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Opioid Self-Administration is Attenuated by Early-Life Experience and Gene Therapy for Anti-Inflammatory IL-10 in the Nucleus Accumbens of Male Rats

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Early-life conditions can contribute to the propensity for developing neuropsychiatric disease, including substance abuse disorders. However, the long-lasting mechanisms that shape risk or resilience for drug addiction remain unclear. Previous work has shown that a neonatal handling procedure in rats (which promotes enriched maternal care) attenuates morphine conditioning, reduces morphine-induced glial activation, and increases microglial expression of the anti-inflammatory cytokine interleukin-10 (IL-10). We thus hypothesized that anti-inflammatory signaling may underlie the effects of early-life experience on later-life opioid drug-taking. Here we demonstrate that neonatal handling attenuates intravenous self-administration of the opioid remifentanil in a drug-concentration-dependent manner. Transcriptional profiling of the nucleus accumbens (NAc) from handled rats following repeated exposure to remifentanil reveals a suppression of pro-inflammatory cytokine and chemokine gene expression, consistent with an anti-inflammatory phenotype. To determine if anti-inflammatory signaling alters drug-taking behavior, we administered intracranial injections of plasmid DNA encoding IL-10 (pDNA-IL-10) into the NAc of non-handled rats. We discovered that pDNA-IL-10 treatment reduces remifentanil self-administration in a drug-concentration-dependent manner, similar to the effect of handling. In contrast, neither handling nor pDNA-IL-10 treatment alters self-administration of food or sucrose rewards. These collective observations suggest that neuroimmune signaling mechanisms in the NAc are shaped by early-life experience and may modify motivated behaviors for opioid drugs. Moreover, manipulation of the IL-10 signaling pathway represents a novel approach for influencing opioid reinforcement.

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INTRODUCTION

Opioid drugs can be effective pain relievers, but they are also highly addictive substances that can lead to drug dependence and carry a significant risk for overdosing. In the United States, overdose deaths from opioids have risen dramatically over the past decade (Calcaterra *et al*, 2013; Rudd *et al*, 2016). Developing solutions to this growing epidemic will be facilitated by a better understanding of the biological and environmental factors that promote vulnerability to, or resilience from, opioid abuse.

Drug addiction is thought to arise from structural and functional adaptations in brain regions associated with motivation and reward, in particular the dopamine-producing neurons of the ventral tegmental area and one of its major targets, the nucleus accumbens (NAc) in the ventral striatum (Robinson and Berridge, 1993). Although the majority of addiction research has focused on identifying the cellular and molecular adaptations of neurons and their associated circuitries (Hyman *et al*, 2006; Lüscher and Malenka, 2011), there is emerging evidence that pathways of neuroimmune signaling (eg, between neurons and glial cells) play an important role in the neurophysiological and behavioral manifestations of acute and repeated exposure to drugs of abuse (Lacagnina *et al*, 2017; Miguel-Hidalgo, 2009).

Glial activity in the context of neuroimmune signaling can be broadly categorized as either anti-inflammatory or pro-inflammatory depending on the cytokines and chemokines expressed and the functional outcomes they support (Dantzer *et al*, 2008; Meyer *et al*, 2008), although their actions can be complex and multidimensional (Ginhoux *et al*, 2016; Ransohoff, 2016). Opioid administration produces pro-inflammatory glial activation via Toll-like

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receptor 4 (TLR4) signaling on microglia and other immunocompetent cells, and the resulting production of cytokines and chemokines may contribute to the reinforcing properties of opioids (Hutchinson *et al*, 2012; Wang *et al*, 2012). In contrast, anti-inflammatory neuroimmune signaling has been shown to attenuate the reinforcing effects of opioids, and its expression in the brain can be profoundly influenced by early-life environmental conditions (Schwarz *et al*, 2011). Specifically, neonatal handling in rats (in which daily, brief separation of pups from the dam promotes increased maternal licking and grooming behavior) (Liu *et al*, 1997) prevents reinstatement of morphine conditioned place preference in adulthood, reduces morphine-induced pro-inflammatory cytokine and chemokine expression in the NAc, and persistently increases gene expression of the anti-inflammatory cytokine interleukin-10 (IL-10) in microglia (Schwarz *et al*, 2011). Because IL-10 is a powerful modulator of pro-inflammatory signaling (Kwilasz *et al*, 2015; Moore *et al*, 2001), these data raise the possibility that environmental conditions early in life may influence liability for drug addiction via changes in IL-10 in the NAc, although several questions remain. First, it is unclear if the 'protective' effect of neonatal handling for opioid conditioned place preference can be extended to a model of opioid self-administration, which more accurately represents some essential features of human drug-taking behaviors (Fernando and Robbins, 2011). Second, the effect of repeated opioid exposure on NAc neuroimmune gene expression is largely unknown. And finally, while changes in IL-10 are associated with handling, it is unknown if direct manipulation of IL-10 is sufficient to influence addiction-related behaviors.

To address these questions, we sought to determine the role of anti-inflammatory signaling, in particular IL-10, in the NAc during self-administration of the opioid remifentanil. Our data demonstrate the following: (1) early-life handling reduces opioid self-administration in adulthood in a drug-concentration-dependent manner, without affecting food or sucrose self-administration; (2) transcriptional profiling of the NAc reveals that neonatal handling persistently increases IL-10 expression and suppresses pro-inflammatory cytokine/chemokine signaling despite repeated opioid exposure; and (3) delivery of IL-10 gene therapy into the NAc of non-handled rats reduces opioid drug-taking without affecting food or sucrose reward, demonstrating specificity to opioid reinforcement. Together, these data implicate NAc anti-inflammatory signaling as an important substrate influencing self-motivated opioid consumption under defined conditions, while providing a novel therapeutic target to exploit in the pursuit of improving treatments for opioid addiction.

MATERIALS AND METHODS

Subjects

Adult male and female Sprague-Dawley rats (males 250–275 g; females 175–199 g; Harlan/Envigo, Dublin, VA) were housed in individually ventilated cages with corn cob bedding and *ad libitum* access to food (Purina Lab Diet 5001) and water. The colony was maintained at 23 °C on a 12 : 12 h light–dark cycle (lights on at 0700 hours), and cages were changed once per week. Rats were housed in same-sex pairs for 1 week before experimental manipulations. For handling experiments, males and females were paired for breeding; when females showed signs of pregnancy, males were removed from the cage. Litters were culled to two females and up to eight male pups per litter on postnatal day 2 (P2), and only males were used in experiments. For plasmid experiments, adult male rats (250–275 g; Harlan/Envigo, Dublin, VA) were pair-housed and given at least 1 week to acclimate to laboratory conditions. All experiments were approved by the Duke University Institutional Animal Care and Use Committee.

Neonatal Handling

Approximately half of the litters were assigned to a brief separation/neonatal handling condition ('Handled') as previously described (Bilbo *et al*, 2007; Schwarz *et al*, 2011). For each handled litter, the dam was removed from the home cage and placed in a clean polycarbonate cage with a securely fastened lid, while pups were moved one at a time to the center of a separate, clean polycarbonate cage with bedding. After 15 min, pups were returned to their home cage, followed by the dams. This handling procedure was conducted once per day between 1400 and 1600 hours from P2 to P20; control litters ('Control') were left undisturbed during this time. On P22, females were culled while all males were weaned and pair-housed with siblings until they reached adulthood (P60). To reduce litter-specific effects, only two to four pups per litter were used in each experiment (mean number of pups from the same litter used per experiment $= 2.5 \pm 0.14$). Adult animals were transferred to the colony room adjacent to behavioral testing rooms and were allowed to acclimate to housing conditions (reverse 12 : 12 light cycle, lights off at 0700 hours) for at least 1 week. All experiments were conducted during the animals' active phase.

