed by a variable AHP (Fig. 3.7C). In 11 neurons, the LTS was followed by a sequence of rhythmic bursts at 0.5-2.2 Hz with intervening AHPs could last for several seconds (Fig. 3.7D).

PVT neurons (n=24) were recorded in voltage clamp so as to quantify the magnitude of the T current giving rise to the LTS. To insure that the T channels were de-inactivated, the protocol applied in the presence of tetrodotoxin (see Fig. 3.7E) was to step the membrane from a -65 mV holding potential to -100 mV for 600 msec, then measure the T current at the offset of the voltage pulse. The frequency distribution of the values of the T currents for this sample of PVT neurons is shown in Fig. 3.7F (mean 331 ± 35 pA; range 106 to 694 pA).

3.2.7 Action potential after-hyperpolarizations (AHPs)

In many excitable cells, action potentials are followed by after-potentials that regulate the excitability of the cell for periods ranging from a few milliseconds to several seconds (Storm, 1990; Bourque et al., 1985). After-potentials that follow single spikes, or a train of spikes, can mediate different forms of feedback regulation of membrane excitability. Potassium channels activated by the action potential mediate outward currents and tend to hyperpolarize the cell, thus generating after-hyperpolarizations

(AHPs) that mediate negative feedback and are the determinants of refractoriness, interspike interval durations and spike timing (Marty, 1989; Vergara et al., 1998). In addition, inward currents evoked by action potentials can generate afterdepolarizations (ADPs) that mediate positive feedback and extra spikes and bursting behaviours (see Yue et al., 2005).

In PVT, different patterns of AHPs, with or without an ADP, followed individual action potentials. This variation most likely reflects the mosaic of neuronal types that comprise the PVT. Examples of AHPs include a fast AHP that decayed monotonically over several tens of milliseconds (Fig. 3.8A), a fast AHP that was followed by a short ADP and a subsequent slow AHP (Fig. 3.8B), and AHPs with fast and slow components (Fig. 3.8C). AHP amplitudes (measured from threshold) ranged from 6.8 to 10.0 mV. Time to peak of the AHP (measured from the peak of the action potential) for the fast AHP was 6.3 ± 0.4 (range 4 to 9.9 ms; n=18) and time to peak of the slow AHP was 46.9 ± 4.7 (range 15 to 103 ms; n= 27).

In many CNS neurons, bursts of action potentials may be followed by AHPs of longer duration (sometimes termed hyperpolarizing afterpotentials, or HAPs; e.g. Bourque et al., 1985). In the present study, depolarizing current injections of increasing levels (20, 40, 60 pA) were applied at resting membrane potentials so as to trigger bursts of tonic action potentials at different frequencies, and the ensuing AHPs evaluated for duration and amplitude (see Fig. 3.9A). As illustrated in Fig. 3.9B, a plot of AHP amplitudes versus the number of action potentials in a train revealed a significant positive relationship ($p < 0.001$; Pearson correlation; correlation coefficient 0.60; n=8 neurons),

presumably because an increasing numbers of spikes causes an increase in intracellular calcium that in turn enhances a calcium-dependent conductance that underlies the AHP.

3.2.8 Spike frequency adaptation (SFA)

In response to sustained supra-threshold inputs or current injections, many classes of neurons exhibit a time-dependent decline in action potential discharge rate (e.g. Michaelis and Chaplain, 1975). This phenomenon, termed spike frequency adaptation or SFA, has a rapid initial phase followed by a slow gradual decline that may continue throughout the duration of firing. Examination of current evoked firing revealed the general characteristics of early and late SFA in the majority of PVT neurons, with some heterogeneity. The latter refers to a subgroup of cells that demonstrated the reverse (see Fig. 3.10$A_{i\text{-}iii}$). The SFA ratio was calculated as *F*init/*F*final (where *F*init is the initial inter-spike interval, and *F*final is the last inter-spike interval). PVT neurons displayed SFA ratios ranging from 1.1 to 3.2 in spikes triggered by a 50 pA current injection lasting for 1 second ; Fig. 3.10$A_{i,\ ii}$). Neurons characterized by a SFA profile did not shift to a non-adapting phenotype during increasing depolarization steps (Fig. 3.10B, C; n = 7 cells tested). The SFA ratio was more pronounced with larger current injections ($P<0.05$; Pearson correlation analysis, Fig. 3.10D; n= 4 cells).

3.2.9 Action potential broadening

Broadening of action potentials during repetitive firing is a widespread phenomenon in neuronal somata, dendrites and axon terminals (e.g. Aldrich et al., 1979; Bourque and Renaud, 1985; Jackson et al., 1991). Intracellular recordings were obtained

from 30 PVT cells and spike trains were elicited by injecting 50 pA depolarizing current pulses lasting for 1 second, evoking an average 13 ± 1 action potentials for each train. In all cells the second action potential in a train was significantly broader than the first ($P<0.0001$, Student's two-tailed paired *t* test; Fig. 3.11A). The spike duration increased by 20 ± 2% from the 1st spike (2.3 ± 0.1 ms) to the 2nd spike (2.8 ± 0.2 ms), accounting for ~55% of the total spike broadening over the duration of the train. While most of the broadening occurred within the initial two to three action potentials (Fig. 3.11Bi, Ci; n=16 cells), the phenomenon in other cells broadened progressively till the end of the train (Fig. 3.11Bii, Cii; n=14). Accompanying the broadening was a reduction in the fast AHP (Fig. 3.11A, 3.12A) and change in the shape of the second spike where the broadening exaggerated a 'shoulder' on the repolarization phase of the action potential (Fig. 3.12B).

Spike broadening has been shown to be frequency dependent in a number of different neurons (Aldrich et al., 1979; Quattrocki et al., 1994; Ma and Koester, 1996; Shao et al., 1999). To test for this property in PVT neurons, action potentials were evoked with 20, 40 and 60 pA depolarizing current injections and broadening compared between the first and second spikes as their interspike interval decreased (see Fig. 3.12A). Action potentials evoked by larger current injections fired at higher frequencies and showed greater broadening (Fig. 3.12A). The mean frequency of discharge between the first two spikes was 6 ± 1 Hz with a 20 pA current injection, 12 ± 3 Hz for a 40 pA current injection and 25 ± 8 Hz for a 60 pA current injection (n =4). In data obtained from 4 neurons, the average action potential half-width increased between the first two

spikes by 17 ± 1 % with a 20 pA current injection, by 29 ± 2% ($P < 0.001$) with a 40 pA current injection and by 50 ± 2% ($P < 0.001$) with a 60 pA current injection (Fig. 3.12C).

Membrane Property	n	Mean ± SEM
Passive membrane properties		
RMP, mV	77	-60.2 ± 0.6
G-conductance, nS (fit – 50 to – 70)	77	1.6 ± 0.1
Action potential (AP) properties		
AP threshold, mV	77	-43.1 ± 0.5
AP amplitude, mV	77	62 ± 1
Half amplitude duration, ms	77	2.0 ± 0.1
AHP peak amplitude, mV	29	13.4 ± 0.7
f-AHP time to peak, ms	18	6.3 ± 0.4
s-AHP time to peak, ms	27	46.9 ± 4.7
I_h, (pA; -110 mV pulse)	58	18.2 ± 2.0
I_R (ratio G-90 to-110/-50 to-70 mV)	77	5.0 ± 0.3
T- current size (pA; -110 mV pulse)	24	-331 ± 35
AP shoulder on repolarization phase	8/33	24%
Tonic firing (RMP, 50 pA pulse)		
Delay to first spike, ms	33	67.7 ± 19.5
First spike amplitude, mV	33	57.9 ± 1.7
First spike threshold, mV	33	-36.9 ± 0.6
First spike half amplitude duration, ms	33	2.2 ± 0.1
Initial firing rate, Hz	33	20.1 ± 1.5
Spike frequency adaptation ratio	30	1.7 ± 0.1
Post train afterhyperpolarization, mV	33	6.8 ± 0.7
Spikes in the train, n	33	13 ± 1
Maximum spike broadening	30	136% ± 4%
Burst firing (-75mV, 50 pA pulse)		
Delay to first spike, ms	33	47.8± 2.9
First spike amplitude, mV	33	59.1 ± 1.8
First spike threshold, mV	33	- 43.5 ± 0.5
First spike half amplitude duration, ms	33	1.6 ± 0.1
Initial firing rate, Hz	33	83.0 ± 7.3
Post train afterhyperpolarization, mV	33	7.1 ± 0.5

Table 3.1. Summary of intrinsic properties of PVT neurons. Results are expressed as mean ± standard error.

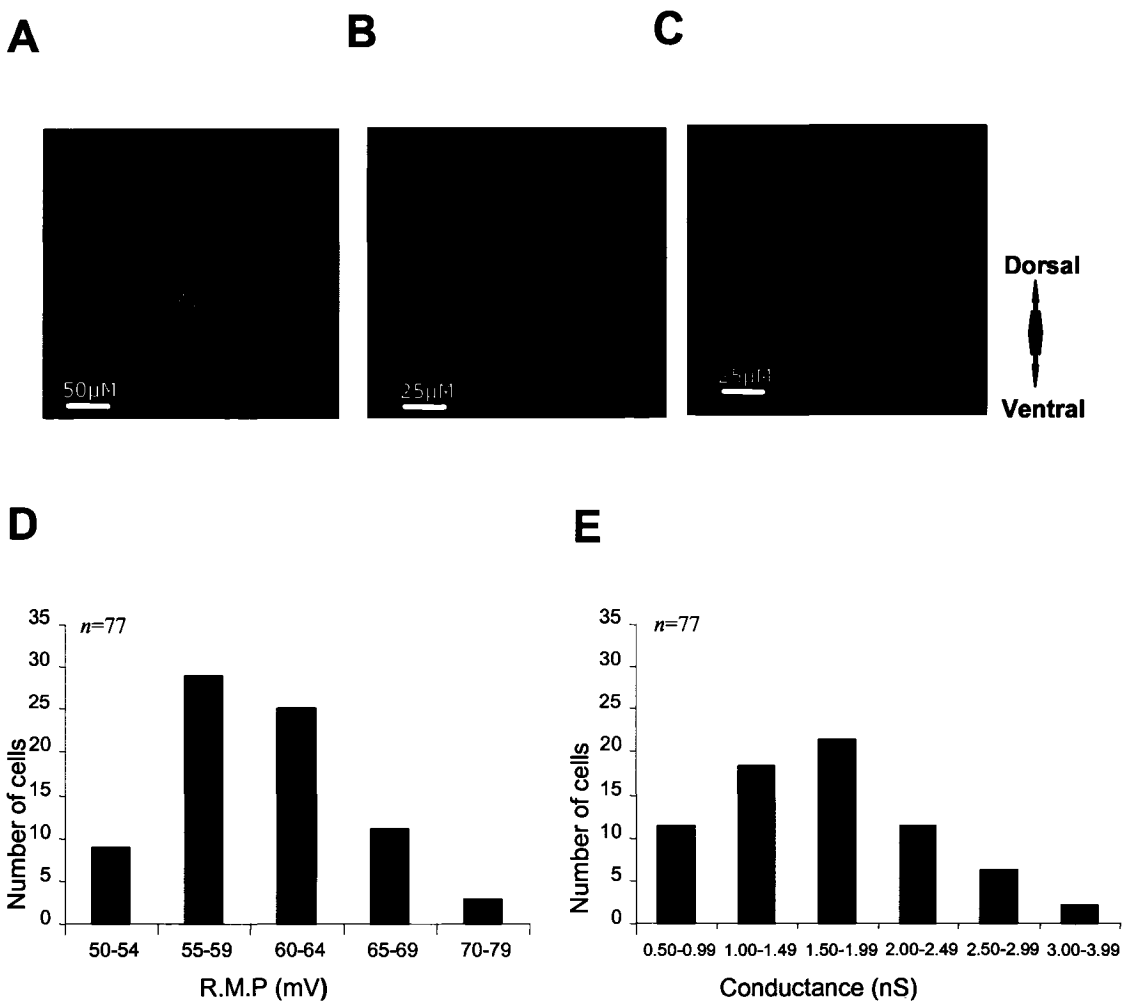

Figure 3.1. Morphology and passive membrane properties. A-C: Lucifer Yellow profiles of 3 representative PVT neurons as visualized with confocal laser scanning microscopy. **D, E:** Frequency histograms of the distribution of resting membrane potential (R.M.P.) and membrane conductances for 77 PVT neurons.

