Subdivision-Specific Responses of Neurons in the Nucleus of the Tractus Solitarius to Activation of Mu-Opioid Receptors in the Rat

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Poole SL, Deuchars J, Lewis DI, Deuchars SA. Subdivision-specific responses of neurons in the nucleus of the tractus solitarius to activation of mu-opioid receptors in the rat. J Neurophysiol 98: 3060–3071, 2007. First published September 26, 2007; doi:10.1152/jn.00755.2007. Microinjection of opioid receptor agonists into the nucleus tractus solitarius (NTS) has differential effects on cardiovascular, respiratory, and gastrointestinal responses. This can be achieved either by presynaptic modulation of inputs onto neurons or by postsynaptic activation of receptors on neurons in specific regions. Therefore we sought to determine whether responses of neurons to activation of opioid receptors were dependent on their location within the NTS. Using whole cell patch-clamp recordings from neurons within the NTS, the mu opioid receptor (MOR) agonist [D-Ala², N-Me-Phe⁴,Gly⁵-ol]-enkephalin (DAMGO, 100 nM) hyperpolarized a proportion of neurons in the medial, dorsomedial and dorsolateral NTS, whereas no postsynaptic responses were observed in remaining subdivisions. DAMGO reduced the amplitude of solitary tract-evoked excitatory postsynaptic potentials (EPSPs) in all neurons tested, regardless of subdivision. The kappa opioid receptor (KOR) agonist U69593 (10–20 μM) also hyperpolarized a small fraction of neurons (6/79) and decreased the amplitude of EPSPs in 50% of neurons. In contrast, the delta-opioid receptor agonist DPDPE (1–4 μM) had no presynaptic or postsynaptic effects on NTS neurons even after preincubation with bradykinin. Anatomical data at the cellular level.

Previous electrophysiological studies in the NTS have shown that ~50% of neurons were hyperpolarized by DAMGO, but there were no postsynaptic effects of delta or kappa opioid receptor agonists, although these drugs were only tested on a low number of neurons (Rhim et al. 1993). Furthermore this study did not define the location of neurons that were responsive to mu-opioid receptor (MOR) activation. There are also discrepancies in the exact role of presynaptic opioid receptors in modulating synaptic transmission onto NTS neurons. In early studies, excitatory postsynaptic potentials (EPSPs) in most NTS neurons were reduced by DAMGO (Rhim et al. 1993); however, studies determining the effects of DAMGO on evoked calcium currents observed decreases in either 50% (Rhim and Miller 1994) or 75% (Endoh 2006) of neurons tested. In another study, DAMGO reduced solitary tract evoked excitatory postsynaptic currents (EPSCs) in all pro-opiomelanocortin (POMC)-expressing NTS neurons (Appleyard et al. 2005). Rhim et al. (1993) also reported that all solitary tract evoked EPSPs were reduced by the kappa agonist U69593 and delta agonist [D-Pen²⁵⁶]-enkephalin (DPDPE); however, in a subsequent paper, the evoked calcium current was decreased by U69593 in only 3 of 15 cells tested, whereas DPDPE was totally without effect (Rhim and Miller 1994). These discrepancies in functional studies are mirrored in anatomical studies because the exact extent and location of opioid receptors in the NTS is an area of dispute (Aicher et al. 2000; Cheng et al. 1996; Nomura et al. 1996). Interestingly, the delta opioid receptor (DOR) is often mainly contained within the cytoplasm, away from the plasma membrane (Arvidsson et al. 1995) and DOR trafficking to the surface must be induced by acute exposure to forskolin or bradykinin before responses are observed (Browning et al. 2004; Patwardhan et al. 2005).

Given that previous studies provide an incomplete picture of opioid receptor mediated responses in the NTS, the aim of this study was to further our understanding of the exact role of opioid receptors on neurons in specific regions. Therefore we sought to determine whether responses of neurons to activation of opioid receptors were dependent on their location within the NTS. Using whole cell patch-clamp recordings from neurons within the NTS, the mu opioid receptor (MOR) agonist [D-Ala², N-Me-Phe⁴,Gly⁵-ol]-enkephalin (DAMGO) into the NTS in freely moving rats increased food intake, the delta and kappa agonists were ineffective in motivating this behavior (Kotz et al. 1997). Such diverse effects may be due to activation of differentially localized pre- or postsynaptic opioid receptors in specific sites within the NTS because there is some degree of topographical organization in the NTS (Altschuler et al. 1991; Ciriello 1983; Finley and Katz 1992; Hayakawa et al. 2001; Housley et al. 1987; Leslie et al. 1982; Zhang et al. 2000). However, this issue has not been fully addressed at the cellular level.

INTRODUCTION

Activation of the opioid receptor family has diverse effects on cardiovascular, respiratory and gastrointestinal autonomic functions (Akil et al. 1984; Burks et al. 1988; Shook et al. 1988, 1990; Waldhoer et al. 2004) with individual responses dependent on the subtype of opioid receptor involved (mu, delta, or kappa) and its location within the CNS. A key site of action is likely to be the nucleus of the tractus solitarius (NTS) in the brain stem, a pivotal integrative center in autonomic regulatory circuits (Spyer 1990).

