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Post-weaning social isolation attenuates c-Fos expression in GABAergic interneurons in the basolateral amygdala of adult female rats

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Abstract

Previous studies have found that adolescent social isolation of rats can lead to an increased anxiety state during adulthood, while chronic anxiety states are associated with dysregulated local GABAergic inhibition within the basolateral amygdala (BL). Therefore, we investigated the effects of post-weaning social isolation of female rats, in combination with a challenge with the anxiogenic drug, *N*-methyl-beta-carboline-3-carboxamide (FG-7142), on a subset of GABAergic interneurons in the BL in adulthood using dual immunohistochemical staining for c-Fos and parvalbumin. Juvenile female rats were reared in isolation or in groups of three for a 3-week period from weaning to mid-adolescence, after which all rats were group-housed for an additional 2 weeks. Group-reared rats and isolation-reared rats injected with FG-7142 had increased c-Fos expression in GABAergic interneurons in the anterior part of the BL compared to group-reared rats and isolation-reared rats, respectively, injected with vehicle. Isolation rearing had a main effect to decrease c-Fos expression in GABAergic interneurons in the anterior part of the BL compared to group-reared rats. These data suggest that post-weaning social isolation of female rats leads to dysregulation of a parvalbumin-containing subset of local GABAergic interneurons in the anterior part of the BL, which have previously been implicated in the pathophysiology of chronic anxiety states. These cellular changes may lead to an increased vulnerability to stress- and anxiety-related responses in adulthood.

Keywords

basolateral amygdala; c-Fos; parvalbumin; FG-7142; immunohistochemistry; isolation-rearing

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1.Introduction

Adverse early life experiences can cause long-lasting alterations to both neural systems and behavior that can result in increased vulnerability to neuropsychiatric disorders such as anxiety and depression in adulthood [for reviews see, 1,2,3,4]. One model of adverse early life experience in rats is social isolation during adolescence. Adult rats exposed to early life social isolation exhibit altered serotonergic activity in various forebrain regions [5–7] that is associated with increased anxiety and fear [8–11].

Post-weaning social isolation alters the sensitivity of serotonergic systems to stress-related stimuli later in adulthood. Our previous studies have shown that social isolation during adolescence in male rats facilitates corticotropin-releasing factor (CRF) -induced increases in extracellular concentrations of serotonin (5-hydroxytryptamine; 5-HT) in the nucleus accumbens (NAc) [7]. In addition, our previous studies also suggest that post-weaning social isolation of male rats causes an up-regulation of CRF type 2 (CRF₂) receptor expression in the dorsal raphe nucleus [DR; 7], a brainstem nucleus that contains topographically organized subsets of serotonergic neurons including a subpopulation of neurons projecting to forebrain circuits that modulate anxiety-related behaviors and anxiety states [12].

The basolateral amygdala (BL) is a complex structure with topographically organized neuronal populations and is enriched with 5-HT containing terminals [13–16]. Neurotransmission in the BL plays an important part in emotional learning and memory [17,18]. Furthermore, several studies suggest that the BL plays an important role in regulating anxiety states [17]. For example, administration of CRF or the CRF-related neuropeptide urocortin 1 (Ucn 1) as well as GABA receptor antagonists into the BL induces anxiety-like behavior in the social interaction test [19–21]. In contrast, administration of midazolam (a benzodiazepine receptor agonist) or blockade of *N*-methyl-D-aspartic acid (NMDA) or non-NMDA glutamate receptors in the BL decreases anxiety-like behavior in the social interaction test [19,22]. Together, these data suggest that the BL is a nodal structure in regulating anxiety-related behavior and anxiety states.

Stress-induced neuronal activity in the BL could be a contributing factor to anxiety states associated with chronic stress. For instance, increases in the excitability of BL neurons are observed following acute CRF receptor activation *in vitro* [23]. Also, repeated injections of subthreshold doses of the CRF-related neuropeptide Ucn 1 into the BL results in a long-lasting anxiety state as measured in the social interaction test and elevated plus-maze [24]. Furthermore, pretreatment with NMDA receptor antagonists blocks this stress-induced chronic anxiety state [24]. These data suggest that synaptic plasticity within the BL plays a critical role in the development of chronic anxiety states.

