THE SOCIAL DEFEAT ANIMAL MODEL OF DEPRESSION SHOWS
DIMINISHED LEVELS OF OREXIN IN MESOCORTICAL REGIONS OF
THE DOPAMINE SYSTEM, AND OF DYNORPHIN AND OREXIN IN
THE HYPOTHALAMUS

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Abstract—Anhedonia is a core symptom of clinical depression. Two brain neuropeptides that have been implicated in anhedonia symptomology in preclinical depression models are dynorphin and orexin; which are concentrated along lateral hypothalamic dopamine reward pathways. These affect regulating neuropeptides modulate each other’s function, implicating an interactive dysfunction between them in anhedonia symptomology. But whether their influences are modified or imbalanced within the hypothalamus or dopamine system in anhedonic preclinical depression models is not yet clear. We used radioimmunoassay to determine this in the rat social defeat model of depression; at a time that anhedonic sexual disinterest was expressed. In tissue samples of the medial prefrontal cortex (mPFC), ventral tegmental area (VTA) and nucleus accumbens, basal dynorphin levels were similar to normal animals. But orexin was reduced in the VTA and mPFC. Also, dynorphin and orexin were both diminished in the hypothalamus which is noteworthy since nearly all hypothalamic orexin cells co-express dynorphin. These findings suggest that orexin and dynorphin function may be imbalanced between the hypothalamus and mesocortical dopaminergic brain regions in depression. © 2012 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: orexin, dynorphin, hypothalamus, mPFC, VTA, anhedonia.

INTRODUCTION

A reduced ability to experience pleasure, termed anhedonia, is a hallmark symptom of depression and is expressed in rodent models of the illness. Various studies suggest that a dynorphin and orexin interactive dysfunction between the hypothalamus and the dopamine reward system might exist in depression; and perhaps cause anhedonia symptoms. Dynorphin and orexin modulate each other’s function (Eriksson et al., 2004; Li and van den Pol, 2006) as well as brain reward mechanisms; though dynorphin typically inhibits and orexin stimulates neural activity (Shippenberg, 2009; Aston-Jones et al., 2010). Dynorphin is also co-expressed in nearly all orexin neurons, which are exclusively located in dorsomedial and lateral regions of the hypothalamus (Chou et al., 2001). Importantly, hedonic behaviors that are commonly diminished in depressed patients, such as the desire to eat and to engage in sexual activity, are controlled by an interaction between the hypothalamus and dopamine system (e.g. see Will et al., 2003b; Hull and Dominguez, 2007). Thus it is noteworthy that the hypothalamus sends dynorphin and orexin neural projections to the three major regions of this system: the ventral tegmental area (VTA), nucleus accumbens, and medial prefrontal cortex (mPFC) (e.g. Fallon et al., 1985; Peyron et al., 1998; Fadel and Deutch, 2002; Baldo et al., 2003). Each of these regions expresses dynorphin and orexin receptors (Marcus et al., 2001; Knoll and Carlezon, 2010) and is implicated in depression susceptibility (see Covington et al., 2010a). Importantly, activation of dynorphin and orexin mechanisms within each also modulates hedonic behavior (Bals-Kubik et al., 1993; Davis et al., 2009; Aston-Jones et al., 2010).

Studies suggest that orexins’ hedonic effect in the VTA might be dampened in depression. VTA orexin release stimulates dopamine function and reward seeking (Aston-Jones et al., 2010). But evidence of diminished cerebrospinal orexin levels, reduced diurnal orexin fluctuations, and deficient VTA dopamine neuron function have all been seen in depressed patients; who commonly show anhedonia (American Psychiatric Association, 2000; Klimek et al., 2002; Salomon et al., 2003; Brundin et al., 2007). Narcolepsy patients also show a high incidence of depression and a diminished VTA reward function (Daniels et al., 2001; Ponz et al., 2010). They show a massive loss in hypothalamic orexin and dynorphin cell expression and cerebrospinal orexin levels as well (Peyron et al., 2000; Crocker et al., 2005).
Diminished hypothalamic orexin measures, including cell number, have similarly been observed in preclinical models of depression that are known to characterize anhedonia and apathy-like symptomology (Allard et al., 2004; Feng et al., 2008; Lutter et al., 2008a). The evidenced decrease in orexin cell number suggests that orexin and dynorphin are likely both diminished in the hypothalamus in depression, and at least orexin diminished in its VTA, accumbens or mPFC terminal regions. There is suggestive evidence in depression models that orexin might be altered in the VTA (Feng et al., 2008). Also, anhedonic depression models show dopamine hypofunction (Miczek et al., 2011a) as would be expected with VTA orexin decreases (Narita et al., 2007; Moorman and Aston-Jones, 2010). But this could also be expected with accumbens dynorphin increases (Shippenberg, 2009) which have been evidenced in some preclinical models but not others (Bjomebekk et al., 2005; Bergstrom et al., 2008; Rubino et al., 2008; Carr et al., 2010).

These orexin and dynorphin alterations could affect reward desire (Aston-Jones et al., 2010; Knoll and Carlezon, 2010). Orexin release in the hypothalamus, as well as orexin and dynorphin release in the VTA, stimulates reward seeking in animals (Singh and Desiraju, 1988; Hamilton and Bozarth, 1988; Mitchell and Stewart, 1990; Muschamp et al., 2007; Aston-Jones et al., 2010; Espana et al., 2010a). Thus, the orexin cell loss described in depression models could diminish reward desire. But the reinforcing or euphoric value of rewards might also be diminished in these models due to their enhanced accumbens dynorphin levels. Orexin or dynorphin administration in the accumbens is aversive to animals (Bals-Kubik et al., 1993; Terashvili et al., 2004; Sharf et al., 2008), as is dynorphin in the mPFC and dynorphin and orexin stress mechanisms in the VTA (Bals-Kubik et al., 1993; Hata et al., 2011).

Thus, changes in the dynamics between orexin and dynorphin function in depressed patients could cause their anhedonia symptomology by disrupting these distinct reward emotional processes. As the above findings suggest, anhedonic symptoms could be caused by an increase in accumbens aversion due to locally enhanced levels of either neuropeptide. But they could also be caused by a decrease in VTA-stimulated reward seeking due to locally diminished levels of either peptide in hypothalamic orexin cell projections to the region. But whether depression models show an imbalance in dynorphin and orexin expression between the hypothalamus and dopamine system is unknown.

