Developmental Changes in Corticotropin Releasing Factor Receptor 1 in the Postnatal Dentate Gyrus

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Developmental Changes in Corticotropin Releasing Factor Receptor 1

in the Postnatal Dentate Gyrus

An honors thesis presented to the
Department of Psychology,
University at Albany, State University of New York
in partial fulfillment of the requirements
for graduation with Honors in Psychology
and
graduation from The Honors College.

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April, 2018
ABSTRACT

Corticotropin releasing factor (CRF) has been established as a key modulator in the stress response. Areas of research have primarily focused on brain regions that control the hypothalamic-pituitary-adrenal (HPA) axis. However, extensive research has yet to be conducted on the CRF receptor 1 (CRFR1) in the dentate gyrus, a region associated with memory functions. Therefore, the purpose of this study is to investigate age-related changes in CRFR1 in the granule layer and the hilus layer of the dentate gyrus in CRFR1 reporter mice at three different age groups; pre-pubertal (p21), adult (p90), and old (22-24 months) age. The results of this study show that CRFR1 labeling did not exist in the granule layer at P21 but appears at moderate to heavy levels at P90 and 22-24 months. Furthermore, CRFR1 labeling in the hilus layer is noticeably visible in all three age groups, with higher levels at P21. Overall, these significant age-related differences at P21 could have implications towards the onset of other childhood-related developments, such as fear memories and possibly even depression.
ACKNOWLEDGMENTS

I would like to thank Dr. Zuloaga for the incredible experience I had being in his lab for two and a half years and for being so willing to take on this thesis with me as well as guide me through the challenging process. The same goes for the graduate students, Zachary Rosinger and Jason Jacobskind, for always being there to help and support me whenever I needed it.

Lastly, I would like to express gratitude to my family for taking such an interest in my research and always motivating me to excel academically and personally.
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INTRODUCTION

Corticotropin-releasing factor (CRF) is a 41 amino acid-containing peptide released primarily in the paraventricular nucleus of the hypothalamus (PVN) (Tenk et al., 2017). It is predominantly known for its mediation of the endocrine, autonomic and behavioral response to stress (Henckens et al., 2016; Arborelius et al., 1999). Alterations in these systems have been linked with many affective disorders such as depression and anxiety disorders (Risbrough & Stein, 2007). In fact, CRF receptor 1 (CRFR1) antagonists have been shown to have anxiolytic and antidepressant-like effects (Dunn, 2016). Compared to CRF receptor 2 (CRFR2), a receptor that has been shown to naturally have anxiolytic effects (Reul & Holsboer, 2002), CRF binds to CRFR1 with high affinity. Additionally, there is a higher distribution of CRFR1 throughout the brain than CRFR2 (Lein et al., 2007). To date, CRFR1 has been localized in numerous regions in the central nervous system (CNS) including the hippocampus, amygdala, hypothalamus, bed nucleus of the stria terminalis (BNST), olfactory bulb and brainstem (Croiset et al., 2000; Merchenthaler et al., 1982).

CRF signaling through CRFR1 has been found to have hippocampus-dependent effects on learning and memory (Blank, 2003). Studies have shown that CRF injected directly into the dentate gyrus led to enhanced fear memory retention in rats (Lee et al., 1993; Ma et al., 1999). On the other hand, however, a study by Chen et al. (2010) showed that introducing NBI 30775, a CRFR1 antagonist, directly into the CA3 was able to restore cognitive functions previously impaired by acute stress. Overall, CRF does seem to have definitive effects on learning and memory, which appear to be region-specific, dose-dependent, and reliant on type of treatment (Blank, 2003; Ma et al., 1999; Chen et al., 2010).
According to research on fear memory, the ability to form such memories are not immediate upon birth. In rats, it has been shown that this ability is still not developed by 25 days of age (Feigley & Spear, 1970). Feigley & Spear found that rats 21-25 days of age had significantly higher retention loss to both a one-way active-avoidance and a passive-avoidance task given 28 days after initial fear conditioning in comparison to rats 60-70 days of age. Rudy & Morledge (1994) replicate this finding in earlier ages by reporting that freezing in p (postnatal) 18 rats during behavioral testing significantly drops off when the retention interval is longer than 24 hours. From this, it can be inferred that fear memories are not sustainable at and before the age of 25 days. This is arguably, in part, due to neural immaturity, since this age is still a critical period of development in the hippocampus. However, in mice, this inability to sustain fear memories no longer exists at P30, thus challenging that maturity has been reached by around this age (Akers et al. 2012).

As reported in past literature, the expression of fear memories has been shown to be enhanced by CRF. However, to what extent CRFR1 mediates the actual development of fear memory during development has yet to be fully explored. Therefore, the purpose of this study was to determine age-related changes of CRFR1 in the dentate gyrus. Ultimately, this research attempts to shed light on if changes in memory function are, in fact, associated with changes in CRFR1 expression.

