



Article

AMPA and NMDA Receptors in Hippocampus of Rats with Fluoride-Induced Cognitive Decline

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Abstract: This experimental study was performed to evaluate the alterations in the expression of a few subunits composing glutamate AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and NMDA (N-methyl-D-aspartate) receptors in the hippocampal cells of Wistar rats in response to long-term fluoride (F⁻) exposure. The animals were given water with background 0.4 (control), 5, 20, and 50 ppm F⁻ (as NaF) for 12 months. The cognitive capacities of rats were examined by novel object recognition (NOR), Y-maze test, and Morris water maze tests. RT-qPCR and Western blotting techniques were used to evaluate the expression of different AMPA and NMDA subunits at transcriptional and translational levels, respectively. Long-term F⁻ poisoning disturbed the formation of hippocampus-dependent working spatial and long-term non-spatial memory. The expression of *Gria1*, *Gria2*, and *Gria3* genes encoding different subunits of AMPA receptors were comparable in hippocampi of control and F⁻-exposed animals, although the levels of both *Grin2a* and *Grin2b* mRNA increased. Long-term F⁻ intake enhanced the ratio of phospho-GluA1/total-GluA1 proteins in subcellular fraction enriched with cytosolic proteins, while decreased content of GluA2 but elevated level of GluA3 were observed in subcellular fraction enriched with membrane proteins. Such changes were accompanied by increased phosphorylation of GluN2A and GluN2B subunits, higher ratios of GluN2A/GluN1 and GluN2B/GluN1 proteins in the cytosol, and GluN2A/GluN2B ratio in membranes. These changes indicate the predominance of Ca²⁺-permeable AMPARs in membranes and a shift between different NMDARs subunits in hippocampal cells of F⁻-exposed rats, which is typical for neurodegeneration and can at least partially underly the observed disturbances in cognitive capacities of animals.

Keywords: rat; hippocampus; AMPARs; NMDARs; gene expression; subcellular distribution



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1. Introduction

Fluoride (F⁻) is ubiquitously distributed in nature due to release from natural (volcanic and geothermal activity, and mineral leaching) and anthropogenic (coal burning, metals, and fertilizers processing) sources [1,2]. In endemic areas, the levels of naturally occurring F⁻ exceeding the permissible limit of 1.5 mg/L [3] are found in water available for daily human needs and farm animal livestock. In developed countries, F⁻-supplementation of drinking water, salt, milk, and dental products is the second important pathway of F⁻ intake. Chronic exposure to F⁻ at industrial sites or accidental poisoning with F⁻-containing chemicals at home are also not rare [1,2]. However, excessive F⁻ consumption induces a spectrum of health disorders, such as dental and skeletal fluorosis and damage to various soft tissues [1,3]. Most important, many recent works raised a concern on possible F⁻ neurotoxicity, including increased risk of neurodegenerative problems such as Parkinson's disease and Alzheimer's disease in adults [4–7], autism spectrum disorders, decreased Intelligence quotient (IQ) score and difficulties with attention in children [8–11], and susceptibility to brain tumors [12]. Other studies, however, claim an absence of thorough clinical investigations and convincing evidence in favor of the association between F⁻

intake and neurological disorders, while the existing findings are insufficient [13,14]. Such controversies highlight the necessity of new research focused on F⁻ neurotoxicity.

F⁻ is known to freely transit the hematoencephalic barrier and accumulate in brain structures to a degree directly reflecting the consumption rate (summarized in [5]). Our previous works [15,16] have shown that the development of chronic fluorosis in rats was accompanied by a decline in cognitive capacities, pathological alterations and death of neurons in CA1 and CA3 hippocampal fields, and altered expression of Ca²⁺-dependent signaling molecules implicated in long-term potentiation (LTP).

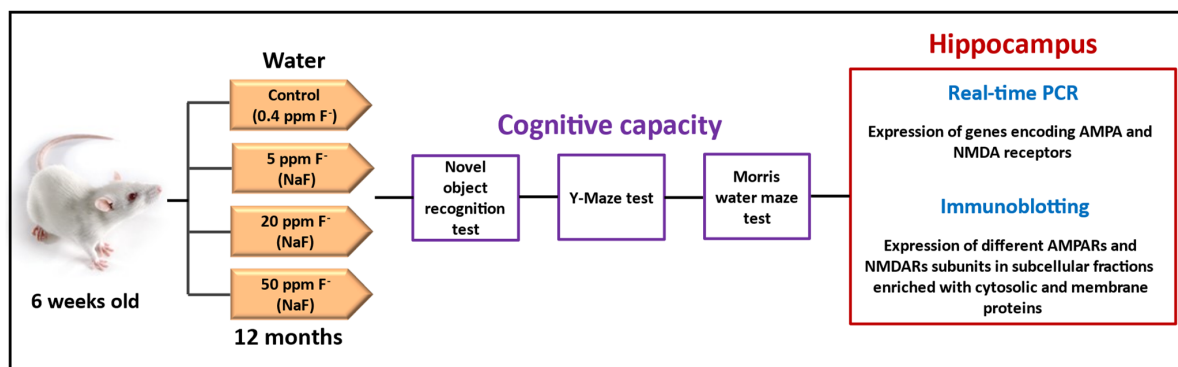
The processes of learning and memory formation are believed to be based on synaptic plasticity, whereas cognitive impairment is associated with perturbed excitatory neurotransmission. F⁻ poisoning alters the levels of glutamate and its receptors in the brains of rodents [17–21]. Glutamate receptors are clustered at postsynaptic membranes and classified by agonist selectivity as ionotropic AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), NMDA (N-methyl-D-aspartate) or kainite receptors, and metabotropic glutamate receptors (mGluR) [22,23]. Tetrameric AMPARs are composed of various combinations of GluA1–GluA4 subunits encoded by *Gria1–Gria4* genes [24–27]. In excitatory neurons of the mature hippocampus, AMPARs predominantly exist as GluA1/2 and GluA2/3 heterodimers at approximately similar ratios. GluA2-containing AMPARs are impermeable to Ca²⁺, whereas that with GluA1 and GluA3 transit Ca²⁺ following activation. Being the central mediators of rapid excitatory glutamatergic synaptic transmission, GluA1-possessing AMPARs are inserted into synapses following LTP and play an important role in learning and memory. GluA2/3-containing AMPARs are not significantly involved in LTP or memory formation but modulates the synaptic strength.

NMDARs are implicated in long-term synaptic plasticity associated with transcription-dependent processes and global protein synthesis. Functional tetrameric NMDARs are highly permeable to Ca²⁺ and composed of two obligatory GluN1 subunits and two GluN2, GluN3 or GluN2/GluN3 subunits. GluN2A and GluN2B are the most abundant regulatory subunits in cognitive-related brain structures. GluN2A-containing NMDARs are located primarily at synaptic sites and are believed to implicate in protective pathways, whereas GluN2B-containing receptors are distributed mainly at extrasynaptic sites and increase neuronal vulnerability. Shifts in the GluN2A/GluN2B ratio underlie both learning and memory, but also different neuropathologies.

The goal of the present study was a thorough evaluation of possible changes in the expression of a few major subunits composing AMPARs and NMDARs in the hippocampus of rats with stable fluorosis at transcriptional and translational levels, as well as their subcellular distribution and phosphorylation state. The work was focused on the hippocampus because it provides the synaptic basis for the initiation, formation, and consolidation of memory.

2. Results

The design of experiments is shown in Scheme 1.



Scheme 1. The timeline of entire experimental procedure.