This study used radioimmunoassay (RIA) to evaluate this possibility in the social defeat animal model of depression; at a time that anhedonic sexual disinterest was expressed. Due to the diminished hypothalamic orexin cell numbers described in this model and the known co-expression of dynorphin in these cells (Chou et al., 2001; Lutter et al., 2008a), we hypothesized that defeated animals would show orexin and dynorphin decreases in the hypothalamus. And since the VTA typically receives a preferentially rich orexin innervation compared to the mPFC and accumbens reward regions (Fadel and Deutch, 2002), we predicted that orexin decreases would also be seen in the VTA. Anhedonia, including sexual disinterest, would not be unexpected after such loss (e.g. Harris et al., 2005; Muschamp et al., 2007; Wang et al., 2009; Moorman and Aston-Jones, 2010; Espana et al., 2010b; McGregor et al., 2011; Thompson and Borgland, 2011). But since dynorphin primarily inhibits dopamine function in the VTA and accumbens, and is co-expressed in cell bodies within the VTA in addition to afferents to the region (Nestler and Carlezon, 2006; Shippenberg, 2009; Knoll and Carlezon, 2010; Panksepp and Watt, 2011), we predicted that basal dynorphin peptide levels would be normal or perhaps enhanced in these regions in this anhedonic depression model.

**EXPERIMENTAL PROCEDURES**

Fig. 1 provides a concise depiction of the experimental design used in this study.

**Animals and housing**

Male Long–Evans rats (N = 39, 8 weeks of age, Harlan, Indianapolis, IN, USA) were socially housed in a light–12 h cycle, lights on 7 AM) and temperature-controlled colony room with food and water available ad libitum. After 3 days of acclimation to the colony room, they received once daily habituation to handling (1 min for 7 days) and two habituation sessions to the proximity test cages employed in this study (10 min each). They were then singly housed and left undisturbed for 28 days before being used as intruder animals or controls in the social defeat paradigm described below. Isolate housing is known to enhance the development and long-term expression of a depression-like phenotype in this paradigm (Ruis et al., 1999; de Jong et al., 2005). All animal procedures were carried out in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and the VA Animal Care and Use Committee.

An established cohort of male Long–Evans rats were used as aggressive resident rats in this study (N = 15; 6–9 months of age). These animals showed a dominant propensity to fight, pin, and occasionally bite a well-known submissive animal that was briefly placed into its homecage. Since Long–Evans males are naturally aggressive once mature as indicated by the literature provided by Harlan (Harlan, Indianapolis), and social isolation enhances aggressiveness, each animal within this cohort had been purchased at 2 months of age and singly housed for 4 months prior to its initial use. They were approximately 3–6 months older and 175–200 g heavier than the above experimental animals that were exposed to social defeat by this cohort.

**Social defeat animal model of depression**

The resident–intruder social defeat paradigm was chosen to model depression since it induces several different types of anhedonia-like symptoms including diminished sexual pursuit (see Nocjar and Panksepp, 2009; Miczek et al., 2011b). Rats that were used as intruder rats (N = 22) were placed in the homecage of an aggressive resident rat during five 30-min social defeat sessions. Control animals (N = 17) were given identically timed sham defeat sessions during which they were exposed to the empty homecage of one of the male aggressive resident rats. Each session was 48–72 h apart and videotaped. Under this social defeat methodology, intruder animals are typically pinned supine by the resident animal (i.e. defeated) within 5–8 min, but remain physically exposed to the aggressive resident animal throughout the 30-min social defeat session (e.g. McLaughlin et al., 2006; Nocjar and Panksepp, 2009). We have found that
the resident animal will not attack after the sessions initial fighting bouts if nearly continuous distanced submission is shown by the intruder in the form of frozen supine, upright or crouched postures. Thus after being quickly defeated and forced to submit, in-truder animals are continuously stressed by the threat of physical aggression if they do not remain submissive across the 30-min session. Although intruder animals appeared psychologically stressed at the end of these sessions (e.g., they screeched and jumped from the resident’s cage when the lid was removed), physical harm (scratches, bites, etc.) was rarely seen. Animals were removed from the study if they experienced significant harm such as scrotal bites, but not after minor harm (slight scratch, ear nip or toe-nail pull) that healed by the next defeat session and thus did not interfere with the animal’s performance.

To show that submission developed in intruder animals, the following behaviors were assessed across defeat sessions: frozen crouch (hunched-back crouching distanced away from the resident rat or directly at the face of the resident rat), rough-and-tumbling fights, defensive uprights (animal upright, towards the resident rat, with 2-paws off of the cage surface), pins (intruder animal supine on its back below the resident rat or against the sidewalls), time to the first pin, and defensive guards (leg kicks or butt or arm push against the resident rat). These measures are typical social defeat assessments (Miczek, 1979; McLaughlin et al., 2006; Walker et al., 2009), except for our measure of frozen crouches directly at the face of the resident rat. In pilot work, we were surprised that some animals cautiously yet repeatedly approached the residents face and froze face-to-face. Because of the vulnerable proximity to the face of the resident animal, this behavior appears defiant while crouching away from the resident appears submissive. But the intruder many times remains frozen in its position if the resident walks away from the intruder at its face. Since others have shown that immobility oriented towards the resident was perhaps due to chronic stress-induced dynorphin release (McLaughlin et al., 2006), we hypothesized that these distinct crouch behaviors might differentially predict dynorphin and orexin neuropeptide change following defeat. Thus, both were measured and further assessed below.

Following social defeat termination, defeated and sham defeated animals were given several behavioral assessments (Experiment 1) or were sacrificed to determine brain neuropeptide expression (Experiment 2) as depicted in the right timeline.

Experiment 1: Behavioral assessments

Our pilot evidence indicated that anhedonia was evidenced in socially defeated animals 2 days after defeat terminated (Nocjar and Panksepp, 2009). But we wanted to validate that our social defeat methodology was aversive and induced a lasting depression-like phenotype in animals. Thus, the following behavioral tests were administered to test this in seven of the above sham defeat control rats and in 12 that were socially-defeated as depicted in Fig. 1.