Self-Administration

Behavioral testing was conducted in operant-conditioning chambers (Med Associates, ENV-008-CT). For remifentanil experiments, overnight training to lever-press for food was performed as previously described (Levin *et al*, 2016). After reaching response criteria ($\geqslant$50 responses in 3 consecutive 30-min sessions), rats were implanted with intravenous catheters as previously described (Hall *et al*, 2015). Briefly, rats were anesthetized with a combination of ketamine (60 mg/kg, i.p.) and dexmedetomidine (0.15 mg/kg, i.p.), the jugular vein was isolated, and a sterile silastic catheter (SAI Infusion Technologies) was inserted and fastened with silk sutures. The opposite end was tunneled subcutaneously to emerge between the scapulae and was attached to an infusion harness (SAI). Rats were given a minimum of 1 week to recover from surgery. Subsequently, rats were allowed to self-administer the opioid remifentanil intravenously on a fixed ratio 1 (FR1) schedule of reinforcement with a 20 s time-out period. Remifentanil was chosen because of its high specificity for μ-opioid receptors and its short half-life, resulting in high rates of responding (Panlilio and Schindler, 2000). Food was temporarily restricted 24 h before the first remifentanil session to motivate initial exploration of the

active lever, but was returned to *ab libitum* access thereafter. Rats received daily 1 h remifentanil sessions for 14 days: 7 days at 0.29 μg/kg and 7 days at 0.9 μg/kg. Rats were saline perfused 14 days after the final session.

For dose–response experiments, animals were allowed to acquire remifentanil at 0.9 μg/kg (1 h daily, FR1, 20 s time-out) without previous food training until responding was stable (defined as <20% variance of infusions received across the previous three sessions, for a minimum of 14 days). Subsequently, rats received daily exposure to one of four concentrations of remifentanil (0.09, 0.29, 0.9, or 2.9 μg/kg) based on previously established doses (Hutchinson *et al*, 2012) in a randomized sequence. To control for order effects, animals from both groups were counterbalanced to receive each dose sequence permutation, and all animals were exposed to all four doses in 4-day cycles. There were no external stimuli indicating which dose would be delivered each day, and the order of doses received in a 4-day cycle did not influence the order received in a subsequent cycle. The dose–response sessions proceeded for 12 days (1 h daily, FR1, 20 s time-out). Rats were saline perfused 24 h after the final session.

For food and sucrose experiments, rats were either food restricted (to 85–90% of free-feeding body weight) and allowed to lever-press for food pellets (TestDiet, 45 mg), or maintained on *ad libitum* diet and allowed to lever-press for sucrose pellets (Bio-Serv, 45 mg, banana flavored). Both conditions received daily 1 h sessions, FR1, 20 s time-out. For all self-administration experiments, the number of drug infusions or pellets acquired per session is reported, along

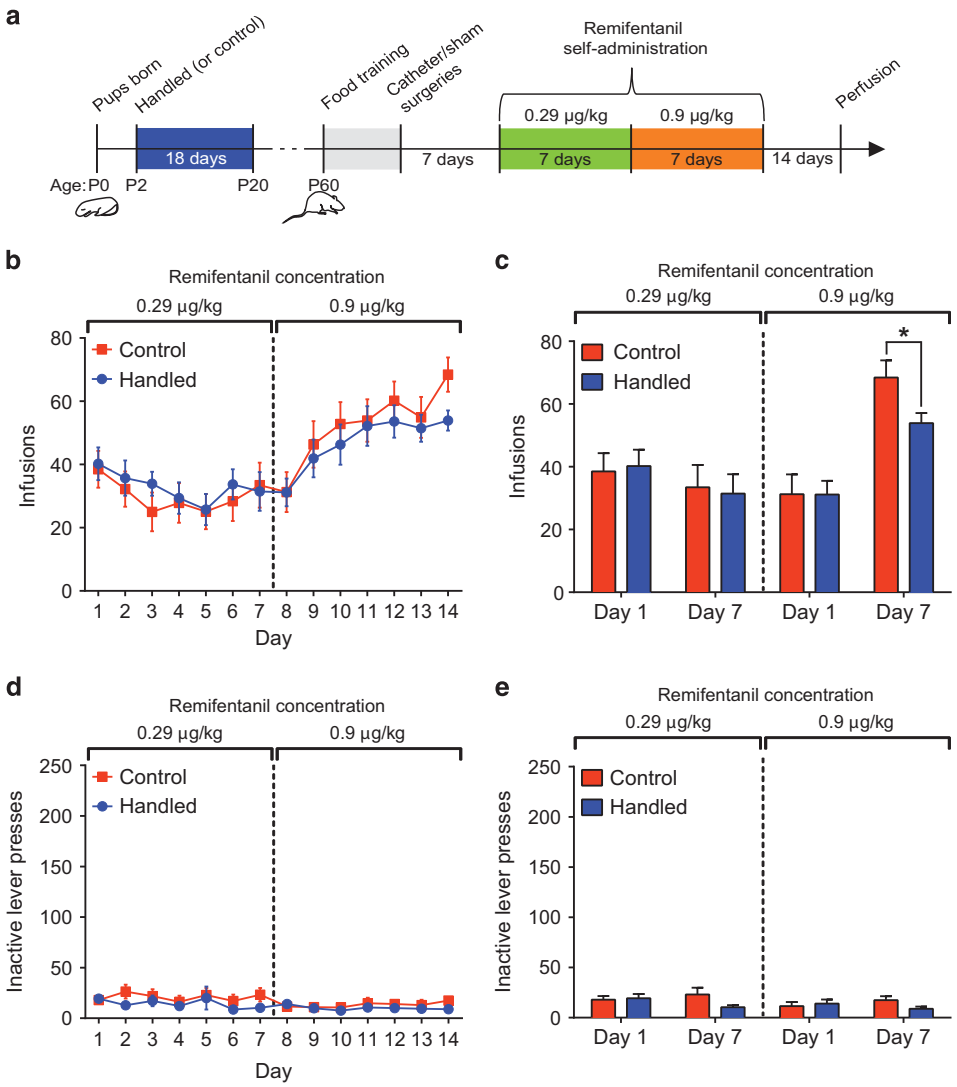


Figure 1 Neonatal handling attenuates remifentanil self-administration in a drug-concentration-dependent manner. (a) Schematic representation of the experimental timeline. Handling was performed by separating dams from their litter for 15 min/day from P2–P20, whereas controls were left undisturbed. Following early-life conditions, adult rats were trained to self-administer intravenous remifentanil ($n = 11–13$/group). (b) The number of intravenous infusions of remifentanil was recorded in daily 1 h sessions (FR1, 20 s time-out) for 14 days: 7 days at 0.29 μg/kg and 7 days at 0.9 μg/kg. (c) Neonatal handling does not influence remifentanil self-administration for adult rats during initial sessions at 0.29 μg/kg when comparing the first and final sessions (day 1 *vs* day 7). In contrast, neonatal handling significantly attenuates the number of remifentanil infusions acquired on day 7 when remifentanil is delivered at 0.9 μg/kg (*$p < 0.05$). (d) Responses on the inactive lever were recorded across sessions. (e) Neonatal handling has no effect on inactive lever pressing when comparing day 1 *vs* day 7 for sessions at both drug concentrations.

with responses on the inactive lever. The ordinate scale for inactive responses is based on matching the scale for active lever responses. See Supplementary Methods for additional information.

Drugs

Remifentanil hydrochloride (Sigma-Aldrich, cat. R1908) was dissolved into 0.9% sterile saline to a concentration of 100 μg/ml and frozen in aliquots at −20 °C, which were thawed and further diluted with 0.9% saline to the required concentration prior to daily self-administration sessions. D-(+)-mannose (Sigma-Aldrich, cat. M6020) was dissolved in 0.9% sterile saline before use.

