EFFECTS OF CALCIUM ON DRINKING FLUOROSIS-INDUCED HIPPOCAMPAL SYNAPTIC PLASTICITY IMPAIRMENT IN THE OFFSPRING OF RATS

Abstract

Objective This study investigated the effects of calcium on fluorosis-induced impairment in learning and memory of offspring rats. Methods Seventy-five newly weaned female Sprague–Dawley (SD) rats were randomly divided into five groups as follows: Control group (Control) drank tap water, and ate the normal diet (calcium content of 0.79%); fluoride group (F) drank 100 mg/L NaF solution, and ate the normal diet; low calcium group (LCa) drank tap water, and ate the low calcium diet (calcium content of 0.063%); low calcium fluoride group (F + LCa) drank 100 mg/L NaF solution, and ate the low calcium diet; high calcium fluoride group (F + HCa) drank 100 mg/L NaF solution, and ate the high calcium diet (calcium content of 7%). After exposing rats to fluoride for three months, male and female rats were mated and 14 and 28 days old offspring were obtained as experimental subjects. Examinations determined the submicroscopic parameters of the synaptic interface and expression levels of specific proteins: doublecortin (DCX) and synaptophysin (p38). Results (1) High fluorosis significantly reduced synapse density, length of synaptic active zone, thickness of postsynaptic density, and led to abnormal changes in the structural parameter of synaptic gap width, which was significantly reduced or increased. High dietary calcium significantly reversed the abnormal changes in structural parameters, and low calcium aggravated these variations. (2) Dietary calcium resulted in nonsignificant effect on expression levels of DCX and p38. Conclusion The results suggested that dietary calcium significantly affected hippocampal synaptic plasticity of offspring of mothers exposed to water fluorosis, but its molecular mechanism may not be related to the expression of DCX and p38 in the brain. The findings also demonstrate the important effects of maternal exposure to water fluorosis on offspring brain functions before water improvement.

Keywords
- calcium • fluorosis • brain development • synapses • DCX • p38

Introduction

Though fluoride is an essential micronutrient to the human body, long-term excessive intake can be toxic to multiple systems, resulting in a condition known as endemic fluorosis. There are three types of endemic fluorosis: coal-burning endemic fluorosis, brick-tea fluorosis, and drinking-water type fluorosis. Drinking-water fluorosis cases are distributed in 28 provinces, provincial cities, and autonomous regions in 1138 counties. This condition seriously harms people’s health. Fluorosis can damage bony organs, such as teeth and bones[1–2]. Excessive fluoride can penetrate the blood–brain barrier then subsequently, brain tissues, thus damaging the central nervous system[3] and changing synaptic plasticity[4]. Previous studies also showed that fluorosis can cause accumulation through placental barrier–brain tissue, resulting in damage in the subsequent generation[5]. Therefore, in endemic fluorosis areas in China, although the fluoride content of drinking water usually follows standards, efforts should center on the effects of maternal exposure to water fluorosis on offspring brain function before water improvement. In this study, SD rats were used to investigate the effects of calcium on drinking-water fluorosis-induced learning-and-memory impairment of offspring rats in order to explore intervention measures for normal brain development in fluorosis areas and to provide basic data for prevention and treatment of endemic fluorosis.