2.1. Brain F⁻ Content

The average F⁻ content in the brains of animals that consumed regular drinking water was 0.43 ± 0.08 µg/g. In the brains of rats exposed to 5 and 20 ppm F⁻, its content increased to 1.23 ± 0.11 ($p = 0.0019$, $n = 5$) and 1.94 ± 0.18 µg/g ($p < 0.0001$, $n = 5$), respectively ($F(2, 12) = 33.61$, $p < 0.0001$) (mean $\pm$ SEM, one-way ANOVA, Bonferroni post-hoc test).

2.2. Influence of Excessive F⁻ Consumption on Memory and Spatial Learning

The non-spatial short-term and long-term memory of animals was evaluated by NOR (novel object recognition) test in 1 h and 24 h after training. The values of the discrimination index (DI) calculated for the 1-h session were comparable for rats from all groups, i.e., they clearly distinguished old and new objects (Figure 1A). In 24 h after training, however, DIs calculated for F⁻-poisoned rats were significantly lower than for control animals ($p = 0.0080$, $p = 0.0008$, and $p < 0.0001$ vs. control for 5F, 20F, and 50F group, respectively), indicating that they were not able to remember old object. These results resemble the data of our previous work [16], which also showed that excessive F⁻ consumption did not affect the formation of short-term memory but impaired long-term memory.

The working memory of rats was estimated by the Y-maze test. The values of spontaneous alternations coefficients calculated for rats given all three F⁻ doses were considerably lower than for control animals ($p = 0.0159$, $p = 0.0286$, and $p = 0.0143$ vs. control for 5F, 20F and 50F group, respectively) (Figure 1B). The total number of arms entries decreased statistically significantly in the groups of animals that consumed 20 and 50 ppm F⁻. These results indicate that acquisition of the spatial task and, accordingly, working memory was impaired by long-term F⁻ exposure.

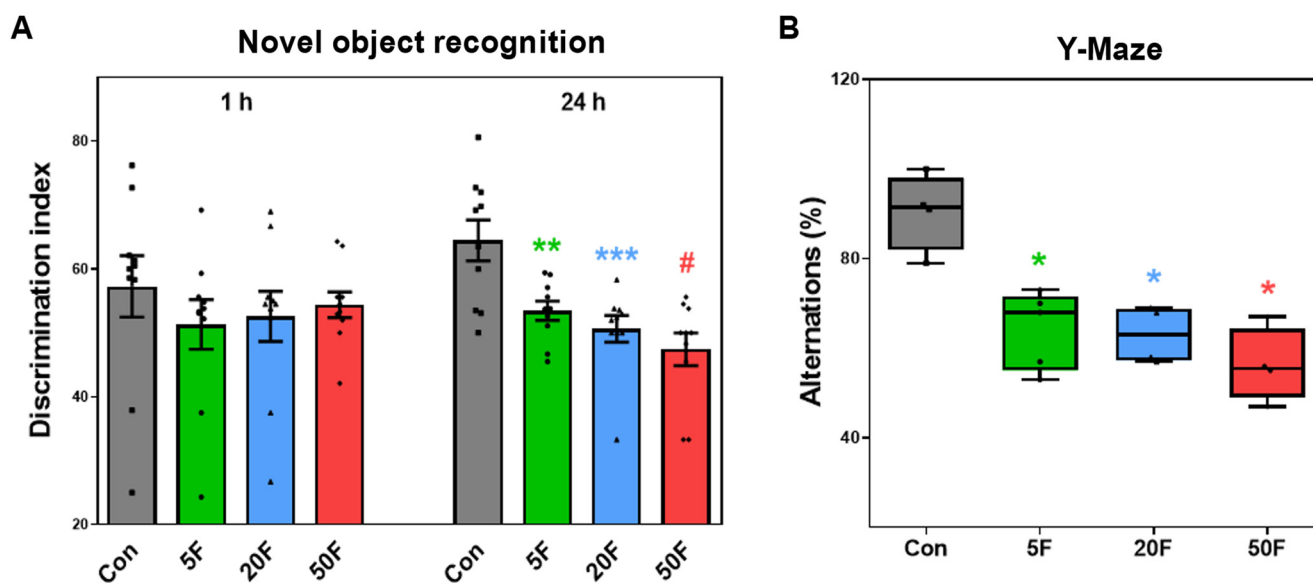


Figure 1. Effects of chronic F⁻ exposure on non-spatial memory. **(A)** Novel object recognition test. Average discrimination indexes calculated for rats from different groups in 1-h and 24-h trials. One-way ANOVA followed by Bonferroni multiple comparison test. Presented are mean values $\pm$ SEM ($n = 10$). **(B)** Y-maze test. Average values $\pm$ SEM ($n = 5$) of spontaneous alternations coefficient for animals from different groups. Mann-Whitney U-criterion. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, # $p < 0.0001$ compared to control. Con—control, 5F—5 ppm F⁻, 20F—20 ppm F⁻, 50F—50 ppm F⁻.

The spatial learning and formation of long-term memory were evaluated by the Morris water maze (MWM) test. On day 1 of the MWM hidden platform acquisition test, the rats from all groups, including the control, needed comparable time to locate the submerged platform (Figure 2A). A progressive decline in the latency of escape to the hidden platform was observed throughout the four-day training. However, two-way

RM-ANOVA revealed a significant effect of group ($F(3, 72) = 7.342; p = 0.0002$) and effect of day ($F(2.753, 161.5) = 11.42; p < 0.0001$), but not significant group by day interaction ($F(9, 176) = 1.568; p = 0.1281$). Further comparison of averages calculated for each day has shown that F⁻-exposed rats needed considerably more time to find the escape platform than control animals. A similar tendency was observed for the distance traveled by rats to reach the platform (Figure 2B), although the statistically significant differences were revealed starting from day 3 only. On the last day of the hidden platform test, all rats from F⁻-treated groups exhibited a longer route before locating the platform, some animals were not able to find the platform at all within a 1-min trial (Figure 2C).