Sexual pursuit reward proximity test. To determine whether the social defeat methodology diminished reward desire, sexual interest was assessed 2 and 14 days after the last social defeat session. Sexual interest was assessed as in our previous reports (see Nocjar and Panksepp, 2002, 2007). In brief, rats were individually placed in a Plexiglas open-field reward proximity chamber that had a wire mesh stimulus cage located in each of its four corners. Two opposite corner stimulus cages contained a hormonally primed sexually-receptive female rat (10 μg estradiol benzoate, 48 h pretest, and 0.5 mg progesterone, 4 h pretest) or a non-receptive female. The stimulus cage wire mesh screening prevented copulation, but allowed assessment of a male animal’s appetitive approach and investigative behavior towards each female target (e.g., time spent pawing and sniffing at the screening). The 10-min test was videotaped, and behavior was later tabulated by an individual that was blind to the animal’s prior defeat experience. Sexual preference scores were calculated by subtracting the total time spent at the stimulus cage that contained the non-receptive female from the total time spent at the cage containing the sexually-receptive female. Since defeat can induce social avoidance (Krishnan et al., 2007; Lagace et al., 2010), time spent at both stimulus cages was used as an assessment of female social interest. Time at these cages plus the time spent at two additional empty stimulus cages in the remaining two corners of the proximity chamber was used as an assessment of stimulus cage exploratory interest, since a generalized stimuli disinterest could affect performance in this task. Vertical locomotion (number of vertical uprights [animal balanced upright on hind paws]) and horizontal locomotion (number of quadrant entries) were also counted across the session since locomotor alterations could also affect performance in this task. Note that chamber quadrants were clearly marked on the chambers floor.

Resident avoidance proximity test. Interest towards the aggressive male resident rat was assessed 4 days after the last social defeat or sham defeat session to see if defeated animals avoided the aggressive resident animal; indicative of conditioned fear. In brief, rats were exposed to a nearly identical reward proximity chamber and methodology as used in the sexual pursuit test above, except that this chamber had only two corner stimulus cages and the test stimuli differed. For this test, one stimulus cage was left empty while the other contained the specific aggressive resident rat that an animal had been physically ex-
posed to, or sham exposed to, during chronic social defeat sessions. The wire mesh screening allowed assessment of a male animal’s appetitive approach and investigative behavior towards the aggressive resident animal (e.g., pawing and sniffing), while protecting it from any aggressive physical attack by the resident animal. Resident preference scores were calculated by subtracting the total time spent sniffing and pawing at the empty stimulus cage from the total time spent at the cage containing the aggressive resident animal. Time spent at both stimulus cages was used to assess stimulus cage exploratory interest. Locomotor behavior was also assessed as in the sexual pursuit task (see above).

**Sucrose preference test.** To test whether social defeat generally and persistently diminished reward desire, a 24-h homecage preference test for 1% sucrose versus plain water was also given to the above rats at approximately 21 days after their last social defeat or sham defeat session. Placement of the sucrose and plain water bottles was counterbalanced between animals. *Sucrose preference scores* were calculated by subtracting the total amount of water consumed from the amount of sucrose consumed. Prior to the test day, rats had been given a 2-h habituation session where they had free access to both liquids within their homecages.

**Forced swim test (FST).** FST immobility was also assessed in these same animals approximately 28 days after social defeat terminated. Immobility during this test is thought to model depression apathy-like symptomology. On the first day, rats were placed in the bucket, and climbing (forelimb thrashing movements directed against the sidewalls of the bucket) was tabulated, as well as immobility latency (time to first expression).

**Experiment 2: Brain dynorphin-A, orexin-A and orexin-B neuropeptide assessment**

Since altered orexin and dynorphin function is implicated in anhedonic behavioral expression (see introduction), and our preliminary findings indicated that anhedonic symptomology was evidenced in socially defeated animals when tested 2 days after the last social defeat session (Nocjar and Panksepp, 2009), we assessed orexin and dynorphin tissue levels at this time in this initial study. As described in Fig. 1, 10 socially defeated and 10 sham defeated animals that had received no other behavioral assessments were sacrificed 2 days post defeat. Their brains were removed and basal neuropeptide levels were determined in extracted tissue samples from the hypothalamus where orexin and dynorphin co-expressing cells are located, and from areas within the dopamine reward system where these cells project.

We used a tissue neuropeptide RIA technique to assess this (see below). RIA is commonly used in published reports of orexin alterations, whether detecting orexin in cerebrospinal fluid (CSF), brain microdialysate or brain tissue, though not in blood (Nishino, 2006). Tissue RIA does not provide a measure of neuropeptide release, but determines the level of available peptide within neuron cell bodies and/or within neuronal vesicles located in axons or axon terminals in a brain tissue sample. RIA is thought to be a sensitive measure, even of CSF orexin peptide levels, under the proper control procedures (Nishino, 2006). In fact, it is commonly thought that orexin A can be sensitively, specifically and reliably detected from all types of samples using either RIA or enzyme-linked immunosorbent assay (ELISA), but RIA is more sensitive than ELISA (Lin et al., 2002). And we have repeatedly detected orexin-B and orexin-A peptide levels, even detected their altered expression, in both cortical and subcortical brain tissue using RIA (Feng et al., 2007, 2008, 2009).

**Tissue dissection.** After decapitation, brains were quickly removed and sliced at 2-mm intervals with the aid of an ice-cold stainless steel brain matrix (ASI Instruments, Warren, MI, USA). Brain slices were placed on an ice-cold anodized aluminum block and the following brain regions were quickly extracted at the following anteroposterior (AP), mediolateral (ML) and dorsoventral (DV) locations: mPFC [AP 3.7–7.7 mm; ML 0–1.0 mm; DV 0.0 to –5.0 mm]; nucleus accumbens [AP 1.7 to –0.3 mm; ML 0.2–2.5 mm; DV –5.5 to –3.0 mm]; hypothalamus [AP –1.3 to –3.3 mm; ML 0–2.2 mm; DV –7.5 to –9.5 mm] and VTA [AP –5.3 to –7.3 mm; ML 0–1.0 mm; DV –7.5 to –8.5 mm]. Each tissue sample was immediately placed in pre-weighed plastic centrifuge tubes, weighed and then frozen at –80 °C until used for RIA neuropeptide assessment.