The BL consists of large pyramidal projection neurons and smaller non-pyramidal interneurons that are primarily GABAergic. A subset of GABAergic interneurons in the BL expresses the calcium binding protein, parvalbumin (PV) and the excitatory 5-HT_{2A} receptor [25]. Activation of 5-HT_{2A} receptors increases inhibitory drive to BL projection neurons [26–28] and 5-HT_{2A} receptor-mediated inhibition of projection neurons is the main effect of 5-HT in the BL [26,29]. In our previous experiments with male rats, FG-7142 increased c-Fos (the protein product of the immediate-early gene, *c-fos*) expression within the PV-ir neurons in multiple subregions of the BL [30]. Based on previous studies demonstrating an important role for BL circuitry in regulating anxiety states, we have postulated that chronic anxiety states in female rats exposed to adolescent social isolation may be due to dysregulation of local GABAergic inhibition in the BL. The objective of this study was to investigate the effects of post-weaning social isolation and a subsequent pharmacological challenge in adulthood with the anxiogenic drug, *N*-methyl-beta-

carboline-3-carboxamide (FG-7142; a partial inverse agonist at the benzodiazepine allosteric site on the γ -aminobutyric acid (GABA)_A receptor), on functional cellular responses in topographically organized subpopulations of PV-expressing GABAergic interneurons in the BL. We hypothesized that post-weaning social isolation in female rats decreases inhibitory activity within the BL during anxiogenic challenge.

2. Materials and Methods

2.1. Animals

Timed-pregnant Sprague Dawley rats (Cat. No.240F; Harlan Laboratories, Indianapolis, IN, USA) arrived on gestational day 12. Upon arrival, dams were individually housed in clear polycarbonate cages (10.25" width $\times$ 18.75" length $\times$ 8" height; Alternative Design, Siloam Springs, AR, USA) containing a one-inch deep layer of bedding (Cat. No. 7090; Teklad Sani-Chips; Harlan Laboratories). The day of birth was designated as postnatal day 0 (PD0). On PD21 (day of weaning), thirty-one female rat pups from 12 litters were pooled and then, using a completely randomized design with 1–2 weanlings from each litter, assigned to one of four treatment groups and housed accordingly. Food (Cat. No. 8640; Teklad 22/5 Rodent Diet, Harlan Laboratories) and tap water, stored in 16 oz reduced-height water bottles (Cat. No. WB16RH; Alternative Designs) with screw lids (Cat. No. FSPCST2.5; AnCare Corp., Bellmore, NY, USA), were available *ad libitum*, and rats were maintained on a 12-h light/dark cycle (lights on at 07:00 h) at room temperature (RT; 22 °C). All animal care was conducted in accordance with the guidelines of the *Guide for the Care and Use of Laboratory Animals*, Eighth Edition (Institute for Laboratory Animal Research, The National Academies Press, Washington, D.C., 2011) and approved by the University of Colorado Institutional Animal Care and Use Committee. All efforts were made to minimize the number of animals used and their suffering.

2.2. Isolation/re-socialization procedure

This procedure was based on a previously published protocol of social isolation/re-socialization [Fig. 1; 7,11,31]. On PD21 (weaning age corresponding to pre-adolescence), female Sprague Dawley rats were housed either individually or in groups of 3 in polycarbonate cages for a 3 week period from PD21 to PD42. Thus, rats were isolated from what is considered preadolescence through early- and most of mid-adolescence [32–34]. After 3 weeks of isolation or group housing, rats were weighed and group-housed (2–3 rats/cage) according to treatment (isolates with isolates, N = 15; group-reared with unfamiliar group-reared rats, N = 16) for a further 20 days (PD42–PD62). This re-socialization following the critical isolation period (pre- to midadolescence) allowed the rats to complete their development from mid-adolescence to early adulthood housed in groups. Furthermore, re-socialization helps serve as a control to ensure that any observed behavioral, neurochemical, or gene expression changes are due to social isolation during a suspected critical period of adolescent development (PD21–PD42) instead of at any other point during early-life. At the end of the 5-week isolation/re-socialization procedure, at a stage considered early adulthood (PD62), rats were challenged with FG-7142 or vehicle.

