I THE NEUROCHEMISTRY OF THE STATES OF SLEEP AND WAKEFULNESS

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Neurochemistry of the preoptic hypothalamic hypnogenic mechanism

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The chapter will summarize our current understanding of the neuronal and neurochemical basis of hypnogenesis. The hypothesis of the localization of a hypnogenic mechanism in the mammalian hypothalamic preoptic area (POA) was first proposed by von Economo more than 70 years ago (von Economo, 1930). This hypothesis has been confirmed by findings that experimental POA lesions suppress sleep, and that electrical, chemical, and thermal POA stimulation induce sleep (reviewed by McGinty & Szymusiak, 2001). Unit recording studies have identified POA neurons that exhibit increased activity during NREM sleep, REM sleep, or both. These *sleep-active* neurons are hypothesized to be the substrate of the hypnogenic mechanism. The past decade has seen substantial progress in the further description of this hypnogenic system; we summarize this progress in this chapter.

Localization of sleep-active neurons within the POA

Studies of sleep-active neuronal discharge across the sleep–wake cycle in freely moving animals provide important information about the hypnogenic process (see below) but, because of sampling limitations, are not suitable for systematic mapping of the exact locations of putative hypnogenic neurons. The application of the c-Fos immunoreactivity (IR) method to map sleep-active neurons has stimulated several advances. C-Fos IR is a marker of neuronal activation in most brain sites; immunohistochemically labeled neurons can be mapped systematically. The localization of c-Fos IR following sustained sleep, but not

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waking at the same circadian time, permits the quantitative mapping of sleep-active neurons.

Initially, it was proposed that sleep-active neurons were concentrated in a small region identified as the ventrolateral POA (VLPO) (Sherin *et al.*, 1996). This finding was based on the fact that, under the conditions of the procedure, few wake-active neurons were present in VLPO. That is, sleep-active neurons were segregated from wake-active neurons, so the presence of c-Fos IR following sleep could be unambiguously attributed to sleep. Subsequently, by using the c-Fos IR method, additional groups of segregated sleep-active neurons were found in the caudal and rostral median preoptic nucleus (MnPN), also a site with little wake-related c-Fos under quiet waking conditions (Gong *et al.*, 2000). The number of cells exhibiting c-Fos IR following sleep was highly correlated with the percentage of sleep during the preceding 2 h. These studies also showed c-Fos IR following sleep throughout dorsal and lateral POA. Sleep-active neurons were also found in these sites with unit recording studies (see below). However, in dorsal and lateral POA, wake-related c-Fos IR was more prominent, so c-Fos IR following sleep might have arisen from some preceding wake-related processes, rather than sleep (but see below).

Phenotype of sleep-active neurons

Most sleep-related c-Fos IR neurons in rostral and caudal MnPN and in VLPO co-localize glutamic acid decarboxylase (GAD), the enzyme marking neurons that synthesize the inhibitory neurotransmitter γ-aminobutyric acid (GABA) (Fig. 1.1). In our initial description, from studies using a polyclonal antibody, 70%–80% of cells expressing c-Fos following high spontaneous sleep co-localized GAD (Gong *et al.*, 2004b). The number of double-labeled cells was highly correlated with percent of sleep in the preceding 2 h in all three sites (Fig. 1.2). In other studies of the MnPN (Modirrousta *et al.*, 2004) and in work from our laboratory using a monoclonal antibody (Gvilia *et al.*, 2006), both the number of c-Fos expressing cells and the percent of c-Fos/GAD double-labeling were somewhat lower, but the co-localization remained significant. Two studies showed that the number of double-labeled cells increased during recovery sleep after sleep deprivation (Gong *et al.*, 2004b; Modirrousta *et al.*, 2004). These studies suggest that additional GABAergic neurons are activated during sleep in association with increased homeostatic drive. This might be one element of a mechanism of sleep homeostasis. Preliminary findings based on retrograde labeling methods showed that many MnPN neurons sending projections to the perifornical lateral hypothalamus (PLH) co-localize GAD (Gong *et al.*, 2004a).

