Toxicology Letters 192 (2010) 324–329

Contents lists available at ScienceDirect

Toxicology Letters

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

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

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ARTICLE INFO

Article history:
Received 14 July 2009
Received in revised form 31 October 2009
Accepted 2 November 2009
Available online 10 November 2009

Keywords:
Fluorosis
ERK1/2
Learning and memory
nAChRs
Rats

ABSTRACT

In order to reveal the mechanism of the decreased ability of learning and memory induced by chronic fluorosis, nicotinic acetylcholine receptors (nAChRs) and the pathway of extracellular signal regulated protein kinase (ERK1/2) were investigated by using the rats fed with different concentrations of sodium fluoride for 6 months. Spatial learning and memory of the rats were evaluated by Morris Water Maze test. The expressions of nAChRs, ERK1/2 and mitogen-induced extracellular kinase (MEK1/2) at protein and mRNA levels were detected by Western blotting and real-time PCR, respectively. The results showed that as compared with controls, the learning and memory capacity in the rats with fluorosis was decreased. The protein expressions of α7 and α4 nAChR subunits in rat brains with fluorosis were decreased by 35% and 33%, whereas the corresponding receptor subunit mRNAs did not exhibit any changes. The increases of phospho- and total-ERK1/2 as well as phospho-MEK1/2 at the protein levels were found in the brains of rats with fluorosis as compared to controls, and no difference of ERK1/2 mRNA was found. In addition, the activation rate of phospho-ERK1/2 was decreased in the brains affected with fluorosis. The modifications of nAChRs and ERK1/2 pathway might be connected with the molecular mechanisms in the decreased capacity of learning and memory of the rats with fluorosis.

1. Introduction

It has been observed that fluoride accumulates in the brains of experimental animals exposed to high doses of the ion for a prolonged period (Guan et al., 1986). The severity of the adverse effects of fluoride on the behavior of rats is directly correlated to the concentrations of fluoride in the plasma and in specific regions of the brain (Mullenix et al., 1995). In addition, the latencies of the pain reaction and conditioned reflex are longer in rats exposed to excessive amount of fluoride in comparison to control animals (Guan et al., 1986). Importantly, the declined level of Intelligence Quotient for children who were born and raised in the areas with endemic fluorosis was observed (Li et al., 1995; Zhao et al., 1996; Lu et al., 2000; Xiang et al., 2003; Trivedi et al., 2007). On the other hand, the change in mental work capacity of adults with chronic fluorosis has been found (Shao et al., 2003). However, the molecular mechanism regarding the action of fluoride on learning and memory is still elusive at present.

Recently, we have reported that the levels of nicotinic acetylcholine receptors (nAChRs) are significantly declined in the brains of rats with fluorosis and in PC12 cells exposed to high concentrations of fluoride (Long et al., 2002; Chen et al., 2003; Shan et al., 2004). The nAChRs are transmitter-gated ion channels composed of α and β subunits (Paterson and Nordberg, 2000). To date, nine different α (α2–α10) and three β (β2–β4) subunits have been cloned in central nervous system. Different α and β subunits or α subunit alone are combined in various ways to form receptor subtypes. In human and animal brains, α4β2 and α7 nAChRs are the major types of the receptors, demonstrating physiological and pharmacological functions and involving in learning and memory-related behaviors and in neuroprotective effects (Couturier et al., 1990; Mousavi et al., 2003).

The mitogen-activated protein kinase (MAPK) cascade has been intensely studied as a primary biochemical pathway through which a variety of extracellular stimuli initiates and regulates processes of cellular transformation (Molna et al., 2006). MAPK cascade is required for hippocampal long-term potentiation (LTP) (Davis et al., 2000; Di Cristo et al., 2001; Sweatt, 2004). In particular, extracellular signal regulated kinases 1 and 2 (ERK1/2) as a critical component of the biochemical cascades that underlie the induction of the procedure of LTP and regulate cellular responses to extracellular stimuli, neuronal differentiation and plasticity (Wu et al., 2001; Goldin and Segal, 2003; Mailliet et al., 2008). ERK1/2 is also activated by neuronal injury (Chu et al., 2004; Zhuang and Schnellmann, 2006), in which activation of ERK1/2 may act as a defensive mechanism that helps to compensate for the deleterious effects of a damaging insult. Furthermore, ERK 1/2 pathway can also activate several substrates (Godeny and Sayeski, 2006).

