Morphine Activates the E Twenty Six-Like Transcription Factor-1/Serum Response Factor Pathway via Extracellular Signal-Regulated Kinases 1/2 in F11 Cells Derived from Dorsal Root Ganglia Neurons

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ABSTRACT

Morphine-induced signaling via opioid receptors (ORs) in dorsal root ganglia (DRG) neurons, the spinal cord, and various brain regions has been shown to modulate gene activity. Hitherto, little attention has been paid to extracellular signal-regulated kinases-1/2 (ERK-1/2)-mediated activation of the serum response factor (SRF) and ternary complex factors (TCFs) such as the E twenty six-like transcription factor-1 (ELK-1) in this context. Using TCF/SRF-dependent reporter gene constructs, a specific ERK-1/2 inhibitor and a dominant-negative ELK-1 mutant, we show herein that morphine activates ELK-1 via ERK-1/2 in DRG-derived F11 cells endogenously expressing μ and δ ORs. Previous studies with glioma cell lines such as NG108-15 cells attributed morphine-induced gene expression to the activation of the cAMP-responsive element binding protein (CREB). Thus, we also analyzed morphine-dependent activation of CREB in F11 and NG108-15 cells. In contrast to the CREB stimulation found in NG108-15 cells, we observed an inhibitory effect of morphine in F11 cells, indicating cell type-specific regulation of CREB by morphine. To obtain data about putative target genes of morphine-induced ELK-1/SRF activation, we analyzed mRNA levels of 15 ELK-1/SRF-dependent genes in cultured rat DRG neurons and F11 cells. We identified the early growth response protein-4 (EGR-4) as the strongest up-regulated gene in both cell types and observed ELK-1 activity-dependent activation of an EGR-4-driven reporter in F11 cells. Overall, we reveal an important role of ELK-1 for morphine-dependent gene induction in DRG-derived cells and propose that ELK-1 and EGR-4 contribute to the effects of morphine on neuronal plasticity.

Introduction

Opioid receptors (ORs) belong to the superfamily of G protein-coupled receptors (GPCRs) and mediate their biological effects via the activation of pertussis toxin (PTX)-sensitive Gi/o proteins (Parolaro et al., 1990). Application of the μ OR (MOP)-selective agonist morphine significantly reduces nociception but also alters gene activity, leading to dependence and tolerance (Li and Clark, 1999; Ammon-Treiber and Höllt, 2005). Spinal cord or brain neurons have been intensively analyzed in this regard (Taylor and Fleming, 2001), but despite the important role of primary sensory or dorsal root ganglia (DRG) neurons in the analgesic actions of morphine, the effects of morphine on gene expression in these neurons are less understood.

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ABBREVIATIONS: OR, opioid receptor; DOP, δ OR; MOP, μ OR; AP-1, activating protein-1; BIM-X, bisindolylmaleimide X; BSA, bovine serum albumin; CamKII, calmodulin-dependent kinase II; Cp, crossing point; CRE, cAMP response element; CREB, CRE binding protein; CREM, CRE modulator; CTAP, d-Phe-Cys-Tyr-d-Trp-Arg-Thr-Pen-Thr-NH2; DAMGO, [d-Ala2-N-MePhe4-glyol-enkephalin; DMEM, Dulbecco’s modified Eagle’s medium; DPDPE, [d-Pen2,d-Pen5]-enkephalin; DRG, dorsal root ganglia; EGR-4, early growth response protein-4; ERK-1/2, extracellular signal-regulated kinases-1/2; p-ERK, phosphorylated-ERK; t-ERK, total ERK; ETS, E twenty six; ELK-1, ETS-like transcription factor; Fos-B, FBJ murine osteosarcoma viral oncogene homolog-B; Fluc, firefly luciferase; FSK, forskolin; eGFP, enhanced green fluorescent protein; GPCR, G protein-coupled receptor; HAT, hypoxanthine-aminopterin-thymidine; HBS, HEPES buffer saline; HEK, human embryonic kidney; KCC-2, K+ /Cl- cotransporter-2; PKA, protein kinase A; PKC, protein kinase C; PTX, pertussis toxin; qRT-PCR, quantitative reverse transcriptase-polymerase chain reaction; REST, repressor element 1-silencing transcription; Scn7a, sodium-activated sodium channel type VII α; SRE, serum response element; SRF, serum response factor; STAT, signal transducers and activator of transcription; TCF, ternary complex factor; YFP, yellow fluorescent protein; PD-184352, 2-(2-chloro-4-iodyophenylamino)-N-cyclopentylmethoxy-3,4-difluorobenzamide.
GPCR-promoted signaling increases gene activity via cAMP response elements (CRE) or serum response elements (SRE). CRE is activated because of its interaction with the CRE binding protein (CREB) (Andrisani, 1999), whereas SRE activity is enhanced after binding to the serum response factor (SRF) and ternary complex factors (TCFs) such as E-twenty six (ETS)-like transcription factor-1 (ELK-1) (Buchwalter et al., 2004). Interactions between CRE and CREB are enhanced after the phosphorylation of CREB by numerous downstream kinases of GPCR signaling such as protein kinase A (PKA), protein kinase C (PKC), calmodulin-dependent kinase II (CamKII), and extracellular signal-regulated kinases-1/2 (ERK-1/2) (Shaywitz and Greenberg, 1999). Likewise, affinity of the ELK-1/SRF/SRE complex is increased after phosphorylation of ELK-1 by ERK-1/2 (Davis, 1995).

