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Long-term changes in reward-seeking following morphine withdrawal are associated with altered N-methyl-D-aspartate receptor 1 splice variants in the amygdala

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Abstract

The NR1 subunit of the NMDA receptor can be alternatively spliced by the insertion or removal of the N1, C1, C2, or C2' regions. Morphine dependence and withdrawal were previously demonstrated to lower N1 and C2' in the accumbens and lower N1, C1, and C2' in the amygdala. Withdrawal has also been demonstrated to increase motivational and anxiety/stress behaviors in rats. We tested the hypothesis that NR1 splicing would be associated with these behaviors during an extended withdrawal period of two months. Motivation was measured using an operant orofacial assay at non-aversive temperatures (37°C) while anxiety and stress were measured by examining this behavior at aversive temperatures (46°C). Lower C1 and C2 expression levels were observed in the amygdala in a subset of the population of withdrawn rats even after two months of morphine withdrawal. These subsets were associated with a hypersensitivity to adverse conditions which may reflect long-term alterations in the withdrawn population.

1. Introduction

Repeated drug administration in humans and animals induces a variety of changes in the nervous system resulting in dependence. In humans, many symptoms occur long after acute withdrawal has ended and can include craving, anxiety, and hypersensitivity to pain (Koob and Le Moal, 2005). Using animal studies, these symptoms may be traced to specific circuit pathways which include the amygdala (AMY) and the nucleus accumbens (NACC) (Crombag et al., 2008; Gardner, 2011; Heinz et al., 2009; Koob, 2009). The long-term

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I have read and have abided by the statement of ethical standards for manuscripts submitted to *Neuroscience* and all authors have approved the final article.

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effects of opiate withdrawal could then result from neuroplastic changes in these areas which propagate addictive behaviors (Duka et al., 2011).

NMDA receptors are proteins in the nervous system capable of inducing long-term effects through the stabilization and reorganization of synapses (Nikonenko et al., 2002) and are therefore prime candidates for stable, drug-induced neuroplastic changes. Alternative splicing of the NR1 subunit of the NMDA receptor is an often overlooked process which can alter this plasticity by modifying the receptors' activation kinetics, phosphorylation, and cellular distribution (Dingledine et al., 1999). The NR1 subunit can exist as eight different isoforms depending on the splicing of exons 5, 21, and 22 (known as N1, C1, and C2 when expressed). N1 and C1 are spliceable cassettes, but C2 and C2' are mutually exclusive C-terminal variants. C2 contains the stop codon for the gene, so if it is spliced out C2' is expressed (Cull-Candy and Leszkiewicz, 2004). Since NR1 splice variants have an effect on synaptic plasticity, it is no surprise that changes in their mRNA and protein levels were previously reported in diseases of long-term synaptic plasticity like chronic pain as well as both cocaine and alcohol abuse (Gaunitz et al., 2002; Loftis and Janowsky, 2002; Prybylowski et al., 2001; Raeder et al., 2008; Winkler et al., 1999; Zhou et al., 2007; Zhou et al., 2006; Zhou et al., 2009). These data together suggest that alternative splicing of NR1 could be a mechanism for inducing long-term drug-induced neuroplastic effects.

Previous studies in our lab demonstrate that some NR1 splice variants are altered after three days of morphine withdrawal in rats. We reported that N1, C1, and C2' decreased significantly in the AMY. N1 and C2' decreased in the NACC as well (Anderson et al., 2012). We set out to examine the hypothesis that these variants are associated with the long-term effects of morphine withdrawal. We predicted that NR1 splice variant levels in most rats would return to pre-drug baselines after an extended two-month withdrawal period but that a subset of these animals would retain the drug-induced conformations based on previous studies in our lab on pain models (Zhou et al., 2009). We further hypothesized that this subset would exhibit long-term changes in motivation, anxiety, and pain behavior due to morphine withdrawal.

2. Experimental Procedures

2.1 Animal care

For all experiments male hairless Sprague-Dawley rats (250–300 g, Charles River, Raleigh, NC) were housed in pairs in 22°C temperature and 31% humidity controlled rooms with a normal 12-hour light/dark cycles (6am–6pm lights on) and had free access to food and water except when fasted prior to testing. These facilities are AAALAC accredited and all procedures were approved by the University of Florida IACUC. Morphine sulfate (15mg/mL, Baxter, Deerfield, IL) was obtained from Webster Veterinary (Devens, MA).

