Published in final edited form as:

Brain Res. 2012 March 14; 1443: 1–17. doi:10.1016/j.brainres.2012.01.005.

Post-weaning social isolation of female rats, anxiety-related behavior, and serotonergic systems

Jodi L. Lukkes¹, Glenn H. Engelman¹, Naomi S. Zelin¹, Matthew W. Hale^{1,2}, and Christopher A. Lowry¹

¹Department of Integrative Physiology and Center for Neuroscience, University of Colorado Boulder, CO 80309, USA

²School of Psychological Science, La Trobe University, Melbourne, VIC 3086, Australia

Abstract

Our previous studies have shown that post-weaning social isolation of male rats leads to sensitization of serotonergic systems and increases in anxiety-like behavior in adulthood. Although studies in humans suggest that females have an increased sensitivity to stress and risk for the development of neuropsychiatric illnesses, most studies involving laboratory rats have focused on males while females have been insufficiently studied. The objective of this study was to investigate the effects of post-weaning social isolation on subsequent responses of an anxiety-related dorsal raphe nucleus (DR)-basolateral amygdala system to pharmacological challenge 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). 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-reared for an additional 2 weeks. We then used dual immunohistochemical staining for c-Fos and tryptophan hydroxylase in the DR or single immunohistochemical staining for c-Fos in the basolateral amygdala. Isolation-reared rats, but not group-reared rats, injected with FG-7142 had increased c-Fos expression within the basolateral amygdala and in serotonergic neurons in the dorsal, ventrolateral, caudal and interfascicular parts of the DR relative to appropriate vehicle-injected control groups. These data suggest that post-weaning social isolation of female rats sensitizes a DR-basolateral amygdala system to stress-related stimuli, which may lead to an increased sensitivity to stress- and anxiety-related responses in adulthood.

Keywords

basolateral amygdala; c-Fos; dorsal raphe nucleus; FG-7142; immunohistochemistry; isolation-rearing

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Please send all correspondence to: Jodi L. Lukkes, Department of Integrative Physiology and Center for Neuroscience, University of Colorado Boulder, Boulder, CO, 80309-0354, USA, Tel: 303-492-8154, Fax: 303-492-0811, jodi.lukkes@colorado.edu.

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1. Introduction

Adverse experience during early development can cause long-lasting alterations in both neural systems and behavior that can manifest as an increased vulnerability to neuropsychiatric disorders such as anxiety and depression in adulthood (Dai et al., 2004; for reviews see, Hall, 1998; Lukkes et al., 2009d; Weiss and Feldon, 2001). One model of adverse early life experience in rats is social isolation during adolescence. Social activity in adolescent rodents is essential for developing an ability to express, and respond appropriately to, intraspecific communicative signals later in life (Meaney and Stewart, 1981; Vanderschuren et al., 1999). Social isolation of rats has the most potent effects on subsequent stress-related behaviors, including anxiety-related behaviors, during a critical phase from weaning (postnatal day 21; PD21) to early adulthood (PD56; Einon and Morgan, 1977; Leng et al., 2004; Weiss et al., 2004a). These changes are long-lasting and persist even after re-socialization (Einon and Morgan, 1977; Kraemer et al., 1984; Leng et al., 2004; Lukkes et al., 2009a; Lukkes et al., 2009c; Weiss et al., 2004b; Wright et al., 1991). Adult rats exposed to early-life social isolation exhibit altered serotonergic activity in various forebrain regions (Fulford and Marsden, 1998; Jones et al., 1992; Lukkes et al., 2009c) that is associated with increased aggression, anxiety, and fear (Einon and Morgan, 1977; Lukkes et al., 2009b; Stanford et al., 1988; Wright et al., 1991).

In the few studies that have investigated the effects of post-weaning social isolation on behavioral measures in female rodents, findings have been inconsistent. However, the limited number of studies that have used female rats suggest that isolation rearing and/or social deprivation appears to increase anxiety-like behavior of female rats (e.g.; Arakawa, 2007; Leussis and Andersen, 2008). For example, female rats isolated from pre- to mid-adolescence, compared to group-reared rats, showed increased latency to emerge into an unfamiliar open-field, decreased center entries and decreased defensive burying (indicative of reduced proactive coping) when tested between P40 and P45 in a state of social deprivation (Arakawa, 2005; Arakawa, 2007; Einon and Morgan, 1977). In addition, a shorter isolation period of female rats from P30–35 also reduced time spent in open arms when tested on the elevated plus-maze (EPM) at P36 (Leussis and Andersen, 2008). In a recent study by Hermes et al. (2011), female rats that were isolated from P19 to P70 exhibited increased anxiety-like behavior in an open-field and social interaction test when compared to group-reared controls (Hermes et al., 2011). Moreover, Weintraub et al. (2010) isolated rats from P30 to P50 and then re-socialized rats until P70 and found that female rats exhibited higher (corticosterone) CORT responses during restraint and during recovery from restraint when compared to group-reared controls (Weintraub et al., 2010). In contrast, Weiss et al. (2004) isolated rats from PD21 until day of EPM testing in adulthood (P91), and found female rats did not exhibit increased anxiety-like behavior, nor was an increase in adrenocorticotrophic (ACTH) or CORT observed in response to a stressor (Weiss et al., 2004b). Similarly, Ferdman et al. (2007) found no effect of 13–14 weeks of social isolation in female rats on anxiety-like behavior in the social interaction test (Ferdman et al., 2007). Methodological differences amongst isolation studies that include differences in onset and duration of isolation as well as strain differences may account for the lack of consistency in relation to the expression of heightened anxiety states.

In the vast majority of post-weaning social isolation studies, rats, typically males, remain in isolation for 4–6 weeks or more (for review, see Fone and Porkess, 2008; Lapid et al., 2003) and are then tested while still in isolation-housed conditions. Thus, rats are tested in a current state of social deprivation in addition to being reared in isolation during post-weaning development (Hall, 1998; Potegal and Einon, 1989). To assess the effects of post-weaning social isolation in the absence of current social deprivation, we and others have developed models of post-weaning social isolation where rats are isolated from weaning

(PD21–22) to mid-adolescence (PD35 or 42) and then re-housed in groups until testing in later adolescence or early adulthood (e.g. Lukkes et al., 2009a; Lukkes et al., 2009b; Lukkes et al., 2009c; Potegal and Einon, 1989; Van den Berg et al., 1999; Fig. 1). This approach has been valuable in determining the long-term effects of isolation rearing on later behavioral and neural measures.

