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Morphine and MK-801 administration leads to alternative NMDAR1 splicing and associated changes in reward seeking behavior and nociception on an operant orofacial assay

Ethan M. Anderson², Arseima Y. Del Valle-Pinero², Shelby K. Suckow², Todd A. Nolan^{3,4}, John K. Neubert^{3,4}, and Robert M. Caudle^{1,2,§}

¹ Department of Oral and Maxillofacial Surgery, University of Florida College of Dentistry, Gainesville, FL 32610, USA

² Department of Neuroscience, University of Florida College of Medicine, McKnight Brain Institute, Gainesville, FL 32610, USA

³ Center for Comprehensive Pain Research, University of Florida, Gainesville, FL, United States

⁴Department of Orthodontics, University of Florida, Gainesville, FL, United States

Abstract

The NMDA receptor plays a large role in opioid-induced plastic changes in the nervous system. The expression levels of its NR1 subunit are altered dramatically by morphine but no changes in its alternative splicing have been reported. Changes in the splicing of the N1, C1, C2, and C2' cassettes can alter the pharmacology and regulation of this receptor. Western blots run on brain tissue from rats made tolerant to morphine revealed altered splicing of the N1 cassettes in the accumbens and amygdala, and the C1 cassette in the amygdala and the dorsal hippocampus. After three days of withdrawal C2'-containing NR1 subunits were down-regulated in each of these areas. These were not due to acute doses of morphine and may represent long term alterations in drug-induced neuroplasticity. We also examined the effects of morphine tolerance on an operant orofacial nociception assay which forces an animal to endure an aversive heat stimulus in order to receive a sweet milk reward. Morphine decreased pain sensitivity as expected but also increased motivational reward seeking in this task. NMDAR antagonism potentiated this reward seeking behavior suggesting that instead of attenuating tolerance, MK-801 may actually alter the rewarding and/or motivational properties of morphine. When combined, MK-801 and morphine had an additive effect which led to altered splicing in the accumbens, amygdala, and the dorsal hippocampus. In conclusion, NR1 splicing may play a major role in the cognitive behavioral aspects especially in motivational reward seeking behaviors.

Keywords

accumbens; amygdala; hippocampus; morphine; NMDA; pain

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§ Corresponding author RMC caudle@ufl.edu Phone: 352-273-6767. EMA ethan241@ufl.edu Phone: 352-273-6761 Fax: 352-392-7609 AYD delvallepinaray@mail.nih.gov Phone: 352-870-8331 SKS suckow@ohsu.edu Phone: 352-214-5052 TAN tnolan@dental.ufl.edu Phone: 352-392-1079 JKN jneubert@dental.ufl.edu Phone: 352-392-1079.

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The N-Methyl-D-Aspartate Receptor (NMDAR) is a well-studied component of drug-induced neural plasticity (Ueda and Ueda, 2009) although its role in tolerance to morphine's analgesic properties is currently in debate. Total NR1 protein expression changes have been demonstrated extensively (Le Greves et al., 1998; Murray et al., 2007; Zhu et al., 1999; Zhu et al., 2003) but no alterations in NR1 splicing in response to morphine have been reported. NR1 is an essential subunit for NMDAR function and can be alternatively spliced to form eight different variants. Each of these variants can have effects on plasticity by modifying the activation kinetics, phosphorylation, and cellular distribution of the NMDA receptor (Dingledine et al., 1999). NR1 has three exons (5, 20, and 21) which can undergo alternative splicing. When expressed these are known as the N1, C1, and C2 cassettes. C2 contains the stop codon for the gene and if it is spliced out, C2' is expressed (Figure 1). Morphine-induced alternative splicing of these cassettes would likely lead to different pharmacological and/or regulatory effects on NMDARs (Zukin and Bennett, 1995) and could provide a mechanism for some aspects of long term chronic drug administration like tolerance and dependence.

NR1 splicing changes have been demonstrated to associate with pain models (Caudle et al., 2005; Prybylowski et al., 2001; Zhou et al., 2009) as well as models of alcohol (Winkler et al., 1999a; Zhou et al., 2007) and cocaine use (Loftis and Janowsky, 2002). Since morphine can have an effect on abuse and pain, modifying NR1 splicing could be a common mechanism by which morphine alters both. For instance, opioid receptor activation causes changes in neural activity in the CNS (Martin et al., 2004) and activity can regulate NR1 splicing (Mu et al., 2003). Based on these findings and others involving the role of NR1 in morphine withdrawal (Manning et al., 1996; Zhu and Ho, 1998) we hypothesized that morphine tolerance and withdrawal would induce alterations in the expression of NR1 splice variants.

Reports suggest that NMDAR antagonism may be able to attenuate morphine tolerance (Shimoyama et al., 2005; Trujillo and Akil, 1991) while others suggest these findings were artifacts of the artificial cut-off times inherent in reflex-based nociception (Carlezon et al., 2000). Indeed, clinical studies have demonstrated that NMDAR antagonists do not alter opioid tolerance in humans (Compton et al., 2008; Galer et al., 2005). Part of the confusion may stem from the fact that NMDAR antagonists like ketamine have been demonstrated to have an additive effect on opioid analgesia (Suzuki et al., 2005; Wadhwa et al., 2001; Weinbroum et al., 2002; Wiesenfeld-Hallin, 1998) which may have an effect on the reflex-based measures of antinociception in animal studies (Carlezon et al., 2000). In order to determine if attenuated tolerance is an artifact of reflex-based measures, we investigated the effects of the NMDAR antagonist MK-801 on morphine tolerance in a non-reflex based operant orofacial thermal pain assay. We hypothesized that MK-801 would attenuate tolerance on our operant assay and would also alter the splicing of NR1. Since MK-801 also alters NR1 splicing (Mu et al., 2003) we hypothesized that this could also be a mechanism for the effects of NMDAR antagonists on long term morphine administration.

2. Experimental Procedures

2.1 Animal care

For all experiments male Sprague-Dawley rats 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 unless otherwise stated. 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 Experiment One

In order to test for morphine-induced changes in NR1 splice variant expression, rats were randomly assigned to a saline (n=7), morphine (n=10), or withdrawal group (n=9). Nociception was assessed using the Plantar Test (Ugo Basile, Collegeville, PA). Rats were placed in a clear chamber for thirty minutes, then an infrared heat stimulus (I.R. setting = 50) was placed under the hind paw and latency to withdraw was recorded. A cut-off time of 32.6 seconds prevented any tissue damage to the foot. Four trials (two per paw with a five minute intertrial interval) were averaged together for each animal as a daily score. Baseline latencies were taken for three days and then rats were given saline injections thirty minutes before testing for three additional days. This last injection day served as the baseline. Twice daily escalating morphine injections (**Table 1**) or equivalent volumes of subcutaneous saline were administered for the next ten days (1-10). Four saline rats and the rats in the morphine group were sacrificed as described below on day 10 within thirty minutes of their last injection. The remaining rats were tested for three more days (11-13) with no injections before being placed in the chamber. After testing, rats were placed in individual empty cages and time spent grooming was recorded for fifteen minutes. These rats were euthanized at the end of day 13.

