J Neurophysiol 89: 2591–2600, 2003;
10.1152/jn.00968.2002.

Excitatory Effects of Hypocretin-1 (Orexin-A) in the Trigeminal Motor Nucleus Are Reversed by NMDA Antagonism

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Submitted 25 October 2002; accepted in final form 14 January 2003

Peever, John H., Yuan-Yang Lai, and Jerome M. Siegel. Excitatory effects of hypocretin-1 (orexin-A) in the trigeminal motor nucleus are reversed by NMDA antagonism. *J Neurophysiol* 89: 2591–2600, 2003; 10.1152/jn.00968.2002. Hypocretin-1 and -2 (Hcrt-1 and -2, also called orexin-A and -B) are newly identified neuropeptides synthesized by hypothalamic neurons. Defects in the Hcrt system underlie the sleep disorder narcolepsy, which is characterized by sleep fragmentation and the involuntary loss of muscle tone called cataplexy. Hcrt neurons project to multiple brain regions including cranial and spinal motor nuclei. In vitro studies suggest that Hcrt application can modulate presynaptic glutamate release. Together these observations suggest that Hcrt can affect motor output and that glutamatergic processes may be involved. We addressed these issues in decerebrate cats by applying Hcrt-1 and -2 into the trigeminal motor nucleus to determine whether these ligands alter masseter muscle activity and by pretreating the trigeminal motor nucleus with a *N*-methyl-D-aspartate (NMDA) antagonist to determine if glutamatergic pathways are involved in the transduction of the Hcrt signal. We found that Hcrt-1 and -2 microinjections into the trigeminal motor nucleus increased ipsilateral masseter muscle tone in a dose-dependent manner. We also found that Hcrt application into the hypoglossal motor nucleus increases genioglossus muscle activity. Pretreatment with a NMDA antagonist (D-(−)-2-amino-phosphonovaleric acid) abolished the excitatory response of the masseter muscle to Hcrt-1 application; however, pretreatment with methysergide, a serotonin antagonist had no effect. These studies are the first to demonstrate that Hcrt causes the excitation of motoneurons and that functional NMDA receptors are required for this response. We suggest that Hcrt regulates motor control processes and that this regulation is mediated by glutamate release in the trigeminal motor nucleus.

INTRODUCTION

Hcrt-1 and -2 are peptides synthesized by neurons in the lateral hypothalamus (De Lecea et al. 1998; Sakurai et al. 1998; Sutcliffe and De Lecea 1999). Hcrt neurons project widely throughout the brain and spinal cord, including brain stem regions involved in sleep and motor control (Nambu et al. 1999; Peyron et al. 1998; van den Pol 1999). Loss of Hcrt-synthesizing neurons and defects in the Hcrt-2 receptor underlie the sleep disorder called narcolepsy, which is characterized by disrupted sleep homeostasis and sudden loss of muscle tone during wakefulness (cataplexy) (Chemelli et al. 1999; Hara et al. 2001; Lin et al. 1999; Peyron et al. 2000; Siegel 1999; Thannickal et al. 2000).

Intracerebroventricular infusion of Hcrt-1 increases locomotor activity in behaving rats (Hagan et al. 1999), and Hcrt-1 and -2 microinjection into midbrain and pontine regions affects hind limb muscle rigidity in decerebrate rats (Kiyashchenko et al. 2001). While Hcrt affects locomotor activity and muscle tone, it is unclear whether it directly affects motoneurons. The trigeminal (V) motor nucleus innervates masseter muscles, which are consistently affected by sleep-dependent hypotonia and cataplexy (Guilleminault 1976; Pedroarena et al. 1994; Soja et al. 1987). Hcrt neurons project to the V motor nucleus and to the hypoglossal (XII) motor nucleus, which innervates the genioglossus (tongue) muscles (Fung et al. 2001). Like the masseter muscles, the genioglossus muscles incur sleep-dependent reductions in muscle tone, which contribute to obstructive sleep apnea (Horner 1996). Because Hcrt neurons exhibit a state-dependent activity pattern (Estabrooke et al. 2001; Kiyashchenko et al. 2002) and because they project to V and XII motor nuclei, we hypothesize that Hcrt is involved in the normal regulation of motoneuronal excitability across the sleep-wake cycle. To assess the role of Hcrt in muscle tone regulation, we tested the hypothesis that microinjection of Hcrt into the V and XII motor nuclei would excite masseter and genioglossus muscles, respectively.

Hcrt binds to and activates G-protein-coupled receptors to affect postsynaptic neuronal activity (Sakurai et al. 1998); however, it may also act presynaptically. van den Pol et al. (1998) reported that Hcrt-1 increases glutamate release in in vitro hypothalamic slices. Similarly, it is suggested that Hcrt acts on presynaptic, glutamatergic laterodorsal tegmental neurons to increase quanta release probability (Burlet et al. 2002). Recent work from this laboratory demonstrates that systemic infusion of Hcrt-1 strongly increases glutamate release in the amygdala (John et al. 2001). Based on these observations, we suggest that Hcrt may not only act directly but may also act indirectly by causing the release of glutamate. To determine if glutamate mechanisms underlie the muscle tone responses to Hcrt-1 application, we pretreated the V motor nucleus with a *N*-methyl-D-aspartate NMDA) antagonist [D-(−)-2-amino-phosphonovaleric acid (D-AP5)] prior to the application of Hcrt-1.

These studies are the first to demonstrate that application of Hcrt causes the excitation of cranial motoneurons and that functional NMDA receptors are required for expression of the

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response. We suggest that changes in Hcrt levels may alter motoneuronal excitability thereby leading to altered muscle tone as seen in cataplexy and during obstructive sleep apnea.

These studies have been presented as a conference abstract (Peever et al. 2002).

METHODS

Animal preparation

The Animal Care Committee at the University of Los Angeles approved all procedures described herein. A total of 17 decerebrate, adult, female cats weighing between 2.5 and 3.8 kg [3.0 ± 0.1 (SE) kg] were used. They were anesthetized with a halothane-oxygen mixture. When cats no longer responded to a firm foot pinch and blink reflexes were absent, tracheotomy, bilateral carotid artery ligation, and femoral artery cannulation were performed. Cats were then placed in a stereotaxic frame (David Kopf Instruments, Los Angeles, CA) and decerebrated using the following procedure. A midline incision was made along the dorsal surface of the cranium and the skin reflected. The connective tissue covering the parietal bones was removed. The dorsal-medial parietal bones were removed, and the dura cut and reflected. All brain structures rostral to the postmammillary-precollicular level were removed by suction, and the cranial cavity was firmly packed with hot, saline-soaked cotton balls. At this point, anesthesia was terminated. To allow access to the V and XII motor nuclei, the rostral occipital bone covering the cerebellum was carefully removed as was the medial tentorium. All exposed bone surfaces were covered with bone-wax. The dura and pia mater covering the cerebellum and pons were carefully removed, and the exposed brain surface was covered with saline-soaked cotton until experiments began. Rectal temperature was monitored and maintained at 38 ± 0.5°C using a custom-built servo-controlled electric heating-pad. Mean arterial blood pressure was recorded from the femoral artery using a blood pressure transducer (Gould, Model P23ID). Data collected from cats in which mean arterial blood pressure remained between 80 and 150 mmHg were analyzed.

