EFFECTS OF FLUORIDE IN DRINKING WATER ON NADPH-DIAPHORASE NEURONS IN THE FOREBRAIN OF MICE: A POSSIBLE MECHANISM OF FLUORIDE NEUROTOXICITY

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SUMMARY: Effects of exposure of Swiss albino adult male mice to 17.6 mg NaF/L in their drinking water for up to 30 days on the NADPH diaphorase (NADPH-d) positive neurons in the forebrain were studied. Histochemical study by the method developed by Hope and Vincent for the distribution of NADPH-d positive neuron cell bodies was conducted in the cerebral cortex, hippocampus, amygdala, caudate putamen, and selected nuclei of the hypothalamus. Counting neuronal cell bodies, their dendritic intersections, and varicosities in the brain of the F-exposed mice showed significant increase in the neuron cell bodies. In the cerebral cortex, amygdala, and caudate putamen, the increase in the number of NADPH-d positive neurons and their dendritic intersections was highly significant ($p<0.05$), but no significant difference was seen in the dendritic branching. A significant increase in the number of varicosities was also observed in the brain of the F-treated mice. These results indicate that excessive F intake caused morphological changes in NADPH-d/NOS (nitric oxide synthase) positive neurons in the brain, thus increasing nitric oxide (NO) synthesis, which is implicated in F-induced neuron cell death. A possible mechanism of F neurotoxicity is thereby suggested.

Keywords: Fluoride neurotoxicity; Mice brain; NADPH-d positive neurons; Nitric oxide synthase (NOS); Nitric oxide and neurons.

INTRODUCTION

Fluoride (F) has been found to have adverse effects on the structure and functions of the animal nervous system.[1,2] Various lines of evidence also indicate that F produces CNS (central nervous system) cellular injury through several mechanisms including free radical generation and excitotoxicity.[3-5] Excitotoxicity stimulates nitric oxide synthase (NOS) activity, causing an increase in intracellular nitric oxide (NO),[6] which combines with superoxide to form peroxynitrite radicals that are implicated in cell death.[7-8] Xu et al.[6] reported that sodium fluoride (NaF) significantly increases NOS activity. Peroxide toxicity and excitotoxicity injury are also associated with selective antioxidant depletion.[9] In the nervous system, NOS reactivity has been consistently co-localized with NADPH-diaphorase (NADPH-d) reactivity.[10] Thus, NOS effects on neurons can be identified with NADPH-d histochemistry.[9-11] NO is a diffusible gaseous free radical, which acts as cellular messenger, serving as a universal modulator of interneuronal communications and is implicated in synaptic plasticity, long-term synaptic potentiation,[10,12,13] and long-term depression.[14] It is also implicated in the pathophysiology of neurodegenerative diseases like Parkinson's, Huntington's, and also in brain ischemia.[15-16] Thus involvement of NO in F toxicity has raised considerable interest for research.

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With these reports in mind, the present study was designed with the specific aim to investigate whether chronic F exposure affects the NADPH-d neuron population in the brain of mice and whether NADPH-d plays a role in F neurotoxicity. The investigation was thus carried out to study the distribution and histochemical and morphometric characterization of NADPH-d positive neurons in the forebrain of mice given NaF in their drinking water.

MATERIALS AND METHODS

Animals: Healthy Swiss albino adult male mice (n = 12, body weight 30±5 g) obtained from Animal House facility, ML Sukhadia University were used for the present study. All protocols were approved by the Institutional Animal Ethical Committee (IAEC, Log. No. 973/ac/06/CPCSEA). Animals were maintained as per institutional norms. The mice were given water *ad libitum* and were housed in an animal house (three per cage) at 22±5ºC with a 12-hr light/dark cycle.

Treatment: The mice were divided randomly into a control (n = 6) and an experimental (n = 6) group. The control animals were given F-free RO water and food prepared in the laboratory as per details of Gold Mohar animal chow Company, India. The experimental animals were given an estimated 8 mg NaF/kg bw/day in their drinking water (17.6 mg NaF/L), based on an average daily water consumption of 10–15 mL. This dosage was selected on the basis of a pilot study and reports available in the literature.

NADPH-d histochemistry: The method of Hope and Vincent was used to investigate the NADPH-d histochemistry.10 The mice were anesthetized with pentobarbital (35 mg/kg), and perfused transcardially with ice chilled saline followed by freshly prepared 4% paraformaldehyde containing 0.1% glutaraldehyde in 0.1 M pH 7.4 phosphate buffer. The brain was dissected out and afterward suspended in same fixative for 1 hr. Following fixation, the brain tissue was washed in phosphate buffer for 1 hr and kept overnight in a series of 10% to 30% sucrose solutions at 4ºC for cryoprotection. Afterward, 45-µm thick coronal sections passing through the forebrain were cut with Vibratome (Leica). Free floating sections were collected in distilled water and rinsed in 0.2 M 7.6 pH phosphate buffer. After rinsing in distilled water, the sections were incubated for 30–60 min in a medium containing 1.0 mg/mL β-NADPH, 0.2 mg/mL NBT (Nitro blue tetrazolium) and 0.6% Triton X-100 in 0.1 M 7.4 pH phosphate buffer (PBS).

