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Delayed Effects of Early Stress on Hippocampal Development

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Early maternal separation has been shown in animal models to produce enduring morphological changes in the hippocampus and other brain structures, which may not become evident until adulthood. Postnatally, the trajectory of overproduction and pruning of axons, dendrites, synapses and receptors shapes the brain between puberty and adulthood. The objective of the study was to ascertain whether this normal trajectory was affected by repeated maternal separation. Rat pups were separated from their mother for 4 h a day between postnatal days 2 and 20 (ISO group), and compared to rat pups that remained with their mother in the animal facilities (AFR group) and were exposed to minimal handling. Immunoreactivity to synaptophysin was quantified in the hippocampus CA1 and CA3, amygdala, and prefrontal cortex using optical densitometry (OD) at 25, 40, 60, 80, and 100 days in male and female rats. Synaptophysin OD increased dramatically in CA1 and CA3 between 25 and 60 days in the AFR group and fell by the same degree between 60 and 100 days, showing the expected sequence of overproduction and pruning. No difference between groups in synaptophysin OD was observed at 25 and 40 days. However, at day 60 synaptophysin was 34–36% lower in CA1 and CA3 of the ISO group, and remained 24–26% lower at 100 days. Early isolation produced no enduring reduction in synaptophysin OD in the amygdala or prefrontal cortex. Overall, these results suggest that early maternal separation produced a regionally specific delayed effect on the structure of the hippocampus by attenuating rates of synaptic development.
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INTRODUCTION

Early stress has been shown in animal models to produce enduring morphological changes in the hippocampus and other brain structures (McEwen, 2000b). Childhood abuse has been associated with reduction in hippocampal volume in adults (Bremner *et al*, 1997; Driessen *et al*, 2000; Stein *et al*, 1997; Vythilingam *et al*, 2002), but not children (De Bellis *et al*, 2001, 1999; Carrion *et al*, 2001). Can early maternal separation produce delayed effects on brain morphology by altering normal developmental trajectories? Prior to puberty, there is a marked overproduction of axons, dendrites, synapses, and receptors (Rakic, 1991). This is followed by a period of rapid pruning and elimination between puberty and adulthood. Up to 50% of synapses and receptors are lost in both cortical (Andersen *et al*, 2000; Huttenlocher, 1979; Lidow *et al*, 1991) and subcortical regions (Seeman *et al*, 1987; Teicher *et al*, 1995). The time course and degree of pruning, however, varies between regions (Andersen *et al*, 2000; Huttenlocher, 1979; Teicher *et al*, 1995). Virtually nothing is known about the effects of maternal separation on this process.

The hippocampus is a brain region that appears to be *especially* vulnerable to the effects of stress. This region has protracted development (Benes *et al*, 1994; Giedd *et al*, 1996), and also has a high density of glucocorticoid receptors in rats. Early exposure to stress or corticosteroids can cause hippocampal remodeling (or atrophy (Sapolsky, 2000)), and is associated with decreases in dendritic branching, vulnerability to subsequent insult, and neurogenesis (Gould *et al*, 2000). High levels of glucocorticoids can be directly neurotoxic to hippocampal pyramidal cells (Sapolsky *et al*, 1991, 1990). Much less is known about the effects of early experience on the development of the prefrontal cortex and the amygdala. The prefrontal cortex has a very protracted ontogeny (Alexander and Goldman, 1978) and is specifically activated by stressors (Bannon and Roth, 1983; Deutch *et al*, 1991), and in primates may have a higher density of glucocorticoid receptors than the hippocampus (Sanchez *et al*, 2000). We have hypothesized that exposure to early maternal separation could affect prefrontal cortical development, possibly resulting in accelerated but attenuated development (Teicher *et al*, 1997). Finally, glucocorticoid receptors are present in the amygdala (Peiffer *et al*, 1991), and maternal separation produces enduring effects on the neurochemical structure of this region (Caldji *et al*, 1998; Barna *et al*, 2003; Ploj *et al*,

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2003a, b). The amygdala plays an integral role in stress responsiveness both directly via changes in the benzodiazepine system (Caldji *et al*, 1998) and indirectly via its corticotropin-releasing hormone (CRH) projections to the locus ceruleus. Changes in maternal care are already reported to increase the CRH mRNA in the amygdala (Barna *et al*, 2003). However, whether early maternal separation can program (or 'direct') synaptic overproduction and elimination during adolescence has never been examined.