2.2 Operant orofacial testing

We measured the effects of morphine administration and withdrawal on motivational reward-seeking behavior and nociception with a reward/conflict operant task first described by Neubert et al. (2005) which is sensitive to morphine (Neubert et al., 2006) and can measure motivation for reward (Anderson et al., 2012). A total of forty rats were fasted for 17 +/- 1hrs (from 5pm the previous night to 9–11am the next day) then placed in a Plexiglas box for ten minute periods and trained to press their faces between two aluminum tubes in order to receive a reward of diluted sweetened condensed milk (2:1 water:milk ratio). These tubes had water continuously flowing through them from a circulating water bath which can be heated to aversive or non-aversive temperatures. Mechanical sensors were attached to the tubes and the feeding bottle so that every time a rat touched its face to the tubes and licked the feeding bottle a recording was taken on DATAQ software (WinDaq Lite Data Acq

DI-194, DATAQ Instruments, Inc, Akron, OH). After six training sessions (three times a week) and one baseline at a non-aversive 37°C the temperature was changed to an aversive 46°C and rats were tested for a baseline session. This was followed by another 37°C and 46°C baseline session. Data from these baseline sessions were averaged together into one 37°C (B37) and one 46°C score (B46) and subjects were separated into two groups while maintaining equal baselines on five measures: Weight, time per contact values at both temperatures, and facial contact times at both 37°C and 46°C. The facial contact time represents the total amount of time the rat makes contact with the heated tube while attempting to obtain the reward. The time per contact is the facial contact time divided by the number of contacts made. It measures the average time of a bout of drinking which alters with the change in temperature and the motivational state of the rat (Anderson et al., 2012). Eight rats were designated to be controls and thirty-two were placed in the morphine treatment group. Next, morphine or an equivalent volume of saline was administered twice daily via subcutaneous injection for ten days in an escalating dosing paradigm as depicted in Table 1 and testing continued every two to three days. After the tenth day all injections were ceased and testing continued for two months. Typically rats were tested for two sessions at 37°C then one session at 46°C each week throughout the withdrawal phase.

2.3 Tissue collection and western blotting

After two months of withdrawal, rats were euthanized by CO₂ inhalation followed by rapid decapitation. Brains were removed and placed in an ice cold Acrylic Rat Brain Slicer Matrix (Zivic Instruments, Pittsburg, PA). Bilateral areas of interest were removed using a 2mm Harris Uni-Core puncher with the Paxinos & Watson rat brain atlas (1998) as a guide. The amygdala (AMY) and nucleus accumbens (NACC) were removed from the slices cut from -2 to -5mm from Bregma. Tissues were placed in 1.5ml tubes and immediately frozen in liquid N₂. Tissue was sonicated with a Sonics Vibra-Cell Sonicator (Danbury, CT, USA) at 60 Amps for 10 seconds in Tissue Disruption Buffer (0.3% SDS, 65mM DTT, 1mM EDTA, 20mM Tris, pH 8.0). Samples were centrifuged at 14,000 rpm for 10 minutes at 4°C. Supernatant was removed and protein concentration was determined by the Bicinchoninic Acid Assay (Pierce Chemical Co., Rockford, IL). A mixture of 20µg of protein, ddH₂O, 5% 2-mercaptoethanol, and 50% 2X Sodium Dodecyl 20% Sulfate buffer was heated in a boiling water bath for 5 minutes, loaded into a 4–20% Tris–glycine gel (Invitrogen, Carlsbad, CA, USA) and run at 80V for 15 minutes then 150V for 45 minutes. Gels were placed in transfer buffer (10% methanol, 48mM Tris, 39mM glycine, pH 9.2) for 30 minutes then transferred onto a Millipore (Bedford, MA, USA) Immobilon-P polyvinylidene fluoride membrane using a Biorad semi-dry transfer device (Hercules, CA, USA). Membranes were blocked in 5% dry milk TTBS buffer (20mM Tris HCL, 0.9% NaCl, 0.05% Tween-20, pH 7.4) for 1 hour. Primary antibodies for NR1 (1:3000, rabbit, Epitomics), N1 and C1 (1:4000, rabbit, Iadarola, NIDCR/NIH), C2 (1:2000, rabbit, Millipore), C2' (1:3000, rabbit, Millipore), and Glyceraldehyde 3-phosphate dehydrogenase (GAP, 1:15000, mouse, Pierce Thermo, Rockford, IL) were placed on membranes on a rotator at 4°C. The next day blots were washed in TTBS 3 times for 10 minutes and secondary antibody was added (anti-rabbit or anti-mouse IgG, HRP-linked, 1:4000, Cell Signaling, Danvers, MA) for 1 hour. Blots were washed 3 times for 5 minutes each and then detected using ECL Plus (Amersham, Pittsburg, PA) and the Carestream Image Station 4000MM (Carestream Health, Rochester, NY). Band density was measured with Carestream Molecular Imaging Software and normalized to GAP levels.

