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Influence of SALVINORIN A and SEX DIFFERENCES on DEPRESSIVE- and ANXIETY-LIKE behaviors in a CHRONIC MILD STRESS paradigm.

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ABSTRACT

Kappa opioid receptors are colocalized with dopamine receptors and are frequently associated with depression, anxiety, stress, and drug use and abuse. KOR antagonism has widely been considered anti-depressive and anxiolytic in animal models while agonism is pro-depressive and anxiogenic. However, recent findings suggest that SalvA, a natural kappa agonist derived from a plant in the mint family, can reduce depressive- and anxiety-like behavior in rats. The current study investigated the effects of chronic mild stress on behavior, attenuation by SalvA, and sex differences. 52 Long-Evans rats, 26 males and 26 females, were exposed to six weeks of CMS. Animals received daily injections of either SalvA or vehicle for the last three weeks of the experiment. Behavioral tests were administered at baseline, three weeks, and six weeks. We predicted that CMS would induce the depressive-like behaviors anhedonia and learned helplessness, as measured by reduced sucrose preference and increased immobility in the forced swim test, respectively. We also predicted that CMS would induce anxiety-like behavior by reducing exploratory behavior and increasing immobility in the open field test. It was predicted that SalvA exposure would attenuate the effects of CMS. Further, we predicted that female rats would be more sensitive to the effects of stress but also show greater response to SalvA. Results showed that CMS did not reliably induce depressive- or anxiety-like behaviors. Because there were no reliable behavioral changes due to stress, the potential antidepressant or anxiolytic effects of SalvA were not apparent. In the male drug group, SalvA appeared to increase anxious behavior in the open field test and depressive-like behavior in the forced swim test, aligning with previous findings that KOR agonists can be anxiogenic and pro-depressive. Additionally, results showed that behavior in the forced swim and open field tests were sex dependent, with males showing more depression- and anxiety-like behaviors than females. More research is necessary to better understand the effects of CMS and SalvA on behavior. Closer examination of the behavioral paradigms measuring depression and anxiety in animals is needed and future research should carefully consider sex differences when employing these behavioral paradigms.

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Like Behaviors in a Chronic Mild Stress Paradigm

The recent opioid crisis is escalating and with that comes the need for closer examination of the comorbid relationships between psychiatric disorders, drug use, and drug abuse. Of recent interest in the field is the role of the kappa opioid system and sex differences in the quest to better understand major depressive disorder, anxiety disorders, and addiction (Wee & Koob, 2010, Freeman, Naylor, Prisinzano, & Woolverton, 2014, Chartoff & Mavrikaki, 2015).

The endogenous opioid system consists of three receptor subtypes: Mu (μ), Delta (δ), and Kappa (κ). Opioid receptors are often colocalized with monoamine receptors, suggesting that opioids play an important role in mood, pleasure, reward, and subsequent influence on mood related disorders. Previously, research has focused on the mu opioid system and the drugs that activate it, as they have increased abuse potential. In opposition to the euphoria common with activation of the mu and delta opioid systems, the kappa opioid system is often referred to as the "anti-reward system". Kappa opioid receptor (KOR) activation has, historically, been linked to feelings of dysphoria, the induction of depressive-like and anxiety-like behaviors, and is commonly associated with stress, pain, drug abuse and other psychopathologies. Stress and pain are well established risk factors for drug abuse and relapse (Bockstaele, Reyes, & Valentino, 2010). Exposure to stress and physical pain can induce the release of the endogenous kappa opioid receptor ligand, dynorphin, in the nucleus accumbens (NAcc) and other brain areas associated with the stress response (Bruchas, Land, & Chavkin, 2010).