Tissue Processing

Rats were saline perfused and the NAc was dissected and rapidly frozen for subsequent mRNA or protein analyses (see Supplementary Methods). For *in vitro* plasmid DNA (pDNA) experiments, cells from whole brains (excluding cerebellum) were cultured either without cell-type-specific isolation or following CD11b microglial isolation as previously described (Williamson *et al*, 2011). Following pDNA behavioral experiments, tissue was fixed in 4% paraformaldehyde, cryoprotected in 30% sucrose, sectioned (40 μm), thaw-mounted directly to Superfrost Plus slides (VWR), coverslipped with Vectashield (Vector), and imaged to confirm infusion site.

Quantitative Real-Time PCR

RNA was isolated using the TRIzol method (Ambion). For tissue collected from rats used in Figure 1, cDNA was synthesized using the QuantiTect reverse transcription kit (Qiagen) and relative gene expression to *Gapdh* was measured using the QuantiFast SYBR Green PCR kit (Qiagen). For tissue collected from rats used in Figure 2, cDNA was synthesized using the RT^2 First Strand kit (Qiagen) and gene expression was quantified with PCR arrays (SABiosciences/Qiagen; cat. PARN-022ZA) using RT^2 SYBR Green ROX master mix (Qiagen). See Supplementary Methods for additional information.

Gene Functional Annotation Clustering Analysis

To assess the functional biological pathways that characterize neonatal handling following remifentanil, the top 20 genes that were up- or downregulated in the PCR array between control and handled rats were uploaded into DAVID functional gene ontology software (https://david.ncifcrf.gov/). All non-housekeeping genes that were expressed in the array served as 'background'. Functional annotation was run, and the top 12 enriched annotation groups (functional clusters) were chosen to represent the group comparisons (Huang *et al*, 2009).

Western Blot

Protein was precipitated from the phenol–ethanol fractions remaining from TRIzol-mediated RNA extraction as described previously (Kopec *et al*, 2017), and the ratio of phosphorylated to total protein was assessed by western blot analysis (see Supplementary Methods).

pDNA Treatments

The pDNA vectors (pDNA-control or pDNA-IL-10) were prepared as previously described (Milligan *et al*, 2006). For *in vitro* pDNA experiments, cultured cells (either dissociated brain tissue or CD11b-isolated cultures) were incubated with D-mannose (5 mg/ml) and pDNA (10 μg/ml) for 2 h. For *in vivo* pDNA treatments, rats underwent stereotaxic surgery to receive bilateral intracranial injections of pDNA and D-mannose into the NAc (7.5 μg/μl pDNA and 50 μg/μl D-mannose). A Hamilton syringe was lowered to the target coordinates (AP: +1.8; ML: ±1.5; DV: −6.8) (Paxinos and Watson, 2007) and 1 μl of pDNA and D-mannose solution was slowly infused (0.1 μl/min). After at least 1 week of recovery, rats were killed for mRNA and protein analysis, or continued to self-administer for remifentanil or food/sucrose as described above. See Supplementary Methods for additional details.

Statistical Analysis

Data were analyzed and depicted using GraphPad Prism 6 software. Behavioral data were analyzed by two-way repeated measures analysis of variance (RM-ANOVA), with early life experience (Control or Handled) or pDNA treatment (pDNA-control or pDNA-IL-10) as the between-subjects factor, and time as the within-subjects factor. Behavioral analyses (RM-ANOVA and *post hoc* comparisons) were *a priori* restricted to the first and final sessions (ie, day 1 *vs* day 7) for all conditions. Sessions for different remifentanil doses were analyzed separately. Dose–response curve data were analyzed by two-way RM-ANOVA, with early life experience as the between-subjects factor and drug concentration as the within-subjects factor. Outcomes of behavioral data were followed by Fisher's protected least significant difference *post hoc* tests when appropriate. Outliers from behavioral assessments were detected by Grubb's test, resulting in the removal of three subjects ($n=1$ control, $n=2$ handled). Normality was tested with D'Agostino-Pearson omnibus normality test. Single-gene PCR data were analyzed by one- or two-way ANOVAs followed by Tukey's or Bonferroni's *post hoc* tests when appropriate, or analyzed by Kruskal–Wallis test followed by Dunn's *post hoc* test for non-normally distributed data. PCR array data and figures were generated with the assistance of software provided by the manufacturer (RT^2 Profiler PCR Array Data Analysis 3.5, SABiosciences/Qiagen), and fold regulation of gene expression was analyzed by two-tailed Student's *t*-test. Western blot and PCR data for *in vivo* pDNA characterization were analyzed by two-tailed Student's *t*-test. The threshold for significance was set at $p<0.05$. All data are presented as mean ± SEM.

RESULTS

Neonatal Handling Attenuates Remifentanil Self-Administration in a Drug-Concentration-Dependent Manner

To test the impact of neonatal handling on a behavioral paradigm of self-motivated opioid drug taking, we measured

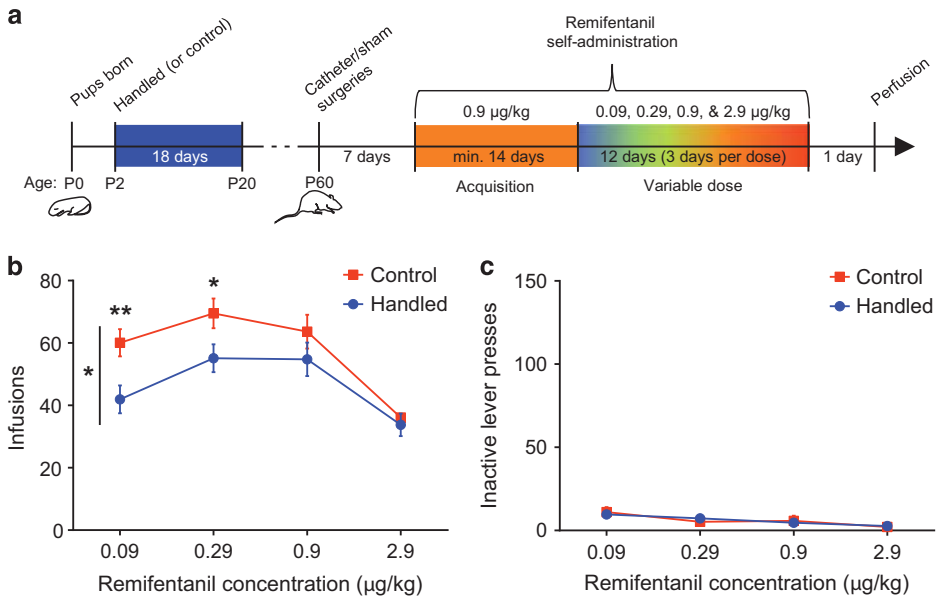


Figure 2 Neonatal handling lowers dose–response curve of remifentanil self-administration. (a) Schematic representation of the experimental timeline. Following neonatal handling or control conditions, adult rats were allowed to acquire remifentanil self-administration at 0.9 µg/kg (1 h daily, FR1, 20 s time-out) until responding was stable (<20% variance across three sessions) for at least 14 days. Subsequent sessions involved randomized access to varying concentrations of remifentanil, which was counterbalanced between groups (four concentrations, three sessions per concentration, across 12 days; $n = 6$–8/group). (b) There is a downward shift in the dose–response curve to remifentanil in rats that received neonatal handling compared with controls (main effect of early-life experience; $^{*}p < 0.05$). *Post hoc* tests revealed that handled rats administer fewer remifentanil infusions per session at the 0.09 or 0.29 µg/kg concentrations relative to controls ($^{*}p < 0.05$, $^{**}p < 0.01$). (c) Handled and non-handled control rats do not show differences in responding on the inactive lever during the dose–response sessions.

