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Effect of Neurotensin on Neurons in the Periaqueductal Gray: An *in vitro* Study

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The effect of neurotensin (NT) on periaqueductal gray (PAG) neurons was measured with extra- and intracellular recording methods in an *in vitro* preparation. Neurons excited by NT were heterogeneously distributed, being preferentially located in a region of PAG rich in NT-like fiber immunoreactivity. The majority of the responsive neurons were located in regions surrounding the aqueduct and the medial part of PAG. In 90% of the neurons, application of NT produced excitation that lasted for more than 2 min, while in the remaining cells, the excitatory effect lasted for less than 1 min. An inhibitory action of NT was rarely observed. Intracellular recordings showed that NT produced a depolarization leading to an increase in the spontaneous activity and multiple spiking with only a slight decrease in membrane resistance. The excitatory effect of NT was observed in neurons that were maintained in a solution containing cobalt. These results support the hypothesis that NT action on PAG neurons is due to the depolarization of the membrane and that this effect is mediated through a postsynaptic mechanism.

Since its discovery by Carraway and Leeman (1973, 1976), neurotensin (NT) has been found to be an important neuropeptide with a wide variety of neuronal effects. Injection of NT into the brain or the ventricular system produces hypothermia (Martin et al., 1980; Chandra et al., 1981; Kalivas et al., 1982a, b), changes in blood pressure (Rioux et al., 1981), and analgesia (Clineschmidt and McGuffin, 1977; Kalivas et al., 1982b; Behbehani and Pert, 1984). Iontophoretic or pressure injection of NT can increase (Young et al., 1978; Miletic and Randic, 1979; Marwaha et al., 1980; Stanzione and Zieglgansberger, 1983; Behbehani and Pert, 1984; Baldino and Wolfson, 1985) or decrease (Guyenet and Aghajanian, 1977) firing rates of neurons in several regions of the brain. Immunohistochemical and receptor binding studies have shown that NT is heterogeneously distributed throughout the CNS (Uhl and Snyder, 1976; Kobayashi et al., 1977; Lazarus et al., 1977; Sanders et al., 1980; Young and Kuhar, 1981; Jennes et al., 1982; Quirion et al., 1982). Of particular interest is the observation that many regions implicated in antinociception and processing of pain information such as the periaqueductal gray (PAG) and the substantia gelatinosa region of the spinal cord (Basbaum and Fields, 1984) contain neurotensinergic neurons and fibers (Young and Kuhar, 1981; Seybold and Elde, 1982). In addition to its direct action, NT can cause release of other neurotransmitters through a presynaptic mechanism. For example, it has been shown that injection of NT into the substantia nigra can cause release of dopamine (Myers and Lee, 1983).

Recently, we have shown that injection of NT into the PAG produces long-lasting analgesia that is associated with an increase in the firing rate of neurons in the PAG (Behbehani and Pert, 1984). Immunohistochemical observations in this laboratory (Shipley et al., 1987) have demonstrated that the distribution of the NT neurons and fibers in the PAG region are not homogenous. On the basis of these observations, it was of interest to determine whether different regions of the PAG respond differently to NT. To test this possibility and to determine if the actions of NT are mediated through a pre- and/or postsynaptic mechanism, we have examined the effect of NT on neurons in different regions of the PAG using an *in vitro* slice preparation.

Materials and Methods

Male Sprague-Dawley rats weighing 120–150 gm were used in all experiments. After decapitation, the brain was removed, and the cerebellum and medulla were excised by transecting the brain at the junction of the superior and inferior colliculi. Two millimeter cuts were made on each side of the sagittal sinus and the slabs between these cuts containing the PAG were cut coronally into 400-μm-thick sections. The slices were incubated in oxygenated physiological saline solution (PSS) with the following composition (mM): NaCl, 124; KCl, 5; $CaCl_2$, 2.5; $MgSO_4$, 1.3; $NaHPO_3$, 26; KH_2PO_4, 1.2; glucose, 10. After 30 min of incubation, one slice was transferred to the recording chamber, placed on a Nylon mesh, and then covered with a second piece of mesh. Throughout the experiment the tissue was superfused with oxygenated PSS at a rate of 3 ml/min. At this rate, the total volume of the chamber (0.6 ml) could be completely exchanged in 30 sec. NT was either applied to the bath or, in some experiments, by pressure injection from a pipette located very near the tip of the recording electrode. In some experiments, PSS containing 3 mM cobalt chloride was used instead of $CaCl_2$ to block presynaptic NT-mediated transmitter release (Clineschmidt and McGuffin, 1977). Glass micropipettes with tip diameter of 1–2 μm and resistance of 10–15 MΩ were used for extracellular recordings, while glass electrodes with a tip diameter of less than 1 μm and resistance of 130–150 MΩ were filled with 3 M potassium acetate and used for all intracellular measurements.