2.3. Challenge with FG-7142

Twenty days following the 3-week isolation procedure, on PD62, rats previously exposed to post-weaning social isolation or group housing received injections of either FG-7142 (N = 16; 7.5 mg/kg, i.p.; Cat No. 0554, Tocris Bioscience, Ellisville, MO, USA) in vehicle (0.9% saline containing 40% 2-hydroxypropyl- β -cyclodextrin (HBC); Cat No. 332607, Sigma-Aldrich, St. Louis, MO, USA) or vehicle alone (N=15) and returned to their home cages. This dose has been shown to increase vigilance and arousal behaviors when tested in a home cage environment, consistent with an increase in an anxiety-like state, 30 to 60 min

following administration of FG-7142 [30,35]. Furthermore, this dose of FG-7142 (7.5 mg/kg) has been shown to increase c-Fos expression in multiple anxiety-related brain regions [35–40] and in parvalbumin-containing GABAergic interneurons in the basolateral amygdala [30]. A 2 hour delay between injection and sacrifice was selected because maximum c-Fos expression occurs between 1 and 3 h following exposure to stress-related stimuli [41]. In addition, previous studies have demonstrated FG-7142-induced c-Fos expression at this time point [30,35–37].

2.4. Dual immunohistochemical detection of parvalbumin and c-Fos in the BL

Two hours following the injections, isolation- and group-reared rats were deeply anesthetized with sodium pentobarbital (90 mg/kg, i.p., Fatal Plus, Vortech, Dearborn, MI, USA) and lavaged to determine stage of estrous cycle with light microscopy. The stage of estrous was determined by examining vaginal cytology with light microscopy. Diestrus was characterized by the presence of leukocytes, proestrus by round nucleated cells, and estrus by cornified, irregularly shaped cells with degenerate nuclei. In each housing condition, all stages of the estrous cycle were represented.

Under anesthesia, rats were perfused transcardially with cold (4 °C) phosphate-buffered saline (0.05 M PBS, pH 7.4), followed by cold (4 °C) 4% paraformaldehyde in 0.1 M sodium phosphate buffer (prepared using 40 g paraformaldehyde, 15 g sucrose, 404 ml 0.2 M Na₂HPO₄, 96 ml 0.2 M NaH₂PO₄, and 500 ml dH₂O, brought to pH 7.4 with sodium hydroxide pellets). Brains were then removed from the cranium, post-fixed overnight in the same fixative at 4 °C, then rinsed 2 times in 0.1 M sodium phosphate buffer (PB; 80.8% 0.1 M Na₂HPO₄·7H₂O, and 19.2% 0.1 M NaH₂PO₄·H₂O), and then immersed in 30% sucrose in 0.1 M sodium phosphate buffer and stored for 2 days at 4 °C. Brains were blocked using a rat brain matrix (RBM -4000C, ASI Instruments, Warren, MI, USA) with a razor blade directly caudal to the mammillary bodies (approximately –5.60 mm bregma). Brains were then flash-frozen with isopentane (cooled between –30 and –40 °C with dry ice) and stored at –80 °C until sectioning. Brains were sectioned at 30 µm using a cryostat and 6 alternate sets of sections were stored in cryoprotectant (30% ethylene glycol, 20% glycerol, 0.05 M PB, pH 7.4) at –20 °C until processed for immunostaining.

One set of alternate sections throughout the BL complex was used for dual immunohistochemical staining for c-Fos (used as a marker of cellular activation) and parvalbumin (which identifies a subset of local inhibitory GABAergic interneurons). Sections were rinsed with 0.05 M PBS for 15 min and then incubated with 0.05 M PBS containing 1% H₂O₂ for 15 min and then rinsed again for 15 min with 0.05 M PBS. Sections were then incubated in 0.05 M PBS containing 0.3% Triton X-100 for 15 min, then incubated in rabbit anti-c-Fos polyclonal antibody in 0.05 M PBS containing 0.1% Triton X-100 (PBST; 1:3,000; Cat. No. PC38, Calbiochem, Gibbstown, NJ, USA) overnight at RT and then rinsed 2 times for 15 min each time in 0.05 M PBS. Sections were then incubated in biotinylated donkey anti-rabbit IgG (1:500; Cat. No. 711-065-152, Jackson ImmunoResearch, West Grove, PA, USA) for 90 min in 0.05 M PBS at RT. Tissue was rinsed in 0.05 M PBS (2 × 15 min) and then incubated in an avidin-biotin-peroxidase complex (ABC; 1:200; Cat. No. PK-6101; Vector Laboratories, Burlingame, CA, USA) for 90 min. Sections were again rinsed in 0.05 M PBS (2 × 15 min) and then placed in peroxidase substrate chromogen (Vector SG, Cat. No. SK-4700, Vector Laboratories) for 15 min in order to visualize c-Fos immunostaining. Sections were then rinsed in 0.05 M PBS (2 × 15 min) to stop the reaction and then in 0.05 M PBS containing 1% H₂O₂ for 15 min. Tissue was rinsed in 0.05 M PBS containing 0.3% Triton X-100 for 15 min, and then incubated with mouse anti-parvalbumin (PV), polyclonal affinity-purified antibody in 0.1% PBST (1:20,000; Cat. No. T8575, Sigma-Aldrich) at RT overnight. Tissue was rinsed in 0.05 M PBS (2 × 15 min) and then incubated in biotinylated donkey anti-mouse IgG in 0.05

