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EGR3 regulates opioid-related nociception and motivation in male rats

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Abstract

Chronic pain can be a debilitating condition, leading to profound changes in nearly every aspect of life. However, the reliance on opioids such as oxycodone for pain management is thought to initiate dependence and addiction liability. The neurobiological intersection at which opioids relieve pain and possibly transition to addiction is poorly understood. Using RNA sequencing pathway analysis in rats with complete Freund's adjuvant (CFA)-induced chronic inflammation, we found that the transcriptional signatures in the medial prefrontal cortex (mPFC; a brain region where pain and reward signals integrate) elicited by CFA in combination with oxycodone differed from those elicited by CFA or oxycodone alone. However, the expression of *Egr3* was augmented in all animals receiving oxycodone. Furthermore, virus-mediated overexpression of EGR3 in the mPFC increased mechanical pain relief but not the affective aspect of pain in animals receiving oxycodone, whereas pharmacological inhibition of EGR3 via NFAT attenuated mechanical pain relief. *Egr3* overexpression also increased the motivation to obtain oxycodone infusions in a progressive ratio test without altering the acquisition or maintenance of oxycodone self-administration. Taken together, these data suggest that EGR3 in the mPFC is at the intersection of nociceptive and addictive-like behaviors.

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Author contributions

S.M., S.A.T., J.A.S., J.X.L., and D.M.D. designed experiments. S.M., S.A.T., J.A.S., M.H., J.C., M.I., and K.W. conducted behavioral experiments. J.A.S. and J.W. generated tissues for RNA sequencing. F.J.S. helped with RNA sequencing analysis. M.K.L. and R.C. generated and provided custom viral constructs. S.M. and S.A.T. performed statistical analyses. S.M. wrote the manuscript with review from D.M.D.

Competing interests

The authors declare no competing interests.

Keywords

Self-administration; Pain; Progressive ratio; Oxycodone; Motivation

Introduction

Chronic or persistent recurring pain has become an enormous global health crisis that incurs profound financial and societal burdens[1,2]. Pain encompasses unpleasant sensory and affective experiences, which often result in restricted activity, opioid dependence, anxiety, depression, and a poorer quality of life[3,4]. Cognitive and emotional appraisal are dysregulated in individuals with chronic pain, concomitant with a heightened negative assessment of pain. The alleviation of pain associated with aversive stimuli can therefore reinforce behaviors that promote the transition to addiction[5]. For example, opioids such as oxycodone are effective therapeutics for analgesia and pain management[6] but can lead to maladaptive neuroplasticity in the mesocorticolimbic dopamine system, resulting in abuse liability[7]. The neurobiological basis of the relationship between antinociception and susceptibility to addiction is not well understood and warrants further examination.

The brain's reward center, including the prefrontal cortex (PFC) in the mesocorticolimbic dopamine system[8], is highly relevant in the context of the affective-motivational need for pain relief. The medial prefrontal cortex (mPFC) continually undergoes transcriptional and morphological plasticity, which underlies the enduring behavioral adaptations in response to chronic pain and opioid exposure[9–13]. Furthermore, neuronal ensembles in the mPFC encode learned associations of drug-paired contexts[14], making it an important region where pain processing and reward mechanisms overlap.

The sensory component of pain is measured through mechanical and thermal thresholds for a painful stimulus whereas affective pain is the aversive, motivational, or emotional aspect of pain[15–18].The sensory dimension identifies the physical characteristics of the noxious stimuli (mechanical stimuli) prompting withdrawal reflexes. On the contrary, the affective-motivational dimension of pain evaluates the emotional component of pain by linking unpleasantness with the noxious stimulus triggering defensive or coping behaviors[19]. Importantly, even if a painful stimulus can elicit similar degrees of pain sensation, they can engage different degrees of pain affect[20]. Additionally, the sensory processing of mechanical pain engages divergent neural substrates and pathways in comparison to the affective dimension of pain[11]. Thus, understanding these varied aspects of pain processing is critical for devising effective pharmacological and psychosocial therapeutic measures against nociception. The mPFC is a critical neural substrate that transforms sensory stimulus and encodes chronification of pain in addition to its association with affective pain processing due to its amygdalar connections[11]. Despite being ostensibly used to study nociception in rodent models, the neural mediators that transduce mechanical nociception remain stubbornly elusive[21] and thus, warrant further investigation in implicated neural substrates.

Early growth response 3 (EGR3) is a master transcriptional regulator expressed in the mPFC and other brain regions. EGR3 typically exhibits an activity-dependent expression profile

and is upregulated by cellular intermediates such as neuregulin1, calcineurin, *N*-methyl-D-aspartate receptors, and neurotrophins[22–26]. In turn, EGR3 modulates numerous downstream targets that regulate processes such as synaptic plasticity, axonal and dendritic extension, and receptor function[25,27], all of which are involved in drug-induced neuroadaptations. EGR3 has therefore been implicated in various psychiatric disorders, and there is emerging evidence that it governs drug-induced maladaptive plasticity[28–31]. How EGR3 in the mPFC regulates the opioid-induced transcriptional programming common to pain processing is currently unknown.