Recording procedures

Bipolar, multistranded, stainless steel electromyographic (EMG) electrodes (~2-mm uninsulated portions exposed and separated by ~1 cm; A-M Systems) were carefully inserted into left and right masseter and genioglossus muscles. EMG signals were amplified (Grass EEG Amplifier, Model 7P511K) and filtered between 30 Hz and 10 kHz. EMG signals were calibrated using a built-in microvolt calibrator. Blood pressure signals were amplified (Grass Low Level DC Amplifier, Model 7P122E) and calibrated using a syphygmomanometer (Labtron). EMG and blood pressure signals were monitored, digitized (Spike 2 Software, 1401 Interface, CED, Cambridge, UK), and stored on a computer (Dell, OptiPlex GX100). EMG signals were integrated off-line in 2-s epochs using a specially written Spike 2 program.

Drugs

Hcrt-1 and -2 (Peptide Institute) were prepared at the beginning of each experiment by dissolving them in artificial cerebral spinal fluid (ACSF, Harvard Apparatus). We used 1, 10, and 100 μM concentrations because it has been shown that they produce measurable changes in muscle tone when injected into the locus coeruleus of rats (Kiyashchenko et al. 2001). D-AP5 and *N*-methyl-D-aspartic acid (NMDA) were purchased from Tocris Cookson (St. Louis, MO), and methysergide maleate (methysergide) was obtained from Sigma RBI (St. Louis, MO). These drugs were dissolved in fresh ACSF to make solutions of the following concentrations: 50 mM D-AP5, 1 mM methysergide, and 10 mM NMDA. These concentrations were chosen because previous studies demonstrate that 50 mM D-AP5 are sufficient to block NMDA channels (Lai and Siegel 1988, 1991), 1 mM methysergide blocks the effect of serotonin application onto XII motoneurons (Kubin et al. 1996), and 10 mM NMDA induces changes in muscle tone when injected into the pontine reticular formation (Lai and Siegel 1991).

Protocol

Experiments began ≥1 h after decerebration. Blood pressure and left/right masseter and genioglossus EMG signals were monitored and recorded during all experimental conditions. A beveled, 25 gauge, 1 μl Hamilton microsyringe (Hamilton, Reno, NV) secured in a micromanipulater (David Kopf instruments) was used to make all microinjections. The tip of the microsyringe was aimed at either the V or XII motor nuclei (Berman 1968). It was considered to be located within the motor nucleus if it caused an increase in baseline EMG activity of the corresponding ipsilateral muscle (see Fig. 1*A*); post hoc histological analysis identified a tract mark within the motor nucleus (see Fig. 2). After probe insertion, ≥10 min elapsed before microinjections were made. If more than one microinjection of Hcrt was made into the same motor nucleus, ≥2 h elapsed before another microinjection was made. When NMDA or serotonin antagonists was applied before Hcrt, they were microinjected into the motor nucleus at the same stereotaxic coordinates at those for Hcrt.

To test our hypotheses, the following manipulations were performed. To verify that microinjection per se had no effect on basal masseter muscle activity, ACSF (0.5 μl) was injected into the V motor nucleus. To demonstrate the excitatory effects of Hcrt-1 and -2 on putative V and XII motoneurons, we unilaterally microinjected 0.5 μl of 100 μM Hcrt-1 or -2 into either the V or XII motor nucleus while monitoring masseter and genioglossus EMG activity. To demonstrate that Hcrt actions were mediated by neurons in the motor nuclei, Hcrt injections were made outside the motor nucleus. To determine whether Hcrt-related glutamate release mediates changes in muscle activity, we unilaterally microinjected 0.5 μl of the glutamate antagonist, D-AP5 (50 mM) into the V motor nucleus immediately prior to microinjection of 0.5 μl of 100 μM Hcrt-1. To demonstrate that the excitatory effects of Hcrt-1 microinjections could be actively reversed, we applied 0.5 μl of 50 mM D-AP5 into the V motor nucleus immediately after the application of 0.5 μl of 100 μM Hcrt-1. To validate that Hcrt-related glutamate release specifically mediates changes in muscle activity, we unilaterally microinjected 0.5 μl of the serotonin antagonist, methysergide into the V motor nucleus immediately before microinjection of 0.5 μl of 100 μM Hcrt-1.

Histology

At the end of each experiment, an iron deposit marked the location of microinjection sites. It was made by positioning a bipolar stimulating electrode at the same stereotaxic coordinates as those for microinjections and then passing a DC current through it for 20 s. Cats were then killed with a overdose of pentobarbital sodium (Nembutal, 50 mg/kg iv). Once a heartbeat could no longer be detected, the brain stem was rapidly dissected and placed in a 100 ml solution of 10% formalin and 30% sucrose in distilled water for ≥3 days. A microtome (Leica, Model SM 2400) was used to cut the brain tissue into 50-μm-thick slices that were stained with Neutral Red and counterstained with potassium ferrocyanide, which permitted detection of iron deposits.

Data analysis

To analyze changes in masseter and genioglossus muscle activities, integrated, bilateral muscle activity was quantified during the following conditions: baseline, that is, 60 s before microinjection; immediately after the completion of microinjection; and for 60 s after the

response returned to baseline (see following text). Response latency was characterized as the period between microinjection and the point at which integrated EMG activity exceeded 2 SD of the baseline mean. Response duration was determined by calculating the period of time that integrated EMG activity remained 2 SD above the baseline mean. Integrated EMG activity returned to baseline conditions levels when it fell below 2 SD of the baseline mean. The percentage change of integrated EMG activity was calculated by dividing the difference of baseline and evoked increase by baseline values and multiplying this factor by 100.

For all comparisons, raw data were used, and differences between groups were considered statistically significant at $P < 0.05$ using two-tailed paired *t*-test (parametric) or Wilcoxon's match-pairs sign-ranked tests (nonparametric). When ANOVA was performed, post hoc comparisons using either the Bonferroni *t*-test (parametric) or Student-Newman-Keuls method (nonparametric) were used to infer statistical significance. Parametric or nonparametric analysis of samples depended on whether the data were normally distributed. The statistical processes used to analyze data are included in the text. All data are expressed as means ± SE Statistical analyses were performed using Sigmastat (Jandel Scientific).

RESULTS

Microinjection locations and control injections

Insertion and penetration of a Hamilton microsyringe into the stereotaxcially defined V motor nuclei (4.0–5.2 posterior, 3.0–5.5 lateral, and 3.5–5.0 ventral to the interaural point) caused a transient (<3 min) burst in the ipsilateral masseter muscle EMG activity (Fig. 1) but had no effect on either contralateral masseter or genioglossus muscle activity. This transient burst of ipsilateral muscle activity was used as a preliminary guide to determine whether the microsyringe was correctly placed within the V motor nucleus. The same approach was used to locate the XII motor nucleus. Similarly, we found that placement of the microsyringe into the stereotaxcially defined XII motor nucleus (12.0–15.5 posterior, 0–2.0 lateral, and 6.0–7.5 ventral to the interaural point) caused a temporary increase in genioglossus muscle activity but was without effect on masseter muscle activity.

The precise anatomical location of microinjection sites was confirmed by postmortem histological observations. Figure 1 shows iron deposits located within the V and XII motor nuclei. In all 17 cats, we found that microinjection sites were located within either the V or XII motor nuclei (Figs. 1 and 2).