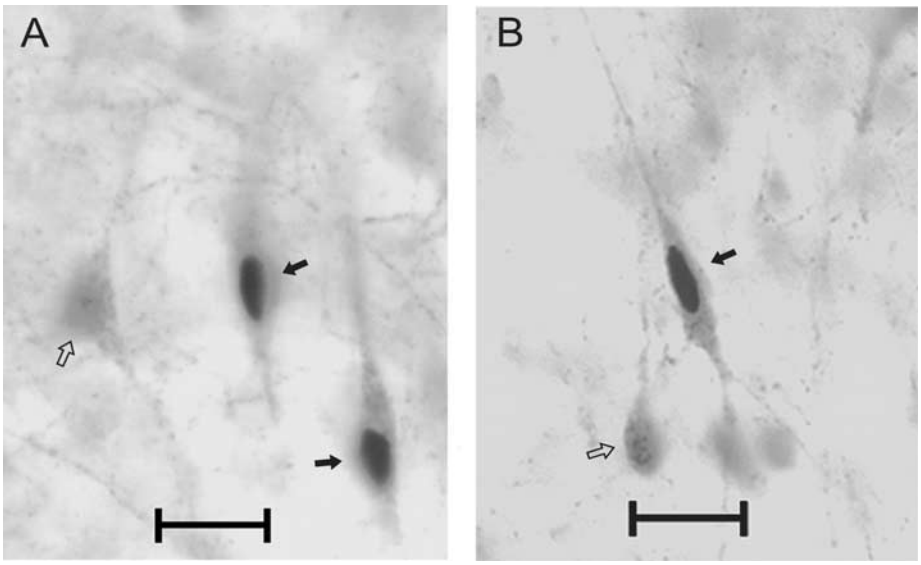

Figure 1.1 Examples of immunostaining for c-Fos IR (dark nuclear staining) and GAD (light cytoplasmic staining) in the MnPN. A higher percentage of GAD-positive cells (arrows) also expressed c-Fos IR (black arrows) following high (A) compared with low spontaneous sleep (B). From Gong *et al.* (2004b). See also Plate 1.

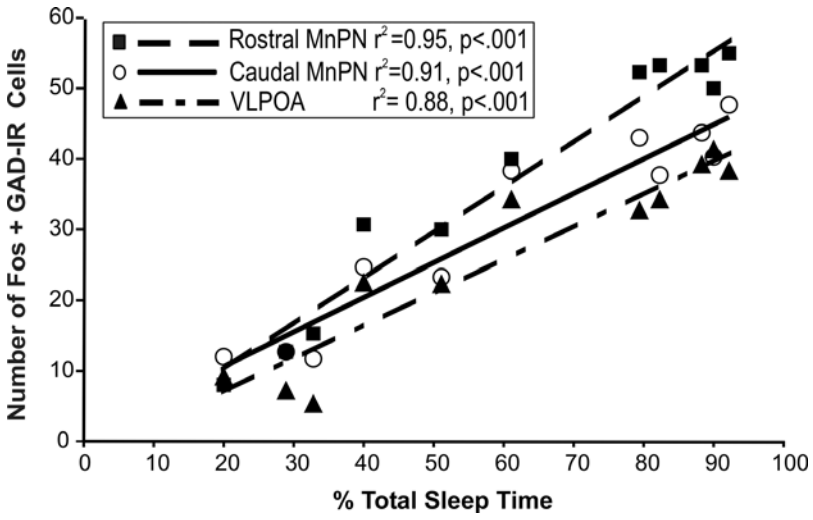

Figure 1.2 Regression analysis between numbers of C-Fos IR + GAD double-labeled neurons and the amount of sleep in the 2 h preceding sacrifice. The numbers of double-labeled cells were highly correlated with the percentage of preceding sleep in VLPO and both rostral and caudal MnPN. From Gong *et al.* (2004b).

