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# The Impact of Postnatal Iron Administration on Memory and Levels of Serotonin and GABA in Hippocampus of Adult Male Rats

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## Keywords

Iron overload; Serotonin; GABA; Memory.

**Abstract**

***Objectives*** Excess iron in the brain has been implicated in the pathogenesis of neurodegenerative disorders. The present study was designed to investigate the effect of postnatal iron administration on memory, hippocampal serotonin (5HT) and γ-aminobutyric acid (GABA) levels and oxidative stress markers in adult male rats. ***Methods*** Thirty pups at age of 7 days weighting 50-52 grams were randomly divided equally into two groups : control (normal pups) and iron overload group treated with ferrous sulphate (3 mg/kg/day,intraperitoneally) for consecutive 21 days. Spatial and avoidance memories were tested using radial arm maze and passive avoidance tests. Hippocamapl levels of 5HT, GABA, serum total antioxidant capacity (TAC), and total peroxide (TP) were determined. Histopathological studies using H&E and Prussian blue stains were done. ***Results*** Iron overloaded rats showed impaired working memory in radial arm maze and impaired avoidance learning in passive avoidance test compared to the controls. Iron overload induced a significant decrease in the hippocampal levels of both 5HT and GABA. Levels of TAC were decreased and total peroxide was increased in iron overload group as compared to control group. Moreover, in contrast to the control group, positive iron depositions in the form of blue particles were detected in different areas of the hippocampus of iron overload group. ***Conclusion*** postnatal iron administration resulted in memory impairment possibly through alternation in hippocampal 5HT and GABA levels and oxidative stress.

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## INTORDUCTION

Iron is an ubiquitous component that plays an essential role in many metabolic functions including brain functions; it has been known to be essential for normal cognitive development and function.[1] Iron is carried by transferrin and transported across the blood–brain barrier mainly by transferrin receptor.[2]

Excessive intake of highly bioavailable forms of iron such as supplemental iron, red meat, and fruit may promote high iron stores and iron overload.[3] Iron overload has been implicated in the pathogenesis of several human neurodegenerative disorders such as Parkinson's disease and Alzheimer's disease resulting in cellular damages.[4] Iron overload, in animals, results in deficits in motor activity, habituation, inhibitory avoidance and working memory both in radial maze and object recognition tests.[3]

The hippocampus is a highly sensitive neural structure that known to be involved in learning and memory. Hippocampal synaptic plasticity is widely assumed to represent a mechanism, by which memory is encoded, consolidated and stored.[5] Fetal and early postnatal life is a period of rapid brain growth and development in most mammals, including humans.[6] It has been shown that, in rodents, the hippocampus is rapidly growing between postnatal day10 and 25.[7] Functionally, in rats hippocampus-dependent memory appears and matures between 3 and 18 month of age. During this period, there is an overall increase in brain iron uptake and utilization.[8] This increased metabolic activity is coincident with extensive dendrite arborization, spine formation, and synaptogenesis[9] as well as the maturation of electrophysiological plasticity.[10] So, the present study was designed to investigate the effect of postnatal iron administration on memory, hippocampal serotonin (5HT) and γ-aminobutyric acid (GABA) levels and oxidative stress markers as possible mechanisms in adult male rats.

## MATERIALS AND METHODS

The experimental protocol was approved by the Institutional Animal Research Committee of the Faculty of Medicine, Assiut University, Egypt that follows the published guidelines and regulations. Pregnant Wistar Albino rats were obtained and maintained in The Assiut University Animal Nutrition and Care House. The animals were maintained in metabolic cages and kept under standard conventional laboratory conditions at a temperature of 22° ±2° C, with a relative humidity of 50 ± 5% and a 12-h/12-h light/dark cycle. They had unlimited access to drinking water ad libitum and rat chow. Each pup was maintained together with its respective mother in a

plastic cage. At the age of 7 days, thirty pups (about 50-52 grams) were randomly divided into two equal experimental groups (each contains 15 pups) as follows:

1-Control group: normal pups received intraperitoneal injection of sodium chloride 0.9% once daily for 21 consecutive days.