1. Materials and methods

1.1 Experimental animals

Weighing 70–90 g each, 75 female rats and 25 newly weaned male Sprague–Dawley rats were procured from the Experimental Animal Center of Zhejiang Province in China. After a week of acclimatization, female rats were randomly divided into five groups with 15 rats per group. These groups were as follows: the control group was provided with tap water (water fluoride <0.2 ppm) and fed the normal diet (calcium content = 0.79%); the fluoride group (F) received 100 mg/L NaF solution (F content = 45 ppm) and fed the normal diet (calcium content = 0.79%); the low-calcium group (LCa) was provided with tap water (water fluoride <0.2 ppm) and fed a low-calcium diet (calcium content = 0.063 %); the low-calcium–fluoride group (F + LCa) was treated with 100 mg/L NaF solution and fed a low-calcium diet (calcium content = 0.063%); and the high-calcium–fluoride group (F + HCa) was provided with 100 mg/L NaF solution and fed a high-calcium diet (calcium content = 7%). All male rats received
tap water (fluoride content <0.2 ppm) and were fed a standard diet, of which the calcium content measured 7.9 g/kg. Tap water was used as solvent in all solutions. After feeding the rats for three months, the fluoride content of the blood and urine served as a success criteria of subchronic fluorosis in this animal model. Male and female rats were mated at a 1:1 ratio. Rat offspring were breastfed after birth until they were mature enough to drink water on their own. Drinking water of the maternal rats remained the same during the lactation period. In this study, newborn rats, 14-day-old rats, and 28-day-old rats were utilized to conduct relevant experiments. Room temperature and humidity were maintained at 25 °C and 50% to 70%, respectively.

1.2 Equipment and reagents
Pathologic Tissue Selected Table, pathological tissue embedding machine, Ultra-Thin Semiautomatic Microtome, pathological tissue dehydrator, bleaching machine, wet box (Jinhua Central Hospital), Olympus optical microscope (Japan), H-7650 Transmission Electron Microscope (Hitachi, Japan), and centrifuge (S417R, 3K18 Eppendorf company, Germany).

PI DCX antibody test kit and synaptic p38 lightning antibody kit (ShangHai HengYuan Biological Technology Co., Ltd.)

1.3 Experimental method
1.3.1 Observation of synaptic ultrastructure in hippocampus
Offspring rats were decapitated. The CA3 area of the hippocampus was separated in offspring rats according to brain stereotaxic. Then, extracted brains were fixed with a 2.5% glutaraldehyde solution and stored in a refrigerator at 4 °C overnight. Samples were fixed overnight and cut into thin slices. Type I astrocytes of gray matter in hippocampal CA3 area were photographed using a JEM-1200EX type electron microscope. Image analysis software Image-Pro Plus 5.1 was used to determine synaptic density, synaptic gap width, thickness of postsynaptic density (PSD), postsynaptic activity band length, and curvature of the synaptic interface. Twenty synapses were analyzed in each group with double blind trials.

The number of synaptic densities per unit area (Nv) was quantitatively analyzed by stereological methods. The formulas used are shown in (1.1) and (1.2):

\[ N_v = \frac{8 \times E \times N_a}{\pi^2} \quad (1.1) \]

where \( E \) corresponds to the average of the reciprocal of PSD length, and \( N_a \) represents the number of synapses per unit area.

\[ N_a = \sum N_x / \sum A_r \quad (1.2) \]

where \( N_x \) corresponds to the number of synapses per image, and \( A_r \) denotes the area of each image.

1.3.2 Immunohistochemical detection
Offspring rats were sacrificed. Samples were collected after perfusion. Coronary frozen sections with a thickness of 35 μm were immunohistochemically stained and counted. Sections were soaked in fresh 3% H₂O₂ for 10 min at room temperature to inactivate endogenous enzyme and then washed twice with phosphate-buffered saline (PBS) for 5 min and goat serum at room temperature for 30 min. Rabbit anti-doublecortin (DCX) (1:500) was added dropwise, and samples were stored in refrigerator at 4 °C overnight. Afterward, sections were washed thrice with PBS for 5 min. Biotin goat anti-rat IgG (1:100) was added dropwise, and samples were incubated at 37 °C for 2 h. Then, sections were washed thrice with PBS for 5 min. Diaminobenzidine–glucose oxidase solution was used and developed under microscopic observation for the timely termination of the reaction, followed by a gradient alcohol dehydration, and addition of transparent xylene. Then, samples were dried and observed by microscopy. Using the Image-Pro Plus image analysis system, each slice was observed and photographed at high magnification (400 x), and its optical density (OD) value was calculated.