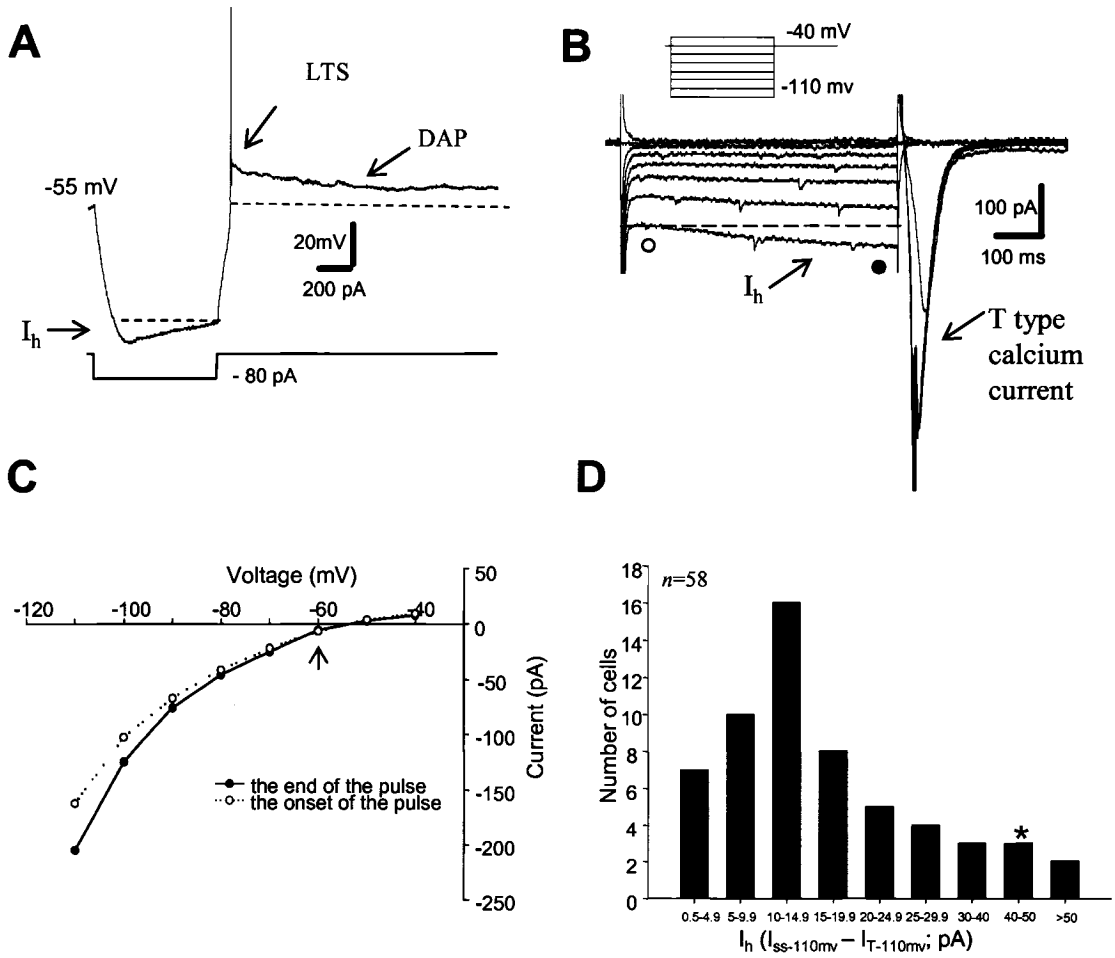

Figure 3.2. Hyperpolarization-activated current (Ih). A: A current clamp trace (above; RMP -55mV) illustrates the I_h-induced depolarizing "sag" in membrane voltage response to a sustained -80 pA hyperpolarizing current pulse (lower trace). On return of membrane potential to the resting level, note the low threshold spike (LTS) with a single superimposed sodium action potential, followed by a long depolarizing afterpotential (DAP; dashed line). **B:** Superimposed traces in voltage clamp recordings from the same neuron illustrate current responses to 8 voltage steps, from –110mV to –40mV. At -110 mV, I_h is measured as the difference between the current at the onset of the pulse (o; dashed red line) and at the end of the pulse (●). A prominent T type current (I_T) occurs at the offset of the pulse. **C:** I-V plots constructed from values taken at the beginning of the pulse (I_t; o) and the 'steady-state' values (I_{ss}; ●) reveal I_h as time-dependent rectification. Convergence of these lines around -45mV (arrow) suggests a non-selective cationic type conductance. **D:** Frequency distribution histogram of I_h for 58 neurons, based on subtraction of I● from IO -110mV. Red star refers to the cell illustrated in B.

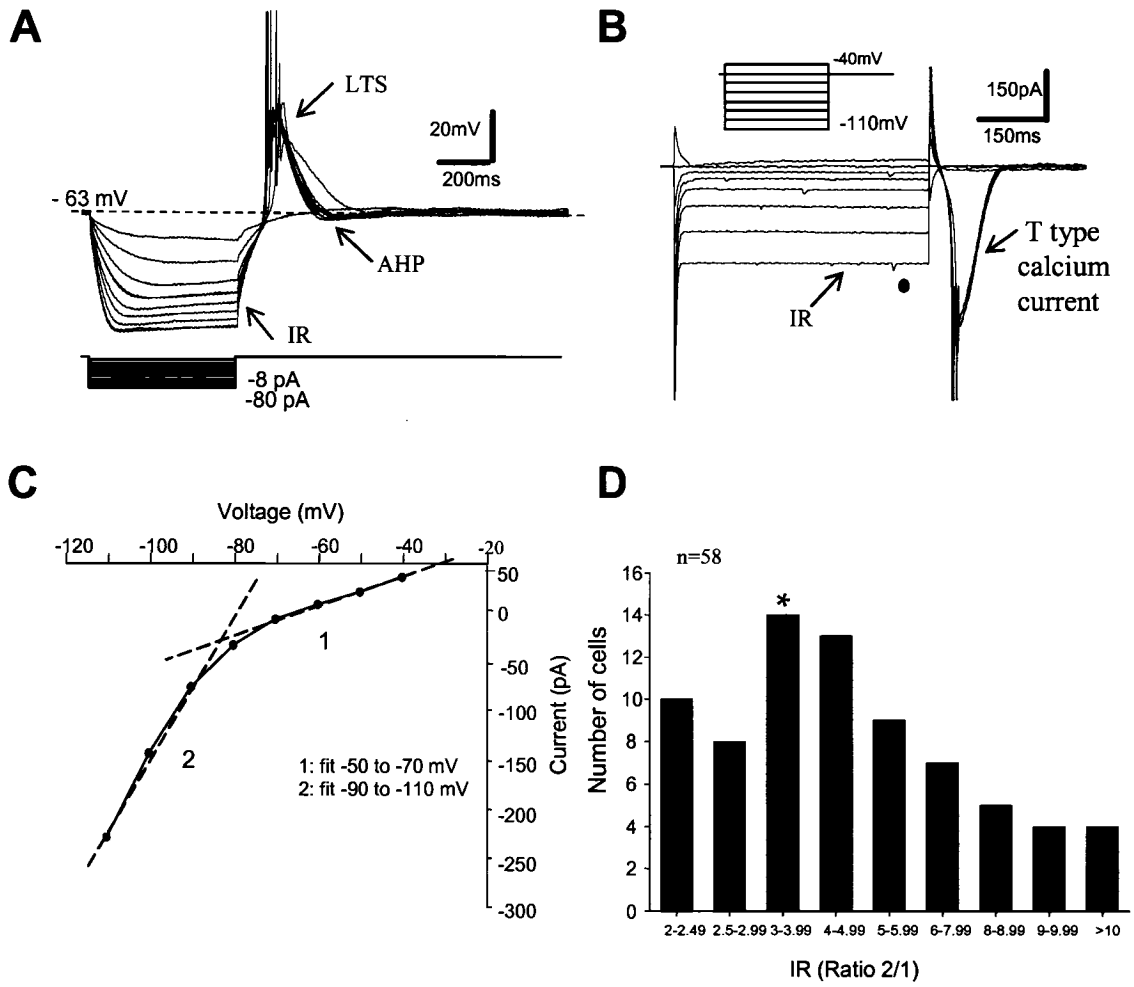

Figure 3.3. Inward rectification (I_R). A: Superimposed current-clamp tracings (above; RMP -63mV) in response to a series of intracellular current pulses (below) reveal time-independent inward rectification (I_R; arrow). In this neuron, a small afterhyperpolarization (AHP) follows the LTS. **B:** I_R is revealed in superimposed current traces in voltage clamp mode from the same neuron in response to a series of voltage pulses from –110mV to –40mV. Measurements are obtained at the symbol (●). **C:** I-V plots constructed from the values taken at steady-state (●) reveal the inward rectification. Values for I_R are determined from the ratio of the line fit in the -90 to -110mV range to that in the -50 to -70mV range (dashed lines 2 / 1). **D:** I_R frequency distribution histogram for 77 cells. The red star refers to the value for the cell illustrated in B.

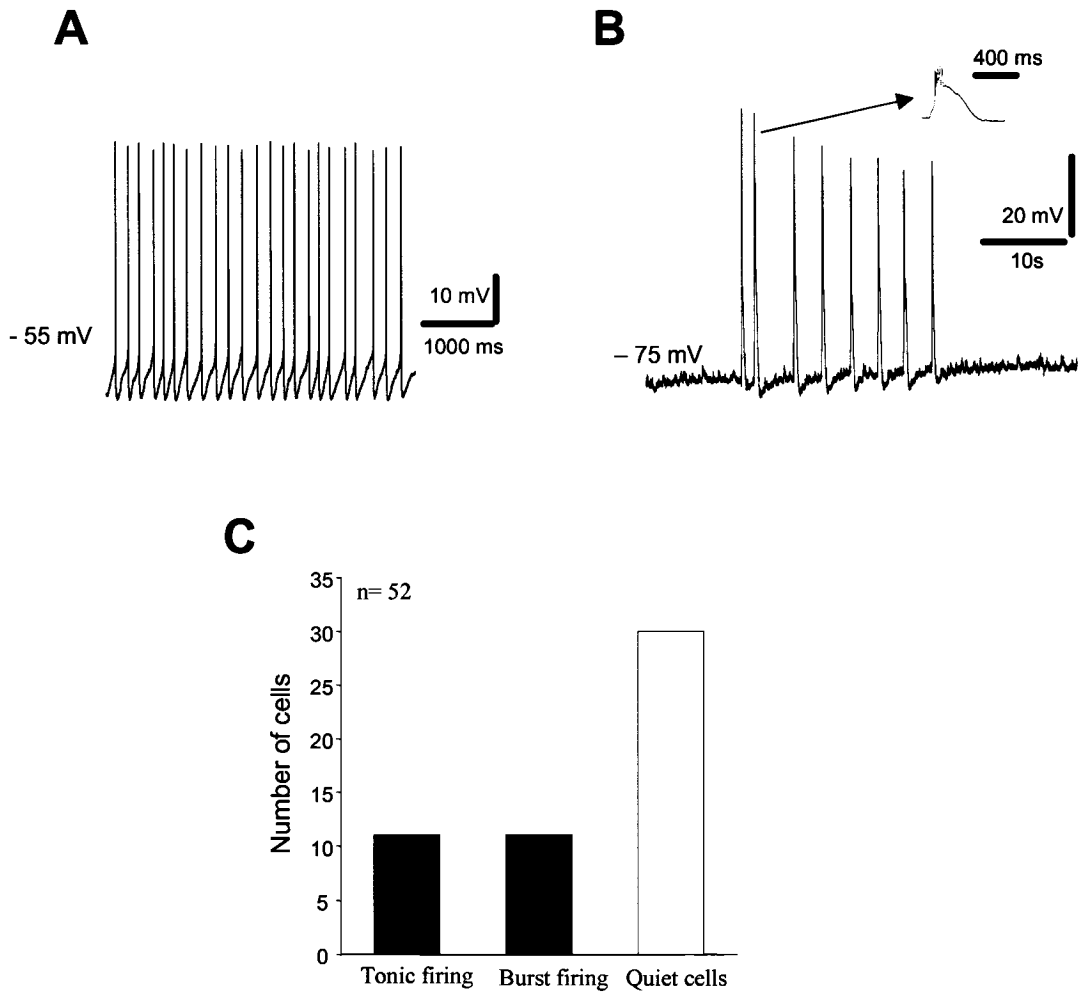

Figure 3.4. Spontaneous activity patterns. A: Current clamp trace from a PVT neuron reveals tonic firing (4.3 ± 0.20 Hz) at a resting membrane potential of -55 mV. **B:** Trace from another PVT neuron with a relatively hyperpolarized resting membrane potential displays a series of spontaneous burst discharges. Note that each burst displays a low threshold spike with superimposed action potentials, and that the second and subsequent bursts are followed by a small afterhyperpolarization. **C:** Histograms depict the distribution of spontaneous activity among 52 PVT neurons: tonic firing (n=11); burst firing (n=11); quiet cells (n=30).

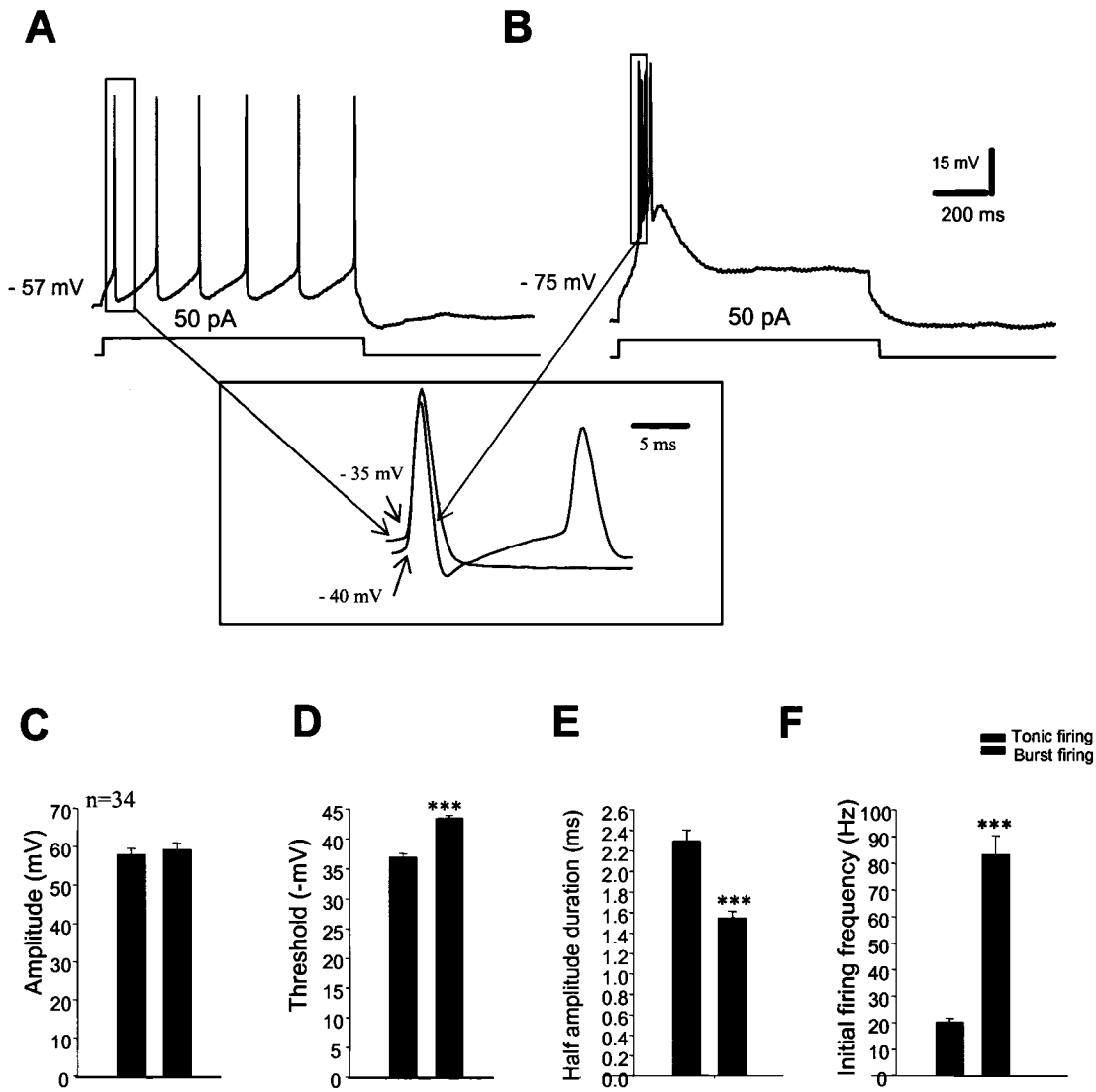

Figure 3.5. Membrane potential-dependent firing properties. A: At resting membrane potential (-57 mV), this neuron displays tonic firing in response to a depolarizing current pulse (0.05 nA, 1s). **B:** After manual adjustment of membrane potential to -75 mV by injection of constant current, the same depolarizing current pulse evokes a low-threshold spike (LTS) superimposed with a crown of action potentials. Inset reveals a more hyperpolarized threshold and shorter duration of the initial action potential for the burst induced response. **C-F:** Histograms for data from 34 neurons treated in this manner reflect initial action potential amplitudes, thresholds, half widths and firing frequency of the first two spikes (*** represents $P < 0.001$).