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. Areas were removed from the slices cut using the following distances from Bregma: nucleus accumbens (NACC), 0 to 2mm; ventral posteromedial thalamus (VPM), amygdala (AMY), and dorsal hippocampus (HIPP) -2 to -5mm; ventral tegmental area (VTA) and periaqueductal gray (PAG), -5 to -8mm; locus coeruleus (LC), -8 to -11mm. Spinal Cord (SC) sections were taken from C1 to C4. 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 ten 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 ten 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 Sulfate buffer was heated in a boiling water bath for five minutes, loaded into a 4-20% Tris-glycine gels (Invitrogen, Carlsbad, CA, USA) and run at 200V for thirty-five minutes. Gels were placed in transfer buffer (10% methanol, 48mM Tris, 39mM glycine, pH 9.2) for thirty 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 TBST buffer (20mM Tris HCL, 0.9% NaCl, 0.05% Tween-20, pH 7.4) for one hour. Primary antibodies for NR1pan (1:1000, rabbit, Epitomics), N1 and C1 (both 1:4000, rabbit, Iadarola, NIDCR/NIH), C2 (1:3000, rabbit, PhosphoSolutions, Aurora, CO), C2' (1:3000, rabbit, Millipore), and Glyceraldehyde 3-phosphate dehydrogenase (GAP, 1:15000, mouse, Pierce Thermo, Rockford, IL) were placed on membranes. After 1-2 nights on a rotator at 4°C, blots were washed in TBST three times for ten minutes each and secondary antibody was added (anti-rabbit or anti-mouse IgG, HRP-linked, 1:4000, Cell Signaling, Danvers, MA) for one hour. Blots were washed three times for five minutes each and then detected using ECL Plus (Amersham, Pittsburg, PA) and Biomax MR film (Kodak, Rochester, NY) or the Carestream Image Station

4000MM (Carestream Health, Rochester, NY). Band density was measured with ImageJ software (National Institutes of Health, Bethesda, MD) for the film or Carestream Molecular Imaging Software.

Rat weights, withdrawal latencies on the Plantar Test, and time spent grooming during withdrawal were analyzed using Two-Way ANOVA with Bonferroni's post-hoc test. Densitometry scores were analyzed with One-Way ANOVA and Bonferroni's post-hoc test. All tests were performed using GraphPad Prism 4 software (La Jolla, CA). For all tests, p-values less than 0.05 were considered significant.

2.3 Experiment Two

Six rats were injected with saline and six were administered the maximal dose of morphine from the previous experiment (60mg/kg). Thirty minutes later the rats were euthanized, tissue was harvested, and western blots were run as described previously. Densitometry scores were analyzed with a two-sample t-test.

2.4 Experiment Three

We measured the effects of morphine on reward-seeking and pain with a reward/conflict operant task first described by Neubert et al. (2005) which is sensitive to analgesics like morphine (Neubert et al., 2006). Hairless Sprague-Dawley rats (250-300 g, Charles River, Raleigh, NC) were used for this experiment as facial hair interferes with the testing procedure (Neubert et al., 2005). These rats were fasted for 20 $\pm$ 1hrs (from 5pm the previous night to 12-2pm the next day) before each training and testing day then placed in a Plexiglas box for twenty minute periods and trained to press their faces between two aluminum tubes in order to receive diluted sweetened condensed milk. 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 two weeks of baseline training (three times a week) 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 a 37°C baseline session. Next, 10mg/kg morphine or an equivalent volume of saline was administered twice daily for ten days and testing continued every two to three days. Rats were randomly separated into four groups: saline injected and tested at 46°C each day (Sal46), saline injected and tested at 37°C each day (Sal37), morphine injected and tested at 37°C each day (Mor37), and morphine injected at 46°C (Mor46). For the Mor46 rats, eight new rats were tested on each day. The rats tested on day 1 were euthanized after testing and eight new rats were used for each subsequent day (eight for 3, eight for 5, etc.). These rats were tested at 37°C to remain trained until the day of their 46°C testing. Pain sensitivity was determined by taking the total amount of time spent making facial contacts and dividing it by the number of facial contacts at an aversive 46°C. This is defined as the time per contact. Analgesics cause the time per contact to increase from the 46°C baseline as rats appear to become insensitive to the heat pain and maintain the facial contact for longer periods of time (Neubert et al., 2006). Data on testing sessions were expressed as a percentage of control of each rat's own baseline values before statistical analysis was performed. Significance ($p < 0.05$) was assessed with four separate Two-Way ANOVA analyses with Bonferroni's post-hoc comparison (Sal37 vs. Sal46, Mor37 vs. Mor46, Sal37 vs. Mor37, and Sal46 vs. Mor46) to isolate the effects of morphine or temperature.

2.5 Experiment Four

Thirty-two hairless rats were trained and baseline measures were established in the same way as Experiment Three. Rats were then randomly split into four groups of eight. Each group received an intraperitoneal injection of either saline or 0.1mg/kg MK-801 hydrogen maleate (Sigma, St. Louis, MO). Thirty minutes later they received a subcutaneous injection of either saline or 10mg/kg morphine. Doses of morphine and MK-801 were equivalent to those reported by Trujillo and Akil (1991). Forty minutes after the second injection rats were tested on the operant task for twenty minutes. Rats were tested for effects on motivation and reward at 37°C on the mornings of days 3 and 5 then tested for pain sensitivity at 46°C on the mornings of days 8 and 10. Rats were euthanized and NACC, AMY, and HIPPO tissue was harvested and western blots were run as previously described forty minutes after a final morphine or saline injection on the evening of day 10. Data were analyzed with Repeated Measures (RM) Two-Way ANOVA with Bonferroni's post-hoc test. Western blots were run as previously described and analyzed with One-Way ANOVA with Bonferroni's post-hoc test.