1.4 Data processing
Data are presented as mean ± standard error (M ± SD). The mean values were determined using the homogeneity of variance test. If the variance passed the homogeneity test, the comparison of means was conducted using a one-way ANOVA. The least significant difference test was applied to conduct multiple comparisons with a significance standard of p < 0.05. All analyses were performed using SPSS 18.0 software.

2 Results
2.1 Statistical results of the synaptic interface ultrastructure in the hippocampal CA3 area of filial rats
2.1.1 Statistical results of the hippocampal synaptic density in CA3 of newborn rats
Figure 1 and Table 1 show the density of the hippocampal CA3 area in newborn rats. Compared with that in the control group, newborn rats in F+LCa group presented significantly decreased hippocampal synaptic density (p<0.05), whereas no significant difference was observed in other groups. Compared with the F group, each group showed no significant difference (p>0.05).

2.1.2 Statistical analysis of synaptic density in the hippocampal CA3 area of 14-day-old rat offspring
Table 2 presents statistical analysis of synaptic density in the hippocampal CA3 area of 14-day-old rat offspring. Compared with that in the control group, male rats in F+LCa group exhibited significantly decreased synaptic density (p<0.05), whereas those of female rats in F group (p<0.05) and in F+LCa group were significantly reduced (p<0.01). Compared with that of the F group, female rats in F+LCa group manifested significantly reduced synaptic density (p<0.05).

2.1.3 Statistical analysis of synaptic density in the hippocampal CA3 area of 28-day-old offspring rats
Table 3 displays results for synaptic density in the hippocampal CA3 area of 28-day-old rat offspring. Compared with that in the control group, male rats in the F group showed significantly decreased synaptic density (p<0.05), whereas significant decreases were
noted in the rat offspring of the F+LCa group (p<0.01) and of female rats in the F and F+LCa groups (p<0.05). Compared with that in the F group, male rats in the F+LCa group presented significantly decreased synaptic density (p<0.01).

2.1.4 Detection and analysis of synaptic interface structural parameters in the CA3 area of hippocampi of newborn rats

Figure 2 and Table 4 provide results from the detection and analysis of synaptic interface structural parameters in the CA3 area of hippocampi of newborn rats. Compared with those in the control group, newborn rats in the F and F+LCa groups showed significantly decreased synaptic active zone length (p<0.05). The width of the synaptic gap significantly increased (p<0.01), whereas Psd thickness decreased significantly in the F+LCa group (p<0.01). Compared with that in the F group, newborn rats in the LCa group revealed significantly reduced synaptic gap width (p<0.05), whereas those in the F+LCa group showed significantly decreased PSD thickness (p<0.01).

2.1.5 Detection and analysis of synaptic interface structural parameters in the CA3 area of hippocampi of 14-day-old rats

Figure 3 and Table 5 display results of detection and analysis of synaptic interface structural parameters in the CA3 area of hippocampi of 14-day-old rats. Compared with that in the control group, male rats in the F+LCa group exhibited significantly decreased synaptic activity band length (p<0.01). The F and F+LCa groups presented significantly increased width of synaptic gap (p<0.01) and significantly decreased PSD thickness (p<0.01). Male rats in the LCa group also displayed significantly decreased PSD thickness (p<0.05). At p<0.05 and p<0.01, the length of the synaptic activity band significantly decreased in female rats in the F group and rats in the F+LCa group, respectively. Female rats in the F+LCa group showed significantly increased width of their synaptic gap (p<0.05). PSD thickness of the F and F+LCa groups decreased significantly (p<0.05), whereas that in the F+LCa group significantly decreased (p<0.01).