Since it is not known where orexin and dynorphin might interact within subregions of the mPFC, accumbens or VTA reward regions, the entire anterior to posterior and medial to lateral expanse of these three reward regions were extracted in this initial study before proceeding to potential subregions of interest within each. Thus, mPFC tissue samples included the entire anterior cingulate, prelimbic and infralimbic subregions. Within this sample were areas that are sensitive to stress, and in particular to stress-induced orexin-B attentional dysfunction (Lambe et al., 2005, 2007). Also included were regions of the anterior cingulate and ventral mPFC that become hypoactive after social defeat stress, and that when respectively lesioned or stimulated induces or reverses depression-like symptoms (Covington et al., 2005; Bissiere et al., 2006; Covington et al., 2010b; Kanarik et al., 2011). Although subregional specificity was lost with the inclusion of all three subregions in our sample, it allowed us to first determine whether neuropeptide change generally occurred in the mPFC after chronic defeat, before implementing studies of subregional involvement.

Nucleus accumbens samples included its shell and core subregions, as well as the medial olfactory tubercles. Care was taken to exclude surrounding areas that are involved in orexin-induced sleep/wake changes such as the medial septum (Berridge et al., 2010). VTA extraction encompassed its rostral and caudal regions, and included the small dopamine cell populations that are stimulated by orexin, and which project to the accumbens shell and mPFC (Vittoz et al., 2008).

Hypothalamus extraction encompassed the anterior hypothalamus back to the most anterior location of the posterior hypothalamic nucleus. Our sample included the lateral, dorsomedial and perifornical nuclei of the hypothalamus where orexin neurons are exclusively located (Nambu, 1999), and at levels where lateral and dorsomedial hypothalamic orexin cells are differentially activated by stress, food and addiction drug cues (see Aston-Jones et al., 2010). But it excluded surrounding areas that are involved in orexin-induced sleep/wake patterns such as the substantia innominata and medial preoptic area (Berridge et al., 2010), although the most rostral end of the sample might have included the most posterior border of the preoptic area.

**Peptide extraction.** Frozen tissue samples were processed using a well-established dry-sample protocol used in our lab for brain tissue peptide extraction (Feng et al., 2007, 2008). In brief, acetic acid (0.5 M) was added to each centrifuge tube containing sampled brain tissue, at a volume equal to 10 times the tissue weight. The centrifuge tubes were then moved to a boiling water bath for 10 min. After removing the tissue blocks, the microtubes were centrifuged for 30 s at 5500 rpm. The remaining supernatants (containing all peptides from the tissue sample) were
air-dried under a hood at 60 °C. The final dried peptide sample extracted from each tissue was subsequently stored at −80 °C until reconstituted for RIA neuropeptide assessment.

RIA assessment of dynorphin-A, orexin-A and orexin-B levels. Standard RIA kits for detecting orexin-A (i[RK-003–30], orexin-B (i[RK-003–32]) and dynorphin-A (i[RK-021–03]) peptide expression were used (Phoenix Pharmaceuticals, Burlingame, CA, USA). Each of these kits provides highly reliable peptide specificity. The orexin-A kit for example does not cross react with orexin-B, and vice versa. Similarly, the dynorphin-A kit does not cross react with dynorphin-B, β endorphin, α-neo-endorphin or enkephalin and detects dynorphin-A in human, rat, mouse and porcine samples. We determined the quality of each kit before experimental assays were conducted by verifying (1) that the binding activity of 100 μl of the 125I peptide solution was within a range of 8500–10000 cpm and (2) that the kits sensitivity ratio was not less than 2.5. The sensitivity ratio was calculated by dividing the cpm value observed at the lowest detection concentration (1 pg/tube) by the cpm value observed at the highest detection concentration (128 pg/tube). Thus, R = CPM1pg/CPM128pg. For example, the mean sensitivity ratio for all orexin-A and -B RIA kits used in this study was 6.02 ± 0.50 and 2.72 ± 0.34, respectively; indicating that they provided a sensitive measure of tissue orexin levels.

Following these determinations, frozen dried peptide samples that had been extracted from the brain tissues collected in this study (see section ‘Peptide extraction’) were reconstituted in RIA buffer at a 1:50 dilution ratio (mg tissue dried peptide sample/μl RIA buffer). These sample stock solutions were then further diluted and preliminary assays were performed to determine the appropriate dilution ratio required to detect orexin-A, orexin-B and dynorphin-A within each of the brain regions assessed in this study. We found for example that the optimal dilution ratio for dynorphin-A was 1:50 in the mPFC, 1:75 in the nucleus accumbens, and 1:150 in the VTA and hypothalamus. Applying these ratios allowed us to sensitively detect dynorphin-A within tissue samples from all regions, although a more concentrated sample solution was required to detect it in mPFC samples for example than hypothalamic or VTA samples.

Once the optimal dilution ratios were determined, levels of all three neuropeptides were then independently determined within each brain tissue sample collected following the manufacturer’s protocol for each neuropeptide (Phoenix Pharmaceuticals, Burlingame, CA, USA). After assay completion, the radioactivity of each sample tube (containing 100 μl of the optimal diluted sample) was determined with a gamma counter (Cobra II Auto Gamma, Packard Instrument Company, Downers Grove, IL, USA). The sample value was compared to a standard curve assessed within the assay, which was generated using a standard protocol formulation. The indicated peptide level was then converted, based on the optimal dilution ratio used, to pg/mg tissue using GraphPad Prism software (San Diego, CA, USA). Since 100 μl of the optimal diluted sample was used in all peptide determinations, sample values were divided by two for example with a 1:50 optimal dilution ratio (mg tissue dried peptide sample/μl RIA buffer) since 2 mg of tissue was needed in 100 μl. Tissue sample assays were carried out in duplicate and the mean of these two measurements was used as data for statistical purposes.

Correlative assessment between hypothalamic dynorphin-A, orexin-A and orexin-B levels and prior social defeat behavioral expression. Immobility during chronic defeat requires dynorphin release (McLaughlin et al., 2006). And notably, defeat causes prolonged activation of orexin cells which typically co-express dynorphin (Chou et al., 2001; see Berridge et al., 2010). Their overstimulation also persistently and detrimentally alters their intracellular expression of orexin and dynorphin (Katsuki et al., 2010). Thus, we wanted to determine whether an animal’s social defeat behavioral expression predicted hypothalamic orexin and dynorphin alteration. To assess this, frozen crouches, fights, uprights and guards that were expressed during the final social defeat session were each correlated with the above hypothalamic orexin-A, orexin-B and dynorphin-A measures.

Statistics

Data are presented as mean ± SEM. Significance was set at p < 0.05. To determine whether submission developed in animals across social defeat sessions, a dependent sample t-test was employed to assess whether submissive and aggressive behavioral expression changed between the first and last social defeat sessions. Then to determine the effects of social defeat on behavior (Experiment 1) and on brain neuropeptide expression (Experiment 2), independent sample t-tests and two-way repeated ANOVAs were used.