3. Results

3.1 Experiment One: Morphine tolerance and withdrawal alter NR1 splicing

Tolerance to the analgesic effects of morphine was measured using a thermal sensitivity assay. No significant differences were found between the groups at baseline. Rats were then injected with escalating doses of morphine (**Table 1**) or an equivalent volume of saline for ten days. A significant effect for morphine ($F(1,150)=36.38$, $p<0.0001$), time ($F(10,150)=6.024$, $p<0.0001$), and their interaction ($F(10,150)=6.104$, $p<0.0001$) were observed with RM Two-Way ANOVA. Latencies returned to levels not significantly different from saline controls during the three withdrawal days: 11-13 (RM Two Way ANOVA, treatment ($F(1,12)=3.559$), time ($F(2,12)=0.1310$), interaction ($F(2,12)=1.921$) (**Figure 2A**). Rats were weighed every other day throughout the procedure and during the ten days of morphine injections a significant effect for morphine was not observed with RM Two-Way ANOVA ($F(1,125)=1.865$, $p=ns$) although a significant effect was observed for time ($F(5,125)=12.21$, $p<0.0001$), and interaction ($F(5,125)=22.40$, $p<0.0001$) likely due to morphine inhibiting weight gain. Morphine had a significant effect on weight during withdrawal (RM Two-way ANOVA: morphine ($F(1,12)=23.35$, $p=0.0004$), time ($F(1,12)=2.091$, $p=ns$), interaction $F(1,12)=6.794$, $p=0.0229$). Post-hoc tests were significant for weight loss on withdrawal days 11 ($p<0.05$) and 13 ($p<0.01$) (**Figure 2B**).

Brain regions were isolated from the rats mentioned above and western blots were run on their tissue. Western blots were probed with antibodies specific for N1, C1, C2, C2', and GAP. A representative western blot of the NACC is presented for twelve rats (four from each treatment group) but all rats were included in the analysis (**Figure 3**). Densitometry scores were normalized by GAP scores as a loading control and expressed as a percentage of control. All scores were analyzed with One-Way ANOVA and Bonferroni's post-hoc test. Decreases in the N1 cassette occurred in the NACC and AMY during the tolerance phase and these levels stayed low during withdrawal ($F(2,23)=5.825$, $p=0.0090$ and $F(2,23)=5.136$, $p=0.0143$ respectively). A decrease in N1 was also observed between the morphine and withdrawn rats in the LC ($F(2,23)=6.820$, $p=0.0047$) (**Figure 4A**). A decrease in the C1 cassette was observed in the HIPPO during the tolerance phase and these levels stayed low during withdrawal ($F(2,23)=6.021$, $p=0.0079$). C1 also decreased in the AMY during withdrawal ($F(2,23)=3.451$, $p=0.0489$) (**Figure 4B**). C2-containing NR1 subunits increased significantly in the HIPPO during the tolerance phase and these levels were decreased when morphine rats were compared to withdrawn ones ($F(2,23)=5.856$, $p=0.0085$) (**Figure 4C**). Lastly, C2'-containing NR1 subunits decreased significantly during withdrawal in the

NACC, AMY, and the HIPP ($F(2,23)=6.564$, $p=0.0056$; $F(2,23)=7.655$, $p=0.0028$; and $F(2,23)=7.921$, $p=0.0024$). C2' levels increased in the VPM during withdrawal as well ($F(2,23)=4.266$, $p=0.0266$) (**Figure 4D**). No significant changes were found in the VTA, PAG, or SC.

The combination of C2 and C2' levels should reflect total NR1 levels therefore we also examined pan-NR1 levels whenever changes in these two splice variants were observed. All scores were analyzed with One-Way ANOVA and Bonferroni's post-hoc test. In the NACC, a significant decrease in total NR1 was observed in withdrawal when compared to the morphine rats ($F(2,23)=3.684$, $p=0.0409$). In the AMY, a decrease in total NR1 was also observed in withdrawal when compared to the morphine rats ($F(2,23)=3.827$, $p=0.0368$). In the HIPP, a significant increase in total NR1 protein occurred in the morphine animals and returned to levels not different from the saline controls after the three days of withdrawal ($F(2,23)=4.051$, $p=0.0311$). No change in total NR1 was observed for the VPM (**Figure 5**).

3.2 Experiment Two: Acute morphine administration does not alter NR1 splice variant expression

We examined the effects of an acute dose (60mg/kg) of morphine on NR1 splice variants in all the areas in which a change was observed during the morphine phase of experiment one. No significant changes were observed in the NACC, AMY, or HIPP for any of the variants tested (for all two-sample t-tests $df=10$ and $p>0.05$) (**Figure 6A-C**).

3.3 Experiment Three: Morphine tolerance alters pain and motivational reward seeking behavior on an operant orofacial nociception assay

The effects of morphine tolerance on an operant orofacial nociception assay were assessed at two different temperatures. As observed in **Figure 7A**, an aversive temperature of 46°C decreases the time per contact values of rats (Paired t-test, $t=4.325$, $df=63$, $p<0.0001$, $n=64$). Subsequent values in **Figure 7B** are expressed as a percent of either this 37°C baseline score or the 46°C baseline score for each individual rat.

Four separate Two-Way ANOVAs with Bonferroni's post-hoc tests were run comparing Sal37 to Sal46, Mor37 to Mor46, Sal37 to Mor37, and Sal46 to Mor46. These results can be found in **Table 2**. The aversive temperature over the five testing sessions resulted in Sal46 rats having lower time per contacts compared to Sal37 rats ($p=0.0248$). A significant effect of temperature was not observed for the Mor37 versus Mor46 rats suggesting thermal pain was not having an effect with this dose of morphine. There was an effect for time ($p<0.0003$) demonstrating that over time tolerance occurred in both groups. Mor37 rats had significantly increased time per contact values compared to Sal37 rats ($p=0.0077$) especially on the first day of injections suggesting that morphine is having more than just an analgesic effect on this assay. Mor46 rats had greatly increased time per contact values when compared to Sal46 controls (likely due to the lower baseline at 46°C) and significant effects were observed for morphine ($p<0.0001$), time ($p<0.0001$), and an interaction ($p<0.0001$) suggesting tolerance to morphine's analgesic effects occurred over time. Significant peak effects were observed on days 1 and 3 with post-hoc tests (**Figure 7B**).

