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SUPPLEMENT

Sleep Circuitry and the Hypnotic Mechanism of $GABA_A$ Drugs

Jun Lu, M.D., Ph.D.[1]; Mary Ann Greco, Ph.D.[2]

[1]Department of Neurology, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA; [2]Behavioral Biochemistry Laboratory, SRI International, Menlo Park, CA

Abstract: Early in the twentieth century, von Economo provided the first evidence linking the hypothalamus with sleep-wake behavior. His studies concluded that the anterior hypothalamus was associated with sleep, whereas the posterior hypothalamus was associated with waking. In the decades following these observations, a wealth of research has shown that an elaborate circuitry comprising a number of brain regions, cell types, and extracellular messengers underlies sleep-wake behavior. In this review, we discuss data generated in the past 10 years that highlight the role of the hypothalamus in sleep-wake behavior and control. In particular, we will focus on the identification of the ventrolateral preoptic nucleus (VLPO) as a sleep center and the hypocretin/orexin cells in the perifornical region of the hypothalamus as constituting a waking center; these two centers are critical for the maintenance of normal sleep-wake architecture, and provide a foundation for our understanding of sleep-wake behavior and its underlying physiology. The data from these and other regions traditionally associated with the sleep-wake cycle have led to a flip-flop switch model of sleep-wake control. The switch is composed of two sets of mutually inhibitory groups of neurons: a sleep group and an arousal group, with the latter modulated by orexin-containing neurons in the lateral hypothalamus. The sleep-promoting GABA (gamma-aminobutyric acid) receptor agonists are a diverse class of drugs, which include barbiturates, benzodiazepines, chloral hydrate, ethanol, and gaseous anesthetics, that have been used to study sleep physiology for many years. Recent studies suggest that these drugs may exert their hypnotic effects in a regionally specific manner. For example, some $GABA_A$ agonists appear to promote sleep by inhibiting the histaminergic cells in the tuberomammillary nucleus and weakly activating the VLPO via agonist binding to the α_1 subunit of $GABA_A$ receptors; whereas, gaboxadol (THIP; 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol) binds to the $\alpha_4\delta$-subunits, potentially promoting sleep by activation of the VLPO. The integration of these data into the flip-flop switch model can be used to better understand sleep-wake control and augment existing therapeutic treatments for sleep disorders.

In many respects, the modern era of sleep research began in 1916, when Baron Constantin von Economo studied patients with a type of viral encephalitis that affected sleep. A comparison of sleep-wake behavior and data obtained from postmortem brain mapping revealed that lesions of the anterior hypothalamus and basal forebrain were associated with severe insomnia, suggesting that these areas of the brain are sleep-promoting centers. In contrast, patients with lesions of the posterior hypothalamus and midbrain exhibited hypersomnolence, suggesting that these regions contain a wake center.[1] In 1946, Nauta confirmed these observations by correlating sleep-wake behavior in rats with surgical damage to preoptic and basal forebrain tissue.[1]

Electroencephalographic recordings (EEG), first described by Berger in 1929, were not utilized in sleep research until the 1950s. Starting in the 1960s, a series of studies combining EEG and electrolytic lesions of the posterior hypothalamus confirmed the existence of the wake-promoting region.[2-4] However, this method was limited: it could not be determined if the insult was to the wake-promoting region itself, to ascending or descending projections from this site, or to both.

An early study by Moruzzi and Magoun showed that electrical stimulation of a large portion of the rostral pontine reticular formation produced a desynchronized EEG in anesthetized cats; this provided evidence for a role of the brainstem in causing wakefulness.[5] In addition, mechanical or electrolytic lesions of the mesopontine reticular formation produced a comatose state.[5-8] The ascending reticular activating system (ARAS), which originates in the rostral pontine reticular formation and passes through the midbrain reticular formation to the thalamus, was thus associated with arousal.

