Published in final edited form as:

Eur J Neurosci. 2008 May ; 27(10): 2629–2642. doi:10.1111/j.1460-9568.2008.06238.x.

Endomorphin-2 is Released from Newborn Rat Primary Sensory Neurons in a Frequency- and Calcium- Dependent Manner

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Abstract

Recent evidence indicates that endomorphins, endogenous mu-opioid receptor (MOR) agonists, modulate synaptic transmission in both somatic and visceral sensory pathways. Here we show that endomorphin-2 (END-2) is expressed in newborn rat dorsal root ganglion (DRG) and nodose-petrosal ganglion complex (NPG) neurons, and rarely co-localizes with brain-derived neurotrophic factor (BDNF). In order to examine activity-dependent release of END-2 from neurons, we established a model using dispersed cultures of DRG and NPG cells activated by patterned electrical field stimulation. To detect release of END-2, we developed a novel rapid capture ELISA, in which END-2 capture antibody was added to neuronal cultures shortly before their electrical stimulation. The conventional assay was effective at reliably detecting END-2 only when the cells were stimulated in the presence of CTAP, a MOR-selective antagonist. This suggests that the strength of the novel assay is related primarily to rapid capture of released END-2 before it binds to endogenous MORs. Using the rapid capture ELISA, we found that stimulation protocols known to induce plastic changes at sensory synapses were highly effective at releasing END-2. Removal of extracellular calcium or blocking voltage-activated calcium channels significantly reduced the release. Together, our data provide the first evidence that END-2 is expressed by newborn DRG neurons of all sizes found in this age group, and can be released from these, as well as from NPG neurons, in an activity-dependent manner. These results point to END-2 as a likely mediator of activity-dependent plasticity in sensory pathways.

Keywords

Activity-dependent release; Dorsal Root Ganglion; Nodose-Petrosal; Opiates

INTRODUCTION

Endomorphin-2 (END-2), a high-affinity mu-opioid receptor (MOR) agonist, regulates sensory transmission at first-order synapses in the spinal cord and brainstem, regions rich in MORs (Arvidsson *et al.*, 1995; Ding *et al.*, 1995; Nomura *et al.*, 1996; Martin-Schild *et al.*, 1998; Aicher *et al.*, 2000, 2001; Abbadie *et al.*, 2002; for review see Zadina *et al.*, 1999). END-2 produces potent analgesic (Stone *et al.*, 1997; Zadina *et al.*, 1997; Goldberg *et al.*, 1998; Soignier *et al.*, 2000; Sakurada *et al.*, 2001) and hypotensive (Champion *et al.*, 1997; Czapla

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et al., 1998) effects. It is expressed by subsets of sensory ganglion cells and localized to dense-core vesicles within central axon terminals of dorsal root (DRG) and nodose ganglion (NG) neurons (Martin-Schild *et al.*, 1999; Wang *et al.*, 2002; Sanderson Nydahl *et al.*, 2004) in proximity to MORs (Martin-Schild *et al.*, 1998; Abaddie *et al.*, 2002; Wang *et al.*, 2003; Silverman *et al.*, 2005). Endogenous END-2, released in the dorsal horn, is thought to act locally to inhibit pain transmission (Chapman *et al.*, 1997; Budai & Fields, 1998; Sanderson Nydahl *et al.*, 2004). However, the precise loci and cellular mechanisms of END-2 action remain elusive.

Besides END-2, primary sensory neurons express a number of peptide neuromodulators, including calcitonin gene-related peptide (CGRP), substance P (SP) and brain-derived neurotrophic factor (BDNF; Pezet *et al.*, 2002; Brain & Cox, 2006). Previous studies showed colocalization of END-2 with CGRP (Pierce *et al.*, 1998; Greenwell *et al.*, 2007) and SP (Martin-Schild *et al.*, 1997, 1998; Sanderson Nydahl *et al.*, 2004; Greenwell *et al.*, 2007). Several lines of evidence point to BDNF as a neuromodulator of afferent transmission in spinal nociceptive (Mannion *et al.*, 1999; Thompson *et al.*, 1999; Lever *et al.*, 2001; Malcangio and Lessmann, 2003) and visceral (Balkowiec and Katz, 2000; Balkowiec *et al.*, 2000) pathways. Recent studies from our laboratory show that MORs dramatically affect the magnitude of BDNF release from dissociated DRG and NG neurons (Scanlin & Balkowiec, 2004), raising the possibility that endogenously expressed END-2 plays a role in regulating BDNF availability at first-order sensory synapses. However, very little is known about *in vivo* interactions of both peptides. To begin exploring cellular substrates for END-2-BDNF interactions, we examined relative distribution of the two peptides in DRG and NG neurons *in vivo*.

Previous studies indicate that END-2 can be released in the dorsal horn by chronic depolarization with elevated extracellular potassium (Lisi & Sluka, 2006), or by electrical stimulation of the dorsal root entry zone (Williams *et al.*, 1999; Dun *et al.*, 2000). However, evidence that END-2 is directly released by primary sensory neurons is lacking, and cellular mechanisms of activity-dependent release of END-2 from neurons are unknown.

To address these issues, we developed an *in vitro* model utilizing patterned electrical field stimulation of dispersed somatic and visceral primary sensory neurons from newborn rats, combined with quantitative analysis of END-2 release by a novel methodology, rapid capture ELISA. Portions of this work have previously been published in abstract form (Scanlin *et al.*, 2006).

MATERIALS AND METHODS

Animals

Postnatal day (P) 1 and P7 Sprague Dawley rats (Charles River Laboratories, Wilmington, MA, USA) were used for this study. All procedures were approved by the Institutional Animal Care and Use Committee of the Oregon Health and Science University, and conformed to the *Policies on the Use of Animals and Humans in Neuroscience Research* approved by the Society for Neuroscience.

Immunocytochemistry

Processing of P1 and P7 DRG and NPG tissue and cultures was performed as previously described (Buldyrev *et al.*, 2006), except that 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, was used as the fixative for the MOR staining of NPG cultures, and the section thickness was 10 μ m.

MOR staining was performed using rabbit anti-MOR (1:1000; Novus Biologicals, Littleton, CO, USA) antibodies overnight at 4°C, followed by sequential room temperature incubations

in: i) goat anti-rabbit biotinylated IgG (1:200; Vector Laboratories, Burlingame, CA, USA) for 1 h, ii) avidin-biotin complex (ABC, 1:100; Vectastain kit, Vector Laboratories) in 0.5 M NaCl in phosphate-buffered saline for 30 min, and iii) diaminobenzidine (DAB) solution (in PBS: 0.3 mg/ml DAB, 0.032% NiCl₂, 0.0075% H₂O₂) for 3–5 min. Control cultures, in which primary antibody was omitted, were completely devoid of staining.

Double END-2/BDNF staining was performed as recently described for double CGRP/BDNF staining (Buldyrev *et al.*, 2006), except that i) rabbit anti-END-2 (1:50; Neuromics, Edina, MN, USA) was used instead of rabbit anti-CGRP, ii) chicken anti-BDNF (Promega Corp., Madison, WI, USA) was used at 1:25 dilution, and iii) both secondary antibodies (donkey anti-chicken IgG-Cy3, Jackson ImmunoResearch, West Grove, PA, USA, and goat anti-rabbit IgG-Alexa 488, Invitrogen, Carlsbad, CA, USA) were used at 1:1000 dilution.

