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Escalated oxycodone self-administration causes differential striatal mRNA expression of FGFs and IEGs following abstinence-associated incubation of oxycodone craving

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Abstract

Addiction to prescribed opioids including oxycodone has reached tragic levels. Herein, we investigated the relevance of fibroblast growth factors (FGFs) and immediate early genes (IEGs) to withdrawal-induced incubation of drug craving following escalated oxycodone self-administration (SA). Rats were trained to self-administer oxycodone for 4 weeks. Seeking tests were performed at various intervals during one month of drug withdrawal. Rats were euthanized one day after the last test and nucleus accumbens and dorsal striata were dissected for use in PCR analyses. Rats given long access (LgA, 9 hours), but not short access (ShA, 3 hours), to drug escalated their oxycodone intake and exhibited incubation of oxycodone seeking during withdrawal. These rats exhibited dose-dependent increases in *fgf2* expression in the dorsal striatum. *Fgfr2* expression was also significantly increased in the striatum in LgA, but not ShA, groups. Similarly, striatal *c-fos* and *junB* mRNA levels showed greater increases in LgA rats. The observations that FGF mRNA levels were more altered in the dorsal striatum than in the NAc of LgA rats suggest that changes in striatal FGF expression may be more salient to incubation of oxycodone craving than alterations in the NAc. Targeting FGF signaling pathways might offer novel strategies against opioid addiction.

Keywords

Opioids; Addiction; Dorsal Striatum; Incubation; Relapse; glia

Introduction

Oxycodone is a semisynthetic agent that is prescribed to patients with chronic moderate to severe pain related to cancer and some neurological disorders (Gaskell et al., 2016; Riley et al., 2008; Schmidt-Hansen et al., 2017). Oxycodone behaves like morphine as an analgesic drug, but it exhibits better bioavailability and a longer half-life (Lugo and Kern, 2004;

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Author Contributions

C.A.B., M.T.M., and M.L. conducted behavioral experiments. C.A.B, M.L., and A.S. performed qRT-PCR experiments. C.A.B and J.L.C wrote the manuscript. J.L.C. supervised the overall project.

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Olkkola et al., 2013; Poyhia et al., 1991; Poyhia et al.; Ruan et al., 2017), making it somewhat more clinically efficacious (Kalso et al., 1991). During the past few years, addiction to prescribed opioids has reached tragic proportions, with many deaths throughout the world attributed to accidental opioid overdoses (Dart et al., 2015; Roxburgh et al.). Many opioid abusers reach those high levels of use because of attempts to combat tolerance to the drug used by increasing the number of tablets that they consume (Ellis et al., 2018), by progressing to inhalation or intravenous routes (Gasior et al., 2016; McCabe et al., 2007), or by switching to more powerful opioids including heroin (Mars et al., 2014; Pouget et al., 2018).

Treatment of opioid-addicted populations can be effective (Ayanga et al., 2016; Stuart et al., 2018) but it has also been fraught with problems including repeated relapses (Lopez-Goni et al., 2014; Nunes et al., 2018; Stuart et al., 2018). To develop more efficacious therapeutic approaches, it is imperative to improve our understanding of the neurobiological underpinnings of addiction to opioid drugs. Therefore, we have begun to use the model of oxycodone self-administration in rats (Blackwood et al., 2018) to query potential molecular and biochemical consequences of opioid exposure. Our behavioral results are consistent with those of other investigators who have reported that rats will escalate their oxycodone intake (Bossert et al., 2018; Mavrikaki et al., 2017; Wade et al., 2015). In addition, we recently found that incubation of oxycodone seeking was related to decreased expression of mu opioid receptors in the dorsal striatum (Blackwood et al., 2018).

In the present study, we investigated the potential role of growth factors and immediate early genes in rats that had shown incubation of oxycodone craving following 4 weeks of forced abstinence from escalated drug self-administration (Blackwood et al., 2018). We focused our attention on the role of fibroblast growth factors (FGFs) (Guillemot and Zimmer, 2011; Ornitz and Itoh, 2015; Reuss and von Bohlen und Halbach, 2003) that, unlike BDNF (Li et al., 2017; Ornell et al., 2018), have not been extensively investigated in addiction models (Doncheck et al., 2018; Even-Chen and Barak, 2018; Flores et al., 1998; Flores and Stewart, 2000). FGFs constitute a family of peptides that influence diverse biological processes such as neuronal proliferation and differentiation in the central nervous system (CNS) (Guillemot and Zimmer, 2011; Ornitz and Ito, 2015; Reuss et al., 2003). Their actions are mediated by binding to transmembrane receptors whose activation occurs through autophosphorylation and activation of intracellular signaling pathways with secondary changes in gene expression (Ornitz and Ito, 2015). These FGF receptors are located in brain regions including the ventral and dorsal striata that are known to be involved in reward pathways (Asai et al., 1993; Fon Tacer et al., 2010; Itoh et al., 1994; Wanaka et al., 1990; Yazaki et al., 1994).

Herein we report that incubation of oxycodone craving during withdrawal from long access to oxycodone self-administration is associated with differential changes in the expression of some FGFs and their receptors in the ventral and dorsal striata of rats. The changes were also more prominent in the dorsal striatum, suggesting that the expression of FGFs in that brain structure may be more relevant to incubation of oxycodone seeking.

Materials and Methods

Subjects

Male Sprague-Dawley rats, (Charles River, Raleigh, NC, USA) weighing 350–400 g before surgery were maintained on a 12-h reversed light/dark cycle with food and water available ad libitum. All procedures were performed according to guidelines outlined in the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (eighth Edition, https://guide-for-the-care-and-use-of-laboratory-animals.pdf) and were approved by the local NIDA (National Institute of Drug Abuse) Intramural Research Program, Animal Care and Use Committee (ACUC).