Table 1. Detection of hippocampal synaptic density in newborn rats (M±SD, n=20)

| Group   | Density (N/μm³) |
|---------|-----------------|
| Control | 0.28±0.17       |
| F       | 0.25±0.28       |
| LCa     | 0.30±0.24       |
| F+LCa   | 0.20±0.10*      |
| F+HCa   | 0.29±0.23       |

Note: * p<0.05, compared to Control group

Table 2. Detection of hippocampal synaptic density in 14 day old rats(M±SD, n=20)

| Group | Density (N/μm³) |
|-------|-----------------|
|       | male rat | female rat     |
| Control | 0.33±0.28 | 0.34±0.37     |
| F       | 0.29±0.28 | 0.27±0.17*    |
| LCa     | 0.31±0.25 | 0.32±0.24     |
| F+LCa   | 0.24±0.22* | 0.20±0.13**# |
| F+HCa   | 0.34±0.31 | 0.33±0.23     |

Note: * p<0.05, ** p<0.01, compared to Control group

# p<0.05, Compared with the fluoride group (F).

Table 3. Detection of hippocampal synaptic density in 28 day old rats(M±SD, n=20)

| Group | Density (N/μm³) |
|-------|-----------------|
|       | male rat | female rat     |
| Control | 0.38±0.28 | 0.36±0.37     |
| F       | 0.31±0.33* | 0.30±0.26*    |
| LCa     | 0.32±0.25 | 0.33±0.35     |
| F+LCa   | 0.22±0.32*** | 0.28±0.42*    |
| F+HCa   | 0.34±0.26 | 0.37±0.41     |

Note: * p<0.05, ** p<0.01, compared to Control group; #* p<0.01, Compared with the fluoride group (F).
Figure 2. Electron microscopic photographs of synaptic interfaces of hippocampal CA3 areas of rats (×100000): A: Control, B: F, C: LCa, D: F+LCa, E: F+HCa

Table 4. Synaptic interface structure of the CA3 area of hippocampi in newborn rats

| Group   | Length of synaptic active zone (nm) | Synaptic gap width (nm) | PSD thickness (nm) | Curvature of synaptic interface |
|---------|------------------------------------|-------------------------|--------------------|-------------------------------|
| Control | 340.50±64.79                       | 16.29±4.64              | 31.64±13.95        | 0.78±0.09                     |
| F       | 286.81±33.27*                      | 24.44±7.70**            | 27.49±9.68         | 0.90±0.05                     |
| LCa     | 327.88±51.74#                      | 17.74±3.91*             | 31.13±12.13        | 0.92±0.06                     |
| F+LCa   | 274.48±66.39*                      | 24.26±10.90**           | 20.32±3.34***      | 0.84±0.09                     |
| F+HCa   | 307.28±41.94                       | 20.98±4.59              | 28.22±3.82         | 0.86±0.09                     |

Note: * p<0.05, ** p<0.01, compared to Control group; # p<0.05, ## p<0.01, Compared with the F group.

Table 5. Synaptic interface structure of the CA3 area of hippocampi in 14 day old rats (M±SD, n=20)

| Group   | Length of synaptic active zone (nm) | Synaptic gap width (nm) | PSD thickness (nm) | Curvature of synaptic interface |
|---------|------------------------------------|-------------------------|--------------------|-------------------------------|
| Male    | Female                             | Male                    | Female             | Male                          | Female                        |
| Control | 330.94±108.31                      | 312.37±73.46            | 15.71±2.21         | 17.66±3.09                    | 33.34±10.56                   | 0.87±0.10                     | 0.83±0.13                     |
| F       | 293.74±51.96                       | 255.62±51.06*           | 21.09±6.74**       | 20.19±4.17                    | 23.39±6.69**                  | 24.26±5.37*                   | 0.83±0.07                     | 0.86±0.10                     |
| LCa     | 327.69±95.44                       | 283.03±50.07            | 18.12±1.93         | 18.51±5.42                    | 25.18±11.23*                  | 26.51±7.21                    | 0.87±0.09                     | 0.77±0.12                     |
| F+LCa   | 231.70±40.36****                   | 244.30±93.31**          | 22.66±4.28**       | 21.73±4.96*                   | 21.99±6.56**                  | 21.30±2.83**                  | 0.82±0.06                     | 0.94±0.04                     |
| F+HCa   | 318.05±55.88                       | 261.67±34.99            | 19.92±4.66         | 20.81±3.15                    | 28.77±6.05*                   | 24.03±5.37*                   | 0.82±0.06                     | 0.83±0.15                     |

