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Facilitating mGluR4 activity reverses the long-term deleterious consequences of chronic morphine exposure in male mice

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Abstract

Understanding the neurobiological underpinnings of abstinence from drugs of abuse is critical to allow better recovery and ensure relapse prevention in addicted subjects. By comparing the long-term transcriptional consequences of morphine and cocaine exposure, we identified the metabotropic glutamate receptor subtype 4 (mGluR4) as a promising pharmacological target in morphine abstinence. We evaluated the behavioral and molecular effects of facilitating mGluR4 activity in abstinent mice. Transcriptional regulation of marker genes of medium spiny neurons (MSNs) allowed best discriminating between 4-week morphine and cocaine abstinence in the nucleus accumbens (NAc). Among these markers, *Grm4*, encoding mGluR4, displayed down-regulated expression in the caudate putamen and NAc of morphine, but not cocaine, abstinent mice. Chronic administration of the mGluR4 positive allosteric modulator (PAM) VU0155041 (2.5 and 5 mg/kg) rescued social behavior, normalized stereotypies and anxiety and blunted locomotor sensitization in morphine abstinent mice. This treatment improved social preference but increased stereotypies in cocaine abstinent mice. Finally, the beneficial behavioral effects of VU0155041 treatment in morphine abstinent mice were correlated with restored expression of key MSN and neural activity marker genes in the NAc. This study reports that chronic administration of the mGluR4 PAM VU0155041 relieves long-term deleterious consequences of morphine exposure. It illustrates the neurobiological differences between opiate and psychostimulant abstinence and points to pharmacological repression of excessive activity of D2-MSNs in the NAc as a promising therapeutic lever in drug addiction.

Keywords

Drug abstinence; opiate; psychostimulant; social behavior; metabotropic glutamate receptor; gene expression

INTRODUCTION

Drug addiction is a chronic psychiatric disorder characterized by loss of control over consumption despite negative consequences [1]. Addicted subjects wanting to quit drug use face a true challenge, as protracted abstinence leads to negative affect, anxiety disorders and social withdrawal [2-4] that increase the propensity for relapse [5]. A better understanding of the long-lasting neurobiological underpinnings of the abstinent state thus appears invaluable to improve relapse prevention [6-8]. Although addiction to most drugs of abuse share similar clinical features, a unitary view of addiction is challenged by different drug-seeking behaviors and brain adaptations across drug classes, particularly for narcotics versus psychostimulants [9-12]. The neurobiological mechanisms underlying abstinence and vulnerability to relapse are thus also likely to diverge between drugs.

In previous reports, we have evidenced common long-term transcriptional adaptations that develop within the central extended amygdala (central EA: central amygdala and bed nucleus of the stria terminalis, CeA and BNST, respectively) upon protracted abstinence to morphine, nicotine, Δ 9-tetrahydrocannabinol (THC) and alcohol [13,14] but not cocaine [14]. These adaptations mostly involved genes with an enriched expression in medium spiny GABAergic neurons (MSNs), a key brain substrate for drug reward and addiction [15-17], and notably 14 genes belonging to a huntingtin (HTT)-related gene network [13,14]. Consistent with shared molecular alterations, protracted abstinence from the aforementioned four drugs induced similar behavioral deficits, including impaired social abilities, motor stereotypies and exacerbated anxiety-like behavior [14,18-20] that were not observed following cocaine abstinence, except for blunted social preference [14]. Remarkably, behavioral deficits evidenced in abstinent from morphine, nicotine, alcohol or THC were similar to those detected in lacking the mu opioid receptor (*Oprm1*^{-/-}), a mouse model of autism [21,22], and, more generally, in most mouse models of this neurodevelopmental disorder [23,24]. Such similarities may reflect common striatal dysfunction and altered reward processing [25,26], possibly involving compromised opioid function [27-29]. Shared neurobiological mechanisms between drug addiction and autism spectrum disorders interestingly argue for the development of novel therapeutic interventions that would benefit to both pathologies, with the caveat that

addiction to cocaine/psychostimulants may diverge from narcotic drugs in their responsiveness to such approaches.

In the present study, we extended our comparison of morphine versus cocaine abstinence-induced transcriptional changes to the caudate putamen (CPu) and nucleus accumbens (NAc), two more brain regions where MSNs represent the main cell type, and analyzed separately the transcriptomes of the CeA and BNST. Not only we confirmed that morphine and cocaine abstinence produce contrasting regulations in gene expression, especially genes with MSN enriched expression, but we detected down-regulated levels of *Grm4* (encoding the presynaptic mGlu4 glutamate receptor - mGluR4) transcripts in the CPu and NAc of morphine, and not cocaine, abstinent mice. Decreased *Grm4* expression retained our attention as another common feature between morphine abstinent and *Oprm1*^{-/-} [21,30], as we evidenced in the latter a rescue of sociability, stereotypic behavior and anxiety-like behavior under chronic facilitation of mGluR4 activity [21]. Activation of mGluR4 was shown to inhibit GABA release by indirect, D2 dopamine receptor-expressing MSNs (D2-MSNs), with therapeutic benefit in Parkinson's disease [31,32]. Interestingly, negative affect in opioid addiction and dependence may involve D2-MSNs rather than direct, D1 dopamine receptor expressing MSNs (D1-MSNs) [33-35]. Here we tested whether chronic administration of VU0155041, a positive allosteric modulator (PAM) and partial agonist of mGluR4, can alleviate the deleterious behavioral and molecular consequences of abstinence to morphine, and presumably not to cocaine. This study suggests that mGluR4 is a promising pharmacological target to treat the long-term behavioral consequences of opioid use disorder (OUD) and further argues for the involvement of distinct neurobiological processes in opiate versus psychostimulant addiction.

METHODS AND MATERIALS

Subjects

In all experiments, we used C57BL6/J male mice aged 8-12 weeks (Charles River, Lyon, France). Mice were group housed and maintained on a 12hr light/dark cycle (lights on at 7:00 AM) at controlled temperature (21±1°C); food and water were available *ad libitum*, except otherwise stated. Mice in the same cage received the

same treatment. All experimental procedures were conducted in accordance with the European Communities Council Directive 2010/63/EU and approved by the Comité d’Ethique pour l’Expérimentation Animale de l’ICS et de l’IGBMC (Com’Eth, 2012-033) and Comité d’Ethique en Expérimentation animale Val de Loire (C2EA-19).

Drugs

Chronic morphine and cocaine treatments were performed as described previously [14] (details in [Supplement 1](#)). Mice were injected twice daily with vehicle (i.p., NaCl 0.9%) or morphine (i.p., escalating doses from 20 mg/kg to 100 mg/kg over 5 days and one injection on day 6 at 100 mg/kg) or cocaine (s.c., 25 mg/kg during 5.5 days). They were left drug-free for 3 weeks before chronic daily treatment with vehicle (NaCl 0.9%) or VU0155041 (Tocris, Bristol, UK; 2.5 or 5 mg/kg, i.p.) started. Behavioral testing started after 8 days of VU0155041 administration, to evaluate chronic effects of treatment. This treatment was maintained for 8 to 15 consecutive days, allowing comprehensive behavioral phenotyping (see time lines in [Figure S1](#)). On testing days, VU0155041 (or vehicle) was administered 30 min before behavioral assays. All compounds were administered in a volume of 10 ml/kg.

Antibodies

In Western blot experiments, primary antibodies targeting GAPDH (Rabbit mAb, ref. 2118; RRID: AB_561053) (1:2000) from Cell Signaling Technology and synaptophysin (mouse SYP antibody [D-4], ref. sc-17750; RRID: AB_628311) (1:2000) from Santa Cruz Biotechnology were used at the indicated dilutions. They were combined with the following secondary antibodies: goat anti rabbit IRDye®800CW (LI-COR®, USA, 926-3221; RRID: AB_621843) (1:15000) and goat anti mouse IRDye®680RD (LI-COR®, USA, 926-68070; RRID: AB_10956588) (1:15000).

