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Structural differences in the hippocampus and amygdala of behaviorally inhibited macaque monkeys

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Abstract

Behavioral inhibition is a temperamental disposition to react warily when confronted by unfamiliar people, objects, or events. Behaviorally inhibited children are at greater risk of developing anxiety disorders later in life. Previous studies reported that individuals with a history of childhood behavioral inhibition exhibit abnormal activity in the hippocampus and amygdala. However, few studies have investigated the structural differences that may underlie these functional abnormalities. In this exploratory study, we evaluated rhesus monkeys exhibiting a phenotype consistent with human behavioral inhibition. We performed quantitative neuroanatomical analyses that cannot be performed in humans including estimates of the volume and neuron number of distinct hippocampal regions and amygdala nuclei in behaviorally inhibited and control rhesus monkeys. Behaviorally inhibited monkeys had larger volumes of the rostral third of the hippocampal field CA3, smaller volumes of the rostral third of CA2, and smaller volumes of the accessory basal nucleus of the amygdala. Furthermore, behaviorally inhibited monkeys had fewer neurons in the rostral third of CA2. These structural differences may contribute to the functional abnormalities in the hippocampus and amygdala of behaviorally

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of California, Davis, and were in accordance with the National Institutes of Health guidelines for the use of animals in research.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

inhibited individuals. These structural findings in monkeys are consistent with a reduced modulation of amygdala activity via prefrontal cortex projections to the accessory basal nucleus. Given the putative roles of the amygdala in affective processing, CA3 in associative learning and CA2 in social memory, increased amygdala and CA3 activity, and diminished CA2 structure and function, may be associated with increased social anxiety and the heritability of behavioral inhibition. The findings from this exploratory study compel follow-up investigations with larger sample sizes and additional analyses to provide greater insight and more definitive answers regarding the neurobiological bases of behavioral inhibition.

Keywords

amygdala; anxiety disorders; associative learning; behavioral inhibition; hippocampus; social behavior

1. | INTRODUCTION

Behavioral inhibition is a disposition to react warily when confronted by unfamiliar people, objects or events, and while first identified in early childhood it has life-long implications for the development and persistence of mood disorders (Fox et al., 2005; Garcia-Coll et al., 1984; Kagan et al., 1987). One of the hallmark characteristics of behaviorally inhibited children is that they are slower to approach strangers and often cease to play, stay quiet, and increase their proximity to the parent or care-giver in situations in which such novelty is introduced (Fox et al., 2005; Garcia-Coll et al., 1984). Since its initial description (Garcia-Coll et al., 1984), the study of this temperamental disposition has grown in importance for researchers and clinicians because of its predictive value for the development of psychopathology later in life. Indeed, several studies have shown that inhibited toddlers and young children tend to be at greater risk of developing anxiety disorders, especially social anxiety (Biederman et al., 1990; Biederman et al., 2001; Chronis-Tuscano et al., 2009; Gladstone et al., 2005; Schwartz et al., 1999). Despite decades of work on its behavioral manifestation, its neural mechanisms remain unclear.

Studies of childhood behavioral inhibition have helped to explain several aspects of this temperament, especially its stability across ages and its heritability. Longitudinal studies have shown that shy and fearful toddlers tend to keep this inhibited profile during childhood (Kagan et al., 1984; Kagan et al., 1988; Pérez-Edgar & Fox, 2005), and that inhibited children often maintain their social wariness into early adulthood (Gest, 1997). However, this inhibited temperament also shows a certain discontinuity, with several internal and external factors (e.g., development of attentional processes, parental care-giving) capable of changing children's behavior and the developmental trajectory of behavioral inhibition (Degnan & Fox, 2007). In addition to its relative stability across the lifespan, behavioral inhibition also demonstrates modest heritability. Children with anxious or depressed parents (i.e., having either anxiety disorders or major depressive disorder) are more likely to be behaviorally inhibited (Biederman et al., 2001). Moreover, twin studies have shown that extremely inhibited behaviors (Robinson et al., 1992) and social anxiety symptoms that may

be related to behavioral inhibition (Warren et al., 1999) are genetically linked and may thus be heritable.

Remarkably, patterns of behavior associated with behavioral inhibition appear to be evolutionarily conserved, opening the possibility of studying behavioral inhibition in animals. In particular, nonhuman primates have great promise in order to decipher causal mechanisms that cannot be studied in humans. Indeed, monkeys show similarities to humans in their temperament (Gosling & John, 1999), and especially in the development and stabilization of inhibited or anxious behaviors (Kalin & Shelton, 1998, 2003). In this exploratory study, we used rhesus monkeys exhibiting a phenotype consistent with human behavioral inhibition to perform quantitative neuroanatomical analyses of brain regions identified via imaging studies in humans.