Intravenous surgery and self-administration training

Animals underwent surgery for insertion of catheters in their jugular veins, essentially as previously described (Cadet et al, 2017; Blackwood et al., 2018). Subcutaneous injections of buprenorphine (0.1 mg/kg) after surgery to relieve pain and allowed the rats to recover for approximately one week before self-administration (SA) training. Rats were trained in self-administration chambers located inside sound-attenuated cabinets and controlled by a Med Associates System (Med Associates, St Albans, VT). Rats (n=38) were randomly assigned to either saline (Sal) (n=8) or oxycodone (n=30) conditions. Oxycodone-assigned rats were trained to self-administer oxycodone-HCL (NIDA Pharmacy, Baltimore, MD) using short and long access paradigms as described (Blackwood et al., 2018). Short access (ShA) rats (n=15) were trained for only one 3-h daily sessions throughout the experiment. Long access (LgA) rats (n=15) were trained to self-administer oxycodone for one 3-h daily session during the first 5 days, followed by two 3-h sessions during days 8–12, and then for three 3-h daily sessions during the last two weeks. Lever presses were reinforced using a fixed ratio-1 with a 20-s timeout accompanied by a 5-s compound tone-light cue. Rats self-administered oxycodone at a dose of 0.1mg/kg per infusion over 3.5-s (0.1 ml per infusion). At the end of each 3-hr session and at the end of the day, the tone-light cue was turned off and the levers retracted. After the last day of training rats were returned to the animal vivarium and individually housed with no access to oxycodone essentially as described previously (Cadet et al., 2017; Blackwood et al., 2018).

Oxycodone seeking tests

To perform cue-induced drug seeking tests, rats were brought back to their corresponding SA chambers on the morning of each test. Drug seeking was assessed at withdrawal day (WD) 5 and 31 under extinction conditions (no oxycodone was made available). The tests consisted of 3-h sessions (Blackwood et al., 2018).

mRNA extraction and quantitative RT-PCR

We euthanized rats 24-h after WD31 for removal of dorsal striata and nucleus accumbens. The dorsal striatum and nucleus accumbens (NAc) were then used for RNA extraction using RNeasy Mini Kit (Qiagen, Valencia, CA). Total RNA (0.5 μg) was reverse-transcribed (RT) with oligo dT primers using Advantage RT-for-PCR kit (Clontech, Mountain View, CA). RT-qPCR was performed as previously described (Cadet et al., 2017) with Roche LightCycler

480 II (Roche Diagnostics, Indianapolis, IN) using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). *B2M* or *GAPDH* were used as reference genes. The results are shown as fold changes calculated as the ratios of normalized gene expression data for oxycodone SA groups compared to the saline group. All quantitative data are presented as means ± SEM. Primer sequences are listed in Table 1. Primer sequences for *fgfr2* recognized transcript variants 1 and 2.

Statistical analyses

We used repeated-measures analysis of variance (ANOVA) to analyze behavioral data, with dependent variable being number of oxycodone infusions on training days and independent variables between subjects (saline, ShA, LgA-L, LgA-H), and within-subject SA day (training days 1–20) factors. Bonferroni post-hoc tests were used to compare reward types (SPSS version 24, IBM, Armonk, NY). Differences in lever pressing during WD3 and WD30 were analyzed by ANOVA. Fisher's PLSD post-hoc tests were used to compare group differences. Biochemical data were analyzed by one-way ANOVA followed by the Fisher's PLSD post hoc test (StatView version 4.0, SAS, to assess Cary, NC). Regression analyses were performed to detect potential correlations between the dose of oxycodone taken and mRNA expression. The null hypothesis was rejected at $p < 0.05$.

Results

Rats exposed to LgA oxycodone self-administration escalate their drug intake over time

Figure 1 shows the results of the behavioral studies. As we previously reported, rats given long access to oxycodone, but not short access to the drug, escalated their intake over a period of 4 weeks (Blackwood et al., 2018). We also found that LgA rats could be divided into two phenotypes, LgA-L and LgA-H, based on the total amount of oxycodone that they self-administered during the 4 weeks of the experiment (Blackwood et al., 2018) (Fig. 1A). Both LgA-L and LgA-H phenotypes, but not the ShA rats, showed incubation of oxycodone seeking after a month of forced abstinence (Fig. 1B).

Effects of oxycodone and withdrawal on FGF mRNA expression

Nucleus Accumbens—Figure 2 shows the effects of oxycodone SA on *fgf1* (Figs. 2A and 2B) and *fgf2* (Figs. 2B and 2D) in the nucleus accumbens (NAc). There were no significant changes in *fgf1* expression [$F(3,33) = 1.48$, $p = 0.238$] (Figs. 2A and 2B). *Fgf2* expression was also not significantly affected in any of the groups [$F = 1.88$, $p = 0.154$] (Figs. 2C and 2D). The results for *fgf8* and *fgf9* are shown in Figure 3. *fgf8* mRNA expression showed a trend towards significance [$F(3, 27) = 2.78$, $p = 0.06$) (Fig. 3A), with post-hoc tests showing significant increases in the ShA oxycodone group in comparison to control and LgA-H groups. Regression analysis revealed a significant positive non-linear relationship of *fgf8* mRNA levels to the amount of oxycodone self-administered (Fig. 3B). *Fgf9* expression was significantly affected in all oxycodone rats [$F(3, 25) = 4.30$, $p = 0.014$] (Fig. 3C), with there being a significant positive correlation between *fgf9* mRNA levels and doses of oxycodone (Fig. 3D).

The effects of withdrawal from oxycodone SA on FGF receptors in the NAc are shown in Figures 4 and 5. *Fgfr1* did not show any significant changes in oxycodone-exposed rats [F (3,31) = 2.46, p = 0.08] (Figs. 4A and 4B). *Fgfr2* mRNA levels were also not significantly affected between the 3 groups of oxycodone [F (3,33) = 1.91, p = 0.15] (Fig. 4C). However, there was a significant negative correlation of *fgfr2* mRNA levels to doses of oxycodone taken (Fig. 4D). The mRNA levels of *fgfr3* [F (3,30) = 1. 70, p = 0.19] (Figs. 5A and 5B) and *fgfr4* [F (3,31) = 0.94, p = 0.43] (Figs. 5C and 5D) in the NAc were not significantly impacted by withdrawal from oxycodone SA.

