Reproductive Toxicology 81 (2018) 108–114

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

Alterations in the memory of rat offspring exposed to low levels of fluoride during gestation and lactation: Involvement of the α7 nicotinic receptor and oxidative stress

Mariana Bartos[a,*], Fernanda Gumilar[a], Cristina E. Gallegos[a], Cristina Bras[a], Sergio Dominguez[a], Nina Mónaco[a], María del Carmen Esandi[b], Cecilia Bouzat[b], Liliana M. Cancela[c], Alejandra Minetti[a]

[a] Toxicology Laboratory, INBIOSUR, Departamento de Biología, Bioquímica y Farmacia, Universidad Nacional del Sur-CONICET, 8000 Bahía Blanca, Buenos Aires, Argentina
[b] INIBIBB, Departamento de Biología, Bioquímica y Farmacia, Universidad Nacional del Sur-CONICET, 8000 Bahía Blanca, Buenos Aires, Argentina
[c] IFEC, Departamento de Farmacología, Universidad Nacional de Córdoba-CONICET, 5000, Córdoba, Argentina

ARTICLE INFO

Keywords:
Fluoride
Development exposure
Memory
Nicotinic receptor
Oxidative stress
Rat offspring

ABSTRACT

Daily exposure to fluoride (F) depends mainly on the intake of this element with drinking water. When administered during gestation and lactation, F has been associated with cognitive deficits in the offspring. However, the mechanisms underlying the neurotoxicity of F remain obscure. In the current study, we investigated the effects of oral exposure to low levels of F during the gestational and lactation periods, on the memory of adult female rat offspring. We also considered a possible underlying neurotoxic mechanism. Our results showed that this exposure reduced step-down latency in the inhibitory avoidance task, and decreased both mRNA expression of the α7 nicotinic receptor (nAChR) and catalase activity in hippocampus.

Our data indicates that low F concentrations administrated during gestation and lactation decrease the memory of 90-day-old female offspring. This suggests that the mechanism might be connected with an α7 nAChR deficit in the hippocampus, induced by oxidative stress.

1. Introduction

The most important factor contributing to fluoride (F) exposure is its content in drinking water [1,2]. In the World Health Organization (WHO) guideline [3] the permissible limit of F in drinking water is 1.5 mg/l. Beneficial effects of F are achieved with low concentrations (0.8–1.2 mg/l) in drinking water and by mixing it with dental paste (1000 ppm and above) [1,4]. However, among the 25 countries that have naturally occurring high F concentrations (> 1.5 mg/l) in groundwater, such as China, India, México and Argentina [5], more than 200 million people suffer from endemic fluorosis [6]. In Argentina, in particular in some areas of the Chaco-Pampean plain, shallow groundwater with very high F concentrations (11.5 mg/l) has been found, which may lead to a potential risk of fluorosis [5].

F exists in drinking water in an ionic form and, following ingestion, rapidly passes through the intestinal mucosa where it interferes with metabolic pathways of living systems [7]. F is a cumulative poison [2]. On average, only 50% of the F ingested by our body each day is excreted through the kidneys while the remaining accumulates in tissues [8,9]. In the organisms of infants and children, about 80–90 % of the absorbed F is accumulated [2]. F is biologically active even at very low concentrations (equal to the 1 ppm in fluoridated drinking water) [10]. F can cross the placenta barrier and diffuse into cord blood [11]. In addition, the significant high F in breast milk indicates the accessibility of fluoride for infants [12,13]. Young individuals are less resistant to the toxic influence of F due to the fact that their defensive mechanisms are not fully developed and the permeability of their blood-brain barrier is higher than among adults [14]. In recent years, scientists have focused on the toxic influence of this element on the nervous system [15]. Epidemiological studies have found that the levels of mental work capacity and the Intelligence Quotient (IQ) are lower for children in the areas with endemic fluorosis as compared to reference areas [16–19]. F accumulation over a period of time has been shown to cause significant neurological damage and neuro-degenerative disorders in animals

* Corresponding author at: Toxicology Laboratory, INBIOSUR, Departamento de Biología, Bioquímica y Farmacia, Universidad Nacional del Sur-CONICET, San Juan 670, 8000 Bahía Blanca, Argentina.
E-mail address: mbartos@criba.edu.ar (M. Bartos).

https://doi.org/10.1016/j.reprotox.2018.07.078
Received 24 January 2018; Received in revised form 3 July 2018; Accepted 12 July 2018
Available online 29 July 2018

[20,21]. Supported by numerous animal experimental studies, the hippocampus has been postulated to be one of the neurotoxic target sites [22–24]. Prolonged exposure to F in the prenatal and postnatal stages of development has a toxic influence on the metabolism of neurons and glia which results in disorders in memory and in learning processes [25–27]. However, the exact mechanisms by which F decreases cognitive and learning abilities and causes memory loss are not clear.

Neuronal nicotinic receptors (nAChRs) are a class of neurotransmitter-gated ion channels present throughout the central nervous system (CNS). nAChRs are involved in cognitive functions, such as learning, memory and attention and executive function, both in humans and in animals [28–31]. Neuronal nAChRs can be homomeric, composed of an α-type subunit, such as α7, or heteromeric, which includes α and non-α subunits. To date, nine different α (α2-α10) and three β (β2-β4) subunits have been cloned in CNS. α7, α4β2, and α3β4 nAChRs are present in the hippocampus [32]. The α7 nAChR has received considerable attention as a consequence of its high expression in the hippocampus and in the neocortex, its ability to form homo-oligomeric receptors, its involvement in several types of learning and memory-related behavior, and its neuroprotective effect [30,33,34]. The key role of the hippocampus in the formation of many forms of memory, including inhibitory avoidance and maze tasks, has been well-documented [35]. Importantly, the α7 nAChR deficit might be connected with functional disorders of the brain and the decreased IQ influenced by fluorosis [36]. Therefore, it is interesting to understand the mechanisms by which the fluorosis produced a decrease in this nAChR.

Oxidative stress is caused by a disturbance in the balance between the synthesis of reactive oxygen species and the activity of anti-oxidative enzymes [37]. The products and inescapable by-products of oxidative metabolism can damage macromolecules like nucleic acids, proteins, and lipids [12]. The CNS is especially sensitive to free radical oxidative damage as it contains high levels of iron, easily oxidisable fatty acids, low antioxidant defense system, and it uses large amounts of oxygen [38]. Moreover the heterogeneity of the developing nervous system, with different cell types and function, makes it more vulnerable to environmental contaminants than the adult nervous system [39]. A certain amount of oxidative damage takes place even under normal conditions; however, the rate of this damage increases during fluorosis, as the efficiency of antioxidative and repair mechanisms decreases leading to oxidative stress in neurons and glia [25,37]. Several previous studies revealed that F induces excessive production of oxygen free radicals and caused a decrease in biological activities of some antioxidant enzymes like catalase (CAT) and glutathione peroxidase (GPx). It also produces lipid peroxidation. As an indicator of the level of lipid peroxidation products malondialdehyde (MDA) is assay as thiobarbituric acid-reactive substance (TBARS) [12,13,20,40–44].

