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DISTRIBUTION OF CORTICOTROPIN-RELEASING FACTOR RECEPTOR 1 IN THE DEVELOPING MOUSE FOREBRAIN: A NOVEL SEX DIFFERENCE REVEALED IN THE ROSTRAL PERIVENTRICULAR HYPOTHALAMUS

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Abstract

Corticotropin-releasing factor (CRF) signaling through CRF receptor 1 (CRFR1) regulates autonomic, endocrine and behavioral responses to stress and has been implicated in the pathophysiology of several disorders including anxiety, depression, and addiction. Using a validated CRFR1 reporter mouse line (bacterial artificial chromosome identified by green fluorescence protein (BAC GFP-CRFR1)), we investigated the distribution of CRFR1 in the developing mouse forebrain. Distribution of CRFR1 was investigated at postnatal days (P) 0, 4, and 21 in male and female mice. CRFR1 increased with age in several regions including the medial amygdala, arcuate nucleus, paraventricular hypothalamus, medial septum, CA1 hippocampal area, and the lateral habenula. Regions showing decreased CRFR1 expression with increased age include the intermediate portion of the periventricular hypothalamic nucleus, and CA3 hippocampal area. We report a sexually dimorphic expression of CRFR1 within the rostral portion of the anteroventral periventricular nucleus of the hypothalamus (AVPV/PeN), a region known to regulate ovulation, reproductive and maternal behaviors. Females had a greater number of CRFR1-GFP-ir cells at all time points in the AVPV/PeN and CRFR1-GFP-ir was nearly absent in males by P21. Overall, alterations in CRFR1-GFP-ir distribution based on age and sex may contribute to observed age- and sex-dependent differences in stress regulation.

Keywords

hypothalamic–pituitary–adrenal axis; corticotropin-releasing factor; corticotropin-releasing factor receptor 1; anteroventral periventricular nucleus; sex difference

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Conflict of interest—None declared.

INTRODUCTION

Corticotropin-releasing factor (CRF) signaling through the G_s-coupled receptor CRF receptor 1 (CRFR1) regulates autonomic, endocrine and behavioral responses to stress (Perrin et al., 1993; Heinrichs et al., 1995; Smith et al., 1998; Subbannayya et al., 2013). Dysregulation of CRFR1 has specifically been linked to several psychiatric disorders including anxiety, depression, and addiction (Chrousos, 2009; Garcia-Carmona et al., 2015; da Silva et al., 2016; Holly et al., 2016; Schussler et al., 2016). Compared to CRF receptor 2 (CRFR2), CRFR1 is expressed at higher levels in the brain (Van Pett et al., 2000; Lein et al., 2007) and has a greater binding affinity to CRF (Perrin et al., 1995). CRFR1 mRNA is expressed throughout the mature rodent brain, and during rodent and human development (Van Pett et al., 2000; Korosi and Baram, 2008; Sandman and Glynn, 2009). Developmental studies on the distribution of CRFR1 are limited to the rat, however, and have primarily investigated the hippocampus, amygdala, cortex, and the paraventricular nucleus of the hypothalamus (PVN) (Avishai-Eliner et al., 1996).

Emerging studies indicate that during pre- and postnatal periods, signaling through CRFR1 exerts lasting effects on the development of several brain regions both during normal development and following early-life stress. Neonatal stress induces alterations in dendritic development, spinogenesis, and synapse formation in the mouse hippocampus and cerebral cortex and these effects are ameliorated by pharmacological antagonism of CRFR1 (Liao et al., 2014; Yang et al., 2015). Recent work by Garcia and colleagues (2014) investigating the role of CRFR1 in normal development of the mouse olfactory bulb suggests that CRF signaling through CRFR1 drives synapse and dendrite formation. Furthermore, early-life stress can produce lasting effects on expression of CRFR1 in several brain regions including the rat amygdala (Grundwald and Brunton, 2015).

CRF-producing cells are expressed in several regions throughout the brain, including the PVN, central amygdala, bed nucleus of the stria terminalis (BNST), Barrington's nucleus, cingulate cortex, hippocampus, accessory bulb of the nucleus accumbens, olfactory bulb, and periaqueductal gray of both mice and rats (Swanson et al., 1983; Aguilera et al., 2004; Kono et al., 2016). CRF has been described in the mouse PVN as early as embryonic day 13.5 (E13.5) and undergoes dynamic fluctuations over perinatal development marked by decreased levels around the time of birth (Keegan et al., 1994). By contrast, in the amygdala there are low levels of CRF during the prenatal period with levels rising during the neonatal period (~Postnatal day (P) 3) (Keegan et al., 1994). Other areas such as the cerebral cortex express no CRF mRNA until P3 (Keegan et al., 1994). Distribution of CRF receptors in adult rats and mice is vast; CRFR1-GFP-ir has been reported throughout the olfactory bulb, cerebral cortex, hypothalamus, hippocampus, amygdala, brainstem regions, and cerebellum (Van Pett et al., 2000; Justice et al., 2008). CRFR2 is also expressed throughout the brain with the highest levels found in the lateral septal nucleus, BNST, medial portion of the amygdala, and dorsal raphe nucleus of mice and rats (Chalmers et al., 1995; Van Pett et al., 2000; Aguilera et al., 2004). Less is known about CRF receptor distribution during development, particularly in the mouse. However, in the rat, large fluctuations in brain CRFR1 have been reported during the neonatal period (Avishai-Eliner et al., 1996). A more comprehensive description of CRFR1 distribution in the neonatal brain will enhance our

understanding of the neural circuitry that regulates early-life stress. In humans, specific CRFR1 polymorphisms have been linked to the onset of adult depression following early-life adversity (Grabe et al., 2010; Laucht et al., 2013). This indicates that CRFR1 expression and function early in life may be critical to development of adult mood disorders.

Sex differences in rats have been reported with males having more CRF-expressing cells than females within the central amygdala (Karanikas et al., 2013). On the contrary, CRF-positive cells are more abundant in the female rat preoptic area and BNST (McDonald et al., 1994; Funabashi et al., 2004). Sex differences in CRFR1 have also been reported and depend upon the region of interest and species. Wealthington et al. (2014) reported greater CRFR1 binding in adult female compared to male rats within the nucleus accumbens, olfactory tubercle, piriform cortex, and the anterior cingulate, while Lim and colleagues (2005) found greater CRFR1 expression in the BNST of female voles. To our knowledge, potential sex differences in CRFR1 expression have not been explored in mice. Such sex differences in the distribution of CRF and its receptors may contribute to observed differences in a variety of stress-related behavioral and hormonal responses reported in both rats and mice (Handa et al., 1994; Jasnow et al., 2006; Zuloaga et al., 2008). Deletion of CRF receptors in mice results in differential effects on anxiety-like behavior in male and female mice (Bale et al., 2002), further supporting the importance of CRFR1 in regulating sex differences in anxiety. In humans, sex differences in distribution and function of CRF and CRFR1 expression may potentially contribute to the etiology of stress-related psychiatric disorders such as anxiety and depression (Bao et al., 2006; Valentino et al., 2012), both of which are more prevalent in women than men (Weissman et al., 1993; Kornstein et al., 2000; Kessler et al., 2005; Seney and Sibille, 2014).

