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Antagonism of α_1-adrenergic and serotonergic receptors in the hypoglossal motor nucleus does not prevent motoneuronal activation elicited from the posterior hypothalamus

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Abstract

The perifornical (PF) region of the posterior hypothalamus plays an important role in the regulation of sleep-wake states and motor activity. Disinhibition of PF neurons by the $GABA_A$ receptor antagonist, bicuculline, has been used to study the mechanisms of wake- and motor activity-promoting effects that emanate from the PF region. Bicuculline activates PF neurons, including the orexin-containing cells that have major excitatory projections to brainstem noradrenergic and serotonergic neurons. Since premotor aminergic neurons are an important source of motoneuronal activation, we hypothesized that they mediate the excitation of motoneurons that results from disinhibition of PF neurons with bicuculline. In urethane-anesthetized, paralyzed and artificially ventilated rats, we found that PF bicuculline injections (1 mM, 20 nl) made after combined microinjections into the hypoglossal (XII) nucleus of α_1-adrenergic and serotonergic receptor antagonists (prazosin and methysergide) increased XII nerve activity by 80% ±16(SE) of the control activity level. Thus, activation of XII motoneurons originating in the hypothalamic PF region was not abolished despite effective elimination by the aminergic antagonists of the endogenous noradrenergic and serotonergic excitatory drives to XII motoneurons and abolition of XII motoneuronal activation by exogenous serotonin or phenylephrine. These results show that a major component of XII motoneuronal activation originating in the posterior hypothalamus is mediated by pathways other than the noradrenergic and serotonergic projections to motoneurons.

Keywords

GABA; noradrenergic neurons; orexin; serotonin; sleep

Introduction

The posterior, lateral hypothalamus plays an important role in the maintenance of vigilance [7]. Pharmacological inhibition of neurons located in the hypothalamic perifornical (PF) region enhances sleep, whereas their activation increases wakefulness [1,29,30]. GABA-containing and sleep-active neurons located in the anterior hypothalamus send axonal projections to the posterior hypothalamic PF region [31]. The PF region is the only site in the brain containing

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neurons that synthesize the excitatory peptides orexins (ORX, also known as hypocretins) [17]. ORX cells are active during wakefulness [8,19,23], and ORX loss causes narcolepsy/cataplexy, a disorder characterized by excessive sleepiness and sudden spells of motor atonia [5,14]. ORX cells have widespread efferent projections that primarily target other wake-related neuronal groups, including brainstem noradrenergic and serotonergic neurons [26].

In urethane-anesthetized rats, microinjections of the $GABA_A$ antagonist, bicuculline, into the hypothalamic PF region activates ORX and other local cells and elicits a systemic response that comprises desynchronized cortical electroencephalogram (EEG), hippocampal theta rhythm, increased hypoglossal (XII) nerve activity and accelerated respiratory rate [21]. A concomitant increase in expression of the immediate early gene, Fos, in pontine noradrenergic neurons suggests that these calls also are activated [21].

Brainstem noradrenergic and serotonergic neurons send axons to all brainstem and spinal motor nuclei, including the medullary XII nucleus [2,15,27]. Data show that the endogenous aminergic drive to XII motoneurons is a major determinant of their wake-related activation [4,10]. This drive is mainly mediated by α_1-adrenergic and type 2 serotonergic receptors [18]. Since ORX activates aminergic neurons [3,16,17,22], we hypothesized that the excitatory motor effects of PF bicuculline are mainly mediated by noradrenergic and serotonergic projections to motoneurons. To test this hypothesis, we assessed the effect of PF bicuculline on XII nerve activity following antagonism of α_1-adrenergic and serotonergic receptors in the XII nucleus. Preliminary results have been published [12].