The aim of the present study was to test the hypothesis that exposure to early maternal separation exerts enduring effects on brain structure by altering either the degree or time course of synaptic overproduction and pruning in the hippocampus, amygdala, and prefrontal cortex. In addition to their apparent sensitivity, these regions were also selected given their importance in memory, executive function, and emotional regulation.

MATERIALS AND METHODS

Subjects

Female multiparous Sprague–Dawley rats (250–275 g, Charles River Laboratories, Wilmington, MA) were bred at McLean Hospital. The day of birth was designated as postnatal day 0 (P0). At 2 days after birth, the litters were culled to 10 pups (five males and five females), and litters were randomly assigned to either an isolation/maternal separation (ISO Group) or animal facility reared group (AFR Group). Pups in the ISO Group were weighed every day and isolated for 4 h per day between P2 and 20, and kept at thermoneutral temperature. This procedure is similar to procedures used by other laboratories (Liu *et al*, 2000; Plotsky and Meaney, 1993). Pups in the AFR Group were not disturbed after day 2, except for routine weekly changes in cage bedding.

Rats were weaned on day 22–23, and group-housed in same-sex caging until the time of killing. Animals were sacrificed at P25, 40, 60, 80, and 100 ± 2 days, as females were always sacrificed in diestrus, which is consistent with our previous studies (Andersen, 2002; Andersen *et al*, 1997) and represents the phase when stress responsiveness may be at its highest point (Figueiredo *et al*, 2002). The selection of ages is based on stages corresponding to childhood, adolescence, young adulthood, and adulthood (Andersen, 2003). An average of $n = 5$–7 rats per condition and sex were used. Data were subsequently collapsed across sex as it did not significantly exert an effect on condition or age.

Perfusion, Histology, and Immunohistochemical Staining

At the appropriate age, subjects were transcardially perfused under sodium pentabarbital anesthesia with 4% paraformaldehyde (PFA), pH 7.4. The brains were removed and post-fixed in 4% PFA solution overnight, then cryoprotected in 20% sucrose in phosphate-buffered saline. Free-floating coronal sections (40 μm) were carefully matched for anatomical location to assure comparison of comparable regions of interest across conditions. Immunoreactivity to synaptophysin, a protein associated with synapses, was used as a our dependent measure (Glantz and Lewis, 1997). Slices were stained for synaptophysin (SVP38, 1 : 1000, Sigma Chemical, St Louis, MO), processed using the standard avidin–biotin technique, and visualized with DAB. Each staining 'run' contained all ages and conditions to account for inter-run variability. Moreover, data were blanked to synaptophysin staining in the corpus callosum within each subject.

Data Analysis

Densitometric analysis of SVP38 was carried out using module IV of the MCID System (Imaging Research Inc., Ontario, Canada) interfaced with a Leica DMRB light microscope, at $\times 5$ magnification, and a Sony CCD camera (model XC 77). For the hippocampal, amygdalar, and prefrontal cortex regions, a box of approximately 2000 pixels was used and placed in the center of the structure. A representative staining is shown in Figure 1. Three measurements were taken for each region, for each hemisphere, plus a background measure. Thus, data for each subject were represented by the average OD of the three sections corrected by the background.

