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Activin Acts with Nerve Growth Factor to Regulate Calcitonin Gene-Related Peptide mRNA in Sensory Neurons

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Abstract

Calcitonin Gene-Related Peptide (CGRP) increases in sensory neurons after inflammation and plays an important role in abnormal pain responses, but how this neuropeptide is regulated is not well understood. Both activin A and Nerve Growth Factor (NGF) increase in skin after inflammation and induce CGRP in neurons *in vivo* and *in vitro*. This study was designed to understand how neurons integrate these two signals to regulate the neuropeptide important for inflammatory pain. In adult dorsal root ganglion neurons, NGF but not activin alone produced a dose-dependent increase in CGRP mRNA. When added together with NGF, activin synergistically increased CGRP mRNA, indicating that sensory neurons combine these signals. Studies were then designed to learn if that combination occurred at a common receptor or shared intracellular signals. Studies with Activin IB receptor or trkA inhibitors suggested that each ligand required its cognate receptor to stimulate the neuropeptide. Further, activin did not augment NGF-initiated intracellular MAPK signals but instead stimulated Smad phosphorylation, suggesting these ligands initiated parallel signals in the cytoplasm. Activin synergy required several NGF intracellular signals to be present. Because activin did not further stimulate, but did require NGF intracellular signals, it appears that activin and NGF converge not in receptor or cytoplasmic signals, but in transcriptional mechanisms to regulate CGRP in sensory neurons after inflammation.

Keywords

inflammation; neuropeptides; ERK; Smad

Hyperalgesia is a key sign of inflammation and is initiated by signals from inflamed tissues that act on sensory neurons to modify molecules involved in pain behaviors. The central mechanism of hyperalgesia involves the hyperexcitability of dorsal horn neurons, which can be attenuated by blocking glutamate receptors (Davis and Inturrisi, 2001, Yashpal et al., 2001), neurokinin receptors (Malmberg and Yaksh, 1992, Thompson et al., 1994) and CGRP receptors (Sun et al., 2004) in the spinal cord. Indeed, alpha-CGRP null mice do not develop hyperalgesia responses to inflammatory insults (Salmon et al., 2001, Zhang et al., 2001). The neuropeptide calcitonin gene related peptide (CGRP) is increased in sensory neurons after peripheral inflammation (Donaldson et al., 1992, Smith et al., 1992, Donnerer et al., 1993, Nahin and Byers, 1994, Seybold et al., 1995, Neumann et al., 1996, Malcangio et al., 1997, Mulder et al., 1997b, Durham and Russo, 1999, Calza et al., 2000, Bulling et al., 2001) and

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CGRP contributes to hyperalgesia (Salmon et al., 2001, Zhang et al., 2001, Sun et al., 2003, Sun et al., 2004, Bird et al., 2006). Thus, CGRP regulation is essential for pain responses to inflammation.

CGRP expression can be stimulated by growth factors such as NGF (Lindsay and Harmar, 1989, Amann et al., 1996) or the cytokine activin A (activin) (Ai et al., 1999, Hall et al., 2001) but whether or how these factors cooperate in regulating the neuropeptide is not known. Following inflammation, both NGF and activin increase in skin (Woolf et al., 1994, Amann et al., 2001, Xu et al., 2005). Exogenous NGF or activin causes prolonged tactile allodynia *in vivo* which may be due to increased pain peptides, including CGRP (Lewin et al., 1993, Xu et al., 2005). Exogenous NGF increases CGRP expression in sensory neurons (Lindsay and Harmar, 1989, Amann et al., 1996, Winston et al., 2001, Cruise et al., 2004). Similarly, activin also increases CGRP-containing neurons in DRG cultures and in the DRG *in vivo* (Cruise et al., 2004, Xu et al., 2005).