Materials and methods

Experiments were performed on 12 adult (320–430 g), male Sprague-Dawley rats obtained from Charles River Laboratories (Wilmington, MA). All animal procedures followed the guidelines established by the National Institutes of Health (NIH Publication 80–23 with subsequent revisions) and were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Animal preparation, recording and microinjections

Rats were pre-anesthetized with isoflurane followed by urethane (1.0 g/kg, i.p.), tracheotomized and had a femoral artery and vein catheterized for arterial blood pressure monitoring and fluid injections, respectively. The right XII nerve was dissected and placed in a cuff electrode for recording [9]. Both cervical vagi were cut to enhance XII nerve activity and make it independent of lung volume feedback. The animal's head was placed in a stereotaxic holder and openings were made in the parietal bones for inserting bicuculline-containing pipette into the posterior hypothalamus on the left side and a recording electrode into the hippocampus on the right side. The caudal medulla was exposed by a posterior fossa craniotomy to insert a glass pipette filled with aminergic antagonists into the right XII nucleus.

The cortical, hippocampal and XII nerve activities were recorded together with end-expiratory CO_2, blood pressure and rectal temperature (35.5–36.5°C), as described previously [21]. The animals were paralyzed with pancuronium bromide (2 mg/kg i.v., Sigma) and artificially ventilated with an air-oxygen mixture (30–60% O_2). The end-expiratory CO_2 was kept constant and sufficient to maintain steady respiratory modulation of XII nerve activity (5–6%). Adequate level of anesthesia was maintained based on stable amplitude and constant rate of inspiratory bursts recorded from the XII nerve, steady blood pressure and slow-wave cortical EEG. If needed, supplemental injections of urethane and pancuronium bromide were administered in 40 mg and 1 mg/kg increments, respectively.

All drugs (Sigma) were diluted from frozen aliquots in 0.9% NaCl before each experiment. We used (−)-bicuculline methiodide, a $GABA_A$ receptor antagonist (l mM); 1-(4-amino-6,7-dimethoxy-2-quinazolinyl)-4-(2-furanylcarbonyl) piperazine hydrochloride (prazosin), an α_1-adrenoceptor antagonist (0.2 mM); methysergide maleate, a broad-spectrum serotonergic receptor antagonist (1.0 mM); phenylephrine, an α_1-adrenoceptor agonist (2 mM); and serotonin creatinine sulfate (5 mM). Prazosin and methysergide were combined in one solution. To mark the PF injection sites, the bicuculline solution contained 2% of Pontamine sky blue dye (ICN Biomedicals, Aurora, OH). Injections sites in the XII nucleus were marked at the end of the experiment by injections of 2% Pontamine.

Microinjections were made from beveled glass pipettes (A-M Systems, Carlsborg, WA; tip diameters of 25–30 μm). The bicuculline pipette was inserted into the hypothalamic PF region aiming at: 3.1 mm caudal to bregma, 1.3 mm lateral to the midline, and a depth of 8.2 mm from the brain surface [25]. Drugs were injected by applying pressure to the fluid in the pipette while monitoring the movement of the meniscus with a calibrated microscope (1 nl resolution). The antagonist mix was injected into the XII nucleus at three sites separated by 0.65 mm in antero-posterior (A-P) direction to cover the entire XII nucleus [10].

Experimental protocol and measurements

After recording of baseline activity, each animal received three injections of the antagonist mix into the XII nucleus (30–40 nl each) made over 5.7 min ±0.4. This was followed 28.9 min ±2.9 later by one bicuculline injection into the PF region (20 nl). The ~30 min delay was chosen because the antagonists exert their maximal effect on XII nerve activity around this time [10].

The magnitude of XII nerve activity was measured from the moving average of the signal (time constant: 100 ms) as the difference between the peak of inspiratory burst and the level of activity during central expiration when activity was minimal or absent. XII nerve activity measured during subsequent stages of the experiment was normalized by its level measured before injections of the antagonist mix into the XII nucleus. The central respiratory rate was determined from XII nerve activity recording. All measurements were derived from 2 min periods at three time points during the experiment: before the antagonist injections into the XII nucleus, after the antagonist injections into the XII nucleus but before bicuculline injection into the PF region, and during the peak of the response to PF bicuculline. The latency of the response to PF bicuculline was measured between the onset of the injection and the time when XII nerve activity started to increase. The power of hippocampal activity in the theta-like frequency range (3–5 Hz) [32] was determined in successive 15 s intervals (Spike-2; CED, Cambridge, UK and Somnologica, MedCare, Buffalo, NY; acquisition rate: 100 Hz).