To verify that microinjection per se had no effect on masseter muscle activity, ACSF was injected into the V motor nucleus. In eight cats, we found that microinjection of ACSF into the V motor nucleus had no effect on ipsilateral masseter muscle activity (paired *t*-test: $P = 0.281$; $t = 1.169$; df = 7; Fig. 3). Therefore we are confident that changes in muscle activity after application of Hcrt are due to the effects of the applied compounds and not due to the mechanical effects of microinjection.

To demonstrate that excitatory effects of Hcrt microinjections are mediated by motoneurons, Hcrt injections were also made outside of V and XII motor nuclei. A total of 17 Hcrt-1 microinjections were made outside the V motor nucleus (Fig. 2*A*), and 7 were made outside the XII motor nucleus (Fig. 2*B*). Hcrt microinjections placed outside the anatomical boundaries of V and XII motor nuclei had no effect on either masseter or genioglossus muscle activities. Accordingly, we conclude that motoneurons mediate the changes in muscle activity after application of Hcrt into V or XII motor nuclei.

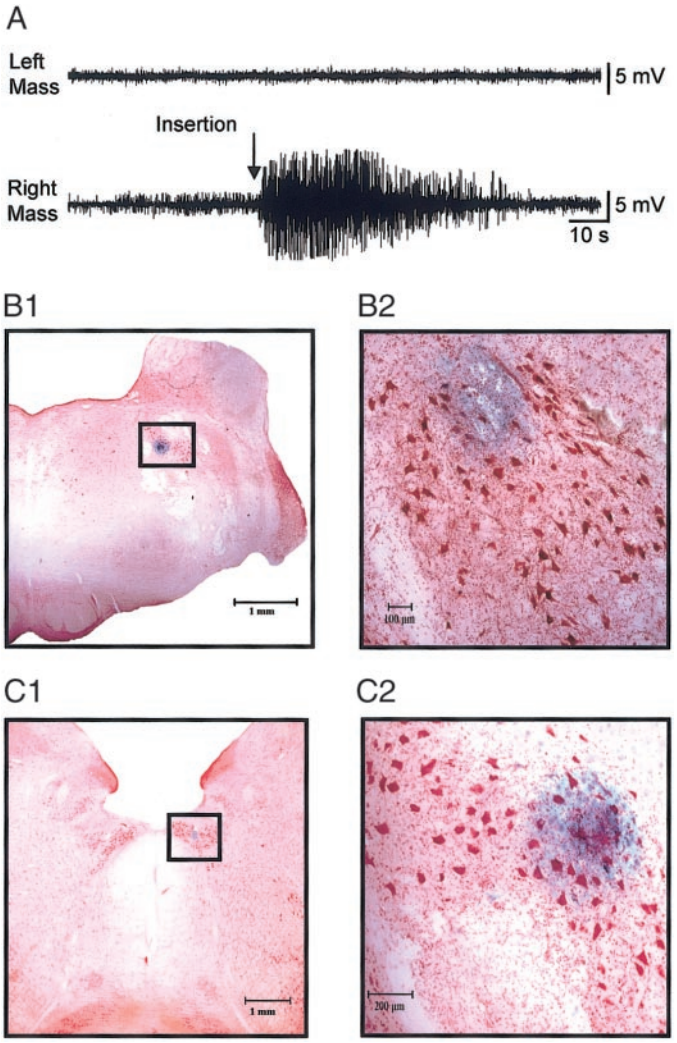

FIG. 1. Activation of ipsilateral masseter activity following insertion of a Hamilton syringe into the V motor nucleus. Histological verification of syringe locations in V and XII motor nuclei. *A*: insertion of a Hamilton syringe into the V motor nucleus unilaterally results in a transient increase of ipsilateral masseter electromyographic (EMG) activity; however, contralateral masseter EMG activity remained unaffected. The same approach was used to identify the stereotaxic location of the XII motor nucleus (data not shown). *B1* and *C1*: microphotographs of V and XII motor nuclei locations, respectively (open boxes). To define microinjection locations, trace amounts of iron were deposited in the V and XII motor nuclei by passing a DC current through a bipolar stimulating electrode at the same stereotaxic coordinates as the Hamilton syringe. *B2* and *C2*: examples of a iron deposits left within the V and XII motor nuclei.

Hcrt-1 and -2 microinjection into the trigeminal motor nucleus

To determine the effect of Hcrt-1 and -2 on the V motor nucleus, we unilaterally microinjected 0.5 μl of 1–100 μM Hcrt-1 or -2 while monitoring masseter and genioglossus muscle activities. A total of 38 Hcrt-1 microinjections were made unilaterally into the V motor nucleus in 11 decerebrate cats. Figure 4 shows how bilateral masseter muscle activity changed after microinjection of 100 μM Hcrt-1 into the V motor nucleus. Microinjection of Hcrt-1 into the V motor nucleus