Here, we demonstrate that oxycodone elicits unique transcriptional signatures in the mPFCs of rats with or without complete Freund's adjuvant (CFA)-induced inflammatory pain. According to a top-down analysis of transcriptional profiling that identified gene alterations under various treatment conditions, we posit that EGR3 is a master transcriptional regulator underlying oxycodone-induced transcriptomic change. We also show that viral and pharmacological manipulation of EGR3 in the mPFC regulates behavioral plasticity pertaining to discrete nociceptive and reward domains.

Materials and Methods

Animals

Male Sprague-Dawley rats (Envigo Laboratories, Indianapolis, IN) weighing 250–275 g were used for all experiments. Animals were housed at 22–25 °C under a 12:12-h reverse light-dark cycle with access to food and water *ad libitum*. All behavioral testing was conducted during the dark cycle. All experiments were conducted in accordance with the Institutional Animal Care and Use Committee (IACUC) of the State University of New York at Buffalo.

Drugs

Oxycodone (generously gifted from the NIDA drug supply program) dissolved in 0.9% sterile saline was administered cumulatively at doses of 0.1, 0.32, 1.0, and 3.2 mg/kg body weight for Von Frey tests. Oxycodone was administered at a dose of 1 mg/kg for place escape avoidance paradigm and locomotor tests, whereas 0.15 mg/kg/infusion (inf) was used for self-administration. To ensure the appropriate dose was used for each animal injection, volumes were adjusted according to the rat's body weight. For self-administration, oxycodone was delivered by syringe pumps, and injection volumes were adjusted daily according to body weight[32].

Cyclosporin (CYSP) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in saline containing 2% dimethyl sulfoxide at a dose of 4 μg/μl, which has been shown to inhibit calcineurin activity. Control animals received 2% dimethyl sulfoxide dissolved in saline (vehicle). CFA was purchased from Thermo Fisher Scientific (Waltham, MA) and dissolved in paraffin oil.

Inflammatory pain induction

To induce inflammatory pain, 0.1 ml of CFA (containing killed *Mycobacterium butyricum*)[33] dissolved in paraffin oil was injected subcutaneously into the rear portion of the plantar side of the right hind paw of each rat under isoflurane anesthesia (2% isoflurane mixed with 100% oxygen at flow rate of 5 L min^{-1}). Rats that served as controls for the inflammatory pain condition were injected with 0.1 mL of sterile saline.

Viral-mediated gene expression

Rats received bilateral intracranial injection of volume-to-volume mixture of AAV-Cre (serotype 2) and a double-floxed inverted open reading frame (DIO) Cre-dependent adeno-associated virus (AAV-serotype 2) vector to express EGR3 fused to EYFP (referred to as AAV-EGR3 in the future). EF1a-DIO vector backbone was obtained from Karl Deisseroth lab. EGR3 was cloned into the DIO framework in AAV serotype 2 (5.1×10^2 genomic particles per μL) by the vector core at UNC Chapel Hill and was provided by the lobo lab. Control rats received AAV-Cre-GFP (referred to as AAV-GFP). Injectors were set at 10° with coordinates described previously[32,34]: AP, +3.2 mm; ML, +1.3 mm; DV, −3.2 mm. Viruses were manually infused over 5 min (0.2 μL/min, total infusion volume of 1 μL), and the needles remained in place for 10 min to ensure diffusion of the AAV. Animals were allowed 14 days for maximal viral expression and recovery before testing. The viral overexpression of EGR3 was timed to mimic the potentiated EGR3 expression in the mPFC observed in rats administered with chronic oxycodone and to mimic the modulation of pain perception in both mechanical and affective pain paradigms. All rats with EGR3 overexpression received CFA for inflammatory pain induction and were assessed for mechanical pain, affective pain, and locomotor activity. For progressive ratio behavior tests (described below), rats underwent viral surgical procedures and received either Cre-inducible AAV-EGR3 or AAV-GFP in the mPFC 14 days prior to jugular catheterization (to ensure maximal expression) to assess active drug taking and motivation behaviors.

Bilateral cannulation and microinjections

To pharmacologically inhibit EGR3, bilateral guide cannulae (C235GS, Plastics One) targeting the mPFC were placed with the following coordinates relative to bregma: AP, +2.9 mm; ML, ±0.6 mm; DV, −3 mm. Animals were then handled daily during the recovery phase to habituate them to the microinjection procedure. Animals were randomly assigned to receive either CSPA (4 μg/μL) or vehicle (2% dimethyl sulfoxide dissolved in saline); 0.5 μL per side was injected bilaterally into the mPFC for 1 min (rate of 0.5μL/min) and then allowed to diffuse for 10 min. Microinjections were repeated daily for 3 days to ensure detection of long-term transcriptional changes. Von Frey, place escape avoidance, and locomotor tests were performed 24 h after the last infusion.

Von Frey test

Weighted von Frey filaments (4, 6, 8, 10, 15, and 26 g; North Coast Medical, Morgan Hill, CA) were used to measure mechanical hyperalgesia[35]. Rats were placed in plastic chambers on a grid (IITC Life Science) that provided access to the bottoms of the rats' hind paws. Von Frey filaments, in order of ascending weight, were applied perpendicular to the plantar

surface of the hind paw from below the grid floor. A withdrawal of the paw from the applied force was considered a positive behavioral response. The mechanical threshold (percent maximal effect) was identified as the lowest force that evoked a positive behavioral response in at least two of three applications to the hind paw. Von Frey responses were measured prior to drug administration, 20 min after each drug dose was delivered, and immediately before the next drug treatment. The test continued until the percent maximal effect at the 26-g filament was reached. Forces greater than 26 g would physically lift an untreated paw and were not considered.