Histology and statistics

At the conclusion of the experiment, the animal received an additional dose of urethane (1 g/kg) and was perfused with cold phosphate-buffered 0.9% NaCl (pH 7.4) followed by 10% formalin. The brain was extracted, postfixed and 50 μm sections were cut in coronal plane through the hypothalamus and in parasagittal plane through the medulla. Sections containing the blue dye were serially mounted and stained with Neutral red.

Following verification that the variables were normally distributed, a paired, two-tailed Student's t-test was used for statistical analysis (SigmaPlot, Jandel, San Rafael, CA). Differences were considered significant when P was less than 0.05. The variability of the means is characterized by the standard error (SE) throughout the report.

Results

Injections of prazosin and methysergide into the XII nucleus

The three injections of the antagonist mix into the XII nucleus reduced XII nerve activity to 24.9% ±6.3 of the pre-antagonist level (n=8, P<0.001); in two experiments, XII nerve activity was abolished at the peak of the response. The depressant effect of the antagonists on XII nerve activity was not associated with any changes in cortical or hippocampal activity or central respiratory rate (45.5 min^{-1} ±1.6 before, and 45.4 min^{-1} ±2.5 after, the antagonists). The initial part of the record in Fig. 1A shows XII nerve activity decline following antagonist injections into the XII nucleus.

Bicuculline injections into the hypothalamic PF region after prazosin and methysergide injections into the XII nucleus

Hypothalamic bicuculline injections made 28.9 min ± 2.9 after the antagonist mix injections into the XII nucleus elicited hippocampal theta rhythm, accelerated the respiratory rate, increased XII nerve activity (Fig. 1A) and activated cortical EEG (not shown; see ref. [21]). The mean latency and duration of the responses were 2.3 min ± 0.6 and 38.5 min ±1.3, respectively. XII nerve activity was significantly increased, from 24.9% ±6.3 to 104% ±16 of its baseline level at the beginning of the experiment (n=8, P<0.01) (Fig. 2A). The average increase from the level of XII nerve activity attained following microinjections of the antagonist mix into the XII nucleus (24.9%) to the peak of the response to PF bicuculline was 80% ±16 (Fig.2A). Following bicuculline, the central respiratory rate increased from 45.4 min^{-1} to 47.7 min^{-1} ±2.2 (P<0.05) (Fig. 2B).

Control experiments

In four rats, the ability of the antagonist mix to block the corresponding receptors was tested by observing the excitatory effects of either serotonin (5 mM, 10 nl; 2 animals) or phenylephrine (2 mM, 10 nl; 2 animals) injected into XII nucleus before, and at different times after, injections of the antagonist mix. The excitatory effects of both agonists were abolished when tested 20–27 min after the antagonists and partially recovered 2.5–3.0 h later (Figs. 1B and C). The long duration of the antagonist mix action was consistent with its long-lasting effect on endogenous aminergic activation of XII motoneurons described previously [10].

Injection sites

Examples of the aminergic antagonist mix and bicuculline injection sites are shown in Figs. 3A and B, respectively. Figure 3C shows the distribution of all PF bicuculline injection sites. They were located at A-P levels −2.80 to −3.80 mm from bregma [25].

Discussion

Contrary to our hypothesis, we found that antagonism of α_1-adrenergic and serotonergic receptors within and around the XII nucleus did not significantly diminish the excitatory effect of hypothalamic bicuculline injections on motoneuronal activity. This shows that the effect of PF bicuculline on XII motoneurons is predominantly mediated by pathways other than those that converge on premotor noradrenergic and serotonergic brainstem neurons with axonal projections to the XII nucleus. Thus, despite compelling neuroanatomical and pharmacological data suggesting that ORX-mediated activation of brainstem aminergic premotor neurons is a major source of wake-related motoneuronal activation [3,16,17,22], our direct assessment of the role of this pathway reveals that it is not essential for mediation of the XII motoneuronal activation from the hypothalamic PF region. Whether this finding also applies to other motoneuronal groups remains to be determined in future studies.