1.1 | Functional abnormalities in the hippocampus and amygdala

Although its behavioral characteristics have been extensively studied, the neurobiological bases of behavioral inhibition are still poorly understood. Neuroimaging studies of children characterized as behaviorally inhibited have shown functional abnormalities in different brain regions, such as the prefrontal cortex (Schwartz et al., 2010; Sylvester et al., 2016), the cingulate cortex (Sylvester et al., 2016), the hippocampus (Blackford et al., 2013), and the amygdala (Blackford et al., 2011; Blackford et al., 2013; Clauss, Seay, et al., 2014; Schwartz et al., 2003).

Among these brain regions, the amygdala has received special attention since it plays a crucial role in the regulation of emotional experience and perception (Lindquist et al., 2012). It has been implicated in mediating unconditioned fear responses (Amaral et al., 2003; Prather et al., 2001), fear learning (Davis, 1992; LeDoux, 2000), novelty detection (Blackford et al., 2010), social information processing (Adolphs, 2003), emotional memory encoding (Richardson et al., 2004), and anxiety (Damsa et al., 2009). Accordingly, Schwartz et al. (2003) found that adults who exhibited a behaviorally inhibited temperament as children had greater amygdala activation in response to the presentation of novel faces. Furthermore, Blackford et al. (2013) showed that amygdala activity is sustained, thus reflecting a slower habituation to novel faces. The amygdala of behaviorally inhibited individuals also presents some alterations in functional connectivity with brain structures including the prefrontal cortex and the insula (Blackford et al., 2014; Clauss, Avery, et al., 2014).

While most studies of behavioral inhibition have focused on possible abnormalities of amygdala function, fewer have considered the hippocampus. Nevertheless, the hippocampus is thought to play an important role in the regulation of fear and anxiety disorders (Canteras et al., 2009; Etkin, 2009; Shin & Liberzon, 2010). Of particular interest, the hippocampus contributes to the association of threat signals processed by the amygdala to specific contexts (Canteras et al., 2009; Richardson et al., 2004), and thus works together with the amygdala to encode affective memories. In addition, the rostral hippocampus has been shown to be essential for the regulation of fear-like behaviors. Indeed, in rodents, a lesion of the ventral hippocampus (which corresponds to the rostral hippocampus in primates) reduces fear behaviors (Bannerman et al., 2003; Kjelstrup et al., 2002; Pentkowski et al., 2006). In

behaviorally inhibited individuals, the hippocampus exhibits some functional abnormalities. Specifically, behaviorally inhibited adult humans fail to exhibit habituation of hippocampal activity across repeated presentations of faces (as was seen in the amygdala), which may reflect a deficit in social learning (Blackford et al., 2013). Furthermore, monkeys with an anxious temperament exhibit an increased activity of the rostral hippocampus when confronted with a threatening situation, in which a human intruder enters the room and approaches their cage (Oler et al., 2010).

In sum, behaviorally inhibited individuals seem to exhibit abnormal activity in both the amygdala and the hippocampus, two highly interconnected brain structures involved in the regulation of fear and associated behaviors (Amaral et al., 2003; LeDoux, 2000; Pitkänen et al., 2000; Pitkänen et al., 2002). To date, however, there is little information about the structural differences that may contribute to the etiology of behavioral inhibition and the predisposition to develop social anxiety disorders.

1.2 | Structural studies of the hippocampus and amygdala

Previous studies using structural magnetic resonance imaging of adolescents or adults who were characterized as behaviorally inhibited children reported no difference in the volume of the whole hippocampus between behaviorally inhibited and control individuals (Schwartz et al., 2015; Sylvester et al., 2016). Studies measuring amygdala volume reported mixed results, with some studies finding a larger amygdala in inhibited individuals (Claus, Seay, et al., 2014; Hill et al., 2010), and others finding no difference (Sylvester et al., 2016). Although no clear conclusion can be drawn from these studies, variations in subject selection might account for some of the discrepancies. Thus, while previous studies provided important data on the volume of the whole amygdala and hippocampus in behaviorally inhibited individuals, they did not investigate potential differences at the level of individual amygdala nuclei or hippocampal regions. Indeed, the amygdala comprises 13 nuclei with different sets of connections and purported functions (Amaral et al., 1992; Balleine & Killcross, 2006; Pitkänen et al., 1997). The hippocampus also comprises several regions, each having distinct morphological features and contributing to different memory functions (Amaral & Lavenex, 2007; Lavenex & Banta Lavenex, 2013; Morris, 2007). As discussed above, higher metabolic activity in the rostral hippocampus triggered by a threatening situation (i.e., human intruder paradigm) is predictive of an anxious temperament in monkeys (Fox et al., 2015; Oler et al., 2010), but no information is available about the structural alterations that may underlie these functional abnormalities.

1.3 | Aim of the study

The aim of this exploratory study was to provide quantitative data on the structural characteristics of the main amygdala nuclei and hippocampal regions in rhesus monkeys exhibiting a phenotype consistent with human behavioral inhibition (Chun & Capitanio, 2016; Gosling & John, 1999; Suomi et al., 2011), at 3.5 months of age. Using stereological methods on Nissl-stained coronal brain sections, we quantified the volumes and neuron numbers of subregions of the hippocampus and amygdala nuclei in five behaviorally inhibited and five non-behaviorally inhibited control monkeys, at 2 years of age. Based on previous functional findings (Oler et al., 2010), we hypothesized that group differences

in structural characteristics should be present in the rostral portion of the hippocampus. In contrast, previous functional studies did not provide specific hypotheses about which amygdala nucleus was more likely to exhibit group differences.