Behavioral experiments

As described previously [14,30], social behavior was explored using the direct social interaction and three-chamber tests, stereotyped/perseverative behavior was assessed by scoring spontaneous motor stereotypies and buried marbles in the marble burying test and by analyzing alternation patterns in the Y-maze, and anxiety-like behavior was evaluated in the novelty-suppressed feeding test. Locomotor

activity was recorded using video-tracking (Viewpoint, Lyon, France). Behavioral assays started on week 4 after cessation of morphine or cocaine exposure, on day 8 of VU0155041/vehicle treatment. On testing days, mice received pharmacological treatment 30 min prior to testing. Detailed protocols can be found in [Supplement 1](#); experimental cohorts are detailed in [Figure S1](#).

Real-time quantitative Polymerase Chain Reaction analysis

Real-time quantitative Polymerase Chain Reaction (qRT-PCR) analysis was performed on brain samples as described previously [14,21,36] (see dissection in [Figure S2](#), supplementary experimental procedures in [Supplement 1](#) and list of probes in [Table S1](#)).

Western blot experiments

Mouse brains were dissected 45 min after social interaction and brain regions of interest were punched out [21,37]. Tissues were processed and protein expression was measured as described previously [37] (see supplementary experimental procedures in [Supplement 1](#)).

Statistics

Statistical analyses were performed using Statistica 9.0 software (StatSoft, Maisons-Alfort, France). For all comparisons, values of $p < 0.05$ were considered as significant. Statistical significance in behavioral experiments was assessed using one or three-way analysis of variance (drug, stimulus, treatment and time effects) followed by Newman-Keuls post-hoc test. Significance of quantitative real-time PCR (qRT-PCR) results was assessed after transformation (if $x < 1$, $y = 1 - 1/x$; if $x > 1$, $y = x - 1$, x : qRT-PCR data) using a two-tailed t-test, and p-value was adjusted using Benjamin-Hochberg correction for multiple testing. Unsupervised clustering analysis was performed on transformed qRT-PCR data [14,21,36] using complete linkage with correlation distance (Pearson correlation) for drug, treatment and brain region (Cluster 3.0 and Treeview software). When used for clustering analysis ([Figure 5A](#)), behavioral data were normalized to vehicle-vehicle condition and transformed using the same formula as qRT-PCR data.

RESULTS

Deregulated expression of MSN marker genes discriminated between morphine and cocaine abstinence

We first compared the long-term consequences of a history of morphine or cocaine exposure at transcriptional level across the CPu, NAc, BNST and CeA, by assessing the expression of a set of 30 candidate genes, including the 14 HTT-related genes regulated by morphine abstinence (*Adora2*, *Arpp21*, *Bcl11b*, *Gcnt2*, *Cnr1*, *Drd1a*, *Fam107a*, *Foxp1*, *Gpr88*, *Hpca*, *Nr4a1*, *Pde10a*, *St8sia3*, *Strip2*)[13], 13 additional marker genes of MSNs identified from the literature (see Table S1) [38-40] and the *Oxt* gene, coding the neuropeptide oxytocin, as a marker of social behavior [30]. We used hierarchical clustering analysis of qRT-PCR data obtained for each drug in each brain region studied (Figure 1, Table S2) to compare gene expression profiles.

In the four regions tested, clustering analysis organized gene expression in three to six clusters (Figure 1A). Convergent effects of morphine and cocaine abstinence were observed in discrete clusters in the CPu (cluster b), NAc (cluster d) and CeA (cluster a) and in three out of six clusters in the BNST (clusters b,c,e). The remaining clusters revealed divergent, when not opposed, influence on gene expression. Interestingly, in a majority of these clusters, HTT-related and MSN marker genes displayed down-regulated expression under morphine conditions contrasting with up-regulated or unchanged expression under cocaine conditions. This pattern of gene expression was observed in the CPu (clusters a and c, 13 genes), NAc (cluster c, 14 genes), CeA (cluster c, 19 genes) and, to a lesser extent, in the BNST (cluster a, 6 genes). Thus, morphine abstinence was associated with down-regulated expression of numerous MSN-enriched genes in the CPu, NAc and CeA, an effect that was not observed under cocaine abstinence. Notably, we noticed seven genes with consistent down-regulated expression in the CPu, NAc and CeA under morphine conditions contrasting with no regulation or upregulation under cocaine abstinence: *Arpp21*, *Drd1a*, *Drd2*, *Hpca*, *Grm4*, *Nr4a1* and *Pde10a* (Figure 1B). Among them, *Grm4* retained our attention for coding mGluR4, which we had previously identified as a promising target to alleviate deficient social behavior.

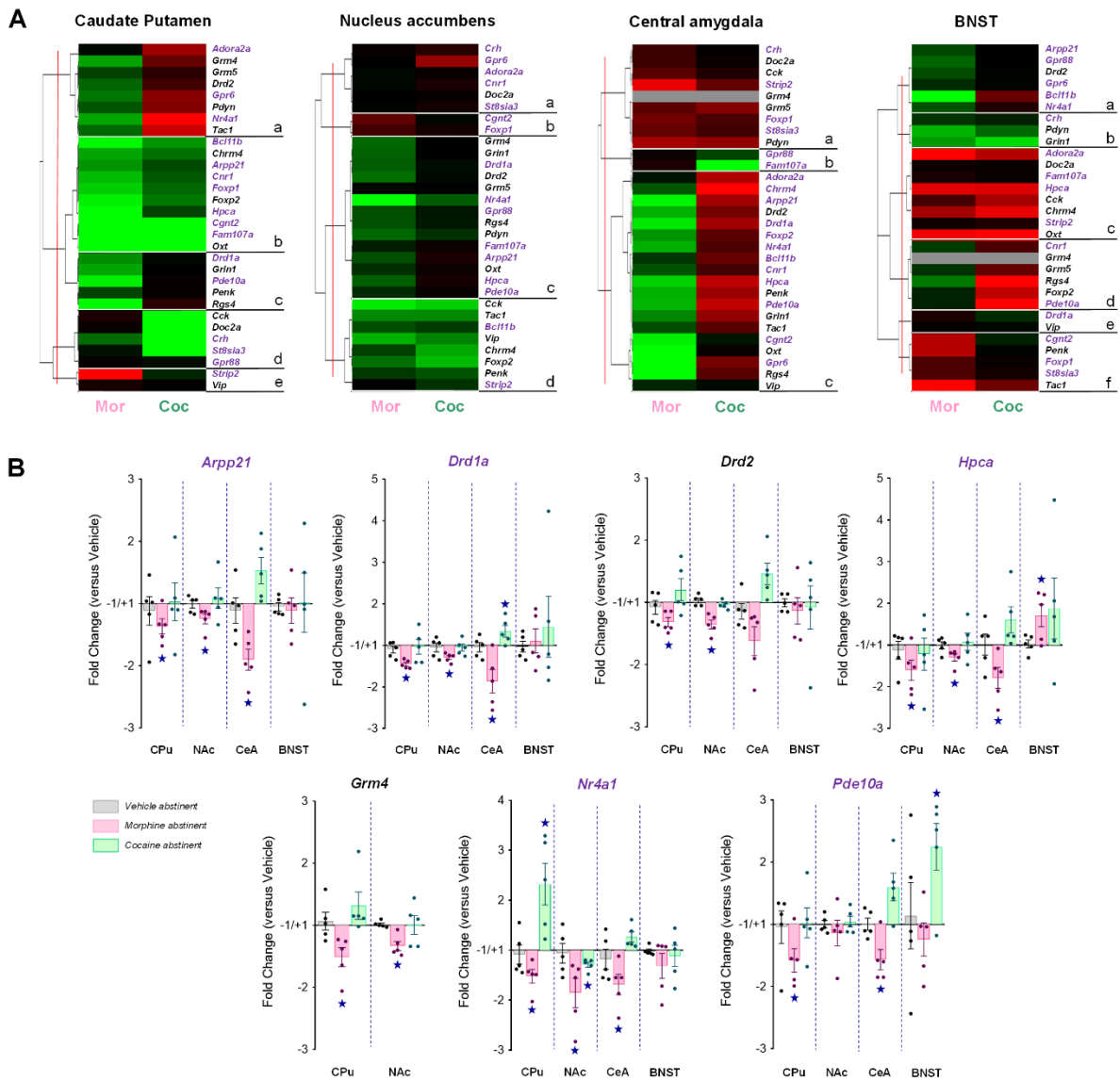


Figure 1. Deregulated expression of MSN marker genes in the CPu, NAc and CeA allows to best discriminate between morphine and cocaine abstinence.

