Psychopharmacology

Antidepressant Activity of Pharmacological and Genetic Deactivation of the Small-Conductance Calcium-Activated Potassium Channel Subtype-3

--Manuscript Draft--

Manuscript Number:	PSPH-D-21-00206R2
Full Title:	Antidepressant Activity of Pharmacological and Genetic Deactivation of the Small-Conductance Calcium-Activated Potassium Channel Subtype-3
Article Type:	Original Investigation
Funding Information:	Canadian Institutes of Health Research — Dr. Francis R. Bambico, Dr. José N. Nobrega Natural Sciences and Engineering Research Council of Canada — Dr. Francis R. Bambico Neuroscience Catalyst (CA) — Dr. Francis R. Bambico, Dr. José N. Nobrega CAMH Discovery Fund Postdoctoral Fellowship — Dr. Mina G. Nashed
Abstract:	Rationale The voltage-insensitive, small conductance calcium-activated potassium (SK) channel is a key regulator of neuronal depolarization, and is implicated in the pathophysiology of depressive disorders. Objective We ascertained whether the SK channel is impaired in the chronic unpredictable stress (CUS) model, and whether it can serve as a molecular target of antidepressant action. Methods We assessed the depressive-like behavioral phenotype of CUS-exposed rats, and performed post-mortem SK channel binding and activity-dependent zif268 mRNA analyses on their brains. To begin an assessment of SK channel subtypes involved, we examined the effects of genetic and pharmacological inhibition of the SK3 channel using conditional knock-out mice and selective SK3 channel negative allosteric modulators (NAMs). Results We found that [125 I]apamin binding to SK channels is increased in the prefrontal cortex and decreased in the hippocampus, an effect that was associated with reciprocal levels of zif268 mRNA transcripts indicating abnormal regional cell activity in this model. We found that genetic and pharmacological manipulations significantly decreased immobility in the forced swim test without altering general locomotor activity, a hallmark of antidepressant-like activity. Conclusions Taken together, these findings link depression-related neural and behavioral pathophysiology with abnormal SK channel functioning, and suggest that this can be reversed by the selective inhibition of SK3 channels.
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Antidepressant Activity of Pharmacological and Genetic Deactivation of the Small-Conductance Calcium-Activated Potassium Channel Subtype-3

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Running Title: Antidepressant Activity of SK3 Inhibition

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Keywords: small-conductance calcium-activated potassium (SK) channel, antidepressant, chronic unpredictable mild stress, prefrontal cortex, SK3 NAM

ABSTRACT

Rationale: The voltage-insensitive, small conductance calcium-activated potassium (SK) channel is a key regulator of neuronal depolarization, and is implicated in the pathophysiology of depressive disorders.

Objective: We ascertained whether the SK channel is impaired in the chronic unpredictable stress (CUS) model, and whether it can serve as a molecular target of antidepressant action.

Methods: We assessed the depressive-like behavioral phenotype of CUS-exposed rats, and performed post-mortem SK channel binding and activity-dependent *zif268* mRNA analyses on their brains. To begin an assessment of SK channel subtypes involved, we examined the effects of genetic and pharmacological inhibition of the SK3 channel using conditional knock-out mice and selective SK3 channel negative allosteric modulators (NAMs).

Results: We found that [^{125}I]apamin binding to SK channels is increased in the prefrontal cortex and decreased in the hippocampus, an effect that was associated with reciprocal levels of *zif268* mRNA transcripts indicating abnormal regional cell activity in this model. We found that genetic and pharmacological manipulations significantly decreased immobility in the forced swim test without altering general locomotor activity, a hallmark of antidepressant-like activity.

Conclusions: Taken together, these findings link depression-related neural and behavioral pathophysiology with abnormal SK channel functioning, and suggest that this can be reversed by the selective inhibition of SK3 channels.

INTRODUCTION

Major depressive disorder is the most common mental illness, with a lifetime prevalence of up to 20% in the general population. It is a debilitating disorder mired by long-term disability and impoverished life quality. Its diagnostic criteria cover a broad range of heterogeneous symptoms with anhedonia and low mood as primary features. Despite fairly uncomplicated diagnostic guidelines and the availability of a number of treatment options, achieving therapeutic efficacy is arduous (Zisook et al., 2009; Malhi and Mann, 2018). Antidepressant medications require at least a month of continuous administration, and only a third of patients achieve remission. The rest are either chronically relapsing or completely resistant, and this oftentimes presents enormous challenges to the care of people with persistent depressive disorder and suicidal patients (Zisook et al., 2009; Bambico and Belzung, 2013; Schramm et al., 2020). Moreover, the precise etiological factors for these symptoms are multifarious.

There is a growing appreciation of the involvement of genetic and epigenetic factors, inflammatory processes and failed neuroplasticity in depression pathogenesis. It is widely acknowledged that chronic stress exposure and associated hormonal cascades powerfully unpack one or more of these factors (Bambico and Belzung, 2013; Price and Duman, 2019). However, current empirical knowledge as to how stress recruits and weighs among pathogenetic mechanisms to determine disease trajectory and progression is rather limited. Based on previous functional neuroimaging studies, computational models have highlighted a role for a widespread disarray in network dynamics and information processing throughout the mood-regulating hubs of the limbic system — the prefrontal cortex (PFC) and its subregions, the hippocampus and the amygdala (Price and Drevets, 2010; Price and Duman, 2019). These forebrain structures harbor a high density of glucocorticoid receptors, and are therefore particularly sensitive to phasic and tonic activity of the hypothalamic-pituitary-adrenal (HPA) axis, which comprises the stress response arsenal of the mammalian nervous system.

The molecular mechanisms mediating stress-induced disarray in activational patterns are not fully understood. Regional cortical metabolic and excitatory disturbances were indicative of impaired expression of inhibitory elements (Gargus, 2006; Faber and Sah, 2007; Faber and Sah, 2010). Among the most heterogeneous and ubiquitous inhibitory ion channels, the small-conductance, calcium-activated potassium channel subfamily (SKC/KCa2) is known to generate the medium afterhyperpolarization of neurons following the action potential peak. Three subtypes of SK channels have been identified and cloned (SK1/KCa2.1 to SK3/KCa2.3) driven by the genes Kcnn1-3. All three subtypes share similar principal molecular architecture as the Shaker-like voltage-gated potassium channels. Each subunit has six membrane-spanning hydrophobic alpha-helical domains. Three associated subunits, the protein phosphatase-2A, casein kinase-2 and calmodulin elements

participate in the allosteric modulation of the channel conductance. The calmodulin subunit is bound to CaMBD, a domain in the C-terminus, and accounts for the sensitivity to calcium transients within intracellular microdomains (Faber, 2009; Faber and Sah 2010; Kshatri et al., 2018). This endows the channel with a unique ability to couple intracellular calcium concentration with low pico-Seimens changes in potassium conductance and membrane potential. Calcium gating via postsynaptic muscarinic or N-methyl-D-aspartate receptors can therefore also modulate SK channel activity (Ngo-Anh et al., 2005; Faber, 2010; Giessel and Sabatini, 2010). This in turn regulates postsynaptic potentials, burst-firing activity, inter-spike interval distribution and spike frequency adaptation. It is through these mechanisms that SK channels mediate some forms of activity-dependent and long-term potentiation-like plasticity that affects limbic behavioural function and stress adaptation (Faber and Sah, 2007, 2010; Faber, 2009; Kshatri et al., 2018).