The mechanisms through which adverse early-life experiences alter vulnerability to stress-related disorders are not clear. One mechanism through which adverse early-life experiences may alter sensitivity to stress-related disorders is through long-term changes in the activity of the global monoaminergic systems, including serotonergic systems, projecting to forebrain circuits that regulate stress- and anxiety-related behaviors (Graeff et al., 1996; Hodges et al., 1987; Maier et al., 1995b; Maier et al., 1995a; Maier and Watkins, 1998; Wise et al., 1972). Early-life 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 alters corticotropin-releasing factor (CRF)-mediated extracellular concentrations of serotonin (5-hydroxytryptamine; 5-HT) in the nucleus accumbens (NAc), a key limbic structure implicated as a substrate for many of the behavioral alterations observed with post-weaning social isolation. In addition, our previous studies also suggest that post-weaning social isolation causes an up-regulation of CRF type 2 (CRF₂) receptor expression in the dorsal raphe nucleus (DR), 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 (Lowry et al., 2005). Increases in CRF receptor binding in the DR have also been described in adult rats exposed to neonatal maternal deprivation (Ladd et al., 1996). The up-regulation of CRF₂ receptor expression in the DR following post-weaning social isolation may underlie the long-term alterations of CRF-induced serotonergic activity in the NAc observed in isolates (Lukkes et al., 2009c). In addition, previous research suggests the involvement of a serotonergic DR-basolateral nucleus of the amygdala (BL) neuronal circuit in the regulation of anxiety states and anxiety-related behavior. For instance, direct microinjection of the CRF₂ receptor agonist urocortin 2 (Ucn 2) into the DR increases c-Fos (the protein product of the immediate-early gene, *c-fos*) expression within serotonergic neurons in the DR and increases extracellular 5-HT concentrations in the BL (Amat et al., 2004). Further studies suggest that increased anxiety-related behavior in a model of learned helplessness is dependent on activation of 5-HT_{2C} receptors within the BL (Christianson et al., 2010). Together, these data support the hypothetical model of a DR-BL neuronal circuit controlling anxiety states.

Based on previous studies demonstrating an important role for DR serotonergic systems in regulating anxiety states, we have hypothesized that sensitization of DR serotonergic neurons is an important mechanism underlying the increased anxiety state associated with early life social isolation. It is not clear if the sensitization of DR serotonergic neurons in social isolates reflects an effect on serotonergic neurons in general or a more selective effect on specific subpopulations of 5-HT neurons, including those that give rise to the DR-NAc and DR-BL systems. The objective of this study was to investigate the effects of post-weaning social isolation following a pharmacological challenge 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 DR serotonergic and non-serotonergic neurons and on c-Fos expression within the BL. We hypothesized that post-weaning social isolation sensitizes a serotonergic DR-BL system to stress-related stimuli, resulting in a stable increase in anxiety state.

2. Results

2.1. Response to novel dark open-field

Rearing differentially affected behavioral response to a 25 min novel dark open-field on Day 1 (Fig. 2). Isolation-reared rats had a greater duration (Fig. 2A; $F_{(1, 29)} = 6.35, p = 0.013$) and frequency (Fig. 2B; $F_{(1, 132)} = 5.52, p = 0.020$) of immobility compared to group-reared rats. No differences were observed between treatment groups in the amount of distance traveled, mean velocity, time spent in the center or the number of entries made into the center of the open-field.

2.2. Acclimation to dark open-field

Neither group-reared nor isolation-reared rats showed evidence of habituation to the novel dark open-field, as evidenced by total distance moved, over a 3 day period (Fig. 3); note that analysis of behavior on Day 1 represents data from the initial *novel dark open-field* exposure described above. Analysis revealed a main effect of *rearing condition* ($F_{(1, 31)} = 6.22, p = 0.013$), but no effect of *day* nor an interaction between *rearing condition* and *day*. On Day 3, isolation-reared rats displayed decreased total distance moved compared to group-reared rats.

2.3. Social interaction test in a familiar dark open-field

No differences between treatment groups were observed in the frequency of social interaction (SI) bouts (Fig. 4A) or the total duration of SI (Fig. 4B). Also, no differences between treatment groups were observed in the frequency of freezing, rearing, approaches, or grooming in the social interaction test (Fig. 4C). Furthermore, no differences between treatment groups were observed in the total duration of freezing, rearing, or grooming (Fig. 4D).

2.4. c-Fos-ir serotonergic neurons in subdivisions of the DR and PnR

Isolation-rearing resulted in increased sensitivity to FG-7142-induced increases in c-Fos expression in serotonergic neurons in subdivisions of the DR (Fig. 5; *rearing condition* $\times$ *drug* $\times$ *brain region* interaction; $F_{(9, 95)} = 2.72, p = 0.035$). Photomicrographs in Fig. 6 illustrate increases in FG-7142-induced c-Fos expression in serotonergic neurons in the DRD (−8.00 mm bregma) in representative rats from each treatment condition. *Post hoc* analysis revealed differences between group-reared and isolation-reared rats. Among isolation-reared rats, FG-7142-injected rats had increased numbers of c-Fos-ir serotonergic neurons in the DRDSh (−8.00 mm bregma), DRV/VLPAG (−8.00 mm bregma), DRC (−8.54 and −9.16 mm bregma), and DRI (−8.54 mm bregma) compared to vehicle-injected controls. In contrast, the only effect of FG-7142 on c-Fos expression in serotonergic neurons in group-reared rats was a decrease in the DRV (−8.00 mm bregma). Among FG-7142-injected rats, c-Fos expression in serotonergic neurons was greater in the DRDSh (−8.00 mm bregma) of isolates when compared to group-reared rats. *Post hoc* analysis also revealed that among vehicle-injected rats, isolation-reared rats had fewer c-Fos-ir serotonergic neurons in the DRD (−7.46 mm bregma), DRV (−8.00 mm bregma), DRV/VLPAG (−8.00 mm bregma), and DRC (−8.54 and −9.16 mm bregma) compared to group-reared controls. Among FG-7142-injected rats, isolation-reared rats had fewer double immunostained neurons compared to group-reared controls in the DRD (−7.46 mm bregma) and DRV (−7.46 mm bregma).