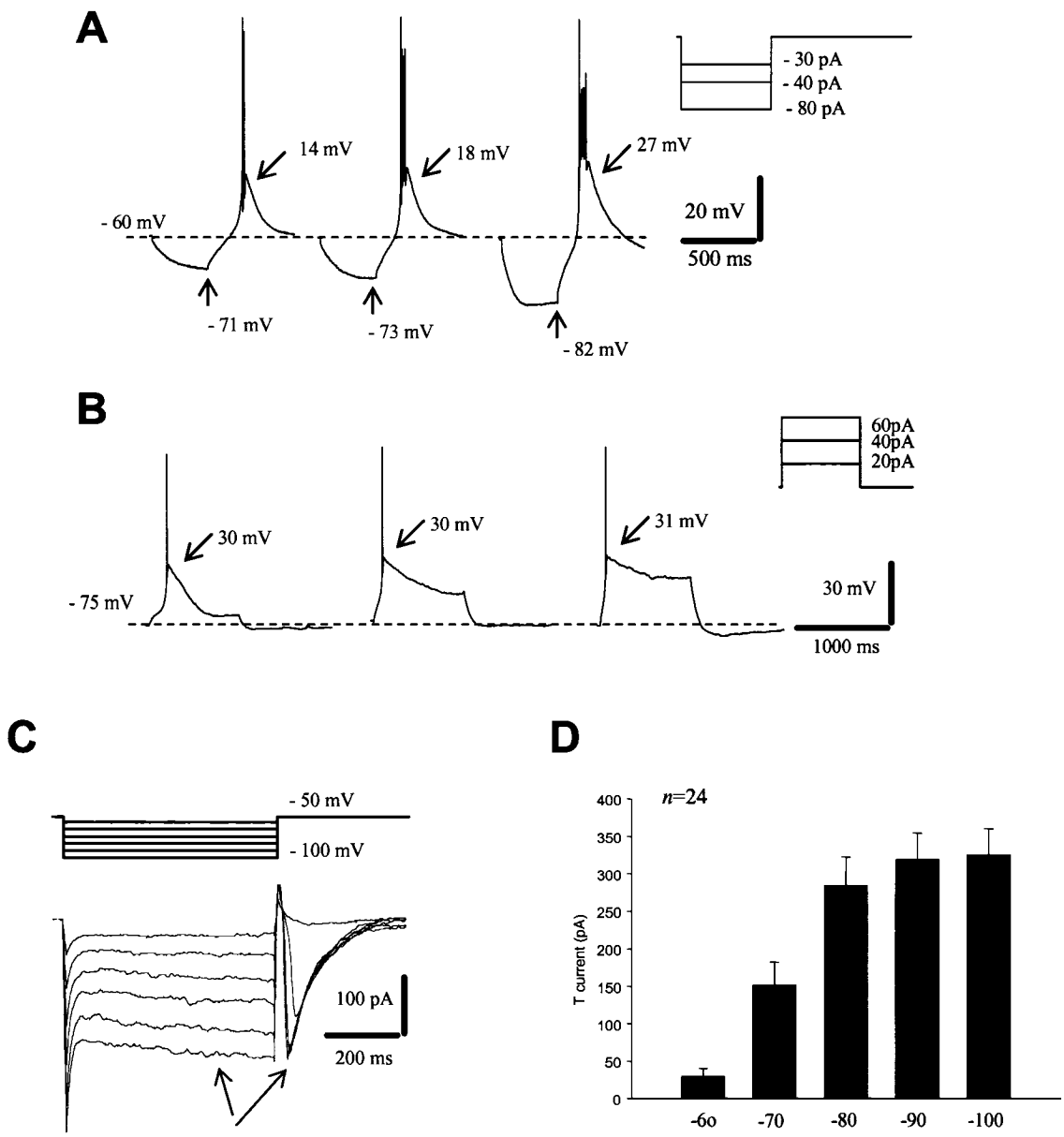

Figure 3.6. LTS and T-current properties. A: Current clamp traces illustrate increasingly larger amplitudes of the LTS evoked at the offset of increasingly larger hyperpolarizing current injections delivered from resting membrane potentials. **B:** By contrast, when held at a hyperpolarized membrane potential, the LTS (around 30 mV) evoked in response to increasingly larger depolarizing current injections remains relatively unchanged in amplitude. **C:** Traces in voltage clamp recordings in TTX illustrate that larger T currents are evoked at more hyperpolarized membrane potentials. **D:** Histogram of voltage clamp data from 24 neurons reflect increasing T currents elicited as membrane potential is stepped to more hyperpolarized levels.

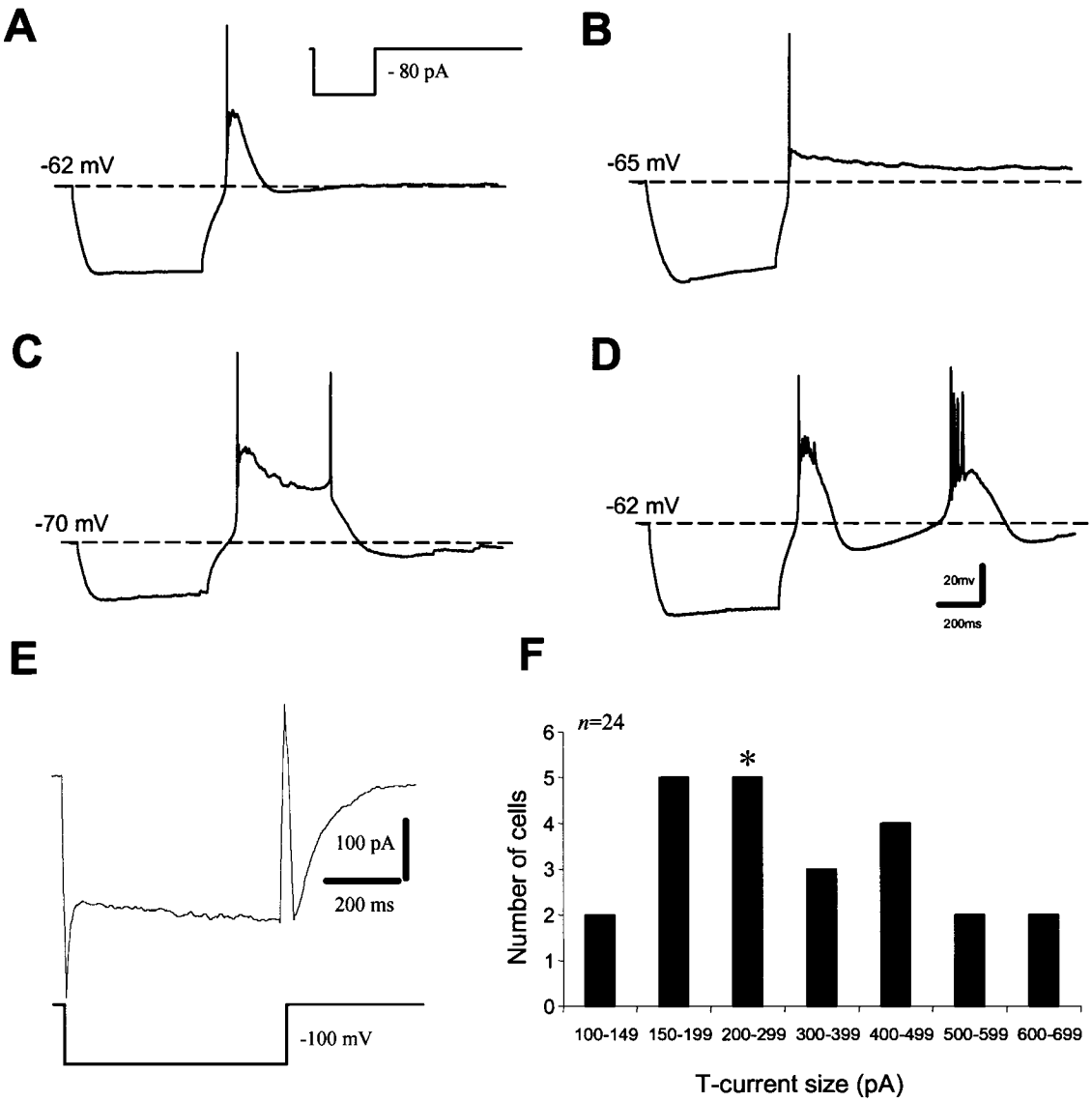

Figure 3.7. LTS and T current properties. A-D: Current clamp traces reflect the heterogeneity in LTS and afterpotential profiles following removal of hyperpolarizing current injections. A: LTS followed by a small afterhyperpolarization (AHP; below the dashed line). **B:** LTS followed by a long duration depolarizing afterpotential (DAP). **C:** LTS followed by an intermediate DAP and subsequent AHP. **D:** LTS with subsequent oscillatory behavior. **E:** T currents were measured in voltage clamp recordings in TTX (V_H -65mV) after removal of a 600 msec step to -100mV, sufficient to de-inactivate most I_T channels. **F:** Frequency distribution of T-current amplitudes for 24 PVT neurons. The red star refers to the value for the cell illustrated in **E**.

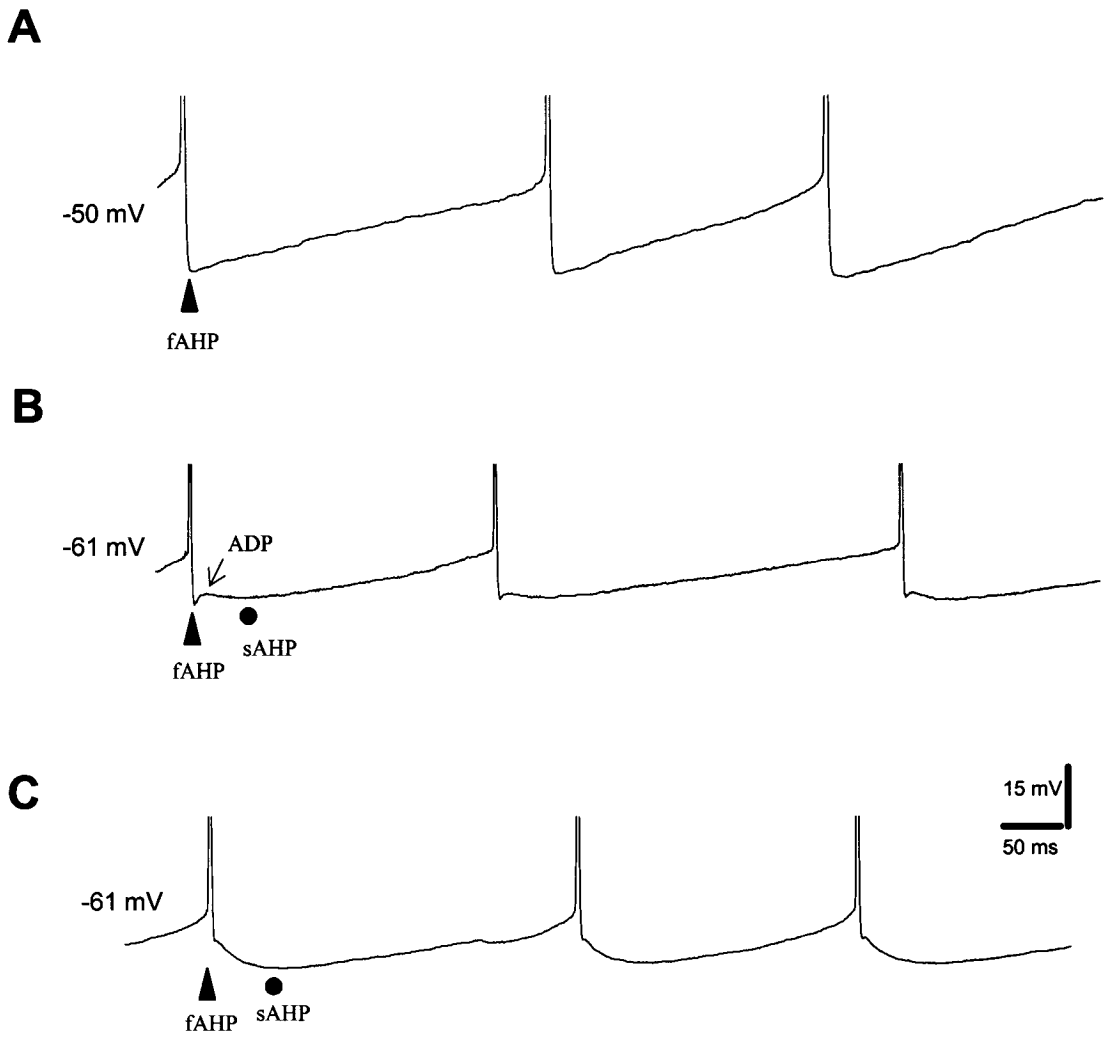

Figure 3.8. Profile of single action potential afterhyperpolarizations (AHPs). A: Sample current clamp trace from a neuron exhibiting a fast AHP (arrowhead) that decays monotonically. **B:** Trace from a neuron that exhibits a AHP with a fast (arrowhead) and slow (closed circle) component intercalated by a afterdepolarization (ADP) (arrow). **C:** Trace from a neuron that exhibits a fast AHP followed by a slow component. Action potentials are truncated.

