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Infralimbic Cortex and Behavioral Responses to Mild Stress after Repeated Social Stress Exposure

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Abstract

Major Depressive Disorder (MDD) is an emotional impairment that can have debilitating consequences for afflicted individuals. Social stress, including isolation or alienation, may be an important factor that contributes to MDD. In two experiments, repeated social defeat was utilized in rodents to induce social stress, modeling the social stressors that are thought to contribute to MDD in humans. Behavioral analyses revealed that dominance and submissive behaviors in the social defeat sessions were quite stable across repeated sessions. Accordingly, there appeared to be little variability between the rats in their social stress experience. This validates the model and allows for the study to draw inferences concerning the impacts of the stress experience from means testing of behavioral and physiological outcomes. The second experiment evaluated the impacts of repeated social defeat on cortical responses to the mild stress of a novel environment. RNAscope was utilized evaluate expression of c-Fos, GAD65, and VGluT1 mRNA in the infralimbic cortex. mRNA for c-Fos was labeled to identify which neurons were active in the infralimbic cortex. This was combined with labels for vGluT1 and GAD65 mRNAs to evaluate if those neurons were excitatory (glutamatergic) or inhibitory (GABAergic). The socially stressed rats exhibited increased mRNA expression for GAD65 in the infralimbic cortex. This suggests increased stress-induced biosynthesis of GABA in inhibitory neurons of the infralimbic cortex.

Introduction

Major Depressive Disorder (MDD) currently affects approximately 280 million people and is the largest contributor to disability worldwide. Symptoms of MDD may include the inability to complete daily tasks and loss of enjoyment in previously pleasurable activities (World Health Organization, 2023). Stressful life events also increase the risk of subsequent depressive episodes (Kendler et al., 1999). This may include social stressors, such as dysfunction in or lack of familial, romantic, or occupational relationships, and may contribute to MDD. Isolation, an

additional symptom of MDD, may contribute to additional social stress upon disease onset, perpetuating a self-reinforcing cycle of worsening depression.

Dysfunctional or lack of social support can trigger pathological changes in neural activity and altered brain function (Kupferberg et al., 2016). Social defeat in a rodent model induces a depressive-like symptomology, resembling MDD in humans, and generates a social conflict between a sexually mature, territorial, dominant resident rat and a younger, submissive intruder rat. The intruder rat is introduced to the resident rat's home cage where they are subjected to aggressive, physical stressors inflicted by the resident, including forced allogrooming, pursuit, attacks, and pinning of the intruder.. Repeated contact between the resident and intruder escalates the dominant behaviors displayed by the resident. Most importantly, the act of pinning is a species-specific behavior that demonstrates the physical dominance of the resident rat and the submission of the intruder (Hollis & Kabbaj, 2014). After dominance/submission is established, a partition is placed between resident and intruder to induce psychological stress on the intruder rat without the risk of injury from continued physical confrontation.After the social defeat session with a dominant rat, intruder rats experience elevated glucocorticoid activity, tachycardia, and hyperthermia. Further, social defeat leads to neurological changes that last for weeks or months after stress (Koolhaas et al., 1997; Miczek et al., 2004).

In social defeat experiments, however, individual differences in dominance behaviors of the resident rats could produce significant differences in the individual experiences of the intruders, and behaviors of the intruder rats during social defeat could reflect differing impacts of the stressor on them. Accordingly, in Experiment 1, we investigated how stable the behaviors of the rats were during social defeat, and how much training is required to reach stability in order to ensure consistent experiences of the intruder rats both within an experiment and from one experiment to the next.

In Experiment 2, we examined the consequences of repeated social defeat on cortical function using RNAscope. The medial prefrontal cortex (mPFC), which includes the prelimbic (PL) and infralimbic (IL) cortices is involved in cognitive processing, emotion regulation, motivation, and social behavior (Minami et al., 2017). Dysfunction of the mPFC has been linked to multiple neurological and psychiatric disorders, including major depressive disorder (Xu et al., 2019). Following repeated restraint stress, significant dendritic atrophy, and spine loss in mPFC

pyramidal neurons can be observed, demonstrating stress induced impairment (Radley et al. 2006).

The focus of this study is the IL; however, it is important to note that both the IL and PL cortices are often associated together but have distinct functions. In fact, stimulation of the IL reportedly suppresses conditioned fear responses, inhibits aggressive behavior, and induces an antidepressant-like response. In addition, the IL has been suggested to be responsible for the acquisition of stress resiliency (Minami et al., 2017). Inactivation of the IL impaired active avoidance behavior, showing its implications in avoiding aversive stimuli (Capuzzo & Floresco, 2020). Inactivation of the IL also reduced immobility during a forced swim test, commonly regarded as a sign of an anti-depressant effect, implicating the IL's role in mood regulation (Slattery et al., 2010).

In a study examining the IL in rats engaged in social interactions, the IL demonstrated increased firing during leaving behavior, specifically when the rat would leave the social interaction, suggesting a role in escape or avoidance of socially threatening situations (Minami et al., 2017). Additionally, stimulation of the IL has been shown to suppress conditioned fear responses, inhibit aggressive behavior, and produce an anti-depressant effect (Faccidomo et al., 2012; Milad et al., 2002; Minami et al., 2017). This infers that firing of the IL was due to stress relief from leaving behavior.

This study further explores the underlying neural mechanisms of the IL after stress induced by social defeat. The neural indicators of social stress on the rodent IL were quantified using RNAscope, an *in situ* hybridization technique that allows us to quantify the expression of up to three specific genes in individual neurons. This technique was utilized to observe the expression of c-Fos, vesicular glutamate transporter 1 (vGluT1), and glutamate decarboxylase (GAD65) mRNAs in individual neurons that are active at the height of the rat's stress response. c-Fos is an immediate early gene that is transiently expressed in neurons that were recently activated and was labelled to identify which neurons were active in the IL cortex during stress exposure. This was combined with labels for VGluT1 and GAD65 mRNAs to evaluate if those neurons were excitatory (glutamatergic) or inhibitory (GABAergic).

Imbalances in the excitatory/inhibitory pathways of the central nervous system is a significant pathological feature of MDD. Clinical studies have shown individuals with MDD

presenting with reduced GABAergic and elevated glutamatergic signaling in the limbic system (Hu et al., 2023). Labeling both excitatory and inhibitory neurons may reveal which population of neurons in the IL are activated (i.e., c-Fos expressing) by stress, and if there are differences in which neurons are responsive after repeated social defeat, when compared with controls that were not socially stressed.

