Hypocretin-1 and GABA in the Pontine Reticular Formation Interact to Promote Wakefulness

by

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DEDICATION

To my parents: Penha and Dad

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PREFACE

Chapter 2, entitled "Wakefulness is Increased by $GABA_A$-Hypocretin Receptor Interaction in the Pontine Reticular Formation" is in press in *Sleep*, 2010.

Appendix, entitled "Neurotransmitters and Neuromodulators Regulating Sleep and Wakefulness." In: Encyclopedia of Behavioral Neuroscience. (Koob, G, Le Moal, M, and Thompson, R, eds), Elsevier Science is in press, 2010.

Appendix, entitled "Sumoylation-Dependent Control of Homotypic and Heterotypic Synergy by the Kruppel-type Zinc Finger Protein ZBP-89" was published in *J Biol Chem*, 2007

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LIST OF ABBREVIATIONS

ACh	acetylcholine
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
ANOVA	analysis of variance
CSF	cerebrospinal fluid
EEG	electroencephalogram
EMG	electromyogram
GABA	γ-aminobutyric acid
G protein	guanine nucleotide-binding protein
GPCR	G protein-coupled receptor
Hcrt-r1	hypocretin receptor-1
Hcrt-r2	hypocretin receptor-2
i.c.v.	intracerebroventricular
HPLC	high performance liquid chromatography
LC	locus coeruleus
LDT	laterodorsal tegmental nucleus
NMDA	N-methyl-D-aspartic acid
NREM	non-rapid eye movement
pmol	picomoles
PPT	pedunculopontine tegmental nucleus
PnO	pontine reticular nucleus, oral part

REM	rapid eye movement
SB-334867	N-(2-Methyl-6-benzoxazolyl)-N″-1,5-naphthyridin-4-yl urea
SEM	standard error of the mean

CHAPTER 1

INTRODUCTION

Since the discovery of the family of hypocretin peptides in 1998, considerable evidence has accumulated showing that hypocretins promote wakefulness. However, few studies have analyzed the interactions between hypocretins and other sleep-wake regulating neurotransmitters/neuromodulators. The pontine reticular nucleus, oral part (PnO) is one brain region important for sleep-wake regulation where the interactions between hypocretin-1, γ-aminobutyric acid (GABA), and acetylcholine (ACh) have been investigated (Bernard et al., 2003; Watson et al., 2008b). Whether these neurochemical interactions mediate the wakefulness promoting effects of hypocretin remains unclear. The main goal of my thesis research was to determine whether the increase in wakefulness caused by administering hypocretin-1 into the PnO is mediated by an interaction between hypocretinergic and GABAergic neurotransmission.

The first section of this chapter provides an overview of the hypocretin system, including discovery of the peptides, structure-function analyses, signal transduction pathways, expression and distribution, and role in regulating sleep and wakefulness. The second section discusses the role of the PnO in regulating the sleep-wake cycle and the principle sleep-wake regulating neurotransmitters in the PnO. The third portion concludes with the overall thesis research question and the specific aims.

THE HYPOCRETIN/OREXIN SYSTEM

Discovery of the Hypocretin/Orexin System

In 1998, two independent research groups simultaneously reported the discovery of hypocretins/orexins. One of the groups characterized an mRNA sequence termed clone 35 that was initially isolated from rat hypothalamic extracts in 1996 (Gautvik et al., 1996). Further sequence analysis revealed that clone 35 encoded a precursor protein that localized in discrete nuclei within the posterior hypothalamus (de Lecea et al., 1998). Due to the hypothalamic origin and the phylogenic relation to the incretin family of peptides, clone 35 was named preprohypocretin (Figure 1.1A**)**. Proteolytic cleavage of preprohypocretin produces two neuropeptides amidated on the carboxyl terminus (hypocretin-1/orexin A and hypocretin-2/orexin B) (Figure 1.1B**)**. The authors concluded that hypocretins are a novel family of neuropeptides that might function to modulate feeding behavior.

The other research group screened for fractions of brain extract that exhibited guanine nucleotide-binding protein (G protein)-coupled agonist activity with the goal of identifying ligands for orphan G protein-coupled receptors (GPCR) (Sakurai et al., 1998). Two hypothalamic peptides elicited an increase in calcium levels in cells stably expressing the orphan GPCR known as HFGAN72. One hypothalamic peptide ligand was named orexin A (hypocretin-1) and the other orexin B (hypocretin-2). HFGAN72 was renamed orexin receptor-1 (hypocretin receptor-1). Orexin receptor-2 (hypocretin receptor-2) was identified by searching for sequences that closely matched orexin receptor-1. After finding that intracerebroventricular (i.c.v.) injection of orexin A in rat caused an increase in feeding behavior the peptides were named orexins, which is derived

from the Greek word for appetite (orexis). Hypocretins and orexins are synonymous and for the purpose of clarity the remainder of this thesis document will use hypocretin nomenclature.

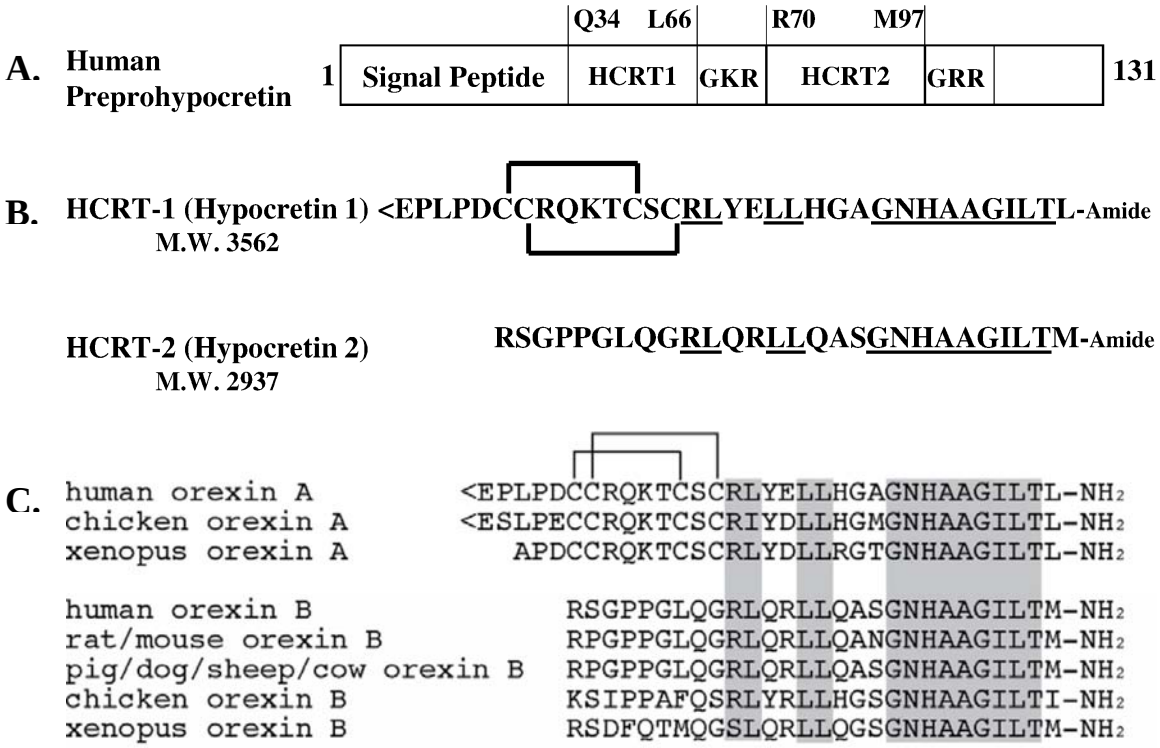

Figure 1.1. Hypocretin Gene and Peptide Structure. (A) Schematic representation of human preprohypocretin showing key structural features. Preprohypocretin is cleaved by prohormone convertases at dibasic residues GKR and GKK to produce hypocretin-1 and hypocretin-2. (B) Hypocretin-1 is a 33 amino acid peptide with two disulfide bonds. Hypocretin-2 is a 29 amino acid linear peptide. Residues underlined are conserved between the two peptides. The C-terminal of both peptides is amidated. (Source: modified from Taheri et al., 2002). (C) Alignment of hypocretin (orexin) sequences across species. Highlighted residues are conserved across species (Source: modified from Ohno and Sakurai, 2008)

Evidence linking hypocretins to the sleep disorder narcolepsy

Although it was hypothesized that hypocretins were mainly involved in feeding behavior, it was soon discovered that hypocretin signaling is also important for maintaining wakefulness. The importance of hypocretin signaling in maintaining wakefulness became evident in 1999 when 1) a mutation in hypocretin receptor-2 (hcrt-r2) caused narcolepsy in dogs (Lin et al., 1999), and 2) preprohypocretin knockout mice exhibited symptoms of narcolepsy (Chemelli et al., 1999). Another study showed that genetic ablation of the preprohypocretin gene in rats also caused a narcoleptic phenotype (Beuckmann et al., 2004). Narcolepsy is a sleep disorder characterized by excessive daytime sleepiness, disrupted nighttime sleep, hypnogogic hallucinations, sudden onset of REM sleep, and cataplexy. The incidence of narcolepsy is 1 in 2000 persons (Mignot, 1998), which is close to the order of incidence for Parkinson's Disease (1:1000) and more than Huntington's Disease (1:5000) (van den Pol, 2000).

Several studies confirmed the contribution of hypocretin deficiency to the pathophysiology of human narcolepsy. Post-mortem brains of narcoleptics lack hypocretinergic neurons (Peyron et al., 2000; Thannickal et al., 2000) and the level of hypocretin-1 in the cerebrospinal fluid (CSF) of narcoleptic patients is undetectable (Nishino et al., 2000; Mignot et al., 2002). Measurement of hypocretin-2 in CSF is difficult (Ripley et al., 2001) and hypocretin-2 appears to be less stable than hypocretin-1 (Kastin and Akerstrom, 1999). Lesion of hypocretin-2-expressing neurons in the lateral hypothalamus of rat causes an increase in sleep and a decrease in wakefulness (Gerashchenko et al., 2001), and the relative loss of hypocretin-2-expressing neurons correlates with the amount of sleep observed (Gerashchenko et al., 2003). The cause of

hypocretin deficiency in narcoleptic patients in not known, but a close association between narcolepsy and the expression of human leukocyte antigen-DQB1*0602 (Mignot, 1997) suggests an autoimmune basis for narcolepsy. Consistent with the autoimmune hypothesis, a recent study showed that narcolepsy is also closely associated with another immune related risk factor, a variant T-cell receptor alpha (Hallmayer et al., 2009).

In order to understand the differential roles of hypocretin receptor-1 (hcrt-r1) and hcrt-r2 in regulating sleep and wakefulness, hcrt-r1, hcrt-r2, and double receptor knockout mice were developed and analyzed for changes in the sleep-wake cycle (Willie et al., 2001; Willie et al., 2003). Hcrt-r2 knockout mice share the same sleep-related phenotypes as the preprohypocretin knockout mice (Willie et al., 2001; Willie et al., 2003). Preprohypocretin knockout mice exhibit symptoms of narcolepsy, including episodes of cataplexy, but have normal amounts of wakefulness and sleep (Chemelli et al., 1999). Hcrt-r1 knockout mice are asymptomatic (reviewed in Willie et al., 2003), suggesting that hcrt-r2 is more important for maintaining wakefulness. However, hcrt-r1/hcrt-r2 double knockout mice exhibit more frequent wake to REM sleep transitions with cataplexy than the hcrt-r2 knockout mice, demonstrating that both hypocretin receptor subtypes are required for the maintenance of wakefulness (Willie et al., 2001; Willie et al., 2003). Hypocretin receptor mutations are rare in humans, but there are patients with single nucleotide polymorphisms in hcrt-r2 (Thompson et al., 2004) that have excessive daytime sleepiness. With the advent of hypocretin receptor antagonists, further studies are exploring the relative importance of hypocretin receptor subtypes in

regulating wakefulness. Hypocretin receptor antagonist studies will be discussed in more detail later in this introduction.

Hypocretin gene and peptide structure

Hypocretin-1 is a 33 amino acid peptide of 3562 Daltons that contains two intramolecular disulfide bonds, and hypocretin-2 is a linear 28 amino acid peptide of 2937 Daltons (Figure 1.1B). Hypocretin-1 and hypocretin-2 share 46% sequence homology and are highly conserved across species, including *Xenopus laevis* (Figure 1.1C). Human, rat, dog, and mouse hypocretin-1 are identical whereas rodent and human hypocretin-2 differ by two amino acids. Secretin, a member of the incretin family, shares a tandem of 7 amino acids with hypocretin-2. Although the sequence homology between secretin and hypocretins is relatively weak, it appears that hypocretins emerged from secretin by genetic rearrangements (Alvarez and Sutcliffe, 2002). Human hypocretin-2 structure was determined using nuclear magnetic resonance spectroscopy and it is structurally similar to neuropeptide Y. Hypocretin-2 consists of a random coil at the amino-terminal followed by two alpha helices that are connected by a short flexible linker (Lee et al., 1999).

Hypocretin receptor pharmacology and signaling pathways

Hcrt-r2 shares 64 % sequence homology with hcrt-r1 (Sakurai et al., 1998). Both receptor genes are highly conserved between human and rat (greater than 94 %). The N-terminus extracellular domain of GPCRs is important for agonist recognition and binding and is highly conserved between hcrt-r1 and hcrt-r2. The intracellular loop III and the C-terminus of GPRCR interact with G proteins and there is weak amino acid sequence identity in these regions of hcrt-r1 and hcrt-r2 (Sakurai et al., 1998). Radioligand binding and calcium mobilization assays determined that hcrt-r2 binds hypocretin-1 and

hypocretin-2 with similar affinities whereas hcrt-r1 is highly selective for hypocretin-1 (Sakurai et al., 1998). Hcrt-r2 is a nonselective receptor for both hcrt-1 and hcrt-2 whereas hcrt-r1 is selective for hypocretin-1.

Hypocretin receptors couple to multiple G proteins and thus to multiple signal transduction pathways. Activation of different types of G proteins (e.g., Gs, Gq, Gi) triggers different signaling cascades and effector proteins. For example, activation of Gs proteins elevates adenylyl cyclase activity to activate protein kinase A (PKA) which results in an increased calcium mobilization and neuronal excitability. Activation of Gq increases calcium conductance and neuronal excitability by activating phospholipases. A number of studies investigated the contribution of different G protein types to either hcrt-r1 or hcrt-r2-mediated changes in membrane conductance, calcium influx, or downstream effectors of G protein receptor activation. Most of the studies used cell lines over expressing either hcrt-r1 or hcrt-r2 (Randeva et al., 2001; Hoang et al., 2003; Zhu et al., 2003; Holmqvist et al., 2005; Johansson et al., 2008). Initially, it appeared that hcrt-r1 coupled to Gq proteins to stimulate phospholipase C and that hcrt-r2 coupled to either Gq or Gi proteins (Zhu et al., 2003). However, hypocretin receptor signaling proved very complex. *In vitro* studies demonstrated that hcrt-r1 couples to Gs, Gq, or Gi proteins, depending on the tissue type and response elicited by hypocretin-1 (Bernard et al., 2003; Hoang et al., 2003; Holmqvist et al., 2005; Bernard et al., 2006). Furthermore, hcrt-r1 differentially coupled to Gs or Gq, depending on the concentration of hypocretin-1 administered (Holmqvist et al., 2005). What this means physiologically is not known. Depending on the region within the rat brainstem, G protein activation showed different pharmacological profiles in response to hypocretin-1 (Bernard et al., 2003, 2006).

Similar to hcrt-r1, hcrt-r2 couples either to Gs, Gq or Gi proteins depending on the tissue type and response elicited by hypocretin-1 (Randeva et al., 2001; Tang et al., 2008).

How hcrt-r2 or hcrt-r1 couple to different G proteins is not well understood, but it is proposed that tissue type, physiological demands (e.g., food deprivation), and receptor density may influence differential coupling (Karteris and Randeva, 2003). In addition to binding different G proteins, studies have alluded to hypocretin receptor dimerization (Ammoun et al., 2003; Hilairet et al., 2003), further complicating hypocretin receptor signaling.

Hypocretin structure activity analyses

Few studies have evaluated the molecular determinants for hcrt-r1 and hcrt-r2 binding. As mentioned previously, hypocretin-1 is selective for hcrt-r1 whereas both hypocretin-1 and hypocretin-2 can activate hcrt-r2. The carboxyl-termini of hypocretin-1 and hypocretin-2 share approximately 73% sequence homology and hypocretin-2 can be thought of as an N-terminally truncated variant of hypocretin-1. Indeed, truncating the N-terminus of hypocretin-1 (residues 14 or 15-33 amino acids remaining) reduced its potency and affinity at hcrt-r1 (Darker et al., 2001). The C-termini of hypocretin-1 and hypocretin-2 are highly conserved and studies show that the C-terminus is important for hypocretin receptor binding and activation (Darker et al., 2001; Ammoun et al., 2003; Lang et al., 2004). Whether the disulfide bonds within hypocretin-1 are important for biological activity is still under debate (Okumura et al., 2001; Lang et al., 2004). Currently, there are modified hypocretin-2 synthetic peptides that are more selective for hcrt-r2 than unmodified hypocretin-2 and hypocretin-1 (Asahi et al., 2003; Lang et al., 2004; Lang et al., 2006). Recently, a hypocretin-1 synthetic peptide was created and is more selective for hcrt-r1 than non-modified hypocretin-1 (Lang et al., 2004).

Hypocretin system neuroanatomy

Hypocretinergic neurons reside in the perifornical, lateral, and posterior hypothalamus in rat (Peyron et al., 1998; Nambu et al., 1999), and the lateral hypothalamus in human (Elias et al., 1998). There are approximately 7000 hypocretinergic neurons in rat lateral hypothalamus (Peyron et al., 1998; Modirrousta et al., 2005) and roughly 20-70,000 hypocretinergic neurons in human lateral hypothalamus (Peyron et al., 1998; Thannickal et al., 2000). The number of hypocretinergic neurons in human brain was estimated using either *in situ* hybridization (Peyron et al., 1998) or immunohistochemistry (Thannickal et al., 2000).

Hypocretin-containing neurons have widespread projections to a number of brain regions and only the cerebellum lacks projections from hypocretinergic neurons (Peyron et al., 1998; Nambu et al., 1999) (Figure 1.2). Hypocretinergic neurons abundantly innervate noradrenergic cells of the locus coeruleus neurons, serotonergic cells of the dorsal raphe, histaminergic cells of the tuberomammillary nucleus, and cholinergic cells of the laterodorsal tegmental/pedunculopontine tegmental nucleus (LDT/PPT) and basal forebrain (Peyron et al., 1998; Nambu et al., 1999). Hypocretins were initially implicated in regulating sleep and wakefulness because hypocretin-containing neurons project to brain areas important for promoting wakefulness, such as the locus coeruleus, tuberomammillary nucleus, and LDT/PPT. In addition to a role in regulating sleep and wakefulness, there is considerable evidence that hypocretins are involved in feeding, temperature regulation, endocrine secretion, and addiction (reviewed in Sharf et al.; Sakurai, 2007; Tsujino and Sakurai, 2009). The hypothalamus, where hypocretinergic neurons are located, is a homeostatic center of control and receives reciprocal projections from multiple brain regions. Hypocretinergic neurons are subject to tight regulation and

Table 1.1 summarizes the neuromodulators and neurotransmitters that influence hypocretinergic activity in the hypothalamus (reviewed in Willie et al., 2001; Tsujino and Sakurai, 2009).

The distribution of hcrt-r1 and hcrt-r2 mRNA closely matches brain regions innervated by hypocretinergic neurons (Trivedi et al., 1998; Marcus et al., 2001). Hcrt-r1 and hcrt-r2 mRNA are distributed in some of the same brain regions, such as the ventral tegmental area and dorsal raphe, but hcrt-r1 and hcrt-r2 mRNA are also differentially distributed in the brain (Trivedi et al., 1998; Greco and Shiromani, 2001; Marcus et al., 2001). Hcrt-r1 mRNA and protein are abundantly expressed in the locus coeruleus and are also found in the anterior and ventro-medial hypothalamic areas, paraventricular thalamic nucleus, pontine reticular formation, hippocampus, LDT/PPT, and the bed nucleus of the stria terminalis (Trivedi et al., 1998; Greco and Shiromani, 2001; Marcus et al., 2001). mRNA for hcrt-r2 is highly expressed in the tuberomammillary nucleus and in other hypothalamic nuclei (Trivedi et al., 1998; Marcus et al., 2001) with lower levels of hcrt-r2 protein present in the dorsal tegmental nucleus and caudal pontine reticular formation (Greco and Shiromani, 2001). The distinct pattern of hypocretin receptor subtype expression suggests different physiological roles for hcrt-r1 and hcrt-r2 in different brain regions.

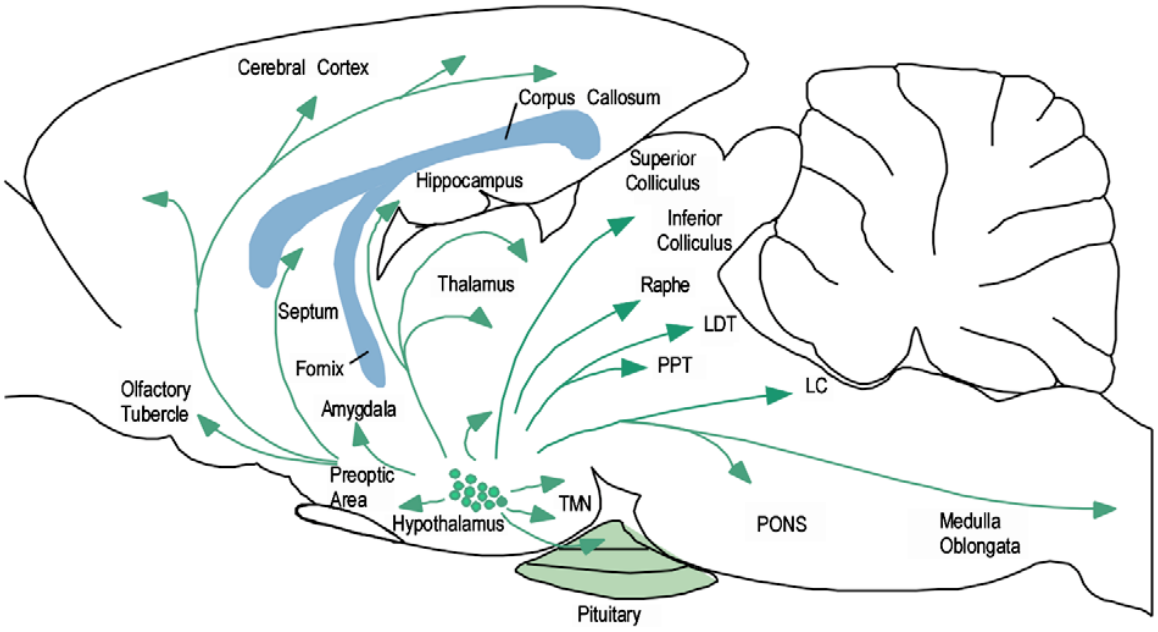

Figure 1.2. Hypocretinergic pathways in the rat brain. Hypocretin neurons originate in the lateral hypothalamus (shown as green circles) and project to a wide range of brain areas. (Source: Ohno and Sakurai, 2008)

Table 1.1

Factors that influence the activity of hypocretin neurons

	Receptor Involved
Excitation	
Glutamate	AMPAR, NMDAR mGluRs
Acetylcholine (muscarinic) (27%)	M3
Ghrelin	GHSR
Cholecystokinin	CCKA
Neurotensin	NTSR2[a]
Vasopressin	V1a
Oxytocin	V1a
Glucagon-like peptide 1	N.D.
CRF	CRFR1
Thyrotropin-releasing hormone	TRH1
ATP	P2X
H^+, CO_2	N.D.
Inhibition	
Glucose	Unknown
GABA	$GABA_A$, $GABA_B$
Serotonin	$5HT_{1A}$
Noradrenaline	$\alpha 2$
Dopamine	$\alpha 2$
Acetylcholine (muscarinic) (6%)	N.D.
NPY	Y1
Enkephalin	μ Opioid receptor
Nociceptin	NOPR
Leptin	OBR
Adenosine	A_1

AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; mGluR, metabotropic glutamate receptor; M3, muscarinic acetylcholine receptor M3; GHSR, ghrelin secretagogue receptor; CCKA, type A cholecystokinin receptor; NTSR2, neurotensin receptor 2; N.D., not determined; CRFR1, CRF-releasing factor 1; TRH1, thyrotropin-releasing hormone receptor 1; NOPR, nociceptin/orphanin FQ receptor; OBR, astrocyte leptin receptor

[a] N. Furutani, T. Abe, M. Hondo, T. Matsuki, N. Tsujino, K. Ichiki, M. Mieda, I. Matsuzaki, H. Takahishi, A. Yamanaka, and T. Sakurai, unpublished observations. (Source: modified from Tsujino and Sakurai, 2009).

Involvement of the hypocretin/orexin system in sleep and wakefulness

Hypocretinergic neurons innervate all major sleep-wake regulating brain areas (Peyron et al., 1998; Nambu et al., 1999; Taheri et al., 1999). The locus coeruleus receives the densest hypocretinergic projections and application of hypocretin-1 has a direct excitatory effect on locus coeruleus neurons (Hagan et al., 1999; Horvath et al., 1999; Bourgin et al., 2000). Neurons in the locus coeruleus fire maximally during wakefulness compared to NREM sleep and REM sleep (Aston-Jones and Bloom, 1981; Berridge and Foote, 1996). The first functional study demonstrating that hypocretins are involved in regulating sleep and wakefulness showed that i.c.v. administration of hypocretin-1 causes an increase in wakefulness and a decrease sleep (Hagan et al., 1999). The increase in wakefulness caused by i.c.v. administration of hypocretin-1 was due to an increase in the duration of wakefulness episodes (Piper et al., 2000). Monoaminergic neurons located in the tuberomammillary nucleus (Bayer et al., 2001; Eriksson et al., 2001; Yamanaka et al., 2002) and dorsal raphe (Brown et al., 2001; Brown et al., 2002; van den Pol et al., 2002) are also excited by hypocretin-1. Cholinergic neurons in the basal forebrain (Eggermann et al., 2001) and LDT/PPT (Burlet et al., 2002; Takahashi et al., 2002; Kim et al., 2009) depolarize in response to hypocretin-1. Hypocretinergic neurons in the lateral hypothalamus discharge at their fastest rates during active wakefulness (Takahashi et al., 2002; Lee et al., 2005; Mileykovskiy et al., 2005) and exhibit bursts of discharge during the phasic muscle twitches characteristic of REM sleep (Takahashi et al., 2002; Mileykovskiy et al., 2005). Higher CSF levels of hypocretin-1 were observed during active waking with movement (Kiyashchenko et al., 2002) and i.c.v. administration of hypocretin-1 increased motor activity in rats (Hagan et al., 1999;

Piper et al., 2000), suggesting that hypocretin-1 is involved in motor activity. Whether the primary function of hypocretin is to enhance motor activity is still debated. Hypocretin-1 levels in the lateral hypothalamus gradually increase throughout the active period and decrease during sleep (Yoshida et al., 2001; Kiyashchenko et al., 2002), further supporting the interpretation that hypocretin-1 promotes wakefulness. Moreover, hypocretin-1 levels in the CSF of dog increased after 24 hours of sleep deprivation (Wu et al., 2002), indicating that the increased hypocretin-1 levels might function to oppose sleep pressure. The activity of hypocretinergic neurons increased after total sleep deprivation (Modirrousta et al., 2005) and after REM sleep deprivation (Verret et al., 2003). Although the activity of hypocretinergic neurons increased after sleep deprivation (Verret et al., 2003; Modirrousta et al., 2005), stimulation of hypocretinergic neurons was not sufficient to keep sleep-deprived subjects awake (Carter et al., 2009). However, stimulation of hypocretinergic neurons during NREM sleep or REM sleep increased the probability of sleep to wake transitions (Adamantidis et al., 2007). Aged rats and mice display fragmentation of wakefulness (Mendelson and Bergmann, 1999) and have decreased hypocretin receptor expression (Terao et al., 2002). Taken together, these studies suggest a role for hypocretin in maintaining wakefulness.

Hypocretin receptor antagonists

Initially, pharmaceutical interest in the hypocretin system was focused on finding a way to administer hypocretin for the treatment of narcolepsy (reviewed in Fujiki and Nishino, 2005). However, there is a growing interest in treating insomnia with hypocretin receptor antagonists (reviewed in Nishino, 2007). In addition to the clinical applications of hypocretin receptor antagonists, subtype-specific antagonists of

hypocretin receptors will be useful to differentiate the physiological roles of hypocretin receptors.

The first hypocretin receptor antagonist, SB-334867 (N-(2-Methyl-6-benzoxazolyl)-N″-1,5-naphthyridin-4-yl urea), was developed by SmithKline Beecham Pharmaceuticals (Smart et al., 2001). Calcium mobilization studies showed that SB-334867 has an affinity of 40 nM at hcrt-r1 and 2000 nM for hcrt-r2. SB-334867 does not show any significant affinities over a panel of 50 GPCRs and ion channels (Smart et al., 2001). A number of studies have used SB-334867 in a variety of behavioral models (reviewed in Upton, 2005). For example, SB-334867 blocks the decrease in resting duration induced by i.c.v. hypocretin-1 (Rodgers et al., 2001), increase in grooming behavior (Duxon et al., 2001), decrease in REM sleep (Smith et al., 2003), increase in thermogenesis (Kiwaki et al., 2004) as well as the increase in antinociception caused by administration of hypocretin-1 to the PnO (Watson et al., 2009). Intraperitoneal injection of SB-334867 alone increased resting time in rats (Rodgers et al., 2001), increased sleep (Smith et al., 2003), and delayed emergence from isoflurane (Kelz et al., 2008) and sevoflurane (Kelz et al., 2008; Dong et al., 2009) anesthesia. SB-408124 (1-(6,8-difluro-methyl-quinoline-4-yl)-3-(4-dimethylamino-phenyl)-urea is another hypocretin receptor-1 antagonist that has an affinity of 6 nM at hcrt-r1 and 2000 nM for hcrt-r2. Few studies have used SB-408124 (reviewed in Upton, 2005). One study showed that subcutaneous administration of SB-408124 in rat did not alter sleep-wake states (Dugovic et al., 2009).

In 2003, the first hcrt-r2 antagonist, N-Acyl 6-7-dimetyoxy-1,2,3,4-tetrahydroisoquinoline was reported (Hirose et al., 2003). This compound has not yet been used for *in vivo* studies. Johnson and Johnson developed another hcrt-r2 antagonist,

JNJ-10397049 (1-(2,4-Dibromophenyl)-3-[(4S,5S)-2,2-dimethyl-4-phenyl-1,3-dioxan-5-yl]urea) (McAtee et al., 2004), that has an affinity of 80 nM for hcrt-r2 and 6000 nM for hcrt-r1. Only two studies have used JNJ-10397049 (Borgland et al., 2008; Dugovic et al., 2009) and one of these studies (Dugovic et al., 2009) showed that subcutaneous administration in rat decreased wakefulness and increased sleep.

A relatively recent study demonstrated that oral administration of the dual hypocretin receptor antagonist almorexant in rat, human, and dog increased sleep (Brisbare-Roch et al., 2007). The sleep-promoting effects were mainly due to blocking hcrt-r2 signaling, depending on the sleep parameter analyzed (Dugovic et al., 2009). Furthermore, the hcrt-r1 antagonist attenuated the increase in NREM sleep caused by the hcrt-r2 antagonist (Dugovic et al., 2009). The mechanisms are not well understood, but hcrt-r1 and hcrt-r2 may differentially alter levels of arousal-promoting neurotransmitters. Hypocretin receptors bind different G proteins and potentially dimerize with other hypocretin receptors or other GPCRs. Furthermore, different sleep-wake regulating brain regions express different densities and subtypes of hypocretin receptors, adding another level of complexity to the mechanisms by which hypocretins are wakefulness promoting.