For the negative control, sections were incubated in a medium prepared without β-NADPH. At the end of incubation, the sections were washed in PBS for 4 hr at 8ºC, placed on glass slides, air dried, and mounted with glycerin jelly. Photomicrographs were taken using an Olympus BX51 microscope using a 4× and 40× objective. Neuroanatomical identification of various areas in each section was done by using the stereotaxic atlas of Franklin and Paxinos.[17]

Quantitative analysis: For neuronal cell count and morphometric analysis, six mice brains were used. From each brain, three consecutive sections passing through each part of the forebrain were examined. For NADPH-d positive cell count, the Image-Pro program (Media Cybernetics) was used. NADPH-d neurons in every tenth serial section of the forebrain region (cerebral cortex, hippocampus,

amygdala, caudate putamen, and hypothalamus) of each control and F-treated mouse was screened. In order to obtain an unbiased estimate of cell numbers, Abercrombie's correction factor was applied to total cell count, which compensates for the over counting of sectioned profiles, using the equation: P = A (M/M + L), where P is the corrected value, A is the raw density measure, M is the thickness of the section (in micrometers), and L is the average diameter of cell bodies along the axis perpendicular to the plane of section. Descriptive statistics were applied to all groups, and differences among groups were evaluated using the t test. Camera Lucida tracing of five neurons from each brain region was carried out at 40× magnification, using an Olympus BX51 microscope. Sholl analysis[18] was performed on all these neurons. At the same magnification, with the aid of a stage micrometer, concentric circles were drawn on tracing paper at 10-µm equivalent intervals. The number of dendritic intersections was counted in successive radial segments of 10-µm distance, taking into consideration the center of the soma as a reference point. The places at which dendrites cross the concentric circles were considered as the points of intersection. The intersections were studied up to a distance of 100 µm.

Varicosity density, defined as the number of swollen or bulbous structure per unit length of process, was estimated by photographing dendrite segments. The exact length of the dendritic segment, as calculated at the proximal part of all the branch orders (magnification of 1000×). The number of swollen structures along that length to express the varicosity density.

Statistical analysis: The data collected from the neuronal cell count, varicosity densities, and number of intersections were analyzed using Prism (GraphPad Software, Inc.). Student's t-test was applied to compare the groups. $p<0.05$ was considered significant.

RESULTS

Distribution and morphological characteristics of NADPH-d positive neurons: Medium- sized multipolar NADPH-d positive neurons were scattered throughout all regions of the cerebral cortex of the control (Figure 1A) and the F-treated mice (Figure 1B).

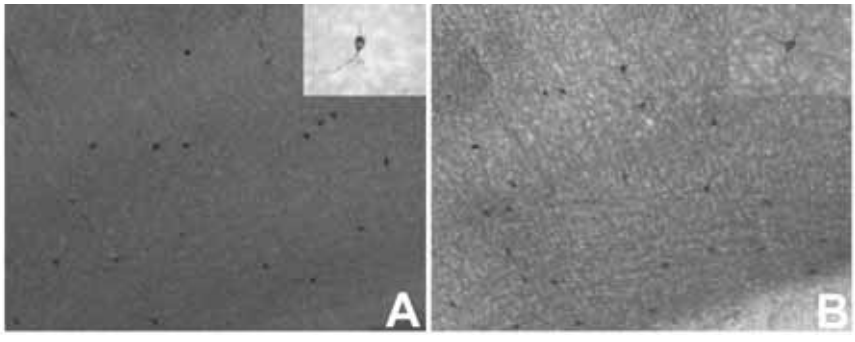

Figure 1A & 1B. NADPH-diaphorase histochemistry of control and fluoride-treated mice brain sections. (A) NADPH-d positive cell bodies in cerebral cortex region of control brain (4×). (B) NADPH-d positive cell bodies in cerebral cortex region of fluoride-treated brain (4×).

NADPH-d positive cell bodies were also observed in the piriform cortex region (Pir) and the dorsal endopiriform claustrum (Den). In the control very few such cells were observed in the hippocampus (Figure 1C), but in the F-treated brain, NADPH-d positive neurons were found in the oriens, pyramidal, and stratum radiatum layers of the hippocampus (Figure 1D).

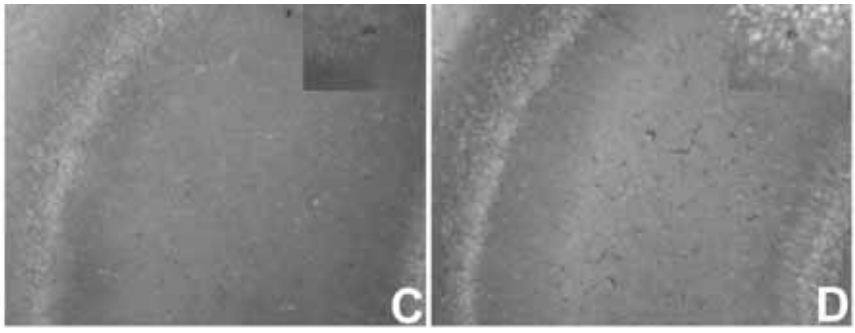

Figure 1C & 1D. NADPH-diaphorase histochemistry of control and fluoride-treated mice brain sections. (C) NADPH-d positive cell bodies in hippocampus (CA1) of control brain. Note few and small neuron cell bodies (4×). (D) NADPH-d positive cell bodies in hippocampus (CA1) of fluoride-treated brain. Note though very few but intensely positive neuron cell bodies observed in LM (Lacunosum moleculare layer) region (4×).

Scattered neuron cell bodies were also observed in the oriens layer (OY), the CA1 area, and the CA3 area of the hippocampus. In the Lacunosum moleculare layer (LM), only a few small sized elliptical cells were found, but dense varicose fibers were present in all seven layers of the hippocampus. Cell bodies showed differential intensity of NADPH-d reactivity. Cell bodies in the cortex and oriens layer of the CA1 area were intensely reactive to NADPH-d for staining, but in other areas examined cell bodies were weakly reactive. In the dentate gyrus, bipolar and tripolar cell bodies and fibers were detected in the polymorphic and moleculare and granular layer. In the amygdaloidal area, darkly stained fibers were observed. In control brain, amygdala region as well, few NADPH-d positive cells were observed (Figure 1E).