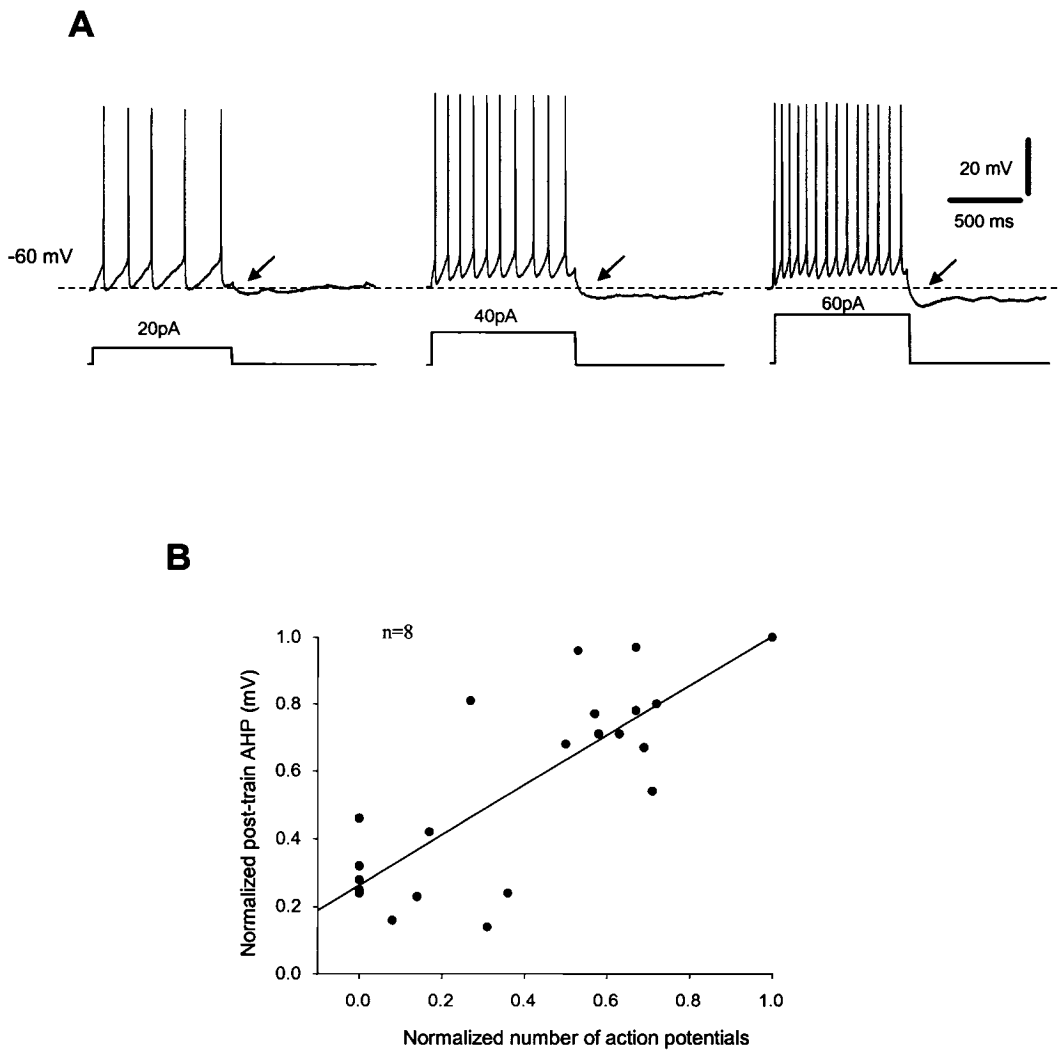

Figure 3.9. Post burst AHPs. A: Current clamp traces (above) at resting membrane potential reveal a train of action potentials evoked by current pulses (below) of 20, 40 and 60 pA. Coincident with an increase in the number of action potentials is an increase in the amplitude of post-burst AHPs (arrows). **B:** When spike numbers and size of the post-train AHP are normalized to their maximum value recorded here at 60 pA, a plot of the number of spikes against the size of the post-train AHP (n= 8 neurons) shows a significant positive relationship ($P<0.001$, Pearson correlation; correlation coefficient 0.60; slope 0.74).

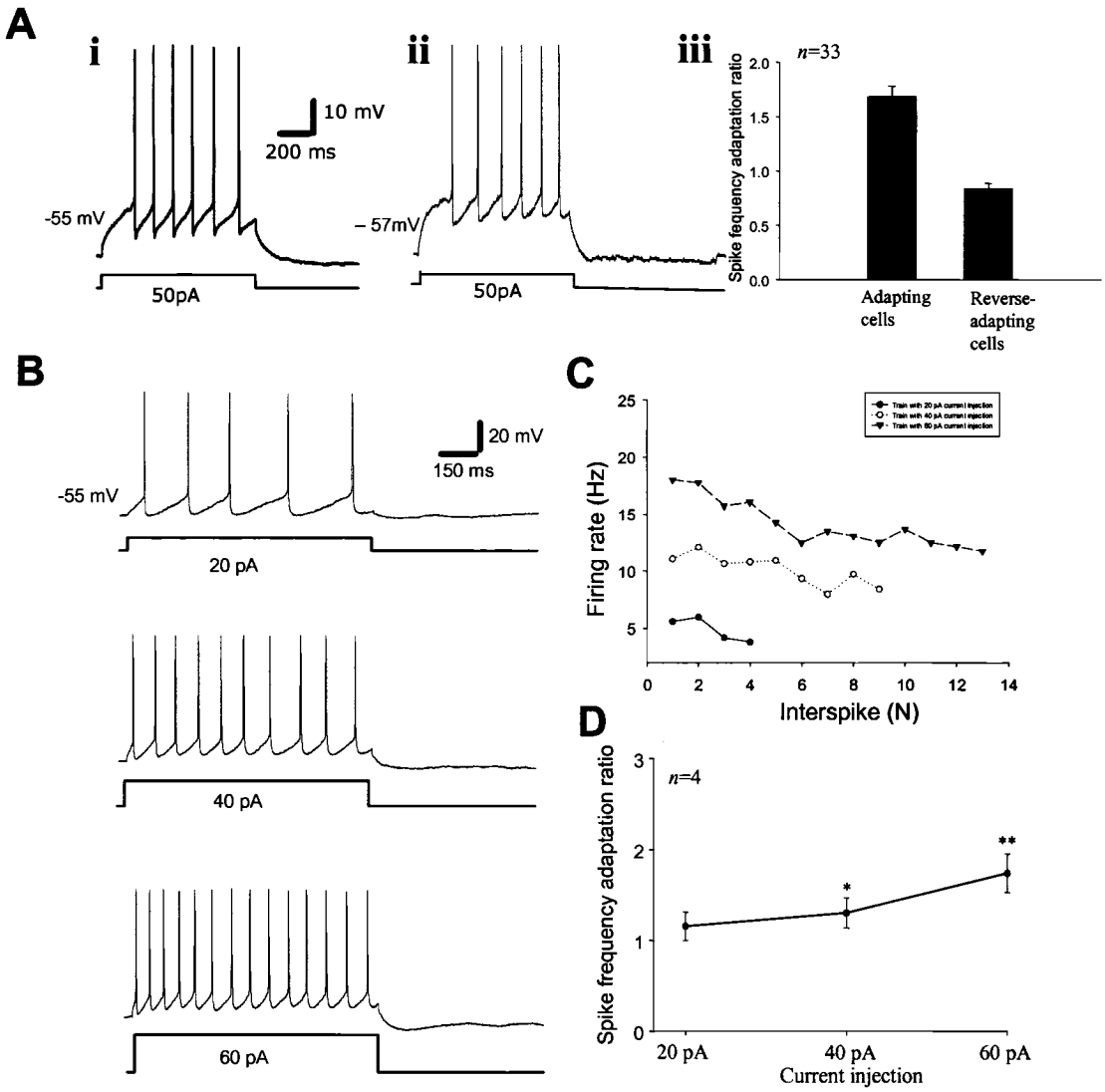

Figure 3.10. Action potential frequency adaptation. Ai: Current clamp trace illustrates modest spike frequency adaptation (SFA) in action potentials evoked by a 50pA current injection. **Aii:** Current clamp trace from a PVT cell that displays 'reverse' SFA to the same stimulus. **Aiii:** Histogram data of the SFA ratio for 28 cells displaying SFA (black) and 5 cells displaying reverse SFA (red). **B:** Traces reflect spike frequency adaptation in action potential trains evoked by 1s current injections of 20, 40 and 60 pA from a resting membrane potential of -55mV. **C:** Plot of the spike frequency for each interspike interval from the current injections shown in B. **D:** Plot of the spike frequency adaptation (SFA) ratio (see text) against the injected currents for 4 neurons. Significant differences are observed between spike trains from different current injections (*P<0.05 and **P<0.01 based on student's two-tailed paired *t* test).

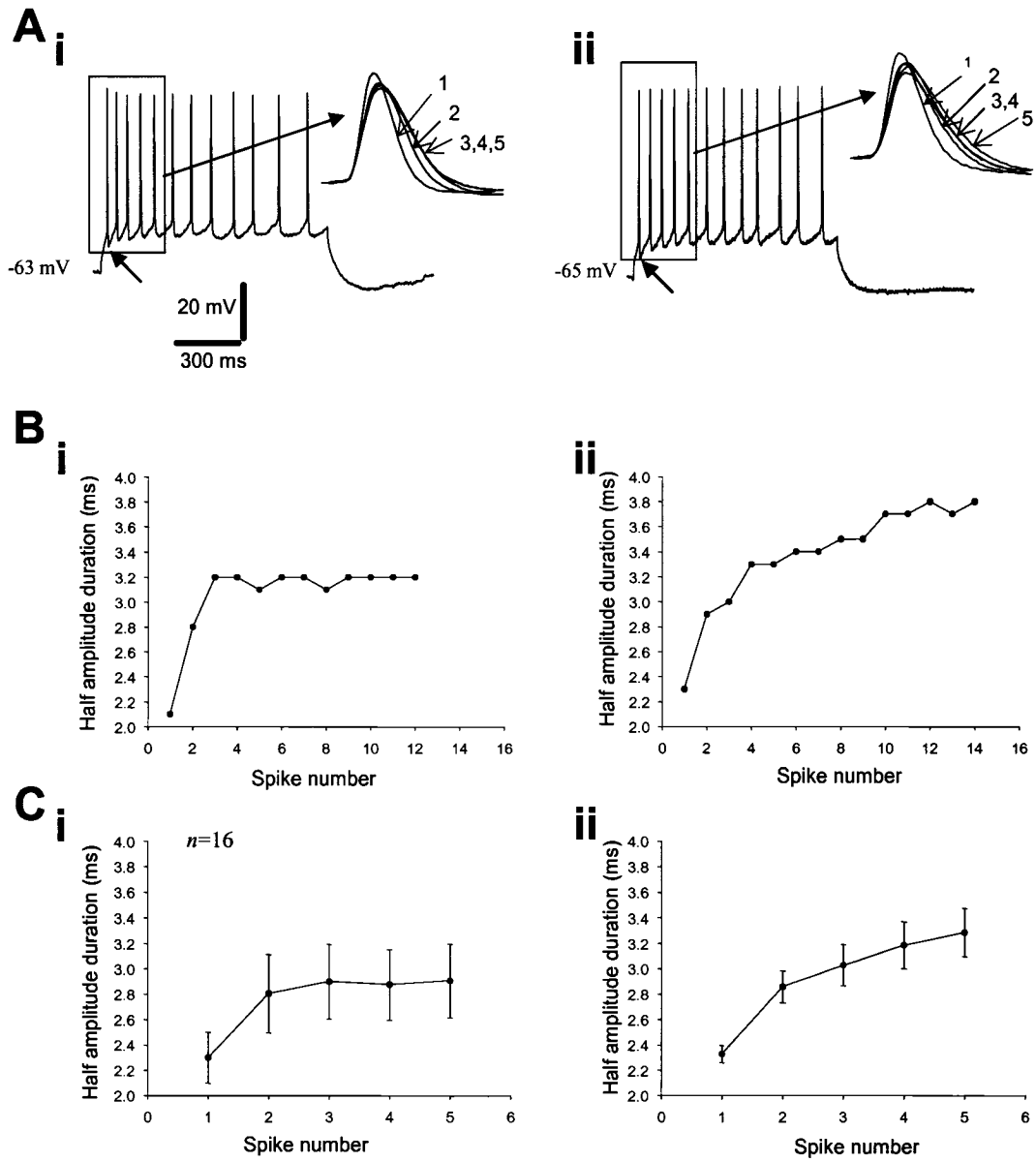

Figure 3.11. Action potential broadening. A: Depolarizing current pulses (50 pA, 1sec) induce trains of action potentials that demonstrate spike frequency adaptation *and* successive spike broadening as reflected in the enlarged superimposed profiles for the first 5 spikes in a train (red boxes). Note the prominent fast AHP (arrow) after the first spike. **B:** Plot of the spike width (measured at 50% amplitude) for the series of action potentials within the trains shown in **A** reveals that the greatest broadening occurs early in the train. **C:** Plot of the change in action potential durations for the first 5 action potentials in 16 neurons tested as in **A**.