Encephalitis, electrical stimulation, and electrolytic lesions, however, have impact on both cell bodies and fibers of passage. Starzl and Magoun showed, in 1951, that lesions of the thalamus did not block EEG desynchronization effects (arousal) produced by electrical stimulation in the midbrain reticular formation, suggesting that some other target of the reticular formation caused cortical activation.[9]

In the 1970s and 1980s, the availability of immunohistochemical techniques led to the identification and characterization of several groups of brainstem neurons that could serve as arousal sites, including the dorsal and median raphe serotoninergic neurons, midbrain dopaminergic neurons, laterodorsal and pedunculopontine tegmental (LDT/PPT) cholinergic neurons, and locus coeruleus (LC) noradrenergic neurons. These cholinergic and monoaminergic neurons send long ascending projections to the thalamus, cerebral cortex, lateral hypothalamus, and basal forebrain (Figure 1).[10] Cholinergic neurons in the LDT/PPT were shown to be highly active during wakefulness and rapid

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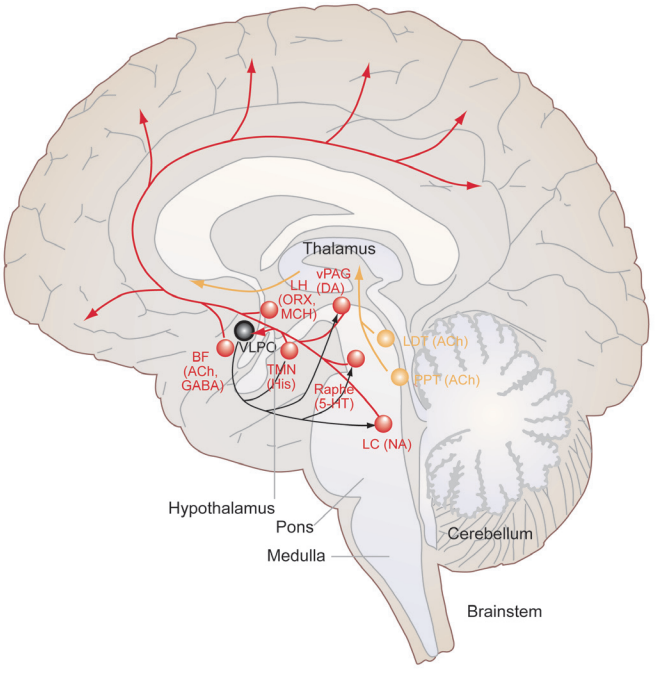

Figure 1—Relationship of the ventrolateral preoptic nucleus (VLPO) and ascending arousal systems. The arousal systems consist of histaminergic neurons within the tuberomammillary nucleus (TMN), dopaminergic neurons of the ventral periaqueductal gray matter (vPAG), serotoninergic neurons within the dorsal raphe nucleus (DRN), noradrenergic neurons of the locus coeruleus (LC), and cholinergic neurons in the laterodorsal and pedunculopontine tegmental (LDT/PPT) nuclei. These systems may influence cerebral cortical activity via four potential routes: direct projections, the basal forebrain relay, the lateral hypothalamus relay, and the thalamus relay. The VLPO and the ascending arousal systems may mutually inhibit each other via the proposed flip-flop switch model of interaction (see Figure 3). Note that LDT/PPT cholinergic neurons and orexinergic neurons do not communicate directly with the VLPO, and LDT cholinergic neurons have a small direct projection to the prefrontal cortex. Reproduced with the permission from Saper CB et al.[71]