Differences were also observed in reward seeking behavior as evidenced by differences in the time spent making facial contacts to receive the reward. No differences were observed between Sal37 and Sal46 rats for facial contact times suggesting that over time neither of these groups changed from their baseline values. No differences were observed between Mor37 and Mor46 rats, suggesting that they spent equivalent times seeking reward. A significant effect of morphine on contact time was observed when comparing Sal37 to Mor37 ($p<0.0001$) suggesting that morphine treated rats spent more time trying to obtain the

reward than saline controls at non-aversive temperatures. Mor46 rats also spent significantly more time making facial contacts than Sal46 rats did ($p<0.0001$). Differences were observed with post-hoc tests on days 3, 5, and 8 suggesting that morphine has a larger effect at aversive temperatures. Time was not significant for any of the morphine treated rats suggesting that rats did not become tolerant to this reward-seeking behavior over the course of the ten day treatment (**Figure 7C**).

Similar results to contact time were observed for the time spent licking to receive the reward. Sal37 rats spent the same amount of time licking as Sal46 rats did and, over time, both increased their licking ($p=0.0077$). Mor46 and Mor37 rats spent equal amounts of time licking and both increased their licking over time ($p=0.0488$). Although Mor37 rats had a trend of spending a larger amount of time licking than Sal37 rats this was not significant, but Mor46 rats spent a much larger amount of time licking than Sal46 rats ($p<0.0001$). This effect changed over time ($p<0.0045$) likely due to the saline rats increasing their licking on day 10. Significant increases in licking for Mor46 rats were observed with post-hoc tests on 3, 5, and 8 (**Figure 7D**).

3.4 Experiment Four: NMDAR antagonism alters morphine-induced motivational reward seeking behavior and pain sensitivity

Hairless Rats were injected with either saline (S, $n=8$), MK-801 (MK, $n=8$), morphine (M, $n=8$), or morphine with an MK-801 pretreatment (MM, $n=8$) and tested on the operant orofacial nociception assay. RM Two-Way ANOVA and Bonferroni's post-hoc test data for all of the following analyses can be found in **Table 3**. On days 3 and 5 of injections all rats were tested at 37°C and a significant difference was only observed for the time ($p=0.0197$), likely due to the increases in the MK-801/morphine group (**Figure 8A**). At 46°C a significant effect was observed for treatment group ($p=0.0411$) and time ($p=0.0036$) but not an interaction on the time per contact values. On day 10 especially the MK-801/morphine group had a significantly higher time per contact values than the MK-801 rats (**Figure 8B**). For total facial contact time at 37°C a significant effect of treatment ($p<0.0001$), time ($p<0.0001$), and an interaction was observed ($p=0.0002$). Again, MK-801/morphine rats had the greatest change in behavior as they spent more time on the facial contact than any other group. On days 3 and 5 MK-801/morphine rats had higher contact times than saline, morphine, and MK-801 rats (**Figure 8C**). Morphine/MK-801 rats spent more time making facial contacts at 46°C as well. Significant effects were observed for treatment ($p<0.0001$), time ($p<0.0001$), and an interaction ($p<0.0001$). On days 8 and 10 MK-801/morphine rats had higher contact times than the other three groups. Also, MK-801 rats had lower facial contact times on day 10 than morphine rats as well (**Figure 8D**). For licking behavior at 37°C a significant effect was observed for treatment group ($p=0.0005$), time ($p=0.0012$), and an interaction between the two ($p=0.0348$). On day 3 the MK-801/morphine group spent more time licking than both saline and MK-801 treated rats. On day 5 MK-801/morphine rats spent more time obtaining reward than saline and morphine rats. Morphine rats also spent more time licking than MK-801 treated rats on day 5 (**Figure 8E**). At 46°C there was a significant effect of group ($p<0.0001$), time ($p<0.0001$), and an interaction ($p=0.0023$) on licking behavior. On day 8 the MK-801 rats licked significantly less than the other three groups. On day 10 the MK-801/morphine rats again had the largest increase in behavior from baseline as they licked more than saline, MK-801, and morphine rats. MK-801 rats also licked less than saline and morphine treated rats on day 10 (**Figure 8F**).

Western blots were run on the NACC, AMY, and HIPPO to determine if NR1 splicing was altered due to morphine and MK-801. Significance was assessed with One-Way ANOVA and Bonferroni's post-hoc test. In the NACC, the N1 cassette was significantly altered by treatment group ($F(3,28)=5.718$, $p=0.0035$) as N1 was slightly decreased in the morphine

and MK-801 groups and these appeared to have an additive effect in the combined group. The MK-801/morphine group had significantly decreased N1 as compared to the saline group ($p<0.01$) and the morphine group ($p<0.05$) (**Figure 9A**). Results were similar for the AMY ($F(3,28)=6.386$, $p=0.0020$) as N1 decreased compared to saline injected rats with both the MK-801/saline treatment ($p<0.05$) and the MK-801/morphine treated animals ($p<0.01$) (**Figure 9B**). No significant changes in N1 occurred in the HIPP (**Figure 9C**). In the NACC, C1 levels also decreased with drug treatment ($F(3,28)=3.568$, $p=0.0265$) and MK-801/morphine treated rats had significantly lower levels than saline treated rats ($p<0.05$) (**Figure 9D**). Similar results again occurred in the AMY with C1 ($F(3,28)=27.80$, $p<0.0001$). When compared to both saline injected and morphine injected rats C1 significantly decreased in the MK-801/saline rats ($p<0.05$, $p<0.05$) and the MK-801/morphine injected rats ($p<0.001$, $p<0.001$). Also, the MK-801/morphine treated rats had lower C1 values than the MK-801/saline treated rats ($p<0.001$) (**Figure 9E**). In the HIPP, C1 splicing also changed with drug treatment ($F(3,28)=10.27$, $p<0.0001$). The MK-801/saline treated rats had lower levels of C1 than the saline rats ($p<0.001$), morphine ($p<0.05$), and MK-801/morphine rats ($p<0.001$) (**Figure 9F**). No change was observed for C2-containing NR1 subunits in the NACC, AMY, or the HIPP (**Figures 9G, 9H, and 9I**). C2'-containing subunits significantly increased in the NACC ($F(3,28)=4.125$, $p=0.0153$) when comparing the MK-801/morphine group to controls ($p<0.05$) (**Figure 9J**). No changes in C2' were observed in the AMY or HIPP (**Figures 9K and 9L**). Finally, NR1pan levels did not differ significantly due to any of the drug treatments for the NACC, AMY, or HIPP (**Figures 9M, 9N, and 9O**).

4. Discussion

In this study we demonstrated that chronic morphine administration and subsequent withdrawal leads to an altered set of NMDARs in several areas of the limbic system. This was not found in acutely treated animals. We also demonstrated that our operant orofacial assay can measure tolerance to morphine's antinociceptive effects as well as morphine's ability to induce reward seeking behavior. We then tested whether MK-801 could attenuate tolerance on this assay. Our findings were that MK-801 had an additive effect on morphine's antinociceptive and reward seeking properties but did not appear to attenuate tolerance. In addition, these two drugs had interesting effects on NR1 splicing in several brain areas, likely due to their ability to alter the excitability of neurons. The end result of these splicing changes will be altered NMDAR activity in the future which may be a mechanism for some of the detrimental effects of long term chronic opioid administration.