Further analysis of the localization of sleep-active neurons within the POA

The numbers of c-Fos/GAD *double-labeled* neurons increased following sleep, compared with waking, in dorsal and lateral POA sites, as well as in MnPN and VLPO (Angara *et al.*, 2004). Sleep-active neurons were also found throughout the lateral POA and in adjacent basal forebrain by using electrophysiological methods (Alam *et al.*, 1995a, 1997; McGinty & Szymusiak, 2001). These findings suggest that the population of sleep-active neurons might be diffusely distributed in the POA, including dorsal and lateral areas, and extend into the basal forebrain. The interpretation is congruent with lesion studies. Some POA lesions that spare the VLPO suppress sleep (Schmidt *et al.*, 2000; John & Kumar, 1998). One study showed a lower correlation between lesion extent and sleep loss after lesions largely sparing VLPO compared with lesions of the VLPO cluster, but this lower correlation seemed to be largely due to cases of *greater* sleep loss than was predicted from lesion extent (see Fig. 1.3 in Lu *et al.*, 2000). Most restricted POA lesions produce partial suppression of sleep. However, very large lesions, encompassing all regions of the POA and adjacent basal forebrain, may produce nearly complete suppression of sleep (McGinty and Sterman, 1968; Sallanon *et al.*, 1989). Thus, most available evidence suggests that sleep-active neurons are distributed in the median, dorsal, lateral, and ventrolateral POA and adjacent basal forebrain.

Details of POA sleep-active neuronal activity

C-Fos IR studies show that increased neuronal activity is correlated with occurrence of sleep, in general. To better evaluate the details of the relationship of neuronal activity and sleep, it is useful to conduct studies of neuronal activity. We have carried out neuronal unit recording studies of VLPO, MnPN, lateral POA, and diagonal band basal forebrain neurons (Suntsova *et al.*, 2002; Szymusiak *et al.*, 1998; Alam *et al.*, 1995a, 1997; Szymusiak & McGinty, 1986). Sleep-active neurons, defined by at least a 20% increase in discharge rate in NREM or REM, constituted 76% of the sample of MnPN neurons and 56% of VLPO neurons, but only about 10% of neurons in lateral POA and DB were sleep-active. These differences are consistent with the differences in the ratios of wake to sleep c-Fos expression in these sites, noted above. Most sleep-active neurons in all sites were slowly discharging during waking, typically doubling discharge rate in NREM sleep (Fig. 1.3). Increases in discharge anticipated EEG synchronization at sleep onset by a few seconds in each site. In MnPN, most sleep active neurons had higher discharge in REM compared with NREM, but in VLPO most neurons had