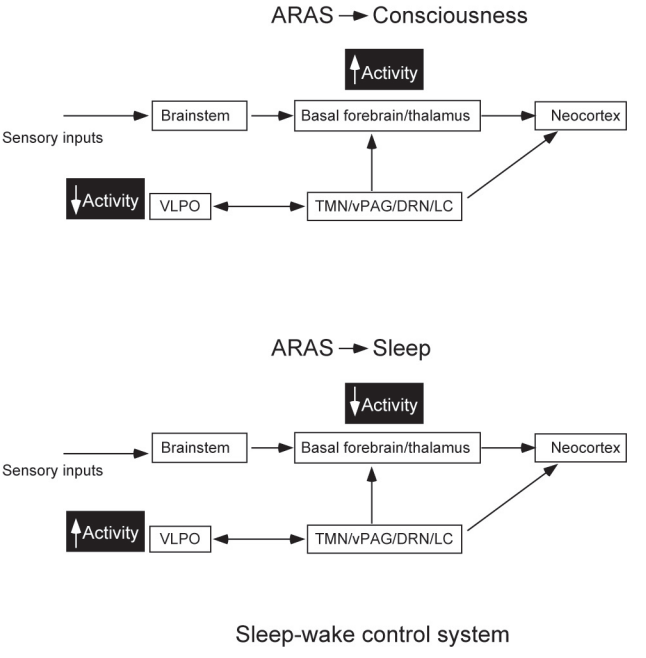

Figure 2—Interaction of the ascending reticular activating system (ARAS) and sleep-wake control system. In this model, the ARAS maintains basic cortical consciousness, whereas the VLPO-monoaminergic systems operate in parallel with the ARAS and influence the ARAS at the basal forebrain/thalamus/neocortex. When the VLPO is active, monoaminergic influences at the basal forebrain/thalamus/neocortex are decreased, resulting in sleep. When the ARAS is impaired caudal to the brainstem, the VLPO-aminergic systems still influence the basal forebrain/thalamus/neocortex, producing coma, but retaining cortical changes associated with the sleep-wake cycle.

eye movement (REM) sleep, and less active during non-REM (NREM) sleep[11-13]; in contrast, the monoaminergic cell groups exhibited their highest discharge during wakefulness, decreased activity during NREM sleep, and virtually no activity during REM sleep.[14-16] The firing pattern of the cholinergic neurons within the LDT/PPT and their projection to the thalamus suggested a role in the characteristic thalamic neuron firing patterns observed during NREM sleep, waking, and REM sleep. Although these data were consistent with a direct relationship between upper brainstem wake-active neurons and changes in cortical activity during waking states, neurotoxic lesions in the reticular formation locations such as the PPT,[17] the oral pontine reticular nucleus,[18] or the LDT[19] failed to replicate the hypersomnolence or comatose states that accompanied electrolytic lesions of this area. This suggests that the many waking areas in the brainstem may allow compensation for the loss of one region, or that the ARAS originates more caudally (Figure 2).

Although earlier studies showed that electrolytic lesions of the posterior lateral hypothalamus resulted in hypersomnolence and stupor,[3,20] lesions of the lateral hypothalamus induced with ibotenic acid or hypocretin-2–saporin produced either a transient effect on or an increase in sleep, indicating that von Economo's encephalitis lethargica was due, at least in part, to a disruption of ascending projections.[21-23]

Hypothalamic Arousal Systems

The tuberomammillary nucleus (TMN), located on the ventrolateral edge of the posterior hypothalamus, contains neurons that co-express histamine and the inhibitory neurotransmitter GABA (gamma-aminobutyric acid), and which project to the cerebral cortex, thalamus, and basal forebrain.[24] TMN neurons exhibit a firing pattern that is most active during wakefulness, decreases during NREM sleep, and falls virtually silent during REM sleep.[25] In mice, genetic ablation of histidine decarboxylase, the enzyme that synthesizes histamine from histidine, resulted in increased sleep fragmentation, increased REM sleep, slower EEG activity during waking, and an inability to maintain wakefulness in a novel environment, but normal amounts of sleep and wakefulness.[26] Acute lesions of the TMN also did not affect total amounts of sleep and wakefulness.[23]

Two research teams in 1998 independently and simultaneously characterized a second cell group within the lateral hypothalamus that is associated with waking and the maintenance of sleep-wake architecture. Cells within the perifornical area surrounding the fornix synthesize orexin, also known as hypocretin, and project to sites throughout the central nervous system (CNS), including the cerebral cortex, basal forebrain, thalamus, brainstem, and spi-

nal column.[27,28] Two distinct orexin receptors activate intracellular signal transduction cascades and show different distribution patterns in the CNS.[29] A series of studies has shown that defects in the signaling system for the excitatory neuropeptide orexin are involved in narcolepsy and cataplexy.[29-34] Detailed analysis of sleep-wake behavior in orexin knockout mice revealed a fragmented sleep pattern and cataplexy, although the total amount of sleep was not altered.[35] Orexin neurons exhibit discharge patterns characteristic of waking neurons in that they are highest during waking, decrease during NREM sleep, and are virtually silent during REM sleep.[36,37] However, few orexin receptors have been found in the sleep-promoting ventrolateral preoptic nucleus (VLPO).[38] This suggests that the orexin system plays a role in the activation and maintenance of wakefulness through its connections to other waking centers.