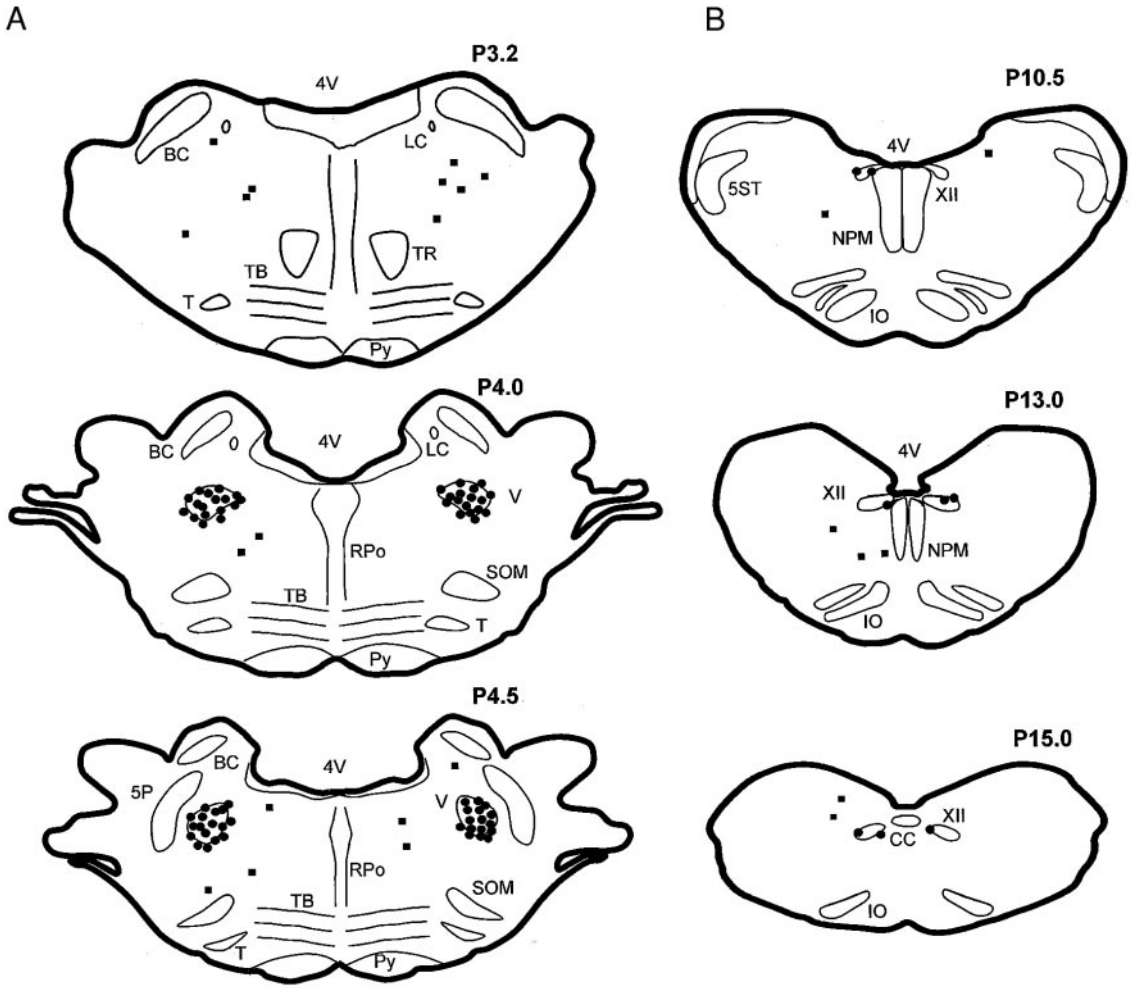

FIG. 2. Group data indicating Hcrt microinjection sites. Distribution of microinjection sites in 17 cats. *A*: ●, Hcrt microinjections sites that caused masseter muscle activity to increase; ■, microinjection sites that had no effect on masseter muscle activity. *B*: ●, Hcrt microinjection sites that caused genioglossus muscle activity to increase; ■, microinjection sites that had no effect on genioglossus muscle activity. P, millimeters posterior to stereotaxic 0 (interaural point). 4V, fourth ventricle; 5P, principal sensory trigeminal nucleus; 5ST, spinal trigeminal tract; AP, area postrema; brachium conjunctivum; CC, central canal; IO, inferior olive; LC, locus coeruleus; NPM, nucleus paramedianus; Py, pyramidal tract; RPo, nucleus raphe pontis; SOM, medial nucleus of the superior olive; T, nucleus of the trapezoid body; TB, trapeziod body; TR, tegmental reticular nucleus; V, trigeminal motor nucleus; XII, hypoglossal motor nucleus.

caused a significant increase in ipsilateral masseter muscle activity that had no effect on either contralateral masseter (Fig. 4) or bilateral genioglossus muscle activities.

Twenty-four Hcrt-2 microinjections (1–100 μM) were made unilaterally within the V motor nucleus in six cats. Hcrt-2 microinjections into the V motor nucleus caused a significant increase in ipsilateral masseter muscle activity (Fig. 4) that had no effect on either contralateral masseter or genioglossus muscle activities.

Hcrt-1 and -2 microinjections into the V motor nucleus increased masseter muscle activity in a dose-dependent manner. After microinjection of 1, 10, and 100 μM of Hcrt-1 into the V motor nucleus, integrated ipsilateral masseter muscle activity significantly increased from baseline levels by: 8.1 $\pm$ 3.2% (Wilcoxon's match-pairs sign-ranked test: $P = 0.031$, $T = 2$, df = 5), 43.2 $\pm$ 23.7% ($P \leq 0.001$, $T = 7$, df = 11), and 81.5 $\pm$ 28.1% ($P \leq 0.001$, $T = 37$, df = 19), respectively (Figs. 4 and 5). Similarly, Hcrt-2 microinjections of 1, 10, and 100 μM into the V motor nucleus caused integrated ipsilateral masseter muscle activity to increase by: 26.9 $\pm$ 18.8% (paired *t*-test: $P = 0.038$, $t = 2.776$, df =3), 61.5 $\pm$ 31.8% ($P = 0.031$, $T = 2$, df = 5), and 74.1 $\pm$ 10.4% ($P \leq 0.001$, $T = 12$, df = 13), respectively (Figs. 4 and 5). The latency of the response also varied in a dose-dependent manner; it changed from 17.5 $\pm$ 7.5 s (1 μM, $n = 6$), 17.7 $\pm$ 6.4 s (10 μM, $n = 12$), and 11.9 $\pm$ 2.3 s (100 μM, $n = 20$) for Hcrt-1 and from 15.3 $\pm$ 8.6 s (1 μM, $n = 4$), 13.5 $\pm$ 5.5 s (10 μM, $n = 7$), and 7.0 $\pm$ 2.5 s (100 μM, $n = 14$) for Hcrt-2 (Fig. 5). The duration of the response also had a dose-dependent time course; it changed from 175.8 $\pm$ 61.3 s (1 μM, $n = 6$), 316.6 $\pm$ 87.0 s (10 μM, $n = 12$), and 1,080.7 $\pm$ 255.0 s (100 μM, $n = 20$) for Hcrt-1 and from 194.8 $\pm$ 101.6 s (1 μM, $n = 4$), 203.7 $\pm$ 76.4 s (10 μM, $n = 6$), and 581.9 $\pm$ 29.5 s (100 μM, $n = 14$) for Hcrt-2 (Fig. 5). We did not detect any significant differences between the action of equimolar concentrations of Hcrt-1 and -2 on masseter muscle activity changes for either percent increase of EMG activity or latency to response (2-way ANOVA: $P = 0.255$ and $P = 0.240$, respectively). However, application of

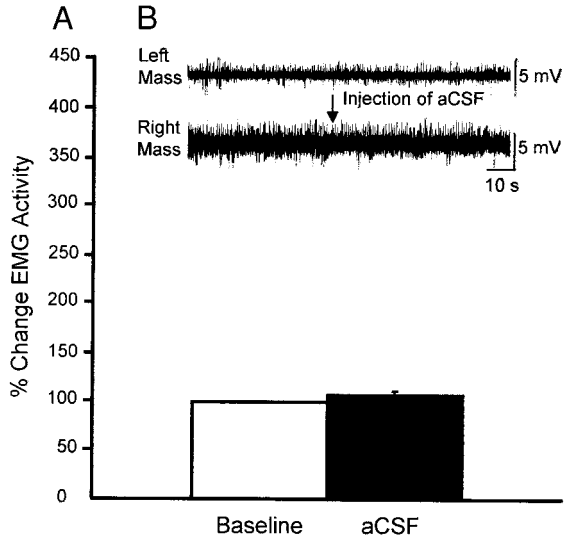

FIG. 3. Effect of microinjection of artificial cerebrospinal fluid (ACSF) into the V motor nucleus on masseter muscle activity. To verify that microinjection per se had no quantifiable effect on masseter (Mass) muscle activity, 0.5 μl of ACSF was microinjected into the V motor nucleus while bilateral masseter muscle activity was monitored (*B*). After application of ACSF into V motor nucleus ($n = 8$), there was no significant change in ipsilateral masseter muscle activity compared with baseline values (*A*).

100 μM Hcrt-1 increased ipsilateral masseter EMG activity for a longer duration than 100 μM Hcrt-2 did (2-way ANOVA: $P = 0.023$, $F = 2.62$, df = 19, 13).