2.5. c-Fos-ir non-serotonergic cells in subdivisions of the DR and PnR

Isolation rearing resulted in an increased sensitivity to FG-7142-induced increases in c-Fos expression in subdivisions of the DR and in the PnR (Table 1; *rearing condition* $\times$ *drug* $\times$

brain region interaction: $F_{(9, 95)} = 2.63, p = 0.031$). *Post hoc* analysis revealed differences between group-reared and isolation-reared rats. Among isolation-reared rats, FG-7142-injected rats had increased c-Fos expression in non-serotonergic cells in the DRV (−8.00 mm bregma), DRC (−8.54 and −9.16 mm bregma), and PnR (−9.16 mm bregma) compared to vehicle-injected rats. In contrast, among group-reared rats, FG-7142-injected rats had decreased c-Fos expression in non-serotonergic cells in the DRV (−7.46 mm bregma) compared to vehicle-injected rats. No differences in c-Fos expression in non-serotonergic cells were observed between group- and isolation-reared, FG-7142-injected rats. However, *post hoc* analysis revealed that among vehicle-injected rats, isolation-reared rats had fewer c-Fos-ir non-serotonergic cells in the DRDc (−8.00 mm bregma), DRV (−8.00 mm bregma), DRC (−8.54 and −9.16 mm bregma), and PnR (−9.16 mm bregma) compared to group-reared controls.

2.6. TPH-ir neurons in subdivisions of the DR and PnR

As expected, the number of TPH-ir cells sampled within each subdivision of the DR varied across subdivisions ($F_{(9, 95)} = 76.68, p < 0.001$; Fig. 5). However, the total number of TPH-immunopositive cells sampled within each subdivision was similar across treatment groups and there was no interaction between subdivision and treatment groups.

2.7. c-Fos-ir cells in subdivisions of the BL

Isolation-rearing resulted in an increased sensitivity to FG-7142-induced increases in c-Fos expression in subdivisions of the BL (*drug*, $F_{(1, 56)} = 17.89, p = 0.001$; *rearing condition*, $F_{(1, 26)} = 0.49, p = 0.493$; *region*, $F_{(6, 56)} = 17.03, p < 0.001$; *drug* $\times$ *region* interaction, $F_{(6, 56)} = 2.50, p = 0.055$; *rearing condition* $\times$ *region* interaction, $F_{(6, 56)} = 2.02, p = 0.108$; *rearing condition* $\times$ *drug* interaction, $F_{(1, 56)} = 5.63, p = 0.027$; *drug* $\times$ *rearing condition* $\times$ *region* interaction: $F_{(6, 56)} = 0.30, p = 0.932$; Fig. 7). Photomicrographs in Fig. 8 illustrate increases in FG-7142-induced c-Fos expression in the caudal BLA (−3.30 mm bregma) in representative rats from each treatment condition. *Post hoc* analysis revealed differences between isolation-reared rats and group-reared rats. Among isolation-reared rats, FG-7142-injected rats, relative to vehicle-injected controls, had increased c-Fos expression in all subdivision of the BL except in the LaVM and BLP (both −3.30 mm bregma). In contrast, there were no effects of FG-7142 on c-Fos expression in group-reared rats in any of the subdivisions of the BL studied. Furthermore, no differences were observed between group- and isolation-reared rats injected with FG-7142. Among vehicle-injected rats, isolation-reared rats had fewer c-Fos-ir cells in the LaDL (−2.12 and −3.30 mm bregma) compared to group-reared controls.

2.8. Correlations between the numbers of c-Fos-ir cells in the BL with c-Fos-ir/TPH-ir neurons in the DR

The numbers of c-Fos-ir cells in the rostral BLA (−2.12 mm bregma) were positively correlated with the numbers of c-Fos-ir serotonergic neurons and non-serotonergic cells in the DRC (serotonergic cells, −9.16 mm bregma, $r = 0.515, p = 0.017$; Fig. 9A; non-serotonergic cells, −8.54 mm bregma, $r = 0.467, p = 0.044$; Fig. 9B). A positive correlation was also observed between the numbers of c-Fos-ir cells in the caudal BLA (−3.30 mm bregma) and the numbers of c-Fos-ir serotonergic neurons in 1) the DRV/VLPAG at −8.00 mm bregma ($r = 0.576, p = 0.010$; Fig. 9C) and 2) the DRI at −8.54 mm bregma ($r = 0.611, p = 0.003$; Fig. 9DC). The numbers of c-Fos-ir serotonergic neurons were highly correlated with the numbers of non-serotonergic c-Fos-ir cell across multiple rostrocaudal levels of the DR (Table 2).

3. Discussion

Social isolation of female rats during a critical period of development sensitized specific subpopulations of serotonergic neurons in the DR to the anxiogenic drug, FG-7142. In contrast to previous studies in male rats, social isolation of female rats did not respond with novelty-induced locomotion or anxiety-like responses in the open-field or social interaction tests. However, when challenged with FG-7142 as adults, isolates responded with increased c-Fos expression in serotonergic neurons in the mid-rostrocaudal and caudal DR, including the DRDSh, DRVl/VLPAG, DRC, and DRI subdivisions, relative to vehicle-injected controls, whereas group-reared rats did not. In addition, among rats treated with FG-7142, isolates responded with greater c-Fos expression relative to group-reared controls in the DRDSh region. Similar responses were observed in the BL, where isolates responded with FG-7142-induced increases in c-Fos expression in most regions of the BL studied, whereas group-reared rats did not. Together, these data suggest that an anxiety-related serotonergic DR-BL system is sensitized in adult female rats previously reared in isolation.

Social isolation of female rats during adolescence did not alter either novelty-induced locomotion or anxiety-like behavioral responses in the open-field or social interaction tests. This contrasts sharply with our previous studies using male rats, in which social isolates responded with increased anxiety-like behavior in these tests. These different outcomes in studies in male and female isolates may be due to methodological differences. Our previous studies in males used the open-field test during the rats' active phase under bright light conditions, whereas the current studies in females used the open-field test during the inactive phase under low light conditions. Thus, the current study used conditions that are known to be less anxiogenic (Bouwknicht et al., 2007; Cunha and Masur, 1978; Hall et al., 2000; Valle, 1970). Alternatively, these differences may be due to sex differences in responses to social isolation. It is possible that inclusion of females at different stages of the estrous cycle compromises our ability to detect rearing-dependent differences in anxiety-related behavior, particularly as anxiety-related behavior varies across the estrous cycle (Fedotova and Ordyan, 2010; Trabace et al., 2011). Although classic indices of anxiety in the open-field (i.e., time spent in center, and number of entries into the center of the open-field) were not affected, isolates responded with a higher duration and frequency of immobility, which is thought to reflect fear-like behavior (freezing; Stanford, 2007) in the open-field, relative to group-reared rats. However, the total distance moved in the open-field did not differ between isolates and group-housed control rats. As immobility accounted for approximately 35 sec and 50 sec of behavior in group-housed and isolation housed rats, respectively, during the open-field exposure (2–3% of total time), it is likely that this difference in immobility (~15 sec, 1% of total time), although significant, was not sufficient to affect total distance moved. Nevertheless, the increase in immobility in female isolates exposed to the open-field, relative to group-reared rats, suggests an increased emotionality.