Data were analyzed with ANOVA (SYSTAT, Evanston, IL) with condition, age, and sex as between-subject factors. Percent differences between the isolation-reared and animal facility-reared groups were presented as mean $\pm$ SEM, with the SEM corrected for the variability in both the experimental and control groups (McLean and Welch, 1971). Sex did not exert any significant effects within these analyses (p's > 0.1), therefore data were collapsed across this variable for all subsequent analyses.

RESULTS

Early maternal separation produced significant changes in expression of synaptophysin in the hippocampus and amygdala, but not the prefrontal cortex. Early maternal separation effects on synaptophysin OD in the hippocam-

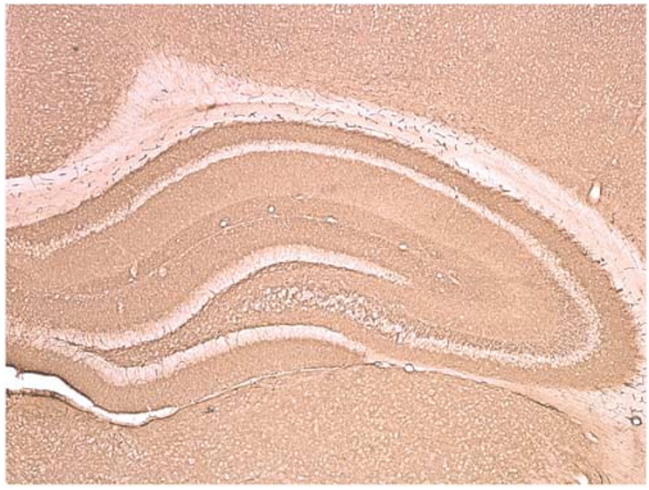

Figure 1 Photomicrograph of the hippocampus stained with synaptophysin (1 : 1000) and visualized with DAB. This image was taken at $\times 5$ magnification. Data measurements are based on a box of approximately 2000 pixels.

pus, but not the amygdala, persisted to the eldest age studied (P100).

Hippocampus

The hippocampus was subdivided into CA1 and CA3 subregions, with both regions demonstrating similar effects. A major effect of isolation stress was observed in CA1 ($F(1,135) = 7.75$, $p < 0.006$) and CA3 ($F(1,135) = 10.02$, $p < 0.002$). Overall synaptophysin was reduced by 17.8% in CA1 and 16.0% in CA3. As illustrated in Figure 2, the effect of isolation stress emerged at 60 days of age. There were no differences between groups at 25 days (CA1: $F(1,32) = 0.56$, $p > 0.4$; CA3: $F(1,32) = 1.03$, $p > 0.3$) or 40 days (CA1: $F(1,28) = 0.55$, $p > 0.4$; CA3: $F(1,28) = 0.19$, $p > 0.6$) of age. However, at day 60 synaptophysin immunoreactivity was reduced by 33.7% ($F(1,24) = 14.84$, $p = 0.0008$) in CA1 and by 35.8% ($F(1,24) = 14.88$, $p = 0.0008$) in CA3. This difference persisted at day 100, the oldest age evaluated (CA1: 26.4% reduction, $F(1,36) = 6.30$, $p = 0.02$; CA3: 24.3% reduction, $F(1,36) = 5.12$, $p = 0.03$).

Evidence for overproduction and pruning was apparent in the AFR group but not the ISO group. Synaptophysin immunoreactivity in CA1 increased by a significant $23.6 \pm 13.2\%$ between P25 and 60 ($t(30) = 2.14$, $p < 0.05$) before falling by $23.4 \pm 7.3\%$ by P100 ($t(34) = 2.51$, $p < 0.02$ in the AFR group. In contrast, the ISO group had a nonsignificant drop of $11.6 \pm 10.3\%$ ($t(26) = 1.12$, $p > 0.2$) between 25 and 60 days, and a further nonsignificant decline between 60 and 100 ($15.0 \pm 9.0\%$ reduction, $t(26) = 1.43$, $p = 0.2$).

