Possible alteration of catecholaminergic transporters in specific brain areas of iron deficit rats

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ABSTRACT

Background: In humans, early ID (iron deficiency) may cause impairment of dopamine (DA) metabolism including DA clearance, transporter density, and dopamine receptor (D1 and D2) densities. Purpose: The present study aims to examine the effects of early ID on the catecholaminergic system within certain brain areas related to attention. Methods: Sprague-Dawley rats were divided into 2 groups; control (CN) fed a diet containing 80 ppm Fe and the iron deficient (ID) fed a diet containing 4 ppm Fe. At the end of study rats were sacrificed and brains were dissected. Catecholaminergic neurotransmitters were estimated in specific brain areas using radioactive ligand techniques. Results: Our results revealed a significant effect of age on DAT levels in the nucleus accumbens (NA), olfactory tubercle (OT), and substantia nigra (SN) but not in the striatum. Specifically, 21-day-old rats had greater DAT levels compared to 45-day-old rats when in the NA, OT, and SN as well as in the OT compared to 75-day-old rats. Additionally, there is a significant age difference on NET levels in the dentate gyrus but not in the frontal cortex or the locus coeruleus. Specifically, NET levels were increased among 45-day-old rats compared to 75-day-old rats. Conclusions: There is a significant age effect on DAT and NET levels in some examined brain areas. These findings are very important as they elucidate the impact of iron deficiency on catecholaminergic systems in the brain. This may explain most of the neurobehavioral sequelae of infantile iron deficiency.

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Introduction

Iron deficiency (ID) is the most prevalent single nutritional disorder worldwide1,2 and is associated with increased risk of delayed mental and motor development.3,4 Iron is necessary for proper myelination, optimal metabolic activity and acts as a co-enzyme for monoamine neurotransmitter synthesis5-7 as well as normal neurotransmitter synthesis and regulation in rats8-10,13-14 and mice.15 Consequently, early life (during infancy) ID is believed to be linked to impaired cognition, altered thermoregulation, neurodegenerative diseases16-19 including Parkinson’s disease,20 restless leg syndrome,21 as well as impaired physical growth.22-23 The particular effects on behavior including cognition may not be totally normalized by iron supplementation.16,24-26

Beard et al.27 reported that ID rats showed more anxiety-like behavior, reduced exploration and a slower habituation rate in a new environment as compared to controls. These behavioral changes accompany changes in dopamine metabolism within the ID rat brain.9 Furthermore, neonatal iron deficiency down-regulates nigrostrial and mesolimbic dopamine receptors.8 This is in accordance with other scientific reports that illustrate the effects of early ID on central dopamine receptor and DA transporter (DAT) densities in rats8,13-14,29-31 and in mice.32-34

Evidence from human imaging studies show that striatal DA neurotransmission is crucial for task performance that requires inhibitory control e.g. card sorting.28 Similar observations in animals showed that striatal DA transmission is essential for any flexible shifting of response.28-32 Additionally, it has been reported that the fronto-striatal system is critical for executive control.38-39 From the neurochemical point of view, it is evident that catecholamines (DA, NE) play a critical role in modulating the prefrontal cognitive function.40 Further, DA depletion in marmosets impairs their ability to maintain attention to one of the perceptual dimension.41 Therefore, the current study examined DA and NE transporter densities within the rat brain at different age periods viz; 21, 45 and 75 days old using radioactive ligand binding.

Methods

Subjects and dietary treatment

The Sprague-Dawley rat breeding stocks were obtained from Harlan Laboratory (Indianapolis, IN). Female breeders were divided into 2 groups; control (CN) fed a diet containing 80 ppm Fe, and the iron deficient (ID) group that fed a diet containing 4 ppm Fe. Male breeders were fed rodent chow (Purina Mills Lab Diet 5001) containing 270 parts per million (ppm) Fe. We purchased Teklad custom diet pellets from Harlan Laboratories which included either TD.09588 iron adjusted diet (80ppm) as a CN diet, or TD. 80396 iron deficient diet (4ppm) as an ID diet. One male and 2 females were placed together for 5 days for mating and breeding purposes. Pregnant dams (confirmed with locating vaginal plug) were then housed alone and checked daily for delivery. Postnatal day 0 (PND0) is the first day pups appeared. Pups out-fostering was done at PND4; pups from ID dams were euthanized. All pups were weaned at PND21 to Purina Rodent Diet (5001), containing 270 ppm iron, ad libitum until the time of sacrifice. All pups were pair-housed in clear plastic shoebox cages measuring 20 cm x 42.5 cm with stainless steel lids. Animals
were given distilled water. The rooms were temperature and humidity-controlled at 22 ± 1°C with an automatic 12/12 h light/dark cycles (light 0600–1800 h). All experimental animals were weighed weekly and monitored closely for health. All experimental protocols complied with the NIH Guidelines and approved by the designated IRB animal committee.

