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TENS stimulates constitutive nitric oxide release via opiate signaling in invertebrate neural tissues

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Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

Summary

Background: There is a major societal concern relating to the addictive properties of analgesic drugs such as morphine with regard to alleviating pain. Because of this, alternative methods of pain relief are, and have been, actively pursued. An extremely promising method for treatment of low to moderate levels of chronic pain in humans is transcutaneous electrical nerve stimulation (TENS).

Material/Methods: All experiments utilized the invertebrate marine bivalve mollusc *Mytilus edulis* pedal ganglia. TENS was achieved using a stimulation apparatus developed by Professor Han of Peking University. TENS experiments employed 2 stimulation protocols: 1) low 2 Hz frequency at 5 mA current, 2) alternating low and high frequencies at 2 and 100 Hz, respectively at 5 mA current. Real-time measurements of nitric oxide (NO), using an amperometric probe, measured NO released into the tissue bath subsequent to TENS.

Results: Pooled *M. edulis* pedal ganglia exposed to TENS demonstrate that stimulation at 2 Hz and 5 mA current promotes time-dependent release of NO. In another experiment, pooled ganglia were stimulated at alternating frequencies of 2 Hz and 100 Hz and 5 mA, which also released NO in a time-dependent manner. Unstimulated control ganglia did not release significant amounts of NO. NO release was antagonized by naloxone and L-NAME exposure, demonstrating that it was receptor and nitric oxide synthase mediated, respectively.

Conclusions: It would appear that TENS stimulates endogenous morphine release since NO release was blocked by naloxone and opioid peptides do not release NO. The present study is highly suggestive of the occurrence of this same mechanism in mammalian neural systems since all biochemical and signaling components are present. Furthermore, it would appear that this process has evolutionary survival value since it occurs in an animal that evolved 500 million years ago.

key words: **TENS • opiate • naloxone • L-NAME • nitric oxide • ganglia • nervous system • invertebrate**

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BACKGROUND

There is a major societal concern relating to the addictive properties of analgesic drugs such as morphine with regard to alleviating pain. Because of this, alternative methods of pain relief are, and have been, actively pursued. An extremely promising method for treatment of low to moderate levels of chronic pain in humans is transcutaneous electrical nerve stimulation (TENS) [1]. TENS is one of the most common forms of electroanalgesia, or pain relief through electrical stimulation, and is used as a method for managing pain caused by various conditions including lower back injury, arthritis, nerve injury and postsurgical trauma [2].

In TENS treatment, a mild and relatively painless electrical current is sent through electrode patches applied to the skin. There are significant studies demonstrating that TENS employs opioid chemical messengers [1,3,4]. Aside from these studies many of the past experiments only involve observations of the effect of electrical acupuncture. For example, a study examined the effectiveness of electroacupuncture in reducing anxiety and pain in human subjects undergoing a colonoscopy procedure [5] and another tested electroacupuncture on the sphincter of the female urethra [6].

The analgesic effects of morphine and endogenous opioids are mediated by activation of opioid receptors [7]. Following opioid binding, many of the intracellular signaling/transduction events are centered on proteins that bind guanosine triphosphate (GTP) and subsequently activate the enzyme adenylate cylase. For opioids, there exist three major types of receptors designated μ (Mu), δ (Delta), and κ (Kappa), each of which recognizes, in its own unique way, morphine, artificially modified forms of morphine such as oxycodone, and a variety of endogenous opioid peptides [7]. A recently published paper has determined that the ability of electroacupuncture (EA) to relieve pain in rats was linked to activation of μ and δ receptors, but not κ, by release of endogenous opioid compounds [8].