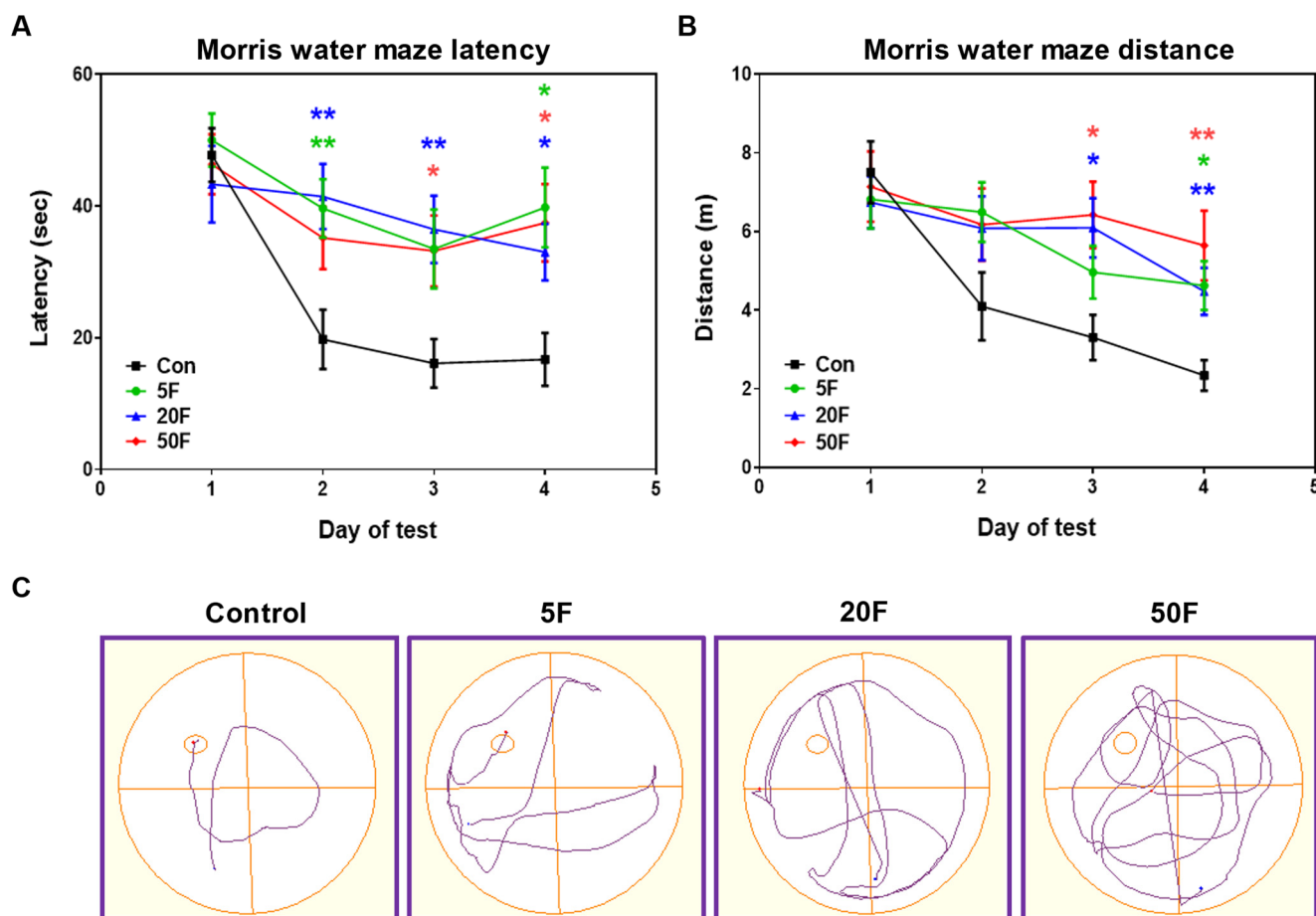


Figure 2. Hidden platform acquisition trial of MWM test. (A,B) Latency and distance traveled by rats to find the platform (means $\pm$ SEM) during four days of training. Two-way RM-ANOVA with Bonferroni post-hoc test. * $p < 0.05$, ** $p < 0.01$ in comparison to control ($n = 10$). (C) Representative trajectories obtained for one rat from each group on 4th day of the hidden platform test.

In the MWM spatial probe test (day 5 without platform), the rats from F⁻-treated groups needed more time to locate the target quadrant and traveled a short distance in this zone (Table 1). The speed of swimming was comparable in all groups of animals on each day of the experiment and on day 5 (Table 1). Thus, although the MWM test did not yield consistent and stable results, it showed that exposure of the rats to excessive F⁻ moderately impaired the processes of visuospatial learning and long-term memory formation.

Table 1. The parameters of swimming obtained in MWM test during the spatial probe trial.

	Control	5F	20F	50F
Time to first entry to target quadrant (s)	5.5 ± 0.6	7.6 ± 0.9 *	8.8 ± 1.0 *	8.1 ± 0.9 *
Time spent in target quadrant (s)	17.8 ± 0.9	15.9 ± 1.7	14.9 ± 1.1 *	16.8 ± 1.6
Total distance (m)	7.1 ± 0.6	5.8 ± 0.6	5.7 ± 0.6	6.9 ± 0.7
Mean speed (m/s)	0.12 ± 0.01	0.10 ± 0.01	0.09 ± 0.01	0.12 ± 0.01
Distance in target quadrant (m)	1.6 ± 0.1	1.0 ± 0.1 **	1.2 ± 0.2 *	1.3 ± 0.2 *
Distance in opposite zone (m)	1.3 ± 0.2	1.7 ± 0.1	2.1 ± 0.3 *	2.6 ± 0.3 **
Number of entries to target quadrant	4.5 ± 0.4	3.3 ± 0.3 *	4.2 ± 0.5	4.5 ± 0.5

Comment: Target quadrant—quadrant in which the hidden platform was placed during the first stage of the test (1–4 days). Opposite zone—quadrant not possessing borders with the target zone. Average values ± SEM are shown, * $p < 0.05$, ** $p < 0.01$ compared to control ($n = 10$).

2.3. Effect of Fluoride on Expression of Subunits Composing AMPA Receptors in Rat Hippocampus

The expression of the *Gria1* gene encoding GluA1 subunits of AMPA receptors was comparable in hippocampal tissues of control and F[−]-poisoned rats (Figure 3A). The content and subcellular distribution of the native form of GluA1 of AMPAR subunits did not change in the cellular fractions enriched with either cytosolic or membrane proteins (further referred to as cytosol or membranes, respectively) (Figure 3B,C). However, the expression of the phosphorylated (Ser845) form of GluA1 subunits increased in the cytosolic fraction of hippocampal cells of animals consumed all F[−] doses ($p = 0.0017$, $p = 0.0016$ and $p = 0.0089$ vs. control for 5F, 20F, and 50F, respectively) (Figure 3B). Accordingly, the ratios of phospho- to total GluA1 protein level in cytosol increased ($p = 0.0039$, $p = 0.0013$, and $p = 0.0011$ for 5, 20, and 50 ppm F[−], respectively) (Figure 3D). In membrane fractions, the amounts of phospho-GluA1 subunits, as well as the phospho/total ratio, remained stable (Figure 3C,E).

The mRNA levels of the *Gria2* gene encoding GluA2 subunits of AMPARs did not differ between the hippocampi of rats who consumed excessive F[−] amounts and that of control animals (Figure 4A). However, the pronounced changes were revealed at the protein level. Thus, the relative content of the GluA2 subunit increased in the cytosolic fraction of the hippocampus of F[−]-exposed animals ($p = 0.0164$, $p = 0.0119$ and $p = 0.0045$ vs. control for 5, 20 and 50 ppm F[−], respectively), but decreased in membranes after consumption of 20 ($p = 0.0007$) and 50 ($p = 0.0037$) ppm F[−] (Figure 4B,C). Similar alterations were observed for phosphorylated (Tyr869/Tyr873/Tyr876) form of GluA2 subunits at 20 ($p = 0.0143$ for cytosol and $p = 0.0417$ for membranes) and 50 ($p = 0.0037$ for cytosol and $p = 0.0232$ for membranes) ppm F[−]. However, the phospho-GluA2/total-GluA2 ratios did not change.

The expression of the *Gria3* gene encoding GluA3 subunits of AMPA receptors was stable in the hippocampal tissues of rats from all groups, including those exposed to high F[−] doses (Figure 5A). However, the protein expression level of GluA3 subunits declined in the cytosolic fraction of hippocampal cells after the impact of 20 and 50 ppm F[−] ($p = 0.0197$ and $p = 0.0135$, respectively) but increased in membranes ($p = 0.0242$ and $p = 0.0087$) (Figure 5B,C).