Hypocretins interacts with multiple neurotransmitters to modulate sleep and wakefulness

Several studies have investigated the brain-region-specific effects of hypocretin-1 and -2 in regulating the sleep-wake cycle. Administration of hypocretin-1 into the locus coeruleus (Bourgin et al., 2000), preoptic area (Methippara et al., 2000), basal forebrain (Espana et al., 2001; Blanco-Centurion et al., 2006), and LDT (Xi et al., 2001) increased wakefulness and decreased sleep. Although hypocretinergic neurons project to all the

major wakefulness promoting brain regions, modulation of sleep and wakefulness is dependent on the interplay and coordination of multiple neurotransmitters.

Histamine is another wakefulness-promoting neurotransmitter that has been shown to interact with hypocretins. For example, intraperitoneal injection of a histamine synthesis inhibitor blocked the increase in wakefulness caused by hypocretin-1 (Yasuko et al., 2010). Histamine levels were significantly lower in the cortex, thalamus, and hippocampus of hcrt-r2 knockout narcoleptic dogs as compared to control dogs (Nishino et al., 2001). Furthermore, a recent report demonstrated that enhancement of histaminergic transmission reduced cataplexy and sleep in narcoleptic dogs (Guo et al., 2009).

Adenosine is a sleep-promoting modulator that has also been shown to interact with hypocretin. Brain levels of adenosine normally increase after sleep deprivation, but lesioning hypocretinergic neurons prevented the increase in adenosine levels after sleep deprivation (Murillo-Rodriguez et al., 2008). Adenosine A1 receptors are expressed on hypocretinergic neurons (Thakkar et al., 2002), and blockade of A1 receptors in the lateral hypothalamus inhibits the sleep-promoting effects of adenosine (Thakkar et al., 2002; Alam et al., 2009).

Glutamate is the major neurotransmitter in the brain and interacts with hypocretin in the hypothalamus. Microdialysis delivery of hypocretin-1 into the substantia innominata increased glutamate levels (Fadel and Frederick-Duus, 2008). Application of hypocretin-1 to the lateral hypothalamus stimulated glutamate release (Li et al., 2002). Microinjection of glutamate into the lateral hypothalamus increased wakefulness (Alam and Mallick, 2008) suggesting that hypocretin and glutamate interact to promote

wakefulness. Antagonist blocking studies are needed to confirm that the interaction between hypocretin and glutamate modulates sleep and wakefulness.

Several studies demonstrate that hypocretin modulates neuronal firing properties (reviewed in Nunez et al., 2009) and the release of other sleep-wake neurotransmitters in a number of brain regions (reviewed in Watson, 2010). All of these neurochemical interactions in different brain regions are important in dictating the resulting behavioral state.

OVERVIEW OF THE PONTINE RETICULAR NUCLEUS, ORAL PART

The ascending reticular activating system

The concept of the ascending reticular formation (ARAS) was first described in 1949 by Moruzzi and Magoun. Until this time, it was thought that sensory stimulation was required for triggering and maintaining wakefulness, and that cessation of sensory stimulation resulted in sleep. Moruzzi and Magoun found that stimulation of the brainstem reticular formation of anesthetized or sleeping cats resulted in electroencephalogram (EEG) signals that were identical to those of wakefulness (Moruzzi and Magoun, 1949). Furthermore, if the brainstem reticular formation was destroyed then sensory stimulation no longer resulted in arousal (Lindsley et al., 1950). Taken together, these seminal studies demonstrated that the reticular formation has the capacity to maintain wakefulness in the absence of sensory stimulation. The ARAS connects the brainstem reticular formation, including the pontine nucleus, oral part (PnO), to areas in the thalamus, hypothalamus, and cortex. The PnO contributes to the generation of

cortical arousal that is characteristic of wakefulness and rapid eye movement (REM) sleep (Lydic and Baghdoyan, 2005). The focus of my studies was the PnO.

The arousal-related activity of the PnO is regulated by the interplay of multiple neurotransmitter systems and brain regions. The PnO receives cholinergic input from the LDT/PPT (Mitani et al., 1988; Shiromani et al., 1988), serotonergic input from the dorsal raphe, nucleus prosupralemniscus (B9 cell group) (Semba, 1993), ventral mesopontine tegmentum, and dorsal mesopontine tegmentum (Rodrigo-Angulo et al., 2000), noradrenergic input from the locus coeruleus and lateral tegmentum (Semba, 1993), histaminergic input from the tuberomammillary nucleus (Semba, 1993), and hypocretinergic input from the lateral hypothalamus (Peyron et al., 1998). The PnO also receives GABAergic projections from the zona incerta (Border et al., 1986), caudal/dorsal hypothalamus (Rodrigo-Angulo et al., 2008), posterior hypothalamus (De La Roza et al., 2004), reticular thalamic nucleus (Reinoso-Suarez et al., 1994), and contralateral PnO (Liang and Marks, 2009). Glutamate is a major signaling molecule in the PnO (Kaneko et al., 1989; Lai et al., 1999), and it has been suggested that one side of the PnO receives glutamatergic projections from the contralateral PnO (Lai et al., 1999).

Cholinergic transmission in the PnO

Multiple lines of evidence support a central role for ACh in cat, rat, and mouse PnO in generating REM sleep (Baghdoyan et al., 1984b; Baghdoyan et al., 1984a, 1987; Baghdoyan et al., 1989; Bourgin et al., 1995; Marks and Birabil, 1998; Coleman et al., 2004; Douglas et al., 2005; Marks and Birabil, 2007). ACh release in the pontine reticular formation is significantly higher during REM sleep than during wakefulness or NREM sleep (Kodama et al., 1990; Leonard and Lydic, 1995, 1997). ACh acts on two

major classes of receptors, nicotinic and muscarinic. There are five muscarinic receptor (mAChR) subtypes (M1, M2, M3, M4, and M5). M1, M3, and M5 receptors are coupled to excitatory Gq proteins whereas M2 and M4 receptors are coupled to inhibitory G-proteins. Receptor audioradiography studies *in vitro* show that M2 receptors represent the major mAChR subtype in the pontine reticular formation (Baghdoyan et al., 1994; Baghdoyan, 1997). Considerable evidence suggests that M2 receptors in the pontine reticular formation are critical for induction of REM sleep (Baghdoyan et al., 1994; Bourgin et al., 1995; Baghdoyan, 1997; Baghdoyan and Lydic, 1999). Cholinergic enhancement of REM sleep is dependent on M2 receptor inhibition of adenylate cyclase and PKA activities (Capece and Lydic, 1997; Marks and Birabil, 2000).

The PnO does not contain cholinergic neurons and receives cholinergic projections from the LDT/PPT (Mitani et al., 1988; Shiromani et al., 1988). Stimulation of the LDT/PPT increases REM sleep (Thakkar et al., 1996) and ACh release in the pontine reticular formation (Lydic and Baghdoyan, 1993). Although it is clear that ACh is important for generating REM sleep there are studies that show administration of high concentrations of cholinomimetics into the PnO increases wakefulness (Bourgin et al., 1995; Marks and Birabil, 2001). The mechanisms by which ACh in the pontine reticular formation increases wakefulness are unknown. Application of the nonspecific cholinergic agonist carbachol either depolarizes or hyperpolarizes PnO neurons (Greene and Carpenter, 1985; Nuñez et al., 1998; Brown et al., 2006; Brown et al., 2008), suggesting that carbachol activates mAChRs located on either GABAergic or non-GABAergic neurons. A recent study demonstrated that carbachol either inhibits or excites GABAergic neurons in the PnO (Brown et al., 2008). One possible explanation is

that GABAergic neurons in the PnO express either M2/M4 receptors or M1/M2/M5 receptors. Activation of M2/M4 receptors on GABAergic neurons in the PnO would result in depolarization whereas activation of M1/M2/M5 receptors on GABAergic neurons would result in hyperpolarization. Whether the ACh-induced increase in wakefulness or REM sleep is related to the differential firing patterns caused by ACh is unknown. These data suggest that ACh and GABA interact to modulate neuronal activity in the PnO, and thus sleep and wakefulness.

GABAergic transmission in the PnO

Studies using light (Mugnaini, 1985; Ford et al., 1995) and electron (Reinoso-Suarez et al., 2001; De La Roza et al., 2004; de la Roza and Reinoso-Suarez, 2006) microscopy demonstrate that neurons in the PnO of cat are immunopositive for GABA. Furthermore, it is estimated that 30% of all synapses in cat PnO are GABAergic (de la Roza and Reinoso-Suarez, 2006). Immunohistochemical studies indicate the presence of GABAergic interneurons in the pontine reticular formation of cat (Brodal et al., 1988; Aas, 1989; de la Roza and Reinoso-Suarez, 2009), primate (Brodal et al., 1988), and rat (Liang and Marks, 2009).

Systemically administered GABAmimetics are well known to elicit sleep or anesthesia. Delivery of a $GABA_A$ receptor agonist directly into the posterior hypothalamus (Lin et al., 1989) increases wakefulness. Increased wakefulness, however, is produced after administration of GABAergic drugs directly into the preoptic area/anterior hypothalamus (Lin et al., 1989). Therefore, GABAmimetics have opposite effects on sleep and wakefulness depending upon their site of action in the brain.

$GABA_A$ receptor signaling in the PnO promotes wakefulness (Camacho-Arroyo et al., 1991; Xi et al., 1999) and suppresses REM sleep (Xi et al., 1999; Sanford et al., 2003; Vazquez and Baghdoyan, 2004; Marks et al., 2008). Increasing or decreasing endogenous GABA levels in the PnO causes an increase or decrease, respectively, in wakefulness (Watson et al., 2008b). Furthermore, GABA levels in the PnO are lower during isoflurane anesthesia than during wakefulness (Vanini et al., 2008).

Intracellular recordings show that rat pontine neurons exhibit inhibitory postsynaptic potentials mediated by $GABA_A$ receptors (Nuñez et al., 1998). Consistent with the finding that GABAergic neurons in the PnO express M2 receptors (Brischoux et al., 2008) are data showing that carbachol either inhibits or excites GABAergic neurons in the PnO (Brown et al., 2008). Further support for the concept that GABA and ACh interact in the PnO is evidence demonstrating that $GABA_A$ receptors in the PnO inhibit ACh release (Vazquez and Baghdoyan, 2004). A recent study demonstrated that the REM sleep enhancement caused by administration of the $GABA_A$ receptor antagonist, gabazine, to the PnO, is dependent on cholinergic transmission (Marks et al., 2008). Taken together, these data suggest that $GABA_A$ receptor signaling in the PnO alters states of sleep and wakefulness, in part, by modulating ACh release. However, the PnO is composed of a heterogeneous population of neuronal types and it is likely that GABA interacts with other neurotransmitters to modulate states of sleep and wakefulness.

Hypocretinergic transmission in the PnO

The first study to investigate the effect of hypocretin in the PnO in modulating sleep and wakefulness was done in 2001. Microinjection of hcrt-r2 antisense into the pontine reticular formation increased REM sleep, suggesting that hypocretins target the

pontine reticular formation to control REM sleep by activating hcrt-r2 (Thakkar et al., 2001). It was later shown that administration of hypocretin-1 into the PnO caused an increase in REM sleep (Xi et al., 2002). However, further studies revealed that the effect of hypocretin-1 in the PnO on sleep and wakefulness is dependent on the behavioral state of the animal at the time of administration. Microinjection of hypocretin-1 during wakefulness in cat (Moreno-Balandrán et al., 2008; Xi and Chase, 2009) and rat (Watson et al., 2008b) caused an increase in wakefulness, whereas microinjection of hypocretin-1 during NREM sleep increased REM sleep (Xi et al., 2002). Administration of hypocretin-1 during NREM sleep does not always cause an increase in REM sleep. For example, microinjection of hypocretin-1 into the LDT or the locus coeruleus during NREM sleep caused an increase in wakefulness (Xi et al., 2001; Xi et al., 2002). Whether microinjection of hypocretin-1 into the LDT or the locus coeruleus during wakefulness will cause an increase in REM sleep is yet to be tested.

The mechanism by which hypocretin-1 in the PnO promotes either wakefulness or REM sleep is not clear. The increase in REM sleep caused by microinjection of hypocretin-1 into the PnO during NREM sleep is dependent on protein kinase C (PKC) activation (Xi and Chase, 2006). Whether PKC activation is also important for the increase in wakefulness caused by hypocretin-1 during wakefulness is unknown. The increase in wakefulness or REM sleep caused by hypocretin-1 is likely to involve the orchestration and coordination of multiple neurotransmitters in specific target neurons.

The PnO expresses hcrt-r1 and hcrt-2 (Greco and Shiromani, 2001) and *in vitro* [^{35}S] guanylyl 5′-(γ-thio) triphosphate ([^{35}S] GTPγS) autoradiography demonstrates that both receptor subtypes are functional in the PnO (Bernard et al., 2003; Bernard et al.,

2006). Furthermore, autoradiography with [^{35}S] GTPγS suggests that at least some hypocretin receptors in the PnO couple to inhibitory G proteins (Bernard et al., 2003; Bernard et al., 2006). Recent immunohistochemical findings show that hypocretin receptors are present on GABAergic neurons in the PnO (Brischoux et al., 2008). Consistent with these findings is the observation that application of hypocretin-1 excites GABAergic neurons in the PnO (Nuñez et al., 2006; Brown et al., 2008). Moreover, delivery of hypocretin-1 to the PnO via microdialysis causes an increase in GABA levels in the PnO (Watson et al., 2008b). Together, these findings demonstrate that hypocretin-1 and GABA interact in the PnO. Whether or not the increase in wakefulness caused by hypocretin-1 (Moreno-Balandrán et al., 2008; Watson et al., 2008b; Xi and Chase, 2009) is dependent on GABAergic transmission is unknown. In addition to GABA, hypocretin-1 interacts with ACh in the PnO. Microdialysis delivery of hypocretin-1 to the PnO increases ACh release in the PnO (Bernard et al., 2003, 2006) by unknown mechanisms.

Glutamatergic transmission in the PnO

Very little is known about glutamatergic regulation of sleep and wakefulness, and few studies have investigated the sleep-related effects of glutamate in the PnO. Immunohistochemical evidence demonstrates that approximately 50% of the pontine reticular formation neurons in cat show glutamate-like immunoreactivity (Lai et al., 1999). Electrophysiological studies demonstrate that glutamate excites pontine reticular formation neurons (Greene and Carpenter, 1985; Nuñez et al., 1998) by activating α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA), kainate, or N-methyl-D-aspartic acid (NMDA) receptors (Stevens et al., 1992).

RESEARCH QUESTION

The goal of my thesis research was to elucidate the mechanisms by which hypocretin-1 administered into the PnO causes an increase in wakefulness. Specific Aims 1 and 2 tested the hypothesis that hypocretin receptors and $GABA_A$ receptors in the PnO mediate the increase in wakefulness induced by hypocretin-1. Specific Aim 3 tested the hypothesis that ACh release in the PnO is modulated by glutamate.

Specific Aim 1 demonstrated that microinjection of hypocretin-1 into the PnO causes a concentration-dependent increase in wakefulness.

Several lines of evidence indicate that hypocretin-1 in the PnO is wakefulness promoting. However, few studies have addressed whether the increase in wakefulness is receptor-mediated. Both hcrt-r1 and hcrt-r2 are localized in the PnO, and hypocretin-1-induced [^{35}S]GTPγS binding is receptor-mediated (Bernard et al., 2003, 2006). Whether the increase in wakefulness caused by hypocretin-1 is concentration-dependent was unknown prior to my studies. Increasing concentrations of hypocretin-1 were administered to the PnO of rat and states of sleep and wakefulness were recorded. Chapter 2 reports the results from these experiments.

This thesis research is the first to show that microinjection of hypocretin-1 into the PnO causes a concentration-dependent increase in wakefulness (Brevig et al., 2010). The increase in wakefulness was caused by decreasing the number and increasing the duration of wakefulness episodes. Microinjection of hypocretin-1 into the PnO caused a concentration-dependent decrease in NREM sleep by decreasing the number and the duration of NREM sleep episodes. As expected, hypocretin-1 produced a concentration-

dependent decrease in REM sleep that was caused by decreasing the number of REM sleep episodes. Lastly, hypocretin-1 decreased in the number of state transitions in a concentration-dependent manner. Results from Aim 1 support the interpretation that the increase in wakefulness caused by hypocretin-1 is receptor-mediated.

Specific Aim 2 determined that the hypocretin-1-induced increase in wakefulness is blocked by the hypocretin receptor-1 antagonist SB-334867 and the $GABA_A$ receptor antagonist bicuculline administered to the PnO.

The results from Aim 1 suggested that the increase in wakefulness caused by hypocretin-1 is receptor-mediated. Aim 2 further determined whether the hypocretin-1-induced increase in wakefulness is mediated by hypocretin receptors. The results are presented in Chapter 2. The hcrt-r1 antagonist SB-334867 was co-administered with hypocretin-1 into the PnO and states of sleep and wakefulness were recorded. The results from Aim 2 demonstrate that the increase in wakefulness was blocked by the hcrt-r1 antagonist SB-334867 (Brevig et al., 2010). Overall, these findings and those from Aim 1 support the conclusion that activation of hypocretin receptors in the PnO increases wakefulness. Future studies to determine whether microinjection of SB-34867 into the PnO causes a concentration-dependent decrease in wakefulness and increase in sleep will be important for determining whether endogenous hypocretin-1 in the PnO functions to promote wakefulness.

The second hypothesis tested in Aim 2 was whether activation of $GABA_A$ receptors is important for mediating increase in wakefulness produced by hypocretin-1. In these studies hypocretin-1 and the $GABA_A$ receptor antagonist bicuculline were co-administered into the PnO. Bicuculline blocked the hypocretin-1-induced increase in

wakefulness, demonstrating that GABAergic transmission in the PnO is at least partly responsible for mediating the hypocretin-1-induced increase in wakefulness. These results are presented in Chapter 2. This is the first study to determine that GABAergic transmission is necessary for hypocretin-1-induced increase in wakefulness.

Specific Aim 3 demonstrated that administration of glutamate into the PnO modulates ACh release in the PnO

The results presented in Aim 3 provide evidence that glutamate modulates ACh release in the PnO. One possible mechanism by which glutamate alters sleep-wake states is by modulating ACh release in the PnO. *In vivo* microdialysis and high performance liquid chromatography coupled with electrochemical detection were used in Chapter 3 studies to detect and quantify ACh. ACh release during dialysis with Ringer's (control) was compared to ACh release during dialysis with glutamate. Administration of glutamate to the PnO decreased ACh release in the PnO. The results from Chapter 3 were used to make implications about the neural circuitry in the PnO that causes the hypocretin-1-induced increase in ACh release. Proposed synaptic models based on results presented in Chapter 3 are discussed in Chapter 4.

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CHAPTER 2

WAKEFULNESS IS INCREASED BY $GABA_A$-HYPOCRETIN RECEPTOR INTERACTION IN THE PONTINE RETICULAR FORMATION

SUMMARY

Hypocretin-1/orexin A administered directly into the oral part of rat pontine reticular formation (PnO) causes an increase in wakefulness and extracellular GABA levels. The receptors in the PnO that mediate these effects have not been identified. Therefore, this study tested the hypothesis that the increase in wakefulness caused by PnO administration of hypocretin-1 occurs via activation of $GABA_A$ receptors and hypocretin receptors.

Hypocretin-1 caused a significant, concentration dependent increase in wakefulness and decrease in rapid eye movement (REM) sleep and non-REM (NREM) sleep. Co-administration of SB-334867 and hypocretin-1 blocked the hypocretin-1-induced increase in wakefulness and decrease in both the NREM and REM phases of sleep. Co-administration of bicuculline and hypocretin-1 blocked the hypocretin-1-induced increase in wakefulness and decrease in NREM sleep caused by hypocretin-1. The increase in wakefulness caused by administering hypocretin-1 to the PnO is mediated by hypocretin receptors and $GABA_A$ receptors in the PnO. These results show for the first time that hypocretinergic and GABAergic transmission in the PnO can interact to promote wakefulness.

INTRODUCTION

The neuropeptides hypocretin-1 and hypocretin-2 (orexin A and orexin B) are synthesized exclusively by neurons in the lateral hypothalamic area (de Lecea et al., 1998; Peyron et al., 1998; Sakurai et al., 1998). Hypocretin deficiency in humans underlies the pathophysiology of narcolepsy, (Peyron et al., 2000; Thannickal et al., 2000) and disruption of hypocretin signaling in mouse (Chemelli et al., 1999), rat (Gerashchenko et al., 2001; Beuckmann et al., 2004), and dog (Lin et al., 1999) leads to narcolepsy-cataplexy. Hypocretinergic neurons project to multiple areas of the brain, including those important for regulating sleep and wakefulness (Peyron et al., 1998). One such area is the pontine reticular nucleus, oral part (PnO) (Peyron et al., 1998; Nuñez et al., 2006). The PnO is the rostral portion of the rodent pontine reticular formation (Paxinos and Watson, 2007) and contributes to the generation of wakefulness and rapid eye movement (REM) sleep (Lydic and Baghdoyan, 2005). Microinjection of hypocretin-1 into rat PnO causes an increase in wakefulness (Watson et al., 2008), and microinjection of hypocretin-1 into cat pontine reticular formation increases the cortically activated states of REM sleep (Xi et al., 2002b) or wakefulness (Moreno-Balandrán et al., 2008; Xi and Chase, 2009).

Administering hypocretin-1 into the PnO may increase wakefulness by modulating the release of arousal-promoting neurotransmitters within the PnO. Direct administration of hypocretin-1 to the PnO of isoflurane-anesthetized rat causes a concentration-dependent increase in both acetylcholine (ACh) release (Bernard et al., 2006) and GABA levels (Watson et al., 2008) within the PnO. Extracellular recording studies of PnO neurons in urethane-anesthetized rat show that iontophoretic application

of hypocretin-1 causes a hyperpolarization that is blocked by prior application of bicuculline (Nuñez et al., 2006). This finding indicates that the hypocretin-1–induced inhibition of PnO neurons is mediated by $GABA_A$ receptors. Identified GABAergic neurons in brainstem slices of mouse PnO have been shown to be excited by hypocretin-1 (Brown et al., 2008), and intracellular recording studies in halothane-anesthetized cat show that hypocretin-1 can also cause direct depolarization of PnO neurons and an increase in PnO neuronal firing rate (Xi et al., 2002b). Numerous studies have demonstrated that GABAergic transmission in the PnO increases wakefulness and inhibits REM sleep (Camacho-Arroyo et al., 1991; Xi et al., 1999; Sanford et al., 2003; Chang et al., 2006; Flint et al., 2007; Marks et al., 2008; Vanini et al., 2008; Watson et al., 2008). The present study provides the first test of the hypothesis that the wakefulness-promoting effects of delivering hypocretin-1 into the PnO are mediated by $GABA_A$ receptors as well as by hypocretin receptors. This hypothesis was evaluated by determining whether (1) microinjection of hypocretin-1 into the PnO causes a concentration-dependent increase in wakefulness, (2) this increase in wakefulness is blocked by coadministration of the hypocretin receptor-1 (hcrt-r1) antagonist SB-334867, and (3) coadministration of the $GABA_A$ receptor antagonist bicuculline also blocks the wakefulness response to hypocretin-1. Portions of these data have been presented as abstracts (Brevig et al., 2008, 2009).

MATERIALS AND METHODS

Chemicals and Drug Solutions

Human hypocretin-1 was purchased from California Peptide Research, Inc. (Napa, CA). Bicuculline methiodide was purchased from Sigma Aldrich (St. Louis, MO) and N-(2-Methyl-6-benzoxazolyl)-N″-1,5-naphthyridin-4-yl urea (SB-334867) was obtained from Tocris Bioscience (Ellisville, MO). Chemicals for Ringer solution (147.0 mM NaCl, 2.4 mM $CaCl_2$, 4.0 mM KCl, 1.0 mM $MgSO_4$, pH 6.0) were acquired from Fisher Scientific (Pittsburgh, PA). All drugs used for the antagonist-blocking studies were dissolved in Ringer solution containing 2% dimethyl sulfoxide, which was purchased from Sigma Aldrich. Drug solutions used for intracranial microinjections were made immediately before use.

Animals, Surgery, and Conditioning of Behavior

Animal experiments were approved by the University of Michigan Committee on Use and Care of Animals and performed in accordance with the US Public Health Service Policy on Humane Care and Use of Laboratory Animals (National Institutes of Health Publication 80-23). Adult (235- to 310-g) male Crl:CD*(SD) (Sprague Dawley) rats (n = 23; Charles River Laboratories, Wilmington, MA) were housed with unlimited access to food and water and kept on a 12-hour light/dark cycle (lights on at 06:00).

Procedures for surgical implantation of recording electrodes and a microinjection guide tube have been described in detail.(Watson et al., 2007; Watson et al., 2008) Briefly, rats were anesthetized with isoflurane (Abbott Laboratories, North Chicago, IL) and implanted with 3 screw electrodes (8IE36320SPCE, Plastics One, Roanoke, VA) for recording the cortical electroencephalogram (EEG). Three pair of EEG electrodes were

placed using the following stereotaxic coordinates relative to bregma: 1.0 mm anterior and 1.5 mm lateral, 2.0 mm posterior and 1.5 mm lateral, and 2.0 mm posterior and 1.27 mm lateral. Two electrodes for recording the electromyogram (EMG) were implanted bilaterally in the dorsal neck muscles. EMG electrodes were assembled from AS632 biomed wire (6 cm length; Cooner Wire Company, Chatsworth, CA) and electrical gold socket contacts (Plastics One, 8IE3630XXXXE). A guide cannula (8IC315GSPCXC, Plastics One) containing a stylet (8IC315DCXXXC, Plastics One) was aimed 3 mm above the left PnO at 8.40 mm posterior to bregma, 1.0 mm lateral to the midline, and 6.2 mm below the skull surface (Paxinos and Watson, 2007). Electrode leads were routed into a 6-pin electrode pedestal (MS363, Plastics One). Dental acrylic (Lang Dental Manufacturing Company, Inc., Wheeling, IL) and 3 anchor screws (MPX-0080-02PC-C, Small Parts, Inc., Miami Lakes, FL) were used to adhere the guide cannula, electrodes, and pedestal to the skull.

Rats recovered from surgery for a minimum of 7 days, during which time they were conditioned to being handled for microinjections and to being tethered (363-441/6, Plastics One) in a recording chamber (Raturn; Bioanalytical Systems, West Lafayette, IN). To determine whether rats were adequately conditioned, a mock microinjection was performed by inserting and removing a microinjector (8IC315IXXXXC, Plastics One). EEG and EMG signals then were recorded for 2 hours. Rats were considered ready to enter the microinjection protocol when the latency to non-REM (NREM) sleep was less than 30 minutes.

Microinjections and Quantification of Arousal States

The day before each microinjection, rats were placed in the recording chambers and tethered overnight. All microinjections were made between 09:30 and 10:30, and

microinjections into the same rat were separated by a minimum of 7 days. Microinjection volume (100 nL) and duration (1 min) were held constant for all drugs, and 2-hour electrophysiologic recordings were started immediately after injection. Two groups of rats were used for this study. The first group (n = 14) received 0, 0.1, 1, 10, and 100 pmol hypocretin-1 (0, 0.36, 3.56, 35.6, and 356 ng, respectively). The order in which the different concentrations of hypocretin-1 were administered was randomized, and not all animals received all concentrations. The second group of rats (n = 9) was microinjected with Ringer solution containing 2% dimethyl sulfoxide (vehicle control), hypocretin-1 (10 pmol), bicuculline (0.2 pmol; 0.1 ng) in combination with hypocretin-1 (10 pmol), or SB-334867 (10 pmol; 3.4 ng) in combination with hypocretin-1 (10 pmol). All rats in the second group received all 4 drug treatments.

EEG and EMG recordings were scored manually in 10-second bins as wakefulness, NREM sleep, or REM sleep using Icelus Acquisition software (Opp, 1998). Previously described methods were used for amplification, digitization, and fast Fourier transform (FFT) analysis of signals (Watson et al., 2007; Watson et al., 2008). Briefly, FFT plots were constructed by analyzing EEG signals in 0.5-Hz increments every 2 seconds for frequencies ranging from 0.5 to 25.0 Hz. Five consecutive 2-second bins were averaged to produce 1 FFT for each 10-second epoch. Sample bins of 10 seconds in duration were averaged over five 1-minute intervals in the first hour following microinjection of Ringer solution and hypocretin-1 (100 pmol/100 nL). Sleep records were scored by 2 investigators, 1 of which was blinded to the treatment condition. Agreement between the 2 scores of greater than 90% was achieved for all records. Dependent measures included the percentage of time spent in wakefulness, NREM sleep,

and REM sleep; the average duration of the longest wakefulness episode from each recording; the average duration of NREM sleep and REM sleep episodes; the number of wakefulness, NREM sleep, and REM sleep episodes; the number of transitions; and the latency to onset of the first episode of NREM sleep and REM sleep.

Histologic Verification of Microinjection Sites

Rats were deeply anesthetized with isoflurane (5%) and decapitated. Brains were immediately removed, blocked, and frozen. The brainstem block was sectioned coronally from caudal to rostral using a cryostat (Leica Microsystems, Nussloch, Germany). Serial sections (40 microns thick) were slide mounted, dried, fixed in paraformaldehyde vapor (80°C), and stained with cresyl violet. Stained tissue sections and a 1-mm calibration bar were digitized using a Super Nikon Coolscan 4000 ED Film Scanner (Nikon Inc, Melville, NY). Microinjection sites were identified and assigned stereotaxic coordinates by comparison with a rat brain atlas (Paxinos and Watson, 2007).

Statistical Analyses

Data sets for all dependent measures were normally distributed. Therefore, data were evaluated by parametric tests and are reported as mean ± SEM. For the concentration-response study, the effects of hypocretin-1 on sleep and wakefulness were determined using a linear mixed model for a randomized incomplete block design. Concentration response data were fit to the equation Y = B+(T-B)/(1+10^((LogEC50-X)×HillSlope)), where B represents the lower limit for the dependent variable (eg, percentage of wakefulness), T is the upper limit for the dependent variable, X is the logarithm of the hypocretin-1 concentration, and Y is the dependent variable. Regression analyses were used to obtain the coefficient of determination (r^2) and calculate the percentage of the response accounted for by the concentration of hypocretin-1 ($r^2 \times 100$).

For the antagonist-blocking study, drug effects on sleep and wakefulness were determined by 1-way analysis of variance (ANOVA) for repeated measures and Dunnett posthoc multiple comparisons test. Because there were so few REM sleep episodes in the first hour after injection, a Poisson regression with a Generalized Estimating Equations model was used to test for drug effects on the number of REM episodes. This approach takes into account the correlation among repeated observations from the same rat. Z-tests were used to determine significant effects, and Bonferroni correction was used for multiple comparisons between drug treatments. Statistical programs used included SAS (release 9.1.3, SAS Institute, Cary, NC) and GraphPad Prism v4.0c for Macintosh. Statistical significance required a p value ≤ 0.05.

RESULTS

Microinjection of hypocretin-1 into the PnO caused a concentration-dependent increase in wakefulness and decrease in sleep

To determine whether the hypocretin-1–induced increase in wakefulness was mediated by hypocretin receptors, increasing concentrations of hypocretin-1 were microinjected into the PnO and the effects on sleep and wakefulness were quantified. Figure 2.1 shows that all microinjection sites used for the concentration-response study were localized to the PnO. The average stereotaxic coordinates (Paxinos and Watson, 2007) for these injection sites were 7.9 ± 0.1 mm posterior to bregma, 8.6 ± 0.1 mm ventral to the top of the skull, and 1.1 ± 0.1 mm from the midline. Figure 2.2 illustrates the sequence of sleep and wakefulness from 2 representative rats in the first hour after PnO microinjection of 2 concentrations of hypocretin-1 relative to control (microinjection of Ringer solution). Hypocretin-1 increased the amount of time spent in wakefulness, decreased NREM sleep, and abolished REM sleep.