Literature is limited regarding the effects of the exposure to low F doses during gestation and lactation on the CNS of the offspring. It is hypothesized that F exposure during gestation and lactation could lead to structural alterations in the neuronal circuit which may later manifest as functional deficits. Since it has been shown previously that female offspring have a greater sensitivity to F effects, compared to male offspring, in neuroconductural studies [45], only the female offspring, of mothers exposed to low F concentrations during the gestation and lactation, were studied. Thus, the purpose of the present work was to study the effect on memory and the underlying effects of the exposure to low levels of F during gestation and lactation of adult female rat offspring. To this end, Wistar rats were exposed to low F concentrations (5 and 10 mg/l) during gestation and lactation. Short-term memory (STM) and long-term memory (LTM) were evaluated by step-down inhibitory avoidance test; the expression level of α7 nAChR mRNA in the hippocampus was determined by real-time PCR; and the antioxidant enzyme activities and lipid peroxidation levels were measured both in the whole brain and in the hippocampus of female adult offspring. To evaluate damage inflicted by oxidative stress, antioxidant enzymes, such as CAT and GPx, along with lipid peroxidation products, such as MDA, were studied as potential biomarkers.

2. Materials and methods

2.1. Materials

Sodium fluoride (NaF) was purchased from Anedra (San Fernando, Argentina).

2.2. Animals

Male and nulliparous female Wistar rats (90–120 days old) were obtained from colonies maintained under specific pathogen-free conditions from our breeding center of the Universidad Nacional del Sur, Bahía Blanca, Argentina. They were maintained under constant temperature (22° ± 1 °C) and under humidity (50–60%) conditions in a 12L:12D cycle (lights on at 7:00 a.m.) and with standard rodent pellet diet and filtered tap water *ad libitum*. In the evening of the proestrus day, they were housed overnight with the male rats. The presence of spermatozoa in the vaginal smears was registered as an index of pregnancy and it was referred to as gestational day 0 (GD 0). Pregnant females were housed individually in cages and were randomly assigned to one of the three following groups: control group (n = 10; filtered tap water), F treated group with 5 mg/l in filtered tap water (n = 10) and F treated group with 10 mg/l in filtered tap water (n = 10), equivalent to doses of 0.6 and 1.2 mg/kg, respectively. Drinking water was changed daily. Dams received the treatment from GD 0 to weaning on postnatal day (PND) 21. Maternal weight gain, food intake and drink consumption were recorded as described before [45]. All pups were weighed and gestation length, litter size and body weight of pups on different PNDs were analyzed as described in Bartos et al., [45]. On PND 21 the female pups of each dam were weaned and housed together according to treatment until PND 90. One female from each litter was randomly selected for the behavioral test, other female from each litter for the α7-AChR expression and another two for the rest of neurochemical determinations in whole brain and in the hippocampus. For memory test we used n = 9–10 per group, and for neurochemical measures we used n = 5 per group. The female offspring that were not utilized in these tests and the full litter of male offspring, were either used for other experiments of our laboratory or were euthanized by using a CO_2 chamber by qualified personnel of our breeding center of Universidad Nacional del Sur.

2.3. Step-down inhibitory avoidance task

Adult female offspring were trained in a step-down inhibitory avoidance paradigm during which stepping-down from a platform presented in a given context was associated with a foot shock, resulting in an increase in the step-down latency. The inhibitory avoidance apparatus was a box with a floor consisting of parallel nonrusting steel bars. A 2.5 cm high platform was placed on the left end of the box. Latency of the rats to step down placing the four paws on the grid was measured. Twenty-four hours prior to training, we conducted a habituation of rats to the new environment, which consisted of placing the rat on the platform and leaving freely explore for 180 s. In the training session, the animals were gently placed on the platform and they received a 0.6 mA, 2 s scrambled foot shock immediately after they stepped down placing their four paws on the grid. Test sessions were carried out 90 min (STM) and 24 h (LTM) after training. They were exactly like the training session, except that the foot shock was omitted. A 180 s ceiling was imposed on test session latency measurements. In the test sessions, step-down latency was used as measure of memory retention [46].

Table 1
Gene-specific primer sets and q-PCR parameters.

Gene name	Primer secuences		Product (bp)
α7 nAChR	Sense	5´-GCTGTACAAGGAGCTGGTCA- 3´	116
	Antisense	5´-TGGATGTGGATGAGAAGAAC- 3´	
GADPH	Sense	5´-TTCACCACCATGGAGAAGGC-3´	221
	Antisense	5´-AGTGATGGCATGGACTGTGGTC-3´	

2.4. Reverse transcription and real time PCR (RT-qPCR) analysis

Total RNA in hippocampal tissue was isolated by TRIZOL reagents (Sigma, Argentina). For each sample 1.5 μg of total RNA was converted into cDNA with Moloney Murine Leukaemia virus reverse transcriptase (MLV-RT; Promega, USA) and random primers (Promega, USA). The gene-specific primer sequences sets and the respective qPCR product sizes are listed in Table 1. Relative quantification of mRNA by qPCR was performed using a Rotor-Gene 6000 (Corbett Reasearch, Australia). The comparative CT method was used to determine the relative gene expression [47]. The α7 nAChR transcript levels were estimated by using the formula $2^{-\Delta Ct}$ where ΔCt represents the difference in Ct values between α7 and GAPDH, the endogenous control gene [48].

2.5. Preparation of brain homogenates

90-day-old female offspring were sacrificed by decapitation. The heads were dissected and brains removed, rinsed briefly in ice cold isotonic saline and were immediately separated into hippocampus [49] or preserved as a complete brain. The whole brain or the hippocampus were homogenized and centrifuged. The supernatant was kept cold further determination of enzymes activities and lipid peroxidation level.

2.5.1. Lipid peroxidation assay

As a level indicator of lipid peroxidation products presented in the whole brain, malondialdehyde (MDA) was assayed as thiobarbituric acid-reactive substance (TBARS) [50]. Finally, the level of MDA was quantitated by a spectrophotometer at wavelength of 532 nm. MDA concentration was expressed as nmol/mg protein.