2-Iron group: received intraperitoneal injection of ferrous sulfate (Fe SO4 7H2O, Sigma Aldrich, France) dissolved in sodium chloride 0.9% at a dose of 3 mg/kg/day for 21 consecutive days. [11]

***Behavioural testing:***

After the end of the experiment (21 days), the behavioural testing for both groups started on the next day as follows:

Eight arm radial maze:

The radial eight-arm maze is a procedure sensitive to deficits in spatial learning performance. The apparatus was radial maze with eight arms (60 cm long, 10 cm wide) radiating from an octagonal shaped central platform (30 cm large). In the training (familiarization; 10 days) phase, first spread food rewards (food cups containing 45 mg sucrose pellet) around the entire maze to encourage exploration (1-2 days). On subsequent days, place food only on the arms; then only at the end of the arms. Finally, food rewards were placed 0.5 cm from the distal part of chosen four arms. At the training phase, animals were placed in the central platform and allowed to enter any of the arms to eat the food reward. After the animal found all pellets or after 10 min had elapsed, it was removed from the maze. An arm entry was registered if the rat placed its four paws within the alley. In the testing phase (5 days), food rewards were placed only in chosen four arms. Re-entries into baited arms were counted as working memory errors. The mean number of errors in each day for 5 consecutive days was used for analysis.[11]

**Passive avoidance test**

The apparatus consisted of two compartments (light and dark) each measuring 22 X 21 X 22 cm. The two compartments were separated by a partition with an opening (8 cm in diameter) at the floor level. The dark compartment's floor consisted of parallel stainless steel rods (0.3 cm in diameter with 1 cm intervals) to produce electric shock. The rat learns to avoid the dark compartment where an electric shock was applied. The test was divided into training and test sessions. In the training session, the rats were allowed to explore the light compartment and a foot electric shock (50 Hz, 5s, 0.2 mA intensity) was applied whenever the rat enters the dark compartment with a maximum three electric shock. The initial latency [IL] to enter the dark chamber was recorded. Five seconds later the rat was removed from the dark chamber and returned to its home

cage. Twenty-four hours later, the retention latency [RL] time was measured in the same way as in the training trial, without application of an electric shock. The latency for entering the dark compartment (four paws in) was recorded and was used for analysis.[12, 13]

***Determination of γ-aminobutyric acid (GABA) and serotonin levels in the hippocampus:***

At the end of the experiment, the rats were decapitated. Brain was removed, rapidly washed with cold phosphate buffered saline (PBS) and the hippocampus was dissected and weighed then immediately frozen in liquid nitrogen and finally stored at -80°C until being assayed for serotonin and GABA. Sample contents of serotonin and GABA were measured by high performance liquid chromatography (HPLC) with fluorescence detection (Agilent 1200 series) as described previously.[14, 15] For GABA and serotonin, 100 µl of hippocampal homogenate was injected to the Zorbax 300 SB-C18 column (4.6 mm × 250 mm, 5µm) under the following conditions: The mobile phase consisted of 1mM disodium hydrogen phosphate buffer, flow rate 1 ml/min and the fluorescence excitation and emission wavelengths were set at $\lambda$ex 340 and $\lambda$em 456 nm, respectively. The resulting chromatogram identifying each neurotransmitter position and area under curve for each sample was compared to that of the standard curve made by Eurochrom HPLC Software, version 1.6.

***Biochemical assessments:***

Serum total antioxidant capacity (TAC) was measured calorimetrically using commercial kit (Bio-Diagnostics, Giza, Egypt).[16] Serum total peroxide (TP) was measured as described by Harma et al.[16] Oxidative stress index, an indicator for oxidative stress was calculated as the percentage ratio of TP to TAC in mM/L.[16]

***Light microscopic examination:***

The hemisphere (containing the hippocampus) was separated from each animal, fixed in 10% neutral buffered formalin, dehydrated and embedded in paraffin. Coronal sections of 5 µm sections were cut and stained by Haematoxylin and Eosin (H&E) for histological study. Prussian blue stain was used for detection of ferric ion salts in hipppocampal tissue. Ferric ion salts have blue color and counter stain was done using eosin stain. [17]

**Statistical analysis**

Data are presented as mean ± standard deviation (SD). The data were analyzed using unpaired t-test. Two-way ANOVA and Student Newman-Keuls Post hoc test were used to examine the effect of iron versus control group across the five testing days on the number of working memory errors using Graph Pad Prism data analysis program (GraphPad software, Inc., San Diego, CA,

USA). A value of $p \leq 0.05$ was considered statistically significant. [18]

## RESULTS

### *Body weight*

At the end of the experiment, there was no significant difference between the final mean body weight of iron group versus control group.