Materials and Methods

Experiment 1: Social Defeat Training

Retired breeder male Long-Evans rats (n=16) and experimentally-naïve six-week old Sprague Dawley rats (n=32) were utilized for the experiment. Being older and larger with previous breeding experience, the Long-Evans rats were housed individually and exhibited territorial, dominant behaviors. Long-Evans rats were housed individually for 14 days before the start of the social defeat sessions to acclimate to their home cage. The Sprague Dawley rats were split into two groups, 16 control rats and two cohorts of 8 "intruders." Being smaller and younger would contribute to them being more submissive and acting as the intruders encroaching on the resident's territory. They were used to train the residents to exhibit dominance behaviors when a novel rat was introduced to the resident's home cage. Sprague Dawley rats were pair-housed for 7-9 days prior to the start of the social defeat sessions. After the first social defeat session, they were housed individually. The control group of Sprague Dawley rats was handled daily and were not exposed to social defeat sessions.

Social Defeat Training Days 1-8

On each of eight consecutive days, a novel intruder rat was introduced to a resident rat's home cage. Each intruder was exposed to a different resident rat each day, once a day. Each social defeat session would last a variable amount of time, depending on the criteria detailed below. Resident/intruder pairing schedules for both cohorts can be found on Table 1a and 1b.

	Resident 1	Resident 2	Resident 3	Resident 4	Resident 5	Resident 6	Resident 7	Resident 8
Intruder 1	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
Intruder 2	Day 8	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Intruder 3	Day 7	Day 8	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Intruder 4	Day 6	Day 7	Day 8	Day 1	Day 2	Day 3	Day 4	Day 5
Intruder 5	Day 5	Day 6	Day 7	Day 8	Day 1	Day 2	Day 3	Day 4
Intruder 6	Day 4	Day 5	Day 6	Day 7	Day 8	Day 1	Day 2	Day 3
Intruder 7	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 1	Day 2
Intruder 8	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 1

Table 1a. Resident/Intruder Social Defeat Pairing Schedule, Cohort 1

	Resident 9	Resident 10	Resident 11	Resident 12	Resident 13	Resident 14	Resident 15	Resident 16
Intruder 9	Day 1	-	-	-	-	-	-	-
Intruder 10	Day 8	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Intruder 11	Day 7	-	Day 1+8	Day 2	Day 3	Day 4	Day 5	Day 6
Intruder 12	Day 6	-	Day 7	Day 1+8	Day 2	Day 3	Day 4	Day 5
Intruder 13	Day 5	-	Day 6	Day 7	Day 1+8	Day 2	Day 3	Day 4
Intruder 14	Day 4	-	Day 5	Day 6	Day 7	Day 1+8	Day 2	Day 3
Intruder 15	Day 3	-	Day 4	Day 5	Day 6	Day 7	Day 1+8	Day 2
Intruder 16	Day 2	-	Day 3	Day 4	Day 5	Day 6	Day 7	Day 1+8

Note: Counterbalancing following the death of Resident 10 prior to day 2 of training caused elimination of Intruder 9; Resident (Res.)

Table 1b. Resident/Intruder Social Defeat Training Pairing Schedule, Cohort 2

Pinning behavior was regarded as complete dominance and occurred when the resident rat tackled the intruder rat into a supine position, their forepaws remaining in contact with the intruder. Pinning ended when contact was broken, typically when the intruder escaped the resident's grasp, or the resident moved away. After three occurrences of the resident pinning the

intruder, or after 10 minutes without the completion of three pins, a wire mesh screen separated the resident and intruder. After an additional 10 minutes, the partition was removed until one more pin occurred, or 10 more minutes passed. After each training session, the intruders were returned to their home cages.

One resident rat in the second cohort died of unknown natural causes, so one intruder rat was removed to adjust the pairing schedule accordingly. The training sessions were recorded using a digital camera set in front of the resident's cage. These recordings were subsequently analyzed for behavior quantification.

Quantitative Analysis of Training Recordings

Video recordings of the social defeat training sessions were analyzed using the Nodulus Observer XT 13 program. Trained, experimentally-blind research assistants scored video recordings referring to defined behaviors noted in Table 2. Behaviors were scored by calculating the ratio of time the resident or intruder spent displaying the specific the behavior compared to the full time of the single session. Ratios were used due to the variable session lengths.

Statistical Analyses

Resident and intruder behaviors were assessed from day 1 to day 7 of social defeat training. Due to a resident rat's death, day 8 was omitted to retain completely novel resident-intruder pairings. Behaviors were sorted into groups (total dominance behavior, total fear-related behavior, total prosocial behavior, and total submissive behavior) to composite related behaviors. A one-way repeated measures analysis of variance (ANOVA) test was used to observe whether social defeat behavior changed across time. Results were considered statistically significant if $p<0.05$. Statistical analyses were conducted using IBM SPSS Statistics 20.0.0.0. and graphs were made using GraphPad Prism (v.10.0.2 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com) software. GraphPad Prism software was used to conduct unpaired, two-tailed t-tests and generate graphs.

Scored behaviors	Operational Definition
Allogroom	One rat grooms the other, usually around the neck and head, but may also include other body parts. Grooming motions include snout contact with body part, with continuous movement of the snout contact. The grooming may include rapid nibbling, while the groomed rat is immobile. Movement of the groomed rat may elicit offensive aggression and kicking behaviors from the groomer. This excludes when one rat is actively pushing down upon the submissive, preventing the submissive rat from moving away (see forced grooming).
Boxing	The resident and intruder rats rear up on the hindlimbs, face to face and push or bat at the other's forelimbs, head, neck and thorax with their forepaws.
Can't See	The Scorer cannot see what behavior the animal is displaying and thus cannot score this section of the video. Ex: the intruder is blocked in the video by the resident.
Defensive Aggression	The aggressor and defensive animals are engaged in physical contact in which the defensive animal is rolling with the aggressor, or pushing the aggressor off, while not in supine or upright posture
Defensive Upright Posture	Rearing up on the hind limbs while facing the aggressor, without physical contact between the rats.
Digging	Using the forepaws and hindpaws to shuffle bedding around the cage.
Escape	Breaking free from any form of physical contact with the aggressor rat, and moving to another location in the cage.
Standing on the Food Hopper	Having both hindpaws located on top of the food hopper within the resident enclosure
Forced Grooming	Allogrooming while actively pushing down upon the submissive's body, head, or neck region. This excludes grooming while the submissive is exhibiting supine posture.
Freezing	Complete immobility for more than 3 sec apart from breathing movements, and movements of the head and neck.
Lateral Kick	Using the hind limbs to kick. May or may not make direct contact with the other rat
Lateral threat	One rat approaches the other from the side or flank, with arched back, and crowds or pushes the other rat.
Mounting	One rat places its forelimbs over the rump of the other rat.
No Screen Interaction	During the screen portion, the rodent is not actively interacting with the screen
Null Behavior	Any time the rat is not engaged in any of the other defined behaviors. The rat may be engaged in locomotor behavior, rearing, self-grooming, environmental sniffing, etc.
Offensive Aggression	Violent contact between the aggressor and the defensive animal. This includes rolling around while in contact with the defensive animal and contact between the snout of the aggressor and the lower back or flanks of the defensive animal. Violent contact specifically excludes sniffing,