M PBS (1:200; Cat. No. E0464, DAKO) for 90 min at RT. Sections were again rinsed in 0.05 M PBS (2×15 min) and then placed in an ABC reagent (1:200; Cat. No. PK-6101, Vector Laboratories) for 90 min at RT and then rinsed 2 more times in 0.05 M PBS for 15 min each. Tissue was then placed in peroxidase substrate, 0.01% 3–3'-diaminobenzidine tetrahydrochloride (Cat. No. D9015, Sigma-Aldrich) in 0.05 M PBS containing 0.005% H_2O_2 for 10–20 min. Finally, tissue was rinsed in 0.05 M PBS for 2×15 min to stop the reaction. Tissue was stored at 4 °C in 0.1 M PB with 0.01% sodium azide until mounted. Tissue was then rinsed briefly with 0.15% gelatin in distilled water and mounted onto slides; cover slips were mounted on the slides using Entellan rapid mounting medium (Cat. No. 14802; EMD Chemicals, Gibbstown, NJ, USA).

2.5. Analysis of c-Fos expression in BL GABAergic and non-GABAergic interneurons

The numbers of c-Fos-ir cells within subdivisions of the BL (Fig. 2) were determined by an observer blind to the treatment conditions. The numbers of c-Fos-immunoreactive/parvalbumin (PV)-immunoreactive (c-Fos-ir/PV-ir) neurons, c-Fos-ir/PV-immunonegative cells, and the total numbers of PV-ir neurons (c-Fos-ir/PV-ir neurons and c-Fos-immunonegative/PV-ir neurons) were determined within specific subdivisions of the BL across two rostrocaudal levels, including the lateral amygdaloid nucleus, dorsolateral part (LaDL) and the basolateral amygdaloid nucleus, anterior part (BLA) at -2.12 mm bregma (Fig. 2A), and the LaDL, lateral amygdaloid nucleus, ventromedial part, (LaVM), lateral amygdaloid nucleus, ventrolateral part (LaVL), BLA, and the basolateral amygdaloid nucleus, posterior part (BLP), at -3.30 mm bregma (Fig. 2B). Round or oval-shaped nuclei with blue-black immunostaining were counted as c-Fos-ir nuclei, and cells with light brown staining throughout the cytoplasm were counted as PV-ir neurons. Cells with both light brown staining of cytoplasm and blue-black staining of nuclei were counted as c-Fos-ir/PV-ir double-immunostained neurons. Data from 3 rats were not included in the analysis due to poor tissue quality.

2.6. Statistical Analysis

Cell count data for numbers of c-Fos-ir/PV-ir neurons, c-Fos-ir/PV-immunonegative cells, and the total number of PV-ir neurons in the BL were analyzed using a linear mixed model analysis using *rearing condition* and *drug* as between-subjects factors and *brain region* as a within-subjects factor. When appropriate, planned pairwise comparisons were conducted using Fisher's protected least significant difference (LSD) tests. All statistical analyses were conducted using PASW Statistics (19.0 for Windows; SPSS Inc., Chicago, IL, USA).

A Grubbs' outlier test was performed prior to the linear mixed model analysis (Grubbs, 1969), and outliers were removed. The Grubbs' test analysis identified 6 outliers out of 565 data points (1.06%) for c-Fos-ir/PV-ir cell counts, 2 outliers out of 565 data points (0.35%) for c-Fos-ir/PV-immunonegative cell counts, and 4 outlier out of 565 data points (0.71%) for the total number of PV-ir GABAergic interneurons sampled in the BL.