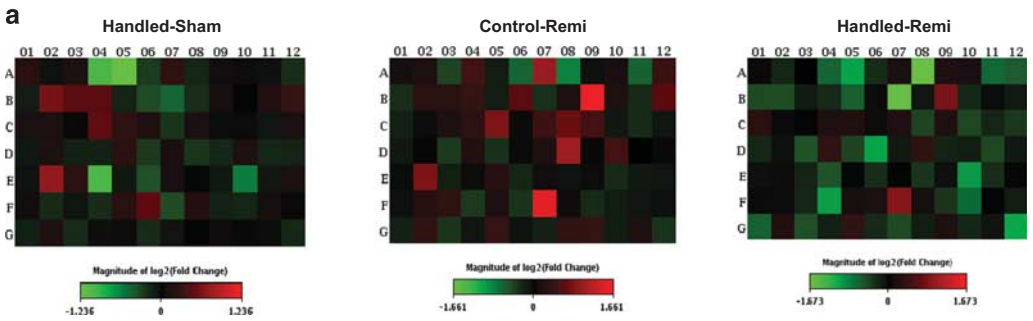
self-administration behavior for remifentanil at two drug concentrations (0.29 and 0.9 µg/kg) for 7 days each (Figure 1a). Remifentanil infusions acquired per session were measured over time (Figure 1b). A two-way RM-ANOVA comparing the first and final sessions for each drug concentration (Figure 1c) revealed no main effect of early-life experience on the number of infusions acquired at 0.29 µg/kg, and no change in behavior over time. In contrast, there was a main effect of time ($F_{1,22} = 79.72$, $p < 0.001$) and a significant interaction between time and early-life experience ($F_{1,22} = 4.61$, $p < 0.05$) for sessions at 0.9 µg/kg. *Post hoc* comparison-revealed controls took significantly more 0.9 µg/kg remifentanil infusions on day 7 compared with handled rats ($p < 0.05$; Figure 1c). Inactive lever responses were also compared as a measure of non-specific lever displacement (Figure 1d). Responses on the inactive lever did not differ across time or between groups for sessions at 0.29 µg/kg. There was a significant interaction for sessions at 0.9 µg/kg ($F_{1,22} = 6.14$, $p < 0.05$); however, *post hoc* comparisons determined there was no difference between groups at either day 1 or day 7 ($p > 0.05$; Figure 1e).

Neonatal Handling Shifts the Dose–Response Function for Remifentanil

These data reveal that neonatal handling influences self-motivated remifentanil consumption, although the response was highly dependent upon drug concentration and emerged on day 7 after the drug concentration was shifted to 0.9 µg/kg. To further investigate the relationship between early-life experience and self-administration behavior as a factor of remifentanil concentration, we trained rats until responding was stable at the 0.9 µg/kg dose and examined the dose–response curve following randomized exposure to four different drug concentrations (Figure 2a). Individual responses for each concentration were averaged across three separate sessions and are depicted as the group mean ± SEM (Figure 2b). A two-way RM-ANOVA revealed that neonatal handling significantly reduced the number of drug infusions acquired (main effect of early-life experience; $F_{1,12} = 4.81$, $p < 0.05$; Figure 2b). *Post hoc* comparisons determined that handled rats acquired fewer remifentanil infusions per session during the 0.09 µg/kg ($p < 0.01$) and 0.29 µg/kg sessions ($p < 0.05$), compared with controls. Interestingly, no group difference was observed for sessions at 0.9 µg/kg, suggesting that the concentration of initial drug exposure and/or number of sessions influenced subsequent drug-taking. The number of infusions acquired was also strongly influenced by drug concentration (main effect of drug concentration; $F_{3,36} = 27.16$, $p < 0.001$), which is consistent with the presumption that rodents modify within-session behavior based on drug received per infusion. Responding on the inactive lever varied by dose (main effect of drug concentration; $F_{3,36} = 8.09$, $p < 0.001$), but we observed no difference between groups for inactive lever pressing (Figure 2c). These results provide evidence that neonatal handling can influence opioid self-administration, and the emergence of this long-lasting effect is strongly dependent upon drug concentration and the sequence of its availability.

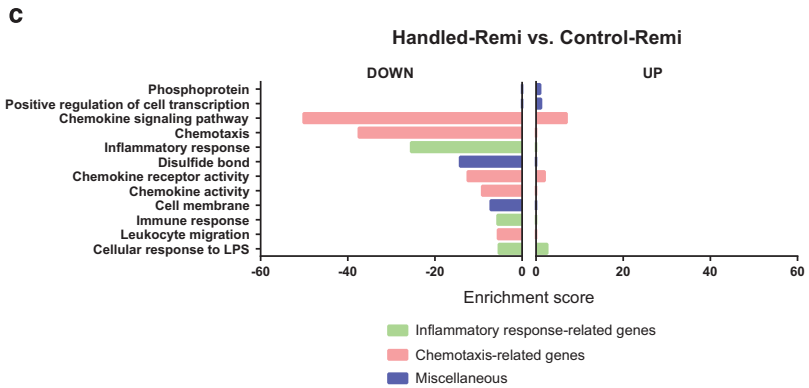
Neonatal Handling Produces an Anti-Inflammatory Environment in the NAc Following Remifentanil Exposure

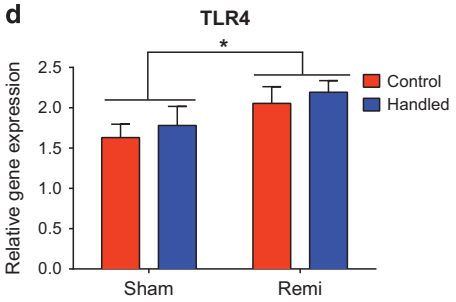
To characterize inflammatory neuroimmune signaling in the NAc, we collected tissue from rats (from Figure 2) and



b

	Gene	Fold regulation	*p* value
Handled-Sham	*Il6*	1.39	0.0364
Control-Remi	*Slit2*	1.32	0.0237
	Ackr2	-1.35	0.0497
	Cx3cr1	-1.3	0.0267
	Ccl17	-1.58	0.0178
Handled-Remi	*Gpr17*	-1.23	0.0403
	Pkrca	-1.14	0.038
	Il16	-1.18	0.0236
	Cmtm3	-1.29	0.0221
	Ccl7	-3.11	0.0225
	Itgb2	-1.28	0.0132
	Tymp	-1.19	0.0096
	Kdr	-1.62	0.0064
	Cx3cr1	-1.45	0.0036
	Tlr2	-1.4	0.002
	Il1b	-2.08	0.0005
	Ccl2	-3.19	0.0006





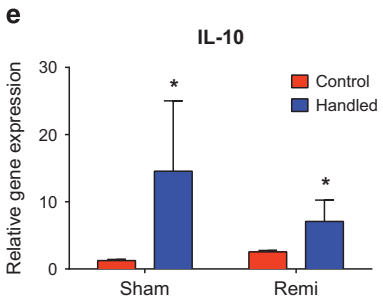


Figure 3 Transcriptional profile of NAc reveals evidence of an anti-inflammatory signature in handled rats. (a) The log2 fold-change plate-layout heat maps of gene expression as assessed by PCR array, compared with Control-Sham ('Control' = non-handled; 'Handled' = neonatal handling; 'Sham' = no remifentanil; 'Remi' = remifentanil self-administration; $n = 6$/group). (b) Fold regulation and *p*-values of significant gene expression changes from PCR array (compared with Control-Sham). After prolonged self-administration of remifentanil, Handled-Remi rats in particular show downregulation of a number of immune-related genes (See Supplementary Table 1 for complete gene list). (c) Functional annotation clustering analysis of gene enrichment scores of the top 20 up- or downregulated genes from array data following self-administration (Handled-Remi compared with Control-Remi) demonstrates robust downregulation of gene expression across most functional clusters. (d) TLR4 mRNA is elevated in NAc of rats that self-administered remifentanil (*$p < 0.05$; Sham: $n = 6$/group; Remi: $n = 11$–13/group). (e) Expression of IL-10 mRNA in the NAc is increased for rats that received neonatal handling (*$p < 0.05$; Sham: $n = 3$–4/group; Remi: $n = 8$–10/group).