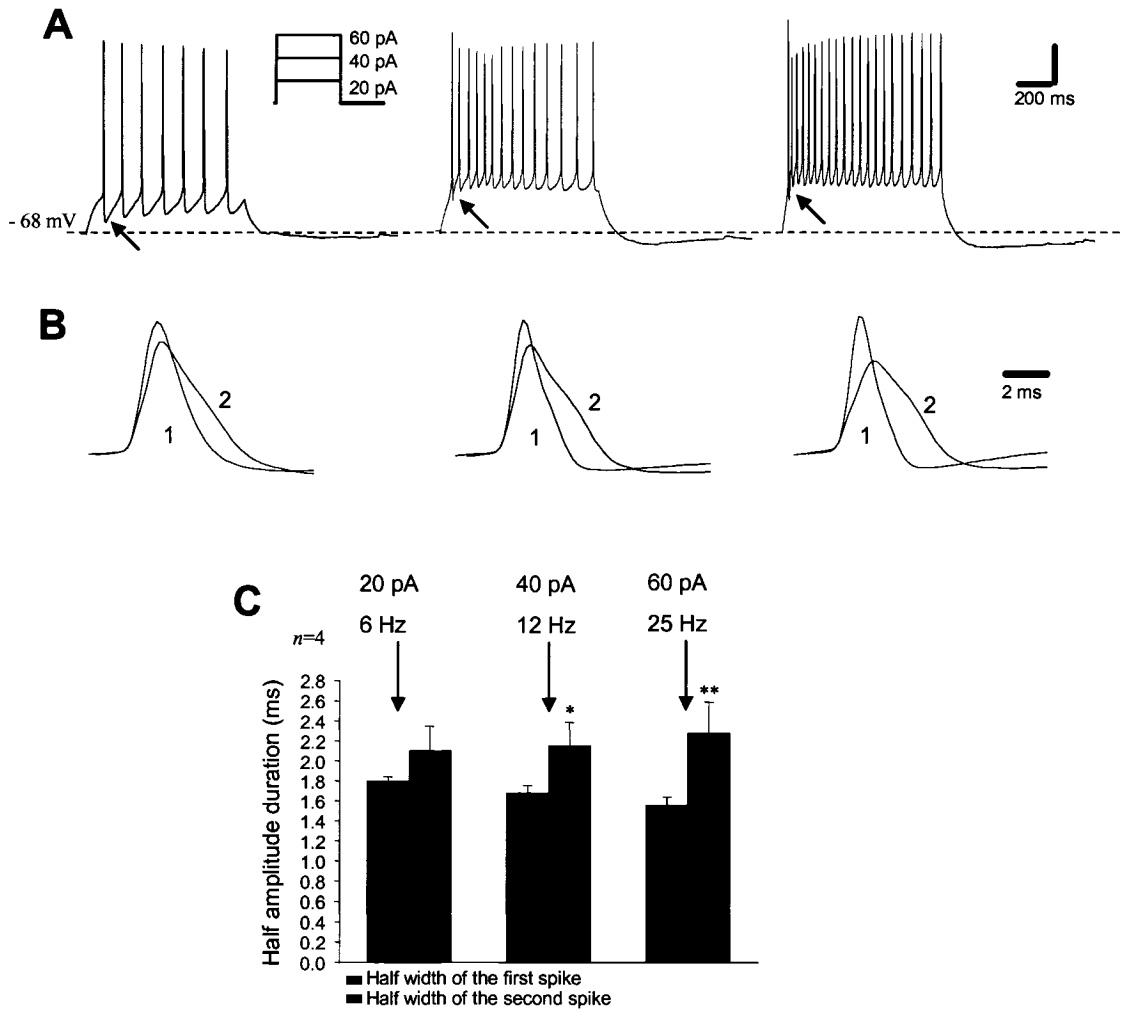

Figure 3.12. Frequency dependent spike broadening. A: Action potential trains were evoked by 1s current injection of 20, 40 and 60 pA. Note the increase in the frequency of action potential discharge with increasing current injections, progressive reduction in the amplitude of the AHPs after the first spoke (arrows), and increase in the amplitude of the post-burst AHP. **B:** The first and second action potentials have been superimposed for each current injection to demonstrate the increasing 'shoulder' on the repolarizing phase of the second spike. **C:** Histogram summary data for 4 neurons of the spike broadening in the first two spikes at different current injections shown in **A** (*P<0.05 and **P<0.01 refer to differences in half width between the initial two spike; Student's two-tailed paired *t* test).

CHAPTER 4

RESPONSE TO EXOGENOUS OREXIN A AND B

4.1 Introduction

The discovery and structural characterization of the orexin peptides in 1998 was quickly followed by the development of markers that allowed for the visualization of the central distribution of orexin neuronal projections as well as the receptors for orexin A and B to be mapped in detail. As mentioned earlier, the PVT is one site that receives a particularly dense distribution of orexin immunoreactive axons throughout its full extent. Neurons in many locations have now been seen to respond to exogenous application of orexin peptides with membrane depolarization (see Chapter 1). The experiments reported in this chapter tested the *hypotheses* that a) orexins would also induce membrane depolarization and firing in PVT neurons by acting at both orexin A and B receptors, and that b) the state dependent properties of PVT neurons could be a determinant in the pattern of firing evoked during these responses. *Objectives* included a) assessment of the magnitude of the response to orexin A and B peptides, b) description of membrane potential-dependent changes in firing, and c) formulation from voltage-current plots about the nature of the conductance(s) mediating the responses to activation of orexin receptors.

4.2 Results

4.2.1 Orexin increases cell excitability: cell attached studies

Initial studies using the cell attached mode tested for a response to bath applied orexin. Initial efforts proved somewhat difficult because many PVT neurons demonstrated little spontaneous activity. Therefore experiments were performed using brief infusions of glutamate (1 mM, 1~2 minutes) in the bath to verify that there was indeed a neuron at the end of the micropipette. As illustrated in Fig. 4.1, 3 of 5 PVT neurons thus recorded were responsive to bath applied orexin A (200 nM), increasing their action potential discharge frequency from 0.15 ± 0.04 Hz to 2.28 ± 0.07 Hz ($P<0.001$, Student's *t* test), a response that typically recovered within 3-4 minutes after washout (Fig. 4.1A and B).

4.2.2 Orexin-induced depolarization: whole cell current clamp observations

From a total of 47 neurons tested for a response to a 2 minute bath application of orexin A and/or orexin B (200 nM), 31 neurons were observed to respond with membrane depolarization. As illustrated in Fig. 4.2, a typical response in otherwise silent neurons was a slowly rising (~ 2 minutes to peak) membrane depolarization that reached a plateau of 7.1 ± 0.6 mV (n=31) with membrane potential slowly returning to the resting level after ~12 minutes. Neurons responded to repeated applications after washout intervals of 30 minutes (n= 12 cells tested), although commonly with smaller magnitudes of response.

The overall magnitudes of first response to 200 nM doses were similar, 7.8 ± 0.7 mV for orexin A (n=19) and 6.1 ± 0.8 mV for orexin B (n=12). This would be consistent with in-situ hybridization data that indicate both OX_1R and OX_2R are present in PVT.

As demonstrated in Fig. 3.5A, PVT neurons display state-dependent firing patterns. Thus it is conceivable that an orexin-induced depolarization might be sufficient to change a neuron from a burst firing mode to a tonic firing pattern. To test this hypothesis in PVT neurons, a transient hyperpolarizing current pulse sufficient to generate a low threshold spike upon return to baseline was applied and the pattern monitored during an orexin-induced depolarization. Indeed, in all 5 cells tested and as illustrated in the amplified inserts in Fig. 4.2A, the low threshold spike disappeared as the membrane potential depolarized under the influence of orexin peptide, and was replaced by tonic firing at the peak of the response.

In Chapter 3 it was mentioned that a minority of PVT demonstrated spontaneous LTS-based burst discharges when recorded in whole cell mode (Fig. 3.4). Only one such neuron was tested with bath applied orexin A (200 nM; Fig. 4.3A). This neuron responded to orexin with a membrane depolarization of ~5 mV, presumably insufficient to induce tonic firing, but sufficient to cause a change in bursting activity such that individual LTS-based bursts became more frequent (from 0.05 Hz to 0.09 Hz values), the afterdepolarizations (DAPs) more prolonged (from 633.2 ± 13.9 ms to 1237.8 ± 49.8 ms; n= 17) with increased number of action potentials on the crest of the DAP (from 4.6 ± 0.3 to 6.9 ± 0.3; n= 17 bursts measured; see Fig. 4.3B).

4.2.3 Orexin-induced inward current: observations in voltage clamp mode

To address membrane conductances, orexin-induced responses were further examined in voltage clamp mode (V_H = -65 mV) in the presence of 1 mM TTX to eliminate sodium-dependent action potentials and synaptic transmission. In 18 / 28 cells tested, orexin 200 nM induced a slowly developing inward current (peak 16.9 ± 1.8 pA) that recovered slowly over 10 mins (e.g. Fig. 4.4A). These results confirm a direct postsynaptic action. Overall, both orexin peptides evoked a similar magnitude of response (17.3 ± 3.4 pA for orexin A (n=8) and 15.1 ± 2.2 pA for orexin B (n=10)).

To evaluate possible ionic conductances involved, voltage current relationships before and during orexin-induced responses were subtracted and the net orexin-induced currents compared. Three different patterns became apparent. One group of 5 neurons demonstrated a net orexin-induced conductance that decreased from a control of 2.0 ± 0.2 to 1.8 ± 0.3 nS ($P< 0.05$, paired t-test) and reversed at -108.0 ± 5.1 mV, a value approximating the potassium equilibrium potential under these conditions (Fig. 4.5). Thus, in neurons displaying such patterns, the orexin-induced inward current and membrane depolarization was likely mediated via reduction in one of more potassium ion conductances. In 3 / 3 PVT neurons tested, barium (1 mM) mimicked the orexin effect by inducing an inward current of -18 ± 6 pA which reversed at -101 ± 2mV, and was associated with a decreased orexin induced inward current (-8.6 ± 1.3 pA; p=0.024) suggesting that the involved conductance is partly barium-sensitive.

By contrast, in another 6 cells, the slope of the net orexin-induced current increased from a control of 2.0 ± 0.3 to 2.2 ± 0.3 nS ($P< 0.05$, paired t-test) with current reversing at -38.0 ± 3.8 mV (e.g. Fig. 4.6). In neurons demonstrating such patterns, the

orexin-induced response appeared to be mediated via an increase in a nonselective cationic conductance. In the remaining 4 cells, the mean net orexin-induced current displayed a non-significant reduction in membrane conductance (from 1.9 ± 0.1 to 1.7 ± 0.1 nS; P= 0.09, paired t-test) and a parallel shift (e.g. Fig. 4.7).

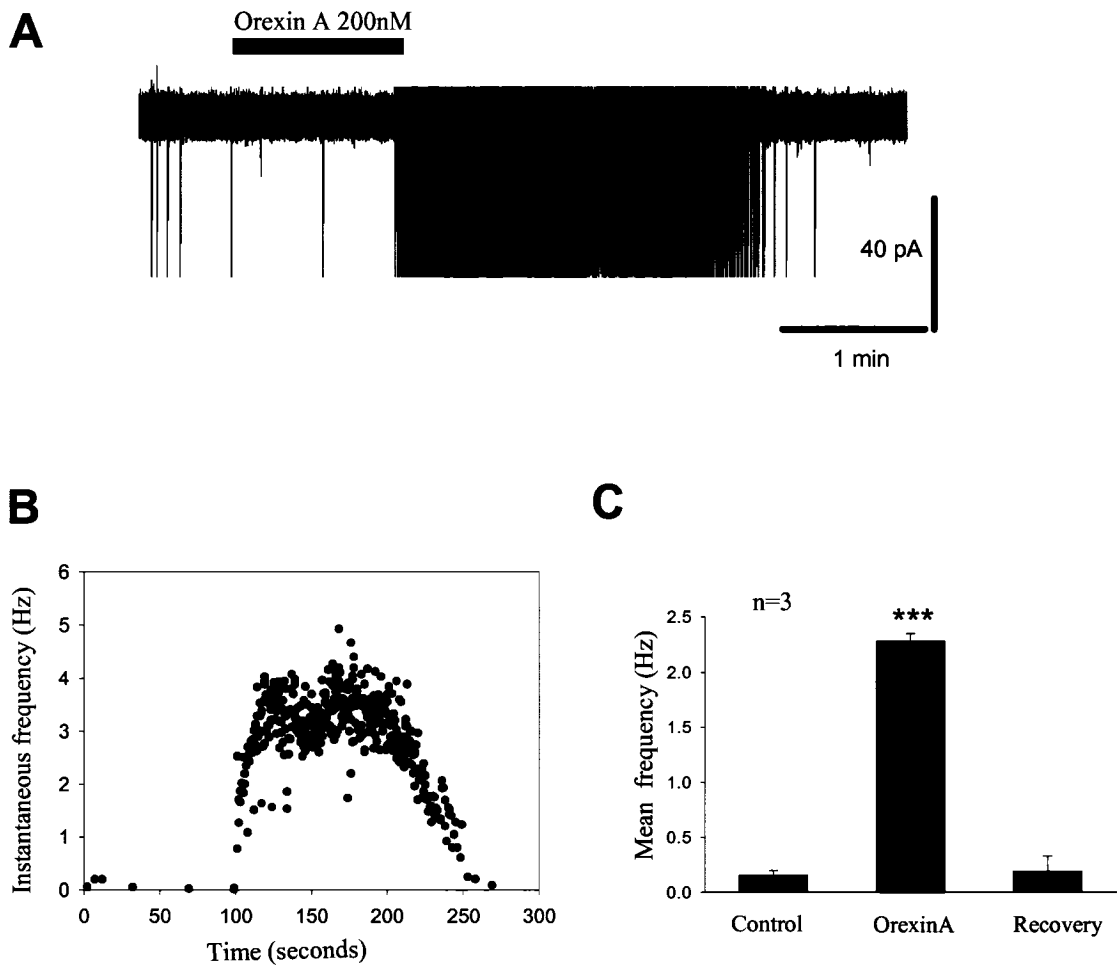

Figure 4.1 Orexin excites a population of PVT neurons. A: Sample of a cell attached recording demonstrates orexin A-induced increase in spike discharges. **B:** Plot of the spike firing frequency during the process of orexin application shown in A. **C:** Histogram data from 3 (out of 5) PVT neurons indicate mean effect of orexin A (200 nM) on spike frequency (***$P < 0.001$, Student's two-tailed paired t test).