	and grooming contacts. Contact may include bites directed at the lower back and flanks.
Pinning	The aggressor stands over the supine submissive rat with forepaws in contact with or above the submissive. Pinning behavior ends when the aggressor breaks physical contact and turns its back or moves away from the submissive or if the submissive escapes.
Screen Interaction	The rodent is actively interacting with the screen during the screened portion of the session. This includes sniffing, biting, climbing on, or rearing up onto the screen.
Social Sniffing	Snout contact between one rat and the body of another rat while stationary. This includes anogenital sniffing but excludes sniffing while in active pursuit (see intruder pursuit and resident pursuit behaviors).
Supine Posture	Laying on the back or side with paws and head raised.
Violence	Violent contact between the snout of the aggressor and the head, throat, or ventrum of the defensive animal. Violent contact specifically excludes sniffing, and grooming contacts. Contact may include bites directed at the head, throat, or ventrum.

Table 2. Social defeat scored behaviors

Experiment 2

Experimentally-naïve 6-week-old male Sprague Dawley rats (n=48) were used in this experiment. The intruder rats (n=24) were used in social defeat sessions and the remaining Sprague Dawley rats (n=24) acted as the control group. The six most consistently behaving resident rats of Experiment 1 were used in Experiment 2.

Social Defeat Sessions

For six consecutive days, a novel intruder rat was introduced to a resident rat's home cage, with every intruder experiencing a different resident each day. The pairing schedule is listed in Table 3. The social defeat sessions followed the same procedure as Experiment 1.

	Resident 1	Resident 2	Resident 3	Resident 4	Resident 5	Resident 6
Intruder 1	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Intruder 2	Day 2	Day 3	Day 4	Day 5	Day 6	Day 1
Intruder 3	Day 3	Day 4	Day 5	Day 6	Day 1	Day 2
Intruder 4	Day 4	Day 5	Day 6	Day 1	Day 2	Day 3
Intruder 5	Day 5	Day 6	Day 1	Day 2	Day 3	Day 4
Intruder 6	Day 6	Day 1	Day 2	Day 3	Day 4	Day 5

Note: This pairing design was used for all 4 cohorts of Experiment 2.

Table 3. Resident/Intruder social defeat pairing schedule – Experiment 2

Complex Environment Testing

After the sixth day of social defeat, each intruder was introduced to a novel complex environment with their original cage-mate for a single session of 120 minutes. Sessions were recorded using a camera placed above the complex environment for subsequent analysis of social interactions and exploratory behaviors. This part of the experiment was conducted by another experimenter and is not reported herein.

Circular Corridor Test

On the second day following the completion of complex environment testing, the rats were placed individually into a novel circular corridor environment for 30 minutes. This was used to evaluate neurobiological responses to a mild stressor after repeated social defeat. The rats were terminated immediately after the circular corridor stress and the brains were collected, frozen in isopentane at -40°C, and stored at -80°C.

RNAscope Imaging

Coronal sections (12 um) of the prelimbic regions and prefrontal cortex were collected from the frozen brains using a freezing cryostat (Leica) and mounted on Superfrost Plus Gold glass slides. The mounted sections were stored at -80°C until processed for RNAscope.

Fluorescent *in situ* hybridization was performed using RNAscope Multiplex Fluorescent Reagent Kit v2 (Cat. # 323100) according to the protocol by Advanced Cell Diagnostics Inc. The kit allows for different fluorophores to be assigned to various channels. Channel 1 (C1) was assigned to c-Fos expression, Channel 2 (C2) was assigned to GAD 65 expression, and Channel 3 (C3) was assigned to vGluT1 expression. These channels were paired with green, yellow, and red fluorophores, marking gene expression in the IL cells.

Images of the IL were taken in a grid approach. A custom CellProfiler approach that optimized the resolution and quality cell counting and colocalization of mRNA transcripts was employed. DAPI was used to identify and label cell nuclei. c-Fos, GAD65, and VGluT1 mRNA transcript molecules are represented as puncta on the images. Using CellProfiler, the puncta were quantified and overlayed onto the DAPI stained cell nuclei. Cells that were identified to be positive for a combination of c-Fos and either GAD65 or VGluT1 were considered colocalized. In the representative images, DAPI is represented in blue, VGluT1 is represented in red, GAD65 is represented in yellow, and c-Fos is represented in green (Figure 4).

Statistical Analysis

Trained and experimentally blind research assistants used CellProfiler to quantify cell bodies and mRNA puncta in images. GraphPad Prism software was used to conduct unpaired, two-tailed t-tests and generate graphs. T-tests were subjected to Welch's correction and results were considered statistically significant if $p<0.05$. Variables from the intruder's IL were compared with the control groups. Variables evaluated included: c-Fos nuclear puncta,GAD65 nuclear puncta, VGluT1 nuclear puncta, c-Fos+ cells, GAD65+ cells, VGluT1+ cells, overlapping GAD65+c-Fos cells, overlapping VGluT1+c-Fos cells, the percentage of c-Fos+ cells that overlapped with GAD65+, the percentage of c-Fos+ cells that overlapped with VGluT1+, and the percentage of c-Fos+ cells not double-labeled.