In the first experiment of this study escalating morphine doses did lead to tolerance and subsequent withdrawal in rats but there were no significant changes in NR1 in areas generally associated with pain (PAG and SC), as determined by Western blot analysis. These results are similar to the findings of Kozela and Popik (2007) and suggest that changes in NR1 splice variation may not have a role in tolerance to morphine's antinociceptive effects in these areas. These treatments were associated with large differences in NR1 splice variant composition in frontal areas more related to higher cognitive functions like motivation, anxiety, and memory.

Two common themes arose in the first experiment. First, it is of interest that N1 in the NACC and AMY and C1 in the HIPP decreased in the morphine rats and did not return to normal levels during withdrawal. This suggests that some morphine-induced changes in splicing may last long after the drug is withdrawn and could be a mechanism for extended alterations in behavior after chronic drug administration. N1 and C1 can be regulated by calcium, membrane depolarization and neuronal activity (Lee et al., 2007; Xie, 2008) therefore these common results may be a compensating mechanism for the increased neuronal activity in these areas during morphine administration and withdrawal (Rasmussen

et al., 1995). In the HIPP however, the morphine induced alteration did not continue through withdrawal. C2 levels rose during the tolerance phase and did return to pre-drug levels during spontaneous withdrawal. The increase in C2-containing NR1 subunits observed in the HIPP could be a counter adaptation to morphine's effects on memory consolidation. Increasing NMDAR activation in the HIPP reverses morphine-induced memory loss (Zarrindast et al., 2011) and over the ten day injection period this cellular adaptation could have occurred in an attempt to offset this morphine effect. Once the morphine is no longer administered and withdrawal ensues, C2 levels could then return to pre-drug levels. Secondly, the NACC, AMY, and HIPP all had decreased C2' in withdrawal and a concomitant decrease in total NR1 protein. Decreases in C2' relative to C2 can occur as a result of increased neuronal activity in culture (Mu et al., 2003). Since the AMY, NACC, and HIPP are all very active during withdrawal (Rasmussen et al., 1995), this suggests that activity-dependent changes in NR1 splicing are occurring in these areas. Decreased levels of N1, C1, and C2' can lead to decreases in NMDAR activation (Cull-Candy and Leszkiewicz, 2004), phosphorylation (Scott et al., 2001a), and neuronal surface expression (Okabe et al., 1999) therefore the likely end result is that after three days of withdrawal from morphine, NMDA receptors are going to be less responsive to glutamate and less capable of future plastic events in the NACC, AMY, and HIPP. These results along with previously reported long-term decreases in C2-containing NR1 subunits following cocaine withdrawal (Loftis and Janowsky, 2002) and long-term increases in C2' after chronic alcohol exposure (Winkler et al., 1999) suggest a common splicing mechanism for chronic drug-induced changes in neuronal plasticity.

NMDAR antagonism has been suggested to attenuate tolerance in spinal-reflex based pain assays like the thermal tail flick (Trujillo and Akil, 1991) and has also been demonstrated to alter NR1 splicing (Mu et al., 2003). We therefore hypothesized that NMDAR antagonism would lead to a different set of NR1 receptors which would be associated with attenuated morphine tolerance. MK-801 leads to increased locomotor activity (Carlsson and Svensson, 1990) and studies in our lab suggest that this interferes with assessing nociception on the Plantar Test (data not shown). Furthermore, spinal reflex-based measures of pain may not be appropriate for measuring the alterations in morphine analgesia and tolerance we expected (King et al., 2007; Morgan et al., 2008; Vincler et al., 2001) with MK-801 pre-administration (Carlezon et al., 2000). We therefore tested for NMDAR antagonist effects on morphine tolerance with an operant orofacial thermal sensitivity assay. This assay is capable of measuring the effects of aversive thermal pain through a decrease in time per contact values and morphine antinociception which increases the time per contact (Neubert et al., 2006). When compared to baseline levels, morphine greatly increased the time per contact values versus Sal46 rats and this reduced over time to levels near baseline confirming that tolerance can be measured with this assay. This effect is not entirely due to the reduction of heat pain by morphine as morphine also had an effect on rats tested at 37°C. This increased effect for Mor37 rats is likely related to the increased reward-seeking behavior demonstrated by increased time spent making facial contacts and time spent licking. This increased reward-seeking behavior is consistent with the fact that mu opioid receptor agonists have previously been demonstrated to induce increases in food intake following inter-accumbal infusion (Katsuura et al., 2011a; Zhang and Kelley, 2002b) possibly due to altered palatability of food rewards (Taha et al., 2009b). These results together demonstrate that morphine injected rats have an increased motivation to obtain the sweet reward and this is likely dependent on morphine's effects on the limbic system.

To further investigate this dual effect of morphine on motivational reward seeking at 37°C and antinociception at 46°C, we examined the effects of a pre-injection of MK-801 on rats administered saline or morphine at both temperatures. Previous results suggested that tolerance to the analgesic effects at 46°C were gone by days 8 and 10, therefore we decided

to test for attenuated tolerance on these days. We also decided to test for changes in motivational reward seeking at 37°C on days 3 and 5 of the injection period. Similar to the findings of Carlezon and Wise (1993), MK-801 potentiated morphine's effects on motivational reward-seeking behavior at 37°C. If examined alone, the higher time per contact values on day 10 at 46°C could be interpreted as attenuated morphine tolerance although studies by Carlezon et al (2000) demonstrate that NMDAR antagonists likely do not attenuate morphine tolerance. Instead it could be a result of MK-801's ability to enhance morphine's antinociceptive properties. Another possibility is that this increase could be a continuation of the change in the motivational reward seeking properties of morphine observed at non-aversive temperatures. Examining this data along with the alterations on days 3 and 5 at 37°C suggests that there is different effect when these two drugs are combined. There is a more complicated relationship than MK-801 simply attenuating morphine tolerance. This suggests that the time per contact values for MK-801/morphine rats at 46°C represent some interaction between these factors.