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doi:10.1016/j.toxlet.2009.11.002

In order to further understand the molecular mechanism of the decreased capacity of learning and memory influenced by fluoride, nAChRs and ERK1/2 pathway were measured by employing rats with chronic fluorosis in the study.

2. Materials and methods

2.1. Materials

Goat polyclonal antibody directed towards α7 and α4 nAChR subunits, and anti-goat IgG conjugated with horseradish peroxidase (Santa Cruz Biotechnology Inc., USA); mouse monoclonal anti-β-actin antibody (Sigma–Aldrich, USA); rabbit polyclonal antibodies directed towards phospho-ERK1/2, phospho-MEK1/2, total-ERK1/2 and total-MEK1/2 (Cell Signalling Technology, USA); anti-rabbit IgG, Hyper Performance Chemiluminescence film and ECL (Enhanced Chemiluminescence) Plus reagent (Amersham, Sweden); QPCR SYBR Green Mix (Infinigen Biotechnology Inc., USA); and all other general chemicals (Sigma–Aldrich, USA) were purchased from the sources indicated.

2.2. Experimental animals

Seventy-two SD (Sprague Dawley) rats (half males and half females, and weighting 90–120 g) were purchased from the Experimental Animal Center in Guizhou, China, and ethical permission for these experiments was obtained from the regional ethical committee for animal studies in Guizhou. The humidity ranged from 30% to 55% and temperature remained between 22 °C and 25 °C. The rats were acclimatized for 1 week in a housing facility before treatment.

The rats were randomly divided into 3 groups, e.g., normal control group (drinking water containing less than 0.5 mg/L of sodium fluoride, NaF), lower fluoride exposed group (drinking water containing 5 mg/L NaF) and higher fluoride exposed group (drinking water containing 50 mg/L NaF). During the study, rats were housed in stainless-steel cages suspended in stainless-steel racks, and the treated water and food were administered to the animals *ad libitum*. The rats were examined at the sixth month after feeding NaF.

2.3. Examination of spatial learning and memory

Spatial learning and memory of the rats were evaluated by using Morris Water Maze test (Morris et al., 1982). The maze consists of a circular pool (180 cm in diameter) with dark walls and filling with tap-water colored by mixing dark ink. An escape platform (9 cm in diameter) made of stainless-steel with dark walls is submerged 0.5 cm below the surface of this water. Each rat was subjected to 4 trials each day with 5–7 min interval of rest between trials for a training period of 5 days. The movement of the rats was monitored with a Videotrack Software (View Point). During the navigation test, the time required to locate the escape platform (escape latency) was determined, and after locating this platform the animal was allowed to sit on it for 2 s. Rats who failed to find platform within 60 s were guided to the platform and then allowed to remain on it for 2 s as well, in which the escape latency in these cases was recorded as 60 s. The 4 trials on each individual day were averaged for statistical analysis. Furthermore, on day 7 when the platform was removed, the time of first crossing the site where the original platform located was recorded. All of these behavioral tests were conducted in a quiet environment with subdued lighting.

2.4. Protein levels of α7 and α4 nAChR subunits and ERK1/2 detected by Western blotting

Brain mixture tissues including the hippocampus and cortex were homogenized with buffer containing 2% Triton X-100 on ice using Telfon homogenizer (Shan et al., 2004). The homogenate was centrifuged at 14,000 × *g* for 40 min at 4 °C, and the protein concentrations of the resulting supernatant were determined. The proteins in the solubilized membrane fraction recovered in the supernatant were separated by 10% SDS-PAGE and then blotted onto polyvinylidene difluoride (PVDF) membranes using a transfer unit (Bio-Rad Inc.). For the quantification of nAChR subunits and ERK1/2 proteins, these PVDF membranes were thereafter incubated with different antibodies (1:1000 dilution) for corresponding proteins, e.g., α7 and α4 nAChR subuints, phospho-ERK1/2, phospho-MEK1/2, total-ERK1/2, and total-MEK1/2, respectively, at 4 °C overnight. After washing the membranes were incubated with HRP-conjugated anti-goat IgG (1:5000) or anti-rabbit IgG (1:3000), respectively, for 60 min. Finally, these membranes were incubated in ECL Plus reagent for 5 min and the signals thus produced visualized by exposure to Hyper Performance Chemiluminescence film.