Results

Firing characteristics of PAG cells in vitro

All PAG neurons that were studied were spontaneously active. The spontaneous firing rates of these cells ranged between 1 to 20 Hz. The mean firing frequency was 4.8 $\pm$ 3 impulses/sec, and the action potentials had a duration of 1–4 msec. Some neurons characteristically fired in short bursts of 2–4 spikes.

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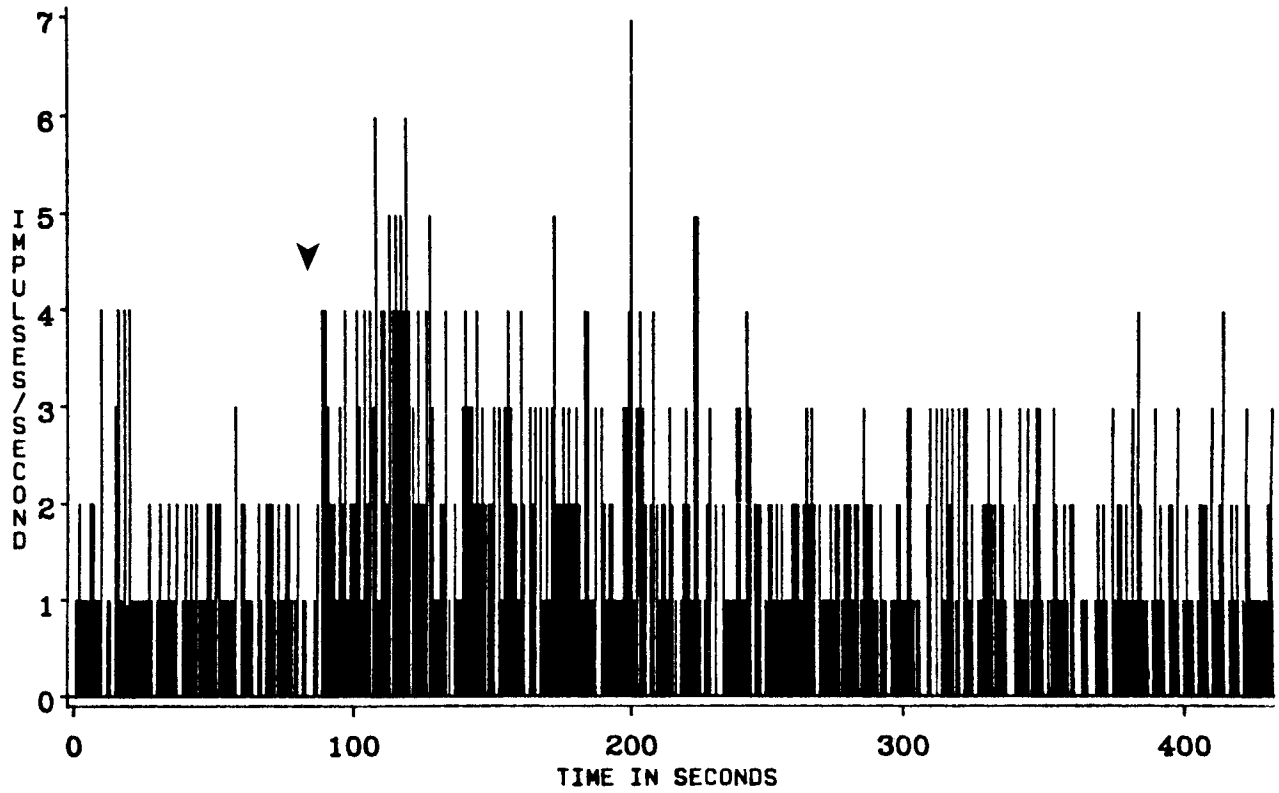

Figure 1. Response of a PAG neuron to 8 nmol of NT applied to the bath for 15 sec. Note that the excitatory response lasted for nearly 3 min.