Place escape avoidance paradigm

Place escape avoidance was used to assess the affective dimension of pain because this paradigm takes into account the past pain felt, the context of pain, and anticipation of future unpleasantness—all of which are vital aspects of the affective-motivational dimension of pain[36]. Briefly, rats were placed on a mesh grid, for experimenter access, and a cage painted half black and half white was placed atop the grid, housing the rat. The right inflamed paw was poked when the rat was on the black side of the chamber, whereas the left paw was poked when the rat entered the white side. The aversive (black chamber) or non-aversive (white chamber) stimulus was administered every 15 s for 30 min to emulate “chronic pain” as previously described[37]. Percent time spent on the white side of the chamber was calculated (white side stimulus/total number of stimuli ´ 100) and used as an indicator of escape/avoidance learning. Oxycodone was administered 20 min before the testing session.

Locomotor activity

An infrared motion-sensor system (AccuScan Instruments, Inc., Columbus, OH) fitted outside transparent plastic cages with Versa Max animal activity software (Omnitech Electronics, Inc., Columbus, OH) was used to measure locomotor activity. Total distance traveled during the 1h test was used as a measure of overall locomotion[38].

Jugular catheterization and patency testing

All rats were implanted with chronic indwelling jugular catheters, as previously described[32]. Briefly, rats were anesthetized using ketamine and xylazine (60 and 5 mg/kg, respectively, intraperitoneally), the right jugular vein was isolated, and the catheter was inserted. The other end of the catheter was fitted to a vascular access harness (Instech, PA). Rats were allowed 5 days to recover from the surgical procedure, and catheter patency was preserved by flushing them daily with 0.2 mL of enrofloxacin (4 mg mL^{-1}) in heparinized saline (50 IU mL^{-1} in 0.9% sterile saline). One day prior to self-administration training, catheter patency was confirmed by loss of muscle tone and righting reflex following an intravenous infusion of ketamine hydrochloride (0.5 mg/kg in 0.05 mL). Only rats with patent catheters were used in behavioral studies.

Oxycodone self-administration and progressive ratio after EGR3 overexpression

Rats were trained to self-administer oxycodone (0.15 mg/kg/inf) for 3 h each day for 10 days in a soundproof operant chamber equipped with active and inactive nose pokes[32]. Responses in the active nose poke resulted in an infusion of oxycodone at fixed ratio 1

that was incrementally increased by 1 each day for 10 days to fixed ratio 10. Responses in the inactive nose poke resulted in no programmed consequences. Following training, the animals were allowed to self-administer oxycodone on a progressive ratio schedule of reinforcement for a maximal session duration of 6 h (day 11). The session for each animal ended if the animal failed to earn an infusion within the last 1 h. The schedule of delivery progressively increased after each infusion in a schedule of 1, 2, 3, 4, 6, 8, 12, 24, 32, 48, etc.[32,39]. After testing, the catheters were flushed with heparin-saline, and the animals were returned to the colony room.

RNA sequencing

Rats received either saline or CFA in their right hind paws and were administered saline or oxycodone (1 mg/kg, intraperitoneally) for 7 days. Thirty minutes after the last drug treatment, animals were killed and the mPFC tissues were collected with 2mm biopsy punches. RNA sequencing was performed as previously described[32]. Briefly, RNA libraries were generated using the Illumina TruSeq RNA library preparation kit, and sequencing was performed on the Illumina HiSeq 2500 system with a 50-cycle single-end flow cell. The RN5 (rat) genome was used for alignments using the TopHat (version 2.0.13) alignment algorithm and the UCSC refGene annotation set. For differential expression analysis, Cuffdiff with default parameters was utilized.

Quantitative PCR

mRNA from tissue punches (2mm punches obtained from the mPFC region) was isolated and purified using TRIzol (Ambion, Austin, TX) and the MicroElute total RNA kit (Omega Bio-tek Inc., Norcross, GA). RNA concentrations were measured on a NanoDrop, and 500 ng was reverse transcribed into cDNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). Reaction mixtures were prepared with iQ SYBR Green Supermix (Bio-Rad Laboratories) and run on an iQ5 system (Bio-Rad Laboratories). Reactions were run in triplicates, and expression was quantified using a relative threshold cycle method with *Gapdh* as a housekeeping gene. The primer sequences are as follows: *Gapdh*, 5′-AACGACCCCTTCATTGAC-3′ (forward) and 5′-TCCACGACATACTCAGCA-3′ (reverse); *Egr3*, 5′-ATGGCTACAGAGAATGTGATGGA-3′ (forward) and 5′-TGGAAGGAGAGTCGAAAGCG-3′ (reverse).