The NAcc plays an important role in stress response, reward, motivation and depressive like behaviors (Pena, 2017). The neurons of the NAcc, primarily dopaminergic (D1 and D2) medium spiny neurons, exhibit coexpression of D1 and dynorphin and agonism of these KORs decreases dopamine levels (Donzanti, Althaus, Payson, & Von Voigtlander, 1992, Ebner, Roitman, Potter, Rachlin, & Chartoff, 2010). KOR activation via stimulation of dynorphinergic neurons in the NAcc core can induce aversive behaviors (Al-Hasani, et al., 2015). KOR agonists also inhibit dopaminergic neurons in the ventral tegmental area, which projects to the NAcc (Margolis, Hjelmstad, Bonci, & Fields, 2003). Chronic KOR activation via a synthetic kappa agonist can induce a significant increase in depressive-like symptoms, including anhedonia, in mice (Dogra, Kumar, Umrao, Sahasrabuddhe, & Yadav, 2016). Salvinorin-A, a natural KOR agonist, has been reported to upregulate DA reuptake and decrease DA release, resulting in pro-depressive effects (Kivell, et al., 2015). Further, KOR activation has been shown to modulate cocaine seeking, underscoring the role of KORs in the pro-addictive components of stress exposure. This system has also been implicated in both physiological and psychological responses to stress (Bruchas, Land, & Chavkin, 2010). In summary, the literature suggests that kappa opioid and dopamine receptors are colocalized in neurons of the NAcc and have modulating effects on each other. Chronic stress can result in the release of dynorphin and subsequent inhibition of dopamine neurons in this area, decreasing dopamine levels and providing a scaffold for the development of mood disorders and drug seeking behaviors.

On the other hand, there is evidence suggesting that activation of KORs can be antidepressant and anxiolytic, providing potential new therapeutic routes in the treatment

of depression and anxiety. One possible explanation for discrepancies in the literature may be related to dosage. For example, lower doses of a KOR agonist produced anxiolytic effects in rats on the elevated plus maze test, where those treated with the agonist had increased open arm entries compared to controls (Privette & Terrian, 1995). A KOR agonist also attenuated learned helplessness, a common symptom of depression, in a foot shock paradigm (Ukai, Suzuki, & Mamiya, 2002). Activation of dynorphinergic neurons in specific regions of the NAcc shell can result in rewarding effects and place preference (Al-Hasani, et al., 2015). A majority of the research on the KOR system utilizes exogenous dynorphin administration, endogenous dynorphin release by streas or pain, or other synthetic KOR agonists and antagonists.

Contemporary trends in the field have turned to the natural, exogenous KOR agonist, Salvinorin-A. While synthetic KOR agonists usually result in pro-depressive effects, SalvA exhibits complex influences on brain chemistry and behavior. Administration of Salvinorin-A can induce both anti- and pro-depressive effects. (Harden, Smith, Niehoff, McCurdy, & Taylor, 2012, Braida, et al., 2009).

Salvia Divinorum is a plant in the mint family. The psychoactive ingredient, Salvinorin-A (SalvA), is reported to be initially dysphoric and hallucinogenic, with increased mood (euphoria) post hallucination in humans (Baggott, Erowid, Erowid, Galloway & Mendelson, 2010). SalvA is thought to be unique because it is the only known naturally occurring KOR agonist, while other kappa agents used in research are synthetic, lab created agonists or antagonists. SalvA has high affinity for and selectively binds to KORs, with little affinity for catecholamine or serotonin receptors, which are

common targets of anti-depressants. Moreover, SalvA is a lipid, whereas most KOR binding ligands are amine agents.

Previous research found that SalvA can produce anxiolytic and anti-depressive effects in animal models using the elevated plus maze, forced swim test, and sucrose preference (Braida et al., 2009, Harden, Smith, Niehoff, McCurdy, & Taylor, 2012). SalvA also has the ability to increase NAcc dopamine release and produce rewarding effects in an intracerebroventricular self-administration and conditioned place preference paradigm, however these effects were dose dependent (Braida, et al., 2007). Together, data suggests that SalvA has unique properties as a KOR agonist, potentially opening new doors for the development of effective treatments for anxiety, depression, and drug abuse. However, it is important to explore the complex actions of SalvA in order to better understand how it can influence behavior and mental health.