Figure 2.3 summarizes the group data for the first hour after injection. ANOVA revealed a significant concentration main effect of hypocretin-1 on the percentage of time spent in wakefulness (Figure 2.3A; $F_{4,32} = 6.51$; $p = 0.0006$), NREM sleep (Figure 2.3B; $F_{4,32} = 6.37$; $p = 0.0007$), and REM sleep (Figure 2.3C; $F_{4,32} = 4.68$; 32; $p = 0.004$) and on the number of episodes of wakefulness (Figure 2.3D; $F_{4,32} = 3.33$; $p = 0.02$) and NREM sleep (Figure 2.3E; $F_{4,32} = 3.38$; $p = 0.02$). The effect of hypocretin-1 on the number of REM sleep episodes approached significance (Figure 2.3F; $z = 9.04$, $p = 0.06$). There was a significant concentration main effect on the duration of the longest wakefulness episode (Figure 2.3G; $F_{4,32} = 6.85$; $p = 0.0004$) and on the average duration of NREM sleep

episodes (Figure 2.3H; $F_{4,30} = 2.75$; $p = 0.046$). There was no significant change in the average duration of REM-sleep episodes (Figure 2.3I). Microinjection of hypocretin-1 into the PnO caused a significant concentration-dependent effect on the latency to onset of NREM sleep (Figure 2.3J; $F_{4,32} = 29.81$; $p < 0.0001$) and REM sleep (Figure 2.3K; $F_{4,21} = 2.97$; $p = 0.043$). Consistent with these effects, the number of state transitions varied significantly as a function of hypocretin-1 concentration (Figure 2.3L; $F_{4,32} = 3.38$; $p = 0.02$). All dependent measures except for the percentage of time spent in REM sleep returned to control levels in the second hour after injection (data not plotted). REM sleep remained decreased ($F_{4,32} = 2.75$; $p = 0.048$) following microinjection of 100 pmol of hypocretin ($p < 0.01$). FFT analysis of EEG signals recorded from 8 rats during the first hour after microinjection showed that, compared with an injection of Ringer solution, hypocretin-1 (100 pmol/100 nL) did not alter EEG power at any frequency.

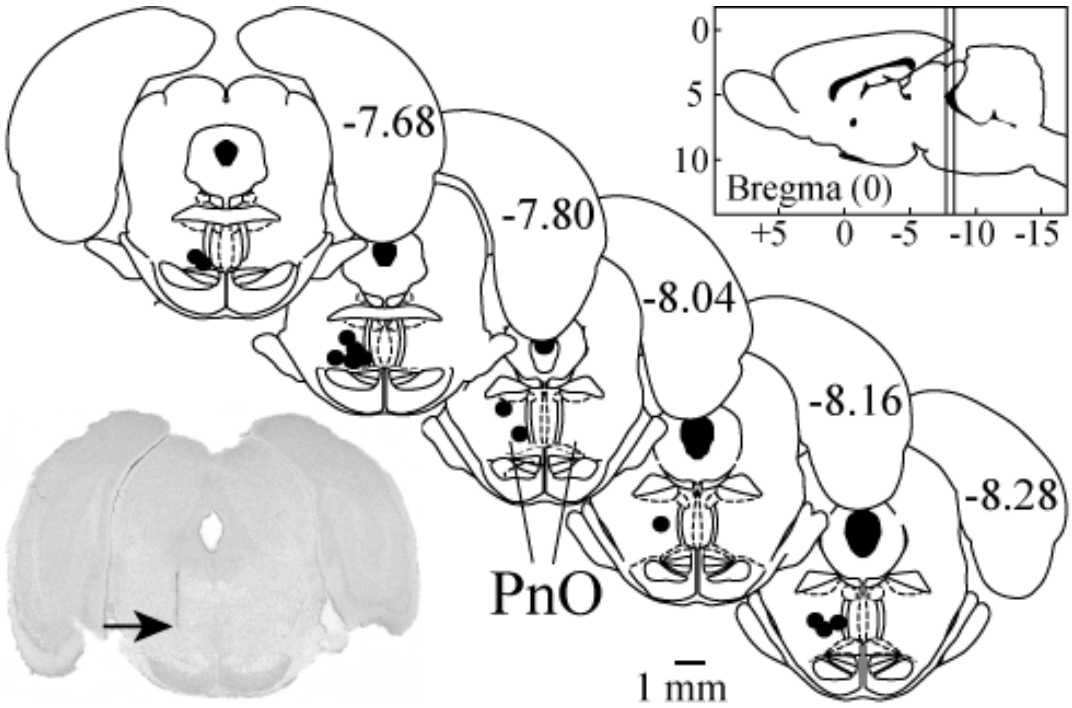

Figure 2.1. All concentrations of hypocretin-1 were injected into the pontine reticular nucleus, oral part (PnO). Microinjection sites (n=14) from the concentration response experiments are represented as black dots on five coronal atlas plates (modified from Paxinos and Watson, 2007). Numbers on the right side of each plate indicate mm posterior to bregma. The sagittal drawing of the rat brain (upper right) contains vertical lines that designate the anterior to posterior range of the microinjection sites, which spanned from 7.6 to 8.3 mm posterior to bregma. The digitized image of a cresyl violet stained section from one rat shows a typical microinjection site (arrow) in the PnO.

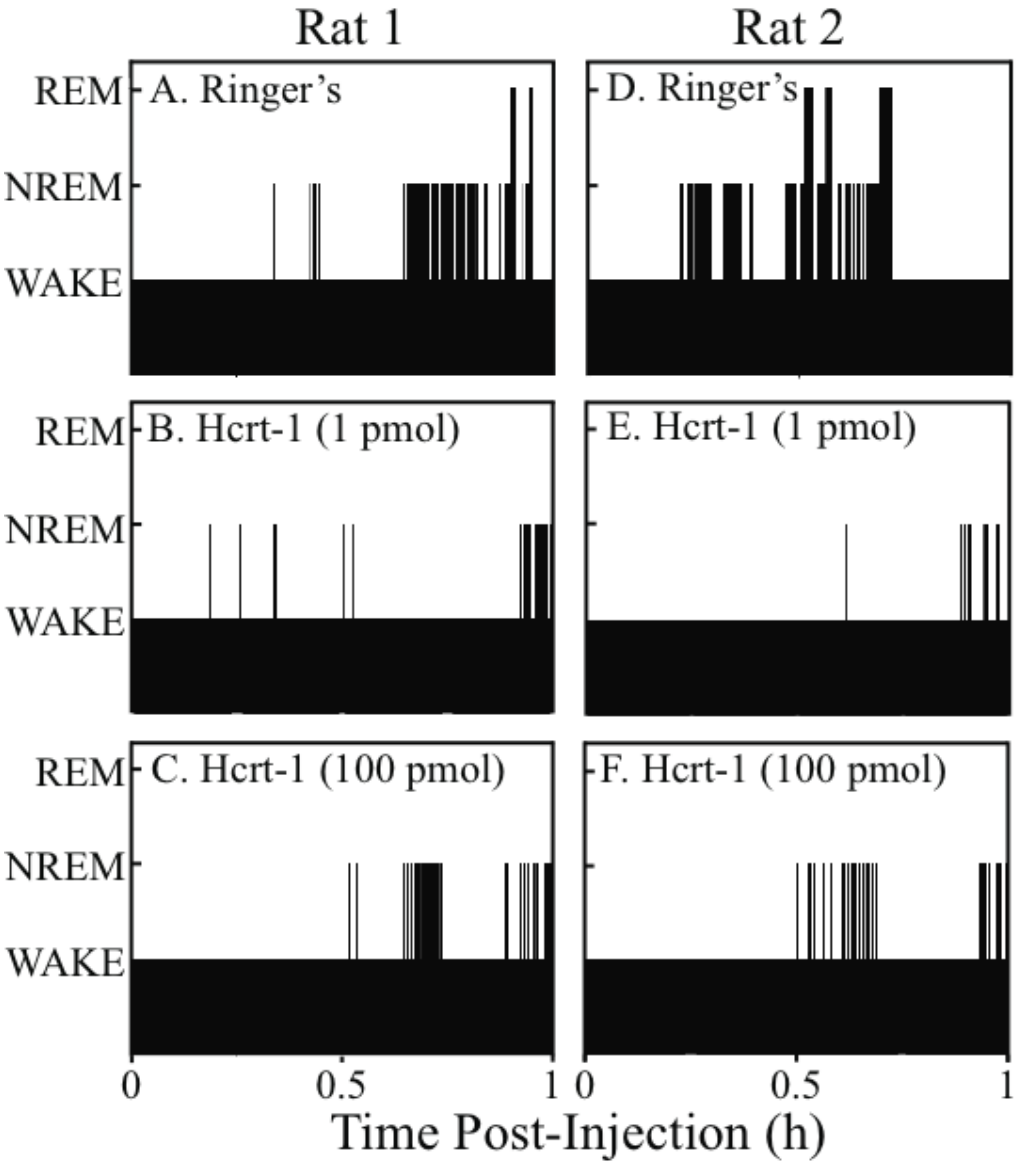

Figure 2.2. Representative time-course plots of wakefulness and sleep are shown for the first hour after microinjection of Ringer solution (vehicle control) or hypocretin-1 (Hcrt-1) into the pontine reticular nucleus, oral part (PnO). The height of the bars indicates the sequence of wakefulness (lowest bars), non-rapid eye movement sleep (NREM) (intermediate bars), and rapid eye movement sleep (REM) (highest bars). Each column shows data from 1 rat.

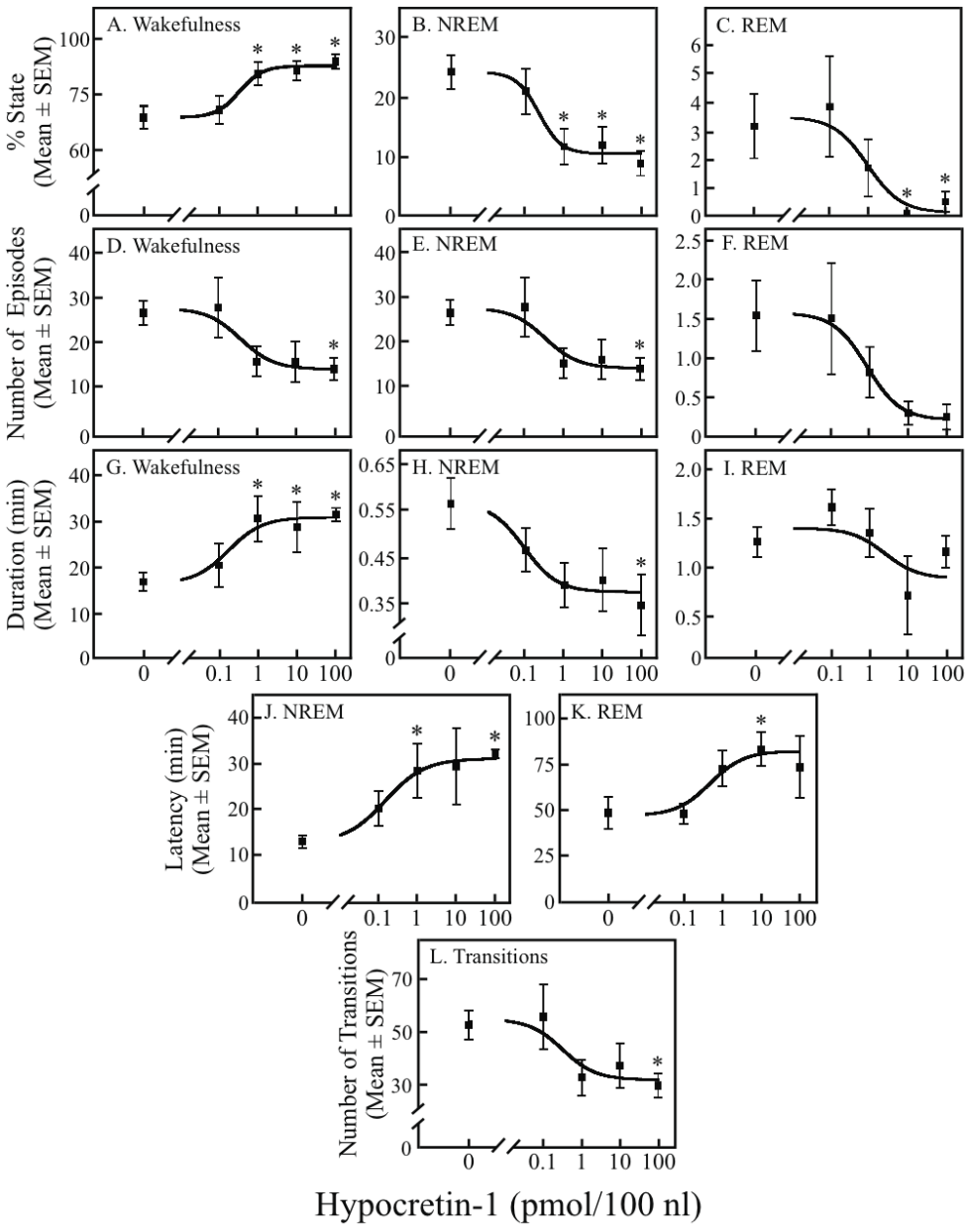

Figure 2.3. Significant concentration-dependent changes in sleep and wakefulness were caused by microinjection of hypocretin-1 into the pontine reticular nucleus, oral part (PnO). Data from 14 rats are plotted for the first hour after injection. Dunnett multiple comparisons tests indicated significant (* $p \leq 0.05$) differences from control (0 pmol/100 nL). For all data sets except rapid eye movement sleep (REM) duration (I), coefficients of determination (r^2) indicated the percentage of the variability accounted for by the concentration of hypocretin-1 was: (A) 98%; (B) 97%; (C) 93%; (D) 91%; (E) 90%; (F) 99%; (G) 95%; (H) 94%; (J) 90%; (K) 93%; (L) 84%. NREM refers to non-rapid eye movement sleep.

Both the hypocretin receptor-1 antagonist SB-334867 and the $GABA_A$ receptor antagonist bicuculline blocked the hypocretin-1–induced increase in wakefulness

To further investigate whether the hypocretin-1–induced increase in wakefulness was mediated by hypocretin receptors, sleep and wakefulness were quantified after coadministering SB-334867 and hypocretin-1. This study also coadministered bicuculline and hypocretin-1 to determine whether GABAergic transmission in the PnO contributed to the hypocretin-1–induced increase in wakefulness. Figure 2.4 documents that all microinjection sites used for the blocking studies were localized to the PnO. The mean stereotaxic coordinates were 7.9 ± 0.1 mm posterior to bregma, 8.9 ± 0.1 mm ventral to the top of the skull, and 1.0 ± 0.1 from the midline. There was no significant difference between the stereotaxic coordinates of injection sites used for the concentration-response study (Figure 2.1) and the receptor-antagonist study (Figure 2.4).

Figure 2.5 plots the time course of wakefulness and sleep recorded from 2 representative rats during the first hour after microinjection of Ringer solution (Figure 2.5A and 2.5E), hypocretin-1 (10 pmol) (Figure 2.5B and 2.5F), SB-334867 (10 pmol) + hypocretin-1 (10 pmol) (Figure 2.5C and 2.5G), and bicuculline (0.2 pmol) + hypocretin-1 (10 pmol) (Figure 2.5D and 2.5H). The hypocretin-1–induced increase in wakefulness and decrease in NREM sleep (Figure 2.5B and 2.5F) were blocked by coadministration of SB-334867 (Figure 2.5C and 2.5G) and by coadministration of bicuculline (Figure 2.5D and 2.5H). SB-334867 also antagonized the decrease in REM sleep (Figure 2.5C and 2.5G), whereas bicuculline did not block the REM-sleep inhibition caused by hypocretin-1 (Figure 2.5D and 2.5H).

The group data are summarized in Figure 2.6. ANOVA revealed that, in the first hour after injection, the percentage of time spent in wakefulness (Figure 2.6A, $F_{3,24} = 5.44$; $p = 0.005$) and NREM sleep (Figure 2.6B, $F_{3,24} = 4.44$; $p = 0.01$) varied as a function of drug treatment. The hypocretin-1–induced increase in the percentage of time spent in wakefulness was blocked by coadministration of either SB-334867 or bicuculline (Figure 2.6A). The decrease in NREM sleep time was reversed by SB-334867 and by bicuculline (Figure 2.6B). A repeated-measures ANOVA adjusted for unequal variances revealed that the percentage of time spent in REM sleep varied significantly as a function of drug treatment (Figure 2.6C, $F_{3,24} = 6.03$; $p = 0.003$). The hypocretin-1–induced decrease in REM sleep was partially reversed by SB-334867 but was not blocked by bicuculline (Figure 2.6C).

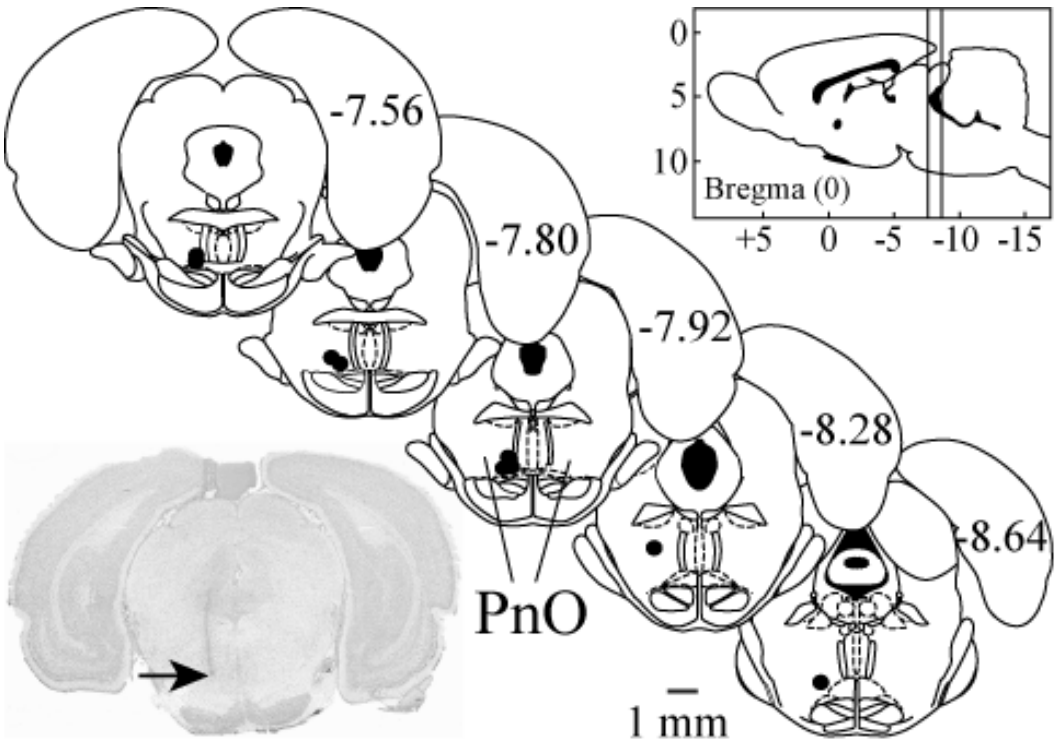

Figure 2.4. SB-334867 and bicuculline were injected into the pontine reticular nucleus, oral part (PnO). Microinjection sites (n = 9) from the antagonist-blocking studies are represented as black dots on 5 coronal atlas plates (modified from Paxinos and Watson, 2007). Numbers on the right side of each plate specify mm posterior to bregma. The vertical lines in the sagittal drawing (upper right) indicate that the microinjection sites spanned from 7.5 to 8.6 mm posterior to bregma. One representative microinjection site (arrow) in the PnO is shown in the tissue section at lower left.

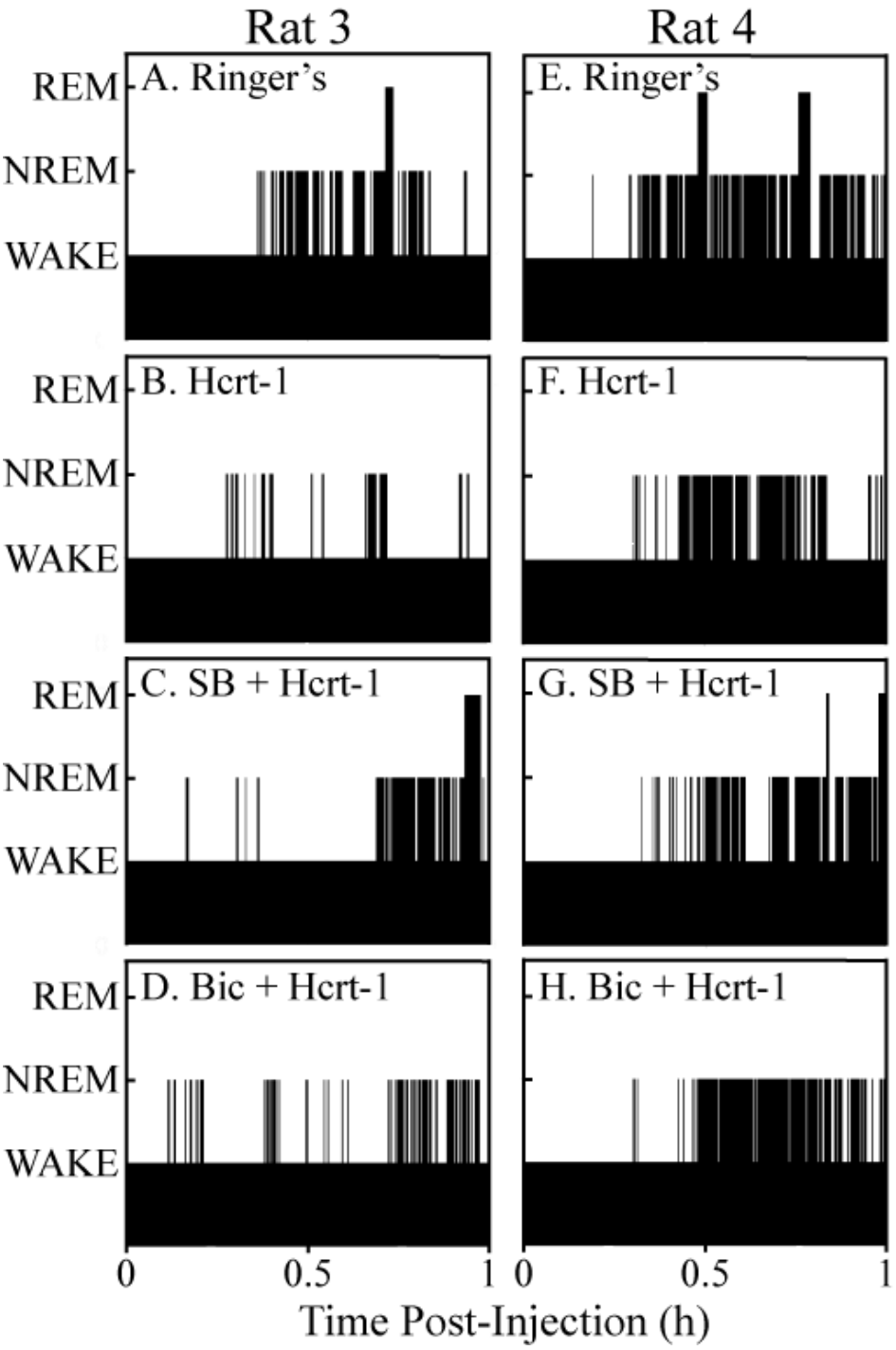

Figure 2.5. Temporal organization of wakefulness and sleep in the first hour after microinjection of Ringer solution (A and E), 10 pmol of hypocretin-1 (B and F), 10 pmol of SB-334867 (SB) + 10 pmol of hypocretin-1 (Hcrt-1) (C and G), and 0.2 pmol of bicuculline (Bic) + 10 pmol of Hcrt-1 (D and H) into the pontine reticular nucleus, oral part (PnO). Each column shows data from 1 rat. Bar height indicates wakefulness (lowest bars), non-rapid eye movement sleep (NREM) (intermediate bars), and rapid eye movement sleep (REM) (highest bars).

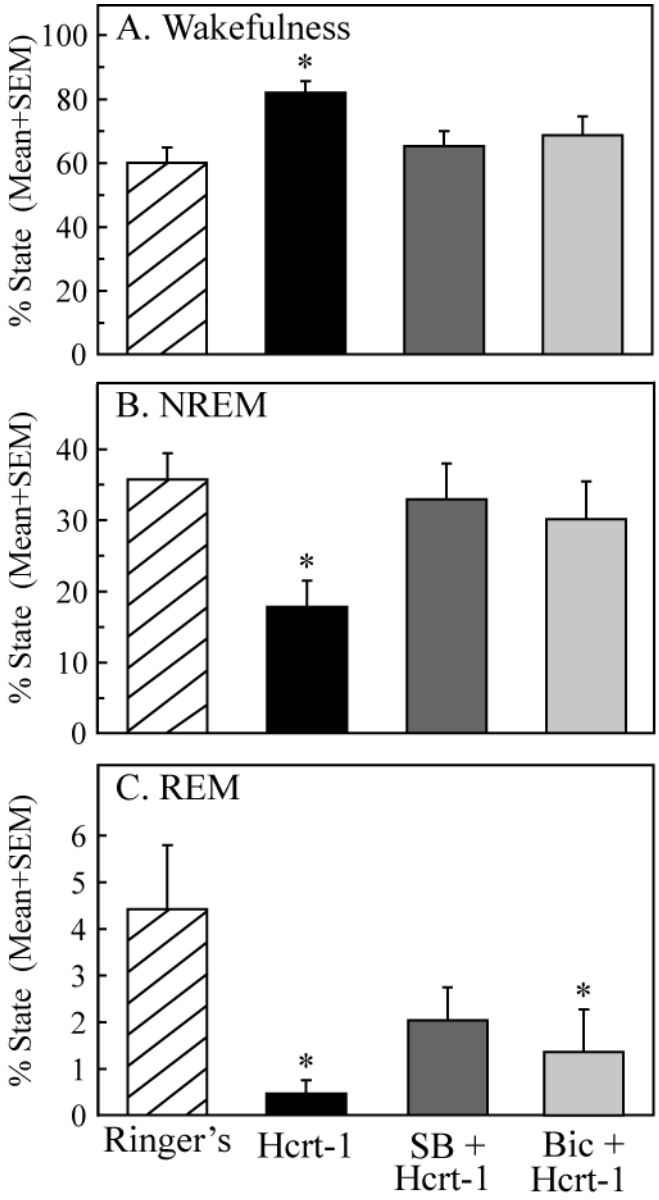

Figure 2.6. Coadministration of SB-334867 (SB) or bicuculline (Bic) blocked the increase in wakefulness and decrease in non-rapid eye movement (NREM) sleep caused by hypocretin-1 (Hcrt-1). Data represent measures from 9 rats during the first hour after injection. Dunnett multiple comparisons tests indicated significant (*$p \leq 0.05$) differences from control (microinjection of Ringer solution).

DISCUSSION

The present study reports, for the first time, that microinjection of hypocretin-1 into the PnO of awake rat caused a concentration-dependent increase in wakefulness that was blocked by coadministration of a hcrt-r1 antagonist. These findings demonstrate that the PnO is one brain region where activating hypocretin receptors can promote wakefulness. An additional novel finding is that the hypocretin-1–induced increase in wakefulness was prevented by administering the $GABA_A$ receptor antagonist bicuculline into the PnO. Considered with data showing that delivering hypocretin-1 to the PnO increases GABA levels in the PnO (Watson et al., 2008) and causes inhibition of some PnO neurons (Nuñez et al., 2006), and with evidence that GABAergic transmission in the PnO is wakefulness promoting (Camacho-Arroyo et al., 1991; Xi et al., 1999; Sanford et al., 2003; Chang et al., 2006; Flint et al., 2007; Marks et al., 2008; Vanini et al., 2008; Watson et al., 2008), the present results support the interpretation that increasing GABAergic transmission in the PnO is one mechanism by which hypocretin-1 increases wakefulness. The results are discussed below relative to the functional roles of hypocretin-1 in rat PnO and the mechanisms by which hypocretin-1 administered to the PnO causes an increase in wakefulness. Limitations of the present study are also considered.

What are the functional roles of hypocretin-1 in the PnO?

The hypocretin peptides have multiple functional roles, one of which is to maintain wakefulness (Ohno and Sakurai, 2008). PnO neurons participate in generating the cortical activation of wakefulness and REM sleep (Steriade and McCarley, 2005), and

microinjection of hypocretin-1 into rat PnO was recently shown to increase the amount of wakefulness (Watson et al., 2008). Determining concentration dependence and antagonist blocking are established approaches for revealing whether or not responses are receptor mediated (Norman, 1979). The present study provides data fulfilling the criteria of concentration dependence (Figure 2.3) and antagonist blocking (Figure 2.6), indicating that hypocretin-1 acts at its receptors in the PnO to increase wakefulness.

Efforts to elucidate functions of hypocretin-1 also have found that microinjecting hypocretin-1 into rat PnO causes an antinociceptive response that is blocked by coadministration of the hcrt-r1 antagonist SB-334867 (Watson et al., 2010). This result demonstrates that the antinociceptive response is mediated by hypocretin receptors. Furthermore, blocking hypocretin receptors in the PnO with SB-334867 in the absence of exogenous hypocretin-1 increases nociceptive responsiveness to a thermal stimulus (Watson et al., 2010). This finding means that endogenous hypocretin-1 in rat PnO is antinociceptive. Microdialysis delivery of hypocretin-1 to rat PnO increases ACh release (Bernard et al., 2006), and ACh in the PnO of cat (Kshatri et al., 1998) and mouse (Wang et al., 2009) is antinociceptive. Future studies can determine whether the antinociceptive effects of hypocretin-1 in rat PnO depend on cholinergic transmission.

By what mechanisms does hypocretin in the PnO increase wakefulness?

Hypocretins are excitatory (de Lecea et al., 1998), and one mechanism by which these peptides are thought to enhance arousal is to activate neurons that drive wakefulness (Tsujino and Sakurai, 2009), thus increasing the release of wakefulness-generating neurotransmitters. Hypocretin-1 delivered to the PnO increases local GABA levels, and GABA in the PnO increases wakefulness (reviewed in Vanini et al., 2010). Pharmacologically increasing GABA levels in the PnO increases wakefulness (Watson et

al., 2008), and endogenous GABA levels in the PnO are significantly greater during wakefulness than during REM sleep (Vanini et al., 2009). GABA levels in the PnO are also greater during wakefulness than during the loss of consciousness produced by the general anesthetic isoflurane (Vanini et al., 2008). Furthermore, loss of endogenous hypocretin increases recovery time from isoflurane anesthesia (Kelz et al., 2008). The present finding that the hypocretin-1–induced increase in wakefulness was blocked by coadministration of bicuculline (Figure 2.6) demonstrates that the wakefulness response is mediated by $GABA_A$ receptors. This finding, therefore, supports the interpretation that the hypocretin-1–induced increase in wakefulness (Figure 2.3) results from enhanced GABAergic transmission in the PnO.

When given systemically to humans or animals, drugs that increase GABAergic transmission produce behavioral states such as sleep (Winsky-Sommerer, 2009) or general anesthesia (Franks, 2008). The mechanisms by which GABAergic drugs cause a loss of waking consciousness are not fully understood but are likely to include inhibition of wakefulness-promoting neurons. For example, the hypnotic eszopiclone may cause sleep by potentiating transmission at $GABA_A$ receptors on pedunculopontine tegmental neurons (Ye and Garcia-Rill, 2009). The mechanisms by which enhancing GABAergic transmission locally in the PnO causes an increase in wakefulness remain to be elucidated. Increased wakefulness is also caused by administering GABAergic drugs directly into the preoptic area/anterior hypothalamus (Lin et al., 1989) or the midbrain reticular formation (Tsuchiya and Fukushima, 1977). Increased sleep, however, is caused by delivering a $GABA_A$ receptor agonist directly into the posterior hypothalamus (Lin et

al., 1989). Therefore, GABAmimetics have opposite effects on sleep and wakefulness depending upon their site of action in the brain (Watson et al., 2008).