Cardiovascular responses are influenced by microinjection of mu, delta, and kappa opioid agonists into the NTS, and the delta agonist additionally increases respiratory rate (Hassen et al. 1983). Gastrointestinal function is also influenced differentially; because microinjection of the mu-opioid agonist...
opioids within the NTS. Because previous discrepancies in results may be due to different recording sites, we mapped the regions of the NTS where activation of mu, kappa, and delta opioid receptor agonists elicited pre- and postsynaptic responses. We demonstrate subdivision specific responses to MOR activation with this electrophysiological data being reinforced by anatomical data at the EM level. Furthermore, we show that kappa agonists can indeed hyperpolarize a small fraction of NTS neurons. This diversity of responses may underlie the differential modulation of autonomic reflexes observed after opioid receptor activation within the NTS.

METHODS

All experiments were performed under UK Home Office License and in accordance with the regulations of the UK Animals (Scientific Procedures) Act 1986.

Male Wistar rats aged 14–21 days were humanely killed by terminal anaesthetizing with sodium pentobarbitone (120 mg/kg, ip) and transcardially perfusing with ice-cold sucrose aCSF, composition (in mM): NaCl (124), NaHCO₃ (26), KCl (3), MgSO₄ (2), NaH₂PO₄ (2.5), CaCl₂ (1) and glucose (10). The brain was removed, terminally anesthetizing with sodium pentobarbitone (120 mg/kg, ip) and in accordance with the regulations of the UK Animals (Scientific Procedures) Act 1986.

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Histology

Following recording, electrodes were very slowly removed from neurons and the slice was immersed in fixative consisting of 4% paraformaldehyde in 0.1 M phosphate buffer for 18 h. Sections were dehydrated and embedded in paraffin, then applied to examine firing properties of the neuron (see (Deuchars et al. 2001b). Following electrophysiological characterization of the neuron, responses to bath applied drugs were determined. Neurons were then fixed and stained with 4% paraformaldehyde in 0.1 M phosphate buffer for 18 h. Sections were dehydrated and embedded in paraffin, then applied to examine firing properties of the neuron (see (Deuchars et al. 2001b)

Drugs used were [D-Ala²,N-Me-Phe³,Gly⁵-ol]-enkephalin (DAMGO; 0.1–1 µM, Sigma), [β-Pen⁵]-enkephalin (DPDPE; 1–4 µM, Sigma), U69593 (10–20 µM), d-Phe-Cys-Tyr-d-Trp-Orn-Thr-Pen-Thr-NH₂ (CTOP; 1 µM, Sigma), nor-Binalorphimine dihydrochloride (nor-BNI; 200 nM), tetrodotoxin (TTX citrate; 1 µM, Tocris), t-AP5 (50 µM) and NBQX disodium salt (10–30 µM). All stock solutions were made using distilled water, excluding U69593 which was made up in 50% ethanol and were stored at −20°C until required. All compounds were then diluted in aCSF and applied into the superfusate at concentrations shown previously to be effective (Browning et al. 2002; Margolis et al. 2003; Milligan et al. 2004; Rhim et al. 1993).

Neuron location

During visualized patch-clamp recordings neurons were identified as belonging to a particular subdivision of the NTS using a combination of previous references which describe the morphology and orientation of neurons and the rat brain atlas stereotaxic co-ordinates (Kalina and Sullivan 1982; Paxinos and Watson 1998; Kalina et al. 1984). Neurons in the commissural subdivision are typically scattered and are small and oval with their axes lying parallel to the dorsal edge of the medulla. In the dorsosolateral subdivision elliptical neurons form a cluster dorsal to the solitary tract, while medial neurons are closely packed and medium sized. Neurons in the intermediate subdivision form a small cluster lying parallel to the solitary tract and neurons in the ventral NTS are medium sized and multipolar (Kalina and Sullivan 1982; Kalina et al. 1984).

Data analysis

Data capture was carried out using either Signal (version 1) and Spike 2 (version 2; Cambridge Electronic Design) or pClamp (version 9, Axon Instruments). The input resistance was calculated for each neuron by measurement of the voltage responses to injected current pulses. Excitatory postsynaptic potential (EPSP) amplitudes were calculated by measuring their peak amplitude from the holding potential and were averaged for ≥10 consecutive sweeps. Changes in EPSP amplitude as a consequence of drug application were expressed as a mean percentage of the control. Assessment of changes in the paired pulse ratio was also carried out (see (Deuchars et al. 2001a). This was calculated by the ratio of the amplitude of the second EPSP to the first EPSP. The paired pulse ratio during drug application was then compared with the control to note any significant change.

The effects of the drugs were tested statistically using the paired Student’s t-test, and differences were considered significant when P < 0.05. A two-sample t-test was used to test for differences in electrophysiological characteristics between groups of neurons and in the size of hyperpolarization for the two concentrations of DAMGO (100 nM and 1 µM), differences were considered significant if P < 0.05. The chi-square test for independence was used to determine whether differences were apparent in the presence of 1-phase or 2-phase afterhyperpolarizations in responsive and unresponsive neurons. Significant differences were evident when P < 0.05.

Histology

Following recording, electrodes were very slowly removed from neurons and the slice was immersed in fixative consisting of 4% paraformaldehyde in 0.1 M phosphate buffer for ≤18 h. Sections were firstly wet mounted and examined under a fluorescence microscope to identify Lucifer yellow filled cells for subsequent localization. Slices were then washed in PBS and incubated for 24–48 h in extravidin-peroxidase (1:250; Sigma), which attaches to the neurobiotin within the neuron and could be visualized with 3′,3′diaminobenzidine (DAB, Sigma) (Deuchars et al. 2001b). Sections were dehydrated and...
mounted in DPX for viewing under the light microscope. This allowed confirmation of their location and enabled more detailed analysis of the basic morphology and size of the recorded neuron.