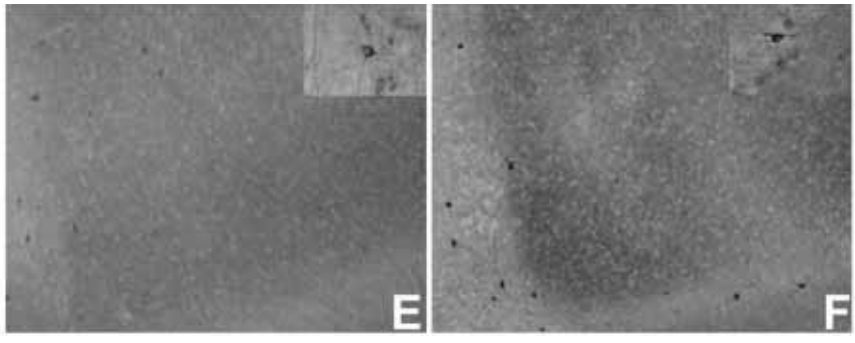

Figure 1E & 1F. NADPH-diaphorase histochemistry of control and fluoride-treated mice brain sections. (E) NADPH-d positive cell bodies in amygdaloidal region of control brain. Note few NADPH-d positive cell bodies in lateral nuclei (4×). (F) NADPH-d positive cell bodies in amygdaloidal region of fluoride-treated brain. Note more intensely positive cell bodies in lateral region and fibrous region in CeM, BMA, BLA, etc. (4×).

A dense network of NADPH-d positive fibers and many medium-sized darkly stained monopolar fusiform, bipolar oval shaped, and tripolar pyramidal shaped cells were observed, especially in basomedial amygdaloidal nucleus (BMA) of the anterior amygdala (Figure 1F) central amygdala nucleus (CeM) in the F-treated brain. Dense varicosities were observed through the entire length of fibers of NADPH-d positive neurons in the basomedial amygdaloidal and central amygdaloidal nucleus of this region. In comparison to control (Figure 1G), medium-sized, moderately stained, monopolar, bipolar, and multipolar cells and a moderately dense fiber network were also observed in the caudate putamen area of the F-treated brain (Figure 1H). Most of the fibers appeared smooth but some showed a few varicosities.

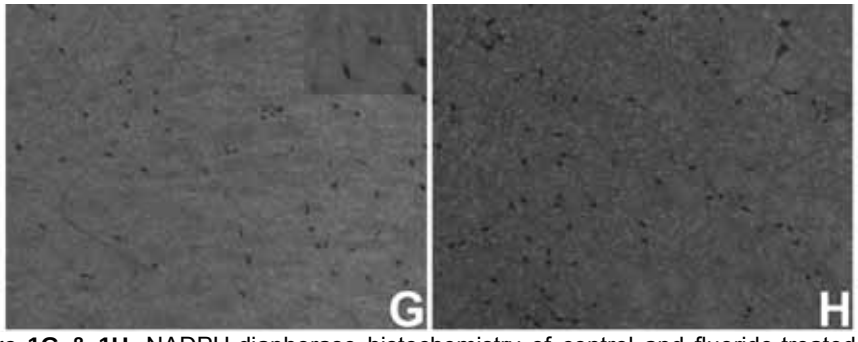

Figure 1G & 1H. NADPH-diaphorase histochemistry of control and fluoride-treated mice brain sections. (G) NADPH-d positive cell bodies in caudate putamen region of control brain (4×). (H) NADPH-d positive cell bodies in caudate putamen region of fluoride-treated brain. Note increase in cell bodies (4×).

NADPH-d positive fibers were also seen in the lateral hypothalamic area of control brain (Figure 1I). The most concentrated region was the dorsal hypothalamic area and the dorsomedial hypothalamic nucleus. These areas also contained numerous medium-sized, mildly stained oval shaped cell bodies. Such staining was absent in the F-treated brain (Figure 1J).

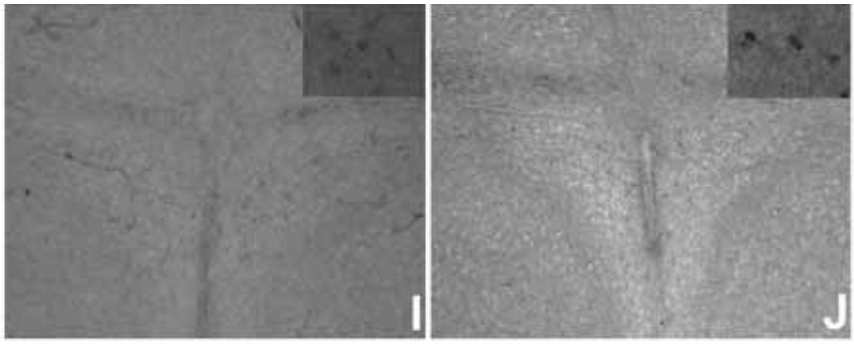

Figure 1I & 1J. NADPH-diaphorase histochemistry of control and fluoride-treated mice brain sections. (I) NADPH-d positive cell bodies in hypothalamus region of control brain (4×). (J) NADPH-d positive cell bodies in hypothalamus region of fluoride-treated brain (4×).