2.4 Data analysis and statistics

Rat weights and behavioral outcomes by group were analyzed with Repeated Measures (RM) Two-Way ANOVA with Bonferroni's post-hoc comparison at several stages: During

morphine injections, during an acute withdrawal stage of sixteen days and an extended withdrawal stage of days seventeen through fifty-eight.

Unpaired t-tests were used to compare total changes in splice variant expression levels between morphine and saline injected animals. F tests were used to examine variance in populations. Frequency distributions were used to place the western blot data into bins then best-fit Gaussian curves were used to determine if separate populations existed within the withdrawn rats. The morphine group was split into two groups (High” and “Low”) based on the expression level of the splice variant being examined. One-Way ANOVA with Bonferroni’s post-hoc tests were used to determine if the groups were different in terms of protein expression levels. RM Two-Way ANOVA analyses with Bonferroni’s post-hoc comparison was used to examine differences in behavioral outcomes for the separated groups. All tests were performed using GraphPad Prism 4 and 5 software (La Jolla, CA). For all tests, p-values less than 0.05 were considered significant.

3. Results

Rats were weighed three times a week throughout the course of the study. No differences in weight were observed during the baseline or injection periods between the saline and morphine treated groups (Figure 1A; $F(1,38)=0.3613$, $p>0.05$). There was a significant effect for time ($F(6,38)=182.2$, $p<0.0001$) and an interaction, ($F(6,38)=12.83$, $p<0.0001$) as both groups tended to gain weight through this period. During the acute withdrawal stage morphine treated rats had significantly decreased weights compared to saline injected rats (Figure 1B; $F(1,38)=12.77$, $p=0.0010$). Significant effects were also observed for time ($F(6,38)=498.0$, $p<0.0001$) and an interaction ($F(6,38)=32.17$, $p<0.0001$) as both groups still tended to gain weight during this period. During the extended withdrawal phase, the morphine treated rats’ weights were not different than the saline treated rats as they had gained back most of the lost weight (Figure 1C; $F(1,38)=0.7006$, $p>0.05$). There was a significant effect for time ($F(6,38)=272.8$, $p<0.0001$) due to both groups gaining weight but no effect for an interaction ($F(6,38)=0.8461$, $p>0.05$).

Morphine administration significantly increased motivational behavior as measured by the time per contact at each dose tested at 37°C (Figure 2A; RM Two-Way, $F(1,217)=30.31$, $p<0.0001$). Post-hoc tests were significant for the 10, 20, and 40 mg/kg doses ($p<0.001$, $p<0.001$, and $p<0.05$). No significant effect was observed for time (RM Two-Way, $F(5,217)=2.151$, $p>0.05$) or an interaction (RM Two-Way, $F(5,217)=2.013$, $p>0.05$) as saline rats had consistent time per contact levels. Morphine also increased the amount of time seeking reward at 37°C as demonstrated by an increase in facial contact times (Figure 2B; RM Two-Way ANOVA, $F(1,217)=15.74$, $p<0.0001$) especially at the 10 and 40 mg/kg doses (post-hoc tests, $p<0.05$). A significant effect was also observed for time as the saline treated rats also increased their contact times each session (RM Two-Way, $F(5,217)=7.334$, $p<0.0001$) albeit at a lesser amount than the morphine treated rats. No interaction was observed (RM Two-Way, $F(5,217)=1.263$, $p>0.05$). Previous studies in our lab demonstrated that morphine was anti-nociceptive at doses of at least 10 mg/kg when tested at the aversive 46°C as well (Anderson et al., 2012).

Figure 3A illustrates that rats during the acute stage of morphine withdrawal had a higher time per contact at 37°C than controls. RM Two-Way ANOVA analysis revealed a significant effect for group ($F(1,38)=6.904$, $p=0.0123$) but not for time or an interaction ($F(3,38)=2.153$, $p>0.05$; $F(3,38)=0.5802$, $p>0.05$). During extended withdrawal this effect on the time per contact levels was no longer evident (RM Two-Way ANOVA; group $F(1,38)=1.587$, $p>0.05$; time $F(3,38)=3.719$, $p<0.0001$; interaction $F(3,38)=0.3613$, $p>0.05$). This suggests that at non-aversive temperatures withdrawing rats have a higher motivation

to obtain the reward than saline controls in the acute stages of withdrawal. Figure 3B illustrates that both groups have similar total facial contact times throughout withdrawal at 37°C. During the acute phase no effect is observed for group (RM Two-Way ANOVA, $F(1,38)=1.008$, $p>0.05$) or time ($F(3,38)=1.252$, $p>0.05$), but an effect is observed for an interaction ($F(3,38)=4.831$, $p=0.0033$). During the extended phase no effect is observed for group ($F(1,38)=0.1752$, $p>0.05$) or an interaction ($F(3,38)=1.691$, $p>0.05$), but a significant effect for time was observed ($F(3,38)=6.641$, $p<0.0001$) suggesting both groups slightly increased their facial contact times over the course of extended withdrawal in non-aversive conditions.