Results

Intruder Behavior

During social defeat training sessions, intruder rat behaviors stabilized by the second session. Total dominance, submission, prosocial, and null behaviors were relatively stable across all eight training days (Figures 1, 2, 3). Intruder rats overall exhibited more submissive behaviors than dominance behaviors (Figures 1, 2). Low levels of allogrooming were observed (Figure 3a). Omitted variables included violence, mounting, lateral kick, and lateral threat due to low frequency of these behaviors.

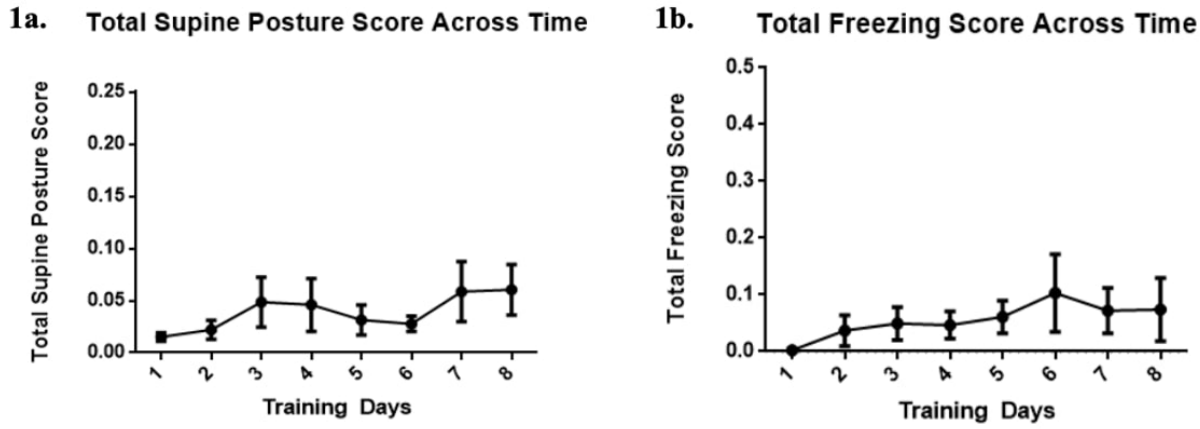

Figure 1. a) Total supine posture scores per day in social defeat training sessions over time in intruder rats (8 days of training). b) Total freezing scores per day in social defeat training sessions over time in intruder rats (8 days of training).

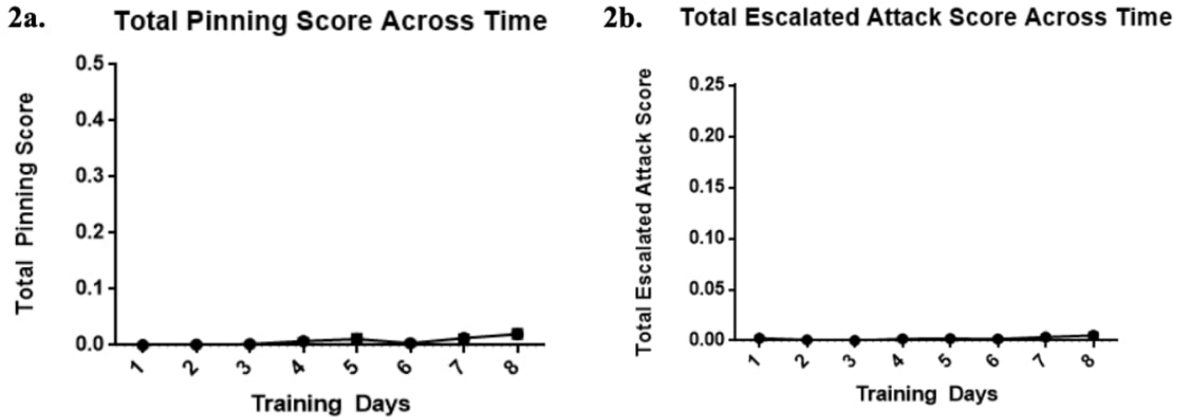

Figure 2. a) Total pinning scores per day in social defeat training sessions over time in intruder rats (8 days of training). b) Total escalated attack scores per day in social defeat training sessions over time in intruder rats (8 days of training).