Dorsal Striatum—Figure 2 also illustrates the effects of oxycodone SA on the mRNA expression of *fgf1* (Figs 2E and 2F) and *fgf2* (Figs 2G and 2H) in the dorsal striatum. Figure 2E shows no significant changes in striatal *fgf1* [F (3, 28) = 1.074, p = 0.376]. There was also no significant correlation between doses of oxycodone and *fgf1* expression (Fig. 2F). In contrast, *fgf2* mRNA levels were increased after withdrawal from oxycodone SA [F (3,26) = 5.227, p = 0.006] (Fig. 2G), with changes in mRNA expression showing significant dose-dependent increases (Fig. 2H). There were no significant changes in striatal *fgf8* expression [F (3,29) = 1.46, p = 0.25] (Figs. 3E and 3F). However, there were significant changes in the expression of striatal *fgf9* [F (3, 29) = 3.63, p = 0.024]. These were due to 57% decreases in the ShA oxycodone group (Fig. 3G). There was also only a weak positive correlation between oxycodone doses and changes in mRNA expression (Fig. 3H).

We also tested the possibility that the expression of striatal FGF receptors might be impacted by oxycodone SA and withdrawal. Figures 4 and 5 illustrate the changes on four FGF receptors (*fgfr1-fgfr4*). There were significant decreases in striatal *fgfr1* mRNA levels [F (3,30) = 5.89, p = 0.003] (Fig. 4E), with significant non-linear dose-dependent effects (Fig. 4F). In contrast, significant increases in *fgfr2* expression [F (3,26) = 4.527, p = 0.011] were observed in both LgA-L and LgA-H groups (Fig. 4G) in a dose-dependent fashion (Fig. 4F). *Fgfr3* mRNA levels were significantly increased in all oxycodone groups [F (3,29) = 4.611, p = 0.009] (Fig. 5F), with significant positive nonlinear relationship to drug doses (Fig. 4F). In contrast, there were no significant changes in *fgfr4* mRNA levels [F (3,30) = 1.75, p = 0.18)] (Figs. 4G and 4H).

Effects of oxycodone and withdrawal on IEG mRNA levels

Nucleus Accumbens—Figure 6 shows the effects of withdrawal from oxycodone SA on *c-fos* (Figs. 6A and 6B) and *fosB* (Figs. 6C and 6D) mRNA levels in the NAc. There were significant [F (3,31) = 3.35, p = 0.032] decreases in *c-fos* mRNA levels, with greater decreases (−40%) occurring in the ShA group (Fig. 6A). *FosB* mRNA levels were also significantly [F (3.31)= 8.30, p = 0.0003] decreased in all 3 oxycodone groups (Fig. 6C), with the ShA group (−45%) showing greater decreases than the LgA-L (−21%) and LgA-H (−39%) groups (Fig. 6C). The decreases in *c-fos* expression did not show any significant relationship to doses of oxycodone taken by the rats (Fig. 6B) while the changes in *fosB* expression showed non-linear relationships to doses (Fig. 6D).

Figure 7 shows the observations for the jun family of IEGs. Figure 7A shows that *c-jun* mRNA levels were significantly [F (3,31) = 12.45, p < 0.0001] decreased in the 3 oxycodone

groups after withdrawal from the drug. The ShA group showed greater decreases (−56%) than the LgA-L (−27%) and the LgA-H (−27%) groups (Fig. 7A). *JunB* mRNA expression was also significantly decreased [F (3,31) = 4.40, p = 0.01] (Fig. 7C), with the changes in ShA (−46%) and LgA-H (−33), but not LgA-L (−16%), being significant (Fig. 7C). *JunD* mRNA levels were also significantly influenced [F (3,29) = 4.81, p = 0.008] by oxycodone withdrawal (Fig. 7E). However, only the ShA group exhibited significant decreases (−45%) in *junD* expression (Fig. 7E). There was no correlation between expression of these 3 IEGs and doses of oxycodone taken (see Figs. 7B, 7D, and 7F).

Dorsal striatum—Figure 6 also illustrates the results of oxycodone withdrawal on striatal expression of *c-fos* (6E) and *fosB* (Fig. 6G) mRNAs. There were significant increases [F (3, 28) = 26.08, p < 0.0001] in *c-fos* expression, with the largest changes being observed in the LgA-L (+5.1-fold) and LgA-H (+3.3-fold). There was also a significant positive correlation between oxycodone doses and *c-fos* expression (Fig. 6F). *FosB* expression was also increased [F (3,30) = 4.21, p = 0.013] in the oxycodone groups, with small significant positive correlation between oxycodone doses and gene expression (Fig. 6H).

In the dorsal striatum, *c-jun* expression was not significantly altered in any group [F (3,23) = 1.53, p = 0.23] (Figs 7G and 7H). In contrast, *junB* mRNA levels were significantly increased [F (3,29) = 3.95, p = 0.018] in both LgA-L (+98) and LgA-H (+99%) (Fig. 7G). There was also a positive correlation between oxycodone doses and *junB* mRNA levels (Fig. 7H). *JunD* expression was also increased [F (3,27) = 3.76, p = 0.022] (Fig. 7H), with no significant correlation between *junD* expression and oxycodone doses (Fig. 7I).

Discussion

The myriad clinical complications of opioid addiction have triggered renewed calls for better therapeutic approaches to pain syndromes and their varied consequences (Boscarino et al., 2010; Rudd et al., 2016). In order to reach these goals, elucidation of the neurobiological adaptions to exposure to these drugs is paramount. Therefore, our laboratory has recently focused its attention on identifying the biochemical and molecular effects of oxycodone exposure since this drug is prescribed by medical professionals for moderate to severe pain (Gaskell et al., 2016; Riley et al., 2008; Schmidt-Hansen et al., 2017). We recently reported that withdrawal from escalated oxycodone intake is accompanied by decreased expression of mu opioid receptor protein in the dorsal striatum of LgA-L and LgA-H rats that exhibited incubation of oxycodone seeking (Blackwood et al., 2018). In this follow-up study, we wanted to know to what extent changes observed in the expression of striatal mu opioid receptor protein might be accompanied by other molecular changes in the brains of these same rats. To do so, we compared the expression of FGFs and IEGs in the nucleus accumbens and dorsal striatum to test the possibility that there might be regional specificity in their responses to withdrawal from oxycodone self-administration. We found that: 1) *fgf2* showed dose-related increases in the dorsal striatum; 2) striatal *fgfr1* expression was decreased in the dorsal striatum; 3) *fgfr2* was increased in the dorsal striatum of all LgA rats that showed incubation of oxycodone seeking; 4) similarly, *c-fos* mRNA levels were substantially increased in the dorsal striatum of LgA rats; and 5) striatal *junB* expression was also increased in the LgA rats. In what follows, we propose that these changes may

potentially be secondary mechanisms that activate signaling pathways that drive incubation of oxycodone craving during abstinence from escalated oxycodone intake.