2 | MATERIALS AND METHODS

2.1 | Animals and histological processing

2.1.1 | Experimental animals—Ten 2-year-old, Indian-origin, unrelated male rhesus monkeys, *Macaca mulatta*, were used for this study. Monkeys were born from multiparous mothers and raised at the California National Primate Research Center (CNPRC). They were maternally reared in 2000 m² outdoor enclosures and lived in large social groups until they were killed. All procedures were approved by the Institutional Animal Care and Use Committee of the University of California, Davis, and were in accordance with the National Institutes of Health guidelines for the use of animals in research.

Behaviorally inhibited (BI) and non-inhibited (control) monkeys were selected via the BioBehavioral Assessment Program at the CNPRC, which was designed to characterize infant rhesus monkeys' biological and behavioral responsiveness. At approximately 3.5 months of age, infants were separated from their mother and placed in individual holding cages (32" (H) × 24" (W) × 26" (D)) for a 25-h period during which several assessments were made (for details on procedures and assessments; see Capitanio, 2017; Golub et al., 2009). Within minutes of arrival (Day 1:09:15) in the testing area, a trained technician recorded the behaviors of the animals in their holding cages. Animals were observed in an identical fashion the next day (Day 2:07:00), 2 h before returning to their mothers and home cages. Exploratory and confirmatory factor analyses revealed two scales that describe the animals' behavioral responsiveness (Golub et al., 2009). The activity scale comprises time spent locomoting, time spent not hanging (from top/side of the cage), the rate of environmental exploration, and whether the animal ate, drank, crouched or not. The emotionality scale includes the rate of cooing, barking, and whether the animal scratched, threatened or lipsmacked. BI monkeys ($n = 5$) were selected as having scores below the mean for activity and emotionality for both Day 1 and Day 2; controls ($n = 5$) were drawn randomly from the rest of the distribution, which comprised a total of 301 animals. The criterion to characterize behavioral inhibition is thus based on factor analyses, which identify more reliable indicators of a trait than individual measurable behaviors (which are therefore not presented here). Behavioral inhibition, defined this way, has been shown to be stable for up to 11 years after the initial assessment (Capitanio, 2019).

2.1.2 | Brain acquisition—At 2 years of age, monkeys were injected with a lethal dose of sodium pentobarbital (50 mg/kg i.v.; Fatal-Plus, Vortech Pharmaceuticals, Dearborn, MI) and perfused with a flush of saline solution (0.9% NaCl) to remove blood from the circulatory system. Immediately after death, that is, cardiac arrest determined by the senior veterinary pathologist at the CNPRC, monkeys' brains were extracted within 30 min, bisected and then the right hemisphere was placed in 4% paraformaldehyde (in 0.1 M phosphate buffer, PB, pH 7.4) for immersion-fixation for 48 h under constant agitation. After fixation, hemispheres were blocked, cryoprotected, and frozen following standard

laboratory protocols (Lavenex et al., 2009). Coronal sections were cut with a freezing sliding microtome (Microm HM 440; Microm Int. GmbH, Walldorf, Germany) into six series at 30 μm and one series at 60 μm . The 60 μm sections were collected in 10% formaldehyde solution in 0.1 M PB (pH 7.4) and post-fixed at 4°C for 4 weeks prior to Nissl staining with thionin. Other series were collected in tissue collection solution and kept at -70°C until further processing.

2.1.3 | Histological processing—The procedure for Nissl-stained sections followed standard laboratory protocols (Lavenex et al., 2009). Sections were removed from 10% formaldehyde solution, thoroughly washed for 2 \times 2 h in 0.1 M PB (pH 7.4), mounted on gelatin-coated slides from filtered 0.05 M PB (pH 7.4), and air-dried overnight at 37°C. Sections were then defatted for 2 \times 2 h in a mixture of chloroform/ethanol (1:1, vol.) and rinsed for 2 \times 2 min in 100% ethanol, 1 \times 2 min in 95% ethanol, and air-dried overnight at 37°C. Sections were then rehydrated through a graded series of ethanol solutions, 2 min in 95% ethanol, 2 min in 70% ethanol, 2 min in 50% ethanol; dipped in two separate baths of dH₂O; and stained for 20 s in a 0.25% thionin (Fisher Scientific, Waltham, MA; catalog No. T-409) solution, then dipped in two separate baths of dH₂O, 4 min in 50% ethanol, 4 min in 70% ethanol, 4 min in 95% ethanol + glacial acetic acid (1 drop per 100 mL of ethanol), 4 min in 95% ethanol, 2 \times 4 min in 100% ethanol, 3 \times 4 min in xylene; and coverslipped with DPX (BDH Laboratories, Poole, United Kingdom).