**Immunohistochemistry**

Male Wistar rats 150–250 g (n = 7) and 18 days old (n = 3) were anesthetized by intraperitoneal injection of sodium pentobarbitone (60 mg/kg) and transcardially perfused with paraformaldehyde (4%, in 0.1M Phosphate buffer) containing either 0.05% or 0.1% glutaraldehyde if the tissue was required for electron microscopy (n = 2). The brains were removed and placed in the same fixative for 12 h at 4–8°C. Following postfixation, brains were washed in phosphate buffered saline (PBS) and 30–50 brains were immersed in Durcupan ACM resin for 12–18 h, mounted on grids, every third grid was examined. At least three ultrathin sections were examined per grid. To eliminate the possibility of double counting of labeled structures on separate grids, every third grid was examined.

Sections required for electron microscopy were permeabilized in 50% ethanol for 30 min at room temperature, washed in PBS then incubated in a H₂O₂ solution for 30 min to block endogenous peroxidase activity. Sections for light microscopy were not treated with 50% ethanol for permeabilization, but had 0.1% Triton X100 added to the primary antibody solution. Tissue processing for light and electron microscope viewing has been described in detail previously (Deuchars et al. 2001a). Briefly, sections were incubated in polyclonal antiserum corresponding to residues 1359–1403 of the carboxy-terminus of the rat mu-opioid receptor (Neuromics, Minneapolis, USA), 1:2000 in PBS. This was localized with secondary avidin peroxidase (1:1500) and visualization with DAB. Sections were treated with 10 mM sodium citrate at 80°C for 30 min. Incubation in the primary antibody solution. For the second antigen retrieval method, instead of incubation in sodium borohydride, sections were treated with 10 mM sodium citrate at 80°C for 30 min.

**Image capture and electron microscopy**

Slides were examined at the light microscope level using a Nikon E600 microscope and images captured using Acquis Image Capture System (Synoptics, Cambridge, UK). These images were adjusted as required using the Corel graphics package version 12. Drawings were constructed using a drawing tube at x40 magnification and were from three representative slices. Sections for light microscopy were dehydrated in increasing concentrations of ethanol and mounted in DPX. Sections for electron microscopy were postfixed in 0.5% osmium tetra oxide and dehydrated in graded ethanol concentrations, 50%, 70%, 90% and 100%. Sections were immersed in Durcupan ACM resin for 12–18 h, mounted on a vibrating microtome (Leica, Milton Keynes, UK) and collected into wells containing PBS.

**RESULTS**

**Responses of NTS neurons to the MOR agonist DAMGO are region specific**

Whole cell patch clamp recordings were obtained from visualized neurons within the NTS. Recordings were performed in current clamp mode so that the firing characteristics of neurons could be assessed. At a holding potential of −60 mV, perfusion of DAMGO (100 nM) evoked a membrane hyperpolarization of 7.4 ± 0.5 mV (Fig. 1A), accompanied by decreases in input resistance in the ventral (v) and intermediate (i) NTS subdivisions at levels rostral (+) and caudal (−) to the obex. Whole neurons which were recovered were mapped.

**Antigen retrieval methods**

As we wanted to visualize MOR immunoreactive cells within the NTS, we used antigen retrieval methods to try to enhance the staining. Prior to incubation in the primary antibody, sections were incubated in 0.1% sodium borohydride for 10 min, washed for 3 x 10 min in PBS then incubated in the primary antibody solution. For the second antigen retrieval method, instead of incubation in sodium borohydride, sections were treated with 10 mM sodium citrate at 80°C for 30 min.