### *Eight-arm radial maze: working memory task:*

Fig. 1 shows the number of working memory errors (numbers of re-entries into baited arms; 4 arms contain food) for the two groups. Over all 5 testing days the mean number of errors was decreased over days in the two groups. Two –way ANOVA revealed that iron group had significantly ($p<0.001$) higher numbers of working memory errors across the 5 testing days compared to control group.

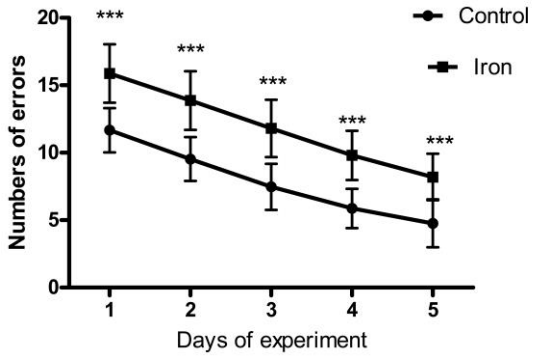


**Fig. (1):** Radial maze learning in albino Wistar rats treated neonatally at the age of 7 day with iron. Animals were treated orally 3mg $Fe^{2+}$ per kg body weight in the post natal days. Two way ANOVA indicated a significant groups effect for number of the errors ($P<0.001$). Values represent mean ±SD. ***$P<0.001$ as iron versus control.

### *Passive avoidance test:*

The results of rat passive avoidance test are reported in Fig. 2. The mean initial latencies did not differ significantly between the control and the iron group. However, after 24 hours, iron group showed significant ($p< 0.001$) shorter latencies to enter to dark compartment compared to control.

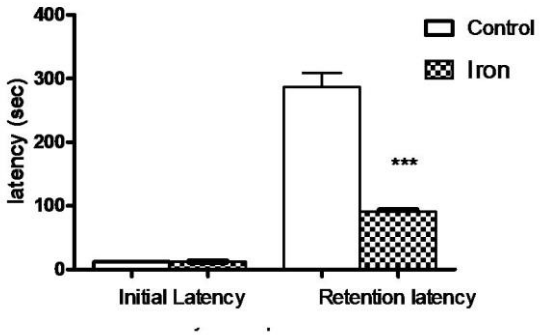


**Fig. (2):** Effect of iron overload 3 mg/kg body weight postnatally in rats at the age of 7 days on passive avoidance test in rats. Values are expressed as mean± SD. *** $P< 0.001$ as iron group versus control.

### *Effect of iron overload on biochemical measurements:*

Table 1 showed that hippocampal GABA levels were significantly lower in iron group compared to control group ($P<0.01$). In addition, iron overload induced a decrease of 5HT in hippocampus compared to control group ($P < 0.01$).

The mean serum levels of TAC were significantly decreased in rats with iron overload group ($P < 0.001$) as compared to control group. In contrast, serum TP levels were significantly increased in iron overload group as compared to control group ($P<0.05$). In addition, oxidative stress index

was significantly increased in iron group as compared to control.

**Table (1):** Mean of hippocampal GABA and serotonin levels, serum total antioxidants (TAC), serum total peroxide (TP) and oxidative stress index (OSI) for control and iron group

| | GABA (mg/l) | Serotonin (mg/l) | TAC (mM/l) | TP (mM/l) | OSI |
|---|---|---|---|---|---|
| **Control group** | 11.34± 0.097 | 0.0046± 0.0011 | 0.93± 0.02 | 0.11± 0.012 | 12.12± 0.01 |
| **Iron group** | 6.165± 0.108*** | 0.0034± 0.0003** | 0.65± 0.07*** | 0.12± 0.01* | 19.55± 0.02*** |

Data are expressed as mean ± SD, *P < 0.05; **P < 0.01; ***P < 0.001 compared to control group.