Similarly, synaptophysin immunoreactivity in CA3 increased by $29.9 \pm 14.0\%$ between P25 and 60 ($t(30) = 2.63$, $p < 0.01$) and fell by $22.8 \pm 7.5\%$ between P60 and P100 ($t(34) = 2.40$, $p < 0.02$) in the AFR group. The ISO group showed a slight fall in synaptophysin immunoreactivity between 25 and 60 days ($7.1 \pm 12.2\%$, $t(26) = 0.61$, $p > 0.5$), and a further nonsignificant decline between 60 and 100 ($8.9 \pm 10.4\%$ reduction, $t(26) = 0.76$, $p > 0.4$).

Amygdala

Quantification of synaptophysin OD in the amygdala (Figure 3a) also revealed significant effects of isolation stress ($F(1,135) = 4.09$, $p < 0.05$), resulting in a modest $6.2 \pm 3.0\%$ reduction in OD. There was a major effect of age ($F(4,135) = 5.55$, $p = 0.004$), which was seen in both groups as a marked increase in synaptophysin after P25. Between 25 and 60 days, synaptophysin OD increased by $16.1 \pm 8.7\%$ ($t(30) = 2.12$, $p < 0.05$) in the AFR group, and

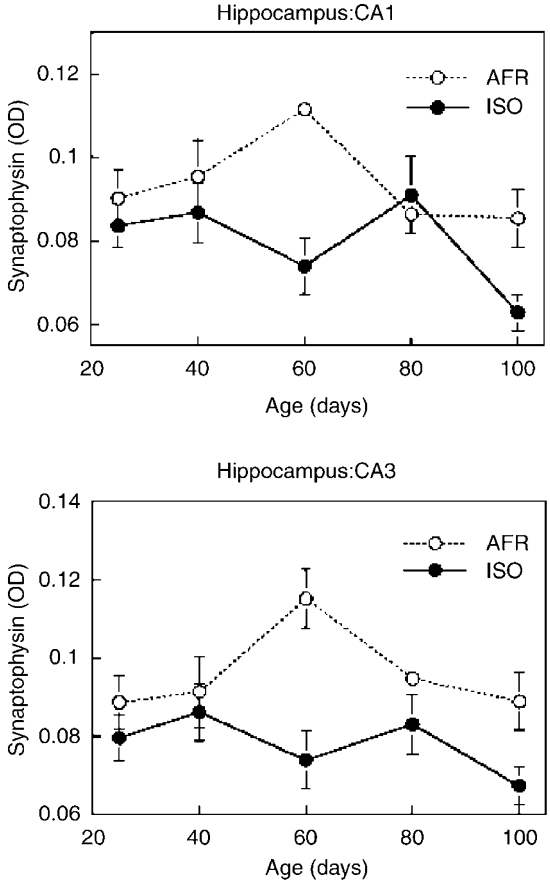

Figure 2 Region of interest analysis of synaptophysin immunolabeling of CA1 (top) and CA3 (lower) of the hippocampus in animal facility reared (AFR; open circles) or isolation (ISO; closed circles) animals across 25, 40, 60, 80, and 120 days of age. Mean ± SEM are presented; average $n = 10$–14 animals at each age.