2.2 | Stereological analyses

2.2.1 | Anatomical boundaries—The nomenclature and cytoarchitectonic organization of the monkey hippocampus and amygdala have been described in detail previously (Amaral et al., 1992; Amaral & Lavenex, 2007; Chareyron et al., 2012; Jabès et al., 2011).

We delineated the hippocampal regions and main amygdala nuclei according to these descriptions. The hippocampal formation was divided into three equal portions along its rostrocaudal axis: rostral, intermediate, and caudal. We included the dentate gyrus, CA3, CA2, CA1 fields of the hippocampus and the subiculum in our measurements. For the amygdala, we considered the six main nuclei: the lateral, paralamina, basal, accessory basal, medial, and central nuclei.

2.2.2 | Volumes—Volume measurements were performed with a 4X Plan Fluor objective (N.A. 0.13) on a Nikon 80i microscope linked to the PC-based StereoInvestigator system (MicroBrightField, Williston, VT), according to the Cavalieri principle (Gundersen & Jensen, 1987). About 30 sections per animal (480 μm apart) were used for the measurements of the dentate gyrus; about 15 sections per animal (960 μm apart) were used for CA3, CA2, CA1, and subiculum. About 15 sections per animal (480 μm apart) were used for the amygdala nuclei. We chose to leave out the most rostral and caudal sections of certain fields in order to avoid a biased sampling resulting from the tangential cut of these extreme regions in coronal sections. The first section of the hippocampus was selected randomly within the first two sections through the dentate gyrus, and the last section was selected based on the presence of a clearly identifiable granule cell layer. The first section of the amygdala was selected based on the clear presence of the lateral, basal and paralamina nuclei, and the last section based on the presence of the medial nucleus.

2.2.3 | Neuron numbers—Neuron number was determined using the optical fractionator method (West et al., 1991; West & Gundersen, 1990). This design-based method allows an estimation of the number of neurons that is independent of volume estimates (Lavenex et al., 2000a, 2000b). We estimated the total number of neurons in the CA3 and CA2 fields of the hippocampus, as well as in the accessory basal nucleus of the amygdala, which exhibited volume differences between BI and control monkeys (see results section). About 15 sections per animal (960 μm apart) were used for CA3 and CA2. About 15 sections per animal (480 μm apart) were used for the accessory basal nucleus of the amygdala. We used a 100X Plan Fluor oil objective (N.A. 1.30) on a Nikon Eclipse 80i microscope (Nikon Instruments, Inc., Melville, NY) linked to the PC-based StereoInvestigator software (MicroBrightField, Williston, VT). All other stereological parameters were the same as in previous stereological studies of the hippocampus (Jabès et al., 2011) and amygdala (Chareyron et al., 2012).

2.3 | Statistical analyses

All sections were coded to allow blind analysis, and the code was broken only after completion of the analyses. All data were checked for normality prior to analysis and transformed if necessary. Data analysis was performed using IBM SPSS Statistics for Macintosh, version 26.0 (IBM Corp. Armonk, NY). Effect size was reported as Cohen's d for independent samples t tests, calculated in Excel: $d = M_1 - M_2/s_{\text{pooled}}$. In our study, the risk of reporting a difference that may not exist (type I error) is not worse than the risk of missing a difference that may exist (type II error). Accordingly, we followed the recommendations of Rothman (1990), who argued that “*not making adjustments for multiple comparisons is preferable because it will lead to fewer errors of interpretation when the data under evaluation are not random numbers but actual observations on nature,*” and Saville (1990), who also argued that a procedure without correction is preferable because it provides greater consistency to compare results between studies.

3 | RESULTS

3.1 | Hippocampus

Given the previous functional findings in monkeys (Oler et al., 2010), we hypothesized that between group differences in hippocampal structure were most likely to be observed in the rostral portion of the hippocampus. We performed a MANOVA with group (BI or control) as the between-subject factor and the volume of the rostral extent of the dentate gyrus, CA3, CA2, CA1, and the subiculum as dependent variables. The group effect neared, but did not reach, conventional levels of statistical significance ($\Lambda = 0.119$, $F_{(5,4)} = 5.911$, $p = .055$, $\eta_p^2 = 0.881$). Univariate analyses revealed no significant group effect on the volume of the rostral dentate gyrus ($F_{(1,8)} = 2.721$, $p = .138$, $\eta_p^2 = 0.254$), rostral CA1 ($F_{(1,8)} = 0.064$, $p = .806$, $\eta_p^2 = 0.008$), or rostral subiculum ($F_{(1,8)} = 2.598$, $p = .146$, $\eta_p^2 = 0.245$). There was, however, a significant group effect on the volume of rostral CA3 ($F_{(1,8)} = 6.105$, $p = .039$, $\eta_p^2 = 0.443$) and rostral CA2 ($F = 12.250$, $p = .008$, $\eta_p^2 = 0.605$). Behaviorally inhibited monkeys had larger rostral CA3 volumes and smaller rostral CA2 volumes than controls (Table 1).