The preabsorption control of anti-END-2 specificity was performed as follows: i) the anti-END-2 antibody (1:50; Neuromics) was mixed with 70 μ M END-2 peptide (Neuromics) in dilution buffer without Triton-X-100 (0.02 M Na₂HPO₄; 0.005 M NaH₂PO₄; 0.5 M NaCl), ii) the antibody/peptide mixture and an equivalent portion of antibody alone were rotated at room temperature for 2 h, followed by overnight rotation at 4°C, and iii) centrifuged at 13,000 rpm for 30 min. Both supernatants containing control and preabsorbed antibody were combined with Triton-X-100 (final concentration 0.3%), and applied to sections for 2 h (Martin-Schild *et al.*, 1997, 1999).

Analysis of immunohistochemistry data

Three ganglia from each age group were analyzed. Four sections per ganglion, equally spaced at a distance of 60 μ m, were imaged with Olympus IX-71 inverted microscope (Olympus America Inc., Center Valley, PA, USA) using either 20 $\times$ (P0 ganglia) or 10 $\times$ (P7 ganglia) objectives, and images were captured with a Hamamatsu ORCA-ER CCD camera (Hamamatsu, Bridgewater, NJ, USA) controlled by either Wasabi (Hamamatsu) or Olympus Microsuite software (v. 5.0, Olympus America Inc). Double END-2/BDNF immunoreactivity was determined by overlaying single-labeled images using CorelDraw (Corel Corp., Ottawa, Canada) for cell size measurements, and Olympus Microsuite (vs. 5.0, Olympus America, Inc.) for cell counts. Cell diameter was determined using ImageJ software (National Institutes of Health, Bethesda, MD, USA; Abramoff *et al.*, 2004). Specifically, the cell perimeter was marked with several points and enclosed surface area was computed and recorded. Average diameter was derived using the formula: diameter = $2\sqrt{(\text{area}/\pi)}$.

The same sections of each ganglion were analyzed independently by two investigators (for cell counts and for cell size measurements). This enabled us to verify cell classification as END-2- or BDNF-immunoreactive by comparing the data on relative proportions of each cell category between both investigators. Only cells with clearly visible nuclear profiles were included in the analysis. A cell was considered END-2- or BDNF-immunoreactive when the fluorescence intensity was clearly above the background. The background level was established independently by each investigator and used for all analyzed images. **Differences between non-immunoreactive (background) and immunoreactive cells were verified by density analysis (Integrated Density / Area; ImageJ), e.g. 177.4 (SE=2.06; n=300) for BDNF-immunoreactive vs. 94.0 (SE=1.54; n=300) for BDNF-non-immunoreactive.**

Cell preparation and culture

DRG and NPG cultures were prepared as recently described by our laboratory (Buldyrev *et al.*, 2006), except that dissociated cells were plated in 96-well polystyrene flat bottom culture-treated plates (Falcon®, BD Biosciences, San Jose, CA, USA) pre-coated with poly-D-lysine (0.1 mg/ml; Sigma, St. Louis, MO, USA), at a density of three DRG or two NPG per well.

DRG and NPG cultures were grown for 3 days in plating medium, consisting of Neurobasal-A medium (Invitrogen) supplemented with B-27 serum-free supplement (Invitrogen), 0.5 mM L-glutamine (Invitrogen), 2.5% fetal bovine serum (HyClone, Logan, UT, USA), 1% penicillin-streptomycin-neomycin antibiotic mixture (Invitrogen), and DRG cultures were supplemented with nerve growth factor (50 ng/ml; Invitrogen).

In experiments designed to rule out a contribution of non-neuronal cells to mechanisms of END-2 release, dissociated DRG and NPG cells (in 0.5 ml of plating medium) were layered on top of a three-layer gradient of Percoll (Sigma; 20%–35%–60%; 1 ml each) and centrifuged for 10 minutes at $800 \times g$ at 4 °C, as previously described by our laboratory (Buldyrev *et al.*, 2006). The two neuron-enriched interfaces ('20%–35%' and '35%–60%') of the three Percoll layers were collected and rinsed twice with culture medium before plating. A vast majority of non-neuronal cells and cell debris form a visible thin layer at the 'plating medium-20%' interface and, therefore, can be easily separated from large-sized neurons (accumulated at the '20%–35%' interface) and medium-to-small-sized neurons (accumulated at the '35%–60%' interface). The selective distribution of different cell types within the Percoll gradient was confirmed by cell type analysis in 3-day cultures derived from individual layers (250 μ l each) of the Percoll gradient (data not shown).

Drug treatment

All drugs were obtained from Sigma. CTAP, ω -agatoxin IVA, and ω -conotoxin GVIA were dissolved in sterile distilled water and used at final concentrations of 1 μ M, 0.4 μ M and 1 μ M, respectively. Nimodipine and thapsigargin were dissolved in ethanol and used at final concentrations of 2 μ M (final concentration of ethanol 0.02%) and 10 μ M (final concentration of ethanol 0.1%), respectively. For conventional ELISA, culture medium was replaced with fresh plating medium, with or without CTAP, 30 min before stimulation. For rapid capture ELISA, culture medium was first replaced with HEPES-buffered physiological solution (HBPS; in mM: 137 NaCl, 5.4 KCl, 2 CaCl₂, 1 MgCl₂, 10 glucose, 10 HEPES), followed by HBPS containing either a drug or its vehicle (control), together with anti-endomorphin-2 polyclonal antibody (Endomorphin-2 EIA kit, Phoenix Pharmaceuticals, Belmont, CA, USA), applied 30 min before stimulation. For removal of extracellular calcium, calcium-free HBPS (in mM: 137 NaCl, 5.4 KCl, 1 MgCl₂, 10 glucose, 10 HEPES, 2 EDTA) was used instead of regular HBPS.

Stimulation of DRG and NPG cultures

Following 3 d *in vitro* and pharmacological treatment, cultures were stimulated with rectangular pulses of 0.5 ms duration and amplitude between 80 and 120 mA, applied across each well of a 96-well plate, as previously described (Buldyrev *et al.*, 2006). In experiments comparing the effects of patterned electrical stimulation with continuous depolarization, KCl (final concentration of 50 mM) was added to four additional culture-containing wells at the beginning of the electrical stimulation period. Control wells were treated with equi-molar concentrations of NaCl.

END-2 immunoassays

To quantify END-2 secretion, END-2 protein released from cells into the medium was measured with both a conventional and a modified (referred to as "rapid capture") competitive sandwich ELISA, using END-2 enzymatic immunoassay (EIA) kit (Phoenix Pharmaceuticals). For the rapid capture ELISA, the concentration of anti-END-2 polyclonal antibody was increased twofold and added directly to cultures before the stimulation. END-2 standards (in duplicate) were prepared in each culture plate at the beginning of stimulation, in either fresh plating medium (conventional ELISA) or HBPS containing anti-END-2 polyclonal antibody (rapid capture ELISA), with and without pharmacological agents, and incubated for the

duration of stimulation. At the end of the stimulation period, experimental samples and standards (50 μ l each) were transferred to the ELISA plate, and anti-END-2 polyclonal antibody with biotinylated END-2 (25 μ l each; conventional assay) or biotinylated END-2 with EIA buffer (Phoenix Pharmaceuticals; 25 μ l each; rapid capture ELISA) were applied, followed by subsequent steps according to the manufacturer's protocol. Absorbance values were read at 450 nm in a plate reader ($V_{\max}$; Molecular Devices, Sunnyvale, CA).