2.5.2. Catalase (CAT) assay

CAT activity of both the whole brain and the hippocampus was assayed following the procedure of Aebi [51] with slight modifications. The catalase activity was measured by calculating the rate of H_2O_2 degradation, the substrate of the enzyme. The enzyme activity was expressed as the rate constant of a first-order reaction (k) related to the protein content.

2.5.3. Glutathione peroxidasa (GPx) assay

GPx activity of both the whole brain and the hippocampus was measured by the method used by Lawrence and Burk [52]. The absorbance was recorded at 340 nm. The enzyme activity was expressed as μmoles of NADPH oxidized per min per mg protein.

2.5.4. Protein assay

Protein content was determined by the micromethod of Bradford [53] with bovine serum albumin (BSA) as the standard protein.

2.6. Statistics

Step-down latencies were presented as mean values ± SEM and their statistical significance was evaluated using the non-parametric followed by Mann-Whitney *U* test

Data of oxidative stress and α7 nAChR mRNA levels were analyzed by one-way ANOVA and the differences between groups were assessed using LSD *post hoc* test. Probability values lower than 0.05 were considered to be significant. All statistical analyses were carried out using software SPSS 21.0 for Windows.

2.7. Ethics

Animal care and handling were in accordance with the internationally accepted standard Guide for the Care and Use of Laboratory Animals [54] as adopted and promulgated by the National Institute of Health. Experimental designs were also approved by the local standard for protecting animal's welfare, Institutional Committee for the Care and Use of Experimental Animals of Universidad Nacional del Sur, Argentina. Number of animal protocols 020/2014 and 021/2014.

3. Results

3.1. Data about the dams and their litters

There were no statistical differences in body weight between the groups of dams on GD0, in maternal weight gain, food intake and drink consumption during the different periods registered, and in gestational length or litter size (Fig. 1 and Table 2), as observed in our previous study [45]. Also, F treatment during gestation and lactation did not affect the body weight of pups at PND 1, 4, 7, 10, 13, 16, 19, 21 and 90 (Table 2). No external malformations were recorded in any of the groups tested.

3.2. Step-down inhibitory avoidance test

To determine the consequences of the exposure to 5 or 10 mg/l F on STM and LTM retention, we examined the performance of female 90-day-old offspring exposed to F, during gestation and lactation.

Latency of the female offspring exposed to 5 mg/l F did not differ from that of control at the 90 min retention test, although it was significantly shorter at the 24 h retention test ($p < 0.05$) (Fig. 2). However, latency of rats exposed to 10 mg/l F during gestation and lactation were significantly shorter in both short- and long-term memory retention ($p < 0.05$).

Thus, 5 mg/l F blocked long-term memory while leaving short-term memory intact. The offspring exposed to 10 mg/l F did not learn to avoid the electric shock, thus demonstrating that the highest F concentration tested affected the two types of memory.

3.3. Expression of α7 AChR subunit mRNA level in hippocampus

To investigate the expression levels of α7 nAChR mRNA in hippocampus of female 90-day-old offspring exposed during gestation and lactation to 5 or 10 mg/l F we performed RT-qPCR.

Fig. 3 shows the relative expression levels of α7 nAChR mRNA in hippocampus. When compared to the control, no changes were observed at the lowest F concentration studied (5 mg/l). However, offspring exposed during gestation and lactation to 10 mg/l F showed statistically significant decreased levels of the α7 nAChR subunit mRNA in hippocampus ($p < 0.05$).

3.4. Oxidative stress in brain of female adult offspring

We next determined the anti-oxidative enzyme activity and lipid peroxidation in complete brain and hippocampus of female adult offspring exposed to low F concentrations.

Fig. 4 illustrates the activities of enzymatic antioxidants, namely CAT and GPx, as well as the MDA content in the complete brain of the control and experimental groups of adult female offspring exposed to 5 and 10 mg/l F during gestation and lactation. Non statistical

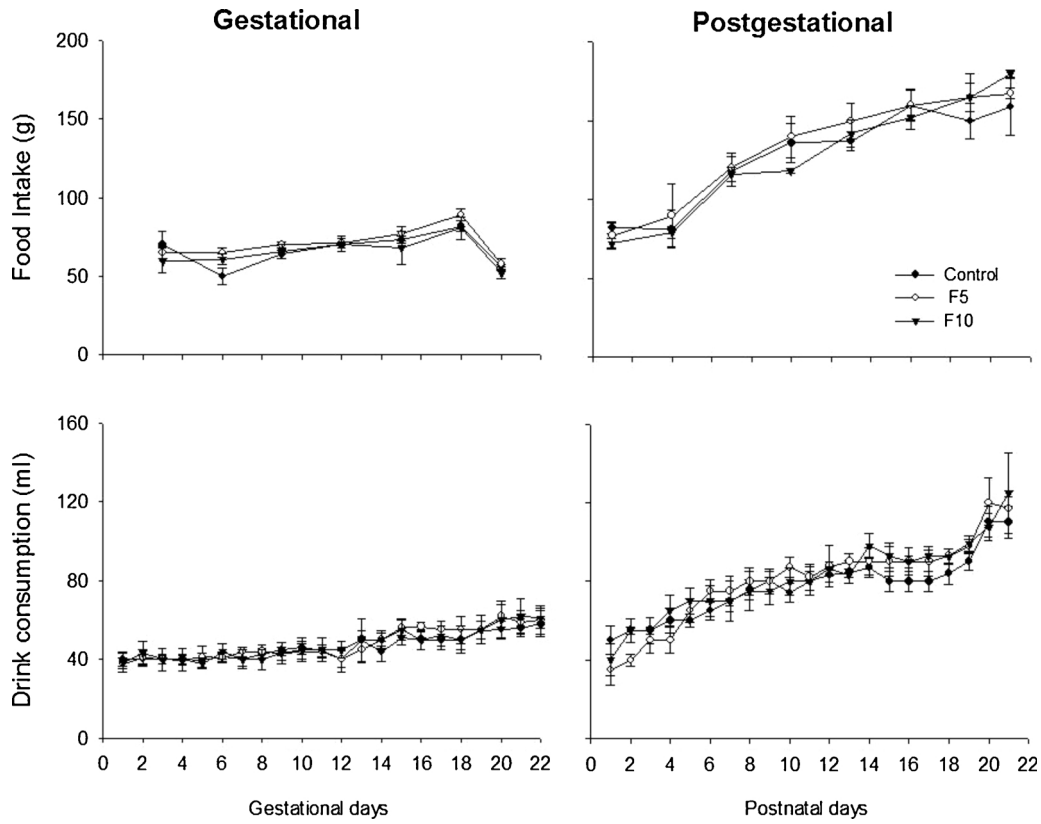

Fig. 1. Food intake and drink consumption of dams exposed to 5 and 10 mg/l F during gestation and lactation period. Food intake of dams was recorded every 3 day whereas drink consumption was recorded daily. All results are expressed as Mean ± SEM of 10 animals per group.