The probability of finding a spontaneously active cell was higher when recording regions surrounding the aqueduct and in the medial parts of the PAG than from its dorsal part. However, when a spontaneously active cell was isolated, no difference in firing rate was observed between the neurons from the different regions of the PAG. Most of the neurons that are included in this study were maintained at room temperature. The bath temperature was increased to 37°C in a few studies, which produced slightly higher spontaneous activity; however, the number of cells recorded under this condition was too small to allow statistical analysis.

Response to NT in normal PSS

NT application usually caused excitatory effects. Of the 72 cells that were studied, NT produced excitation in 57 cells, inhibition in 2 cells, and no effect on the other cells. Two types of excitatory responses were noted: (1) In most neurons (52/57) NT caused an increase in firing rate that lasted up to 3 min. A typical response is shown in Figure 1. In this cell, NT was added to the bath at a concentration of 8 nmol over a period of 15 sec. As shown in Figure 1, the NT effect began within 20 sec after its application and lasted for 150 sec. (2) In 5 of the 57 neurons, the response to NT was short-lasting. Figure 2 shows the response of a cell in this category. The activity of this cell increased within 20 sec after NT application, but the excitation effect lasted for less than 1 min. The anatomical distribution of neurons that were recorded within the PAG is shown in Figure 3. It should be noted, however, that, in a majority of the slices, the probability of finding a spontaneously active cell in these regions was higher than the probability of finding similar type neurons in the dorsal PAG. For this reason, only 9 cells in the most dorsal part of PAG (the region corresponding to Hamilton's dorsal PAG region) were examined. In this population, 6 cells were excited and 3 cells did not respond to NT.

Response to NT in the presence of cobalt

In order to determine if the effect of NT was mediated through a presynaptic mechanism, slices were perfused with PSS in which the calcium had been replaced with 3 mM cobalt (PSS/Co). The responses to NT before and after cobalt application were examined in 10 cells; the effects of NT were evaluated only in the presence of cobalt in another 12 cells. Usually, NT was applied after the tissue had been perfused with PSS/Co for 5 min, although in 2 cells NT was applied after 15 min of PSS/Co. In the majority of the neurons, prolonged exposure to PSS/Co caused an increase in the firing rate and reduction in the size of the action potential. In 2 cells, the firing rate was reduced to 10% of the rate before cobalt application. However, in all of the neurons treated with cobalt, NT caused excitation with a duration that was not significantly different from the cells that had been exposed to normal PSS. Figure 4 shows the response of the same neuron shown in Figure 1 to NT after the slice had been incubated in PSS/Co for 6 min. In this cell, the excitation caused by NT was indistinguishable from that produced by the peptide when the slice was incubated in normal PSS.

Intracellular recording experiments

Intracellular recordings were made from 20 neurons located in the medial part of the PAG and in regions surrounding the cerebral aqueduct. Within this population, 6 cells had membrane potentials between −50 and −65 mV and could be held long enough to measure their response to NT. Twelve cells had