No obvious differences were observed between groups during the acute withdrawal phase for the time per contact levels at 46°C as both groups were heavily inhibited by the aversive heat stimuli (Figure 3C; group $F(1,38)=0.08164$, $p>0.05$; time $F(3,38)=2.939$, $p>0.05$; interaction $F(3,38)=1.020$, $p>0.05$) or the extended withdrawal phase (Figure 3C, group $F(1,38)=0.7164$, $p>0.05$; time $F(3,38)=0.1076$, $p>0.05$; interaction $F(3,38)=0.3139$, $p>0.05$). Facial contact times between groups were similar during the acute phase and both changed over time (Figure 3D; group $F(1,38)=1.677$, $p>0.05$; time $F(3,38)=4.992$, $p=0.0092$; interaction $F(3,38)=0.1988$, $p>0.05$). During the extended phase however, an effect was observed for group as the withdrawn rats had consistently and significantly lower facial contact times at 46°C (Figure 3D; group $F(1,38)=5.123$, $p=0.0294$; time $F(3,38)=0.3425$, $p>0.05$; interaction $F(3,38)=0.4801$, $p>0.05$) demonstrating that alterations in sensitivity to aversive stimuli can be detected by this assay for up to two months after chronic morphine withdrawal.

After euthanizing and harvesting tissue western blots were run and t-tests and F-tests were used to examine differences between the saline and withdrawn groups. As demonstrated in Table 2, no overall densitometry differences in the means were observed in the total NR1 subunit or any splice cassettes. F-tests revealed that the variances were altered in the AMY (for the N1, C1, and C2 cassettes) and the NACC (C1 cassette). We hypothesized that the differences in variances between these groups could be due to there being more than one population of rats in the withdrawn group. One of these groups may be more sensitive to the aversive stimuli at 46°C and the other may be less sensitive to it.

In the AMY, the C1 cassette of all withdrawn animals was not significantly different from controls (Figure 4A, unpaired t-test $t(37)=1.084$, $p>0.05$). However, the withdrawn group did have a larger variance ($F(30,7)=5.959$, $p=0.0203$). Densitometry data was plotted into a frequency distribution with bins of five percentage points as compared to controls. A Gaussian best fit for one population has an R^2 of 0.6509 (Figure 4B), but fitting the data to two populations gives R^2 values of 0.8650 (Figure 4C). An extra sum of squares comparison of fits was also performed and a $p<0.0001$ was given in favor of the two population fit over the one population fit ($F(3,28)=14.80$). This further suggests that two distinct populations of withdrawn rats exist in our study. These animals were thus separated into a “Low C1” and a “High C1” group based on the two population distribution. Throughout the extended withdrawal period, the Low C1 group had lower facial contact times at 46°C than either the High C1 or control groups (Figure 4D). RM Two-Way ANOVA was significant for a group effect ($F(2,111)=9.92$, $p=0.0309$) but not for time ($F(3,111)=0.56$, $p>0.05$) or an interaction ($F(6,111)=0.61$, $p>0.05$) throughout this phase. These data indicate that lower C1 levels in the AMY are associated with inhibited motivational behavior during aversive conditions in a subset of the withdrawn population.

Also in the AMY, the C2 cassette of all withdrawn animals was not significantly different from controls (Figure 5A, unpaired t-test $t(37)=1.029$, $p>0.05$). However, the withdrawn group had a larger variance ($F(30,7)=8.020$, $p=0.0082$). Densitometry data was plotted into a

frequency distribution with bins of ten percentage points as compared to controls. A Gaussian best fit for one population has an R^2 of 0.791 (Figure 5B), but fitting the data to two populations gives R^2 values of 0.9934 (Figure 5C). An extra sum of squares comparison of fits was performed and a $p < 0.0001$ was given in favor of the two population fit over the one population fit ($F(3,6)=60.85$). This strongly suggests that two distinct populations of withdrawn rats exist in our study, a “Low C2” and a “High C2” group. Throughout the extended withdrawal period, the Low C2 group had lower facial contact times at 46°C than either of the other groups (Figure 5D). RM Two-Way ANOVA was significant for a group effect ($F(2,108)=3.717$, $p=0.0341$) but not for time ($F(3,108)=0.5438$, $p>0.05$) or an interaction ($F(6,108)=0.3540$, $p>0.05$) throughout this phase. These data indicate that lower C2 levels in the AMY are also associated with inhibited motivational behavior during aversive conditions in a subset of the withdrawn population.