In order to determine whether the volumetric differences observed in rostral CA3 and rostral CA2 were driven by differences in neuron numbers, we counted neurons in those areas. The larger volume of rostral CA3 in the BI group was not associated with more neurons, since the number of neurons in that region did not differ between BI and control groups ($t_{(8)} = 0.026$, $p = .980$, $d = 0.0167$; BI group: $M = 654,993$, $SD = 83,727$; control group: $M = 653,206$, $SD = 125,464$). In contrast, the smaller volume of rostral CA2 in the BI group was associated with a smaller number of neurons in that region in the BI group ($t_{(8)} = 2.511$, $p = .036$, $d = 1.588$; BI group: $M = 39,883$, $SD = 9058$; control group: $M = 53,825$, $SD = 8492$). For the sake of completeness, we also performed further exploratory MANOVAs on the intermediate and caudal portions, respectively. There was no group effect in either MANOVA, nor were any of the univariate analyses significant (Supporting Information 1).

3.2 | Amygdala

Amygdala nuclei volumes (Table 2) were subjected to a MANOVA with group (BI or control) as the between-subject factor. The group effect was not significant in the MANOVA ($\Lambda = 0.192$, $F_{(7,2)} = 1.203$, $p = .526$, $\eta_p^2 = .808$), and there were no group differences in the univariate analyses on the volumes of the lateral ($F_{(1,8)} = 0.368$, $p = .561$, $\eta_p^2 = 0.044$), basal ($F_{(1,8)} = 0.064$, $p = .806$, $\eta_p^2 = 0.008$), paralaminar ($F_{(1,8)} = 0.284$, $p = .608$, $\eta_p^2 = 0.034$), medial ($F_{(1,8)} = 0.45$, $p = .837$, $\eta_p^2 = 0.006$), central ($F_{(1,8)} = 0.003$, $p = .957$, $\eta_p^2 = 0.0004$), or other nuclei ($F_{(1,8)} = 0.815$, $p = .393$, $\eta_p^2 = 0.092$). However, despite the overall MANOVA being not significant, there was a strong difference between groups in the volume of the accessory basal nucleus ($F_{(1,8)} = 12.821$, $p = .007$, $\eta_p^2 = 0.616$), which survived a very stringent Bonferroni correction.

Given this finding, we estimated neuron numbers in that nucleus in order to determine whether the volume differences were driven by differences in neuron numbers. The number of neurons in the accessory basal nucleus was not significantly different between the two groups ($t_{(9)} = 1.816$, $p = .107$, $d = 1.148$; BI group: $M = 769,508$, $SD = 104,266$; control group: $M = 878,527$, $SD = 84,584$).

3.3 | Volumes of brain structures predict group membership

Unsurprisingly, a MANOVA performed on the volumes of rostral CA3, rostral CA2 and the accessory basal nucleus of the amygdala revealed statistically significant group differences ($\Lambda = 0.144$, $F_{(3,6)} = 11.908$, $p = .006$, $\eta_p^2 = 0.856$), since the univariate analyses of all three variables revealed differences between the BI and control animals. Given the significant MANOVA, we anticipated that a discriminant function analysis would likely be significant. However, we did not anticipate that a combination of those three variables would be able to perfectly classify all subjects. One function with an eigen-value of 5.954 accounted for 100% of the variance ($\Lambda = 0.144$, $\chi^2_{(3)} = 12.606$, $p = .006$), and classified 5 out of 5 subjects as belonging to the BI group and 5 out of 5 subjects as belonging to the control group.

As a means by which to understand the consistency of the findings across subjects, as suggested by the discriminant function analysis, we analyzed the relationships between the volumes of the three brain regions exhibiting differences between BI and control monkeys (Figure 1). The volume of rostral CA3 was inversely related to the volume of the accessory

basal nucleus ($R^2 = 0.67$; $F_{(1,8)} = 16.141$, $p = .004$). The volume of rostral CA2 was not related to the volume of the accessory basal nucleus ($R^2 = 0.25$; $F_{(1,8)} = 2.703$, $p = .139$), or the volume of rostral CA3 ($R^2 = 0.05$; $F_{(1,8)} = 0.426$, $p = .532$).

4 | DISCUSSION

This exploratory study aimed to provide quantitative data on the structural characteristics of the hippocampus and amygdala in behaviorally inhibited rhesus macaque monkeys. Compared to controls, BI monkeys had a larger volume of the rostral third of CA3 and a smaller volume of the rostral third of CA2, as well as a smaller volume of the accessory basal nucleus of the amygdala. Interestingly, the rostral third of CA2 contained fewer neurons in BI monkeys than in control monkeys. In addition, the volume of the rostral third of CA3 was inversely related to the volume of the accessory basal nucleus. No group differences in other regions of the hippocampus or nuclei of the amygdala were observed.