***Histopathological results:***

Light microscopic examination of haematoxylin and eosin stained sections of control group revealed that the hippocampus has interlocking structure of C shaped Ammon's horn (cornu ammonis) and V shaped dentate gyrus (DG). Ammon's horn or hippocampus proper includes four fields; CA1, CA2, CA3 and CA4 (Fig.3a). Iron treated rats showed obvious histopathological changes in CA1 and DG. Most pyramidal neurons in CA1 appeared shrunken with irregular outlines, darkly stained cytoplasm and dense ill defined nuclei. The affected neurons were surrounded by clear zone due to retraction of their soma. Dentate gyrus contained numerous apoptotic cells with irregular outlines, darkly stained pyknotic nuclei and deeply stained cytoplasm (Fig. 3).

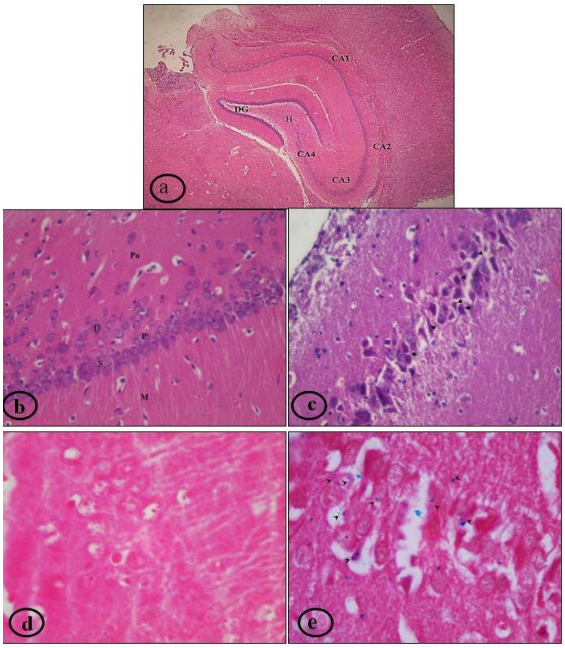


**Fig. (3): a**: A photomicrograph of a section in the hippocampus of the control group showing the interlocking structure of C-shaped Ammon's horn and V-shaped dentate gyrus (DG) and their laminar organizations (H&E x 40).
**b:** A photomicrograph of a section in CA1 field of the control group showing its layers; polymorphic (Po), Pyramidal (P) and molecular (M) layers. Pyramidal cells are arranged into densely packed superficial layer (S) and loosely arranged deep layer (D) (H&E x 400).
**c :** A photomicrograph of CA1 field of iron treated group showing most pyramidal cells having irregular outline with dense nuclei and deeply stained cytoplasm (arrow heads) (H&E x400).
**d:** A photomicrograph of a section in CA1 field of the control group showing negative reaction for iron particles (Prussian blue stain x 1000).
**e:** A photomicrograph of CA1 field of iron treated group showing positive reaction to iron particles in its layers (arrow heads) (Prussian blue stain x1000).

**Histochemical results:**

In contrast to the control group, positive iron depositions in the form of blue particles were detected in CA1 and DG areas of the hippocampus of iron group after Prussian blue staining (Fig.4).

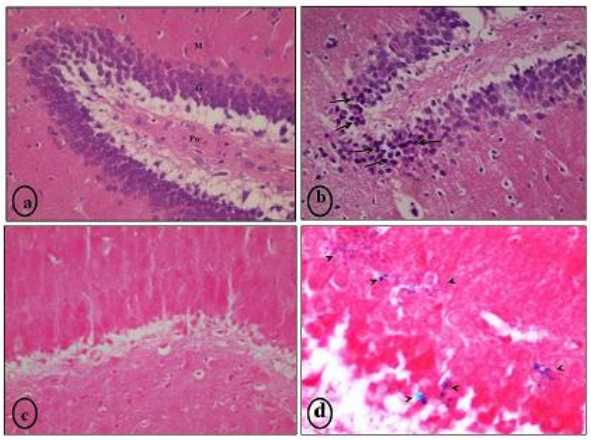

**Fig. (4) a:** A photomicrograph of a section in DG of the control group showing its layers including outer molecular (M), middle granule (G) and inner polymorphic (Po) layers. The granule cells appear small and closely packed (H&E x 400).
**b:** A photomicrograph of DG of iron treated group showing many granule cells became shrunken with darkly stained cytoplasm and dense nuclei (arrows) (H&E x400)
**c:** A photomicrograph of a section in DG of the control group showing negative reaction for iron particles (Prussian blue stain x 1000).
**d:** A photomicrograph of DG of iron treated group showing positive iron particles in all layers (arrow heads) (Prussian blue x1000).