**FIG. 1.** Subdivision specific hyperpolarization of nucleus tractus solitarius (NTS) neurons on application of [d-Ala², N-Me-Phe⁴, Gly⁵-ol]-enkephalin (DAMGO). A: DAMGO (100 nM) hyperpolarized neurons, accompanied by decreases in input resistance in the medial (M), dorsomedial (A2), and dorsolateral (A3) NTS. B: maps showing the location of neurons responding to DAMGO (filled star) and those unresponsive to DAMGO (filled circle). These were constructed from drawings of brain stem sections showing the approximate location of NTS subdivisions at levels rostral (+) and caudal (−) to the obex. Whole neurons which were recovered were mapped. AP, area prostrema; CC, central canal; c, central NTS; com, commissural NTS; dl, dorsolateral NTS; dm, dorsomedial NTS; DVN, dorsal vagal motor nucleus; in, intermediate NTS; is, interstitial NTS; lat, lateral NTS; Pc, pars compacta; Sg, substantia gelatinosa; v, ventral NTS; vlat, ventrolateral NTS; m, medial NTS; TS, tractus solitarius; 4V, 4th ventricle. C: application of DAMGO had no postsynaptic effect in the ventral (C1), intermediate (C2), and commissural (C3) NTS.
a significant decrease in the input resistance from 1.5 ± 0.09 to 1.1 ± 0.07 GΩ (P < 0.0005) in 28% of neurons (50/178) in the NTS. At 1 μM DAMGO the amplitude of the hyperpolarizing response was also 7.4 ± 0.5 mV (n = 5, not shown) and was accompanied by a significant decrease in input resistance from 1.3 ± 0.22 GΩ to 0.8 ± 0.14 GΩ (P < 0.05, n = 5). The magnitude of hyperpolarization induced by DAMGO at 1 μM was not significantly different from that elicited by 100 nM DAMGO (P > 0.99) suggesting that 100 nM DAMGO evokes near maximal responses. The effect of DAMGO was reversible on washout and repeated doses of DAMGO had similar effects, showing that there was no desensitization in response on repeated application. The first application of DAMGO induced a hyperpolarization of 4.7 ± 0.4 mV with the second application inducing a hyperpolarization of 4.6 ± 0.9 mV (n = 5, not shown). There were no significant differences in the magnitude of hyperpolarization (P > 0.88). The likelihood of the neuron to respond to the postsynaptic actions of DAMGO depended on its location within the NTS (Fig. 1). Hyperpolarizing responses were observed only in the medial (43% of neurons (34/80), Fig. 1A1), dorsomedial (21% of neurons (6/29), Fig. 1A2) and dorsolateral (53% of neurons (10/19), Fig. 1A3) subdivisions. Neurons in the ventral (n = 10, Fig. 1C1), intermediate (n = 16, Fig. 1C2), commissural (n = 12, Fig. 1C3), dorsal (n = 5) and lateral (n = 7) NTS were unresponsive. Neurons unresponsive to 100 nM DAMGO were subsequently shown to be unresponsive to 1 μM DAMGO (n = 14) to further strengthen the separation of responsive and unresponsive neurons. This lack of effect in specific regions was mirrored in our immunohistochemical data which described low densities of immunopositive neurons in regions where no postsynaptic responses were observed (see following text).

Following electrophysiological recordings, neurons were filled with neurobiotin and subsequently recovered. The mapping of recovered cells confirmed the restricted location of responsive cells to the medial, dorsomedial and dorsolateral subdivisions of the NTS (Fig. 1B). Examples of filled neurons are shown in Fig. 2, the morphology of recovered neurons in each subdivision was similar to that of neurons previously described in these subdivisions, (Kalina and Sullivan 1982; Kalia et al. 1984).

Hyperpolarizations induced by DAMGO were maintained in TTX (1 μM, n = 5, Fig. 3A). The magnitude of the hyperpolarization and decrease in input resistance induced by DAMGO in the presence of TTX were not significantly different from the hyperpolarization and input resistance prior to TTX (P > 0.05), thus suggesting a direct action on postsynaptic receptors, although it cannot be ruled out that DAMGO might be affecting neurotransmitter release onto these neurons in a TTX-insensitive manner. DAMGO mediated hyperpolarizations were antagonized by the specific MOR antagonist CTOP (1 μM) indicating that this effect was mediated through a selective action at the MOR (n = 5, Fig. 3B and 3C). Using values representing the response as a percentage of the control, responses to CTOP alone (100.04 ± 0.74%) and CTOP and DAMGO (100.46 ± 0.17%) were not significantly different from the control response (P > 0.1, Fig. 3D). There were no significant changes (P > 0.1) in input resistance from control values (1.7 ± 0.44 GΩ) with application of CTOP (1.8 ± 0.46 GΩ) or CTOP and DAMGO (1.8 ± 0.47 GΩ).

Effects of DAMGO on EPSPs were observed throughout the NTS

To determine whether there was any regional specificity in the presynaptic responses to DAMGO, twin-pulse stimuli were applied to the tractus solitarius at 100–400 ms intervals to evoke pairs of EPSPs. If a significant change in the paired pulse ratio occurred then this would be consistent with a presynaptic site of action. DAMGO reduced the peak amplitude of pairs of evoked EPSPs (n = 39); EPSP 1 was reduced to 47.7 ± 2.8% of control and EPSP 2 to 61.8 ± 3.4% of control (Fig. 4). This decrease in amplitude was accompanied by a significant increase in the paired pulse ratio from 0.71 ± 0.03 to 0.94 ± 0.05 (P < 0.0005, Fig. 4D1). These presynaptic effects were observed in all neurons tested, regardless of their location in the NTS and their postsynaptic response to DAMGO (Fig. 4). At this holding potential (-70 mV), these EPSPs were mediated by action at non-NMDA receptors since NBQX (10–30 μM), but not AP-5 (50 μM), abolished these EPSPs (n = 5, Fig. 4B2).

CTOP (1 μM) blocked the reduction in peak amplitude seen on application of DAMGO and also the increase in paired pulse ratio (n = 5, Fig. 4C2). CTOP itself had no significant effect on the amplitude of either EPSP or indeed the paired pulse ratio (P > 0.1, Fig. 4D2).

The decrease in EPSP peak amplitude and increase in the paired pulse ratio suggests that DAMGO could exert its presynaptic actions through a reduction in neurotransmitter release.

The mu-opioid receptor location correlates with electrophysiological observations

NTS neurons which were responsive and unresponsive to DAMGO were recovered subsequent to electrophysiological recordings. Responsive neurons were present only in the medial, dorsomedial and dorsolateral subdivisions of the NTS (Fig. 1B).