An interest in therapeutically targeting the different SK channel subtypes has gained traction in recent years. First, there has been a growing appreciation of the distinct functional effects associated with SK1, SK2 and SK3 channels (Strøbaek et al. 2006; Lujan et al. 2009; Deignan et al. 2012). Second, previous preliminary human studies have implicated the SK3 channel in depressive and cognitive disorders and in aging (Chandy et al 1998; Jones et al. 2002; Ujike et al. 2001; Tomita et al. 2003). Third, SK1-3 are expressed in structures implicated in depression and in rapid antidepressant response, e.g., the cingulate cortex and the serotonin-producing raphe nuclei (Stocker and Pedarzani 2000; Tacconi et al. 2001; Sailer et al. 2002; Sailer et al. 2004). Fourth, evidence from preclinical animal models of depression has pointed towards SK3 overexpression or hyperactivity in the raphe and prefrontal cortex (Sargin et al. 2016; Qu et al. Theranostics 2020; Bambico et al. 2020). Lastly, while the prototypical, naturally occurring subtype-nonselective SK ligand, apamin, has a narrow therapeutic window, later development of compounds with variable SK subtype affinities have yielded better safety profiles favoring therapeutics (Shakkottai et al. 2001; Strøbæk et al. 2006; Sorensen et al. 2008).

In a rodent chronic unpredictable stress (CUS) model, drug-mediated blockade of SK channel conductance, instigated by muscarinic receptor inhibition, effectively led to depolarization-induced plasticity detected in the prelimbic (PrL) subregion of the medial prefrontal cortex (mPFC). This effect was associated with a rapid antidepressant-like response (Bambico et al., 2020). By contrast, stress-induced glucocorticoid release modulates calcium mobilization and rapidly enhance the transcription and expression of SK channels via glucocorticoid type II receptors (Shipston et al. 1996; Tian et al., 1998; Levitan et al., 1991). In addition, evidence for epigenetic regulation under pathological conditions has also been recently found (Cadet et al., 2017). As such, SK channels and associated elements may serve as therapeutic molecular targets for rapid and effective relief of stress-induced conditions such as depression.

Here we examined the possibility that increased activity of SK channels could serve as an intermediary mechanism that links depression-related pathophysiology induced by CUS exposure to impaired neuronal transmission in the PFC. Using SKC conditional knock-out mice and pharmacological approaches, we ascertained that targeting SK3 can indeed convey potent antidepressant activity.

MATERIALS AND METHODS

Animals

All procedures conformed to the guidelines of the Canadian Council of Animal Care, the Canadian Institutes of Health Research and the Institutional Animal Care Committee of the Centre for Addiction and Mental Health (CAMH). All rats were obtained from Charles River (Ontario, Canada) and weighed 210-220 grams at the start of experiments. Rats were single-housed and kept under standard vivarium conditions (12-hour light–dark cycle, lights on at 07:30; temperature at 20 ± 2 °C; 50–60% relative humidity) Adult male Fischer 344 rats (n=8 per group) were used to understand CUS pathophysiology as they are most responsive to this paradigm (Wu and Wang, 2010). Adult male Sprague-Dawley rats (n=4-8 per group) were used for the drug infusion experiments in the absence of any stressors. This experimental strategy allowed us to first establish the involvement of SK channels in the neurobiological mechanism underlying a stress-induced depressive-like state. To this end, CUS animals were assessed on a comprehensive battery of behavioural tests, followed by investigation of SK channel levels in key brain regions using [^{125}I]apamin autoradiography. Subsequent to establishing the involvement of SK channels in the CUS model, novel SK channel negative allosteric modulators (NAMs) were screened for antidepressant-like activity using the forced swim test (FST). While the FST is often used as part of a battery of tests in chronic stress models of depression, the primary utility of this test is in its predictive validly. Indeed, the FST alone (in the absence of other stressors) is acutely sensitive to compounds with known antidepressant effects in humans, as well as being insensitive to ineffective compounds (Willner, 1984; Can et al., 2012). Thus, while the FST does not represent construct or face validity for depressive phenotypes, it is the gold standard behavioural test for screening novel compounds for potential antidepressant-like effects (Commons et al., 2017). Using the FST in this way allowed us to efficiently screen multiple novel NAMs at various doses and establish their potential antidepressant utility through a predictive assay. Mice harboring mutations in the SK3 (Kcnn3) gene were also used. The embryos were cryo-recovered at Jackson Laboratory (CT, USA): B6.129S4- Kcnn3^{tm1Jpad}/J; tTA-tetO. The tetracycline-controlled trans-activator protein (tTA) as well as the tetracycline operator (*tetO*; also called tetracycline-responsive element [TRE] or tet-

operator) is inserted upstream of the translation initiation site into the 5' UTR of the Kcnn3 locus. This conditional mutation allows for blockade of SK3 expression by administration of tetracycline (or its analog doxycycline [dox]) in SK3 tTA homozygotes. The SK3 tTA colony was maintained by inbreeding sexually mature (12 weeks or older) male and female WT or heterozygous mice. Breeding pairs were maintained for a 30-week rotation before being retired. Offspring were weened at 4 weeks of age, at which point male and female offspring were housed separately to a maximum of 5 mice per cage. After weening, tissue samples were obtained from the tails of each animal for RT-PCR genotyping. Male offspring were used for behavioural experiments (WT n=10, Het n=11, Hom n=13) during adulthood (12-24 weeks). Two weeks prior to behavioural testing, WT and homozygous mice were given *ad libitum* access to chow containing 0.0625% dox (Catalogue #1813583-203; TestDiet, St. Louis, MO, USA), which delivers 2-3 mg/day of dox. This diet was maintained throughout behavioural testing. The dose of dox chosen is commonly used in similar conditional mutation models where the target tissue has poor penetration (i.e., the brain) to ensure maximal induction (Redelsperger et al., 2016). For these experiments, the genetic manipulation was the only form of intervention with no stressors applied to the mice. For this set of experiments, we conceptualized the genetic KO of SK3 similar to a drug intervention, with FST and novelty-induced hypophagia (NIH) assessments as valid and sensitive predictors of antidepressant-like response of SK3 KO.

Chronic Unpredictable Stress (CUS) paradigm

Rats were subjected to mild, unpredictable and uncontrollable stressors as described previously (Willner, 2005; Bambico et al., 2020). Figure 1a describes the timeline of procedures. After four days of acclimatization following arrival, the animals were exposed to a bottle of sucrose solution (1% w/v) ad libitum for three days. This was then followed by a discrimination training, where the sucrose bottle was accompanied by a water bottle. The final SP measurements were used as baseline.

To induce depression-relevant behaviours, three or four stressors were given daily for five weeks. The combination of stressors is based on stress intensity/duration and sequence unpredictability. For example, in the first week, the following schedule was used:

Day 1: 09:00-12:00 - cage tilt (3 hours), 15:00-17:00 - cold room (2 hours), 20:00-23:00 - high-frequency sound (3 hours), 20:00-08:00 - food deprivation (12 hours).

Day 2: 11:00 – intraperitoneal saline injection (acute), 14:00-17:00 - novel environment (3 hours), 19:30-19:30 - light cycle reversal (lights on for 24 hours).

Day 3: 09:00-12:00 - predator odor (3 hours), 15:00-15:30 - restraint (30 min), 18:30-21:30 - static noise (3 hours), 23:00-07:30 – stroboscopic light (8.5 hours).

Day 4: 09:00-12:00 – high-frequency sound (3 hours), 15:00-18:00 – novel environment (3

hours), 21:00-09:00 – water in cage (12 hours).
Day 5: 12:00-15:00 – cage tilt (3 hours), 18:00 – intraperitoneal saline injection (acute), 21:00-09:00 – empty water bottle (12 hours).
Day 6: 11:00-14:00 – predator odor (3 hours), 17:00-20:00 – stroboscopic light (3 hours), 23:00-23:30 – restraint (30 min).
Day 7: 09:00-11:00 – cold room (2 hours), 14:00-17:00 – static noise (3 hours), 20:00-08:00 – cage tilt (12 hours).
Note that in the aforementioned schedule, an additional allowance of one hour was allotted for any logistical requirements and preparations needed for the stress exposures. The complete list of stressors, their duration, combinations and descriptive details are presented in Table 1 (previously employed or modified after Bambico et al., 2019; Bambico et al., 2020). No stressors were given during the behavioural tests. Stress-naïve controls (CTR) were left undisturbed in a separate room. Behavioural data for CTR and CUS animals were analyzed offline using an automated behavioural videotracking/quantitation system (Videotrack, Life Sciences, Canada).