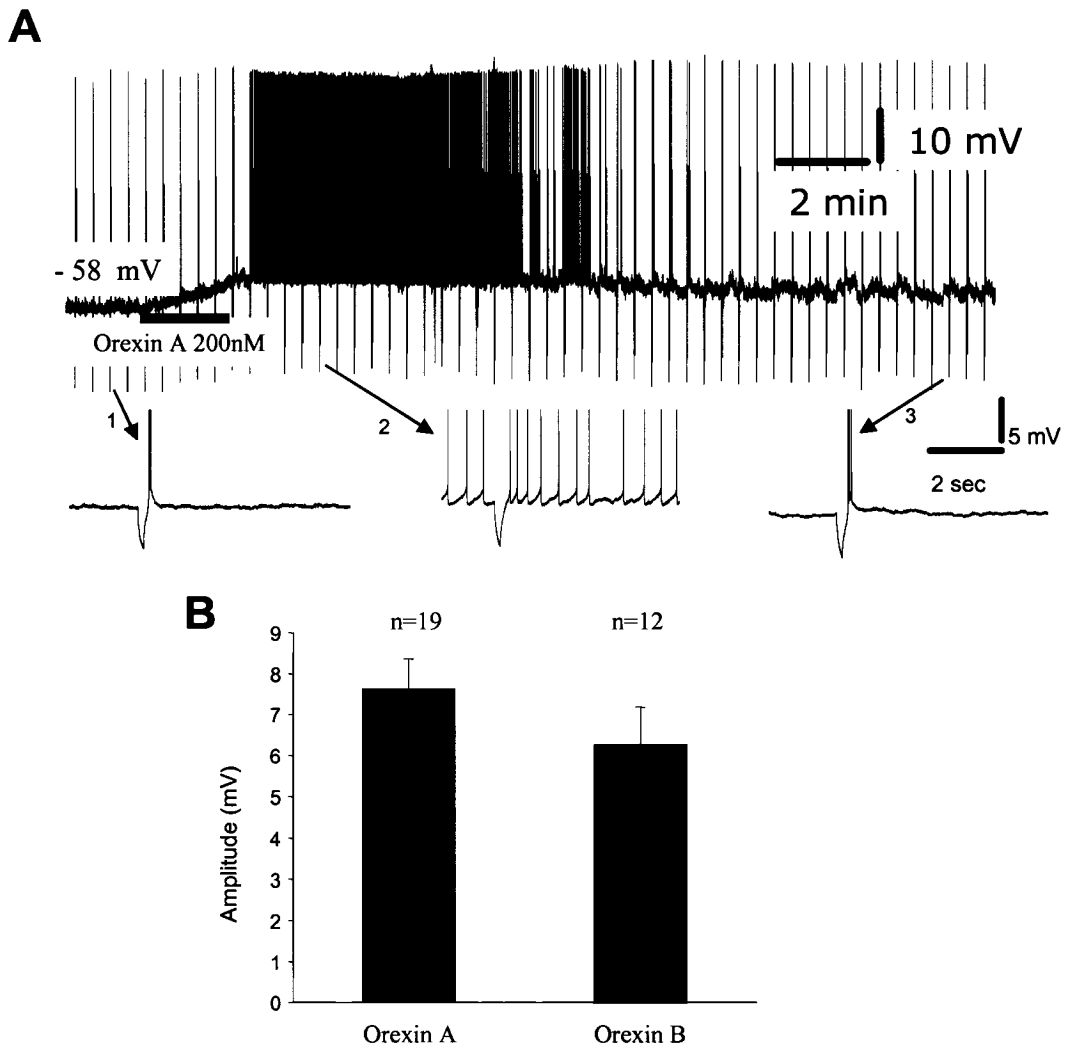

Figure 4.2 Orexin-induced depolarization changes firing patterns. A: Current clamp trace from an anterior PVT neuron illustrates an orexin-induced (red bar) slowly rising and prolonged membrane depolarization (resting membrane potential -58 mV). Vertical lines represent transient hyperpolarizing current injections (250ms; -60pA; 0.04 Hz) that trigger a LTS and burst of action potentials prior to drug application (see below, expanded trace #1). During the orexin-induced depolarization, the LTS is inactivated and the cell displays tonic firing (expanded trace #2). The LTS recovers upon return of the membrane potential to resting levels (expanded trace #3). **B:** Histograms depict similar amplitudes of membrane depolarization for orexin A (200 nM; n=19, black bar) and orexin B (200 nM; n=12, red bar).

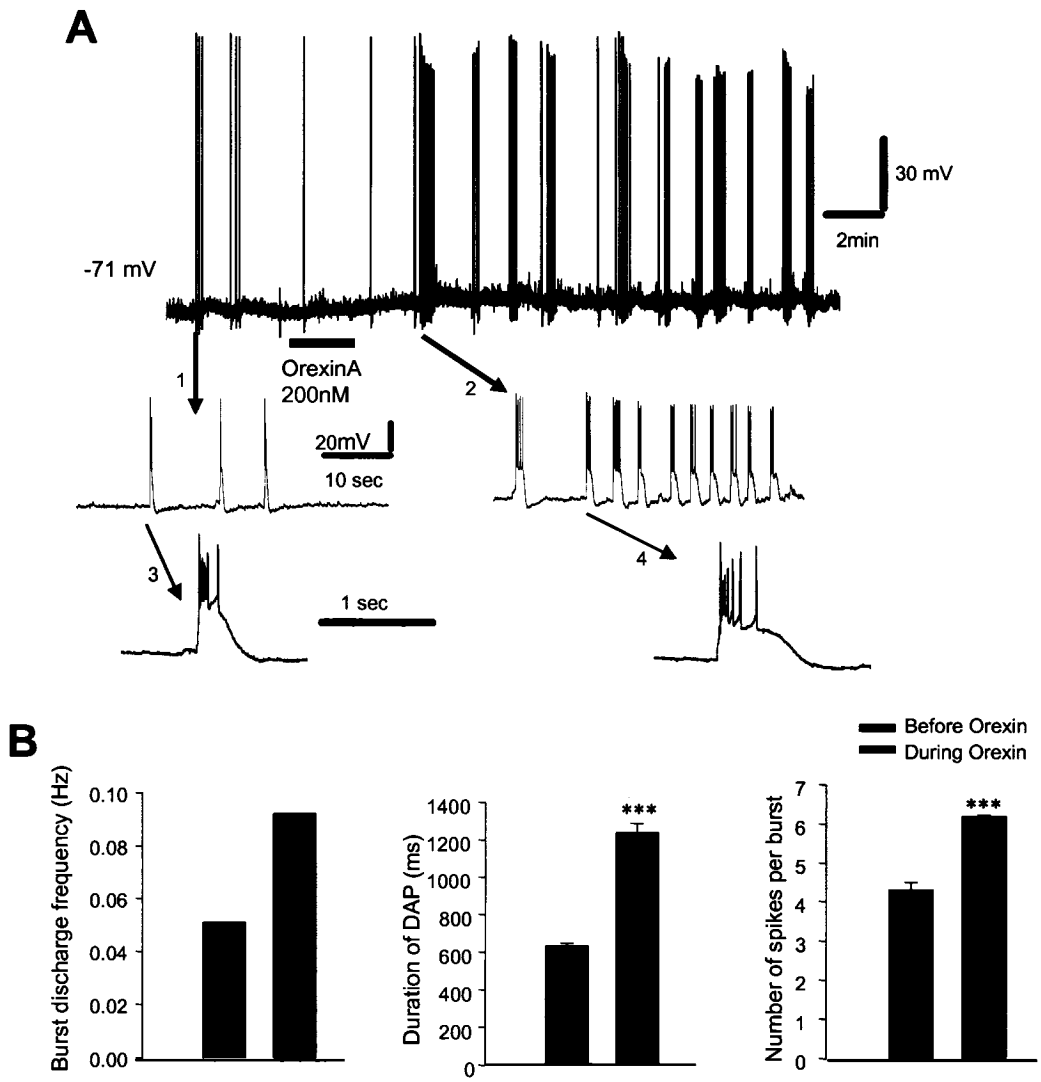

Figure 4.3 Orexin-induced depolarization enhances spontaneous burst discharges. **A:** Whole-cell current clamp recording from an anterior PVT neuron (RMP -71 mV) that displays spontaneous LTS-dependent burst discharges at rest (arrows 1 and 3). Application of orexin A (200nM, 2.3 minutes) is followed by a 5 mV membrane depolarization (5 mV), and changes the LTS-dependent burst discharges by increasing their frequency and width of the depolarizing afterpotential (DAP) such that each now induces a larger number of sodium spikes at a higher frequency (arrows 2 and 4). **B:** Histograms of pooled data from 17 burst discharges of this neuron illustrate the orexin-induced increase in frequency of burst discharge, mean duration of the DAP and number of spikes per burst (***$P<0.001$ based on student's two-tailed paired *t* test).

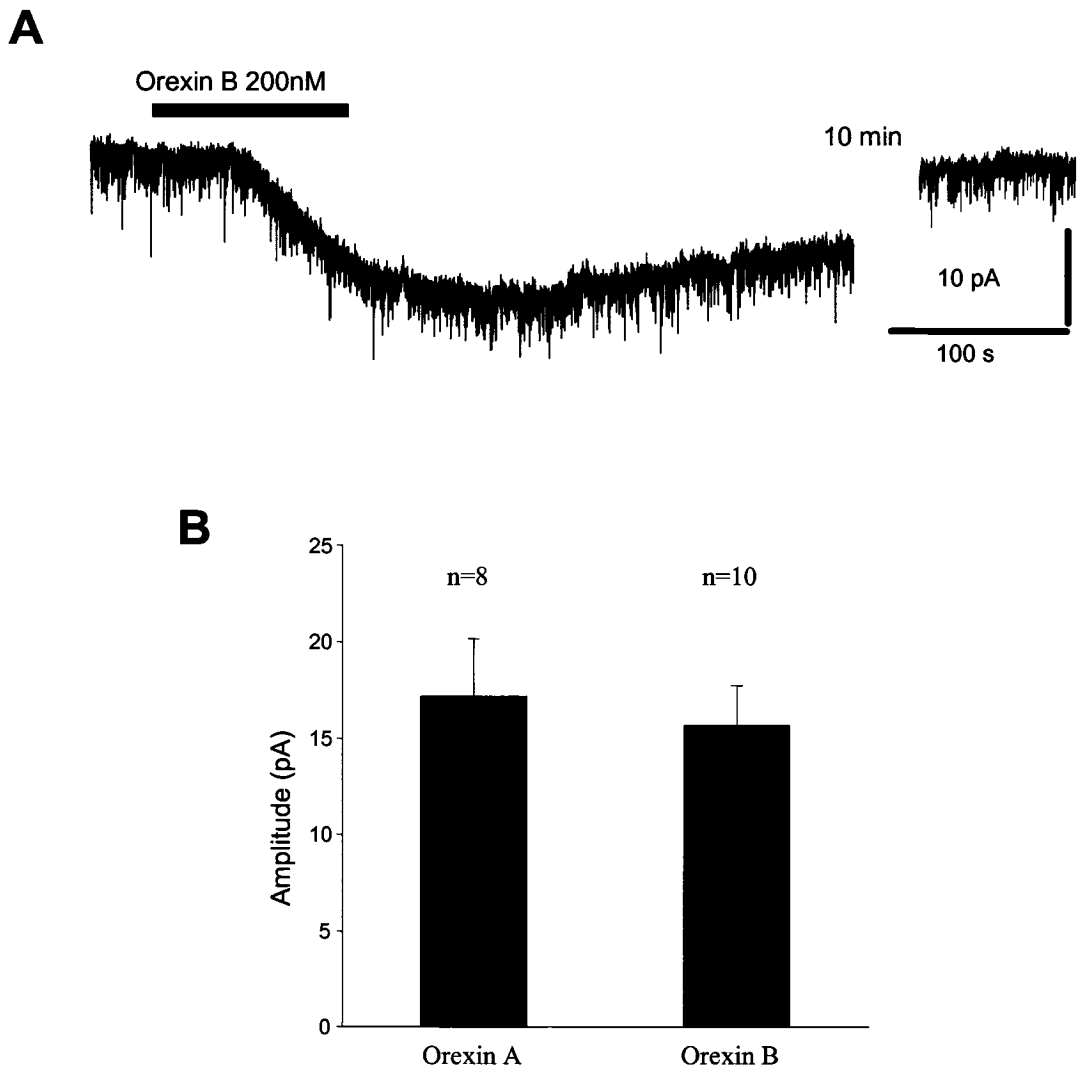

Figure 4.4 Orexin induces inward currents. A: Voltage-clamp trace (Vh = -50mV) illustrates slow inward current subsequent to bath applied orexin (200 nM) for 2 minutes. **B:** Histograms for a population of cells thus tested indicate no difference in the magnitude of the inward currents induced by orexin A (200 nM; n=8, black bar) or orexin B (200 nM; n=10, red bar).

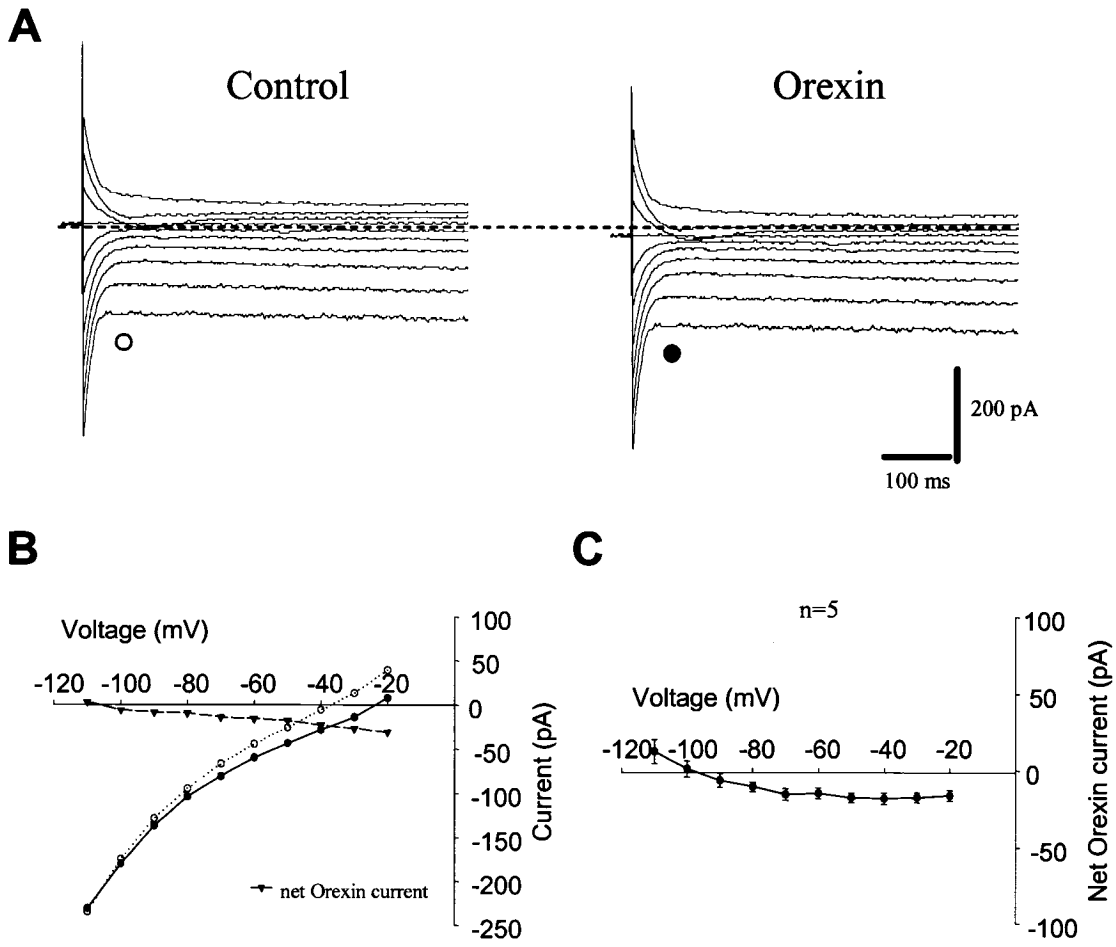

Figure 4.5 Net orexin A-induced current that reverses close to -100 mV. A: Voltage-clamp recordings obtained in the presence of TTX before (left) and at the peak (right) of an orexin-induced response (200 nM). **B:** I-V plots constructed from values taken at the beginning of the pulse (open and closed symbols, respectively) and the net orexin-induced current (red triangles) illustrate reduction in current and reversal near -110 mV. **C:** Mean net orexin (A or B) current for 5 PVT neurons that display a similar response profile with reduction in membrane conductance and reversal close to the potassium equilibrium potential.