2.5. mRNA levels of α7 and α4 nAChR subunits, and ERK1/2 determined by quantitative real-time PCR

Total RNA in brain tissue was isolated by Trizol reagents (Invitrogen, USA). For each sample 3 μg of total RNA were converted into first-strand cDNA using the first-strand cDNA synthesis kit (Promega, USA) and oligo-dT primers according to the protocol recommended by the manufacturer. The real-time PCR primers for target transcripts were designed using the complete cDNA sequences deposited in GenBank (accession numbers: NM_012832.2 for α7 nAChR, NM_ 024354.1 for α4 nAChR NM_ 053842.1 for ERK1/2, NM_ 031144.2 for β-actin) (Table 1). The quantitative real-time PCRs were performed using the ABI PRISM 7300 Sequence Detection System (Applied Biosystems, USA) and analyzed with GeneAmp7300 SDS software. Real-time PCR reactions were performed with the Universal TaqMan 2× PCR mastermix (Infinigen Biotechnology Inc., USA) in a 20 μL reaction volume for α7 nAChR, α4 nAChR and β-actin; for ERK1/2 and β-actin, the quantitative real-time PCR reactions were carried out in a 20 μL volume containing 2 μL of first-strand cDNA, 2× Hotsybr PCR Reaction Mix (Infinigen Biotechnology Inc., USA), and 1 Mm of forward and reverse primers. The detailed procedure was performed as the manufacturer's protocol. The thermal cycling conditions included 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Melting curve analysis was always performed after the amplification to check PCR specificity. The ABI7300 software was used to analyse data and calculate CT (threshold cycle) values. The α7 nAChR, α4 nAChR and β-actin transcript levels were estimated by using the formula $2^{-\Delta\Delta CT}$ where ΔCT represents the difference in CT values between target and housekeeping assays.

2.6. Statistical analysis

The results were expressed as mean values ± SD for different groups. These means were examined for statistically significant differences employing ANOVA; correlation between the level of ERK1/2 activation rate and probe test was analyzed by correlation test; and data for navigation test by repeated measure in the SPSS11.5 software (SPSS Inc., USA).

3. Results

3.1. Spatial learning and memory

The mean values of escape latency were shown in Table 2. The obviously prolonged escape latency was detected in the rats fed with higher concentration of fluoride as compared with controls, showing a decreased spatial learning capacity of these animals with fluorosis. During the probe trail, rats with fluorosis took longer time to cross the platform area than controls (Fig. 1).

3.2. Expression of α7 and α4 nAChRs subunits at protein level

As determined by Western blotting, levels of the α7 nAChR subunit protein (Fig. 2A) in brains were significantly decreased by 23% in lower fluoride exposed group and by 35% in higher fluoride exposed group as compared to control animals. For the protein level of α4 nAChR subunit (Fig. 2B), the decreases by 17% in lower fluoride exposed group and by 33% in higher fluoride exposed group were observed as compared to controls.