(A) We used qRT-PCR to compare the expression of 30 candidate genes, among which 14 previously identified HTT-related genes (purple characters), 13 additional MSN marker genes and *Oxt* as a marker transcript of social behavior, in the CPu, NAc, CeA and BNST under morphine and cocaine abstinence conditions compared to their saline counterparts. Hierarchical clustering organized gene expression data in three to six clusters per region. In a majority of these clusters, HTT-related and MSN marker genes displayed down-regulated expression under morphine conditions contrasting with up-regulated or unchanged expression under cocaine conditions. This pattern of gene expression was

observed in the CPu (clusters a and c), NAc (cluster c), CeA (cluster c) and, to a lesser extent, in the BNST (cluster a). **(B)** Seven MSN marker genes displayed consistent down-regulated expression in the CPu, NAc and CeA under morphine conditions contrasting with no regulation or upregulation under cocaine abstinence, among which *Grm4*, coding for the mGlu4 receptor. Gene expression data are expressed as fold change versus vehicle abstinent for each condition (mean \pm SEM, n=5 per group). Comparison to vehicle group: $p < 0.05$ (two-tailed t test, p-value adjusted using Benjamin-Hochberg correction for multiple testing). qRT-PCR data used for clustering are displayed in Table S2.

Chronic VU0155041 administration relieved social behavior deficits in morphine and cocaine abstinent mice

We then tested whether facilitating mGluR4 activity by chronically administering a PAM of mGluR4, VU0155041 (2.5 or 5 mg/kg), in morphine abstinent mice would rescue their behavioral deficits. For comparison purpose, we administered the same treatment (VU0155041, 5 mg/kg) to cocaine abstinent mice.

In the direct social interaction test ([Figure 2A](#)), morphine abstinent mice displayed a marked deficit in social interaction that was dose-dependently normalized by VU0155041 treatment (partially at 2.5 mg/kg, completely at 5 mg/kg), as evidenced by restored time (abstinence x dose interaction: $F_{2,57}=56.2$, $p < 0.0001$), number (abstinence x dose: $F_{2,57}=28.5$, $p < 0.0001$) and duration of nose contacts (abstinence x dose: $F_{2,57}=70.1$, $p < 0.0001$), as well as number of following (abstinence x dose: $F_{2,57}=13.3$, $p < 0.0001$) and grooming episodes (abstinence x dose: $F_{2,57}=20.7$, $p < 0.0001$), especially after a social contact (abstinence x dose: $F_{2,57}=35.3$, $p < 0.0001$). In contrast, cocaine abstinent mice behaved similarly as vehicle abstinent mice in this test, and VU0155041 treatment had no significant impact on their social behavior, except for an increase in their number of following episodes (abstinence x treatment: $F_{1,28}=8.9$, $p < 0.01$).

In the three-chamber test ([Figure 2B](#)), morphine abstinent mice failed to show preference for interacting with the mouse rather than the toy, as evidenced by similar time spent interacting with both stimuli, and longer duration of their close interactions with the toy than with the mouse. Chronic administration of VU0155041 at 5 mg/kg restored social preference in morphine abstinent mice, rescuing longer time (stimulus x abstinence x treatment: $F_{2,86}=7.4$, $p < 0.01$) and duration of close contacts (stimulus x abstinence x treatment: $F_{2,86}=23.3$, $p < 0.0001$) with the mouse versus the toy. In cocaine abstinent mice, altered social preference was evidenced by a reduction in the difference between close contact duration with the toy versus the mouse. Chronic

VU0155041 significantly shortened the duration of interactions with the toy in these mice. Thus, chronic facilitation of mGluR4 activity relieved social deficits in both morphine and cocaine abstinent mice.

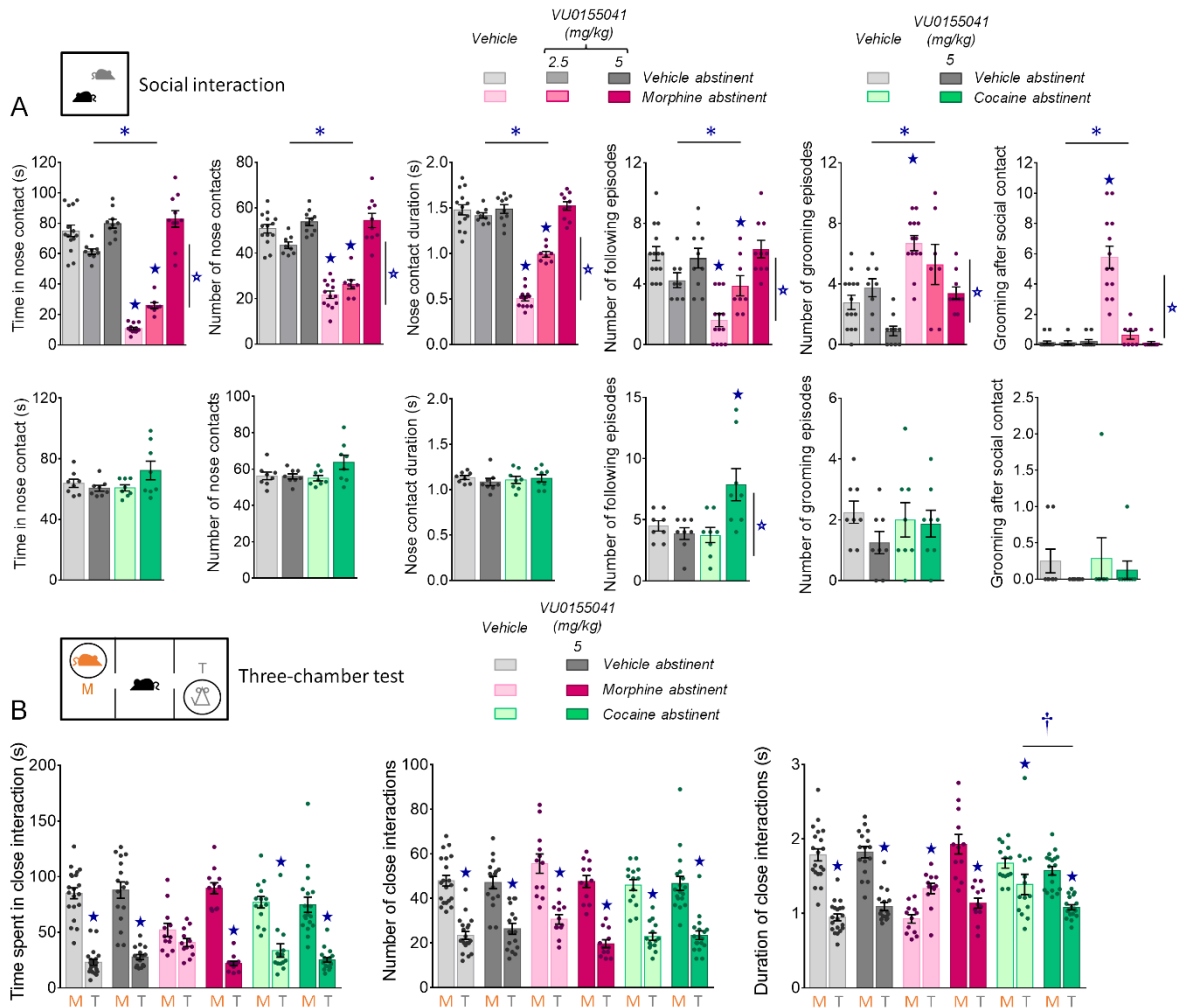


Figure 2. Effects of chronic VU0155041 administration on social behaviors in morphine and cocaine abstinent mice.