Hypocretin-1 increases ACh release in the PnO (Bernard et al., 2006), and increasing cholinergic transmission in the PnO may contribute to the mechanism by which PnO administration of hypocretin-1 increases wakefulness. Cholinergic transmission in the PnO promotes the EEG activation characteristic of both wakefulness and REM sleep (Lydic and Baghdoyan, 2005; Steriade and McCarley, 2005). Future studies are required to determine whether the increase in wakefulness caused by delivering hypocretin-1 into the PnO can be blocked by coadministering a cholinergic receptor antagonist.

The hypocretin-1–induced increase in wakefulness was comprised of an increase in the duration of the longest wakefulness episode and a decrease in the number of wakefulness episodes (Figure 2.3). The increase in wakefulness was accompanied by a decrease in NREM sleep and REM sleep. EEG power was not altered by hypocretin-1, demonstrating similarity to spontaneously occurring wakefulness, NREM sleep, and REM sleep with respect to the trait of EEG activity. Hypocretin-1 decreased the percentage of time spent in NREM sleep by decreasing both the number and duration of NREM sleep episodes. The hypocretin-1–induced consolidation of wakefulness into longer episodes and the decrease in the number of state transitions is consistent with the finding that narcoleptic patients (Taheri et al., 2002; Scammell, 2003) and mice lacking hypocretin (Mochizuki et al., 2004; Zhang et al., 2007) show fragmentation of sleep and wakefulness. These results also support a recent modeling study predicting that

hypocretin-1 preferentially acts on long episodes of wakefulness (Diniz Behn et al., 2008).

Effects of hypocretin-1 on wakefulness varies with behavioral state and microinjection site

The sleep-wake response to microinjecting hypocretin-1 into cat pontine reticular formation depends on the behavior state of the cat when the drug is administered, the site of injection within the pontine brainstem, and the amount of hypocretin-1 injected. Microinjecting hypocretin-1 (125 pmol/250 nL; 450 ng) into the oral part of cat pontine reticular formation during NREM sleep increases REM sleep (Xi et al., 2002a), whereas administering hypocretin-1 (125 pmol/250 nL; 450 ng) into the same region during wakefulness produces an increase in wakefulness (Xi and Chase, 2009). Microinjection of hypocretin-1 (2 to 20 pmol/20 nL; 7 to 71 ng) into a more dorsal region of cat pontine reticular formation (referred to as peri-locus coeruleus α) during wakefulness causes a concentration-dependent increase in wakefulness and decrease in NREM sleep and REM sleep, whereas the same concentrations of hypocretin-1 delivered to the ventral part of cat pontine reticular formation cause a selective inhibition of REM sleep and no change in the amount of wakefulness or NREM sleep (Moreno-Balandrán et al., 2008). The dissimilar findings between these 2 studies in cat are reconciled by noting that different brain regions were microinjected with different amounts of hypocretin-1 in different microinjection volumes (Xi et al., 2002a; Moreno-Balandrán et al., 2008).

Species-specific responses to PnO microinjection of hypocretin-1 in rat and cat have been discussed in detail (Watson et al., 2008). For the present report and a previous study (Watson et al., 2008) using rat, all microinjections were made during wakefulness, and hypocretin-1 caused an increase in wakefulness (Figure 2.3). Rat microinjection sites

in the present study (Figures 2.1 & 2.4) are in homologous regions of cat pontine reticular formation that produced either increases in wakefulness when hypocretin-1 was injected during wakefulness (Xi and Chase, 2009) or selective decreases in REM sleep (Moreno-Balandrán et al., 2008). Hypocretin-1 did significantly decrease REM sleep in the present study and also decreased NREM sleep (Figure 2.3). The reasons for these differences are not known but could include differences in microinjection sites, hypocretin receptors, and afferent hypocretin terminals, in addition to the use of different microinjection volumes and amounts of hypocretin-1.

Limitations, Conclusions, and Potential Clinical Significance

One unexplained finding of the present study is that bicuculline did not block the hypocretin-1–induced decrease in the amount of REM sleep (Figure 2.6C). This finding was unexpected because bicuculline alone delivered to the PnO of rat (Sanford et al., 2003; Marks et al., 2008), cat (Xi et al., 1999; Vazquez and Baghdoyan, 2004), and mouse (Chang et al., 2006) increases REM sleep. Pontine reticular formation administration of bicuculline increases local ACh release (Vazquez and Baghdoyan, 2004), and the increase in REM sleep that occurs by administering $GABA_A$ receptor antagonists to the PnO is blocked by the muscarinic cholinergic antagonist atropine (Marks et al., 2008). Hypocretin-1 also increases ACh release in the PnO (Bernard et al., 2006), and PnO administration of cholinomimetics significantly increases REM sleep (reviewed in Lydic and Baghdoyan, 2008). These data support the interpretation that GABAergic transmission in the PnO inhibits REM sleep, in part, by inhibiting ACh release. A higher concentration of bicuculline or a $GABA_B$ receptor antagonist may be required to reverse the hypocretin-1–induced decrease in REM sleep.

The experiments reported here did not identify the hypocretin receptor subtype or subtypes in the PnO that mediate the wakefulness response to PnO administration of hypocretin-1 (Figure 2.3). Hypocretins signal through two subtypes of GPCRs, hcrt-r1 and hcrt-r2, also called OX_1R and OX_2R (reviewed in Ohno and Sakurai, 2008). Hcrt-r1 is selective for hypocretin-1, whereas hcrt-r2 does not distinguish between hypocretin-1 and hypocretin-2 (Sakurai et al., 1998). Rat PnO contains both hypocretin receptor subtypes (Greco and Shiromani, 2001), and hcrt-r1 and hcrt-r2 in the PnO each contribute to the hypocretin-induced increase in ACh release within the PnO (Bernard et al., 2006). Hcrt-r2 is present on GABAergic neurons in rat PnO (Brischoux et al., 2008), suggesting that hcrt-r2 mediates the increase in PnO GABA levels caused by hypocretin-1 (Watson et al., 2008), as well as the hypocretin-1–induced hyperpolarization of PnO neurons (Nuñez et al., 2006). SB-334867 has a 50-fold higher affinity for hcrt-r1 than for hcrt-r2 (Duxon et al., 2001), and the finding that the hypocretin-1–induced increase in wakefulness was blocked by SB-334867 (Figure 2.6A) supports the interpretation that the wakefulness response was mediated by hcrt-r1. Coadministration of SB-334867 did not return REM sleep to control levels (Figure 6C), suggesting that hcrt-r2 may mediate the decrease in REM sleep.

Data from the present study (Figures 2.3 & 2.6) demonstrate that exogenous hypocretin-1 activates hypocretin receptors in rat PnO to increase and consolidate wakefulness. Whether endogenous hypocretin in rat PnO normally functions to promote wakefulness awaits the demonstration that antagonizing hypocretin receptors in the PnO causes a concentration-dependent decrease in wakefulness.

Despite the limitations discussed above, the present data show for the first time that activating hypocretin receptors in rat PnO causes an increase in wakefulness that is mediated by GABAergic transmission at $GABA_A$ receptors. This novel finding demonstrates that hypocretinergic and GABAergic transmission can interact in the pontine reticular formation to increase wakefulness. An extensive body of evidence supports the interpretation that one physiologic role of hypocretins is to maintain wakefulness (reviewed in Ohno and Sakurai, 2008). Neuronal systems regulating sleep and wakefulness are anatomically distributed and neurochemically heterogeneous, and the present data suggest that the PnO is one region where hypocretin-1 and GABA interact to increase periods of uninterrupted wakefulness and inhibit sleep. In addition to its wakefulness-promoting actions, hypocretin-1 has antinociceptive effects (Bingham et al., 2001), which also can be evoked from the PnO (Watson et al., 2010). Opioids are the most widely used drugs for the treatment of pain, and negative side effects include disruption of the sleep-wake cycle (Lydic and Baghdoyan, 2007). GABA levels in the PnO are decreased by opioids (Watson et al., 2007) and increased by hypocretin-1 (Watson et al., 2008). Studies of $GABA_A$-receptor point-mutated mice showing that systemic alterations of transmission at $GABA_A$ receptors can be antinociceptive (Zeilhofer et al., 2009) encourage continuing efforts to localize GABAergic modulation of nociception to specific brain regions. The present demonstration of a wakefulness-promoting interaction between hypocretinergic and GABAergic neurotransmission in the PnO indicates the importance of determining whether coadministering hypocretin receptor agonists with opioids can provide analgesia without disrupting wakefulness.

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CHAPTER 3

GLUTAMATE ADMINISTERED TO RAT PONTINE RETICULAR NUCLEUS, ORAL PART DECREASES ACETYLCHOLINE RELEASE

SUMMARY

Immunohistochemical studies have indicated that neurons in the pontine reticular nucleus, oral part (PnO) are immunopositive for glutamate (Kaneko et al., 1989; Lai et al., 1999). Maximal glutamate levels in the PnO occur during wakefulness (Watson et al., 2008), suggesting a causal link between glutamate release in the PnO and the generation of wakefulness. ACh is a principal modulator of sleep and wakefulness and a number of studies show that cholinergic transmission in the PnO increases REM sleep or wakefulness (revewied in Lydic and Baghdoyan, 2008). To further understand the neurochemical mechanisms by which glutamate in the PnO affects sleep-wake states, *in vivo* microdialysis was used to test the hypothesis that glutamate modulates ACh release in the PnO. Dialysis delivery of glutamate caused a decrease in ACh release in the PnO. The findings presented here are consistent with the interpretation that one mechanism by which glutamate alters sleep and wakefulness is by modulating cholinergic transmission in the PnO.

INTRODUCTION

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system yet little is known about the role of glutamate in sleep cycle control. State-dependent changes in glutamate levels suggest that depending on the brain area, glutamate promotes either wakefulness or REM sleep (Brevig and Baghdoyan, 2010). Administration of the glutamate agonist kainate into the dorsal raphe (Sakai and Crochet, 2001) or the periaqueductal gray (Crochet et al., 2006) causes an increase in wakefulness. Depending on the dose, glutamate causes either an increase in REM sleep or an increase in wakefulness when microinjected into the pedunculopontine tegmental nucleus (PPT) (Datta and Siwek, 1997; Datta et al., 2001a). One brain area that contributes to the generation of wakefulness and REM sleep is the PnO (Lydic and Baghdoyan, 2005). The biosynthetic enzyme for glutamate, glutaminase, was immunohistochemically localized to the reticular formation (Kaneko et al., 1989), and approximately 50% of pontine reticular formation neurons in cat show glutamate-like immunoreactivity (Lai et al., 1999). Glutamate excites pontine reticular formation neurons (Greene and Carpenter, 1985; Nuñez et al., 1998) and intracellular recordings demonstrate that the PnO contains functional α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA), kainate, and N-methyl-D-aspartic acid (NMDA) receptors (Stevens et al., 1992). Few studies have investigated the role of glutamate in the PnO in regulating sleep and wakefulness. The levels of glutamate in rat PnO are higher during wakefulness than during sleep (Watson et al., 2008). Administration of the glutamate agonists kainate and AMPA, but not NMDA, into a region dorsal to the PnO (peri-locus coeruleus alpha) causes an increase in

REM sleep (Onoe and Sakai, 1995). Taken together these findings suggest that glutamatergic transmission in the PnO might increase wakefulness or REM sleep.

One possible mechanism by which glutamate in the PnO modulates wakefulness and REM sleep is by modulating ACh release. Several lines of evidence indicate that ACh is a principal modulator of sleep-wake states (reviewed in Lydic and Baghdoyan, 2008). Spontaneous ACh release in the cortex is high when the cortex is activated, such as during REM sleep and wakefulness (Marrosu et al., 1995). ACh release in the pontine reticular formation is significantly increased during REM sleep (Kodama et al., 1990; Leonard and Lydic, 1995, 1997), and administration of cholinomimetics to the pontine reticular formation causes a REM sleep-like state (Baghdoyan et al., 1984b; Baghdoyan et al., 1984a, 1987; Baghdoyan et al., 1989; Imeri et al., 1994; Bourgin et al., 1995; Marks and Birabil, 1998, 2007).

A number of studies show that glutamate modulates ACh release, and depending on the brain region glutamate agonists cause either an increase (Kurosawa et al., 1989; Giovannini et al., 1995; Ikarashi et al., 1998; Moor et al., 1998; Fournier et al., 2004) or a decrease (Giovannini et al., 1995; Materi and Semba, 2001; Neigh-McCandless et al., 2002; Hernandez et al., 2003; Del Arco et al., 2008) in ACh release. Both systemic administration and microdialysis delivery of a NMDA receptor antagonist to cat pontine reticular formation cause a decrease in ACh release in cat pontine reticular formation (Lydic and Baghdoyan, 2002). These findings suggest that glutamate modulates ACh release in cat pontine reticular formation. The present study tested the hypothesis that microdialysis delivery of glutamate to rat PnO modulates ACh release in the PnO.

MATERIALS AND METHODS

Chemicals and Animals

Glutamate (Fluka Biochemika Buchs, Switzerland) was dissolved in Ringer's solution (147 mM NaCl, 2.4 mM $CaCl_2$, 4.0 mM KCl, 10 μM neostigmine bromide (Sigma-Aldrich, St. Louis, MO), pH 5.8-6.2). Salts for Ringer's solution were acquired from Fisher Scientific (Pittsburgh, PA). Animal experiments were approved by the University of Michigan Committee on Use and Care of Animals. Adult (235 to 250 g) male Crl:CD*(SD) (Sprague Dawley) rats (n=5; Charles River Laboratories, Wilmington, MA) were housed with unlimited access to food and water and kept on a 12-hour light/dark cycle (lights on at 0600).

Surgical preparation, microdialysis, and high performance liquid chromatography with electrochemical detection.

The time-course of the dialysis experiments is shown schematically in Figure 3.1A. Rats were anesthetized with 3.0% isoflurane (Abbott Laboratories, North Chicago, IL) in O_2 and placed in a Kopf Model 962 small animal stereotaxic instrument with a Model 920 rat anesthesia mask (David Kopf Instruments, Tujunga, CA). Following placement in the stereotaxic instrument, the delivered isoflurane concentration was decreased to 1.8 %. A water blanket kept at 37°C was used to maintain core body temperature. The core body temperature and delivered isoflurane concentration were measured using a CardiocapTM/5 monitor (Datex-Ohmeda, Madison, WI). A scalp incision was made to expose the skull and a craniotomy for the CMA/11 dialysis probe (cuprophane membrane, 1 mm in length, 0.24 mm in diameter, 6 kDA cut-off; DMA Microdialysis, North Chelmsford, MA) was made at stereotaxic coordinates 8.4 mm anterior to bregma and 1.0 mm lateral to the midline (Paxinos and Watson, 2007).

Following insertion of the dialysis probe, the delivered concentration of isoflurane was reduced to 1.5 % and held constant for the remainder of the experiment. The levels of ACh were allowed to stabilize before collecting control samples. The microdialysis probe was perfused continuously with Ringer's solution (vehicle control) at a constant 2.0 µl/min flow rate. Five control samples (25 µl/12.5 minutes per dialysis sample) were collected during dialysis with Ringer's solution before glutamate delivery was initiated by turning a CMA/110 liquid switch. Five dialysis samples were collected during microdialysis with glutamate (1 mM). CMA/11 dialysis probes are estimated to deliver 5% of the dialysate drug concentration, and in the present study the estimated concentration of glutamate delivered to the PnO was approximately 500 µM. After the collection period, the scalp wound was closed using sutures. The animal was placed in a cage and kept under a heat lamp until ambulatory.

The percent of ACh recovered by the dialysis probe was determined *in vivo* before and after every experiment. The dialysis probe was placed in a solution of known ACh concentration and five dialysis samples were collected and analyzed for ACh. The pre- and post-experiment probe recoveries were calculated and only experiments in which pre and post-experiment probe recoveries did not change significantly in the direction of the drug effect were included into the final data set.

Quantification of ACh

Each dialysis sample was injected into a high performance liquid chromatography system (Bioanalytical Systems, West Lafayette, IN) and carried through the system in 50 mM Na_2HPO_4 mobile phase (pH 8.5) at a flow rate of 1 ml/min. ACh was converted proportionally into hydrogen peroxide by an immobilized enzyme reactor column. The

hydrogen peroxide was detected by a platinum electrode (+0.5 V applied potential) in reference to an Ag+/AgCl electrode. The chromatographic signal was digitized and quantified using ChromoGraph software (Bioanalytical Systems). The amount of ACh in each dialysis sample was determined by comparing chromatographic peak area produced by the dialysis sample to a seven point standard curve (0.05-1.0 pmol). Differences in ACh before and during glutamate administration were evaluated using a paired two-tailed t-test.

Histological identification of dialysis probe sites

Three days after microdialysis experiments, rats were deeply anesthetized with isoflurane (5%) and decapitated. Brains were immediately removed, frozen, and sectioned coronally (40 microns thick) from caudal to rostral using a cryostat (Leica Microsystems, Nussloch, Germany). Serial sections were slide mounted, dried, fixed in paraformaldehyde vapor (80°C), and stained with cresyl violet. Stained tissue sections and a 1 mm calibration bar were digitized using a Super Nikon Coolscan 4000 ED Film Scanner (Nikon Inc, Melville, NY, USA). Microdialysis probe sites were identified and assigned stereotaxic coordinates by comparison with a rat brain atlas (Paxinos and Watson, 2007).

RESULTS

Microdialysis delivery of glutamate to the pontine nucleus oral part (PnO) decreased ACh release in the PnO

The effect of glutamate on ACh release is shown in Figure 3.1B-C. Figure 3.1B illustrates the time-course of ACh release during one typical experiment. Each bar represents sequential ACh samples collected for 12.5 minutes. Figure 3.1C summarizes data from five microdialysis experiments. Glutamate significantly decreased ACh release by an average of 23.5 % ($t=5.0$, $df=4$, $p=0.007$). Histological data confirming that all dialysis sites were restricted to the PnO are shown in Figure 3.2. The average stereotaxic coordinates (Paxinos and Watson, 2007) for these injection sites were 8.3±0.2 mm posterior to bregma, 9.0±0.15 mm ventral to the top of the skull, and 0.9±0.12 mm from the midline.

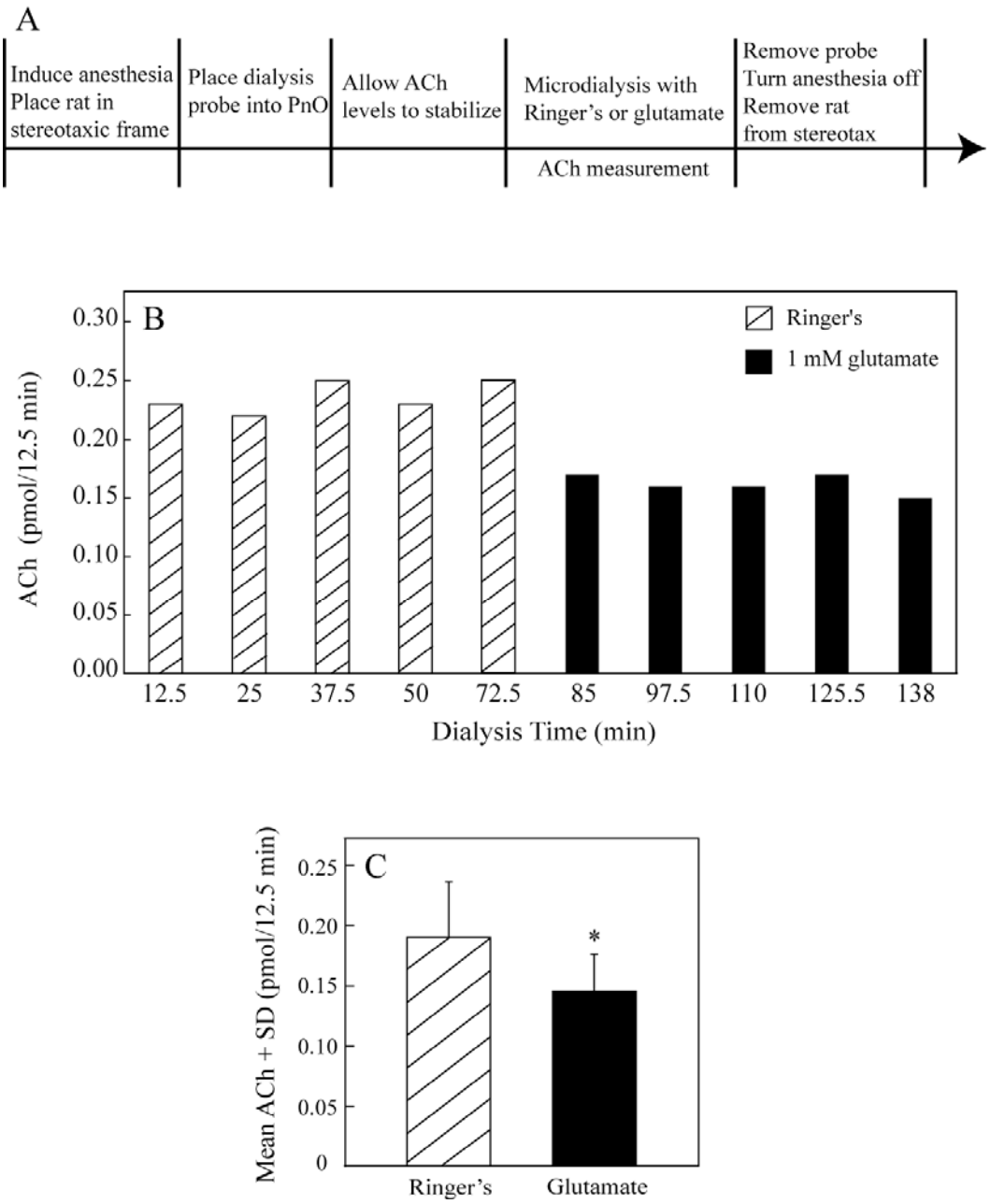

Figure 3.1. Dialysis delivery of glutamate into the PnO decreased ACh release. (A) Schematic timeline of microdialysis experiments. The experimental manipulations are shown on the top row and the bottom row designates when the dependent measure was acquired. (B) Time course of the glutamate-induced decrease in ACh release from one representative microdialysis experiment. Each bar represents ACh levels plotted in sequential 12.5 minute intervals. Hatched bars indicate ACh content in samples collected during dialysis with Ringer's (control) and solid bars represent ACh release during dialysis with glutamate. (C) Data were averaged across time for control (Ringer's) samples and for glutamate samples for each experiment, and then across experiments (n=5) to show that glutamate caused a significant (*p=0.007) decrease in ACh release.

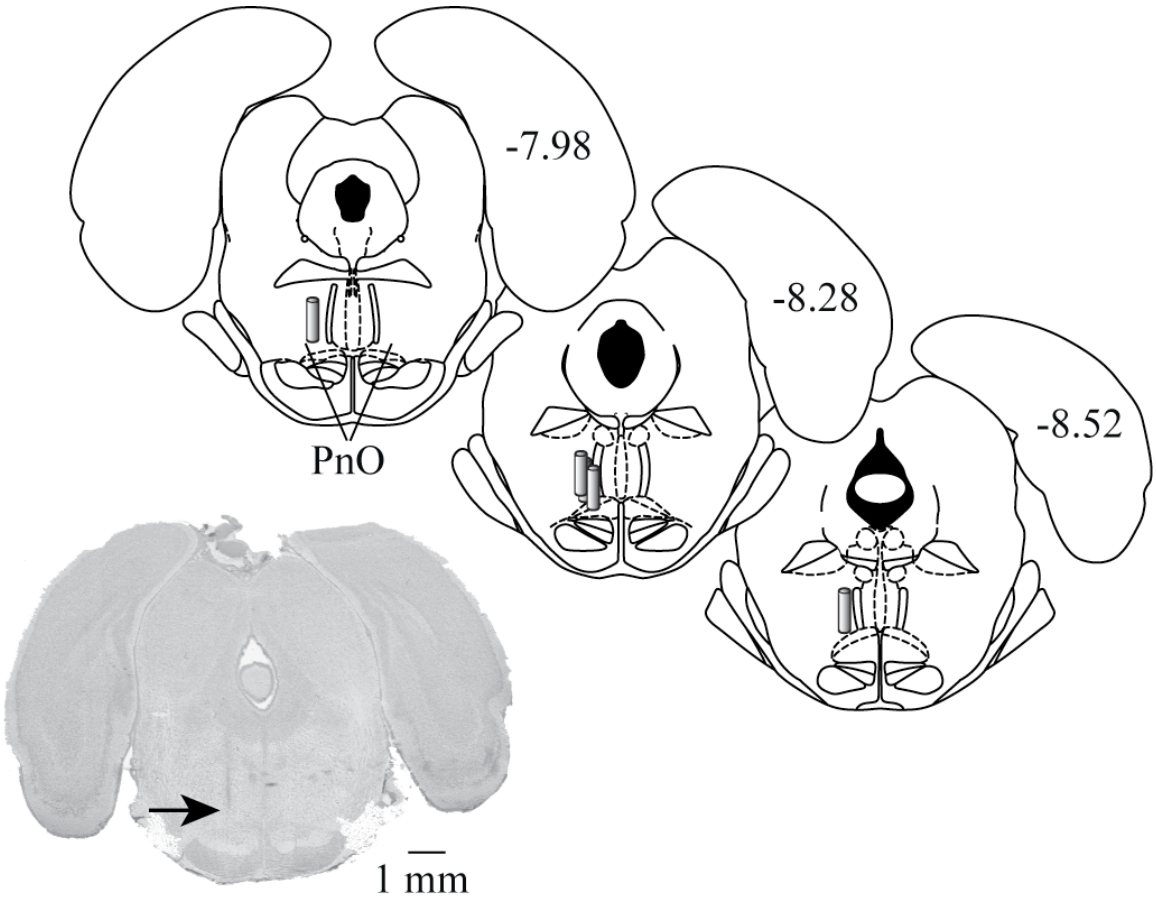

Figure 3.2. All microdialysis sites were located in the PnO. Microdialysis sites (n=5) are represented as cylinders on three coronal atlas plates (modified from (Paxinos and Watson, 2007)). Each cylinder is drawn to scale and corresponds to the 1 mm length and 0.24 mm diameter of the dialysis probe membrane. Numbers on the right side of each plate indicate mm posterior to bregma. The cresyl violet stained section from one rat illustrates a representative dialysis probe site in the PnO. The most ventral portion of the 1 mm dialysis membrane is indicated by the arrow.

DISCUSSION

The present study shows that microdialysis delivery of glutamate to rat PnO caused a decrease in ACh release in the PnO. The present results encourage future studies aiming to identify which glutamate receptor subtypes modulate ACh release in the PnO. Future experiments could also determine whether glutamate in the PnO causes a concentration-dependent decrease in ACh release. In order to address whether endogenous glutamate modulates ACh in the PnO, future studies may use glutamate receptor antagonists.

Role of glutamate receptor subtypes in modulating ACh release

Few studies have investigated the relative importance of specific glutamate receptor subtypes in modulating ACh release. Blockade of NMDA receptors in the pontine reticular formation of isoflurane-anesthetized cat caused a decrease in ACh release in the pontine reticular formation (Lydic and Baghdoyan, 2002). These findings suggest that activation of NMDA receptors might cause an increase in ACh release in the pontine reticular formation. Although the present and previous (Lydic and Baghdoyan, 2002) results appear contradictory, there are a number of studies showing that depending on which glutamate receptor subtype is stimulated, ACh release is either decreased or increased. For example, ACh release in the striatum of awake, freely moving rat is increased by activation of NMDA receptors, whereas activation of non-NMDA receptors causes a decrease in ACh release (Anderson et al., 1994; Giovannini et al., 1995; Ikarashi et al., 1998). Inhibition of NMDA receptors (Neigh-McCandless et al., 2002; Zmarowski et al., 2007) or AMPA and kainate receptors (Neigh-McCandless et al., 2002) in the nucleus accumbens of awake rat causes an increase in ACh release in the cortex. In the

medial septum, NMDA receptors appear to be more important in mediating the increase in ACh release in the hippocampus than AMPA or kainate receptors (Moor et al., 1998). Stimulation of AMPA receptors in the basal forebrain of awake, freely moving rats (Fadel et al., 2001) and urethane-anesthetized rats (Fournier et al., 2004) causes an increase in ACh release in the cortex. It is evident from these studies that depending on the brain region, activation of specific glutamate receptor subtypes causes either an increase or a decrease in ACh release.

The mechanism(s) by which activation of different glutamate receptor subtypes cause either increased or reduced ACh release is unknown. One possible explanation is that the synaptic location (e.g., GABAergic or glutamatergic neurons) of the specific glutamate receptor subtypes dictates whether ACh release is increased or decreased. One can also speculate that the relative expression level of specific glutamate receptor subtypes determines whether glutamate increases or decreases ACh release. One possible explanation why glutamate in the PnO did not increase ACh release is that there are more functional non-NMDA receptors in the PnO, and activation of these receptors overrides the effect of activating NMDA receptors, resulting in decreased ACh release.

Role of glutamate receptor subtypes in modulating sleep and wakefulness

Activation of specific glutamate receptor subtypes might produce different effects on the sleep-wake cycle. Seminal studies demonstrated that kainate receptors in the PPT are key for generating REM sleep, whereas NMDA receptors in the PPT are key for generating wakefulness (Datta et al., 2001b; Datta, 2002; Datta et al., 2002). Electrical stimulation of the LDT/PPT can cause either an increase in wakefulness (Steriade, 1993) or an increase in REM sleep (Thakkar et al., 1996). Additionally, electrical stimulation

of the LDT/PPT causes an increase in ACh release in the pontine reticular formation (Lydic and Baghdoyan, 1993). Depending on the concentration administered to the pontine reticular formation, cholinergic agonists can cause either an increase in REM sleep or wakefulness (Baghdoyan et al., 1989; Bourgin et al., 1995; Marks and Birabil, 2001). Whether activation of NMDA receptors in the PPT causes a further increase in ACh release compared to activation of kainate receptors in the PPT is not known.

Evidence that glutamate interacts with GABA to decrease ACh release

The observed inhibitory effect of glutamate in the PnO on ACh release is likely to involve GABAergic receptors. One study demonstrated that the inhibitory effect of non-NMDA receptors on ACh release in the striatum was reversed by the $GABA_A$ receptor antagonist bicuculline (Giovannini et al., 1995). These findings encourage future studies to test the hypothesis that co-administration of bicuculline and glutamate to the PnO will block the glutamate-induced decrease in ACh release in the PnO. Consistent with the notion that glutamate interacts with GABA to inhibit ACh release in the PnO is that bicuculline causes an increase in ACh release (Vazquez and Baghdoyan, 2004), demonstrating that GABAergic transmission in the PnO inhibits ACh release in the PnO. Although glutamate in the PnO might interact with GABA to inhibit ACh release, the potential involvement of glycine in the PnO should not be disregarded (Camacho-Arroyo and Tapia, 1992; Hasegawa et al., 2000).

In addition to demonstrating that glutamate in the PnO modulates ACh release, the results from Chapter 3 might also provide insight into the synaptic mechanisms by which administration of hypocretin to the PnO increases both ACh release (Bernard et al., 2003, 2006) and GABA levels in the PnO (Watson et al., 2008). As previously

mentioned in the introduction, several studies show that hypocretin-1 and glutamate interact (Li et al., 2002; Alam and Mallick, 2008; Fadel and Frederick-Duus, 2008). However, it is unknown whether hypocretin-1 and glutamate interact in the PnO. However, one can speculate that hypocretin might interact with glutamate to increase both ACh release and GABA levels in the PnO. Synaptic models for how hypocretin-1, glutamate, GABA, and ACh interact are discussed in Chapter 4.

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CHAPTER 4

CONCLUSION

This Ph.D. thesis research investigated the mechanisms by which administration of hypocretin-1 to the PnO promotes wakefulness and also tested the effect of administration of glutamate to the PnO on ACh release. The results are consistent with the interpretation that hypocretin-1 in the PnO promotes wakefulness by activating hypocretin receptors and $GABA_A$ receptors, and that glutamate in the PnO modulates ACh release. These results are discussed below in the context of possible synaptic mechanisms by which hypocretin-1 promotes wakefulness. Possible future studies and clinical implications are also discussed.