To quantify END-2 cellular content, post-stimulation cell layers from rapid capture ELISA experiments, or intact ganglia dissected from newborn rats, were immediately transferred to prechilled lysis buffer (20 mM Tris buffer, pH 7.4, 137 mM NaCl, 1% Nonidet-P40, 10% glycerol, 1 mM PMSF, 0.5 mM sodium vanadate, 10 μ M aprotinin, and 100 μ M leupeptin) diluted 1:1 in EIA buffer, mechanically ground, and sonicated on ice using a microprobe sonicator (Sonicator 3000, Misonix, Inc., Farmingdale, NY, USA). END-2 standards were also prepared in the lysis : EIA (1:1) buffer, and transferred simultaneously with samples to the ELISA plate, followed by steps of the conventional END-2 ELISA.

END-2 levels were calculated from the standard curve prepared for each plate, using SOFTmax PRO v. 4.3 software (Molecular Devices). END-2 standards included concentrations ranging 0–100000 pg/ml in initial experiments and 0–1000 pg/ml in following experiments. The standard curves were fitted to 4-parameter logistics and the linear portion of the curve ranged from 10–30 pg/ml to 300–500 pg/ml. The quantities of END-2 in experimental samples were within the linear range of the standard curve. For control wells in which polyclonal anti-END-2 was omitted, absorbance values were not significantly different from the absorbance of blank wells. Samples were compared using ANOVA followed by Duncan's multiple comparison procedure, and $p < 0.05$ was considered significant. Data are expressed as mean $\pm$ standard error.

RESULTS

Endomorphin-2 (END-2) immunoreactivity is present in DRG neurons as early as postnatal day 1

END-2 expression is well-documented in adult lumbar DRG neurons (Martin-Schild *et al.*, 1997; Sanderson Nydahl *et al.*, 2004) and sensory afferent fibers in the spinal cord (Martin-Schild *et al.*, 1997, 1998). However, it is currently unclear whether END-2 is expressed in early postnatal DRG neurons, since there is no evidence for its expression in afferent fibers prior to P7 in rat (Barr & Zadina, 1999a, 1999b).

In order to approach the issue of END-2 expression in early postnatal DRG neurons, we examined END-2 immunoreactivity in sections of lumbar (L4/5) DRGs derived from P1 and P7 rats. To verify the specificity of the END-2 immunolabeling, we first performed several control experiments, including preabsorption of the primary antiserum with END-2 peptide (Fig. 1a). We found that, even though overnight incubation of the primary antiserum at its final working concentration *without* the peptide decreased to some extent the intensity of END-2 staining (Fig. 1a, Control), the preabsorption of the antiserum with its cognate peptide abolished the staining (Fig. 1a, Preabsorbed). The higher background staining observed with the preabsorbed, compared to control, antiserum is likely due to the presence of some of the antibody-peptide complexes (Fig. 1a, Preabsorbed). To control for non-specific binding of the secondary antibody, the primary antiserum was omitted resulting in an abolition of the staining (Fig. 1b).

We counted END-2-immunoreactive (IR) cells in sections from 3 P0 and 3 P7 L4/5 rat DRGs. Twelve sections, equally spaced at a distance of 60 μ m, from three ganglia (four sections per ganglion) were analyzed per age group. Only cells containing a nuclear profile were included in the analysis. END-2-non-IR cells were identified based on the presence of a nuclear profile

and a clearly delineated plasma membrane. The results of this analysis indicate that 12.0% of P0 and 16.3% of P7 DRG neurons express END-2 immunoreactivity (Table 1; Fig. 1c, d).

Next, we examined the cell size distribution of END-2-IR, compared to END-2-non-IR, cells, using the same ganglia that were used for cell counts. Three-hundred END-2-IR and 300 END-2-non-IR neurons (100 per ganglion per group) were randomly chosen in 8–11 sections of each ganglion. The cell size was determined as described in the Methods section. Our data indicate that the END-2-IR population in neonatal DRG is not limited to neurons of small size and, instead, spans almost the entire range of cell sizes found in this age group (Fig. 1e, f; Beland & Fitzgerald, 2001). Interestingly, these data resemble the previously described distribution of opioid receptor immunoreactivity in rat DRG during the first postnatal week (Beland & Fitzgerald, 2001), and suggest that the opioid system plays a modulatory role in both nociceptive and non-nociceptive pathways in early postnatal animals. A similar pattern of END-2 immunoreactivity in P0 DRG was observed using polyclonal rabbit-anti END-2 (1:100) obtained from a different source (Novus Biologicals).

To quantify the total END-2 content in DRGs *in vivo*, we employed END-2 ELISA of freshly harvested L4 DRG lysates. We found that END-2 immunoreactivity was present as early as postnatal day 1 (5.03 ± 0.51 pg/ganglion; 17.68 ± 1.79 pg/mg of tissue, $n=30$ ganglia). At the end of the first postnatal week, END-2 content per ganglion remained unchanged (4.98 ± 0.32 pg/ganglion at P7, $n=30$ ganglia, $p>0.05$). However, when expressed per gram of tissue, there was a significant decrease in END-2 content between P1 and P7 (11.87 ± 0.75 pg/mg of tissue at P7, $n=30$ ganglia, $p<0.05$). Considering the fact that the END-2 ELISA is based on different antibodies than those used for END-2 immunohistochemistry, this result provides independent confirmation that significant levels of END-2 are present in the newborn DRG. Together, these data provide the evidence for END-2 immunoreactivity in rat DRG neurons as early as P1.

END-2-immunoreactive and BDNF-immunoreactive neurons comprise two mostly distinct subpopulations of the neonatal DRG

DRG neurons express several neuropeptides, such as brain-derived neurotrophic factor (BDNF; Lever *et al.*, 2001), calcitonin gene-related peptide (CGRP; Brooks *et al.*, 2004) and substance P (SP; Holz *et al.*, 1988; Lever *et al.*, 2003), that are thought to modulate transmitter release acting at the presynaptic site (Sanderson Nydahl *et al.*, 2004). Previous studies have demonstrated that in adult rat DRG, 95% of END-2-immunoreactive (END-2-IR) cells also show SP immunoreactivity (Sanderson Nydahl *et al.*, 2004). Our recent studies indicate that modulation of MORs affects the magnitude of activity-dependent release of BDNF from cultured DRG neurons (Scanlin & Balkowiec, 2004). Since there are no published data regarding relative localization of END-2 and BDNF in sensory neurons, we have begun investigating the co-localization of both peptides in DRG *in vivo*. Sections of 3 P0 and 3 P7 lumbar (L4/5) DRGs double-immunostained for END-2 and BDNF were used for this analysis. The analysis was performed independently by two investigators of the study, and the results are summarized in Table 1 and Figure 2. Our data indicate that only a small subset of newborn DRG neurons expresses BDNF (15.8% based on the cell counts summarized in Table 1; 14.0% derived from the cell size analysis presented in Fig. 2b). The percentage of END-2-IR cells that also show BDNF immunoreactivity increases by P7 (24.5% based on the cell counts summarized in Table 1; 19.7% derived from the cell size analysis presented in Fig. 2c, Chi-square = 3.44, 1 degree of freedom, $p<0.05$). Similar to the cell size distribution of END-2-IR cells (Fig. 1), neurons that show double END-2/BDNF immunoreactivity are distributed across the entire size range (Fig. 2 b, c). Together, these data indicate that during the first week of postnatal development, END-2-IR and BDNF-IR DRG neurons represent two largely non-overlapping populations.