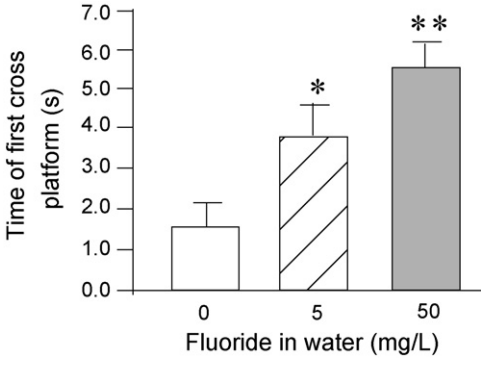

Fig. 1. The time of first crossing the site where the original platform located from the rats. Spatial learning and memory were tested in a Morris Water Maze involving a circular pool 180 cm in diameter. On day 7 in which the platform was removed, the time of first cross the site where the original platform located was recorded. The values are shown as the means ± SD of 10 cases. $^{*}P < 0.05$ and $^{**}P < 0.01$ in comparison to the control value, as determined by analysis of one-way ANOVA.

Table 1
Sequences of the primers employed for amplification of mRNAs of α4 and α7 nAChR subunits, ERK1/2 and β-actin by real-time PCR.

mRNA	Sequences	Length (bp)
α7 nAChR	5′-TAATACGACTCACTATAGGGAGGAAGAGGCCCGGAGAGGACAA-3′ 5′-CGCCACATACGACCCCAGAG-3′	274
α4 nAChR	5′-TAATACGACTCACTATAGGGAGGGCGAGGCCGGCATCTTGAGT-3′ 5′-GCTGGGCACATGCTGGACAC-3′	207
ERK1/2	5′-CTACACGCAGCTGCAGTACAT C-3′ 5′-GTGCGCTGACAGTAGGTTTGA-3′	472
β-Actin	5′-GCCATCCTGCGTCGTGACCTG-3′ 5′-CATTTGCGGTGCACGATGGAG-3′	560

Table 2
Performance of rats with fluorosis in the navigation test (s) ($\bar{x} \pm s$).

Groups	*N*	First day	Second day	Third day	Forth day	Fifth day	Sixth day
Control	10	53.8 ± 14.2	39.3 ± 6.9	19.1 ± 9.1	8.9 ± 6.0	8.3 ± 3.4	4.8 ± 2.7
Lower fluoride	10	59.9 ± 0.16	46.0 ± 8.0^{A}	24.0 ± 2.7^{A}	13.8 ± 5.7^{A}	8.9 ± 5.3^{A}	7.4 ± 4.1^{A}
Higher fluoride	10	60 ± 17.4	36.9 ± 16.8	37.7 ± 12.9^{AB}	16.5 ± 7.8^{AB}	19.7 ± 7.6^{AB}	12.2 ± 5.7^{AB}

"A" means the data as compared to controls, $P < 0.05$; "B" as compared to the group with lower fluoride in drinking water, $P < 0.05$.

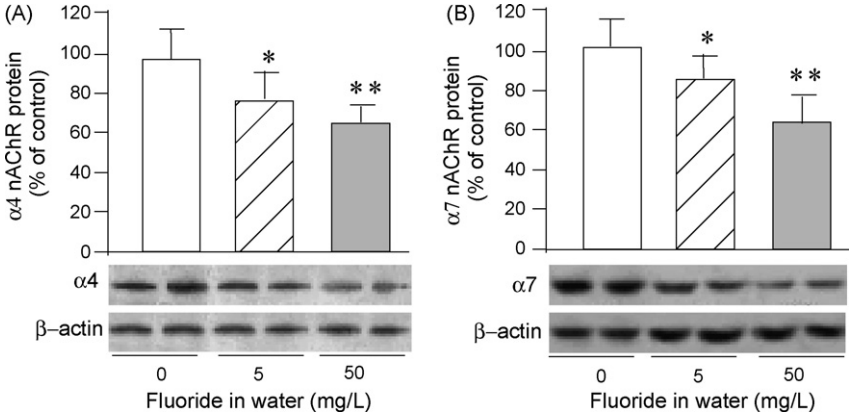

Fig. 2. Expression of nAChR α4 and α7 subunits at protein level in brains from the rats with fluorosis. The α4 and α7 nAChR subunits at protein level were detected by Western blotting. The values are shown as the means ± SD of 10 cases. $^{*}P < 0.05$ and $^{**}P < 0.01$ in comparison to the control value, as determined by analysis of one-way ANOVA.