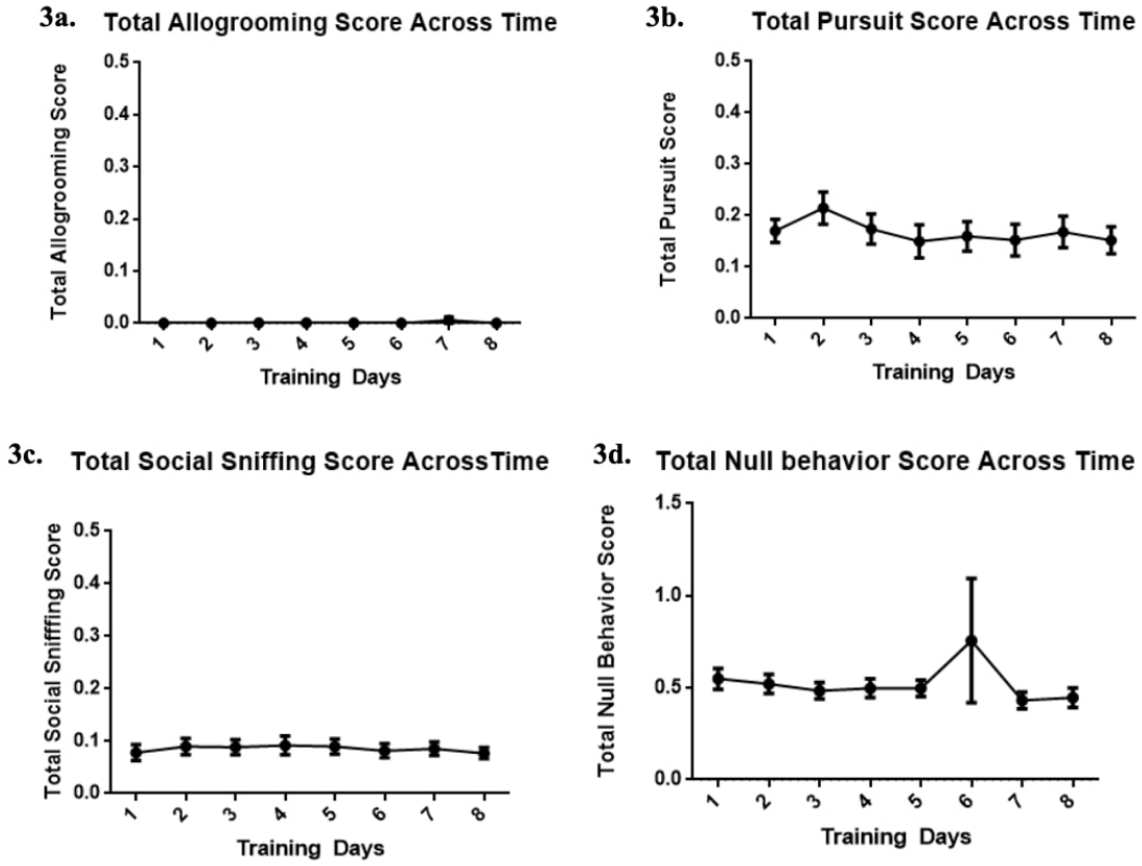

Figure 3. a) Total allogrooming scores per day in social defeat training sessions over time in intruder rats (8 days of training). b) Total pursuit scores per day in social defeat training sessions over time in intruder rats (8 days of training). . c) Total social sniffing scores per day in social defeat training sessions over time in intruder rats (8 days of training). d) Total null behavior scores per day in social defeat training sessions over time in intruder rats (8 days of training).

RNAscope Gene Expression of the Infralimbic Cortex

There was a significantly greater number of the GAD65 Cell Bound Puncta [$t(45.59)=2.752$, $p<0.01$], and greater percentage of c-Fos cells that overlapped with GAD65 [$t(47.66)=2.850$, $p<0.01$] in the cells of the infralimbic cortex of the intruder rats compared to the control group (Table 4, Figures 5, Figure 13). There were no significant differences between intruder and control rats in c-Fos Positive (+) Cells, GAD65 Positive (+) Cells, or VGluT1 Positive (+) Cells. There was no significant difference between glutamatergic cell bound puncta, or in overlapping cells with GAD65 + c-Fos or VGluT1 + c-Fos. There was no significant difference in the percentage of c-Fos+ cells that overlapped with VGluT1+ or the percentage of cFos+ cells not double-labeled within the infralimbic cortex (Table 4).

Variable	Unpaired two-tailed independent samples t-test results
GAD65 Cell Bound Puncta **	$t(45.59) = 2.752$, $p<0.01$^
vGluT1 Cell Bound Puncta	$t(48.97) = 0.1234$, $p>0.05$^
c-Fos Cell Bound Puncta	$t(48.50) = 0.08080$, $p>0.05$^
GAD65 Positive (+) Cells	$t(40.33) = 0.9258$, $p>0.05$^
vGluT1 Positive (+) Cells	$t(41.04) = 0.6378$, $p>0.05$^
c-Fos Positive (+) Cells	$t(40.74) = 1.443$, $p>0.05$^
Overlapping Cells with GAD65 + c-Fos	$t(48.99) = 1.381$, $p>0.05$^
Overlapping Cells with vGluT1 + c-Fos	$t(38.86) = 1.565$, $p>0.05$^
% of c-Fos + Cells co-expressing GAD65 **	$t(47.66) = 2.850$, $p<0.01$^
% of c-Fos + Cells co-expressing cGluT1	$t(35.47) = 0.2011$, $p>0.05$^
% of c-Fos + Cells not expressing Co-Label	$t(42.94) = 1.680$, $p>0.05$^

Note: ^ denotes the Welch's correction was used; Asterisks (**) denote $p<0.01$.