The effects of morphine on gene regulation have been attributed to the phosphorylation of CREB and the concomitant activation of CRE (Maldonado et al., 1996; Widnell et al., 1996; Li and Clark, 1999; Zhou and Zhu, 2006). Glial-derived NG108-15 or Neuro2A cells have extensively been used to study opioid-induced signaling (Blume, 1978; Chakrabarti et al., 1995). In these cell lines morphine exerts its effects on CRE via CamKII- or PKC-mediated phosphorylation of CREB (Bilecki et al., 2000, 2004). However, given that opioids engage signaling pathways that both increase (activation of ERK-1/2, CamKII, and/or PKC) and decrease CREB activity (PKA inhibition via G_\text{i/o}), the net effect of such opposing morphine effects on CRE activity might differ in distinct cell types. ERK-1/2 play a central role in GPCR-dependent gene induction, and downstream targets of ERK-1/2 such as ELK-1 have been reported to play an important role in the gene regulation of adult DRGs (Kerr et al., 2010) and mediate the central effects of other abusive drugs such as cocaine (Besnard et al., 2011). Hence the ERK-1/2/ELK-1/SRF pathway emerges as a likely alternative by which morphine could induce gene expression in the absence of CREB activation.

So far, surprisingly little is known about the role of the ERK-1/2/ELK-1/SRF pathway in morphine-induced gene expression. In MOP-overexpressing cells inconsistent observations have been made, because both etorphine and morphine induced ERK-1/2 phosphorylation in HEK293 cells, but only etorphine activated an ELK-1-driven reporter gene construct (Zheng et al., 2008), whereas in MOP-overexpressing Chinese hamster ovary cells morphine-induced activation of an ELK-1 reporter was observed (Shoda et al., 2001). Signaling pathways responsible for ELK-1 activation in different OR-overexpressing cell models have not been sorted out, and to our knowledge activation of the ELK-1/SRF pathway by morphine in DRG neurons or other endogenous expression systems has not been described.

F11 cells are regarded as an authentic cell model for DRG neurons (Franel et al., 1987) and endogenously express δ OR (DOP) and MOP subtypes (Fan et al., 1993). Thus, to investigate morphine-dependent gene induction in a DRG-derived cell line and answer the question of whether morphine regulates ELK-1/SRF in an endogenous cell system, we analyzed the effects of opioids on various reporter gene constructs in F11 cells. First, we found that morphine significantly enhances ELK-1/SRF signaling via ERK-1/2 in F11 cells, and second, comparing the CREB/CRE pathway in NG108-15 and F11 cells, we observed that morphine increases CREB activ-
HBS buffer for 30 min at room temperature. After cell harvesting in HBS without BSA, – 1 × 10⁷ cells per well were seeded in 96-well plates, and total luminescence was measured in a FLUOstar Omega plate reader (BMG, Offenburg, Germany) at 37°C. HBS, 0.02% methanol HBS, or HBS with the corresponding ligand was automatically injected after 5 s. Total emission was measured at 1-s intervals. Luminescence was normalized by defining the first ratio (1 s) measured as 100%.

cAMP Accumulation. To determine agonist-promoted cAMP accumulation – 200,000 cells were seeded in 12-well plates coated with 0.1% poly-l-lysine 24 h before the experiment and labeled in serum-free HAM’s F12 medium containing 2 μCi/mL of [³H]adenine for 16 h. Cells were stimulated for 30 min at 37°C in DMEM containing 1 μM 3-isobutyl-1-methylxanthin, forskolin (FSK) when indicated, and various concentrations of different ligands. The reaction was terminated by removing the medium and adding ice-cold 5% trichloroacetic acid. [³H]ATP and [³H]cAMP were then purified by sequential chromatography using dowex-resin/aluminum oxide columns, and radioactivity was measured in a β-counter.

Firefly Luciferase Reporter Gene Assays. For the detection of TCF/SSRF activity a reporter gene construct containing an AP-1 site (TGAGTCA) for c-Fos/c-jun, a cis-inducible element (TTCCCGT-CAA) for STAT and an ETS/SRE site (GAGTGCATTAGGA-CATC) for TCF/SSRF, or a reporter solely containing five repeats of the ETS/SRE-site were used. Note that the ETS site binds ELK-1, SRF accessory protein-1, and neuroepithelial transformation gene-1. Twenty-four hours after transfection, cells were seeded in 24-well plates coated with 0.1% poly-l-lysine. After 24 h, cells were serum-starved for 16 h and then stimulated in serum-free HAM’s F12 medium or DMEM containing various ligands for 6 h. The dominant negative ELK-1 mutant (REST/ELK-1-ßC) has been described previously (Stefano et al., 2007) and was kindly provided by Dr. Gerald Thiel (Universität des Saarlandes, Homburg, Germany). To monitor CRE activity, a pAD-CRE-firefly luciferase (Fluc) plasmid containing six CRE sites was transfected into F11 or NG108-15 cells. The p2NXL reporter (kindly provided by Dr. Refugio Garcia-Villegas, Cinevast-Pin, Mexico City, Mexico) has been described previously (Garcia-Villegas et al., 2009) and contains the coding sequence of the Fluc under the control of the minimal promoter of the mouse sodium-activated sodium channel type VI α (Scn7a) that harbors a binding site for EGR-4 and an E box. Cells were lysed, and Fluc activity was determined by using a luciferase reporter system (Promega, Mannheim, Germany) according to the manufacturer’s protocol by using a FLUOstar Omega plate reader (BMG). If reporter gene activity of distinct transcription was compared, overall transcription efficacies were controlled by co-expression of a plasmid encoding soluble YFP under the control of a constitutively active promoter. YFP emission (excitation, 480; emission, 520) was determined by using the software supplied with the LightCycler 480. Sequences of primer pairs used are given in Table 1.

