ROLE OF THE DYNORPHIN/KOR SYSTEM IN MEDIATING THE STRESS RESPONSE IN MICE

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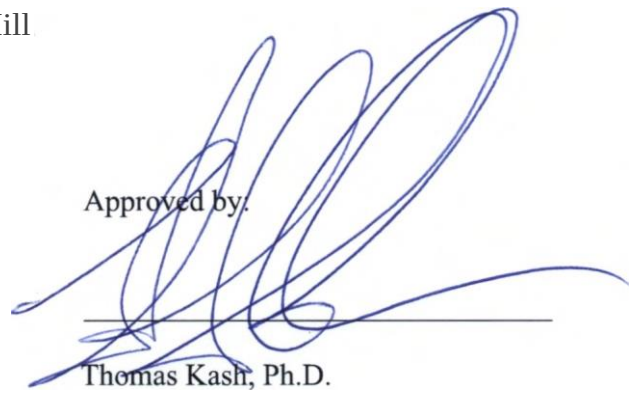

ABSTRACT

Alcohol and drug abuse represent two of today's greatest unsolved public health problems. The dynorphin/kappa opioid receptor (KOR) system has been shown to mediate the increased rewarding effects of drugs of abuse and the increased anxiety and stress seen during alcohol withdrawal. In the present study, we examined the role of the dynorphin/KOR system in the Bed Nucleus of the Stria Terminalis (BNST) in stress related behaviors using an animal model. We stereotaxically injected mice with an inducible caspase construct to selectively destroy dynorphin cells in the BNST and tested mice using the elevated plus maze (EPM), open field, and forced swim stress (FSS) behavioral paradigms. Our results show that under basal, non-stress conditions, ablating dynorphin from the BNST does not produce significant changes in behavior. However, dynorphin and KORs may play an important role in mediating behavior following a stressful event (FSS). Using Designer Receptors Exclusively Activated by Designer Drugs (DREADDs), we show that functionally inhibiting dynorphin neurons in the BNST may have an anxiogenic effect under stressful conditions faced in the FSS-EPM behavioral paradigm. These results, along with an ongoing set of behavioral experiments with the DREADD mice, may provide novel insight for the role of the dynorphin/KOR system in mediating the stress response relevant to alcohol and drug use disorders.

INTRODUCTION

Alcohol, drug use, and anxiety disorders represent significant unsolved public health problems. Looking at alcohol specifically, 3.8% of all global deaths in 2004 were attributed to alcohol (Rehm, *et al.* 2009). Societal costs in the United States associated with these alcohol use

disorders were an estimated $148 billion in 1992 and rose to $223.5 billion by 2006 (Harwood, Fountain, & Livermore, 1998; Bouchery *et al*. 2011). A high co-morbidity has been shown between alcoholism and anxiety disorders, suggesting the possible presence of common mechanisms (Swendsen, 1998; Grant & Harford, 1995).

Several brain regions have been implicated in these disorders; among them is the Bed Nucleus of the Stria Terminalis (BNST). The BNST is a critical output region of the extended amygdala that receives cortical and limbic projections and itself projects to multiple brain regions involved in drug-seeking behaviors, alcohol withdrawal, and anxiety behaviors (Silberman and Winder, 2013). Several members of the endogenous opioid system – a system known to mediate emotional and behavioral responses to stress – are expressed in the BNST and may be implicated in these behaviors. Among those broadly expressed is the kappa opioid receptor and its ligand dynorphin (Poulin, 2009).

Several studies have demonstrated a significant role of the dynorphin/KOR system in stress responses, addiction-related behaviors, and anxiety disorders (Bruchas, Land & Chavkin 2010; Knoll & Carlezon 2010). Stress causes a cascade leading to the release of dynorphin, which binds the kappa opioid receptor (Nabeshima, *et al*. 1992). Activation of the dynorphin/KOR system has been shown to mediate the increased rewarding effects of cocaine and the increased anxiety and stress seen during alcohol withdrawal (McLaughlin, *et al*. 2003; Schank *et al.* 2012). Furthermore, chronic stress is known to increase the likelihood of relapse into drugs and alcohol-seeking behavior (Sinha 2001) and can increase the risk of depression in addition to lowering overall quality of life (Brown *et al*. 1989).