utilized PCR arrays to broadly analyze gene expression of 84 cytokine/chemokine-related genes (Figure 3a). As a baseline drug-naive control group, NAc tissue was also collected from rats that underwent jugular isolation surgery but received no remifentanil ('Sham'). Transcriptional profiling of the NAc revealed decreased expression of pro-inflammatory cytokine and chemokine genes in handled rats that received remifentanil (ie, Handled-Remi), compared with non-handled controls (ie, Control-Sham); 12 genes investigated were significantly downregulated, including CX3CR1, TLR2, IL-1β, and CCL2 (Figure 3b and Supplementary Table 1), which represent suppression of key messengers and receptors mediating pro-inflammatory responses. Non-supervised hierarchical clustering of all non-housekeeping genes grouped rats primarily by early-life experience (Supplementary Figure S1), indicating a shared organization of neuroimmune gene regulation influenced by handling. Functional annotation clustering analysis of gene enrichment scores from array data comparing handled against control rats after self-administration revealed shared patterns of gene downregulation across a variety of functional clusters related to chemokine signaling and inflammatory regulation (Figure 3c). Although observing gene enrichment for these functional clusters was not surprising (as the gene set was pre-determined from the array), the consistent pattern of neuroimmune gene downregulation in Handled-Remi rats

was striking and suggestive of an anti-inflammatory environment in the NAc.

The anti-inflammatory neuroimmune phenotype in Handled-Remi rats could be driven by *de novo* down-regulation of pro-inflammatory signaling networks, or handling could increase expression of anti-inflammatory signaling molecules, which in turn suppress transcription of pro-inflammatory mediators. To disambiguate these possibilities, we first measured TLR4 expression in the NAc of equivalently aged rats (from Figure 1) 14 days following cessation of self-administration, to investigate persistent changes in gene regulation. TLR4 is a critical initiator of pro-inflammatory responses, and there is considerable evidence that opioid compounds induce pro-inflammatory responses via TLR interactions, including TLR4 (Bachtell *et al*, 2015; Eidson *et al*, 2017; Wang *et al*, 2012). We found a significant main effect of drug treatment on TLR4 mRNA ($F_{1,32} = 4.26$, $p < 0.05$) but no effect of early-life experience (Figure 3d); thus, remifentanil increases NAc expression of TLR4 regardless of early-life conditions. We next compared gene expression of anti-inflammatory IL-10, which broadly inhibits the expression of pro-inflammatory mediators released downstream of TLR ligation (Kishore *et al*, 1999; Saraiva and O'Garra, 2010). Consistent with previous work (Schwarz *et al*, 2011), we found IL-10 mRNA was elevated in the NAc of handled rats (Figure 3e), even after repeated remifentanil exposure ($p < 0.05$; Kruskal–Wallis and Dunn's test). Collectively, these results converge to support the notion that the early-life maternal environment can influence drug-induced cytokine/chemokine gene expression patterns in the NAc, which is characterized in part by increased anti-inflammatory gene expression.

Plasmid IL-10 Gene Therapy Increases IL-10 and Modifies Downstream Signaling

We next tested the hypothesis that genetic manipulation of anti-inflammatory IL-10 in the NAc is sufficient to attenuate opioid reinforcement in normal control rats. Naked pDNA encoding rat IL-10 (Figure 4a) has previously been delivered to the spinal cord to augment IL-10 transcription and effectively suppress glial pro-inflammatory cytokine production (Ledeboer *et al*, 2007), and delivery of D-mannose as a transgene adjuvant along with pDNA-IL-10 greatly improves plasmid transfection from a single injection (Dengler *et al*, 2014). To confirm the efficacy of pDNA-IL-10 treatment, we first measured IL-10 gene expression *in vitro* with dissociated rat brain cell cultures. Incubation with pDNA-IL-10+D-mannose increased IL-10 mRNA ($F_{3,8} = 42.13$, $p < 0.001$), which was not observed with D-mannose alone or pDNA-control+D-mannose (Figure 4b). Furthermore, when microglia ($CD11b^+$ cells) were isolated and cultured separately from $CD11b^-$ cells, pDNA-IL-10+D-mannose treatment increased IL-10 mRNA to a greater extent in the $CD11b^+$ population ($F_{3,15} = 4.01$, $p < 0.05$; Figure 4c). This suggests that, under *in vitro* conditions, microglia primarily (although not exclusively) internalize the pDNA vector and increase IL-10 transcription.

To manipulate IL-10 *in vivo*, we stereotaxically delivered pDNA (IL-10 or control) with D-mannose bilaterally into the NAc (Figure 4d). One week later, NAc and adjacent striatal tissue was isolated and processed for mRNA and protein analyses. Treatment with pDNA-IL-10 significantly increased IL-10 mRNA compared with controls ($t_8 = 2.43$, $p < 0.05$; Figure 4e). We additionally precipitated protein from the majority of the same tissue samples and measured the phosphorylation state of extracellular signal-related kinase (ERK), a member of the mitogen-activated protein kinase (MAPK) family that is well characterized both for its involvement in the induction of IL-10 following pattern recognition receptor ligation on macrophages and dendritic cells (Saraiva and O'Garra, 2010), as well as being downstream of IL-10 signaling itself in the brain (Pereira *et al*, 2015). The ratio of phosphorylated ERK1/2 to total ERK1/2 was significantly decreased *in vivo* 1 week after rats received a single bilateral injection of pDNA-IL-10 into the NAc, compared with pDNA-control ($t_6 = 8.13$, $p < 0.001$; Figure 4f). These data demonstrate that the method of pDNA-IL-10 and D-mannose delivery utilized here, when administered *in vitro* and *in vivo*, effectively and persistently alters the transcriptional activity of IL-10 and functionally influences the phosphorylation state of a critical kinase for IL-10 signaling.

Plasmid IL-10 Delivery in the NAc Attenuates Remifentanil Self-Administration

To address the behavioral outcome of IL-10 gene therapy, we stereotaxically delivered pDNA (IL-10 or control) with D-mannose bilaterally into the NAc and measured self-administration for remifentanil at two drug concentrations (0.29 and 0.9 μg/kg) for 7 days each (Figure 5a). Remifentanil infusions acquired per session were measured over time (Figure 5b). A two-way RM-ANOVA comparing the first and final sessions for each drug concentration revealed a main effect of time on remifentanil consumption at 0.29 μg/kg ($F_{1,16} = 6.90$, $p < 0.05$), which we suspect was related to increased food seeking on day 1. Importantly, there was no effect of pDNA treatment at this dose (Figure 5c). In contrast, at 0.9 μg/kg there was a main effect of time ($F_{1,16} = 28.03$, $p < 0.001$) and a significant interaction between test day and pDNA treatment ($F_{1,16} = 11.06$, $p < 0.01$). *Post hoc* comparisons revealed pDNA-IL-10 treatment significantly reduced remifentanil consumption on day 7 compared with pDNA-control treatment ($p < 0.05$; Figure 5c). Responding on the inactive lever did not differ across sessions or between groups (Figure 5d and e).