Immunoblotting

2mm mPFC tissue punches of each rat were homogenized with 50 μL of RIPA buffer containing proteinase and phosphatase inhibitors. Protein concentrations were determined by Bradford protein assay. Immunoblotting was performed by loading 30 μg of protein per lane into 4–20% gradient Tris-SDS polyacrylamide gels. After electrophoresis, proteins were then transferred to nitrocellulose membranes and blocked with Rockland buffer. Membranes were incubated in primary antibody (mouse anti-Egr3, 1:300; Santa Cruz Biotechnology, Dallas, TX) diluted in Rockland blocking buffer overnight at 4 °C. Membranes were incubated with antibodies to GAPDH (1: 10,000; Cell Signaling Technology, Danvers, MA) for 1 h at room temperature (22–25 °C) to ensure equal protein loading. Membranes were then washed in Tris-buffered saline with 0.1% Tween-20 and incubated with appropriate

IRDye secondary antibodies (1: 5,000; LI-COR, Inc., Lincoln, NE) diluted in Rockland blocking buffer at room temperature for 1 h. Membranes were imaged on a LI-COR system and quantified by densitometry analysis with ImageJ software (National Institutes of Health, Bethesda, MD). Expression in each lane was normalized to its loading control and treatment condition.

Data analysis

All statistical analyses were performed with Prism (GraphPad Software Inc., La Jolla, CA). Either a Student's *t* test or two-way ANOVA was used to determine significance followed by Tukey's *post hoc* analysis as appropriate. Significance was defined as a two-tailed *P* value of <0.05, and data are presented as means and standard errors.

Results

Oxycodone elicits unique transcriptional signatures in the mPFC

RNA sequencing was performed on mPFC tissues from rats with or without chronic inflammatory pain induced by CFA (Fig. 1a). A total of 145 genes were significantly modulated in the chronic inflammatory state. There was an upregulation of biological processes involved in the ensheathment of axons/neurons, myelination, glial cell development, and oligodendrocyte differentiation (Fig. 1b). Administration of oxycodone to animals with chronic inflammation significantly altered 326 genes, with an upregulation in the expression of genes involved in cellular communication and synaptic signaling and a downregulation in processes pertaining to myelination and neuron ensheathment (Fig. 1c). By contrast, administration of oxycodone by itself altered the expression of 132 genes, including those involved in responses to endogenous stimuli, metabolism, cell differentiation, and cellular development (Fig. 1d). The mRNA transcript profile for oxycodone administered to animals in a state of chronic pain revealed a general suppression of genes in processes related to cell differentiation, tissue development, the MAPK pathway, metabolism, and behavior (Fig. 1e). In total, 54 genes were uniquely altered between the oxycodone and in CFA with oxycodone treatments (Table 1).

Oxycodone upregulates *Egr3* in the mPFC

Notably, oxycodone increased *Egr3* expression irrespective of the inflammatory state. Oxycodone administration to animals that received CFA or saline increased in the amount of *Egr3* expressed as FKPM (two-way ANOVA: pain effect, $F_{(1,32)} = 0.9979$, $P > 0.05$; drug effect, $F_{(1,32)} = 22.01$, $P < 0.001$; interaction, $F_{(1,32)} = 0.08879$, $P > 0.05$, $n = 9$/group) and fold change (two-way ANOVA: pain effect, $F_{(1,32)} = 1.626$, $P > 0.05$; drug effect, $F_{(1,32)} = 18.81$, $P < 0.002$; interaction, $F_{(1,32)} = 1.070$, $P > 0.05$, $n = 9$/group) (Fig. 1f, g).

EGR3 overexpression in the mPFC potentiates the effect of opioids on mechanical pain without altering affective pain

To examine how EGR3 affects oxycodone-induced antinociception, we performed a Von Frey test for mechanical analgesia in animals with EGR3 overexpression in the mPFC (Fig. 2a, b). There was a significant difference in the mechanical threshold at the 1.0-mg/kg dose, suggesting that EGR3 potentiates mechanical pain relief (two-way ANOVA: drug effect,

$F_{(3,40)} = 20.78$, $P < 0.0001$; virus effect, $F_{(1,40)} = 5.470$, $P < 0.05$; interaction, $F_{(3,56)} = 1.159$, $P > 0.05$; $n = 6$/group) (Fig. 2c). No significant differences were observed between EGR3-overexpressing (AAV-EGR3-AAV) and control (AAV-GFP) rats for mechanical threshold at the 0.1-, 0.32-, and 3.2-mg/kg doses. To assess affective responses to pain, animals were evaluated in the place escape avoidance paradigm; a locomotor test was used as a behavioral control. EGR3 overexpression did not alter the preference for the white side (non-painful stimulus-paired side) of the place escape avoidance chamber ($t_{10} = 1.540$, $P > 0.05$; $n = 6$/group) or overall locomotion ($t_{10} = 0.8506$, $P > 0.05$; $n = 6$/group) (Fig. S1a, b)

Pharmacological inhibition of EGR3 in the mPFC attenuates the effect of opioids on mechanical pain