Activin and NGF can induce transcriptional changes in sensory neurons, but are thought to utilize discrete intracellular signals (Durham and Russo, 2003, Cruise et al., 2004). For example, activin binds the activin receptor complex, and stimulates Smad2/3 phosphorylation and translocation. The Smad heteromeric complex, in conjunction with other nuclear binding proteins, then regulates the transcription of target genes (Attisano et al., 1996, Heldin et al., 1997, Massague and Gomis, 2006). By contrast, NGF has been reported to regulate CGRP promoter activity through ERK-MAP kinase pathways (Freeland et al., 2000, Durham and Russo, 2003). However, other reports demonstrate that these ligands converge on common intracellular signals (Kretschmar et al., 1997, Lutz et al., 2004, Bao et al., 2005, Imamichi et al., 2005, Zhang et al., 2005). Therefore, the aim of this study was to obtain a molecular understanding about how activin and NGF act together to increase CGRP in sensory neurons after inflammation.

EXPERIMENTAL PROCEDURES

Primary neuron culture

Primary cultures of adult DRG lumbar neurons were prepared from 8–10 week old Sprague Dawley rats (Charles River, Wilmington, MA) (Cruise et al., 2004). For CGRP mRNA induction assays, cells were plated at 2.1×10^3 cell/cm² and allowed to attach overnight in defined neurobasal medium (Gibco-BRL, Gaithersburg, MD; with B27 medium supplement, penicillin–streptomycin 1:200, 3 mM glutamine). Defined medium was chosen as adult DRG neurons are neurotrophin independent and do not require NGF for survival (Lindsay, 1988), and the absence of serum restricts glial proliferation. Other studies demonstrate robust neuronal survival and differentiation under these conditions (Cruise, 2004). Reagents were added the following day (day1) and included human recombinant activin A (Activin, R&D Systems, Minneapolis, MN) and nerve growth factor (NGF, Austral Biologicals, San Ramon, CA). For pharmacological experiments, cultures were pretreated with SB431542 (Sigma, St Louis, MO), K-252a, U0126, SB203580 or SP600125 (Calbiochem, La Jolla, CA) for one hour, followed by activin, NGF or combination treatment. All drugs were dissolved in dimethyl sulfoxide (DMSO), such that the final concentration of DMSO ranged from 0.02% (K252a) to 0.08% (U0126) and control wells or those with ligand alone each contained 0.08% DMSO vehicle. DMSO at these concentrations has no effect on CGRP expression (Data not shown). The culture medium was changed every other day, except for cultures used for pharmacological experiments that were treated daily, and collected on day 5. For Western blot assay, cells were plated at 5.2×10^3 cell/cm² in neurobasal medium for four hours, before specific ligands were added for 0.5–60 minutes. Four hours plating was chosen to reduce any cell proliferation, and to identify cell signals that were not modified by extended *in vitro* cultures.

RNA isolation, cDNA synthesis and Quantitative real time PCR

RNA isolation of DRG cultures was performed with RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA quantity was determined using 260nm absorbance. Extracted RNA was treated with DNase to remove genomic DNA and confirmed by testing in real-time PCR reactions with all sets of the primers. First-strand cDNA synthesis was performed according to Xu et al (2005). Two-step SYBR green PCR reaction was performed using an iCycler (Bio-Rad laboratories, Hercules, CA) according to Xu et al (2005). The following PCR primers were used. Rat alpha-type CGRP: forward, 5'-aaccttagaaagcagcccaggcatg-3'; reverse, 5'-gtgggcacaaagttgtccttcacca-3' with an expected 246bp fragment. (a generous gift from Dr. Andy Russo, University of Iowa); Rat GAPDH, forward, 5'-tcaaggctgagaatgggaag-3'; reverse, 5'-tactcagcaccagcatcacc-3' (Becker JC et al., 2003) with an expected 103bp fragment. GAPDH was used as the internal control. In general, each variable was run in triplicate and repeated twice to obtain a value in an experiment, and at least three independent experiments were performed.