While the dynorphin/KOR system has been implicated in many of the aforementioned disorders, its full role and specific mechanisms in the stress response and stress-induced

behaviors are not well understood. Better understanding these mechanisms may provide critical information about therapeutic targets involving the KOR and treatment strategies for alcoholism, drug addiction, and anxiety disorders.

In the present study, we examine the necessity of the dynorphin/KOR system in stress related behaviors using an animal model. We selectively ablated dynorphin from the BNST through stereotaxic injection of an inducible caspase construct. This Cre-inducible caspase can only be cleaved by TEV, which is also packaged in the AAV vector. By injecting this construct into a dynorphin-Cre mouse, we can selectively induce cell apoptosis only in those neurons expressing Cre. Additionally, we are undertaking a set of experiments using Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) to alter the excitability of Cre neurons (in a dynorphin-Cre mouse) during stress. These DREADDs are excitatory or inhibitory engineered G-protein coupled receptors that are exclusively activated by inert synthetic ligands (Armbruster, *et al.* 2007). We stereotaxically injected an inhibitory Gi coupled DREADD receptor into the BNST, which when activated by its synthetic ligand clozapine-N-oxide (CNO), causes hyperpolarization and decreased excitability of these Cre neurons, functionally inhibiting them. We hypothesize that upon induced apoptosis of dynorphin-Cre neurons (via flex caspase3) or functional inhibition of dynorphin-Cre neurons (via DREADDs) in the BNST, external stressors will unable to activate the local BNST KOR system, resulting in a less anxious phenotype as compared to control mice.

METHODS

Animals used

Adult male dynorphin-Cre mice were generated by the Krashes lab using recombineering techniques as previously described (Krashes, *et al*. 2014). These dynorphin-Cre mice were crossed to wild-type littermates to produce the offspring used in this study. All mice used were genotyped to affirm whether they were Cre+ before experimentation. Cre+ males were used for all experiments excluding the Forced Swim-EPM experiments, for which wild-type dynorphin-Cre male mice were used. Mice were housed in cages with 2-3 littermates, water, and rodent chow. The housing room had a 12-hour light/dark cycle. All procedures were conducted in compliance with the Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of the University of North Carolina.

Stereotaxic Surgery

Mice were anesthetized using inhaled isoflurane delivered through a stereotax. Mice were placed on a heating pad to assist with thermoregulation. The mice were stereotaxically injected with 500 nL DIO-hChR2 (control) or 500nL flex capase3 (experimental) bilaterally to the BNST (coordinated from Bregma: +0.90 medial/lateral, +0.27 anterior, -4.25 ventral). The surgical incision was closed using VetBond. The mice were subcutaneously injected with 1.0 ml 0.9% saline after surgery to assist with blood loss and recovery. Mice were provided with acetaminophen in their water pre- and post-operatively. Mice were allowed to recover for 10 days before performing behavioral experiments. N=9 control mice and n=11 flex caspase mice were injected by Sakibul Huq.

Designer Receptors Exclusively Activated by Designer Drugs (DREADDs)

Mice were stereotaxically injected, as described previously, with 300 nL AAV8/hSyn-DIO-mCherry (control) or 300 nL AAV8/hSyn-DIO-hM4D (Gi)-mCherry bilaterally to the BNST (coordinated from Bregma: +0.80 medial/lateral, +0.29 anterior, -4.25 ventral). Coordinates and injection volume were adjusted from previous experiments to prevent spill-over of injected substance into neighboring brain regions. N=8 control mice and n=8 DREADD mice were injected with appropriate substances by Sakibul Huq and Nicole Capik. Mice were allowed to recover for three weeks to allow DREADD receptors to express. They were injected with the synthetic ligand for the hM4D receptor, clozapine-N-oxide (CNO), 45 minutes before undergoing the Forced Swim Stress-Elevated Plus Maze (FSS-EPM) test described below.

Elevated Plus Maze (EPM)

An elevated plus maze was used that contains four arms elevated 1m above the floor. Two of the arms are open, meaning that there are no walls protecting the mice from falling off, and two arms are closed, with 7cm high walls protecting the mice from falling off. A small square central area exists at the intersection of the four arms. Mice were transported one-by-one from the main lab into the behavior room. Each mouse was placed in the central area and its movement was recorded for five minutes using cameras above the experimental apparatus. All elevated plus maze tests were performed by Sakibul Huq.