To test whether EGR3 bidirectionally affects oxycodone-induced antinociception, we pharmacologically inhibited NFAT, an upstream regulator of EGR3[40], by bilaterally injecting CYSP, a calcineurin/NFAT inhibitor, into the mPFC (Fig. 2d). CYSP prevents NFAT from translocating to the nucleus and activating EGR3. Intra-mPFC inhibition of EGR3, through NFAT, attenuated the relief of mechanical hyperalgesia elicited by 3.2 mg/kg oxycodone (two-way ANOVA: drug effect, $F_{(3,56)} = 21.07$, $P < 0.0001$; virus effect, $F_{(1,56)} = 8.216$, $P < 0.05$; interaction, $F_{(3,56)} = 1.159$, $P > 0.05$; $n = 8$/group) (Fig. 2e). CYSP did not affect the preference for the white side of the place escape avoidance chamber ($t_{(14)} = 0.6079$, $P > 0.05$) or overall locomotion ($t_{(14)} = 1.1085$, $P > 0.05$) (Fig. S1c, d), suggesting that suppression of EGR3 activity does not influence affective pain. The downregulation of EGR3 via NFAT inhibition was confirmed by quantitative PCR ($t_{(11)} = 2.057$, $P = 0.03$) and Western blotting ($t_{(11)} = 2.969$, $P = 0.01$) (Fig. 2f, g)

EGR3 overexpression increases the rewarding effects of oxycodone

To determine if EGR3 alters the rewarding effects of opioids, we employed a volitional model of oxycodone self-administration (Fig. 3a). Virus-mediated overexpression of EGR3 in the mPFC did not alter the acquisition of oxycodone self-administration, according to the mean number of infusions of 0.15 mg/kg oxycodone ($F_{(9, 180)} = 0.5802$, $P > 0.05$; $n =$ 10/group) (Fig. 2b). However, EGR3 overexpression in the mPFC increased the total number of infusions earned ($t_{(18)} = 2.164$, $P = 0.04$) and the breakpoint ($t_{(18)} = 2.24$, $P = 0.03$) during a 6-h progressive ratio test (Fig. 3c, d), indicating that EGR3 regulates the motivational domain of reward processing.

Discussion

Opioids are currently amongst the most commonly prescriped drugs for pain relief and analgesia[6,41]. However, because they modulate the mesolimbic dopamine system, repeated use of opioids can lead to dependence and addiction[42,43]. The idea that chronic pain can induce affective-emotional behaviors implicates pain in reward processing, learning, and goal-oriented functions. Many studies have examined alterations in gene expression caused by chronic pain[44–46] or drugs of abuse[47,48], but potential transcriptomic changes from exposure to oxycodone in a state of chronic inflammatory pain is not well characterized. Here, we elucidate a comprehensive understanding of the distinct transcriptomic signatures unique to oxycodone exposure in the presence or absence of chronic inflammatory pain,

revealing the transcriptional regulation at the intersection of oxycodone exposure and oxycodone exposure during pain.

The expression of genes involved in oligodendrocyte differentiation and myelination was elevated by inflammation-induced nociception, highlighting the important role of oligodendrocytes in the nociceptive state. The differentiation and activity of oligodendrocyte precursor cells is increased by neural activity and experiences[49], whereas dysregulated function of these cells is associated with decreased motor activity[50–52], depression-like behavior[53], and neuroinflammatory responses[54,55]. Prior studies showed that opioids such as methadone and buprenorphine increase the expression of myelin-related genes in early development[56,57]. Furthermore, oligodendrocyte precursor cell differentiation and transcripts for myelination increase with heroin self-administration[32]. Conversely, there was a decrease in genes related to myelination and glial cell differentiation in rats with CFA-induced chronic inflammation such that the expression of genes upregulated by chronic pain was reversed by oxycodone, showing that the opioid can counter the effects of the nociceptive state at the transcriptional level. Oxycodone administered to animals with chronic inflammation also upregulated genes associated with cell-to-cell communication and synaptic signaling. However, exposure to oxycodone in the absence of inflammation upregulated genes in processes that play vital roles in synaptic plasticity[58], memory formation[59], glutamatergic receptor regulation[60], signal transduction, cell differentiation, and responses to environmental stress[61], representing a transcriptomic profile that may be unique to oxycodone only exposure[62,63].

Preclinical and clinical evidence demonstrate that stressful stimuli, including pain, activate EGR proteins[64–67]. Immediate early genes such as *Egr3* underlie gene-environment interactions and mediate neuronal activity underlying higher-order processes such as learning, cognition, and reward mechanisms[31]. EGR3 has also been implicated in modulating drug-induced maladaptive plasticity that is influenced by sex and cell-type specificity[29,30]. However, it is not clear how EGRs regulate the mechanical or emotional aspects of pain transmission or how such regulation intersects reward and motivation. We focused on EGR3 because it influences immunomodulation, synaptic plasticity, cellular differentiation, and glial cell dynamics, which are modified by oxycodone treatment, making it a suitable candidate for investigation underlying nociception and reward modalities. We found that overexpression of EGR3 in the mPFC increased the mechanical pain threshold, whereas inhibition of EGR3 decreased it. This is consistent with the dual role of mPFC in pain processing, both exerting top-down control and receiving nociceptive inputs[68–71]. For example, neuromodulation of this region attenuates mechanical pain[72], and mPFC projection neurons regulate pain threshold[73].