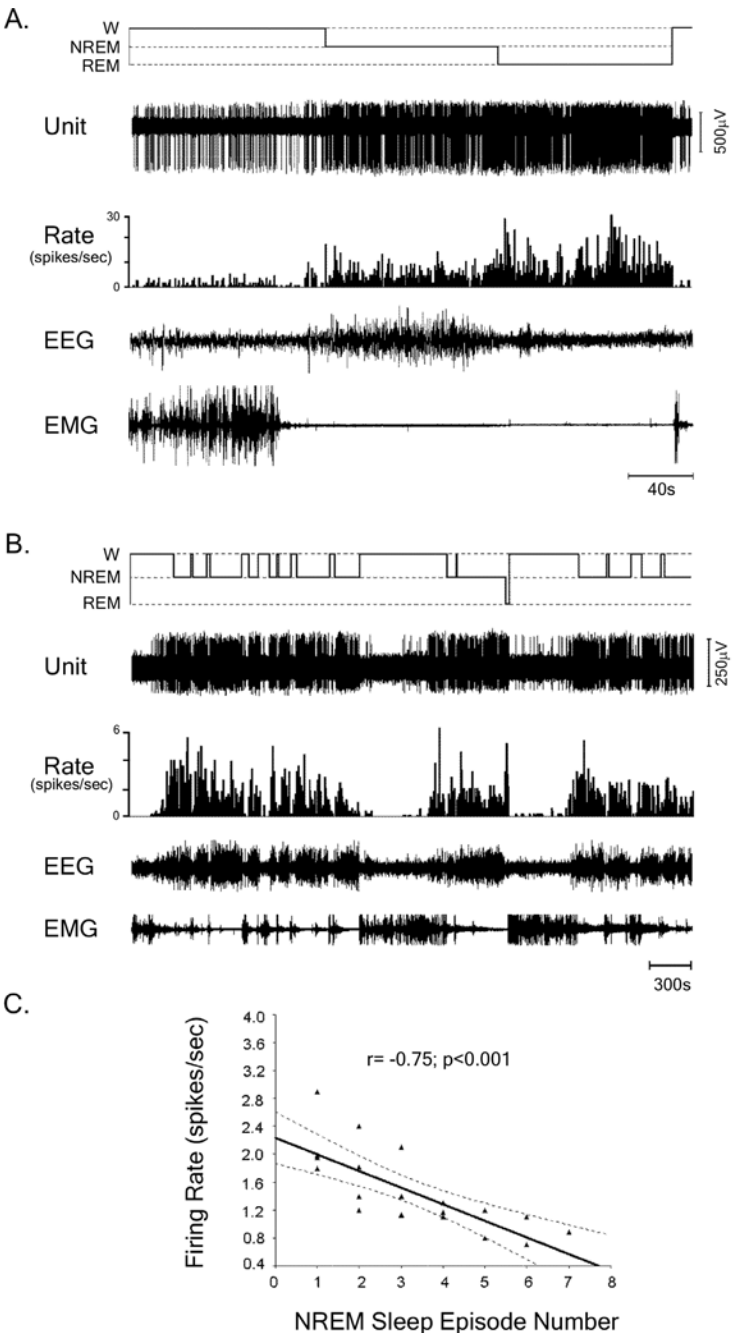

Figure 1.3 (A) Example of MnPN sleep-active neuron. Discharge increases coincident with occurrence of slow wave activity and increases further in REM sleep (right side of sample). (B) Compressed record showing rate histogram across several bouts of NREM sleep. (C) Analysis of a population of such cells showed that MnPN sleep-active neurons exhibited highest discharge during initial NREM episodes and showed progressively declining discharge during subsequent bouts, possibly correlated with sleep drive. From Suntsova *et al.* (2002).

similar or lower discharge in REM, compared with NREM. There were additional differences between the sites. In MnPN, discharge tended to be higher at the beginning of a sustained sleep period, and gradually decline during successive NREM epochs within a period of sustained sleep. In VLPO, discharge progressively increased within a sustained sleep epoch, in association with increasing slow wave activity.

We have also recorded neuronal discharge in several wake-promoting neuronal groups, including the PLH (Alam *et al.*, 2002), histaminergic tuberomammallary nucleus (TMN) of the posterior hypothalamus (PH) (Steininger *et al.*, 1999), dorsal raphe nucleus (Guzman-Marin *et al.*, 2000), and basal forebrain (Szymusiak & McGinty, 1986). The putative histaminergic neurons of the TMN, the putative serotonergic neurons of the dorsal raphe nucleus, and the putative hypocretinergic neurons of the PLH exhibit a wake-active, NREM- *and* REM-off pattern of discharge, changing reciprocally across the W–NREM–REM cycle compared with the typical discharge pattern of the majority of MnPN neurons. Other wake-active neurons in the PH, PLH, and basal forebrain exhibit lower rates in NREM, but higher rates in REM, a discharge pattern that is reciprocal to many VLPO neurons, as well as subsets of MnPN and lateral POA neurons. Thus, the different subtypes of sleep-active neurons may play distinct roles in sleep micro-architecture, and these types may be regionally segregated within the POA.