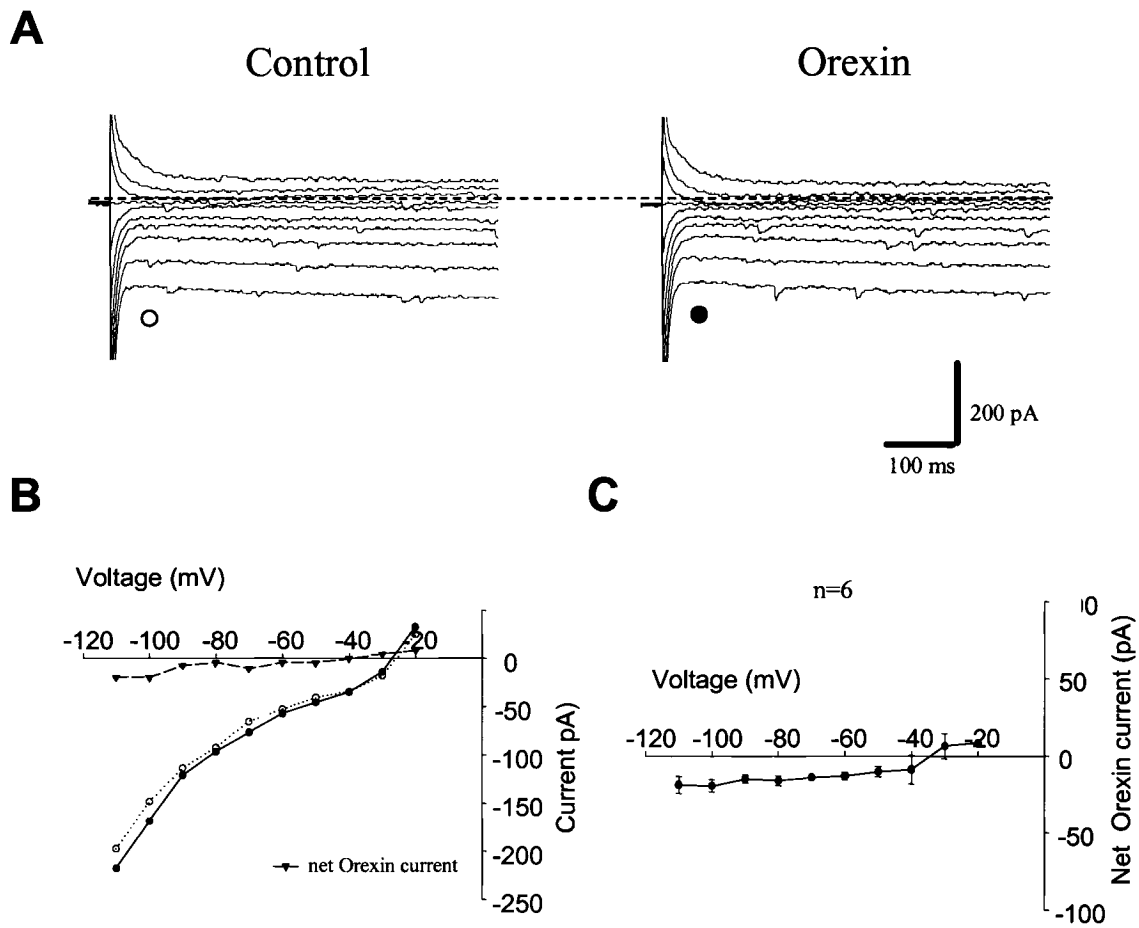

Figure 4.6 Net orexin B-induced current that reverses near -40 mV.
A: As per the protocol applied in Fig 4.5, voltage clamp recordings under control and at the peak of the orexin-induced response. **B:** I-V plots and net orexin-induced current for this neuron reveals reversal close to -40mV. **C:** Mean net (orexin A or B) current for 6 PVT neurons indicates increase in membrane conductances with reversal near -35 mV.

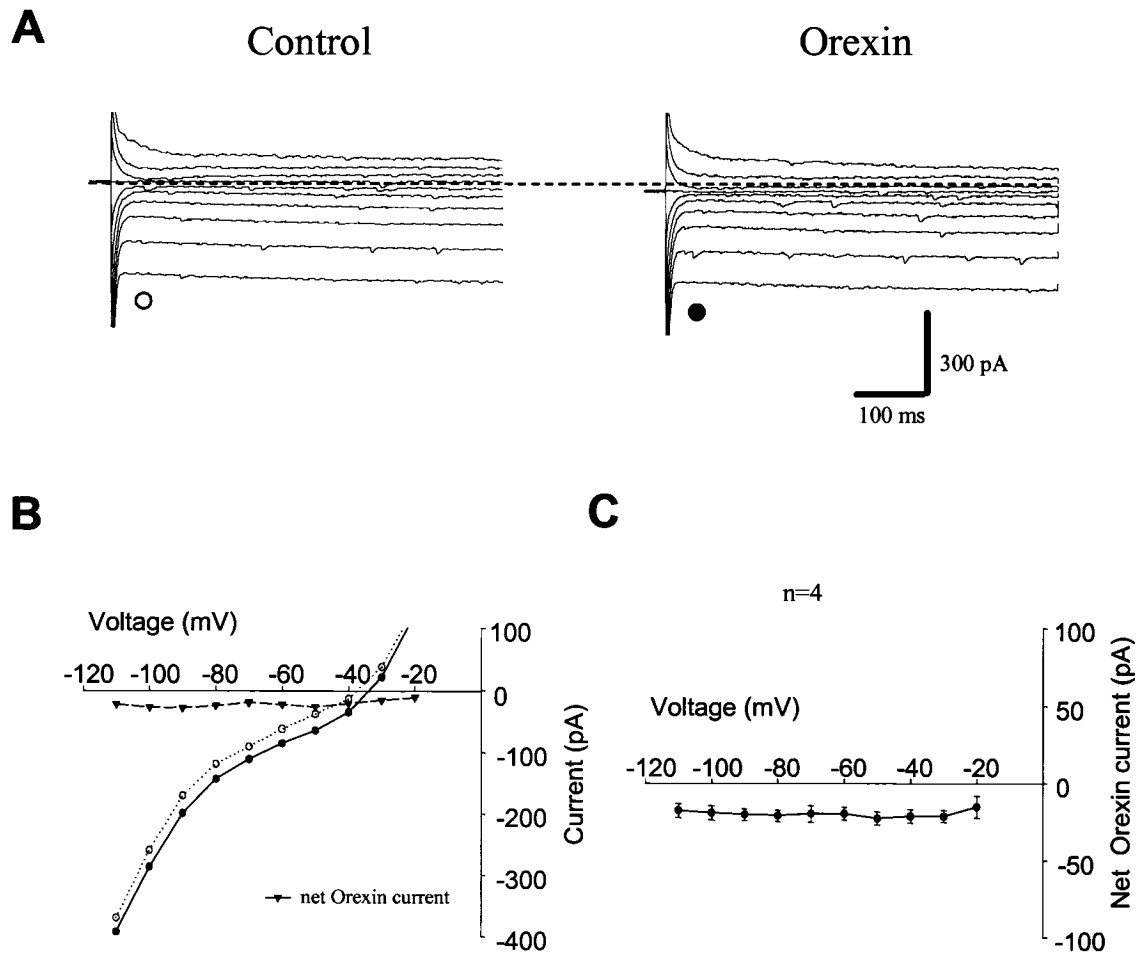

Figure 4.7 Net orexin A-induced current with no reversal within the voltage range tested. A, B: Using the same recording protocol and measurements as depicted in Figures 4.5 and 4.6, the I-V lines remain parallel (parallel shift) with no reversal. **C:** Mean net orexin (A or B) current for 4 neurons lacks a reversal in the voltage range tested (20 to 110 mV).

CHAPTER 5

DISCUSSION

5.1 Discussion of properties of thalamic paraventricular neurons.

Deta present here are the first report on the intrinsic conductances specific to PVT, the important relay between hypothalamic and brain stem structures and telencephalic areas. Data indicate that PVT, for the most part represent a homogeneous population with respect to expression of active intrinsic membrane conductances that include: time-dependent and time-independent inward rectification, state-dependent burst or tonic firing patterns, T-type currents and low threshold spikes, action potential after hyperpolarizations, spike frequency adaptation and spike broadening.

5.1.1 Time-dependent inward rectification (I_h)

Time dependent inward rectification was first discovered more than 20 years ago (Brown and Difrancesco, 1979). Because its hyperpolarization-activated properties were considered unique, it received the term If (f for funny) in cardiac (Brown and Difrancesco, 1979) and Iq (q for queer) in hippocampal neurons (Halliwell and Adams, 1982). Now termed I_h, this conductance is recognized as a ubiquitous component of a wide variety of neuronal and non-neuronal cells. I_h is a mixed sodium/potassium conductance whose activation produces an inward current that slowly depolarizes the membrane (Pape, 1996) and is believed to underlie a depolarizing "pacemaker" potential observed in many cells throughout the brain, including the thalamus and the cortex (Huguenard and McCormick, 1992; McCormick and Bal, 1997; Robinson and Siegelbaum, 2003). Thus it seems that I_h may contribute to the oscillatory activity found

in PVT thalamocortical neurons. The expression of I_h is correlated with the expression of types 2 and 4 hyperpolarization –activated cyclic nucleotide-sensitive cation nonselective (HCN) mRNAs, which are highly expressed in anterodorsal thalamus (Santoro et al., 2000). In addition to pacemaker activity, physiological roles ascribed to I_h include control and limitation of resting potential, control of membrane resistance and dendritic integration, and regulation of synaptic transmission (reviewed in Robinson and Siegelbaum, 2003).

5.1.2 Time independent inward rectification (I_R)

Inwardly rectifying potassium channels (Kir), first found in skeletal muscle, can conduct more current when the membrane potential is hyperpolarized than when it is depolarized from the K^+ equilibrium potential by an same amount, even with equal K^+ concentrations ($[K^+]$) on both sides of the membrane (Katz, 1949; Hodgkin and Horowicz, 1959; Hagiwara and Takahashi, 1974; Hagiwara et al., 1976). These K^+ channels accomplish numerous biological tasks (Hille, 2001) such as controlling the resting membrane potential, regulating cardiac and neuronal electrical activity, secretion of neurotransmitters and hormones, and maintaining electrolyte balance. Some inwardly rectifying K^+ channels can be modulated by external signals, such as hormones, neurotransmitters through the activation of specific G-proteins, and the internal metabolic state of the cell (Jan and Jan, 1997). The 15 mammalian genes products related to the Kir family have been classified into seven subfamilies (Kir 1–7), encoded by at least 12 genes (Doupnik et al., 1995). In-situ hybridization studies show that the Kir channel subunits are differentially expressed in the mammalian CNS (Bredt et al., 1995; Karschin

et al., 1996). Kir4 and Kir7.1 channels are strong inwardly rectifying channels involved in setting the membrane potential, buffering extracellular potassium and modulating the waveform of the action potential in neurons. Kir3 is also strong rectifier and activated by Gβγ. The majority of PVT neurons display modest inward rectification, and channels from the Kir1, 2, 4 or 7 families may contribute to rectification in these neurons.

5.1.3 Membrane potential-dependent firing properties

All PVT cells exhibit distinct membrane potential-dependent activity patterns – tonic firing and burst firing, key features of many thalamocortical neurons (Bal et al., 1995; Crunelli et al. 1989; Jahnsen and Llinas, 1984a, b). Studies show that the firing mode depends on the activation state of voltage-gated, T-type Ca^{2+} channels in the soma and/or dendrites (Llinas and Jahnsen, 1982; Steriade and Llinas, 1988; Sherman and Guillery, 1996). At depolarized membrane potentials (>~ -60 mV, for about 50~~100ms), I_T is inactivated and the neuron responds to an excitatory input (e.g., an EPSP) with a single spike, or to a sustained depolarizing pulse with tonic firing. On the other hand, hyperpolarization (< ~ -65 mV, maintained for about 50~~100 ms) removes this inactivation, so I_T is de-inactivated and response on return to resting levels or to a suprathreshold depolarization will be a T current dependent low-threshold Ca^{2+} spike (LTS) crowned with a burst of action potentials (Sherman, 1996; Reinagel et al., 1999).

Both tonic and burst firing modes efficiently relay information to the cortex in behaving animals, but have markedly different consequences for information processing: in tonic mode, a cell's firing rate faithfully and linearly reflects the amplitude and duration of an excitatory input; in contrast, burst mode produces a less linear relay of

information, but the information relayed has greater detectability because of a greater signal-to-noise ratio and stronger activation of postsynaptic cortical targets. Therefore, those firing mode strongly affects the nature of the signal that is relayed to the cortex (Sherman, 2001).

Although bursting is relatively rare during full wakefulness and more common during slow-wave sleep, drowsiness or certain pathological states, evidence of burst firing, together with tonic firing has been reported in various species during waking behavior (Sherman, 2001). Recent data from rodents, cats and monkeys suggest that burst firing serves as a wake-up call for the detection of novel, previously unattended stimuli in the wakeful state. For PVT, whether the burst mode appears in the wakeful state requires further testing. What is apparent here is that orexin receptor activation appears capable of attenuating these state-dependent intrinsic membrane properties, thereby influencing the patterns of activity of these cells. While the present analysis was conducted on neurons at a circadian time corresponding to the rat quiet period (daylight), it would be interesting to perform the same experiments on neurons at a circadian time corresponding to the animal's active period (in the dark). One might speculate that the intrinsic properties of these thalamocortical neurons will be different, that more neurons will be in a state of depolarization, and perhaps firing in a spontaneous tonic mode. Whatever the situation, it is likely that firing patterns play an important functional role within individual basal-ganglia-thalamocortical circuits (Lu et al., 1993; McCormick, 1992; Sherman, 1996; Sherman and Guillery, 1996).

5.1.4 Ion channels mediating the low threshold spike

Due to heterogeneity of channels and lack of selective blockers, it remains a challenge to decipher the channels and subunits mediating the low threshold spike (Ertel and Ertel, 1997). Cloning of three subunits α1G, α1H and α1I (Klugbauer et al., 1999; Williams et al., 1999), also termed Cav3.1, Cav3.2, Cav3.3 (Cribbs et al., 1998; Perez-Reyes et al., 1998; Lee et al., 1999b) has facilitated analyses of the biophysical properties (distinctive voltage dependence, kinetics, and single channel conductance) of this T-type Ca^{2+} channel family (Carbone and Lux, 1984; Huguenard, 1996). PVT has higher levels of α1G expression, low levels of α1I expression and no detectable α1H expression (Talley et al., 1999). Further classification of the subunit specificities within PVT neurons may result from discovery or development of selective antagonists and/or by utilizing a single-cell molecular approach.