In vehicle-injected rats, isolates had lower numbers of c-Fos-positive serotonergic neurons relative to group-reared rats in a number of subregions of the DR, suggesting a hyposerotonergic tone in social isolates. This difference was observed widely throughout the DR for serotonergic neurons, including rostral DRD, mid-rostrocaudal DRV and DRVl, and the DRC. Also among vehicle-injected rats, isolates had lower numbers of c-Fos-positive non-serotonergic neurons relative to group-reared rats in a number of subregions of the DR including the mid-rostrocaudal DRDc and DRV, the caudal DRC and PnR. These regions are not considered specific to stress- or anxiety-related responses (Hale and Lowry, 2011; Lowry et al., 2005), and this difference may reflect a more global difference in baseline serotonergic function. This could result, for example, from an increased expression of inhibitory 5-HT_{1A} autoreceptors in isolates, increases in other inhibitory mechanisms, such as local GABAergic inhibition (Jolas et al., 2000), or decreases in excitatory glutamatergic

or noradrenergic input (Crawford et al., 2011; Vandermaelen and Aghajanian, 1983). The effects of social isolation on 5-HT_{1A} receptor expression or function in the DR, or local GABAergic inhibition, have not been studied previously in female rats and would be an important objective for future studies.

Although we did not detect differences between isolates and group-reared rats in anxiety-like behavior in the open-field and social interaction tests under the conditions used, isolates responded to challenge with the potent anxiogenic drug, FG-7142, with activation of a subset of serotonergic neurons in the DR, whereas group-reared rats did not. It is not clear why group-reared female rats did not respond to FG-7142 with increased c-Fos expression in serotonergic neurons, as previous studies using males did find FG-7142-induced increases in c-Fos expression in DR serotonergic neurons (Abrams et al., 2005). However, previous studies using males involved social isolation for 36–48 h prior to treatment, which may have sensitized the rats to subsequent challenge with FG-7142. Regardless, our findings suggest that anxiety-related serotonergic systems are sensitized in female isolates, relative to group-reared controls. Isolates responded with increased c-Fos expression in serotonergic neurons in the mid-rostrocaudal and caudal DR, a region that has previously been implicated in serotonergic responses to anxiogenic drugs (Abrams et al., 2005), anxiety-related neuropeptides (Staub et al., 2005; Staub et al., 2006), and stress- or anxiety-related stimuli (Gardner et al., 2005; Grahn et al., 1999). In particular, among isolation-reared rats, FG-7142 treatment increased c-Fos expression in the DRDSh, DRV/LVPAG, and DRC, and DRI (all located in the mid-rostrocaudal and caudal DR) but notably not the rostral DRD, and not the DRV at any level studied. Similar effects were observed in male rats treated with FG-7142, with increases in the mid-rostrocaudal DRD and the DRC, but not the rostral DRD, and not the DRV at any level studied (Abrams et al., 2005). Thus, the regional pattern of c-Fos activation in serotonergic neurons in group-reared but briefly isolated male rats and female rats exposed to social isolation during adolescence was very similar.

The apparent hyposerotonergic tone under baseline conditions in isolates contrasts with the increased excitability of serotonergic neurons in isolates following challenge with FG-7142. This dichotomy could arise if multiple regulatory mechanisms are altered by adolescent social isolation. For example, isolation could result in increased tonic local GABAergic input to serotonergic neurons (or decreased tonic excitatory glutamatergic or noradrenergic input), while at the same time, isolation could result in decreased 5-HT_{1A} autoreceptor function, resulting in exaggerated responses following challenge with FG-7142. Dysregulation of 5-HT_{1A} autoreceptor binding and function has been implicated in the etiology of stress-related neuropsychiatric disorders. For instance, reduced 5-HT receptor binding in the DR has been found in patients with unipolar or bipolar depression, panic disorder, and social anxiety disorder.

Among rats treated with FG-7142, isolates responded with greater c-Fos expression in serotonergic neurons relative to group-reared controls in a single region, the DRDSh. The DRDSh region has been suggested previously to be an FG-7142-sensitive region and an important component of a serotonergic DR-BL circuit involved in regulation of anxiety states (Abrams et al., 2005; Hale et al., 2008b; Hale et al., 2008a; Lowry et al., 2005). The DRDSh region contains a large number of serotonergic neurons projecting to the BL, as well as other regions involved in the control of anxiety-related behavior and anxiety states, such as the medial prefrontal cortex, bed nucleus of the stria terminalis, and dorsal hypothalamic area (Abrams et al., 2005; Commons et al., 2003; Lowry et al., 2005). Our data are consistent with the hypothesis that early life social isolation sensitizes anxiety-related serotonergic systems in the DRDSh to anxiogenic stimuli.

Within the amygdala, isolates responded with FG-7142-induced increases in c-Fos expression in most regions of the BL studied. In contrast, group-reared rats did not. The greatest increases in FG-7142-induced c-Fos expression were found in the BLA at both -2.12 and -3.30 mm bregma. This subdivision of the BL has unique projections in comparison to other BL subdivision in that it projects to the BLP, the caudate putamen, and the nucleus accumbens (Swanson and Petrovich, 1998). 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 (Ghashghaei and Barbas, 2002; Swanson and Petrovich, 1998). A neural circuit consisting of the orbitofrontal cortex, the BL, and the nucleus accumbens has been suggested by earlier studies to encode information in relation to both aversive and appetitive events (Holland and Gallagher, 2004). More specifically, this neural circuit involving the BLA may be important in the regulation of approach behavior in conflict situations or in anxiety-related tests, such as the open-field and social interaction tests (Day et al., 2001; Gray and McNaughton, 2003; Hale et al., 2006). For instance, the BLA and BLA-projecting neurons in the lateral entorhinal cortex, ventral subiculum, and CA1 region of the ventral hippocampus are selectively activated following exposure of rats to a mild anxiogenic challenge, such as exposure to the open-field (Hale et al., 2008b; Hale et al., 2006). In the current study, we found increased numbers of c-Fos-ir serotonergic neurons within the DRDSh and increased numbers of c-Fos-ir cells in the BLA of isolation-reared rats injected with FG-7142. As mentioned previously, the DRDSh projects to the BLA and is important in the regulation of anxiety states. 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. The numbers of c-Fos-ir neurons in the BL were positively correlated with the numbers of c-Fos-ir serotonergic neurons in subregions of the caudal DR, including the DRVl/VLPAG, DRC, and DRI, regions that are known to give rise to projections to the BL (Abrams et al., 2005). Furthermore, the numbers of c-Fos-ir cells in the rostral BLA were positively correlated with the numbers of c-Fos-ir non-serotonergic cells in the DRC. These correlations are consistent with the hypothesis that a DR-BL system plays a role in increased sensitivity to anxiogenic challenges.