Self-Administration of Natural Rewards is not Altered by Neonatal Handling or Plasmid IL-10 Delivery in the NAc

To determine if the effect of neonatal handling on remifentanil administration is due to a generalized decrease in reward valuation, we assessed self-administration behavior for food or sucrose pellets in separate groups of handled and control rats from the same litters used for remifentanil (Supplementary Figure S2a). The number of pellets acquired per session rose dramatically over time both for food (Supplementary Figure S2b) and sucrose (Supplementary Figure S2e). There was a significant main effect of time for rats acquiring food ($F_{1,13} = 69.22$, $p < 0.001$) and sucrose ($F_{1,9} = 12.29$, $p < 0.01$). However, there was no effect of early-life experience on food (Supplementary Figure S2c) or

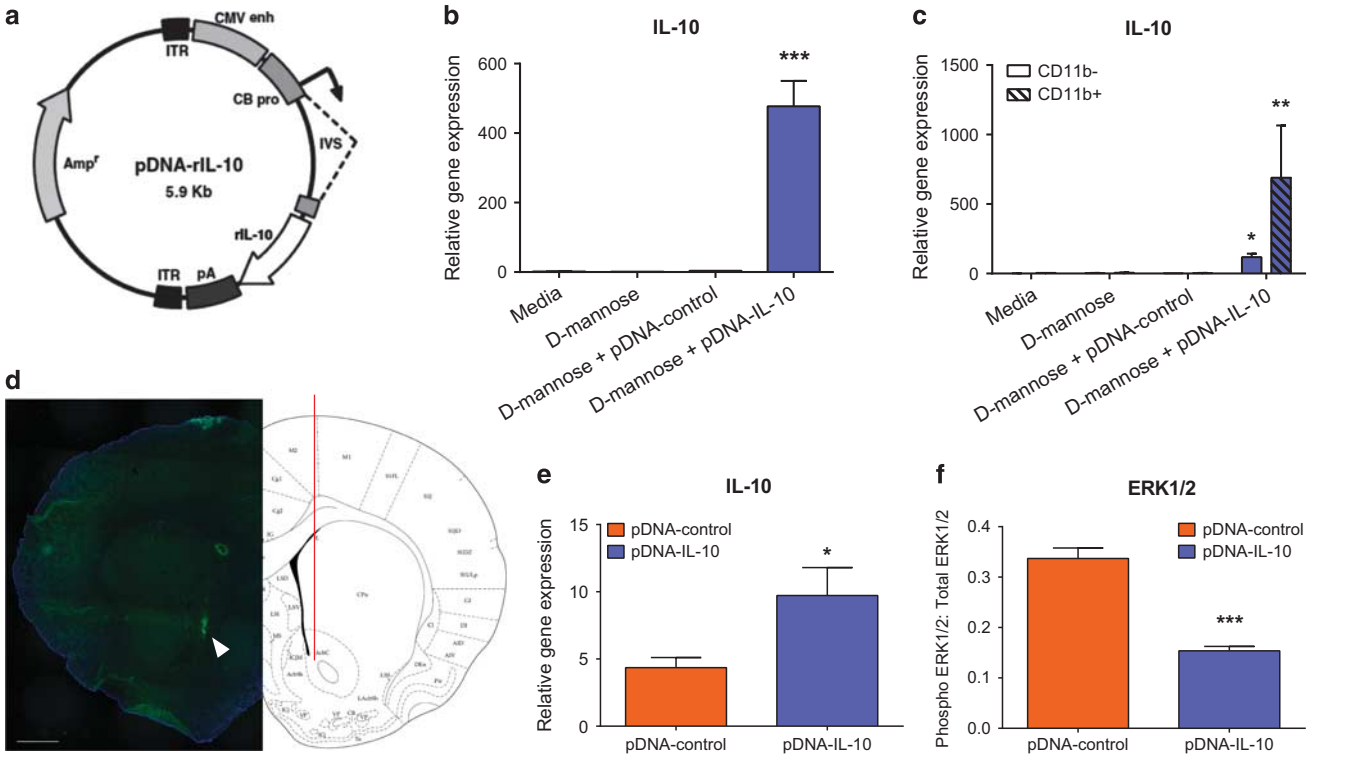


Figure 4 Naked pDNA encoding rat IL-10 delivered with D-mannose increases IL-10 expression *in vitro* and *in vivo*. (a) Design of plasmid vector (adapted from Milligan *et al*, 2006). (b) Using dissociated cell culture, incubation with D-mannose+pDNA-IL-10 for 2 h markedly increases IL-10 mRNA expression compared with D-mannose+pDNA-control or D-mannose alone (***$p<0.001$, Tukey's *post hoc* test; $n=3$/group). (c). Isolated microglia (CD11b$^+$ cells) show a greater increase in IL-10 mRNA following 2 h incubation with D-mannose+pDNA-IL-10, compared with the CD11b$^-$ population (*$p<0.05$ main effect of pDNA-IL-10 treatment; **$p<0.01$ compared with CD11b$^-$ population, Bonferroni's *post hoc* test; $n=2$–3/group). (d) Left panel: Representative image of pDNA infusion site in NAc. Images acquired at × 10 magnification (Zeiss, Axio Imager) and tiling achieved using Zen 2 software (Zeiss); DAPI in blue, scale bar = 1 mm. White arrowhead indicates infusion site, observed near the anterior commissure. Right panel: Representation of the stereotaxic coordinates for pDNA treatment into NAc based on the Paxinos and Watson atlas. Red line indicates path of Hamilton syringe. (e) IL-10 mRNA is elevated in striatal tissue 1 week following pDNA-IL-10 treatment (*$p<0.05$; $n=5$/group). (f) ERK1/2 activation (ratio of phosphorylated ERK1/2 to total ERK1/2 protein) is decreased in the striatum 1 week following pDNA-IL-10 treatment (***$p<0.001$; $n=4$/group).

sucrose (Supplementary Figure S2f) self-administration, and no differences between groups in their responding on the inactive lever during trials for food (Supplementary Figure S2d) or sucrose (Supplementary Figure S2g).

Similarly, food and sucrose self-administration was investigated following pDNA treatment (Supplementary Figure S3a). Responding for both food and sucrose was robust across sessions (Supplementary Figure S3b and e). The number of food pellets acquired per session increased over time ($F_{1,8}=9.87$, $p<0.05$), but there was no effect of pDNA treatment on food intake (Supplementary Figure S3c). The number of sucrose pellets acquired did not change over time (presumably due to high initial responding as a result of previous food sessions), and was not affected by pDNA treatment (Supplementary Figure S3f). In addition, responding on the inactive lever did not differ across sessions or between groups for either food (Supplementary Figure S3d) or sucrose (Supplementary Figure S3g). These data suggest that neither handling nor pDNA-IL-10-induced attenuation of opioid self-administration generalize to food or sucrose.

DISCUSSION

Our results reveal that the early-life maternal environment can influence later-life opioid self-administration in a drug-concentration-dependent manner. Moreover, we demonstrate that neonatal handling induces an anti-inflammatory transcriptional environment in the NAc after repeated remifentanil exposure and specifically increases anti-inflammatory IL-10 expression. We thus hypothesized that IL-10 expression may be a novel resilience factor for addiction liability. In support of this, direct administration of pDNA overexpressing IL-10 into the NAc of rats reduced self-administration of remifentanil in a dose- and time-dependent manner, without affecting the response to natural rewards. Importantly, these data are the first to our knowledge to demonstrate that gene therapy for an anti-inflammatory cytokine targeting a brain region critical for reward processing can influence opioid drug-taking behaviors.