It has been well-established that women are more often diagnosed with major depression than men. Previous findings in animal models of chronic mild stress (CMS) revealed that dopamine and 5-HT activity were decreased in female, but not male, rats (Dalla, et al., 2005, Dalla, et al., 2008). These results suggested that females may show increased sensitivity to CMS and therefore, the onset of depressive-like symptoms. In contrast, one study reported that female rats were less sensitive to the depressive-like effects of a KOR agonist (Russell, et al., 2015). These depressive effects were not due to sex differences in pharmokinetics, as concentration of the KOR agonist in the brain did not differ. Moreover, intracranial self-stimulation, another common measure of responsiveness to reward, in female rats did not differ depending on stage of the estrous cycle, suggesting circulating gonadal hormones were not responsible for the sex

differences in sensitivity to effects of the KOR agonist. Recent findings revealed increased expression of the DA precursor, tyrosine hydroxylase, in the female ventral tegmental area. This finding in conjunction with evidence of enhanced dopamine synthesis may suggest a resiliency to dopamine suppression by KOR activation in females (Conway, et al., 2019). Research from Walker et al., 2000, suggested that increased dopamine in the female NAcc may also be a protective factor against KOR activation induced dopamine inhibition.

Although depression and anxiety are more often reported in women than in men, the majority of animal studies are conducted on male rodents. The main reason for this discrepancy is the estrous cycle and hormonal fluctuations at its different stages are often considered a potential confound. A 2014 meta-analysis of 293 animal studies indicated that female mice were no more variable than males, regardless of estrous cycle stage (Prendergast, Onishi, & Zucker, 2014). I argue that conducting research on female rodents as they naturally cycle increases the translational reliability to humans.

The aim of the current study was to close the gap in the literature concerning the role of kappa opioids in mood disorders and addiction. Specifically, the current study investigated the unique KOR agonist SalvA and its potential as an anti-depressant and anxiolytic agent. It also examined the influence of sex differences on the development of depressive-like behaviors as well as recovery from these behaviors after SalvA exposure.

Hypothesis 1: I predicted that chronic mild stress would induce depressive-like and anxiety-like behaviors in rats.

1a. Chronic mild stress would increase immobility in the forced swim test compared to baseline.

1b. CMS would increase immobility and time spent in the periphery in the open field test compared to baseline.

1c. CMS would reduce sucrose preference compared to baseline preference.

Hypothesis 2: I predicted the unique features of SalvA would make it effective in reversing the effects of CMS.

2a. SalvA would attenuate immobility in the forced swim test.

2b. SalvA would reduce immobility and time spent in the periphery in the open field test.

2c. SalvA would restore sucrose preference.

Hypothesis 3: I predicted that female rats would show an exaggerated response to CMS but also increased recovery via SalvA compared to males.

Methods:

Experimental design

A total of 52 Long-Evans rats, 26 males and 26 females, were individually housed as per standard laboratory procedure. Rats were assigned to one of four groups: male control, male drug, female control, or female drug. Main factors for the 2 x 2 x 3 factorial analysis were sex (male or female), drug condition (drug or vehicle) and timepoint of behavioral tests (baseline, week three, and week six). Behavioral data was collected at baseline, three weeks, and six weeks. Stressors were applied randomly for the full six-week experiment while drug or vehicle were administered only during the final three-week period. There was a total of seven replications of the study.

Procedures

Chronic Unconditioned Mild Stress: Chronic mild stress is a common method used to induce depressive- and anxiety-like behaviors in animals (Taylor, Cabrera, & Hoffman, 2014). All animals received the same stressors for six weeks. Common stressors in the chronic mild stress paradigm were used. Stressors included food deprivation, water deprivation, home cage tilted at a 45° angle, bedding wet with 200ml of water, and placement in the soiled cage of another rat. Alteration of normal day/night light cycles, overnight illumination, and continuous illumination were also used as stressors. All stressors were maintained for between 12 and 24 hours.

Behavioral Measures

Forced Swim Test: The forced swim test is a behavioral measure that assesses learned helplessness, a common symptom of MDD. Learned helplessness is displayed by animals as immobility in the forced swim test (Bogdanova, Kanekar, D'Anci, & Renshaw, 2013). To assess learned helplessness, animals were placed in a 60.96cm x 25.4cm x 25.4cm clear glass cylinder with 45.72cm of room temperature (~25°c) water. All animals were exposed to the testing environment for six minutes as an acclimation period the day before baseline. Animal movements were recorded, and videos were analyzed to determine the percent of time spent immobile during the test.