Since both low C1 and low C2 levels led to inhibited behavior at 46°C we attempted to determine if they were the same population of animals. Pearson’s correlation gave a $p=0.0961$ and an R^2 value of 0.09 (Figure 6). This indicates that the loss of either C1 or C2 alone could be associated with enhanced sensitivity at 46°C.

Also in the AMY, the N1 cassette of all withdrawn animals was not significantly different from controls (Figure 7A, unpaired t-test $t(37)=0.8959$, $p>0.05$). However, the withdrawn group did have a larger variance ($F(30,7)=6.831$, $p=0.0135$). Densitometry data was plotted into a frequency distribution with bins of ten percentage points as compared to controls. A Gaussian best fit for one population has an R^2 of 0.7709 (Figure 7B), but fitting the data to two populations gives R^2 values of 0.8731 (Figure 7C). An extra sum of squares comparison of fits was also performed and a $p=0.0470$ was given in favor of the two population fit over the one population fit ($F(3,13)=3.491$). These animals were thus separated into a “Low N1” and a “High N1” group based on the two population distribution. No differences were observed between these groups for facial contact times at 46°C (Figure 7D). (RM Two-Way ANOVA, group ($F(2,108)=2.540$, $p=n.s.$) but not for time ($F(3,108)=0.56$, $p>0.05$) or an interaction ($F(6,111)=0.61$, $p>0.05$) throughout this phase.

Finally, C1 cassette of all withdrawn animals in the NACC was not significantly different from controls (Figure 8A, unpaired t-test $t(37)=0.3431$, $p>0.05$). However, the withdrawn group did have a larger variance ($F(30,7)=4.681$, $p=0.0408$). Densitometry data was plotted into a frequency distribution with bins of five percentage points as compared to controls. A Gaussian best fit for one population has an R^2 of 0.7305 (Figure 8B), but fitting the data to two populations gives R^2 values of 0.8015 (Figure 8C). An extra sum of squares comparison of fits was also performed and a $p=0.0054$ was given in favor of the two population fit over the one population fit ($F(3,41)=4.888$). These animals were thus separated into a “Low C1” and a “High C1” group based on the two population distribution. Similar to findings for N1 in the AMY, no differences were observed between these groups for facial contact times at 46°C (Figure 8D). (RM Two-Way ANOVA, group ($F(2,111)=2.610$, $p>0.05$) but not for time ($F(3,111)=0.4979$, $p>0.05$) or an interaction ($F(6,111)=0.9481$, $p>0.05$) throughout this phase.

4. Discussion

Altered NR1 splice variants remained in the AMY for at least two months following repeated morphine administration. While these altered splice variants were not observed in all rats, the subsets that did retain them were more sensitive to aversive, painful conditions. These long-term alterations in NR1 splice variants could play a role in the negative long-term effects of morphine withdrawal like increased anxiety and stress.

The effects of morphine administration and withdrawal were analyzed in two different ways: by total group changes and by examining individual differences based on each rat's NR1 splice variant expression. Morphine had several effects that were observed in the entire group. As expected, total group effects were observed for weight loss during the acute, but not extended withdrawal phase. This demonstrates that the morphine treated rats were dependent (Goode, 1971). Also, group effects were observed on the operant orofacial assay during morphine administration. Increases in time per contact values and facial contact times demonstrate morphine's ability to alter motivation for the reward at non-aversive temperatures (Anderson et al., 2012). This is likely due to morphine's enhancement of taste palatability (Doyle et al., 1993; Katsuura et al., 2011; Taha et al., 2009; Zhang and Kelley, 2002). This group effect continued during acute withdrawal as morphine treated animals had higher facial contact times and significantly higher time per contact values. A similar increase in food reward seeking during withdrawal has been reported (Nocjar and Panksepp, 2007) and this could be an attempt to compensate for the averseness of withdrawal or could be due to the associations made between morphine and the reward during previous pairings. The subsequent decrease over time could be attributed to an extinction of this behavior (Schmidt et al., 2005; Marlatt, 1990; Hayes and Gardner, 2004).