Data Analysis. Data obtained by Western blotting were analyzed by using ImageJ (National Institutes of Health, Bethesda, MD). Statistical significance of differences was assessed by Student’s t test (between two groups) or one-way analysis of variance and Tukey’s honest significance post hoc test (between more than two groups).

**Results**

**Opioid Receptor Expression in F11 Cells.** F11 (rat dorsal root ganglia neurons × mouse neuroblastoma) cells are generally regarded as a suitable cell model for analyzing DRG neuron-specific signaling (Francel et al., 1987; Boland et al., 1991; Puttfarcken et al., 1997; McIlvain et al., 2006; Jung and Miller, 2008) and endogenously express DOP and MOP but not κ opioid receptor subtypes (Fan et al., 1992, 1993). MOP-specific agonists such as DAMGO increase intracellular calcium concentrations in these cells. In line with these prior findings, we detected 367 ± 92 fmol/mg specific OR binding sites in the F11 cell pool studied (Fig. 1A) and observed that DAMGO (Fig. 1B) or morphine (Fig. 1C) induced intracellular calcium transients. Thus, F11 cells may serve as a suitable model system for analyzing the effects of morphine on the ELK-1/SSRF pathway.

**Morphine-Induced TCF/SSRF-Driven Protein Expression in F11 Cells.** ERK-1/2 have been shown to be targets of morphine-dependent signaling and play a critical role in gene induction, because they are dominant upstream regulators of TCFs and CREB. Thus, we first analyzed effects of morphine on ERK-1/2 phosphorylation. We observed a rapid and transient increase in ERK-1/2 phosphorylation with a peak at 2.5 min and a subsequent decline thereafter (Fig. 2, A and C). In accord with previous studies (Parolaro et al., 1990), Go protein-proteins are required for morphine-induced signaling, because treatment of F11 cells with PTX completely blocked morphine-promoted phosphorylation of ERK-1/2 (Fig. 2, B and C).

To investigate morphine-dependent gene induction in F11 cells, we took advantage of a reporter gene construct that encodes a promoter containing cis elements for STAT, TCF/SSRF, and AP-1 (for details see Materials and Methods). Expression of this reporter construct revealed that DAMGO, morphine, and the DOP-specific agonist DPDPE induced luciferase expression to an extent comparable with serum stimulation (Fig. 3A), indicating that morphine enhanced gene expression in F11 cells via STAT, TCF/SSRF, or AP-1. To narrow down the cis element responsible for morphine-induced reporter activation, we next expressed a reporter gene construct containing five repeats of TCF/SSRF binding sites.
but no consensus sites for STAT or AP-1. Morphine induced significant luciferase expression via TCF/SRF similar to the DAMGO effect and more pronounced than the gene expression induced by DPDPE (Fig. 3B). Thus, we provide the first evidence for morphine-induced gene expression via TCF/SRF in neuronal, DRG-derived cells. Morphine activates both MOP and DOP, but exhibits selectivity toward the MOP subtype (Fukuda et al., 1995). Accordingly, the morphine concentration used (1 μM) was assumed to activate mainly the MOP. To test this hypothesis, we applied CTAP, a highly selective MOP antagonist (Pelton et al., 1986), and measured ligand-induced TCF/SRF reporter activity. Indeed, we observed a significant reduction in DAMGO- and morphine-, but not DPDPE-induced reporter activity (Fig. 3C), indicating that morphine mediates its effects in F11 cells via the MOP subtype.

**Morphine-Induced Activation of TCF/SRF in F11 Cells Required ERK-1/2 and ELK-1 Activation.** The ETS site of the TCF/SRF-driven reporter used herein contains a binding site for ELK-1, SRF accessory protein-1, or neuroepithelial transforming gene-1 (Price et al., 1995). In addition to ERK-1/2, other kinases such as c-Jun N-terminal kinase have been shown to activate TCFs (Yordy and Muise-Helmicks, 2000), and even TCF-independent SRF activation mediated by the monomeric G protein Rho and myocardin-related transcription factors has been demonstrated (Hill et al., 1995; Posern and Treisman, 2006). Therefore, to further dissect the signaling pathway underlying morphine-induced activation of the TCF/SRF reporter and elucidate the role of ERK-1/2, we tested the impact of an ERK-1/2 inhibitor (PD-184352) and a dominant-negative ELK-1 mutant (REST/ELK-1-C) on morphine-dependent TCF/SRF reporter activation (Stefano et al., 2007). PD-184352 significantly inhibited basal, serum and even stronger morphine-promoted reporter activity (Fig. 4A), indicating that ERK-1/2 have a major contribution to the effects of morphine on the TCF/SRF reporter. In line with this notion, we observed increased phosphorylation of ELK-1 after 2 to 5 min
(first peak) and 20 to 30 min (second peak) of morphine stimulation, an effect that was again blocked by PD-184352 (Fig. 4B). Coexpression of the REST/ELK-1-6C mutant and the TCF/SRF reporter significantly inhibited basal reporter activity, although coexpression of the ELK-1 mutant did not affect the overall transfection efficiency of a plasmid encoding soluble YFP (Fig. 4C), indicating that ELK-1 activity is generally required for TCF/SRF-driven reporter activity in F11 cells. In addition, depressing ELK-1 activity by expression of a dominant negative mutant precluded morphine-promoted ETS/SRE activation (Fig. 4C), indicating that ELK-1 is required for morphine-promoted activation of the TCF/SRF reporter.