Previous studies have documented a potential role for BDNF in substance use disorders in humans and in animal models of addiction to drugs including opioids (Ornell et al., 2018). For example, BDNF protein expression is decreased in the NAc of heroin-injected rats (Li et al., 2017) whereas BDNF mRNA levels were increased in the locus coeruleus (LC) by repeated injections of morphine (Numan et al., 1998). Morphine treatment also increased BDNF protein levels in the frontal cortex (FC) and striatum of rats (Bachis et al., 2017). However, much less has been written about the members of the FGF family of trophic factors in relation to addiction and most studies have focused mainly on the effects of psychostimulants including cocaine and amphetamines on *fgf2* gene expression (Even-Chen and Barak, 2018).

FGFs constitute a family of, at least, 23 growth factors that are involved in important biological processes including embryonic and neural development, tissue repair, regulation of endocrine functions, and adult neurogenesis, among others (Carter et al., 2015; Guillemot and Zimmer, 2011; Imamura, 2014; Itoh, 2010; Mudo et al., 2009) FGFs exert their functions by binding to five FGF receptors (FGFR1–5) that are very structurally similar (Porta et al., 2017; Sleeman et al., 2001). FGFR1–3 are widely expressed in the brain (Asai et al.; Yazaki et al., 1994), FGFR4 shows low levels of brain expression (Fon Tacer et al., 2010; Itoh et al., 1994) while FGFR5, a soluble protein, has not been reported in rodent brains. Importantly, FGFs and their receptors are located in brain regions such as the ventral and dorsal striatum, hippocampus, and the frontal cortices (Eckenstein et al., 1994; Itoh et al., 1994; Woodward et al., 1992; Yazaki et al., 1994), brain regions that represent nodal points in reward pathways that are involved in addiction processes (Koob and Volkow, 2016). The location of FGFs and FGFRs in these structures suggests that these signaling pathways and their downstream targets might be involved in brain health and diseases including substance use disorders (SUDs). Indeed, evidence in human and animal studies has accumulated to support the idea of FGF2 involvement in major depressive disorders and anxiety (Turner et al.). Of significant importance are the observations that *fgf2*, *fgfr2*, and *fgfr3* mRNA levels are co-downregulated in the brains of human patients who suffered from major depressive disorders (MDD) (Evans et al., 2004), suggesting co-regulation of these genes in MDD patients.

As mentioned above, a role for the FGF signaling pathway in addiction has also been suggested in the case of psychostimulants focusing almost always on *fgf2* expression (Even-Chen and Barak, 2018). Specifically, repeated cocaine injections increased *fgf2* mRNA levels in the frontal cortex and striatum of adult (Fumagalli et al., 2006) and in the hippocampus of adolescent (Giannotti et al., 2013) rodents. *Fgf2* mRNA expression is also increased in the NAc following cocaine conditioned place preference (CPP) (Doncheck et al., 2018). Animals bred for greater drug seeking behaviors showed increased FGF2 expression in the dentate gyrus of the hippocampus and neonatal injection of FGF2 increased cocaine self-administration by adult rats (Turner et al., 2009). Moreover, a single *fgf2* injection on post-natal day 2 increased cocaine sensitization in rats bred for low responses for drugs (Clinton et al., 2012). The neonatal FGF2 injection was also reported to

increase *fgf2* mRNA expression in the core of the NAc (Clinton et al., 2012). Similar to cocaine, injections of amphetamine also impact FGF2 expression in the brain (Flores et al., 1998; Flores and Stewart, 2000), with significant increases in FGF2 protein levels having been reported in dopaminergic cell bodies and projection areas (Flores and Stewart, 2000). Other drugs including nicotine and alcohol can also influence *fgf2* mRNA expression in the brain (Belluardo et al., 2008; Even-Chen and Barak, 2018) (Even-Chen and Barak, 2018). Those observations are consistent with our present observations that a month withdrawal from oxycodone SA is associated with changes in the expression of striatal *fgf2* mRNA levels, with the long access groups showing greater increases (see Figure 2). These changes are of interest in view of the observations of increased expression of *fgfr2* mRNA levels in the two LgA groups because FGF2, a mostly astrocytic protein, can interact with all the FGF receptors including FGFR2 (Porta et al., 2017), whose mRNA expression has been detected in the dorsal striatum (Asai et al., 1993). Importantly, because FGF2 and FGFR2 are mainly located in glial cells (Asai et al., 1993; Miyake et al., 1996; Woodward et al., 1992), these results also suggest the possibility that glial cells can play important roles in mediating incubation of oxycodone craving in rats and/or relapse to oxycodone abuse in humans. The observations of concomitant increased striatal *fgf2* and *fgfr2* mRNA levels also suggest potential secondary or tertiary feed-forward mechanisms wherein upregulation of both the ligand and its receptor might lead to potentiated behavioral responses (incubation) during withdrawal from oxycodone self-administration. Our observations are consistent with the report that *fgf2* and *fgfr2* mRNA levels are co-downregulated in the brains of MDD patients (Evans et, 2004). Co-regulation of another trophic factor, BDNF, and its receptor, TrkB, has also been reported after electroconvulsive treatment (ECT) (Nibuya et al., 1995), suggesting that trophic factor-induced neuroadaptions to exogenous stimuli may involve feed-forward mechanisms in some cases. These feed-forward mechanisms in the case of incubated oxycodone seeking might include plastic responses generated by *fgf2* that is known induce neuroplastic changes in the CNS (Zechel et al., 2010). It is worthwhile noting that the rats that showed incubation also showed higher striatal *c-fos* and *JunB* mRNA levels than the ShA group that did not show incubation, thus implicating *fgf2*, *fgfr2*, *c-fos, and junB* in a mu opioid receptor-dependent pathway that is associated with augmented cue-induced drug seeking after prolonged abstinence. This idea will be tested in future experiments. It is also important to note, in relationship to potential effects of FGF2 in the brain, that the RNA expression of *fgfr1,* another FGF2 receptor which is located in neurons (Asai et al., 1993), was downregulated in all oxycodone groups, suggesting that activation of neuronal FGFR1 by FGF2 might have led to different adaptations than activation of glial FGFR2. The data also suggest that FGFR1 might not be directly involved in the manifestation of incubated behaviors since the ShA rats that did not show incubation of oxycodone seeking after prolonged abstinence also showed decreased *fgfr1* mRNA expression (compare patterns of expression of the *fgfrs* in Figures 4G and 5G, respectively). Thus, our data also support the idea that regulation of FGFRs by their ligands may be cell type-specific (glial vs neurons) based on different intracellular mechanisms activated by these receptors.