Since many neuropathological conditions are accompanied by shifts in levels of different subunits composing AMPARs, we calculated the ratio between the expression of *Gria* genes and of GluA subunits composing Ca²⁺-impermeable and Ca²⁺-permeable receptors. This analysis did not reveal any changes in the *Gria1*/*Gria2* mRNA ratio in the hippocampal cells of rats consumed 5 and 20 ppm F[−] but showed its statistically significant elevation at 50 ppm F[−] ($p = 0.0433$) (Supplementary Figure S1A). The GluA1/GluA2 ratio for both native and phosphorylated forms of proteins remained unaltered in the cytosolic fraction (not shown) but considerably enhanced in membranes of animals from 20F ($p = 0.0219$ and $p = 0.0040$ for total and phosphorylated forms, respectively) and 50F ($p = 0.0498$ and $p = 0.0136$) groups (Supplementary Figure S1C). *Gria3*/*Gria2* gene expression ratio did

not change as well (Supplementary Figure S1B), although GluA3/GluA2 ratio decreased in cytoplasm ($p = 0.0070$, $p = 0.0005$ and $p = 0.0002$ for 5, 20 and 50 ppm F^- , respectively) (Supplementary Figure S1E), but increased in membrane fractions of cells after intake of 20 ($p = 0.0003$) and 50 ($p = 0.0058$) ppm F^- (Supplementary Figure S1F). Besides, chronic F^- poisoning led to a rise in the GluA3/GluA1 ratio in hippocampal membranes of rats given 20 and 50 ppm F^- ($p = 0.0413$ and $p = 0.0081$) (Supplementary Figure S1D).

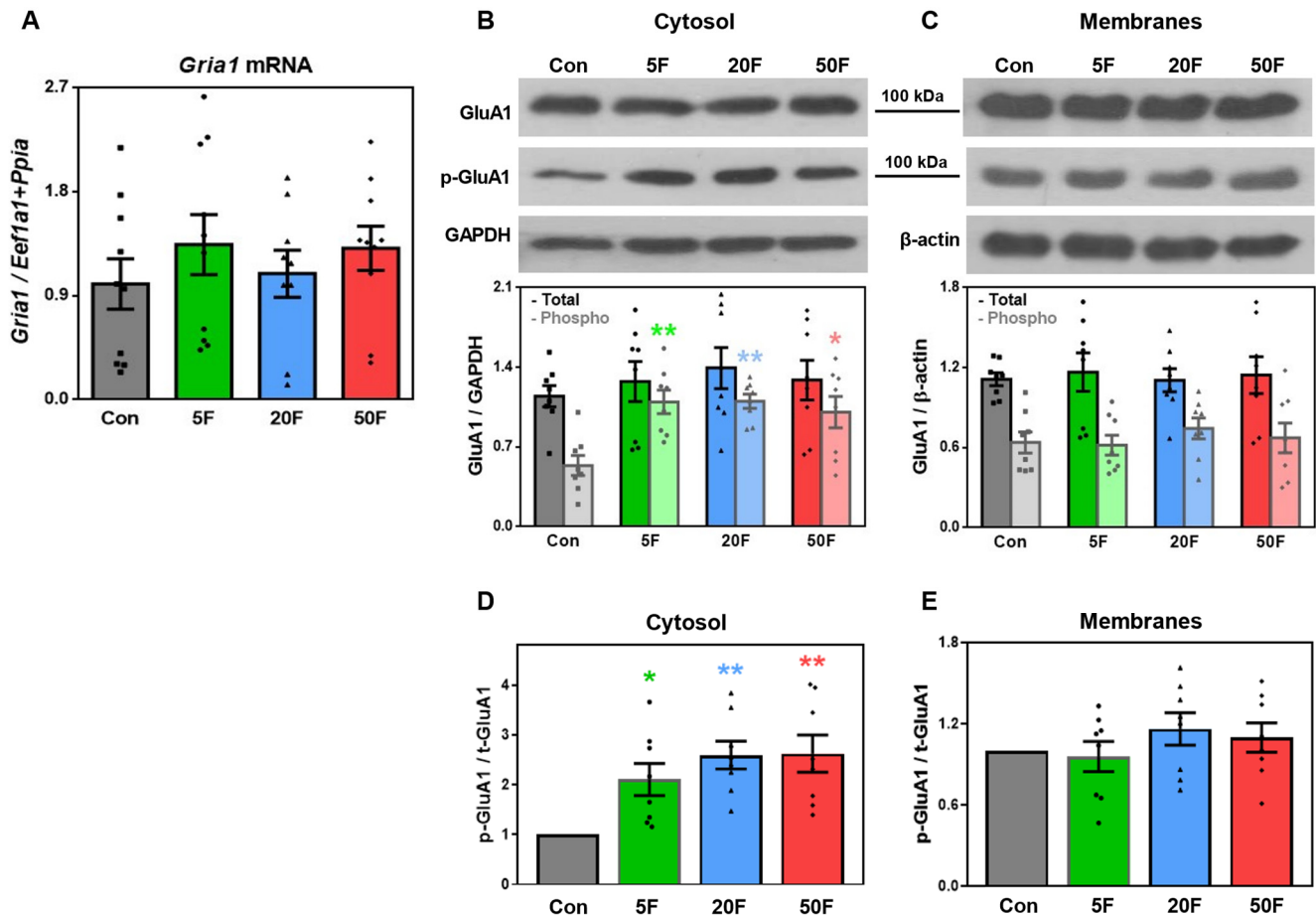


Figure 3. Expression of GluA1 subunits of AMPARs in hippocampus of F^- exposed rats at transcriptional and translational levels. (A) Relative expression of *Gria1* gene in hippocampus of rats consumed different F^- doses normalized to a pair of reference genes (*Eef1a1+Ppia*) (average values $\pm$ SEM, $n = 10$). (B,C) Changes in the protein content of native and phosphorylated (Ser845) forms of GluA1 subunit in the cytosol and membranes of hippocampal cells, respectively. Shown are typical immunoblots and average values $\pm$ SEM ($n = 8$). * $p < 0.05$, ** $p < 0.01$ in comparison to corresponding (native or phosphorylated) control. (D,E) Changes in phospho-GluA1/total GluA1 ratios in cytosolic and membrane fractions. The data are expressed relative to p-GluA1/t-GluA1 ratio of respective controls, which is taken as 1 in each experiment (means $\pm$ SEM, $n = 8$). * $p < 0.05$, ** $p < 0.01$ in comparison to control ratios. One-way ANOVA followed by Bonferroni multiple comparison test.