In the current study, we investigated potential sex differences in, and the development of the CRFR1 system using bacterial artificial chromosome (BAC) transgenic mice reporting expression of CRFR1, with green fluorescent protein (GFP; BAC transgenic CRFR1-GFP mice; Justice et al., 2008). Our findings indicate that CRFR1 expression is dynamic throughout neonatal development, with sex- and age-dependent differences.

EXPERIMENTAL PROCEDURES

Animals

BAC transgenic CRFR1-GFP mice were used in this study (Justice et al., 2008). Male and female CRFR1-GFP mice were mated and brains of 30 offspring (n = 15 per sex) were collected at multiple time points for use in this study. 10 animals per age group (n = 5 per sex; P0, 4, 21) were used for the study, comprising the three developmental time points. Specific ages were selected based on previous reports that indicated major developmental changes in a variety of genes (including CRF) across comparable developmental time points (Cao and Patisaul; 2013; Keegan et al., 1994; Zuloaga et al., 2014). Mice were maintained under a 12/12 L/D cycle (lights on at 0700), with food and water available *ad libitum*. Mice were geno-typed by PCR; using the nucleotide sequences: CCT ACG GCG TGC AGT GCT TCA GC forward and CGG CGA GCT GCA CGC TGC GTC CTC reverse EGFP350 primers. Actin470 was used as a control gene. All procedures were approved by the

University at Albany Institutional Animal Care and Use Committee and were in accord with National Institutes of Health guidelines.

Perfusion and tissue processing

On the day of birth (postnatal day 0; P0) and P4, mice were cryoanesthetized by placing them on crushed ice. Mice were then intracardially perfused with 5 ml (P0) or 10 ml (P4) of 4% phosphate-buffered paraformaldehyde. P21 mice were overdosed with a 100 mg/kg ketamine/xy lazine/acepromazine cocktail prior to perfusion with 15 ml of 4% paraformaldehyde. Brains were excised and stored in 4% paraformaldehyde for 24 h at 4 °C. Brains were then placed into a 30% sucrose solution, and stored at 4 °C. P0 brains were sectioned through the coronal plane using a cryostat (Microm HM505E, MICROM international GmbH, Walldorf, Germany) at 50 μm into two alternate series, P4 were sectioned at 40 μm into two series, and P21 were sectioned at 40 μm into three series. P0 tissue is fragile, and therefore, P0 brains were sectioned at 50 instead of 40 μm to preserve integrity. Tissue was stored in a cryopreservative solution at 4 °C until immunohistochemistry was performed.

Immunohistochemistry

For visualization of CRFR1-GFP-ir, sections were rinsed in phosphate-buffered saline (PBS; pH 7.6), incubated in 1% hydrogen peroxide and 0.4% Triton-X in PBS (PBS-TX) for 10 min. Next, tissue was re-rinsed in PBS and incubated in 4% normal goat serum (NGS) in PBS-TX for 1 h. Tissue was then incubated overnight in primary antisera for GFP (1:10,000, rabbit, Life Technologies, A6455, Carlsbad, CA, USA). The following day, tissue was rinsed in PBS then incubated in biotinylated goat anti-rabbit antisera in PBS-TX (1:500; Vector Laboratories, Burlingame, CA, USA) for 1 h. After, tissue was rinsed in PBS and then placed in avidin–biotin complex (ABC Elite kit, Vector Laboratories). Tissue was then rinsed again in tris-buffered saline (TBS) and placed for 10 min in diaminobenzidine as the chromagen for visualization of CRFR1-GFP-positive cells. Sections from wild-type brains were utilized as negative controls to assess CRFR1-GFP-ir. No labeling was found in these sections.

Microscopic analysis of CRFR1 distribution

Assessment of CRFR1-GFP-ir distribution was conducted on a Nikon 80i microscope equipped with a digital camera. The Allen Institute mouse brain atlas (Lein et al., 2007) was used to identify P21 brain areas and an atlas of the developing mouse brain was used for P0 and P4 brains (Paxinos et al., 2007). Three independent investigators qualitatively assessed CRFR1-GFP density and intensity independently within selected brain regions using a rating scale. Density and intensity scores generated by this assessment were equally weighted and averaged. These scores were then used to generate a composite score for each brain region within an individual mouse ($n = 5$ per age/sex). Values were applied on the following scale utilized in a previous study (Zuloaga et al., 2014). −, No label; +/−, minimal but still noticeable labeling intensity/density of cells; +, low labeling intensity/density of cells; ++, moderate labeling intensity/cell density; +++, high labeling intensity/cell density; ++++, very high labeling intensity/cell density.

Quantitative assessment was performed within brain regions (AVPV/PeN, periventricular nucleus of the hypothalamus-intermediate portion (PeN/Int), and arcuate nucleus) identified as showing sex or developmental differences. These quantifications were performed to confirm visual ratings in terms of cell density. Images of the AVPV/PeN, PeN/Int, and arcuate nucleus were captured on a Nikon 80i microscope using a 20× objective. Fixed area frames were placed around the anatomical boundaries of the AVPV/PeN (rectangle), arcuate nucleus (triangle), and PeN/Int (rectangle) as identified by the Allen Atlas (P21) and the developing mouse brain (P0 and P4) (Paxinos et al., 2007). CRFR1-GFP-ir cells were counted using ImageJ software to estimate CRFR1-GFP-ir cells/mm^2. Two bilateral regions were used for quantification within the AVPV/PeN, arcuate nucleus, and PeN/Int. These analyses were utilized to estimate cell density within these selected regions. A 2-way ANOVA was used for statistical analyses with age and sex as factors ($n = 5$ per age/sex). Effect sizes were calculated for all significant ANOVAs using eta squared. Significant main effects/interactions were further analyzed using Bonferroni's corrected *T*-tests. Significance level was set at $p \leq 0.05$ and data are shown as means ± SEM.

RESULTS

CRFR1 distribution in the postnatal mouse forebrain

Hypothalamus.—A striking sex difference in CRFR1-GFP-ir distribution was found in mice at P0, P4, and P21 in the AVPV/PeN with females showing a dense cluster of CRFR1-GFP-ir cells along the 3rd ventricle (Fig. 1; Table 1). This cluster was largely absent in males. The rostral medial preoptic area (MPOA), ventral to the anterior commissure was stable between P0 and P21, with moderate clustering of CRFR1-GFP-ir (Table 1). In the PVN CRFR1-GFP-ir was relatively low at all time points assessed although there was an apparent increase between P4 and P21 (Table 1; Fig. 2G–I). The region directly dorsal to the PVN (peri-PVN) showed moderate levels of labeling throughout postnatal development (Table 1). The PeN/Int, which lies ventral to the caudal PVN contained moderate labeling at P0 and P4, but gradually became sparse by P21 (Table 1; Fig. 3A–C). Unlike the AVPV/PeN, no sex differences were present. Within the lateral hypothalamus, modest labeling was present throughout development with no apparent changes between P0 and P21 (Table 1; Fig. 2G–I).