All cell count data are expressed as means + S.E.M. All brain regions were included in each linear mixed model analysis. Statistical significance was accepted when $p < 0.05$.

3. Results

3.1. Mean c-Fos-ir GABAergic interneurons in subdivisions of the BL

Rearing condition and FG-7142 both had main effects to alter the numbers of c-Fos-ir/PV-ir neurons within the BL, while the effect of drug was dependent on brain region (Fig. 3; *rearing condition*: $F_{(1, 79)} = 8.35$, $p = 0.008$; *drug*: $F_{(1, 79)} = 24.52$, $p < 0.001$; *brain region*: $F_{(6, 79)} = 20.55$, $p < 0.001$; *rearing condition drug* $\times$ *drug* interaction: $F_{(1, 79)} = 2.83$, $p =$

0.105; *rearing condition* $\times$ *brain region* interaction: $F_{(6, 79)} = 1.54, p = 0.213$; *drug* $\times$ *brain region* interaction: $F_{(6, 79)} = 6.73, p < 0.001$; *rearing condition* $\times$ *drug* $\times$ *brain region* interaction: $F_{(6, 79)} = 1.83, p = 0.143$). Photomicrographs in Fig. 4 illustrate FG-7142-induced c-Fos expression in PV-ir GABAergic interneurons in the BLA (−3.30 mm bregma) in representative rats from each treatment condition.

3.1.1. FG-7142 effects—Among group-reared rats, FG-7142-injected rats had increased numbers of c-Fos-ir/ PV-ir GABAergic interneurons in the LaDL (−2.12 mm and −3.30 mm bregma) and BLA (−2.12 mm and −3.30 mm bregma) compared to vehicle-injected controls. The only effect of FG-7142 on c-Fos expression in PV-ir GABAergic interneurons in isolation-reared rats was an increase in the BLA (−3.30 mm bregma).

3.1.2. Rearing effects—Among vehicle-injected rats, isolation-reared rats had less c-Fos expression in PV-ir GABAergic interneurons in the LaVM (−3.30 mm bregma) compared to group-reared controls. Among FG-7142-injected rats, c-Fos expression in PV-ir GABAergic interneurons was decreased in the LaDL (−2.12 mm and −3.30 mm bregma), BLA (−3.30 mm bregma) and BLP (−3.30 mm bregma) of isolates when compared to group-reared rats.

3.2. c-Fos-ir PV-immunonegative cells in subdivisions of the BL

Rearing condition and FG-7142 both had main effects to alter the numbers of c-Fos-ir/PV-immunonegative neurons within the BL, while the effects of both drug and rearing condition were dependent on brain region (Figure 5; *rearing condition*: $F_{(1, 79)} = 47.15, p < 0.001$; *drug*: $F_{(1, 79)} = 22.49, p < 0.001$; *brain region*: $F_{(6, 79)} = 18.91, p < 0.001$; *rearing condition* $\times$ *drug* interaction: $F_{(1, 79)} = 2.26, p = 0.146$; *rearing condition* $\times$ *brain region* interaction: $F_{(6, 79)} = 4.08, p < 0.001$; *drug* $\times$ *brain region* interaction: $F_{(6, 79)} = 3.65, p = 0.010$; *rearing condition* $\times$ *drug* $\times$ *brain region* interaction: $F_{(6, 79)} = 1.70, p = 0.166$).

3.2.1. FG-7142 effects—Among group-reared rats, FG-7142-injected rats had increased c-Fos expression in PV-immunonegative cells in the LaDL (−2.12 mm bregma), BLA (−2.12 mm and −3.30 mm bregma), and BLP (−3.30 mm bregma) compared to vehicle-injected rats. Among isolation-reared rats, FG-7142-injected rats had increased c-Fos expression in PV-immunonegative cells in the BLA (−3.30 mm bregma) and BLP (−3.30 mm bregma) compared to vehicle-injected rats.