Open Field Testing: Emotionality is considered organic, expressive reactions to a situation. Open field activity and emotionality are related, as emotional rats are less active and exploratory than non-emotional animals (Doyle & Yule, 1959). The open field test is a behavioral measure of both general locomotor activity and anxiety-like behaviors (Bowman, Ferguson, & Luine, 2002). Animals that exhibit increased anxiety-like

symptoms are expected to spend more time around the perimeter of the apparatus compared to the center. Additionally, immobility is a commonly used passive response in animals to threatening stimuli (Archer, 1976). Changes in immobility in studies of anxiolytics confirm that immobility is a valid model of fear and anxiety (Hard, Engel, Larsson, & Musi, 1986). Animals were placed in the center of a 67cm x 67cm x 42.5cm clear acrylic apparatus and allowed to explore freely during a 10-minute trial. Data was recorded using Anymaze© software to determine the ratio of time spent in the perimeter versus the center of the apparatus and time spent immobile during the 10-minute trial. All animals were exposed to the open field apparatus for 30 minutes to get acclimated to the testing environment prior to baseline.

Anhedonia: Anhedonia, a common symptom of major depressive disorder, is characterized by motivational dysregulation and decreased feelings of reward (Taylor, Cabrera, & Hoffman, 2014). Anhedonia was measured using sucrose preference, whereas reduced preference for sucrose water is associated with increased anhedonic behavior. Bottles of 1% sucrose were prepared with 10g of granulated white cane sugar per 1 liter of water. Sucrose water was presented alongside tap water in identical bottles, zip tied together, and placed on the cage lid 5cm apart with nozzles at equal level to assure equal ease of access. Animals were allotted access to both bottles for 24 hours. All animals were sucrose naive and were exposed to the sucrose solution for a 24-hour acclimation period before baseline testing.

Materials

Salvia in a powdered form (Sigma Aldrich, St. Louis, Missouri, USA) was solubilized 75% dimethyl sulfoxide and 25% saline solution (0.9%) for injection. The
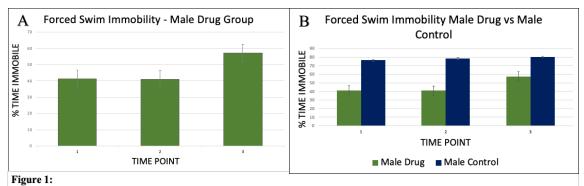
solution was injected subcutaneously daily for three weeks at 0.3mg per kg/ body weight.

Control Animals received saline injections.

Statistical Analyses

Forced Swim Test

A 3-way ANOVA was conducted to determine the effects of sex, drug group, and time point on immobility in the forced swim test, as measured by percent of time spent immobile during the last 240 seconds of the swim period. There was a simple main effect of time point, $F(1, 48) = 4.921$, $p < 0.01$. Post-hoc analyses revealed a significant difference between baseline ($M = 41.32$ $SD = 26.71$) and week 6 ($M = 57.29$ $SD = 22.60$) in the male drug group, $p < 0.05$ and between week 3 ($M = 41.04$ $SD = 26.71$) and week 6 ($M = 57.29$ $SD = 22.60$) in the male drug group, $p < .05$ (see Figure 1A).

There was a simple main effect of drug group, $F(1, 48) = 8.798$, $p < 0.01$. Post-hoc analyses revealed a significant difference between the male control group ($M = 76.19$ $SD = 17.46$) and the male drug group ($M = 41.32$ $SD = 26.71$) at baseline, $p < 0.001$, between the male control group ($M = 77.98$ $SD = 24.33$) and the male drug group ($M = 41.04$ $SD = 26.71$) at week 3, $p < 0.001$, and between the male control group ($M = 80.06$ $SD = 24.29$) and the male drug group ($M = 57.29$ $SD = 22.60$) at week 6, $p < 0.05$ (see figure 1B).

Figure 1:
A: Male rats that received SalvA displayed more learned helplessness at week 6 than at basline or week 3.
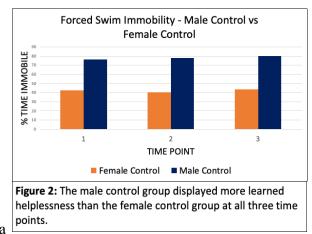**B:** Male rats in the control group displayed more learned helplessness than those in the drug group at baseline, week 3, and week 6.