Table 2
Data of dams and their litters.

	Control group n = 10	5 mg/l F n = 10	10 mg/l F n = 10
Body weight of dams (g)			
GD 0	280.0 ± 13.2	275.4 ± 12.6	281.0 ± 12.9
Body weight gain of dams (g)			
GD 0-3	13.2 ± 1.5	15.0 ± 1.0	15.5 ± 0.9
GD 3-6	8.0 ± 1.7	7.3 ± 0.7	7.5 ± 0.5
GD 6-9	11.3 ± 1.5	13.2 ± 1.4	9.8 ± 1.3
GD 9-12	16.9 ± 1.5	13.7 ± 2.2	16.5 ± 2.0
GD 12-15	16.8 ± 1.3	18.6 ± 2.4	14.8 ± 2.5
GD 15-18	31.5 ± 3.0	35.0 ± 2.9	34.0 ± 3.4
GD 18-20	29.2 ± 3.2	28.5 ± 2.5	30.5 ± 5.2
GD 0-20	122.0 ± 4.5	115.6 ± 5.4	131.5 ± 7.2
Length of gestation (days)	22 ± 0	22 ± 0	22 ± 0
Litter size			
Female	5.3 ± 0.9	6.4 ± 0.9	5.9 ± 0.9
Male	5.0 ± 0.6	7.2 ± 0.6	6.0 ± 0.9
Total	10.5 ± 1.0	12.8 ± 2.2	11.3 ± 2.1
Body weight gain of offspring			
PND1	6.5 ± 0.3	6.0 ± 0.2	6.3 ± 0.5
PND4	9.0 ± 0.5	10.4 ± 0.2	9.4 ± 0.3
PND7	12.0 ± 0.7	13.1 ± 0.5	14.0 ± 0.4
PND10	18.0 ± 0.6	17.5 ± 0.2	18.5 ± 0.8
PND13	22.3 ± 1.2	23.3 ± 1.0	21.0 ± 0.8
PND16	28.0 ± 2.2	28.9 ± 1.5	29.2 ± 1.4
PND19	32.0 ± 2.5	33.4 ± 1.5	35.0 ± 1.6
PND21	40.2 ± 2.5	39.5 ± 1.3	38.6 ± 3.1
Body Weight PND90 (g)			
Female	193 ± 7.0	196 ± 9.5	197 ± 7.5

Values are mean ± SEM. GD (gestational day) PND (postnatal day).

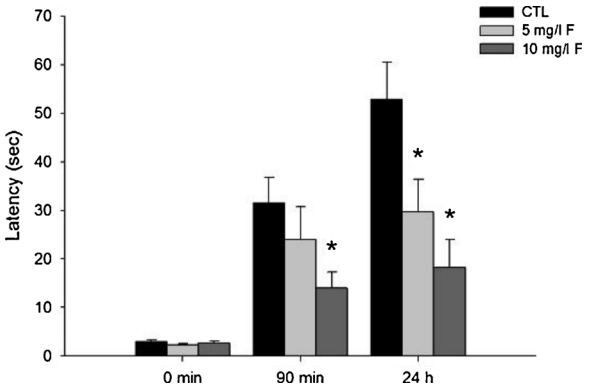

Fig. 2. Latencies measured in the step-down inhibitory avoidance test during the training session (0 min) and the test sessions for the evaluation of STM (90 min after training) and LTM (24 h after training) in the adult female offspring exposed to 5 and 10 mg/l F. Data represent the mean ± SEM latency in seconds of 9–10 animals per group. *p < 0.05, compared to control group.

significance was detected in the experimental groups compared with the controls (Fig. 4A–C), both in the CAT and GPx activity as in the MDA content in the complete brain.

We subsequently continued the analysis of oxidative stress in the hippocampus. Here, we only analyzed CAT and GPx activities since they showed a slight, although non-statistically significant, reduction in the whole brain homogenate (Fig. 4A and B). As shown in Fig. 5A, when offspring were exposed to 5 and 10 mg/l F during the gestation and lactation we observed a decreased CAT activity in hippocampus ($p < 0.001$) compared to the respective control. One-way ANOVA revealed significant differences in groups, $F_{(2,14)} = 30.72$, $p < 0.001$. Non-statistically significant changes were observed in the GPx activity in this brain area when offspring were exposed to either F

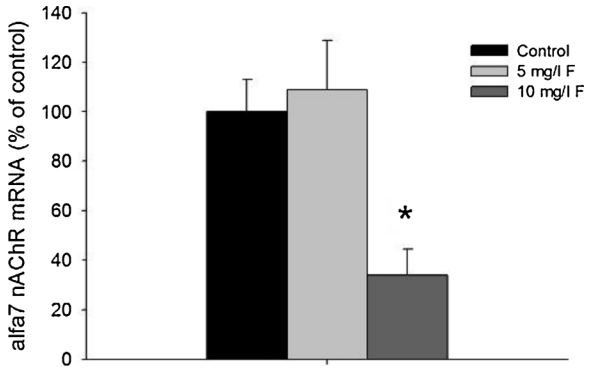

Fig. 3. Expression of nAChR α7 subunit at mRNA level in hippocampus from 90-day-old female offspring exposed during gestation and lactation to 5 and 10 mg/l F. Quantitative real-time PCR was performed to determine mRNA level. The values are shown as the means ± SD of 5 rats. *p < 0.05, compared to control group.

concentrations during gestation and lactation (Fig. 5B).

4. Discussion

Fluoride toxicity in humans has been an area of intense research in the past 2–3 decades [55,56]. With the availability of ample data demonstrating its effect on the CNS [1,2,8,22], it is increasingly important to understand the molecular events leading to nervous system alterations. The exposure begins with F passing through the placenta to the fetus [14] and it continues during infancy through F-containing milk and drinking water. Moreover, the immaturity of excretory or enzymatic systems in developing animals may favor F accumulation [12,57]. The CNS during development is highly sensitive to the influence of F due to its weak protective mechanisms. In the childhood period, exposure to this element may therefore cause permanent damage to the function of all brain structures [39,58]. As previously reported [45], we demonstrated that early exposure to low F concentrations during gestation and lactation does not affects neither maternal weight gain during pregnancy nor gestational length, litter size, or body weight of rat pups on different PNDs as shown in Fig. 1 and Table 2.