Behavioural Testing

Sucrose preference test (SPT). After 24 hr of water deprivation both CUS and CTR rats were individually placed in a test cage. They were allowed to discriminate and select between two drinking bottles for one hour, one containing 1% sucrose solution (w/v) and the other tap water (see Bambico et al., 2019; Bambico et al., 2020). The position of the bottles on the cage top cover was switched midway to minimize directional bias. SPT measurements were carried out once a week for five weeks. A sucrose preference index was defined as a ratio of sucrose intake to total fluid intake. The SPT was not conducted during other post-CUS tests.

Forced swim test (FST). Rats were placed in a 25-27^{o}C water-filled Plexiglas cylinder (20 cm diameter, 50 cm high, water depth of 20 cm) as described previously (Porsolt et al., 1977; Bambico et al., 2007). Passive and active coping behaviour (frequency and duration of immobility, swimming and climbing episodes) were recorded for 5 min after 15-min pre-exposure 24 hr earlier. The FST was conducted towards the end of the light phase and under minimal anxiogenic conditions (Bambico et al., 2007). Animals were then removed from the water cylinder, dried with a towel and placed in a cage over a heat source. The videotracking system was calibrated to consider the rat to be immobile when making only movements necessary to keep the head above the water, exert slow limb movements during swimming, and more forceful struggling during climbing. A similar procedure was used to test mice in the FST. The water-filled cylinders for mice were 18 cm in diameter and 30 cm high (filled to 20 cm). Passive and active coping behaviour for

mice was recorded for 4 minutes with no pre-exposure, and analysed offline using the videotracking system.

Novelty-suppressed feeding test (NSFT). The procedure was as described earlier (Bodnoff et al., 1989; Bambico et al., 2020). Rats were placed in a novel chamber (80x80x50 cm) or home cage after 36 hr of food deprivation. Latency to approach the center and feed was recorded after 12 regular chow food pellets were placed at the center of arena. Cut-off time was 600 s.

Novelty-induced hypophagia test (NIH). The NIH is a modification of the NSFT that replaces standard chow with a sweetened palatable food, and eliminates the need for food deprivation while similarly assessing anxiety-like behaviours (Dulawa et al., 2004). 24h prior to testing, mice were habituated to eating a palatable sweetened food (Froot Loops; Kellogg's, MI, USA) by placing 2 Froot Loops for each animal in the home cage. On the test day, mice were placed in a novel chamber (50x50x30 cm) or home cage and latency to approach the center and feed was recorded after 4 Froot Loops were placed at the center of arena. Cut-off time was 300 s.

Social interaction test (SIT). As described in Bambico et al. (2020), an unfamiliar conspecific was placed in a plastic grid cage (30 cm^3) against the wall on one end of an arena measuring 80x80x15 cm. The test animal was placed in the opposite end of the arena, and the amount of time the animal spent in areas distal and proximal to the cage was recorded. The total time spent investigating the partner animal through sniffing and contact with the cage was also assessed.

Open Field Test (OFT). As described in Bambico et al. (2020), after 5 min of habituation, locomotor activity (total distance travelled) was recorded for 5 min in a 50x50x30 cm polycarbonate open field chamber (Med Associates Inc., St. Albans, Vermont). Rats and mice underwent identical protocols on the OFT.

Pharmacological interventions

Three compounds from the series originally described in Sorensen et al. (2008) were chosen on the basis of their reported affinity for SK3 receptors and synthesized anew. We refer to these as ABD1114 (Sorensen compound 42, reported IC_{50} = 61 nM); ABD1115 (compound 37, reported IC_{50} = 34 nM); ABD1144 (compound 34, reported IC_{50} = 17 nM). Chemical structures (Sorensen et al, 2008) are shown in Figure 2. To ascertain their potential antidepressant activity, these compounds were administered directly into the cerebral ventricles immediately prior to behavioural tests. The intracerebroventricular (ICV) route was initially chosen in order to bypass potential brain

penetration problems. All compounds were dissolved in vehicle containing 5% polyethylene glycol (PEG), 5% Tween® 80, and 0.25% dimethyl sulfoxide (DMSO). ICV cannulae were surgically implanted targeting the left ventricle: anteroposterior (AP) = -1.0 mm, mediolateral (ML) = 2.0 mm to the left, dorsoventral (DV) = 3.5 mm from the skull surface. Following 1 week of post-surgical recovery, rats received drug or vehicle infusions with a 2 μl volume delivered over 3 min through a 27-gauge needle connected to an infusion pump. The needle was left in place for an additional 3 min to allow complete diffusion of the infusate. Behavioural testing commenced 5 minutes after drug infusion. The concentration of each compound was adjusted such that all infusions were delivered at the same volume of 2 μl in vehicle. In the initial dose-response set of experiments, the range of doses were estimated based on IC_{50} values and common ranges used in ICV drug administration. Based on this set of experiments, a re-test of ABD1114 and ABD1115 was conducted at an increased dose.

Biochemical Experiments

After determining the time course of CUS-induced behavioural effects, a separate cohort of CTR and CUS animals was used to assess CUS-induced brain changes. Rats were sacrificed by decapitation and brains rapidly removed, frozen over dry ice and stored at -80 °C. Unfixed coronal brain sections (20 μm) cut on a Leica cryostat at -20 °C, thaw-mounted onto Fisher Superfrost™ slides VWR, Mississauga, ON), and stored at -20 °C until processing.

SK channel binding (autoradiography). For quantitative autoradiography, slides were thawed and incubated for 30 min at 4 °C with 100 pM [^{125}I]apamin (2200 Ci/mmol, New England Nuclear) in 100 mM Tris-HC1 buffer (pH 7.4) containing 0.1% bovine serum albumin. Non-specific binding was defined by 1 μM unlabeled apamin in adjacent slides. Incubated sections were serially washed with buffer (4 °C), TCA and distilled water (4 °C). They were then dehydrated in 70% ethanol and air dried (10 sec). The slides were then exposed to Kodak BioMax film at 4 °C for 1 week along with calibrated radioactivity standards to convert optical densities on film into μCi/gT.

In situ hybridization of zif268, an immediate early gene marker of neuronal cell activity. In situ hybridization proceeded as previously described (Mansourian et al., 2020; Volle et al., 2018). Briefly, prior to hybridization sections were fixed in 4% paraformaldehyde for 5 min at room temperature and rinsed in 10 mM phosphate buffer (pH 7.4) containing 150 mM NaCl (2 x 2 min). Slides were then incubated overnight at 60 °C with [^{35}S]UTP labeled riboprobes (200,000 cpm/μL prepared by *in vitro* transcription using consensus promoter sequences for T7 RNA polymerase and cDNA sequences complementary to bases 660–679 (5′- tcacctatactggccgcttc-3′) and bases 1062–

1043 (5'- aggtctccctgttgttgtgg-3') according to GenBank # NM_012551). The sections were then washed in 4× SSC at 60 °C, immersed in RNase A solution (Sigma Aldrich, St. Louis, USA) dehydrated in 70% ethanol, and air-dried. The slides were then exposed to Kodak BioMax film for 6 days at 4 °C along with calibrated radioactivity standards.

Film analyses. Films for SKC binding or in situ hybridization were developed in an automated Konica SRSX-101A film processor. Quantification was performed on coded films using an MCID Elite system (InterFocus Imaging, Linton, UK). Brain regions of interest were defined according to the atlas of Paxinos and Watson (1998). Standard curves obtained from calibrated radioactivity standards were used to convert raw optical density values to radioactivity levels in microcuries per gram of tissue (μCi/gT). Densitometric readings were first averaged across all sampling windows in a section and then across all sections to produce a final density value for each region for each animal.

Statistical Analysis

The data are presented as mean ± standard error of the mean (SEM), and subjected to t-tests, one-way or two-way mixed design analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons, as appropriate. For SPT and FST, treatment groups were between-subjects factor and time (week or day) as a within-subjects factor. For NSFT, treatment groups were between-subjects factor and environment (novel vs. home) as within-subjects factor. A p value $\leq$ 0.05 was considered as statistically significant.