END-2 is expressed in newborn sensory neurons from the nodose-petrosal ganglion complex

Not only has END-2 been well established as a modulator of analgesic effects in spinal pathways, but also of blood pressure- and heart rate- lowering effects through autonomic pathways (Champion *et al.*, 1997; Czapla *et al.*, 1998; Kasamatsu *et al.*, 2004; Viard & Sapru, 2006). Therefore, we next examined END-2 immunoreactivity in the P1 and P7 nodose-petrosal ganglion complex (NPG), which contains cell bodies of visceral sensory neurons critical for the control of the cardiovascular system. Three P1 and five P7 NPGs were processed for END-2/BDNF double-immunohistochemistry according to the same protocols that were used for staining of DRGs. The distribution of END-2 and BDNF immunoreactivity was examined in all sections of the ganglion complex. Unlike BDNF-immunoreactive cells, which were present throughout majority of the sections, END-2-IR cells were for the most part located within the junction between two ganglia forming the complex at both ages examined (Fig. 3, showing P7). Moreover, only a few END-2-IR cells also expressed BDNF immunoreactivity (Fig. 3), and this was true for both P1 and P7. Our results show that END-2 immunoreactivity is not restricted to sensory neurons of somatic origin but also present in NPG visceral sensory neurons, where it is likely to play a specific role by serving only a distinct subpopulation of these neurons.

Endogenous MORs sequester released END-2, impeding detection by conventional ELISA

We next sought to determine whether END-2 can be released from DRG and NPG neurons by activity, using patterned electrical field stimulation of dissociated newborn cultures as a model. We examined the effects of 1-h stimulation with various frequencies (5–100 Hz) and patterns (continuous and bursting). However, our attempts to reliably and reproducibly detect END-2 release by conventional ELISA have failed due to low levels of END-2 in the samples. This was particularly the case for DRG cultures, whose samples (control and stimulated alike) were consistently below the sensitivity limits of the ELISA (data not shown). Although samples derived from NPG cultures in general showed higher levels of END-2 compared to DRG-derived samples, less powerful stimuli, such as chronic depolarization or low-frequency electrical stimulation, here too yielded END-2 levels that were either below or barely above the sensitivity limit of the assay (Fig. 4b, 6 Hz Continuous, white bar).

We first addressed the possibility that rapid degradation of END-2 by endogenous proteases contributes to the difficulties detecting END-2 in the cell supernatant. Indeed, known amounts of exogenous END-2, incubated for 1 h in DRG culture-conditioned medium, showed significantly lower levels of detected END-2 compared with samples of the same amount of exogenous END-2 incubated in fresh medium (data not shown). Addition of 1 μ M aprotinin, which inhibits numerous serine proteases, or 1 μ M actinin, inhibitor of multiple enkephalin-degrading proteases including aminopeptidase M, did not improve END-2 detectability (data not shown). However, previous studies have documented that, in addition to serine- and aminopeptidases, metallo-proteases and membrane-bound dipeptidyl peptidase IV contribute to END-2 degradation (Shane *et al.*, 1999; Tömböly *et al.*, 2002; Janecka *et al.*, 2006).

Since virtually all neurons in our cultures express MOR, the receptor for END-2 (Fig. 4a), we hypothesized that our inability to detect released END-2 by conventional ELISA, which utilizes cell supernatants, is related to the presence of MORs. In addition to the possibility of a rapid binding of released END-2 to these receptors, MOR activation could lead to the inhibition of END-2 release (Moises *et al.*, 1994; Rusin & Moises, 1995; Wilding *et al.*, 1995; Wu *et al.*, 2004). To test this hypothesis, we introduced a highly selective MOR antagonist, CTAP (1 μ M), to P1 NPG culture 30 min before the stimulation. Consistent with our hypothesis, electrical stimulation (6 Hz continuously, and 72-Hz bursts of 4 pulses delivered at 6 Hz; Liu *et al.*, 1998, 2000; Chen *et al.*, 1999) in the presence of CTAP resulted in significantly higher

levels of detected END-2 release as compared to the levels detected in the absence of CTAP (Fig. 4b). Moreover, CTAP treatment resulted in a significant increase in the basal, unstimulated release (302.22 ± 5.16 pg/ml with CTAP *versus* 207.19 ± 7.82 pg/ml without CTAP, $n=4$, $p<0.01$). Pretreatment with $1 \mu\text{M}$ CTAP of DRG cultures stimulated with 4-pulse bursts at 20 Hz intra-burst frequency, delivered once every 800 ms, also resulted in a significant increase in the levels of detected END-2 release (108.29 ± 1.11 pg/ml with CTAP *versus* 21.70 ± 11.92 pg/ml without CTAP, $n=4$, $p<0.05$). Together, these data demonstrate that native MORs significantly contribute to the limited ability of conventional ELISA to detect release of END-2 from primary sensory neurons.

Novel rapid capture ELISA methodology offers dramatic improvement in detection of released END-2

There is increasing evidence that MOR signaling pathways are linked to other cell functions, including transmitter release. For example, in rat DRG neurons, MORs inhibit P/Q- and N-type calcium channels (Moises *et al.*, 1994; Rusin & Moises, 1995; Wilding *et al.*, 1995; Wu *et al.*, 2004). These channels are mainly localized to presynaptic terminals of these neurons, modulate neurotransmitter release, and contribute to anti-nociception (Holz *et al.*, 1988; Diaz & Dickenson, 1997; Matthews & Dickenson, 2001; Heinke *et al.*, 2004; Murakami *et al.*, 2004). Therefore, despite the promising results obtained using MOR blockade during stimulation of cultures, we sought to find a means of increasing END-2 detectability without altering the MOR signaling.

These investigations led us to develop a novel END-2 ELISA methodology which involves addition of the primary antibody to the culture medium during stimulation, enabling rapid capture of the released END-2 for subsequent detection. We applied this methodology to reliably and reproducibly quantify endogenous END-2 release from cultured DRG and NPG sensory neurons, while minimizing loss of released END-2 due to potential binding by endogenously expressed MORs, MOR-mediated feedback inhibition of END-2 release, and/or degradation by endogenous proteases.