However, our findings revealed that the exposure during gestation and lactation to 5 and 10 mg/l F reduced step-down latency in the inhibitory avoidance task in female adult offspring. This impairment in the performance of the step-down inhibitory avoidance task is an effect on memory that might be related to the cognitive and memory alterations in people exposed to F in drinking water. The memory impairment is probably a consequence of the accumulation of F in different parts of brain, especially in hippocampus [12,59,60]. Emotionally arousing experiences create long-term memories that are initially labile, but over time become insensitive to disruption through a process known as consolidation. One-trial fear-motivated learning tasks, such as step-down inhibitory avoidance, have largely contributed to the knowledge of consolidation process [61]. It is known that the differential sensitivity to F neurotoxicity in different brain regions is due to preferential F accumulation and also to alteration of biochemical or cellular processes that are uniquely associated with each particular region [12]. It has been shown that NaF causes neurotoxicity *in vitro,* principally at hippocampal neurons [24]. Histopathological examination of the brain, has confirmed that the hippocampus is the region most affected by F intoxication [59]. Accumulation of F in the nervous system affects the synthesis of neurotransmitters, enzyme activities, receptor expression and neuronal plasticity [21]. The hippocampus is involved in learning and memory. The inhibitory avoidance task is a behavioral test broadly used to evaluate the effects of drug treatment on aversive-memory [46]. Step-down inhibitory (passive) avoidance learning in the rat triggers biochemical events in the hippocampus that are necessary for the retention of this task [46]. It involves learning how to not step down from a platform in order to avoid a mild foot shock. A single learning experience starts a cascade of events, which can lead to different forms of memory: short-term memory that lasts minutes to hours and long-term memory that lasts days, weeks, and even a lifetime [62]. Memory is not a unitary function, it depends on the integrated activity of various brain structures and neurotransmitter systems and it involves multiple receptors, postsynaptic mechanisms, and signal transduction pathways [63]. Among the many neurotransmitter systems, ACh (Acetylcholine) mediates its effects impinging on a variety of nAChR and muscarinic (mAChR) receptors present in the hippocampus, which suggests their involvement in the mechanisms of learning and memory [64].

We found a significantly decreased expression of α7 subunit mRNA in the hippocampus of 90-day-old female offspring exposed to 10 mg/l F. Previous research carried out on rats exposed chronically to 30–100 ppm of F for 7 months showed a significant reduction in nAChRs [65], suggesting that a decrease in the number of nAChRs may play an important role in the mechanism by which F causes dysfunction of CNS. Alterations of nAChRs in association with fluorosis may result from the changes at different steps in receptor synthesis (e.g., transcription, translation and/or post-translational modifications) and in receptor turnover (including membrane insertion). In the present study we showed that the transcription process is affected, which would result in lower levels of α7 receptors at the membrane surface.

The basal forebrain cholinergic complex comprising medial septum, horizontal and vertical diagonal band of Broca, and nucleus basalis of Meynert provides the mayor cholinergic projections to the cerebral cortex and hippocampus. The cholinergic neurons of this complex have been described to undergo moderate degenerative changes during aging and in neurodegenerative diseases, resulting in cholinergic hypofunction that has been related to the progressing memory deficits [66,67]. It appears that hippocampal ACh may not only modulate specific computational function of the hippocampus but it also contributes to the functional coordination of multiple memory systems in a task-dependent manner [66]. In a similar way, pharmacological studies conclusively demonstrate that blockade or stimuli of cholinergic receptors

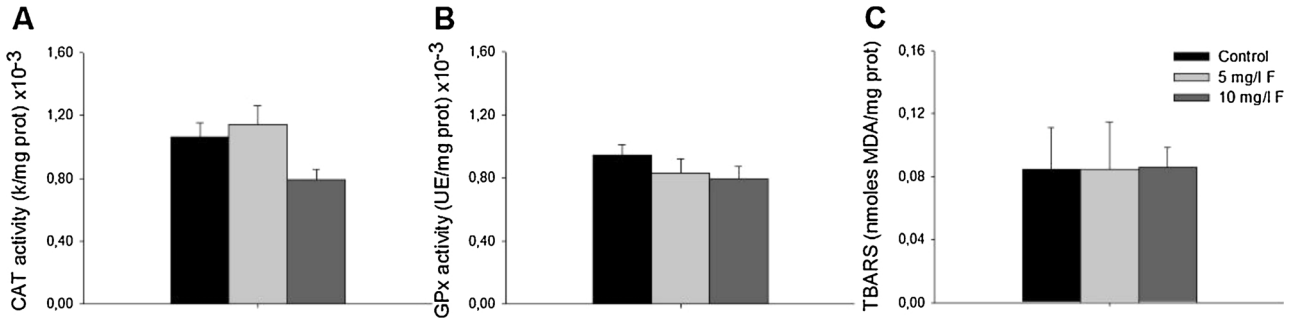

Fig. 4. Activities of CAT (A) and GPx (B), and MDA content (C) in the complete brain homogenates of adult female offspring exposed to 5 and 10 mg/l F during the gestation and lactation. The values are shown as the means ± SD of 5 rats per group.

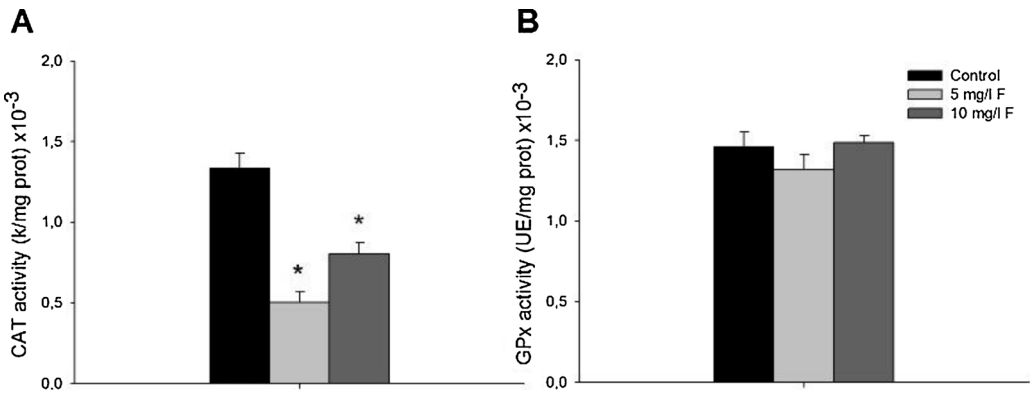

Fig. 5. Activities of CAT (A) and GPx (B) in the hippocampus of 90-day-old female offspring exposed to 5 and 10 mg/l F during the gestation and lactation. The values are shown as the means ± SD of 5 rats per group. *p < 0.001, compared to control group.

by drugs impairs or enhance the encoding of memory [68]. Therefore, the decrease of the α7 nAChR expression in hippocampus could be involved, at least in part, in the loss of memory.