3.3. Expression of ERK1/2 at protein level

The level of the phospho-ERK1/2 and total-ERK1/2 proteins was increased in brains of the rats with fluorosis than controls (Fig. 3). The activating rate by calculating the gray scales of phospho-ERK1/2 as compared to total-ERK1/2 was significantly decreased in brains of the rats with fluorosis (Fig. 4). The increase of phospho-MEK1/2 was observed in brains of the rats with fluorosis as compared to controls, but no change in total-MEK1/2 was detected (Fig. 5). Furthermore, a negative correlation ($r = -0.364$, $P < 0.05$)

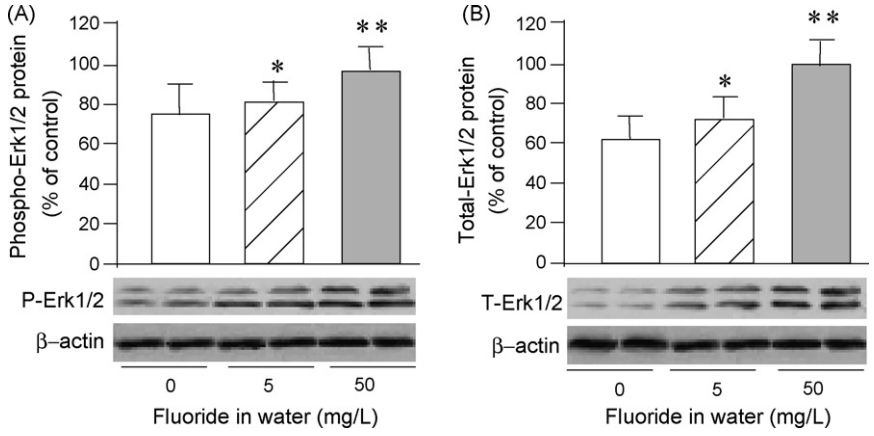

Fig. 3. The expression of the phospho-ERK1/2 and total-ERK1/2 at protein level in brains from the rats with fluorosis. The phospho-ERK1/2 and total-ERK1/2 at protein level were detected by Western blotting. The values are shown as the means ± SD of 10 cases. $^{*}P < 0.05$ and $^{**}P < 0.01$ in comparison to the control value, as determined by analysis of one-way ANOVA.

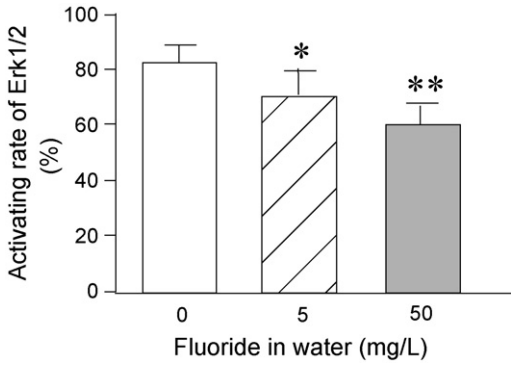

Fig. 4. Activation rate of ERK1/2 in brains from the rats with fluorosis. The activation rate of ERK1/2 was evaluated by compared the gray densities between phospho-ERK1/2 and total-ERK1/2. The values are shown as the means ± SD of 10 cases. $^{*}P < 0.05$ and $^{**}P < 0.01$ in comparison to the control value, as determined by analysis of Pearson's correlation.

Table 3
mRNAs of α7 and α4 nAChR subunits, and ERK1/2 by real-time quantitative PCR ($\bar{x} \pm s$).

mRNA	Group	ΔCT	$2^{-\Delta\Delta CT}$
α7 nAChR	Control	27.20	7.28E−09 ± 3.86E−09
	Lower concentration group	29.44	1.32E−08 ± 1.52E−08
	Higher concentration group	27.11	8.93E−09 ± 7.60E−09
α4 nAChR	Control	27.02	7.28E−09 ± 3.86E−09
	Lower concentration group	25.09	1.12E−08 ± 1.65E−08
	Higher concentration group	26.94	6.35E−09 ± 8.63E−09
ERK1/2	Control	4.17	3.29E−06 ± 1.44E−06
	Lower concentration group	4.58	3.18E−06 ± 1.25E−06
	Higher concentration group	0.20	2.53E−06 ± 2.35E−06

between the ERK1/2 activating rate and time of first cross the platform area was found in the rats fed with higher concentration of fluoride (data not shown).