Efferents from sleep-active neurons

POA neurons are the source of efferents to several established arousal systems. The VLPO is the source of a strong projection to the histaminergic neurons of the ventrolateral posterior hypothalamus (Sherin *et al.*, 1998). The dorsal raphe nucleus receives afferents from the lateral POA and MnPN as well as VLPO (Zardetto-Smith & Johnson, 1885; Peyron *et al.*, 1998; Steininger *et al.*, 2001). The VLPO and dorsolateral POA send projections to the posterior lateral hypothalamus (Steininger *et al.*, 2001; Yoshida *et al.*, 2006; Sakurai *et al.*, 2005). In these semiquantitative analyses, the medial and lateral POA, and MnPN, as well as VLPO, have the highest density of projections to the PLH field. There is evidence that hypocretin/orexin-containing neurons of the PLH receive direct projections from VLPO GABAergic neurons (Sakurai *et al.*, 2005). The locus coeruleus also receives POA projections (Steininger *et al.*, 2001). Thus, it is likely that projections from the POA sleep-active neurons to arousal systems are GABAergic, at least in part. In support of this hypothesis, we have found that stimulation of the MnPN produces short-latency inhibition of PLH neurons (Fig. 1.4). Train stimulation evoked EEG synchronization.

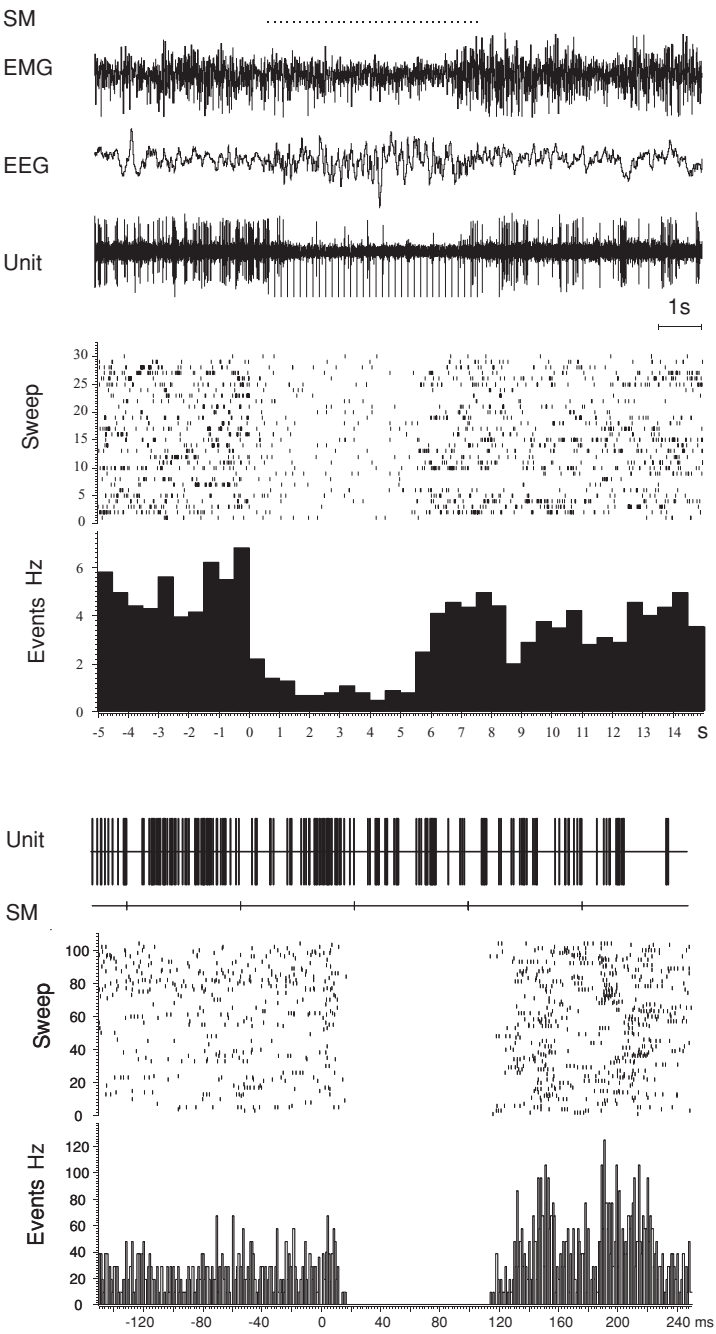

Figure 1.4 Effects of MnPN stimulation on EEG patttern and PLH neuronal activity. Upper: An MnPN 6 s stimulus train suppressed PLH neuronal discharge, evoked EEG synchronization, and reduced EMG activity. The sweep display shows effects of successive stimulus trains. The events display shows averaged PLH neuronal discharge rate in conjunction with train stimulation. Lower: Raster plot showing neuronal discharge during single pulse stimulation. In this example, PLH neuronal activity was inhibited with a latency of about 10 ms; inhibition lasted about 110 ms. Activation of MnPN neurons during NREM sleep would result in suppression of PLH neuronal activity.

GABA release and GABAergic control of arousal systems