Within the caudal portion of the hypothalamus CRFR1-GFP-ir was dense in the dorsomedial hypothalamus of both sexes at P0, P4, and P21 (Table 1; Fig. 3A–C). Ventromedial hypothalamus immunolabeling was minimal and confined to the ventrolateral division (Table 1; Fig. 3A–C). The P0 and P4 arcuate nucleus contained sparse CRFR1-GFP-ir, though labeling became extensive at P21 (Fig. 3A–F). Males at P0 and P21 appeared to have slightly more CRFR1-GFP-ir within the anterior hypothalamus (Table 1). Ventral to the arcuate, little to no CRFR1-GFP-ir was localized in the median eminence (ME) at any age (Fig. 3A–F). Just lateral to the arcuate, the tuberal nucleus exhibited modest labeling with a small increase from P0 to P4, which returned to approximately P0 levels by P21 (Table 1). In the suprachiasmatic nucleus (Table 1), CRFR1-GFP-ir cells were moderate and relatively stable across development. In contrast, however, CRFR1-GFP-ir was absent in the supraoptic nucleus (Table 1).

Cerebral cortex.—CRFR1-GFP-ir distribution within the cerebral cortex showed dramatic variation across P0, P4, and P21 time points (Table 1; Fig. 4A–C). At P0 (Fig. 4A)-positive cells were concentrated in the middle cortical areas. At P4 (Fig. 4B), labeling was strongest in the outer and deep layers of the cortex with a less densely populated cell group found in the middle area between those layers. At P21 (Fig. 4C) CRFR1-GFP-ir cells were distributed throughout the cortex; high density within the middle and outer middle layers, and less within the inner cortical layers.

Hippocampus and habenula.—CRFR1-GFP-ir cells were prominent in the CA1, CA3, and dentate gyrus regions of mice at all developmental time points examined. However, the intensity of labeling appeared to be weaker at P21 compared to P0 and P4 within CA3 and increased in the P21 CA1 (Table 1; Fig. 4D–F). Labeling within CA1 and CA3 was highest in the pyramidal cell layers (Fig. 4D–F), and within the dentate gyrus, labeling was highest within the hilus (Fig. 4G), with no immunoreactivity found in the granule cell layer. The lateral habenula (Table 1) displayed clear CRFR1-GFP-ir cells at P4 and P21 with lesser labeling found at P0. The medial habenula showed no CRFR1-GFP-ir labeling (Table 1).

Amygdala and BNST.—In the P21 amygdala (Table 1; Fig. 4J), the greatest levels of CRFR1-GFP-ir cells were in the medial and basomedial divisions. In the medial region, at P0 and P4, there was slightly less labeling than P21 (Fig. 4H–J). At P0, P4, and P21, the overall highest labeling within the amygdala was found in the basomedial division (Table 1). Moderate labeling was seen in the central nucleus (largely confined to the medial division), the lateral nucleus, and the cortical nucleus (Fig. 4H–J). Within the lateral division, there is sparse labeling at P0, which then increased by P4 and persisted at P21 (Fig. 4H–J). The anterior division of the basolateral amygdala contained very few immunoreactive cells, at all ages. The BNST showed extensive labeling at all time points with no apparent sex differences, though there was a subtle drop in expression by P21 (Table 1; Fig. 2D–F).

Septum and diagonal bands.—The medial septum exhibited extensive expression of CRFR1 at all developmental ages in the mouse brain, with slightly less labeling at P0 (Table 1; Fig. 2A–C). Lateral septum CRFR1-GFP-ir levels were low to moderate at time points (Table 1; Fig. 2A–C). CRFR1-GFP-ir was consistently expressed in the diagonal band with a slight increase from P0 to P4, where it remained constant by P21 (Table 1; Fig. 2A–C).

CRFR1-GFP quantification within selected brain regions

CRFR1-GFP-ir cells were quantified within the AVPV/PeN, PeN/Int, and arcuate nucleus. A two-way ANOVA in the AVPV/PeN indicated a significant main effect of age ($F(2, 24) = 8.71$, $p \leq 0.001$, $\eta_p^2 = 0.270$) and sex ($F(1, 24) = 21.08$, $p \leq 0.001$, $\eta_p^2 = 0.327$) (Fig. 5A). Subsequent post hoc tests revealed a greater number of CRFR1-GFP-ir cells in females than males at P0 ($p \leq 0.01$), P4 ($p \leq 0.001$), and P21 ($p \leq 0.01$) (Fig. 5A). A two-way ANOVA of PeN/Int CRFR1-GFP-ir cells revealed a significant age effect ($F(2, 24) = 9.20$, $p \leq 0.001$; $\eta_p^2 = 0.417$), with a greater number at P0 and P4 compared to P21 (Fig. 5B). However, there was no sex effect or interaction within the PeN/Int. A two-way ANOVA of arcuate CRFR1-GFP-ir cells revealed a significant age effect ($F(2, 24) = 24.11$, $p \leq 0.001$; $\eta_p^2 = 0.654$), with

greater CRFR1-GFP-ir cells at P21 compared to earlier ages (Fig. 5C). No sex effect or interaction was found in the arcuate nucleus. All sampled distributions met the criteria for normal distribution and equal variances.

DISCUSSION

Due to the lack of reliable antibodies targeting CRFR1, we used a validated mouse model (Justice et al., 2008); BAC-based CRFR1-GFP mice. Regional CRFR1 distributions reported at P21 in this study were similar to a previous study using adult CRFR1-GFP mice (Justice et al., 2008), indicating that forebrain CRFR1 distribution patterns are largely established, though possibly not complete, by this time point. Understanding how CRFR1 changes over development is vital. Mapping the distribution of CRFR1 during development may provide insight into how CRF signaling can impact brain development and behavior. Previous reports indicate that CRF signaling via CRFR1 during early development contributes to dendrite and synapse formation in the hippocampus, cerebral cortex, and olfactory bulb (Garcia et al., 2014; Liao et al., 2014; Yang et al., 2015). Whether, these effects also occur within other brain regions, and their critical periods for affecting dendrite formation and synapto-genesis are unknown. However, for all regions, changes reported in expression patterns across development reflect a maturation of the CRFR1 system and likely indicate periods during which behavioral and hormonal responses associated with CRF signaling are also developing and maturing.

Moreover, CRFR1 and CRF are implicated in the pathophysiology of anxiety and depression (Nemeroff and Vale, 2005). Both conditions exhibit a sex difference in diagnosis, with females outnumbering males, post-puberty. However, diagnoses prior to the onset of puberty are reversed from adult rates: males outnumber females in diagnoses (Hankin, 2002). It is possible that differences in stress regulation throughout development, as CRFR1 expression changes, contribute to the relationship between biological sex and diagnosis prevalence. This may partially explain the change in anxiety and depression prevalence rates by sex surrounding a period of development that includes fluctuations in circulating sex hormones. Due to clear implications for stress and mental health, there is a need for investigating and expanding upon sex differences in CRFR1 expression throughout the developing forebrain. For these reasons, we investigated the distribution of CRFR1-GFP-ir in the developing male and female mouse forebrain. Throughout the fore-brain, we report that a number of regions displayed developmental differences, and identified sex differences in discrete brain nuclei.