Independent sample t-tests were employed in Experiment 1 to determine whether defeated versus sham defeated animals differed in the resident avoidance test (resident preference scores, locomotor scores and stimulus cage exploration), submissive preference test, or last FST (immobility, swimming and climbing duration). They were also used in Experiment 2 to determine whether these groups differed in neuropeptide expression.

Repeated measures ANOVAs were used when multiple tests of a behavior were being compared between these groups. Thus, a two-way repeated measures ANOVA (day × group) was used to determine whether defeated versus sham defeated animals differed during the first and second sexual pursuit tests (sexual pursuit scores, female social interest, motor exploration, and stimulus cage exploration), or differed across the two FSTs (% of session immobile, swimming and climbing) or four body weight tests (weight gain post defeat). If a significant interaction between test day and group was found, post-hoc analysis of main effects was further explored using post-hoc pairwise comparisons adjusted for multiple comparisons (p < [0.05 divided by the number of comparisons]).

And finally, to determine whether an animal’s social defeat behavioral expression predicted hypothalamic orexin and dynorphin peptide changes, Pearson’s product correlations (r) were conducted between social defeat behaviors (crouching behaviors, fights, uprights and guards) and subsequent hypothalamic neuropeptide expression (orexin-A, orexin-B and dynorphin-A). The proportion of the variance in neuropeptide level attributable to each behavior (r²) was also calculated.

RESULTS

Chronic social defeat behavior

As seen in the left graph in Fig. 2a, intruder animals spent more time in a frozen submissive crouch posture while in the presence of the aggressive resident animal by the final defeat session (t[21] = 4.79, p < 0.001; see Total Frozen Crouch). Two types of frozen crouch behavior were assessed in animals as depicted in the right half of this graph (for their behavioral description, see section ‘Social defeat animal model of depression’). Frozen crouch postures distanced away from the resident animal increased across defeat sessions (t[21] = 3.79, p < 0.01), while crouch behaviors at the face of the resident animal increased minimally across defeat (p = 0.20). The right graph in Fig. 2a shows that animals also engaged in fewer fights (t[21] = 3.48, p < 0.003), defensive uprights (t[21] = 2.52, p < 0.03) and guards (t[21] = 3.34, p < 0.004) by the last defeat session. Thus, behavioral submission in the presence of the aggressive resident animal increased across social defeat sessions.
Note that frozen crouch distanced away from the resident animal was related to hypothalamic neuropeptide decreases seen 2 days after defeat terminated (see section ‘Relationship between social defeat behavioral expression and hypothalamic levels’ below and Fig. 6 b).

Experiment 1: Behavioral study test performance

Sexual pursuit tests conducted 2 and 14 days after defeat. As seen in the left graph in Fig. 3a, sexual pursuit was diminished in defeated animals 2 days and 14 days after the last social defeat exposure compared to normal controls (overall group effect: $F[1,17] = 9.101$, $p < 0.009$; and no interaction with test day: $F[1,17] = 0.57$, $p = 0.46$). But as seen in the right graphs in Fig. 3a, defeated animals spent a similar amount of time investigating the two stimulus cages that contained the female animals as did normal controls (no overall group effects: $F[1,17] = 2.14$, $p > 0.05$ and $F[1,17] = 0.04$, $p > 0.05$, respectively; and no group × day interaction in either test), as seen in the right graphs in Fig. 3a. These findings indicate that the diminished interest shown by defeated rats towards the sexually receptive female animal was not due to a decreased interest in the stimulus cage, a locomotor decrement or a decreased social interest towards females during this test. Thus, social defeat induced lasting sexual anhedonia in animals.

Resident avoidance proximity test conducted 4 days after defeat. Fig. 2b shows the interest expressed by defeated and normal sham defeated controls towards the aggressive resident animal when assessed 4 days after defeat (left graph). Opposite to controls, defeated animals avoided the stimulus cage that contained the aggressive...
resident rat that they had been exposed to during chronic defeat ($t_{(17)} = 2.93, p < 0.01$). As indicated in the right graph in Fig. 2b, defeated animals did not spend significantly less time exploring the chambers two stimulus cages during the test ($t_{(17)} = 1.72, p = 0.10$). But their vertical and horizontal locomotor counts were diminished during the test ($t's_{(17)} = 2.51$ and $2.34$ respectively, and $p's < 0.03$) in stark contrast to their normal locomotion shown during the sexual pursuit tests (see Fig. 3a). These findings of diminished approach towards the aggressive resident animal, of decreased open-field locomotor expression, but normal exploratory interest in the stimulus cages during the test, suggest that conditioned fear developed in defeated animals.

Sucrose preference test conducted 21 days after defeat. Fig. 3b shows that the 24-h sucrose preference scores of defeated animals were diminished compared to controls when tested 21 days after social defeat terminated ($t_{(11)} = 3.08, p < 0.02$). Note that three of the initial animals in this study were sacrificed before this test was conducted, and the data from three additional animals were lost due to technical difficulties with the task. Nonetheless, this finding indicates that the anhedonia expressed by defeated animals when tested 2 days after social defeat (see sexual anhedonia expression in Fig. 3a) was still evident in this depression model nearly 3 weeks later.

As seen in the right graph in Fig. 3b, normal sham defeated controls and socially defeated animals gained weight after social defeat sessions terminated (overall day effect $F_{(3,42)} = 57.99, p < 0.001$). But, defeated animals showed a non-significant trend towards faster weight gain than normal sham controls ($group \times weight$ interaction: $F_{(3,42)} = 2.65, p = 0.06$).

FST conducted 28 days after defeat. Fig. 4 shows the immobility, swimming, and climbing behavior shown by defeated rats and normal sham defeated controls during two forced swimming tests that were conducted 24 h apart (FST1 and FST2) approximately 28 days after defeat.
terminated. As indicated in Fig. 4a (left graph), both defeated animals and normal controls showed a shorter latency to become immobile in FST2 than FST1 (overall test effect: $F_{[1,13]} = 16.57$, $p < .01$; and no test × group interaction). But defeated rats showed shorter immobility latencies overall (overall group effect: $F_{[1,13]} = 8.30$, $p < .02$; and no test × group interaction: $F_{[1,13]} = 0.43$, $p = .52$).