During extended withdrawal morphine treated rats no longer had significantly higher motivational scores at 37°C, but they did have significantly lower facial contact times at 46°C. This suggests that these rats have an increased sensitivity to the aversive stimulus over time. Many animal and human studies suggest that a long lasting hypersensitivity to pain may exist after chronic opioid administration (Angst and Clark, 2006) so it is possible that this is reflected here. However, in the operant orofacial assay pain sensitivity is generally determined by examining a rat's time per contact at aversive temperatures (Neubert et al., 2005; Neubert et al., 2006). Hypersensitivity should be reflected as a decrease in the time per contact values compared to controls at 46°C. We did not observe any changes in the time per contact values at 46°C for either the acute or extended period so it may be that hypersensitivity is not the cause of this long-term behavior. We did observe that the withdrawn rats' total facial contact time was significantly lower than the saline treated rats. This indicates that the withdrawn rats spent less time seeking reward at more aversive temperatures, suggesting they may be more sensitive to aversive conditions than controls. One well-characterized aspect of withdrawal is increased stress and anxiety which could be responsible for this increased sensitivity (Avila et al., 2008; Castilho et al., 2008; Schulteis et al., 1998). Although hypersensitivity to pain is a possible explanation for the inhibition during aversive conditions over time, we suspect that a better explanation lies in the modulation of the emotional response to the stimulus, not the painfulness of it.

As glutamatergic activity and NMDA receptors are both linked to emotional responses to pain, negative emotional states, and anxiety in the AMY, (Ansah et al., 2010; Cabral et al., 2009; Glass et al., 2008; Spuz and Borszcz, 2012) we examined this area for changes in NR1 subunits. We observed altered NR1 splicing in the AMY in a subpopulation of withdrawn rats that was associated with behavioral changes on our operant task. The AMY is a region responsible in part for pain processing as NMDA receptors in the AMY contribute to the antinociceptive properties of morphine (Manning and Mayer, 1995b; Manning and Mayer, 1995a). It is also important for the positive and negative emotional aspects of morphine use (Hou et al., 2009) as measured by morphine-induced conditioned place preference (Rezayof et al., 2007) and withdrawal-induced conditioned place aversion (Glass et al., 2008; Stinus et al., 1990). Plastic effects in the negativity of opioid withdrawal have been linked to glutamatergic activity in the extended amygdala and can remain in place for over a month using conditioned place preference tasks (Reti et al., 2008) so this area is a prime candidate for being responsible for inhibited reward-seeking during extended withdrawal.

NR1 splicing in the AMY was predictive of motivational behaviors in aversive conditions in our study. One subset of rats retained low levels of C1 in the AMY. A different subset had lower C2 levels. Both of these expression levels predicted lower average facial contact times throughout the withdrawal period and both of these effects would result in less active NMDA receptors in the AMY. It should be noted that seven of the eight animals in the Low C1 group were also in the Low C2 group suggesting that these groups are not completely distinct. The C1 insert has three serines which can be phosphorylated in vivo by PKC and PKA (Tingley et al., 1997). The C1 region also contains many binding areas for PSD-associated proteins like neurofilament-L, yotai, and calmodulin which can alter the stability of NMDA Receptors in the postsynaptic density (PSD) (Cull-Candy and Leszkiewicz, 2004). It is likely that this subset of rats have fewer NMDA receptors anchored in the synapse or lower levels of phosphorylation. NR1 subunits with lower levels of C2 may allow less calcium into the cell (Rameau et al., 2000). Like the animals with lower C1 expression, less active NMDA receptors would be expressed in the AMY. These morphine-induced splicing changes in the NMDA receptor may be responsible for generating and/or propagating this long-term sensitivity to aversive conditions.

The C1 changes observed could be due to the interactions between NMDA receptors and mu opioid receptors (MORs) in the central nucleus of the AMY (CeA). About 40% of neurons in the CeA are hyperpolarized by MOR agonists like morphine (Zhu and Pan, 2004) and these agonists reduce the probability of glutamate release presynaptically (Zhu and Pan, 2005). This relationship between NMDA receptors and MORs is likely responsible for the inhibition of the aversiveness of opioid withdrawal as demonstrated by the injection of NMDAR antagonists into the CeA (Watanabe et al., 2002) or the deletion of NR1 within the CeA (Glass et al., 2008). In the CeA, as well as many other brain areas, an upregulation of the cAMP pathway and CRE-dependent gene expression has been demonstrated to occur during naltrexone-precipitated withdrawal (Bie et al., 2005; Shaw-Lutchman et al., 2002). A link has also been reported between C1 and the NMDA receptor's ability to induce gene expression via a CRE-dependent mechanism (Bradley et al., 2006). While the presence or absence of C1 does not alter receptor inactivation, these lower C1 levels could be an adaptation in response to the activity of a subset of MOR and NMDA receptor containing cells in the CeA during withdrawal (Glass et al., 2008; Glass et al., 2009; Glass, 2010; Zhu and Pan, 2004; Zhu and Pan, 2005). Our previous data suggests that the C1 changes found in the long term withdrawn subset occurred within three days of withdrawal (Anderson et al., 2012). This was an effect for all withdrawn animals however. This posed the question of whether or not these splice variants return to normal or not. Interestingly, it looks as if it does recover to control levels in some animals. The animals that are left with abnormal C1 levels also have behavioral side effects that could be related to anxiety and reactions to stress.