The most apparent sex difference in CRFR1 was found in the AVPV/PeN with females showing greater numbers of CRFR1-GFP-ir cells from P0-P21. In P21 males, cells expressing CRFR1 were few and scattered in this region with no clear nucleus present. This finding expands upon existing evidence for sexual dimorphisms of other cell phenotypes within the AVPV/PeN (Simerly, 1989; Simerly et al., 1997; Semaan et al., 2010; Brock et al., 2015; Scott et al., 2015). Specifically, tyrosine hydroxylase (TH) and kisspeptin are two phenotypes of which expression in this region are sexually dimorphic (F > M) (Semaan et al., 2010; Poling and Kauffman, 2013; Scott et al., 2015). There is also an overall volumetric sex difference in the AVPV following the same pattern (F > M) (Kanaya et al., 2014).

Interestingly, the sex difference in the AVPV/PeN is apparent at P0 and persists through P21 and into adulthood (P60; data not shown). This indicates that the CRFR1 sex difference appears prior to sexual dimorphisms in both TH and kisspeptin AVPV/PeN populations (Waters and Simerly, 2009; Poling and Kauffman, 2013) and may be similarly regulated by perinatal gonadal hormone secretions. Gonadal steroid hormone receptors (estrogen receptor alpha and beta (ERα, ERβ), and androgen receptor (AR)) are highly expressed within this brain area, suggesting these cells may be vulnerable to effects of developmental gonadal secretions (Kanaya et al., 2014; Zuloaga et al., 2014). Further investigation into gonadal steroid hormones in the establishment and maintenance of the CRFR1 sex difference within the AVPV/PeN is warranted. The AVPV/PeN region is known to regulate ovulation as well as sexual and maternal behaviors (Simerly et al., 1997; Scott et al., 2015). Furthermore, cell phenotypes within the AVPV/PeN change depending upon sexual/maternal experience (Scott et al., 2015). CRF signaling at CRFR1 in the AVPV/PeN may provide a mechanism through which stress acts to impact related behaviors. The AVPV/PeN of the rat contains neurons that project to a number of brain areas that regulate reproductive and stress-related behaviors as well as hormone release (vascular organ of the lamina terminalis, medial preoptic area, ventral lateral septum, arcuate nucleus, PVN, and periaqueductal gray) (Gu and Simerly, 1997). It is possible that AVPV/PeN CRFR1 projections to these regions may regulate sexually dimorphic patterns of behavior and hormonal functions.

Many brain regions showed marked changes in CRFR1 through postnatal development. The arcuate nucleus exhibited an increase in expression by P21 in comparison to P0 and 4. The arcuate is involved in many biological functions, including glucose intake, cardiovascular regulation, and hormone release. The arcuate regulates these functions via neuroendocrine neuron signaling to the pituitary gland and subsequent release of various hormones (Palkovits, 2008; Sapru, 2014; Hussain et al., 2015). The arcuate nucleus is also responsive to both psychological stress and CRF administration. Arcuate neurons expressing kisspeptin are inhibited by CRF (Li et al., 2010); such inhibition provides the potential for stress to modulate sexual behaviors, as kisspeptin signaling modulates gonadotropin-releasing hormone (GnRH) secretion. Another hypothalamic nucleus, the PVN, is critical for the release of CRF and activation of the HPA axis. In the PVN, CRFR1 levels are generally low, with a slight developmental increase in CRFR1-GFP-ir between P4 and P21. However, when compared to adult levels reported in Justice et al., 2008, and in contrast to most other brain regions, P21 PVN CRFR1 levels do not appear to be fully developed, with fewer cells present than in adulthood. Previous studies indicate CRF levels in the PVN fluctuate across the perinatal period and into adulthood in the mouse; just before birth levels are low and adult levels are nearly reached by P14 (Keegan et al., 1994). PVN CRFR1 levels increase by P21, and therefore may be responsive to the increase in circulating CRF within the area which also increases during this developmental phase (Keegan et al., 1994). PVN CRF neurons have recently been demonstrated to project to and stimulate PVN CRFR1-containing cells (Ramot et al., 2017). The anterior hypothalamus appeared to have a sex difference which at P21 showed elevated CRFR1-GFP-ir in males compared to females. This sex difference in CRFR1 may have implications for sexually dimorphic effects of stress on anterior hypothalamus functions including thermoregulation and sleep regulation (Boulant, 2000; Deschenes and McCurry, 2009; McGivern et al., 2009), which are impacted in

conditions such as anxiety or depression. However, whether this sex difference in anterior hypothalamic CRFR1 persists into adulthood has yet to be determined.

Across development, CRFR1-GFP-ir within the MPOA was stable between P0 and P21. The MPOA is known to have anatomical sex differences (Morris et al., 2004), and influence sexual behavior in rodents (Nutsch et al., 2016). Although, no obvious sex differences in receptor protein were apparent, previous work in rats has demonstrated a sex difference in MPOA CRF, with females exhibiting a higher density (McDonald et al., 1994; Funabashi et al., 2004). It is possible that CRFR1 cells in the MPOA, and other regions, might become sexually dimorphic in adulthood since sex differences in select brain regions, including the MPOA, are influenced by pubertal and adult gonadal hormone levels (Morris et al., 2004). The MPOA has been indicated as a site for hypothalamic–pituitary–gonadal (HPG) and HPA axis interactions. Specifically, androgen and estrogen binding to their cognate receptors in the MPOA are hypothesized to modulate central CRF and arginine vasopressin release via projections to PVN neurons (Williamson and Viau, 2007; Williamson et al., 2010). There is abundant expression and sex differences in the distribution of AR, ERα, and ERβ in the MPOA so it is possible that CRFR1-expressing cells co-localize these receptors and may also do so in a sexually dimorphic fashion.

In the BNST, there was a moderate developmental decline in CRFR1-GFP-ir from P0 to P21, however, levels were relatively high throughout. Although we did not find an obvious sex difference in CRFR1 density in the BNST of developing mice, sex differences in CRF have been reported in the BNST. In the oval nucleus of the BNST, CRF expression is greater in female compared to male rats (Funabashi et al., 2004). In voles, there are also reports of a sex difference in which males have significantly increased CRFR2 binding compared to females (Lim et al., 2005). Moreover, in rat models, a similar sex difference has been reported with increased CRFR2 receptor binding in the male BNST (Wealthington et al., 2014). CRFR2 deletion leads to increased anxiety-like behaviors; it may be that CRFR2 is partially necessary for inhibition of stress-related responses (Kishimoto et al., 2000; Reul and Holsboer, 2002). Together these findings suggest the BNST may be a central region through which CRF can exert sexually dimorphic effects on stress-like behavior, although sex-dependent effects may rely primarily on signaling via CRFR2 and not CRFR1.