Studies to date have not undertaken a full analysis of the localization of the MOR in different subdivisions of the NTS. Therefore to determine the postsynaptic location of MORs in these subdivisions, immunoreactivity for this receptor was examined in the NTS. Antigen retrieval methods improved the visibility of labeled neurons. Tissue from rats aged 18 day and
adult rats was examined and the distribution of MOR-immunoreactivity in the NTS was comparable. This was to be expected since previous studies have also shown similar levels of DAMGO binding in the NTS at these two ages (Xia and Haddad 1991). Drawings were constructed from three representative slices in the older rats (Fig. 5). At the level primarily used for electrophysiological recordings (Fig. 5A2) MOR immunoreactive neurons were observed in the medial, dorsomedial and dorsolateral subdivisions of the NTS. Since previous immunohistochemical studies have not examined the subcellular localization of the MOR in the dorsal NTS, the dorsolateral NTS was examined at the electron microscope level. Electron microscopy confirmed that the MOR is located at both presynaptic (Fig. 6F and G) and postsynaptic (Fig. 6H) sites in this region and that MOR immunoreactivity could also be seen presynaptically and postsynaptically at the same synapse (Fig. 6C-E). To quantify this further, 104 MOR immunoreactive structures were identified in the dNTS. 62 (60%) of these labeled structures were terminals onto unlabeled dendritic elements, 18 (17%) were labeled terminals onto labeled dendrites, 16 (15%) were labeled axons en passage and 8 (8%) were synaptic connections where the postsynaptic structure but not the presynaptic terminal was labeled.

**Cell characteristics**

(Zhu and Pan 2004) and (Santos et al. 2004) found that specific populations of neurons within the central nucleus of the amygdala and substantia gelatinosa respectively responded to applications of DAMGO. These neurons displayed different firing patterns, action potential characteristics and input resistance. In contrast, (Rhim et al. 1993) found no differences in

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**Fig. 3.** The hyperpolarization induced by DAMGO is through direct action at postsynaptic mu-opioid receptors. The hyperpolarization mediated by DAMGO persisted in the presence of TTX (1 µM), suggesting that this effect is mediated through a direct action at postsynaptic receptors (A). The mu opioid receptor (MOR) antagonist D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂ (CTOP, 1 µM), prevented the hyperpolarization mediated by DAMGO, confirming that these effects are mediated through action at the MOR, examples shown in a medial (B) and dorsolateral (C) neuron. D: pooled data to show the hyperpolarization elicited by DAMGO (100 nM and 1 µM) as a percentage of control membrane potential. Responses to both 100 nM and 1 µM were significantly different from the control (P < 0.005). There was no significant difference in the size of response elicited by 100 nM and 1 µM DAMGO (P > 0.50, 2-sample t-test). CTOP antagonised the DAMGO-mediated hyperpolarization. Values shown as a percentage of control.

**Fig. 4.** The presynaptic actions of DAMGO are ubiquitous. The presynaptic actions of DAMGO were seen in all subdivisions of the NTS, regardless of their postsynaptic response to DAMGO. A1: this medial NTS neuron exhibited both postsynaptic and presynaptic responses to DAMGO. A2: example of a neuron from a subdivision where postsynaptic responses to DAMGO were not observed. In this intermediate NTS neuron, DAMGO reduced the amplitude and increased the paired-pulse ratio in pairs of evoked excitatory postsynaptic potentials (EPSPs). B1: DAMGO reduced the amplitude and increased the paired-pulse ratio in this medial NTS neuron. In the same neuron (B2), these EPSPs were abolished on application of NBQX, AP-5 having no effect (n = 5), indicating these were elicited by action of glutamate on AMPA/kainate receptors. C1: in this dorsolateral NTS neuron, the presynaptic effects of DAMGO were blocked by MOR antagonist CTOP (1 µM, C2), confirming that these effects are mediated through action at the MOR. D1: pooled data showing that the increase in paired-pulse ratio seen with DAMGO was significantly different to the control (P < 0.0001). D2: pooled data to show that CTOP itself had no effect on the paired-pulse ratio and inhibited the increase in paired-pulse ratio induced by DAMGO.
the general properties for neurons within the NTS that were responsive to postsynaptic actions of DAMGO and those that were unresponsive.

To compare the electrophysiological and morphological characteristics of neurons responsive and unresponsive to postsynaptic actions of DAMGO, we took samples of cells from each group in the medial, dorsomedial and dorsolateral subdivisions (Table 1). In the dorsolateral subdivision there were significant differences between the afterhyperpolarization (AHP) amplitude ($P < 0.05$) and duration ($P < 0.05$) in responsive and unresponsive neurons (Fig. 7B). The AHP amplitude was $25.1 \pm 3.1$ mV compared with $16.8 \pm 2.0$ mV and the duration was $65.4 \pm 8.4$ ms compared with $38.2 \pm 5.9$ ms in responsive ($n = 7$) and unresponsive ($n = 7$) neurons respectively. In neurons of dorsomedial subdivision there was a significant difference ($P < 0.05$) in AHP duration ($37.5 \pm 8.3$ ms in unresponsive neurons ($n = 5$)) compared with $63.8 \pm 7.8$ ms in responsive neurons) but no significant difference in AHP amplitude ($P > 0.1$, Fig. 7C). Action potential amplitude, duration, half-width and cell input resistance were not significantly different in responsive and unresponsive neurons in the dorsolateral and dorsomedial subdivisions ($P > 0.1$, Fig. 7). In cells within the medial subdivision of the NTS, a two-sample $t$-test revealed no significant differences between action potential duration, amplitude or half-width and no significant difference between AHP duration and amplitude ($P > 0.1$, $n = 10$, Fig. 7A). There was also no significant difference in input resistance ($P > 0.5$). The chi-square test was used to determine if any differences existed in the occurrence of 1-phase or 2-phase AHPs in neurons responsive or unresponsive to DAMGO. In the dorsolateral subdivision there was a significant difference from the expected ($P < 0.05$), responsive neurons were more likely to have 1-phase AHPs and unresponsive neurons were more likely to have 2-phase AHPs. In the dorsomedial and medial subdivisions there was no significant difference ($P > 0.5$). Morphological analysis of responsive and nonresponsive neurons of the medial NTS revealed no significant differences in soma size ($P > 0.5$) and no consistent differences in soma orientation (Table 2, see Fig. 2 for representative filled neurons).
Neurons of the NTS were unresponsive to the delta opioid agonist DPDPE