There was a simple main effect of sex, $F(1, 48) = 15.060$, $p < 0.001$. Males ($M = 63.50$ $SD = 1.90$) spent significantly more time immobile than females ($M = 42.70$ $SD = 1.40$). There was no significant 3-way interaction of sex by drug group by time point, $F(2, 96) = 0.409$, $p = 0.665$. There was no 2- way interaction of sex by time point, $F(2, 96) = 0.867$, $p = 0.423$ or drug group by time point, $F(2, 96) = 2.041$, $p = 0.135$.

There was a significant 2-way interaction of sex by drug group, $F(1, 48) = 10.724$, $p < 0.01$. Post-hoc analyses revealed a significant difference between the male control group ($M = 76.19$ $SD = 17.46$) and female control group ($M = 42.26$ $SD = 15.79$) at baseline, $p < 0.001$, a

Figure 2: The male control group displayed more learned helplessness than the female control group at all three time points.

significant difference between the male control group ($M = 77.98$ $SD = 24.33$) and female control group ($M = 40.18$ $SD = 11.65$) at week 3, $p < 0.001$, and a significant difference between the male control group ($M = 80.06$ $SD = 24.29$) and female control group ($M = 43.45$ $SD = 20.95$) at week 6, $p < .001$ (see figure 2).

Open Field Test

A 3-way ANOVA was conducted to determine the effects of sex, drug group, and time point on the open field test, measured as time spent in the periphery compared to the center of the apparatus. There was a significant main effect of sex, $F(1, 48) = 5.689$, $p < 0.05$. There was a significant difference between the male control group ($M = 592.14$ $SD = 13.14$) and the female control group ($M = 579.06$ $SD = 20.97$) at baseline, $p < 0.01$ (see figure 3A).

There was a significant main effect of time point, $F(2, 96) = 9.649$, $p < 0.001$. There was a significant difference between baseline ($M = 579.06$ $SD = 20.97$) and week 3 ($M = 589.21$ $SD = 11.49$) in the female control group, $p < 0.01$, a significant difference between baseline ($M = 579.06$ $SD = 20.97$) and week 6 ($M = 588.95$ $SD = 8.51$) in the female control group, $p < 0.01$, and a significant difference between baseline ($M = 586.23$ $SD = 15.35$) and week 6 ($M = 595.72$ $SD = 72$) in the male drug group, $p < 0.05$ (see figure 3A,B).

There was no significant 3-way interaction of sex by drug group by time point $F(2, 96) = 1.464$, $p = 0.236$. There was no 2- way interaction of sex by time point, $F(2, 96) = 0.241$, $p = 0.786$. There was no 2- way interaction of drug group and time point $F(2, 96) = 0.119$, $p = 0.888$. There was no 2-way interaction of sex and drug group $F(1, 48) = 3.613$, $p = 0.063$.

A 3-way ANOVA was conducted to determine the effects of sex, drug group, and time point on immobility in the open field test, measured as time spent immobile compared to total time in the open field apparatus. There was a significant main effect of sex $F(1, 48) = 9.891$, $p < 0.01$. Post-hoc analyses revealed a significant difference
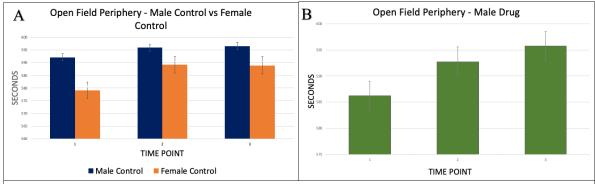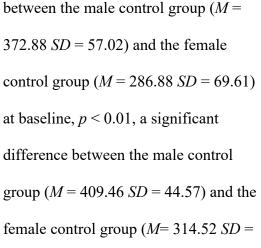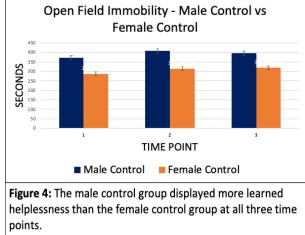