An unexpected result was that MK-801 injected rats remained at baseline levels of the time per contact values, total contact time, and time spent licking throughout testing. This was not anticipated as MK-801 was demonstrated to increase the preference for food reward using the conditioned place preference test (Yonghui et al., 2006) and also increases food intake in rats (Burns and Ritter, 1997). On the other hand, MK-801 reduced motivation on progressive ratio schedules (Buffalo et al., 1994) therefore it could be interfering with the motivational factors necessary for reward-seeking behavior on our task. Another possibility is that MK-801 may be interfering with cognition in this task. The nature of operant tasks is that a functioning cortex is needed. MK-801 alone may have interfered with the rats' ability to change their behavior on this assay over time.

Since morphine injected into the NACC increases reward seeking behavior (Katsuura et al., 2011b;Taha et al., 2009a;Zhang and Kelley, 2002a) we investigated this area for changes in NR1 splicing. Both the N1 and C1 cassettes appeared to decrease with MK-801 or morphine alone, but when both drugs were co-administered statistical significance was achieved. No change in C2 expression was detected. NMDAR antagonism and morphine also tended to increase C2' and when administered together C2' increased significantly. NR1pan levels did not alter significantly. This is likely due to the much larger amount of C2 in this area than C2' according to studies examining NR1 mRNA (Winkler et al., 1999b). If this is true for NR1 protein as well, then C2' levels can increase in neurons without having a large measurable effect on NR1pan western blots. Together these changes suggest that the co-administration of morphine and MK-801 leads to modulation of glutamatergic signaling in the NACC. Mu opioid receptors are most often found on different neurons than NR1 subunits in co-localization studies on NACC shell neurons (Gracy et al., 1997b). This suggests that separate groups of neurons in this motivational area would be inhibited with morphine and NMDAR antagonists. Thus, when the two drugs are combined the NACC would be highly activated. This increase in activity could initially be responsible for the potentiating effects of MK-801 on morphine-induced motivation reward seeking. Over ten days of injections this increased activation of the NACC would likely result in long term compensatory NR1 splice variant adaptations (Lee et al., 2007). Indeed, the neurons in MK-801/morphine rats have less responsive NR1 subunits than the control NR1 subunits (Cull-Candy and Leszkiewicz, 2004) supporting this hypothesis. These results demonstrate that the NACC may be less responsive to glutamatergic activation in the future and could play a role in drug-induced changes in behavior associated with this area.

Differences in N1 and C1 splicing were also observed in the AMY in a similar pattern to the changes in the NACC. The MK-801 treated rats had lower levels of both splice variants and the MK-801/morphine treated rats had the lowest. These decreases in N1 and C1 in the

AMY are likely due to changes in activity due to the drug treatments. Their similarity to the changes observed in the NACC are likely due the fact that Mu opioid receptors and NMDA receptors are found co-localized on GABAergic neurons in the AMY (Glass et al., 2008) in a comparable way to the accumbens' GABAergic medium spiny neurons (Gracy et al., 1997a). Other similarities between these areas are that MK-801 has been demonstrated to increase Fos-like immunoreactivity in both the AMY and NACC (Carr and Kutchukhidze, 2000). Once again, this alteration in neuronal activity may lead to similar splicing patterns in both of these areas for the MK-801 treated rats.

In the HIPP, splicing changes due to drug treatment were only observed in the C1 cassette which decreased in both the morphine treated and MK-801 treated groups. The lowest levels of C1 were found in the MK-801 treated rats and these rats also likely had problems learning on our operant task. On days 8 and 10 every group except those treated with MK-801 alone had responding rates that were increased from baseline. This stagnant behavior by the MK-801 rats could represent impaired learning over time. Lower C1 levels may reflect NMDA receptors that are less capable of synaptic plasticity (Scott et al., 2001b) which could be a mechanism for the effects on learning on our operant testing. This behavior was not the case for the morphine treated rats that also had lowered C1 levels, however suggesting that other factors like morphine's effect on motivational reward seeking behavior may have an overshadowing role on the behavior in learning in this task. Interestingly, when MK-801 and morphine were co-administered these changes appeared to be blocked and were at levels not significantly different from those of the saline controls. This blocking effect of MK-801 on morphine-induced C1 changes could be a mechanism for MK-801's ability to block the acquisition of morphine-induced conditioned place preference, a behavior dependent on HIPP functioning (Zarrindast et al., 2007).

It should be noted that some of the splice variant changes we report here may be dose dependent. The escalating dose paradigm used in experiment 1 result in much higher amounts of morphine being administered over time than the 10mg/kg twice daily dose paradigm in experiments 3 and 4. As a result more subtle effects are observed in our western blots. The N1 changes in the NACC and AMY and the C2 changes in the HIPP did not obtain statistical significance though there were similar trends between the two data sets. The C1 cassette changes observed in the HIPP did occur in both dosing paradigms however, so this change may be especially sensitive to morphine administration.

Conclusions

Protein expression levels and splicing of the NR1 subunit of the NMDAR change with repeated morphine injections and subsequent withdrawal in the NACC, AMY, and HIPP. Morphine also increases motivational reward seeking behavior in rats and this is potentiated by a pretreatment with MK-801. When combined, MK-801 and morphine, alter NR1 splicing in the NACC which will likely result in changes in motivational reward seeking behavior in the future.

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Abbreviations

AMY   amygdala

GAP	glyceraldehyde 3-phosphate dehydrogenase
HIPP	dorsal hippocampus
LC	locus coeruleus
Mor37	rats tested at 37°C and injected with morphine
Mor46	rats tested at 46°C and injected with morphine
NACC	nucleus accumbens
NMDAR	N-Methyl-D-Aspartate Receptor (NMDAR)
NR1	NMDAR1 subunit of the NMDA receptor
NS	non-significant
PAG	periaqueductal gray
RM	repeated measures
SC	spinal cord
Sal37	rats tested at 37°C and injected with saline
Sal46	rats tested at 46°C and injected with saline
VPM	ventral posteromedial thalamus
VTa	ventral tegmental area

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Highlights

Morphine tolerance and withdrawal induces altered NMDA receptor splicing
NMDA receptor antagonism potentiates motivational reward seeking behavior
MK-801 potentiates morphine induced NMDAR splicing in the accumbens

Figure 1. NMDA receptor subunit 1 alternative splicing

N1 and C1 are spliceable cassettes, but C2 and C2' are mutually exclusive and each contain a stop codon. Black boxes represent non-spliceable exons.