As seen in the right graph in Fig. 4a, the percentage of time that defeated rats and normal controls spent immobile during these tests increased from FST1 to FST2 (overall test day effect: $F_{[1,13]} = 8.49$, $p < .02$; and no test × group interaction). But defeated animals showed a higher percentage of time immobile overall in both FST measures (overall group effect: $F_{[1,13]} = 15.06$, $p < .003$; and no group × test interaction effect: $F_{[1,13]} = 0.01$, $p = .90$). And although the percentage of time spent swimming was not significantly different between defeated rats and normal controls (no overall group effect: $F_{[1,13]} = 1.90$, $p = .19$; although the group × test interaction was marginal: $F_{[1,13]} = 3.84$, $p = 0.07$ and an overall test effect was seen: $F_{[1,13]} = 4.81$, $p < 0.05$), their percentage of time spent climbing during these tests did differ overall (overall group effect: $F_{[1,13]} = 6.21$, $p < 0.03$; and no group × test interaction or main test effect). Thus as typically seen in this paradigm, immobility was shown sooner and at a higher level in FST2. But defeated rats showed more immobility and a faster latency to become immobile. They also showed less climbing during these tests overall.

The actual duration of the above behaviors during FST2 is plotted in Fig. 4b. Immobility duration during FST2 differed between defeated and normal controls ($t_{[13]} = 2.47$, $p < .03$). Also, non-significant trends of a diminished swimming duration ($t_{[13]} = 2.08$, $p = .05$) and climbing duration ($t_{[13]} = 1.74$, $p = .10$) was seen. Thus, socially defeated rats showed higher immobility in the final FST, and a trend towards lower swimming and climbing.

Note that the data were lost from one defeated animal due to a video-recording technical error during the test. And of course, data were not available from the same three defeated animals that were sacrificed prior to the sucrose test (see above).

**Experiment 2: Neuropeptide study RIA determinations**

The typical tissue extraction areas used in this study are depicted by the hatched regions in Fig. 5 (anterior extent of samples (Paxinos and Watson, 1998)). Samples were collected 2 days after the last sham or social defeat session since defeated animals typically show anhedonic sexual disinterest at this time (see Fig. 2; Nocjar and Panksepp, 2009).

**RIA determinations of dynorphin-A, orexin-A and orexin-B within the mPFC, nucleus accumbens, VTA and hypothalamus.** As seen in Fig. 6a, basal levels of orexin-A and orexin-B were diminished in the mPFC of socially de-
feated animals compared to sham defeated controls (t’s [13–14] = 2.24 and 3.29 respectively, p’s < 0.05); but dynorphin levels were not altered (t[16] = 0.65, p = 0.95). In the nucleus accumbens, persistent basal neuropeptide alterations were not evidenced (t’s [13–15] = 0.19, 0.15 and 0.33, p’s = 0.85, 0.88 and 0.71 respectively). In the VTA, basal levels of orexin-B were significantly diminished (t[16] = 2.60, p < 0.02) and a similar non-significant trend was apparent for orexin-A (t[17] = 1.79, p = 0.09), while dynorphin-A alterations were not evidenced (t[18] = 0.63, p = 0.54). However, dynorphin-A, orexin-A and orexin-B levels were all diminished in the hypothalamus of defeated animals (t’s [13–14] = 2.58, 2.19 and 3.53 respectively, p’s < 0.05). Thus, orexin-A and orexin-B were diminished in mesocortical regions of the dopamine reward system, and both orexin peptides as well as dynorphin-A were diminished in the hypothalamus in the social defeat model of depression.

Relationship between social defeat behavioral expression and hypothalamic levels of dynorphin-A, orexin-A and orexin-B neuropeptides. As seen in Fig. 6b, hypothalamic orexin-B levels shown two days after defeat terminated were negatively and significantly correlated with the animals distanced crouch behavior during defeat exposure (frozen crouch away from the resident animal, r = −0.82, p = 0.04). Although not shown for orexin-A (r = −0.50, p = 0.24, r² = 0.25), a similar non-significant trend was shown between dynorphin-A and this crouch behavior (r = −0.68, p = 0.09, r² = 0.46).

The relationship between hypothalamic neuropeptide expression and other social defeat behaviors was not significant: correlation coefficients between dynorphin-A, orexin-A or orexin-B and crouch at the residents face (r = −0.10, 0.40, and −0.11; respectively), fights (r = −0.07, 0.24, and −0.03; respectively), and uprights (r = −0.26, −0.17, and −0.30; respectively). And the proportion of the variance in neuropeptide level attributable to each of these behaviors (r²) was 24% at best. Note that correlations with guard behaviors were not conducted because only one animal expressed this behavior during the final defeat session.

These findings indicate that a prolonged distanced crouch response predisposes orexin loss in this depression model. The similar trend between this behavior and dynorphin loss supports further exploration of this relationship since our hypothalamus tissue sample not only included orexin and dynorphin co-expressing cells in the dorsal hypothalamus, but also included other ventral hypothalamus dynorphin cell populations (Chou et al., 2001).

**DISCUSSION**

We found anhedonic sexual disinterest in the rat social defeat model of depression when assessed 2-days after termination of defeat. Importantly, orexin peptide levels were diminished in the dopamine reward system at this time and both orexin and dynorphin levels were decreased in the hypothalamus. The model also showed a lasting generalized depressive phenotype. Sexual pursuit
and sucrose intake remained diminished for at least 3 weeks, and apathy-like behavior in the FST was still evident one month after defeat terminated. This is the first report of diminished sexual interest or motivation in a depression model other than our preliminary findings (Nocjar and Panksepp, 2009), although enhanced FST apathy and diminished sucrose intake and copulation have all been previously seen (e.g. see Sugiura et al., 1997; Rygula et al., 2005; Lutter et al., 2008b; Haenisch et al., 2009; Ito et al., 2009; Miczek et al., 2011b). Our findings also provide the first direct evidence of a potential orexin dysfunction in mesocortical regions of the dopamine system in depression.