Brain dissection and sectioning

The same procedure as described in Burhans et al was followed.11–12 The animals at 21, 45 and 75 days of age. The numbers of animals by sex (m/f) allocated to each age group by dietary condition for DAT ligand binding were 15, 24 and 11 rats with age of 21d, 45d and 75d respectively. While the numbers of animals for NET ligand bindings were 14, 17 and 11 rats with age of 21d, 45d and 75d respectively. There were uneven numbers of males and females for each experiment due to the limited availability of samples.

The brains were removed from the skull and then divided mid sagittally on ice. The brains were divided into 2 hemispheres, however, only the right hemisphere were used. The right hemisphere was placed in isopentane cooled by dry ice and stored at −80°C until tissue sectioning within 4–5 months of harvest. Serial sagittal sections of 20 µm thickness were obtained, starting at the midline at −19°C using a Leica CM1950 cryostat (Leica Microsystems GmnH, Germany). The sections were placed on gelatin-coated slides with 2 sections per slide. These sections included individual brain regions specific for DAT; striatum, nucleus accumbens (NA), substantia nigra (SN), and olfactory tubercle (OT); and others specific for NET; frontal cortex (FC), dentate gyrus (DG), and locus coeruleus (LC). These brain regions were identified according to the mouse brain atlas by Swanson (1998). Gelatin coated slides were prepared by immersion in 0.95% gelatin (VWR International, West Chester, PA) and 0.0014% chromium (III) potassium sulfate (Alfa Aesar, War Hill, MA). After that the slides were dried at room temperature overnight, placed in sealed plastic bags, and stored at refrigerator until ligand binding.

DA transporter ligand binding

DA transporter ligand binding was performed as reported by Burhans et al.11 and Andrews et al.44 [3H]-RTI-55 was purchased from Perkin Elmer (Boston, MA). The slides were incubated in a solution of [3H]-RTI-55 (1098.7 µCi/ml, 2200 Ci/mmol) and protease inhibitor cocktail (DIC) diluted in a phosphate buffer (50 mM Na2HPO4; 50 mM NaHPO4; pH 7.4) 1:10 for 90 min at 4°C. We added 10 µM fluoxetine hydrochloride (Eli Lilly, Indianapolis, IN) to block serotonin transporter binding. Thus the presence of GBR 12935 (1 µM) and fluoxetine hydrochloride (10 µM) were essential for non-specific binding. The slides were washed 3 times in ice-cold fresh phosphate buffer for 5 min each, after the incubation period. Following the final wash, the slides were quickly dipped once in ice-cold double distilled H2O to desalt the tissue and dried by a steady flow of air overnight at room temperature. DAT slides and an autoradiographic [3H] Microscale (Amersham Biosciences, Picataway, NJ) were exposed Kodak BioMax MR-1 film (Amersham Biosciences, Picataway, NJ) at 4°C for 10 weeks.

Quantification of transporter ligand binding

The procedures as described in Burhans et al were followed.11 Ligand binding slides were quantified using NIH Image (Bethesda, MD). The standard curve was based on the level of radioactivity of the microscale on the day the film was developed. The average amount of the bound radio-ligand was measured by NIH Image using the standard curve and the Rodbard prediction equation. For each individual rat, the amount of transporter was obtained by calculating the average of the specific binding sections (2 sections per slide) and subtracting the average of non-specific binding sections (2 sections per slide). Data was then averaged across treatment groups. The original data was expressed as nanoCuries, however, the final binding values were reported in femtomoles (fmol) of bound radioligand (refer to the appendix section for data conversion). It is important to note that not all sections were used to determine receptor binding because of folding, tears, etc., which explains the inconsistency of the animal numbers through various age groups. Furthermore, we repeated the ligand binding for some animals, which reduced the number of available slides for the subsequent binding experiment. This explains why the 21-day-old group includes only females in the following analyses.