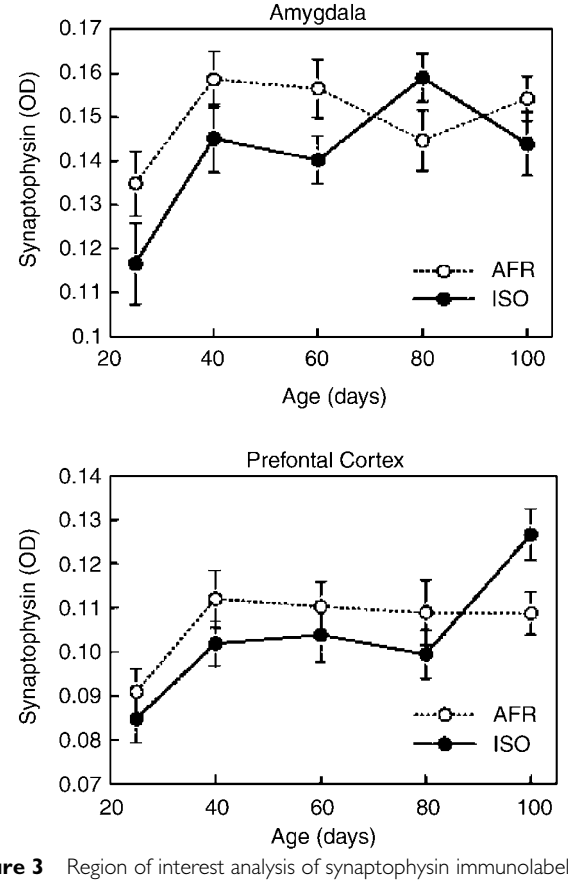

Figure 3 Region of interest analysis of synaptophysin immunolabeling of the amygdala (top) and the prefrontal cortex (lower) in animal facility reared (AFR; open circles) or isolation (ISO; closed circles) animals across 25, 40, 60, 80, and 120 days of age. Mean ± SEM are presented; average $n = 10$–14 animals at each age.

increased by 20.3 ± 12.0% ($t(26) = 2.01$, $p = 0.05$) in the ISO group. However, in contrast to the hippocampus, there was no decline between 60 and 100 days. Synaptophysin OD on day 100 was within 3% of day 60 values for the AFR groups ($t(34) = 0.27$, $p > 0.7$) and the ISO group ($t(26) = 0.38$, $p > 0.7$).

Prefrontal Cortex

Unlike the hippocampus and amygdala, there was no significant main effect of isolation stress ($F(1,122) = 0.59$, $p > 0.4$). There was, however, a major effect of age ($F(4,122) = 7.85$, $p = 0.00001$). Both groups had a marked rise in synaptophysin OD after P25. On average, OD was 21.7 ± 7.6% greater on day 60 than day 25. Between days 25 and 80, there was a trend for synaptophysin OD to be lower in the ISO group than the AFR group (7.6 ± 3.9% reduction, $t(108) = 1.80$, $p = 0.07$). However, at P100 synaptophysin OD was 16.1 ± 6.8% ($t(36) = 2.98$, $p = 0.005$) greater in the ISO animals than in the AFR group.

DISCUSSION

The results of this study suggest that early maternal separation had two main effects. First, early maternal separation reduced the overall synaptophysin levels in hippocampus relative to the AFR control groups. Second, and perhaps of greater interest, is the time course and nature of the effect. Early maternal separation appeared to prevent the normal overproduction of synapses in hippocampus, but not the amygdala or prefrontal cortex. This time-course difference has never been demonstrated previously, as other studies have examined only adult systems to illustrate the impact of stress (de Kloet *et al*, 1996; Lehmann *et al*, 2002; Matthews *et al*, 2001). Together, these data suggest that early maternal separation has protracted regionally specific effects that occur long after the stressor has been removed.

The finding of protracted isolation effects is in stark contrast to the effects in adult animals, where dendritic remodeling within the hippocampus reverses within 7–10 days after removal of 21 days restraint stress (McEwen, 2000a). The data presented in Figure 2 suggest that early life events set in motion a series of adverse events that lead to the progressive loss of hippocampal synapses rather than an immediate loss of density. Moreover, the effect of early maternal separation persisted for at least 80 days after cessation of the stressor.

These observations provide a possible explanation for the observation that childhood abuse has been found to be associated with reduced hippocampal volume in adults (Bremner *et al*, 1997; Driessen *et al*, 2000; Stein *et al*, 1997; Vythilingam *et al*, 2002), but not children (De Bellis *et al*, 2001, 1999; Carrion *et al*, 2001). Based on these findings, early stress-induced alterations in human hippocampal size should not be apparent until at least early adulthood.