High-frequency bursting patterns of electrical stimulation are significantly more effective at releasing END-2 from nodose-petrosal ganglion complex (NPG) sensory neurons compared to depolarizing concentrations of KCl

Using the highly sensitive rapid capture ELISA methodology, we first sought to determine whether END-2 is released from NPG visceral sensory neurons in an activity-dependent manner. A vast majority of previous studies of activity-dependent release of END-2 and other neuropeptides used elevated extracellular potassium to stimulate the cells. Although treatment with 40 mM KCl results in a marked increase in phospho-cAMP-response element binding protein (pCREB) staining (Balkowiec & Katz, 2000), a marker of neuronal depolarization (Ghosh *et al.*, 1994; Moore *et al.*, 1996), elevated KCl has been shown to be relatively ineffective at releasing some neuropeptides (Balkowiec & Katz, 2000). Therefore, we compared the effects of 1-h exposure to 50 mM extracellular KCl with 1-h electrical field stimulation with two protocols, both known to induce plastic changes at first-order synapses in the brainstem *nucleus tractus solitarius* (Liu *et al.* 1998, 2000; Chen *et al.*, 1999): (1) a continuous stimulation at 24 Hz, and (2) 4-pulse bursting stimulation with 72-Hz bursts (24 Hz average frequency), on END-2 release from 3-d cultures of newborn NPG neurons. The average stimulation frequency and, consequently, the total number of delivered pulses were the same for both electrical stimulation protocols.

Both electrical stimulation and exposure to depolarizing concentrations of KCl resulted in significant increases in release of END-2 (equimolar NaCl control: 172.91 ± 26.16 pg/ml, KCl: 292.29 ± 25.96 pg/ml, $n=3$, $p<0.05$; unstimulated control: 35.96 ± 6.48 pg/ml, 24-Hz

continuous: 154.57 ± 28.61 pg/ml, $n=12$, $p<0.001$). However, the magnitude of END-2 release in response to the bursting stimulation protocol was significantly larger compared to both, the effect of KCl and 24-Hz continuous stimulation (72-Hz bursts: 267.76 ± 28.70 pg/ml, KCl: 119.38 ± 25.96 pg/ml, 24-Hz continuous: 118.61 ± 28.61 pg/ml, $p<0.05$; Fig. 5). Together, these data indicate that END-2 is released from NPG neurons in an activity-dependent manner and, therefore, can be considered as a mediator of synaptic plasticity in visceral sensory pathways.

High-frequency patterns of electrical stimulation that are known to induce spinal LTP are significantly more effective at releasing END-2 from DRG neurons than low-frequency stimulation

The magnitude of the release of some endogenous neuropeptides, such as BDNF, from primary sensory neurons is regulated by the frequency and pattern of stimulation (Balkowiec & Katz, 2000; Lever *et al.*, 2001; Buldyrev *et al.*, 2006). To determine what mechanisms regulate activity-dependent secretion of END-2 from DRG neurons, we examined the relationship between the pattern of stimulation and the magnitude of endogenous END-2 release from 3-d cultures of newborn DRG. The magnitude of release of other peptides depends on the total number of pulses delivered (Lundberg *et al.*, 1986; Iverfeldt *et al.*, 1989; Franck *et al.*, 1993; Nemeth *et al.*, 1999) and the temporal pattern in which the pulses are applied (Cazalis *et al.*, 1985; Lundberg *et al.*, 1986; Whim & Lloyd, 1989, 1994; Balkowiec & Katz, 2000, 2002; Lever *et al.*, 2001; Buldyrev *et al.*, 2006). We designed three stimulation protocols to compare the relative efficacy of low- *versus* high-frequency stimulation, and continuous *versus* bursting stimulation: i) continuous stimulation at 5 Hz; ii) continuous stimulation at 20 Hz; and iii) bursts of 100 pulses at 100 Hz, delivered once every 5 sec (20 Hz average frequency), a frequency that corresponds to high, LTP-evoking activity in spinal pathways (Randic *et al.*, 1993; Liu & Sandkuhler, 1997). In protocols (ii) and (iii), the total number of pulses delivered was equal, but the intra-burst frequency and inter-burst interval were varied. The magnitude of END-2 release was compared among these stimulation protocols applied to P1 DRG cultures over a 1-h period. END-2 levels were measured by the rapid capture ELISA methodology. 20-Hz continuous stimulation and 100-pulse at 100 Hz bursting stimulation (20 Hz average), both resulted in high levels of released END-2 compared to unstimulated controls (20 Hz, 91.78 ± 13.05 pg/ml, $n=16$, $p<0.001$; 100 Hz, 116.06 ± 20.07 pg/ml, $n=12$, $p<0.001$; Fig. 6a), whereas 5 Hz continuous stimulation did not evoke significant release of the peptide (2.28 ± 3.00 pg/ml, $n=12$; Fig. 6a). Notably, tetanic stimulation at 20 Hz, a protocol that has been shown to induce a lower magnitude of LTP compared with 100-Hz stimulation (Liu & Sandkuhler, 1997), resulted in comparable levels of END-2 release. These data indicate a correlation between frequency of stimulation and/or total number of delivered pulses, but not stimulation pattern, and the magnitude of END-2 release from DRG neurons. Moreover, high levels of activity known to be effective at inducing spinal LTP are also highly effective at evoking END-2 release.

Consistent with the observation that high-frequency stimulation releases large quantities of END-2 from P1 DRG neurons, a reduction in cellular content of END-2 was readily detected following 100-Hz bursting stimulation (62.49 ± 2.79 pg/culture in non-stimulated controls, $n=8$, *versus* 15.72 ± 2.57 pg/culture following the stimulation, $n=10$; $p<0.001$; Fig. 6b).

To rule out the possibility that activity-dependent END-2 release occurs through a mechanism involving non-neuronal cells present in our cultures, we compared the levels of END-2 release from our standard, mixed DRG cultures with the release from neuron-enriched cultures. In these cultures, a vast majority of non-neuronal cells were removed by selective plating of the neuron-rich fractions of a Percoll density gradient (Buldyrev *et al.*, 2006). Electrical stimulation at 100 Hz (100-pulse bursts; 20 Hz average) resulted in END-2 release from both

mixed and neuron-enriched cultures, with no significant differences between these two (mixed, 138.83 ± 25.94 pg/ml, $n=8$; neuron-enriched, 122.98 ± 34.39 pg/ml, $n=8$, $p=0.7694$). Therefore, the removal of non-neuronal cells did not affect END-2 release by patterned electrical stimulation. To support this, there were no differences in total levels of END-2 between mixed (61.81 ± 1.41 pg/culture, $n=3$) and neuron-enriched (66.37 ± 0.72 pg/culture, $n=3$) cultures, strongly suggesting that non-neuronal cells do not contribute to END-2 release in our model.