A third lateral hypothalamic group that may be involved in sleep regulation consists of cells that are intermingled with orexin neurons and express the peptide melanin-concentrating hormone (MCH).[39] MCH neurons show activation of c-Fos, an immediate early gene, during periods of high levels of REM sleep,[40] and mice with double knockouts of the MCH and orexin neurons display more severe narcolepsy than animals in which only the orexin gene is deleted (Willie JT and Yanagisawa M, personal communication). MCH cells have projections that are almost identical to those of the neighboring orexin neurons, but their action is likely to be inhibitory, and many of them also contain GABA. Hence, because MCH cells also appear to have an activity profile that is opposite that of the orexin neurons, they may in fact reinforce a similar type of functional response.

Finally, it is likely that at least one additional lateral hypothalamic cell type is involved in arousal, for which classification has been elusive. As described above, a selective lesion of the TMN or loss of orexinergic neurons does not affect the total amount of sleep, but large, nonselective, cell-specific lesions of the lateral hypothalamus induced with ibotenic acid do increase the total amount of sleep.[21]

Preoptic Sleep Center

Insight into the identity of the hypothalamic sleep center proposed by von Economo proceeded more slowly than for the wake-active centers. Studies have shown that electrical stimulation of the putative sleep area in cats increased sleep while electrolytic lesions of the basal forebrain decreased sleep.[41,42] Kainic acid–induced lesions of the preoptic area and a large portion of the basal forebrain produced insomnia in cats,[43] as did acute suppression of the preoptic region by the GABA agonist muscimol.[44] Extracellular recordings from this area showed that 24% of cells were sleep-active (~50% of recorded cells were wake-active), and most of the sleep-active neurons were located in the ventral basal forebrain.[45] In 1989, Sallanon and colleagues induced focal ibotenic acid lesions in cats, mostly in the preoptic area, and found that cats with lesions in the ventral part of the lateral preoptic area had the greatest reductions in both NREM and REM sleep over a long period.[46] These pioneering studies showed that sleep was actively controlled by a sleep center in the preoptic area, although the identity of the sleep-promoting cells was not established.

In 1996, Sherin and colleagues[47] used c-Fos, a cellular marker of neuronal activity, to identify hypothalamic cells that project to the TMN in the hypothalamus, a region associated with waking.[48-50] Their investigation revealed that a cluster of neurons in the VLPO expressed c-Fos during sleep, but not during wakefulness, thus providing evidence that this region contained sleep-active cells.[47] Later studies showed that VLPO neurons projected to and received projections from the TMN and the brainstem monoaminergic systems, including the dorsal (DRN) and median raphe (MnRN) serotoninergic neurons and the LC noradrenergic neurons.[51,52] Additional inputs to the VLPO were identified from the circadian control system, as well as from the infralimbic cortex and the lateral septum.[52]

Cells within the VLPO were found to contain the inhibitory neurotransmitters GABA and galanin.[51] In situ analysis using galanin mRNA showed regional homologies of the VLPO in mice, cats, primates, and humans, indicating that the VLPO is conserved across mammalian species.[53] Electrophysiologic analysis in freely moving rats indicated that VLPO cells fired two to three times faster during sleep than during waking,[54] and were activated by the somnogens adenosine and prostaglandin D2.[55-57] Lesions of the VLPO in rats, induced with ibotenic acid, caused severe insomnia, marked by reductions in both NREM sleep (by almost 55%) and delta power (by about 70%)[58] that lasted up to 3 months (Lu J, Saper CB, unpublished data, 2006). These animal models provide intriguing insight into the mechanisms associated with long-term sleep loss. Recent studies have shown that with progressive sleep loss, rats develop deficits in hippocampal learning and retention in the Morris water-maze test[59] and hippocampal long-term potentiation that appears to be impaired by adenosine buildup.[60]