While early maternal separation was associated with persistent decrease in synaptophysin OD in the hippocampus, this was not the case in either the amygdala or prefrontal cortex. Further, early maternal separation appeared to exert a dramatic suppressive effect on synaptic overproduction in the hippocampus, but did not appear to affect this process in the other brain regions. This suggests that there may be important regional differences in the factors that regulate the trajectories of synaptic development.

Interestingly, the effects of early maternal separation on synaptophysin immunoreactivity in the hippocampus may be the first evidence of any environmental influence on the normal developmental process of overproduction and pruning in a nonsensory system. We had previously reported that neither the postnatal removal of gonadal hormones (Andersen *et al*, 2002) nor chronic antagonism of NMDA receptors by MK-801 (Teicher *et al*, 2003) affected the overproduction and pruning of dopamine receptors in the striatum. This study measured synaptophysin, which may be under different regulatory control. It is unlikely that gonadal hormones play a significant role in decreased synaptophysin because no sex differences were observed. The role of glutamate, however, cannot be ruled out with certainty in this study. Glutamate mediates activity-dependent pruning of dendrites and synapses via NMDA receptor activation and calcium influx in a number of brain regions, including the sensorimotor cortex and the cortex (Kozlowski *et al*, 1997; Nelson *et al*, 1990; Rabacchi *et al*, 1992). Studies examining the influence of MK-801 administration during the overproduction and pruning phase in animals with a history of maternal separation are ongoing.

The opposite approach may also be taken: increased maternal interactions in the AFR group may offer some form of protection relative to those in the ISO group. Studies on maternal arched back nursing from Meaney's group strongly suggest that greater attention by the dam mediates enhanced resiliency in the pups later in life by programming the HPA system through the GABA (Caldji *et al*, 1998) and arginine–vasopressin systems (Anisman *et al*, 1998). Part of this resiliency may be mediated by the 'normal' overproduction and pruning of synapses during adolescence, which was observed in synaptophysin measures in the AFR group.

One limitation of the present study is the estimation of synaptic density using synaptophysin immunoreactivity quantified by optical densitometry. Electron microscopy has been used as a definitive technique to quantify synapse numbers or density, but this approach is costly, labor intensive, and technically difficult (Calhoun *et al*, 1996). Synaptophysin-based techniques have been used with increasing frequency to estimate synaptic density in clinical (Eastwood *et al*, 1995; Glantz and Lewis, 1997; Vawter *et al*, 2002) and preclinical studies (Mazer *et al*, 1997; Saito *et al*, 1994). While several studies have used optical density as a means of analyzing immunohistochemical preparations (Glantz and Lewis, 1997), great care needs to be taken to provide very consistent levels of staining across conditions and across analytical runs. We carefully matched samples and counterbalanced all ages and conditions between analytical runs to avoid potential confounding effects. Our methodology was sufficiently rigorous and robust to detect important developmental differences in synaptophysin OD in controls that were in accord with Golgi studies (Norrholm and Ouimet, 2000). Stereological assessment of synaptophysin-labeled presynaptic boutons may provide an even better estimate of synaptic density than optical

densitometry (Calhoun *et al*, 1996), and these findings should be verified using alternative quantitative techniques and additional markers.

Overall, these findings show that early isolation maternal separation exerts an enduring effect on hippocampal development, and suggest that the effect may be time-dependent and arises as a consequence of an arrested phase of synaptic overproduction. Enduring alterations in synapse formation in the hippocampus may provide a new understanding for the enhanced vulnerability of individuals with a history of childhood abuse to develop depression (Heim and Nemeroff, 2001; Putnam, 2003; Teicher, 2002; Wise *et al*, 2001) or post-traumatic stress disorder (Robin *et al*, 1997; Yehuda *et al*, 2001; Zaidi and Foy, 1994) later in life.

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