Calcium influx through voltage-activated calcium channels contributes to mechanisms of endogenous END-2 release evoked by patterned electrical stimulation

To begin characterizing cellular mechanisms underlying activity-dependent release of endogenous END-2 from DRG neurons, we next examined the role of extracellular calcium and voltage-activated calcium channels. In the presence of calcium, 100-Hz bursting stimulation resulted in a highly significant release of END-2 (control: 65.15 ± 13.63 pg/ml, stimulated: 140.89 ± 15.88 pg/ml, $n=8$, $p<0.01$). In sister cultures stimulated in the absence of extracellular calcium, END-2 release was present (control: 9.62 ± 3.17 pg/ml, stimulated: 45.87 ± 8.73 pg/ml, $n=8$, $p<0.01$), but significantly reduced ($p<0.05$; Fig. 7a). We also pretreated the cultures with a cocktail of L-, N- and P/Q-type voltage-activated calcium channel blockers (2 μ M nimodipine, 1 μ M ω -conotoxin, and 0.4 μ M ω -agatoxin) for 30-min. Similar to the effects of the removal of extracellular calcium, END-2 release evoked by 100-Hz bursting stimulation still took place in the presence of the blockers (control with blockers: 1.88 ± 1.37 pg/ml, stimulated with blockers: 23.74 ± 4.01 pg/ml, $n=4$, $p<0.01$), but was significantly reduced ($p<0.05$; Fig. 7a). Together, these data indicate that calcium entry through voltage-activated calcium channels contributes to END-2 release from DRG neurons evoked by patterned electrical stimulation.

Calcium mobilization from intracellular stores triggers END-2 release from DRG neurons

Previous studies have demonstrated that in addition to calcium influx, calcium released from intracellular stores may be involved in mechanisms of neuropeptide secretion (Canossa *et al.*, 2001, Balkowiec & Katz, 2002, Buldyrev *et al.*, 2006). Therefore, we next examined the effect of calcium release from intracellular stores by thapsigargin, an inhibitor of endoplasmic reticulum Ca^{2+} -ATP-ase, applied in the presence and absence of extracellular calcium, on END-2 release from DRG neurons. One-hour treatment with 10 μ M thapsigargin resulted in a significant release of END-2 (vehicle: 62.63 ± 21.82 pg/ml, thapsigargin: 207.19 ± 24.81 pg/ml, $n=4$, $P<0.01$; Fig. 7b) that was unaffected by removal of extracellular calcium (vehicle: 55.45 ± 29.83 pg/ml, thapsigargin: 206.31 ± 12.37 pg/ml, $n=4$, $P<0.01$; Fig. 7b). These data indicate that mechanisms of END-2 release from DRG neurons may involve calcium mobilization from intracellular stores.

DISCUSSION

The present study demonstrates that END-2 is expressed by newborn DRG and NPG neurons of all sizes found in this age group (Beland & Fitzgerald, 2001), and rarely co-localizes with BDNF. The amount of endogenous END-2 released from DRG neurons depends on the total number of delivered pulses and is regulated by stimulus frequency. Namely, high-frequency continuous or bursting stimulation is disproportionately more effective at evoking the release than either stimulation at low frequency or exposure to elevated (50 mM) extracellular potassium. Moreover, calcium influx through voltage-activated calcium channels contributes to activity-dependent END-2 release, which can also be triggered by calcium mobilization from intracellular stores. In addition, we provide evidence for activity-dependent release of END-2 from visceral sensory neurons in the nodose-petrosal ganglion complex (NPG). Together, our data show that endogenous END-2 is released from primary sensory neurons in response to

physiologically-relevant patterns of electrical stimulation, including those known to induce plastic changes at sensory synapses.

Since the identification of END-2 by Zadina and colleagues in 1997, only a few studies have approached the regulation of END-2 availability in sensory pathways. Microprobes coated with anti-END-2 antibody were used in order to demonstrate that electrical stimulation of the dorsal root fibers evokes release of endogenous END-2 from the spinal cord *in vitro* (Williams *et al.*, 1999; Dun *et al.*, 2000). A more recent study utilized high-performance liquid chromatography to detect a modest increase in END-2 levels in the cerebro-spinal fluid from the spinal cord perfused *in vivo* with depolarizing concentrations of KCl (100 mM; Lisi & Sluka, 2006). However, none of the previous studies have demonstrated that END-2 can be directly released from primary sensory neurons nor characterized the cellular mechanisms of the release. To our knowledge, our study is the first to utilize a reliable and highly sensitive assay to quantitatively assess levels of END-2 release and, in turn, to examine the cellular mechanisms mediating activity-dependent release of END-2.

The major obstacle in studies examining END-2 release has been a limited ability to detect the peptide, which is released in the picogram range. Our finding that native MORs are interfering with END-2 detection may, at least in part, explain previous difficulties with detecting the peptide. Indeed, detection of released END-2 by our rapid capture ELISA methodology is greatly enhanced compared to conventional ELISA. With rapid capture ELISA, we were able to quantify release of endogenous END-2 from dissociated neurons using sample volumes as small as 50 μ l. Moreover, our present data demonstrate that KCl-induced depolarization, used in some previous studies, is significantly less effective at evoking release of END-2 from dissociated primary sensory neurons compared to high-frequency bursting patterns of electrical field stimulation. These data suggest that an additional, different signaling pathway is activated selectively by bursting patterns of electrical stimulation to evoke END-2 release. In this context, our invention of END-2 rapid capture ELISA methodology combined with electrical field stimulation is of particular importance, as it represents a powerful model to effectively evoke and quantify END-2 release from neurons and, in turn, probe cellular mechanisms underlying regulation of the release process.

Although opioid modulation of spinal sensory transmission and plasticity, such as long-term potentiation (LTP), has been investigated, the relationship between the patterns of activity and the magnitude of release of endogenous opioids at sensory synapses remains unclear. In general, mediators of synaptic plasticity are thought to act by altering calcium thresholds and/or adaptation of secretory responses. While key molecules that link membrane potential with control of release have not been identified, likely mediators are presynaptic receptors and calcium channels. Thus, a peptidergic candidate of activity-dependent and synapse-specific modulation should act locally, and its release and action should be controlled only by selective patterns of neuronal activity. Studies of the subcellular distribution of END-2 have shown that the peptide is localized to dense-core vesicles in sensory axon terminals (Martin-Schild *et al.*, 1999; Wang *et al.*, 2002, 2003; Sanderson Nydahl *et al.*, 2004; Silverman *et al.*, 2005). Our evidence demonstrates that END-2 is released from primary afferents in an activity-dependent manner. Specifically, patterns of activity known to induce spinal LTP are highly effective at releasing END-2. In turn, released END-2 most likely activates presynaptic MORs coupled to voltage-gated calcium channels (Moises *et al.*, 1994; Wilding *et al.*, 1995; Rusin & Moises, 1995, 1998; Hamra *et al.*, 1999; Wu *et al.*, 2004). Together, our current findings support a role for END-2 as a peptide mediator of synaptic transmission and plasticity in sensory pathways.

Our results demonstrate that high-frequency bursts are not significantly more effective in releasing END-2 from DRG neurons than the same number of stimuli delivered as a continuous

train. A hallmark study by Lundberg *et al.* (1986) demonstrated an approximately four-fold increase in Neuropeptide Y release upon stimulation of sympathetic nerves with 20-Hz bursts compared to 2-Hz continuous stimulation of the same total number of pulses. Other neuropeptides, such as BDNF, also show more apparent, compared to END-2, pattern-sensitivity of the release, with high-frequency bursting stimulation being dramatically more effective compared to low-frequency continuous or bursting stimulation protocols (Balkowiec & Katz, 2000, 2002; Lever *et al.*, 2001; Buldyrev *et al.*, 2006). Our data also show that discharge of calcium from intracellular stores by thapsigargin, an antagonist of endoplasmic reticulum Ca^{2+} -ATP-ase, leads to END-2 release. Thus, mobilization of calcium from intracellular calcium stores may also be involved in activity-dependent release of END-2. Even though there are limitations in extrapolation of data from dissociate cultures to the intact system, we believe that our results may help shed light on the role of END-2 in activity-dependent modulation of sensory transmission. Moreover, based on the present findings, we propose that highly active synapses, in contrast to weakly active synapses, are more likely to meet threshold requirements for END-2 release and, in turn, activation of MOR autoreceptors which possibly modulate classical neurotransmitter and/or neuropeptide release leading to regulation of LTP.