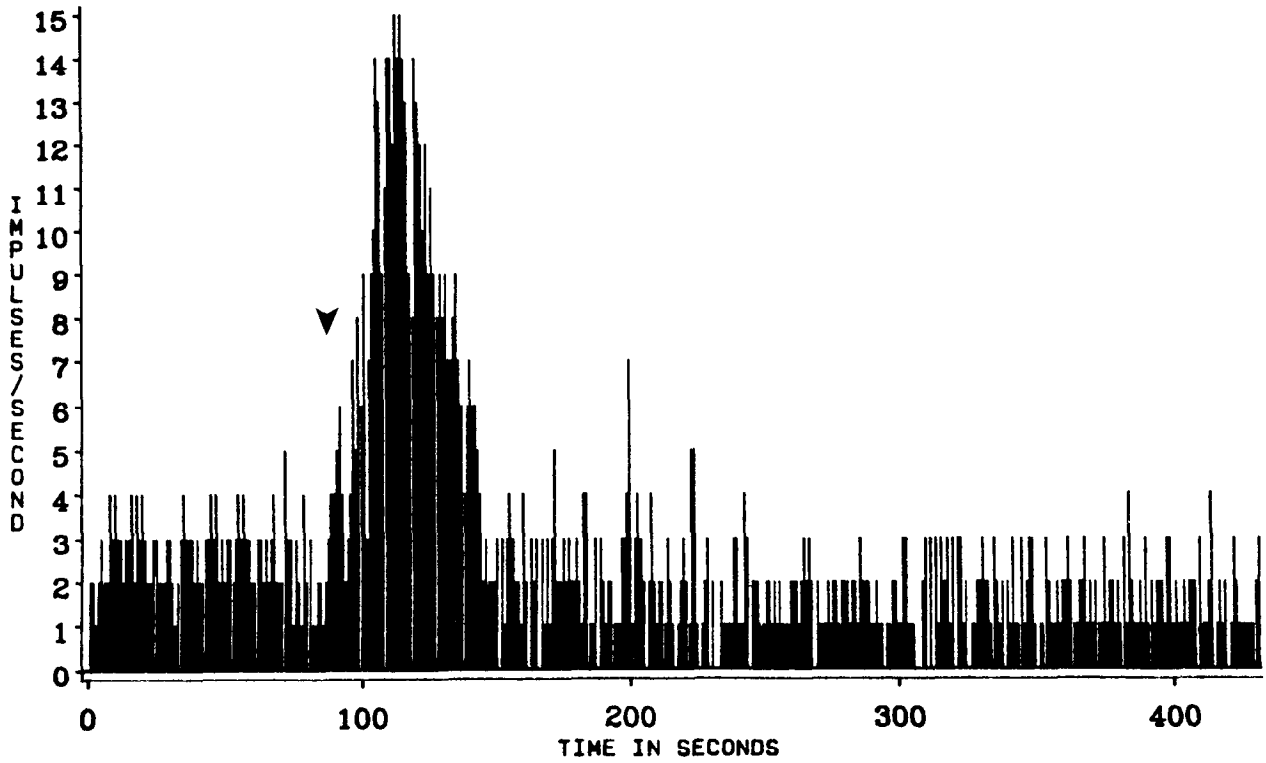

Figure 2. Excitatory response of a cell (different from that shown in Fig. 1) to 8 nmol of NT. Note that the effect of NT lasted for less than a minute.

resting potentials between −30 and −40 mV. These neurons showed no sign of injury and were stable for more than 5 min. Two additional neurons had unstable resting potentials and were excluded from further study. All neurons were spontaneously active and had a firing rate of 0.2–14 impulses/sec. The majority of the cells produced discrete single action potentials; only 3 cells consistently displayed bursts of spikes.

The 6 neurons with stable resting potentials between −50 and −65 mV were examined in greater detail. Five of these had a resting resistance that ranged between 20 and 55 MΩ, and one cell had a resistance of 200 MΩ. Excitatory postsynaptic potentials with amplitude of 5–12 mV were detected in all recordings from these neurons. The majority of these EPSPs were followed by an action potential, and only in a few cases were EPSPs detected in isolation. Figure 5 shows a typical response from a cell in this category. As shown in this figure, NT application caused a gradual depolarization of approximately 6 mV and significantly increased the firing rate of the cell without any significant change in the membrane resistance. In this group of cells, bath application of NT at concentration of 0.8 μM consistently caused a depolarization that ranged from 5 to 17 mV and produced an increase in firing rate and multiple spike activity in all these neurons. Membrane depolarization produced by NT lasted from 90 to 170 sec, the median duration of the depolarization being 145 sec.