Cell Type-Specific CREB Regulation by Morphine in F11 and NG108-15 Cells. Having established that morphine initiates gene induction via ERK-1/2 and the ELK-1/SRF/SRE pathway, we wondered whether morphine would additionally activate CREB and CRE in F11 cells. Because cAMP-dependent protein kinase A (PKA) is the major activator of CREB, we measured the effects of morphine on forskolin-induced cAMP accumulation and found that DAMGO, morphine, and DPDPE significantly reduced FSK-induced cAMP accumulation in a PTX-sensitive manner (Fig. 5A). To assess the influence of morphine-dependent inhibition of cAMP production on CREB activity, we next used a reporter gene construct that contained six CRE sites. Although we did not observe increased CREB activity in response to a 4-h stimulation by DAMGO or morphine, the CRE reporter strongly reacted to FSK, which increases cAMP levels independently of OR (Fig. 5B). In contrast, 16 h after ligand stimulation significant inhibition of CREB by morphine and DAMGO was detectable (Fig. 5D). These data contrast with previously observed opioid-induced phosphorylation/activation of CREB in other cell lines (Bilecki et al., 2000, 2004). Therefore, we included NG108-15 cells in our experiments. We noted that in analogy to the situation in F11 cells DAMGO, morphine, and DPDPE significantly reduced cAMP levels in NG108-15 cells (Fig. 5C). However, in accord with previous reports, expression of the DOP subtype is predominant compared with the MOP (Gomes et al., 1999). It is noteworthy that in NG108-15 cells morphine transiently increased CRE activity after 4 h, but did not show a stimulatory effect after 16 h (Fig. 5, B and D), suggesting cell type-specific differences in the regulation of CREB. Next, we analyzed the effects of PD-184352 and BIM-X (a PKC inhibitor) on morphine-promoted CRE activity. Whereas PD-184352 further enhanced the inhibitory effects of morphine on CRE in F11 cells (Fig. 5E), BIM-X blunted the activating effects in NG108-15 cells (Fig. 5F), indicating that ERK-1/2 may contribute to CRE activation by morphine in the former cell model, whereas PKC is indispensable for CRE stimulation in the latter. However, ERK-1/2-mediated CRE activation seems to be masked by dominant G_{i/o}-mediated inhibition of the cAMP/PKA CREB pathway by morphine in F11 cells (summarized in Fig. 9).

Morphine Does Not Activate the ELK-1/SRF Pathway in NG108-15 Cells. Given cell type-specific differences in morphine-promoted activation of CREB, we next wondered whether similar to the situation in F11 cells morphine activates the TCF/SRF pathway in NG108-15 cells. As shown in Fig. 6A, stimulation of NG108-15 cells with 1 μM or even 10 μM morphine failed to induce TCF/SRF reporter activity, whereas serum and DPDPE significantly activated the reporter, indicating cell type-specific differences in morphine-promoted TCF/SRF activation. This is of particular interest because 10 μM morphine similarly affected cAMP production compared with 1 μM DPDPE (Fig. 6B), indicating that the inability of morphine to activate TCF/SRF in NG108-15 cells is not simply caused by its weaker potency to activate the DOP.

Morphine Induces Expression of Early Growth Response Protein-4 in Cultured Rat DRG Neurons and F11 Cells. So far, our data suggest that morphine-dependent gene induction is mediated primarily by ERK-1/2 and the ELK-1/SRF/SRE complex in F11 cells. To gain first insight into a putative physiological role of this pathway, we aimed to identify target genes that are upregulated via ELK-1/SRF in DRG neurons after morphine stimulation. In detail, we determined mRNA levels of 15 genes known to be regulated by SRF in neurons (Knoll and Nordheim, 2009) by performing qRT-PCR experiments with cDNAs obtained from cultured rat DRG neurons. As summarized in Table 1 and Fig. 7A, stimulation of DRG neurons with morphine (1 μM) for 1 h significantly upregulated mRNA levels of Fos-B, SRF, and EGR-4, with EGR-4 being the strongest induced gene. To verify the role of ERK-1/2 in this process, we analyzed the effects of PD-184352 on morphine-promoted EGR-4 induction. The ERK-1/2 inhibitor completely blocked EGR-4 induction (Fig. 7C), indicating an important role for ERK-1/2 in this process. Next, we performed identical qRT-PCR experiments with F11 cells and found that similar to DRG neurons morphine significantly induced mRNA expression of Fos-B and EGR-4 and additionally of EGR-2 and CRE modulator-2 (CREM-2) (Table 1; Fig. 7B). It is noteworthy that similar to DRG neurons EGR-4 appeared as the strongest induced gene, indicating similar effects of morphine on gene expression in F11 cells and cultured DRG neurons. In addition, compatible with our data obtained with DRGs, PD-184352 also blocked EGR-4 induction in F11 cells (Fig. 7D). Thus ERK-1/2-mediated expression of EGR-4 by morphine seems to be a common event in DRGs and DRG-derived cells.

