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Research report

Modulation of 5-HT release in the hippocampus of 30-day-old rats exposed in utero to protein malnutrition

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Abstract

Previous in vivo microdialysis studies have shown increased spontaneous release of 5-HT in the hippocampus of adult behaving rats exposed to prenatal protein malnutrition. Furthermore, behavioral studies have shown that adolescent rats (PD30) that have been prenatally protein malnourished demonstrate an increased sensitivity to the benzodiazepine chlordiazepoxide (CDP). Given this altered sensitivity to benzodiazepines in adolescent malnourished rats, the present study was designed to test the hypothesis that the increased release of 5-HT in the hippocampus is present in adolescent rats and that this release is modulated by CDP. An altered release of 5-HT at PD30 would suggest an early developmental change associated with prenatal malnutrition. PD30 rats were implanted with microdialysis probes into the dorsal hippocampus and 5-HT release was monitored before and after administration of CDP. As previously reported in adult rats, release of 5-HT was significantly elevated in the dorsal hippocampus of PD30 rats as compared to well-nourished 30-day-old controls. Administration of CDP did not affect the release of 5-HT from the hippocampal formation of well-nourished rats but significantly decreased the elevated release of 5-HT in the malnourished rats. Following CDP, 5-HT release in the malnourished rats was at the same levels as release in well-nourished animals. Benzodiazepines have been reported to decrease extracellular 5-HT in stressed rats but not in unstressed rats. Thus, the elevated 5-HT release in the hippocampus in rats exposed to prenatal protein malnutrition may be associated with an increased response to stress. These data support other data that prenatal protein malnutrition alters the response to stressful stimuli possibly through changes in the GABAergic and/or serotonergic systems.

Theme: Development and regeneration

Topic: Nutritional and prenatal factors

Keywords: Serotonin; In vivo microdialysis; Chlordiazepoxide

1. Introduction

We have previously investigated the raphé-hippocampal 5-HT system in normal and prenatally malnourished, behaving adult rats [20,22,26]. Previous studies have shown increased basal levels of 5-HT in the hippocampus of malnourished rats [4,20] with an enhanced response in 5-HT release following electrical stimulation of the median raphé nucleus [20]. The etiology of this increase in malnourished animals is still unknown.

One function of the serotonergic forebrain system is as an integral part of the stress response [7,8,14,36]. Forebrain release of serotonin increases in response to acute stressors, whereas extracellular levels of 5-HT decline in chronic stress [1,13,17,29]. Forebrain serotonin levels are also increased in anxiety, which may be a behavioral component of the stress response. Benzodiazepines such as chlordiazepoxide (CDP) are anti-anxiety drugs that modulate GABA effects in the CNS. CDP and other benzodiazepines decrease the release of 5-HT in brains of stressed animals. Tonkiss et al. [34] have shown that 30-day-old malnourished rats show a decreased response to the amnestic effects of chlordiazepoxide (CDP) in the Morris Water Maze. This suggests that the sensitivity of

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malnourished animals to benzodiazepines is decreased, which may relate to alterations in $GABA_A$ receptors in the hippocampus, an area of the brain involved in learning and memory [18,37].

In the present study we sought to examine if basal release of 5-HT in the hippocampal formation is increased in the behaving juvenile rat at age 30 days, and if 5-HT release in the hippocampal formation is modulated by CDP. To assess this we have used in vivo microdialysis coupled with HPLC to determine the release of 5-HT from the dorsal hippocampus of behaving, 30-day-old rats prior to and following administration of CDP.

2. Materials and methods

2.1. Subjects and diet

Male Sprague–Dawley rats were exposed to a diet consisting of either 25% casein (well-nourished) or 6% casein (malnourished) in utero as described in Morgane et al. [24,25]. Briefly, female Sprague–Dawley rats were fed a diet of either 6% casein or 25% casein 3 weeks prior to mating and throughout pregnancy. At birth animals were cross-fostered to well-nourished (25% casein) dams. At 21 days of age (p21) rats were weaned from the dams and housed with same-sex littermates. Animals were maintained with ad lib 25% rat chow and water. The vivarium was maintained on a reversed light/dark cycle (lights on 19:00 h, lights off 07:00 h). Only one animal from any litter was used in the present study.