The cerebral cortex underwent many developmental changes in CRFR1 expression; CRFR1 expression peaked in the medial cortical layers by P21, while the outer layers peak by P4. The distribution of CRFR1 in the cortex for the P0 group by comparison, was more scattered, indicating dynamic changes in distribution between each of these postnatal time points. The scattered densities of CRFR1 at P0 in the cortical layers, compared with the P21 cortex, suggest that CRFR1+ cells may migrate within the cortex during postnatal development. Alternatively, certain populations of CRFR1-positive cells in the cortex may either undergo apoptosis or decrease their expression of CRFR1 as the mouse cortex develops. The rat forebrain also undergoes large shifts in CRFR1 expression during development. Cortical CRFR1 mRNA in the rat has been reported to peak by P2, and drops from P4 to P16, at which point mRNA expression levels are maintained into adulthood (Avishai-Eliner et al., 1996). In the mouse, CRFR1 labeling in the cortex appears prior to CRF, which does not appear until P3 (Keegan et al., 1994). The presence of CRFR1 in the

cortex prior to CRF-producing cells in the same region suggests that in early development, the cortex can respond to CRF, though local cortical CRF signaling is not likely.

The CA1 hippocampal area was the forebrain region with the greatest overall CRFR1-GFP-ir density. Developmental changes were also most notable in CA3 where high levels are found at P0 and P4, but decrease by P21. By comparison, in rats, CA3 shows moderate CRFR1 mRNA in adulthood, and expression fluctuates throughout development, peaking at ~300–600% of adult levels by P6 (Avishai-Eliner et al., 1996). Therefore, our findings in the mouse CA3 indicate similar CRFR1 expression during postnatal development, with peak intensity during the early neonatal period and a substantial drop thereafter. The hippocampus is known to regulate the HPA axis, primarily via glucocorticoid binding to glucocorticoid receptors; negative feedback decreases CRF release from the PVN (Herman et al., 2016). The hippocampus is also linked to regulation of emotional behaviors such as anxiety and depression, both of which are more prevalent in women than men (Kornstein et al., 2000; Kessler et al., 2005). In adult rats (P98), a sex difference in CRFR1 has been reported in which females show greater CRFR1 receptor binding in CA3 compared to male rats (Wealthington et al., 2014). Although no apparent sex differences were detected at any development ages within either CA1, CA3 or the dentate gyrus, it remains possible that sex differences may develop later. In the present study, mouse dentate gyrus CRFR1 levels were lower across all ages compared to CA1 and CA3. Dentate gyrus immunoreactivity was almost exclusive to the hilus region, with little to no expression in the granule cell layer. In contrast, CRFR1 immunoreactivity has been reported in the granule cell layer of the adult dentate gyrus (Rissman et al., 2012). CRF signaling in the dentate gyrus is involved in learning and consolidation of fear memories (Blank et al., 2003; Thoeringer et al., 2012). Therefore, an absence of CRFR1 in the granule cell layer may impact development of fear-associated memories early in life (Akers et al., 2012).

Hippocampal CRF levels also fluctuate throughout postnatal development. CRF-expressing neurons progressively increase throughout the hippocampus and peak by P11–18, at which point they drop to adult levels (Chen et al., 2001). Chen and colleagues (2001) further report a transiently expressed early postnatal phenotype resembling that of Cajal-Retzius cells, which are known to drive hippocampal development before they migrate to the cortex. The developing hippocampus appears highly sensitive to CRF as chronic CRF exposure in development leads to adult hippocampal dysfunction, including cognitive impairment in mice and rats (Ivy et al., 2010; Liao et al., 2014). Furthermore, *in vitro* studies show that CRF administration reduces dendritic arborization of hippocampal cells and antagonism of CRFR1 reverses this effect (Ivy et al., 2010). These shifts in both CRF and CRFR1 during the postnatal period might reflect sensitive periods in which CRF and stress can affect development of the hippocampus and associated behaviors.

In addition to the hippocampus, amygdala sub-regions showed changes in CRFR1 expression throughout development. These changes were most apparent in the medial and lateral divisions of the amygdala where an increase in CRFR1 occurred at each time point. The lateral amygdala relays information to the basolateral amygdala via excitatory afferent pathways, from which projections are sent to the central amygdala (Shekhar et al., 2005), leading to behavioral and/or emotional responses. Increased CRFR1 within this region

indicates a developmental enhancement of CRF signaling to this region and may reflect developmental changes in emotion regulation. The rodent medial amygdala receives inputs from the olfactory bulb and controls social and sexual behavior (Cushing et al., 2008; Sano et al., 2016). Again, these developmental increases in CRFR1 may reflect increasing CRF regulation of these functions prior to puberty. Central amygdala CRFR1-GFP-ir is stable between P0 and P21 and is largely localized within the medial sub-division with moderate/weak labeling in the lateral division which contains dense CRF and glucocorticoid receptor immunoreactive cells. Stable levels in central amygdala CRFR1 during postnatal development match a similar pattern of development reported in the rat brain (Avishai-Eliner et al., 1996). Unlike CRFR1, central amygdala CRF mRNA has been found to fluctuate through postnatal development. CRF levels are low around the time of birth and steadily rise to approximate adult levels by P14 (Keegan et al., 1994). CRFR1 in the central amygdala has been linked to a number of behaviors and functions including anxiety, alcohol dependence, and nociception which have been shown to be sex dependent (Haramati et al., 2011; Rouwette et al., 2012; Koob, 2014). However, the absence of clear sex differences in central amygdala CRFR1 indicates that sex differences in CRFR1 expression alone cannot account for these behavioral differences, although it remains possible that sex differences may be subtle or emerge post-puberty. CRF-producing cells have been reported to be sexually dimorphic in the central amygdala with greater numbers in female compared to male rats (Karanikas et al., 2013). This sex difference in CRF may be linked to differential susceptibility to alcohol- and stress-related disorders (Karanikas et al., 2013).

Overall, we report that expression of CRFR1 is dynamic during postnatal development in the mouse and is sexually dimorphic in discrete regions. The present data will aid further studies exploring developmental stress and sex differences in behavioral and hormonal stress responsivity. Altogether, these data will enhance our understanding of how CRF signaling might sex-specifically affect brain function and behavior during postnatal development.

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Abbreviations:

BAC	bacterial artificial chromosome
BNST	bed nucleus of the stria terminalis
CRF	corticotropin-releasing factor
CRFR1	CRF receptor 1
GFP	green fluorescent protein
PBS	phosphate-buffered saline
PVN	paraventricular nucleus
TH	tyrosine hydroxylase