Orexin and dynorphin levels were diminished in the hypothalamus

Although stress can acutely enhance hypothalamic orexin cell activation (Winsky-Sommerer et al., 2004; Harris and Aston-Jones, 2006; Furlong et al., 2009; Berridge et al., 2010; Johnson et al., 2010; Nollet et al., 2011), a growing literature with depression animal models suggests that orexin cell function is likely diminished in depression. In the social defeat model used in the current work, decreased hypothalamic pre-proorexin mRNA and orexin cell count and activation have all been reported (Lutter et al., 2008a). And we show that orexin-A and orexin-B peptide levels are diminished. Hypothalamic decreases in orexin peptide and orexin cell size have also been seen in the neonatal clomipramine and Wistar–Kyoto depression models (Allard et al., 2004; Feng et al., 2008). Hypothalamic orexin1 receptor expression is also diminished (Allard et al., 2004). And we provide the first evidence in preclinical depression models that both orexin and dynorphin peptides are decreased in the hypothalamus, which is noteworthy since dynorphin is co-expressed in nearly all hypothalamic orexin cells (Chou et al., 2001).
Further work is needed however to determine the location of the dynorphin loss since our hypothalamic tissue sample included orexin cells as well as other ventral hypothalamic cell populations that contain dynorphin (see Chou et al., 2001; Harthoom et al., 2005). But several pieces of evidence suggest that dynorphin was likely lost from orexin cells. First, our defeated animals showed higher weight increases than controls after defeat sessions terminated; an effect seen with orexin cell ablation and loss of both peptides (orexin/ataxin-3 transgenic mice (Nishino et al., 2000; Hara et al., 2001; Mieda et al., 2004; Crocker et al., 2005)) but not when orexin is lost but dynorphin remains in these cells (i.e., orexin deficient mice (Willie et al., 2001)). Second, orexin cell decreases have been previously reported in socially defeated animals (Lutter et al., 2008a). Third, prolonged activation of orexin neurons, as would occur under prolonged defeat stress (see Berridge et al., 2010), diminishes both orexin and dynorphin in hypothalamic orexin cells (Katsuki et al., 2010). And finally, we found that sexual pursuit and hypothalamic orexin and dynorphin peptide levels were all diminished 2 days after defeat terminated. In a similar temporally related fashion, castration decreases copulation and orexin cell survival which would ablate both orexin and dynorphin (Chou et al., 2001; Muschamp et al., 2007).

The hypothalamic orexin and dynorphin decreases seen in our social defeat depression model suggest that hypothalamic function may be vastly dysregulated in depression. Decreased hypothalamic orexin function diminishes reward seeking (Aston-Jones et al., 2010), and our depression model expressed anhedonia. But locally decreased levels of orexin and dynorphin could also dampen the activity of hypothalamic neuropeptide Y cells and cells that contain melanin concentrating hormone (Li and van den Pol, 2006); disturbing feeding (Dryden et al., 1996; TRitos et al., 2001; Chen et al., 2002; Chaffer and Morris, 2002; Bayer et al., 2002; Li and van den Pol, 2006). The diminished dynorphin levels could also disinhibit the remaining functional orexin cells (Li and van den Pol, 2006), dysregulating sleep and arousal, metabolism and energy balance (Horvath et al., 1999; Hagan et al., 1999; Bourgin et al., 2000; Seeley and Woods, 2003). Notably, disturbances in all have been seen in depression.

Interestingly, chronic social defeat induces immobility in animals by enhancing dynorphin release (McLaughlin et al., 2006). Although it is not known whether hypothalamic orexin and dynorphin co-expressing cells are involved in this dynorphin effect, chronic defeat causes prolonged activation of these cells which detrimentally affects their intracellular dynorphin and orexin expression (Berridge et al., 2010; Katsuki et al., 2010). Thus, we hypothesized that social defeat behavior might predict hypothalamic orexin and dynorphin loss in this study. Behaviors that were rarely shown during chronic defeat (fighting, uprights, guards and crouches at the residents face) did not predict neuropeptide loss likely due to low variability caused by their rare expression. But an animal’s propensity to submissively crouch distanced from the resident animal predicted orexin loss in this depression model. A significant relationship with dynorphin loss was not seen (−0.68, p = 0.09), but this was perhaps due to the inclusion of other ventral hypothalamus dynorphin cell populations in our sample. Further assessment of this relationship should be conducted.

**Orexin levels in mesocortical regions of the dopamine system were diminished**

Social distress predisposes depression in humans and animals alike ( Bjorkqvist, 2001; Huhman, 2006; see Miczek et al., 2011a) and appears to have a strong detrimental effect on dopaminergic brain regions. Several of the disturbances reported implicate orexin dysfunction.

For example, dampened mPFC function has been seen in anhedonic socially defeated animals and in depressed patients (e.g. see Covington et al., 2005; Mayberg et al., 2005; Bissiere et al., 2006; Konarski et al., 2007; Covington et al., 2010b; Kanarik et al., 2011). Cognitive and memory deficits (von Frijtag et al., 2000; Narayanan et al., 2011; Yu et al., 2011), as well as altered mPFC gliogenesis (Czeh et al., 2007), diminished brain-derived neurotrophic factor (BDNF) expression (Miczek et al., 2008), chromatin remodeling (Hinwood et al., 2011), and diminished pyramidal excitation and synaptic neuroplasticity have also been reported (Covington et al., 2005; Leuissus and Andersen, 2008). We show that this model has diminished mPFC levels of orexin-A and orexin-B. Locally decreased orexin-B function could cause the cognitive, pyramidal and neuropsychological deficits and dampened mPFC function described in this model (see Huang et al., 2006; Borgland et al., 2006; Wise, 2006; Lambe et al., 2007). By triggering glutamate release, orexin-B enhances excitability of pyramidal cells in the mPFC (Lambe and Aghajanian, 2003; Lambe et al., 2005; Lambe et al., 2007). And notably, pyramidal cell stimulation in the ventral mPFC diminishes depressive symptoms in animals, while their inhibition in the rostral anterior cingulate induces these symptoms (Bissiere et al., 2006; Covington et al., 2010b).

And our demonstrated mPFC orexin-A decreases could at least partially cause anhedonia in this depression model. Orexin-A has been implicated locally in reward pursuit (Davis et al, 2009) and it stimulates deep neuronal layers of the mPFC that are implicated in reward seeking (McFarland and Kalivas, 2001; Bayer et al., 2004; Xia et al., 2005). Note that we hypothesized that anhedonia could also be caused by enhanced mPFC levels of dynorphin, since it locally induces dysphoria in animals (Bals-Kubik et al., 1993). But normal levels of the peptide were expressed in the area as seen also in the prefrontal cortex of depressed patients (Peckys and Hurd, 2001).