Importantly, it has recently been demonstrated that morphine can be endogenously made by normal healthy animal cells, including human immune cells [9,10]. In effect, morphine has always been thought of as a drug, not a natural regulatory factor found in very low concentrations in animal cells. Complementing this work is the discovery and cloning of a sub-type of the μ-opioid receptor, μ3, that is located on nervous, vascular and immune cells, and only recognizes morphine, not endogenous opioid peptides [11–13]. A unique aspect of the μ3 opioid receptor is that following morphine binding, cellular activation is mediated by production of the free radical gas nitric oxide (NO) [14]. NO is a signaling molecule which also possesses neurotransmitter properties through its stimulation of the intracellular enzyme guanylate cyclase [15,16]. In the present report, we demonstrate that TENS stimulates the production of constitutive NO, which also has been associated with analgesia [17].

MATERIAL AND METHODS

Preparation of invertebrate nervous tissue

All experiments utilized the invertebrate marine bivalve mollusc, *Mytilus edulis,* collected from the local waters of Long Island Sound and maintained under laboratory conditions for three weeks, prior to experimental use [18]. Animals were kept in artificial seawater (Instant Ocean, Aquarium Systems) at a salinity of 30 PSU and at a temperature of 18°C [19]. Nervous tissues from 10 to15 *Mytilus edulis* pedal ganglia were excised and pooled in a 1.5 ml siliconized microcentrifuge tube and placed into wet ice prior to TENS treatment.

TENS treatment

Pooled *Mytilus edulis* pedal ganglia were suspended and gently pelleted in 1 ml of sterile phosphate buffered saline (PBS) in the same 1.5 ml siliconized microfuge tube. TENS was achieved using a patented stimulation apparatus developed by Professor Han of Peking University. The plastic electrode covering of the TENS unit was removed to reveal the electrode wires, one of which was positioned to contact the pooled nervous tissue preparation, the other to contact the side of the microfuge tube. Prior to TENS, a NO-selective nanoprobe was carefully lowered into position 1 mm above the pooled ganglia preparation and equilibrated for 5 minutes prior to TENS. TENS experiments employed 2 stimulation protocols: 1) low 2Hz frequency at 5mA current, 2) alternating low and high frequencies at 2 and 100 Hz, respectively at 5mA current. Real-time measurements of NO released into the tissue bath subsequent to TENS were performed as described below.

In order to demonstrate that the amperometric responses following TENS were from genuine NO molecules, the selective cNOS inhibitor N-nitro-L-arginine methyl ester (L-NAME) was used to selectively block NO production following TENS. Pooled *M. edulis* pedal ganglia were incubated beforehand for 20 min in 1 mL of PBS containing 10 μM of L-NAME. After 20 min of incubation, 30 min of TENS stimulation at alternating frequencies of 2Hz and 100 Hz and 5mA current was started at 0 minutes.

Real-time measurement of NO concentration

NO released from nervous tissues preparations was quantified using an Apollo 4000 Free Radical Analyzer with an NO-selective amperometric nanoprobe [20] and proprietary software (World Precision Instruments, Sarasota, Fl, as described in [21]). S-nitroso-N-acetyl-DL-penicillamine (SNAP), a NO donor, was used to construct a standard curve, ranging from 0 to 100 nM (Figure 1). Real-time NO measurements were calculated by extrapolation of amperometric values to nM using the linearized SNAP-generated standard function and later transferred to Sigma-Plot (Systat Software Inc, Richmond, CA) for statistical analysis.

RESULTS

An experiment utilizing 15 pooled *M. edulis* pedal ganglia demonstrates that a TENS stimulation at 2 Hz and 5 mA current promotes time-dependent release of NO, peaking at 4 nM in the phosphate buffer bath solution between 2 and 12 min and returning to baseline at 30 min (Figure 2, upper bold trace). The lower dotted trace represents NO release from equivalent pooled ganglia incubated under identical conditions without TENS stimulation. In unstimulated ganglia, probe drift occurred and relative negative

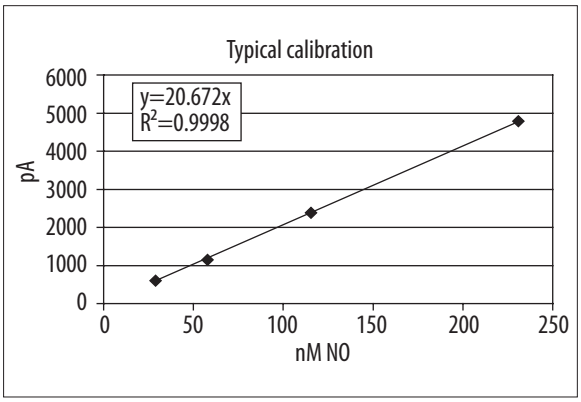

Figure 1. Representative calibration for amperometric nanoprobe.