Studies using microdialysis showed increased GABA release during sleep, including in REM sleep in the dorsal raphe nucleus (Nitz & Siegel, 1997a) and locus coeruleus (Nitz & Siegel, 1997b), and in NREM sleep in posterior hypothalamus (Nitz & Siegel, 1996). We have studied the role of GABA in control of PLH neurons including hypocretin/orexin-containing neurons in relation to sleep–wake state (Alam *et al.*, 2005). Administration of the GABA antagonist bicuculline (BIC) by microdialysis in PLH suppressed NREM and REM sleep and increased c-Fos IR labeling in hypocretin-positive neurons (Fig. 1.5). These neurons express GABA receptors (Moragues *et al.*, 2003). In the dorsal raphe nucleus, application of the GABA agonist muscimol increased REM sleep, and application of a GABA antagonist, picrotoxin, suppressed REM sleep. These studies suggest that GABA is tonically facilitating both NREM sleep and REM sleep by inhibiting hypocretin neurons, dorsal raphe serotonergic neurons, and other arousal systems. One source of the GABA released during NREM in the PLH as well as the dorsal raphe nucleus and locus coeruleus could be POA and basal forebrain sleep-active GABAergic neurons. In support of this hypothesis are findings that MnPN electrical stimulation inhibits PLH neuronal discharge (Fig. 1.3) and that inactivation of basal forebrain neurons with a GABA agonist, muscimol, increases c-Fos expression in PLH neurons, including orexin-containing neurons (Satoh *et al.*, 2003).

Thermosensitive component of the POA hypnogenic system

Local POA warming by 1–2 °C by means of a water-perfused 'thermode' can trigger NREM sleep onset, increase NREM within sustained sleep, and increase EEG slow wave activity within sustained NREM sleep (reviewed by McGinty & Szymusiak, 2001). Effects of local POA warming are mediated by responses of warmth-sensitive neurons (WSNs) that are identified within the POA. WSNs exhibit brisk increases in discharge in response to small changes in local temperature, more than is expected on the basis of metabolic considerations. WSNs typically constitute 10%–20% of neurons encountered; most POA neurons are unresponsive to small changes in local temperature. We previously showed that a majority of WSNs are sleep-active. Sleep-active WSNs also increase discharge a few seconds before EEG synchronization at sleep onset (Alam *et al.*, 1995a, 1997). Since activation of these neurons by local POA warming is sufficient to initiate and maintain NREM sleep, and these neurons are also activated before and during spontaneous sleep, we can hypothesize that the activation of sleep-active WSNs facilitates spontaneous NREM sleep. The activation of