Note: * p<0.05, ** p<0.01, compared to Control group; # p<0.05, ## p<0.01, Compared with the F group.
Figure 3. Electron microscopic photographs of the synaptic interface of the hippocampal CA3 region of 14 day old rat offspring (x100000)
A: Control male  B: Control female  C: F male  D: F female  E: LCa male
F: LCa female  G: F+LCa male  H: F+LCa female  I: F+Hca male  J: F+Hca female
with the F group, male rats in the F+LCa group exhibited significantly decreased length of their synaptic activity band (p<0.01), whereas the thickness of their PSD increased significantly in the F+HCa group (p<0.05). No significant changes were observed in synaptic parameters in female rats (p>0.05).

2.1.6 Detection and analysis of synaptic interface structural parameters in the CA3 area of hippocampi of 28-day-old rats

Figure 4 and Table 6 display results from the detection and analysis of the synaptic interface structural parameters in the CA3 area of hippocampi of 28-day-old rats. Compared with the control group, male rats in the F and F+LCa group presented significantly decreased synaptic activity band length (p<0.01). The width of their synaptic gap significantly increased in the F (p<0.05) and F+LCa groups (p<0.01). PSD thickness decreased significantly in the F+LCa group (p<0.05). Male rats in the F and F+LCa groups displayed significantly decreased length of their synaptic activity band (p<0.01). The width of the synaptic gap significantly increased in the F+LCa group (p<0.01). PSD thickness was significantly reduced in the F, F+LCa, and F+HCa groups (p<0.05). Compared with that in the F group, the length of the synaptic activity band significantly increased in male rats of the LCa and F+HCa groups (p<0.01), whereas that in the F+LCa group significantly decreased (p<0.05). PSD thickness decreased significantly in male rats of the F+LCa group (p<0.05). The length of the synaptic activity band significantly increased in female rats in the LCa and F+HCa groups (p<0.01). The thickness of the PSD increased significantly in the LCa group (p<0.05).

2.2 Statistical test results on DCX and synaptophysin (p38) in rat offspring brains

2.2.1 Observational results of the expression of hippocampal DCX and p38 in newborn rats

Figures 5 and 6 and Table 7 show observational results of DCX and p38 expression in hippocampi of newborn rats. Closely arranged positive cells appeared in the brains of newborn rats in each group. As shown in Table 8, compared with that in the control group, no significant difference existed in expressions of DCX and p38 protein in fetal rats (p>0.05). Compared with the F group, no significant difference was observed in expression of DCX and p38 protein in fetal mice (p>0.05).

2.2.3 Observational results of the expression of hippocampal DCX and p38 in 28-day-old rat offspring

Table 9 presents observational results of the expression of hippocampal DCX and p38 in 28-day-old mice (p>0.05).

3 Discussion

The hippocampus is closely related to learning and memory and emotional behavior. Synapses are one of the key structures of neural signal...
Figure 4. Electron microscopic photographs of the synaptic interface of the hippocampal CA3 region of 28 day old rat offspring (×100000)
A: Control male  B: Control female  C: F male  D: F female  E: LCa male
F: LCa female  G: F+LCa male  H: F+LCa female  I: F+Hca male  J: F+Hca female
transmission. This process is easily affected by age, medicine, environment, and other factors. Synaptic plasticity serves as the basis of learning and memory; it mainly deals with the probability of synapse occurrence and morphological structural changes with stimulation. Synaptogenesis is an important process of brain development, and synapse structure and characteristics determine transmission efficiency of synaptic information.