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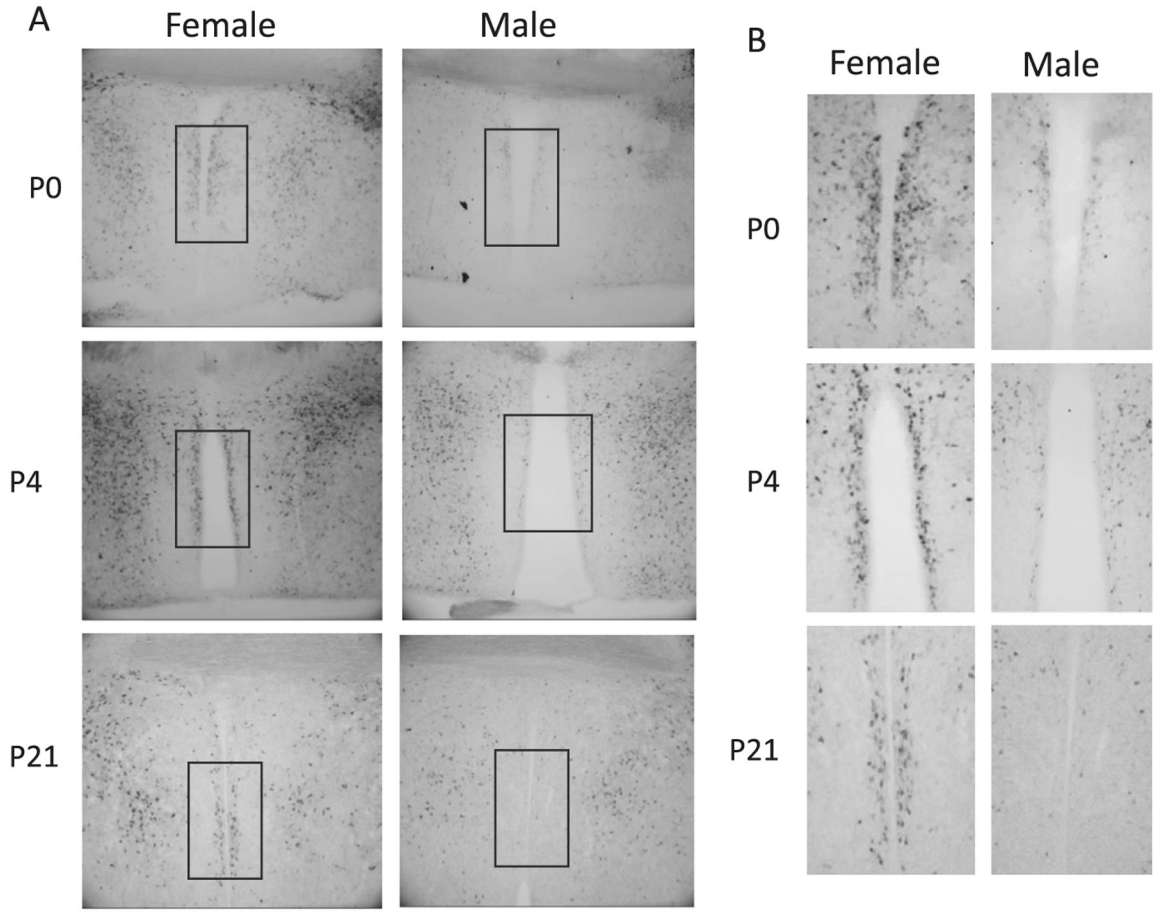

Fig. 1.

CRFR1-GFP expression within the rostral region of the anteroventral periventricular nucleus (AVPV/PeN). (A) Representative images from female and male AVPV/PeN during developmental periods P0, P4, and P21. (B) High magnification images illustrating the AVPV/PeN for female and male subjects during the listed developmental periods. The dense clustering of CRFR1-GFP-ir can be seen clearly near the 3rd ventricle of females but not males (right). $n = 5$ per age/sex.

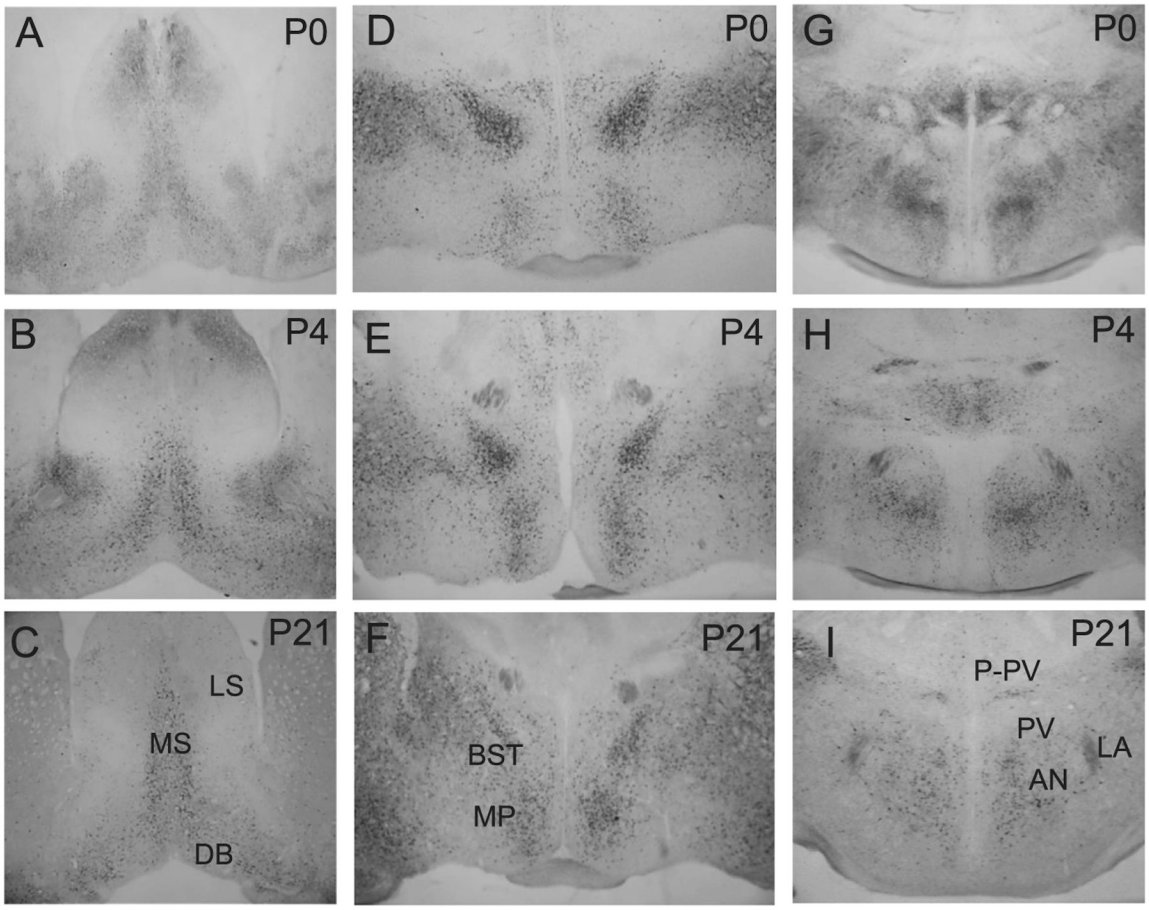

Fig. 2.

CRFR1-GFP expression in septal and hypothalamic regions. (A–C) Representative images of the lateral septum, medial septum, and diagonal band for P0, P4, and P21. Note that distribution within the medial septum increased slightly across development. The lateral septum slightly decreased by P21, in comparison to earlier ages. The diagonal band increased slightly from P0 to P4, but leveled off at P4, with no visual differences from P4 to P21. (D–F) Representative images from the BST and the medial preoptic area at ages P0, P4, and P21. (G–I) Representative images for the nucleus just dorsal to the paraventricular hypothalamus (P-PV), the paraventricular hypothalamus, the anterior hypothalamus, and the lateral hypothalamus at ages P0, P4, and P21. Within the anterior hypothalamus, there was a slight decrease in CRFR1-GFP-ir density between P4 and P21. MS; medial septum, LS; lateral septum, DB; diagonal band, BST; bed nucleus of the stria terminalis, MP; medial preoptic area, P-PV; nucleus dorsal to the paraventricular hypothalamus, PV; paraventricular hypothalamus, AN; anterior hypothalamus, LA; lateral hypothalamus.