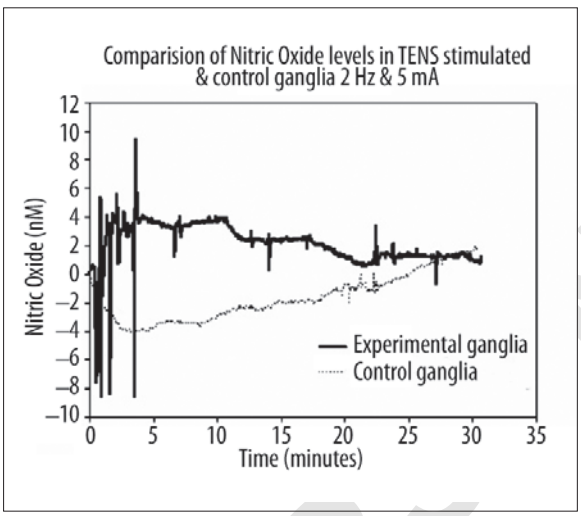

Figure 2. Measurement of NO release via nanoprobe from 15 excised *M.edulis* ganglia *in vitro* with and without TENS treatment at 2Hz and 5 mA for 30 minutes.

NO concentration values were observed that slowly returned to baseline at 30 min.

In another representative experiment, 10 pooled *M. edulis* pedal ganglia were stimulated at alternating frequencies of 2 Hz and 100 Hz and 5m, which also released NO in a time-dependent manner between 25 to 30 min (Figure 3, upper bold trace; Table 1). In contrast to the TENS experiments employing 2 Hz stimulation at 5 mA (Figure 2), alternating 2 Hz and 100 Hz stimulation promoted a different pattern of NO release: a slowly developing time-dependent increase in NO concentration in the tissue bath that remained elevated at the 30 min time point. Unstimulated control ganglia yielded negative NO concentration values that were essentially unchanged over 30 min (dotted trace).

To determine whether 30 min of TENS stimulation at alternating frequencies of 2 Hz and 100 Hz and 5 mA current promoted NO release due to non-selective tissue damage, a second phase of 2 Hz and 100 Hz stimulation was performed on previously stimulated pooled *M. edulis* pedal ganglia. As depicted in Figure 4, TENS stimulation at alternating frequencies of 2 Hz and 100 Hz and 5mA current promoted time-dependent release of NO from 10 pooled *M. edulis* pedal ganglia, reaching a maximal concentration of 8 nM between 25 to 30 min. Following the termination of TENS at 30 min, NO concentrations in the tissue bath were observed to return to baseline. After a 10 min rest period, a second 30 min phase of TENS was started. As depicted in Figure 4, the second phase of TENS stimulation at alternating frequencies of 2 Hz and 100 Hz and 5mA current promoted time-dependent release of NO from the same 10 pooled *M. edulis* pedal ganglia, reaching a maximal concentration of 4 nM at later time points. This experiment provides initial proof that TENS releases NO by selective stimulation of neurons, not by irreversible tissue damage.

As observed in Figure 5 (and Table 1), TENS stimulation of pooled *M. edulis* pedal ganglia in the presence of 10 µM L-NAME did not produce increased NO release into the tissue bath. This demonstrated that the amperometric responses accurately measured genuine NO released into the bath solution, not artifact.