The mPFC region is also heavily involved in encoding the value of reward and action-outcome associations[74,75] through its modulatory effects on inhibitory control, a phenomenon that is disrupted in pain processing and drug addiction[11,76]. Overexpression of EGR3 in the mPFC increased the motivation to self-administer oxycodone in a progressive ratio schedule of delivery, which is a classic method to evaluate the reinforcing effects of drugs[77] and aids in parsing out the motivational aspect of addiction. A potential explanation for this finding is the regulation of glutamatergic and GABAergic synaptic plasticity by

EGR3 (by regulating the transcription of receptor subunits)[78,79], which could fine-tune the neurotransmission to and from the mPFC[11]. Furthermore, BDNF, an ubiquitous neurotrophic factor that is known to be altered by exposure to drugs of abuse and pain[80,81], regulates NFAT[82] and EGR3[24] and could be an underlying mechanism of action observed in this study. However, EGR3 also influences other neuroadaptations such as myelination and mitochondrial transcriptomics that are essential to drug-induced plasticity[32,83,84].

It is important to consider that the experiments were conducted with mPFC region that includes both prelimbic (PrL) and infralimbic (IL) cortexes. Both PrL and IL have been reported to have distinct functionalities underlying addiction and pain processing[85–91]. Thus, it could be possible that Egr3 changes observed here reflect the net change in both the regions or changes in one region over the other. Additionally, it is noteworthy that inhibition of NFAT pathway through CYSP can have widespread effects through alteration of calcium dynamics[92] and immune signaling[93] mechanisms that might have contributed to the behavioral effects.

In summary, this study identifies EGR3 as a modulator of opioid-related plasticity underlying both reward and nociceptive processing. We propose the NFAT-EGR3 pathway in the mPFC as a cellular mediator in regulating mechanical nociception and motivation. Future studies will aim to better unravel the distinct and/or common downstream targets of EGR3 through which such functional consequences are achieved. Because sex differences are thought to play a vital role in behavioral responding influenced by EGR3[30], future studies should also evaluate the effects in female rats. Our study also indicates that the transcriptomics of other immediate early genes in mesolimbic dopamine substrates should be examined to understand the plasticity events driving maladaptive behaviors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Data availability

The datasets generated during and/or analyzed during the present study are available from the corresponding author on reasonable request.

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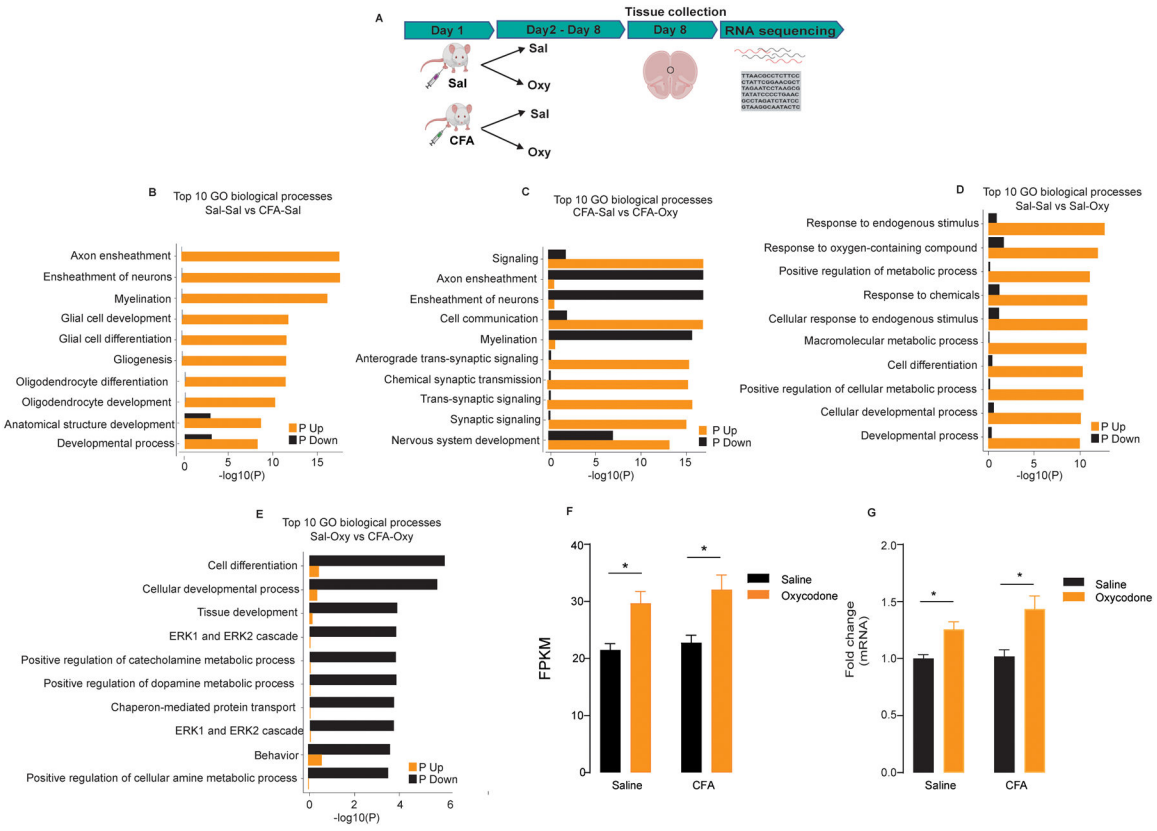

Figure 1. RNA sequencing reveals discrete transcriptomics for altered biological processes based on nociceptive induction and treatments.