(A) In the direct social interaction test, facilitation of mGluR4 activity in morphine abstinent mice (top row, $n=8-14$ per group) dose-dependently normalized social interaction parameters. In cocaine abstinent mice (bottom row, $n=8-9$ per group), VU0155041 at the dose of 5mg/kg had no significant effect on social behavior, except for an increase in the number of following episodes. **(B)** In the three-chamber test, chronic administration of VU0155041 at 5 mg/kg restored social preference in morphine abstinent mice. In cocaine abstinent mice, the difference between close contact duration with the toy and close contact duration with the mouse was significantly increased by mGluR4 PAM treatment. Results are shown as scatter plots and mean \pm sem. Asterisk: abstinence effect (morphine/cocaine versus vehicle), open stars: treatment effect (VU0155041 versus vehicle), solid star: abstinence x treatment interaction, dagger: stimulus (toy versus mouse) x abstinence x treatment, comparison to

close contact duration with the toy in vehicle abstinent mice (two-way ANOVA or three-way ANOVA with stimulus as repeated measure, followed by Newman-Keuls post-hoc test); $p < 0.05$.

Chronic VU0155041 treatment normalized stereotypic behavior and anxiety-like behavior in morphine abstinent mice

We further evaluated the effects of chronic VU0155041 treatment by assessing its influence on other, non-social, long-term behavioral adaptations detected in morphine abstinent mice. We first assessed stereotyped/perseverative behavior in drug abstinent mice. Chronic VU0155041 administration (2.5 and 5 mg/kg) decreased rearing in morphine-exposed mice (abstinence x dose: $F_{2,56}=7.8$, $p < 0.01$), dose-dependently reduced grooming in both morphine and vehicle groups (abstinence: $F_{1,56}=242.1$, $p < 0.0001$; dose: $F_{2,56}=12.3$, $p < 0.0001$), increased burying behavior in vehicle abstinent mice (abstinence x dose: $F_{2,56}=3.3$, $p < 0.05$) and finally suppressed circling and head shakes in morphine abstinent mice while increasing the occurrence of these behaviors in vehicle controls (*Circling* - abstinence x dose: $F_{2,56}=29.1$, $p < 0.0001$; abstinence x dose: $F_{2,56}=29.1$, $p < 0.0001$; *Head shakes* - abstinence x dose: $F_{2,56}=25.9$, $p < 0.0001$) (Figure 3A). In vehicle and cocaine abstinent mice, chronic VU0155041 treatment (5 mg/kg) had no effect on cocaine abstinence-induced increase in rearing frequency (abstinence: $F_{1,30}=7.4$, $p < 0.05$) but increased the frequency of circling episodes (treatment: $F_{1,30}=7.2$, $p < 0.05$).

When exploring a Y-maze, morphine abstinent mice displayed impaired spontaneous alternation, mostly due to increased number of perseverative same arm returns. Chronic mGluR4 PAM administration dose-dependently restored spontaneous alternation (abstinence x dose: $F_{2,57}=8.6$, $p < 0.001$) by suppressing same arm returns (abstinence x dose: $F_{2,57}=39.5$, $p < 0.0001$); no effect of the treatment was detected in vehicle abstinent mice (Figure 3B). In this test, cocaine-exposed mice showed an increase in spontaneous alternation that was suppressed by chronic VU0155041 (abstinence x dose: $F_{1,30}=4.3$, $p < 0.05$) by normalizing the number of alternate arm returns (abstinence x dose: $F_{1,30}=4.2$, $p = 0.05$).

In the marble-burying test (Figure 3C), chronic VU0155041 reduced the number of marbles buried by morphine abstinent mice to the level of non-treated vehicle abstinent mice but increased this number when administered to vehicle abstinent mice (abstinence x dose: $F_{2,57}=7.8$, $p < 0.01$). Similarly, VU0155041 increased marble burying in cocaine and vehicle abstinent mice (treatment: $F_{1,30}=86.8$, $p < 0.0001$).

We assessed anxiety-like behavior in abstinent mice using the novelty-suppressed feeding test (Figure 3D). Chronic mGluR4 PAM administration normalized latency to feed and food intake in morphine abstinent mice since the lower dose (abstinence x dose: $F_{2,57}=10.5$, $p<0.001$), with no effect in vehicle or cocaine abstinent mice. In the latter, latency to feed was reduced in cocaine abstinent mice (abstinence: $F_{1,30}=17.5$, $p=0.001$).

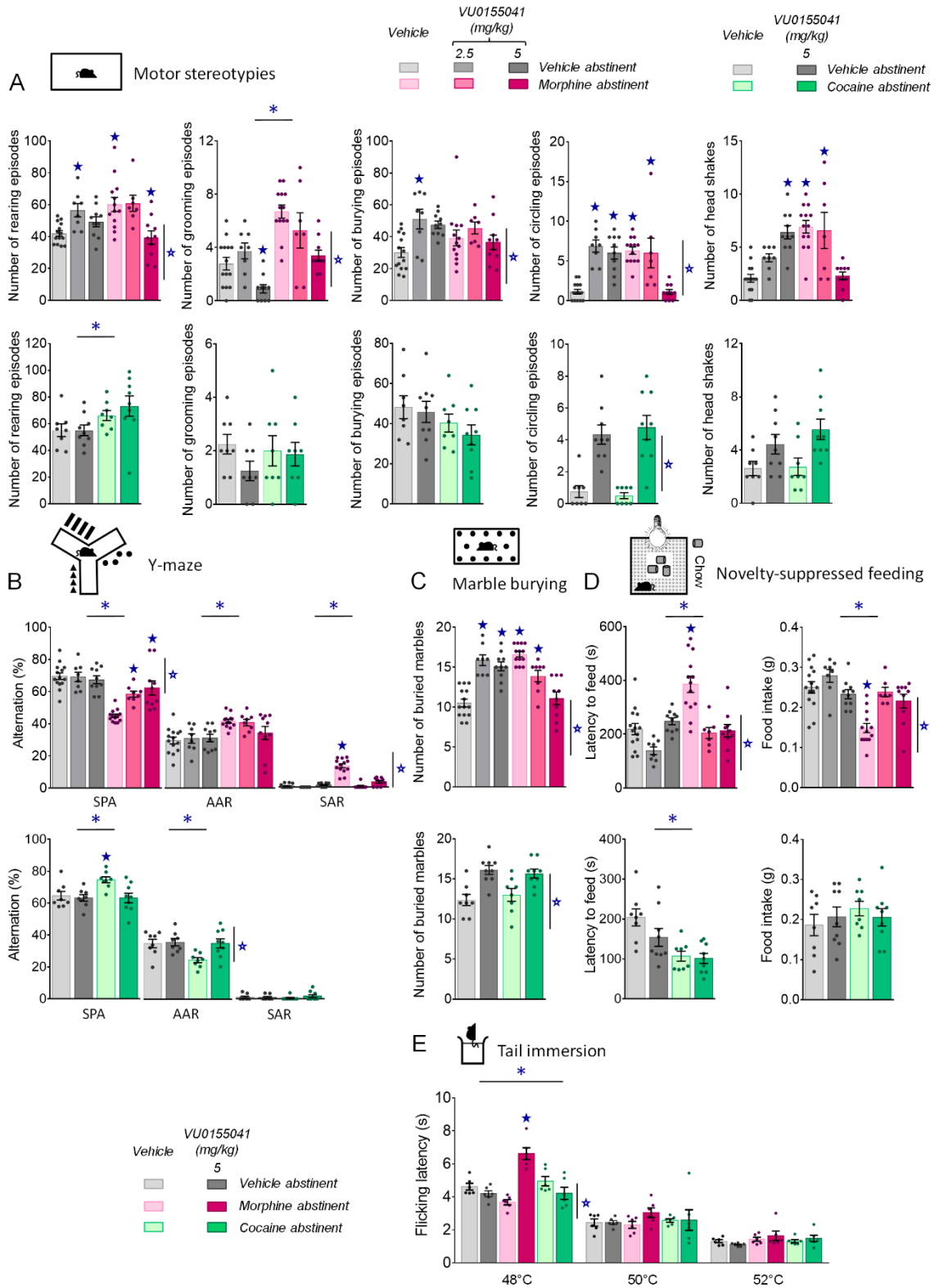
Finally, we evaluated nociceptive thresholds in morphine and cocaine abstinent mice under vehicle or VU0155041 treatment (5 mg/kg). Morphine abstinent mice showed a tendency for decreased nociceptive thresholds at 48°C, a temperature at which chronic mGluR4 PAM demonstrated analgesic effects in morphine-exposed mice only (abstinence x treatment: $F_{2,30}=28.5$, $p<0.0001$) (Figure 3E).