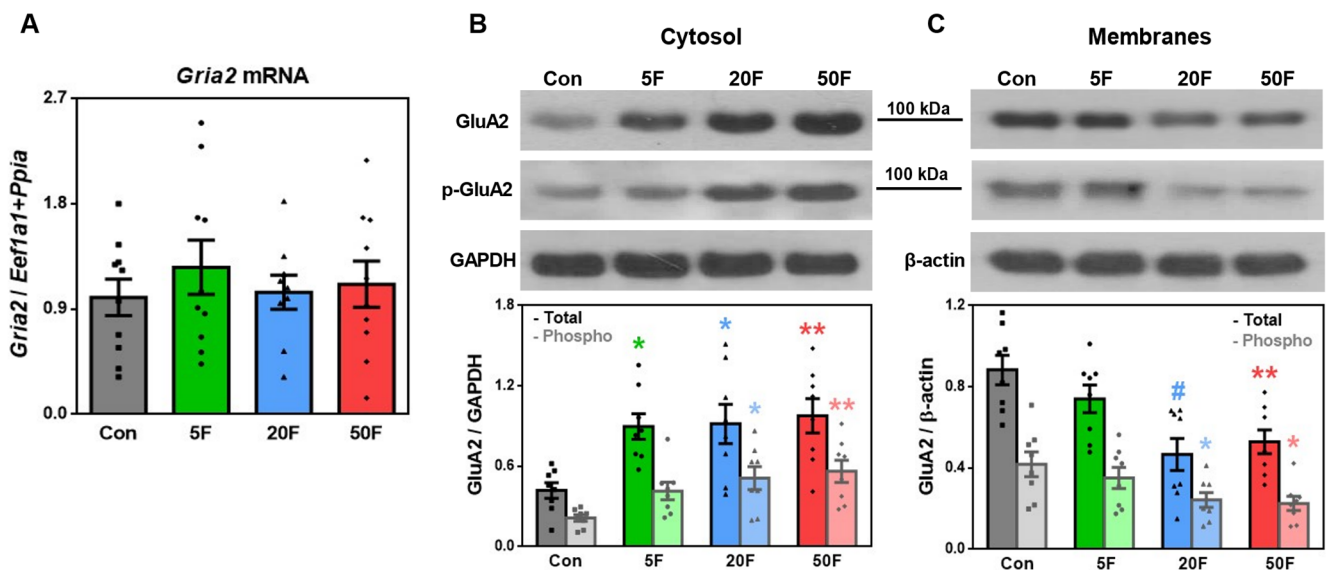


Figure 4. mRNA and protein levels of GluA2 subunits of AMPARs in the hippocampus of F^- poisoned rats. **(A)** Expression of *Gria2* gene in hippocampus of rats exposed to different F^- amounts (average values $\pm$ SEM, $n = 10$). **(B,C)** Alterations in the protein content of native and phosphorylated (Tyr869/Tyr873/Tyr876) forms of GluA2 subunits in the cytosol and membranes of hippocampal cells, respectively. Typical immunoblots and average values $\pm$ SEM ($n = 8$). * $p < 0.05$, ** $p < 0.01$, # $p < 0.001$ in comparison to controls.

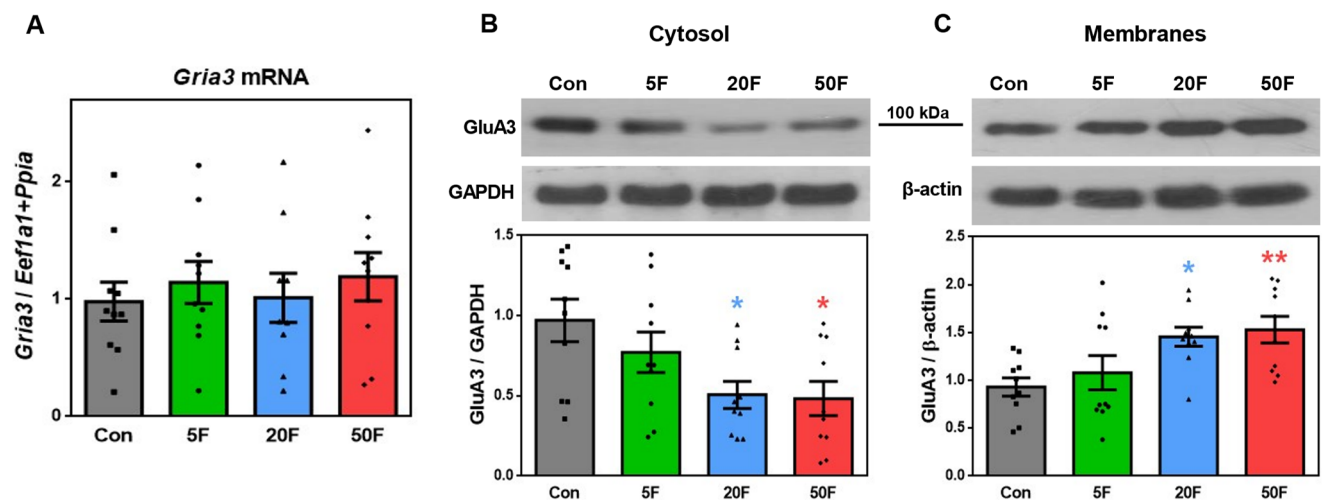


Figure 5. Effect of F^- on expression of GluA3 AMPARs subunits in rat hippocampus. **(A)** Expression of *Gria3* gene in hippocampus of animals consumed different F^- doses (averages $\pm$ SEM, $n = 10$). **(B,C)** Typical immunoblots and average values $\pm$ SEM for GluA3 protein levels in cytosol and membranes ($n = 8$). * $p < 0.05$, ** $p < 0.01$ in comparison to control.

2.4. Effect of Fluoride on Expression of Subunits Composing NMDA Receptors in Rat Hippocampus

The expression level of the *Grin1* gene encoding the GluN1 subunit of NMDA receptors did not respond to excessive F^- consumption by the animals (Figure 6A). Accordingly, increasing F^- concentrations did not change the subcellular distribution of GluN1 subunits. The relative content of both native and phosphorylated (Ser890) GluN1 forms in cytosolic and membrane fractions of hippocampal cells (Figure 6B,C), as well as phospho/total ratios, were stable as well.

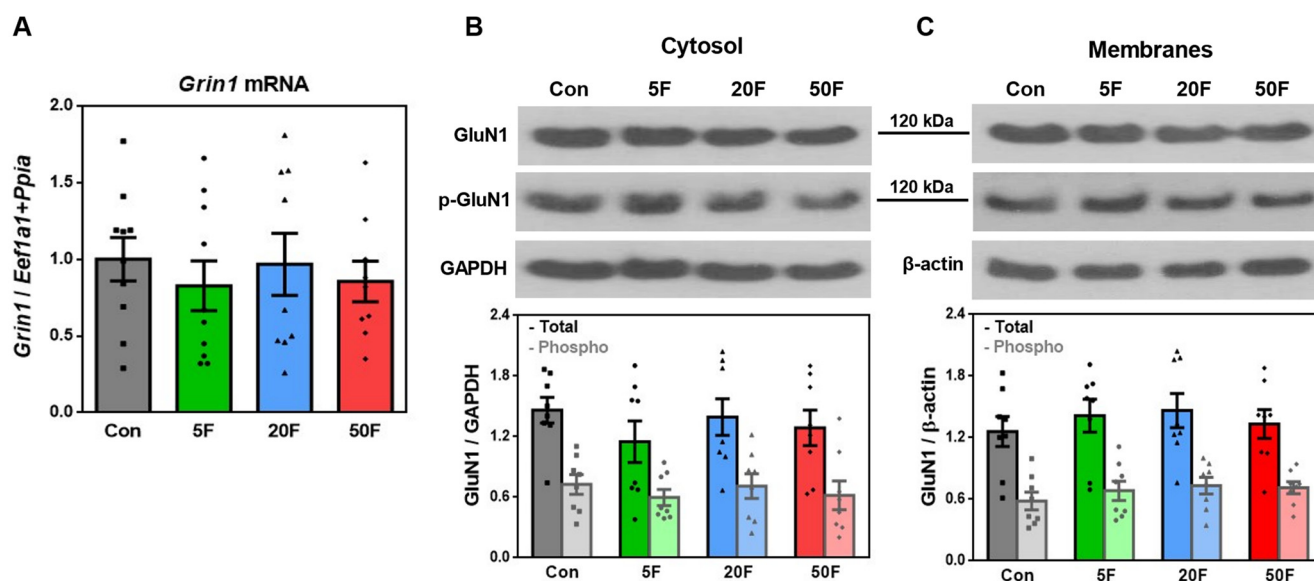


Figure 6. mRNA and protein levels of GluN1 subunits of NMDARs in hippocampal cells. **(A)** Relative expression of *Grin1* gene in the hippocampus of rats consumed different F^- doses normalized to that of a pair of reference genes (*Eef1a+Ppia*). Average values $\pm$ SEM ($n = 10$). **(B,C)** Protein expression of native and phosphorylated (Ser890) forms of GluN1 subunit in cytosolic and membrane fractions, respectively. Shown are typical immunoblots and average values $\pm$ SEM ($n = 8$).