Western Blot

Western immunoblot of DRG cultures was performed as described (Cruise et al., 2004). Proteins were denatured in 4x NuPAGE® LDS Sample buffer (Invitrogen, Carlsbad, CA) containing 0.5% BME at 70 °C for 10 minutes and 5 µg total protein was separated on the precast 4% stacking, 10% separating SDS-PAGE gel (Bio-Rad laboratories) and transferred to nitrocellulose membranes. The blots were blocked in 5% skim milk for 1 hr and incubated overnight at 4 °C in appropriate primary antibody diluted in 5% BSA-TBST solution: rabbit anti-phospho Smad2 (1:250, Cell Signaling), mouse anti-Smad2, rabbit anti-phospho ERK1/2, rabbit anti-phosphop38, rabbit anti-p38, rabbit anti-phospho JNK, rabbit anti-JNK (1:1000; Cell Signaling), rabbit anti-ERK2 (1:20,000, Santa Cruz Biotechnology). Secondary antibodies used were: goat anti-rabbit IgG or goat anti-mouse IgG (1:2500, Jackson ImmunoResearch). Primary antibody was omitted in control studies, and specific signal was absent. Western immunoblot films well below saturation were quantified on a Versadoc scanner in arbitrary units, and data presented from at least two independent experiments.

Statistical analyses

The relative gene expressions were calculated with Gene Expression Macro supplied by Bio-Rad laboratories. Generally, the Ct of CGRP was normalized against that of GAPDH in each sample and fold changes of RNA levels was calculated by $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001), in which the relative changes of genes of interest in the experimental group was calculated as the ratio of normalized data over control group. This analysis was performed for all variables in a study, and at least three independent experiments were compared. The unpaired *t* test was used for two group comparisons and one-way ANOVA followed by Bonferroni/Dunn's test was used for multiple group comparisons. Data are presented as mean $\pm$ SEM and confidence indicated with asterisks. ****p*<0.0001, ***p*<0.001, **p*<0.05 (Statview 4.1 software, Abacus Concepts, Inc., Berkeley, CA).

RESULTS

Activin acted synergistically with NGF to increase CGRP mRNA in adult DRG neurons

To test if activin and NGF ligands acted in parallel or together, each ligand was added alone and in combination, and CGRP mRNA was assayed. The addition of high levels of NGF (100ng/ml) did not increase CGRP mRNA at 2 or 6 hours (data not shown) but increases were detected at 24 hours and CGRP mRNA levels continued to increase each day in this NGF concentration (Fig. 1A gray bars). While CGRP mRNA levels appear to decline with time without added NGF (Doughty et al., 1991, Shadiack et al., 2001) neuronal cell survival is known

to remain high in these cultures (Omri and Meiri, 1990, Cruise, 2004), and indeed, GAPDH mRNA levels detected by real-time PCR remain similar (data not shown). From these data, we chose to examine CGRP regulation at 4 days *in vitro* to obtain robust and reproducible CGRP mRNA increases. To learn more about NGF actions, neurons were then treated with different concentrations of NGF and CGRP mRNA was quantified (Fig. 1B). Maximal CGRP mRNA induction of 8-fold occurred at 10 ng/ml NGF, and 2.5 ng/ml NGF produced half maximal neuropeptide induction. By contrast, neurons treated with 1–50 ng/ml activin alone did not increase CGRP mRNA (Fig. 1C). To learn if activin modulated NGF-induced CGRP mRNA synthesis, adult DRG cultures were then treated with activin in the presence of NGF concentrations that produced half-maximal stimulation of CGRP. When added together, activin at each concentration tested increased CGRP mRNA to levels above that induced by NGF or activin alone (Fig. 1D). These data suggest that activin and NGF or their downstream signals cooperate in some fashion to synergistically increase CGRP.