In conclusion, chronic mGluR4 PAM administration relieved motor stereotypies, perseverative behavior and excessive anxiety-like behavior and produced analgesia in morphine abstinent mice, with little or no effect in vehicle or cocaine abstinent mice.

Figure 3. Chronic facilitation of mGluR4 signaling relieved motor stereotypies, perseverative behavior, elevated anxiety and lowered nociceptive thresholds in morphine abstinent mice.

(A) Under morphine versus vehicle conditions (upper row, $n=8-14$ per group), morphine abstinent mice displayed spontaneous stereotyped grooming, circling and head shakes that were dose-dependently suppressed by chronic VU0155041 administration. Under cocaine versus vehicle conditions (lower row, $n=8-9$ per group), this treatment increased the frequency of circling episodes. **(B)** In the Y-maze test, chronic VU0155041 administration in morphine abstinent mice (upper panel) normalized their pattern of exploration by suppressing same arm returns. Under cocaine versus vehicle conditions (lower panel), mGluR4 PAM suppressed cocaine abstinence-induced increase in spontaneous alternation by restoring the number of alternate arm returns to vehicle values. **(C)** In the marble-burying test, chronic VU0155041 dose-dependently normalized the number of marbles buried by morphine abstinent mice while increasing this number in vehicle abstinent controls; similarly this treatment increased marble burying in vehicle and cocaine abstinent mice. **(D)** In the novelty-suppressed feeding test, VU0155041 normalized the latency to eat in the arena and food intake of morphine-abstinent mice in the home cage. Latency to feed was reduced in cocaine abstinent mice. **(E)** In the tail immersion test, at 48°C, nociceptive thresholds tended to be decreased in morphine abstinent mice treated with vehicle; VU0155041 was analgesic at this temperature and in morphine abstinent mice only. No effect were detected at 50 and 52°C. Asterisk: abstinence effect (morphine/cocaine versus vehicle); open star: treatment effect (VU0155041 versus vehicle); solid star: abstinence x treatment interaction (two-way ANOVA followed by Newman-Keuls post-hoc test);

$p < 0.05$. Abbreviations: AAR: alternate arm returns; SAR: same arm returns; SPA: spontaneous alternation.



Chronic mGluR4 facilitation inhibited locomotor sensitization to morphine exposure

Then, we assessed morphine and cocaine locomotor sensitization in mice made abstinent from morphine or cocaine and chronically treated with VU0155041 (5 mg/kg) or vehicle. In morphine abstinent mice treated with vehicle, we observed that a single injection of morphine (10 mg/kg) four weeks after cessation of chronic morphine exposure induced a greater locomotor response than in vehicle abstinent mice, demonstrating locomotor sensitization. Chronic VU0155041 administration significantly blunted, although not completely suppressed, this conditioned response (Figure 4A, left panel), as shown by diminished total distance travelled after morphine injection compared to vehicle-treated mice (right panel) (65-180 min; abstinence x treatment: $F_{1,31}=4.8$, $p<0.05$; time x abstinence: $F_{23,713}=4.4$, $p<0.0001$). In cocaine abstinent mice, an acute injection of cocaine (15 mg/kg) induced detectable locomotor sensitization over the first 60 min of the test; chronic VU0155041 treatment delayed the peak of cocaine-induced locomotor activity but did not significantly decrease cocaine-induced locomotion (65-120 min; time x abstinence: $F_{11,352}=3.0$, $p<0.001$; time x treatment: $F_{11,352}=5.4$, $p<0.0001$) (Figure 4B). In conclusion, VU0155041 administration was able to dampen morphine-induced locomotor sensitization in morphine abstinent mice.

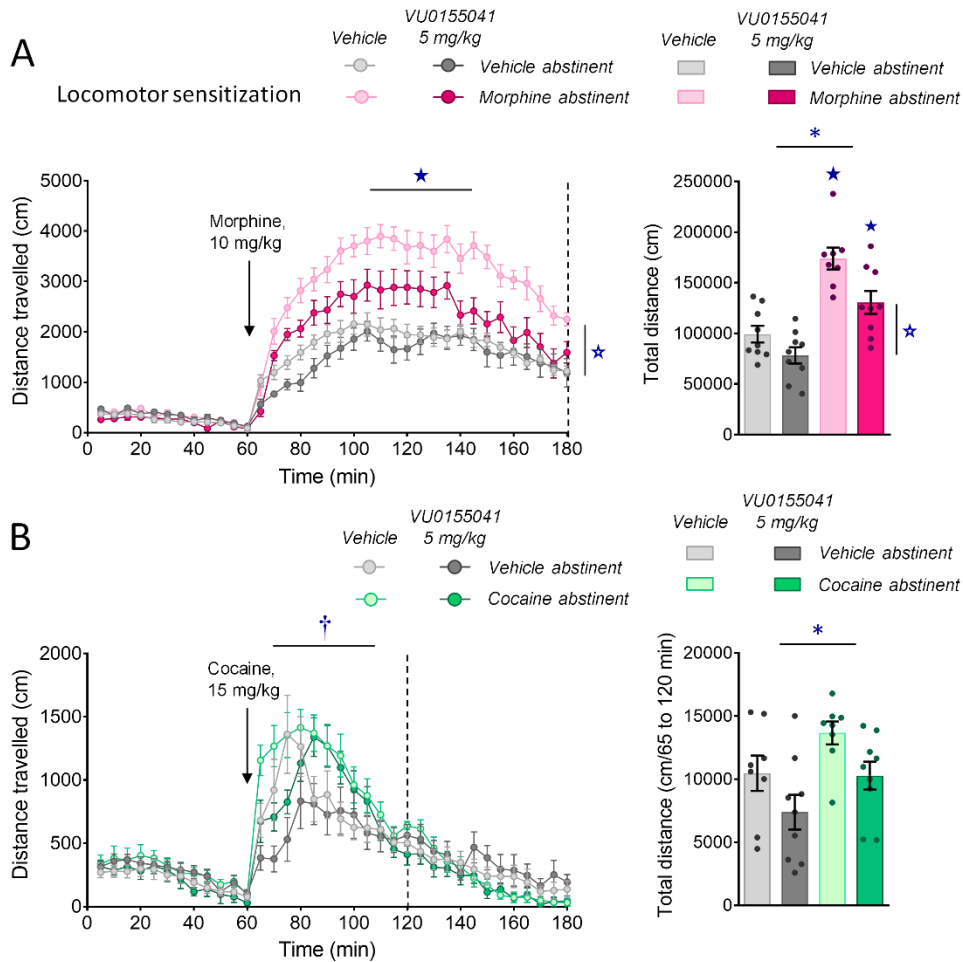


Figure 4. Chronic VU0155041 treatment inhibits morphine-induced locomotor sensitization.

(A) Morphine abstinent mice ($n=8-9$ per group) displayed greater locomotor response to acute morphine (10 mg/kg) than vehicle abstinent mice and this sensitization was significantly decreased upon chronic VU0155041 (5 mg/kg, left panel) as shown by diminished total distance travelled after morphine injection compared to vehicle-treated mice (right panel). **(B)** In cocaine abstinent mice ($n=8-9$ per group), acute cocaine (15 mg/kg) induced detectable locomotor sensitization during the first 60 min of the test; chronic VU0155041 (5 mg/kg) delayed the peak of locomotor activity (left panel) without decreasing cocaine-induced locomotion (right panel). Results are shown as scatter plots and/or mean \pm sem. Asterisk: abstinence effect (morphine/cocaine versus vehicle); open star: treatment effect (VU0155041 versus vehicle); dagger: time \times treatment interaction; solid star: abstinence \times treatment interaction (two- or three-way ANOVA followed by Newman-Keuls post-hoc test); $p < 0.05$.