Recently, it has been determined that histaminergic neurotransmission in hippocampus appears critical to provide the brain with the plasticity necessary for LTM but not STM of step-down inhibitory avoidance test and the cyclic adenosine monophosphate (cAMP) signaling pathway is a key step in memory consolidation processing [61,69]. Considering that long-term memory was affected in female adult offspring exposed to both F concentrations, it would be interesting to investigate whether or not this parallel system is affected in offspring after being exposed to low F concentration during gestation and lactation.

Numerous analyses carried out on cell cultures [2] and animal models [15,65,70] confirmed that F accumulation in the brain leads to the increase of the concentration of reactive oxygen species, the decrease of the activity of antioxidative enzymes and the increase of the intensity of lipid peroxidation [37]. A marked increase in oxidative stress, lipid peroxidation and a decrease in the activity of antioxidant enzyme were found in brain regions of rats chronically exposed to 100 mg/l F through drinking water [71]. Furthermore, chronic F exposure (100–200 mg/l) has been found to increase neuronal lipid peroxidation and to decrease the activity of antioxidant enzymes in 3 generations [72]. Starting from conception, the time and magnitude of oxidative stress and the pre and post-natal environment (antioxidant or pro-oxidant) will influence the degree and magnitude of neuronal developmental deficits [12]. About this, previous research has indicated that exposure to high F concentration (500 mg/l) in mice during gestation and lactation can induce to functional changes, metabolic disorders, and histopathological alterations of some organs, and that these changes are due to F-induced oxidative stress of both mothers and their pups [73]. Here we showed that exposure to low levels of F during gestation and lactation produces a decreased of the CAT activity in the hippocampus compared to the respective control. Previous studies involving PC12 cells treated with different concentrations of F (0.1–100 ppm) for 48 h, and in rats exposed to high doses of fluoride (30 and 100 ppm) in their drinking water for 7 months, have shown a decrease in the expression of different nAChRs subunits (α3 and α7) [25]. The F toxicity was mostly prevented by a pretreatment with antioxidant. It is suggested that the F-induced oxidative stress may be involved in deficit of nAChRs in the brain [25]. Other studies with SH-SY5Y cells exposed to F (0.05–5 μM) have also confirmed this hypothesis [15]. Therefore, it is plausible that oxidative stress induced by low F concentration during the gestation and lactation plays an important role in the mechanism of down-regulation of expression of α7 nAChR mRNA and in memory impairment in exposed offspring.

5. Conclusions

Taken together, our findings lead us to conclude that exposure to the two F concentrations tested during gestation and lactation can adversely affect the offspring. F exposure during gestation and lactation, even at low concentrations, alters parameters of the central nervous system functionality, in particular the formation of memory. The persistent decrements in intelligence documented in children, adolescents, and young adults exposed during early life to F may presage the development of neurodegenerative disease later in life. Among the mechanisms postulated to mediate these effects, increased oxidative stress would produce changes in diverse macromolecules that affect the nAChRs expression in the hippocampus of the female adult offspring.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This research was supported by grants from the *Secretaría General de Ciencia y Tecnología* from the *Universidad Nacional del Sur* (UNS; Grant number PGI 24/B223), the National Research Council (CONICET; Grant number PIP 11220130100393CO), and National Agency for the Promotion of Science and Technology of Argentina (ANPCyT; Grant number PICT 2013 939 and PICT 2015 0941). We are grateful to Translator Natalia Difilippo for her technical english assistance.

References

[1] E. Perumal, V. Paul, V. Govindarajan, L. Panneerselvam, A brief review on experimental fluorosis, Toxicol. Lett. 223 (2) (2013) 236–251.
[2] K. Dec, A. Lukomska, D. Maciejewska, K. Jakubczyk, I. Baranowska-Bosiacka, D. Chlubek, A. Wasik, I. Gutowska, The influence of fluorine on the disturbances of homeostasis in the Central nervous system, Biol. Trace Elem. Res. 177 (2) (2017) 224–234.
[3] WHO, Guidelines for Drinking-Water Quality, World Health Organization (WHO), Geneva, 2011.
[4] A.I. Ismail, H. Hasson, Fluoride supplements, dental caries and fluorosis: a systematic review, J. Am. Dent. Assoc. 139 (11) (2008) 1457–1468.
[5] H.B. Nicolli, J. Bundschuh, C. Blanco Mdel, O.C. Tujchneider, H.O. Panarello, C. Dapena, J.E. Rusansky, Arsenic and associated trace-elements in groundwater from the Chaco-Pampean plain, Argentina: results from 100 years of research, Sci. Total Environ. 429 (2012) 36–56.
[6] M. Amini, K. Mueller, K.C. Abbaspour, T. Rosenberg, M. Afyuni, K.N. Moller, M. Sarr, C.A. Johnson, Statistical modeling of global geogenic fluoride contamination in groundwaters, Environ. Sci. Technol. 42 (10) (2008) 3662–3668.
[7] S. Chouhan, S.J. Flora, Effects of fluoride on the tissue oxidative stress and apoptosis in rats: biochemical assays supported by IR spectroscopy data, Toxicology 254 (2008) 61–67.
[8] O. Barbier, L. Arreola-Mendoza, L.M. Del Razo, Molecular mechanisms of fluoride toxicity, Chem. Biol. Interact. 188 (2) (2010) 319–333.
[9] D. Kanduti, P. Sterbenk, B. Artnik, Fluoride: a review of use and effects on health, Materia socio-medica 28 (2) (2016) 133–137.