In the hypothalamic area, the NADPH-d positive neuron cell bodies were observed in the paraventricular nucleus (Pa), the nucleus of the horizontal limb of the diagonal band (HDB), the parvocellular nucleus (PaPc), and the lateral

hypothalamic area (LH). In some areas including the CeM, GP (Globus pallidus), etc., moderately stained processes were also observed. Moreover, positive cell bodies were noticed in the putamen area. Thus results showed that F treatment significantly increased the number of NADPH-d positive neurons in most of the areas of the brain compared to the control group.

Neuronal cell count: NADPH–d positive neuron cell bodies were counted in specific areas of the cerebral cortex (piriform cortex region), sub regions of hippocampus, various nuclei of amygdale, caudate putamen area and nuclei of hypothalamic region of the control and the F-treated group. The mean NAPDH-d positive neuronal count of each area is presented in Figure 2.

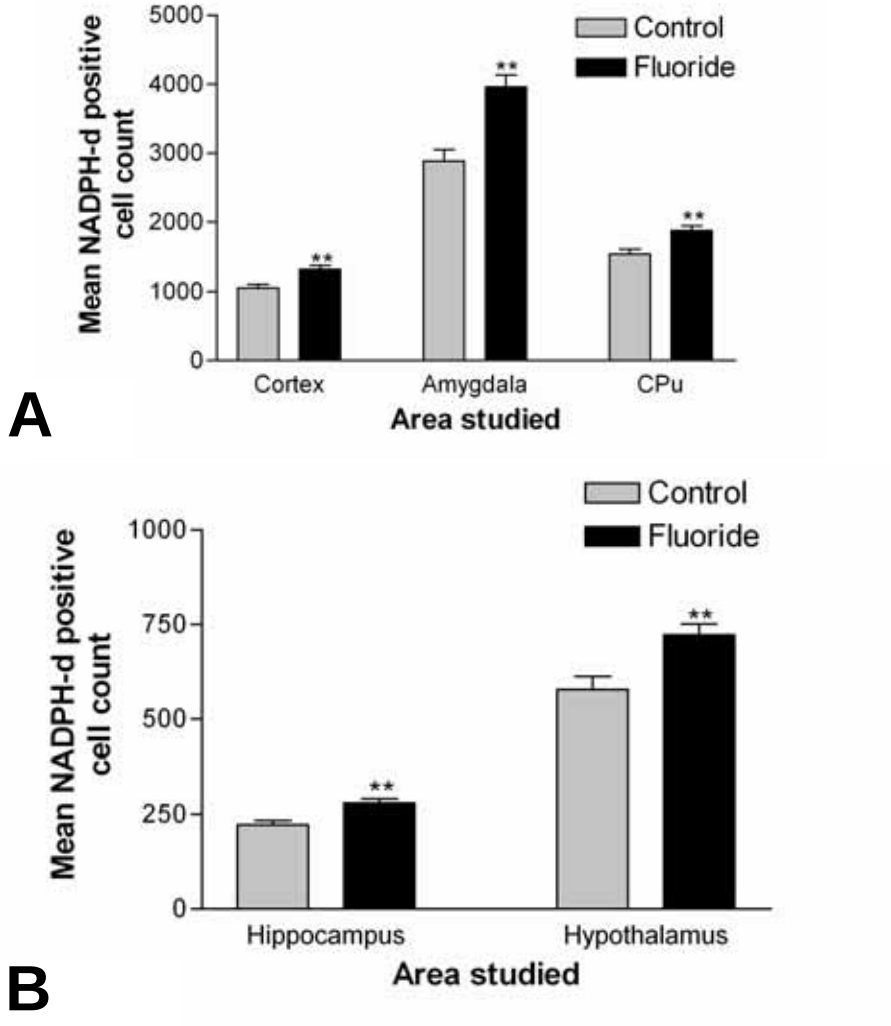

Figure 2. (A) Comparisons of mean of NADPH-d positive cell count in cerebral cortex (piriform cortex region), amygdala (central amygdaloidal nucleus (CeM), basomedial amygdaloidal nucleus (BMA)) and caudate putamen region of control and fluoride-treated mice brain. (B) Comparisons of mean of NADPH-d positive cell count in hippocampus (CA1 region), hypothalamus (paraventricular nucleus (Pa) and parvocellular nucleus (PaPc)) region of control and fluoride-treated mice brain.

In all the areas studied, a significant increase in the number of NADPH-d positive cell bodies was observed in the F-group compared to the control. The amygdale and hypothalamus, however, had more significant increase.

Branching pattern: A non-significant change in the number of branching points was observed in all the areas of the F-treated brain (Figure 3d).

Dendritic intersections: The complexity of dendritic trees in the F-treated and control brains was assessed using the Sholl analysis.[17] Observations clearly indicate an increase in the dendritic intersections (Figures 3 and 4).