RESULTS

CUS increased depressive-like reactivity

CUS, a well-established etiological model, reproduced a cluster of depressive-like behaviours (n=8 per group). CUS animals displayed an anhedonia-like reduction in sucrose consumption in a 1-hr preference test (SPT) (sucrose preference, SP score ~60% after 3-5 weeks of exposure vs. ~90% in CTR $p<0.01$; Figure 1b). CUS animals also exhibited an anxious depressive-like sensitivity to novelty in the NSFT as indicated by an increased latency to feed in a novel environment (+150% of CTR, $p<0.01$ (Figure 1c), while both CUS and CTR animals behaved similarly in the home cage environment. In the forced swim test (FST), CUS animals showed prolonged passive-like immobility (+100% of CTR; $p=0.005$; Figure 1d) and had significantly lower total swimming during the test (50% of CTR; $p = 0.005$; Figure 1d). We observed decreased social behaviour in CUS animals in the social interaction test (SIT). Time spent investigating a novel conspecific in the proximal area (-30% of CTR), as well as contact duration (-57% of CTR) were

also significantly lower in CUS animals (p=0.042 and p=0.013, respectively) (Figure 1e).

CUS altered SK channel binding and zif268 mRNA levels in the limbic forebrain

Exposure to CUS induced upregulation of [^{125}I]apamin binding to SK channels in the prelimbic (PrL) subregion of the mPFC (37%) (p=0.006) and hippocampal CA1 region (17%) (p<0.036), respectively (Figure 2a; n=6-8 animals per group; 2-4 sections/structure; 5-10 sampled micro-areas/structure). No differences in other brain areas were observed between CTR and CUS animals.

In the PrL mPFC, CUS-exposed animals had significantly lower levels of *zif268* mRNA than non-stressed controls (p<0.05; Figure 2b). However, in the dorsal hippocampus (dHPC) and ventral hippocampus (vHPC), *zif268* mRNA expression was significantly higher in CUS animals compared to the CTR animals. The rest of the brain areas had similar expression of *zif268* mRNA in both CUS and CTR animals (n=6-8 animals per group; 2-4 sections/structure; 5-10 sampled micro-areas/structure).

Genetic or pharmacological inhibition of SK3 decrease immobility in the FST

Conditional KO in SK3 tTA mice induced an antidepressant-like phenotype (Figure 3) consistent with what has previously been published for this mutant strain (Jacobsen et al. 2008). In the FST indicated significant genotype effects ($F_{2,33}$ = 4.88, p= 0.014; Figure 3a), with significantly lower immobility durations in SK3 homozygotes (hom, n=13) when compared to the wildtypes (WT, n=10) and heterozygotes (het, n=11) (p<0.005 and 0.05, respectively). In a 5-min open field test, homozygous mice showed a non-significant 25% increase as compared to wildtypes (p = 0.09; Figure 3b). In male SK3 homozygotes, this potential increase in ambulatory activity may be explained by light conditions during testing. It was previously shown that male SK3 homozygotes show increased ambulatory activity under 20 lux lighting, but decreased ambulatory activity under 100 lux lighting, when compared to wild-types (Jacobsen et al. 2008). Consistent with an antidepressant-like phenotype, homozygotes also showed a non-significant decrease in latency to feed in the novel environment phase of the NIH (63% of wild-type; p = 0.07; Figure 3c), while both homozygotes and wild-types behaved similarly in the home cage environment.

Experimental compounds with high affinity for the SK3 channel subtype were tested in the FST 5 min after ICV infusions. ABD1144 infusion resulted in dose-dependent decreases in immobility in the FST at nanogram doses (Figure 4d). ABD1114 infusions resulted in decreases in immobility in the FST in a non-dose-dependent manner at picogram doses (Figure 4b), whereas ABD1115 given in picogram amounts resulted in a subtle downward trend but without statistical significance (Figure 4c). Given its lack of effects at sub-nanogram doses, the experiment with ABD 1115 was then repeated at 10 ng in a fresh cohort of animals, using ABD1114 given at the same dose as a positive control. One-way ANOVA on immobility duration in the FST confirmed significant treatment effects ($F_{2,20}$=19.57, p<0.01, n=7 per group) with significantly lower immobility durations in both drug treatment groups (Figure 5a) when compared to vehicle-treated controls. In

this re-test, ABD1144 was not included as it did not initially show a significant effect at 10 ng, with significant effects for this compound being observed at 30-100 ng. As in previous trials, these treatments in the re-test did not result in locomotor activity changes in the open field (Figure 5b), again suggesting that antidepressant-like effects of these compounds in the FST were not due to unspecific motor activation by the compounds.

DISCUSSION

The main findings of this study were as follows. First, rats displaying depression-relevant symptoms after exposure to chronic unpredictable stress showed increased binding to SK channels in limbic regions of the brain as measured by [^{125}I]apamin receptor autoradiography. Since [^{125}I]apamin does not distinguish between the three SK channel subtypes, subsequent work focused on the SK3 subtype as a possible candidate, based on preliminary work on SK transgenic mice and on SK3 being implicated in depressive and related psychiatric disorders (Tomita et al., 2003; Jacobsen et al. 2008; Smolin et al., 2012; Imbrici et al., 2013). Mice with partial or total genetic ablation of SK3 expression in brain were found to have a depression-resistant phenotype when subjected to the forced swim test (FST) and novelty-induced hypophagia (NIH) test. When experimental compounds with high affinity for the SK3 subtype were tested, they were found to induce antidepressant-like effects when administered directly into the brain ventricular system.

CUS and other pro-depressive paradigms have been shown to variably alter the activity of limbic forebrain neuronal subpopulations, increasing for example the excitability of amygdaloid principal neurons (Buffalari and Grace, 2009) and brainstem noradrenergic neurons (Jedema and Grace, 2003) and decreasing those of ventral tegmental area (VTA) dopaminergic neurons (Moore et al., 2001; Chang and Grace, 2014) and dorsal raphe serotonergic neurons (Bambico et al., 2009; Veerakumar et al., 2014). While the exact molecular mechanisms underlying these differential effects remain a matter of conjecture, there are indications that they invoke a complex interplay of stress-induced channelopathies. Since SK channels, known to regulate neuronal activity, are abundantly expressed in regions involved in emotion regulation and stress adaptation, notably the PFC and the hippocampus (Stocker and Pedarzani, 2000), we characterized their distribution patterns in these and other brain regions. We found CUS effects that are consistent with known region-dependent neurophysiological abnormalities in depression-related phenotypes. We have also determined that pharmacological and genetic deactivation of SK3 could lead to antidepressant-like effects.

CUS exposure resulted in depressive- and anxiety-like behaviours, which were accompanied by increases in overall SK channel binding in key corticolimbic regions. This impact of CUS is likely

attributable to increased allostatic load and hyperactivation of the HPA stress axis. The resultant modulation of glucocorticoids, including corticosterone, has been shown by others to instigate glucocorticoid receptor II-mediated intracellular cascade leading to the synthesis and insertion of potassium channels or their subunits into the plasma membrane (Shipston et al., 1996; Tian et al., 1998; Levitan et al., 1991). Because of the ubiquitous expression of potassium channels and their direct involvement in neurotransmission, it is no surprise that they have been intimately implicated in stress-related disorders such as depression, as also confirmed by preclinical genetic (Liou et al., 2009; Smolin et al., 2012; Imbrici et al., 2013) and behavioural studies (Heurteaux et al., 2006; Sargin et al., 2016; Qu et al., 2019; Bambico et al., 2020).