The mRNA level of the *Grin2a* gene encoding GluN2A subunit of NMDA receptors significantly increased in the hippocampus of rats from 20F ($p = 0.0331$) and 50F ($p = 0.0045$) groups in comparison to control (Figure 7A). Accordingly, exposure of animals to 20 and 50 ppm F^- enhanced the expression of both total ($p < 0.0001$ and $p = 0.0008$) and phosphorylated (Tyr1246) ($p < 0.0001$ and $p = 0.0059$) forms of GluN2A subunits in the cytosol. The cytosolic phospho-GluN2A/GluN2A ratio increased as well ($p = 0.0035$ and $p = 0.0300$ for rats from the 20F and 50F groups, respectively) (Figure 7D). In membranes, the changes in total protein content occurred following intake of all three F^- doses ($p = 0.0009$, $p = 0.0092$, and $p = 0.0214$ for 5F, 20F, and 50F, respectively) (Figure 7B,C), while the level of phosphorylated form enhanced only in the cells of rats from 50F group ($p = 0.0019$). Phospho-GluN2A/total-GluN2A ratios increased in hippocampal cells of all animals consumed excessive F^- ($p = 0.0042$, $p = 0.0268$ and $p = 0.0268$ for 5, 20 and 50 ppm F^- , respectively) (Figure 7E).

The expression of the *Grin2b* gene encoding the GluN2B subunit of NMDA receptors was unchanged in the hippocampus of rats exposed to 5 ppm F^- but considerably increased in the cells of animals given 20 and 50 ppm F^- ($p = 0.0084$ and $p = 0.0184$ vs. control, respectively) (Figure 8A). These changes were accompanied by a rise of both total and phosphorylated (Tyr1472) forms of GluN2B subunits in the cytosol of hippocampal cells of rats given 20 ($p = 0.0013$ and $p < 0.0001$ for native and phospho-GluN2B, respectively) and 50 ($p = 0.0001$ for GluN2B and $p < 0.0001$ for phospho-GluN2B) ppm F^- , as well as by phospho-GluN2B/total-GluN2B ratios ($p = 0.0039$ and $p = 0.0032$ for 20 and 50 ppm F^-) (Figure 8B,D). No alterations were revealed in the membranes of F^- -poisoned animals (Figure 8C,E).

The mRNA content of the *Grin3a* gene was comparable in hippocampal cells of control rats and animals treated with 5 and 20 ppm F^- but slightly increased in the cells of rats from the 50F group ($p = 0.0364$) (Supplementary Figure S2).

Grin2a/Grin2b expression ratios did not change in the hippocampus of rats exposed to excessive F^- , while *Grin2a/Grin1* ($p = 0.0219$) and *Grin2b/Grin1* ($p = 0.0442$) ratios increased at 20 ppm F^- (Supplementary Figure S3A,C,E). The ratio between native GluN2A/GluN2B subunits in the cytosol was unaltered as but significantly increased in membranes of hippocampal cells from all F^- -poisoned rats ($p = 0.0014$, $p = 0.0198$ and $p = 0.0465$ for 5F,

20F and 50F, respectively), while that of phosphorylated forms enhanced in hippocampi of animals given 50 ppm F^- ($p = 0.0006$) (Supplementary Figure S3B). In contrast, the changes in the GluN2A/GluN1 ratio were observed predominantly in the cytosolic fraction of cells obtained from rats given 20 ($p = 0.0217$ and $p = 0.0228$ for native and phosphorylated forms, respectively) and 50 ($p = 0.0490$ for total and $p = 0.0426$ for phospho-forms) ppm F^- (Supplementary Figure S3D,F). The shift in GluN2B/GluN1 ratio was statistically significant in the cytosol of hippocampal cells of rats exposed to 20 ($p = 0.0015$ for phospho-form) and 50 ($p = 0.0402$ and $p = 0.0007$ for total and phospho-forms, respectively) ppm F^- .