A second group of sleep-active neurons in the median preoptic nucleus (MnPO) was described following the identification of the VLPO.[61] Similar to VLPO cells, a subset of MnPO neurons expresses c-Fos and discharges more rapidly during NREM and REM sleep.[62-64] Interestingly, unlike VLPO neurons, the MnPO cells also fire faster during prolonged wakefulness (which increases sleep pressure).[63] Many of the MnPO neurons that were c-Fos–positive during sleep also contain GABA and project to the lateral hypothalamus, including the perifornical region that contains orexin cell bodies and is associated with wakefulness.[62,63] However, there is currently no evidence that lesions of the MnPO affect sleep, and it is not clear whether the arousal systems have afferents to the MnPO. In addition, the MnPO has been implicated in the regulation of body temperature, through long descending projections to the raphe pallidus in the ventral medulla,[65,66] and in fluid homeostasis, through projections to the paraventricular nucleus and supraoptic nucleus of the hypothalamus.[67,68] It is unclear whether the cells in the MnPO that are c-Fos positive during sleep are responding to body temperature, which also falls during sleep; sleep-active cells and neurons associated with c-Fos expression following administration of hypertonic saline appear to belong to separate populations in the MnPO.[67] Afferent connections of the MnPO are from the subfornical organ, the paraventricular nucleus of the hypothalamus, the parabrachial nucleus, the nucleus of the solitary tract, and the ventrolateral medulla; all of these areas have been implicated in cardiovascular regulation.[69]

The Flip-Flop Switch Model of Sleep-Wake Regulation

A flip-flop switch model of sleep-wake regulation has been recently proposed.[70-72] A flip-flop circuit contains two sets of

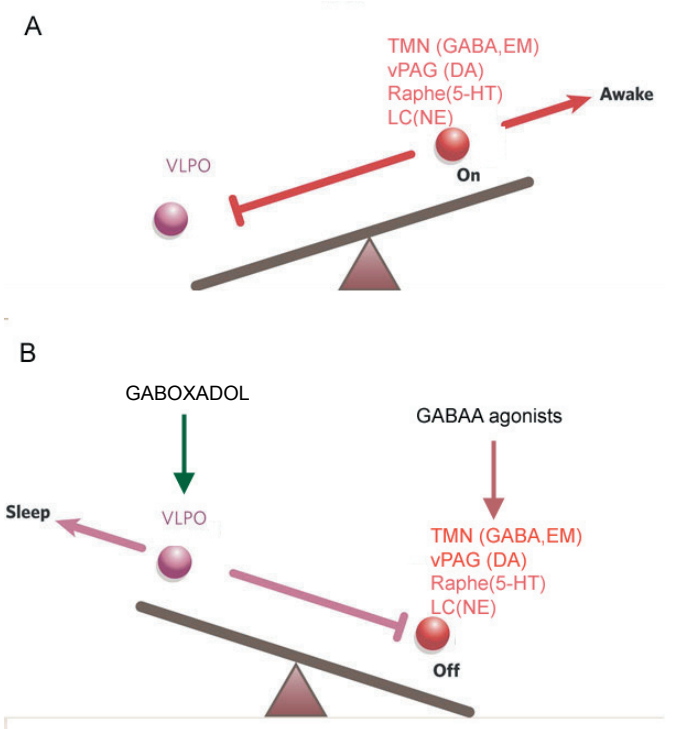

Figure 3—The flip-flop switch model for sleep-wake control, a potential locus targeted by $GABA_A$ receptor agonist drugs to produce sedative effects. **a.** Small, stimulatory perturbation of the arousal side (right side) of the switch would reinforce (disinhibit) itself through inhibition of the VLPO, resulting in a stable arousal state. **b.** In contrast, a small stimulation of the VLPO by endogenous sleep agents such as adenosine would lead to disinhibition of VLPO neurons and inhibition of the arousal side. $GABA_A$ receptor agonist drugs appear to promote sleep by directly inhibiting the TMN via binding to $GABA_A$ receptors that contain the α_1 subunit. Gaboxadol, a partial $GABA_A$ receptor agonist, may promote sleep (sedation) by activating the VLPO via binding to $GABA_A$ containing the α_4 subunit in GABAergic input sources projecting the VLPO, such as the prelimbic cortex and lateral septum. Modified with permission from Saper CB et al.[71]

mutually inhibitory elements. When the activation of one side is slightly stronger, there is increased inhibition of the weaker side. This inhibition further tips the balance of the switch towards the stronger side as it receives increased activation through disinhibition of afferent inputs. Flip-flop switches make sharp state transitions; this property may explain the suddenness of falling asleep or waking up. The sleep side is the VLPO and the arousal side includes TMN histaminergic neurons, DRN serotoninergic neurons, ventral periaqueductal gray (vPAG) dopaminergic neurons,[73] and LC noradrenergic neurons. Each side of the switch inhibits the other. The VLPO neurons contain GABA and galanin, an inhibitory peptide, and project to the neuronal groups of the arousal systems, whereas the TMN, LC, DRN, and vPAG dopaminergic neurons all project to the VLPO, and norepinephrine, serotonin, and dopamine have been shown to inhibit VLPO neurons in in vitro slice recordings.[74,75] Although histamine does not affect VLPO activity,[74] TMN neurons also contain GABA and endomorphin, both of which are inhibitory neurotransmitters in the VLPO (Figure 3).