VTA hypofunction has also been seen in anhedonic socially defeated animals (Miczek et al., 2011a) similar to other stress-induced depression models and depressed patients (Di Chiara and Tanda, 1997; Klimek et al., 2002). Diminished BDNF expression is also seen in the area (Miczek et al., 2008). Note that prolonged uncontrollable or continuous social distress appears necessary to induce both anhedonia and dampened BDNF and VTA function in the social defeat depression model; milder exposures to weeks of brief rescued social distress
actually enhances reward seeking and VTA function (Miczek et al., 2008, 2011a). Our findings provide a potential mechanism for the VTA hypofunction evidenced in this model and for the anhedonia it would induce.

For example, the hypothalamus sends a prominent orexin projection to the VTA which when stimulated promotes effort and reward motivation, including sexual interest (Muschamp et al., 2007; Aston-Jones et al., 2010; example reviews Espana et al., 2010a; Thompson and Borgland, 2011). Orexin directly activates dopamine cells in this region, although perhaps mainly by an extrasynaptic mechanism (Narita et al., 2006; Balcita-Pedicino and Sesack, 2007; Vitoz et al., 2008). Orexin also magnifies glutamatergic drive to the area (Borgland et al., 2008; Moorman and Aston-Jones, 2010). Particularly important is orexin’s diurnal amplification of mPFC glutamatergic stimulation of the VTA; an effect thought to diurnally promote motivational arousal (Moorman and Aston-Jones, 2010). Although dynorphin levels appeared minimally affected, our sexually-anhedonic defeated animals showed diminished VTA orexin expression which the above evidence indicates could cause VTA hypofunction and anhedonia symptomology.

In fact, several pieces of evidence implicate VTA orexin hypofunction in the sexual disinterest evidenced in depression. For example, orexin activates mesolimbic dopamine cells that are typically stimulated by exposure to an estrous female (Pfaus et al., 1990; Narita et al., 2006). Activation of these cells also purportedly triggers sexual pursuit (Mas et al., 1990; Louilot et al., 1991; Damsma et al., 1992; Hull and Dominguez, 2007); and orexin simultaneously activates these cells and stimulates sexual pursuit (Muschamp et al., 2007). Thus, VTA orexin loss could induce sexual anhedonia in depression by causing dopamine hypofunction. In support of this, we found decreased VTA orexin levels in a sexually-anhedonic depression model that expresses VTA dopamine hypofunction (Miczek et al., 2011a) and a copulatory disinterest that is reinstated by dopamine treatment (Sugiura et al., 1997).

A final hypothesis that was proposed in this study was that orexin and dynorphin enhancements might be seen in the accumbens in anhedonic socially defeated animals. Dynorphin locally inhibits accumbens dopamine release (see Nestler and Carlezon, 2006; Knoll and Carlezon, 2010; Alcaro and Panksepp, 2011). And notably dopamine hypofunction is seen in the area in this depression model (Miczek et al., 2011a). Furthermore, both orexin and dynorphin appear to induce dysphoria in the accumbens (Bals-Kubik et al., 1993; Terashvili et al., 2004; Sharf et al., 2008). Although evidence suggests that anhedonia could be due to changes in kappa receptor sensitivity or post-synaptic influences of dynorphin within the accumbens (Bruchas et al., 2007; Mu et al., 2011), we show that it is likely not due to changes in basal levels of dynorphin or orexin in the area.

Basal accumbens orexin changes have not been assessed in depression models prior to this study. But several labs have assessed dynorphin alterations. Unless animals were female, most concur with our finding of unaltered basal dynorphin in the area. This was shown regardless of whether the depressive-like phenotype was a natural genetic trait or induced by adolescent drug exposure or by chronic mild stress in adulthood (Bjomebekk et al., 2005; Bergstrom et al., 2008; Rubino et al., 2008). The dynorphin measure also did not matter. Prodynorphin mRNA was normal within soma throughout the accumbens in two of these depression models (Bjomebekk et al., 2005; Bergstrom et al., 2008). Also, RIA determination of accumbens tissue neuropeptide levels, which was used in the current study, showed normal dynorphin A in male anhedonic animals as we found (Rubino et al., 2008). Thus, dynorphin synthesis within accumbens cell soma as well as dynorphin-A peptide within local cells and afferents to the region appear normal in depression models.

But ELISA neuropeptide assessment detected accumbens dynorphin-A enhancements in the Wistar-Kyoto depression model (Carr et al., 2010), and similar to RIA, ELISA detects neuropeptide levels throughout the cell. Perhaps accumbens dynorphin enhancement is specific to the Wistar–Kyoto model, which differs from most depression models in its resistance to antidepressant treatment (Lopez-Rubalcava and Lucki, 2000; Tejani-Butt et al., 2003; Will et al., 2003a).

**Conclusion and relevance to depression**

This study demonstrates that an imbalance in orexin and dynorphin affective interactions between the hypothalamus and dopamine system may exist in depression. Our described mPFC and VTA orexin loss in the social defeat depression model indicates that orexin cell populations to mesocortical regions of the dopamine system may be particularly sensitive to social distress. Also, since such orexin loss could dampen both stimulus-induced and diurnal motivational arousal (Moorman and Aston-Jones, 2010), it implicates mesocortical orexin dysfunction in the anhedonia and apathy expressed by this preclinical model and in depressed patients. The orexin and dynorphin loss we describe in the hypothalamus could also cause extensive emotional dysregulation.

Unfortunately, little is known about orexin and dynorphin affective interactions. Further work that decipher opens the behavioral effect of these imbalances may clarify their interactive role in brain emotional processing related to depression. Subregional assessment of the areas addressed in this study is also needed to better localize these roles. However, we do know that orexin functional decreases can cause emotional instability (Scott et al., 2011) as clearly seen in our orexin-deficient socially defeated animals. Orexin functional increases also ameliorate depressive-like symptomology in preclinical depression models and depressed patients (DeMet et al., 1999; Lutter et al., 2008a; Ito et al., 2009). Although it will be challenging to determine how our reported orexin and dynorphin decreases parallel or compound other effects seen in this depression model including numerous gene and neuropeptide synthesis alterations (Panksepp and Watt, 2011; Miczek et al., 2011b), more effective depression treatment will not be developed without further understanding of such multifactorial interactions.
CONTRIBUTORS

Drs. Nocjar and Panksepp contributed to the experimental design, while all authors contributed to the analysis, interpretation of the results and in the writing of the manuscript. Dr. Nocjar was responsible for conducting all animal behavioral work and brain tissue extraction, and Drs. Zhang and Feng for conducting the RIA analysis.

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