METHODS

Animals

This study consisted of BAC transgenic CRFR1-GFP mice that were maintained on a 12/12 light/dark cycle (lights on at 0700), with food and water made available ad libitum. CRFR1-GFP mice were mated and the brains of 44 offspring (n = 22 per sex) were excised at three different
time points for the purposes of comparing age groups. Time points were chosen to represent pre-pubertal (p21), adult (p90), and old (22-24 months) age. All procedures have been approved by the University at Albany Institutional Animal Care and Use committee and were in accordance with the National Institutes of Health guidelines.

**Perfusion and Tissue Processing**

Mice were euthanized via cervical dislocation followed by decapitation. Perfusion consisted of 30 ml of 4% paraformaldehyde. Storage of excised brains persisted for 24 hours in a solution of 4% paraformaldehyde at 4 °C. Afterwards, brains were submerged into a solution of 30% sucrose and remained at the original 4 °C. A cryostat (Microm HM505E, MICROM international GmbH, Walldorf, Germany) was used to section the brains into three series through the coronal plane at 40 μm. Until immunohistochemistry was performed, all tissue remained at 4 °C in a cryopreservative solution.

**Immunohistochemistry**

In order to remove the cryopreserve, tissue was rinsed in phosphate-buffered saline (PBS; pH 7.6) and allowed to soak in a solution of 1% hydrogen peroxide and 0.4% Triton-X in PBS (PBS-TX) for 10 minutes. After re-rinsing in PBS, the tissue was allowed to incubate for one hour in 4% normal goat serum (NGS) in PBS-TX. Tissue was then transferred to a primary antiserum for GFP (1:10,000, rabbit, Life Technologies, A6455, Carlsbad, CA, USA) to be incubated overnight. The next day, tissue was once again rinsed in PBS and then immersed in biotinylated goat anti-rabbit antisera in PBS-TX (1:500; Vector Laboratories, Burlingame, CA, USA) for one hour. After
rinsing with PBS one last time, the tissue was placed in avidinbiotin complex (ABC Elite kit, Vector Laboratories) for 60 minutes and then rinsed with tris-buffered saline (TBS). For visualization of CRFR1-GFP-positive cells, tissue was placed in diaminobenzidine for 10 minutes. For negative controls, sections from wild-type brains were utilized to assess GFP-ir. Lastly, the tissue was mounted on slides and coverslipped with Permount (Fisher Scientific, Fairlawn, NJ, USA).

**Analyses**

A Nikon 80i Eclipse microscope was used to evaluate the density of CRFR1-GFP-ir under 20x magnification. Bilateral images were captured within 2 brain sections for each brain. The Allen Institute mouse brain atlas was used to identify brain regions. Brain regions examined include both the granule and hilus layer of the dentate gyrus (Bregma -2.06 to -2.30). All cell quantifications were performed through the Image J software with the aid of a fixed frame (rectangle). All statistical analyses were performed using a two-way ANOVA with age (p21, p90, and 22-24 months) and sex (male and female) as variables. Additional post hoc analyses were performed using the Bonferroni correction.

**RESULTS**

**Granule Cell Layer**

A two-way ANOVA indicated a significant main effect of age (F(2,28) = 1093.69, p < 0.001). Subsequent post hoc tests revealed a profound lack of immunoreactivity of CRFR1 at P21, in comparison to P90 (p < .001) and 22-24 months (p < .001) in which moderate to heavy
levels of CRFR1 expression was observed (Figure 1a-b). Labeling from P90 to 22-24 months remained consistent and no significant effects of sex or interaction between sex and age were found.

**Figure 1a.** CRFR1-GFP-ir cells were quantified in the granule layer of the dentate gyrus at P21, P90, and 22-24 months. A significant age difference was observed, in which profoundly more immunoreactivity was found in P90 and 22-24 months in comparison to P21. *Indicates significant difference between P21 and all other age groups, p <0.001.

![Granule](image)

**Figure 1b.** Representative images of the dentate gyrus from P21 (left) and P90 (right). High magnification images clearly illustrate a profound increase in CRFR1-GFP-ir in the granule layer from P21 to P90. GCL, granule cell layer.
Hilus Cell Layer

A two-way ANOVA indicated a significant main effect of age (F(2,28) = 6.50, p < 0.01). Subsequent post hoc tests revealed a greater number of CRFR1-GFP-ir cells at P21, in comparison to P90 (p < .05) and 22-24 months (p < .01) which observed a gradual decrease (Figure 2). From P90 to 22-24 months, labeling remained unchanged and no significant effects of sex or interaction between sex and age were detected.

Figure 2. CRFR1-GFP-ir cells were quantified in the hilus layer of the dentate gyrus at P21, P90, and 22-24 months. A significant age difference was observed, in which more immunoreactivity was found at P21 in comparison to P90 and 22-24 months. *Indicates significant difference between P21 and all other age groups, p <0.01.