Hcrt-1 microinjection into the hypoglossal motor nucleus

To demonstrate the excitatory effects of Hcrt-1 on motor activity, we unilaterally microinjected 0.5 μl of 100 μM Hcrt-1 into the XII motor nucleus while monitoring masseter and genioglossus EMG activity. A total of 12 Hcrt-1 microinjections were made into XII motor nucleus unilaterally in six decerebrate cats. Figure 6 shows how bilateral genioglossus muscle activity changed after microinjection of 100 μM Hcrt-1 into the XII motor nucleus. After microinjection of Hcrt-1 into the XII motor nucleus, ipsilateral genioglossus muscle activity increased by 41.1 ± 9.7% above baseline values (Bonferroni *t*-test: $P < 0.05$, $t = 2.447$, df = 5). The latency and duration of the response were 10.0 ± 2.7 and 231.8 ± 108.7 s, respectively. Compared with pretreatment values, contralateral genioglossus muscle activity was unaffected by Hcrt-1 application (paired *t*-test: $P = 0.185$, $t = 1.440$, df = 5; Fig. 6). The change in genioglossus muscle activity elicited by application of 100 μM Hcrt-1 into XII motor nucleus was not statistically different from the response elicited by application of 100 μM Hcrt-1 into V motor nucleus (Mann-Whitney rank sum test: $P = 0.563$, $U = 58$, df = 19,5).

NMDA antagonist into the trigeminal motor nucleus

To determine whether Hcrt-mediated glutamate release regulates motor nucleus excitability and hence changes in masseter muscle activity, the glutamate antagonist, D-AP5 was microinjected into the V motor nucleus immediately prior to microinjection of Hcrt-1. In five cats, eight unilateral microinjections of D-AP5 (0.5 μl) into the V motor nucleus had no significant effect on integrated ipsilateral masseter muscle activity (1-way ANOVA: $P = 0.489$, $F = 1.00$, df = 7, 7; Fig. 7). Unlike Hcrt-1 application alone, 100 μM Hcrt-1 microinjection did not increase ipsilateral masseter muscle activity after NMDA channels were blocked by prior application of D-AP5 (1-way ANOVA: $P = 0.285$, $F = 1.64$, df = 7, 7; Fig. 7).

To demonstrate that the excitatory effects of Hcrt-1 microinjections could be actively reversed, we applied 0.5 μl of 50 mM D-AP5 into the V motor nucleus immediately after the application of 0.5 μl of 100 μM Hcrt-1. In three cats, three microinjections of 100 μM Hcrt-1 into the V motor nucleus significantly increased basal masseter muscle activity by 115.0 ± 37.1% (Bonferroni *t*-test: df = 2; $t = 1.080$; $P < 0.05$; Fig. 8). This effect was reversed by application of D-AP5; within 103.3 ± 5.2 s of applying D-AP5 ipsilateral masseter muscle activity returned to within baseline levels (Fig. 8). Application of D-AP5 caused a significant reduction in the

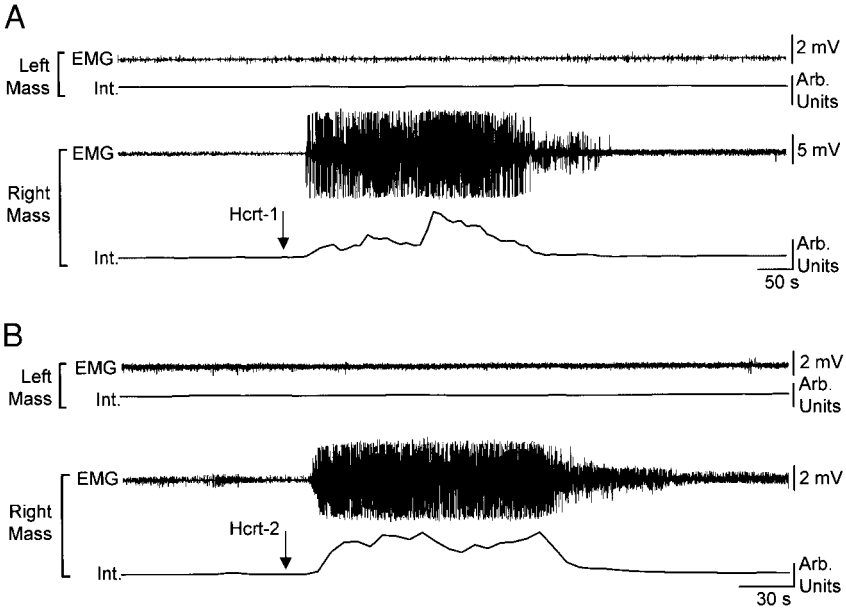

FIG. 4. Changes in masseter muscle activity after microinjection of Hcrt-1 and -2 into the V motor nucleus. A unilateral microinjection of either Hcrt-1 (*A*) or Hcrt-2 (*B*) into the V motor nucleus caused an increase in ipsilateral masseter muscle EMG activity; contralateral muscle activity remained unchanged. *Top*: raw EMG activity; *bottom*: integrated (int.) muscle activity and are in arbitrary units (Arb. Units). ↓, the times at which either Hcrt-1 or -2 was microinjected.

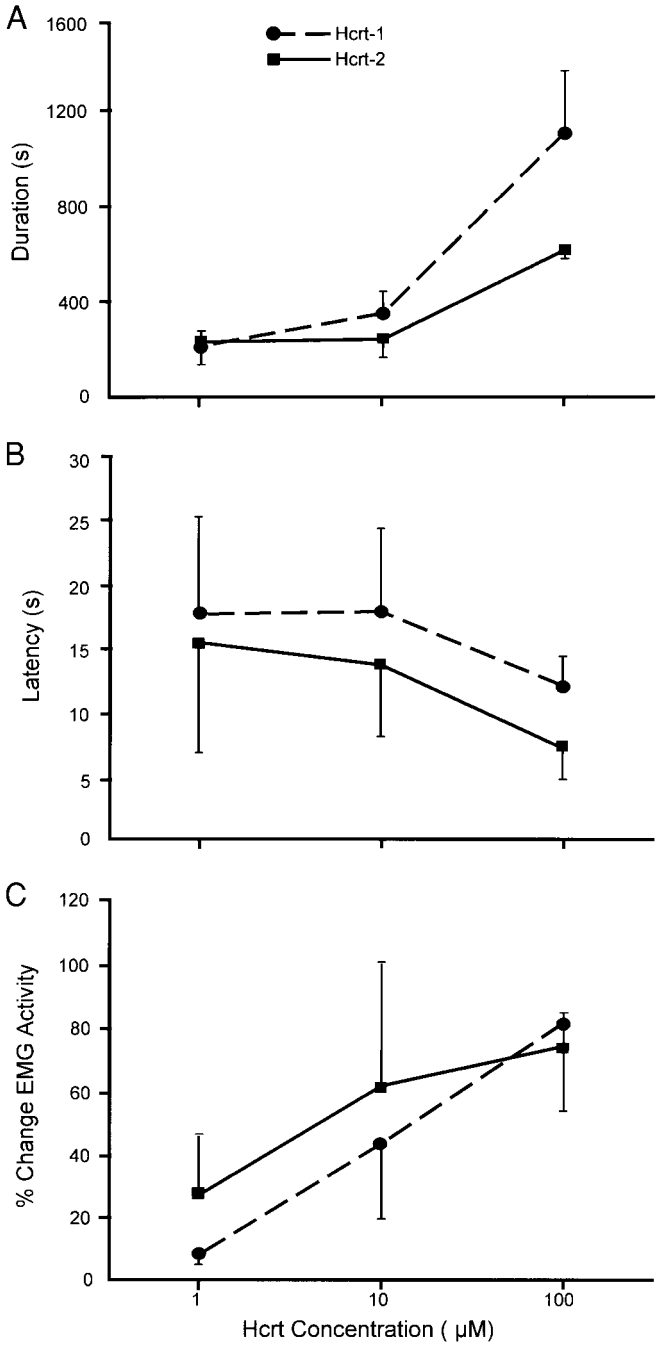

FIG. 5. Does-response effects of Hcrt-1 and -2 application into the V motor nucleus. Microinjection of 1, 10, and 100 μM of Hcrt-1 and -2 into the unilateral V motor nucleus result in a dose-dependent increase in integrated ipsilateral masseter muscle activity compared with baseline values; response latency and duration also occur in a dose-dependent manner.

duration of the Hcrt-1 response compared with Hcrt-1 application alone (Mann Whitney rank sum test: $P = 0.025$; $U = 50$, df = 19,3). Application of 100 μM Hcrt-1 alone caused ipsilateral masseter muscle tone to increase for 1,080.7 ± 255.0 s (see Fig. 5); however, with application of D-AP5, the Hcrt-1 response only lasted 410.0 ± 94.4 s (Fig. 8).