Previous studies suggested that dietary calcium significantly affect hippocampal synaptic plasticity of offspring whose mothers were exposed to water fluorosis. This result not only agrees with the experimental results on the central nervous system of early-stage newborn mice but also with those for the placental barrier. Such findings also showed sensitivity of the synaptic ultrastructure of the hippocampus, length of synaptic active zone, thickness of postsynaptic dense material, and width of the synaptic gap to fluoride exposure. However, further research is required to determine the molecular mechanism of how fluoride exposure influences hippocampal synaptic plasticity.

Swingle and Pfiffner’s hormone DCX is a microtubule-associated protein. In mammalian developmental stages, DCX is widely used in central nervous system processes, such as migration of neuronal cell bodies, leading
processes, and differentiation of axons, which act as markers of migrating and immature neurons\(^{17}\). In the development of the human brain, DCX is primarily expressed in the fetal cerebral cortex and can be detected at nine weeks of gestation. Peak expression level is observed at 12–20 weeks of gestation, with neuronal migration as the most frequent stage, and then decreases gradually\(^{18}\). In this experiment, after immunohistochemical detection, no significant difference was observed in expression levels of DCX protein in rat offspring in each group. p38 is one of the specific markers of synaptic terminals, participating in release of neurotransmitters, such as Ca\(^{2+}\)-dependent acetylcholine, and is closely related to synaptic plasticity. Expression and quantity of p38 immune products accurately reflects distribution and density of\(^{19}\) synapses, respectively. Human brain synapses mainly develop in late pregnancy and early postnatal development. However, p38 can be detected during rat birth, but its expression initially starts at low levels before gradually increasing. p38 peaks at four days after birth, when the synapse is in a rapid development stage; then, its expression decreases gradually; p38 expression levels in 30-day-old rats is similar to that in adult rats\(^{20}\). In this study, immunohistochemistry detected no significant difference in the expression levels of p38 in each of the offspring rat groups. The molecular mechanism of how maternal fluoride exposure affects the hippocampal synaptic plasticity of the offspring may not be affected by expression of hippocampal DCX and p38 protein. Our previous studies showed that changes in the expression of PSD-95 after exposure to fluoride may be one of the mechanisms of brain damage induced by fluoride\(^{21}\). Further studies should investigate the molecular mechanism of the maternal effects of fluoride exposure on hippocampal synaptic plasticity in their offspring.

Currently, no specific drug is available for endemic fluorosis because of its complex pathogenesis. However, researchers are focusing on studies of fluoride-resistant drugs, for example, vitamin C and the trace element selenium\(^{22–23}\). The relationship between calcium and fluorosis also became an important topic of research. Calcium is an important intracellular messenger. Through different methods, calcium activates signal transduction and regulates and controls physical reactions, such as neurotransmitter delivery. After long-term intake of excessive fluoride, fluorine directly attacks oxygen molecules, interferes with normal metabolism of oxygen\(^{24–25}\), and results in the formation of excessive oxygen free radicals. Excessive oxygen radicals attack cell membranes and affect cell calcium pools, such as calcium ion channels and intracellular endoplasmic reticulum, causing an imbalance in calcium metabolism. This study is based on methods reported in literature\(^{26}\), wherein rats with high-calcium and low-calcium diets were exposed to fluoride. The results indicated that high dietary calcium fluoride exposure can reverse decreases or significantly decrease hippocampal synaptic density, length of synaptic activity area, and maternal-induced PSD thickness of offspring and reverse abnormal changes, such as decreases in synaptic gap width and in synaptic interface structural parameters. However, low dietary calcium induced abnormal changes in synaptic interface structure in hippocampi of rat offspring. These results suggest that the maternal effects of fluoride exposure on brain cells of rat offspring in drinking-water type fluorosis are important. Appropriate levels of dietary calcium maybe one of the effective measures for prevention and control of normal brain development of rat offspring in endemic fluorosis areas.

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