As mentioned earlier, all rats given long access to oxycodone (LgA-L and LgA-H) showed similar increases in incubation of oxycodone seeking that was associated with decreases in striatal mu receptors (Blackwood et al., 2018). These observations had suggested that striatal

opioid receptor mechanisms might, in part, be responsible for some of neurobiological drivers of incubation of oxycodone seeking in rodents and/or relapses in human addicted to opioid drugs. Our findings of increase striatal *fgf2* mRNA levels in animals that showed incubation of oxycodone craving are consistent with reports of interactions of mu and FGF receptors (Belcheva et al., 2002; Di Liberto et al., 2014) and suggest that interactions of striatal Mu and FGF receptors might constitute links in the chain (s) of downstream molecular events that occur at various intervals in this model of oxycodone use disorder. This idea is supported that the observation that *fgfr1*-dominant negative glioma cells showed attenuated Mu receptor-induced ERK activation (Belcheva et al., 2002). This reasoning is also supported by the demonstration that FGF2 can influence the expression of Mu receptors in rats (Turner et al., 2019). Moreover, some of the proposed mechanisms for Mu/FGF receptor interactions may involve FGF signaling via Src kinases (Auciello et al., 2013; Cunningham et al., 2010) that appear to play significant roles in opioid withdrawal (Zhang et al., 2017). Furthermore, the proposal that activation of Mu receptors can lead to cleavage and shedding of FGF2 by matrix metalloproteinases is also of interest to this discussion (Di Liberto et al., 2014). The resulting increase in FGF2-induced stimulation of FGFRs after Mu receptor activation could potentially serve as a feed-forward mechanism to further potentiate activation of Mu receptors since these receptors are located at the site of convergence of Mu and FGF signaling pathways (Belcheva et al., 2002).

The differential increase in FGF2 expression is also interesting in relationship to data obtained from rats that were bred to show high responses to a novel environment (bHR) (Piazza et al., 1990). bHR animals self-administer amphetamine more than low response (bLR) rats (Piazza et al., 1990). The bHR rats also have higher levels of *fgf2* mRNA in the hippocampus in comparison to bLR rats (Perez et al., 2009). Interestingly, administration of cocaine differentially impacted *fgfr1* mRNA expression in the brains of these two phenotypes (Turner et al., 2008). Specifically, repeated injections of cocaine (15 mg/kg) for 7 days decreased *fgfr1* mRNA expression in the hippocampus of bHR (not bLR) rats but increased *fgfr1* mRNA levels in the prefrontal cortex of bLR (not bHR) rats (Turner et al., 2008). It is also noteworthy that bHR rats that have higher *fgf2* mRNA expression also exhibited higher *mu* opioid receptor mRNA expression in their brain when compared to bLR rats (Turner et al., 2019). Importantly, injection of FGF2 early in life normalized *mu* opioid receptor expression in adult bLR rats (Turner et al., 2019). Taken together with our previous demonstration of altered Mu opioid expression in animals that showed incubation of oxycodone craving (Blackwood et al., 2018) and the papers discussed here, our present results provide further support for the idea that FGF signaling pathways might play a role in the behavioral manifestations of incubation during drug withdrawal.

In summary, we have found that incubation of oxycodone seeking after withdrawal from escalated drug self-administration is accompanied by increased expression of *fgf2* and *fgfr2* mRNAs in the rat striatum. These changes are also associated with greater increases in striatal *c-fos* and *junB* mRNA levels in incubated rats. In contrast, there were similar increases in *fosB* expression in all rats exposed to either escalated or non-escalated drug intake. Patterns of mRNA changes in the nucleus of these rats did not correspond to incubation of oxycodone seeking in LgA rats. In contrast to our results, *fgf2* and *fgfr2* mRNA levels have been reported to be decreased in the brains of MDD patients (Evans et

al., 2004). Thus, it is intriguing to suggest that MDD and incubation of craving may represent behavioral manifestations of opposite FGF-mediated neurobiological phenomena. Finally, because changes in *fgf2* expression had been observed in other models of drug abuse, the present observations suggest that manipulation of FGF2-dependent signaling pathways may offer potential novel strategies towards curbing addiction to psychostimulant and/or opioid drugs.