In our hands, the observed SK channel overexpression in the PrL, with hyperpolarizing consequences, was associated with a significant decrease in *zif268* mRNA, indicating cell hypoactivity. This corroborates other studies that show decreased expression of immediate early gene products of cell activation and cell firing activity in the PrL and other related corticolimbic regions using various chronic depression paradigms (Matsuda et al., 1996; Westenbroek et al., 2003; Bambico et al., 2009; Rosenkranz et al., 2010). Some aspects of PrL neurotransmission, e.g., pyramidal excitation and LTP-like plasticity, have been linked to activity-dependent neuroplasticity, neurotrophic repair, gliogenesis and network reorganization (Price and Duman, 2020). CUS-induced SK channel and *zif268* effects likely undermine activity-dependent plasticity, and are possibly crucial contributing factors to cortical atrophy and synaptic shrinkage associated with depression and stress-related symptoms (Hains et al., 2009; Radley et al., 2013; Negrón-Oyarzo et al., 2014). In turn, SK channel-induced PrL deactivation could weaken PFC inhibitory control over depression/stress-related hyperexcitability of the infralimbic cortex (considered a homologue of the mPFC in rodent), HPA axis (Vertes et al., 2004; Covington et al., 2010; Fuchikami et al., 2015; Hare and Duman, 2020; Radley et al., 2013), as well as other limbic-modulating structures, e.g., monoaminergic (Bambico et al., 2009) and amygdaloid nuclei (Holmes, 2008). CUS-induced SK channel and *zif268* effects could therefore conceivably initiate the progressive allostatic overload that is observed in depression, underscoring the chronic and relapsing nature of the disorder. These data lead to the notion that SK channel activation may serve as molecular switch that triggers the progressive deterioration produced by rumination and impaired appraisal of controllability, adaptation and flexibility (Granon et al., 2000; Amat et al., 2005; Maier and Watkins, 2010; Varela et al., 2012).

Contrary to the effects observed in the PrL, CUS exposure resulted in an increase rather than a decrease in hippocampal *zif268* expression. While we do not have a definite explanation for this observation, it is possible that this region-specific effect may have occurred as a result of differential SK channel distribution across different cell subpopulations and cell compartments

(dendritic versus somatic localization) which are functionally linked to divergent excitatory-inhibitory biochemical pathways (Bock et al.,2019). For instance, since hippocampal GABAergic neurons copiously contain SK channels, SK overexpression could result in decreased GABA transmission and increased overall intrinsic hippocampal excitability. Indeed, it has been shown that elevated hippocampal activity, particularly the ventral hippocampal – accumbal projections can predict stress/depression-related vulnerability (Muir et al., 2020).

Having established that impaired PFC SK channel activity may be crucial to depressive-like symptoms, it seems reasonable to suggest that SK channels may be targeted to elicit an antidepressant response. Because of the channel's direct involvement in the regulation of neuronal activity, the use of SK channel inhibitors can potentially elicit a fast and robust antidepressant activity. Furthermore, the pico-Siemens conductance of the channel will forge better dose-dependent titratability of therapeutically relevant effects on regional metabolic activity and activity-dependent plasticity. Preliminary genetic studies have indicated that among the three SK channel subtypes, SK3 has been shown to be involved in the cognitive and affective symptoms of stress-related disorders, including bipolar disorder and schizophrenia (Chandy et al 1998; Jones et al. 2002; Ujike et al. 2001; Tomita et al., 2003; Smolin et al., 2012; Imbrici et al., 2013). Moreover, rodent models of chronic depression, e.g., chronic isolation, lead to overexpression of SK3 channels corticolimbic structures, e.g., the midbrain raphe (Sargin et al., 2016). Indeed, the current data show that doxycycline treatment of SK3 tTA mice, which nullified the expression of SK3 channels completely in homozygotes and partially in heterozygotes, displayed a depression-resistant phenotype in the FST and NIH, consistent with previous reports (Jacobsen et al. 2008). Complete SK3 deletion in homozygotes led to significantly shorter FST immobility duration when compared to their WT counterparts, as well as a trend towards decreased latency to feed in the NIH.

In the FST, we tested the antidepressant activity of three SK channel-acting drugs with preferential affinities for SK3 over SK1 and SK2 (Sorensen et al. 2008). ICV infusion of the SK3 negative allosteric modulators (NAMs), ABD1114, ABD1115 and ABD1144 led to dose-dependent attenuation of immobility when compared to the vehicle. As locomotor activity in the OFT was unaffected, this enhancement in FST activity suggests robust antidepressant activity.

There are indications that SK3 channels are expressed in PFC (PrL) and hippocampal GABAergic interneurons and in monoaminergic neurons, without any significant extent of overlap to SK1 and SK2 channels (Tacconi et al., 2001; Sailer et al., 2002; Martin et al., 2017). Both GABAergic and monoaminergic neurotransmission are compromised in depressive disorders (Bambico and Belzung, 2014; Malhi and Mann, 2018; Prevot and Sibille, 2020). We therefore surmise that SK3-NAMs' antidepressant action may be mediated by these mechanisms. The PFC-raphe-hippocampal/amygdala circuit has been implicated in depression. Antidepressant stimulation

of serotonergic neurons in the raphe nucleus results in increased serotonin in the hippocampus, amygdala and PFC (Holmes, 2008; Price and Drevets, 2010; Bambico and Belzung, 2013; Hare and Duman, 2020). More recently, stimulation of PrL glutamatergic neurons and deactivation of the same in IL have been proposed to mediate the rapid antidepressant action of ketamine (Fuchikami et al., 2015). We propose that an antidepressant response via SK3 channel antagonism can be achieved by stimulation of the PFC-raphe-hippocampal/amygdala circuit in a number of ways. First, depression-like pathophysiology is associated with increased raphe SK3 channels, decreasing the intrinsic excitability of serotonergic neurons (Sargin et al., 2016). Therefore, SK3 inhibition conversely activates these neurons, increasing or normalizing the release of serotonin in postsynaptic sites in the hippocampus, amygdala and PFC. Second, IL pyramidal activity is hyperactive in depression models (Fuchikami et al., 2015; Price and Duman, 2020) leading to aberrant glutamatergic activation of downstream limbic projection sites. SK3 channel inhibition in IL GABAergic interneurons activates them, enhancing GABA release and inhibiting IL pyramidal neurons. In turn, decreased glutamatergic input from the IL to raphe local GABAergic interneurons will disinhibit and stimulate serotonergic neurons. Third, in parallel, decreased glutamatergic input from the IL to the PrL will stimulate resident PrL pyramidal neurons, which could curtail the cognitive symptoms associated with depression. Lastly, although not directly tested in the current set of experiments, we hypothesize that in chronic stress models, SK3-NAMs will directly act on SK channels that are overexpressed in the PrL, likewise normalizing PrL activity. We have previously shown that intra-PrL infusion of apamin resulted in a rapid antidepressant action (Bambico et al., 2020) suggesting a paramount role of this PFC structure in this effect of SK antagonism. Recapitulating this local dorsomedial PFC/PrL effect with SK3 NAMs is under way. We also cannot preclude possible contributions of SK3 in other corticolimbic structures, e.g., the nucleus accumbens, which may be involved in the hedonic disturbances in depression (Bambico and Belzung, 2013). Indeed, SK3 is prominent in subcortical areas, notably in the striatum and nucleus accumbens (Stocker and Pedarzani 2000; Tacconi et al. 2001; Sailer et al. 2002; Sailer et al. 2004), and our data on apamin binding and *zif268* mRNA levels have shown non-significant but noticeable changes in these and other limbic structures.

The concerted action on these multiple antidepressant-related pathways could convey robust therapeutic effects. To build upon our current results, the pharmacodynamics and pharmacokinetics of SK3-NAMs should also be explored for potential utility in systemic administration routes at reasonable doses. Future investigations should explore the most promising SK3-NAMs, as well as SK3 homozygotes, in chronic stress models to further validate the construct and face validity of targeting SK3 channels for antidepressant-like effects.

In summary, the current findings suggest that impaired activity-dependent plasticity and

decreased PFC neurotransmission after CUS exposure may be instigated by abnormal overexpression of SK channels, likely via genomic mechanisms linked to prolonged glucocorticoid receptor activation. The antidepressant activity observed after genetic and pharmacological SK3-NAM-mediated deactivation of SK3 channels points towards the feasibility of developing novel therapeutics for the treatment of depression and other stress-related disorders.