Data analysis

Experimental data was expressed as fmol of bound radioligand. The values represent mean ± SEM. The total numbers of examined animals were 14, 17, and 10 representing 21 d, 45 d, and 75 days old rats respectively. The distribution of data was examined for outliers and for normal distribution (>3 SD from the mean), but nothing needed to be removed for DAT and NET data. The transporter densities were subjected to multivariate analysis of variance (MANOVA) for two between-subject variables (diet, age), and multiple dependent variables for DAT (STR, NA, OT, SN) and NET (FC, DG, LC). Statistical significance was determined at α = 0.05. Tukey’s HSD post-hoc analyses were used when appropriate. All data were analyzed using SYSTAT 12 (SYSTAT Software, Inc., USA).

Results

DA transporters

MANOVA reveals a significant age effect on DAT levels in the nucleus accumbens (NA), olfactory tubercle (OT), and substantia nigra (SN) [F(2,31) = 7.54, p<0.05; F(2,31) = 23.22, p<0.05; F(2,31) = 12.32, p<0.05] respectively but not in the striatum. Specifically, 21 day old rats had higher DAT levels compared to 45 day old rats in the NA, OT and SN (p<0.05 for all regions) as well as in the OT compared to 75 day old rats (p<0.05). There was no main effect for diet and no diet-age interactions (see Table 1 Figure 1).
Table 1: DAT ligand binding (125I-RTI-55) in four brain regions of Sprague-Dawley rats at the age of 21 days

| Group | Striatum | Nucleus accumbens | Olfactory tubercle | Substantia nigra |
|-------|----------|-------------------|-------------------|-----------------|
| CN    | 13.59 ± 1.87 (n = 8) | 10.54 ± 1.04* (n = 8) | 7.10 ± 0.76* (n = 8) | 6.17 ± 1.37* (n = 7) |
| ID    | 17.34 ± 4.05 (n = 7) | 14.14 ± 3.14* (n = 7) | 7.46 ± 0.85* (n = 7) | 4.16 ± 0.95* (n = 6) |

*Significant difference from 45 day old rats, p<0.05.
CN: control, ID: iron deficient. Table of mean ± SEM.
Concentrations in fmol RTI-55.

**Discussion**

The current experiments yielded numerous interesting findings vis-à-vis dietary iron deficiency and brain functioning early in life. The first finding is that there is a significant age effect on DAT levels in the nucleus accumbens (NA), olfactory tubercle (OT), and substantia nigra (SN) but not in the frontal cortex or the locus coeruleus. Specifically, NET levels were higher among 45 day old rats compared to 75 day old rats (p<0.05). There was no main effect for diet and no diet-age interaction on any of the dependent variables (see Table 2; Figure 2).

**Norepinephrine transporter**

MANOVA revealed a significant age difference on NET levels in the dentate gyrus (F(2,35) = 4.00, p<0.05) but not in the frontal cortex or the locus coeruleus. Specifically, NET levels were higher among 45 day old rats compared to 75 day old rats (p<0.05). There was no main effect for diet and no diet-age interaction on any of the dependent variables (see Table 2; Figure 2).

**Results of current studies are consistent with the postnatal developmental pattern of DAT throughout different age groups.** For instance, within the same dietary group e.g. CN or ID, current DAT results showed a trend for DAT levels to be high at 21 days of age, after that, DAT levels declined at the age of 45 day old and finally elevated at the age of 75 day old. Such a pattern is nearly similar to the postnatal development of dopamine D1 receptors that increase in their level in rat striatum to a maximal level at PND35-40, followed by significant elimination of excessive receptors (pruning) to stable levels sustained into adulthood. This supports the time selection for induction of ID during the critical window of dopamine system differentiation (PND4-21) as the age of onset of the dietary iron deficiency may have an important impact on how much and where brain iron is lost, and on the possible reversibility with subsequent iron repletion. In contrast, there was no such pattern in NET levels, which explained by the presence of another developmental time window for NET that differs from DAT. Another explanation for this discrepancy is that NET might compensate to some extent for the reduction in DAT levels.