VU0155041 treatment rescued the expression of MSN marker genes prominently in the NAc

To gain insight into the molecular mechanisms involved in beneficial effects of VU0155041 treatment, we evaluated its effects on transcriptional regulations induced by morphine abstinence in the CPu, NAc and CeA, where gene expression was the most contrasted between morphine and cocaine abstinence. This experiment was performed following a session of social interaction (Figure S3A), to allow correlations between gene expression and social behavior. We focused on the seven MSN marker genes highlighted in Figure 1 (blue characters in Figure 5A), in addition to *Oxt* and *Avp* coding for the social neuropeptides oxytocin and vasopressin, *Fos* as a marker of neuronal activity, and *Bdnf* (coding for brain derived Neurotrophic factor - BDNF) and *Syp* (coding for the vesicular protein synaptophysin) as markers of synaptic plasticity.

We performed hierarchical clustering analysis of qRT-PCR data for each brain region to visualize the influence of VU0155041 treatment on gene expression (Figure 5A, Table S3). Overall, transcriptional profiles in morphine abstinent mice treated with VU0155041 (morphine-VU0155041) were closer to those of VU0155041-treated vehicle abstinent (vehicle-VU0155041) mice than vehicle-treated morphine abstinent (morphine-vehicle) mice. This was particularly true in the CPu and NAc, where gene expression under morphine-VU0155041 and vehicle-VU0155041 conditions were the most similar. In the CPu, this pattern was mostly driven by an increase of gene expression under VU0155041 treatment (clusters a and b). In the NAc, the expression profile was dominated by a down-regulation under morphine-vehicle conditions (clusters a, b, c) that was rescued by chronic VU0155041. The modulation of social parameters (time in nose contact and following episodes) across treatment conditions followed a similar pattern and clustered with the expression of MSN marker genes (all but *Drd2*), *Fos*, *Avp* and *Oxt* in the NAc (clusters a and b). In the CeA, rescued expression under VU0155041 treatment was observed in a single cluster (cluster b), also gathering social parameters. Together, these results indicate that VU0155041 treatment rescued morphine-induced down-regulation of MSN marker gene expression prominently in the NAc, an effect that was tightly correlated with the behavioral improvements observed in morphine-VU0155041 mice.

Focusing on MSN marker genes, down-regulated expression under morphine-vehicle conditions was confirmed in the NAc (but not the CPu) for *Drd1a* and *Hpca*,

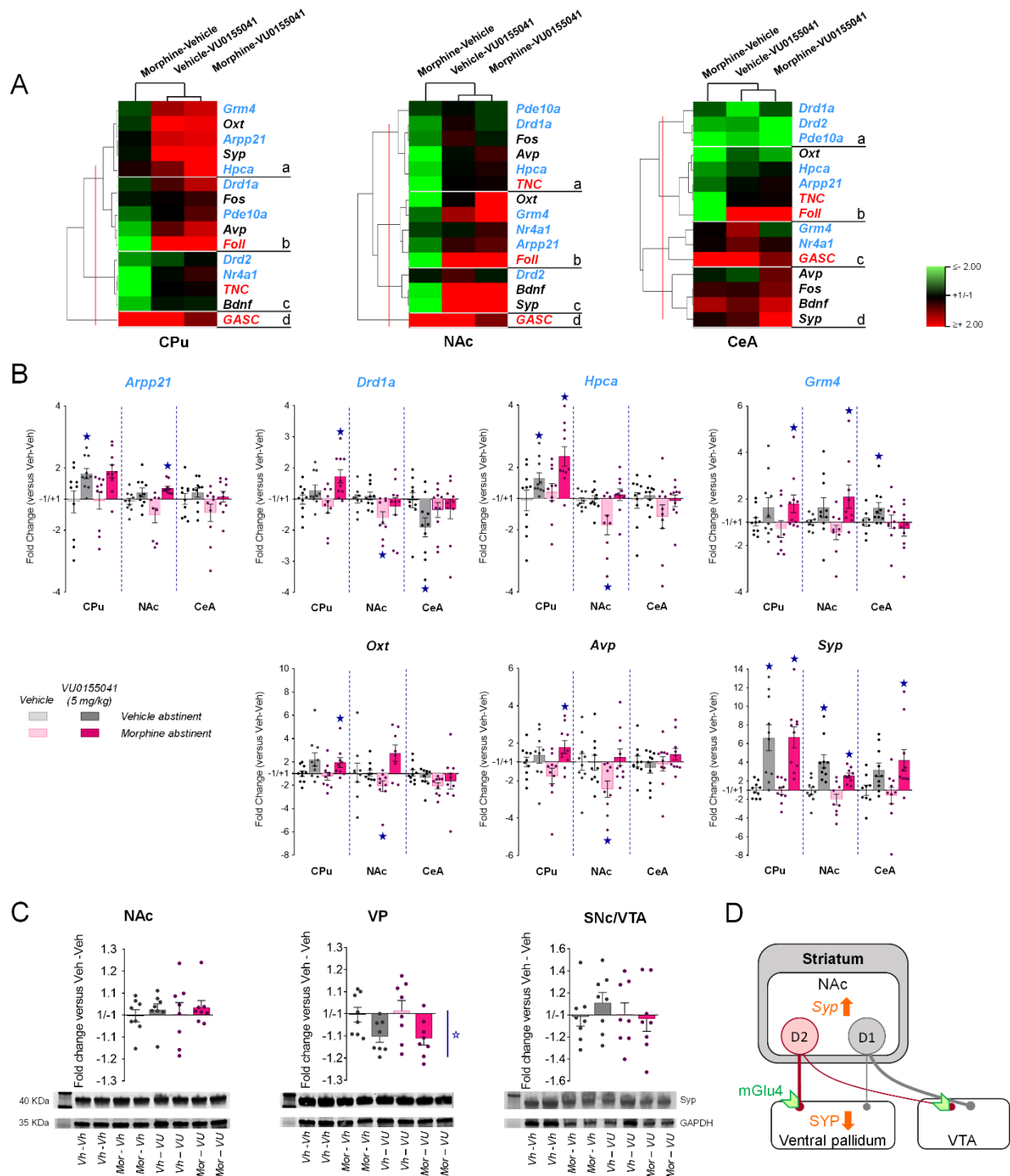
while it failed to reach significance for *Arpp21*, *Gmr4*, *Nr4a1* and *Pde10a*. VU0155041 rescued (*Drd1a*, *Hpca*) or increased (*Arpp21*, *Grm4*) the expression of these genes. Interestingly, the expression of *Oxt* and *Avp* was also decreased in the NAc of morphine-vehicle mice, and chronic mGluR4 PAM treatment similarly rescued their expression. Finally, VU0155041 induced *Syp* expression in the three regions tested, in both vehicle and morphine abstinent animals.

Intrigued by *Syp* expression data in the striatum, especially in the NAc, we assessed synaptophysin (SYP) protein levels using western blotting. We dissected out the NAc, as the main substrate of long-term opiate-induced behavioral deficits, its projection sites: ventral pallidum (VP) and ventral tegmental area (VTA), and the neighboring CPu. At behavioral level, we verified again that chronic VU0155041 rescued morphine abstinence-induced deficit in social interaction (Figure S3B). At molecular level, we failed to detect modifications of SYP protein levels in the NAc, CPu and SNc/VTA (Figures 5D, S3B, S4 and S5). In the VP, however, we measured a significant decrease in SYP immunoreactivity. Thus beneficial effects of chronic VU0155041 were associated with a decrease in SYP levels in the VP, the main projection site of NAc D2-MSNs (Figure 5D).

Figure 5. Transcriptional regulations induced in the striatum and amygdala of morphine abstinent mice by chronic facilitation of mGluR4 signaling.