3.4. Expression of α7 and α4 nAChR subunits, and ERK1/2 at mRNA level

No significant changes in the mRNA abundances of α7 and α4 nAChR subunits, and ERK1/2 were found in brains of the rats with fluorosis as compared to controls (Table 3).

4. Discussion

To test whether the fluoride concentrations used in the study influences memory formation and storage, the Morris water maze task was performed. We observed that during the 6 days of the navigation tests, the animals with fluorosis performed poorly than controls, which indicated that fluoride may influence the spatial learning. At the same time the rats with fluorosis took a significant longer time than controls to cross the previous location of the platform in probe trail, suggesting an impairment of long-term memory. These results were coincident with the published data relating learning and memory with fluorosis (Li et al., 1995; Zhao et al., 1996; Trivedi et al., 2007).

In our previous results, we hypothesized that the mechanism regarding to declined capacity of learning and memory resulted from fluorosis may have a connection to the changes of cholinergic nervous system (Long et al., 2002; Chen et al., 2003; Shan et al., 2004). Importantly, nAChRs play major roles in cognitive process such as learning and memory and also have the neuroprotective function (Paterson and Nordberg, 2000). The nAChRs containing α4 subunit in combination with β2 subunit are among the most abundant, high-affinity nicotine binding sites in the mammalian brain. The α7 nAChR has received considerable attention for the involvement in several types of learning and memory-related behavior (Levin and Simon, 1998; Hogg et al., 2003). The novel α7 nAChR agonist improved working and recognition memory (Boess et al., 2007) and knockout of α7 subunit caused significant deficits in spatial discrimination (Curzon et al., 2006; Levin et al., 2009). The α4β2 may be relevant to brain reward circuits that could plausibly contribute to cognitive learning (Dehaene and Changeux, 1997), improve this effect enhanced by nicotine treatment (Davis et al., 2007), and prevent the suppressive action of β-amyloid peptide on the hippocampal LTP (Wu et al., 2008). The results in the study showed a significant decline of α7 and α4 nAChR subunit proteins in rat brains influenced by fluorosis, suggesting that the changed nAChRs may focus in the mechanism of the decreased capacity of learning and memory of these animals.

It has been indicated that one important signaling pathway linked to learning and memory is the ERK1/2 pathway (Sweatt, 2004). Phosphorylated ERK1/2 has been observed in the hippocampus after training in hippocampal dependent tasks (Atkins et al., 1998; Giovannini, 2006). The inhibitor of ERK1/2 activation led to a blockade of long-term fear memory formation (Selcher et al., 2003), and the memory retention and retrieval depend on activation of ERK1/2 pathway (Izquierdo et al., 2001). It has been indicated that