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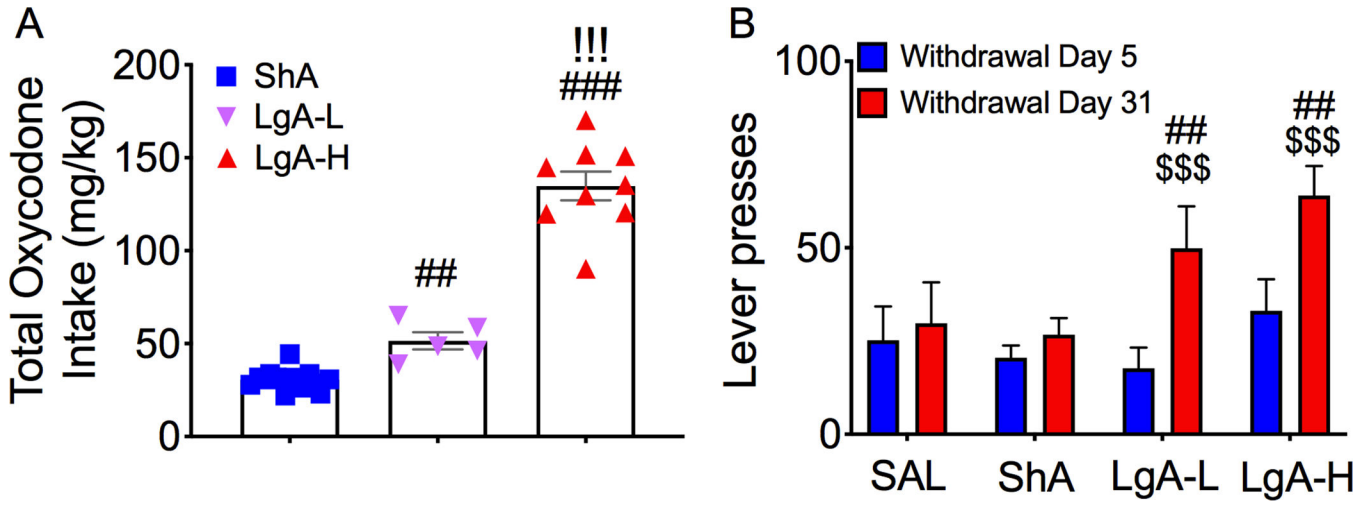

Figure 1. Differential total oxycodone intake and drug seeking behavior in rats given short or long access to the drug.

(A) LgA-L and LgA-H rats showed greater total oxycodone intake than ShA rats. (B) LgA-L and and LgA-H rats showed higher drug seeking behavior on WD31 than on WD5. The values represent means ± SEM. Key to statistics: ##, ### = $p < 0.01, 0.001$, respectively, in comparison to ShA rats; !!! = $p < 0.001$, in comparison to LgA-L rats. $ $ $ = $p < 0.001$, in comparison to withdrawal day 5.

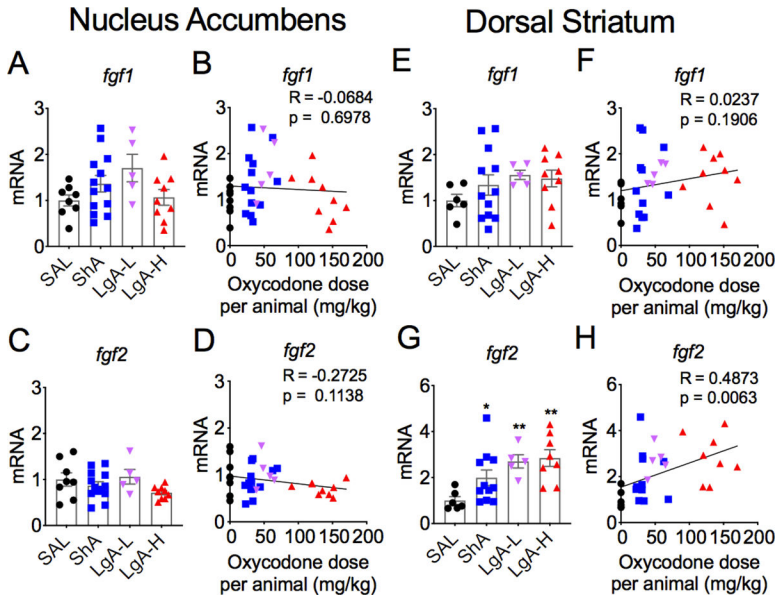

Figure 2. Incubation of oxycodone seeking is accompanied by increased striatal *fgf2* mRNA expression.

(A-B) Fgf1 mRNA expression showed no significant changes in the nucleus accumbens and (E-F) the dorsal striatum. (C-D) NAc *fgf2* mRNA levels were not affected after incubation. (G-H) Striatal *fgf2* mRNA expression was increased in an oxycodone dose-dependent fashion. The values in the bar graphs represent means ± SEM (n=5–14 animals per group). Note the differences in scales on the Y-axis. Key to statistics: *, ** = $p < 0.05$, 0.01 respectively, in comparison to saline rats.

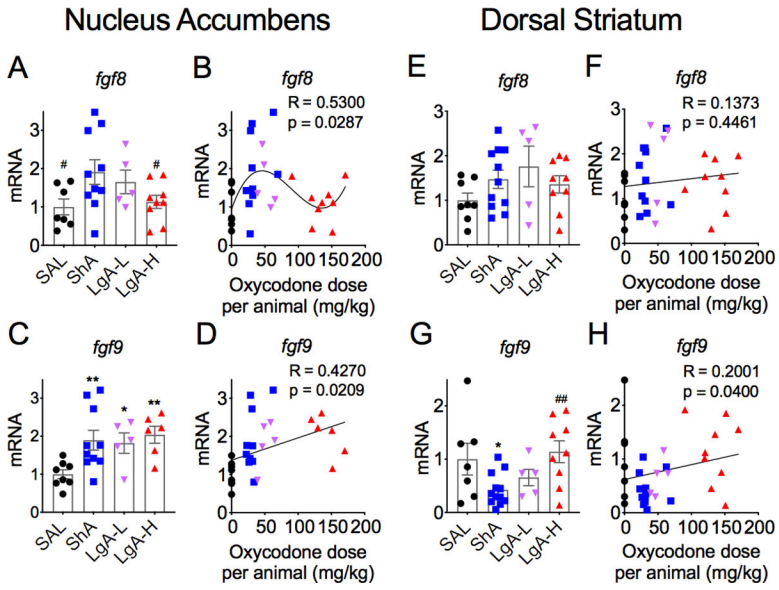

Figure 3. Differential *fgf8* and *fgf9* mRNA levels in the nucleus accumbens and dorsal striatum.