Activin effects with NGF on CGRP induction required the activin receptor

Because activin's actions occurred only in the presence of NGF, it seemed possible that activin was acting on the NGF receptor itself. To test if activin effects required functional activin or NGF receptors, pharmacological inhibitors were added to specifically block the activation of each receptor. Each inhibitor was tested initially after a single ligand was added. NGF (2.5 ng/ml) induction of CGRP mRNA was largely blocked with the addition of K252a (200 nM), as expected as this drug is a potent inhibitor of the NGF receptor, trkA kinase (Koizumi et al., 1988). Some residual CGRP induction remained, but higher concentrations of K252a (400 nM) could not be used as they were toxic (data not shown). By contrast, the addition of SB431542 (10 μ M) had no effect on NGF-induced CGRP mRNA, as it is a competitive ATP binding site kinase inhibitor of ActR IB (Laping et al., 2002). To address which receptors were required to mediate the synergistic effects of activin in combination with NGF, drugs were added in the presence of 20 ng/ml activin plus 2.5 ng/ml NGF that produces robust CGRP expression. The addition of K252a partially blocked activin plus NGF effects, but some CGRP induction remained after drug action (Fig. 2). The activin receptor inhibitor SB431542 also partially blocked CGRP induction by the ligand combination, but less effectively. Increased levels of SB431542 (20 μ M) did not further decrease activin plus NGF effects (data not shown) suggesting that the dose administered was maximally effective. These data suggest that each cognate receptor contributed to the CGRP mRNA induction when these ligands were presented at the same time.

Activin or NGF-mediated intracellular signals were not augmented by the other ligand

The synergistic stimulation of CGRP mRNA by activin and NGF results from intracellular signals initiated by the ligands and receptors. To test which intracellular signals were stimulated by each ligand alone or in combination, adult DRG were acutely isolated for four hours before stimulation with ligand, and phospho-specific antibody reagents were used to assay intracellular signals over minutes. To learn which concentration of activin maximally stimulated intracellular signals, activated pSmad2 was assayed at one hour. As expected, Smad2 activation increased with added activin, and maximal stimulation was reached at 6 ng/ml activin (Fig. 3A). This phospho-Smad2 reagent detects Smad2 only when dually phosphorylated at serine 465 and serine 467, and detects phosphorylated Smad3 at its equivalent site. The present studies revealed one band with a molecular mass consistent with Smad2 phosphorylation. To learn more about the temporal stimulation of Smad2 by activin, 5 ng/ml activin was added to neurons, and phosphorylated Smad2 was evaluated at subsequent times. Activin resulted in maximal stimulation of pSmad2 at one hour, and sustained levels of pSmad2 were still present at 2 hour (Fig. 3C). By contrast, similar activin concentration and temporal stimulation studies showed no effect on phospho-ERK (pERK) stimulation (Fig. 3B,

3D). These observations indicate that activin stimulates pSmad2 at nanomolar concentrations and that stimulation by activin results in a long-lived pSmad2 signal.

The activity of major protein kinases after addition of each ligand or their combination was assayed. As expected, activin (20 ng/ml) addition for 5 or 15 min stimulated pSmad2 while NGF (50 ng/ml) did not (Fig. 4A). By contrast, NGF addition for 5 or 15 min stimulated pERK2 activation but activin had no effect on this kinase activity (Fig. 4B). In addition, NGF addition resulted in modest activation of p38 but not JNK (Fig. 4C, D) in adult sensory neurons, but activin had no effect on these kinases. These data support the activation of traditionally expected intracellular pathways by each ligand, when added alone.

To test if activin increased NGF-mediated intracellular signals, neurons were treated with both 20 ng/ml Activin plus 50 ng/ml NGF for 15 minutes. The two ligands added together stimulated pSmad2 to levels achieved by activin alone, and pERK2 to levels stimulated by NGF alone (Fig. 4A, B). Thus, the synergistic stimulation of CGRP mRNA seen when activin and NGF were added together was not mediated by additional stimulation either of these kinase pathways. In addition, several studies were designed to test if presentation of one ligand first altered responses to the second. For example, neurons were treated with one ligand for 5 mins or 4 hours and followed with 15 mins stimulation by the second ligand. Such “priming” by activin or NGF did not demonstrate any effect on the later treatment (data not shown). Thus, in combination, NGF and activin appear to increase the classical signaling pathways associated with these ligands in adult DRG neurons, and no obvious cross talk between these two signaling pathways was observed.