Morphine-Promoted Activation of an EGR-4-Driven Reporter Requires ELK-1 Activity in F11 Cells. To validate our data obtained with qRT-PCR experiments, we next set out to analyze the effects of morphine on EGR-4-promoted gene induction. It has been reported that expression of the sodium-activated sodium channel Scn7a in DRG neurons is regulated by a minimal promoter containing a binding site for EGR-4 (García-Villegas et al., 2009). Thus, we took advantage of a reporter gene construct (p0.2NxL) that contains this promoter sequence and analyzed the effects of morphine on EGR-4-driven reporter activity in F11 cells. We found that morphine transiently increased EGR-4-dependent reporter activity (Fig. 8), indicating that morphine induced the expression of biologically active EGR-4. The promoter of the EGR-4 gene has been reported to contain CRE and SRE sites (Croby et al., 1992; Holst et al., 1993). To narrow down the cis element responsible for morphine-promoted EGR-4 expression in F11 cells, we coexpressed the dominant negative mutant of ELK-1 and the p0.2NxL reporter. Morphine failed to increase EGR-4-dependent protein expression under these conditions (Fig. 8), indicating that ELK-1 activation is responsible for morphine-promoted EGR-4 induction in
F11 cells. Thus, we identified EGR-4 as the first gene to be up-regulated by morphine via ELK-1 in DRG-derived cells.

**Discussion**

Morphine has been shown to induce cellular adaptations that occur on the posttranslational or transcriptional level and lead to tolerance and dependence (Li and Clark, 1999; Gintzler and Chakrabarti, 2000; Chakrabarti et al., 2001; Ammon-Treiber and Höllt, 2005). Activation of CREB and ELK-1/SRF are probably the most common pathways by which GPCR-activating drugs affect gene expression. Although ELK-1 and SRF have been shown to modulate neuronal plasticity (Yu and Yezierski, 2005; Ji et al., 2009; Knöll and Nordheim, 2009; Besnard et al., 2011; Klinger et al., 2011), surprisingly little attention has been paid to ELK-1/ SRF in the context of morphine-induced neuronal plasticity, which contributes to drug tolerance/dependence. Therefore, we analyzed the effects of morphine on ELK-1/ SRF-mediated gene regulation in F11 cells endogenously expressing the DOP and MOP subtype. We observed morphine-induced activation of the ELK-1/ SRF pathway and thus show for the first time that morphine is able to modulate gene expression via ELK-1/ SRF in F11 cells and separate these cells from established cell models such as NG108-15 cells that show morphine-promoted CREB activation and no effects on ELK-1/ SRF (Fig. 9).

Considering putative signaling pathways responsible for morphine-induced activation of ELK-1/ SRF in F11 cells, it is noteworthy that in MOP-overexpressing HEK293 cells opioids have been shown to activate ERK-1/2 via PKC and β-arrestins (Zheng et al., 2008). PKC-dependent activation of
ERK-1/2 occurred rapidly (2–5 min) and did not alter ELK-1 activity, whereas β-arrestin-promoted activation of ERK-1/2 occurred at later time points (20–30 min) and increased ELK-1-driven reporter activity. Commensurate with the established inability of morphine to recruit β-arrestins to the MOP in HEK293 cells or other cell lines (Keith et al., 1996), it consequently failed to activate ELK-1 despite its robust PKC-mediated activation of ERK-1/2. In F11 cells, inhibition of PKC activity by BIM-X failed to block morphine-promoted ERK-1/2 activation (data not shown), suggesting that ERK-1/2 and thus also ELK-1/SRF are differentially regulated in HEK293 and F11 cells. Given that morphine-promoted ERK-1/2 activation occurred rapidly (after 2.5 min), we assume that β-arrestins are not involved. However, further studies are required to determine a putative role for β-arrestins in morphine-promoted ELK-1 activation in F11 cells. At this point, we postulate that unidentified, PTX-sensitive signaling pathways are responsible for the morphine-promoted ELK-1/SRF activation via ERK-1/2 in F11 cells.