Injections of bicuculline into the hypothalamic PF region of urethane-anesthetized rats elicit cortical and hippocampal activation, increased motoneuronal discharge, and accelerated central respiratory rhythm [21]. At the bicuculline injection site, many cells are activated, as indicated by increased Fos expression in ORX-containing and other neurons [21]. Since brainstem noradrenergic and serotonergic cells are major efferent targets of hypothalamic ORX neurons [22,26] and themselves send dense projections to motor nuclei [2,15,27], one would expect the motoneuronal activation in response to PF bicuculline to be mediated to a major extent by noradrenergic and serotonergic afferents to motoneurons. To assess this construct, we quantified the activation of XII motoneurons that occurs in response to PF bicuculline following pharmacological blockade of adrenergic and serotonergic receptors in the XII nucleus. We chose to use this model system because the location and shape of the XII nucleus and the presence of consistent spontaneous activity in XII motoneurons under anesthesia make it particularly suitable for an antagonist microinjection study. We previously developed and characterized the methodology of antagonist microinjections into the XII nucleus [10,11], and our present control experiments (Figs. 1B and C) demonstrate that this approach results in a complete antagonism of relevant aminergic receptors. We also previously quantified XII nerve activation by PF bicuculline in experiments without antagonist injections into the XII nucleus using the same animal model [21]. In that study, XII nerve activity was increased by PF bicuculline by 100% ±24 of its pre-bicuculline level.

In the present study, bicuculline was injected into PF region after injections of α1-adrenergic and serotonergic antagonists into the XII nucleus. These injections reduced XII nerve activity to 24.9% of the control level, which was identical to the decrease obtained in the same animal model in our previous study (24.9% ±3.6) [10]. The aminergic antagonist injections created a new, lower baseline level of XII nerve activity against which we then assessed the effect of PF bicuculline (Fig. 2A). When measured relative to the new baseline, the magnitude of XII nerve activation following PF bicuculline would be as high as 260% ±98 for the 6 rats in which some activity remained after the antagonists. This is considerably more than the 100% increase that we previously observed without the aminergic antagonists [21], but one should note that the baseline used in this method of quantification was reduced which causes an overestimation of the magnitude of the excitatory effect of PF bicuculline. A more conservative approach that is also compatible with our previous study is to express the XII nerve activity increase elicited by PF bicuculline relative to XII nerve activity baseline prior to any pharmacological interventions. Using this approach, we determined that PF bicuculline increased XII nerve activity in the presence of the aminergic receptor antagonists in the XII nucleus by 80% ±16. This is only slightly less than the 100% ±24 activation produced by PF bicuculline in our previous study without the aminergic antagonists [21], and not significantly different from that activation ($P = 0.46$). Thus, the antagonists had lesser effect on the bicuculline response than would be expected based on the hypothesis that brainstem noradrenergic and serotonergic cells with projections to motor nuclei mediate a major portion of motoneuronal activation that emanates from the PF region of the posterior hypothalamus.

If brainstem premotor noradrenergic and serotonergic neurons mediate only a small fraction of motoneuronal activation from the PF region of the posterior hypothalamus, one needs to consider other pathways. Those that may be proposed based on the existing neuroanatomical and pharmacological data include direct excitatory projections of ORX neurons to motoneurons [13,22,33], ORX-mediated activation of hypothalamic histaminergic neurons that may, in turn, activate motoneurons [24,34], brainstem cholinergic neurons [20,28], or other non-adrenergic/non-serotonergic premotor neurons that are excited by pathways that descend from the posterior, lateral hypothalamus [6]. Since PF bicuculline also excites many non-ORX cells in the PF region [21], such neurons may mediate motoneuronal activation from the posterior hypothalamus through pathways that use transmitters other than those classically considered important for wake-related motor activation.