The one hour duration of patterned electrical stimulation used in our study is significantly longer than standard LTP-inducing protocols. However, the levels of endogenous END-2 released during shorter stimulation periods were either variable or below minimal detection limits of the ELISA (data not shown). One likely explanation of this phenomenon would be low levels of END-2 available for release. The total level of END-2 in our model was about 60 pg per culture, and only a small fraction (approximately 20%) was released during 1 hour of the most effective, high-frequency bursting stimulation (about 12 pg per culture; derived from the data presented in Fig. 6a). A need for this relatively long duration of stimulation is consistent with previous studies using 1-hr stimulation protocols in order to detect another neuropeptide that is known for its scarcity, BDNF (Balkowiec & Katz, 2000, 2002; Buldyrev *et al.*, 2006). Current studies in our laboratory show that the same 1-hr stimulation protocol leads to more than 10-fold higher levels of CGRP, compared to BDNF, release from dissociated cultures of P1 trigeminal ganglia (Martin & Balkowiec, unpublished observations). Consistent with previous studies of CGRP release (Malcangio & Bowery, 1996; Malcangio *et al.*, 1997), a significantly shorter duration of stimulation (10 min) was sufficient to detect CGRP, but not BDNF, release in our *in vitro* model of P1 trigeminal neurons (Martin & Balkowiec, unpublished observations). Thus, it is likely that END-2, similar to BDNF, is released at disproportionately lower levels compared to some other neuropeptides, such as CGRP. Our observations also suggest that, in our model, release of END-2 from DRG and NPG neurons occurs over a prolonged period of stimulation. The relatively long duration of the stimulation is consistent with neuropeptide release occurring during repetitive electrical stimulation, where diffuse elevation of intracellular calcium favors release of large dense-core vesicles (LDCVs; Verhage *et al.*, 1991), and slow emptying of peptide content from LDCVs during exocytosis (Brigadski *et al.*, 2005; Buldyrev *et al.*, 2006; for review see: Lessmann *et al.*, 2003). Interestingly, a study by Lever and colleagues (2001) suggests that there is a considerable delay in BDNF release, compared to substance P and glutamate, in the dorsal horn in response to electrical stimulation of DRG afferents. Again, this draws attention to the possibility that END-2 release could also occur at a slower rate compared to some other neuropeptides.

Our study shows that END-2 is expressed by a subpopulation of nodose-petrosal ganglion complex (NPG) visceral sensory neurons and can be released from these neurons by activity. Although the cardiovascular effects of opiates described in earlier studies (Champion *et al.*, 1997; Czapla *et al.*, 1998; Kasamatsu *et al.*, 2004; Viard & Sapru, 2006) could potentially be accounted for by the inhibition of the sympathetic tone at the level of the rostral ventrolateral medulla (Aicher *et al.*, 2001; Llewellyn-Smith *et al.*, 2001; Stornetta *et al.*, 2001; Guyenet *et al.*, 2002), there is also substantial evidence that MOR agonists modulate calcium currents in

NPG primary sensory neurons (Rusin & Moises, 1998), including the baroreceptor population (Hamra *et al.*, 1999). Moreover, END-2 has been localized to primary afferent terminals in the medial *nucleus tractus solitarius* (NTS) of the lower brainstem (Silverman *et al.*, 2005), the primary central target of baroreceptor afferents. Our current data together with the earlier evidence raise the possibility that the first-order sensory synapse in the NTS is another likely site of activity-dependent modulation of cardiovascular reflexes by opioids (Wang & Li, 1988; Gordon, 1990; Kasamatsu *et al.*, 2004; Viard & Sapru, 2006).

Our studies were conducted with 3-d-old cultures of newborn DRG and NPG neurons, allowing us characterize the mechanisms of presynaptic release of END-2. Notably, removal of target neurons does not significantly alter maturation of functional synapses or neuropeptide release *in vitro* (Suarez-Roca & Maixner, 1995; Qin *et al.*, 2005). Moreover, the release of END-2 from cultured neurons was found to be electrical stimulation pattern-sensitive and cell type-specific, consistent with the observation that mechanisms governing the release of a particular peptide are retained by neurons in culture in the absence of target tissues (Whim & Lloyd, 1994). Thus, the pattern sensitivity of END-2 release observed in the present study is likely to arise from factors intrinsic to the neuron. In addition, previous studies using similar stimulus protocols as those used in our study have shown no significant effect on cell survival (Balkowiec & Katz, 2000), suggesting that the release is unlikely to be attributable to damage of cells by electrical stimulation.

Although all the available data suggest that END-2 is released from axon terminals of sensory neurons, we cannot rule out the possibility that END-2 is in part released from non-synaptic sites, such as the cell soma, as has been shown for calcitonin gene-related peptide and substance P (Huang & Neher, 1996; Zhang & Zhou, 2002; Ouyang *et al.*, 2005). END-2 could also be released along axons, as recently demonstrated for BDNF (Ng *et al.*, 2007). Somatic secretion would most likely act in an autocrine or paracrine manner to modulate the activity of DRG neurons and, thereby, the transmission of sensory inputs to the spinal cord.

In conclusion, the present study shows that primary sensory neurons release END-2 in response to patterns of electrical stimulation known to induce plastic changes at sensory synapses, suggesting that END-2 is a mediator of early developmental and/or adult activity-dependent plasticity in sensory pathways. Given that transient, repetitive electrical stimulation used in our study resembles patterns of nerve activity *in vivo*, this model provides new opportunities for defining physiological mechanisms of END-2 release and, consequently, END-2 roles in synaptic development and function.

Acknowledgements

The authors thank Hui-ya Hsieh for help with Percoll purification of DRG cultures, and Jessica L. Martin for advice on digital image analysis. This work was supported by American Heart Association (0230095N) and National Heart, Lung, and Blood Institute (HL076113) grants to A.B; E.A.C. was supported in part by M. J. Murdock Charitable Trust.

ABBREVIATIONS

BDNF	brain-derived neurotrophic factor
CGRP	calcitonin gene-related peptide
CTAP	H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH ₂
DRG	

	dorsal root ganglion
EIA	enzyme immunoassay
END-2	endomorphin-2
HBPS	HEPES-buffered physiological solution
IR	immunoreactive
L	lumbar
LDCV	large dense-core vesicle
LTP	long-term potentiation
MOR	mu-opioid receptor
NPG	nodose-petrosal ganglion complex
NTS	<i>nucleus tractus solitarius</i>
P	postnatal day
pCREB	phospho-cAMP-response element binding protein
SP	substance P

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Figure 1.