To demonstrate that glutamate increases muscle activity in a similar manner to Hcrt-1 and that its excitatory effects could be reversed with glutamate antagonists, we applied 10 mM of the glutamate agonist, NMDA (0.5 μl) into the unilateral V motor

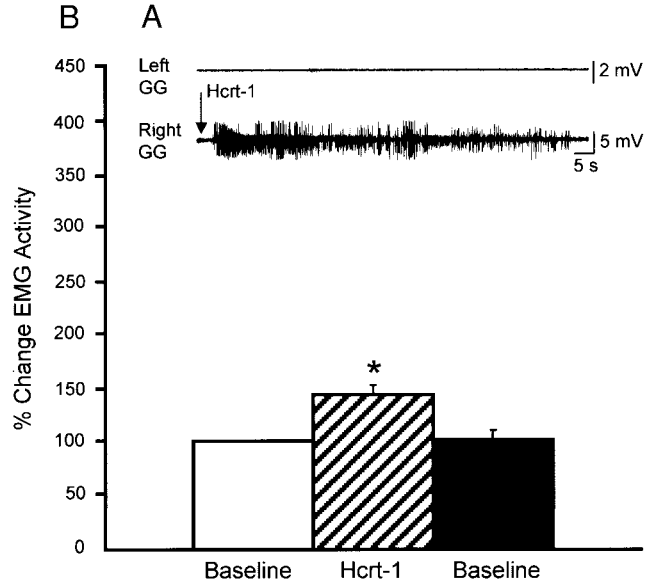

FIG. 6. Changes in genioglossus (GG) muscle activity following microinjection of Hcrt-1 into the XII motor nucleus. *A*: a representative recording of GG muscle activity after microinjection of Hcrt-1 (↓) unilaterally into the XII motor nucleus ($n = 6$). *B*: Hcrt-1 application into the XII motor nucleus significantly increased ipsilateral GG muscle activity within 10.0 ± 2.7 s with a return to baseline conditions after 231.8 ± 108.7 s. *, a statistically significant ($P < 0.05$) increase compared with baseline.

nucleus, and then immediately applied 50 mM D-AP5 to reverse its effects. In four cats, seven microinjections of NMDA caused integrated ipsilateral masseter muscle activity to significantly increase by 252.3 ± 69.7% (Student-Newman-Keuls method: $P < 0.05$, df = 6). Application of D-AP5 reversed this excitation; within 55.7 ± 14.3 s, ipsilateral muscle activity

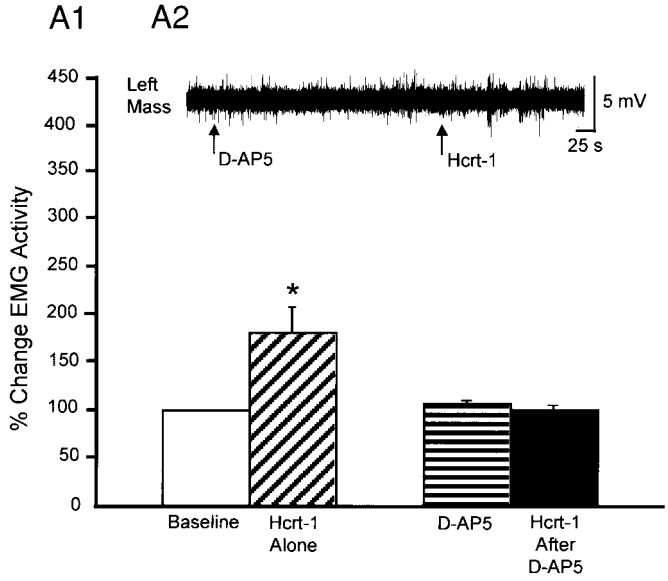

FIG. 7. Pretreatment with D-AP5 blocked the effect of Hcrt-1 microinjection into the V motor nucleus. Microinjection of D-AP5 (*A1*) into the V motor nucleus unilaterally had no effect on baseline masseter muscle activity; however, it did abolish the increase in masseter muscle activity evoked by microinjecting 100 μM Hcrt-1 into the V motor nucleus. *, a statistically significant ($P < 0.05$) increase compared with baseline. *A2*: an example recording, which demonstrates that microinjection (↑) of D-AP5 into the V motor nucleus before Hcrt-1 ($n = 8$) abolished the increase in masseter muscle activity.

returned to baseline levels (Student-Newman Keuls method: $P > 0.05$, df = 6; Fig. 8).

Serotonin antagonist into the trigeminal motor nucleus

To validate that Hcrt-dependent glutamate release mediates muscle activity with some specificity, we unilaterally microinjected methysergide into the V motor nucleus immediately prior to microinjection of Hcrt-1. In three cats, five microinjections of 1 mM methysergide unilaterally into the V motor nucleus had no effect on integrated masseter muscle activity (Fig. 9). Unlike glutamate antagonists, which blocked the effects of Hcrt-1, application of methysergide had no effect on the Hcrt-1 response. We found that 0.5 μl microinjection of 100 μM Hcrt-1 caused integrated ipsilateral masseter muscle activity to increase significantly by 105.9.5 ± 54.2% (Student-Newman-Keuls method: $P < 0.05$, df = 4; Fig. 9). The latency and duration of the response were 9.8 ± 5.7 and 442.6 ± 251.9 s, respectively. Pretreatment with methysergide had no statistical effect on the magnitude (Mann-Whitney rank sum test: $P = 0.209$, $U = 67$, df = 19, 5), duration (unpaired t-test: $P = 0.240$, $t = 1.319$, df = 23) or latency (Mann-Whitney rank sum test: $P = 1.0$, $U = 67$, df = 19, 5) of the Hcrt-1 response alone (comparisons were made between Hcrt-1 alone and Hcrt-1 after methysergide application).