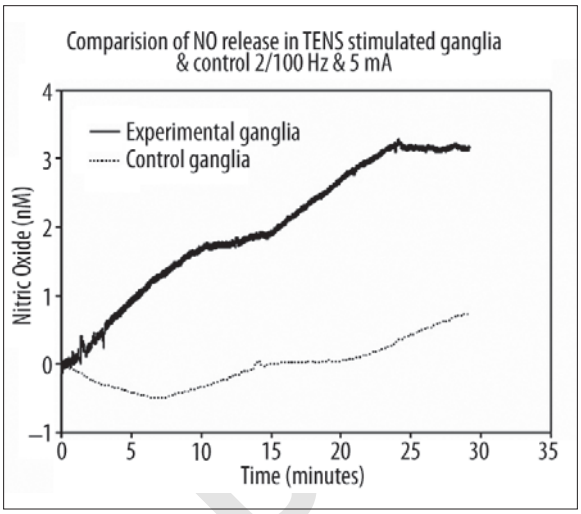

Figure 3. Real time amperometric probe representation of spontaneous NO release from 10 excised *M. edulis* pedal ganglia treated at 2/100Hz and 5 mA for 30 minutes.

Table 1. Nitric Oxide release from 10 excised *M. edulis* pedal ganglia replicated 4 times and treated at 2/100Hz and 5 mA (electrical stimulation = ES) for 30 minutes. The peak height of NO release was combined from the replicates and the SEM determined. Drugs were administered as described in the text. All values compared to the ES value were significant at the $P<0.01$ level of significance as determined via a Two way Analysis of Variance. Control was compared to ES, and ES was compared to ES + L-Name and ES + Naloxone. L-NAME and Naloxone alone produced no NO changes, which remained within the control range.

Treatment	NO levels nM (±SEM)
Control	0.1±0.1
ES	6.4±1.2*
ES + L-NAME (10^{-4} M)	0.5±0.1*
ES + Naloxone (10^{-6} M)	0.6±0.2*

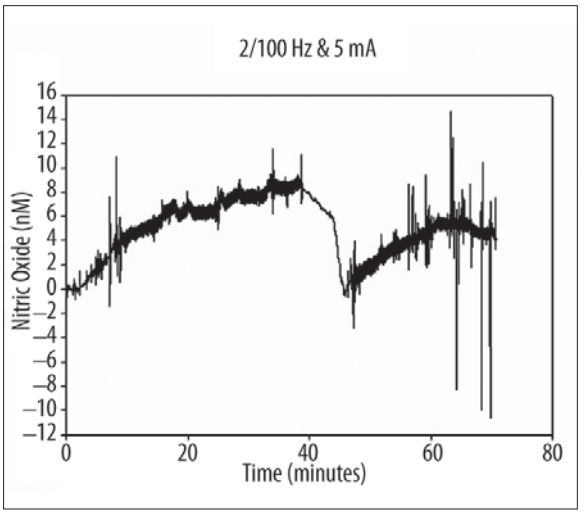

Figure 4. Real time amperometric probe representation of spontaneous NO release from 10 excised *M. edulis* pedal ganglia treated at 2/100Hz and 5 mA for 30 minutes, then stopped and restimulated.

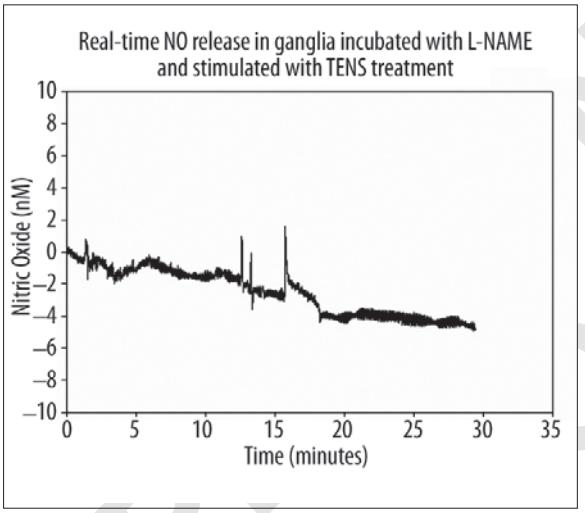

Figure 5. Real time amperometric probe representation of spontaneous NO release from 10 excised *M. edulis* pedal ganglia incubated with L-NAME for 20 minutes and stimulated at 2/100Hz and 5 mA for 30 minutes.