F tests were also significant for C1 in the NACC and N1 in the AMY but we did not observe any associations between these two splice variant expression levels and any behavioral effect. This suggests that although C1 and C2 levels in the AMY may be responsible for the inhibition of behavior at 46°C, these effects are unrelated to N1 alterations in the AMY. The NACC is responsible in part for aversive effects of opioid withdrawal (Williams et al., 2001). Therefore it is possible that alterations of NMDA receptors in this area could be responsible for the inhibition of motivation at 46°C. Our findings for the NACC do not support this hypothesis as we observed no association between N1 levels in the NACC and any behaviors measured by our operant assay. The inhibited behavior at aversive temperatures is more clearly associated with C1 and C2 expression in the AMY.

It is possible that within an untreated group of animals like the saline group, there is actually a two population distribution based on differences in the levels of NMDA receptor splice

variants similar to the one we found in the morphine group. We believe that the small variance of the saline samples suggests that this is not the case. The F test demonstrates that we have a sufficient N to suggest there is a difference in the variances between the saline and the withdrawn populations. If there were two populations within the saline group, they would likely have a much larger variance and the F test would not have been significant. There is certainly a chance for an error to occur with this F-test, but this is less than a 5% chance as shown by the p-values in Table 2.

This is not the first report of long-term alterations in NR1 splice variants and drug use. Ethanol causes long-term alterations in striatal N1 (Raeder et al., 2008), accumbal C1 (Zhou et al., 2007), and hippocampal C2' (Winkler et al., 1999). Changes in striatal C2-containing NR1 subunits also occurred in extended cocaine withdrawal (Loftis and Janowsky, 2002). Altered spinal cord NR1 splicing has also been observed in other chronic conditions like animal models of chronic pain (Gaunitz et al., 2002; Prybylowski et al., 2001; Zhou et al., 2009). Taken together, these studies suggest that NR1 splicing could be a common mechanism for long-term adverse effects caused by drug administration.

In the human population, changes in motivation, anxiety, and adverse reactions to stress are hallmarks of the dependent population (Koob and Le Moal, 2005). These drug-induced behaviors can be partially localized to the AMY (Crombag et al., 2008; Gardner, 2011; Heinz et al., 2009; Koob, 2009). If these changes in the AMY are similar in people they could contribute to the long-term changes in anxiety found in many dependent patients. New non-drug-related behaviors would be more difficult to learn as the NMDA receptors in the AMY would not function at pre-drug levels. Also, since motivational factors and stress are common causes of relapse in the human addicted population (Gardner, 2011; Koob, 2009) altered NR1 splicing could be a marker for an increased propensity to relapse. The regulatory factors of NR1 splicing are not fully understood yet, more research is needed in this area as these splice variants could provide molecular targets for the treatment of long-term alterations in opioid-induced changes in motivation and anxiety.

Conclusions

NR1 splice variant expression level changes are observed in the amygdala in a subset of the population of dependent rats even after two months of morphine withdrawal. These subsets had associated changes in motivational behavior and/or hypersensitivity to adverse conditions which may reflect long-term alterations in the withdrawn population.

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Glossary

AMY	amygdala
NACC	nucleus accumbens
NMDA Receptor	N-methyl-D-aspartate receptor
NR1	subunit 1 of the NMDA Receptor
B37	baseline measures at 37°C
B46	baseline measures at 37°C
CeA	central nucleus of the amygdala

MOR**mu opioid receptor****Reference List**

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Highlights

Acute opiate withdrawal increases motivational behavior in non-aversive conditions.

Extended opiate withdrawal inhibits motivational behavior in aversive conditions.

The C1 and C2 cassettes of NMDAR1 in the amygdala are associated with inhibition.

Figure 1. Weights

A. No differences in weight were observed at baseline or during morphine administration. **B.** Morphine injected rats had significantly lower weights during the acute withdrawal phase. **C.** No differences in weight were observed during extended withdrawal. For all graphs: saline N=8, morphine N=32.

Figure 2. Morphine 37°C behavior

A. Morphine treated rats had significantly higher time per contact values throughout the administration period. Post-hoc tests were significant for the 10, 20, and 40 mg/kg doses ($p < 0.001$, $p < 0.001$, and $p < 0.05$). **B.** Morphine also increased the facial contact times during the administration period especially at the 10 and 40 mg/kg doses (post-hoc tests, $p < 0.05$). For both graphs: saline $N=8$, morphine $N=32$.