Cell type-specific gene regulation by morphine raises an important question about the molecular determinants responsible for these differences. It is noteworthy that F11 cells express DOP and MOP but not κ opioid receptor subtypes (Fan et al., 1993), whereas NG108-15 cells almost exclusively express the DOP (Gomes et al., 1999). In line with this overall situation, the effects of DAMGO on cAMP production were much more pronounced in F11 compared with NG108-15 cells. Thus, cell type-specific activation of ELK-1/

**Fig. 4.** A, F11 cells were transfected with a reporter gene construct containing five repeats of the TCF/SRF site, pre-stimulated or not with PD-184352 (30 min; 10 μM), and then stimulated with serum (20%), DAMGO, or morphine (1 μM) for 6 h. Data of three independent experiments performed in quadruplicate were compiled by subtracting the average signals of basal condition (with or without PD-184352) from those of ligand-stimulated cells. Then ligand-induced signals in the absence of PD-184352 were defined as 100%, and ligand-induced effects were calculated as percentages. Finally, the effects of PD-184352 on ligand-induced stimulation were determined by subtracting these values from 100. ††, p < 0.01, significant difference between ligand-treated cells and basal. † , p < 0.01, significant difference to zero. B, F11 cells were treated or not for 30 min with 10 μM PD-184352 and then stimulated with 1 μM morphine for various periods of time. Cells were analyzed by Western blotting using an antibody specific for p-ELK-1. One representative immunoblot is shown. Data were quantified by setting values of unstimulated cells (with or without PD-184352) as 100%. Data obtained after 2.5 and 5 min (first peak) or after 20 and 30 min (second peak) of morphine stimulation obtained for three independent stimulations were pooled and are shown in the diagram as the mean ± S.E.M. † , p < 0.05, a significant difference to zero. ††, p < 0.01, difference between basal and PD-184352-treated cells. C, F11 cells were transfected with the TCF/SRF reporter construct. A plasmid encoding soluble YFP served as a control for transfection efficiency, and a plasmid encoding the dominant negative REST/ELK-1-ΔC mutant was used to silence ELK-1. Cells were treated with 1 μM morphine for 6 h. YFP signals were identical under both conditions. Left, one representative experiment is shown as ratios of YFP and luciferase signals (1000×). Right, data of four independent experiments were compiled by defining signals of basal condition (in the absence of REST/ELK-1-ΔC) as 100%. ††, p < 0.01, significant difference to unstimulated cells.
SRF might reflect exclusive morphine-induced ELK-1/SRF activation by MOP receptors. This assumption is supported by the finding that a MOP-specific antagonist blocked morphine-induced ELK-1/SRF activation in F11 cells. However, the DOP-specific agonist DPDPE was able to activate ELK-1/SRF in NG108-15 cells, indicating that DOPs are functionally linked to this pathway. Because 10 μM morphine did not activate ELK-1/SRF, although they similarly affected cAMP production compared with 1 μM DPDPE, it seems that DOP-mediated activation of ELK-1/SRE is distinctly regulated by different agonists in NG108-15 cells. Future investigations are required to clarify the exact mechanisms responsible for ligand-specific activation of ELK-1/SRF via DOP in NG108-15 cells.

With regard to cell type-specific morphine-promoted signaling, it is also of note that fusion of neuroblastoma NG18TG-2 cells with rat DRG neurons created the F11 cell line (Fan et al., 1992) and with glioma cells (C6-BU-1 cells) created the NG108-15 cell line (Augusti-Tocco and Sato, 1969). Regulation of gene activity might generally differ in cells of different origins. Fundamental differences among both cell types are first reflected by our finding that a PKC inhibitor reduced morphine-induced CREB activation in NG108-15 but not in F11 cells, whereas inhibition of ERK-1/2 affected morphine-promoted

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**Fig. 5.** A and C, FSK-induced (5 μM) cAMP accumulation in F11 (A) or NG108-15 cells (C) treated or not with PTX (50 ng/ml for 16 h) was measured after stimulation of the cells with 1 μM DAMGO, morphine, or DPDPE for 30 min. Data of three to five independent experiments were compiled by calculating ligand-induced inhibition of FSK-induced cAMP accumulation in percentages. ***, p < 0.001, significant difference to zero. ††, p < 0.01, significant difference between PTX-treated and untreated cells.** B and D, F11 or NG108-15 cells were transfected with a CRE reporter gene construct and stimulated with FSK (5 μM), DAMGO, or morphine (each 1 μM) for 4 h (B) or 16 h (D). Data of three to five independent experiments performed in quadruplicate were compiled by defining basal values as 100% and are given as ligand-induced changes in percentages. ***, p < 0.01, significant difference to zero. †, p < 0.05, significant difference between F11 and NG108-15 cells.††, p < 0.01, significant difference between inhibitor-treated and untreated cells (basal).
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CREB activation in the latter but not in the former cell line. Second, it was remarkable that short FSK stimulation (4 h) activated CRE stronger in F11 cells, and prolonged stimulation (16 h) was more efficacious in NG108-15 cells (Fig. 5, B and D), suggesting that PKA regulates CREB activity in NG108-15 cells with slower kinetics compared with F11 cells. In line with its selectivity toward the MOP subtype, the impact of morphine (1 μM) on the cAMP pathway was more robust in F11 than in NG108-15 cells. Thus, the weak effects of morphine on cAMP/PKA in NG108-15 cells might allow morphine-promoted PKC activation to activate CREB, despite the inhibitory actions of morphine on CREB caused by PKA inhibition. In contrast, the fast and strong effects of morphine on cAMP/PKA sufficiently inhibited CREB activity and thus obscured morphine-promoted CREB activation via ERK-1/2 in F11 cells. Therefore, we postulate that weak and belated effects of morphine on PKA activity are responsible for transient CRE activation in NG108-15 cells, whereas profound and fast effects of morphine on PKA curtail CRE activation in F11 cells (Fig. 9).