3.1. Conclusions

Together, these data suggest that an anxiety-related serotonergic DR-BL circuit is sensitized in adult female rats previously reared in isolation. These data are consistent with clinical studies demonstrating decreased expression of autoinhibitory 5-HT_{1A} receptor expression (Drevets et al., 1999; Nash et al., 2008), and increased brain serotonin turnover in patients with anxiety disorders, including panic disorder (Esler et al., 2007).

4. Experimental Procedures

4.1. Animals

Timed-pregnant Sprague Dawley rats (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, housed according to treatment groups. 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.

4.2. 5-week isolation/re-socialization procedure

The procedure in this manuscript was based on a previously published protocol of social isolation/re-socialization (Lukkes et al., 2009a; Lukkes et al., 2009b; Lukkes et al., 2009c). On PD21 (weaning age corresponding to pre-adolescence), female Sprague Dawley rats were housed either individually or in groups of 3 in polycarbonate cages as described above for a period of 3 weeks during a time of pre-adolescent (PD21) to mid-adolescent (PD42) development. 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 housed with unfamiliar group-reared rats, N = 16) for a further 2 weeks (PD42–PD56). This re-socialization following the critical isolation period (pre- to mid-adolescence) allowed the rats to complete their development from mid-adolescence to early adulthood. At the end of the 5-week isolation/re-socialization procedure, when rats were in early adulthood (P56), they were used in the experiments. Behavioral testing was performed during the light cycle from 8:00 a.m. to 6:00 p.m. Testing was done in a randomized design that ensured rats in any given treatment group were not preferentially tested at one time of day. Fig. 1 is a schematic illustrating the experimental timeline of the experiment.

4.3. Exposure to a novel open-field arena

Two days following the 5-week isolation/re-socialization procedure, each of the thirty-one female (n = 15–16/group) rats was placed in a novel dark (~11 lux) plastic square open-field (100 cm width × 100 cm length × 30.48 cm height) illuminated by red light for 25 min to assess anxiety-like behavior. A digital video camera (Sony Handycam, DCR-HC52, Sony Corporation of America, New York, NY, USA) using mini DV digital tape (Sony DVM-60PRL; 360 minute Premium Mini DV Tape, TapeStockOnline, Anaheim, CA, USA) was fixed above the arena to record behavior. For analysis of anxiety-related behavior in the novel open-field, a virtual center square (50 cm × 50 cm) was defined. Tapes were analyzed using a video observation system (Ethovision 6.0 XT, Noldus Information Technology, Wageningen, The Netherlands). The behaviors scored for general locomotion were total distance moved in the arena and mean velocity of movement. The behaviors scored as measures of anxiety-like behavior were time spent in center, and number of entries into the center of the open-field. The behaviors scored as fear-like behavior were frequency and duration of immobility (<2.5% movement).

4.4. Habituation to a novel dark open-field

To determine habituation to the dark open-field, rats were placed individually in the dark open-field for 3 consecutive days (day 1 described above in Section 4.3, and two additional days) for 25 min. Total distance moved (cm) was determined using Ethovision 6.0 XT for 25 minutes each day over the three day acclimation period.

4.5. Exposure to a social interaction test in a familiar open-field arena

Following the three day acclimation period, rats were exposed to a 25 min social interaction test. This involved placement of the rat in the same open-field, illuminated with red light (~11 lux), with an unfamiliar, size and age-matched female conspecific. At the start of the test, rats were placed on opposite sides of the test arena towards the center facing each other. Each unfamiliar conspecific was used only 2 times per day (once with an isolation-housed

experimental rat, and once with a group-housed experimental rat using a completely randomized design). The maximum number of times an unfamiliar conspecific was used was 3 times. Video footage of the social interaction test was then scored by an observer blind to treatment, using Noldus *The Observer*, ver. 5.0 software. The frequency and duration of social interaction were analyzed for the 30 min testing period. Social interaction is defined as behavior that is initiated by the test rat including sniffing, following, grooming, kicking, mounting, jumping on, wrestling and boxing with, crawling over or under the partner. In addition, the latency to first approach the unfamiliar conspecific, the frequency and duration of rearing, freezing, approach, and grooming, were also scored. Immediately following the 30 min social interaction test, 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.

4.6. Challenge with FG-7142

Twenty-four hours following the social interaction test, 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 replaced in their home cages. Our lab, as well as many other labs, has found increased anxiety-like behavior 30 to 60 min following administration of FG-7142 (Abrams et al., 2005; Evans et al., 2006; File and Pellow, 1984; Otter et al., 1997; Salchner et al., 2006; Singewald et al., 2003). This dose of FG-7142 (7.5 mg/kg) has been shown to increase c-Fos expression in multiple anxiety-related brain regions, increase anxiety-like behavior, and increase serotonergic metabolism within multiple stress-related brain regions (Abrams et al., 2005; Evans et al., 2006; File and Pellow, 1984; Otter et al., 1997; Salchner et al., 2006; Singewald et al., 2003). We did not conduct behavioral tests as the maximal behavioral effects are observed 30–60 min following FG-7142 treatment, and behavioral testing at this time point would have been a confound for interpreting drug-induced c-Fos expression at 120 min.

4.7. Dual immunohistochemical detection of tryptophan hydroxylase and c-Fos in the DR

Two hours following FG-7142 or vehicle injections, rats previously reared in isolation and group-reared rats were deeply anesthetized with sodium pentobarbital (90 mg/kg, i.p., Fatal Plus, Vortech, Dearborn, MI, USA) and lavaged a second time to determine stage of estrous cycle. Under anesthesia, rats were perfused transcardially with phosphate-buffered saline (0.05 M PBS, pH 7.4) at RT, 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 razorblade directly caudal to the mammillary bodies (approximately –5.60 mm bregma) to ensure a consistent coronal plane of sectioning. 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 μ m 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 brainstem raphe complex was used for dual immunohistochemical staining for c-Fos (used as a marker of cellular activation) and tryptophan hydroxylase (TPH; the rate-limiting enzyme in the biosynthesis of serotonin; used as a marker of serotonergic neurons). Sections were rinsed 2 times with 0.05 M PBS. Sections were then incubated with 0.05 M PBS containing 1% H₂O₂ for 15 min, then rinsed 2 times (15 min each) with 0.05 M PBS, and then incubated in 0.05 M PBS containing 0.3% Triton X-100 for 15 min. Sections were 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 18 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 then incubated with sheep anti-tryptophan hydroxylase (TPH), polyclonal affinity-purified antibody in 0.1% PBST (1:12,000; Cat. No. T8575, Sigma-Aldrich) at RT overnight and then rinsed in 0.05 M PBS (2 × 15 min) before being incubated in biotinylated rabbit anti-sheep IgG in 0.05 M PBS (1:200; Cat. No. PK-6106, Vector Laboratories) 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. SK-4700, Vector Laboratories), in 0.05 M PBS containing 0.005% H₂O₂ for 10–20 min and then rinsed in 0.05 M PBS 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).