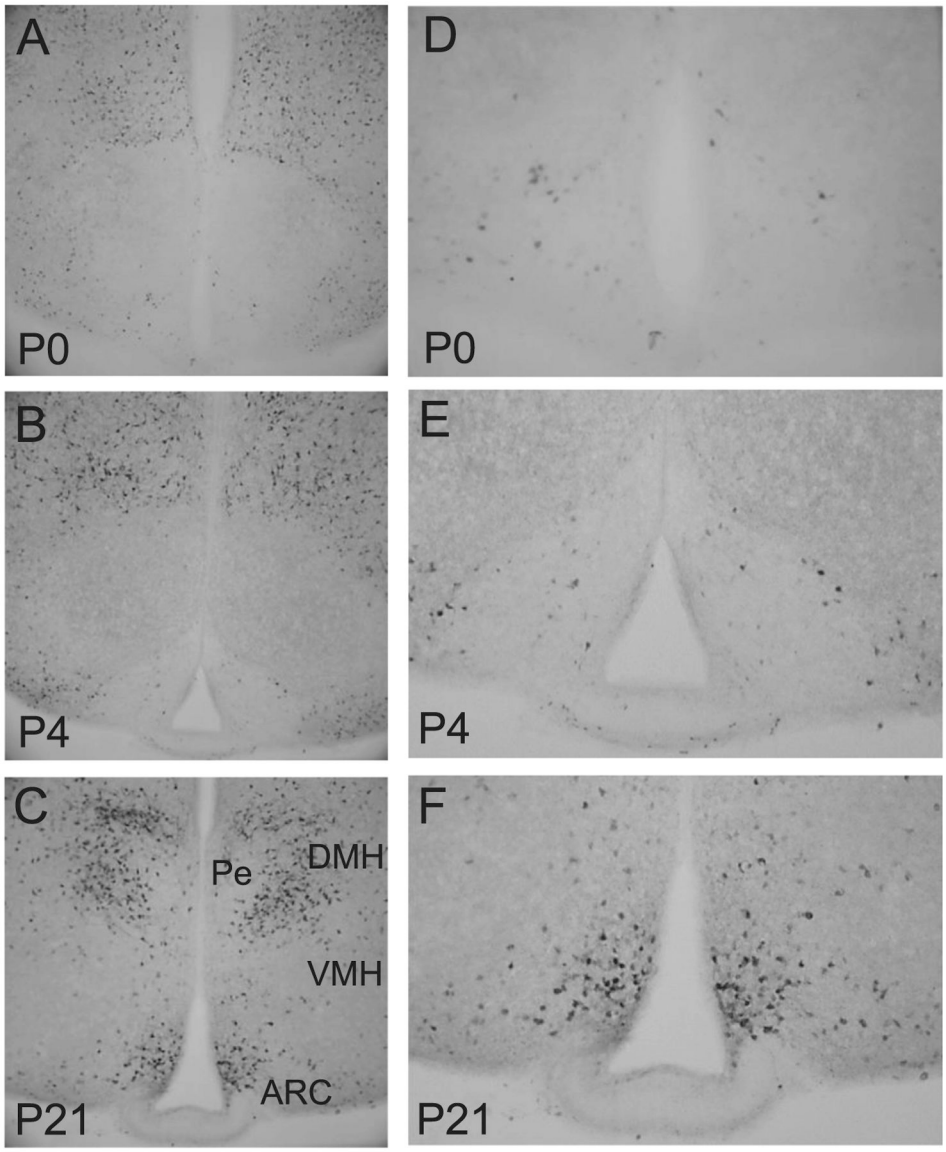

Fig. 3.

CRFR1-GFP expression in the caudal hypothalamus. (A–C) Representative images of the intermediate periventricular nucleus, dorsomedial hypothalamus, ventromedial hypothalamus and the arcuate nucleus at (A) P0, (B) P4, and (C) P21. Within the intermediate region of the periventricular nucleus, there was a decrease in CRFR1-GFP-ir from P4 to P21. There were slight increases in CRFR1-GFP-ir for the dorsomedial hypothalamus across developmental windows measured. Little labeling was seen within the ventromedial hypothalamus across ages. Importantly, the arcuate nucleus displayed a dramatic increase in labeling between P4 and P21 (D–F). Pe; intermediate periventricular nucleus, DMH; dorsomedial hypothalamus, VMH; ventromedial hypothalamus, ARC; arcuate nucleus of the hypothalamus.

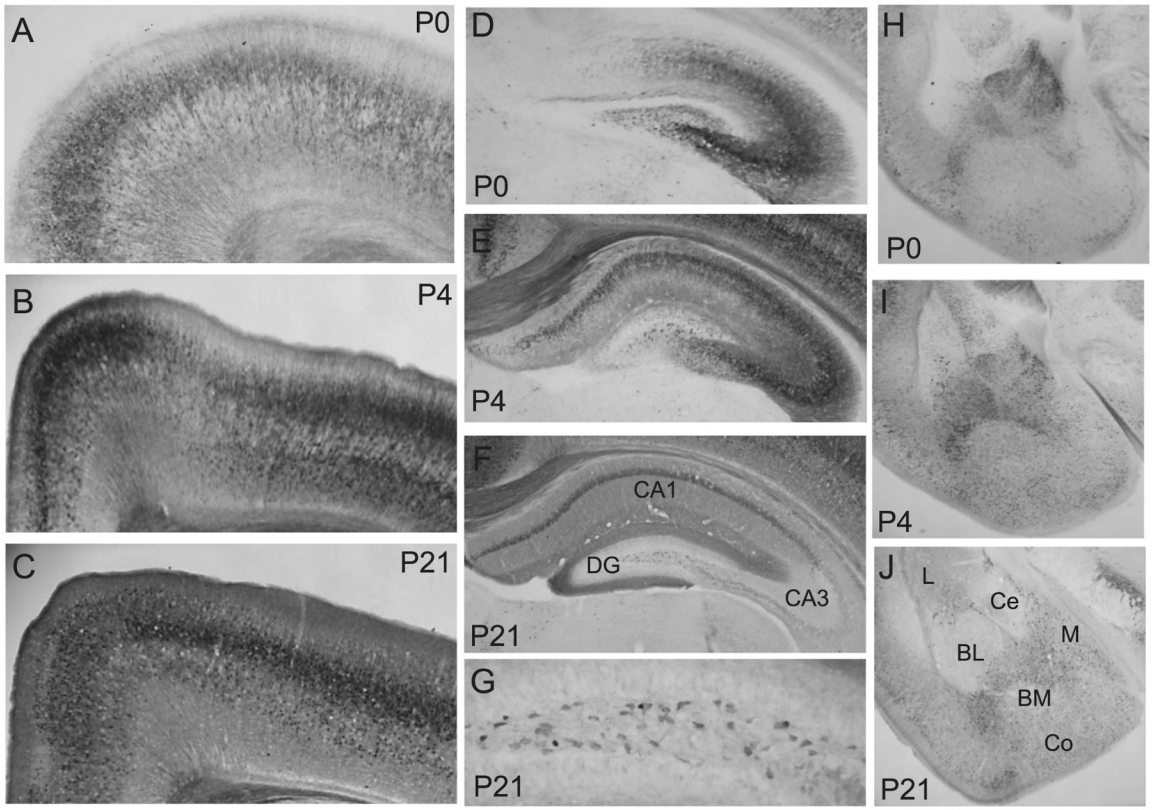

Fig. 4.