(A-B) *fgf8* mRNA expression is increased in the ShA rats in comparison to control and LgA-H rats. (C-D) NAc *fgf9* mRNA levels are increased after exposure to oxycodone. (E-F) Striatal *fgf8* mRNA expression showed no significant changes after oxycodone and withdrawal. (G-H) Striatal *fgf9* mRNA levels are decreased in the ShA rats. The values in the bar graphs represent means ± SEM (n=5–15 animals per group). Note the differences in scales on the Y-axis. Key to statistics: *, **, *** = $p < 0.05$, 0.01, 0.001, respectively, in comparison to saline rats; #, ## = $p < 0.05$, 0.01, respectively, in comparison to ShA rats.

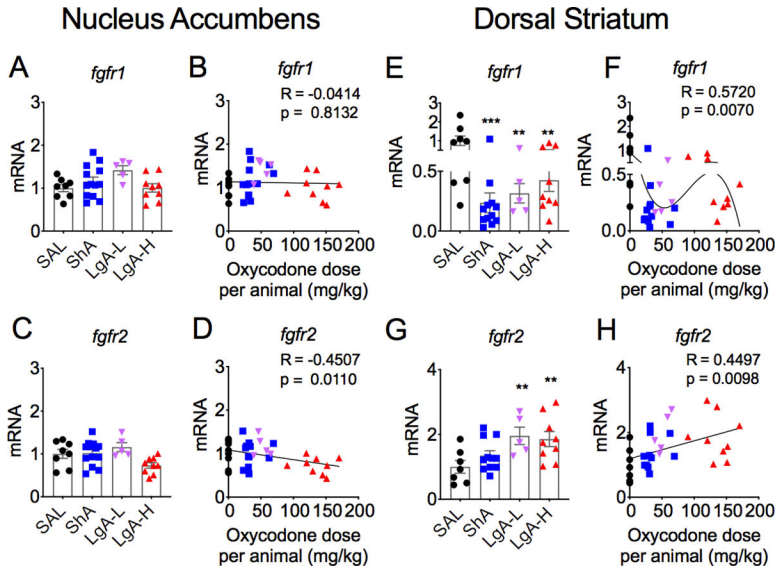

Figure 4. Differential mRNA expression of *fgfr1* and *fgfr2* in the NAc and dorsal striatum after withdrawal from oxycodone.

(A-B) *fgfr1* and (C-D) *fgfr2* mRNA levels showed no significant changes in the NAc. (E-F) Striatal *fgfr1* mRNA levels are decreased in all oxycodone rats. (G-H) Striatal *fgfr2* mRNA expression showed dose-dependent increases in LgA-L and LgA-H rats. The values in the bar graphs represent means ± SEM (n=5–15 animals per group). Note the differences in the scales in the Y-axis in G and H for *fgfr2* expression in comparison to others. Key to statistics: **, *** = $p < 0.01$, 0.001, respectively, in comparison to saline rats.

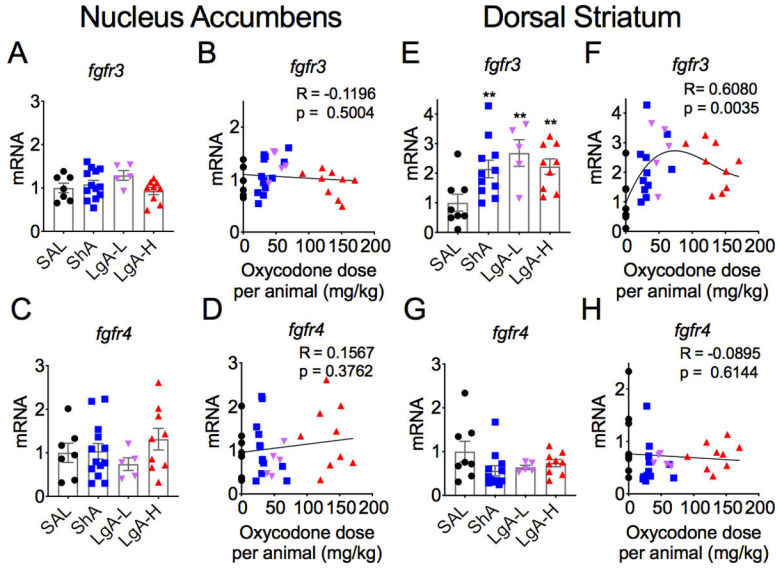

Figure 5. Increased *fgfr3* mRNA expression in the dorsal striatum.

(A-B) *fgfr3* and (C-D) *fgfr4* mRNA levels are not affected by oxycodone withdrawal in the NAc. In contrast, (E-F) there were significant increases in striatal *fgfr3* mRNA expression in all oxycodone rats whereas (G-H) striatal *fgfr4* mRNA levels were not significantly impacted. The values in the bar graphs represent means ± SEM (n=5–15 animals per group). Note the differences in scales on the Y-axis for *fgfr3* in comparison to the others. Key to statistics: ** = $p < 0.01$, in comparison to saline rats.

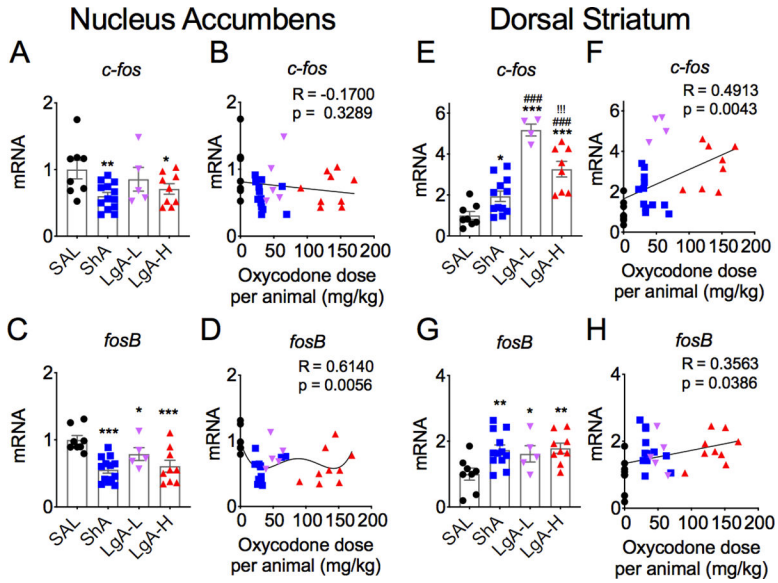

Figure 6. Differential changes in *c-fos* and *fosB* mRNA levels in the NAc and dorsal striatum after oxycodone withdrawal.