In a second approach to test if NGF-initiated signals were necessary for the effects of activin addition, pharmacological inhibitors were used to learn which signaling pathways were required for CGRP mRNA induction. Initial tests were designed to learn which signaling pathways were required for NGF alone to induce CGRP mRNA. Previous reports suggest that NGF signals through the ERK pathway to regulate CGRP mRNA expression (Freeland et al., 2000, Durham and Russo, 2003). However, the potent and specific inhibitor U0126 of the ERK1/2 pathway (Favata et al., 1998, Portis and Longnecker, 2004) decreased NGF mediated CGRP mRNA induction by less than half (Fig. 5A). Higher concentrations of U0126 (10, 20 and 40 μ M) did not further reduce NGF mediated induction, and 60 μ M U0126 caused cell death (data not shown). In other studies, CGRP expression can be regulated through JNK and p38 pathways (Bowen et al., 2006). To test the role of these signals, SB203580 (20 μ M), a highly specific inhibitor of p38 MAP kinase (Lee et al., 1994, Shin et al., 2007) was used, but this reagent only slightly decreased NGF induced CGRP mRNA (Fig. 5A). Similarly, the addition of SP600125 (10 μ M), a potent inhibitor of JNK (Bennett et al., 2001, Clerk et al., 2002), again only slightly decreased NGF induced CGRP mRNA (Fig. 5A). These data suggest that each pathway contributes partially to NGF mediated signals that increase CGRP mRNA. Cells were then treated with combinations of inhibitors. While the ERK pathway appeared to bear the largest role in CGRP induction, the addition of inhibitors to p38 and JNK showed reductions that suggested these pathways also contributed to mRNA induction. The maximum inhibition of CGRP induction by NGF alone was obtained only when ERK and p38 or JNK pathways were blocked or all three pathways were blocked together (Fig. 5A). These data suggest that ERK is the predominant signaling pathway used by NGF to regulate CGRP mRNA but that all three MAPK pathways can combine to regulate CGRP expression by NGF.

The signaling pathways used by activin in combination with NGF to stimulate CGRP expression are similar to those used by NGF alone. Again, inhibition of the MAPK pathway by U0126 partially decreased CGRP induction by the ligand combination, but the most robust inhibition was obtained only by inhibiting ERK plus p38 or JNK pathways (Fig. 5B). In combination, these data indicate that activin addition does not further stimulate NGF

intracellular kinases, but that NGF-stimulated kinase activities are required for both ligands to act together. These observations suggest that the induction of CGRP mRNA by activin in combination with NGF include cooperative mechanisms in the nucleus.

DISCUSSIONS

Hyperalgesia is a cardinal sign of inflammation, and CGRP has been shown to play important role in initiating and maintenance abnormal pain after inflammation. Both activin and NGF increase in skin after inflammation and can regulate CGRP to contribute to abnormal pain. The present study identifies for the first time that activin in combination with NGF synergistically stimulates CGRP induction, suggesting that the presence of both ligands in inflamed skin has potent effects on neuropeptide regulation. This study demonstrates that each cognate receptor is stimulated and parallel intracellular signals are initiated, but that activin synergistic action requires the function of an intact NGF signaling cascade. These data rule out various cytoplasmic cross-talk mechanisms and strongly suggest that activin stimulatory actions with NGF include cooperative mechanisms in the nucleus.

A serum-free adult sensory neuron culture system was used to study CGRP mRNA regulation. Unlike those in the embryo, adult DRG neurons are neurotrophin independent and do not require NGF for survival (Lindsay, 1988, Lindsay and Harnmar, 1989, Kimpinski et al., 1997, Winston et al., 2001, Dodge et al., 2002). Further, the absence of serum restricts glial proliferation, and allows the assay of specific ligand stimulation. We have demonstrated robust neuronal survival and differentiation of adult sensory neurons under these conditions (Cruise, 2004). Although glial proliferation is reduced without serum, many flat cells were apparent in cultures at 4 days in vitro. Despite this concern, only neurons but not glia show phospho-Smad2 (pSmad2) staining in the nucleus after activin stimulation (Cruise et al., 2004), and others have shown that functional activin receptors are present in peripheral neurons but not glia (Kos et al., 2001). These data suggest that neurons are the major activin responsive cells in our studies. Similarly, Schwann cells do not express trkA but only common neurotrophin receptor p75 (NTR), p75 only transiently activates ERK1/2 in some cell lines (Lad and Neet, 2003), so it is likely that the ERK activation we detected in the culture after NGF stimulation is from neurons but not glia.