Figure 3. Behavioral differences between acute versus extended withdrawal at 37°C and 46°C

A. Morphine treated rats had significantly higher time per contact values at 37°C during acute withdrawal, but not extended withdrawal. **B.** No differences were observed in the facial contact times for acute or extended withdrawal at 37°C. **C.** No differences were observed in the time per contact values for acute or extended withdrawal at 46°C. **D.** No differences were observed for morphine treated rats during acute withdrawal at 46°C for facial contact times. During extended withdrawal however, significantly lower facial contact times were observed for morphine treated rats. For all graphs: saline N=8, morphine N=32.

Figure 4. AMY C1 changes are associated with long-term responses to aversive stimuli
A. No differences in the means were observed in the C1 cassette of withdrawn animals and controls but more variance is found in the withdrawn group. **B.** One Gaussian population explains some of the data. **C.** Two Gaussian populations best explain the data. **D.** Significantly lower facial contact times were observed for the altered group during the extended withdrawal period at 46°C. **E.** Representative blot of the C1 levels of three saline, high C1, and low C1 rats in the AMY. All bands illustrated are from the same gel and blot. For A: Saline N=8, Morphine N=39. For E: Saline N=8, High C1 N=23, Low C1 N=8.

Figure 5. AMY C2 changes are associated with long-term motivation and responses to aversive stimuli

A. C2 expression levels in the withdrawn animals were not significantly different from controls but more variance is found in the withdrawn group. **B.** One Gaussian population explains some of the data. **C.** Two Gaussian populations best explain the data. **D.** Low C2 rats had lower facial contact times at 46°C over time. **E.** Representative blot of the C2 levels of three saline, high C2, and low C2 rats in the AMY. All bands illustrated are from the same gel and blot. For A: Saline N=8, Morphine N=31. For D: Saline N=8, Low C2 N=21, High C2 N=10.

**A trend is observed for C1 and C2
densitometry values to correlate significantly**

Figure 6. AMY C2 and C1 levels approach significant a significant correlation
Decreased C1 and C2 levels may be found in the same rats although these populations are not exactly the same.

Figure 7. AMY N1 changes are not associated with long-term motivation and responses to aversive stimuli

A. N1 expression levels in the withdrawn animals were not significantly different from controls but more variance is found in the withdrawn group. **B.** One Gaussian population explains some of the data. **C.** Two Gaussian populations best explain the data. **D.** No differences were observed for lower facial contact times at 46°C over time between the groups. For A: Saline N=8, Morphine N=31. For D: Saline N=8, Low N1 N=23, High N1 N=8.

Figure 8. NACC C1 changes are not associated with long-term motivation and responses to aversive stimuli

A. NACC C1 expression levels in the withdrawn animals were not significantly different from controls but more variance is found in the withdrawn group. **B.** One Gaussian population explains some of the data. **C.** Two Gaussian populations best explain the data. **D.** No differences were observed for lower facial contact times at 46°C over time between the groups. For A: Saline N=8, Morphine N=31. For D: Saline N=8, Low N1 N=22, High N1 N=9.

Table 1

Escalating morphine doses

Day	Morphine Dose
B37, B46	0 mg/kg
1–2	5 mg/kg
3–4	10 mg/kg
5–6	20 mg/kg
7–8	40 mg/kg
9–10	60 mg/kg

These are the doses of morphine that were injected over time for the morphine and withdrawn groups. Control animals received equivalent volumes of saline on each of these days. B37 and B46 are baseline days for those temperatures.

Table 2

T and F-tests for NR1 total and all splice variants in the NACC and AMY.

	NACC		AMY	
	t-test	F-test	t-test	F-test
NR1	t(37)=1.333, p=n.s.	F(30,7)=1.506, p=n.s.	t(37)=0.3249, p=n.s.	F(30,7)=2.649, p=n.s.
N1	t(37)=0.1500, p=n.s.	F(30,7)=2.817, p=n.s.	t(37)=0.8959, p=n.s.	F(30,7)=5.959, p=0.0135
C1	t(37)=0.9601, p=n.s.	F(30,7)=4.681, p=0.0408	t(37)=1.084, p=n.s.	F(30,7)=6.831, p=0.0203
C2	t(37)=1.969, p=n.s.	F(30,7)=3.517, p=n.s.	t(37)=1.029, p=n.s.	F(30,7)=8.020, p=0.0082
C2'	t(37)=0.7447, p=n.s.	F(30,7)=3.246, p=n.s.	t(37)=0.5824, p=n.s.	F(30,7)=2.499, p=n.s.