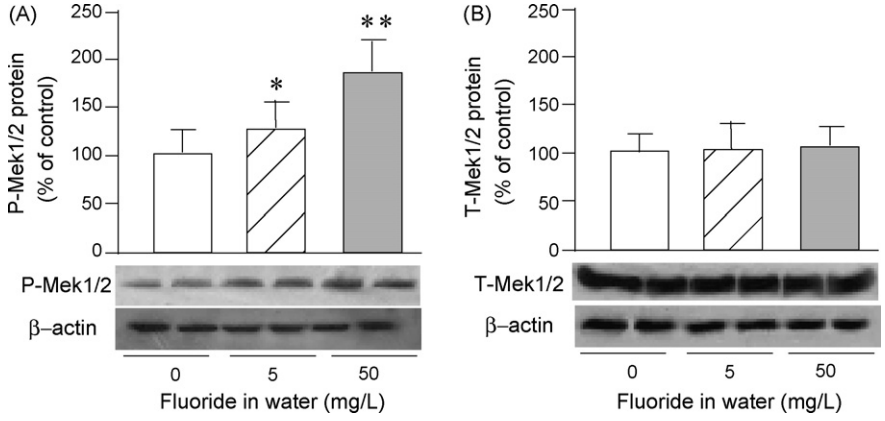

Fig. 5. Expression of phospho-MEK1/2 and total-MEK1/2 at protein level in brains from the rats with fluorosis. The phospho-MEK1/2 and total-MEK1/2 at protein level were detected by Western blotting. The values are shown as the means ± SD of 10 cases. $^{*}P < 0.05$ and $^{**}P < 0.01$ in comparison to the control value, as determined by analysis of one-way ANOVA.

NaF exposure can induce phosphorylation of ERK1/2, which may involve the induction of apoptosis (Karube et al., 2009; Thrane et al., 2001). Upregulations of total-ERK1/2 and phospho-ERK1/2 in the brains of rats with fluorosis were observed in the present study, which was consistent with these data pointed above. On the other hand, the depression of the activation of ERK1/2 was found in the study, which might be a mechanism resulted in changed learning and memory capacity. Furthermore, the overexpression of total-ERK1/2 and phospho-ERK1/2 in rats with fluorosis might likely be a result for compensation with decreased activating rate of ERK1/2 in order to sustain a normal level of memory. Interestingly, a direct correlation between the activating rate of ERK1/2 and rat performance of probe trial detected, suggesting that the decreased activating rate of ERK1/2 may connect with the lower learning and memory.

MEK1/2, a dual specificity kinase, phosphorylates threonine and tyrosine residues of MAP kinase, thus leading to the activation of MAP kinase (Boulton et al., 1991; Crews et al., 1992). The mammalian MEK1/2 displays high substrate selectivity towards MAP kinase in vitro (van Hemert et al., 2001). Some study showed neuronal MEK1/2 is important for normal fear conditioning in mice (Shalin et al., 2004). Dorsohippocampal injections of the MEK1/2 inhibitors U0126 and PD98059 immediately after the non-reinforced trials prevented ERK1/2 activation, which was dissociable from potential actions on memory retrieval or reconsolidation (Fischer et al., 2007). In our findings, the expression of phospho-MEK1/2 in the rat brains with fluorosis was increased as compared to controls, which may result in the high level of phospho-ERK1/2 for a compensation of the decreased activating rate of ERK1/2.

Importantly, the involvement of specific nAChR subtypes in the regulation of Ras/MEK/ERK signaling pathway has been investigated (Schuller et al., 2003; Chernyavsky et al., 2005) and definitive evidence of activation of the Ras/MEK/ERK signaling through α7 nAChR was obtained (Arredondo et al., 2006). Many recent studies also indicated that nAChRs were involved in phosphorylation of ERK1/2 in brains or in cultured neuronal cells (Bitner et al., 2007; Nakayama et al., 2006). In the present study, α7 and α4 nAChR subunits at protein level in the brains of rats with fluorosis were significantly decreased, which might be connected to the lower activating rate of ERK1/2, suggesting that the down-regulated process of ERK1/2 activating rate was due to the inhibited expression of the receptors induced by high-fluoride.

In conclusion, the declined capacity in spatial learning and memory in rats with fluorosis, the decreased expressions of α7 and α4 nAChR subunits, the increases of phospho-MEK1/2 and both phospho-ERK1/2 and total-ERK1/2, and the lower activating rate of phospho-ERK1/2 in brains of the rats with fluorosis were detected, in which these changes may be resulted from the post-transcription levels of these proteins instead of corresponding mRNAs. Since the functions of nAChRs and ERK1/2 pathway are mainly related to cognition, the modifications of nAChRs and ERK1/2 pathway might be involved in the molecular mechanisms in the decreased capacity of learning and memory of the rats with fluorosis.

Conflict of interest statement

There are none.

Acknowledgments

This work was financed by grants from the Foundation in the China National Natural Scientific Foundation (30760224), the Foundation in the Ministry of Science and Technology of China (S2010GR0928), the Government Foundations in Guizhou Province of China and the Foundation of Guiyang Medical College.

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