We evaluated the effects of chronic VU0155041 treatment (5 mg/kg) on transcriptional changes induced by morphine versus vehicle abstinence in the CPu, NAc and CeA. We focused on the seven MSN marker genes (blue characters) highlighted in Figure 1, *Oxt* and *Avp* coding for the social neuropeptides oxytocin and vasopressin, *Fos* as a marker of neuronal activity, and *Bdnf* and *Syp* as markers of synaptic plasticity. **(A)** Clustering analysis organized gene expression in 4 clusters per region. In the CPu and NAc, chronic VU0155041 restored (CPu: clusters b and c; NAc: clusters a, b,c) or increased (CPu: cluster a) the expression of most genes tested, which clustered with social parameters. In the CeA, rescued expression under VU0155041 treatment was observed in a single cluster (cluster b). **(B)** Among MSN marker genes highlighted in Figure 1B, *Arpp21*, *Drd1a* and *Hpca* displayed significant down-regulation in the NAc, but not *Gmr4*, *Nr4a1* or *Pde10a*. VU0155041 rescued the expression of *Drd1a* and *Hpca* and increased the expression of *Arpp21* and *Grm4* in this region. Similarly, down-regulated expression of *Oxt* and *Avp* in the NAc of morphine-vehicle mice was rescued by VU0155041 treatment. *Syp* expression was increased upon VU0155041 exposure in the three regions tested. **(C)** Western blot analysis failed to detect modifications of synaptophysin (SYP) protein levels in the NAc, CPu and VTA but revealed a significant decrease in SYP immunoreactivity in the VP, **(D)** which represents the main projection area of NAc D2-MSNs. Gene (n=9-10 per group) and protein (n=8 per group) expression data are expressed as fold change versus vehicle - vehicle group

for each condition (scatter plots and mean \pm SEM). Comparison to vehicle - vehicle group: star $p < 0.05$ (two-tailed t test, p-value adjusted using Benjamin-Hochberg correction for multiple testing). Foll: number of following episodes, GASC: grooming after social contact, TNC: time in nose contact. qRT-PCR data used for clustering are displayed in Table S3.



DISCUSSION

Transcriptional regulation of MSN marker genes in the NAc allows best discriminating between morphine and cocaine abstinence

We previously evidenced persistent and contrasted transcriptional regulations in the central EA of 4-week morphine versus cocaine abstinent mice for a set of HTT-related genes known for their enriched expression in MSNs [13,14]. In the present study, we extended our investigation to a wider collection of MSN marker genes and to *Oxt*, coding for the social neuropeptide oxytocin. First, we revealed that gene expression patterns in the CeA were more discriminative than those in the BNST to distinguish between morphine and cocaine abstinence. Then, we found enduring transcriptional modifications in two more brain regions where MSNs represent the main cell type, CPu and NAc. Overall, modified expression of MSN maker genes, belonging or not to the HTT-centered network, allowed to discriminate between morphine and cocaine abstinence conditions in the CPu, NAc and CeA. This result indicates that abstinence from both drugs significantly impacted MSN population, but in different, if not opposite, directions. A majority of MSN marker genes displayed common down-regulated expression under morphine condition; they were indifferently genes with enriched expression in D1- (*Drd1a*, *Pdyn*, *Tac1*) or D2-MSNs (*Drd2*, *Foxp1*, *Penk*, *Grm4*, *Rgs4*) or in both MSN populations (*Arpp21*, *Hpca*, *Nr4a1*, *Pde10a*, *Rgs4*) [38,39]. Thus, prolonged abstinence from morphine, and not cocaine, inhibited the expression of a set of genes in D1- and D2-MSNs. These data substantiate the notion of differential neurobiological substrates involved in opiate versus psychostimulant addiction [9,14,41,42].

The seven MSN-enriched genes *Arpp21*, *Drd1a*, *Drd2*, *Hpca*, *Grm4*, *Nr4a1* and *Pde10a*, [38,43-45] captured our interest for displaying highly discriminative patterns of expression between morphine and cocaine conditions and sharing consistent down-regulation in the CPu, NAc and CeA. *Arpp21* codes for Cyclic AMP regulated phosphoprotein 21 (ARPP-21) so called regulator of calmodulin signaling (RCS) that modulates striatal calcium and dopamine signaling [46,47]. When phosphorylated, ARPP-21 amplifies D1 but attenuates D2 dopamine receptor signaling in striatal MSNs [47]. *Drd1a* and *Drd2* genes code for the dopamine receptors D1 and D2, respectively. D1 receptor couples Gs proteins and stimulates the activity of *Drd1a*

expressing MSNs; conversely, D2 receptor is coupled to Gi/o proteins, inhibiting the activity of *Drd2* expressing MSNs [48]. *Hpcal4* encodes the neural calcium sensor protein hippocalcin, a main contributor of the slow afterhyperpolarization (sAHP) [49,50]. In morphine abstinent rats, sAHP is attenuated in the NAc MSNs, increasing the excitability of a subset of MSNs [51], possibly D2-MSNs [34]. *Grm4*, preponderantly expressed in D2-MSNs [52], encodes the presynaptic mGlu4 glutamate receptor, negatively coupled to adenylyl cyclase, which decreases excitability and reduces neurotransmitter release at axon terminals [53]. *Nr4a1* is a nuclear receptor/immediate early gene and marker of striosomes, gathering most D1 MSNs in the CPu. Altered *Nr4a1* expression compromises transcription of multiple members of the D1 receptor signaling cascade [54]. *Pde10a* encodes the cyclic nucleotide phosphodiesterase 10A (PDE10A) that regulates cGMP and cAMP signaling cascades and striatal function [55,56]. Inhibition of PDE10A stimulates the activity of MSNs, with D2-MSNs being more sensitive to this effect [57,58]. Therefore, down-regulated expression of the seven above genes in morphine abstinent mice potentially favored the activity of D2 over D1 MSNs, tilting the striatofugal balance towards excessive D2 MSN tone. Altered striatofugal balance, notably in the NAc, represents a key cellular mechanism in drug addiction [17,59,60]. Recent work has elegantly evidenced, by measuring dopamine-induced cAMP signaling in the NAc, that, along repeated opioid exposure, this balance progressively shifts towards preponderant D2-MSN activity, maintained during abstinence [61], as previously observed in morphine withdrawal [34]. Exacerbated D2-MSN activity could drive the negative affect associated to morphine dependence and withdrawal, increasing the risk of relapse [33-35]. Here, we hypothesized that pharmacological intervention allowing to dampen D2-MSN activity, as by facilitating mGluR4 activity, should relieve long-term behavioral alterations in morphine abstinent mice.

Chronic facilitation of mGluR4 activity relieves behavioral deficits and dampens locomotor sensitization in morphine abstinent mice

Morphine abstinent mice display a dramatic alteration of their social behavior [14,18,20,62-64]. Chronic administration of the mGluR4 PAM VU0155041 dose-dependently normalized social interaction to vehicle-abstinent levels and restored the preference for making more and longer contacts with the mouse versus the toy in the three-chamber test. Chronic VU0155041 similarly restored social behavior in the

Oprm1^{-/-} mouse model of autism, together with the associated activation of the reward circuit [21]. Social interactions are pleasurable events in humans and rodents and therefore activate the brain reward circuit [27,65,66]. Exacerbated/prolonged D2-MSN tone, notably in the NAc, as observed in morphine abstinent mice [34,61], was shown to induce aversion [67-69]. Hence, excessive D2-MSN activity in morphine abstinent mice may compromise social interactions by hampering their rewarding properties, and chronic VU0155041 treatment would restore social behavior by normalizing this activity. Prior studies have evidenced relieving effects of the serotonin reuptake inhibitor fluoxetine [18] and oxytocin analog carbetocin [20] on social deficit in morphine abstinent mice. Remarkably, oxytocin promotes social reward by triggering long-term depression (LTD) at NAc D1 and D2-MSN neurons, an effect that requires serotonin release [70]. Shared beneficial effects of VU0155041, fluoxetine and carbetocin on social behavior in morphine abstinent mice may thus involve common inhibition of D2-MSNs.

Besides social impairments, morphine abstinent mice display motor stereotypies, stereotypic marble burying and perseverative behavior in the Y-maze, as previously reported [14]. Here we show that chronic VU0155041 administration, at the highest dose, suppressed these behaviors. Similarly, VU0155041 alleviated stereotypies in *Oprm1*^{-/-} [21] and relieved stereotypies in mice lacking the *Elfn2* gene (*Elfn2*^{-/-}), which also display autistic-like symptoms [71]. Interestingly, stereotypic behavior was shown to involve NAc D2-MSNs in another mouse model of autism, Neuroligin-3 knockout mice [72]. Hence, VU0155041 may reduce stereotypies in morphine abstinent mice by repressing D2-MSN activity.