3.2.2. Rearing effects—*Post hoc* analysis revealed that among vehicle-injected rats, isolation-reared rats had decreased c-Fos expression in the LaDL (−3.30 mm bregma) and LaVM (−3.30 mm bregma) compared to group-reared controls. Among FG-7142-injected rats, c-Fos expression was decreased in the LaDL (−2.12 mm bregma), BLA (−2.12 mm bregma and −3.30 mm bregma), LaVL (−3.30 mm bregma), LaVM (−3.30 mm bregma) and BLP (−3.30 mm bregma) of isolates when compared to group-reared rats.

3.3. PV-ir neurons in subdivisions of the BL

As expected, the number of PV-ir cells sampled within each subdivision of the BL varied across subdivisions; however, there were no effects of either rearing condition or FG-7142, and no interactions between rearing condition or FG-7142 and brain region, on the numbers of PV-ir cells (Figure 3; *rearing condition*: $F_{(1, 79)} = 4.04, p = 0.056$; *drug*: $F_{(1, 79)} = 1.75, p = 0.20$; *brain region*: $F_{(6, 79)} = 92.84, p < 0.001$; *rearing condition* $\times$ *drug* interaction: $F_{(1, 79)} = 2.54, p = 0.124$; *rearing condition* $\times$ *brain region* interaction: $F_{(6, 79)} = 1.76, p = 0.151$; *drug* $\times$ *brain region* interaction: $F_{(6, 79)} = 2.03, p = 0.101$; *rearing condition* $\times$ *drug* $\times$ *brain region* interaction: $F_{(6, 79)} = 1.05, p = 0.422$).

4. Discussion

Social isolation of female rats during a critical period of development had a main effect to decrease c-Fos expression in PV-expressing GABAergic interneurons. Group-reared rats challenged with FG-7142 as adults responded with increased c-Fos expression in PV-expressing GABAergic interneurons in a majority of BL subdivisions, including the rostral and caudal LaDL and BLA when compared to vehicle-injected controls. In contrast, isolation-reared rats failed to respond to FG-7142 with increased c-Fos expression in PV-expressing GABAergic interneurons in the rostral LaDL and BLA, and responded with an attenuated increase in c-Fos expression in the caudal BLA. These findings may be dependent on an overall decreased excitability of the PV-expressing subpopulation of GABAergic interneurons under both basal and FG-7142-challenged conditions. In non-PV-expressing cells, c-Fos expression was increased following FG-7142 administration in group-reared rats in the rostral LaDL, rostral and caudal BLA, and the BLP. In several cases, i.e., the rostral LaDL and BLA and the caudal BLA and BLP, the effects of FG-7142 on c-Fos expression in non-PV-expressing cells were attenuated or absent in isolation-reared rats. Again, these findings may be dependent on an overall decreased excitability of the non-PV-expressing cells under both basal and FG-7142-challenged conditions. Together, these data suggest that post-weaning social isolation of female rats leads to decreased excitability of a stress-related subpopulation of local inhibitory PV-expressing GABAergic interneurons within the BL, a nodal structure controlling anxiety states.

Group-reared rats challenged with FG-7142 as adults responded with increased c-Fos expression in PV-expressing GABAergic interneurons in a majority of BL subdivisions, including the rostral and caudal LaDL and BLA, but not the caudal LaVL, LaVM, or BLP, when compared to vehicle-injected controls. These findings are consistent with a previous study showing that FG-7142 increased c-Fos expression in PV-expressing GABAergic interneurons in the rostral and caudal LaDL and BLA, but not in the caudal LaVL, LaVM, or BLP of male rats [30]. Thus, the pattern of FG-7142-induced increases in c-Fos expression in PV-expressing GABAergic interneurons in the BL was identical in male and female rats that were group-reared during adolescence. In contrast, social isolation of female rats during a critical period of development resulted in an attenuation (caudal BLA) or prevention (rostral and caudal LaDL and rostral BLA) of FG-7142-induced increases in c-Fos expression in PV-expressing GABAergic interneurons. The overall main effect of rearing condition may reflect an overall decrease in excitability of PV-expressing GABAergic interneurons in the BL under both basal and FG-7142-challenged conditions. These effects are consistent with previous studies showing that stress exposure, or priming with stress-related neuropeptides, decreases GABAergic inhibitory postsynaptic potentials in BLA projection neurons, in association with development of a chronic anxiety-like state [24,42].