Endomorphin-2 (END-2) is expressed by a subset of postnatal day (P) 1 and P7 rat dorsal root ganglion (DRG) neurons. (a) Micrographs of adjacent 10- μ m sections of P7 lumbar DRG treated with END-2 antiserum following its overnight incubation at the working concentration in the absence (Control) or presence (Preabsorbed) of 70 μ M END-2 peptide. The 'Preabsorbed' image is characterized by a complete lack of END-2-immunoreactive cell bodies; scale bar 100 μ m; (b) A micrograph of a single 10- μ m section of P7 lumbar (L4/5) DRG from a control experiment in which the primary END-2 antiserum was omitted. This section is adjacent to the section presented in (d) and was processed in the same experiment; scale bar 200 μ m; (c, d) Micrographs of a single 10- μ m section of P1 (c) and P7 (d) L4/5 DRG

immunostained for END-2. END-2-immunoreactive cell bodies can be seen throughout each section. White boxes delineate the area of the inset, which is presented at higher magnification to the right of each image; scale bars 200 μm for the main images, and 50 μm for the insets; **(e, f)** Cell size distribution of END-2-immunoreactive (END-2-IR; white bars) compared with END-2-non-immunoreactive (END-2-non-IR; black bars) neurons from P1 **(e)** and P7 **(f)** L4/5 DRG. Three-hundred END-2-IR and 300 END-2-non-IR neurons were randomly chosen in 3 ganglia (100 neurons per group per ganglion) for each age.

Figure 2.

END-2-immunoreactive (IR) and BDNF-IR neurons from neonatal rat lumbar (L4/5) DRG constitute mostly non-overlapping populations. (a) Micrographs of a single 10- μ m section of P7 DRG double-immunostained for END-2 and BDNF; scale bar 200 μ m; (b, c) Cell size distribution of neurons double-labeled for END-2 and BDNF (END-2-/BDNF-IR; black bars) as a portion of the END-2-IR population (END-2-IR; white bars), in P1 (b) and P7 (c) L4/5 DRG. The same population of three-hundred END-2-IR cells presented in Figure 1 was used for this analysis.

Figure 3.

END-2 is expressed by a subset of rat nodose-petral ganglion complex (NPG) neurons, largely non-overlapping with BDNF-immunoreactive cells. (a) Micrographs of a single 10- μ m section of P7 NPG double-immunostained for END-2 and BDNF; NG, nodose ganglion; PG, petrosal ganglion; CN IX, cranial nerve IX (glossopharyngeal); CN X, cranial nerve X (vagus); scale bar 500 μ m; (b) A higher magnification view of the region outlined in (a) by the white box; scale bar 200 μ m. Especially large number of END-2-immunoreactive cell bodies can be seen within the junction between NG and PG, the region deficient in BDNF-positive cells.

a **b** **Figure 4.**

Inhibition of mu-opioid receptors (MOR) enhances the magnitude of detected END-2 release. **(a)** MOR immunoreactivity in dissociated cultures of P1 nodose-petrosal ganglion complex (NPG) neurons; **(b)** Mean above control levels of END-2 release from dissociated cultures of P1 NPG in response to 1-h continuous stimulation at 6 Hz (6 Hz Continuous) or bursting stimulation with 4 pulses at 72 Hz delivered once every 166.7 ms (72 Hz Bursts), in the absence (white bars; 6 Hz Continuous, n=8 cultures; 72 Hz Bursts, n=4 cultures) or presence (grey bars; 6 Hz Continuous, n=14 cultures; 72 Hz Bursts, n=10 cultures) of 1 μ M CTAP, a selective MOR antagonist. END-2 was detected using a conventional competitive sandwich ELISA; * p<0.05.

Figure 5.

Endogenous END-2 is released from P1 nodose-petrosal ganglion complex (NPG) neurons *in vitro* in an activity-dependent fashion. Mean levels of END-2 released from NPG cultures during 1-h treatment with 50 mM KCl (50 mM KCl, n=3 cultures), and during 1-h electrical field stimulation with biphasic rectangular pulses of 0.5 ms duration, delivered continuously at 24 Hz (24 Hz Continuous, n=12 cultures) or as 72-Hz bursts of 4 pulses once every 166.7 ms (72 Hz Bursts, n=4 cultures). The cultures were stimulated under the rapid capture ELISA conditions, i.e. in the presence of END-2 capture antibody. Bars represent END-2 release above unstimulated controls; *p<0.05.

Figure 6.

Endogenous END-2 is released from P1 DRG neurons *in vitro* in a frequency-dependent manner. **(a)** Mean above control levels of END-2 released from DRG cultures during 1-h electrical field stimulation with biphasic rectangular pulses of 0.5 ms delivered either continuously at 5 Hz (5 Hz Continuous, n=12 cultures) and 20 Hz (20 Hz Continuous, n=16 cultures), or as 100-Hz bursts of 100 pulses once every 5 sec (100 Hz Bursts, n=12 cultures). The cultures were stimulated under the rapid capture ELISA conditions; **(b)** Mean levels of END-2 cellular content in the cell layer of P1 DRG cultures following 100-Hz bursting stimulation with 100 pulses per burst delivered once every 5 sec (100 Hz Bursts, n=10 cultures)

compared to unstimulated control levels (Control, n=8 cultures), as detected by conventional ELISA; *** $p < 0.001$.

Figure 7.

Calcium influx through voltage-activated calcium channels and calcium mobilization from intracellular stores contribute to activity-dependent END-2 release from P1 DRG neurons. (a) Mean levels of END-2 released in P1 DRG cultures during 1 h of electrical field stimulation with biphasic rectangular pulses of 0.5 ms, delivered as 100-Hz bursts of 100 pulses once every 5 sec, in the presence (100 Hz Bursts, n=8 cultures) or absence (100 Hz Bursts / Ca²⁺-free, n=8 cultures) of extracellular calcium, or in the presence of a cocktail of voltage-activated calcium channel antagonists consisting of 2 μ M Nimodipine, 1 μ M ω -Conotoxin GVIA and 0.4 μ M ω -Agatoxin IVA (100 Hz Bursts / Nim/Ctx/Agtx, n=4 cultures); (b) END-2 release from P1 DRG neurons can be evoked by calcium mobilization from intracellular stores,

independent of extracellular calcium. Mean levels of END-2 released during 1-h application of 10 μ M thapsigargin, an inhibitor of endoplasmic reticulum Ca^{2+} -ATP-ase, in the presence (Thaps 2mM Ca^{2+}) or absence (Thaps Ca^{2+} -free) of extracellular calcium (n=4 cultures). Bars represent END-2 release above unstimulated controls; * $p<0.05$.

Quantification of END-2-immunoreactive (IR) cells and co-localization of END-2 with brain-derived neurotrophic factor (BDNF) in postnatal day 1 and 7 lumbar (L4/5) DRG

Twelve sections, equally spaced at a distance of 60 μ m, from three ganglia (four sections per ganglion) were analyzed for each age group.

Postnatal day	Total number of cells analyzed	Number of END-2-IR cells	Percentage of END-2-IR cells out of total number of cells analyzed	Number of END-2 / BDNF-IR cells	Percentage of BDNF-IR cells out of END-2-IR cells
1	1315	158	12.0%	25	15.8%
7	1826	298	16.3%	73	24.5%