DISCUSSION

The results of this study found little to no CRFR1 expression in the granule layer at P21, and then a maturation of cells expressing CRFR1 by P90 which persisted into 22-24 months. An
opposite effect was found in the hilus layer, where the greatest amount of CRFR1 expression was found at P21, which decreased by P90 and remained unchanged into 22-24 months. This decrease in CRFR1 may reflect either the loss of cells within the hilus or decrease in expression within existing cells, either of which is possible due to the significant changes the hippocampus undergoes during postnatal development. Lastly, a sex difference between CRFR1 expression was assessed without finding anything statistically significant.

A lot of research has been done on the CRF receptor and its mediation in stress responses, but little work has focused on how this receptor mediates fear memory, specifically in the dentate gyrus. Fear memories are widely recognized for their association with post-traumatic stress disorder (PTSD). Therefore, this present study sought to look further into age-related changes in CRFR1 in both the granule and hilus cell layers and speculate the link between the emergence of CRFR1 in the granule layer and the ability to generate sustainable fear memories.

Firstly, the dentate gyrus, specifically the granule layer, is especially unique due to its capability of adult neurogenesis in both humans and many other species (Masiulis et al., 2011). Such a process was for a long time not thought possible in humans but has thus far been found to occur in the dentate gyrus and olfactory bulb. It is still a matter of discussion as to what purpose neurogenesis plays in these specific regions. So far, there has been a lot of research pointing to CRF as a possible mediator in this process. Reported by Alonso et al. (2004), 53% of granule cell neurogenesis were reduced following chronic mild stress, which was then reversed following repeated administration of SSR125543A, a CRFR1 antagonist. Additionally, neurogenesis may relate to the appearance of CRFR1. Rather than the already existing cells in the dentate gyrus beginning to express CRFR1 later in life, there is the alternate possibility that it is the new cells that are born through neurogenesis that are the cells containing CRFR1.
This study reported that CRFR1 did not become expressed until some point after P21. Past literature, as discussed above, showed that fear memories don’t appear to be fully mature by P25 (Feigley & Spear, 1970). This research speculates that the emergence of CRFR1 cells in the dentate gyrus may be linked to the maturation of fear memories.

One possible explanation to be considered for the lack of CRFR1 at P21 is the constant neuronal turnover occurring at this age, which leads to a greater proportion of immature neurons. Because of this, an alternative reason for the inability to form sustainable fear memories at this age is due to the constant remodeling of hippocampal circuitry. Without stable neuronal connections, memories can still be established, but will ultimately be forgotten as new input and output networks are made (Frankland et al., 2013). Neurogenesis is also thought to play a role in this process because it results in the continuous integration of newly generated neurons, which leads to even more altered hippocampal circuitry. In fact, by increasing neurogenesis in the dentate gyrus, it has been shown to induce increased forgetting of contextual fear memory (Gao et al., 2018). At some point throughout this development, the results of this study show that CRFR1 will appear, either through neurogenesis or expression in already existing cells. One speculation is that the expression of CRFR1 may act as a stabilizing mechanism, resulting in mature, and therefore, sustainable fear memories.

Lastly, as stated previously, CRF has been implicated in the development of many affective disorders such as depression and anxiety. It is estimated that granule cells in the dentate gyrus may start expressing CRFR1 only a couple days after P21 (Akers et al. 2012). In human equivalence, this would be around childhood. Although depression is most often diagnosed in adulthood, it can actually still develop in as early as childhood. An interesting implication that arises from this is whether the emergence of CRFR1 in the granule cells not only mediates fear
memories, but also the development of depression. CRF is mostly known for mediating disorders like depression and stress by affecting regions that regulate the HPA axis. However, the previously discussed study by Alonso et al (2014) showed that, not only did the CRF antagonist inhibit the reduction of neurogenesis in the granule layer, but it also decreased depressive-like symptoms. Although the study didn’t specifically draw a parallel, the evidence can still raise a connection between depression and neurogenesis. Moreover, multiple studies have indicated that antidepressant medication contributes to the promotion of granule cell neurogenesis, which is a topic of current debate over how antidepressant medications have their effects (Krzak et al, 2017; Segi-Nishida, 2017). Therefore, it could be postulated that the signaling of CRF through CRFR1 has an indirect link to depression through the mediation of neurogenesis in the granule cell layer of the dentate gyrus.

Moving forward, more research would need to be done to determine the specific days in which CRFR1 becomes expressed in the granule layer of the dentate gyrus, and then specifically compare this to when fear memories start emerging in order to substantiate a link between CRFR1 and fear memories. Most research that has investigated CRFR1 and fear memories have been on rats. However, the research that has been done using mice have shown that the ontogeny of fear learning may occur at an earlier age in comparison to rats. For this reason, it would be beneficial for further studies looking into this link to utilize a mice model. Ultimately, what this current study showed was that there may be implications towards the expression of CRFR1 and other behavioral developments, which if understood further, could lead to a better comprehension for disorders like PTSD, anxiety, and depression.
REFERENCES