Figure 3:
A: The male control group showed more anxiety-like behavior then the female control group at baseline. The female control group showed more anxiety-like behavior at week 3 and week 6 than at baseline.
B: The male drug group showed more anxiety-like behavior at week 6 than at baseline
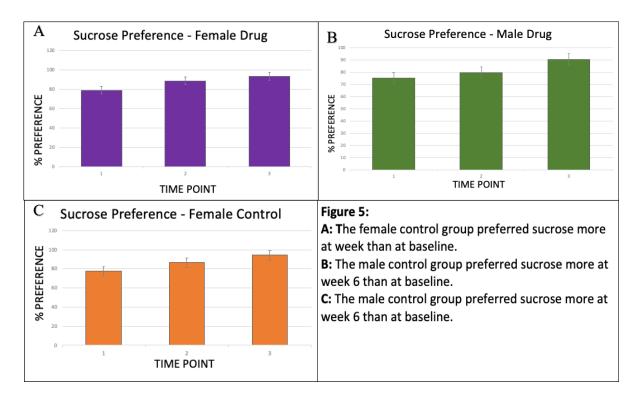
between the male control group ($M =$ 372.88 $SD = 57.02$) and the female control group ($M = 286.88$ $SD = 69.61$) at baseline, $p < 0.01$, a significant difference between the male control group ($M = 409.46$ $SD = 44.57$) and the

Figure 4: The male control group displayed more learned helplessness than the female control group at all three time points.

female control group ($M= 314.52$ $SD =$ 137.27) at week 3, $p < 0.01$, and a significant difference between the male control group ($M = 396.00$ $SD = 62.05$) and the female control group ($M = 318.58$ $SD = 101.56$) at week 6, $p < 0.05$ (see figure 4).

There was a significant main effect of time point, $F(2, 96) = 5.039$, $p < 0.01$. Post-hoc analyses revealed a significant difference between baseline ($M= 322.72$ $SD = 83.63$) and week 3 ($M = 361.01$ $SD = 95.15$) and between baseline ($M = 322.72$ $SD = 83.63$) and week 6 ($M = 354.15$ $SD = 81.03$), both $p < 0.01$. There was no significant main effect of drug group, $F(1, 48) = 0.226$, $p = 0.636$. There was a no significant 3-way interaction of sex by drug group by time point $F(2, 96) = 1.573$, $p = 0.212$. There was no 2- way interaction of sex by time point $F(2, 96) = 0.637$, $p = 0.532$, drug group by time point $F(2, 96) = 0.136$, $p = 0.873$, or sex by drug group $F(1, 96) = 3.536$, $p = 0.066$.

Sucrose Preference

A 3-way ANOVA was conducted to determine the effects of sex, drug group, and time point on sucrose preference, measured as amount of sucrose solution consumed compared to tap water. There was no main effect of sex, $F(1, 48) = 0.815$, $p = 0.371$ or drug group, $F(1, 48) = 1.492$, $p = 0.228$.

There was a significant main effect of time point, $F(2, 96) = 13.792$, $p < 0.001$.

Post-hoc analyses revealed a significant difference between baseline (M = 77.57 SD = 14.73) and week 6 (M = 95.38 SD = 6.48) in the female control group, $p < 0.01$, between baseline (M = 74.97 SD = 20.10) and week 6 (M = 90.36 SD = 9.46) in the male drug group, $p < 0.05$, and between baseline (M = 78.96 SD = 8.13) and week 6 (M = 93.24 SD = 9.20) in the female drug group, $p < 0.05$. There was no significant 3-way interaction of sex by drug group by time point, $F(2, 96) = 1.218$, $p = 0.300$. There was no 2-way interaction of sex and drug group, $F(1, 48) = 1.989$, $p = 0.165$, sex by time point, $F(2, 96) = 0.415$, $p = 0.661$, or drug group by time point, $F(2, 96) = 0.267$, $p = 0.766$.

Figure 5:
A: The female control group preferred sucrose more at week than at baseline.
B: The male control group preferred sucrose more at week 6 than at baseline.
C: The male control group preferred sucrose more at week 6 than at baseline.

Discussion

This study investigated the development of depressive- and anxiety-like behaviors after exposure to chronic mild stress and recovery with SalvA, as well as sex differences impacting these behaviors.