We and others have demonstrated that NGF increases the mRNA and protein levels of CGRP and CGRP release in adult DRG cultures (Lindsay and Harnmar, 1989, Southall and Vasko, 2000, Winston et al., 2001). In our cultures, 2.5 ng/ml NGF increases CGRP mRNA around 3 fold after 4 days. By comparison, CFA (complete Freund's adjuvant) intraplantar injection increases CGRP mRNA around 50% in L5 DRG at day 3 (Mulder et al., 1997a), probably because NGF mRNA only slightly increases in the first week after CFA injection (Malin et al., 2006). In addition, we have shown that subcutaneous activin administration increased CGRP protein expression in the innervating DRG neurons (Xu et al., 2005), but the present data suggest that activin alone cannot regulate CGRP mRNA in isolated neurons. These studies suggest either that NGF or factors with similar permissive activity are available *in vivo*. While it is possible that activin also regulates CGRP at the translational level, this seems unlikely (Viney et al., 2004). NGF levels in undamaged skin appear sufficient to allow activin effects to be apparent, and both NGF mRNA and protein are present in adult smooth muscle, hair follicle sheath cells, keratinocytes, and hypodermal fibroblasts (Hasan et al., 2000). Confirmation of this notion will require further study *in vivo*.

This study confirms that NGF regulates CGRP expression in adult DRG cultures (Lindsay and Harnmar, 1989, Winston et al., 2001), and begins to untangle the signaling cascades that underlie its effects in primary neurons. In previous reports, NGF activated a 1250bp rat CGRP promoter that contains proximal cyclic AMP- and ras-responsive regions and a distal enhancer (Durham

and Russo, 2003). In promoter-reporter studies with transfected trigeminal neurons or PC12 cells, NGF mediated activation was totally blocked by PD98059 or U0126, which suggests that NGF signals through ERK to drive the CGRP promoter (Freeland et al., 2000, Durham and Russo, 2003). By contrast, in the present report, U0126 blocked perhaps half the NGF effect, and further inhibition was achieved only by blocking either p38 or JNK in addition to ERK. Although p38 is normally involved in stress responses, NGF can signal through p38 pathway to regulate Transient Receptor Potential Vanilloid subtype 1 (TRPV1) after inflammation (Ji et al., 2002). Furthermore, we could not totally block CGRP mRNA induction with all three MAPK pathway blockers.

There are at least three differences between the previous promoter/reporter assays and the present study. An obvious difference is that the promoter assay was performed within 2 hours but our experiments lasted 4 days. Thus, it was possible that U0126 degraded in our culture over time. To address this potential concern, pERK activation was tested in fresh DRG cells that were treated with medium from 4 day U0126 treated cultures, and very low pERK levels were detected, indicating the drug remained active (data not shown). A second difference is that the promoter assay tested the activation of a 1250bp rat CGRP promoter but the present study utilized the entire CGRP mRNA. It is possible that the 1250bp rat CGRP promoter contains elements responding to ERK but not additional elements required to respond to p38 or JNK signaling pathways. However, TNF- α has been shown to activate the 1250bp rat CGRP promoter through p38 and JNK signaling pathways in transfected trigeminal neurons, which suggest this mini CGRP promoter does contain elements needed to respond to p38 or JNK signaling pathways (Bowen et al., 2006). The last difference is the promoter assay was done in trigeminal cultures and we used DRG cell cultures, and a cell type difference may account for the signaling observed.