Acknowledgments

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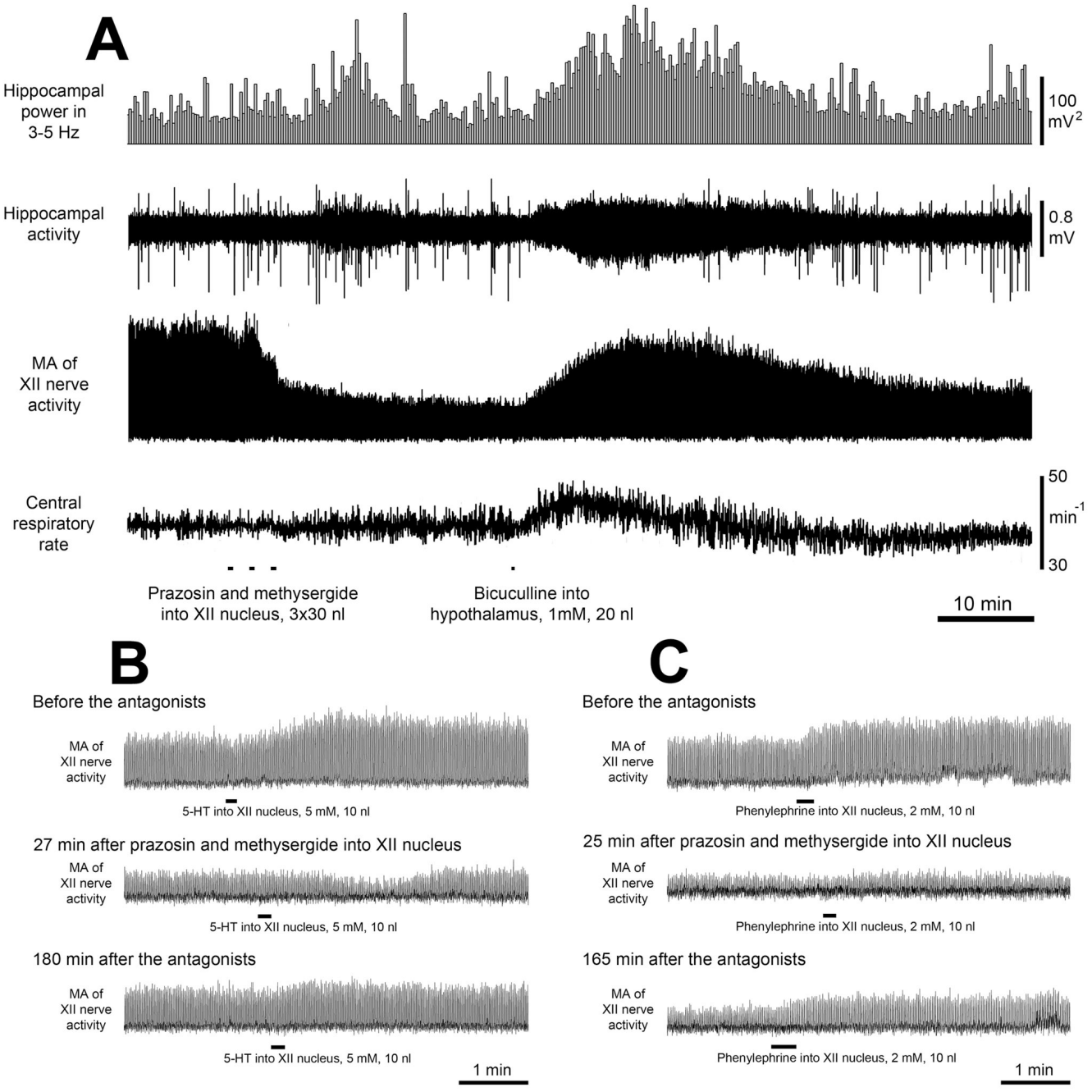

Figure 1. Example of the effect of combined prazosin and methysergide injections into the XII nucleus followed by bicuculline injection into the perifornical (PF) hypothalamus on XII nerve activity (A), and control experiments conducted to verify the effectiveness of the aminergic antagonists (B and C)

A: Three injections of the antagonist mix into the XII nucleus depress XII nerve activity, whereas bicuculline injected into the hypothalamic PF region 29 min later increases it, elicits hippocampal theta rhythm and accelerates the central respiratory rate. **B:** excitatory effect of serotonin (5-HT) injected into the XII nucleus (top trace) is abolished by the antagonist mix (middle trace) and then partially recovers ~3 h later (bottom trace). **C:** excitatory effect of

phenylephrine injected into the XII nucleus (top trace) is also abolished by the antagonist mix (middle trace) and then partially recovers ~3 h later (bottom trace). MA – moving average.