The first hypothesis was that chronic mild stress (CMS) would induce depressive and anxiety-like behaviors in rats. I predicted that CMS would increase immobility in the forced swim test compared to baseline. This was not the case, as there was no significant difference between baseline and week 3 for any group, implying that learned helplessness was not displayed after three weeks of CMS. There were also no significant differences between week 3 and week 6 for either control group, indicating no effect of CMS on immobility. In the male drug group, there was a difference between baseline and week 6 and week 3 and week 6, however, it is impossible to determine if the differences seen were due to the effects of stress or SalvA exposure.

There were, however, sex differences in the forced swim test. Results showed that the male control group spent more time immobile than the female control group at baseline, week 3, and week 6. This may suggest that males show higher trait immobility at baseline and may be more sensitive to the effects of CMS. The sex differences in the forced swim test seen in the present study are not in agreement with the third proposed hypothesis that females would be more sensitive to the effects of CMS. This finding is, however, consistent with previous research showing that females are more active in the forced swim test than males (Alonso, Castellano, Afonso, & Rodriguez, 1991). Other studies have concluded that, in response to stressful stimuli, females use more active responses while males use more passive behavior, like immobility, which may explain the sex differences seen here (Padilla, Barrett, Shumake, & Gonzalez-Lima, 2009).

I predicted that SalvA would attenuate immobility in the forced swim test. In contrast to my hypothesis, there were no significant differences within the female drug group. The male drug group spent significantly more time immobile at week 6 than week

3 and at baseline. Because there was no change in immobility between baseline and week 3, the change between week 3 and week 6 cannot reliably be attributed to SalvA. In short, there was no effect of CMS to be reversed by SalvA. In fact, the increase in immobility between weeks 3 and 6 and might suggest anxiogenic instead of anxiolytic properties of SalvA in males. Another possible explanation is that the drug had no effect and the increase in immobility was due to the three additional weeks of CMS.

There were between group differences in the forced swim test. The male control group spent more time immobile than the male drug group at baseline, week 3 and at week 6. The former is an unexpected difference, specifically because the two male groups showed behavioral differences before the drug was administered. This difference in baseline anxiety between male groups is interesting and deserves further exploration.

The validity of the forced swim test as a measure of depressive behavior has been questioned in the literature. Forced swimming is sometimes used as a stressor as opposed to as a test of the effects of stress. For example, a 2016 review concluded that forced swimming is a stressor and immobility is a passive and adaptive evolutionary response to cope with that acute stress. The authors suggested that the forced swim test should not be used to identify depression rat models (Kloet & Molendijk 2016).

I hypothesized that CMS would increase periphery time and immobility time in the open field test compared to baseline. The female control group spent more time in the periphery at week 3 and at week 6 than at baseline. Although this agrees with my hypothesis that CMS would increase anxiety-like behaviors over time, there was no significant increase from week 3 to week 6. Habituation to chronic stress is dependent on multiple factors, including stress severity (Herman, 2013). Because we used a mild stress

paradigm, it is plausible that the female control group reached a point of habituation after week 3. Although there was a trend towards increased periphery time, there were no significant differences between baseline and week 3 or week 3 and week 6. As such, it is unclear whether the increase in anxiety-like behavior between baseline and week 6 was due to the effects of CMS or SalvA. Additionally, the female drug group spent more time in the periphery after CMS exposure but it did not reach significance.

The male control group spent more time in the periphery of the open field than the female control group at baseline, which may suggest a higher baseline level of anxiety in males. This finding is contrary to my hypothesis, based on previous findings, that females would be more sensitive to the effects of CMS. However, it is concurrent with research reporting male rats spent more time immobile and were more anxious in the open field test than females (Kokras, et al., 2011). Another study found that male rats exposed to CMS showed reduced exploratory activity while females displayed increased exploratory behaviors (Horst, Wichmann, Gerrits, Westenbroek, & Lin, 2009).

The hypothesis that SalvA would reduce anxiety in the open field test was rejected. There were no significant reductions in time in the periphery or immobility after drug exposure. The male drug group spent more time in the periphery at week 6 than at baseline. This might suggest that either SalvA produced and anxiogenic effect in males or the effects of CMS weren't apparent until closer to six weeks of stress exposure. The female drug group showed less time in the periphery after SalvA exposure, but it did not reach significance.