DISCUSSION

Using a decerebrate cat preparation, we demonstrate that application of Hcrt-1 and -2 unilaterally into the V motor nucleus caused a dose-dependent increase in ipsilateral masseter muscle tone and that Hcrt-1 caused a similar increase in

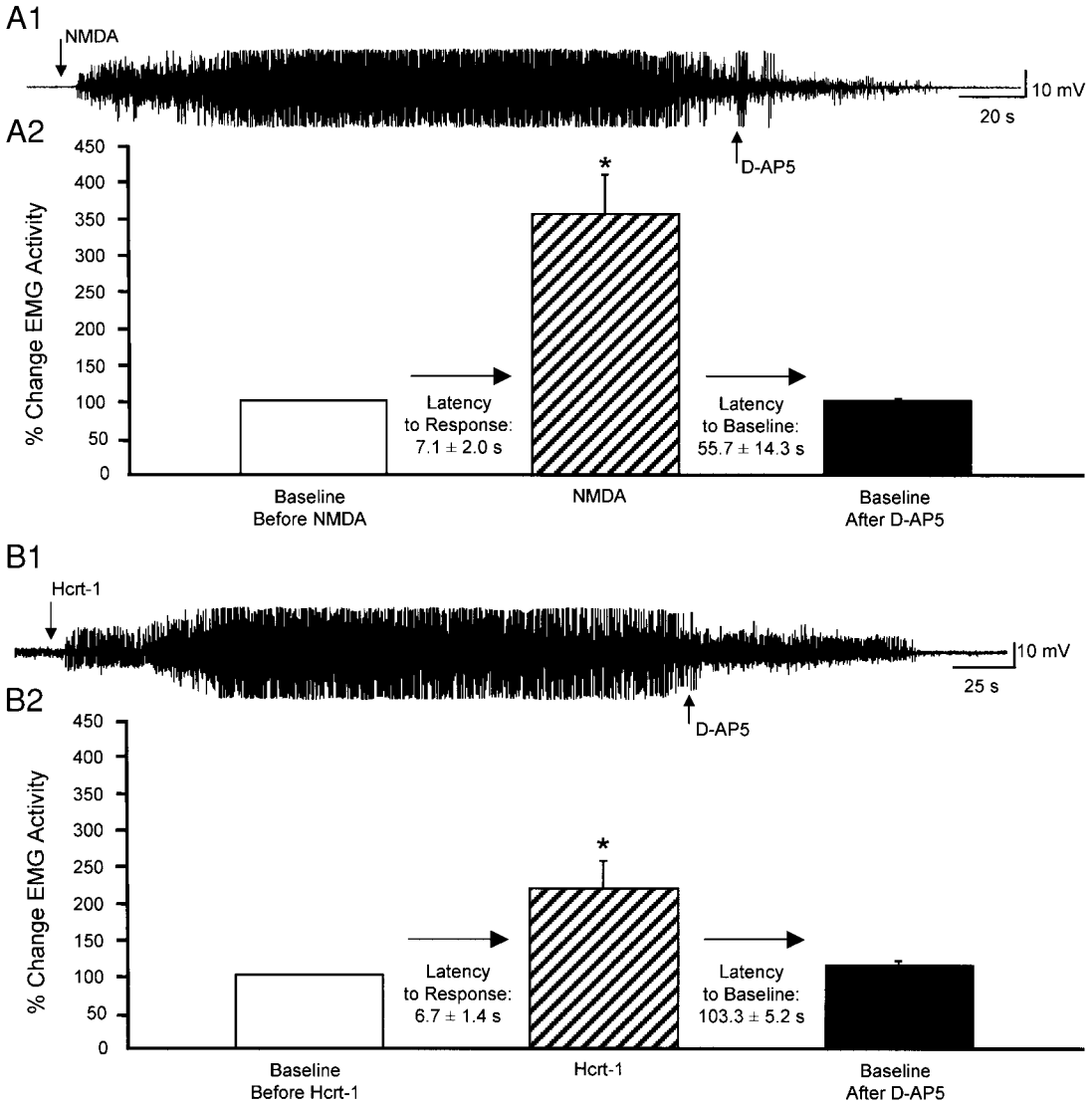

FIG. 8. The excitatory effects of *N*-methyl-D-aspartate (NMDA) and Hcrt-1 are abolished after application of D-(−)-2-amino-phosphonovaleric acid (D-AP5) into the V motor nucleus. The EMG traces shown in *A1* and *B1* demonstrate that application of either NMDA or Hcrt-1 into the V motor nucleus cause an increase in masseter muscle activity; however, microinjection of D-AP5 into the V motor nucleus quickly reduced masseter muscle activity to baseline values. At a latency, ipsilateral integrated masseter muscle activity significantly increased following application of NMDA (*A2*, $n = 7$) or Hcrt-1 (*B2*, $n = 3$) into the V motor nucleus; this excitatory response was rapidly reversed after D-AP5 was microinjected into the V motor nucleus. *, a statistically significant ($P < 0.05$) increase compared with baseline.

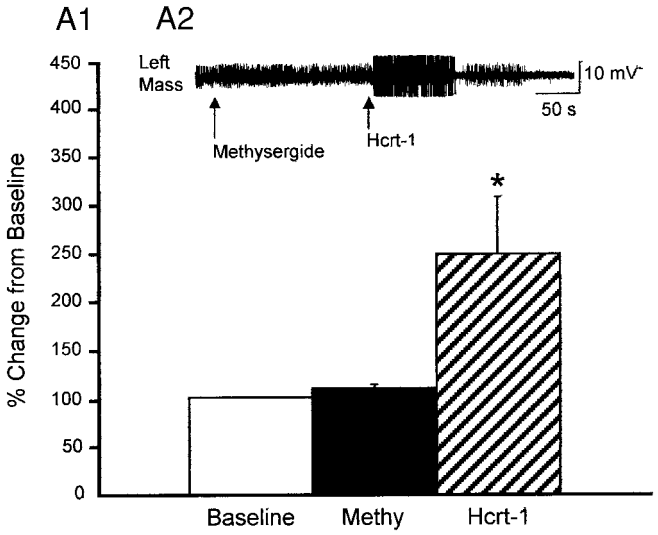

FIG. 9. Pretreatment with methysergide does not reverse the masseter muscle response to Hcrt-1 microinjection into the V motor nucleus. Application of the serotonin antagonist, methysergide, into the unilateral V motor nucleus had no effect on baseline masseter muscle activity (*A1*, $n = 5$). Note: this injection did not affect the increase in masseter muscle activity evoked by microinjecting Hcrt-1into the V motor nucleus. *A2*: an example recording showing that the expected Hcrt-1 response is preserved even when the V motor nucleus has been pretreated with methysergide. *, a statistically significant ($P < 0.05$) increase compared with baseline.

genioglossus muscle tone when applied to the XII motor nucleus. However, blockade of NMDA channels abolished the excitatory response to Hcrt-1 application. We conclude that Hcrt exerts a facilitatory effect on muscle tone when applied to these motor nuclei and that functional NMDA receptors are required for the expression of this effect.

Hypocretin and motor output

Our findings demonstrate that Hcrt facilitates excitatory processes within motor nuclei. Four lines of evidence support this claim. First, our histological observations clearly show that Hcrt microinjections were made within V or XII motor nuclei. Second, microinjections of Hcrt into brain structures immediately adjacent to the V or XII motor nuclei had no effect on masseter or genioglossus activity. Third, Hcrt application unilaterally into the V motor nucleus only affected ipsilateral masseter muscle activity and was without affect on either contralateral masseter or genioglossus muscle activities. The pontine inhibitory area and locus coeruleus are motor-related areas that are in close proximity to the V motor nucleus. Chemical and electrical stimulation of these areas consistently produces *bilateral* muscle activity changes (Lai and Siegel 1988; Lai et al. 1989). If Hcrt microinjections spread to these motor-related areas, then they would undoubtedly alter muscle tone bilaterally and would also affect both masseter and genioglossus muscle activities. Because such a response was never observed, we conclude that microinjections only affected neurons within the target area. Four, because careful electrophysiological studies demonstrate that Hcrt-1 and -2 depolarize neurons in multiple brain regions, including hypothalamus (van den Pol et al. 1998), locus coeruleus (Ivanov and Aston-Jones 2000), basal forebrain (Eggermann et al. 2001), and laterodorsal tegmental nucleus (Burlet et al. 2002), we propose that Hcrt also depolarizes V and XII motoneurons.