Open Field (OF)

An open field chamber within a black box containing an LED light was used; the chamber was a large white Plexiglas square box (without a ceiling) measuring 50cm x 50cm x

25cm. Mice were transported one-by-one from the main lab into the behavior room. Each mouse was placed into the center of the chamber and its movement was recorded for 30 minutes using cameras above the experimental apparatus. All open field tests were performed by Sakibul Huq.

Forced Swim Stress (FSS)

Forced swim cylinders consisting of transparent Plexiglas measuring 20cm in diameter x 40 cm high containing water (25^{o}C) filled halfway to the top were used. Mice were transported one-by-one from the main lab into the behavior room. Each mouse was placed into the water in the cylinder and its movement (mobility vs. immobility) was recorded for six minutes using cameras above the experimental apparatus. Two mice were run at a time; the two cylinders used were separated by an opaque black wall. After each trial, the mice were placed under a heat lamp for four minutes or until dry. All FSS tests were performed by Sakibul Huq.

Forced Swim-Elevated Plus Maze (FSS-EPM)

Mice were placed in Forced Swim Stress cylinders, as described previously, for 5 minutes and allowed to rest under a heat lamp for 4 minutes. Mice were then immediately placed on the Elevated Plus Maze for 5 minutes and analyzed as described previously using EthoVision software. All FSS-EPM experiments were performed by Sakibul Huq.

CNO Injection and FSS-EPM

N=5 dynorphin-Cre DREADD mice and n=4 control mice were given systemic intraperitoneal (IP) injections of clozapine-N-oxide (CNO) 45 minutes prior to the FSS test in the

FSS-EPM behavioral paradigm. CNO was administered at 10 ml/kg. All CNO injections and subsequent FSS-EPM experiments were performed by Sakibul Huq.

Preparation of Brain Slices

Perfusions were conducted as previously described by the Kash Lab (Li, *et al*. 2013). All perfusions were performed by Alexis Kendra.

All brains were sliced into 45μm slices using a vibratome. Slices were placed in well plates containing 50% Phosphate Buffered Saline (PBS)/50% glycerol solution. All slicing was performed by Sakibul Huq.

Immunohistochemistry using Cre staining is currently being performed on all brain slices to verify the success of stereotaxic surgeries. The IHC protocol for Cre staining was piloted and designed by Ayumi Nakamura in the lab of Mohanish Deshmukh (UNC Neurobiology). The protocol involves a primary antibody step, a secondary antibody step, and mounting onto slides with DAPI. All immunohistochemistry will be performed by Nicole Capik.

Analysis of Data

Elevated plus maze, open field, forced swim stress, and FSS-EPM tests were analyzed using Ethovision software. The software tracks the movement of the mice during the different behavioral testing paradigms and exports the raw data to Microsoft Excel. Excel was then used to create the graphs shown in the "Results" section. The forced swim stress experiments were analyzed by Sakibul Huq. The elevated plus maze, open field, and FSS-EPM experiments were analyzed by Sakibul Huq and Nicole Capik.

RESULTS

Figures 1-5 below represent the data gathered from the behavioral paradigms used to test for differences between control and experimental mice using the flex caspase and DREADD technological approaches. Sample sizes (n values) are shown on the columns in the figures. In Figure 1, the time spent in the open arms of the elevated plus maze is measured for control (n=9) and experimental (n=11) mice. The control mice spent an average of 40.7 seconds (out of 300 seconds) in the open arms, while the experimental mice spent an average of 44.6 seconds in the open arms. These times are almost equal, showing no difference between the control and experimental groups.

Figure 2 displays the amount of time spent in the center of the Open Field chamber for the control (n=8) and experimental (n=10) mice. In the center of the chamber, the control mice spent an average of 96.4 seconds while the experimental mice spent an average of 123.8 seconds (out of 1800 seconds). There is no statistically significant difference between the control and experimental groups.

Figure 3 displays the percent time spent immobile in the Forced Swim Stress cylinder for the control (n=8) and experimental (n=11) mice. The control mice were immobile 74.5% of the time on average while the experimental mice were immobile for 71.3% of the time on average. There is no statistically significant difference between the control and experimental groups.