Neonatal Handling and Opioid Reinforcement

Early-life experience (eg, stress or neglect) is one of the strongest predictors of substance abuse later in life (Dube *et al*, 2003; Felitti *et al*, 1998), though little is known about the long-lasting neurobiological mechanisms that shape an individual's vulnerability or resilience for drug addiction. We demonstrate here that a reasonably simple manipulation of neonatal handling from P2 to P20 has a subtle yet significant

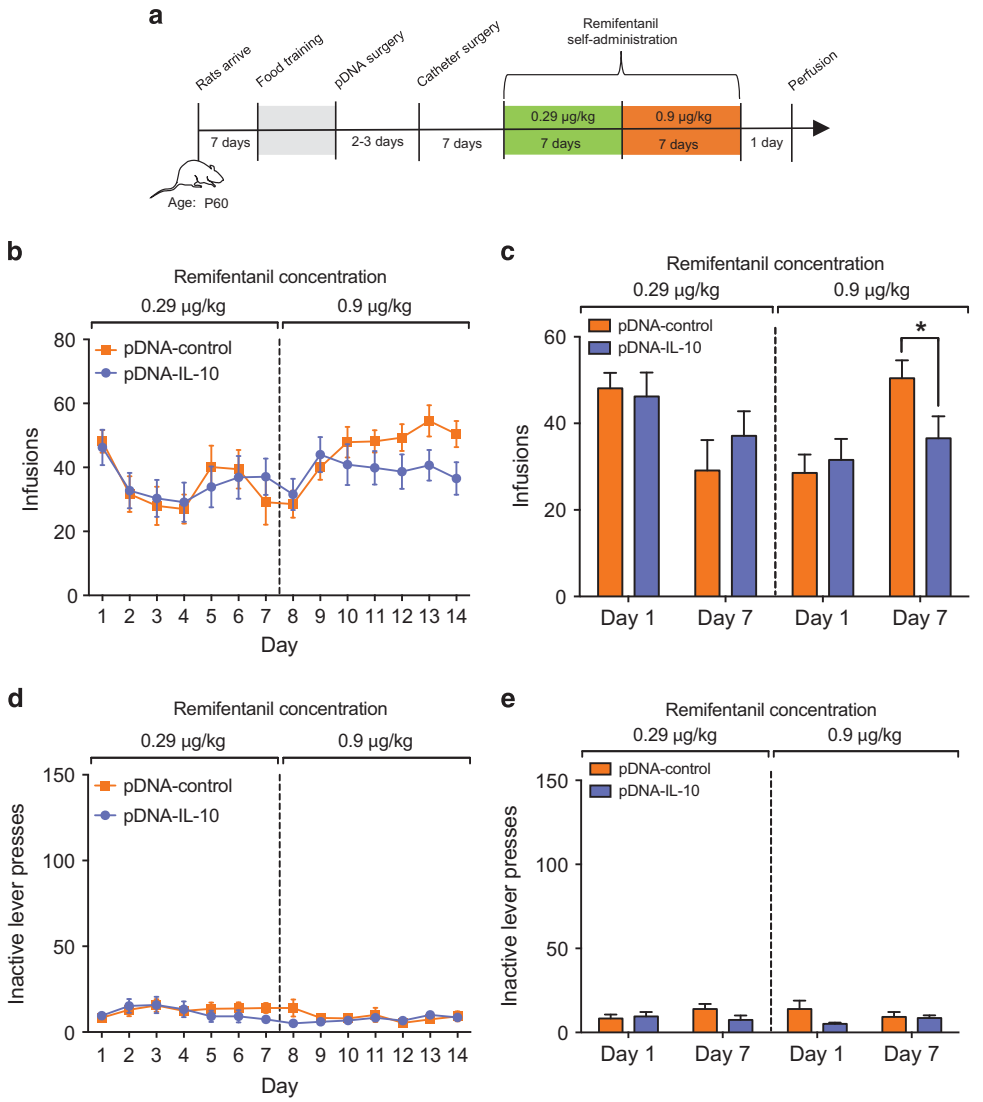


Figure 5 Naked pDNA encoding rat IL-10 delivered into NAc reduces remifentanil self-administration. (a) Schematic representation of the remifentanil self-administration experimental timeline. Adult rats received bilateral stereotaxic delivery of D-mannose along with pDNA-control or pDNA-IL-10, and remifentanil self-administration behavior was observed ($n = 9$/group). (b) The number of intravenous infusions of remifentanil was recorded in daily 1 h sessions (FR1, 20 s time-out) for 14 days: 7 days at 0.29 µg/kg and 7 days at 0.9 µg/kg. (c) pDNA-IL-10 treatment does not influence remifentanil self-administration during initial sessions at 0.29 µg/kg, although infusions per session did decrease from day 1 *vs* day 7. In contrast, pDNA-IL-10 significantly attenuated the number of remifentanil infusions acquired on day 7 when remifentanil is delivered at 0.9 µg/kg, compared with pDNA control (*$p < 0.05$). (d) Responses on the inactive lever were recorded across sessions. (e) Inactive lever responding during remifentanil sessions did not differ between groups.

effect on motivational behaviors for the fast-acting opioid remifentanil, attenuating the number of infusions acquired in a drug-concentration-dependent manner and resulting in a downward deflection of the dose–response curve. The enduring effects of neonatal handling on rodent physiological and behavioral sequelae have been known for decades (Levine, 1967), and many authors have proposed that variations in maternal care during this critical period of postnatal development (both naturally occurring variations and those induced by the experimenter through home-cage disruption) can influence neural circuit function throughout the lifespan (Curley and Champagne, 2016; Korosi and Baram, 2010; Meaney, 2001). Previous investigations have found neonatal handling to reduce cocaine and ethanol consumption (Moffett *et al*, 2006; Ploj *et al*, 2003), but its effect on opioid drug-taking was unknown. Here we report that neonatal handling attenuates remifentanil self-administration under defined conditions. Interestingly, the effect of handling was typically revealed when the remifentanil concentration was altered from the dose received in early sessions, implying a strong dose-dependent relationship (further discussion below). To this end, handling appears to shift the dose–response curve for remifentanil, where lower concentrations of drug are less reinforcing in the handled group. It is unlikely this response is due to a difference in tolerance, as the infusion rate at these lower doses did not fluctuate across sessions (Supplementary Figure S4); however, formal measures of drug tolerance were not assessed and cannot be entirely ruled out.

Neuroinflammatory Signaling and Opioid Reinforcement

The neuronal circuits projecting to and from the dopamine receptor-expressing neurons in the NAc have been extensively characterized for their role in motivation and reward learning (Hyman *et al*, 2006). Drug-induced synaptic plasticity in NAc medium spiny neurons is thought to contribute to the persistence of addiction behavior (Enoksson *et al*, 2012; Graziane *et al*, 2016; Hearing *et al*, 2016; Pascoli *et al*, 2014), and μ-opioid receptor-expressing neurons in the NAc are also critical for the maintenance of remifentanil consumption (Cui *et al*, 2014). More recently, microglial cytokine signaling in the NAc has been associated with synaptic and behavioral responses to morphine (Schwarz and Bilbo, 2013) as well as cocaine (Lewitus *et al*, 2016). On the basis of these collective observations, we focused our characterization of neuroimmune gene expression to the NAc; however, it remains to be determined if the transcriptional results observed here are specific to the NAc. Distinct glial and neuroimmune contributions to morphine tolerance and withdrawal have been identified in the ventral tegmental area (Taylor *et al*, 2016), periaqueductal gray (Eidson *et al*, 2017), and spinal cord (Burma *et al*, 2017), suggesting that neuroimmune adaptations occur across distributed neural circuits and contribute to diverse outcomes associated with repeated opioid exposure.

Our transcriptional analyses suggest that cytokine and chemokine signaling within the NAc, including pathways associated with TLRs, may represent important neural-glial communication associated with opioid reinforcement. For instance, we found that remifentanil self-administration increases TLR4 expression in the NAc regardless of early-life experience. Opioids can interact with the lipopolysaccharide-binding pocket of myeloid differentiation factor-2 (MD-2), leading to TLR4 oligomerization and subsequent pro-inflammatory cytokine and chemokine transcription (Wang *et al*, 2012). Non-specific modulators of inflammatory signaling (eg, ibudilast) as well as specific disruption of TLR4 signaling reduces the rewarding effects of opioids, potentially through reduced NAc dopamine transmission (Bland *et al*, 2009; Hutchinson *et al*, 2012). The contribution of TLR signaling to addiction-like behavior is supported by studies in which disruption of TLR4 signaling prevents morphine and oxycodone conditioned place preference and reduces remifentanil self-administration (Hutchinson *et al*, 2012). Similarly, chronic antagonism of TLR4 with (+)-naltrexone reduces cue-induced heroin self-administration following withdrawal (Theberge *et al*, 2013). Given that TLR4/MD-2 signaling may also underlie behavioral responding to cocaine (Northcutt *et al*, 2015), TLR activity could represent a unifying mechanism regulating the immune responses to drugs of abuse, and inhibition of the pro-inflammatory downstream products may alter drug reinforcement.