Overall, we provide evidence that morphine increases ELK-1/SRF-dependent gene induction in an endogenous cell system derived from DRG neurons. In line with this notion, morphine increased mRNA levels of Fos-B and EGR-4 (alias NGFI-C or pAT133) not only in F11 cells but also in cultured embryonic DRG neurons. The Fos-B and the EGR-4 promoter has been reported to contain SRE and CRE sites (Crosby et al., 1992; Lazo et al., 1992; Holst et al., 1993). Thus, morphine-induced expression of both proteins could be mediated either by TCF/SRF or CREB. Because morphine did not activate CRE and a dominant-negative ELK-1 mutant blocked morphine-induced activation of an EGR-4-driven reporter in F11 cells, we propose that ERK-1/2-mediated activation of the ELK-1/SRF/SRE pathway is responsible for the effects of morphine on EGR-4 expression.

With regard to a putative physiological relevance of morphine-induced effects on ELK-1 and CREB, it is interesting that previous studies revealed a switch from cell proliferation to differentiation associated with neurite outgrowth in F11 cells after ELK-1 inhibition and concomitant CREB activation (Ghil et al., 2000; McIlvain et al., 2006). Therefore,

![Figure 6](image-url)

**Fig. 6.** A, NG108-15 cells were transfected with a reporter gene construct containing five repeats of the TCF/SRF site and stimulated with serum (20%), DPDPE (1 μM), or morphine (1 or 10 μM) for 6 h. Data of three to five independent experiments performed in quadruplicate were compiled by defining signals of basal condition as 100%. **B**, FSK-induced (5 μM) cAMP accumulation in NG108-15 cells was measured after stimulation of the cells with 1 μM DPDPE or 1, 5, or 10 μM morphine for 30 min. Data of three to five independent experiments were compiled by calculating ligand-induced inhibition of FSK-induced cAMP accumulation in percentages.

**TABLE 1**
Morphine-promoted gene induction in serum-starved F11 cells or cultured rat DRG neurons stimulated for 1 h with 1 μM morphine (MOR) as monitored by qRT-PCR

<table>
<thead>
<tr>
<th>Gene Number</th>
<th>Name</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Cultiuated DRG Neurons</th>
<th>F11 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_011442.2</td>
<td>Actb</td>
<td>gccctctggccacctgagaa</td>
<td>tctcttggaagcctggacq</td>
<td>21.22 ± 1.15</td>
<td>20.84 ± 0.85</td>
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<tr>
<td>NM_022197.2</td>
<td>Fos-C</td>
<td>gggacatgtctcttcacctac</td>
<td>gatctgcttacaaagctcttg</td>
<td>31.33 ± 0.97</td>
<td>30.29 ± 1.38</td>
</tr>
<tr>
<td>NM_001013146.1</td>
<td>Egr-1</td>
<td>cagctgctggaaagctgagac</td>
<td>gatctgcttacaaagctcttg</td>
<td>32.67 ± 1.12</td>
<td>31.06 ± 1.16</td>
</tr>
<tr>
<td>NM_001108902.1</td>
<td>Srf</td>
<td>gcacactcttatccggaaga</td>
<td>atgtggccacccacagtt</td>
<td>32.50 ± 1.46</td>
<td>30.19 ± 1.09</td>
</tr>
<tr>
<td>NM_021836.2</td>
<td>Jun-B</td>
<td>ggactgaccaacctcagcc</td>
<td>gatctgcttacaaagctcttg</td>
<td>30.05 ± 0.78</td>
<td>28.48 ± 0.54</td>
</tr>
<tr>
<td>NM_0125151.2</td>
<td>Cbf-b</td>
<td>ccagctgctggaaagctgagac</td>
<td>gatctgcttacaaagctcttg</td>
<td>30.55 ± 2.97</td>
<td>28.39 ± 1.45</td>
</tr>
<tr>
<td>NM_054633.1</td>
<td>Egr-2</td>
<td>ctacccgtggaagacctg</td>
<td>aatgtggccacccacagtt</td>
<td>33.38 ± 2.1</td>
<td>30.75 ± 1.40</td>
</tr>
<tr>
<td>NM_014708.1</td>
<td>Egr-3</td>
<td>caactcctccgcaagcagg</td>
<td>gatctgcttacaaagctcttg</td>
<td>32.99 ± 2.9</td>
<td>30.91 ± 0.84</td>
</tr>
<tr>
<td>NM_019137.1</td>
<td>Egr-4</td>
<td>gctcttctcttcaactcctc</td>
<td>gatctgcttacaaagctcttg</td>
<td>36.15 ± 1.67</td>
<td>31.64 ± 0.67</td>
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<tr>
<td>NM_024388.1</td>
<td>Nur-77</td>
<td>aacgctgctggaaagctgagac</td>
<td>gatctgcttacaaagctcttg</td>
<td>29.35 ± 0.65</td>
<td>28.70 ± 0.78</td>
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<tr>
<td>NM_001106069.1</td>
<td>Lh-b</td>
<td>cagctgctggaaagctgagac</td>
<td>gatctgcttacaaagctcttg</td>
<td>30.02 ± 1.56</td>
<td>28.70 ± 0.69</td>
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<tr>
<td>NM_017334.1</td>
<td>Crem-2</td>
<td>gctcttctcttcaactcctc</td>
<td>gatctgcttacaaagctcttg</td>
<td>29.59 ± 1.74</td>
<td>28.47 ± 0.89</td>
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<tr>
<td>NM_00110860.1</td>
<td>Crem-3</td>
<td>aacgctgctggaaagctgagac</td>
<td>gatctgcttacaaagctcttg</td>
<td>35.59 ± 0.72</td>
<td>34.14 ± 1.56</td>
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<tr>
<td>NM_153826.1</td>
<td>Npas-4</td>
<td>aacgctgctggaaagctgagac</td>
<td>gatctgcttacaaagctcttg</td>
<td>36.55 ± 1.16</td>
<td>34.38 ± 1.20</td>
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<tr>
<td>NM_021837.2</td>
<td>Cyr-61</td>
<td>aacgctgctggaaagctgagac</td>
<td>gatctgcttacaaagctcttg</td>
<td>28.24 ± 1.96</td>
<td>27.33 ± 2.00</td>
</tr>
<tr>
<td>NM_022266.2</td>
<td>Ctcf</td>
<td>gctcttctcttcaactcctc</td>
<td>gatctgcttacaaagctcttg</td>
<td>23.15 ± 1.78</td>
<td>22.17 ± 1.08</td>
</tr>
</tbody>
</table>
Fig. 7. A and B, qRT-PCR experiments were performed with four independent cDNAs derived from serum-starved cultured DRG neurons (A) or three cDNAs in the case of F11 cells (B) stimulated or not with morphine (1 h, 1 μM) using specific primers for 15 distinct genes as indicated in Table 1. The name of the analyzed gene is listed under the corresponding bar. p values indicate the differences to zero. Cp values of each measurement were normalized by first subtracting data obtained for actin from those obtained for the corresponding gene in untreated or morphine-treated cells. Then data obtained after morphine treatment were subtracted from those of basal conditions and are given as the mean ± S.E.M. **, p < 0.01; *, p < 0.05, significant difference to zero. Genes with p < 0.05 are shown as black bars. C and D, DRGs (C) and F11 cells (D) treated or not with PD-184352 (30 min, 10 μM) are shown and analyzed as described above. ††, p < 0.01, significant difference between untreated and PD-184352-treated cells.
Morphine has the propensity to counteract neurite outgrowth in F11 cells by activating ELK-1/SRF and inhibiting CREB. This is of further interest when considering morphine-induced expression of the CREM-2 in F11 cells (Fig. 7B), which has been reported to inhibit CREB (Foulkes et al., 1991), and thus could further enhance the inhibitory effects of morphine on cell differentiation.