The findings in Chapter 2 are the first to show that microinjection of hypocretin-1 into rat PnO causes a concentration-dependent increase in wakefulness by increasing the duration of wakefulness episodes and by decreasing the number of wakefulness episodes (Fig. 2.3 A,D,G) (Brevig et al., 2010). These results support a recent modeling study that predicts hypocretin-1 preferentially acts to consolidate wakefulness (Dinz Behn et al., 2008). Furthermore, the hypocretin-1-induced consolidation of wakefulness into longer episodes is consistent with the finding that narcoleptic patients (Taheri et al., 2002; Scammell, 2003) and mice lacking hypocretin (Mochizuki et al., 2004; Zhang et al., 2007) show fragmentation of sleep and wakefulness.

Having demonstrated that administration of hypocretin-1 to the PnO causes a concentration-dependent increase in wakefulness (Fig 2.3A) (Brevig et al., 2010), additional experiments were designed to determine whether the increase in wakefulness is caused by activation of hypocretin receptors in the PnO. These studies used the hcrt-1 antagonist SB-334867 to block the hypocretin-1-induced increase in wakefulness (Fig. 2.6). SB-334867 has a 50-fold higher affinity for hcrt-r1 than hcrt-r2, (Smart et al., 2001), supporting the interpretation that hcrt-r1 mediates the wakefulness response. Whether endogenous hypocretin-1 in the PnO normally functions to promote wakefulness is unknown. Future experiments could address this question by determining whether blocking hypocretin receptors causes a concentration-dependent decrease in wakefulness.

Brain regions that regulate sleep and wakefulness differentially express hcrt-r1 and hcrt-r2, and in some of these brain regions the expression level of hcrt-r1 and hcrt-r2 also varies. The relative importance of hypocretin receptor subtypes in regulating sleep and wakefulness is not well understood. Systemic administration of SB-334867 increased sleep (Smith et al., 2003) and resting duration (Duxon et al., 2001), indicating that hcrt-r1 activation is important in regulating sleep and wakefulness. In contrast, subcutaneous administration of another hcrt-r1 antagonist, SB-408124, did not alter sleep-wake states (Dugovic et al., 2009). Whether these differences are due to differences in the compounds and/or the routes of administration is unknown. Subcutaneous administration of a hcrt-r2 antagonist JNJ-10397049 caused a decrease in wakefulness and an increase in sleep (Dugovic et al., 2009). Future experiments may determine the relative contribution of hypocretin receptor subtypes in different brain

regions in regulating sleep and wakefulness by using specific hypocretin receptor agonists and antagonists.

Multiple lines of evidence demonstrate that hypocretins and GABA functionally interact. Hypocretin-1 excites GABAergic neurons in the brainstem reticular formation (Li et al., 2002; Brown et al., 2008), substantia nigra (Korotkova et al., 2002), and the medial septum (Wu et al., 2002). Inhibition of neurons in the PnO caused by hypocretin-1 application is blocked by bicuculline (Nuñez et al., 2006), suggesting that the inhibition is mediated by $GABA_A$ receptors. Further evidence supporting this interpretation is that hypocretin receptors are localized on GABAergic neurons in the PnO (Brischoux et al., 2008). Hypocretin and GABA also have been shown to interact in the lateral hypothalamus. The increase in wakefulness caused by lateral hypothalamic administration of the $GABA_A$ receptor antagonist bicuculline correlates with increased activity of hypocretinergic neurons (Alam et al., 2005). Hypocretin and GABA do not always interact as shown by a study in which administration of hypocretin-1 to the substantia innominata did not alter GABA levels (Fadel and Burk, 2009). The results in Chapter 2 are the first to report that GABAergic transmission in the PnO is necessary for the hypocretin-1-induced increase in wakefulness (Fig 2.6). Narcoleptic patients without disrupted nighttime sleep display higher GABA levels in the medial prefrontal cortex than narcoleptic patients with disrupted nighttime sleep (Kim et al., 2009), suggesting that GABA might act to reduce sleep disturbances. Few studies have evaluated the functional importance of another class of GABA receptors, the metabotropic $GABA_B$ receptors, in relation to narcolepsy. Selective deletion of $GABA_B$ receptors on hypocretinergic neurons in mice results in sleep fragmentation that is more severe than in

hypocretin knockout mice (Matsuki et al., 2009), suggesting a role of $GABA_B$ receptors in maintaining wakefulness.

Treatments for narcolepsy target the symptoms and not the pathophysiology of the disease, hypocretin deficiency (Fujiki and Nishino, 2005). An ideal treatment for narcolepsy would be to replace hypocretin similar to the way L-DOPA is used to treat Parkinson's disease. However, the therapeutic use of hypocretin-1 to treat narcolepsy is limited because the ability of peptides to penetrate the central nervous system is restricted. Therefore, it is important to develop drugs that target the downstream effectors of hypocretin. Results from the Chapter 2 studies further our understanding of how hypocretin increases wakefulness and contribute to the rationale design of more selective drugs to treat narcolepsy.

Although glutamate is a major neurotransmitter in the PnO (Lai et al., 1999), little is known about the effects of glutamate in this brain region on sleep and wakefulness. A recent report suggests a causal relationship between glutamate levels in the PnO and wakefulness (Watson et al., 2008a). One possible mechanism by which glutamate alters sleep-wake states is by modulating ACh release in the PnO. The findings in Chapter 3 are the first to report that administration of glutamate to the PnO decreases ACh release in the PnO (Fig. 3.1). Administration of the NMDA receptor antagonist, ketamine, also causes a decrease in ACh release in the PnO (Lydic and Baghdoyan, 2002). Results from Aim 3 studies support further investigation of the role of glutamate in modulating ACh release in the PnO. Concentration-response studies would help determine whether glutamate could cause a biphasic, concentration-dependent change in ACh release.

Cataplexy is the sudden loss of muscle tone that is observed in both narcoleptic patients and in animal models of narcolepsy. Administration of the muscarinic receptor agonist carbachol to the PnO produces cataplexy that is mediated by M2 receptors (Reid et al., 1994). However, administration of the glutamate receptor agonists AMPA and kainate into the PnO does not produce cataplexy (Reid et al., 1994). These results are in line with the Chapter 3 results showing that glutamate in the PnO inhibits ACh release in the PnO.

The mechanisms by which glutamate in the PnO decreases ACh release are unknown, but may involve activation of GABAergic neurons. GABA inhibits the release of ACh in a number of brain regions (Moor et al., 1998; Materi and Semba, 2001), and blocking $GABA_A$ receptors enhances ACh release in the PnO (Vazquez and Baghdoyan, 2004; Marks et al., 2008). Administration of the $GABA_A$ receptor antagonist bicuculline reversed the inhibitory effect of glutamate on striatal ACh release (Giovannini et al., 1995). These findings encourage future studies that will test the hypothesis that co-administration of bicuculline and glutamate to the PnO blocks the glutamate-induced decrease in ACh release in the PnO.

Results presented in Chapters 2 and 3 provide novel insight into possible synaptic mechanisms by which hypocretin-1 increases both GABA levels (Watson et al., 2008b) and ACh release (Bernard et al., 2003). How can hypocretin-1 in the PnO increase both GABA levels and ACh release when evidence suggests that GABAergic transmission in the PnO inhibits ACh release (Vazquez and Baghdoyan, 2004; Marks et al., 2008)? Further adding to the conundrum are data demonstrating that GABA in the PnO suppresses REM sleep whereas ACh release in the PnO enhances REM sleep. However,

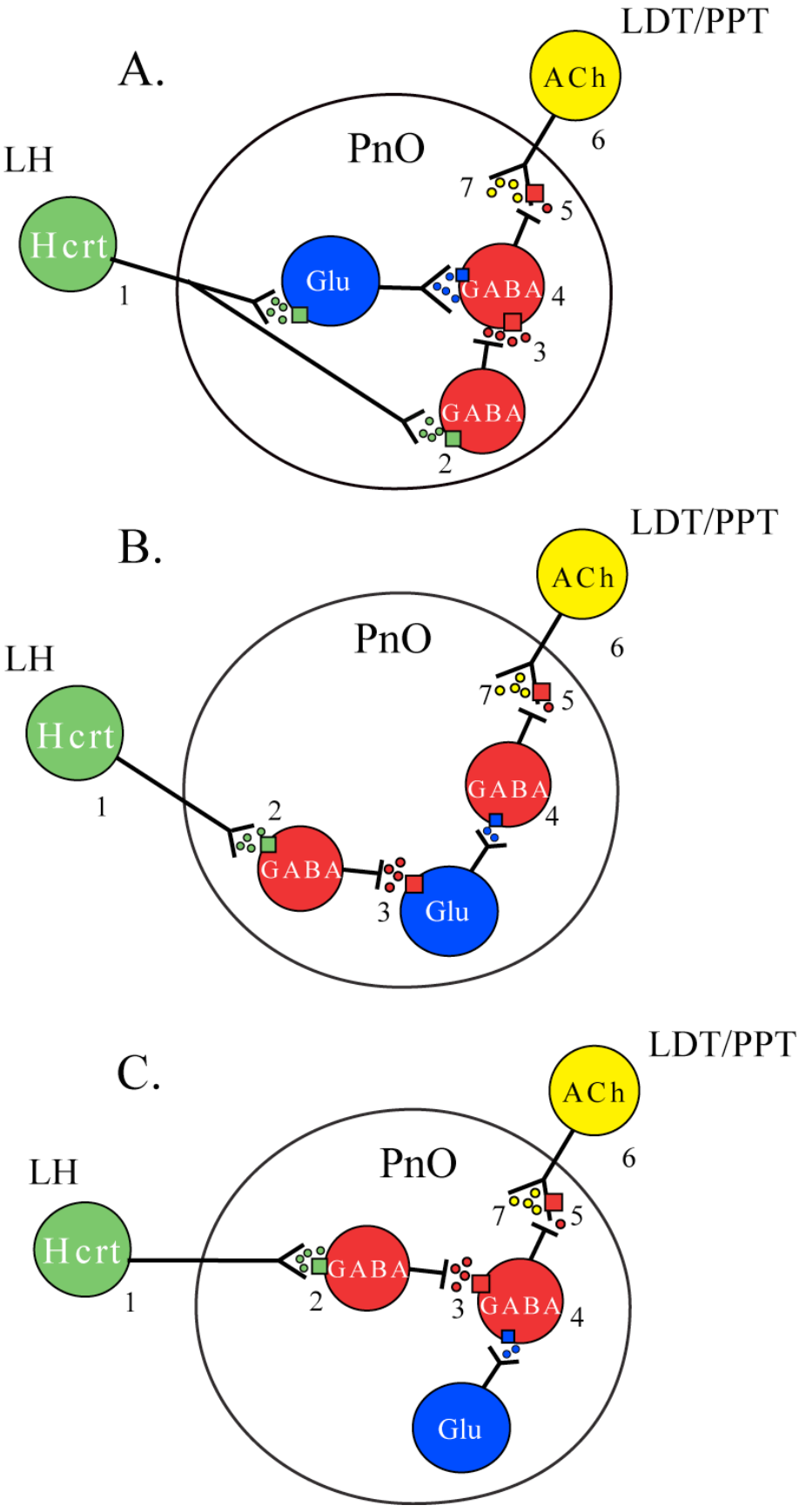
administration of cholinomimetics at high concentrations to the PnO can cause an increase in wakefulness (Bourgin et al., 1995; Marks and Birabil, 2001).

The simplest explanation for how hypocretin-1 increases ACh release is that hypocretin receptors may be located presynaptically on cholinergic terminals. However, I propose three synaptic models that integrate the present Ph.D. thesis findings with previous functional and immunohistochemical studies. These models each propose that hypocretin-1 increases ACh release in the PnO by interacting with both GABA and glutamate (Fig 4.1). Although it is not known whether hypocretin-1 and glutamate interact in the PnO, several studies have shown that hypocretin-1 and glutamate interact in other brain regions (Kodama et al., 2002; Li et al., 2002; Alam and Mallick, 2008; Fadel and Frederick-Duus, 2008; Tose et al., 2009a, b). In Model A, hypocretin release in the PnO simultaneously stimulates both GABA and glutamate release. The released GABA then acts on postsynaptic GABA receptors on a GABAergic interneuron, which inhibits GABA release, resulting in increased ACh release. The released glutamate caused by hypocretin-1 in the PnO activates postsynaptic glutamate receptors on a GABAergic interneuron to increase GABA levels, resulting in decreased ACh release. Model A assumes that the increase in GABA levels caused by hypocretin-1 overrides the effect of increased glutamate release. In Model B, hypocretin release in the PnO stimulates GABA release. Activation of postsynaptic GABA receptors on a glutamatergic neuron results in disfacilitation of a separate GABAergic neuron and therefore, an increase in ACh release. Similar to Model B, hypocretin-1 stimulates GABA release in Model C. GABA activates postsynaptic GABA receptors on a GABAergic interneuron that synapses on presynaptic cholinergic terminals. Inhibition of

the GABAergic interneuron will cause an increase in ACh release. The PnO is composed of a heterogeneous population of neuronal types with many different synaptic connections. The proposed models in Figure 4.1 are not the only possible synaptic connections by which hypocretin-1 can increase both GABA levels and ACh release.

Uncovering the mechanisms mediating the hypocretin-induced increase in wakefulness will pave the way to other pharmaceutical targets that will benefit patients with sleep disorders, such as narcolepsy or insomnia. Therefore, it is important to develop selective hypocretin receptor agonists and antagonists as well as learn more about hypocretin receptor signaling across states and brain regions.

Figure 4.1. Three possible synaptic models in which hypocretin-1 in the PnO increases both ACh release and GABA levels. The models are based on research described in this Ph.D. thesis and on results from previous studies. The numbers shown on the models indicate citations. **1**. Hypocretin neurons innervate the PnO (Peyron et al., 1998). **2.** Hypocretin receptors localize on GABAergic neurons in the PnO (Brischoux et al., 2008). **3.** Hypocretin increases GABA levels in the PnO (Watson et al., 2008b). **4**. Not all GABAergic neurons in the PnO contain hypocretin receptors (Brischoux et al., 2008). **5**. It is not known whether there are $GABA_A$ receptors on cholinergic neurons, however, the $GABA_A$ receptor antagonist, bicuculline causes an increase in ACh release in the PnO (Vazquez and Baghdoyan, 2004). **6.** Cholinergic neurons in the LDT/PPT project to the PnO (Shiromani et al., 1988). **7.** Hypocretin increases ACh release in the PnO (Bernard et al., 2003, 2006). It is not known if hypocretin and glutamate (Glu) interact in the PnO. Therefore, each model shows either hypocretin in the PnO increasing **(A)**, decreasing **(B)**, or **(C)** having no effect on Glu levels. The finding that glutamate in the PnO decreases ACh release (Chapter 3) supports the interpretation that glutamate receptors localize on GABAergic neurons, and is shown in all of the proposed models.


A.
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ACh
PnO
6
LH
7
5
Hcrt
GABA
2
3
GABA
4
1
Glu

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APPENDIX

NEUROTRANSMITTERS AND NEUROMODULATORS REGULATING SLEEP AND WAKEFULNESS

Synopsis

Wakefulness, NREM sleep, and REM sleep are modulated by complex interactions between multiple neurotransmitters and neuromodulators acting in many different brain areas. This chapter selectively highlights progress in understanding the role of selected neurotransmitters regulating states of sleep and wakefulness.

1. Definitions

EEG electroencephalogram; the electrical activity of the cerebral cortex measured by placing electrodes on the skull. The different stages of sleep are defined, in part, by characteristic pattern of cortical electrical activity

GABA gamma-aminobutyric acid; the major inhibitory neurotransmitter in the brain. The binding of GABA to its receptor on the surface of a neuron reduces the probability that the neuron will discharge

LDT/PTT laterodorsal and pedunculopontine tegmental nuclei; a brainstem nucleus located at the border between the midbrain and pons which contains cholinergic neurons that play an important role in the generation of REM sleep

NREM non-rapid eye movement; the majority of sleep time is spent in NREM sleep, which is divided into four stages. Stages 1 and 2 are lighter states of sleep, whereas stages 3 and 4 are deeper and more resorative. Stages 3 and 4 together are also

called slow wave sleep because the cortical electroencephalogram is characterized by slow frequency (0.5 to 4 Hz) waves. Normal sleep is characterized by an orderly progression from Stages 1 to 4 and then back up to Stage 2, from which REM sleep is entered.

pontine an adjective meaning of the pons; normally used to describe neurons that are located in the part of the brainstem called the pons

Pontine reticular formation PRF; a group of brainstem neurons that forms the pontine component of the ascending reticular activating system

2. Neurotransmitters Neuromodulators Regulating Sleep

Sleep neurobiology aims to elucidate the mechanisms by which states of sleep and wakefulness are generated by the brain. Great progress in achieving this aim has been made since the discovery of rapid eye movement (REM) sleep in 1953 by Eugene Aserinsky and Nathanial Kleitman. A mechanistic understanding is emerging from studies that have identified sleep related brain regions, neurotransmitters, and receptors. This review highlights some of the key neurotransmitters involved in the process of sleep cycle control, with an emphasis on sleep state dependent changes in neurotransmitter release. Figure 1 shows the location of the neurons that synthesize these neurotransmitters, and schematizes some of their projection pathways relevant for sleep.

2.1. Acetylcholine (ACh) was the first neurotransmitter discovered and is an important mediator of wakefulness, REM sleep, motor control, and the autonomic system. The brain contains two major cholinergic projection systems, both of which play a role in generating states of sleep and wakefulness. ACh-containing neurons in the basal

forebrain project to the neocortex and hippocampus, where acetylcholine functions to activate the electroencephalogram (EEG), mediate focused attention, and consolidate memories. Cholinergic neurons in the basal forebrain fire fastest during wakefulness and REM sleep, and discharge more slowly during non-REM (NREM) sleep. This discharge pattern of basal forebrain cholinergic neurons is consistent with the acetylcholine release pattern measured in the cerebral cortex (**Table A.1**). The degeneration of basal forebrain cholinergic neurons that occurs in Alzheimer's disease contributes to memory loss, daytime sleepiness, and disrupted nighttime sleep in these patients. A second group of acetylcholine containing neurons is found in the laterodorsal and pedunculopontine tegmental nuclei (LDT/PPT) of the brain stem. LDT/PPT neurons project up to the thalamus, hypothalamus, and basal forebrain, as well as down to numerous brain stem nuclei, including the pontine reticular formation. One group of cholinergic LDT/PPT neurons discharges selectively during REM sleep, consistent with the finding that acetylcholine release in the pontine reticular formation is greatest during REM sleep (**Table A.1**). A second group of LDT/PPT neurons fires fastest during wakefulness and REM sleep, consistent with the pattern of relatively high ACh release in the thalamus during wakefulness and REM sleep and relatively low thalamic ACh release during NREM sleep (**Table A.1**).

Table A.1. State Dependent Changes in Acetylcholine Release Vary According to Brain Region

Brain Region	Acetylcholine Release	Species	Reference
cerebral cortex	Wake = REM > NREM	cat	Science 192:161-162, 1971
DTF	REM > Wake = NREM	cat	Neurosci Letts 114: 277-282, 1990
caudate nucleus	Wake = REM > NREM	cat	ibid
NMC	Wake = REM > NREM	cat	Brain Res 580: 348-350, 1992
NPM	REM > Wake = NREM	cat	ibid
thalamus	Wake = REM > NREM	rat	J Neurosci 14: 5236-5242, 1994
cerebral cortex	Wake $\geq$ REM > NREM	cat	Brain Res 671:329-332, 1995
hippocampus	REM > Wake > NREM	cat	ibid
PRF	REM > Wake = NREM	cat	J Neurosci 17: 774-785, 1997
basal forebrain	REM > Wake > NREM	cat	Am J Physiol Regul Integr Comp Physiol 280:598-601, 2001

Table A.1 abbreviations: DTF (dorsal tegmental field, part of the pontine reticular formation), NPM (nucleus paramedianus, part of the medullary reticular formation), NMC (nucleus magnocellularis, part of the medullary reticular formation), PRF (pontine reticular formation)

Although acetylcholine release during sleep and wakefulness differs according to brain region, one consistent finding is that in all of the brain regions analyzed, acetylcholine levels are greatest when the EEG is activated, such as during REM sleep and wakefulness. During NREM sleep when the EEG is deactivated, acetylcholine release is at its lowest (**Table A.1**).

Acetylcholine exerts its effects by activating two major classes of receptors called nicotinic and muscarinic. Nicotinic receptors are ligand-gated ion channels and exist as 17 subtypes. Activating nicotinic receptors in the thalamus contributes to cortical activation by suppressing slow wave activity characteristic of NREM sleep. There are five muscarinic receptor subtypes. M1, M3, and M5 receptors are coupled to excitatory G proteins whereas M2 and M4 receptors are coupled to inhibitory G proteins. Acetylcholinesterase hydrolyzes acetylcholine to choline and acetic acid. Nerve gases inhibit acetylcholinesterase to increase acetylcholine levels, which leads to muscle paralysis and death.

Additional insights concerning the endogenous neurotransmitter regulating sleep have come from administering drugs into specific brain regions of experimental animals while recording sleep and wakefulness. Delivering drugs into the brain is possible, in part, because the brain has no pain receptors. Thus, sleep can be studied humanely in animals following drug administration into specific brain regions. These types of studies provide insights into the sleep related functions of specific brain regions and receptor subtypes localized within those brain regions. For example, pontine reticular formation microinjection of drugs that mimic the effects of acetylcholine causes an increase in REM sleep. These data fit well with the observation that acetylcholine release in the pontine reticular formation is increased during REM sleep (**Table A.1**). In the pontine reticular formation, drugs that are effective for increasing REM sleep includ those that inhibit the breakdown of endogenous acetylcholine and drugs that activate M2 muscarinic cholinergic receptors. Studies in humans have shown that administering low doses of acetylcholinesterase inhibitors also causes an increase in REM sleep. Taken together, these data support the interpretation that the cholinergic transmission in the pontine reticular formation contributes to the generation of REM sleep.

2.2. **Serotonin** is a monoamine neurotransmitter that contributes to the regulation of appetite, emotion, vascular smooth muscle tone, and wakefulness. Serotonin is synthesized by the raphé nuclei of the brain stem and these nuclei project widely to the rest of the brain. The dorsal raphé nucleus has been most thoroughly studied with respect to sleep and wakefulness. The firing rate of serotonergic dorsal raphé neurons is fastest during wakefulness, slower during NREM sleep, and slowest during REM sleep,

suggesting that serotonin promotes wakefulness and is inhibitory to REM sleep. Fourteen serotonin receptor subtypes have been identified, and are expressed throughout the brain and periphery. Clinically, serotonin receptor agonists are used for treating migraine, headache, and preventing nausea. Serotonin reuptake inhibitors, which block uptake of serotonin from the synapse to increase serotonin levels, are used to treat depression. A consistent finding across most brain regions studied to date is that serotonin levels are highest during wakefulness, lower during NREM sleep, and lowest during REM sleep (**Table A.2**). These measures of serotonin release support the interpretation that high serotonin levels are important for wakefulness, and that a decrease in serotonin release promotes sleep.

Table A.2. Serotonin Release is Greater during Wakefulness than during Sleep, Independent of Brain Region

Brain Region	Serotonin Release	Species	Reference
PRF	Wake > NREM > REM	cat	Neurosci Res 18:157-70, 1993
dorsal raphé	Wake > NREM > REM	rat	Neuroscience 83:807-814, 1998
frontal cortex	Wake > NREM > REM	rat	ibid
dorsal raphé	Wake > NREM > REM	cat	Brain Res 648: 306-312, 1994
PPT	Wake > NREM > REM	cat	Sleep Res Online 2:21-27, 1999
amygdala	Wake > NREM = REM	cat	Brain Res 860:181-189, 2000
locus coeruleus	Wake > NREM > REM	cat	ibid
hippocampus	Wake > NREM > REM	rat	Eur J Neurosci 17:1896-1906, 2003

Table A.2 abbreviations: PRF (pontine reticular formation) PPT (pedunculopontine tegmental nucleus)

2.3. Norepinephrine promotes wakefulness and is an important mediator of autonomic function and muscle tone. Neurons that synthesize norepinephrine are concentrated in the brain stem nucleus called the locus coeruleus. These neurons project rostrally to the forebrain and cerebral cortex, as well as caudally to the brain stem and spinal cord. Similar to the serotonergic neurons described above, noradrenergic neurons fire at their fastest rates during wakefulness, slow their discharge during NREM sleep, and cease firing during REM sleep. This "Wake-on/REM-off " discharge pattern is unique to monoaminergic neurons and is consistent with the wakefulness promoting role of norepinephrine. As a result of the unique state dependent discharge pattern, norepinephrine release is higher during wakefulness than during sleep in all brain regions studied (**Table A.3**).

Table A.3. Norepinephrine Release is Greater during Wakefulness than during Sleep, Independent of Brain Region

Brain Region	Norepinephrine Release	Species	Reference
locus coeruleus	Wake > NREM > REM	cat	Brain Res 860:181-189, 2000
amygdala	Wake > NREM > REM	cat, rat	Brain Res 860:181-189, 2000; J Korean Med Sci 17:395-399, 2002
nucleus accumbens	Wake > NREM = REM	rat	J Neurosci Res 81:891-899, 2005
prefrontal cortex	Wake > NREM = REM	rat	ibid

Clinically, norepinephrine has many uses including the treatment of shock because it acts as a vasoconstrictor. Norepinephrine binds to and activates α and β receptors. There are two α noradrenergic receptors, α1 and α2. The α2 receptors function as autoreceptors to regulate the release of norepinephrine. The α2 receptor agonist dexmedetomidine is used frequently in the intensive care unit setting to provide sedation. Xylazine is an α2 receptor agonist used clinically in veterinary practice for sedation. Preclinical studies have shown that dexmedetomidine inhibits the firing of wakefulness promoting locus coeruleus neurons.

2.4. Histamine is a monoamine synthesized in the tuberomammillary nucleus of the posterior hypothalamus. These neurons send projections throughout the brain, including the sleep-promoting anterior hypothalamus, the arousal promoting LDT/PPT, and the neocortex. Similar to the other monoamines serotonin and norepinephrine, histaminergic neurons fire with a wake-on/REM-off discharge pattern and function to promote wakefulness. Histamine also modulates the immune system and gastric acid secretion. Histamine is synthesized by the enzyme histadine decarboxylase. Knock out mice lacking the gene responsible for histamine synthesis show slow frequencies in the EEG during wakefulness, suggesting that they are less alert than control mice. In addition,

mice that cannot synthesize histamine do not show the normal increased wakefulness in response to a novel environment.

There are three known subtypes of histamine receptors, H1-H3, which are coupled to G proteins. H1 and H2 receptor antagonists are used clinically to block allergic responses and gastric acid secretions, respectively. First generation antihistamines cause drowsiness, and H1 receptor knockout mice show a decreased waking response to the arousal promoting peptide hypocretin (orexin). Less is known about H3 receptor subtype function except that it is an autoreceptor and modulates the release of histamine. Histamine release has only been studied in a few brain regions during sleep and is highest during wakefulness, similar to serotonin and norepinephrine (**Table A.4**).

Table A.4. Histamine Release is Greater during Wakefulness than during Sleep

Brain Region	Histamine Release	Species	Reference
preoptic hypothalamic area	Wake > REM > NREM	cat	Neuroscience 114:663-670, 2002
prefrontal cortex	Wake > Sleep	rat	Neurosci Res 49:417-420, 2004

Inhibiting tuberomammillary neurons with the $GABA_A$ receptor agonist muscimol causes a decrease in wakefulness and an increase in sleep, consistent with a wakefulness promoting role for histamine and a sleep-promoting role for GABA. Microinjection of an H1 receptor agonist into the LDT also causes an increase in wakefulness, as does administering histamine into the basal forebrain. Consistent with these finding are data showing that increasing histamine levels in the tuberomammillary nucleus by blocking the enzymatic degradation of histamine also increases wakefulness and decreases both NREM sleep and REM sleep. In general, the wakefulness promoting effects of histamine

are mediated by the H1 receptor, whereas increases in sleep are produced by drugs that activate H2 or H3 receptors.

2.5. Dopamine is the precursor of norepinephrine and mediates mood, motor activity, addiction, and the secretion of prolactin. The D1 family (D1, D5) of dopamine receptors is coupled to stimulatory G proteins to activate adenlycyclase. The D2 family (D2, D3, D4) of dopamine receptors is coupled to inhibitory G proteins that inhibit adenylcyclase. Similar to norepinehprine, dopamine is used clinically to treat shock. Dopamine neurons of the substantia nigra selectively degenerate in Parkinson's Disease, which is characterized by excessive daytime sleepiness and disrupted nighttime sleep. Dopamine receptor agonists are widely used to treat Parkinson's disease. The firing rates of dopaminergic neurons in the ventral tegmental area and substantia pars compacta do not vary across sleep-wake states. However, dopamine levels do vary across sleep-wake states in the frontal cortex, nucleus accumbens, and spinal cord (**Table A.5**). The locus coeruleus appears to not be a target region for dopaminergic regulation of arousal state as dopamine levels do not show state specific changes.

Table A.5. State Dependent Changes in Dopamine Release Vary According to Brain Region

Brain Region	Dopamine Release	Species	Reference
locus coeruleus	Wake = NREM = REM	cat	Brain Res 860:181-189, 2000
frontal cortex	Wake > NREM = REM	rat	J Neurosci Res 81:891-899, 2005
nucleus accumbens	Wake = REM > NREM	rat	ibid
spinal cord	Wake = NREM > REM	cat	J Neurophysiol 100:598-608, 2008

2.6. Hypocretins (also known as orexins) are neuropeptides discovered by separate groups in 1998 and play diverse roles in physiology by regulating arousal, feeding, nociception, energy homeostasis, neuroendocrine and cardiovascular functions. The two

subtypes of hypocretins, hypocretin-1 (orexin A) and hypocretin-2 (orexin B) are synthesized in the lateral, posterior, and perfornical hypothalamus and project widely throughout the brain. There are two receptor subtypes, hypocretin receptor-1 and hypocretin receptor-2. Hypocretin receptor-2 binds both peptides equally, whereas hypocretin receptor-1 is selective for hypocretin-1. Hypocretin deficiency underlies the sleep disorder narcolepsy, which is characterized by excessive daytime sleepiness and disrupted nighttime sleep, and can be accompanied by cataplexy, or the sudden loss of muscle tone.

One normal, physiological role of the hypocretins is the promotion of wakefulness. Hypocretin levels are greater in the hypothalamus and the basal forebrain during wakefulness and REM sleep (when the EEG is activated) when compared to NREM sleep (**Table A.6**). These data fit nicely with the increase in firing rate of basal forebrain neurons observed during wakefulness and the increase in activity seen from PET imaging in the basal forebrain. Hypocretinergic neurons in the lateral hypothalamic area also increase firing rate during wakefulness. Interestingly, hypocretin levels in locus coeruleus (wake promoting region) do not vary across sleep-wake states even though this is where the most hypocretin-1 receptors are located. Hypocretin levels are greater during wakefulness than during sleep in the lateral hypothalamus and medial thalamus (**Table A.6**).

Table A.6. Hypocretin Release is Lowest during NREM Sleep

Brain Region	Hypocretin/Orexin Levels	Species	Reference
lateral hypothalamus	Wake > Sleep	rat	Eur J Neurosci 14:1075-1081, 2001
hypothalamus	Wake = REM > NREM	cat	J Neurosci 22:5282-5286, 2002
basal forebrain	Wake = REM > NREM	cat	ibid
locus coeruleus	Wake = NREM = REM	cat	ibid

Microdialysis studies suggest a causal relationship between neuromodulator levels and states of wakefulness, NREM sleep, and REM sleep. Pharmacological approaches further describe the sleep-related roles of these sleep modulators in different brain regions. Understanding the interactions between sleep modulatory neurochemical systems in different brain regions as well as deciphering the effects of specific neurotransmitter receptor subtype on sleep-wake states will pave the way for better pharmacological therapeutics for disorders of sleep and mental health.