[10] P. Connett, 50 Reasons to Oppose Fluoridation, (2012) http://fluoridealert.org/articles/dr-paul-connett-responds-to-wichitans-for-healthy-teeth/.
[11] D.K. Fassman, Prenatal fluoridation. A literature review, N. Y. State Dent. J. 59 (6) (1993) 47–51.
[12] P.M. Basha, N. Madhusudhan, Pre and post natal exposure of fluoride induced oxidative macromolecular alterations in developing central nervous system of rat and amelioration by antioxidants, Neurochem. Res. 35 (7) (2010) 1017–1028.
[13] R. Niu, X. Xue, Y. Zhao, Z. Sun, X. Yan, X. Li, C. Feng, J. Wang, Effects of fluoride on microtubule ultrastructure and expression of Tubalpha1a and Tubbeta2a in mouse hippocampus, Chemosphere 139 (2015) 422–427.
[14] L.L. Needham, P. Grandjean, B. Heinzow, P.J. Jorgensen, F. Nielsen, D.G. Patterson Jr., A. Sjodin, W.E. Turner, P. Weihe, Partition of environmental chemicals between maternal and fetal blood and tissues, Environ. Sci. Technol. 45 (3) (2011) 1121–1126.
[15] Q. Gao, Y.J. Liu, Z.Z. Guan, Oxidative stress might be a mechanism connected with the decreased alpha 7 nicotinic receptor influenced by high-concentration of fluoride in SH-SY5Y neuroblastoma cells, Toxicol. In Vitro 22 (4) (2008) 837–843.
[16] X.S. Li, J.L. Zhi, R.O. Gao, Effect of fluoride exposure on intelligence in children, Fluoride 28 (1995) 189–192.
[17] Y. Lu, Z.R. Sun, L.N. Wu, X. Wang, W. Lu, S.S. Liu, Effect of high fluoride water on intelligence in children, Fluoride 33 (2000) 74–78.
[18] Q. Xiang, Y. Liang, L. Chen, C. Wang, B. Chen, X. Chen, M. Zhou, Effect of fluoride in drinking water on children's intelligence, Fluoride 36 (2003) 84–94.
[19] Q.Q. Tang, J. Du, H.H. Ma, S.J. Jiang, X.J. Zhou, Fluoride and children's intelligence: a meta-analysis, Biol. Trace Elem. Res. 126 (1–3) (2008) 115–120.
[20] K. Chirumari, K.R. Pratap, Dose-dependent effects of F on the hippocampus and neocortex of rat brain, Fluoride 40 (2007) 101–110.
[21] M. Bhatnagar, P. Rao, A. Saxena, R. Bhatnagar, P. Meena, S. Barbar, A. Chouhan, S. Vimal, Biochemical changes in brain and other tissues of young adult female mice from fluoride in their drinking water, Fluoride 39 (2006) 280–284.
[22] P.J. Mullenix, P.K. Denbesten, A. Schunior, W.J. Kernan, Neurotoxicity of sodium fluoride in rats, Neurotoxicol. Teratol. 17 (2) (1995) 169–177.
[23] M. Bhatnagar, P. Rao, J. Sushma, R. Bhatnagar, Neurotoxicity of fluoride: neurodegeneration in hippocampus of female mice, Indian J. Exp. Biol. 40 (5) (2002) 546–554.
[24] M. Zhang, A. Wang, T. Xia, P. He, Effects of fluoride on DNA damage, S-phase cell-cycle arrest and the expression of NF-kappaB in primary cultured rat hippocampal neurons, Toxicol. Lett. 179 (1) (2008) 1–5.
[25] K.R. Shan, X.L. Qi, Y.G. Long, A. Nordberg, Z.Z. Guan, Decreased nicotinic receptors in PC12 cells and rat brains influenced by fluoride toxicity–a mechanism relating to a damage at the level in post-transcription of the receptor genes, Toxicology 200 (2–3) (2004) 169–177.
[26] A.L. Choi, G. Sun, Y. Zhang, P. Grandjean, Developmental fluoride neurotoxicity: a systematic review and meta-analysis, Environ. Health Perspect. 120 (10) (2012) 1362–1368.
[27] J. Lee, Y.E. Han, O. Favorov, M. Tommerdahl, B. Whitsel, C.J. Lee, Fluoride induces a volume reduction in CA1 hippocampal slices via MAP kinase pathway through volume regulated anion channels, Exp. Neurobiol. 25 (2) (2016) 72–78.
[28] M.S. Thomsen, H.H. Hansen, D.B. Timmerman, J.D. Mikkelsen, Cognitive improvement by activation of alpha7 nicotinic acetylcholine receptors: from animal models to human pathophysiology, Curr. Pharm. Des. 16 (3) (2010) 323–343.
[29] J.J. Ballesta, C. del Pozo, J. Castello-Banyuls, C.C. Faura, Selective down-regulation of alpha4beta2 neuronal nicotinic acetylcholine receptors in the brain of uremic rats with cognitive impairment, Exp. Neurol. 236 (1) (2012) 28–33.
[30] K.T. Dineley, A.A. Pandya, J.L. Yakel, Nicotinic ACh receptors as therapeutic targets in CNS disorders, Trends Pharmacol. Sci. 36 (2) (2015) 96–108.
[31] J. Corradi, C. Bouzat, Understanding the bases of function and modulation of alpha7 nicotinic receptors: implications for drug Discovery, Mol. Pharmacol. 90 (3) (2016) 288–299.
[32] L.M. Teles-Grilo Ruivo, J.R. Mellor, Cholinergic modulation of hippocampal network function, Front. Synaptic Neurosci. 5 (2013) 2.
[33] C. Gotti, F. Clementi, Neuronal nicotinic receptors: from structure to pathology, Prog. Neurobiol. 74 (6) (2004) 363–396.
[34] B. Lendvai, F. Kassai, A. Szajli, Z. Nemethy, alpha7 nicotinic acetylcholine receptors and their role in cognition, Brain Res. Bull. 93 (2013) 86–96.
[35] L.A. Izquierdo, D.M. Barros, M.R. Vianna, A. Coitinho, T. de David e Silva, H. Choi, B. Moletta, J.H. Medina, I. Izquierdo, Molecular pharmacological dissection of short- and long-term memory, Cell. Mol. Neurobiol. 22 (3) (2002) 269–287.
[36] D. Paterson, A. Nordberg, Neuronal nicotinic receptors in the human brain, Prog. Neurobiol. 61 (1) (2000) 75–111.
[37] K.L. Zhang, D.D. Lou, Z.Z. Guan, Activation of the AGE/RAGE system in the brains of rats and in SH-SY5Y cells exposed to high level of fluoride might connect to oxidative stress, Neurotoxicol. Teratol. 48 (2015) 49–55.
[38] D.A. Butterfield, Oxidative stress in neurodegenerative disorders, Antioxid. Redox. Signal. 8 (11–12) (2006) 1971–1973.
[39] P. Grandjean, P.J. Landrigan, Developmental neurotoxicity of industrial chemicals, Lancet 368 (9553) (2006) 2167–2178.
[40] Y.P. Reddy, S.K. Tiwari, A.P. Shaik, A. Alsaeed, A. Sultana, P.K. Reddy, Effect of sodium fluoride on neuroimmunological parameters, oxidative stress and antioxidative defenses, Toxicol. Mech. Methods 24 (1) (2014) 31–36.