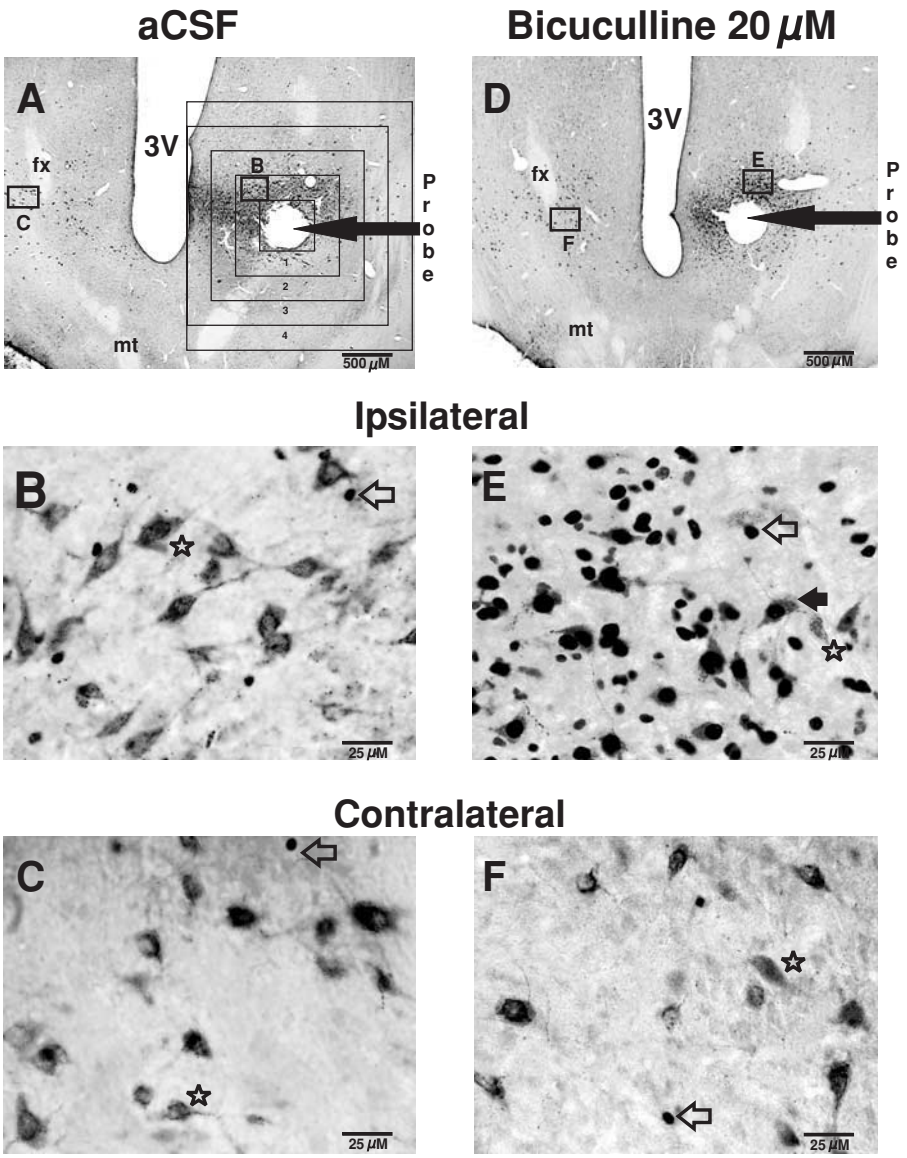

Figure 1.5 Effects of local microdialytic administration of the GABA antagonist bicuculline (BIC) on c-Fos expression in PLH cells. (A, D) Horizontal sections around the hole left by microdialysis membrane. A grid (A) around the probes was used to quantify effects of drug as a function of distance from the membrane. (B, E) Effects of aCSF (B) or BIC (E) on c-Fos IR (small dark spots) and orexin/hypocretin (gray cytoplasmic label) immunostaining. BIC greatly enhanced the numbers of cells exhibiting c-Fos IR. Some orexin/hypocretin-labeled cells were double-labeled. There were no changes contralaterally (C, F), showing that effects were not due to behavioral changes. From Alam *et al.* (2005).