Table 4. RNAscope Statistical Analysis

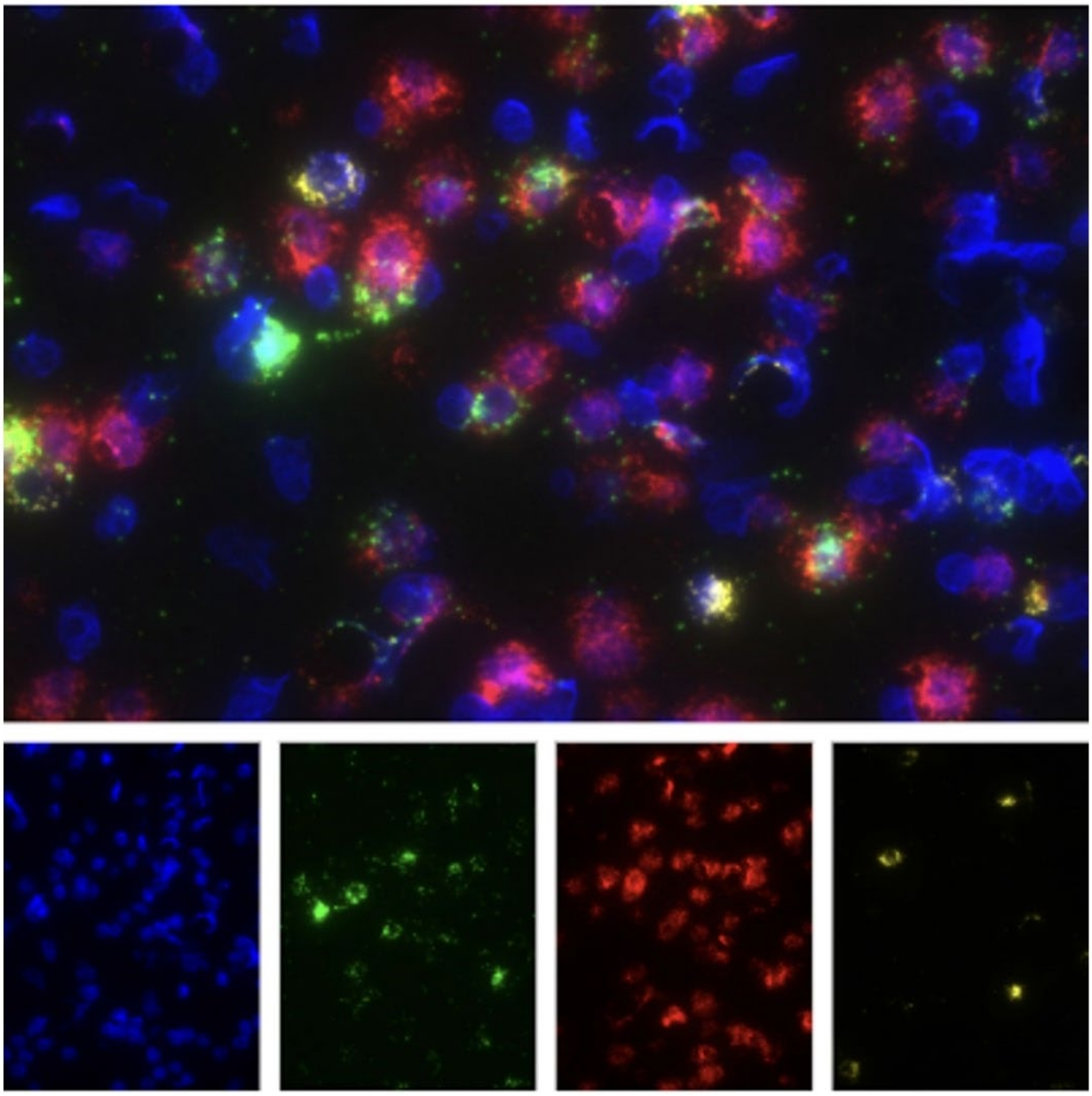

Figure 4. RNAscope representative sample images. Top image: Composite image of all channels overlayed. Bottom images, left to right: DAPI channel layer, c-Fos channel layer, vGluT1channel layer, GAD65 channel layer.

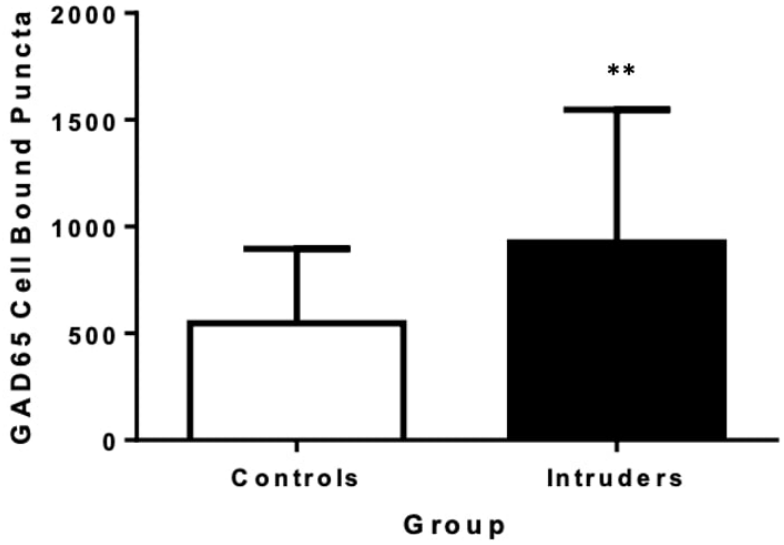

Figure 5. Comparison of GAD65 cell bound puncta between control and intruder groups. Asterisks (**) denote $p<0.01$.

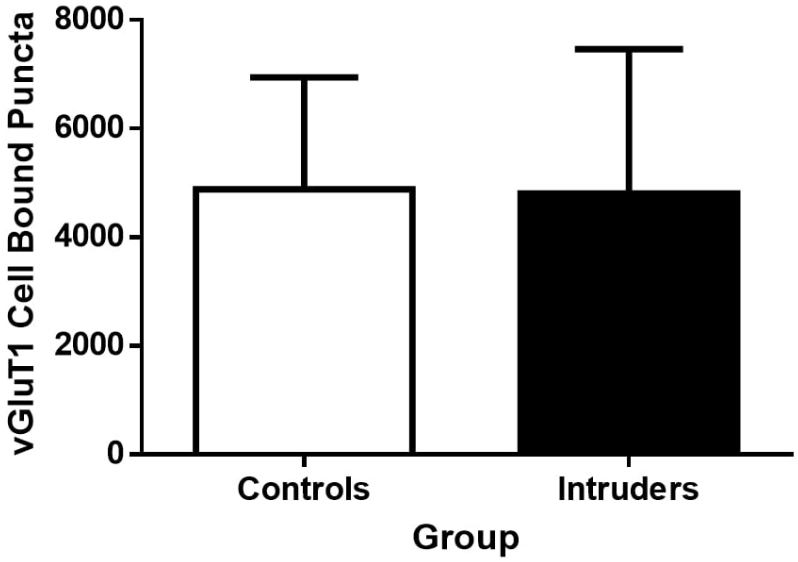

Figure 6. Comparison of vGluT1 cell bound puncta between control and intruder groups.

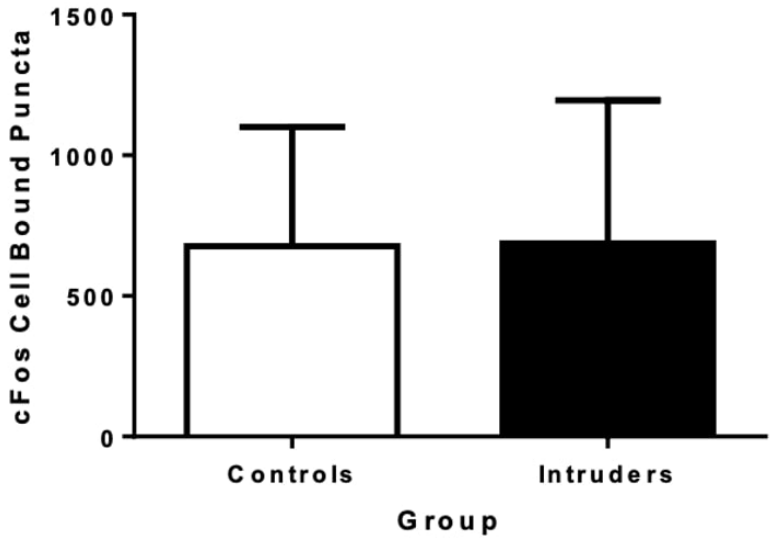

Figure 7. Comparison of c-Fos cell bound puncta between control and intruder groups.