Funding and Disclosure

This research was supported by awards from the Neuroscience Catalyst program (Toronto) (FRB and JNN), the Canadian Institutes of Health Research (FRB and JN) and the National Science and Engineering Research Council of Canada (FRB). M.N. was additionally supported by a CAMH Discovery Fund Post-doctoral Fellowship.

Conflict of Interest: None declared.

Acknowledgments

We thank J. Li, U. Mumtaz, S. Khan, S. Sivaruban, M. Billyard, E. Hauck, D. Oleinichenko, Michael Coombs and Lucas Francis Fowler for technical assistance at different stages of the work.

Author Contributions

MGN and FRB performed the main experiments and analysed the data. SW, RR, DN, MW, JZ and HL assisted in conducting some aspects of the experiments. SW, NH and OCD assisted in data curation. IRG contributed in drug synthesis. FRB and JNN wrote the manuscript. MGN, IRG and JNN revised the manuscript.

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Figure Captions

Figure 1: Top: CUS protocol and sucrose preference test (SPT) results. (a) Time line of procedures. Dy0-Dy12 (Day0-12, sucrose discrimination training), Wk0-Wk4 (Week0-Week4), chronic unpredictable stress (CUS) exposure), with once-a-week, 1-hr SPT. Dy0-Dy2, behavioural tests (coat state measurements, NSFT, SPT, OFT, FST, SPT). Dy2-Dy5, zif268 in situ hybridization (ISH) and SK channel (SKC) autoradiography (ADR). (b) Progression of SP (%) from baseline (Wk0) to Wk5. Data points and bars indicate mean SP±SEM; **, $p<0.01$; n=8 per group.

Bottom: CUS effects on other depression-relevant behaviour; comparison between controls (CTR) and stressed (CUS) animals. (c) Latency to feed (cut-off, 600s) in the novel environment and home cage. (d) Immobility, swim and climbing duration in the forced swim test (FST). (e) Social investigation with caged conspecific, measured as total duration of visits within the area proximal to chamber and physical contact (sniffing) with conspecific. Bars represent mean±SEM; *$p<0.05$; **, $p<0.01$; n=8 per group.

Figure 2: (a) CUS effects on [^{125}I]apamin binding to SK channels. Left: CUS increased SK channel binding in the prelimbic (PrL) medial prefrontal cortex (mPFC) and in the hippocampal CA1 subregion. Right: Representative photomicrographs of CUS and CTR brain sections showing the PrL and the cingulate (Cg) subregions of the mPFC (top row) and the hippocampus (bottom row). Bars represent means ± SEM; +$p<0.05$, ++$p<0.01$; n=6-8 animals/group; 2-4 sections/structure; 5-10 sampled micro-areas/structure.

(b) CUS effects on *zif268* mRNA levels. In situ hybridization for *zif268* mRNA showed that CUS decreased mRNA levels in the prelimbic (PrL) and increased levels in dorsal and ventral hippocampi (dHPC, vHPC). Bars represent mean ± SEM; *p<0.05; n=6-8 animals/group; 2-4 sections/structure; 5-10 sampled micro-areas/structure.

Figure 3: Depression-resistant phenotype in SK3 tTA mice after doxycycline-induced knockout of SK3 channels; comparison between wild-type (WT) and homozygous mice (Hom). (a) Total immobility in the forced swim test (FST). (b) Total distance traveled in the open field test. (c) Latency to feed in the novel environment and home cage during the novelty-induced hypophagia test (NIH). Bars represent means ± SEM; ***p<0.001; n=10-13 per group.

Figure 4: (a) Structure of test compounds: negative allosteric modulators (NAM). (b, c, d) Antidepressant-like activity of SK3 channel negative allosteric modulators (SK3-NAM) in rats. Total immobility duration the forced swim test (FST)after intracerebroventricular infusion of varying concentrations of SK3-NAMs: ABD1114, ABD1115 and ABD1144. Insets: Distance travelled (cm) in the open field. Bars represent means ± SEM; **, p<0.01; ***p<0.001; n=4-8 per group.

Figure 5: Re-test of two SK3 channel negative allosteric modulators (SK3-NAM) in the forced swim test (FST) at optimal doses. Left: Total immobility duration of after intracerebroventricular infusion of 10 ng of ABD1114 road 1115. Right: Distance travelled (cm) in the open field. Bars represent means ± SEM; ***p<0.001; n=7 per group.

Figure 1

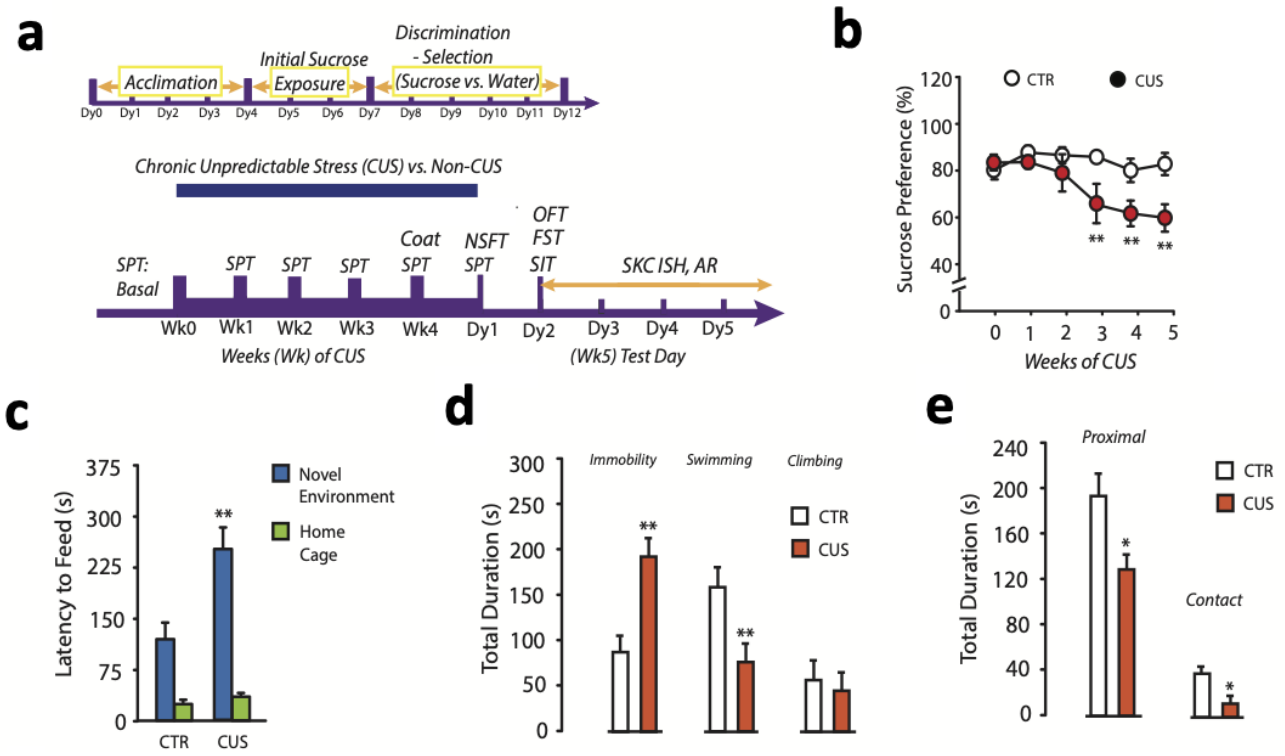
a
Acclimation
Initial Sucrose Exposure
Discrimination - Selection (Sucrose vs. Water)
Dy0 Dy1 Dy2 Dy3 Dy4 Dy5 Dy6 Dy7 Dy8 Dy9 Dy10 Dy11 Dy12
Chronic Unpredictable Stress (CUS) vs. Non-CUS
SPT: Basal
SPT
SPT
SPT
Coat SPT
NSFT SPT
OFT FST SIT
SKC ISH, AR
Wk0 Wk1 Wk2 Wk3 Wk4 Dy1 Dy2 Dy3 Dy4 Dy5
Weeks (Wk) of CUS
(Wk5) Test Day
b
CTR
CUS
Sucrose Preference (%)
120
100
80
60
40
0
**
**
**
0 1 2 3 4 5
Weeks of CUS
c
Latency to Feed (s)
375
300
225
150
75
0
**
Novel Environment
Home Cage
CTR
CUS
d
Total Duration (s)
300
250
200
150
100
50
0
Immobility
Swimming
Climbing
**
**
CTR
CUS
e
Total Duration (s)
240
200
160
120
80
40
0
Proximal
Contact
*
*
CTR
CUS