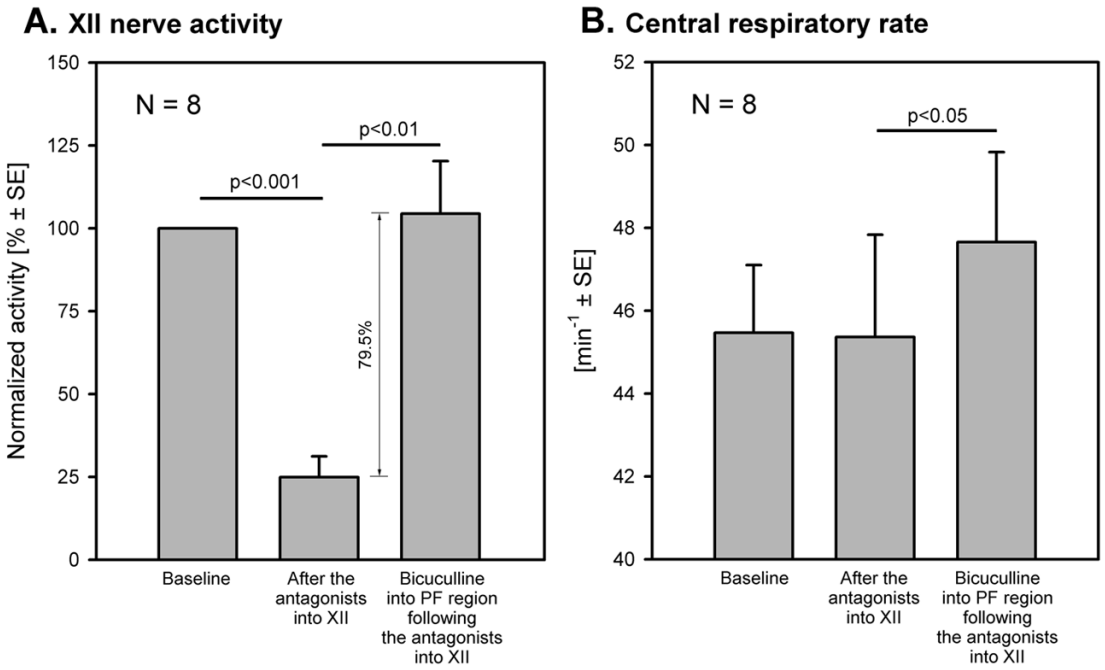

Figure 2. Mean effects of the combined injections of prazosin and methysergide into the XII nucleus and then bicuculline into the perifornical hypothalamus on XII nerve activity and central respiratory rate

A: the antagonist mix injected into the XII nucleus reduced XII nerve activity to 24.9% of its pre-antagonists baseline level. The subsequent hypothalamic injections of bicuculline increased XII nerve activity by 80% of the same baseline level despite antagonism of adrenergic and serotonergic receptors in the XII nucleus. **B:** the central respiratory rate was not affected by the antagonist injections into the XII nucleus but was increased by the subsequent hypothalamic bicuculline injection.