Discussion

The data obtained from these experiments indicate that the PAG region can be successfully studied in an *in vitro* preparation. PAG neurons maintained at room temperature were spontaneously active and in many respects displayed discharge properties indistinguishable from those obtained in an *in vivo* preparation. At room temperature, the firing rate of the PAG cells averaged about 4.8 Hz; this rate is slightly lower than the average firing rate of PAG neurons in an *in vivo* preparation, which in our experiments average about 7 Hz. Three conditions may be responsible for this difference. First, the temperature of

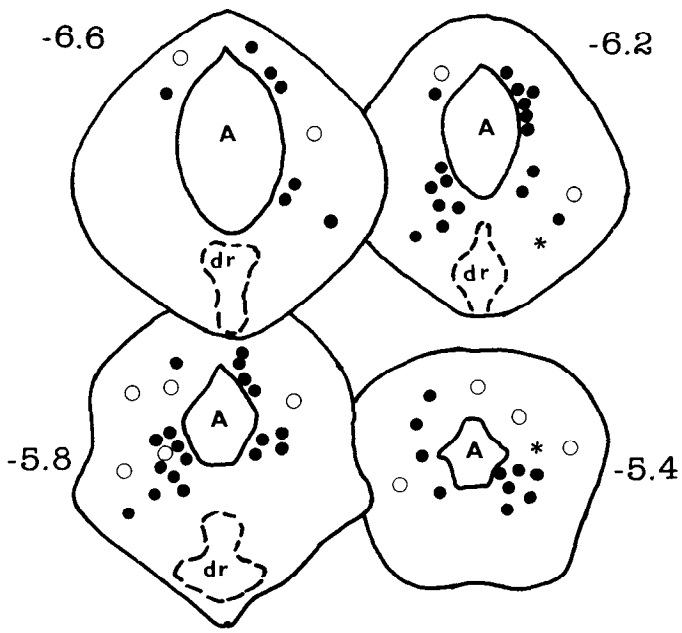

Figure 3. Distribution of neurons recorded within the PAG. *Closed circles* depict neurons that were excited and *open circles* those cells that did not respond; *asterisks* show the location of cells that were inhibited by NT. As indicated, neurons that were excited by NT were located in the regions surrounding the aqueduct and the medial part of the PAG.