2.2. Stereotaxic surgery

Twenty-nine-day-old rat pups were anesthetized with pentobarbital (50 mg/kg ip). Guide cannulae (CMA 12, CMA/Microdialysis AB, Acton, Mass.) were implanted into dorsal hippocampus using the coordinates from Sherwood and Timiras [30] for 21- and 39-day-old pups. Initial surgeries were done using two rat pups to determine the proper co-ordinates of AP −3.3; L +2.3 and DV −1.4 from bregma. Similar coordinates were used by Bronzino et al. [6] in 30-day-old rat pups. Skullcaps were made first by applying dental acrylic to the exposed skull in the area next to where the probe would be placed. The skullcap was removed after it had dried and then was reattached to the skull with cyanoacrylate glue. The hole for the guide cannula was then drilled, the guide cannula lowered into place and more dental acrylic added to complete the skullcap. This skullcap was successfully in place for the day of the experiment in approximately 75% of animals.

2.3. In vivo microdialysis

During surgery, a 2 mm CMA 12 probe (CMA/Microdialysis AB, Acton, MA) was slowly lowered through the guide cannula into the dorsal hippocampus over a 3 min period. Overnight the probe was perfused with artificial cerebrospinal fluid (artCSF) at a rate of 0.1 μl/min. The artCSF consisted of 147 mM NaCl, 1.26 mM $CaCl_2$, 2.5 mM KCl, 1.18 mM MgCl in sterile water. ArtCSF contained 1 mM sertraline (Pfizer Pharmaceuticals, Groton, CT), a selective 5-HT reuptake inhibitor, to stabilize levels of extracellular 5-HT. The animal was placed in a large Plexiglas bowl with a collar attached by a guide wire to a suspension arm. ArtCSF was perfused through the probe using a CMA/Microdialysis Syringe pump and a 1.0 ml syringe. The microdialysis bowl was placed in a sound attenuating chamber with a fan running continuously for air exchange and as a source of white noise. The tubing from the microdialysis probe was run through a liquid swivel and collected externally to the chamber using a fraction collector. The awake animal system, syringe pump and fraction collector were purchased from CMA/Microdialysis (N. Chelmsford, MA).

The morning following implantation of the probe, the perfusion rate was increased to 1 μl/min and basal samples were collected every 20 min for 3 h. All experiments were started at 08:00–08:30 h with lights off at 07:00 h. Saline (0.9%, 5 ml/kg, i.p.) was then administered and three additional 20-min samples were collected. CDP (5.6 mg/kg. i.p., Sigma, St. Louis, MO) was administered and samples were collected for an additional 3 h.

2.4. Analysis of 5-HT

Samples were analyzed immediately by high-performance liquid chromatography (HPLC) with electrochemical detection (HPLC–ECD). The system was a ESA Choulochem II using a 3 μM 4.6×100 mm C_{18} column (Microsorb MV, Varian, Walnut Creek, CA). The mobile phase consisted of 0.1 M disodium phosphate, 15% acetonitrile, 10 mM EDTA, and 1.5 mM octanesulfonic acid at a pH of 5.4. This allowed for the measurement of 5-HT with a sensitivity of 0.5 fmol/20 μl sample. The area under the curve for samples was compared using computer software Chromperfect (Justice Laboratory Software, Palo Alto, CA) with a regression analysis of AUC for three authentic standards (10^{-9}, 5×10^{-10}, 10^{-10} M) injected onto the column at the beginning of each experimental day to determine the levels of 5-HT. 5-HT was verified by examining the voltammogram for standards against that determined using microdialysis samples.

2.5. Histology

At the completion of each study, the animal was perfused transcardially with formalin under pentobarbital anesthesia and the brain removed. The brain was sectioned and Nissl stained for verification of the probe placement (Neuroscience Associates, Knoxville, TN).