The flip-flop model predicts that homeostatic forces act on the switch by pushing it closer to its transition point. When the number of neurons on either side of the switch is reduced, it is more difficult for homeostatic forces to overcome the remaining stronger side. As a result, the switch continuously rides closer to its transition point, and there are more transitions in both states. This phenomenon is seen in rats with VLPO lesions that have more sleep bouts, but an ~50% decrease in total sleep time, because the sleep bouts are shortened. These rats can initiate but cannot maintain sleep because the number of remaining VLPO neurons is insufficient to keep the arousal systems silent for long.[58]

We would expect that lesions of arousal systems would have the opposite outcome: more and shortened waking bouts, but more frequent awakenings. In this model, the orexin neurons are not part of the switch, because VLPO neurons do not have orexin receptors. Thus, loss of orexin neurons weakens the arousal side of the switch, because it has an excitatory effect via orexin receptors on neurons in the TMN (OX2 receptors), LC (OX1 receptors), DRN (OX1 and OX2 receptors), and vPAG dopaminergic neurons (OX1 and OX2 receptors).[38] Once this tonic excitatory input is removed, the weakened arousal side is more easily pushed closer to the transition point, resulting in more transitions and fragmentations.[35] As a result, loss of the orexin influence produces more transitions into sleep and sleep fragmentations, but it does not alter total amounts of sleep.

Among the ascending arousal systems, only lesions of vPAG dopaminergic neurons and the lateral hypothalamus result in the predicted reductions of total wakefulness with more transitions into wakefulness.[73,76] As noted earlier, the cell type in the lateral hypothalamus that provides this potent promotion of arousal has not yet been characterized.

Although both the ARAS and VLPO-monoaminergic system are involved in regulation of sleep-wake behaviors, the relationship between them is unclear. We propose that ARAS is a fundamental structure involved in the maintenance of consciousness, whereas the VLPO-monoaminergic system that runs in parallel with ARAS controls sleep (Figure 2). The two systems converge at the basal forebrain, thalamus, and neocortex. If damage to the ARAS occurs at the sub-basal forebrain and thalamus, VLPO-monoaminergic control can still reach to the neocortex.

In the decades since von Economo's initial observations, a more complete understanding of the circuitry that underlies sleep-wake control has emerged. This research is particularly important because of the high prevalence of insomnia. Insomnia occurs in about one of every three American adults and is often treated with $GABA_A$ agonists.[77] This necessitates an understanding of how these drugs interact with the endogenous sleep system. In the following section, we review the effects of $GABA_A$ agonists on regions associated with sleep-wake behavior.

Neuronal Substrates of Sedative Mechanisms of $GABA_A$ Drugs

We have already discussed that the inhibitory effects of GABA are mediated by the activation of two types of GABA receptors: ionotropic receptors that are ligand-gated channels ($GABA_A$ and $GABA_C$ receptors) and metabotropic receptors that are G-coupled proteins acting by activation of second messenger systems ($GABA_B$ receptors).[78,79]

To examine the effects of $GABA_A$ agonists on the endogenous sleep-wake systems, Nelson and colleagues monitored c-Fos expression in the CNS after systemic drug administration. Administration of subanesthetic doses of gaboxadol, pentobarbital, alcohol, propofol, chloral hydrate, urethane, isoflurane, muscimol,