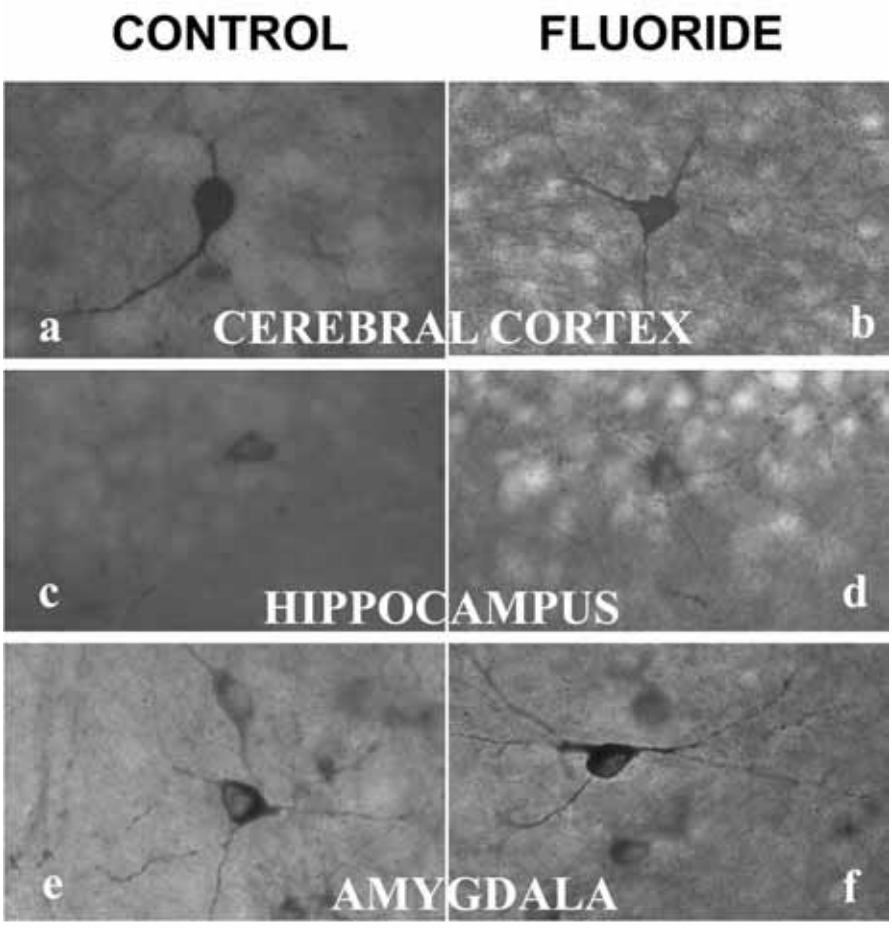

Figure 3a-3f. NADPH-d positive neurons in cerebral cortex, hippocampus, and amygdala regions from control (a, c, e) and fluoride-treated (b, d, f) mice brain at higher magnification (40×).

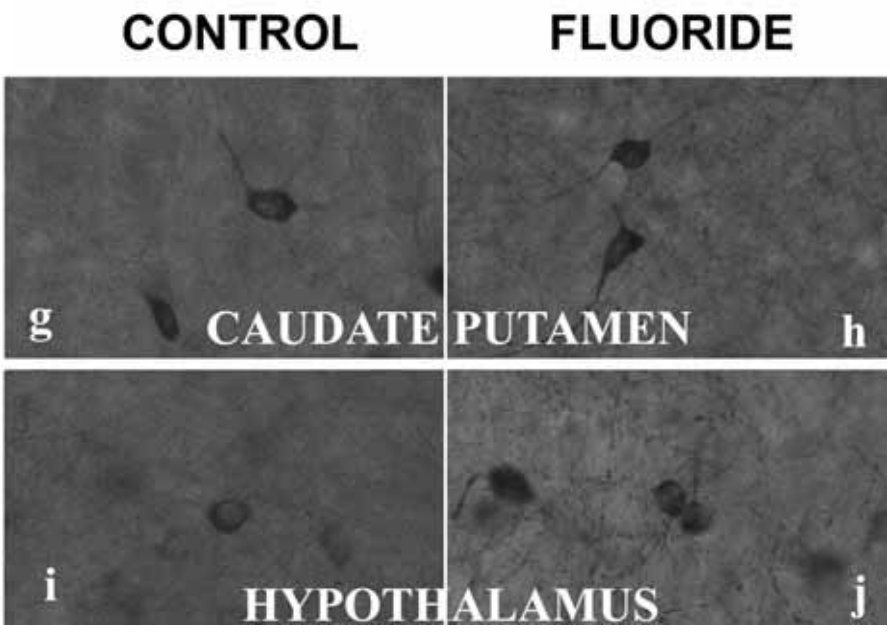

Figure 3g-3j. NADPH-d positive neurons in caudate putamen and hypothalamus regions from control (g, i) and fluoride-treated (h, j) mice brain at higher magnification (40×).