Despite the promising view of TLR4 as a master regulator of opioid-induced neuroinflammation, some conflicting reports have emerged regarding its function in opioid-induced behavioral effects, including morphine withdrawal and remifentanil self-administration (Mattioli *et al*, 2014; Tanda *et al*, 2016). In light of these observations, alternative immune signaling pathways may also be recruited to mediate drug-induced inflammation. TLR2, for instance, has also been implicated in mediating microglial inflammatory responses to opioids (Zhang *et al*, 2011), cocaine (Liao *et al*, 2016), and ethanol (Fernandez-Lizarbe *et al*, 2013), and we observed TLR2 gene downregulation specifically in handled rats following remifentanil. There were additional unexpected findings from the transcriptional profiling, such as significant elevations of IL-6 in drug-naive handled rats. Given the pro- and anti-inflammatory properties of IL-6 in classic- and trans-signaling (Scheller *et al*, 2011), it is difficult to predict the functional outcomes this would support. Although the precise mechanisms of opioid-induced neuroinflammation remain unclear, further research aimed at cell-type-specific perturbations of neuroimmune signaling may help resolve these issues.

The Role of IL-10 in Opioid Reinforcement

We confirm that neonatal handling produces a persistent elevation in IL-10 gene expression in the NAc, consistent with previous reports (Schwarz *et al*, 2011). IL-10 is a potent inhibitor of TLR-mediated signaling and can directly inhibit cytokine transcription and translation (Kishore *et al*, 1999). The selectivity to which IL-10 can inhibit TLR4 is further demonstrated by the characterization of microRNA-146b, which is induced by IL-10 and negatively regulates TLR4-mediated pro-inflammatory cytokine and chemokine induction (Curtale *et al*, 2013). Thus, we predicted that elevated expression of IL-10 may be sufficient to suppress the downstream signaling pathways of TLR activation in response to opioids, either directly inhibiting TLR activity or indirectly influencing gene expression patterns of other critical cytokines and chemokines, including those identified in the PCR array. The data derived from the functional pathway analysis supports this assumption, as functionally related inflammatory genes from the PCR array show a strong, consistent pattern of downregulation in the NAc from handled rats.

On the basis of the data presented here, we predicted that augmentation of IL-10 expression in non-handled rats would recapitulate the behavioral results induced by neonatal handling. Because we sought to determine if IL-10 upregulation itself is sufficient to influence behavior, regardless of early-life experience, we chose to treat only non-handled subjects. We administered naked pDNA-IL-10 due to its proven efficacy for gene therapy while avoiding some immunogenicity concerns from viral transfection (Milligan *et al*, 2006; Yin *et al*, 2014). Although transfection efficiency of pDNA is a major challenge, D-mannose as a transgene adjuvant greatly improves transgene expression, potentially by stimulating phagocytosis (Dengler *et al*, 2014). Indeed, we provide evidence that pDNA-IL-10 treatment with D-mannose increases IL-10 mRNA both *in vitro* and *in vivo*. IL-10 mRNA was upregulated *in vivo* 1 week following treatment and was accompanied by changes in ERK protein phosphorylation, suggestive of persistent changes at the levels of cytokine transcription and downstream effectors (Pereira *et al*, 2015). Whether these pDNA-induced changes in IL-10 and ERK phosphorylation persist throughout self-administration awaits further exploration.

Following confirmation of our pDNA-IL-10 treatment paradigm, we present novel evidence that direct gene

manipulation of an anti-inflammatory cytokine can influence opioid reinforcement. Interestingly, this response was drug-concentration-dependent in a manner similar to the neonatal handling condition. We predict that microglia may play a critical role in these outcomes, given that acute morphine administration profoundly increases IL-10 transcription primarily in microglial cells of the NAc (Schwarz *et al*, 2013). Previous analysis of isolated NAc microglia determined that neonatal handling results in epigenetic alterations to the DNA methylation state of IL-10 (Schwarz *et al*, 2011), which may explain the remarkably persistent effects of maternal care on IL-10 expression into adulthood. Likewise, we found *in vitro* treatment with pDNA-IL-10 significantly increases IL-10 mRNA expression to a greater extent in the $CD11b^+$ microglial population. This suggests that cellular internalization of the pDNA vector occurs primarily in microglia. However, additional experiments are required to determine the cellular localization of the IL-10 transgene *in vivo*. Although it is currently unclear if other immunocompetent cells of the central nervous system (such as astrocytes or meningeal T cells) are influenced by or contribute to the behavioral consequences observed following neonatal handling or pDNA manipulation, our results are consistent with the interpretation that anti-inflammatory signaling in the brain can influence behavioral responses to opioids.

Limitations and Conclusions

There are limitations inherent in experimental design that are worth considering. Although the handling conditions were identical preceding remifentanil self-administration (Figures 1 and 2), there are important differences in remifentanil exposure between the experiments, including number of sessions, doses received, and the sequence of its availability. There is considerable evidence that the extent of drug exposure (in terms of dose and frequency of exposure) can impact subsequent behavioral and molecular responses to the drug (Ahmed *et al*, 2000; Mendrek *et al*, 1998; Robinson and Kolb, 2004). Thus, we cannot rule out the possibility that differences in the quantity and duration of remifentanil exposure could explain some of the behavioral or transcriptional effects observed. For instance, handled rats on the dose–response curve show attenuated drug intake during sessions at lower concentrations (0.09 and 0.29 μg/kg), whereas the significant effect of handling was revealed only when the dose was increased from 0.29 to 0.9 μg/kg. This suggests that the concentration of intravenous remifentanil received per infusion during initial sessions shapes the outcome of responding to subsequent changes in drug concentration, and this outcome can be influenced by early-life environmental conditions. Indeed, there is evidence that drug dose and session time during training or early sessions can influence drug consumption in subsequent trials for abused substances, including cocaine and heroin (Mandt *et al*, 2012; Martin *et al*, 1998). Although these differences in experimental design prevent direct comparisons between results, the data converge on a hypothesis of anti-inflammatory signaling in the NAc as a characteristic of early-life experience that influences opioid reinforcement, which was elaborated upon by pDNA-IL-10 experiments. Further delineation of how early-life experience or IL-10 gene therapy influences opioid intake over time in a drug-concentration-dependent manner is a topic of considerable interest that warrants further characterization.

An additional caveat to note is that differences in cytokine and chemokine gene expression in Handled-Remi rats (Figure 3) could be a consequence of decreased drug exposure, as opposed to handling *per se*. Future experiments employing yoked, non-contingent delivery of remifentanil could offer additional insight into the molecular consequences that occur when drug intake is controlled. Moreover, attenuation of remifentanil drug-taking could potentially be explained by altered motivation to positive reinforcers, or a behavioral deficit to operant conditioning. However, this notion was not supported by the data, as neither handling nor pDNA-IL-10 treatment influenced food or sucrose self-administration. Although the experimental timeline differed from rats receiving remifentanil (ie, no jugular catheterization surgery), the results suggest that handling and intracranial pDNA-IL-10 treatment do not suppress motivation for palatable rewards.

In conclusion, utilizing both environmental and genetic manipulations aimed at altering IL-10 expression, we identify an anti-inflammatory mechanism that is associated, under certain conditions, with resilience against the reinforcing properties of opioids. These data support the expanding literature implicating neuroimmune signaling from glia and other sources as important modulators of behavioral outcomes associated with drug addiction (Lacagnina *et al*, 2017). Future research aimed at modifying immune responses with increased precision might offer additional insights to improve therapeutics for those already suffering from opioid abuse.

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