Anatomical tracing data illustrate that in cats, Hcrt neurons make synaptic connections with V and XII motoneurons (Fung et al. 2001). In rats, in situ hybridization histochemistry demonstrates the presence of Hcrt receptor mRNA expression in brain stem regions that correspond to V and XII motor nuclei (Marcus et al. 2001). Furthermore, Volgin et al. (2002) found that identified XII motoneurons express mRNA that encodes the Hcrt-2 receptor. Given Hcrt projections to cranial motoneurons and receptor expression on them, in combination, with the results presented here, we purpose that Hcrt plays a permissive role in regulating motoneuronal excitability.

We found no consistent difference in the masseter muscle tone response to Hcrt-1 compared with Hcrt-2, although the effects of Hcrt-1 lasted longer than Hcrt-2 did but only at the highest dosage (100 μM). In decerebrate rats, Kiyashchenko et al. (2001) reported that Hcrt-1 and -2 microinjections into the locus coeruleus produced similar increases in muscle tone. There are two identified Hcrt receptors: HcrtR1 and HcrtR2; however, other as yet unidentified subtypes could exist. In rats, Marcus et al. (2001) reported that HcrtR1 is expressed in the V motor nucleus and HcrtR2 in the XII motor nucleus. HcrtR1 is 30 times more selective for Hcrt-1, whereas HcrtR2 is nonselective (Sakurai et al. 1998). Hence, the expression of HcrtR1 in the V motor nucleus may explain why the duration of muscle tone increase was significantly longer at 100 μM for Hcrt-1 than for Hcrt-2. Furthermore, steep concentration gradients produced by microinjection techniques may attenuate the effects produced by relatively small differences in receptor response.

Interaction of glutamate and hypocretin

Application of Hcrt-1 caused an increase in masseter muscle tone when applied to the V motor nucleus. This response, however, was blocked with pretreatment of D-AP5, and was actively reversed by D-AP5 application. Based on these observations, we suggest that functional NMDA channels are required for the expression of the Hcrt response. These are the first data to demonstrate a functional link between the excitatory effects of Hcrt and glutamatergic processes. Indeed, blockade of NMDA receptors within V motor nuclei nullifies the excitatory Hcrt response on masseter muscle activity, indicating that glutamatergic pathways are critical for Hcrt function. We propose that Hcrt-1 may act on presynaptic receptors located on glutamatergic axons and modulate motoneuronal activity by presynaptic glutamate release. Two lines of evidence support this contention. First, both Hcrt and glutamate-containing presynaptic terminals are found within the V motor nucleus (Bae et al. 1999; Fung et al. 2001). Second, Hcrt has been shown to modulate amino acids release. In in vitro hypothalamic slices, van den Pol et al. (1998) reported that in the absence of synaptic transmission, Hcrt application increases the release of glutamate. In anesthetized rats, intravenous administration of Hcrt-1 alters glutamate release in the amygdala, which receives dense Hcrt projections, but has no affect on glutamate release in the cerebellum, a region virtually devoid of Hcrt projections (John et al. 2001). Similarly, Kodama and Kimura (2002) demonstrate that systematic Hcrt-1 application increases glutamate release within the locus coeruleus in behaving rats. The most parsimonious explanation of these findings would be that Hcrt modulates the presynaptic release of

glutamate. We suggest that Hcrt binds to presynaptic receptors to liberate glutamate, thus activating NMDA receptors on V motoneurons to cause motoneuronal excitation and increased masseter muscle activity.

While the response to Hcrt-1 was blocked and reversed by glutamate antagonists, the broad-spectrum serotonin antagonist, methysergide did not neutralize the muscle tone response to Hcrt-1. Accordingly, we suggest that Hcrt processes are not mediated or dependent on serotonin receptors at the level of the V motor nucleus. Furthermore, we are confident that the dosage of methysergide was sufficient to fully block serotonin receptors and that this could not explain that lack of effect. We used higher methysergide concentrations than those previously reported to block serotonin activity in both the V and XII motor nuclei (Kubin et al. 1992; Okabe and Kubin 1996; Ribeiro-do-Valle et al. 1991). We propose the Hcrt acts on presynaptic glutamatergic terminals within the V motor nucleus to regulate glutamate release onto motoneurons, thus altering their excitability via NMDA receptors.

Hypocretin and motor control regulation

The extensive distribution of Hcrt projections throughout the CNS, coupled with recent behavioral studies, suggests that Hcrt probably regulates multiple physiological processes. Indeed, the Hcrt system is implicated in variety of homeostatic processes like feeding, energy metabolism and sleep function (Siegel 1999); however, compounding evidence strongly links the Hcrt system with motor regulation. Recent work from this laboratory demonstrates that there is a pronounced association between CSF Hcrt concentration and motor activity in behaving dogs. Wu et al. (2002) reported that CSF Hcrt concentrations are highly correlated with motor activity levels. They also reported that sleep deprivation leads to increased CSF Hcrt concentrations; however, it is tightly correlated with the level of motor activity during the deprivation procedure rather than with the amount of sleep loss.

Obstructive sleep apnea is a major sleep disorder in which muscle tone plays a key role. It is caused by collapse of the upper airways due, in part, to sleep-dependent reductions in pharyngeal muscle tone (Kubin et al. 1996). Sleep-dependent reductions in muscle tone occur because sleep-related neurons project to motoneurons and by active inhibition and disfacilitation reduce their excitability (Horner 1996). Hcrt neurons project to both the V and XII motor nuclei, which innervate upper airway related muscles affected by sleep hypotonia. Hcrt neuronal activity appears to vary as a function of arousal state. Estabrooke et al. (2001) reported that Hcrt neurons are relatively more active in wakefulness than in sleep, and we recently found that Hcrt concentrations in the lateral hypothalamus and in basal forebrain are greater during wakefulness than during slow-wave sleep (Kiyashchenko et al. 2002). Therefore withdrawal of Hcrt neuronal inputs during slow-wave sleep may lead to disfacilitation of motoneuronal excitability and reduced pharyngeal muscle tone, thus contributing to obstructive sleep apnea during this state. Hcrt administration may have important clinical effects; it might be useful in the treatment of obstructive sleep apnea and cataplexy because it could minimize the loss of muscle tone associated with these conditions. Indeed, John et al. (2000) demonstrate that systemically administered Hcrt-1 produced a dose-dependent reduction in cataplexy in canine narcoleptics.

In summary, we suggest that the Hcrt system is intimately involved in motor regulation, and it contributes to cataplexy and sleep-dependent muscle atonia. We provide the first evidence that Hcrt alters motoneuronal excitability in a glutamate-dependent manner. This latter observation is of physiological importance because it indicates that a major function of Hcrt may be to regulate presynaptic glutamate release.

We thank B. Nienhuis and O. Lyamin for technical advice.

This work was supported by the Medical Research Service of the Department Veterans Affairs and National Institutes of Health Grants NS-14610, HL-41370, and HL-60296. J. H. Peever holds a postdoctoral fellowship sponsored by the National Sleep Foundation.

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