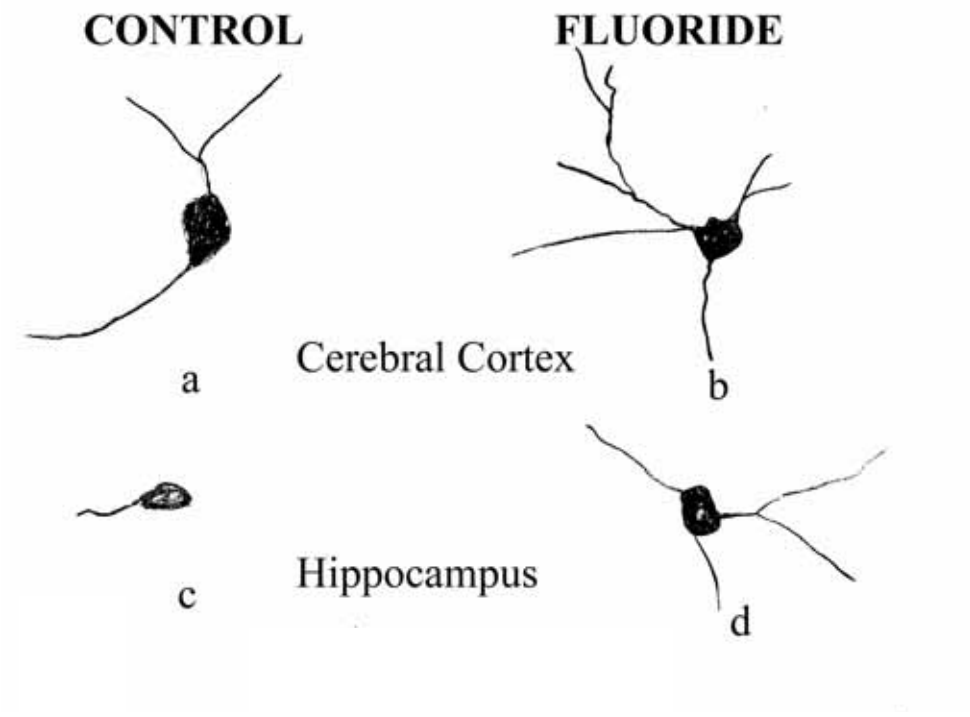

Figure 4a-4d. Camera Lucida tracings of NADPH-d positive neurons in cerebral cortex and hippocampus regions from control (a, c) and fluoride-treated (b, d) mice brain.

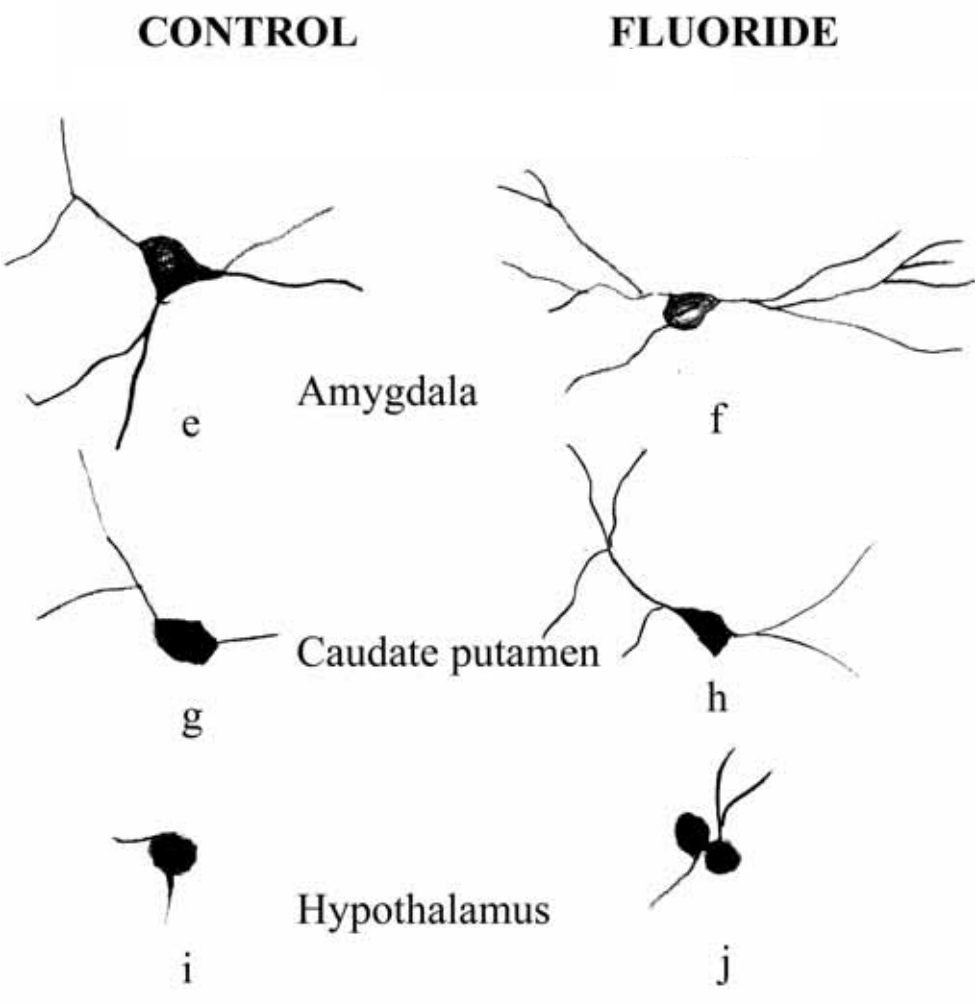

Figure 4e-4j. Camera Lucida tracings of NADPH-d positive neurons in amygdala, caudate putamen, and hypothalamus regions from control (e, g, i) and fluoride-treated (f, h, j) mice brain.

Results are expressed as the mean number of dendritic intersections ±SD, summarized in Figure 5A-5D).