(**a**) Experimental timeline for RNA sequencing of the mPFC under various treatment conditions. (**b**) *mRNA* transcript profile comparing saline-treated rats that did not receive CFA to rats that received CFA. (**c**) *mRNA* transcript profile comparing CFA-treated rats that received either saline or oxycodone. (**d**) mRNA transcript profile comparing saline versus oxycodone treatments in rats that did not receive CFA. (**e**) *mRNA* transcript profile comparing oxycodone-treated rats that did not receive CFA to rats that received CFA. (f) *Egr3* transcript changes expressed as fragments per kilobase of exon per million mapped fragments (FKPM) between CFA and saline rats that received either oxycodone or saline. (**g**) *mRNA* expression of *Egr3* expressed in fold change between CFA and saline rats that received either oxycodone or saline. All data are presented as means ± SEMs with statistical significance (*) at $P < 0.05$. * represents significant differences between oxycodone groups of saline and CFA with their respective controls (saline + saline and saline + CFA)

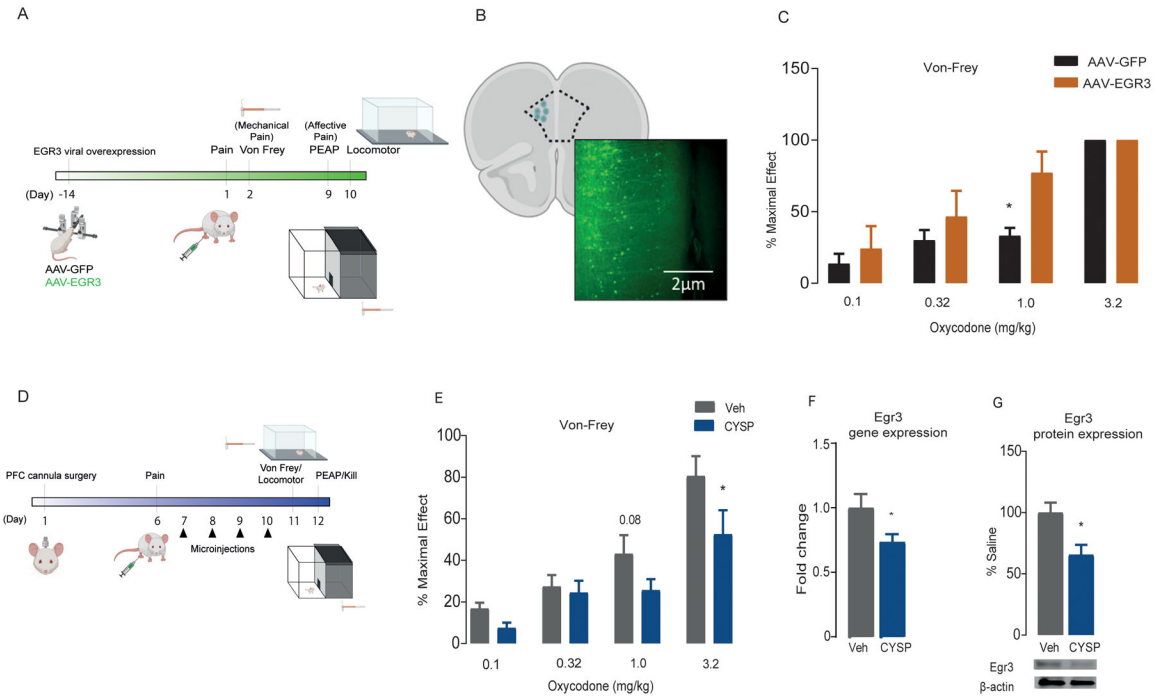

Figure 2. EGR3 in the mPFC regulates mechanical nociception.

(**a**) Experimental timeline for EGR3 overexpression in the mPFC followed by nociceptive and locomotor behavior tests. (**b**) Anatomical placement of viral infection with AAV-cre in combination with AAV-DIO-EGR3 (referred to as AAV-EGR3) and representative image (10x) of AAV infection in the mPFC. (**c**) Percent maximal effect determined through Von Frey filament test for mechanical hyperalgesia at increasing doses of oxycodone in rats that were microinjected in the mPFC with AAV-GFP or AAV-EGR3. * represents significant differences between AAV-GFP and AAV-EGR3 in % maximal effect at 1 mg/kg dose (**d**) Experimental timeline for bilateral CYSP injections into the mPFC followed by nociceptive and locomotor behavior tests. (**e**) Percent maximal effect determined through Von Frey filament test for mechanical hyperalgesia at increasing doses of oxycodone in vehicle- and CYSP-microinjected rats. * represents significant differences between vehicle and CYSP in % maximal effect at 3.2 mg/kg dose EGR3 gene (**f**) and protein (**g**) expression in the mPFC in vehicle and CYSP-microinjected rats. * represents significant differences between vehicle and CYSP in EGR3 expression. All data are presented as means ± SEMs with statistical significance (*) at $P < 0.05$.