4.1 | Hippocampal differences between BI and control monkeys

The rostral third of CA3 was larger in BI monkeys than in control monkeys, but this volumetric difference was not related to a difference in neuron number. Other morphological characteristics, such as neuropil volume or number of glial cells, may account for this volumetric difference. This increase in volume of rostral CA3 may reflect an increased intrinsic or extrinsic connectivity, possibly linked to a more prominent activity in BI individuals. Indeed, as mentioned earlier, the rostral hippocampus of BI monkeys exhibits increased metabolic activity in response to threats (Oler et al., 2010). Furthermore, in rats facing a predator, behavioral inhibition correlates positively with CA3 activity (Qi et al., 2010). Since repeated activation of a brain structure can be associated with an increase in volume (May et al., 2007; Taubert et al., 2012), the increased volume of rostral CA3 could be related to increased activity in novel situations considered to be threatening.

The implication of CA3 in behavioral inhibition is not new, since this particular region of the hippocampus has been considered a central component of a “behavioral inhibition” system (Gray, 1982). Considering the unique contributions of CA3 to associating experiences (Rolls et al., 2005), rapid contextual learning (Nakashiba et al., 2008), and approach-avoidance behaviors (Schumacher et al., 2018), it would be of great interest to further specify the morphological changes that underlie the increased volume of rostral CA3 in BI individuals, as well as to understand how such morphological changes may contribute to the functional changes linked to behavioral inhibition.

While the rostral third of CA3 was larger, the rostral third of CA2 was smaller and had a smaller number of principal neurons in BI monkeys compared to controls. The difference in neuron number between BI and control monkeys is particularly intriguing, with respect to the heritability of behavioral inhibition. Indeed, the number of CA2 pyramidal neurons is already established by birth (Jabès et al., 2011), suggesting that BI individuals may present a genetic predisposition to have fewer neurons in rostral CA2. Interestingly, two studies showed that glucose metabolism of the rostral hippocampus is not only predictive of an anxious temperament, but also heritable (Fox et al., 2015; Oler et al., 2010). Considering the CA2-driven feedforward inhibition of CA3 (Boehringer et al., 2017), a lower number

of neurons in CA2 might contribute to higher activity levels in the rostral hippocampus (and possibly its expansion), and constitute one of the heritable characteristics underlying behavioral inhibition.

On a functional level, structural alterations of CA2 in BI individuals may have important implications for the way they process and react to novel social stimuli. Indeed, recent studies have begun to unravel some of the unique properties and functions of CA2, including its roles in social memory (Hitti & Siegelbaum, 2014; Stevenson & Caldwell, 2014), aggressive behaviors (Leroy et al., 2018; Pagani et al., 2015), novelty detection (Wintzer et al., 2014), and in the regulation of hippocampal excitability (Boehringer et al., 2017). In the context of behavioral inhibition, impaired CA2 function may contribute to blunting the habituation process when facing a novel social situation, as well as the recognition of a familiar one. Indeed, it has been shown that impairing CA2 function suppresses social habituation and recognition in rodents (Hitti & Siegelbaum, 2014; Stevenson & Caldwell, 2014). Accordingly, the hippocampus of BI individuals exhibits sustained activity (i.e., slow habituation) to novel faces (Blackford et al., 2013), indicating a reduced ability to familiarize one-self to new people. A slow hippocampal habituation has also been linked to higher levels of social fearfulness (Avery & Blackford, 2016). Moreover, socially inhibited individuals exhibit less improvement than controls in the recognition of faces after repeated exposures, indicating an impairment in face learning (Avery et al., 2016). Future studies are needed to determine whether the structural abnormalities we observed in rostral CA2 are indeed a heritable characteristic and whether CA2 dysfunction may be directly related to increased hippocampal activity and the slow social habituation and recognition in BI individuals.

4.2 | Smaller accessory basal nucleus of the amygdala in BI monkeys

While the amygdala has been broadly implicated in behavioral inhibition (Clauss, Seay, et al., 2014; Hill et al., 2010), findings from existing structural or functional studies of the amygdala in BI individuals did not lead to clear hypotheses about differences in specific nuclei. We found that BI monkeys had a smaller accessory basal nucleus of the amygdala than control monkeys. This smaller volume was not accompanied by a lower number of neurons. This suggests that other structural changes, such as neuropil volume (contributed to by dendritic length and the number of synaptic connections) or number of glial cells, might underlie this volumetric difference. Although little is known about the specific function(s) of the accessory basal nucleus, as most functional studies have generally considered the more broadly defined basolateral complex, its connectivity with other amygdala nuclei and other brain structures puts it in a prime position to modulate contextual fear responses (Amaral et al., 2003). Its substantial connectivity with the prefrontal cortex, cingulate, insula and hippocampal formation makes it an important hub for the regulation of amygdala activity (Amaral et al., 1992; Petrovich et al., 1996; Pitkänen et al., 2000, 2002). Through its excitatory projections to the central and medial nuclei (Pitkänen et al., 1997), the accessory basal nucleus may also modulate amygdala output toward the periaqueductal gray and hypothalamus that in turn trigger endocrine, autonomic and defensive responses.