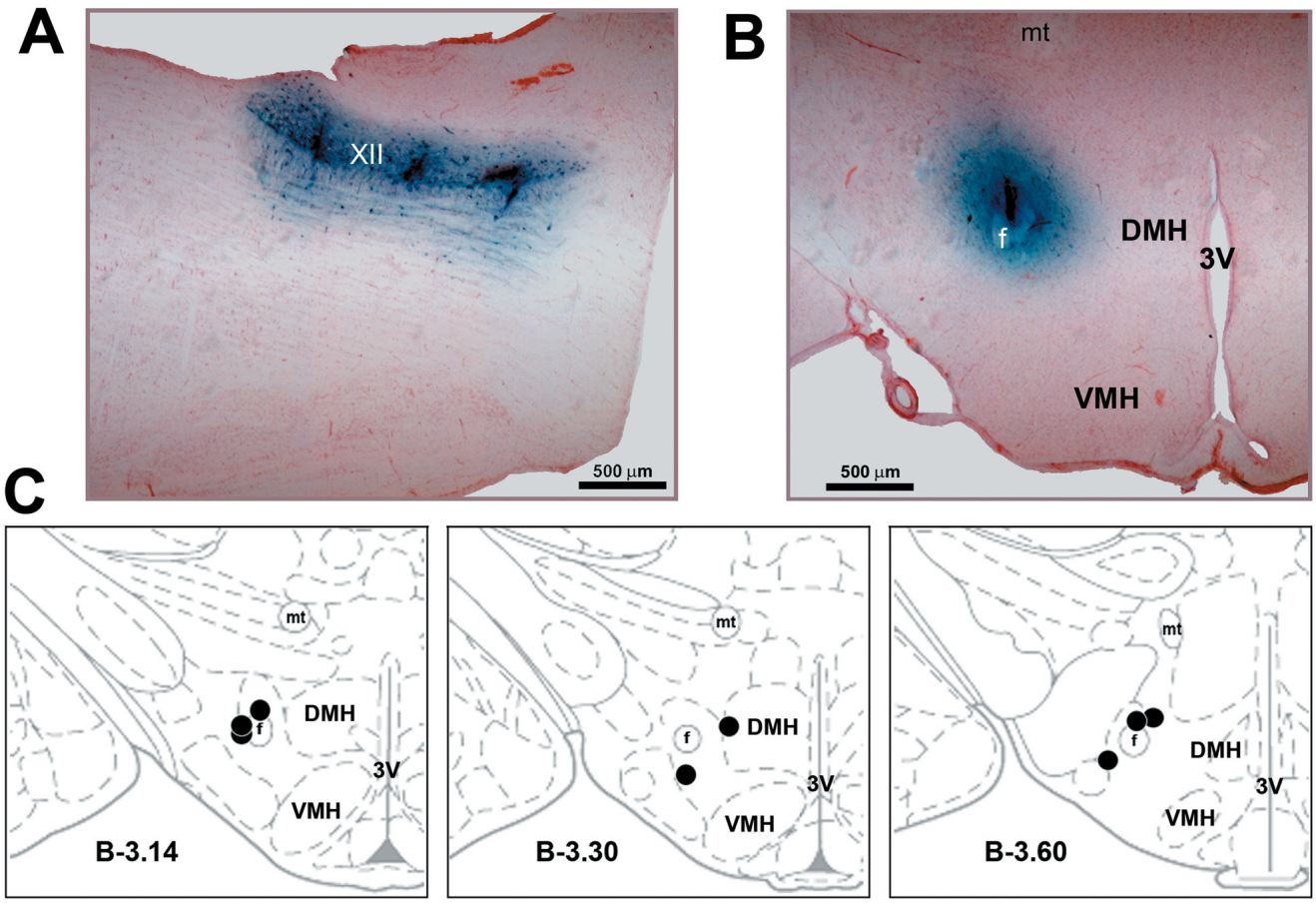

Figure 3. Medullary and hypothalamic injection sites

A: three antagonist mix injection sites in the XII nucleus shown in a parasagittal plane. **B:** hypothalamic bicuculline injection site shown in coronal plane. The sites shown in A and B are from the experiment illustrated in Fig. 1A. **C:** the distribution of all bicuculline injection sites superimposed onto the closest standard cross-sections from a rat brain atlas [25]. Abbreviations: 3V – third ventricle; DMH – dorsomedial hypothalamic nucleus; f – fornix; mt – mammillothalamic tract; VMH – ventromedial hypothalamic nucleus; XII – hypoglossal nucleus.