The effects of activin in combination with NGF on CGRP mRNA was abolished by the specific pharmacological blocker SB431542 that inhibits activin receptor like kinases (Laping et al., 2002). Activin receptors phosphorylate Smad2/3 (Attisano et al., 1996, Heldin et al., 1997) that interacts with common Smad4 and translocates to the nucleus to alter transcription. In addition to this classical pathway, TGF-beta family members have been shown to activate alternative pathways in different cell systems including ERK, JNK and p38 MAP kinases (Kretschmar et al., 1997, Bao et al., 2005, Imamichi et al., 2005, Zhang et al., 2005). In our hands, NGF activated MAPK pathways and transiently activated the Akt but not PKC pathway (data not shown), which suggests that it is unlikely Akt or PKC signals are involved in NGF regulating CGRP mRNA (data not shown). Furthermore, we did not see any activation of MAPK pathways by activin in adult DRG cells. Instead, the present data suggest that activin functions through its own receptor in the presence of NGF to increase CGRP mRNA without further activating NGF signaling pathways. Indeed, Smad 2/3 phosphorylation is detected and Smad2 translocation into the nucleus has been observed in these neurons after activin addition (Cruise et al, 2004). The Smad 2/3 complex with Smad 4 is thought to alter gene expression and may interact with smad-response elements (SRE) composed of an palindromic sequence GTCTAGAC (Zawel et al., 1998). The rat CGRP promoter region has multiple such sequences located upstream of the transcription start site, although it is not known if they are utilized. It is possible that activin stimulated Smadcomplexes bind to SREs that function with NGF signal recruited transcription factors to further increase CGRP transcription.

Although we did not measure CGRP release in the cultures, it is known that NGF increases CGRP release in adult DRG cultures (Southall and Vasko, 2000). Further, CGRP can auto-regulate its expression through a G-protein-coupled receptor activated protein kinase A pathway (Zhang et al., 2007) that may be reflected in residual CGRP mRNA induction in the presence of multiple inhibitors.

Our data suggest that activin in combination with NGF stimulates CGRP mRNA expression and this effect requires an intact NGF receptor and intracellular signaling, but independently stimulates the activin receptor and Smad signals. We interpret these results to indicate that this activin synergy with NGF occurs not in the cytoplasm, but at the promoter level. It will be important and interesting to identify any changes in the transcriptional complex that regulates CGRP after inflammation. This report provides a new mechanism for CGRP regulation that highlights activin as a candidate for new therapeutics to treat abnormal pain.

Acknowledgments

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Abbreviations

activin	activin A
CFA	complete Freund's adjuvant
CGRP	calcitonin gene-related peptide
DMSO	dimethyl sulfoxide
NGF	nerve growth factor
TRPV1	transient receptor potential vanilloid subtype 1

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Fig. 1.

Activin acts with NGF to induce CGRP mRNA in adult DRG cultures.

Adult sensory neurons were maintained in serum free medium without NGF overnight followed by A. NGF (100ng/ml, labeled N) treatment for 1–4 days. After NGF treatment, CGRP mRNA increased each day in culture (one-way ANOVA $*p < 0.05$, $***p < 0.0001$, values compared to D1 NGF). B. Increased concentrations of NGF for 4 days stimulated CGRP induction with maximal stimulation at 10 ng/ml (all values significantly increased above 1 ng/ml NGF, $p < 0.0001$). C. Different concentrations of Activin did not increase CGRP mRNA after 4 days. D. In the presence of half-maximal concentration of NGF at 2.5 ng/ml, activin (5–50 ng/ml) synergistically increased CGRP mRNA compared to NGF alone $***p < 0.0001$, $*p < 0.05$. In each case, quantitative real-time PCR Ct values for CGRP were normalized to Ct values of the housekeeping gene GAPDH as in (Livak and Schmittgen, 2001), to reflect mean $\pm$ SEM of fold changes of CGRP mRNA. Three independent experiments were compared.

Fig. 2.

Activin effects on CGRP mRNA occur through its own receptor.