(A-B) *c-fos* mRNA levels are significantly decreased in the NAc of ShA and LgA-H rats. (C-D) *fosB* mRNA levels are decreased in the NAc of all oxycodone rats. (E-F) There were greater increases in striatal *c-fos* mRNA in the LgA-L and LgA-L rats in comparison to the ShA group. (G-H) Striatal *fosB* mRNA expression is increased to the same degree in all oxycodone rats. The values in the bar graphs represent means ± SEM (n=5–15 animals per group). Note the differences in scales on the Y-axis for each separate sub-figure, with striatal *c-fos* and *fosB* showing greater magnitude of changes after oxycodone withdrawal. Key to statistics: *, **, *** = $p < 0.05$, 0.01, 0.001, respectively, in comparison to saline rats; ##, ### = $p < 0.01$, 0.001, respectively, in comparison to ShA rats; !!! = $p < 0.001$, in comparison to LgA-L rats.

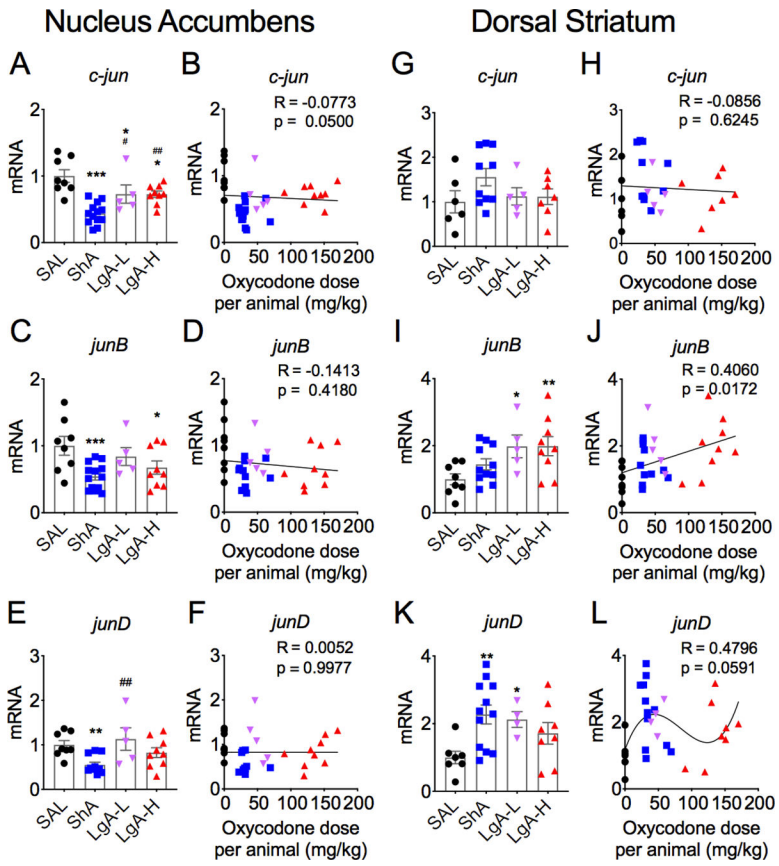

Figure 7. Differential mRNA expression of jun family members in the NAc and dorsal striatum after oxycodone withdrawal.

(A-B) *c-jun* mRNA levels are decreased in all oxycodone rats. (C-D) jun-B mRNA levels are decreased in ShA and LgA-H rats. (E-F) NAc *junD* mRNA expression is decreased in ShA rats. (G-H) Striatal *c-jun* mRNA levels are not impacted by oxycodone withdrawal whereas (I-J) *junB* mRNA expression is increased in both LgA-L and LgA-H groups. (K-L) *junD* mRNA levels are increased in the striatum of ShA and LgA-L rats. The values in the bar graphs represent means ± SEM (n=5–15 animals per group). Note the differences in the scales on the Y-axis for the 3 mRNAs, with striatal *junB* and *junD* showing greater magnitude of changes in expression. Key to statistics: *, **, *** = $p < 0.05, 0.01, 0.001$, respectively, in comparison to saline rats; #, ## = $p < 0.05, 0.01$, respectively, in comparison to ShA rats.

Table 1.

List of Primer Sequences used in RT-PCR.

Gene Name	Forward	Reverse
c-fos	GGGCAAAGTAGAGCAG	CTCTTTCAGTAGATTGGCA
fosB	GAAGCTGGAGTTCATGC	ATGGGCTTGATGACAGA
c-jun	TTGCCCCAACAGATCC	GCTGCGTTAGCATGAG
junB	TCTTTCTCTTCACGACTACA	CTAGCTTCAGAGATGCG
junD	CCTGGAGGAGAAAGTCAAGACC	GTGGCTGAGGACTTTCTGTTTG
fgf1	AGAAAGCCATCTTGTTTCTC	GAAGCACTGCTTACAAATTCA
fgf2	GATCCCAAGCGGCTCTA	ACACTCCCTTGATGGACA
fgf8	TACATGGCCTTTACCCGCAAG	CGGGTAGTTGAGGAACTCGAAG
fgf9	AAACTGAGTCTGGAAGGCGAAT	GCAAAGCTTGGGGATCATTTCA
fgfr1	GGAGAAGAAACTGCACG	ACC ACA GAG TCC ATT ATG A
fgfr2	TTTCAACTCTGCTGTCCGATGA	CATCTTGGGATGAGGACTCTGG
fgfr3	CGGTGACATCAACTGACGAGT	GCTAGGGTCCGCGTAAACAT
fgfr4	AGATGATGAAGCTAATCGG	TTTCTTATAGTAGTCGATGTGG
b2m	GATCTTTCTGGTGCTTGT	AGCTCAATTTCTATTTGAGGT
gadph	CCTTCTCTTGTGACAAAGTG	CCCATTTGATGTTAGCGG