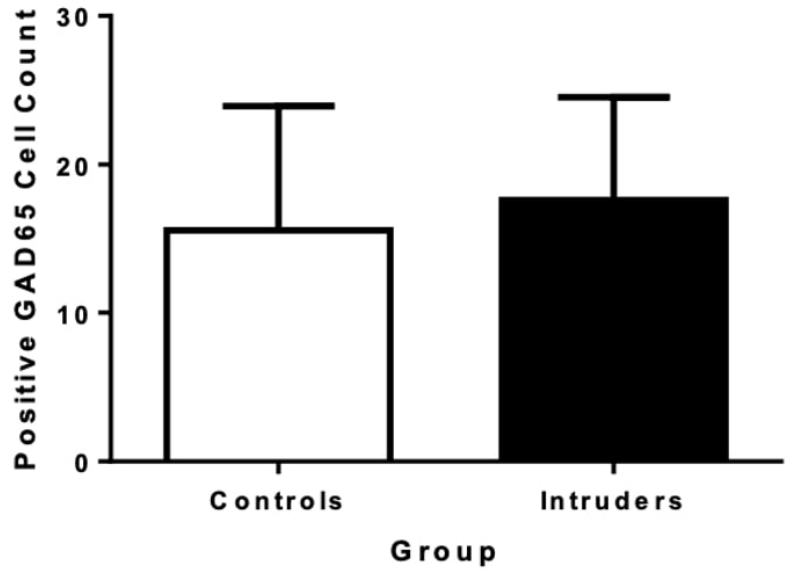

Figure 8. Comparison of positive GAD65 (+) cell count between control and intruder groups.

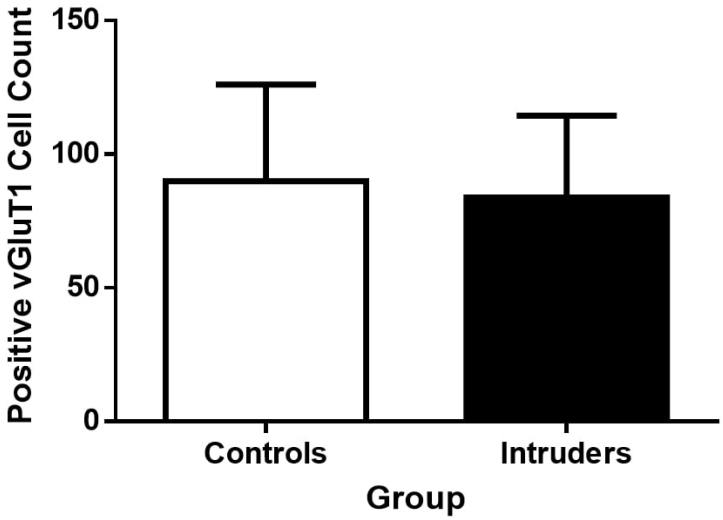

Figure 9. Comparison of positive vGluT1 (+) cell count between control and intruder groups.

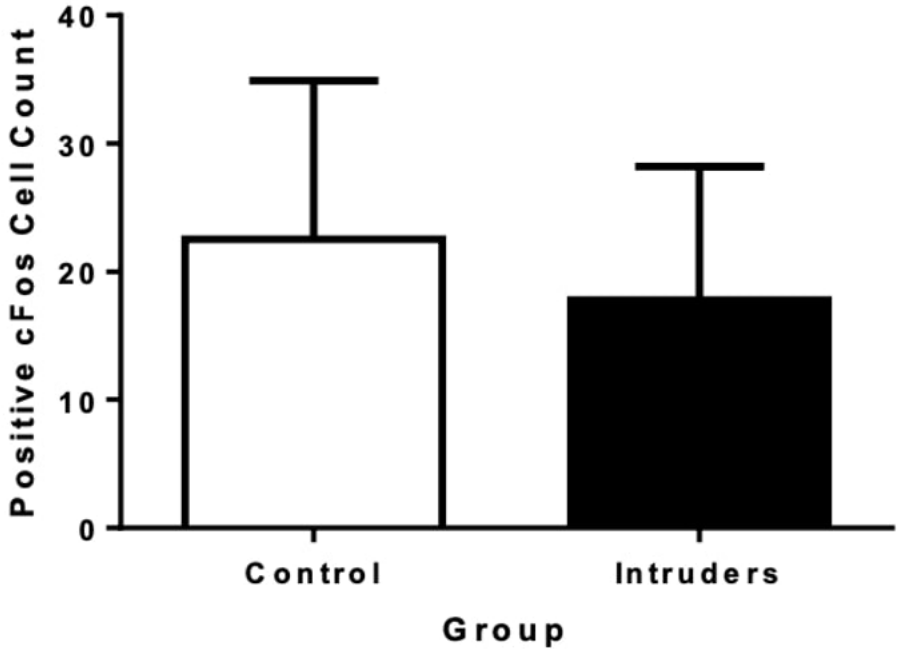

Figure 10. Comparison of positive c-Fos (+) cell count between control and intruder groups.

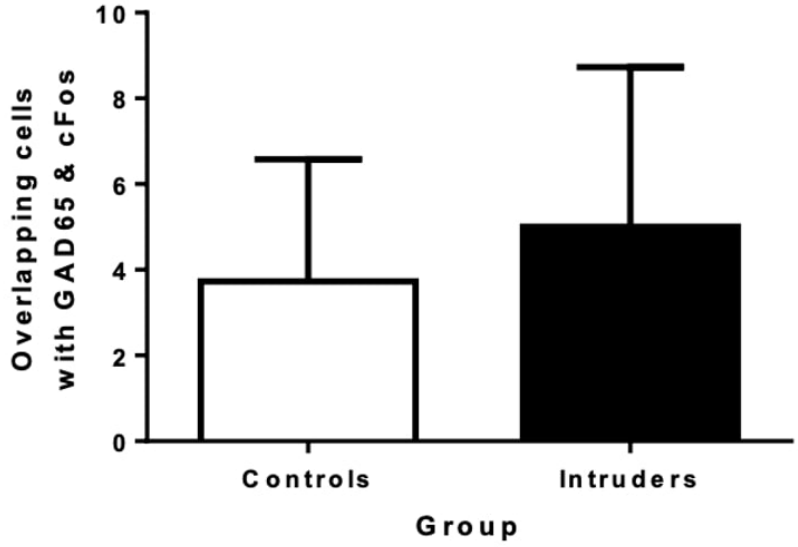

Figure 11. Comparison of number of overlapping cells of GAD65 and c-Fos between control and intruder groups.