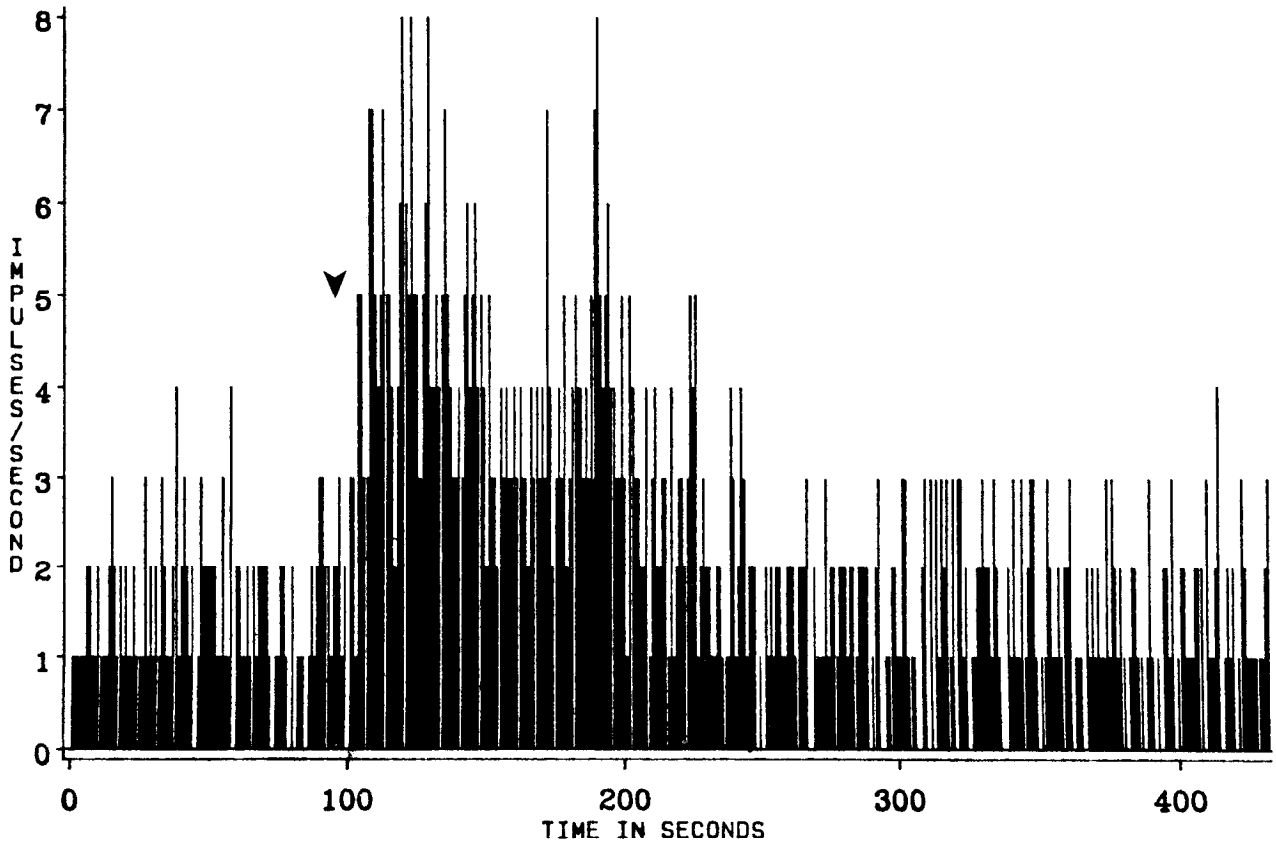

Figure 4. Response of the same neuron shown in Figure 2 to 8 nmol of NT after the slice was perfused with a PSS solution containing 3 mM of cobalt. Note that the average baseline firing rate was slightly lower than the baseline firing rate in normal PSS. However, NT-mediated excitation in this cell had the same time course and magnitude as the response observed in the presence of PSS.

the *in vitro* preparation was lower than the body temperature that had been maintained in our *in vivo* experiments. In the experiments in which the slice temperature was raised to 37°C, there was a 34% increase in the firing rate. However, since the number of records at this higher temperature was insufficient for statistical analysis, it is impossible to determine whether the temperature was the primary factor contributing to the lower spontaneous firing rate. Second, these results could be attributed

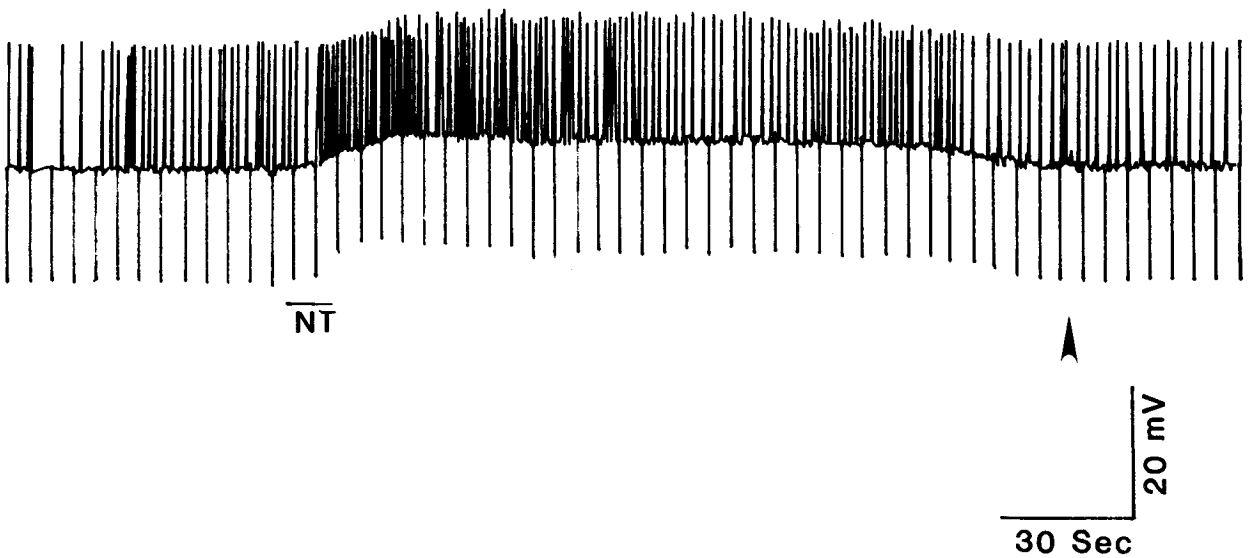

Figure 5. Effect of NT on membrane potential of a PAG cell. Final concentration of NT was 8 nmol. This cell had a resting membrane potential of −58 mV and spike height of 63 mV. During the excitatory phase, cell fired in bursts of 2–4 spikes. There was a slight decrease in membrane resistance, which was measured by passing 0.5 nA, 100 msec hyperpolarizing pulse at 5 sec intervals. Isolated EPSPs (*arrows*) indicate that the recording was from the soma rather than from the axon of this cell. Note that the actual spike height has been truncated as a result of the limited frequency response of the chart recorder.