3. Results

Probes were inserted through the guide cannula into the dorsal hippocampus on the day prior to the experiment and were perfused at a rate of 0.1 μl/min overnight. Stable release of 5-HT was achieved within 1 h of increasing the perfusion rate of the probe. At the time that these basal levels were achieved, all animals were in a quiet-waking state characterized by lying on their side in the cage with their eyes open, but without activity. We have shown in conscious animals in a quiet-waking state, 3 h after probe insertion, that 5-HT release in the dorsal hippocampus is decreased by addition of the Na^+-channel blocker tetrodotoxin TTX and by deletion of Ca^{2+} from the CSF producing declines of 5-HT of 75 and 65%, respectively [22]. All probes were placed selectively within the dorsal hippocampal formation as shown in Fig. 1.

Basal release of 5-HT in the hippocampus of 30-day-old rats was significantly elevated in animals exposed prenatally to protein malnutrition (Fig. 2, $t(22)=2.8369$, $P<0.05$). Administration of CDP reduced levels of 5-HT in the hippocampal formation of malnourished rats whereas 5-HT levels in the hippocampus of well-nourished rats were unaffected by CDP (Fig. 3). There was a significant effect of diet on the release of 5-HT from the hippocampal formation (One-way ANOVA, $F(1,109)=8.129$, $P<0.05$). There was no effect of time ($F(9,109)=0.426$, $P=0.91$) or an interaction between factors (diet×time, $F(9,109)=0.995$, $P=0.45$). This indicates that the differences in groups are attributed to diet but because the release was not different between groups for most of the experiment, i.e. after CDP, the differences were minimal for time and the interactions between groups.

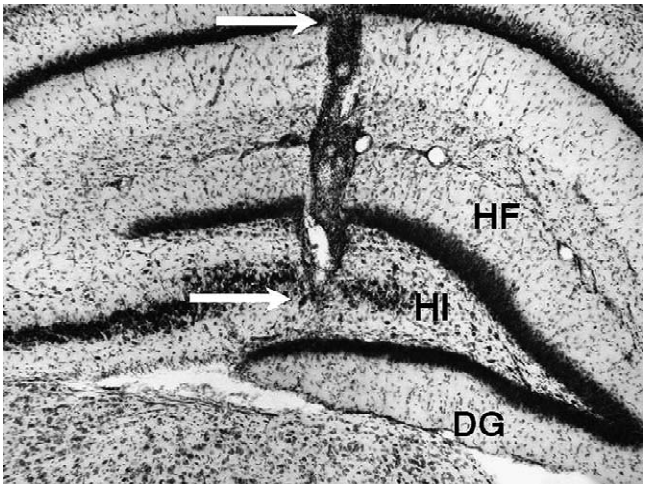

Fig. 1. Histological frontal section through the dorsal hippocampal formation (HF) showing track of microdialysis probe with tip centered in hilus (HI) of dentate gyrus (DG). The extent of the active membrane region of the microdialysis probe (2 mm) is indicated by black arrows. The active membrane extended through stratum oriens, the stratum pyramidal of the CA1 area of the hippocampal formation, stratum radiatum, molecular layer and the tip placed into the hilus of the dentate gyrus.

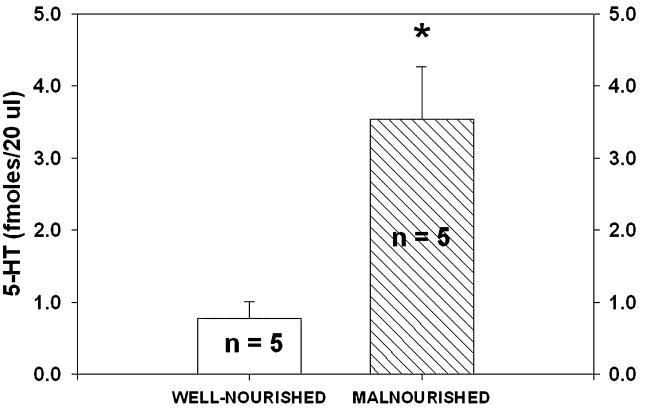

Fig. 2. Basal release of 5-HT in the dorsal hippocampal formation of 30-day-old rats exposed to prenatal protein malnutrition (malnourished, $n=5$) or well-nourished controls (well-nourished, $n=5$). Basal release was significantly increased in malnourished animals as compared to controls. * Significantly different from control group, t-test, $P<0.05$.