zolpidem, and allopregnanolone (neurosteroid) induced a slow-wave EEG pattern and increased delta power (0.5 Hz to 4 Hz), as well as increased c-Fos expression in VLPO neurons.[80,81] The number of c-Fos–positive neurons in the VLPO varied with the specific drug administered, but was consistently less than half of the number observed during spontaneous sleep. The one drug that did not fit this profile was gaboxadol: administration of gaboxadol (5 mg/kg) resulted in VLPO c-Fos expression that was similar to that observed during natural sleep. All of the $GABA_A$ agonist drugs tested, however, consistently and completely suppressed c-Fos expression in the TMN. The cerebral cortex showed an overall low level of c-Fos expression, consistent with the sleep-like behavior exhibited by treated animals. Expression of c-Fos in the other ascending arousal systems, however, was not always suppressed. For example, all of the $GABA_A$ agonist drugs tested produced marked c-Fos expression in the LC. Alcohol administration also induced c-Fos expression in the ventral tegmental dopaminergic neurons, whereas alcohol and choral hydrate induced c-Fos expression in the hypothalamic paraventricular nucleus and supraoptic nucleus. These results suggest that $GABA_A$ agonists engage the endogenous sleep-wake systems at variable sites in the wake-promoting centers and the sleep-promoting VLPO, although with lower activation of the VLPO than is achieved by spontaneous sleep. At anesthetic doses, $GABA_A$ agonists, including gaboxadol, would further slow down and flatten the EEG and suppress c-Fos expression in the entire CNS, including sleep-wake control systems, as predicted. Thus, interaction of $GABA_A$ agonists with the endogenous sleep system is dose-dependent.

Injection of the $GABA_A$ receptor antagonist gabazine into the TMN (and surrounding area) blocked the hypnotic effects produced by systemic administrations of pentobarbital, muscimol, and propofol.[81] Because $GABA_A$ agonists only moderately activate the VLPO, but totally suppress c-Fos expression in the TMN, the sedating effects of these drugs may predominately target and inhibit TMN activity. The partial $GABA_A$ agonist (on $\alpha_1\beta_3\gamma_{2s}$ subunits) gaboxadol promotes NREM sleep and increases delta power, but unlike most $GABA_A$ agonists, it does not suppress REM sleep.[82-84] In mice, gaboxadol appears to alter patterns of slow-wave EEG.[85] These results suggest that the hypnotic effects of gaboxadol may occur, at least in part, through activation of the VLPO (Figure 3). Consistent with this idea, preliminary data show that lesion of the VLPO attenuates the sedative effects of gaboxadol (Vogel V, Saper CB, Lu J, unpublished data, 2006). Clinically, in two randomized, double-blind, placebo-controlled crossover studies, gaboxadol was well tolerated and demonstrated efficacy in shortening the latency to sleep onset, increasing sleep intensity and quality, and decreasing the number of awakenings and the amount of intermittent wakefulness, without affecting next-day cognitive performance in both young and elderly healthy individuals.[86,87]

Molecular Sedative Mechanisms of $GABA_A$ Agonist Drugs

Rudolf and Mohler et al have studied the effects of α-subunit mutations of the $GABA_A$ receptor on the sedative, anxiolytic, amnestic, and muscle-relaxant effects of benzodiazepine drugs. Experiments conducted in mice with specific mutations in GABA α subunits demonstrated that the sedative effect of benzodiazepines is mediated largely by α_1 subunits,[88-90] whereas the anxiolytic effect is mediated via α_2 subunits.[91] Compared with benzodiazepines and other $GABA_A$ agonists, gaboxadol has a lower affinity for α_4, δ, and α_1 subunits, acting as a partial $GABA_A$ agonist.[92] $GABA_A$ $\alpha_4\delta$-containing receptors are predominately located extrasynaptically and perisynaptically,[93-95] which contributes to tonic inhibitory effects seen in the hippocampus,[96] thalamus,[97] and cerebral cortex.[98] The TMN contains α_1 subunits,[93,94] consistent with the hypothesis that GABA agonists inhibit TMN activity by binding to the α_1 subunit of $GABA_A$ receptors. As some GABAergic sources projecting to the VLPO,[52] such as the prelimbic cortex and lateral septum, contain $\alpha_4\delta$ receptors,[94,99] gaboxadol may act on $\alpha_4\delta$ receptors there to mediate activation of the VLPO. These receptor subtypes are also present in the cerebral cortex.[98] Collectively, these results indicate that $GABA_A$ agonists exert their hypnotic effects in site- and receptor-subunit–specific manners. The further elucidation and characterization of these aspects of $GABA_A$-agonist binding, particularly how gaboxadol activates the VLPO, may provide a basis for the development of new therapeutic strategies to treat sleep disorders.

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