to the effect of anesthesia. Since all *in vivo* recordings that have been reported in our laboratory have been performed on rats under urethane or chloral hydrate anesthesia, it is possible that the anesthetics cause a change in the spontaneous firing rate of the cells in this region. Third, it is possible that excitatory afferents to the PAG are severed during the preparation of the slices. Unfortunately, our current experiments cannot distinguish between these possibilities.

The qualitative characteristics of the NT responses in the *in vitro* preparation were very similar to the responses observed when NT was administered in the *in vivo* preparation. The only major difference noted was the duration of changes produced by the peptide. In the *in vivo* preparation, many neurons were excited by NT for a 3–8 min period. In the current study, we did not observe any response that lasted for more than 3 min. This difference may be due to the rapid washing of the slices in the *in vitro* situation compared with the slower clearance of NT *in vivo*. Nonetheless, it is significant that long-lasting responses were observed in the present experiments, indicating that NT produces effects that have a significantly longer duration of action than expected from classical transmitters.

The cells that were responsive to NT were not uniformly distributed throughout the PAG region. There was a tendency for the responsive neurons to be distributed close to the cerebral aqueduct and in the region corresponding to the medial region of the PAG according to Beitz (rat) and Hamilton (cat). This distribution closely correlates with the distribution of the NT-containing nerve fibers and terminals, as described in the companion article (Shipley et al., 1987). This observation further emphasizes the anatomical data (Hamilton, 1973; Beitz, 1985; Shipley et al., 1987) and suggests that the PAG region cannot be considered as a single homogenous entity.

The response to NT observed in the present study was similar to the response observed from neurons in the hypothalamus (Baldino and Wolfson, 1985) and in the bed nucleus of the stria terminalis (Sawada et al., 1980). These responses are, in turn, different from the effect of neurotensin on the guinea pig ileum (Williams et al., 1979) in that we never observed neurons that responded by inhibition followed by excitation. This suggests that the central effect of NT may be different from its effect in the intestine.

In the current study we observed 2 different types of response to NT: one short (40–60 sec) and one long (more than twice as long) excitation. This difference could not be attributed to the location of the cells within the slice. Both types of neurons were located at a depth of 170–210 μm from the surface of the slice. Furthermore, the onset latency of excitatory response was similar for cells with short- and long-lasting responses. It is possible that the short-lasting effect of NT represents an extreme end of a response continuum. Since the number of neurons that showed this type of a response was small, it is not possible to establish a bimodal distribution for response duration. This observation is similar to the effect of NT on PAG neurons recorded with *in vivo* preparations (Sanders et al., 1980) and leads us to speculate that more than one type of receptor may mediate the excitatory effect of NT. The distribution of neurons within the PAG that were responsive to NT was not uniform.

The results of the intracellular experiments showed that the excitatory effect of NT is associated with a depolarization of the membrane with only a slight decrease in membrane resistance. The ionic mechanism of this action of NT is not known. However, a slight increase in sodium permeability could have caused this response. More detailed experiments under voltage-clamp conditions will be required to elucidate the ionic basis of the excitation produced by NT. In these experiments we recorded from numerous cells that had low, but stable, resting potentials. Some of these cells could be held for more than 10 min. It is not clear if these neurons were in some way damaged or if, indeed, there are cells that have low resting potentials. Further pharmacological experiments will be needed to clarify this point.

Finally, the excitatory effect of NT was nearly identical when the slices were incubated in normal PSS or in PSS containing cobalt. This would suggest that the excitation responses observed in this report and in our previous studies (Behbehani and Pert, 1984) do not result from the action of NT on transmitter release from other presynaptic terminals, but rather that the potent, prolonged excitatory action of this intriguing peptide is mediated by a postsynaptic mechanism.

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