Primary adult DRG neurons were plated in serum-free medium overnight and pretreated with drugs K252a (200 nM, 0.02% DMSO) or SB431542 (10 μM; 0.04% DMSO) for 1 hour, followed by activin (20 ng/ml) or NGF (2.5 ng/ml) or both for 4 days. All wells contained 0.08% DMSO. Inhibition of trkA kinase with K252a reduced NGF stimulation (**p<0.0001, values compared to 2.5 ng/ml NGF). When activin and NGF were added at the same time, synergistic stimulation of CGRP occurred (**p<0.0001, values compared to 2.5 ng/ml NGF) and this stimulation was partially reduced by inhibition of either trkA kinase with K252a or activin receptor kinase with SB431542 (### p<0.0001, values compared to 2.5 ng/ml NGF plus 20 ng/ml activin). However, K252a was less effective on the NGF and activin combined treatment (p<0.05, values compared to 2.5 ng/ml NGF plus K252a). In each case, quantitative real-time PCR Ct values for CGRP were normalized to Ct values of the housekeeping gene GAPDH as in (Livak and Schmittgen, 2001), to reflect mean±SEM of fold changes of CGRP mRNA. Three-eight independent experiments were compared.

Fig. 3.

Activin signaling pathways in the adult sensory neurons.

A. Activin increases pSmad2 activation in adult sensory neurons. Adult sensory neurons were plated in serum-free neurobasal medium for 4 hours, followed by different concentrations of activin for an hour. Representative blots of pSmad2 and a reprobe for Smad2 in the same samples are shown. (n=2) B. Activin does not activate pERK. Representative blots of pERK2 and ERK2 reprobe are shown. ERK 2 antibody also has low detection for ERK1. C. Activin (5 ng/ml) stimulates long-lasting pSmad2 activation; representative blots of pSmad2 and Smad2 reprobe. D. Activin (5ng/ml) does not activate pERK, representative blots of pERK2 and ERK2 reprobe.

Fig. 4. Activin stimulates pSmad2 while NGF stimulates pERK2 in adult sensory neurons. Activin (20 ng/ml) or NGF (50 ng/ml) or both were added to adult DRG neurons for 5 or 15 minutes to evaluate stimulation of intracellular signaling pathways. A. Activin activates pSmad2 but NGF does not. Activin and NGF in combination does not further increase phosphorylation of Smad2 compared to 15 mins activin treatment ($p > 0.05$; $n = 3$). B. NGF increases pERK2 but activin does not. Activin and NGF in combination does not further increase phosphorylation of ERK2 compared to NGF treatment ($p > 0.05$; $n = 3$). Representative blots of pSmad2 and a reprobe for Smad2, and a second blot for pERK2 and a reprobe for ERK2 in the same samples are shown. C. In similar studies, the p38 pathway was slightly activated by NGF (* $p < 0.05$, when 5 mins NGF is compared to 5 mins activin treatment) but not activin ($n = 3$). D. The JNK pathway was not activated after NGF or activin treatment. ($n = 3$). Representative blots of p-p38 and a reprobe for p38, and a second blot for pJNK and a reprobe for JNK in the same samples are shown.

Fig. 5.

NGF and activin signal through multiple MAPK pathways to regulate CGRP expression.

A. NGF alone or B. Activin plus NGF. Primary adult DRG cultures were plated in serum-free medium overnight and pretreated with drugs UO126 (40 μ M) to inhibit ERK, SB203580 (20 μ M) to inhibit p38, or SP600125 (10 μ M) to inhibit JNK for 1 hour, followed by activin (20 ng/ml) or NGF (2.5 ng/ml) or both for 4 days (vehicle contained 0.08% DMSO, see Methods). Data represent quantitative real-time PCR, corrected for GAPDH expression and reflects the mean $\pm$ SEM of fold changes of CGRP mRNA in 3 experiments. *** p <0.0001, ** p <0.001, * p <0.05 was defined with one-way ANOVA followed by Bonferroni/Dunn's test with

comparison to 2.5 ng/ml NGF and ### $p < 0.0001$, # $p < 0.05$ was obtained from comparing to 2.5 ng/ml NGF plus 20 ng/ml Activin. ($n \geq 3$ for each condition).