Considering the putative physiological relevance of our findings in terms of morphine tolerance/dependence, it is noteworthy that Fos-B and its stable isoform ΔFos-B have been recognized as sustained molecular switches of addiction to cocaine and other abusive drugs in brain neurons (Nestler et al., 1999, 2001), suggesting that putative morphine-promoted gene regulation in vivo.

Fig. 8. F11 cells were transfected with the p0.2NxL reporter construct containing a binding-site for EGR-4 (EGR-4-BS) and an E box. A plasmid encoding soluble YPF served as a control for transfection efficiency, and a plasmid encoding the dominant-negative REST/ELK-1-3C mutant was used to silence ELK-1. Cells were treated with morphine (1 μM) for 1, 2, 3, and 4 h. YPF signals were identical under both conditions. Data of six experiments performed in quadruplicate were compiled by defining signals of basal condition as 100%. **, p < 0.01, significant difference to zero.

In conclusion, we reveal an as-yet-unappreciated role of the ELK-1/SRF pathway in morphine-promoted gene induction. It seems that this new pathway plays a dominant role in DRG-derived cells and links MOP subtypes to other transcription factors such as Fos-B and EGR-4. EGR-4 in turn activates CREB via blockade of PKA overcomes PKC-mediated activation of CREB. As a net effect, CRE is activated. Morphine does not activate CREB via blockade of PKA overcomes PKC-mediated activation of CREB. As a net effect, CRE is activated. Morphine does not activate CREB. Inhibition of CREB via blockade of PKA does not mask PKC-mediated activation of CREB. As a net effect, CRE is activated. Morphine does not activate CREB and inhibits PKA activity. Inhibition of CREB via blockade of PKA does not mask PKC-mediated activation of CREB. As a net effect, CRE is activated. Morphine does not activate CREB and inhibits PKA activity. Inhibition of CREB via blockade of PKA overcomes ERK-1/2-mediated activation of CREB. As a net effect, CRE is inhibited. However, morphine-promoted ERK-1/2 activation leads to the activation of the ELK-1/SRF complex and thus to the induction of genes containing TCF/SREs in their promoter regions, for instance, EGR-4.
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Authorship Contributions

Participated in research design: Rothe, Boekhoff, Gudermann, and Breit.

Conducted experiments: Rothe, Solinski, and Breit.

Contributed new reagents or analytic tools: Breit.

Performed data analysis: Rothe, Boekhoff, and Breit.

Wrote or contributed to the writing of the manuscript: Gudermann and Breit.

References