CRFR1-GFP expression within the cerebral cortex, hippocampus, and amygdala. (A–C) In the cerebral cortex CRFR1 immunoreactivity changed dramatically during postnatal development. At P0, labeling was greatest within middle cortical layers while at P4 there were dense and defined CRFR1 layers in both outer and deep cortical layers. By P21 density was greatest in outer and middle cortical layers. Overall, CRFR1-GFP-ir at P0 was somewhat lower than the other ages across regions of cortex. (D–F) Representative images of the hippocampus for P0, 4, and 21, respectively. There was a gradual increase in CA1 pyramidal layer density throughout developmental time points assessed, while there was a stark decrease in immunoreactivity within the CA3 region from P0 to P21. CRFR1-GFP-ir also decreased slightly by P21 within the dentate gyrus. (G) Representative high magnification image of the hilus, the most densely labeled portion of the dentate gyrus. (H–J) Representative pictures of the amygdala at P0, 4, and 21. There was a gradual increase in CRFR1-GFP-ir within the LA and MeA between P0 and P21. Labeling was generally higher in the basomedial amygdala than in the other regions of the amygdala, throughout development. DG, dentate gyrus; L, lateral amygdala; M, medial amygdala; Ce, central amygdala; BM, basomedial amygdala; BL, basolateral amygdala; CO, cortical amygdala.

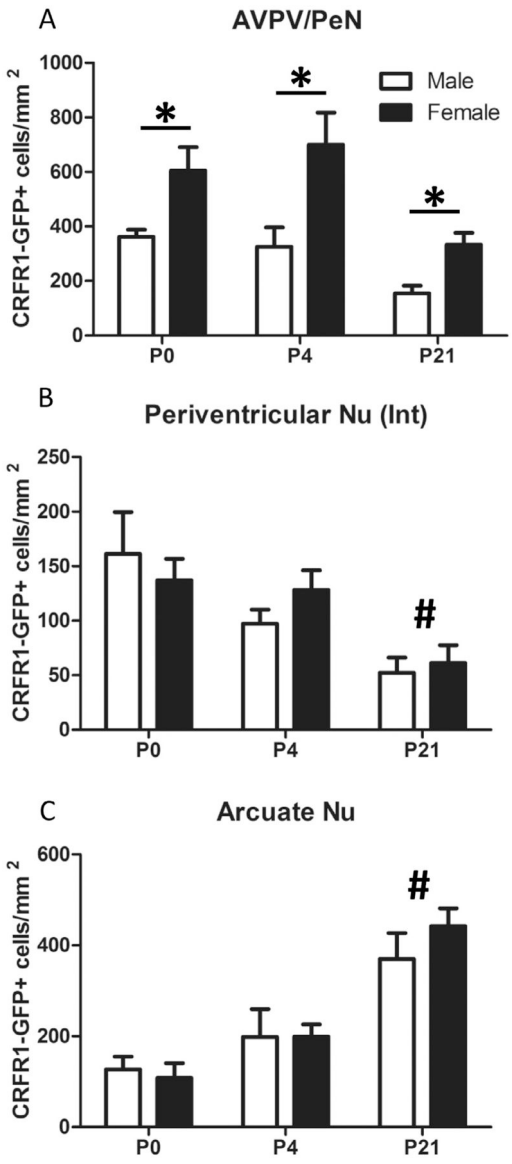

Fig. 5.

Quantification of CRFR1-GFP-ir within selected brain regions. CRFR1-GFP-ir cells were counted in selected brain regions of P0, P4, and P21 mice. (A) In the AVPV/PeN, there were significant sex differences at all age groups, with females having more CRFR1-GFP-ir than males. (B) Within the intermediate division of the periventricular nucleus, there were no sex differences, but there were significantly fewer CRFR1-GFP-ir cells, regardless of sex, by P21, in comparison to P0 and P4. Finally, within the arcuate, there was a significant increase in immunoreactivity at P21 in comparison to P0 and 4. *Indicates sex difference, $p < 0.01$. #Indicates significant difference between P21 and other age groups, $p < 0.01$. Shown as means ± SEM. AVPV/PeN; anteroventral periventricular nucleus of the hypothalamus, periventricular nucleus (Int); periventricular nucleus of the hypothalamus, intermediate division.

Table 1.

Distribution of CRFR1-GFP-ir cells in the developing mouse forebrain. Density and intensity of CRFR1-GFP-ir was rated visually during developmental periods P0, P4, and P21, as having −, No label; +/−, minimal but still noticeable labeling intensity/density of cells; +, low labeling intensity/density of cells; ++, moderate labeling intensity/cell density; +++, high labeling intensity/cell density; ++++, very high labeling intensity/cell density

	P0		P4		P21	
Brain region	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
Outer cortical layers	+ +	++	+++	+++	++	++
Middle cortical layers	+ +	++	++	++	++++	++++
Inner cortical layers	+	+	+++	+++	++	++
Medial septum	+	+	++	++	++	++
Lateral septum	+	+	++	++	+	+
Diagonal band	+	+	++	++	++	++
Bed Nucleus of the Stria Terminalis, principal nucleus	+++	+++	+++	+++	++	++
Lateral amygdaloid nucleus	+/−	+/−	+	+	+	+
Basolateral amygdaloid nucleus	+	+	+	+	+	+
Basomedial amygdaloid nucleus	+++	+++	+++	+++	+++	+++
Central amygdaloid nucleus	++	++	++	++	++	++
Medial amygdaloid nucleus	+/−	+/−	+	+	+ +	+ +
Cortical amygdaloid nucleus	++	++	++	++	++	++
CA 1	+++	+++	+++	+++	++++	++++
CA 3	++++	++++	++++	++++	++	++
Dentate gyrus	++	++	++	++	++	++
Medial habenular nucleus	–	–	–	–	–	–
Lateral habenular nucleus	+	+	++	++	++	++
Suprachiasmatic nucleus	++	++	++	++	++	++
Medial preoptic area	++	++	++	++	++	++
Anteroventral periventricular nucleus	+	+++	+	+++	+/−	++
Periventricular hypothalamic nucleus (Intermediate)	+	+	+	+	+/−	+/−
Peri-paraventricular hypothalamic nucleus	++	++	++	++	++	++
Paraventricular hypothalamic nucleus	+/−	+/−	+/−	+/−	+	+
Supraoptic nucleus	–	–	–	–	–	–
Anterior hypothalamus	+++	++	+++	+++	++	+
Lateral hypothalamic area	+	+	+	+	+	+
Arcuate nucleus	+/−	+/−	+	+	+++	+++
Dorsomedial nucleus	++	++	++	++	++	++
Tuberal nucleus	+	+	++	++	+	+
Ventromedial hypothalamic nucleus	+/−	+/−	+/−	+/−	+	+