4.8. Immunohistochemical detection of c-Fos in the BL

One set of alternate sections throughout the BL complex was used for single immunohistochemical staining for c-Fos. Methods were identical to those described in Section 4.7 above for immunostaining of c-Fos in the dorsal raphe nucleus, but excluded steps for immunostaining of TPH.

4.9. Analysis of c-Fos expression in DR serotonergic and non-serotonergic neurons

Sections were analyzed by an observer blind to the treatment conditions. The numbers of c-Fos-immunoreactive/TPH-immunoreactive (c-Fos-ir/TPH-ir) neurons, c-Fos-ir/TPH-immunonegative cells, and the total numbers of TPH-ir neurons (c-Fos-ir/TPH-ir neurons and c-Fos-immunonegative/TPH-ir neurons) were determined within subdivisions of the rostral (−7.46 mm bregma), mid-rostrocaudal (−8.00 mm bregma), and caudal (−8.54 mm bregma; −9.16 mm bregma) regions of the DR (Fig. 5). Subdivisions and anatomical levels of the DR were determined using a stereotaxic atlas of rat brain (Paxinos and Watson, 1998) and an atlas of TPH immunoreactivity in rat brain (Abrams et al, 2004). 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 TPH-ir neurons. Cells with both light brown staining of cytoplasm and blue-black staining of nuclei were counted as c-Fos-ir/TPH-ir double-immunostained neurons. Analysis of immunostaining in the DR was performed in specific subdivisions of the DR, including the dorsal raphe nucleus, dorsal

part (DRD) and dorsal raphe nucleus, ventral part (DRV) at -7.46 mm bregma, the DRD core (DRDc, as defined by Abrams et al., 2005), DRD shell (DRDSh, as defined by Abrams et al., 2005), DRV, and dorsal raphe nucleus, ventrolateral part/ventrolateral periaqueductal gray (DRVl/VLPAG) at -8.00 mm bregma, the dorsal raphe nucleus, caudal part (DRC) and dorsal raphe nucleus, interfascicular part (DRI) at -8.54 mm bregma, and DRC, and pontine raphe nucleus (PnR) at -9.16 mm bregma (Fig. 5). Data from 1 rat was not included in the analysis due to poor tissue quality.

4.10. Analysis of c-Fos expression in the BL

The numbers of c-Fos-ir cells within subdivisions of the BL (Fig. 7) were determined by an observer blind to the treatment conditions. Analysis of c-Fos immunostaining was performed in 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, 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. Round or oval-shaped nuclei with blue-black immunostaining were counted as c-Fos-ir nuclei. A camera lucida system attached to an upright Leica DMLS microscope with a C plan 10X objective lens (Leica Mikroskopie und Systeme GmbH, Wetzlar, Germany) was used to count c-Fos-ir cells. The microscope projected the image of the c-Fos-ir nuclei onto paper and the projected image was then traced by an experimenter blind to the treatment groups. An adjacent Nissl-stained coronal section (at approximately -2.12 and -3.30 mm bregma) for each c-Fos-immunostained rat brain section was also drawn using the camera lucida system. The microscope was used to project the image of the BL onto acetate, which was then traced by the experimenter. The Nissl-stained section was used to generate separate templates that delineated the subdivisions of the left and right BL, using anatomical criteria described by Hale et al., 2006. Landmarks, such as the apex of the BL, the external capsule marking the lateral border of the complex, and blood vessels, were used to align the template with the c-Fos-immunostained section. The template for each individual rat brain section was placed over the camera lucida drawing of c-Fos-ir nuclei in the adjacent section from the same rat and the number of c-Fos-ir cells in each subdivision was counted. Data from 2 rats were not included in the analysis due to poor tissue quality.

4.11. Statistical Analysis

The effect of rearing condition on open-field behaviors (anxiety-related behaviors) on day 1 was analyzed using Student's *t*-tests while habituation over the 3 day habituation period (total distance moved) was analyzed using analysis of variance (ANOVA) with repeated measures with *rearing condition* as a between-subjects factor and *day* (Days 1–3) as a within-subjects factor. For the social interaction test, the effects of *rearing condition* on anxiety-like behavioral measures were analyzed using Student's *t*-tests. Statistical significance was accepted when $p < 0.05$.

Cell count data for numbers of c-Fos-ir/TPH-ir neurons, c-Fos-ir/TPH-immunonegative cells, and the total number of TPH-ir neurons in the DR and c-Fos-ir nuclei in the BL were analyzed using separate multifactor analysis of variance (ANOVA) with repeated measures 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 Grubb's outlier test was performed prior to all multifactor ANOVAs (Grubbs, 1969), and outliers were removed. For the open-field behaviors Days 1–3, 105 out of 4920 data points

were excluded (2.0% of total data). For analysis of behavioral data from the social interaction test on Day 4, 9 out of 150 data points for duration were excluded (6.0% of total data) and 4 out of 150 data points for frequency were excluded (2.7% of total data). The Grubb's test analysis identified 10 outliers out of 350 data points (2.9%) for c-Fos-ir/TPH-ir cell counts, 5 outliers out of 350 data points (1.4%) for c-Fos-ir/TPH-immunonegative cell counts, and 1 outlier out of 350 data points (0.3%) for the total number of serotonergic neurons sampled in the DR. In the BL, 1 out of 210 data points (0.5%) was excluded for c-Fos-ir cell counts.

Replacement data for the repeated measures ANOVAs were calculated using the Petersen method (Petersen, 1985). Replacement data were not included in *post hoc* analyses and are not represented in tables or in graphical representation of the data. All behavioral and cell count data are expressed as means $\pm$ S.E.M. All brain regions were included in each overall multifactor ANOVA with repeated measures. Statistical significance was accepted when $p < 0.05$.

Acknowledgments

C.A. Lowry is supported by an NSF CAREER Award (NSF-IOS #0845550) and a 2010 NARSAD Young Investigator Award. G.H. Engelman was supported by a Bioscience Undergraduate Research Skills and Training (BURST) fellowship, and an Undergraduate Research Opportunities Program (UROP)/Howard Hughes Medical Institute (HHMI) Individual Grant funded by the Biological Sciences Initiative (BSI) through a grant from the Howard Hughes Medical Institute (HHMI). N.S. Zelin was supported by a BURST fellowship, and an UROP Assistantship Grant funded by the BSI. The project described was supported by Award Numbers F32MH084463 (JLL) and R01MH086539 (CAL) from the NIMH. C.A. Lowry has consulted for Enlight Biosciences. The authors certify that they have no other actual or potential conflicts of interest in relation to this article, nor do they have a financial relationship with the organization that sponsored the research. The authors have full control of all primary data and agree to allow the journal to review the data if requested.