The most robust increases in c-Fos expression in PV-expressing GABAergic interneurons, in both isolation-reared and group-reared rats, were observed in the BLA at rostral and caudal levels, consistent with previous studies showing that anxiogenic stimuli strongly activate the BLA subregion of the BL. For example, exposure of male rats to the open-field test strongly increases c-Fos expression in PV-expressing neurons and non-PV-expressing cells in the caudal BLA [43]. This is likely due to specific afferent input to this region of the BL during challenge with anxiogenic stimuli, including the lateral entorhinal cortex, CA1 region of the ventral hippocampus, and subiculum [44]. This subdivision of the BL has a unique pattern of projections in comparison to other BL subdivision in that it projects to the BLP, the caudate putamen, and the nucleus accumbens [15]. In addition, the BLA has unique reciprocal connections with the frontal, parietal and cingulate cortices as well as input from the lateral subdivisions of the BL and the orbitofrontal cortex [15,45]. These

findings suggest that the BLA subregion of the BL is an important node in the neural circuits controlling anxiety-related behavior and anxiety states.

In contrast to the widespread effects of FG-7142 to increase c-Fos expression in PV-expressing GABAergic interneurons, isolation-reared rats failed to respond to FG-7142 with increased c-Fos expression in PV-expressing GABAergic interneurons in the rostral LaDL and BLA, and responded with an attenuated increase in c-Fos expression in the caudal BLA. This may have been due to a main effect of social isolation to decrease c-Fos expression in the PV-expressing GABAergic interneurons, and a decreased baseline c-Fos expression in social isolates, relative to group-reared rats, although this comparison did not reach statistical significance in all subregions of the BL. As mentioned above, decreased GABAergic inhibition of BLA projection neurons has been associated with amygdala priming and the development of chronic anxiety-like states in rats [24]. These findings raise the possibility that decreased GABAergic inhibitory tone within the BLA may also contribute to the chronic anxiety-like state induced by adolescent social isolation [e.g.; 7,11,31,46,47].

In non-PV-expressing cells, c-Fos expression was increased following FG-7142 administration in group-reared rats in the rostral LaDL, rostral and caudal BLA, and the BLP. The neurochemical phenotype of these non-PV-expressing cells in the BL could be either other populations of GABAergic inhibitory interneurons, or glutamatergic projection neurons. The BL includes large pyramidal projection neurons and smaller non-pyramidal interneurons that are primarily GABAergic. Subsets of GABAergic interneurons in the BL are based on the amount of calcium-binding proteins and include subpopulations of PV-expressing neurons, calbindin-expressing neurons, somatostatin-expressing neurons and small bipolar and bitufted interneurons that exhibit extensive co-localization of calretinin and cholecystokinin [48–52]. In several cases (i.e., the rostral LaDL and BLA and caudal BLA and BLP), the effects of FG-7142 on non-PV-expressing cells were attenuated or even absent in isolation-reared rats. Again, this may have been due to a general effect of social isolation to decrease c-Fos expression in the non-PV-expressing cells, and a decreased baseline c-Fos expression in social isolates, relative to group-reared rats, although this comparison did not reach statistical significance in all subregions of the BL. The implications of isolation rearing on these non-PV-expressing cells will require further study.

5. Conclusions

These data suggest that post-weaning social isolation of female rats interferes with the normal, adaptive activation of a subpopulation of local inhibitory GABAergic interneurons within the BL by stress-related stimuli, which may lead to an increased vulnerability to stress- and anxiety-related responses in adulthood. Chronic anxiety states in female rats exposed to adolescent social isolation may be due to dysregulation of resilience mechanisms involving serotonergic activation of 5-HT_{2A} receptor expressing GABAergic interneurons in the BL.

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Fig. 1.

Diagram illustrating the experimental timeline. White box indicates time of group-or isolation-rearing. Light gray box represents re-socialization period. Dark gray indicates that animals were injected with vehicle (veh) or FG-7142 on postnatal day (PD) 62. Black box represents time at which perfusion and brain extraction were initiated.

Fig. 2.