The delta agonist DPDPE had no postsynaptic effects on neurons in all subdivisions of the NTS ($n = 34$, not shown), of these $3/22$ neurons tested exhibited a postsynaptic response to DAMGO (not shown). The amplitude and paired pulse ratio of pairs of evoked EPSPs ($n = 7$, not shown) were also unaffected by application of $1–4 \mu M$ DPDPE ($P > 0.1$) of control values, EPSP 2; $92.2 \pm 5.3\% (P > 0.1)$ of control, PPR $0.7 \pm 0.1$ to $0.6 \pm 0.1 (P > 0.1)$. Four of these neurons were tested and found to be responsive to presynaptic actions of DAMGO (not shown). Since activation of the MOR may increase DOR receptor trafficking to the membrane surface (see Cahill et al. 2001 and discussion), 12 neurons were exposed to DAMGO for 3–5 min prior to application of DPDPE. Following this preexposure to DAMGO, neurons were still unresponsive to the postsynaptic actions of DPDPE. (Patwardhan et al. 2005) have also demonstrated that preincubation of rat trigeminal ganglia with bradykinin for 15 min results in rapid trafficking of the DOR to the plasma membrane and functional competence of these receptors. In the present

TABLE 1. Electrophysiological characteristics of NTS neurons responsive and unresponsive to DAMGO

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<th>Subdivision</th>
<th>Response to DAMGO</th>
<th>Action Potential</th>
<th>AHP</th>
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<td></td>
<td></td>
<td>Duration, ms</td>
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<tr>
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<td>M</td>
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<tr>
<td>M</td>
<td>No</td>
<td>5.3 ± 0.4</td>
<td>2.5 ± 0.2</td>
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Mean values = SE showing action potential duration, amplitude, and half- width and afterhyperpolarization (AHP) amplitude and duration. The shape of the AHP after an action potential, i.e. whether they were single component (1 phase) or had two components differentiated by the slope of the decay phase (2-phase) were also determined for both [d-Ala²,N-Me-Phe⁷,Gly⁵-ol]-enkephalin (DAMGO) responsive and unresponsive neurons in the dorsomedial (DM), dorsolateral (DL), and medial (M) nucleus tractus solitarii (NTS). In the dorsomedial subdivision, there is a significant difference ($P < 0.05$) in AHP duration in unresponsive neurons compared to responsive neurons. In the dorsolateral subdivision, there were significant differences in the AHP amplitude ($P < 0.05$) and duration ($P < 0.05$) in responsive and unresponsive neurons.
study preincubation of slices in 20 μM bradykinin for 15–30 min prior to DPDPE application did not result in an inhibitory response to DPDPE (2 μM, n = 9), even though responses to DAMGO were still observed (not shown).

The kappa opioid agonist U69593 has presynaptic and postsynaptic effects on neurons of the NTS

The kappa opioid receptor (KOR) agonist U69593 hyperpolarized 6/79 neurons in the NTS (Fig. 8A). The magnitude of this hyperpolarization was 9.7 ± 1.5 mV (10–20 μM). Neurons unresponsive to 20 μM also did not respond to 40 μM U69593. A decrease in input resistance from 1.3 ± 0.17 to 0.9 ± 0.15 GΩ was also observed. Responsive neurons were located in the medial (3/27), dorsomedial (1/11), dorsolateral (1/10) and intermediate (1/9) subdivisions. Twenty two neurons in other subdivisions (commissural, ventral, dorsal and lateral) were unresponsive to U69593. Of neurons hyperpolarized by DAMGO and subsequently tested for a response to U69593, 3/10 were also hyperpolarized by U69593. Thus U69593 failed to induce a response in seven neurons which were hyperpolarized by DAMGO. In addition, one neuron was not affected by DAMGO but was hyperpolarized by U69593 therefore it is unlikely that U69593 was acting nonselectively at MORs.

U69593 also reduced the amplitude and increased the paired pulse ratio of evoked EPSPs (8/19, Fig. 8B). 10–20 μM U69593 significantly (P < 0.05) decreased the first EPSP to 39.7 ± 9.6% and the second EPSP to 63.1 ± 10.0% of the control (n = 8). This decrease in amplitude was accompanied by an increase in paired pulse ratio. Addition of 10–20 μM U69593 significantly increased the paired pulse ratio from 0.77 ± 0.07 to 1.62 ± 0.36 (n = 8, P < 0.05). The KOR antagonist nor-BNI (200 nM) antagonized the reduction in peak amplitude seen on application of U69593 (10 μM, n = 4, Fig. 8C1). U69593 reduced the EPSP amplitude to 44.2 ± 1.5% of the control amplitude while in the presence of nor-BNI and U69593, the EPSP amplitude was 105.0 ± 7.4% of the control amplitude (Fig. 8C2). This further suggests that U69593 is indeed acting at the KOR. Responsive neurons were located throughout the NTS, with no correlation to subdivisions.