Figure 4 displays time spent in the open arms and entries into the open arms for wild-type mice that underwent the FSS-EPM behavioral paradigm. Unlike data shown in Figures 1-3, no surgeries were performed on these mice. As seen in Figure 4a, Control mice spent 76.2 seconds in the open arms, and FSS mice spent 51.8 seconds in the open arms, on average. This is not a statistically significant difference. Figure 4b shows that control mice entered the open arms 8.2

times, and FSS mice entered the open arms 3.25 times, on average. These results are statistically significant ($p<0.05$) for the control vs. FSS groups.

Figure 5 shows FSS-EPM data for DREADD and control mice that underwent the FSS-EPM behavioral paradigm. The control mice spent an average of 60.0 seconds in the open arms of the EPM, while the DREADD mice spent an average of 38.0 seconds in the open arms of the EPM. The control mice entered the open arms an average of 7.0 times, while the DREADD mice entered the open arms an average of 3.6 times; this was a statistically significant difference ($p<0.05$).

Elevated Plus Maze Time in Open Arms

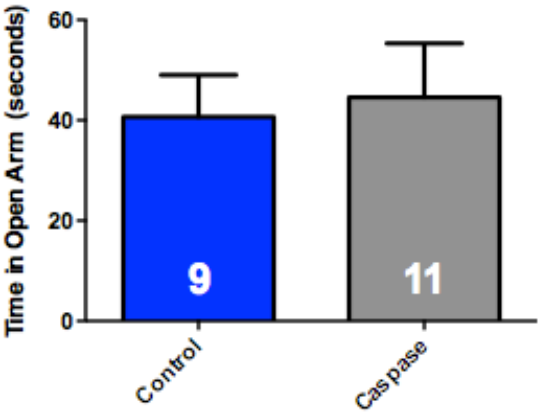

Figure 1. Time spent in the open arms of the elevated plus maze for control (n=9) and experimental (n=11) mice.

Open Field Time Spent in Center of Chamber

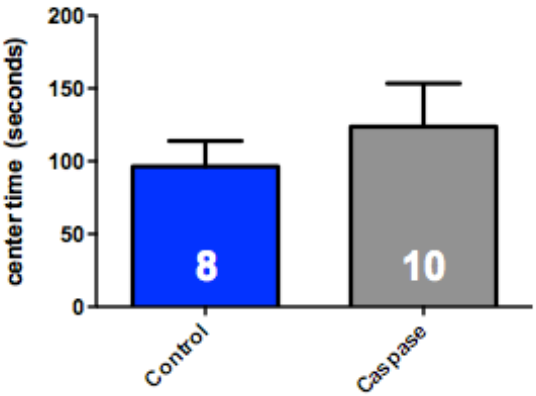

Figure 2. Time spent in the center of the open field chamber for control (n=8) and experimental (n=10) mice