4. Discussion

As reported by our laboratory in previous studies of adult animals [20], basal release of 5-HT in the hippocampus of 30-day-old rats was significantly elevated in animals exposed prenatally to protein malnutrition (Fig. 2). It should be noted that release in this context would also include changes in reuptake and metabolic changes as well as synaptic release of 5-HT. Increased levels of 5-HT in young animals previously exposed to prenatal malnutrition

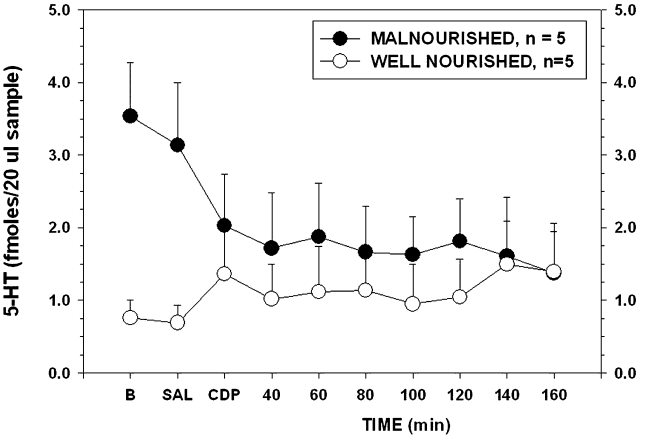

Fig. 3. 5-HT release from the dorsal hippocampus of 30-day-old prenatally protein malnourished animals (malnourished, $n=5$) or 30-day-old well-nourished controls (well-nourished, $n=5$). B, baseline determinations over 1 h period (three samples); SAL, three 20 min samples collected following the injection of 1 ml/kg saline, i.p.; CDP, 5.6 mg/kg chlordiazepoxide was injected i.p. at the beginning of this 20 min collection period. There was a significant difference between malnourished rats and well-nourished controls ($P<0.05$).

have been reported by other groups using the same model [9,11,16,19,27,31–33].

Resnick and Morgane [27] showed that throughout development from days PD 0 through 30, serotonin levels in the brain are elevated in malnourished animals. Chen et al. [10] further showed that potassium stimulated release of 5-HT from hippocampal slices of malnourished animals was increased compared to control. Of interest is the finding that basal release of 5-HT in hippocampal slices was not different in malnourished animals, suggesting that increased release of 5-HT in the conscious animal is the result of changes in the more widely distributed serotonergic system of the brain and is not inherent to the circuitry of the isolated hippocampal slice. Mokler et al. [21] have shown that the GABAergic responses to 5-HT in the hippocampal slice are not altered by prenatal protein malnutrition. Bath application of 5-HT in the hippocampal slice produces a 5-HT_3 mediated increase in GABA release from interneurons. This excitatory effect of 5-HT is not altered in malnourished animals. Thus, an increased release of 5-HT in the hippocampus of prenatally malnourished animals would lead to an enhanced inhibition of the principal cells and, hence, enhanced inhibition in the trisynaptic circuit, a finding suggested by the experiments of Bronzino et al. [5,6].

In contrast with the findings of enhanced 5-HT release in the hippocampal formation are the findings of Blatt et al. [4] showing that malnourished animals have decreased 5-HT fibers in the hippocampus, decreased 5-HT reuptake sites and decreased 5-HT_{1A} receptors, suggesting that there is decreased serotonergic innervation of the hippocampus in malnourished animals. These combined alterations of the serotonergic innervation of the forebrain suggest early and extensive changes in the serotonergic systems of the brain following malnutrition. Recent work, however, has shown that the birth dates of 5-HT neurons in the midbrain raphé nuclei are unchanged by prenatal protein malnutrition [15]. Thus, there may be alterations in the growth and maturation of the serotonergic system. Diaz-Cintra et al. [12], however, have shown that at 30, 90 and 220 days of age, malnourished rats have increased dendritic branching and dendritic spines in only the multipolar cells in the dorsal raphé nucleus. Further research is necessary to determine the factors that are involved in these changes.