Figure 2

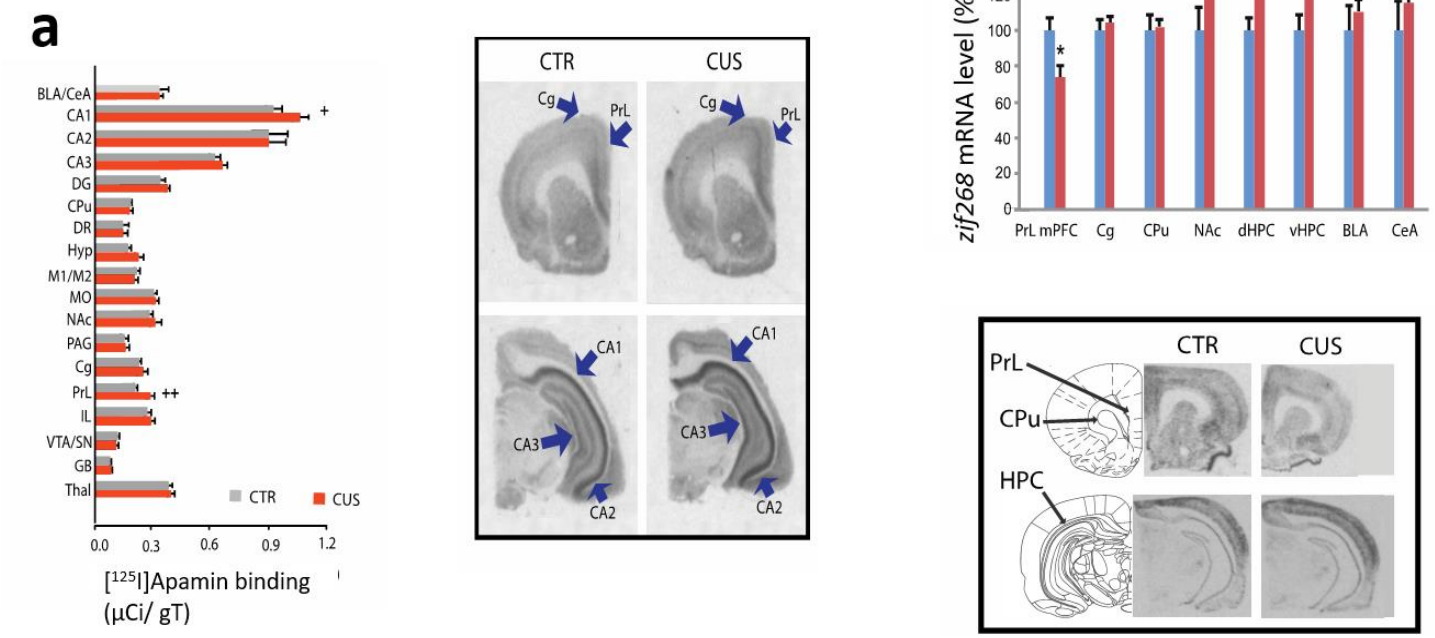
a
BLA/CeA
CA1
CA2
CA3
DG
CPu
DR
Hyp
M1/M2
MO
NAc
PAG
Cg
PrL
IL
VTA/SN
GB
Thal
+
++
CTR
CUS
0.0
0.3
0.6
0.9
1.2
[125I]Apamin binding
(µCi/ gT)
CTR
CUS
Cg
PrL
CA1
CA3
CA2
b
zif268 mRNA level (% of control)
180
140
120
100
80
60
40
20
0
PrL mPFC
Cg
CPu
NAc
dHPC
vHPC
BLA
CeA
*
PrL
CPu
HPC

Figure 3

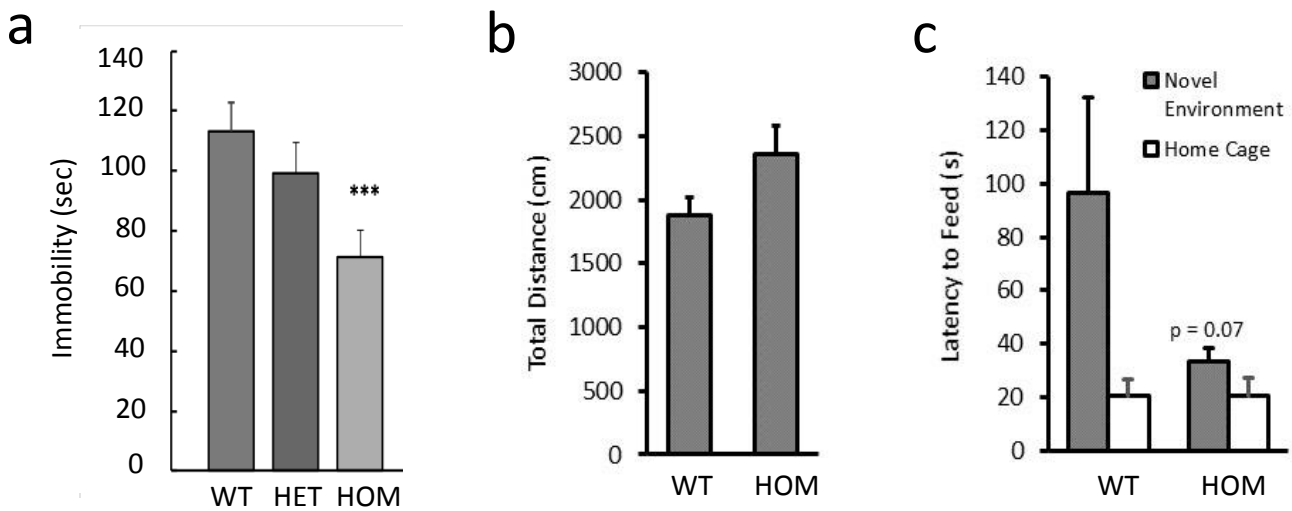
a
Immobility (sec)
140
120
100
80
60
40
20
0
***
WT
HET
HOM
b
Total Distance (cm)
3000
2500
2000
1500
1000
500
0
WT
HOM
c
Latency to Feed (s)
140
120
100
80
60
40
20
0
Novel Environment
Home Cage
p = 0.07
WT
HOM