**DISCUSSION**

These data support the idea that opioid receptors are concentrated in different regions of the NTS. First we demonstrate that activation of postsynaptic mu-opioid receptors hyperpolarized a proportion of neurons only within the dorsomedial, dorsolateral and medial NTS. These observations were supported by our immunohistochemical data showing high levels of immunopositive neurons in these regions compared with the rest of the NTS. Second, activation of presynaptic MORs reduced EPSP amplitudes in all neurons tested regardless of their location in the NTS. Third, activation of KORs hyperpolarized a small fraction of NTS neurons and decreased the amplitude of EPSPs in 48% of neurons tested. Finally, no DOR-mediated effects were observed in the NTS even after preincubation with bradykinin, which can induce trafficking to the membrane. This heterogeneity in the responses of neurons to activation of opioid receptors may reflect differences in the roles of the neurons affected and lends support to the idea that opioid receptors can influence select neurons to varying degrees. Thus the pre- and postsynaptic distribution of mu-opioid receptors may ultimately differentially influence autonomic functions.

**Location of the mu opioid receptor within the NTS**

We found that neurons hyperpolarized by DAMGO were restricted to the medial, dorsomedial, and dorsolateral NTS, which was confirmed after recovery of these neurons. This study extends the work from previous laboratories where the locations of neurons hyperpolarized by DAMGO were not determined, even though only a proportion of NTS neurons were affected (Rhim et al. 1993). In our study, the distribution of MOR-mediated responses and the location of MOR-immunoreactive cell bodies correlate within the NTS. At the electron microscope level we demonstrated MOR immunoreactivity at pre- and postsynaptic sites in the dorsolateral NTS. Moreover, postsynaptic densities were apposed to presynaptic densities that were both MOR immunoreactive. Such a strong influence from these receptors was confirmed by electrophysiological recordings where we observed both pre- and postsynaptic effects of DAMGO on a proportion of dorsolateral NTS neurons. Our results in the dorsolateral NTS differ from previous studies on the cellular location of the MOR in the medial NTS (Aicher et al. 2000; Cheng et al. 1996; Glass and Pickel 2002; Nomura et al. 1996) where the majority of MOR labeling was concentrated in different regions of the NTS. First we demonstrate that activation of postsynaptic mu-opioid receptors hyperpolarized a proportion of neurons only within the dorsomedial, dorsolateral and medial NTS. These observations were supported by our immunohistochemical data showing high levels of immunopositive neurons in these regions compared with the rest of the NTS. Second, activation of presynaptic MORs reduced EPSP amplitudes in all neurons tested regardless of their location in the NTS. Third, activation of KORs hyperpolarized a small fraction of NTS neurons and decreased the amplitude of EPSPs in 48% of neurons tested. Finally, no DOR-mediated effects were observed in the NTS even after preincubation with bradykinin, which can induce trafficking to the membrane. This heterogeneity in the responses of neurons to activation of opioid receptors may reflect differences in the roles of the neurons affected and lends support to the idea that opioid receptors can influence select neurons to varying degrees. Thus the pre- and postsynaptic distribution of mu-opioid receptors may ultimately differentially influence autonomic functions.

**TABLE 2. Morphology of medial NTS neurons responsive and unresponsive to DAMGO**

<table>
<thead>
<tr>
<th>Subdivision</th>
<th>Response to DAMGO</th>
<th>Lateral to Medial Length, μm</th>
<th>Dorsal to Ventral Length</th>
<th>Soma Area, μm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medial</td>
<td>Yes</td>
<td>15.1 ± 1.4</td>
<td>12.6 ± 1.5</td>
<td>183 ± 20.3</td>
</tr>
<tr>
<td>Medial</td>
<td>No</td>
<td>17.2 ± 1.2</td>
<td>11.4 ± 0.7</td>
<td>198 ± 23.2</td>
</tr>
</tbody>
</table>

Mean values showing soma size (± SE) in recovered neurons that were responsive and unresponsive to DAMGO.
presynaptic. However, because we observed pre- and postsynaptic responses in the same neuron in medial, dorsolateral, and dorsomedial NTS, this, coupled with our electron microscopic data, suggests that neurons expressing postsynaptic MORs are always also influenced by presynaptic MORs.

Neurons responding to activation of postsynaptic MORs display different characteristics

Our studies showed differences in the properties of some of the NTS neurons responding to activation of postsynaptic MORs. In the dorsolateral NTS, neurons with postsynaptic responses to DAMGO had significantly larger AHP amplitudes and durations than unresponsive neurons, whereas responsive neurons in the dorsomedial subdivision had a significantly longer AHP. Such neuron-specific responses to DAMGO have been previously reported by Zhu and Pan (2004) and Santos et al. (2004) in the central nucleus of the amygdala and substantia gelatinosa, respectively. This observation that neurons in the NTS with specific electrophysiological characteristics respond to MOR activation may aid in the functional identification of neurons in future studies. There were no morphological characteristics exhibited exclusively by neurons with postsynaptic MORs. This may be expected because the morphology of neurons in different NTS regions tends to be heterogeneous and does not necessarily reflect function (Deuchars et al. 2000; Paton et al. 2000, 2001). A similar lack of morphological differences in neurons exhibiting postsynaptic responses to DAMGO has also been reported in the central nucleus of the amygdala (Zhu and Pan 2004).