Administration of CDP reduced the release of 5-HT in the hippocampal formation of malnourished rats whereas 5-HT release in the hippocampus of well-nourished rats was unaffected by CDP (Fig. 3). These data are in agreement with those from microdialysis studies that show that benzodiazepines reduce elevated release of 5-HT in brains of rats during stress but not during periods of non-stress [38]. 5-HT release in the ventral hippocampus was monitored by in vivo microdialysis during exposure to an anxiety-provoking environment, the elevated plus maze. 5-HT levels were not affected by diazepam prior to exposure to the plus maze. Exposure to the plus maze elevated extracellular 5-HT, which was reduced to control values after administration of diazepam [38]. Similarly, Rex et al. [28] have reported that 5-HT release, as determined by microdialysis, is increased in the frontal cortex of guinea pigs exposed to the elevated plus maze, and that this increase in 5-HT is reduced by diazepam. Thus, benzodiazepines reduce extracellular levels of 5-HT in stressed animals.

Malnourished rats have been shown to have a diminished behavioral response to the effects of CDP in the Morris Water Maze [34]. Furthermore, rats exposed to prenatal malnutrition show a decrease in anxiety and an increase in impulsiveness as determined using an elevated T maze test [2,3]. In a study examining the reactivity of malnourished rats to chronic unpredictable stress, malnourished animals show a decreased response to the stressors [35]. Finally, PD11 but not PD7 rat pups exposed to prenatal malnutrition show a enhanced sensitivity to CDP in the suppression of ultrasonic vocalizations (J. Tonkiss, personal communication). Thus, behavioral data presents a profile of animals that are less stressed, less anxious and less reactive to stress, in contrast with the present neurochemical data that suggests that malnourished animals are more reactive to stressful situations.

Other alterations in the function of the hippocampal formation have been shown in animals exposed to prenatal malnutrition. Bronzino et al. [6] showed that malnourished animals, using this same paradigm of malnutrition, show a significant impairment of long-term potentiation in the hippocampal formation.

In the present study, the effects on 5-HT release in the hippocampal formation are similar to findings of 5-HT release in stressed animals [7,8,14,36]. Well-nourished rats show a release of 5-HT that is unaffected by the administration of CDP. In contrast, malnourished rats show an enhanced release of 5-HT in the hippocampal formation that is decreased to the levels seen in well-nourished rats after CDP. This supports our view that malnourished rats are stressed. A number of factors may be involved in this effect. The surgery was performed 18 h prior to the beginning of the microdialysis experiment. Animals were initially housed with littermates and following surgery were placed alone in a microdialysis chamber. The microdialysis room was quiet and the chambers were in sound-attenuating boxes. The animals were not handled following the surgery and the vials were collected remotely (outside of the boxes). If the elevated levels of 5-HT in the malnourished animal were due to stress, this would suggest that the malnourished animal is more sensitive to stress given that the well-nourished controls do not appear to be stressed, as shown by 5-HT levels in the hippocampus. Our previous studies have also shown elevated basal release of 5-HT in the hippocampal formation and the median raphé nucleus [20,23]. In these experiments, animals were not exposed to stressful environments again suggesting that the malnourished animal is more easily stressed.

The present experiments and previous experiments have shown that malnourished rats respond to stress differently than well-nourished animals depending upon the measurement used to evaluate the stress. In some stressful situations, including the present experiment, malnourished animals react with an enhanced response to stress, whereas in other experiments malnourished animals demonstrate a decreased response to stress. These differences need to be more thoroughly explored to determine how the stress response is altered and to understand the underlying mechanisms of these responses in the malnourished animal.

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