Figure 4

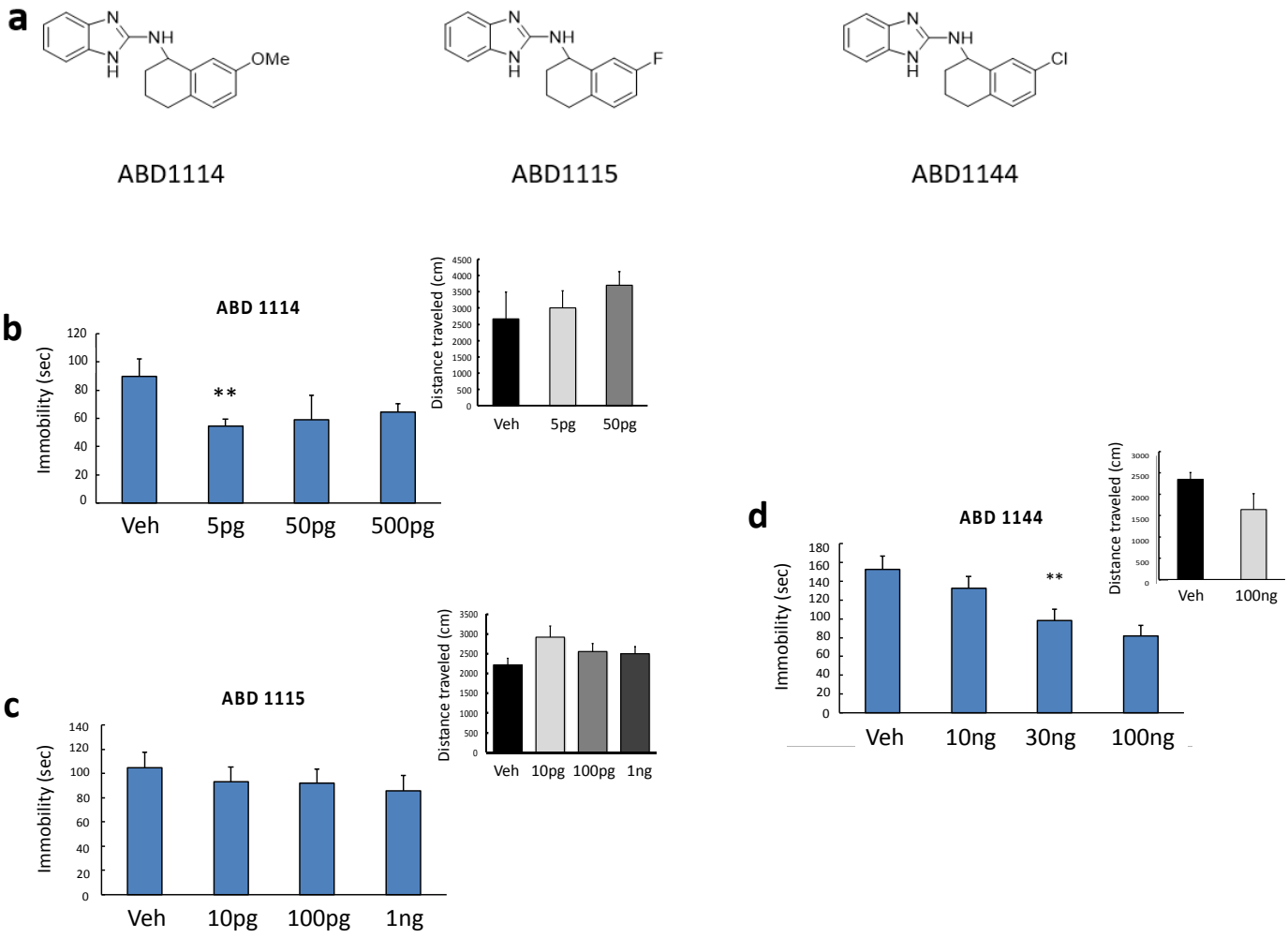
a
ABD1114
ABD1115
ABD1144
b
ABD 1114
Immobility (sec)
Veh
5pg
50pg
500pg
Distance traveled (cm)
Veh
5pg
50pg
c
ABD 1115
Immobility (sec)
Veh
10pg
100pg
1ng
Distance traveled (cm)
Veh
10pg
100pg
1ng
d
ABD 1144
Immobility (sec)
Veh
10ng
30ng
100ng
Distance traveled (cm)
Veh
100ng

Figure 5

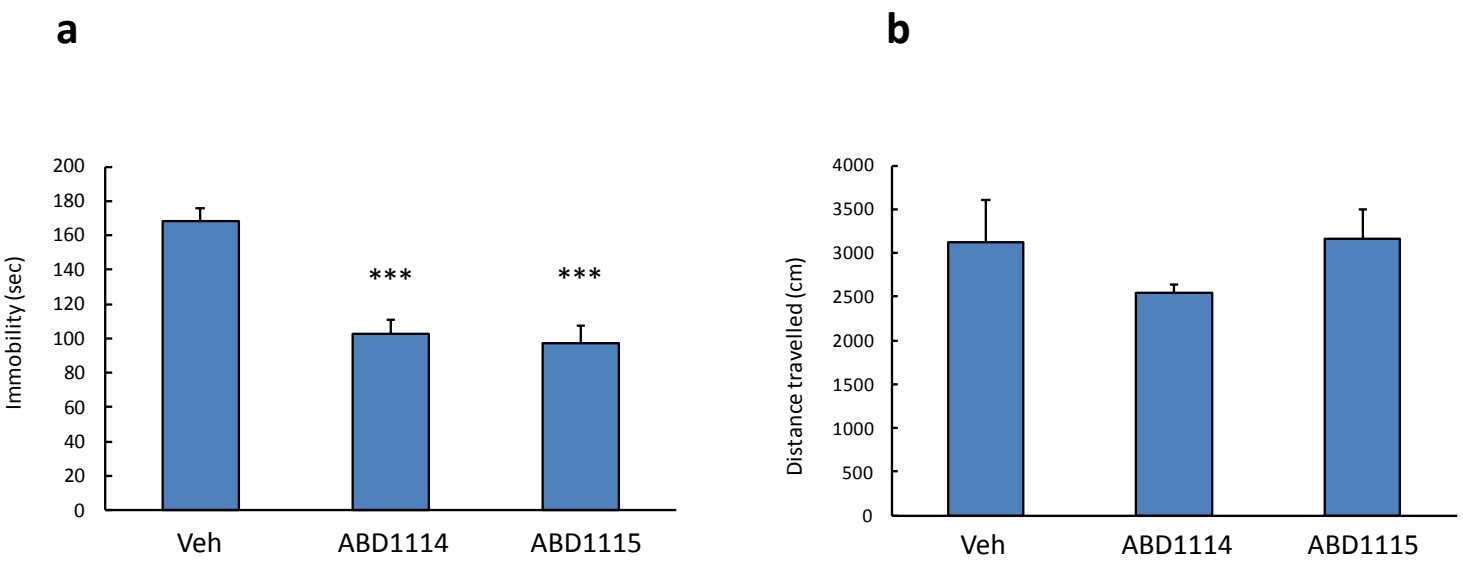
a
b
Immobility (sec)
200
180
160
140
120
100
80
60
40
20
0
***
***
Veh
ABD1114
ABD1115
Distance travelled (cm)
4000
3500
3000
2500
2000
1500
1000
500
0
Veh
ABD1114
ABD1115

Table 1. Descriptions of stressors used in the CUS paradigm.

Stressor	Description	Notes
1. Cage tilt	Home cages were tilted at ~45º for 3-12 hours.	Can be overnight or during the day
2. Cold room	Animals were kept in an empty cage at 4 ºC for 2 hours	Not grouped with #9 or 12
3. Food deprivation	Food pellets were withdrawn from the home cage for 12-18 hours.	Not grouped with #13
4. High frequency sound	Animals were subjected for 3 hours to high frequency noise generated by an ultrasonic rodent pest repeller (Victor ®).	
5. Intraperitoneal injections	Animals received intraperitoneal saline injections with a 25-gauge needle.	
6. Light cycle reversal	Light-dark cycle reversed for 24 hours	24 hours
7. Novel environment	Animals were displaced to an unfamiliar room, then placed back to the housing room after 3 hours	
8. Predator odor	One tablespoon of sand with fox urine was placed in the home cage and removed after 3 hours.	
9. Restraint	Animals were immobilized by a plastic restraining cone (Harvard Apparatus) for 30 minutes in the home cage at room temperature	Not grouped with #2 or 12
10. Static noise	Animals were subjected to static radio noise for 3 hours.	
11. Stroboscopic lighting	Animals were subjected to stroboscopic lights for 3 to 12 hours in a dark environment.	
12. Water in cage	Five hundred milliliters of water (~10 ºC) were placed in the home cage. The cage was cleaned and dried after 12-18 hours.	Not grouped with #2 or 9; overnight only
13. Water deprivation/empty bottle	Water bottles were withdrawn from the cage lids for 12-18 hours.	Not grouped with #3