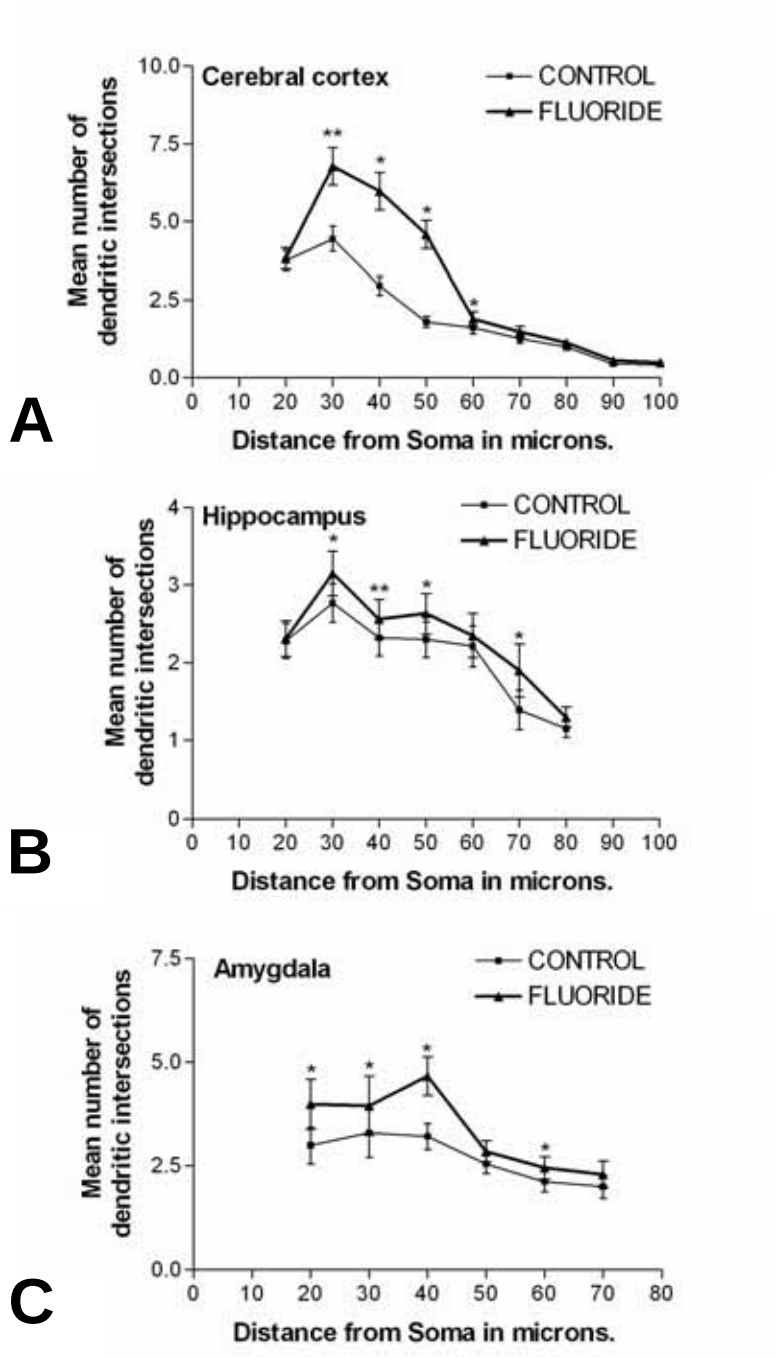

Figure 5A-5C. Dendritic intersections (Sholl analysis) of NADPH -d positive neurons. (A) cerebral cortex (Pir) region, (B) hippocampus region, (C) amygdala in control and fluoride-treated brain of mice. Each point represents the mean of 5-7 neurons from each mice (SD not shown). Note significant increase in the dendritic intersection at 20, 30, 40, 50, 60, 70 or 80 µm distance from the soma respectively. Mice treated with 8 ppm sodium fluoride. Control versus fluoride. *p < 0.05, **p < 0.05. (t- Test).

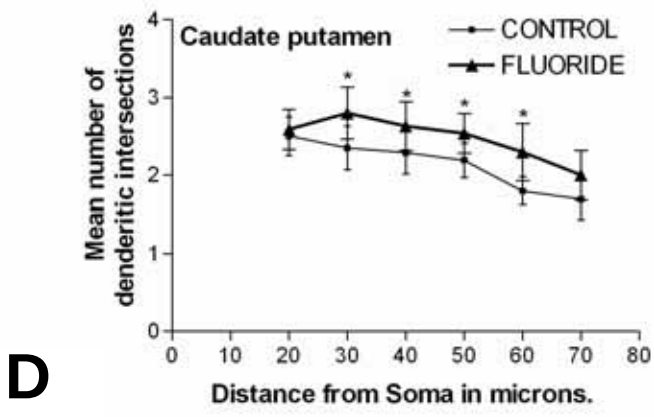

Figure 5D. Dendritic intersections (Sholl analysis) of NADPH -d positive neurons. (D) caudate putamen region in control and fluoride-treated brain of mice. Each point represents the mean of 5-7 neurons from each mice (SD not shown). Note significant increase in the dendritic intersection at 20, 30, 40, 50, 60, 70 or 80 µm distance from the soma respectively. Mice treated with 8 ppm sodium fluoride.
Control versus fluoride. *p < 0.05, **p < 0.05. (t- Test).

Statistical analysis showed a significant increase in the dendritic intersections in the cerebral cortex, hippocampus, caudate putamen, and amygdale of the F-group at various distances from the cell body. Data are summarized in Table 1.

Table 1. Increase in dendritic intersections at various distances from the cell body in selected brain areas (Sholl analysis)[a]

Area	30 µm	40 µm	50 µm	60 µm	70 µm
Cerebral cortex	0.0083	Nc	Nc	Nc	Nc
Hippocampus	0.0334	0.0117	0.0432	0.0467	0.0146
Caudate putamen	0.0308	0.0168	0.0286	0.0136	Nc
Hypothalamus	Nc	Nc	Nc	Nc	Nc
Amygdala	0.0404	0.0117	0.0428	0.0467	Nc

[a]Data represent p values. Nc = no change

Dendritic varicosities: Dendritic varicosities were studied in three NADPH-d positive neurons traced from each control and F-treated brain area. The actual spacing of varicosities in individual dendritic segments varied greatly. The individual dendritic segment has zero to four varicosities in the control and zero to seven varicosities in the F group. As the varicosities were visualized only partially,

their components were not analyzed further. The analyzed data are summarized in the Table 2.