We next verified that conflict anxiety-like behavior was exacerbated in morphine abstinent mice, as evidenced by increased latency to eat in the novelty-suppressed feeding test [14]. From the lowest dose we tested, chronic VU0155041 normalized anxiety-like behavior, as previously observed in *Oprm1*^{-/-} and *Elfn2*^{-/-} mice [21,71]. This result is consistent with previous reports of facilitating mGluR4 activity having anxiolytic effects in mice [73-75] and rats [76]. In addition, VU0155041 treatment produced analgesia only in morphine abstinent mice whose tendency for lowered nociceptive thresholds is reminiscent of withdrawal symptoms [77]. Stimulation of mGluR4 produces analgesia under chronic pain or inflammatory conditions only [78,79]. Interestingly, opioid-induced hyperalgesia was shown to involve an activation of the glutamatergic system [80] that may represent the neurobiological substrate for

analgesic effects of VU0155041 in morphine abstinent mice. Finally, morphine-exposed mice displayed significant locomotor sensitization to morphine after prolonged abstinence, consistent with previous findings [14,81], which was dampened by VU0155041. Morphine-induced sensitization involves D1 receptor activation in striatal D1-MSNs [81-83], whose activity may be impacted by mGluR4 either directly in D1-MSNs [84,85] or through corticostriatal terminals forming synapses at D1-MSNs [86]. However, an effect of VU0155041 at D2-MSNs cannot be ruled out, as they also contribute to drug-induced sensitization [87,88].

Collectively, blunted social behavior, stereotyped behavior, elevated anxiety-like behavior and low nociceptive thresholds in morphine abstinent mice fit with clinical reports of social withdrawal, impaired cognitive flexibility and high comorbidity with anxiety disorder and depression in patients with a history of chronic opioid exposure [89-96]. Importantly, such negative affect, combined with conditioned effects of opioids, are considered as the two major causes of relapse in Opioid Use Disorder [97,98]; they were both significantly alleviated in morphine abstinent mice under VU0155041 treatment. However, this was observed at distinct, single time points for each test, social interaction being the exception (D8 and D15, Figure S3A and B). The kinetics of chronic VU0155041 effects over days of administration and after cessation of treatment will deserve further investigation.

In contrast with morphine, cocaine abstinence had little impact on sociability, as previously shown [14], and VU0155041 treatment either preserved or even improved this behavior. Chronic VU0155041, however, induced motor stereotypies (circling, head shakes, marble burying) in these mice as it did in vehicle abstinent mice, possibly by tilting the D1/D2-MSN balance towards D1-MSN activity, which triggers stereotypic behaviors [99]. Contrary to morphine abstinence, cocaine abstinence was associated with decreased anxiety in the novelty-suppressed feeding test [14]; chronic facilitation of mGlu4 activity had no influence on this response. Finally, VU0155041 failed to significantly reduce cocaine-induced locomotor sensitization; however, this failure may be due to the weak amplitude of cocaine- compared to morphine-induced response. These results further illustrate the neurobiological differences between opiate and cocaine abstinence.

VU0155041-induced transcriptional changes in the NAc are coherent with a repression of D2-MSN activity and restauration of social reward

We proposed above that mGluR4 PAM treatment relieved social behavior in morphine abstinent mice by repressing D2-MSN and boosting D1-MSN activity in the NAc. Our qPCR results in morphine abstinent mice treated or not with VU0155041 were consistent with this hypothesis. Indeed, VU0155041 restored or increased expression of *Arpp21*, *Drd1a*, *Hpca* and *Grm4* in the NAc, where their respective encoded proteins can either dampen D2-MSN activity or facilitate D1-MSN activity. Interestingly, these transcriptional regulations were tightly correlated with pro-social behaviors. NAc *Bdnf* expression tended to be decreased in our experiments, as previously shown under prolonged morphine abstinence [100], while VU0155041 tended to increase this expression. Reduced BDNF signaling in the NAc would decrease GABAergic activity specifically in D1-MSNs [101], favoring excessive D2 tone. Furthermore, *Bdnf* and *Syp* displayed very similar expression patterns in the NAc and, in a lesser extent, in the CPu, consistent with the demonstrated ability of the former in triggering expression of the second [102,103]. Globally, VU0155041 treatment induced an increase in *Syp* mRNA levels, most notably in the striatum. As regards the NAc, this phenomenon appears a compensatory mechanism, as SYP protein levels were decreased in the VP, the main projection area of NAc D2-MSNs. The mGlu4 receptor is located presynaptically at axon terminals where its activation represses neurotransmitter release. Thus, reduced expression of SYP in the VP could have resulted from the inhibitory action of mGluR4 PAM treatment on GABA release from indirect MSNs in this region. These data point to repression of D2-MSN activity in the NAc as a key substrate supporting social effects of mGluR4 facilitation in morphine abstinent mice.

The NAc is a hub region for the integration of reward processes [104-106], a key brain substrate in drug addiction [107,108] and finally a critical substrate for social behavior. Consistent with their pleasurable properties, social stimuli activate the NAc in humans [109,110] and this activation is blunted in patients with ASD [27], depression [111], schizophrenia [112] or OUD [113] who display impaired social behavior. In rodents, activating neuromodulator or neuropeptide receptors in the NAc stimulates or drives social behavior [70,114-116] and models of ASD, schizophrenia or depression show abnormal volume, connectivity and/or function of the NAc [117-122]. In our study, *Fos* expression induced in the NAc by social exposure was dampened in morphine abstinent mice, suggesting blunted social reward, and rescued upon VU0155041 treatment, as we previously observed in *Oprm1*^{-/-} mice

[21]. Moreover, NAc expression of *Oxt* and *Avp* transcripts, plausibly transported from the hypothalamus [123], was restored by mGluR4 PAM treatment. The former gene codes for oxytocin, critically involved in mediating social reward in the NAc [70,124]; the role of vasopressin (encoded by *Avp*) in the NAc remains poorly understood, but vasopressin dysregulation has been highlighted in autism [125]. Together, these results argue for a restoration of social reward under VU0155041 treatment, likely through a resetting the D1/D2-MSN balance in the NAc. Further investigations will be required, however, to confirm this hypothesis and also to explore the role of NAc and extra-NAc mGluR4 populations in regulating social behavior. Of note, *Grm4* expression was not down-regulated upon VU0155041 administration, suggesting limited tolerance to pharmacological treatment.

One ought to point out some limitations to our study. The low sample number of mice allocated to each experimental condition in transcriptome analyses resulted in lower statistical power that may lie behind the lack of significance of some regulations of gene expression, despite consistent expression patterns between independent experiments (Figures 1 and 5). Moreover, this study was performed in male mice only, to allow direct comparisons with previous reports [13,126] and one should thus be cautious regarding transposition to females. In *Oprm1^{-/-}* mice, however, VU0155041 was as efficient to relieve deficient social behavior, stereotypies and anxiety in females as in males [21], which bodes well for beneficial effects in female morphine abstinent mice and for potential clinical applications.

Conclusion

The present study reports that facilitating glutamate mGluR4 activity can relieve the deleterious long-term behavioral and molecular consequences of opiate, but not cocaine, exposure. These results highlight the neurobiological differences between opiate and psychostimulant abstinence, arguing against a unitary view of drug addiction [9,41]. They also substantiate the view of negative affect in opiate abstinence as resulting from excessive activity of striatal D2-MSNs, more specifically in the NAc [33-35,61]. In this context, pharmacological compounds repressing striatopallidal activity appear as promising candidates for the treatment of OUD, possibly by restoring “liking” [127] for non-drug, notably social, stimuli. More broadly, opiate addiction sharing striking phenotypic and neurobiological features with other diseases considered as “reward deficiency syndromes”, such as autism or

depression [27,121,128,129], our work opens promising avenues towards the development of common therapeutic strategies for these pathologies.

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Author contributions

J.A.J.B., J.G., L.P.P. and J.L.M. designed the experiments. J.A.J.B., L.P.P., J.G., T.L., A.L. and J.L.M. performed behavioral experiments. J.A.J.B., Y.C. and L.P.P. performed qRT-PCR experiments. Y.C. performed western blot experiments. J.A.J.B., L.P.P., J.G., Y.C. and J.L.M. analyzed the data. J.A.J.B. and J.L.M. wrote the article. All authors discussed the results and commented on the manuscript.

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