Abbreviations

FG-7142	N-methyl-beta-carboline-3-carboxamide
DR	dorsal raphe nucleus
DRD	dorsal raphe nucleus, dorsal part
DRV	dorsal raphe nucleus, ventral part
DRVL	dorsal raphe nucleus, ventrolateral part
VLPAG	periaqueductal gray, ventrolateral part
DRI	dorsal raphe nucleus, interfascicular part
DRC	dorsal raphe nucleus, caudal part
PnR	pontine reticular formation
PD	postnatal day
BL	basolateral nucleus of the amygdala
BLA	basolateral amygdaloid nucleus, anterior part
BLP	basolateral amygdaloid nucleus, posterior part
LaDL	lateral amygdaloid nucleus, dorsolateral part
LaVM	lateral amygdaloid nucleus, ventromedial part
LaVL	lateral amygdaloid nucleus, ventrolateral part

ir	immunoreactive
EPM	elevated plus-maze
NAc	nucleus accumbens
TPH	tryptophan hydroxylase
CRF	corticotropin-releasing factor
CRF₂ receptor	corticotropin-releasing factor type II receptor
GABA	γ -aminobutyric acid
5-HT	5-hydroxytryptamine

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Research Highlights

1. Isolation-rearing of female rats sensitizes an anxiety-related circuit
2. Social isolation increased FG-7142-induced c-Fos in a subset of serotonergic neurons
3. Social isolation increased FG-7142-induced c-Fos expression in the amygdala

Fig. 1.
Schematic illustrating experimental timeline and sequence of procedures.

Fig. 2.

Graphs representing behavioral responses of isolates and group-reared rats during exposure to a novel dark open-field test for 25 min on Day 1. Graphs illustrate (A) duration and (B) frequency of immobility during the test. Data are presented as means $\pm$ S.E.M. *, $p < 0.05$ compared to group-reared control rats, Fisher's Protected Least Significant Difference (LSD) test. Sample size, $n = 15$ for isolation-reared and $n=16$ for group-reared treatment groups.

Fig. 3.

Graphs illustrating the effects of post-weaning social isolation on total distance traveled during acclimation to the test environment (Days 1–3). Graph represents total distance traveled during a daily 25 min exposure to an open-field over 3 consecutive days. Day 1 represents data from the same test illustrated in Fig. 2. Data are presented as means $\pm$ S.E.M. *, $p < 0.05$ compared to group-reared control rats, Fisher's Protected Least Significant Difference (LSD) test. Sample size, $n = 15$ for isolation-reared and $n=16$ for group-reared treatment groups.

Fig. 4.

Graphs illustrating the effects of post-weaning social isolation on behavior in the social interaction test conducted on day 4. Graphs represent A) the total number SI bouts, and B) total duration (sec) of SI, C) the total number of behavioral events (freezing, rearing, approach, and grooming), and D) duration (sec) of behavioral events (freezing, rearing, and grooming) in the 25 min social interaction test on day 4 in a familiar dark open-field following the 3 day acclimation period. Data are presented as means $\pm$ S.E.M. Sample size, $n = 15$ for isolation-reared and $n=16$ for group-reared treatment groups.

Fig. 5.

Graphs demonstrating the effects of a pharmacological challenge 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 saline, following the 5-week isolation/re-socialization protocol, on c-Fos expression in serotonergic neurons in the dorsal and pontine raphe nuclei. A–D) Photomicrographs illustrate subdivisions of the midbrain and pontine raphe complex analyzed at four rostrocaudal levels; subdivisions are outlined by dashed lines and based on a stereotaxic atlas of the rat brain (Paxinos and Watson, 1998). Graphs are organized into columns representing the rostrocaudal levels of A) –7.46 mm bregma, B) –8.00 mm bregma, C) –8.54 mm bregma, and D) –9.16 mm bregma. E) Closed bars indicate mean numbers of c-Fos-ir/tryptophan hydroxylase (TPH)-ir neurons and open bars indicate mean numbers of TPH-ir neurons sampled in each subdivision. Abbreviations: Aq, cerebral aqueduct; bv, blood vessel; DRC, dorsal raphe nucleus, caudal part; DRD, dorsal raphe nucleus, dorsal part; DRDSh, dorsal raphe nucleus, dorsal shell part; DRDc, dorsal raphe nucleus, dorsal core part; DRI, dorsal raphe nucleus, interfascicular part; DRV, dorsal raphe nucleus, ventral part; DRV/VLPAG, dorsal raphe nucleus, ventrolateral part/ventrolateral periaqueductal gray; mlf, medial longitudinal fasciculus; PnR, pontine raphe nucleus; xscp, decussation of the superior cerebellar peduncle. Data are presented as means $\pm$ S.E.M. *, $p < 0.05$ versus group housing condition, within the same drug treatment condition, +, $p < 0.05$ versus vehicle-treated control within the same housing condition, Fisher's Protected Least Significant Difference (LSD) test. Sample size, $n = 6$ for group-reared/vehicle, $n = 9$ for group-reared/FG-7142, $n = 7$ for isolation-reared/vehicle, and $n = 8$ for isolation-reared/FG-7142. Scale bar, 200 μ m.

Fig. 6.

Photomicrographs illustrating effects of post-weaning social isolation and pharmacological challenge with FG-7142 on immunohistochemical staining of c-Fos (blue) and tryptophan hydroxylase (TPH; brown) in the mid-rostrocaudal dorsal raphe nucleus (DR; -8.00 mm bregma). Photomicrographs of sections from representative rats in each treatment group are illustrated as follows: **A, B**, group-reared/vehicle-treated group ($n = 6$); **C, D**, group-reared/FG-7142-treated group ($n = 9$); **E, F**, isolation-reared/vehicle-treated group ($n = 7$), and **G, H**, isolation-reared/FG-7142-treated group ($n = 8$). Black boxes in **A, C, E**, and **G** indicate regions shown at higher magnification in panels **B, D, F**, and **H**, respectively. Black boxes in **B, D, F**, and **H** indicate regions shown at higher magnification in insets in the lower right corner of each panel. Arrows indicate non-serotonergic c-Fos-ir cells, white arrowheads indicate TPH-ir/c-Fos-immunonegative neurons, and black arrowheads indicate double immunostained c-Fos-ir/TPH-ir neurons. For abbreviations, see Fig. 3 legend. Scale bar, **A, C, E**, and **G**, $200\ \mu\text{m}$; **B, D, F**, and **H**, $40\ \mu\text{m}$; insets, $20\ \mu\text{m}$.