Table 2. Dendrite varicosities as means±SD in the control and F-treated mice brain

Sample features	Control			Fluoride		
	Total	Average (µm)	Range (µm)	Total	Average (µm)	Range (µm)
Dendrite segment (n=12)	52	4.3±2.0	2–8	91	8.0±2.0	3–10
Varicosities/ dendrites segment	23/12	1.9±1.4	0–4	61/12	5.1±1.4	0–7
Intervaricosity spacing	52/23	2.3	0.5–3.3	91/61	1.5	0.5–3.9

DISCUSSION

Enzyme nicotinamide adenine dinucleotide diaphorase (NADPH-d) positive neurons are a subpopulation of inhibitory neurons in the mammalian central nervous system.[19] Earlier distribution of NADPH-d has been described in mice brain.[20-22] These neuron cell bodies were observed in all the areas of the forebrain (with brain sections passing through Bregma –0.94 mm to –4.04 mm according to Franklin and Paxinos in their Mice Brain Atlas[17]) including the hippocampus, thus demonstrating varied morphology, i.e., poorly ramified bipolar neurons to highly ramified multipolar neurons as reported earlier.[11] The present study for the first time also demonstrated that NADPH-d positive cell bodies and fibers are present throughout the forebrain region of F-treated mice. Interestingly, in the cerebral cortex, hippocampus, amygdale, caudate putamen, and hypothalamic nuclei of F-treated mice brain, a significant increase in number of NADPH-d positive neurons as well as the enzyme reaction intensity was observed as compared to the control brain. These neuron cell bodies were of two types, intensely stained Golgi type I appearance and small weakly stained neurons. Morphological characterization of these cell bodies in each region studied showed a significant increase in the number of intersections in F treated brain when compared with control brain. The change was more significant in the hippocampus, cerebral cortex, and amygdala. In certain areas like the hypothalamus, dendritic arborization was poor but in the cerebral cortex, hippocampus, amygdala, and caudate putamen, complex dendritic arborization was observed. Neurons showing weak enzyme reactivity showed less dendritic arborization, but those with intense reactivity to staining showed more dendritic arborization. In addition to dendritic branching, dendritic varicosities were also examined in three NADPH-d positive neurons traced from each control and F-treated brain area. The actual spacing of varicosities in individual dendritic segments varied greatly. The individual dendritic segment had zero to four varicosities in the control and zero to seven varicosities in the F group.

Dendrites are vulnerable to neuronal injury, particularly during pathological conditions. As excitatory dendritic contacts are made predominantly on dendritic arbours, the dendrites have been proposed to be the initial sites of glutamate-mediated excitotoxic injury, which is characterized by formation of varicosities along the length of dendritic arbour.[23,24] These changes have been observed previously in various pathological conditions including hypoxia, epilepsy, brain tumour, aging, and in neurodegenerative diseases such as Parkinson's and Alzheimer's.[25]

Earlier it has been reported that dendritic varicosities result from osmotic swelling due to ionic balances imposed by glutamate receptor activation.[25] Increased NADPH-d intensity associated with increased dendritic branching and varicosities in F treated brain indicates injurious effects of F. Further investigation is required in order to ascertain why NADPH-d neurons undergo such dynamic change in morphology in response to F. It seems likely that an increased extracellular influx of Ca^{++} reported during formation of varicosities and intersections may activate NOS and thus release NO, which ultimately can cause injury to neurons.[26] Xu et al.[6] have also shown that NaF significantly increases NOS (nitric oxide synthase) activity. This NOS increase releases NO, which combines with superoxide radicals to form peroxynitrites,[27] a highly toxic radical responsible for neuronal injury, a phenomenon seen here in Figure 6.

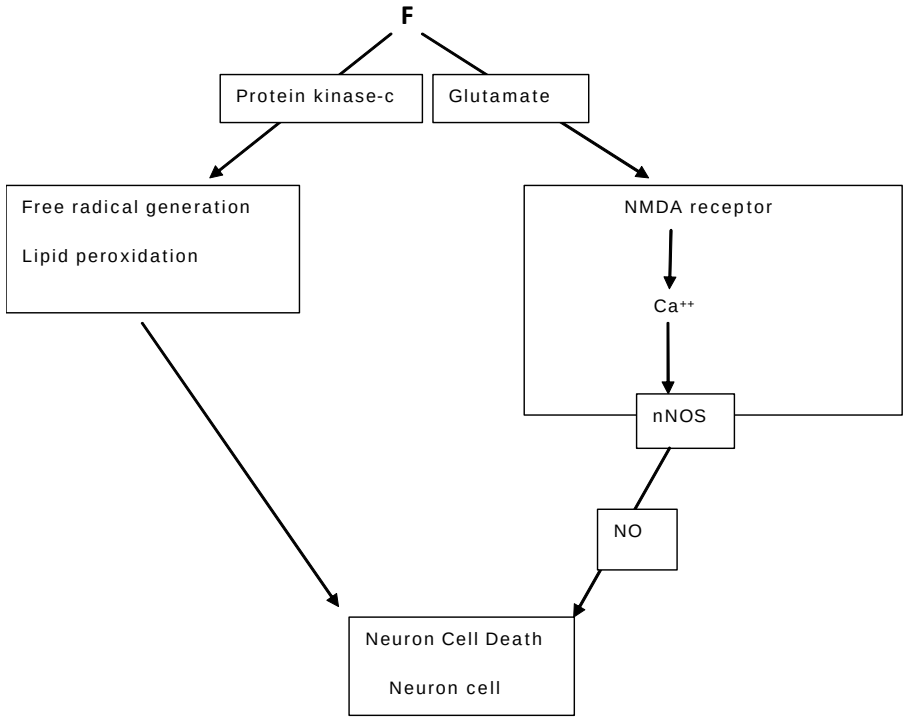

Figure 6. Mechanism of F-induced neurotoxicity.

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