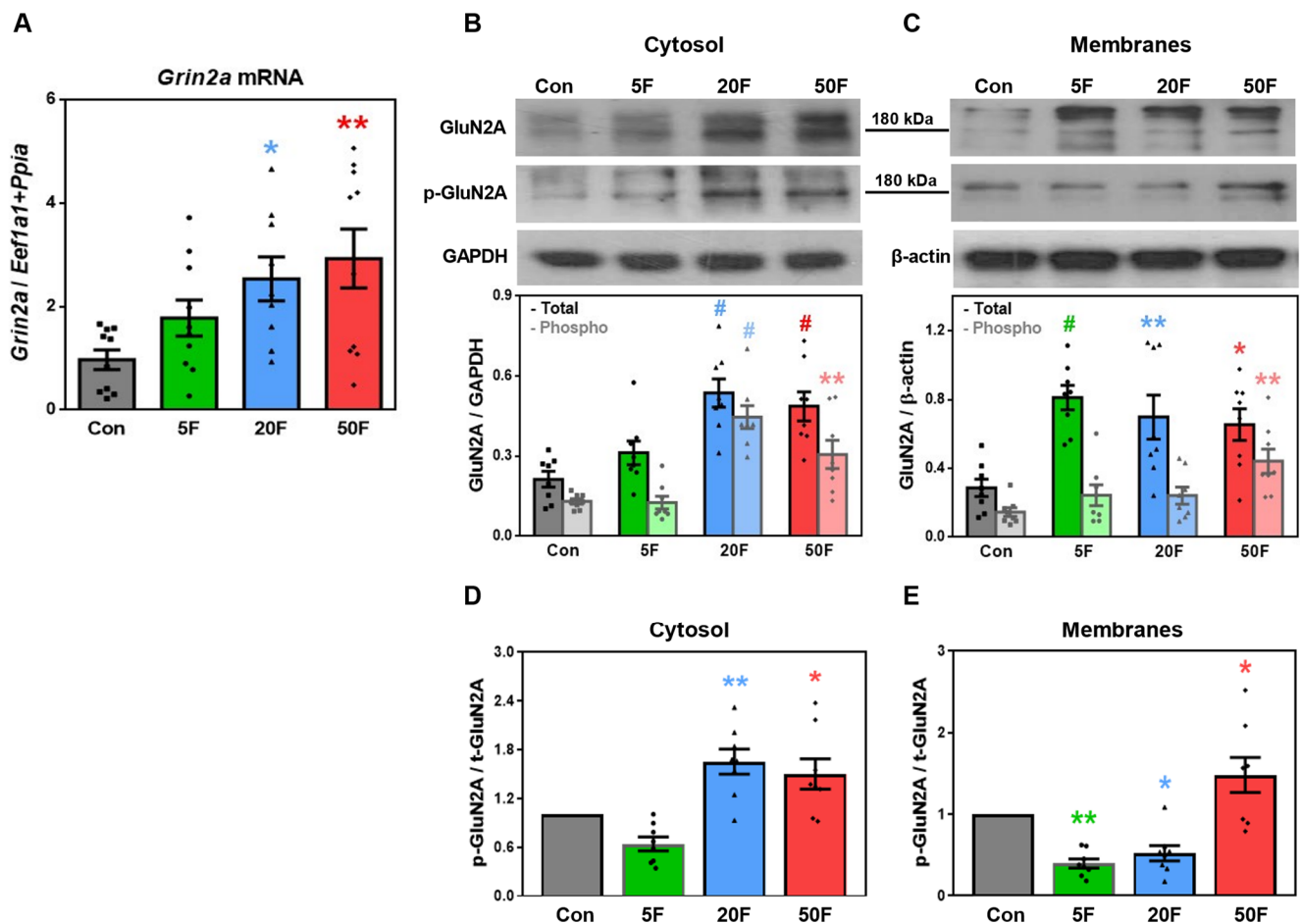


Figure 7. mRNA and protein content of GluN2A subunits of NMDARs in hippocampal cells. (A) Expression of *Grin2a* gene in hippocampus of rats consumed different F^- doses. Means $\pm$ SEM ($n = 10$). (B,C) Levels of native and phosphorylated (Tyr1246) forms of GluN2A subunit in the cytosolic and membrane fractions. Typical immunoblots and average values $\pm$ SEM ($n = 8$). (D,E) Phospho-GluN2A/total GluN2A ratios in cytosolic and membrane fractions (means $\pm$ SEM, $n = 8$). * $p < 0.05$, ** $p < 0.01$, # $p < 0.001$ compared to corresponding control.

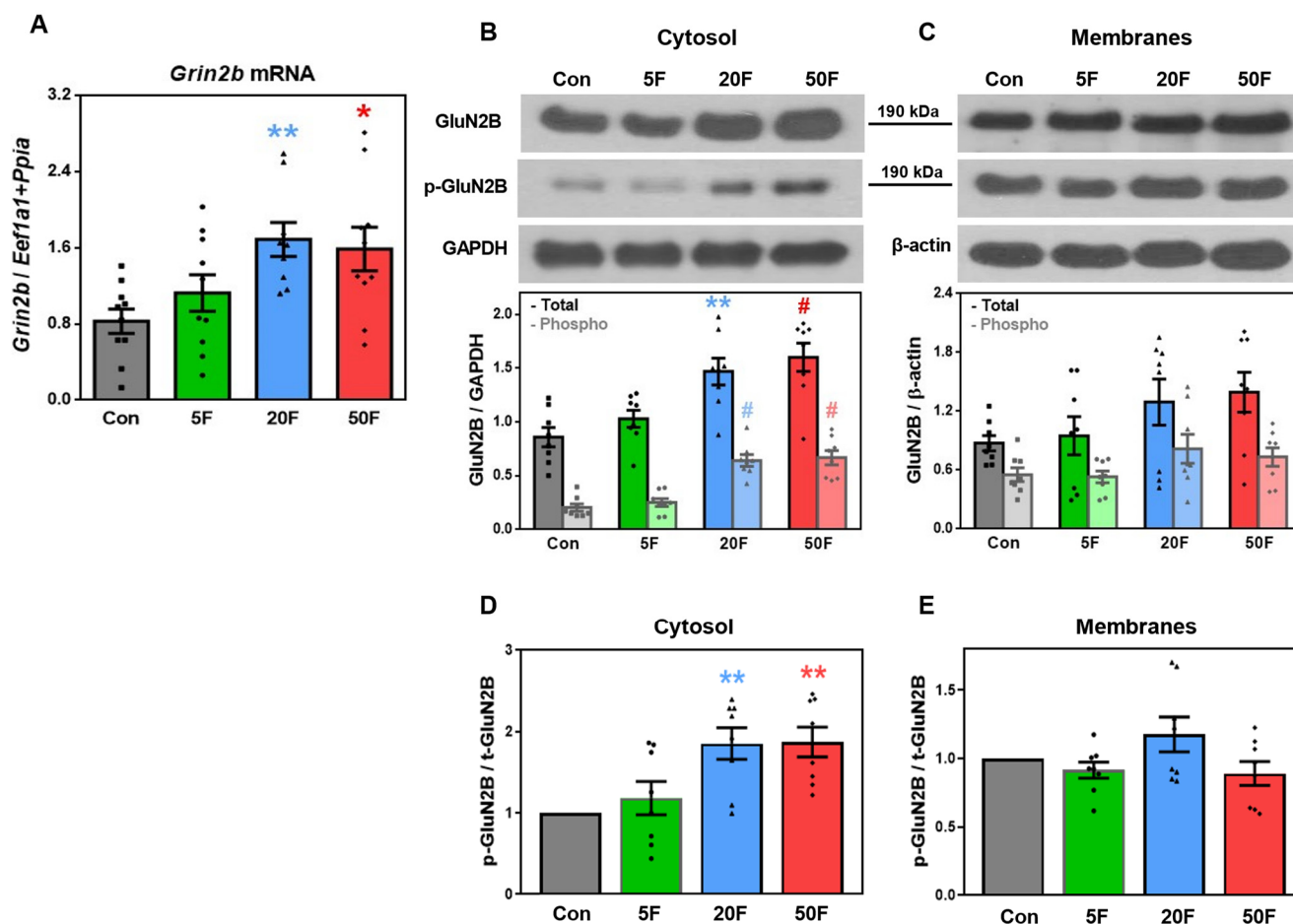


Figure 8. Expression of GluN2B subunits of NMDARs in hippocampal cells of F-exposed rats at transcriptional and translational levels. (A) Levels of *Grin2b* gene mRNA (mean values $\pm$ SEM, $n = 10$). (B,C) Content of native and phosphorylated (Tyr1472) forms of GluN2B subunit in cytosolic and membrane fractions. Shown are typical immunoblots and average values $\pm$ SEM ($n = 8$). (D,E) Changes in phospho-GluN2B/total GluN2B ratios in cytosolic and membrane fractions (means $\pm$ SEM, $n = 8$). * $p < 0.05$, ** $p < 0.01$, # $p < 0.001$ compared to control.

3. Discussion

The results of the present study show a complicated picture of alterations in the expression of different subunits composing AMPA and NMDA glutamate receptors in hippocampus of F-exposed rats at both transcriptional and translational levels (Figures 3–8).

Changes in synaptic AMPAR number and subunit composition are the primary mechanisms underlying synaptic plasticity and cognitive capacities [28–30]. On the other hand, subcellular AMPAR redistribution, such as the replacement of Ca^{2+} -impermeable GluA2-containing AMPARs with Ca^{2+} -permeable GluA1-dominant AMPARs at synapses, is observed in many neuropathological conditions [30]. Thus, endocytosis of GluA2-containing AMPARs and facilitated delivery of GluA2-lacking receptors to synaptic sites was found in the spinal cord of rats with peripheral nerve injury [31] and diabetic neuropathy [32] or in hippocampal neurons after ischemic insult [33]. Internalization of AMPARs associated with LTD (long-term depression) and synaptic failure has been linked with Alzheimer's disease (AD) [34]. Disturbed trafficking and reduced AMPAR number were observed at synaptic and extrasynaptic membranes of different types of neurons in P301S tau and APP/PS1 transgenic mouse AD models [35,36]. Stress-induced behavior changes were associated with the insertion of Ca^{2+} -permeable AMPARs in the amygdala [37], while decreased AMPAR levels were revealed in different brain regions of schizophrenic patients [38]. The

dysfunction of GluA3-containing AMPARs at least partially underlies amyloid- β -triggered memory deficits [39], mild cognitive decline [40], and aggressive behavior [41].