Fig. 7.

Graphs illustrating the effects of a pharmacological challenge with *N*-methyl-beta-carboline-3-carboxamide (FG-7142) or saline following the 5-week isolation/re-socialization protocol on c-Fos-ir cells at two rostrocaudal levels of the basolateral amygdala. Photomicrographs of Nissl-stained rat brain sections illustrating subregions of the basolateral amygdala selected for analysis. Photomicrographs illustrate the basolateral amygdala at A) -2.12 mm bregma and B) -3.30 mm bregma. Dashed and solid lines were imported directly from a standard stereotaxic atlas (Paxinos and Watson, 1998) and overlaid onto the photomicrographs. C) Data are presented as means $\pm$ S.E.M. *, $p < 0.05$ versus group housing condition, within the same drug treatment condition, +, $p < 0.05$ versus vehicle-injected group within the same housing condition, Fisher's Protected Least Significant Difference (LSD) test. Sample size, $n = 6$ for group-reared/vehicle, $n = 7$ 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; ec, external capsule; ic, internal capsule; LaDL, lateral amygdaloid nucleus, dorsolateral part; LaVL, lateral amygdaloid nucleus, ventrolateral part; LaVM, lateral amygdaloid nucleus, ventromedial part; lab, longitudinal association bundle; opt, optic tract. Scale bar, 500 μ m.

Fig. 8.

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 the basolateral amygdala (−3.30 mm bregma). A, C, E, G) Nissl-stained sections with overlaid templates from representative rats in each treatment group: A) group-reared/vehicle-treated group (n = 6); C), group-reared/FG-7142-treated group (n = 7); E) isolation-reared/vehicle-treated group (n = 7), and G) isolation-reared/FG-7142-treated group (n = 7). B, D, F, H) Adjacent c-Fos-immunostained sections with overlaid templates from the same rats. Small black boxes in **B**, **D**, **F**, and **H** indicate regions of the basolateral amygdala shown at higher magnification in the insets in the lower right-hand corner of each panel. Arrows indicate c-Fos-immunoreactive cell nuclei. Scale bar, **A–H**, 200 μm; **B**, **D**, **F**, and **H**, insets, 50 μm.

Fig. 9.

Graphs illustrating correlations between c-Fos expression in the basolateral amygdaloid nucleus, anterior part (BLA) and c-Fos expression in serotonergic neurons in subregions of the dorsal raphe nucleus (DR). A) Scatter and line plot showing a positive correlation between the numbers of c-Fos-immunoreactive (ir) cells in the rostral BLA and the numbers of c-Fos-ir/tryptophan hydroxylase-ir neurons in the DR, caudal part (DRC), at -9.16 mm bregma. B) Scatter and line plot showing a positive correlation between the numbers of c-Fos-ir cells in the rostral BLA and the numbers of c-Fos-ir/tryptophan hydroxylase-immunonegative neurons in the DR, caudal part (DRC), at -8.54 mm bregma. C) Scatter and line plot showing a positive correlation between the numbers of c-Fos-ir cells in the caudal BLA and the numbers of c-Fos-ir/tryptophan hydroxylase-ir neurons in the DR, ventrolateral part/ventrolateral periaqueductal gray (DRVL/VLPAG), at -8.00 mm bregma. D) Scatter and line plot showing a positive correlation between the numbers of c-Fos-ir cells in the caudal BLA and the numbers of c-Fos-ir/tryptophan hydroxylase-ir neurons in the DR, interfascicular part (DRI), at -8.54 mm bregma.

Table 1

Effects of social isolation on FG-7142-induced c-Fos expression in non-serotonergic cells in the midbrain raphe complex

Region	Rostrocaudal level (mm bregma)	Group/Vehicle	Group/FG-7142	Isolate/Vehicle	Isolate/FG-7142
DRD	-7.46	56 ± 11.3	52.3 ± 5.6	38.3 ± 5.9	61.7 ± 9.8
DRV	-7.46	23.0 ± 4.3	10.0 ± 0.9 ⁺	15.8 ± 3.2	22.7 ± 5.4
DRDSH	-8.00	12.6 ± 0.5	15.8 ± 2.6	15.6 ± 5.0	17.3 ± 2.0
DRDe	-8.00	2.8 ± 0.7	1.3 ± 0.5	1.0 ± 0.4 [*]	2.5 ± 0.7
DRV	-8.00	8.8 ± 3.6	7.8 ± 2.5	2.7 ± 0.5 [*]	8.0 ± 1.7 ⁺
DRV/VLPAG	-8.00	77.4 ± 7.6	83.4 ± 14.2	84 ± 11.2	81.5 ± 6.8
DRC	-8.54	21.4 ± 2.9	17.5 ± 2.3	11.8 ± 1.5 [*]	17.5 ± 1.6 ⁺
DRI	-8.54	9.6 ± 1.5	11.9 ± 1.8	6.1 ± 1.4	9.2 ± 1.0
DRC	-9.16	19.4 ± 1.8	19.4 ± 1.7	10.7 ± 1.6 [*]	25.0 ± 1.5 ⁺
PhR	-9.16	13.6 ± 0.8	13.0 ± 0.9	9.1 ± 1.5 [*]	16.2 ± 1.7 ⁺

* , $P < 0.05$ group versus isolation-rearing condition, within the same drug treatment condition.⁺ , $P < 0.05$ vehicle versus FG-7142 drug treatment effect, within the same housing condition.

Table 2

Correlations of numbers of c-Fos-ir/TPH-ir neurons with numbers of c-Fos-ir/non-TPH-ir cells across multiple subdivisions of the DR

Rostrocaudal level (mm bregma)	Region	<u>c-Fos-ir/TPH-ir neurons vs. c-Fos-ir/non-TPH-ir cells</u>	
-7.46	DRD	$r = 0.689$	$p < 0.001$
-7.46	DRV	$r = 0.486$	$p = 0.006$
-8.00	DRDSh	$r = 0.600$	$p < 0.001$
-8.00	DRDc	$r = 0.773$	$p < 0.001$
-8.00	DRV	$r = 0.603$	$p < 0.001$
-8.00	DRV/LVPAG	$r = 0.826$	$p < 0.001$
-8.54	DRC	$r = 0.831$	$p < 0.001$
-8.54	DRI	$r = 0.432$	$p = 0.017$
-9.16	DRC	$r = 0.895$	$p < 0.001$
-9.16	PnR	$r = 0.675$	$p < 0.001$