2.7. GABA is the major inhibitory neurotransmitter in the brain and is synthesized from glutamate. Unlike the neurotransmitters discussed above, GABA is released from glial cells as well as from neurons. These glial cells, or astrocytes, are in close proximity to synapses and modulate synaptic transmission. Furthermore, GABA transporters reside on both neurons and astrocytes, so GABA is taken up into astrocytes.

GABA binds to three types of receptors, $GABA_A$, $GABA_{\mathbf{B}}$, and $GABA_C$. $GABA_A$ and $GABA_C$ receptors are ligand-gated ion channels and $GABA_B$ is a G protein-coupled receptor. The $GABA_A$ receptor has binding sites for a variety of molecules that produce sedation, sleep, or general anesthesia. Benzodiazepines also act via the $GABA_A$ receptor to reduce anxiety. Almost all brain regions contain GABAergic interneurons. The basal forebrain also contains GABAergic projection neurons, which provide input to the cerebral cortex. GABA levels in the cortex during sleep and wakefulness have not been measured. GABAergic neurons in the anterior hypothalamus send projections to the wakefulness promoting monoaminergic neurons in the posterior hypothalamus, locus coeruleus, and dorsal raphé, as well as the cholinergic LDT/PPT neurons. GABA levels

in the dorsal raphé, locus coeruleus, and posterior hypothalamus are higher during sleep than during wakefulness (**Table A.7**). GABA levels in the LDT/PPT have not been measured during sleep and wakefulness, but GABAergic interneurons in the LDT/PPT are active during REM sleep. The $GABA_A$ receptor agonist muscimol causes sleep when microinjected directly into the posterior hypothalamus, but causes wakefulness when delivered directly into the anterior hypothalamus. These findings are consistent with the interpretation that GABA in these brain areas contributes to sleep generation by inhibiting wakefulness promoting neurons.

Table A.7. State Dependent Changes in GABA Levels

Brain Region	GABA Levels	Species	Reference
dorsal raphe	REM > Wake = NREM	cat	Am J Physiol 273: R451-R455, 1997
locus coeruleus	REM > NREM > Wake	cat	Neuroscience 78: 795-801, 1997
posterior hypothalamus	NREM > Wake = REM	cat	Am J Physiol Regul Comp Physiol 271:1707-1712, 1996
spinal cord	REM > Wake = NREM	cat	J Neurophysiol 100:598-608, 2008

GABA levels in the pontine reticular formation are greater during wakefulness than during NREM sleep, REM sleep, or general anesthesia. These data indicate that GABA in the pontine reticular formation contributes to the generation of wakefulness. A wakefulness promoting role for GABA is also supported by data showing that wakefulness is either increased or decreased by drugs that either increase or decrease, respectively, pontine reticular formation GABAergic transmission. GABA and acetylcholine interact in the pontine reticular formation to regulate REM sleep. Activation of $GABA_A$ receptors in the pontine reticular formation decreases REM sleep, in part, by decreasing acetylcholine release. Similarly, blocking $GABA_A$ receptors in the pontine reticular formation increases acetylcholine release and increases REM sleep. In summary, GABAergic inhibition either inhibits or promotes wakefulness, depending

upon the brain region. In brain regions that promote wakefulness, GABAergic inhibition functions to increase sleep. In brain regions that promote sleep, GABAergic inhibition contributes to an increase in wakefulness.

2.8. Glutamate is the major excitatory neurotransmitter in the brain and is the precursor to GABA. Similar to GABA, glutamate is synthesized and released from neurons and glial cells. Glutamate exhibits its actions by activating ionotropic NMDA, AMPA, or kainate receptors and metabotropic glutamatergic receptors. Little is known about the role of glutamate in sleep cycle control. Glutamate is excitatory, and in most brain areas, glutamate levels are highest during wakefulness or REM sleep, which are both activated brain states (**Table A.8**).

Table A.8. Glutamate Levels Vary Across the Sleep Wakefulness Cycle in a Brain Region Specific Manner

Brain Region	Glutamate Levels	Species	Reference
NMC	REM > NREM = Wake	cat	Brain Res 780:178-181,1998
NPM	Wake= NREM = REM	cat	ibid
NAcc	Wake > NREM = REM	rat	J Neurosci Res 81:891-899, 2005
prefrontal cortex	Wake = NREM = REM	rat	ibid
oFC	REM > Wake > NREM	rat	Arch Med Res 38:52-55, 2007
spinal cord	REM > NREM = Wake	cat	J Neurophysiol 100:598-608, 2008
PH/TMN	Wake = REM > NREM	rat	Am J Physiol Regul Integr Comp Physiol 295:R2041-R2049, 2008

Table A.8 abbreviations: NPM (nucleus paramedianus), NMC (nucleus magnocellularis), NAcc (nucleus accumbens, oFC (orbital frontal cortex), TMN (tuberomamillary nucleus)

The results from studies that have microinjected glutamatergic drugs into the brain and quantified the effects on sleep and wakefulness have shown that glutamate promotes either wakefulness or REM sleep, depending on the brain region where glutamate is administered. Glutamate at lower doses delivered directly into the PPT causes REM sleep and at higher doses causes wakefulness. The increase in REM sleep is mediated by kainate receptors and the increase in wakefulness results from activating

NMDA receptors. Furthermore, activating the metabotropic glutamate subtype II receptor decreases wakefulness and increases NREM sleep. These data demonstrate that endogenous glutamate is an important regulator of arousal and suggest that in the PPT, activation of kainate and NMDA receptors is important for inducing sleep whereas activating metabotropic subtype II receptors is important for inducing wakefulness. Administration of glutamate to the dorsal raphé or basal forebrain increases wakefulness and decreases sleep.

Ketamine is an NMDA receptor agonist and is used clinically to produce a state of dissociative anesthesia. The term dissociative arose from the finding that when ketamine is used for surgical procedures, sensory information reaches the cortex but is not accurately perceived as painful, thus causing a dissociation between objective and subjective experience. When given intravenously or directly into the pontine reticular formation, ketamine suppresses REM sleep and decreases the release of the REM sleep promoting transmitter acetylcholine.

2.9. Adenosine is a purine nucleoside that is derived from the breakdown of ATP, and adenosine has many physiological functions. The function of adenosine most relevant for this volume is as an endogenous promoter of sleep. There are four adenosine receptor subtypes, A1, A2a, A2b and A3, which are G-protein-coupled receptors. A1 and A3 receptors inhibit adenyl cyclase activity whereas A2a and A2b receptors activate adenyl cyclase. One of the most widely used legal drugs in the world is caffeine, which promotes wakefulness and increases alertness by blocking adenosine receptors.

Adenosine levels in the brain increase during wakefulness and decrease during sleep (**Table A.9**).

Table A.9. Adenosine Levels Increase during Wakefulness and Decrease during Sleep

Brain Region	Adenosine Levels	Species	Reference
basal forebrain	Wake > NREM = REM	cat	Neuroscience 99:507-517, 2000
cortex	Wake > NREM = REM	cat	ibid
thalamus	Wake > NREM = REM	cat	ibid
preoptic area	Wake > NREM = REM	cat	ibid
dorsal raphé	Wake = NREM = REM	cat	ibid
PPT	Wake = NREM = REM	cat	ibid

Table A.9 abbreviations: PPT (pedunculopontine tegmental nucleus)

Adenosine levels in different brain regions have been quantified during sleep deprivation and subsequent recovery sleep. These studies show that changes in adenosine levels during sleep deprivation and recovery sleep were different depending on the brain region analyzed. For example, adenosine levels in the cortex and basal forebrain accumulate during prolonged wakefulness and fall during recovery sleep. In contrast, adenosine levels do not increase in the dorsal raphé or pedunculopontine tegmental nucleus during sleep deprivation. Furthermore, adenosine acting on A1 receptors inhibits wakefulness promoting neurons in the basal forebrain. Adenosine also inhibits wakefulness promoting histaminergic and hypocretinergic neurons in the posterior and lateral hypothalamus, and increases the activity of anterior hypothalamic GABAergic neurons that promote sleep.

Pharmacological approaches have provided novel evidence that adenosine is sleep promoting. Adenosine A1 receptor antagonists delivered directly into the pontine reticular formation or the prefrontal cortex shorten recovery time from general anesthesia, whereas A1 receptor agonists increase anesthesia recovery time. Administering adenosine into the lateral dorsal tegmental nucleus decreases wakefulness and increases

sleep. Adenosine A1 receptor activation in the pontine reticular formation increases REM sleep and decreases wakefulness. Microinjection of A2a agonist into the pontine reticular formation increases acetylcholine release and increases REM sleep. Taken together, current data demonstrate that although adenosine levels are highest during wakefulness (**Table A.9**), adenosine is a sleep promoting agent.

3. Concluding Remarks

Sleep is important for many physiological functions, including the immune system, cognition, learning and memory, and mental health. As of 2006, over 70 million Americans suffer from sleep related disorders, costing an estimated 15.9 billion dollars (http://www.sleepmed.md/page/1896). The elderly population is increasing, and the incidence of sleep disorders increases with aging. Understanding how sleep is regulated by neurotransmitters and neuromodulators, and discerning how these transmitter and modulator system interact will improve the medications prescribed for sleep disorders. In 2008 a study demonstrated that the inverse histamine-3 receptor agonist tiprolisant reduces excessive daytime sleepiness in narcoleptic patients and in hypocretin deficient mice. Modafinil is another drug used to reduce excessive daytime sleepiness, and although the mechanism of action is not well understood modafinil acts through multiple neurotransmitter systems. Olanzapine is an atypical antipsychotic that improves sleep in schizophrenics and acts at several neurotransmitter receptors. Future studies will need to focus more on elucidating interactions between multiple neurotransmitter systems and on interacting sleep related brain regions.

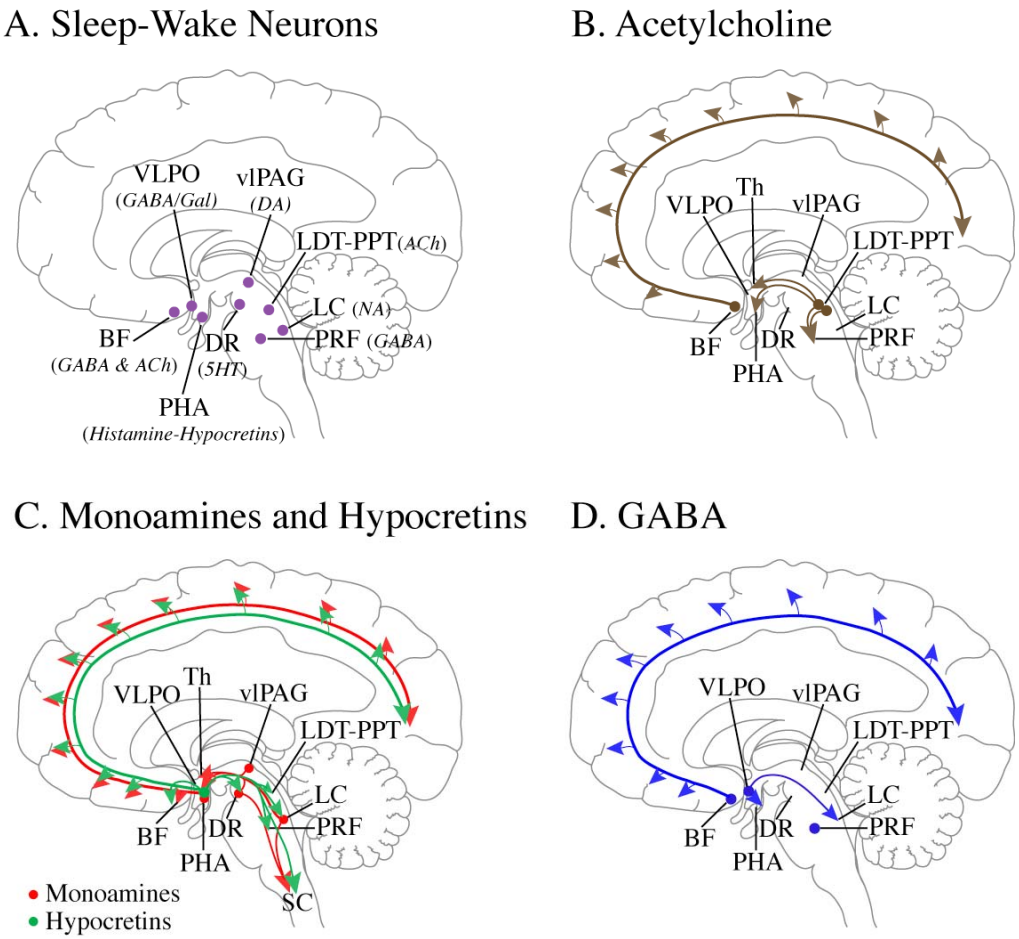

Figure A.1. Localization of the major neuronal groups and pathways regulating sleep and wakefulness on a drawing of the human brain. A shows the major sleep-wake related neurons and their neurotransmitters. B indicates cholinergic projections from the basal forebrain and the brainstem. C schematizes the monoaminergic and hypocretinergic wakefulness promoting neurons and their projections. D draws the projections from GABAergic neurons in the basal forebrain and preoptic area. Abbreviations: ACh, acetylcholine; BF, basal forebrain; DR, dorsal raphe nucleus; DA, dopamine; Gal, galanin; LC, locus coeruleus; LDT-PPT, laterodorsal and pedunculopontine tegmental nucleus; NA, noradrenaline; PHA, posterior hypothalamic area; SC, spinal cord; 5-HT, serotonin; Th, thalamus, vlPAG, ventrolateral periaqueductal gray; VLPO, ventrolateral preoptic area. From: Vanini G, Baghdoyan HA, and Lydic R. Relevance of sleep neurobiology for cognitive neuroscience and anesthesia. In: *Awareness and Consciousness in Anesthesia*, edited by GA Mashour, Oxford University Press, in press, 2009.

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During the three years I spent in Dr. Jorge Iñiguez-Lluhí's laboratory as a graduate student, my research focused on transcriptional regulation by the Small Ubiquitin-like Modifier (SUMO) family of proteins. One of my first projects in the lab was to determine whether the Krüppel-type zinc finger binding protein ZBP-89 was SUMO-modified *in vitro.* The published manuscript shown below includes the results from *in vitro* SUMOylation assays that I performed under the mentorship of Dr. Jorge Iñiguez-Lluhí.

SUMOYLATION-DEPENDENT CONTROL OF HOMOTYPIC AND HETEROTYPIC SYNERGY BY THE KRUPPEL-TYPE ZINC FINGER PROTEIN ZBP-89

Sergey Chupreta, Holly Brevig, Longchuan Bai, Juanita L. Merchant and Jorge A. Iñiguez-Lluhí

SUMMARY

The Krüppel-like transcription factor ZBP-89 is a sequence-specific regulator that plays key roles in cellular growth and differentiation, especially in endodermal and germ cell lineages. ZBP-89 shares with other members of the Sp-like family an overlapping sequence specificity for GC rich sequences in the regulatory regions of multiple genes. Defining the mechanisms that govern the intrinsic function of ZBP-89 as well as its competitive and non-competitive functional interactions with other regulators is central to understand how ZBP-89 exerts its biological functions. We now describe that post-translational modification of ZBP-89 by multiple SUMO isoforms occurs at two conserved synergy control motifs flanking the DNA binding domain. Functionally, SUMOylation does not directly alter the ability of ZBP-89 to compete with other Sp-like factors from individual sites. At promoters bearing multiple response elements however, this modification inhibits the functional cooperation between ZBP-89 and Sp1. Analysis of the properties of ZBP-89 in cellular contexts devoid of competing factors indicates that although on its own it behaves as a modest activator, it potently synergizes with heterologous activators such as the glucocorticoid receptor. Notably, we find that when conjugated to ZBP-89, SUMO exerts a strong inhibitory effect on such synergistic

interactions through a critical conserved functional surface. By regulating higher-order functional interactions, SUMOylation provides a reversible post-translational mechanism to control the activity of ZBP-89.

INTRODUCTION

Eukaryotic ZBP-89 (also known as BERF-1, BFCOL1 or ZNF148) is a widely expressed Krüppel-type transcription factor that harbors an N-terminal cluster of four (three canonical C2H2 and a variant C2H2) zinc-finger motifs. *In vivo* experiments indicate that ZBP-89 plays important roles in the generation and maintenance of specific cell lineages since haploinsufficiency causes infertility in mice due to apoptosis of germ cells (Takeuchi et al., 2003) whereas knockdown of ZBP-89 in zebrafish disrupts early steps in hematopoiesis (Li et al., 2006). ZBP-89 also appears to play important roles in the gastrointestinal tract since mice exclusively expressing a splice variant lacking the first 127 residues display increased sensitivity to colitis (Law et al., 2006b), whereas intestinal overexpression of ZBP-89 activates apoptosis and mitigates neoplasia burden in ApcMin/+ mice (Law et al., 2006a). Functionally, ZBP-89 has been most often linked to transcriptional inhibition of genes such as those for gastrin, vimentin or ornithine decarboxylase (ODC) (Merchant et al., 1996; Law et al., 1998a; Law et al., 1998b; Wieczorek et al., 2000; Park et al., 2003; Boopathi et al., 2004). ZBP-89 shares with Sp1 and other Sp-like factors the ability to recognize GC-rich sequences in target genes. This overlapping DNA recognition has led to a competitive model of inhibition where ZBP-89 represses gene transcription by displacing proteins such as Sp1 or Sp3 (Merchant et al., 1996; Cheng et al., 2000). Thus, an analysis of the proximal ODC gene promoter revealed that Sp1 and ZBP-89 bind to GC elements in a mutually exclusive manner (Law et al., 1998b). In other cases, ZBP-89 appears to inhibit by binding to DNA independently of Sp1 (Zhang et al., 2003). In addition to its role in transcriptional

inhibition, ZBP-89 has been implicated as a modest positive regulator of a set of genes including p21, lymphocyte-specific protein-tyrosine kinase (lck) and stromelysin (Ye et al., 1999; Yamada et al., 2001; Malo et al., 2005). In the case of the p21Waf1 promoter, recruitment of the coactivator p300 appears to contribute to activation of this gene (Bai and Merchant, 2000). The overlap in DNA binding specificity with widely expressed Sp-like factors however, makes the mechanistic analysis of ZBP-89 function particularly challenging. Broad dissection of ZBP-89 suggests that distinct regions are involved in negative and positive regulation with repressive and transactivating functions in the N-terminal and C terminal regions, respectively (Hasegawa et al., 1997; Passantino et al., 1998). How these still poorly defined functions are selectively employed in different contexts and how ZBP-89 engages in competitive and non-competitive functional interactions with other factors remain poorly understood.

An emerging mechanism to regulate higher order interactions among transcription factors involves the function of Synergy Control or SC motifs (Iñiguez-Lluhí and Pearce, 2000). These short regulatory sequences, which we first identified in the glucocorticoid receptor (Iñiguez-Lluhí and Pearce, 2000); are present in multiple factors across a wide range of families. SC motifs are distinct from transcriptional activation functions and often map to negative regulatory regions. Functionally, SC motifs limit the synergistic transcriptional output from complexes assembled at multiple response elements without altering the activity of a regulator from a single site (Iñiguez-Lluhí and Pearce, 2000; Subramanian et al., 2003; Komatsu et al., 2004). Mechanistically, we and others have demonstrated that SC motifs operate in an autonomous manner by serving as sites for reversible post-translational modification by members of the Small Ubiquitin-like

Modifier (SUMO) family of proteins (Poukka et al., 2000; Holmstrom et al., 2003; Subramanian et al., 2003; Komatsu et al., 2004).

The mammalian SUMO family consists of four genes designated SUMO1-4. SUMO2 and 3 are closely related whereas SUMO1 shares ~ 48% identity to either SUMO2 or 3 (Saitoh and Hinchey, 2000; Su and Li, 2002). A more recently identified gene encodes a fourth isoform very similar to SUMO2/3 (Bohren et al., 2004). SUMO proteins are structurally related to ubiquitin, and the analogous SUMO modification pathway is carried out by a distinct, SUMO-specific set of enzymes. Following translation, processing by SUMO proteases removes C-terminal residues in SUMO to expose a conserved di-glycine motif (Johnson, 2004). Notably, SUMO4 harbors a Pro residue at position 90, which prevents initial processing by known SUMO protease enzymes and subsequent conjugation (Owerbach et al., 2005). Whether this member functions through non-covalent interactions only remains to be determined. After this initial cleavage, SUMO is then activated in ATP-dependent manner by the heterodimeric E1-activating enzyme SAE1/SAE2. The thioester-linked SUMO is then transferred to the SUMO specific E2-conjugating enzyme Ubc9 which in turn, recognizes specific substrates and catalyzes the formation of an isopeptide bond between SUMO and the target lysine. This step is facilitated by SUMO E3 ligases such as RanBP2 and members of the protein inhibitor of activated STAT (PIAS) family (Sachdev et al., 2001; Pichler et al., 2002; Chun et al., 2003). Finally, SUMO conjugation is reversed through the isopeptide activity of SUMO specific proteases (Yeh et al., 2000). Recent functional and structural analysis by our group (Chupreta et al., 2005) indicates that once conjugated, individual SUMO isoforms mediate their distinct effects through a conserved surface that

interacts with Val/Leu rich motifs in target proteins (Song et al., 2004; Reverter and Lima, 2005; Lin et al., 2006; Rosendorff et al., 2006). The nature of the SUMO-interacting proteins responsible for SUMO-dependent transcriptional inhibition remains poorly defined but may include HDACS and DAXX (Yang and Sharrocks, 2004; Lin et al., 2006) as well as other proteins (Rosendorff et al., 2006). Although the role of SC motifs and SUMOylation in the regulation of the synergistic effect of potent transcriptional regulators such as the glucocorticoid receptor is becoming better understood (Holmstrom et al., 2003; Chupreta et al., 2005), the impact of this regulatory mechanism on factors that are generally viewed as dominant-negative or "decoy" relatives of other factors remains largely unexplored.

In the present study, we identify and characterize two synergy control motifs that flank the zinc-finger cluster DNA-binding domain of ZBP-89. We find that the conserved lysines (K115 and K356) within these SC motifs serve as the main sites for conjugation by members of the SUMO family. By taking advantage of cellular contexts that harbor or are devoid of Sp-like factors, we demonstrate that the SC motifs do not alter the ability of ZBP-89 to displace Sp-like factors. Rather, we find that by recruiting SUMO to ZBP-89, the SC motifs potently limit the intrinsic ability of ZBP-89 to synergistically activate transcription from reiterated binding sites (homotypic synergy) and to cooperate with nearby-bound heterologous factors (heterotypic synergy).

MATERIALS AND METHODS

Expression plasmids – An N-terminal, hexa-histidine-tagged, CMV-driven human ZBP-89 expression plasmid was generated in two steps. Oligonucleotides 5'-CTAGCGGCACCATGGA-GCATCACCACCATCATCATG-3' and 5'-GA-

TCCATGATGATGGTGGTGATGCTCCATG-GTGCCG-3', which contain a starting ATG codon within a consensus Kozak's sequence followed by a hexa-histidine-tag were annealed and ligated into the BamHI and NheI sites of pcDNA3.1 (+) to yield pcDNA3.1 (+)-His. Human ZBP-89 cDNA was then PCR amplified using forward primer 5'-GATGGATCCATGA-ACATTGACGACAAACTGGAAGG-3' and reverse primer 5'-CATCTCGAGTTAGCCAA-ACGTCTGGCCAGTTGTG-3'. The resulting fragment was digested with BamHI and XhoI and ligated into the same sites of pCDNA3.1(+)-His to yield pcDNA3.1 (+)-His-ZBP-89. Single and double point mutations that replaced Lys 115 and 356 with Arg were prepared using the QuikChange™ site-directed mutagenesis approach using pcDNA3.1 (+)-His-ZBP-89 as a template. N-terminal HA-tagged ZBP-89 expression plasmids were generated by ligating the 3092 bp BamHI/XbaI fragment from pcDNA3.1(+) His ZBP-89 into the same sites of pcDNA3.1(+) HA (Holmstrom et al., 2003). The expression vector for the non-cleavable fusion of HA-SUMO1 at the N terminus of ZBP-89 K115/356R was generated through excision of the HA-SUMO portion from pcDNA3 HA-SUMO1(-Gly) Gal4 (Holmstrom et al., 2003) as an NheI/BamHI fragment and ligation into the same sites of pcDNA3.1(+) His-ZBP-89 K115/356R. Fusions to HA-SUMO2, HA-SUMO2 K33E/K42E and SUMO3 were generated the same way using the corresponding pcDNA3 HA-SUMO (-Gly) Gal4 vectors (Holmstrom et al., 2003; Chupreta et al., 2005). The RSV-driven control (pS6R) and WT rat GR (p6R GR) expression vectors as well as the CMV-driven (pCDNA3) expression vectors for HA tagged SUMO1, SUMO2 and SUMO3 are described in (Iñiguez-Lluhí et al., 1997) and (Holmstrom et al., 2003). pCMV β-gal is a CMV-driven β-galactosidase expression vector and was used to correct for transfection efficiency.

To generate ZBP-89 *Drosophila* expression plasmids, driven by the actin 5C promoter, two oligonucleotides 5'-GATCAGCTAGCGA-ATTCTCTAGAA-3' and 5'-AGCTTTCTAG-AGAATTCGCTAGCT-3' containing NheI and XbaI restriction sites were annealed and ligated into the HindIII and BamHI sites of pA5C (kindly supplied by Dr. Thomas Kornberg, University of California, San Francisco) to yield pA5C*. NheI/XbaI fragments containing His-tagged WT or mutant ZBP-89 were excised from the corresponding pcDNA3.1 (+)His-ZBP-89 plasmids and ligated into the same sites of pA5C* to yield pA5C His-ZBP-89 and pA5C His-ZBP-89 K115/356R respectively. Non-cleavable fusions of HA SUMO isoforms at the N terminus of ZBP-89 K115/356R were generated by insertion of NheI/BamHI fragments exactly as described above for the mammalia expression of rat GR (pA5C GR) was generated by transferring the GR coding region (as a BamHI fragment) from p6RGR into the BamHI site of pA5C. pPac Sp1 (Courey and Tjian, 1988) and pActin β-gal (gift of Dr. Ken Cadigan, Univ. Michigan) are expression plasmids for Sp1 and β-galactosidase respectively and are driven by the actin 5C promoter.

Reporter plasmids –The pΔODLO 02 reporter plasmid where a minimal *Drosophila* distal alcohol dehydrogenase promoter (-33 to +55) drives the luciferase gene is described in (Subramanian et al., 2003). Luciferase reporter plasmids bearing one or more ZBP binding sites were generated as follows: The oligonucleotides 5'-GAGCCCCG-CCCCTCCCCCGCA-3' and 5'-GATCTGCGG-GGGAGGGGCGGG-3', containing a single ZBP-89 binding site from the ODC promoter (-114 to -100) (Law et al., 1998b) were annealed and ligated into the BamHI and BglII sites of pΔODLO 02 to yield pΔ$(ODC)_1$-Luc. Similar ligation of oligonucleotides 5'-

GATCCTGTACAGGATG-TTCTA-3' and 5'-GATCTAGAACATCCTGT-ACAG-3' bearing a GRE from the tyrosine aminotransferase gene (Jantzen et al., 1987) yielded $p\Delta(TAT)_1$-Luc. Reporter plasmids containing multiple ZBP-89 or GR binding sites were generated as follows: Ligation of independent BseRI/BglII and BamHI/BseRI fragments of $p\Delta(ODC)_1$-Luc yielded $p\Delta(ODC)_2$-Luc. The same operation applied to $p\Delta(ODC)_2$-Luc and $p\Delta(TAT)_1$-Luc yielded $p\Delta(ODC)_4$-Luc and $p\Delta(TAT)_2$-Luc. Ligation of BseRI/BglII and BamHI/BseRI fragments of $p\Delta(ODC)_1$-Luc and $p\Delta(ODC)_2$-Luc respectively yielded $p\Delta(ODC)_3$-Luc. The reporter plasmid bearing one ZBP-89 site upstream of a single glucocorticoid response element ($p\Delta(ODC)_1(TAT)_1$-Luc) was generated by ligation of BseRI/BglII and BamHI/BseRI fragments from $p\Delta(ODC)_1$-Luc and $p\Delta(TAT)_1$-Luc respectively. The same strategy using $p\Delta(ODC)_2$-Luc and $p\Delta(TAT)_2$-Luc yielded $p\Delta(ODC)_2(TAT)_2$-Luc. The pODC-Luc reporter plasmid harbors the region from –400 to +79 of the human ornithine decarboxilase gene in the pGL2 plasmid (Promega, Madison, WI).

The *Drosophila* vector for *Cell Culture and Transfections* – Human embryonic kidney 293T cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 5% fetal bovine serum (FBS). Human AGS gastric carcinoma cells were cultured in DMEM media containing 10% FBS. *Drosophila melanogaster* S2 cells were grown in Schneider's medium (GIBCO) supplemented with 10% heat-inactivated FBS at 25 °C. Except for data in Fig 2B, all cell lines were transfected by using Lipofectamine™ and plus™ reagent (Invitrogen). For *in vivo* SUMOylation experiments, 293T cells ($2x10^6$) were seeded onto 10 cm plates. After 24 h, cells were co-transfected with 3 μg of His-tagged ZBP-89 expression plasmids and 7 μg of HA-

SUMO expression plasmids or empty vector as indicated in Fig. 3. Cells were harvested 24 h after transfection in 0.75 ml of urea lysis buffer (8 M urea, 0.5 M NaCl, 45 mM Na_2HPO_4, 5 mM NaH_2PO_4, 10 mM imidazole, pH 8.0) and sonicated. Lysates were incubated with 0.1 ml of 50% Ni^{2+}-NTA-agarose (Qiagen) equilibrated in lysis buffer for 1 h at room temperature with rocking. The resin was then washed three times with 10 bed volumes of wash buffer 1 (8 M urea, 0.4 M NaCl, 17.6 mM Na_2HPO_4, 32.4 mM NaH_2PO_4, 10 mM imidazole, pH 6.75) and two times with wash buffer 2 (buffer 1 with 150 mM NaCl and no urea). Proteins were eluted by boiling in 50 µl 2X Laemli sample buffer, resolved by 7.5% SDS-PAGE and processed for immunoblotting as described below.

For *in vivo* SUMOylation in AGS cells, $2x10^6$ cells were seeded in 10 cm plates and transiently transfected using the calcium phosphate method with the constructs indicated in the figure legend. 40 hours post-transfection, cells were lysed for 10 min on ice with 0.75 ml of high salt lysis buffer (20 mM Hepes, pH 7.5, 5 mM EDTA, 1 mM EGTA 1% NP40, 400 mM NaCl, 20 mM N-ethylmaleimide (NEM), and 1 tablet per 10 ml of buffer of EDTA-free protease inhibitors (Roche). After 5 minutes, NEM was quenched by addition of DTT to 40 µM final and a further 2 min. incubation. Five percent of the sample was reserved for further analysis, and the remaining cell extracts were incubated with monoclonal HA-11 antibody (Covance, Berkeley, CA) and protein-A agarose (Invitrogen) at 4 °C overnight. The immuno- precipitates were washed four times in low salt lysis buffer (high salt lysis buffer containing 200 mM NaCl), and eluted in 2X Laemli sample buffer. Samples were resolved by 7.5% SDS-PAGE and processed for immunoblotting as described below.