Figure 2. The effects of morphine on thermal sensitivity, weight, and grooming behavior
 Significance for each analysis was assessed with Repeated Measures Two-Way ANOVA. A) The latency to withdraw from a heat stimulus increased from saline injected controls (n=6) on days 1-10 when morphine was injected (n=11). Significant effects on hindpaw latency for morphine, time, and their interaction were observed. Latencies of withdrawn rats (n=5) returned to levels not significant from saline injected rats (n=3) during the three day withdrawal period (days 11-13). B) A significant effect for morphine was not observed during the injection period but a significant effect was observed for time and an interaction. Weight decreased significantly during morphine withdrawal. Significant effects on weight loss were observed for morphine, time, and their interaction. The data presented is the mean

+/- SEM, an * indicates a p-value < 0.05 for Bonferroni's post-hoc test and ** indicates a p-value < 0.01.

Figure 3. A representative blot of NR1 changes in the nucleus accumbens due to morphine
Sample western blots illustrating results from 12 of the 26 animals used in this study. Each row contains data from one antibody (NR1pan, N1, C1, C2, C2', or GAP).

Figure 4. Quantification of NR1 changes in the brain due to morphine tolerance and withdrawal
 A) N1 decreased significantly in the morphine animals and did not return to baseline levels during withdrawal in both the nucleus accumbens (NACC) and the amygdala (AMY). N1 also decreased in the locus coeruleus (LC) during withdrawal. B) C1 decreased significantly in the dorsal hippocampus (HIPP) during the morphine phase and these levels did not return to pre-drug levels during withdrawal. C1 also decreased in the AMY during withdrawal. C) C2 increased significantly in the HIPP during the tolerance phase. These levels dropped significantly during withdrawal as compared to the morphine rats. D) C2' decreased significantly during withdrawal in the NACC, AMY, and the HIPP. C2' levels increased in the ventral posterior nucleus of the thalamus (VPM) during withdrawal. No significant changes were found in the ventral tegmental area (VTA), periaqueductal gray (PAG), or spinal cord (SC) for any cassettes. The data presented is the mean $\pm$ SEM. An * represents a significant result in Bonferroni's post-hoc test when compared to the saline group and a # represents significance between the morphine and withdrawn groups. Significance for all analyses was assessed with One Way ANOVAs and Bonferroni's post-hoc tests.

Figure 5. Quantification of NR1 total protein changes in the brain due to morphine tolerance and withdrawal

A significant decrease in total NR1 was observed in the NACC and the AMY in withdrawal when compared to the morphine rats. In the HIPPO, a significant increase in total NR1 protein occurred in the morphine animals and returned to normal levels after the 3 days of withdrawal. No change in total NR1 was observed for the VPM. The data presented is the mean $\pm$ SEM. An * represents a significant result in Bonferroni's post-hoc test when compared to the saline group and a # represents significance between the morphine and withdrawn groups. Significance for all analyses was assessed with One Way ANOVAs and Bonferroni's post-hoc tests.

Figure 6. An acute dose of morphine does not alter NR1 splice variation

No significant changes were found in the A) NACC, B) AMY, or C) HIPP due to an acute dose of morphine. The data presented is the mean $\pm$ SEM. Significance was assessed with two-sample t-tests (for all, $df=10$) for each cassette in each area with $p<0.05$ being considered significant.

Figure 7. Morphine alters pain and reward-seeking behavior at aversive and non-aversive temperatures on the operant orofacial nociception assay

A) An increased temperature is aversive as shown by the decrease in time per contact values from all tested rats 37°C baseline to their own 46°C baseline. **B)** Aversive temperatures decreased the time per contact values for saline treated rats. Morphine increased the time per contact values at both temperatures (Mor37 vs Sal 37 on day 1 and Mor46 vs Sal37 on days 1 and 3) and tolerance occurred to this over time. **C)** Saline treated rats had similar facial contact times. The two morphine treated groups also had similar facial contact times. Morphine treated rats spent more time making facial contacts than saline controls at both temperatures. This behavior is most evident for morphine treated rats at 46°C on days 3, 5,

and 8. None of these contact times changed over time suggesting no tolerance to the morphine treated groups over the course of 10 days. **D)** Both saline treated groups spent the same amount of time licking and both increased their licking over time. Mor46 and Mor37 rats spent equal amounts of time licking and both increased their licking over time as well. Mor46 rats spent a much larger amount of time licking than Sal46 rats especially on days 3, 5, and 8. This effect changed over time likely due to the saline rats increasing their licking on day 10. Significance for each analysis was assessed with Two-Way ANOVA and Bonferroni's post-hoc test. N=8 for each time point for each group. These results can also be found in **Table 2**. The data presented is the mean $\pm$ SEM, an * indicates a p-value < 0.05 for Bonferroni's post-hoc test, ** indicates a p-value < 0.01 , and *** indicates a p-value < 0.001 .

Figure 8. Morphine and MK-801 alter pain and reward-seeking behavior on the operant orofacial nociception assay at aversive and non-aversive temperatures

A) On days 3 and 5 of injections all rats were tested at 37°C and a significant difference was only observed for the time ($p=0.0197$), likely due to the increases in the morphine and the MK-801/morphine groups. **B)** At 46°C a significant effect was observed for treatment group and time on the time per contact values. On day 10 the MK-801/morphine group had a significantly higher time per contact values than the MK-801 rats. **C)** For total facial contact times at 37°C a significant effect of treatment, time, and an interaction was observed. MK-801/morphine rats had the greatest change in behavior as they spent more time on the facial contact than any other group. On days 3 and 5 MK-801/morphine rats had higher contact times than saline, morphine, and MK-801 rats. **D)** At 46°C significant effects were observed for treatment, time, and an interaction. Morphine/MK-801 rats spent more time making facial contacts than any other group on days 8 and 10. Also, MK-801 rats had lower facial contact times on day 10 than morphine rats as well. **E)** For licking behavior at 37°C a significant effect was observed for treatment group, time, and an interaction between the two. On day 3 the MK-801/morphine group spent more time licking than both saline and MK-801 treated rats. On day 5 MK-801/morphine rats spent more time obtaining reward than saline and morphine rats. Morphine rats also spent more time licking than MK-801 treated rats on day 5. **F)** At 46°C there was a significant effect of group, time, and an

interaction between the two on licking behavior. On day 8 the MK-801 rats licked significantly less than the other three groups. On day 10 the MK-801/morphine rats licked more than saline, MK-801, and morphine rats. MK-801 rats also licked less than saline and morphine treated rats on day 10. Results from all Repeated Measures Two-Way ANOVAs and Bonferroni's post-hoc tests can also be found in **Table 3**. The data presented is the mean $\pm$ SEM, an * indicates a p-value < 0.05 for Bonferroni's post-hoc test, ** indicates a p-value < 0.01 , and *** indicates a p-value < 0.001 . Each group has an N=8. B37 stands for baseline measures at 37°C and B46 are baseline measures at 46°C.