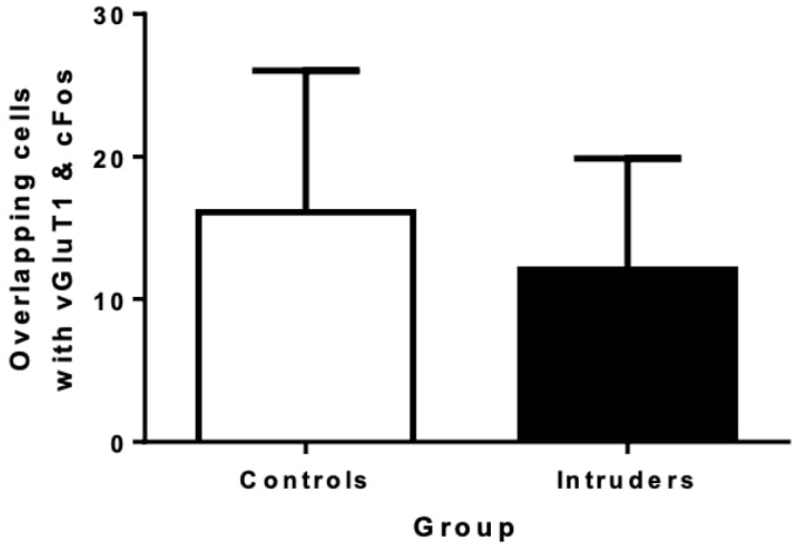

Figure 12. Comparison of number of overlapping cells of vGluT1 and c-Fos between control and intruder groups.

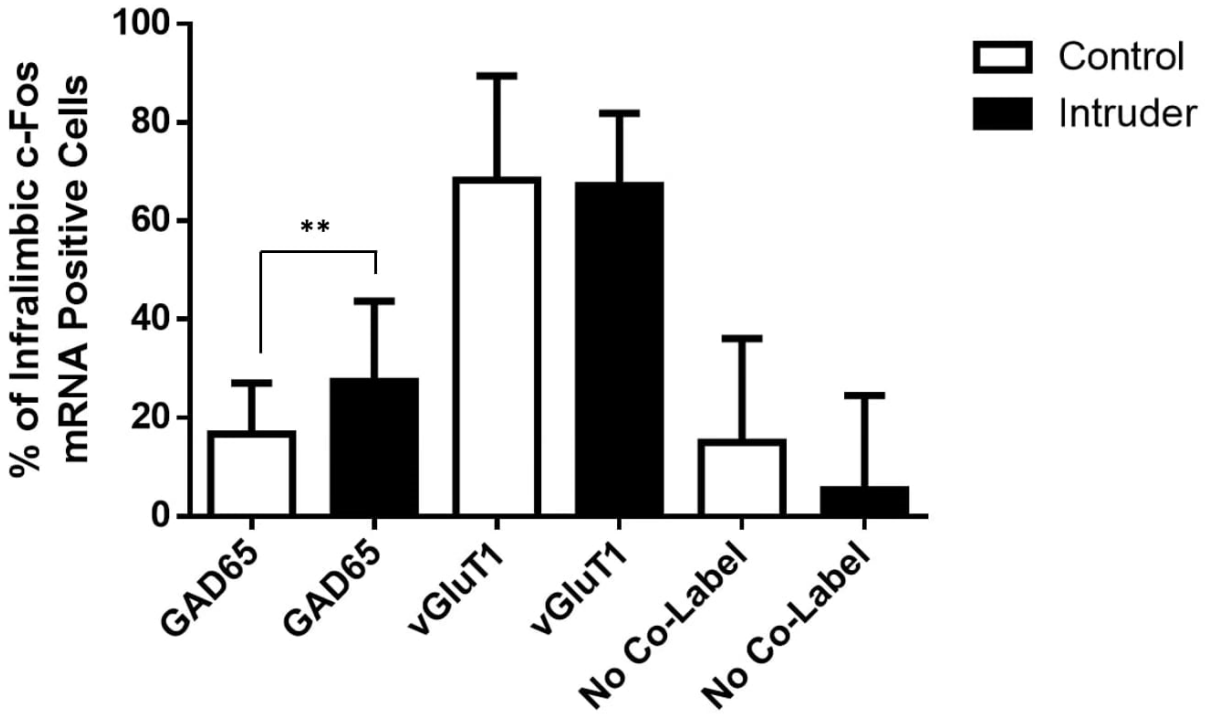

Figure 13. Comparison of the percentages of IL c-Fos mRNA positive cells that overlap with GAD65 or vGluT1, and c-Fos mRNA positive cells that do not overlap between the control and intruder groups. Asterisks (**) denote $p<0.01$.

Discussion

In Experiment 1, after the first social defeat session, submissive behaviors slightly increased and pursuit slightly decreased. By the second session, all behaviors of the intruder rats were relatively stabilized. Accordingly, it seems that the intruder rats learned to submit in the first training session and generalized that learning to additional encounters with larger territorial rats. Alternatively, the stabilization of the intruders' behaviors by Day 2 could indicate that the resident rats became more efficient in their expression of dominant behaviors during the first training session and maintained those stable behaviors throughout the seven repeated social defeat sessions. Over the entirety of the social defeat sessions, the intruder rats displayed more submissive behaviors than dominant behaviors, confirming that the intruder rats behaved as subordinates to the territorially dominant residents, a relationship in which we would expect them to be socially stressed. The intruders also exhibited low levels of allogrooming, which may also indicate high levels of stress in the environment.

Intruders exhibiting significantly more GAD65 Cell Bound Puncta indicates that repeated exposure to social stress caused an upregulation of inhibitory signaling in the IL and potentiation of long-term effects. As the IL is implicated in fear suppression and stress relief, the elevated GABAergic signaling could indicate that the socially defeated rats experience heightened fear and elevated stress. However, these possibilities would need to be evaluated with additional behavioral testing. The percentage of c-Fos cells that overlapped with GAD65 positive cells being significantly higher in intruders than controls demonstrates that repeatedly-defeated intruder rats had heightened sensitivity to acute mild stress, by selectively activating inhibitory signaling in the IL. There was not a similar elevation in overlap of c-Fos and vGluT1, strongly supporting the idea that the repeated stress produced a selective increase in stress-responsive activation of inhibitory circuits, not excitatory circuits. Additionally, the activation of inhibitory neurons may contribute to avoidance behaviors associated with IL function.

There is still much to be revealed about the underlying mechanisms of the IL in this domain. Future research should be done to investigate the social behaviors of female rats, as this study was limited to males. Also, due to GAD65 and vGluT1 being late response genes, the study may benefit from exposing rats to social stress for longer than one week. Lastly, characterizing what

behavioral phenotypes are driven by increased activity of the IL will contribute to a more comprehensive understanding of the IL's function in relation to MDD.

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