## DISCUSSION

The present study showed that administration of iron at a dose of 3 mg/kg to rat pup (at day 7 after birth and for subsequent 21 days) caused significant cognitive impairment in all the animals. Iron treatment disrupts the spatial memory (assessed by the eight-arm radial maze) and avoidance memory (assessed by the passive avoidance task). These findings suggest that this period is a critical brain sensitivity period to iron overload. These findings are in agreement with previous studies that reported that administration of iron to rodents in the neonatal period induces persistent memory deficits.[19] This could be explained that the young brain is particularly sensitive to iron excess due to an immature iron permeability of the blood–brain barrier. [11]

Previous studies demonstrated that, the neural deficits were observed when iron was administrated on postnatal days 10–12, therefore suggesting a critical brain sensitivity period to iron overload. [20,21] In contrast to our results, Maaroufi et al. [11] reported that administration of iron (3 mg of FeSO4 per kg of body weight) during 21 consecutive days to adult rats didn't cause alterations in the water maze and radial maze tasks. This may be explained by the age differences between the present study and those of Maaroufi et al. [11]

In the present study, histopathological results may explain the impairment of memory of iron treated rats as they have obvious histopathological changes mainly in CA1 and DG in the form of shrunken of pyramidal neurons in CA1 with irregular outlines, darkly stained cytoplasm and dense ill defined nuclei. In addition, dentate gyrus contained numerous apoptotic cells with irregular outlines, darkly stained pyknotic nuclei and deeply stained cytoplasm. These changes were more obvious in crest than the limbs.

Although in previous experiments with a long-lasting increase in iron levels in the basal ganglia iron content within the

hippocampus following neonatal iron treatment has not been determined.[22] The histological results of the present study indicated positive iron depositions in the form of blue particles in different areas of the hippocampus of iron-treated rats.

The hippocampus and the prefrontal cortex have been known as main targets of serotoninergic neurons of the raphe nuclei. Interestingly, the serotoninergic system has been involved in modulation of memory processes. [23]. Moreover, Calabrese et al.[24] reported that serotonin is particularly critical for proper wiring of neural circuits and play an impressive role in neurodevelopmental disorders.

In the present study, hippocampal levels of serotonin and GABA were measured to determine whether iron overload had a direct impact on the hippocampus. The results revealed that rats treated with iron had lower hippocampal levels of serotonin and GABA compared to controls. The present findings may be explained by Wilson and Molliver [25] who reported that 5-HT regulates the excitability of glutamatergic cortical neurons, and of their cholinergic afferents by means of presynaptic inhibitory contacts. In addition, the glutamatergic and cholinergic neurotransmitter systems play an important role in memory formation, but there is increasing evidence showing that serotonin functionally interacts with these systems, and thereby has the ability to play a modulatory role in memory functions.[26] Furthermore, Lehmann et al.[27] reported that loss of cholinergic and serotonergic transmission causes persistent memory impairments in rats, as compared with the loss of cholinergic transmission alone, thus there may be a link between our biochemical findings and the behavioral deficits. In addition, our results may be supported by previous work of Marchetti et al.[28] who demonstrated amelioration of associative memory in rats due to usage of 5-HT4 agonist. Moreover, administration of 5-HT4 receptor antagonists induced amnesia in the mouse passive avoidance test to the same extent comparable to that induced by the amnesic drugs [29], confirming our results.

Previous study indicated that iron accumulation in the brain induces neurodegenerative disorders via oxidative stress mechanisms.[30] In the present study, the results revealed decreased serum TAC and increased TP levels and hence increased OSI in iron group compared to control rats. In previous in vitro study, addition of exogenous $Fe^{2+}$ resulted in stimulation of lipid peroxidation ranging from 10-fold in cortex to 20-fold in hypothalamus homogenates.[31] In addition, de Lima et al. [(32)] reported that memory impairment due to excess iron was correlated to brain oxidative damage in young rats after short term iron administration.

Nevertheless, Budni et al.[33] found that neonatal iron overload results in alterations associated with memory dysfunction in the hippocampus, including increases in oxidative stress.

## CONCLUSIONS

As observed in the present study, postnatal iron administration appears to induce memory impairment; it is possible that iron overload causes memory impairment through alternation in hippocampal serotonin and GABA levels and oxidative stress.

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