For functional assays, AGS cells ($2x10^4$) were seeded (0.5 ml) onto 24-well plates. Cells were transfected 24 h later with the indicated amounts of ZBP-89 expression plasmids. To control for promoter dosage effects, cells treated with sub-maximal amounts of ZBP-89 expression vector also received the required amount of empty expression vector to maintain equimolar doses of expression plasmid. Cells also received 25 ng of the indicated luciferase reporter plasmid and 25 ng of pCMV β-gal. For Fig.5D, cells also received 20 ng of p6RGR expression plasmid. In all cases, the total amount of DNA was supplemented to 0.3 μg/well with pBSKS(+). For experiments including GR, media was supplemented 16 h post-transfection to 10 nM dexamethasone (Dex) or vehicle (0.1% ethanol). Cells were lysed 36 h after transfection and luciferase and β-galactosidase activities were determined as described (Iñiguez-Lluhí et al., 1997). Functional assays in S2 cells were carried out by seeding in 24-well plates (8×10^4 cells/well, 0.5 ml) followed 24 h later by transfection with the amounts of expression plasmids indicated in the figure legends. All conditions contained equimolar amounts of each type of expression vector to control for promoter dosage effects. Cells also received 50 ng of the indicated luciferase reporter plasmid and 50 ng of pActin β-gal. For the GR experiments, transfections included 100 ng of pA5C GR and cells were stimulated with 100 nM Dex for the last 24 hr. The total amount of DNA was supplemented to 0.3 μg/well with pBSKS(+). Cells were harvested 48 h post-transfection and processed as described above.

Immunoblotting – For the *in vivo* SUMOylation experiments, immunoblots were probed with primary mouse anti tetra-His (Qiagen, USA), rabbit anti-ZBP-89 antiserum or HA-11 (Covance, Berkeley, CA) monoclonal antibodies followed by goat anti mouse or

donkey anti-rabbit IgG peroxidase conjugated (Bio-Rad) secondary antibodies. ZBP-89 and GR expression levels were confirmed by western blotting. Cells were transfected as described for the functional assays and lysed in 1X SDS PAGE sample buffer containing 150 mM NaCl 36 h after transfection, resolved on 7.5% SDS-PAGE gels and processed for immunoblotting. Mammalian transfection samples were probed with HA-11 (Covance, Berkeley, CA) monoclonal antibody followed by goat anti mouse IgG peroxidase conjugate (Bio-Rad) as secondary. For transfections in *Drosophila* cells, ZBP-89 and derivatives were detected with an anti ZBP-89 rabbit serum followed by secondary goat anti-rabbit IgG peroxidase conjugate (Bio-Rad). GR was detected using BuGR2 (Covance, Berkeley, CA) primary and goat anti-mouse IgG peroxidase conjugate secondary antibodies. Images were captured in a Kodak Image Station 440 CF using Super Signal West Femto substrates (Pierce). All the experiments were performed at least thrice with similar results

Protein-protein interaction and in vitro SUMO conjugation assays – ZBP-89 forms or luciferase were synthesized *in vitro* using the T7-TNT Quick™ Coupled Transcription-Translation system (Promega) in the presence of [^{35}S] methionine using pCDNA3.1(+) His-ZBP-89 or Luciferase plasmids as templates. Binding reactions (50 µl) were performed at 4 °C and contained 1.2 nmol of GST or GST-UBC9 fusion protein (Subramanian et al., 2003) bound to 20 µl of glutathione-Sepharose 4B (Amersham Biosciences) in binding buffer (50 mM NaCl and 1 mg/ml bovine serum albumin) and 10 µl of radiolabeled proteins. After 1 h incubation with rocking, the resin was washed four times with 0.1% Nonidet P-40 in phosphate-buffered saline. Samples were eluted by boiling in 40 µl of SDS-PAGE sample buffer, resolved on 7.5% SDS-PAGE gels and

radioactive proteins were visualized using a PhosphorImager screen (Amersham Biosciences).

SUMOylation reactions were carried out in 20 µl of 50 mM Tris (pH 7.5), 5 mM $MgCl_2$ and in presence (as indicated) of 1 µg of GST SAE2/SAE1, 5 µg of GST hUBC9, 5 µg of His-SUMO-1GG (Subramanian et al., 2003) and 5 µl of *in vitro* translated [^{35}S] labeled His-ZBP-89 WT or its SUMOylation deficient mutants or luciferase control as indicated. Reactions were initiated by the addition of an ATP regeneration system (final concentrations: 10 units/ml creatine kinase, 25 mM phosphocreatine, 5 mM ATP) and pyrophosphatase (0.6 units/ml final). Samples were incubated at 30° C for 90 min and reactions were terminated by addition of 20 µl disruption buffer (50 mM Tris-HCl, pH 6.8, 1.67% SDS, 10% glycerol, 0.24 M β-mercaptoethanol, 0.015% bromophenol blue) and boiling for 5 min. Samples were resolved by 7.5% SDS-PAGE and radioactive proteins were visualized as above.

RESULTS

ZBP-89 interacts with Ubc9 and is a target of SUMOylation at two conserved SC motifs – Bioinformatic analysis of ZBP-89 revealed the presence of two sequences resembling synergy control motifs located in the N-terminal half of the protein surrounding the zinc-finger DNA binding domain (Fig. A.2.1). Analysis of the sequence conservation in the SC motif regions shows that the cardinal features of SC motifs, including flanking proline residues, are conserved across vertebrate species, including distant teleost fish. In contrast, surrounding sequences and the third and non-essential position of the SC motif are more variable, suggesting the presence of a selective pressure for the conservation of the SC motifs and their associated functions. SC motifs

serve as sites for SUMOylation through their direct interaction with Ubc9. Notably, we recently isolated Ubc9 in a yeast two hybrid screen using ZBP-89 as bait (data not shown). To confirm this interaction, we carried out *in vitro* interaction assays using immobilized GST-Ubc9 fusion protein as an affinity resin. As can be seen in Fig. A.2.2A, *in vitro* transcribed and translated ZBP-89 is efficiently retained on this matrix but no binding is observed to an equimolar amount of GST. In arginines (ZBP-89 mut) did not interfere with this interaction (Fig. A.2.2A, right).

The identification of Ubc9 as an interaction partner of ZBP-89 coupled with the presence of well-conserved synergy control motifs within its sequence strongly suggested that this factor is likely to be subject to SUMO modification. To examine this possibility directly, we transiently transfected human AGS gastric carcinoma cells with expression vectors for HA-tagged SUMO3 and ZBP-89 (Fig. A.2.2). Immunoblotting of cell extracts with the anti-ZBP-89 antibody reveals the expression of both endogenous as well as exogenous ZBP-89 (middle panel). Similarly, probing with an anti-HA antibody demonstrates the incorporation of SUMO3 into cellular proteins (lower panel). Analysis of HA immunoprecipitates from cells expressing both ZBP-89 and HA SUMO3 revealed the presence of a ~160 kDa slower mobility form in addition to smaller amounts of unmodified ZBP-89 (Fig. A.2.2B top panel). The upper band is consistent with SUMO modification whereas the unmodified material probably derives from dimerization of modified and non-modified forms of ZBP-89. Notably, small amounts of a similar slow-migrating species are also detected from cells expressing exclusively HA-SUMO3, suggesting that endogenous ZBP-89 is also modified. To confirm and characterize the SUMOylation of ZBP-89, we expressed N-terminally His-tagged WT ZBP-89 and HA-

tagged SUMO1, SUMO2, or SUMO3 in HEK 293T cells. Cells were lysed under denaturing conditions (8M Urea) in an effort to preserve the SUMOylation status of cellular proteins and to disrupt non-covalent interactions. The cleared lysates were subjected to Ni^{2+} chelate chromatography to isolate his-tagged ZBP-89, and samples were resolved by SDS-PAGE and analyzed by western blotting. As can be seen in Fig. A.2.3A, co-expression of ZBP-89 with any of the three SUMO isoforms resulted in the appearance of a ~160 kDa HA immunoreactive band. This species was not observed when either SUMO or ZBP-89 were expressed alone and is indicative of a SUMO-modified form of ZBP-89. As in the case of other transcription factors, (Johnson, 2004; Hay, 2005) the stoichiometry of modification appears to be relatively low since the SUMO-modified contrast, firefly luciferase, which lacks sequences resembling SC motifs, did not bind to either matrix. Consistent with the known binding properties of Ubc9, replacement of the lysine residues within the SC motifs by form was difficult to detect with the ZBP-89 directed antibodies we examined. Although we have not analyzed the linkage topology, the large apparent size of this form may be consistent with a single SUMO modification since branched SUMO-modified proteins often run anomalously in SDS-PAGE. Moreover, ZBP-89 may be particularly prone to such anomalous behavior since the full-length protein migrates substantially slower than the predicted 89 kDa (Fig. A.2.3B). Analysis of crude cell lysates revealed that SUMO conjugates as well as free SUMO accumulate to substantially higher levels in the case of SUMO2 and 3 when compared to SUMO1 (see supplemental Figure 1 and (Subramanian et al., 2003)). Nevertheless, comparable levels of ZBP-89 SUMOylation were observed with all three SUMO isoforms. Whether this reflects a relative preference for SUMO1

modification of ZBP-89 remains to be explored in more detail. To examine the role of the putative SC motifs in ZBP-89 SUMOylation, samples were isolated from cells expressing ZBP-89 mutants bearing a Lys to Arg substitution in either the first (K115R), second (K356R) or both SC motifs (K115/356R). As can be seen in Fig. A.2.3A, each substitution did not substantially reduce the ~160 kDa form whereas the double mutant essentially eliminated it. Probing of the same samples with an anti-His-tag antibody (Fig. A.2.3B), indicated that neither the expression of SUMO isoforms, nor the presence of the mutations alter the expression or recovery of ZBP-89. Taken together, the results strongly support the idea that *in vivo* ZBP-89 is a target of SUMO modification, that all three SUMO isoforms can be conjugated to ZBP-89, and that the main sites of modification correspond to the proposed SC motifs.

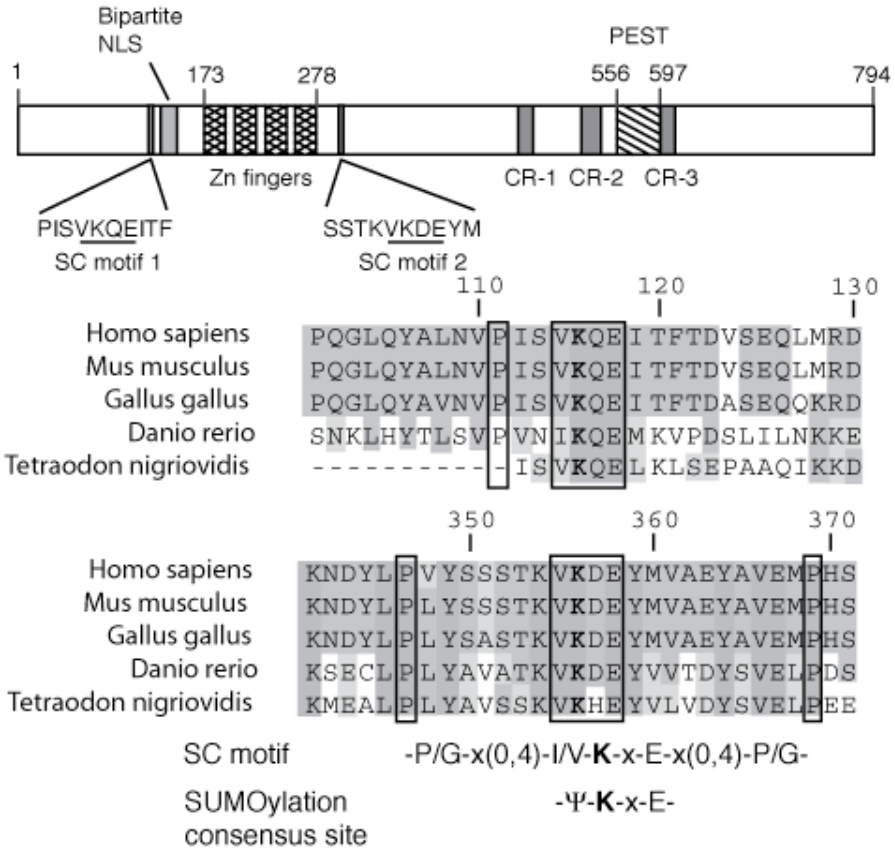

Figure A.2.1. *ZBP-89 harbors two conserved Synergy Control motifs* Top: schematic representation of human ZBP-89 highlighting key structural features. CR1-3: Conserved Regions 1 to 3. The two Synergy Control (SC) motifs flanking the DNA binding domain are shown underlined. Bottom: alignment of ZBP-89 sequences centered on the first and second SC motifs. Numbering corresponds to the human sequence. The core SC motifs as well as flanking proline residues are boxed. Lysines (K115 and K356) targeted for SUMOylation are bold. The consensus sequence for SC motifs and SUMOylation sites are shown below the alignment.

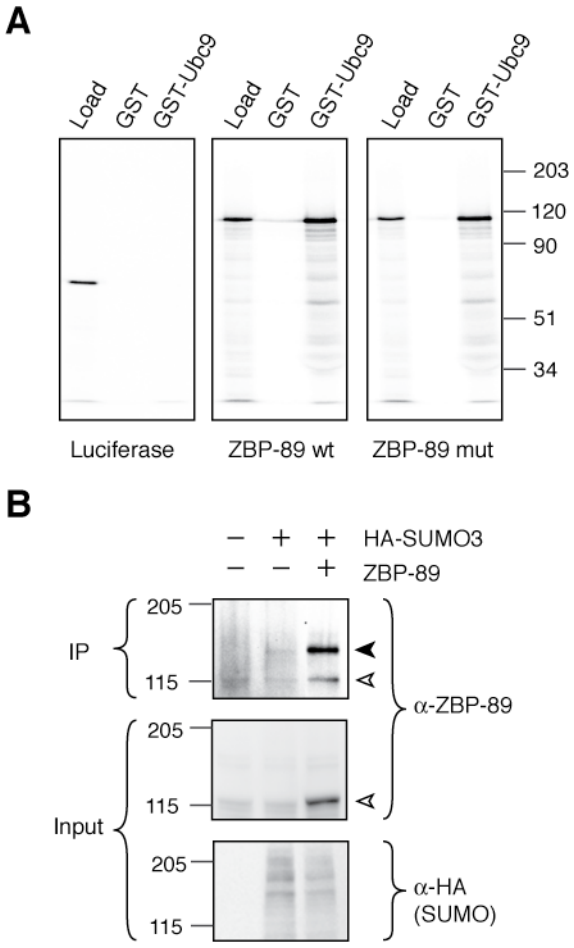

Figure A.2.2. *ZBP-89 interacts with Ubc-9 and is a target of SUMOylation.* A) *In vitro* interaction between ZBP-89 and Ubc9. Purified GST or GST-Ubc9 fusion proteins bound to glutathione-sepharose beads were incubated with the indicated *in vitro* translated and [^{35}S]-labeled proteins and processed as described in Materials and Methods. ZBP-89 mut corresponds to the K115R/K356R double mutant. Load corresponds to 10% of applied material. B) *In vivo* SUMOylation of ZBP-89. AGS cells were transfected with expression plasmids pCDNA3.1(+)-His ZBP-89 WT (WT), pCDNA3 HA-SUMO3 or the corresponding empty vectors as indicated. Cells were lysed after 24 hrs and extracts immunoprecipitated with anti HA antibodies as described in Materials and Methods. Extracts and immunoprecipitates were processed for immunoblotting with anti HA (bottom panel) or anti-ZBP-89 antibodies. Open and closed arrowheads respectively, indicate the positions of intact and SUMO-modified ZBP-89.

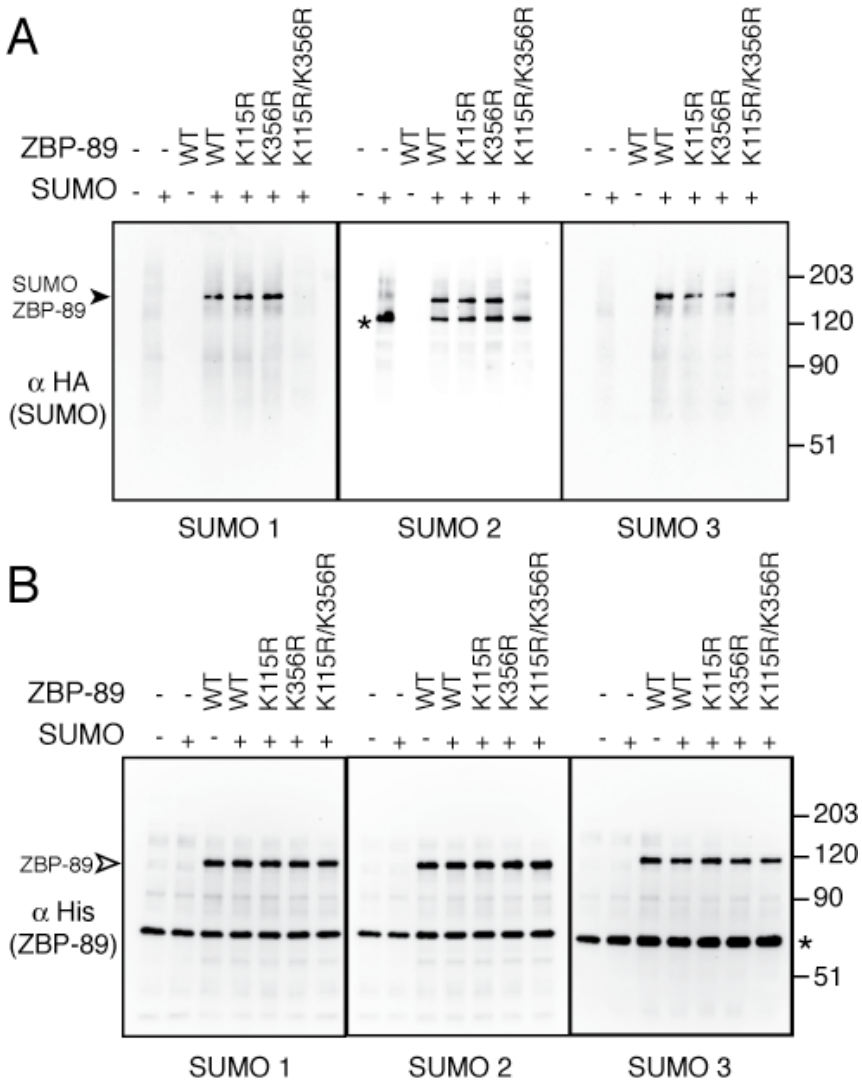

Figure A.2.3. *In vivo modification of ZBP-89 by different SUMO isoforms requires intact SC motifs.* Human 293T cells were transfected as described in Materials and Methods with either control pCDNA3.1(+) vector (-), pCDNA3.1(+)-His ZBP-89 WT (WT), or pCDNA3.1(+)-His ZBP-89 single (K115R and K356R) and double (K115R/K356R) mutants along with pCDNA3 HA-SUMO1, 2 or 3 as indicated. Cells were lysed 24 h after transfection under denaturing conditions. Proteins were purified using Ni^{2+}-NTA resin as described in "Materials and Methods" and processed for immunoblotting with anti HA (panel A) and anti hexa-His (panel B) antibodies. The positions of intact and SUMO-modified ZBP-89 are indicated by open and closed arrowheads respectively whereas the stars indicate the position of nonspecific bands.

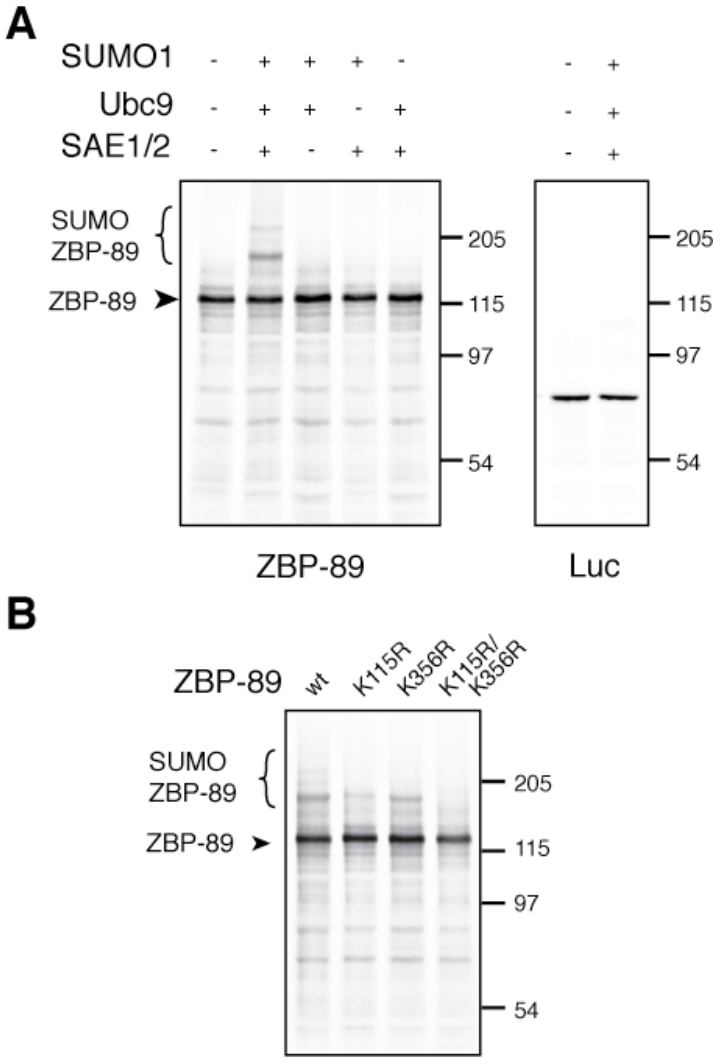

Figure A.2.4. *The SC motifs in ZBP-89 are SUMO-modified in vitro.* A) *In vitro* translated [^{35}S]-labeled ZBP-89 WT (left) or luciferase control protein (right) were used as substrates for *in vitro* SUMOylation reactions in the presence or absence of the indicated purified proteins: His-SUMO1, GST-Ubc9 and GST-SAE1/SAE2 as described in Materials and Methods. Samples were resolved by SDS-PAGE and visualized using a PhosphorImager. B) *In vitro* translated [^{35}S]-labeled ZBP-89 WT (WT) and the indicated mutants were subjected to *in vitro* SUMOylation reactions in the presence of all required components. In both panels, the positions of intact and SUMO-modified ZBP-89 are indicated by the arrowhead and bracket respectively.

To further investigate the mechanism of ZBP-89 SUMO modification, we employed a reconstituted *in vitro* SUMOylation assay consisting of purified recombinant SUMO1 as well as E1 (SAE1/SAE2) and E2 (Ubc9) enzymes. SUMOylation targets consisted of *in vitro* translated [^{35}S]-labeled proteins. As in the *in vivo* experiments, *in vitro* synthesized His-tagged ZBP-89 migrated with an apparent Mr of ~110 kDa (Fig. A.2.4A). Incubation with all SUMOylation machinery components led to the generation of slower migrating bands of ~160 and 220 kDa consistent with mono- and di-SUMOylated forms of ZBP-89. Omission of the E1 or E2 enzymes or SUMO itself during the reaction prevented the appearance of the slowly migrating species (Fig. A.2.4A), indicating that they represent specific covalent modification of ZBP-89 by SUMO1. In contrast, *in vitro* transcribed firefly luciferase, which lacks sequences related to SC motifs, did not serve as substrate (Fig. A.2.4A, right). Similar results were obtained with SUMO2 (not shown). In contrast to WT ZBP-89 and in agreement with the *in vivo* experiments, the higher molecular weight species are absent in the case of the K115R/K356R mutant, in which both potential SUMOylation sites are disrupted (Fig. A.2.4B). Interestingly, the Lys to Arg substitution at position 115 produces a more significant reduction in SUMOylation than that observed with the substitution at position 356, suggesting a preferential targeting of Lys 115 under our *in vitro* conditions. This mild bias however, was not detected in the *in vivo* experiments. Taken together the above results argue strongly that the lysine residues within the first and second SC motifs of ZBP-89 (Lys115 and Lys356) serve as the main sites for conjugation of all three SUMO isoforms both *in vivo* and *in vitro*.

The SC motifs in ZBP-89 exert a SUMO-dependent inhibitory effect only at compound response elements – Given our previous characterization of SC motifs in other factors, we anticipated that the functional effects of the SC motifs in ZBP-89 would be restricted to contexts where multiple instances of one or more SUMOylated factors are recruited to independent sites on DNA (Iñiguez-Lluhí and Pearce, 2000; Holmstrom et al., 2003). To determine the functional role of the SC motifs in ZBP-89, we generated HA-tagged expression vectors for the K115/356R double mutant (ZBP-89 mut) as well as for a non-cleavable fusion between SUMO1 and the ZBP-89 mutant lacking its own SUMOylation sites (SUMO1-ZBP-89 mut). The former plasmid generates a ZBP-89 form with disabled SC motifs (due to the loss of SUMO modification), whereas the latter mimics a persistently SUMOylated form of ZBP-89. The fusion strategy is particularly useful since it allows for selective recruitment of SUMO to a specific factor without interfering with the endogenous complement of SUMO modified proteins. Moreover, in the cases where it has been examined in detail, the fusions accurately recapitulate the principal effects of SUMOylation (Holmstrom et al., 2003). We first compared the activity of the SUMOylation mutant and SUMO-fused forms to WT ZBP-89 at reporter genes bearing either one or three copies of a GC site from the ornithine decarboxilase (ODC) gene (Law et al., 1998b). Sp1 and ZBP-89 bind this element in a mutually exclusive manner and ZBP-89 antagonizes the effects of Sp1 through a displacement mechanism (Law et al., 1998b). In human AGS gastric carcinoma cells, these reporters display substantial basal activity compared to a control reporter lacking ODC sequences (Fig. A.2.5A and B). This activity is mainly attributable to endogenous Sp1 (Law et al., 1998b). Transfection of increasing doses of expression plasmid encoding ZBP-89 led to a dose-dependent

inhibition of both promoters. At the reporter containing a single ZBP-89 binding site (Fig. A.2.5A), where presumably only one protein can be present at a time, the SUMOylation mutant and the SUMO fusion displayed comparable activities to WT ZBP-89, indicating that SUMOylation does not appear to influence the ability to bind the response element and displace endogenous Sp1-like factors. The comparable activities are also consistent with the equivalent expression levels of the three forms (Fig. A.2.5 inset). At the reporter bearing three copies of the element however, (Fig. A.2.5B) we consistently observed that at intermediate doses, the SUMOylation deficient ZBP-89 was a weaker inhibitor whereas the SUMO1 fused ZBP-89 was a stronger inhibitor compared to WT ZBP-89. Given that the intact proximal promoter of the ODC gene harbors multiple GC rich elements, we anticipated that ZBP-89 SUMOylation would also influence transcriptional inhibition at the native promoter. As seen in Fig. A.2.5C, this appears to be the case since the inhibition pattern is similar to that observed at the simpler $(ODC)_3$ reporter. In addition, we obtained similar results in the case of the proximal gastrin promoter (not shown). Given that at both the natural and $(ODC)_3$ reporters Sp1-like factors and ZBP-89 can be recruited simultaneously through occupancy of independent sites, the data indicate that SUMOylation of ZBP-89 exerts a negative influence on the transcription complexes that assemble at compound response elements.

If ZBP-89 SUMOylation acts to inhibit mixed or heterotypic complexes, this modification should also influence the activity of unrelated activators bound nearby. To examine this point, we took advantage of the well-described synergistic cooperation between CACCC box binding factors such as Sp1 and the glucocorticoid receptor (Strähle et al., 1988; Müller et al., 1991). As can be seen in Fig. A.2.5 D, the activity of a

target gene bearing a single ODC element upstream of a single glucocorticoid response element from the tyrosine aminotransferase gene [$(ODC)_1$-$(TAT)_1$] is substantially higher upon activation of the glucocorticoid receptor with dexamethasone, than in its absence (endogenous Sp-like factors alone). The activity is also higher when compared to a reporter bearing only a single TAT element (GR activity alone, data not shown). Expression of ZBP-89 in this context resulted in a dose-dependent inhibition of activity. Notably, the SUMOylation deficient ZBP-89 was a weaker inhibitor as evidenced by a rightward shift in the dose-response. This difference is due to loss of SUMOylation since fusion of SUMO1 to the ZBP-89 mutant restored its inhibitory potential. A similar pattern, with slightly more pronounced differences was observed at a reporter bearing two copies of each response element (not shown). Taken together, the above results indicate that SUMOylation of ZBP-89 modulates its ability to regulate transcription in mammalian cells, mainly by altering cooperation with other factors. Thus, at compound response elements, complexes including SUMOylated ZBP-89 are less conducive to transcriptional activation.

ZBP-89 antagonizes Sp1 through displacement and SUMOylation-dependent inhibition of synergy at compound response elements in Drosophila S2 cells – As mentioned above, the effects of ZBP-89 in mammalian cells likely depend on both its intrinsic transcriptional properties as well as on its ability to compete for response element occupancy with other factors. To evaluate the role of SUMOylation in each of these contexts and to define more clearly the intrinsic properties of ZBP-89, we turned to *Drosophila* Schneider S2 cells. This model has proven extremely valuable to study Sp-like factors since, unlike most mammalian cells, they are functionally devoid of this class

of regulators (Courey and Tjian, 1988). To determine whether it is possible to recapitulate the effects observed in mammalian cells, we examined the ability of ZBP-89 to inhibit Sp1 driven activity at promoters bearing one or three copies of the ODC response element (Fig. A.2.6). As expected, in the absence of Sp1, the activity of these reporters was negligible (indistinguishable from the background signal from untransfected cells). Expression of Sp1 led to a robust activation of these reporters with the activity at the $(ODC)_3$ reporter being nearly four-fold higher than that of the reporter bearing a single site. As in mammalian cells, expression of ZBP-89 led to a dose-dependent inhibition of activity. At the reporter with a single ODC response element, we did not detect significant differences between WT ZBP-89 and the SC motif mutant or the SC motif mutant SUMO-1 fusion (Fig A.2.6A). These data parallel the observations in mammalian cells (Fig. A.2.5A) and further support that SUMOylation of ZBP-89 does not alter its ability to compete with Sp1 for occupancy of the response element. In the case of the reporter bearing three response elements (Fig A.2.6B), disruption of the SC motifs in ZBP-89 reduced, whereas the SUMO1 fusion enhanced the ability of ZBP-89 to inhibit transcription. The data reiterate the observations in mammalian cells (Fig. A.2.5B) and indicate that SUMO modification of ZBP-89 reduces the transcriptional output of complexes assembled at compound response elements. Clearly, the ability to recapitulate the ZBP-89 mediated transcriptional effects obtained in mammalian cells validates Schneider S2 cells as an adequate model to probe the effects of SUMO modification on ZBP-89 mediated transcriptional effects.