Photomicrographs of parvalbumin- (PV-)/c-Fos-immunostained sections illustrating subregions of the basolateral amygdaloid complex selected for analysis. Photomicrographs illustrate the basolateral amygdaloid complex at A) -2.12 mm bregma and B) -3.30 mm bregma. Dashed lines, demarcating subdivisions of the basolateral amygdaloid complex were imported directly from a standard stereotaxic atlas of the rat brain (Paxinos and Watson, 1998) and overlaid onto the photomicrographs. Abbreviations: BLA, basolateral amygdaloid nucleus, anterior part; BLP, basolateral amygdaloid nucleus, posterior part; ec, external capsule; ic, internal capsule; LaDL, lateral amygdaloid nucleus, dorsolateral part; LaVL, lateral amygdaloid nucleus, ventrolateral part; LaVM, lateral amygdaloid nucleus, ventromedial part. Scale bar: $250\ \mu\text{m}$.

Fig. 3.

Graphs illustrating the effects of a pharmacological challenge, following a post-weaning social isolation/re-socialization protocol, with the partial inverse agonist at the benzodiazepine allosteric site on the γ -aminobutyric acid (GABA)_A receptor, *N*-methyl-beta-carboline-3-carboxamide (FG-7142, a potent anxiogenic drug) or vehicle on numbers of c-Fos-ir/parvalbumin (PV)-ir neurons (black-lined bars) and total numbers of PV-ir neurons (gray-lined bars) at two rostrocaudal levels of the basolateral amygdaloid complex. Data are presented as means + S.E.M. *, $p < 0.05$ group versus isolation housing condition, within the same drug treatment condition, +, $p < 0.05$ vehicle versus FG-7142 drug treatment effect within the same housing condition, Fisher's protected least significant difference (LSD) test. Sample size, $n = 6$ for group-reared/vehicle, $n = 8$ for group-reared/FG-7142, $n = 7$ for isolation-reared/vehicle, and $n = 7$ for isolation-reared/FG-7142. Abbreviations: BLA, basolateral amygdaloid nucleus, anterior part; BLP, basolateral amygdaloid nucleus, posterior part; LaDL, lateral amygdaloid nucleus, dorsolateral part; LaVL, lateral amygdaloid nucleus, ventrolateral part; LaVM, lateral amygdaloid nucleus, ventromedial part.

Fig. 4.

Photomicrographs illustrating effects of post-weaning social isolation and pharmacological challenge with *N*-methyl-beta-carboline-3-carboxamide (FG-7142) on immunohistochemical staining of c-Fos (dark blue-black-stained cell nuclei) in parvalbumin (brown/orange cytoplasmic staining) neurons in the basolateral amygdaloid complex (−3.30 mm bregma). Low power photomicrographs showing the basolateral amygdaloid complex of rats from each treatment group, including A) group-reared/vehicle (n = 6), B) group-reared/FG-7142 (n = 8), C) isolation-reared/vehicle (n = 7), and D) isolation-reared/FG-7142 (n = 7). Black boxes in A–D are shown at higher magnification in E–H. E–H) Higher power photomicrographs showing the basolateral amygdaloid nucleus. Black boxes in E–H are shown at higher magnification in insets in the lower right corner of each panel. Black arrows indicate c-Fos-ir/PV-immunonegative cells, white arrowheads indicate c-Fos-immunonegative/PV-ir neurons, and black arrowheads indicate c-Fos-ir/PV-ir (double-immunostained) neurons. Scale bar: A–D, 250 μm; E–H, 100 μm, insets, 25 μm.

Fig. 5.

Graphs illustrating the effects of a pharmacological challenge, following a post-weaning social isolation/re-socialization protocol, with FG-7142 or vehicle on numbers of c-Fos-ir/PV-immunonegative cells at two rostrocaudal levels of the basolateral amygdaloid complex (-2.12 mm and -3.30 mm bregma). Data are presented as means + S.E.M. *, $p < 0.05$ group versus isolation housing condition, within the same drug treatment condition, +, $p < 0.05$ vehicle versus FG-7142 drug treatment effect within the same housing condition, Fisher's protected least significant difference (LSD) test. Sample size, $n = 6$ for group-reared/vehicle, $n = 8$ for group-reared/FG-7142, $n = 7$ for isolation-reared/vehicle, and $n = 7$ for isolation-reared/FG-7142. Abbreviations: BLA, basolateral amygdaloid nucleus, anterior part; BLP, basolateral amygdaloid nucleus, posterior part; LaDL, lateral amygdaloid nucleus, dorsolateral part; LaVL, lateral amygdaloid nucleus, ventrolateral part; LaVM, lateral amygdaloid nucleus, ventromedial part.