**Beyond the desire to replicate previous findings, the current study sought to relate our findings to the impaired performance of ID rats in ASST.** Contrary to what was expected significant dietary effect on DAT and/or NET levels within several examined brain regions, the data revealed no significant dietary effect on DAT and NET levels. However, it was reported a significant age effect on DAT and NET levels. As reported before ID rats performed poorly in ASST as compared to CN at 45 day old with performance improvement at 65 day old age after MePh treatment. Although, there is a restoration of systemic iron dependent proteins like hemoglobin (Hb) and hematocrit (Hct), the impact of ID on the central nervous system is likely irreversible in this model. This explained by the fact that the effects of ID *in utero* or during lactation (i.e. preweaning)
Table 2: NET ligand binding ([H]-nisoxetine) in three brain regions of Sprague-Dawley rats at the age of 45 days

| Group | Frontal cortex | Dentate gyrus | Locus coeruleus |
|-------|----------------|---------------|-----------------|
| CN    | 46.27 ± 4.69   | 72.61 ± 13.59 | 60.01 ± 10.17   |
|       | (n = 8)        | (n = 8)       | (n = 8)         |
| ID    | 57.48 ± 10.82  | 91.90 ± 10.94 | 61.22 ± 5.45    |
|       | (n = 9)        | (n = 9)       | (n = 9)         |

*Significant difference from 75 day old rats, p<0.05. CN: control; ID: iron deficient. Table of mean ± SEM. Concentrations in fmol [H]-Nisoxetine.

Fig. 2: NET ligand binding ([H]-nisoxetine) in dentate gyrus of Sprague-Dawley rats at different ages. *Significant difference from 75 day old rats, p<0.05. CN = control; ID = iron deficient.

appear to be irreversible in terms of DA metabolism in rats\(^9\) as well as in mice.\(^2\) Furthermore, it is apparent that cognitive impairment may not be attributed to a single neurotransmitter, but rather, alterations and interactions of several systems in different brain regions. Additionally, it is hard to explain the poor performance of ID rats reported in previous published article\(^44\) based on catecholaminergic levels in specific brain area per se; nonetheless, several brain areas are responsible for this poor performance. For instance, there was no significant dietary effect on striatal DAT levels. Despite that, there is evidence that variations in baseline striatal DA synthesis capacity alter individual human performance in reversal learning.\(^46\)

Study Limitations

Needless to say, the current study has important methodological limitations. First, there was not enough biological brain samples to represent an equal number of males and females. Given that females are largely refractory to the effects of iron deficiency on DA receptors,\(^13-14\) and male rats showed a greater effect of ID on DAT levels than did the female rats.\(^12\) Combining data from both male and female animals may affect the final conclusions. Second, the study did not measure 5-HT transporter levels which might show some sort of compensation to the reduction of DAT or NET levels\(^47\) and also it was hard to measure DAT in PFC because of low density levels. Lastly, the 75 days old rats received methylphenidate (Meph) treatment for 15 days which might affect the results by inducing up regulation of DA and NE within the brain through inhibition of their reuptake.\(^49\) Additionally, Meph blocks the DA and the NE transporter molecules however, it improves ID rat performance in ASST. Such improvements may be attributed to the possible reductions in regional cerebral blood flow in some of the fronto-parietal circuit with enhancement of the efficiency of information processing.\(^50\) Also, it is possible that MePh, via its actions on catecholamines, boosts signal-to-noise in PET.\(^50\) There is evidence that striatum is the most sensitive area in the brain to the DA-depleting amphetamine MePh,\(^51\) which might explain the non-significant effect of diet on striatum at the age of 75 days.

It is worth emphasising more that while comparing the current ligand binding data with that of Burhans et al.,\(^11\) it should be taken cautiously as they used 21 days old Sprague-Dawley rats and sacrificed males and females after 5 or 8 weeks of dietary treatment respectively. Additionally, they made rats iron deficient post-weaning while in the current model rats are ID at PND4 with out fostering to ID dams (i.e. lactational).

Conclusions

In summary, early ID in rats alters many monoaminergic-mediated behaviors, including learning, spatial memory, and other complex tasks. Such changes might be irreversible despite the fact that there is a restoration of peripheral and/or central iron. Moreover, the current report examined only DAT and NET levels however, other neurotransmitter systems may also be affected by early ID, and these systems need further attention in subsequent studies. It could be argued that levels of monoamine transporters are weak predictors of the alterations in attention and animal performance in ASST. Future studies measuring monoamine transporter activities may highlight the effects of brain iron deficiency on various neural pathways with further defining the functional ramifications.

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