There were sex differences in the open field test. The male control group spent more time immobile than the female control group at baseline, week 3, and week 6. This

might suggest that males have a higher baseline level of anxiety. There is also the potential that males were more susceptible to the effects of stress than females, which would oppose my hypothesis that females would be more vulnerable to CMS. However, previous research reported that non-stressed males are less active than females in the open field test (Broadhurst, 1957, Denenberg & Morton, 1964). The finding that male controls spent more time in the periphery than female controls at week 3 and week 6 could also be a result of the habituation to stress by females discussed earlier.

I predicted that CMS would result in anhedonic behavior as measured by reduced sucrose preference. This hypothesis was rejected. The female control group preferred sucrose more at week 6 than at baseline. There was a trend towards a greater preference for sucrose at week 3 than at baseline, but the difference failed to achieve statistical significance. Overall, the female control group showed an increase in sucrose preference from baseline after 3 and 6 weeks of stress, opposite to what was expected. The male drug group also preferred sucrose more at week 6 than at baseline, again, in the opposite direction of our hypothesis.

Because results showed an overall trend towards increase in preference instead of reduced preference, there was no apparent effect of CMS to be reversed by SalvA. Thus, the hypothesis that SalvA would recover sucrose preference was rejected. According to a study by Forbes, Stewart, Matthews, and Reid (1996), food deprivation was necessary to decrease sucrose consumption in a CMS paradigm. Decreased consumption was also associated with reduced body weight. Findings indicated that reduced sucrose preference was independent of the stress paradigm and it was concluded that sucrose preference may not be a valid measure of reward responsiveness. Additionally, results of a study by

Wang, et al., 2009, suggested that in the chronic mild stress model of depression, it takes three to five weeks for anhedonic behavior to normalize. Thus, expected results may have been attained if the paradigm was longer than six weeks and animals were exposed to SalvA for longer than three weeks.

In the present study, CMS failed to produce depressive-like behavior in the forced swim test and sucrose preference test. CMS produced anxiety-like behavior in the open field test but only in the female control group and it was not maintained after three weeks. Because there was no reliable effect of stress, there was no change in behavior for SalvA to reverse. In the female drug group, there was a trend towards an increase in anxiety in the open field after CMS and then a return to baseline after SalvA exposure, but unfortunately it did not reach significance. So, it appears that SalvA failed to reliably reverse immobility in the forced swim test, reduce anxious behaviors in the open field test, and restore sucrose preference when in fact the fault may fall on the CMS paradigm.

CMS has a history of poor reproducibility and inter-laboratory reliability (Deussing, 2006, Nestler, Gould, & Manji, 2002). Although we followed a schedule of stressors similar to those in other, successful studies, it is possible that our stressors were not intense enough to produce the desired results. Future direction should focus on perfecting the CMS paradigm in order to reliably produce a depressed state in animals.

Because CMS failed to produce the expected behaviors, it is difficult to determine if any true drug effects were seen. Based on previous findings that SalvA can be anxiolytic at low doses, we chose to use a low dose. The chosen dose may have been too low to produce an effect. Further research should be carried out on the dose dependency of SalvA. In addition, anhedonic behavior takes more than three weeks to normalize after

introduction of an antidepressant agent. Therefore, extending the paradigm past six weeks would give increased exposure to the drug and may increase the chance of more significant drug effects.

The effects of SalvA on behavior in animal models has been hotly debated. In this case, and specifically for the male drug group, the data seemed to coincide with other labs that found SalvA to be pro-depressive and anxiogenic. Future research should continue to examine the complex nature of SalvA and the kappa opioid system as a whole in relation to stress, depression, anxiety. It is also necessary to better understand the intricate relationship between sex differences and behavior in the chronic mild stress paradigm. The critique of the validity of the forced swim and sucrose preference test combined with issues surrounding reproducibility of the chronic mild stress paradigm highlight the need to further explore which paradigms are most successful at inducing and measuring depressive- and anxiety-like behavior in animal models.

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