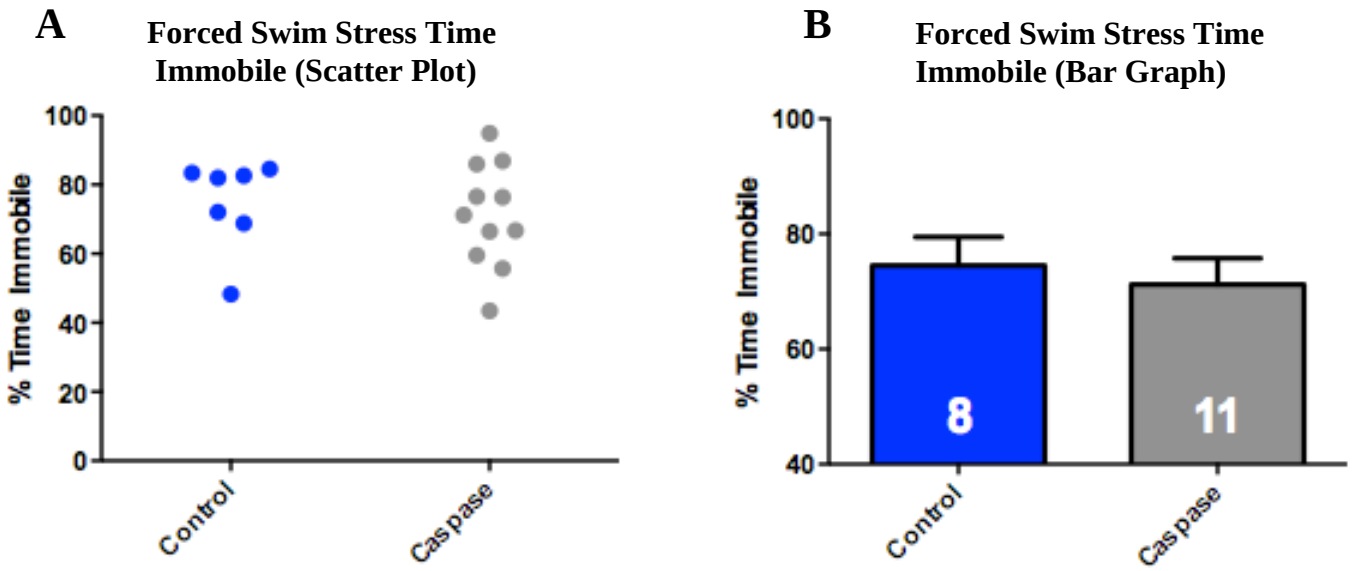

Figure 3. Scatter plot (A) and bar graph (B) of percent time spent immobile in FSS cylinder for control (n=8) and experimental (n=11) mice.

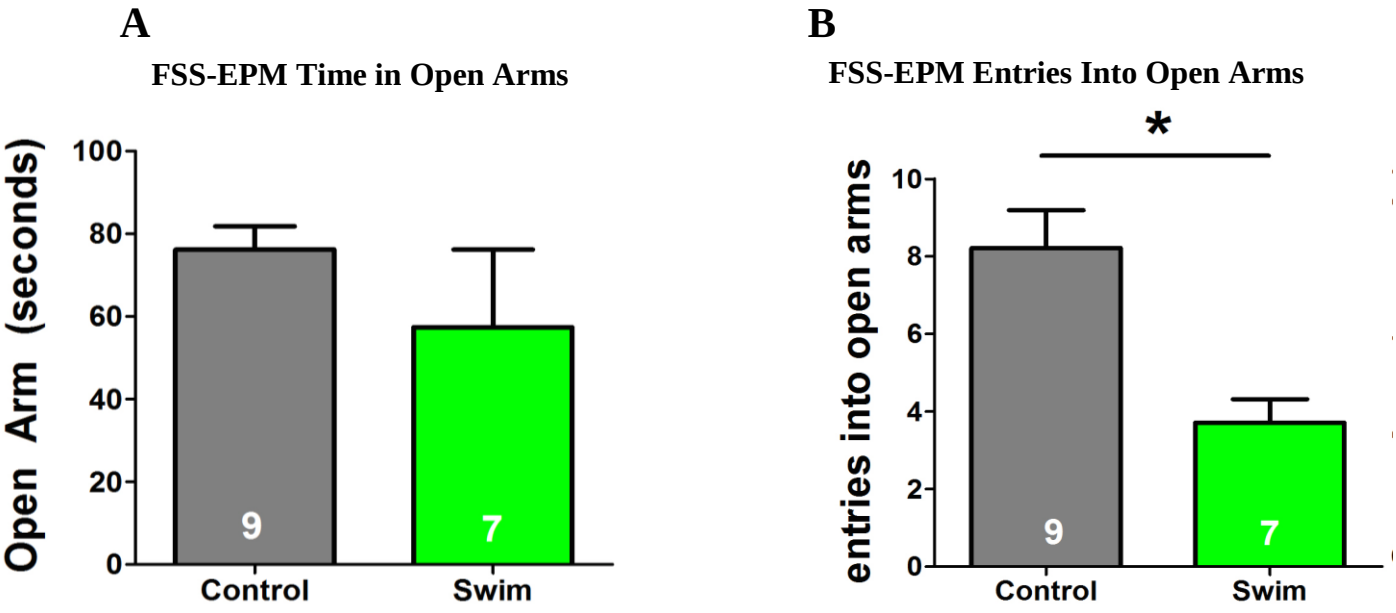

Figure 4. Time spent in the open arms (A) and entries into the open arms (B) of the EPM for control (no FSS) and FSS-EPM mice.