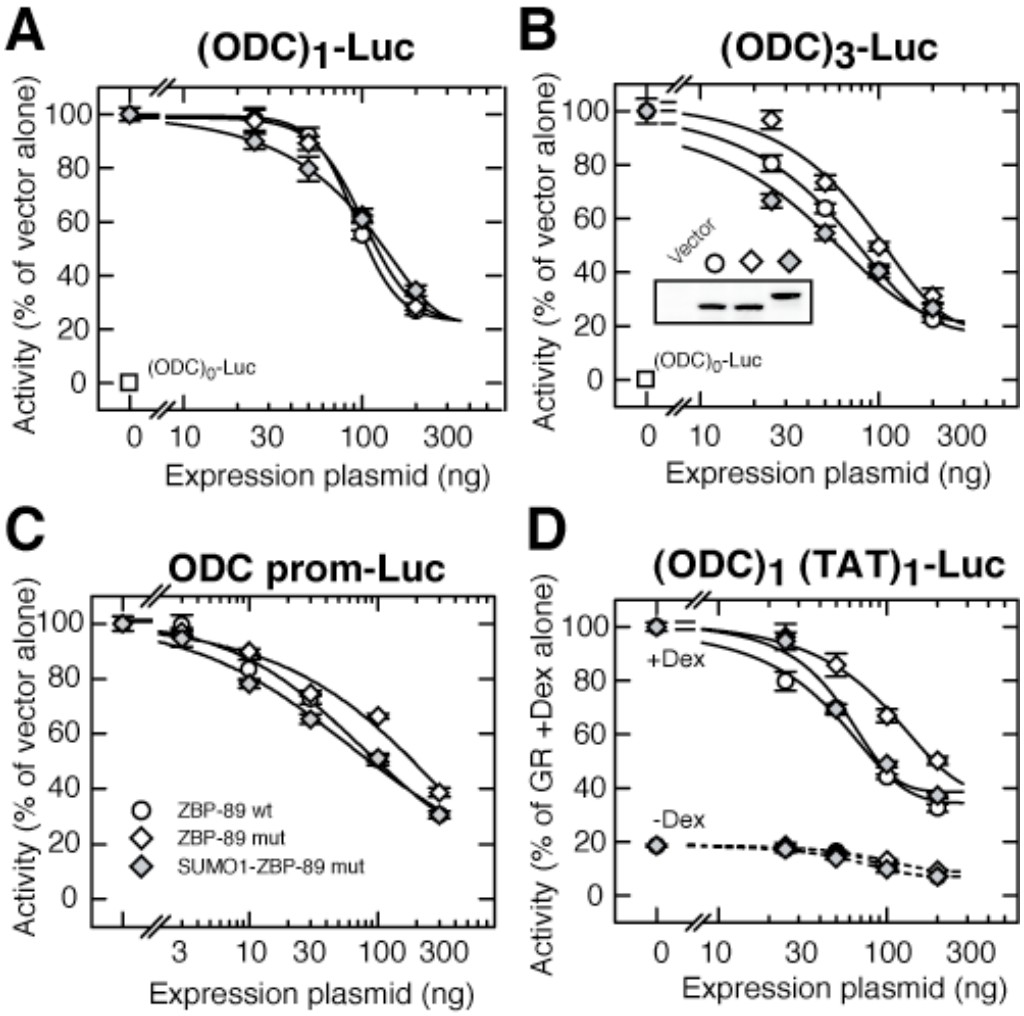

Figure A.2.5. *In mammalian cells, SUMOylation of the SC motifs in ZBP-89 influence its inhibitory activity in a promoter-dependent manner.* AGS cells were co-transfected as described in Materials and Methods with the indicated amounts of expression vectors for HA-WT ZBP-89 (open circles), the K115R/K356R double mutant that disrupts both SC motifs (open diamonds) or for an N-terminal fusion of SUMO1 to the double mutant ZBP-89 (shaded diamonds). Cells were co-transfected with luciferase reporter plasmids driven by one A) or three B) ZBP-89 binding sites from the ODC gene or by the proximal ODC promoter C). AGS cells were co-transfected with the indicated amounts of ZBP-89 expression plasmids and a constant amount (20 ng) of GR expression plasmid together with a reporter plasmid containing one copy each of both ZBP-89 (ODC) and GR (TAT) binding sites. Cells were treated 24 h after transfection with 10 nM dexamethasone (+Dex) or vehicle (-Dex) and processed as described in "Materials and Methods". The data represent the average ± SEM of at least three independent experiments performed in triplicate and are expressed as a percentage of the activity in the absence of ZBP-89 (panels A, B and C) or GR + Dex activity alone (panel D). The corresponding reference values for each reporter are: pΔODC$_1$-Luc (0.300 ± 0.009), pΔODC$_3$-Luc (0.618 ± 0.041), pODC-Luc (0.996 ± 0.042) pΔODC$_1$TAT$_1$-Luc (0.996 ± 0.042). The inset shows the expression levels of the ZBP-89 variants at the dose of 100 ng.

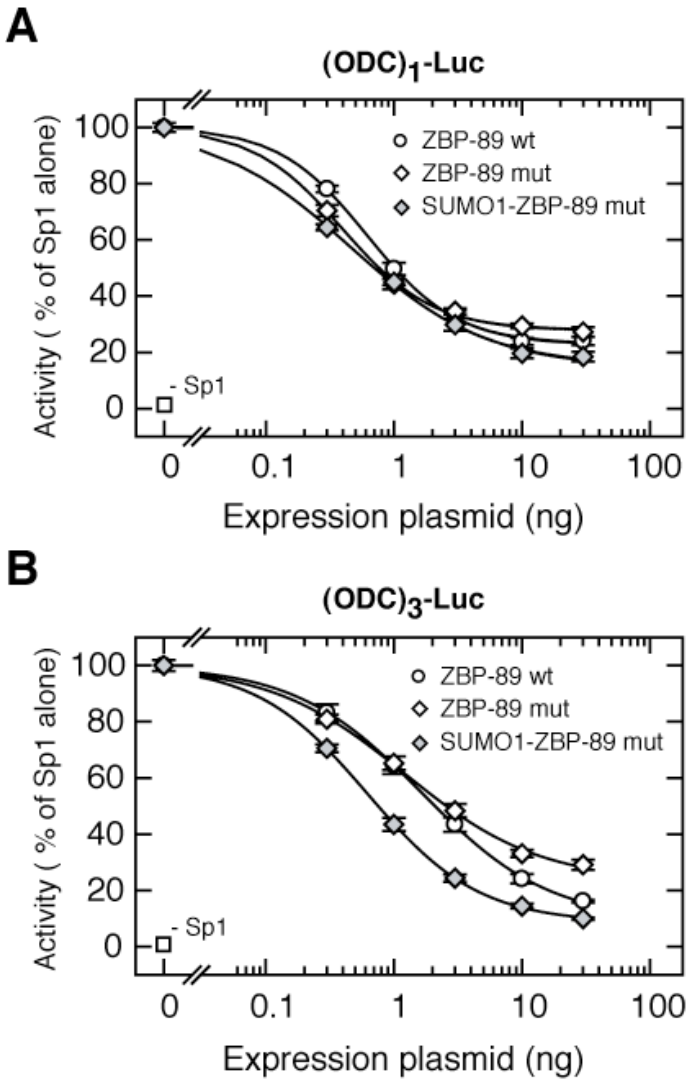

Figure A.2.6. SUMO modification enhances the ability of ZBP-89 to antagonize Sp1 in Drosophila cells only at promoters harboring multiple binding sites. Drosophila S2 cells were co-transfected with the indicated amounts of expression vectors for WT ZBP-89 (open circles), the K115R/K356R double mutant that disrupts both SC motifs (open diamonds), or for an N-terminal fusion of SUMO1 to the double mutant ZBP-89 (shaded diamonds). Cells received a constant amount (10 ng) of Sp1 expression plasmid and luciferase reporter plasmids containing one ($p\Delta ODC_1$-Luc, A) or three ($p\Delta ODC_3$-Luc, B) ZBP-89 binding sites from the ODC gene. Data represent the average ± SEM of at least three independent experiments performed in triplicate and are expressed as a percentage of the activity in the presence of Sp1 alone, which amounted to 2.44 ± 0.18 and 9.39 ± 0.71 for $p\Delta ODC_1$-Luc and $p\Delta ODC_3$-Luc respectively.

In the absence of Sp1, ZBP-89 functions as an activator whose synergy potential is restricted by SC motifs – The functional antagonism evident in Figs A.2.5 and A.2.6 indicates that ZBP-89 can compete with Sp1 for common binding sites. The substantial residual activity at the highest doses of ZBP-89 expression plasmid however, suggested that ZBP-89 may possess intrinsic transcriptional activation properties that could be modulated by SUMO modification. To probe this question directly, we examined the activity of ZBP-89 in the absence of competing factors (S2 cells) at reporter genes harboring from zero to four copies of the ODC response element. As can be seen in Fig. A.2.7, expression of WT ZBP-89 caused a ~3-fold activation of the reporter bearing a single ODC response element. Notably, the presence of additional response elements did not lead to significant enhancements in activity (Fig. A.2.A.2.7, closed bars). This indicates that ZBP-89 can function as a modest activator, but its ability to engage in homotypic synergistic interactions is severely limited. Consistent with our model of synergy control, disruption of the SC motifs in ZBP-89 did not affect its activity at a single site, but led to a substantial enhancement at reporters bearing multiple sites (Fig. A.2.7, hatched bars). Notably, the expansion of the number of response elements results in synergistic effects since the activity is higher than predicted from purely additive effects (dashed lines). Taken together, these results indicate that ZBP-89 is capable of activating transcription, and that the SC motifs in ZBP-89 limit its homotypic synergy potential at compound response elements. Given the much greater relative activity of Sp1 (>10 fold compared to ZBP-89), it is therefore clear how in cellular contexts where Sp1 is present, replacement by ZBP-89 leads to a lower overall activity.

SUMO isoforms mediate the functional effects of the SC motifs through a distinct functional surface – Disruption of the SC motifs in ZBP-89 leads to both loss of SUMOylation and to a loss of synergy control. This suggests that SC motifs exert their effects by serving as SUMO acceptor sites. Moreover, the ability of the SUMO1 fusion to recapitulate the functional effects of SC motifs indicates that recruitment of SUMO isoforms to ZBP-89 leads to the observed functional effects. To probe the ability of individual SUMO isoforms to regulate ZBP-89 function, we generated N-terminal fusions of all three SUMO isoforms to the SUMOylation-deficient SC motif mutant of ZBP-89 and examined their promoter-dependent activities in S2 cells. At a promoter harboring a single ZBP-89 binding site (Fig. A.2.8A), both the WT (solid bar) and SC motif mutant (hatched bar) forms of ZBP-89 displayed identical activities (~3 fold over basal). In contrast, at the promoter bearing three response elements, disruption of the SC motifs in ZBP-89 led to substantially greater (~2.6 fold) activation compared to WT ZBP-89 (Fig. A.2.8B). Fusion of either SUMO1, 2 or 3 to the SC mutant ZBP-89, which mimics the SUMOylated form of ZBP-89, led to a dramatic inhibition of activity at the promoter bearing three response elements (Fig. A.2.8B shadowed hatched bars). Such severe inhibitory effects of SUMO isoforms suggest that even at the low stoichiometry of modification observed *in vivo*, SUMOylation could have profound consequences on activity. Interestingly, the SUMO fusions also led to a striking inhibition of activity at the promoter bearing a single response element (Fig. A.2.8A, shaded hatched bars). The fact that the WT and SC motif mutant have comparable activities at this promoter may indicate that the stoichiometry of modification may not be sufficiently high to cause an effect in this context. It is also possible that the N-terminal fusions do not fully

reproduce all of the properties of ZBP-89 when SUMOylated at its endogenous SC motifs. The results from both promoters however, clearly indicate that ZBP-89 activity is exceedingly sensitive to the inhibitory effects of all three SUMO isoforms. Through a detailed analysis, we have recently identified a highly conserved surface in SUMO that is responsible for its inhibitory properties on multiple transcription factors (Chupreta et al., 2005). Disruption of this SUMO effector surface by substitution of two critical basic residues (Lys 33, Lys 42 in SUMO2) by glutamic acid dramatically affects the ability of SUMO to inhibit transcription (Chupreta et al., 2005). Importantly, and in contrast to WT SUMO2, fusion of the K33/42E SUMO2 mutant to ZBP-89 failed to inhibit activity at the $(ODC)_1$ promoter and was substantially less inhibitory than WT SUMO2 at the $(ODC)_3$ promoter (Fig. A.2.8 A and B). Since all SUMO fusions are expressed at comparable levels, these results indicate that the inhibitory properties of SUMO on ZBP-89 depend on its critical effector surface and are not simply due to steric hindrance effects.

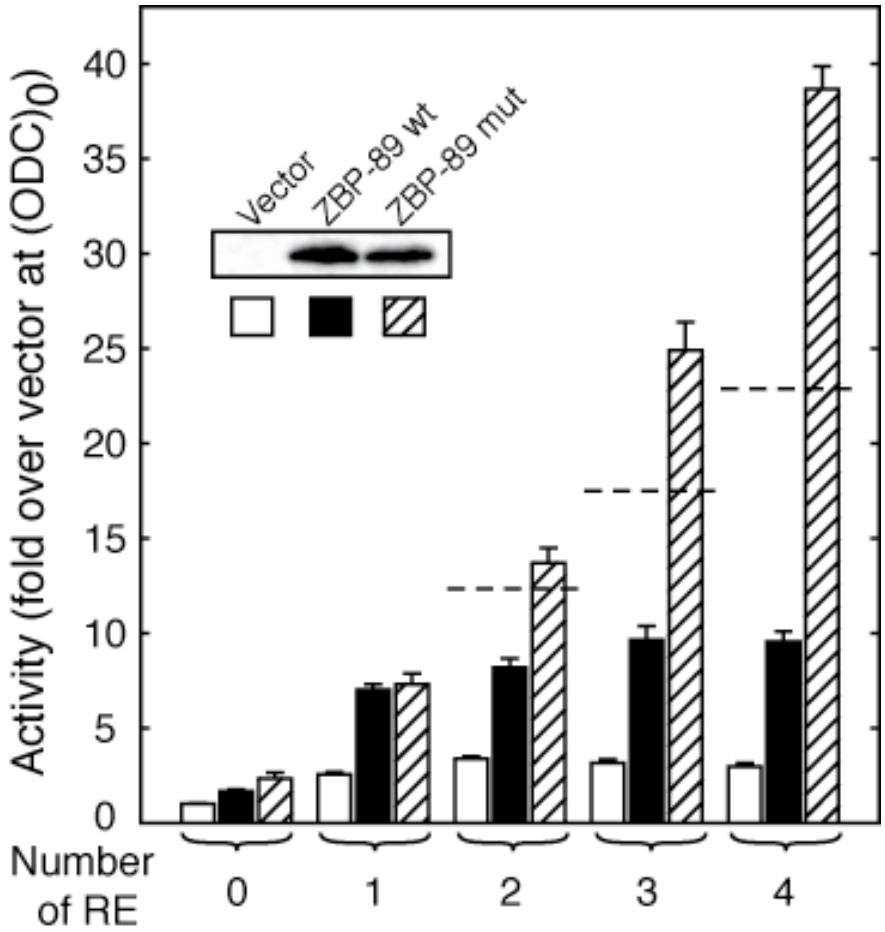

Figure A.2.7. *In the absence of competing factors, homotypic synergistic activation by ZBP-89 is strongly inhibited by SC motifs.* Drosophila S2 cells were co-transfected with 100 ng expression vectors for ZBP-89 WT (solid bars), ZBP-89 SC motif double mutant (K115R/K356R, hatched bars) or equimolar amounts of control vector (pA5C, open bars) together with luciferase reporters harboring the indicated numbers of ZBP-89 response elements (RE). Data represent the average ± SEM of at least three independent experiments performed in triplicate and are expressed as a fold induction over the activity obtained for the vector alone at the p$\Delta(ODC)_0$-Luc reporter (0.29 ± 0.02). The dashed Reference lines indicate the predicted activity assuming simple additive effects. The inset shows the expression levels of the ZBP-89 variants.

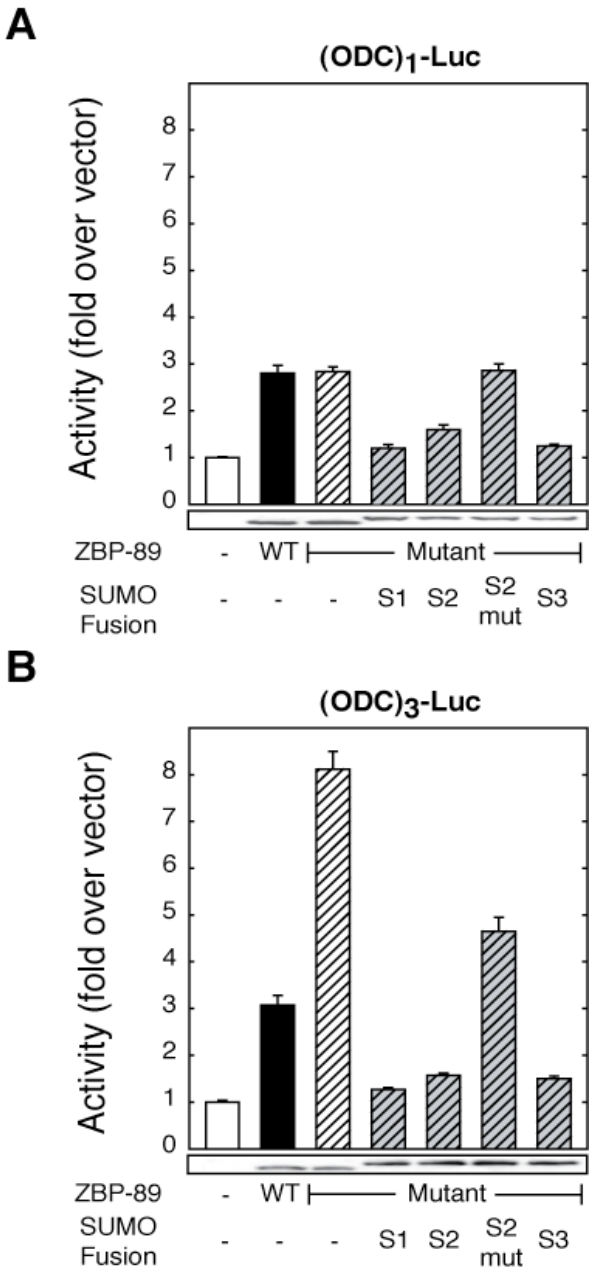

Figure A.2.8. *SUMO isoforms inhibit ZBP-89 activity through a critical effector surface. Drosophila* S2 cells were co-transfected with 100 ng of control vector (pA5C, open bars) or expression vectors for ZBP-89 WT (solid bars), ZBP-89 SC motif double mutant (K115R/K356R, hatched bars) or ZBP-89 SC motif mutant fused to the indicated SUMO isoforms (shadowed hatched bars). The SUMO2 mutant harbors the K33E and K42E mutations. Reporter plasmids were pΔ$(ODC)_1$-Luc (panel A) and pΔ$(ODC)_3$-Luc (panel B). Data represent the average ± SEM of at least three independent experiments performed in triplicate and are expressed as a fold induction over the activity obtained for the vector alone, which amounted to 1.03 ± 0.06 and 1.47 ± 0.08 for pΔ$(ODC)_1$-Luc and pΔ$(ODC)_3$-Luc respectively. The expression of the individual ZBP-89 variants as determined by immunoblotting is shown below the corresponding bars.

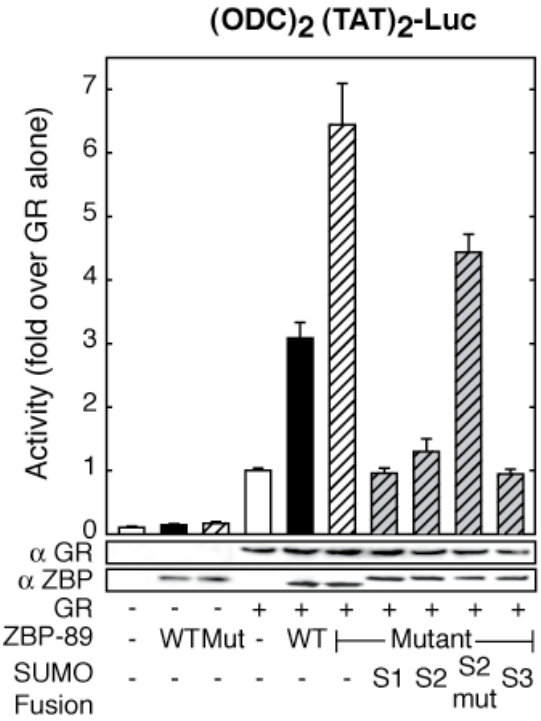

Figure A.2.9. *SUMOylation of ZBP-89 potently inhibits its ability to synergize with GR.* *Drosophila* S2 cells were co-transfected with 100 ng of control vector (pA5C, open bars) or expression vectors for ZBP-89 WT (solid bars), ZBP-89 SC motif double mutant (K115R/K356R, hatched bars), or ZBP-89 SC motif mutant fused to the indicated SUMO isoforms (shadowed hatched bars). Cells also received 200ng expression vector for WT GR as indicated and the pΔ(ODC)$_2$(TAT)$_2$-Luc reporter. The SUMO2 mutant harbors the K33E and K42E mutations. Cells were treated with 100 nM dexamethasone 24 h after transfection, and were processed as described in Materials and Methods. Data represent the average ± SEM of at least three independent experiments performed in triplicate and are expressed relative to the activity obtained for GR alone (10.03 ± 2.01). The expression of the individual ZBP-89 variants and GR as determined by immunoblotting is shown below the corresponding bars.

SUMOylation of the SC motifs in ZBP-89 limits its ability to engage in heterotypic synergy with other activators – The above analysis indicates that ZBP-89 can function as an activator, and that modification by SUMO is an effective mechanism to inhibit its ability to self-synergize (homotypic synergy). To examine both the ability of ZBP-89 to cooperate with other activators and the role of SUMOylation in such heterotypic synergy, we examined in S2 cells the functional interaction between WT GR and ZBP-89 at a promoter bearing two ZBP-89 binding sites upstream from two GREs (Fig. A.2.9). In the absence of GR, the WT and mutant forms of ZBP-89 displayed a modest pattern of activity similar to that observed at the $(ODC)_2$-Luc reporter. Exclusive expression of GR, however, resulted in substantial ligand-dependent activation (~7 fold higher than WT ZBP-89). Notably, co-expression with WT ZBP-89 led to a three-fold enhancement of activity compared to GR alone. Thus, although ZBP-89 is a modest activator, it is able to synergize very effectively with GR. Furthermore, disruption of the SC motifs in ZBP-89 led to an even more substantial (~6.5 fold) enhancement of GR activity. This indicates that, like in the case of homotypic interactions, the SC motifs strongly inhibit the heterotypic synergy potential of ZBP-89. Remarkably, although fusion of any of the three SUMO isoforms to the SC motif deficient ZBP-89 does not affect expression, this maneuver essentially eliminates the amplifying effect on GR activity since the resulting activity is comparable to that of GR alone. Furthermore, the inhibitory effect of SUMO requires an intact effector surface since fusion of the K33E/K42E SUMO2 mutant is much less effective (Fig. A.2.9, S2 mut). Taken together, these results indicate that, by serving as sites of post-translational modification for SUMO, the SC motifs in ZBP-89

play an important role in controlling the transcriptional activity of this ubiquitous regulator.

DISCUSSION

The Synergy Control Motifs in ZBP-89 are sites for SUMO modification – Our finding that ZBP-89 interacts with Ubc9 and that the two SC motifs in ZBP-89 correspond to the main sites of SUMO modification extends the impact of the synergy control mechanism to this family of transcription factors. The very recent demonstration of SUMO modification of other distantly related Krüppel type transcription factors (Karène et al., 2005; Wei et al., 2006; Lee et al., 2007), as well as Sp1 (Spengler and Brattain, 2006), indicates that it is a widespread mechanism to regulate this broad class of regulators. Although all three SUMO isoforms can be conjugated to ZBP-89, the fact that SUMO2 and 3 accumulate to substantially higher levels than SUMO1 argues for a preference for SUMO1 modification *in vivo*. Although the intranuclear localization of ZBP-89 has not been defined, the distinct subnuclear targeting of isoform-specific SUMO proteases suggests that ZBP-89 may accumulate in regions enriched in SUMO2/3 specific proteases such as the nucleoli (Nishida et al., 2000; Gong and Yeh, 2006). Initial analysis of GFP fusions indicates that both WT and SC mutant ZBP-89 localize exclusively to the nucleus (not shown). Likewise, the fact that the apparent preference for SUMOylation of K115 over K356 *in vitro* is not observed *in vivo* may be due to the actions of cellular activities such as E3 ligases that are absent in the *in vitro* assay. Although lysine residues can be targets for multiple modifications, we do not observe alterations in the steady-state levels of ZBP-89 upon disruption of the SC motifs, suggesting that they are unlikely to contribute to ubiquitination-dependent degradation. Furthermore, the ability of SUMO

fusions to reverse the effects of SC motif disruption argues strongly that SUMOylation is responsible for the function of the SC motifs.

ZBP-89 as an activator and synergy partner regulated by SUMOylation – Although in most cases ZBP-89 appears to inhibit transcription of target genes, functional data suggests that it can exert a positive effect on transcription in certain contexts (Bai and Merchant, 2000; Yamada et al., 2001; Bai and Merchant, 2003) (Bai and Merchant, 2000; Yamada et al., 2001; Bai and Merchant, 2003). Whether this is due to a displacement mechanism or an intrinsic activation function remains unresolved. We have obtained evidence that ZBP-89 can function as a direct albeit weak activator by taking advantage of S2 cells, which provide a cellular context devoid of competing factors. This analysis is consistent with data derived from Gal4 DBD fusions to recruit ZBP-89 domains since Gal4 fusions to C-terminal fragments of ZBP-89 activate transcription from reiterated Gal4 sites (Hasegawa et al., 1997). In contrast to most activators, increasing the number of response elements did not lead to enhanced transcription. This lack of synergy was relieved by disruption of the SC motifs indicating that SUMOylation exerts a strong inhibitory effect on ZBP-89's ability to engage in homotypic synergy. Such behavior can be viewed as an extreme example of the synergy control mechanism observed in other activators such as GR, ETS-1 (Iñiguez-Lluhí and Pearce, 2000) and C/EBPα (Subramanian et al., 2003) and is akin to the properties of Sp3 (Ross et al., 2002; Sapetschnig et al., 2002). Notably, although on its own ZBP-89 appears to be a weak activator, its remarkable ability to cooperate with other factors such as GR indicates that it has the capacity to exert quite substantial effects on activation when embedded in a complex promoter. SUMOylation may therefore modulate the proposed cooperation

between ZBP-89 and JKTBP1 in the activation of the cytochrome c oxidase Vb gene (Boopathi et al., 2004). In this view, ZBP-89 may serve a similar function to the "accessory factors" that are required for full glucocorticoid induction of metabolic genes such as PEPCK (Wang et al., 2004; Cassuto et al., 2005). Consistent with a role in the regulation of synergistic transcription, the ability of ZBP-89 to cooperate with GR is inhibited by SUMO modification. These data extend the role of SUMO modification to include heterotypic cooperation between different factors and suggests that SUMOylation is likely to be an important mechanism to regulate the concerted output of the transcription factor complexes that assemble at natural enhancers. In contexts where ZBP-89 competes with other factors for a common site, the final functional effect of ZBP-89 will therefore depend not only on the relative intrinsic activities of the competing factors but also on their SUMOylation-regulated ability to cooperate with additional factors bound nearby.

Inhibitory effect of ZBP-89 SUMOylation depends on recruitment to multiple binding sites and a critical surface in SUMO In most cases, ZBP-89 functions as an inhibitor of transcription and displacement of stronger activators from GC rich sites is likely to be an important and widespread mechanism. Notably, SUMOylation does not alter the ability to compete with Sp1 at a single site. In contrast, at promoters bearing multiple sites where ZBP-89 can engage in heterotypic interactions with Sp1, ZBP-89 SUMO modification limits transcriptional activation. This is consistent with the role of SUMOylation as a mechanism to regulate higher-order interactions among transcription factors. It is important to note that many sequence-specific factors are targets of SUMOylation and therefore at complex promoters there is substantial redundancy in

SUMO mediated inhibition. Consistent with this notion, our analysis of the effects of SC motifs in the glucocorticoid receptor indicate that the most dramatic effects of loss of SUMOylation are observed when all potential SUMOylation sites in a complex are disrupted (Iñiguez-Lluhí and Pearce, 2000; Holmstrom et al., 2003). The recent demonstration that Sp1 is also subject to SUMO modification (Spengler and Brattain, 2006) suggests that the relatively modest effect of disrupting the SUMOylation sites in ZBP-89 that we observe in mammalian cells may be due to a reduction, but not elimination of all potential SUMOylation sites at the promoter. In addition, the analysis of Gal4 DBD fusions suggests that ZBP-89 harbors an intrinsic repressor function in the N-terminal region (Hasegawa et al., 1997; Passantino et al., 1998). The implicated domain spans the two SC motifs and in the case of the vimentin gene, a direct repression mechanism involving HDAC recruitment has been invoked (Zhang et al., 2003; Wu et al., 2007). Whether the SC motifs are sufficient to account for this activity or additional direct repressive functions are present in this N-terminal domain remains to be determined. In the case of KLF-8 however, Wei *et al.* argue that SUMOylation *attenuates* the ability of KLF-8 to repress transcription at the KLF-4 promoter (Wei et al., 2006). Given the overlap in sequence specificity with other GC box binding proteins however it is difficult to determine whether this is due to a direct influence on an intrinsic repressive function or to factor displacement and altered cooperation effects. Our data however, clearly indicate that the SUMOylation-dependent effects of SC motifs are restricted to promoter contexts where functional interactions involving multiple response elements take place. Based on our mechanistic analysis, we have proposed that SUMO-dependent recruitment of inhibitory cofactors may require a multivalent SUMO-target

interaction. Conversely, the cofactors or machinery that mediates the inhibitory effects of SUMO may be able to antagonize only the activity emanating from stable complexes such as those that assemble at reiterated sites. In this view, SUMO may inhibit from a single site as long as the stoichiometry is high and the DNA-activator binding is stable. The reduced activity of the ZBP-89 SUMO fusion at a single site may be an example of this scenario. As in essentially all other examples of SUMOylation effects on transcription factors, this modification can exert a substantial effect even when the steady-state stochiometry of modification that can be detected is low. Although the exact mechanism is unclear, it is possible that SUMOylation facilitates the establishment of an altered functional state (by facilitating or disrupting protein interactions for example). Once established, such a state may not require persistent SUMOylation for its maintenance. Through an extensive, structurally based mutagenesis analysis, we (Chupreta et al., 2005) and others (Rosendorff et al., 2006) have identified a key conserved surface in SUMO responsible for its inhibitory effects on transcription. X-ray (Reverter and Lima, 2005) and NMR structural analysis (Song et al., 2004) indicates that this effector region in SUMO interacts with Val/Leu-rich SUMO binding motifs in context-specific target proteins. In this regard, certain Histone Deacetylases (Yang and Sharrocks, 2004) and DAXX (Lin et al., 2006), which harbor such motifs have been implicated in SUMO dependent inhibition of transcription. It is likely however, that additional processes are at play since we have been unable to demonstrate a role for these proteins in SC motif function. It is clear however, that the effects of SUMO on ZBP-89 do rely on this conserved functional surface of SUMO.

The substantial impact of SUMOylation on the activity of ZBP-89 argues that it is likely to contribute to its regulation *in vivo*. Given the important role of ZBP-89 for germ line and hematopoietic lineages, it is possible that developmental changes in ZBP-89 SUMOylation could contribute to the temporally regulated and cell-type restricted activity of this otherwise mostly ubiquitous factor. The recent demonstration that variations in the levels and activity of SUMOylation machinery components can accompany cellular differentiation programs (Jones et al., 2006) lends support to this view. Identifying the proteins and signals that regulate ZBP-89 SUMOylation and interaction with other modifications will thus be informative. Over-expression of ZBP-89 leads to cell-cycle arrest and apoptosis both in cell culture (Bai and Merchant, 2001) and in intestinal tissues upon targeted transgenic expression (Law et al., 2006a). A direct interaction between the Zn-finger region of ZBP-89 and p53 likely contributes to this effect (Bai and Merchant, 2001) (Bai and Merchant, 2001). Interestingly, disruption of the SC motifs did not affect the ability of ZBP-89 to induce cell-cycle arrest (not shown). This suggests that SUMOylation may not interfere with p53 interactions or that arrest may depend on ZBP-89 transcriptional effects that involve displacement mechanisms insensitive to SUMOylation. The influence of SUMOylation on transcription factor driven cell cycle effects appears to depend on the nature of the factor since SUMOylation of KLF-8 appears to inhibit the ability of this factor to promote entry into G1 (Wei et al., 2006). We have recently shown that a splice variant of ZBP-89 generated by alternative promoter usage yields an N-terminally truncated isoform. Since the first SC motif is absent in this form, alterations in SUMOylation may contribute to the shortened lifespan and increased sensitivity to dextran-sulfate-sodium-induced colitis displayed by mice

exclusively expressing this N-terminally deleted form (Law et al., 2006b). Taken together, our analysis indicates that by modulating the ability of ZBP-89 to engage in higher order functional interactions, SUMOylation offers a context-dependent post-translational mechanism to regulate the activity of this multifunctional transcription factor.

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