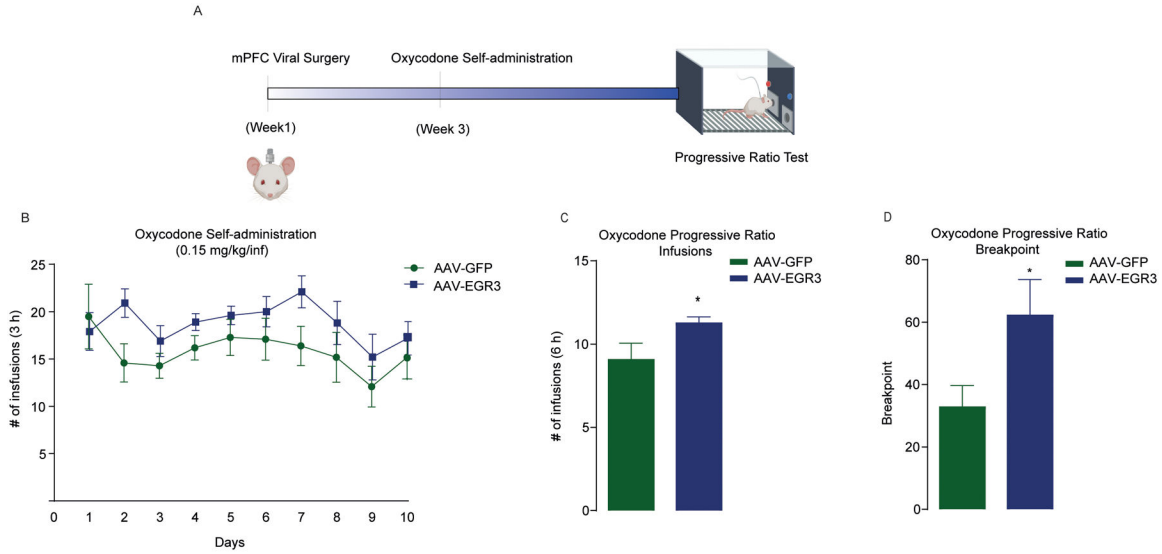

Figure 3. EGR3 overexpression in the mPFC increase the rewarding effects of oxycodone.

(**a**) Experimental timeline for EGR3 overexpression in the mPFC followed by oxycodone self-administration and progressive ratio behavior. Numbers of infusions (**b, c**) and breakpoints (**d**) in a 6-h progressive ratio test in rats microinjected with AAV-GFP or AAV-EGR3. * represents significant differences between AAV-GFP and AAV-EGR3 in number of infusion and breakpoint. All data are presented as means ± SEMs with statistical significance (*) at $P < 0.05$.

Table 1:

List of uniquely altered genes between oxycodone and oxycodone with CFA treatment.

Genes	P-Value	FDR
Chchd10	7.81E-18	1.01E-13
Tbcb	8.57E-14	5.57E-10
Sepw1	5.66E-13	2.45E-09
Cetn3	3.07E-12	9.97E-09
Echs1	2.01E-10	5.22E-07
Clip3	2.87E-10	6.22E-07
Scn4b	3.36E-10	6.25E-07
Sulf1	4.05E-10	6.59E-07
Apold1	6.85E-09	9.89E-06
Myh11	2.50E-08	3.24E-05
Sccpdh	2.96E-08	3.50E-05
Mx2	4.77E-08	4.91E-05
Mif	4.91E-08	4.91E-05
Acta2	6.31E-08	5.86E-05
Cyr61	6.88E-08	5.96E-05
Hapln4	2.53E-07	0.000205
Cdhr1	3.05E-07	0.000233
Grcc10	4.00E-07	0.000289
Col11a2	9.59E-07	0.000656
Chid1	2.20E-06	0.001432
Vwa5b2	3.30E-06	0.001989
Fcho1	3.37E-06	0.001989
Col27a1	6.02E-06	0.003386
Hsp90aa1	6.25E-06	0.003386
Ucma	1.23E-05	0.006393
Stac2	1.28E-05	0.006393
Foxp2	1.48E-05	0.006987
Vwa3a	1.52E-05	0.006987
Brd2	1.56E-05	0.006987
Syt2	1.77E-05	0.00769
Miat	1.88E-05	0.007878
Btg2	2.10E-05	0.00855
Tagln	2.30E-05	0.009071
Hprt1	2.42E-05	0.00926
Scn1b	2.62E-05	0.00975
LOC690918	3.50E-05	0.012643
Ube2b	3.61E-05	0.01268

Genes	P-Value	FDR
Ptgds	4.00E-05	0.013698
Rn5-8s	4.74E-05	0.015446
Atf3	4.85E-05	0.015446
Ndufa1	4.87E-05	0.015446
Chrna5	6.03E-05	0.018652
RT1-M5	7.82E-05	0.023659
RGD1562161	8.35E-05	0.024275
RGD1566401	8.40E-05	0.024275
Kcnv2	0.000118	0.033238
Nnat	0.000128	0.035342
Nxph3	0.000159	0.043066
Kcnk4	0.000172	0.0456
Anxa6	0.000176	0.0458
Tyrp1	0.000186	0.047388
Npy	0.000193	0.047388
Aldoart2	0.000194	0.047388
Cdh1	0.000197	0.047388