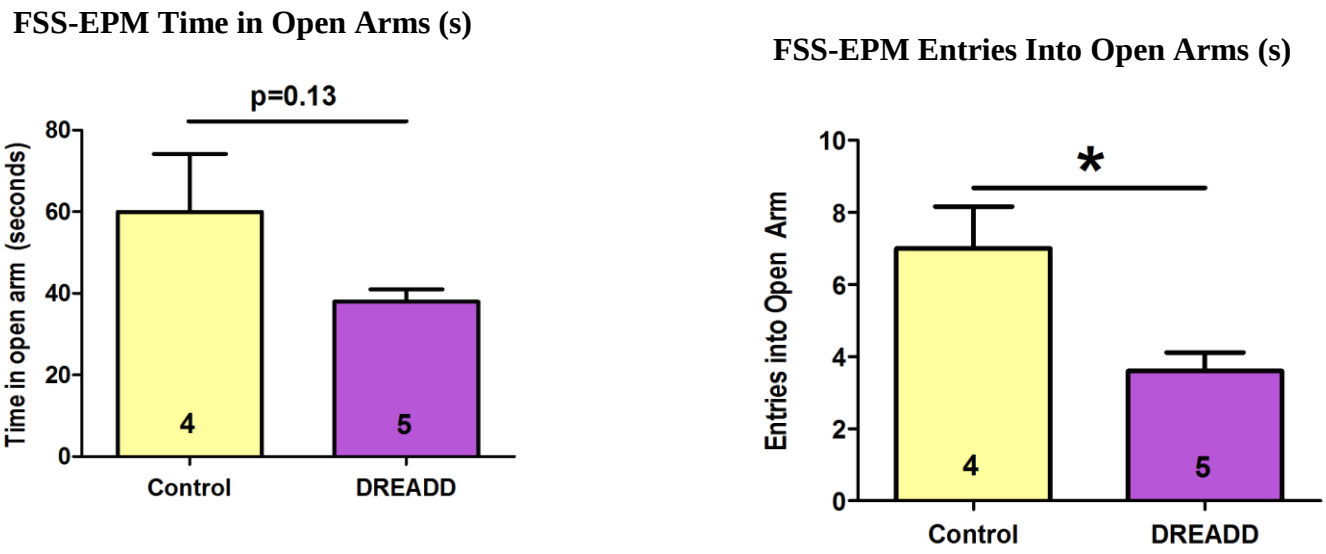

Figure 5. Time spent in the open arms (A) of the EPM and entries into the open arms of the EPM (B) for DREADD and control mice in the FSS-EPM paradigm.

DISCUSSION

The results of the study thus far have interesting implications for the role of the dynorphin/KOR system in the BNST in mediating the stress response in mice.

Cre staining will be used to verify the success of stereotaxic surgeries for the control (hChR2) and flex-capase3 mice. We want to see a lack of Cre stain in the injection site – that is, no cell bodies where the caspase was injected. The protocol is currently being improved to ensure accuracy of the stain (data not shown). Verification of the brains could affect the representation of data – that is, some mice shown may be valid while others may prove invalid due to unsuccessful apoptosis of dynorphin-Cre neurons.

The elevated plus maze testing paradigm takes advantage of the natural tendency of mice to explore novel environments; mice tend to avoid open areas, especially when they are brightly lit, favoring darker, more enclosed spaces (Bailey & Crawley, 2009). Mice that spend more time in the open arms tend to be bolder and are indicative of a less anxious and stressed phenotype.

The data in Figure 1 show no differences in the amount of time spent in the open arms between the experimental and control groups. Additionally, no difference was seen in entries into the open arms between these two groups (data not shown). This data alone does not support our hypothesis that ablation of dynorphin would cause a less anxious phenotype in the experimental mice.

The open field test provides a way to systematically assess novel environment exploration and general locomotor activity and screen for anxiety-related behavior in rodents (Bailey & Crawley 2009). Mice that spend significantly more time exploring the unprotected center area demonstrate anxiolytic-like baseline behavior (Prut & Belzung, 2003). The data in Figure 2 show no significant difference in time spent in the center (and corners) of the open field chamber between the experimental mice (without dynorphin) and control mice. Thus, the open field data alone are not telling of the role of the dynorphin/KOR system in mediating the stress response.