Recent studies on resting-state brain functional connectivity in humans have shown that BI young adults exhibit reduced amygdala connectivity with prefrontal cortices (Blackford et al., 2014; Roy et al., 2014). More specifically, the basolateral amygdala of BI individuals exhibits a decreased functional connectivity with the ventromedial and dorsolateral prefrontal cortices (Blackford et al., 2014). A smaller accessory basal nucleus observed in our BI monkeys may similarly reflect a decrease of amygdala connectivity with the prefrontal cortex, and especially with the medial prefrontal cortex (mPFC) with which it shares substantial connections (Amaral et al., 1992). Consequently, a decreased connectivity between the mPFC and the accessory basal nucleus may lead to more inhibited behaviors. Future studies are needed to confirm a causal functional disconnection between the mPFC and the accessory basal nucleus in BI individuals, and also investigate other potential changes in the functional connectivity of this nucleus, for instance with the striatum and cingulate cortex (Blackford et al., 2014; Roy et al., 2014; Taber-Thomas et al., 2016).

4.3 | A smaller accessory basal nucleus is linked to a larger rostral CA3

Based on the previous discussions on the accessory basal nucleus and rostral CA3, it is possible that a smaller accessory basal nucleus may be associated with a more active amygdala, which may, in turn, impact the activity and structure of rostral CA3. Indeed, CA3 receives its major amygdala inputs from the basal nucleus, but also from the accessory basal nucleus either directly or via the entorhinal cortex (Amaral et al., 1992; Pitkänen et al., 2000, 2002). Interestingly, persistent excitatory projections from the amygdala to the rostral hippocampus may contribute to maintaining inhibited behaviors. Indeed, studies in rodents have demonstrated that activation of the basolateral projections to the ventral hippocampus decreases exploration and social behaviors, whereas inhibition of those projections increases exploration and social behaviors (Felix-Ortiz et al., 2013; Felix-Ortiz & Tye, 2014).

Furthermore, an increased activity of excitatory projections from the basolateral amygdala to the rostral hippocampus might contribute to the vulnerability to develop anxiety disorders. We already mentioned that amygdala-hippocampus interactions support emotional memory encoding, including fear-related memories. Contextual fear conditioning requires precisely an interaction between those two structures (Canteras et al., 2009; LeDoux, 2000). Interestingly, in rats, electrical stimulation of the basomedial and basolateral nuclei of the amygdala facilitates the induction of long-term potentiation in the dentate gyrus (Ikegaya et al., 1996), suggesting that amygdala projections enhance memory encoding in the hippocampus. Considering the role of the amygdala in threat detection (Amaral et al., 2003), its capacity to facilitate the acquisition of fear memories is essential to form memories more effectively in dangerous situations. However, for BI individuals exhibiting an overactive amygdala even in secure environments, too much excitatory input to the hippocampus could make them more prone to fear conditioning. Consistent with this idea, recent studies have shown that BI individuals are faster than controls at associative learning tasks, such as the conditioned eyeblink response (Allen et al., 2014; Caulfield et al., 2013; Myers et al., 2012).

4.4 | Consistency across animals, despite small sample sizes

A limitation of this study was the small number of animals in each experimental group. While this may be considered a small sample size for behavioral or imaging work in rodents

or humans, it is a fairly robust sample size for this type of quantitative neuroanatomical analyses performed in monkeys. It is critical to note, then, that the observed differences between BI and control monkeys were consistent across individuals, which suggests that the effects are biologically meaningful. In particular, in the discriminant function analysis, which uses the outcome variables to predict the groups and is not constrained to a linear combination of the variables, one function explained all of the variance and correctly classified all of the subjects into the correct group. This level of classification accuracy is not a foregone conclusion, even following a significant MANOVA, as we have seen for example when using social behavior to predict lesion group membership (Bliss-Moreau et al., 2013).

One additional consideration is that we considered BI as a between-subject factor, despite the fact that the behavioral measures used to categorize monkeys as BI or non-BI were continuous. While we hoped that an analysis of the variables used to categorize monkeys as behaviorally inhibited (activity and emotionality on Days 1 and 2 of the Biobehavioral Assessment) might be possible, the distribution of those variables and their low variance within each group precluded that possibility. A future direction for this work would be to perform quantitative neurobiological analyses of animals that vary more substantially along the activity/emotionality dimension rather than treating BI as a categorical variable. That would require a much larger sample than was available for this study and make the stereological study very difficult to carry out using nonhuman primates.