Figure 9. Morphine and MK-801 co-administration alters NR1 splice variation in the nucleus accumbens

A) N1 was significantly decreased in the morphine and MK-801 group as compared to the saline group and the morphine group. B) N1 decreased in both the MK-801 and the MK-801/morphine group compared to saline treated rats in the AMY. C) No significant changes in N1 occurred in the HIPP. D) C1 levels decreased in the morphine and MK-801 group in the NACC. E) In the AMY, C1 significantly decreased in the MK-801 group compared to both the saline and morphine group. The MK-801/morphine group also had decreased C1 compared to the saline, morphine, and MK-801 groups. F) In the HIPP, C1 decreased in the MK-801 group when compared to all other groups. G) No change was

observed for C2-containing NR1 subunits in the NACC. H) No change was observed for C2-containing NR1 subunits in the AMY. I) No change was observed for C2-containing NR1 subunits in the HIP. J) C2' increased in the NACC when comparing the MK-801/morphine group to the saline group. K) No changes in C2' were observed in the AMY. L) No changes in C2' were observed in the HIP. M) NR1pan levels did not change with any treatment in the NACC. N) NR1pan levels did not change with any treatment in the AMY. O) NR1pan levels did not change with any treatment in the HIP. The data presented is the mean $\pm$ SEM, an *, **, *** indicates a p-value less than 0.05, 0.01, and 0.001 for Bonferroni's post-hoc test when compared to the saline/saline group. A # and ### indicates a p-value less than 0.05 and 0.001 for Bonferroni's post-hoc test when compared to the saline/morphine group. A @ and @@@ indicates a p-value less than 0.05 and 0.001 for Bonferroni's post-hoc test when compared to the MK-801/saline group. Significance was assessed with One Way ANOVAs. N=8 for each group.

Table 1**Escalating morphine doses**

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.

Day	Morphine Dose every 12 hours
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
11-13	0 mg/kg

Table 2

Two-Way ANOVA values for Figure 7

Analysis	Group	Time	Interaction	Day 1	3	5	8	10
B	S37 vs. S46	F(1,70)=4.591 p=0.0248	F(4,70)=1.149 p=ns	F(4,70)=1.918 p=ns	ns	ns	ns	ns
	M37 vs. M46	F(1,70)=2.553 p=ns	F(4,70)=6.000 p=0.0003	F(4,70)=1.032 p=ns	ns	ns	ns	ns
	S37 vs. M37	F(1,70)=7.535 p=0.0077	F(4,70)=0.8588 p=ns	F(4,70)=1.036 p=ns	*	ns	ns	ns
	S46 vs. M46	F(1,70)=45.01 p<0.0001	F(4,70)=8.366 p<0.0001	F(4,70)=7.841 p<0.0001	***	ns	ns	ns
C	S37 vs. S46	F(1,70)=0.4016 p=ns	F(4,70)=1.477 p=ns	F(4,70)=1.147 p=ns	ns	ns	ns	ns
	M37 vs. M46	F(1,70)=0.7322 p=ns	F(4,70)=1.519 p=ns	F(4,70)=0.2285 p=ns	ns	ns	ns	ns
	S37 vs. M37	F(1,70)=18.48 p<0.0001	F(4,70)=1.413 p=ns	F(4,70)=0.4721 p=ns	ns	ns	ns	ns
	S46 vs. M46	F(1,70)=60.63 p<0.0001	F(4,70)=0.5867 p=ns	F(4,70)=1.325 p=ns	***	*	**	**
D	S37 vs. S46	F(1,70)=0.6081 p=ns	F(4,70)=3.778 p=0.0077	F(4,70)=0.4857 p=ns	ns	ns	ns	ns
	M37 vs. M46	F(1,70)=3.878 p=ns	F(4,70)=2.519 p=0.0488	F(4,70)=0.3475 p=ns	ns	ns	ns	ns
	S37 vs. M37	F(1,70)=2.097 p=ns	F(4,70)=2.411 p=ns	F(4,70)=0.3293 p=ns	ns	ns	ns	ns
	S46 vs. M46	F(1,70)=27.80 p<0.0001	F(4,70)=4.155 p=0.0045	F(4,70)=1.335 p=ns	*	*	**	ns

Bonferroni post-hoc tests results: an

* indicates a p-value < 0.05

** indicates a p-value < 0.01

*** indicates a p-value < 0.001.

Table 3

Repeated Measures Two-Way ANOVA values for Figure 8

Analysis	Group	Time	Interaction	SvM	SvMK	SvMM	MvMK	MvMM	MKvMM
A Time Per Contact	ns	P=0.0197	ns	3-ns	3-ns	3-ns	3-ns	3-ns	3-ns
37°C	F(3,56)=1.55	F(2,56)=4.21	F(6,56)=1.60	5-ns	5-ns	5-ns	5-ns	5-ns	5-ns
B Time Per Contact	p=0.0411	p=0.0036	ns	8-ns	8-ns	8-ns	8-ns	8-ns	8-ns
46°C	F(3,56)=3.14	F(2,56)=6.23	F(6,56)=1.98	10-ns	10-ns	10-ns	10-ns	10-ns	10-ns
C Facial Contact Time	p<0.0001	p<0.0001	p=0.0002	3-ns	3-ns	3-ns	3-ns	3-ns	3-ns
37°C	F(3,56)=13.63	F(2,56)=17.32	F(6,56)=5.37	5-ns	5-ns	5-ns	5-ns	5-ns	5-ns
D Facial Contact Time	p<0.0001	p<0.0001	p<0.0001	8-ns	8-ns	8-ns	8-ns	8-ns	8-ns
46°C	F(3,56)=18.50	F(2,56)=27.71	F(6,56)=7.97	10-ns	10-ns	10-ns	10-ns	10-ns	10-ns
E Time Spent Licking	p=0.0005	p=0.0012	p=0.0348	3-ns	3-ns	3-ns	3-ns	3-ns	3-ns
37°C	F(3,56)=8.16	F(2,56)=7.61	F(6,56)=2.46	5-ns	5-ns	5-ns	5-ns	5-ns	5-ns
F Time Spent Licking	p<0.0001	p<0.0001	p=0.0023	8-ns	8-ns	8-ns	8-ns	8-ns	8-ns
46°C	F(3,56)=11.93	F(2,56)=19.32	F(6,56)=3.95	10-ns	10-ns	10-ns	10-ns	10-ns	10-ns

Bonferroni post-hoc tests results: an

* indicates a p-value < 0.05

** indicates a p-value < 0.01

*** indicates a p-value < 0.001.