Possible functional significances of region specificity of MOR responses

Our electrophysiological and anatomical data report postsynaptic MORs only in the medial, dorsomedial, and dorsolateral regions of the NTS. Many studies support a role for these subdivisions in cardiorespiratory control. Tracing
studies report a specific band of afferent inputs from aortic baroreceptor (Ciriello 1983), carotid body (Finley and Katz 1992), lungs (Donoghue et al. 1982), and chemoreceptor (Donoghue et al. 1984) afferents lying across the medial, dorsomedial, and dorsolateral regions of the NTS, the regions where we observed our postsynaptic effects with MOR activation. Baroreceptive neurons were located in the dorsomedial and medial NTS (Deuchars et al. 2000), whereas chemoreceptive neurons were more common in the dorsolateral and medial NTS (Paton et al. 2001). Phenylphrine-induced hypertension resulted in a band of fos-labeled neurons in medial, dorsomedial, and dorsolateral subdivisions (Chan and Sawchenko 1998; Mayne et al. 1998), as did formalin injection into the lung complementing the distribution of pulmonary afferent fibers (Xie et al. 1998). Indeed this region is often referred to as the cardiorespiratory region (e.g., see Paton et al. 1993), although it must be noted that there is a moderate degree of afferent input to these regions from superior laryngeal (Altschuler et al. 1989) and stomach wall (Leslie et al. 1982), and thus there may be some degree of heterogeneity with respect to function of neurons. However, our report shows that only a proportion of neurons in the medial, dorsolateral, and dorsomedial regions are influenced by postsynaptic MORs, which could still fit therefore with a specific role of these neurons in cardiorespiratory control. This suggestion is further supported by our observation that responsive neurons exhibited different firing characteristics.

**DOR agonist DPDPE has no effect on neurons of the NTS**

In the NTS, discrepancies exist in reports detailing the extent and distribution of the DOR. Reports of low levels of DOR mRNA (Mansour et al. 1993, 1994) or DOR binding (Quirion et al. 1983) contrasts with others describing very strong DOR labeling (Arvidsson et al. 1995; Goodman et al. 1980; Kivell et al. 2004). Any DORs present are likely to be D1 opioid receptors because the NTS was devoid of deltorphin binding sites (Mennicken et al. 2003). We observed no pre- or postsynaptic effects on application of DPDPE to neurons in any of the NTS subdivisions, similar to previous observations (Rhim et al. 1993). This lack of effect may be due to DOR location because in the NTS, DOR was mainly contained within the cytoplasm, away from the plasma membrane (Arvidsson et al. 1995). Chronic application of morphine can increase DOR density at the cell surface, an effect blocked by the MOR antagonist CTOP and absent in MOR knockout mice, demonstrating that the MOR is required for DOR targeting to the cell membrane (Cahill et al. 2001; Morinville et al. 2003). Opioid receptor trafficking to the membrane can be induced after 5 min of forskolin exposure (Browning et al. 2004), whereas redistribution of DORs from the cytoplasm to the cell membrane by bradykinin exposure took ~1.5 min (Patwardhan et al. 2005). In our study, pretreatment with either DAMGO or bradykinin (receptors known to be localized in the NTS) (Caligione et al. 1996; Chen et al. 2000; Privitera et al. 2003) failed to induce DOR postsynaptic responses. This suggests that such a mechanism was not responsible for the lack of effects observed here and may support those studies that show little or no DORs in the NTS.

**Kappa agonist U69593 has pre- and postsynaptic effects in the NTS**

A previous study in the NTS had reported no postsynaptic effects with the KOR agonist U69593 at 10 μM (Rhim et al. 1993) although only seven neurons were tested with the drug. This is pertinent because in this study, a small proportion of neurons were hyperpolarized by U69593. In fact, U69593 hyperpolarized just 7.5% of neurons in the NTS, compared with the 28% that were hyperpolarized by DAMGO. The fact that this small proportion of neurons was affected however may be significant since the average hyperpolarization observed was over 9 mV, twice the amplitude of that elicited with DAMGO. Only a percentage of neurons was responsive to both DAMGO and U69593 and this, along with the high degree of selectivity of U69593 at KORs (Emmerson et al. 1994; Lahti et al. 1985) and the observation that presynaptic effects were blocked by a KOR antagonist, indicates that the effect is due to a selective action at KORs. This subpopulation of neurons express KORS postsynaptically that may exert a powerful but selective inhibition of NTS neurons. In the caudal NTS dense KOR1 immunoreactivity has been demonstrated in axons (Mansour et al. 1996), indicating a predominantly presynaptic site of action which fits with our findings of few postsynaptic receptor mediated responses. In keeping with this, we have also shown that U69593 reduced the amplitude and increased the paired pulse ratio of EPSPs in ~50% of NTS neurons suggesting a more widespread distribution of presynaptic KORS.

In summary, the electrophysiological and morphological data from these studies provides a comprehensive report of the role of the three opioid receptors in the control of NTS neuronal activity. These data highlight a selective role for opioid receptors in the control of NTS excitability.

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