5.1.5 Afterhyperpolarizations (AHPs)

In all PVT neurons, action potentials are followed by afterhyperpolarizations (AHPs), important determinants of neuronal excitability and firing patterns. In general, three overlapping kinetic components of AHPs (ie. fast, medium and slow) can be distinguished, generated by three broad families of calcium-activated potassium channels, called BK, SK, and IK channels (e.g. Sah, 1996).

A fast AHP (fAHP) immediately following the action potential typically lasts 1-10 ms, and is due to the activation of I_C, a current carried by the large conductance voltage- and Ca^{2+} -dependent K^+ channels termed BK channels (Lancaster and Adams, 1986; Shao et al., 1999; Sah and Faber, 2002). Following the fAHP, cells may display a

prolonged hyperpolarization phase lasting for hundreds of milliseconds, possibly seconds. These slower calcium-activated potassium currents may be separated into two distinct components, the medium AHP (mAHP) and the slow AHP (sAHP) (Sah, 1996).

A component of the mAHP is a current carried by small conductance Ca^{2+}-activated and voltage independent K^+ channels, termed SK channels, which are sensitive to the bee venom apamin and which decay with time constants of ~ 200 ms (Stocker et al., 1999; Gerlach et al., 2004). Four SK channel family genes have been cloned, with three expressed in CNS: SK1, SK2, and SK3 (Ishii et al., 1997; Joiner et al., 1997; Stocker and Pedarzani, 2000). The channels underlying the mAHPs in PVT neurons remain to be characterized. The CNS distribution of SK channels reveals that anterior PVT neurons express all three while posterior PVT expresses a high level of SK3, but low levels of SK1 and SK2 (Stocker and Pedarzani, 2000). This implies a functional difference between neurons within the same nucleus.

The channels underlying the sAHP are voltage independent K^+ channels (I_K) whose activation requires a rise in cytosolic calcium, typically by Ca^{2+} influx through voltage-dependent Ca^{2+} channels (Lancaster and Adams, 1986). Different from SK channels, they are not blocked by apamin (Stocker et al., 1999; Bowden et al., 2001; Gerlach et al., 2004). In addition, transgenic study showed that none of the SK channels underlie the slow AHP (Bond et al., 2004). Further efforts are necessary for identification of channels underling slow AHP.

As for physiological roles, since fAHPs contribute to the action potential repolarization, inactivation of BK channels underling it might be responsible for the spike broadening during repetitive firing (Lancaster and Nicoll, 1987; Storm, 1990; Sah, 1996).

The mAHP, mediated by activation of small conductance (SK) channels, controls action potential firing frequency in a number of cell types including hippocampal and cortical pyramidal neurons (Schwindt et al., 1988; Stocker et al., 1999; Pedarzani et al., 2001). However, this current appears to have little role in spike frequency adaptation (Faber and Sah, 2002). By contrast, the sAHP is widely acknowledged to be the main determinant of spike frequency adaptation (Madison and Nicoll, 1982; Faber and Sah, 2002). Although the channels underlying slow AHP is not known (Sah and Faber, 2002) they are negatively modulated by a variety of transmitters such as glutamate, 5HT, noradrenaline and acetylcholine, almost all of which depress the sAHP and reduce spike frequency adaptation (Nicoll, 1988; Schwindt et al., 1988; Faber and Sah, 2002).

5.1.6 Post-train afterhyperpolarization (post-train AHP)

In PVT neurons, as in other central neurons, a depolarizing current evokes a train of action potentials that is followed by a prolonged AHP, sometimes termed "hyperpolarizing afterpotentials", or HAPs (e.g. Bourque et al., 1985). Previous studies indicate that Ca^{2+} activated K^+ channels contribute to these post-burst AHPs, since reducing Ca^{2+} influx or intracellular Ca^{2+} accumulation attenuates their magnitude (Bourque et al., 1985; Thomson, 1984; Thomson and West, 1990). In the present study, the amplitude of the post-burst AHP was positively correlated with the number of action potentials in the burst, probably because of accumulating intracellular Ca^{2+} from Ca influx during the action potential.

Mechanically, currents underlying medium and slow AHPs may both contribute to the post-burst AHP (Storm, 1990; Sah, 1996). It was noted that in some neurons (e.g.

hippocampus), post-train AHPs appear to be mediated by apamin-insensitive, voltage-independent and Ca^{2+} dependent K^+ currents (Lancaster and Nicoll, 1987; Lorenzon and Foehring, 1992) while in other neurons (e. g. subthalamic nucleus) these AHPs involve apamin-sensitive, voltage-independent and Ca^{2+} dependent K^+ currents (Storm, 1990; Sah, 1996; Hallworth, 2003). For PVT neurons, considering the varied duration and amplitude of post-train AHPs, a heterogeneity of calcium-activated potassium conductances may be involved, and issue yet to be addressed.

5.1.7 Spike frequency adaptation (SFA)

Spike-frequency adaptation (SFA), the decrease of firing rates during a sustained current injection, is a prominent feature of neural dynamics. SFA has been observed in neurons of various systems from several species (Michaelis and Chaplain, 1975). In mammals, SFA has been observed in rodent motoneurons (Granit et al., 1963; Sawczuk et al., 1995), hippocampal CA1 pyramidal cells (Lancester and Nicoll, 1987; Madison and Nicoll, 1984), and pyramidal cells of the piriform cortex (Barkai and Hasselmo, 1994). In the neocortex of rodents, SFA has been identified in most pyramidal neurons, some of which are bursting neurons (Connors and Gutnick, 1990; Mason and Larkman, 1990; McCormick et al., 1985).

Various ionic currents appear to contribute to the development of SFA in different neurons. Most common are: the calcium-gated potassium medium and slow AHPs (Madison and Nicoll, 1984; Avoli et al., 1994; Lorenzon and Foehring, 1992); the M-current (I_M), a slow-activating non-inactivating voltage-sensitive potassium current

(Madison and Nicoll, 1984; McCormick et al., 1993); fast sodium current inactivation (Michaelis and Chaplain, 1975; Schwindt and Crill, 1982; Melnick et al., 2004).

5.1.8 Spike broadening

Broadening of action potentials, an increase in the spike duration during repetitive firing, is seen in neurons from a wide variety of invertebrate and vertebrate species, both in vitro and in vivo. Spike broadening occurs in all compartments of the neuron: soma (Aldrich et al., 1979; Ma and Koester, 1996; Shao et al., 1999), dendrite (Andreasen and Lambert, 1995) and axon terminal (Jackson et al., 1991; Geiger and Jonas, 2000). Various mechanisms may underly spike broadening. In invertebrates, where it was first studied, broadening is frequency dependent and is due to cumulative inactivation of voltage-gated K^+ currents (Aldrich et al., 1979; Quattrocki et al., 1994; Ma and Koester, 1996). A similar mechanism has been described in various mammalian neurons, including mossy fibre terminals in the hippocampus (Geiger and Jonas, 2000), magnocellular hypothalamic neurons (Bourque and Renaud, 1985) and pituitary neurosecretory terminals (Jackson et al., 1991). These K^+ currents include slowly inactivating, delayed rectifier type currents (Aldrich et al., 1979; Geiger and Jonas, 2000) and low threshold, rapidly inactivating A-type K^+ currents (Giese et al., 1998; Ma and Koester, 1996; Sakurai et al., 2006). In hippocampal pyramidal neurons, inactivation of a Ca^{2+}-activated K^+ current mediated by rapidly inactivating large conductance BK channels contributes to spike broadening (Halvorsrud et al., 1999; Shao et al., 1999; Greffrath et al., 2004). An enhanced activation of voltage-gated Ca^{2+} channels late in the train may also be a contributing factor (Aldrich et al., 1979). In PVT neurons, more than

40% broadening happened in the second spike. Furthermore, the fast after-hyperpolarization (fAHP), which represents a continuation of the BK-dependent spike repolarization, reduces most rapidly in the second spike in amplitude during high-frequency firing (Lancaster and Nicoll, 1987; Borg-Graham, 1987; Storm, 1990; Borg-Graham, 1998). Therefore, the inactivation of BK channels is likely to be a key factor in spike broadening in PVT neurons.

Action potential broadening has an important role in many aspects of neuronal activity. Since Ca^{2+} influx during action potentials largely occurs during the repolarization (Llinas et al., 1982), spike broadening has a large impact on the amount of Ca^{2+} influx and the resultant increase in cytoplasmic free Ca^{2+} may in turn regulate a range of cellular processes (Clapham, 1995), including Ca^{2+}-dependent ion channels, enzymes, second messenger cascades, gene transcription, and release of transmitters or hormones (Jackson et al., 1991; Byrne and Kandel, 1996; Sabatini and Regehr, 1997).

5.2 Discussion of Response to Exogenous Orexins

5.2.1 Orexin receptors in PVT neurons

The present study demonstrates that exogenous orexin A and B both act postsynaptically to depolarize a population of PVT neurons. The slow onset and prolonged pattern of response is typical of central neuronal responses to the application of various neuropeptides e.g. vasopressin (Kolaj and Renaud, 1998) and angiotensin (Oz et al., 2001). The fact that both orexin A and orexin B can depolarize PVT neurons with similar magnitudes agrees with the in situ hybridization histochemistry data of Marcus et al (2001) who reported almost identical binding for their OX_1R and OX_2R probes in

PVT. The observation that both orexin A and B can induce depolarization in a subpopulation of PVT neurons implies that some of these PVT neurons express *both* receptors. While a full dose-response analysis was not attempted here, the present observations contrast with other studies on midline thalamic neurons where the authors proposed that the excitatory action of orexin was mediated primarily by OX_2 receptors based on the relative potencies of orexin A versus orexin B (Bayer et al., 2002; Ishibashi et al., 2005; Huang et al., 2006). It is interesting to note that other studies have independently shown agreement between electrophysiological data and in situ hybridization data. For example, the excitatory action of orexin on tuberomammillary neurons was reported to be mediated by OX_2 receptors (Bayer et al., 2001; Eriksson et al., 2001) a structure that appears to express only OX_2R mRNA (Marcus et al., 2001). By contrast, the excitatory actions of orexin on locus coeruleus neurons is reported to be mediated by OX_1 receptors (Bourgin et al., 2000), a structure that expresses mainly OX_1R mRNA (Marcus et al., 2001)

5.2.2 Orexin-induced conductances

Orexin receptors have been shown to be coupled to G proteins (Sakurai, 1999). In PVT, analyses of I-V relationships and net orexin-induced currents indicate that the downstream effect of their activation can result in inward currents that involve two different conductances, reduction in one or more potassium conductances and/or increase in a putative non-selective cationic conductance. As reviewed in Chapter 1, this is similar to orexin-induced conductances observed in other neurons e.g. locus coeruleus (Murai and Akaike, 2005) and nucleus tractus solitarius (Yang and Ferguson, 2003). In those

neurons that display a parallel shift in their net orexin-induced current, one might speculate that this arises because both types of conductance are involved. This possibility remains for investigation. Additional investigations are required to characterize which of various types of potassium and non-selective cationic conductances, and downstream signaling pathways are engaged by these orexin receptors in PVT neurons.

In terms of further investigations, given that PVT neurons demonstrate an innervation from the suprachiasmatic nucleus, the brain's biological clock, and that PVT neurons display circadian rhythmicity in their immediate early gene activation patters, there is the possibility that the 'arousal' activity induced by orexins will differ over the day-night continuum. Thus, it may be informative to compare PVT neurons for their intrinsic properties and their responses to orexins under reversed day-night conditions i.e. to use slices from animals acclimatized to a reversed light-dark cycle. Also, since PVT contains a mosaic of neurons that have differing axonal projections (e.g. accumbens versus amygdala versus prefrontal cortx), their intrinsic properties and/or complement of orexin receptors may be different. In the long term therefore, the introduction of suitable retrograde tracers together with electrophysiology of neurons labeled according to their axonal targets may reveal some unique differences among the PVT neuronal population that can be related to their innervation.

5.2.3 Functional implications

PVT together with other thalamic midline nuclei has long been considered a component of the 'ascending reticular activating system", exerting a major influence on sleep, wakefulness and arousal. Indeed, PVT is innervated by monoaminergic inputs that

include histamine, dopamine, noradrenaline, and serotonin fibers (Cornwall and Phillipson, 1988; Otake and Ruggiero, 1995; Panula et al., 1989; Rico and Cavada, 1998), all of which are considered to be involved in the promotion and maintenance of wakefulness (Jones, 2003; Siegel, 2004). Orexin is now well known for its role in maintaining wakefulness, and its lack results in the sleep disorder known as narcolepsy (Nishino et al., 2000; Sutcliffe and de Lecea, 2002). Anatomically, PVT receives a dense orexin innervation, in sharp contrast with the lack of orexin innervation in the sensory relay thalamic nuclei as well as the mediodorsal nucleus and the habenular complex (Kirouac, 2005). Furthermore, orexin release is consistent with circadian alertness, highest during active wakefulness and REM sleep, lower during quiet wakefulness and lowest during slow-wave sleep (Kiyashchenko et al., 2002). PVT can also be activated by a wide range of stress paradigms (Brown et al., 1992; Deutch et al., 1995; Young and Deutch, 1998). Thus, orexin may exert a state-dependent action on PVT neurons to promote thalamocortical activity. As the activity of thalamocortical relay neurons is state-dependent, any interaction between their intrinsic properties and synaptic connections is likely to influence their firing patterns. As illustrated in the present study, exogenous (and presumably endogenous) orexin is capable of inducing changes in burst and tonic firing in PVT. In this instance, since the outflow from PVT includes substantial projections to limbic related regions, notably prefrontal cortex, accumbens and amygdala, it would appear that the orexinergic innervation of PVT is, in a sense, a "wakeup call" from the lateral hypothalamus to 'regulate' activity in basal-ganglia-thalamocortical circuits.

Increased activity of PVT neurons by their orexin innervation may influence several functions ascribed to PVT. For example, aside from a role in arousal and

regulation of the sleep-wakefulness cycle (Bentivoglio, 1991; Watts, 1987), PVT appears to have a role in viscerolimbic functions (Cardinal, 2002; Pennartz, 1994; Walker, 2003), energy homeostasis (Freedman and Cassell, 1994; Otake, 2005), autonomic function (Bhatnagar and Dallman, 1999; Van der Werf et al., 2002) and motivated behaviors (Cardinal et al., 2002; Christakou et al., 2004; Kelley, 2004; Salamone et al., 2005).

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