The hypothesis that TENS releases an endogenous opioid compound, which was responsible for the NO release, was also examined by exposing pooled neural tissue to the opioid receptor blocker naloxone (Figure 6; Table 1). 30 min of TENS stimulation at alternating frequencies of 2 Hz and 100 Hz and 5mA current in the presence of 1 μM naloxone did not produce increased NO released into the tissue bath, demonstrating that NO released into the bath solution following TENS was stimulated by the release of an endogenous opioid/opiate chemical signal.

Discussion

TENS at 2 Hz and 5 mA and alternating at 2/100Hz and 5 mA increased the ganglionic release of NO. The stimulated release of NO was observed to be higher using the

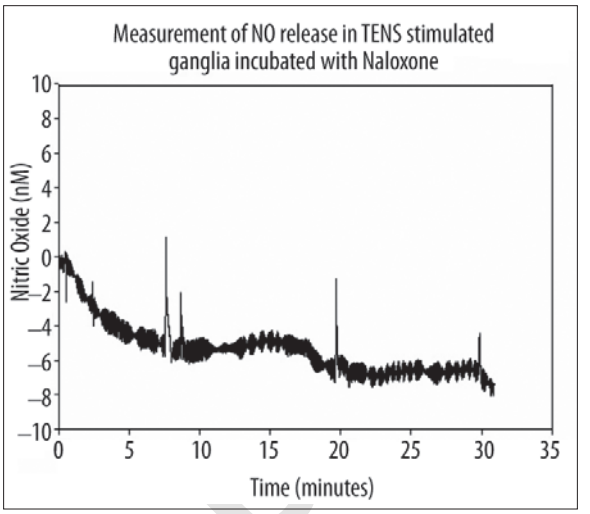

Figure 6. Real time spontaneous NO release from 10 excised *M. edulis* pedal ganglia incubated with Naloxone and stimulated with TENS treatment.

TENS protocol of alternating frequencies of 2/100Hz and 5mA. Important control experiments indicated that: 1) *M. edulis* nervous ganglia can be rechallenged for stimulation and therefore are not damaged by TENS treatment of 2/100 Hz and 5 mA, and 2) L-NAME, an inhibitor of cNOS, decreased NO levels, demonstrating that real NO was released. Finally, incubation with naloxone, a selective opioid receptor blocker, prevented NO release from pooled *M. edulis* pedal ganglia, proving that, in the other experiments without the inhibitor, TENS stimulated the release of endogenous opiate/opioid signaling molecules, which released NO. We are thus able to show that TENS-induced NO production is linked with the activation of opioid receptor sites within the ganglia. This study relates to the ability of alternating low and high frequency TENS stimulation at 2 and 100 Hz to relieve pain in humans [1] and indicates that NO release may play a genuine role in pain control procedures, see [17].

The major hypothesis regarding whether TENS stimulated the release of endogenous opioid/opiate chemical messengers, which triggered NO release, was demonstrated indirectly via naloxone blockade of TENS-stimulated NO release. In past studies using *M. edulis* tissues, we have demonstrated the presence of various opioid peptides and their precursors (see [22]). Recently, we have also demonstrated that these tissues contain and are able to synthesize morphine [9]. Additionally, *M. edulis* neural tissues only respond to morphine in stimulating NO release and various opioid peptides, e.g., methionine enkephalin, do not exhibit this action [21,23], which suggests the presence of the μ3 opiate receptor subtype [24].

Conclusions

Taken together, it would appear that TENS stimulates endogenous morphine release, since NO release is blocked by naloxone and opioid peptides do not release NO. Thus, the present study is highly suggestive of the occurrence of this same mechanism in mammalian neural systems since all biochemical and signaling components are present.

Furthermore, it would appear that this process has evolutionary survival value since it occurs in an animal that evolved 500 million years ago.

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