In the forced swim stress test, mice typically show exploratory behavior during the first two minutes of the test but become quite immobile during the last four minutes of the test (from which data are analyzed). Immobility was originally considered to be evidence that mice had learned that escape was impossible and had given up hope (Castagne *et al.* 2009). Many experiments have since shown that immobility is reduced by a wide range of clinically active antidepressant drugs, and the test is now commonly used as a screen for depressive states and antidepressant activity (Hascoet & Bourin, 2009). There is no significant difference seen in Figure 3 between the control and experimental groups, indicating that no anxiety or depressive phenotype is seen in one group of mice compared to the other.

It is important to note that the first two behavioral paradigms – the EPM and open field – were performed under basal, non-stress conditions, since the EPM and open field tests are not particularly stressful. Since we saw no difference between the experimental and control mice under basal, non-stress conditions, we wanted to see whether we could produce a different phenotype between our groups under stressful conditions using these same behavioral paradigms, since stress-induced activation of the KOR is supported in the literature (Bruchas, Land, & Chavkin, 2010).

To achieve this goal, we ran a cohort of wild-type mice through FSS immediately followed by EPM, as described in the methods above. Here, the control mice did not undergo FSS before EPM, while experimental mice were subjected to FSS followed by EPM. As seen in Figure 4, no significant difference was seen between control and experimental mice in time spent in the open arms of the EPM, but a statistically significant difference was seen between the control and experimental mice in entries into the open arms, with the control mice entering the open arms an average of 4.95 more times. This showed that the FSS test was able to serve as a significant stressor in the mouse's life and caused it to show a more anxious phenotype in the EPM as compared to control (no FSS) mice. This also served as a control to show that we were able to produce a stressful phenotype in our lab using the protocol specifications described above.

Next, we wanted to run another round of behavioral assays under these stressful conditions using a second technological approach – Designer Receptors Exclusively Activated by Designer Drugs (DREADDs). These DREADDs are special G-protein coupled receptors, and the ones we used were Gi-coupled inhibitory receptors. After being stereotaxically injected with these DREADDs or control virus, we ran these mice through a similar round of behavior under

stressful conditions (using the FSS-EPM protocol). Forty-five minutes before each experimental mouse was placed in the forced swim cylinder, it was intraperitoneally injected with the inert ligand for these receptors, clozapine-N-oxide (CNO), allowing us to specifically lower the excitability of dynorphin-Cre neurons in the BNST. Both control (control virus surgically injected) mice and experimental mice (inhibitory DREADD receptor surgically injected) were intraperitoneally injected with CNO to control for the stressful injection before behavior; since CNO is inert, it had no effect on the surgery control mice and simply served as an injection control. As displayed in Figure 5, there is no significant difference seen between the control (no DREADD) and experimental (DREADD) groups in time spent in the open arms of the EPM. Interestingly, there is a statistically significant difference between the control and experimental groups in entries into the open arms, with the control groups entering the arms 3.4 times more, on average. Notably, the control groups are actually showing the less anxious phenotype here, and the DREADDs seem to be having an anxiogenic effect.

This anxiogenic effect of functionally inhibiting dynorphin in the BNST is the opposite effect than what we expected in our hypothesis, and it goes contrary to existing evidence in the field. Importantly, the existing literature deals with the effects of activating and inactivating KORs systemically, and here we are only looking at the dynorphin/KOR system in the BNST. Our results suggest that functionally inhibiting dynorphin in this region could have an anxiogenic effect rather than the expected anxiolytic effect.

It is important to note that we have a small sample size with this DREADD data, and future behavioral assays need to be performed to confirm this effect. These mice will be run through Novelty-Suppressed Feeding and the Light/Dark Box tests to test for a stress phenotype, and following these tests, another round of mice will be injected with DREADDs and run

through the FSS-EPM, Novelty-Suppressed Feeding, and Light/Dark Box behavioral paradigms to increase the sample size.

Ultimately, the mechanism we are trying to understand is simply one piece of a larger puzzle in understanding the circuits underlying the stress response and alcoholism in mice. Though we may be moving towards an exciting discovery about the dynorphin/KOR system in the BNST, we don't know exactly where these dynorphin neurons project and what effects they might have on downstream brain regions. Thus, in addition to running more mice through the DREADD surgeries and behavioral paradigms discussed above, we will also perform a tracer study to see where these dynorphin neurons in the BNST project. Through all these studies, we may take an important step towards understanding the brain circuitry involved in the stress response and alcoholism and may eventually inform clinical and pharmacological treatment strategies for some of our most pressing public health concerns.

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