[41] B.H. Zhou, J. Zhao, J. Liu, J.L. Zhang, J. Li, H.W. Wang, Fluoride-induced oxidative stress is involved in the morphological damage and dysfunction of liver in female mice, Chemosphere 139 (2015) 504–511.
[42] S.F. Nabavi, S.M. Nabavi, S. Habtemariam, A.H. Moghaddam, A. Sureda, M. Mirzaei, Neuroprotective effects of methyl-3-O-methyl gallate against sodium fluoride-induced oxidative stress in the brain of rats, Cell. Mol. Neurobiol. 33 (2) (2013) 261–267.
[43] O.L. Adebayo, P.D. Shallie, B.A. Salau, E.O. Ajani, G.A. Adenuga, Comparative study on the influence of fluoride on lipid peroxidation and antioxidants levels in the different brain regions of well-fed and protein undernourished rats, J. Trace Elem. Med Biol. 27 (4) (2013) 370–374.
[44] D. Shanthakumari, S. Srinivasalu, S. Subramanian, Effect of fluoride intoxication on lipidperoxidation and antioxidant status in experimental rats, Toxicology 204 (2-3) (2004) 219–228.
[45] M. Bartos, F. Gumilar, C. Bras, C.E. Gallegos, L. Giannuzzi, L.M. Cancela, A. Minetti, Neurobehavioural effects of exposure to fluoride in the earliest stages of rat development, Physiol. Behav. 147 (2015) 205–212.
[46] I. Izquierdo, J.H. Medina, Memory formation: the sequence of biochemical events in the hippocampus and its connection to activity in other brain structures, Neurobiol. Learn. Mem. 68 (3) (1997) 285–316.
[47] T.D. Schmittgen, K.J. Livak, Analyzing real-time PCR data by the comparative C(T) method, Nat. Protoc. 3 (6) (2008) 1101–1108.
[48] L. Dionisio, M. Jose De Rosa, C. Bouzat, C. Esandi Mdel, An intrinsic GABAergic system in human lymphocytes, Neuropharmacology 60 (2-3) (2011) 513–519.
[49] G. Paxinos, C. Watson, The Rat Brain in Stereotaxic Coordinates, 6th ed., (2007).
[50] C.J. Beuret, F. Zirulnik, M.S. Gimenez, Effect of the herbicide glyphosate on liver lipoperoxidation in pregnant rats and their fetuses, Reprod. Toxicol. 19 (4) (2005) 501–504.
[51] H. Aebi, Catalase in vitro, Methods Enzymol. 105 (1984) 121–126.
[52] R.A. Lawrence, R.F. Burk, Glutathione peroxidase activity in selenium-deficient rat liver, Biochem. Biophys. Res. Commun. 71 (4) (1976) 952–958.
[53] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[54] Guide for the Care and Use of Laboratory Animals, 8th ed., The National Institute of Health, 2011.
[55] A.A.f.T.S.a.D. Registry), Toxicological Profile for Fluorides, Hydrogen Fluoride, and Fluorine (Update), 2003.
[56] L. Valdez-Jiménez, C. Soria Fregozo, M.L. Miranda Beltrán, O. Gutiérrez Coronado, M.I. Pérez Vega, Efectos del flúor sobre el sistema nervioso central, Neurología 26 (2011) 297–300.
[57] W. Zheng, M. Aschner, J.F. Ghersi-Egea, Brain barrier systems: a new frontier in metal neurotoxicological research, Toxicol. Appl. Pharmacol. 192 (1) (2003) 1–11.
[58] P. Grandjean, P.J. Landrigan, Neurobehavioural effects of developmental toxicity, Lancet. Neurol. 13 (3) (2014) 330–338.
[59] H.S. El-lethey, M.M. Kamel, I.B. Shaheed, Neurobehavioral toxicity produced by sodium fluoride in drinking water of laboratory rats, J. Am. Sci. 6 (2010) 54–63.
[60] A.W. Burgstahler, J.C. Calanhoun, Neurotoxicity of fluoride, Fluoride 29 (1996) 57–58.
[61] F. Benetti, C.R. Furini, J. de Carvalho Myskiw, G. Provensi, M.B. Passani, E. Baldi, C. Bucherelli, L. Munari, I. Izquierdo, P. Blandina, Histamine in the basolateral amygdala promotes inhibitory avoidance learning independently of hippocampus, PNAS 112 (19) (2015) E2536–42.
[62] J.L. McGaugh, Time-dependent processes in memory storage, Science 153 (3742) (1966) 1351–1358.
[63] M.G. Giovannini, D. Lana, G. Pepeu, The integrated role of ACh, ERK and mTOR in the mechanisms of hippocampal inhibitory avoidance memory, Neurobiol. Learn. Memory 119 (2015) 18–33.
[64] D. Lana, F. Cerbai, J. Di Russo, F. Boscaro, A. Giannetti, P. Petkova-Kirova, A.M. Pugliese, M.G. Giovannini, Hippocampal long term memory: effect of the cholinergic system on local protein synthesis, Neurobiol. Learning Memory 106 (2013) 246–257.
[65] Y.G. Long, Y.N. Wang, J. Chen, S.F. Jiang, A. Nordberg, Z.Z. Guan, Chronic fluoride toxicity decreases the number of nicotinic acetylcholine receptors in rat brain, Neurotoxicol. Teratol. 24 (6) (2002) 751–757.
[66] J. Micheau, A. Marighetto, Acetylcholine and memory: a long, complex and chaotic but still living relationship, Behav. Brain Res. 221 (2) (2011) 424–429.
[67] R. Schliebs, T. Arendt, The cholinergic system in aging and neuronal degeneration, Behav. Brain Res. 221 (2) (2011) 555–563.
[68] M.E. Hasselmo, The role of acetylcholine in learning and memory, Curr. Opin. Neurobiol. 16 (6) (2006) 710–715.
[69] G. Martel, R. Jaffard, J.L. Guillou, Identification of hippocampus-dependent and hippocampus independent memory components in step-down inhibitory avoidance tasks, Behav. Brain Res. 207 (1) (2010) 138–143.
[70] Y.J. Liu, Q. Gao, C.X. Wu, Z.Z. Guan, Alterations of nAChRs and ERK1/2 in the brains of rats with chronic fluorosis and their connections with the decreased capacity of learning and memory, Toxicol. Lett. 192 (3) (2010) 324–329.
[71] T. Kaur, R.K. Bijarnia, B. Nehru, Effect of concurrent chronic exposure of fluoride and aluminum on rat brain, Drug Chem. Toxicol. 32 (3) (2009) 215–221.
[72] P.M. Basha, P. Rai, S. Begum, Evaluation of fluoride-induced oxidative stress in rat brain: a multigeneration study, Biol. Trace Elem. Res. 142 (3) (2011) 623–637.
[73] H. Bouaziz, F. Croute, T. Boudawara, J.P. Soleilhavoup, N. Zeghal, Oxidative stress induced by fluoride in adult mice and their suckling pups, Exp. Toxicol. Pathol. 58 (5) (2007) 339–349.