5 | CONCLUSION

This exploratory study is the first quantitative evaluation of the structure of the hippocampus and amygdala in a robust animal model of behavioral inhibition, the rhesus monkey. We found consistent differences between BI and control monkeys with respect to the volumes of the rostral hippocampal fields CA3 and CA2, and the accessory basal nucleus of the amygdala, as well as group differences in neuron numbers in rostral CA2. Based on these structural findings and what is currently known about the function(s) of these brain regions, one can make several hypotheses that will require further testing. First, a smaller accessory basal nucleus might reflect a lower inhibitory influence of the mPFC on amygdala circuits, leading to an overactive amygdala and more passive defensive behaviors. Second, an overactive amygdala may in turn increase neuronal activity in the rostral hippocampus and contribute to an increase in the volume of CA3. High levels of activity of the excitatory projections from the amygdala to the hippocampus would also promote more inhibited and less social behaviors. Finally, fewer neurons in rostral CA2 may reflect a heritable predisposition that affects both hippocampal activity and the way novel social stimuli are processed, which may contribute to impaired social learning in BI individuals. The findings from this exploratory study compel follow-up investigations with larger sample sizes and additional analyses to provide greater insight and more definitive answers regarding the neurobiological bases of behavioral inhibition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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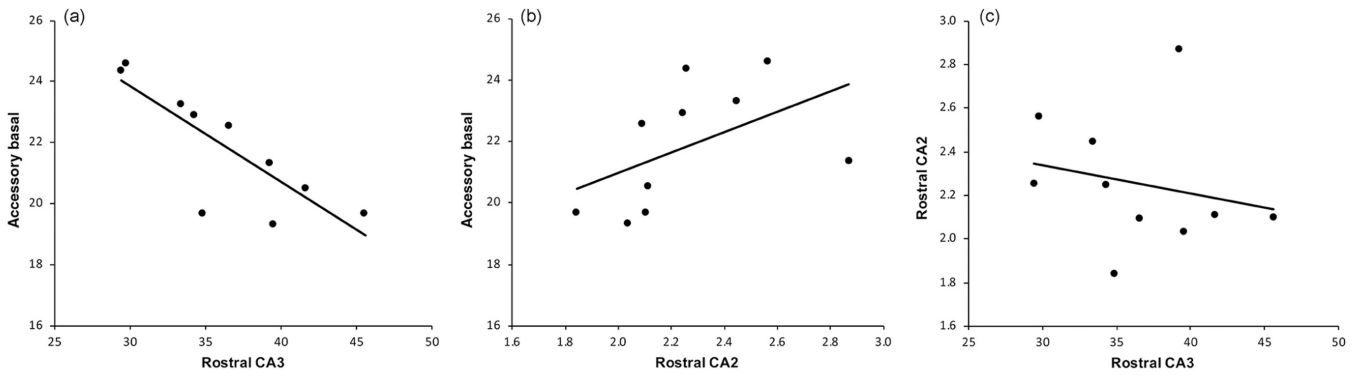


FIGURE 1.

Relationships between the volumes (mm^3) of the accessory basal nucleus, rostral CA3, and rostral CA2. (a) Accessory basal nucleus and rostral CA3 ($\text{CA3} = -2.126 \times \text{AB} + 82.85$). (b) Accessory basal nucleus and rostral CA2 ($\text{CA2} = 0.076 \times \text{AB} + 0.609$). (c) Rostral CA2 and rostral CA3 ($\text{CA2} = -0.013 \times \text{CA3} + 2.731$). See main text for details

TABLE 1

Volume (in mm³; mean \pm *SD*) of distinct hippocampal regions in behaviorally inhibited (BI) and control monkeys

Volume	BI	Control
<i>Rostral</i>		
Dentate gyrus	39.66 \pm 6.18	32.99 \pm 6.59
CA3	39.67 \pm 4.23	33.23 \pm 4.00
CA2	2.04 \pm 0.11	2.48 \pm 0.26
CA1	25.61 \pm 1.83	25.14 \pm 1.40
Subiculum	12.79 \pm 0.56	11.47 \pm 1.73
<i>Intermediate</i>		
Dentate gyrus	20.85 \pm 3.93	18.36 \pm 3.94
CA3	13.19 \pm 1.42	10.78 \pm 2.47
CA2	2.27 \pm 0.13	2.00 \pm 0.46
CA1	25.90 \pm 4.32	23.73 \pm 2.57
Subiculum	9.26 \pm 1.89	9.16 \pm 1.95
<i>Caudal</i>		
Dentate gyrus	13.11 \pm 1.77	11.96 \pm 3.57
CA3	8.73 \pm 0.83	8.07 \pm 2.14
CA2	1.80 \pm 0.26	1.86 \pm 0.23
CA1	22.88 \pm 2.60	23.28 \pm 2.72
Subiculum	9.83 \pm 1.62	8.68 \pm 2.25

TABLE 2

Volume (in mm³; mean \pm *SD*) of the main amygdala nuclei in behaviorally inhibited (BI) and control monkeys

Volume	BI	Control
Lateral	31.41 \pm 4.99	33.32 \pm 4.95
Basal	38.42 \pm 7.48	39.49 \pm 5.83
Paralaminar	4.33 \pm 0.86	4.60 \pm 0.74
Accessory basal	20.35 \pm 1.31	23.30 \pm 1.30
Medial	4.76 \pm 0.50	4.83 \pm 0.58
Central	4.68 \pm 0.82	4.70 \pm 0.70
Other nuclei	65.79 \pm 5.92	68.61 \pm 3.70

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