The negative impact of F^- on cellular AMPARs has been reported in a few early works. Depressed LTP in hippocampal microglial cells following exposure of rats to 120 ppm F^- for 12 weeks was associated with decreased content of GluA2 subunits [42]. The level of *Gria2* mRNA was reduced in the brains of mouse pups exposed to 25–100 ppm NaF prenatally and during lactation, although no changes were observed for GluA1 [43]. In contrast, total cellular levels of GluA1 and GluA2 AMPAR subunits in mice hippocampus and cortex did not respond to combined prenatal/P90 exposure to 25 ppm F^- [21]. In this study, we observed considerable alterations in the levels of different AMPAR subunits in subcellular fractions. An increased GluA2 protein content in the cytosol probably indicates intracellular retention of this subunit (Figure 4). In contrast, changes in the location of GluA3 subunits can be linked with membrane insertion (Figure 5). Such subcellular redistribution of GluA2 and GluA3 at the background of stable GluA1 expression (Figure 3) suggests the shift in the ratio between Ca^{2+} -permeable and Ca^{2+} -impermeable AMPARs in membranes (Supplementary Figure S1) probably leading to the prevalence of Ca^{2+} -permeable AMPARs at synaptic sites. Moreover, such changes are typical for neurodegenerative processes. However, the stable expression of corresponding *Gria* genes indicates that all alterations in AMPAR levels in the rat hippocampus are provided exclusively by their post-translational modifications.

Phosphorylation of GluA subunits is another mechanism regulating AMPAR biophysical properties and functions, as well as their trafficking to synapses or endocytosis [25,44–46]. The study on mice with mutations at GluA1 Ser845 and Ser831 confirmed an important role of phosphorylation in learning and motivated behavior [47]. Ser845-GluA1 phosphorylation is believed to control the recruitment of this subunit to membranes for synaptic insertion or removal from synapses during synaptic plasticity, while Tyr876-GluA2 phosphorylation prevents endocytosis. Acute stress, declined cognitive capacities, and depressive disorders are accompanied by phosphorylation of both GluA1 (Ser845 and Ser831) and GluA2 (Ser880) subunits in different brain regions [48–50]. The altered ratio between native and phosphorylated GluA1 subunits underlie memory impairment in the APP/PS1 mouse AD model [51]. In our work, an enhanced phospho/total GluA1 ratio revealed in the cytoplasm of hippocampal cells of F^- -poisoned animals point to activation of this subunit (Figure 3). Ser845-GluA1 phosphorylation might facilitate the insertion of GluA1-containing AMPARs to postsynaptic density or indicate the formation of a reserve pool of these molecules. An increased level of phosphorylated GluA2 subunits in the cytosol but its declined amount in membranes with the stable phospho/total GluA2 ratio is probably entirely linked with their subcellular redistribution (Figure 4). Overall, such changes suggest an impaired intracellular GluA1 and GluA2 subunit trafficking, contributing to an altered ratio of various GluA subunits in membrane AMPARs.

Similar to AMPARs, an overwhelming body of evidence centered on NMDARs as inductors of synaptic plasticity, with the expression of GluN1 and GluN2A subunits increasing during memory formation and consolidation [52–54]. Despite this favorable role, the changes in the GluN2A/GluN2B ratio can induce excitotoxicity and implicate a wide range of neurological disorders [55]. Increased GluN2A/GluN2B ratio in amygdala neurons of transgenic mice before fear learning significantly disrupted long-term memory consolidation [56]. Changed GluN2A/GluN2B ratio following the knockdown of the *Grin2a* gene led to impaired contextual fear-conditioning memory and increased seizure susceptibility [57]. Methamphetamine-induced schizophrenia in rats was accompanied by decreased expression of *Grin* genes in different brain regions [58]. Mutations in the *GRIN2B* gene are associated with intellectual disability, developmental delay, motor impairments, autism spectrum disorder, and epilepsy in humans [59]. The changed ratio between GluN2A- and GluN2B-containing NMDARs, their allosteric modulations, disturbed trafficking, or impaired interaction with AMPARs are well-acknowledged events in epileptogenesis [60–62]. On the other hand, a few works [63–65] suggested the neuroprotective function of hippocampal astrocytic NMDARs since pharmacological antagonism of GluN2A and GluN2B

or knockdown of *Grin2a* gene exacerbates β -amyloid (A β)-induced synaptotoxicity and cognitive decline.

The data describing an F^- impact on NMDAR functions are controversial. Using three experimental systems (brains of adult rats given 50 ppm F^- for 6 months, their offspring sacrificed during early postnatal life, and primary cultured hippocampal neurons of neonatal rats treated with 5 and 50 ppm F^- for 48 h), Wei et al. [66] showed that gene and protein expression of GluN1 and GluN2B subunits enhanced, GluN2A did not change, and GluN3A decreased. A similar increase of NMDAR1 mRNAs and protein was described in the hippocampus of rat pups exposed to 150 mg/L NaF prenatally and until PND28 [20]. Upregulation of GluN2A and downregulation of GluN2B protein expression was observed in the hippocampus of mice exposed to 25 ppm F^- prenatally/up to P90 [21]. In contrast, down-regulation of NMDAR mRNA or protein was reported in the brains of offspring mice treated with 50–100 ppm F^- for 90 days [67]. Microglia-induced neuroinflammation in the hippocampus of rats given 120 ppm F^- for 12 weeks was associated with a decline in NMDA2B protein [42].

In our study, in contrast to AMPARs, F^- -induced changes in the expression of NMDARs subunits in rat hippocampus occurred at both transcriptional and translational levels (Figures 6–8). Overexpression of *Grin2a* and *Grin2b* genes suggests enhanced synthesis, while increased phosphorylation of GluN2A and GluN2B subunits can be associated with their intensive trafficking. However, elevated *Grin2a/Grin1* and *Grin2b/Grin1* ratios, cytosolic GluN2A/GluN1 and GluN2B/GluN1 ratios, and membrane GluN2A/GluN2B ratio (Supplementary Figure S3) indicate the severe disturbances in synaptic processes in response to F^- exposure. In spite of both GluN2A and GluN2B subunits being upregulated, an increase in membrane GluN2A/GluN2B ratio might be linked with the insertion of newly synthesized GluN2A-dominant NMDARs to membranes and internalization of GluN2B-containing receptors. Increased phosphorylation of GluN2B (Tyr1472) subunits in the cytosol can facilitate their association with postsynaptic density protein PSD-95 or stabilize the extrasynaptic location of GluN2-containing NMDARs. However, stable expression of obligatory GluN1 subunit at the background of increased expression of GluN2A and GluN2B subunits might be associated with altered expression of other minor NMDAR subunits, which were not examined in our study.

Overall, we assume that changes in the ratio between Ca^{2+} -permeable and Ca^{2+} -impermeable AMPARs subunits and between GluN2A and GluN2B subunits in membrane NMDARs induce excitotoxicity and at least partially underly an impairment in cognitive capacities of F^- -poisoned animals (Figures 1 and 2). Fluoride ions are known to form stable complexes with metal ions such as Mg^{2+} , Mn^{2+} , Al^{3+} , and Fe^{3+} [68], which theoretically allows them to interact with enzymes possessing these metals in active centers. Thus, F^- directly binds with the structural and catalytic Mg^{2+} in glycolytic metalloenzyme enolase [69]. Besides, F^- is a potent inhibitor of protein phosphatases [70]. Although these effects have been shown in vitro and the doses of F^- sufficient for triggering such reactions in vivo are not established, we assume that effects of F^- on NMDARs in the rat hippocampus are associated with its binding to Mg^{2+} , while increased receptors phosphorylation is at least partially due to impaired dephosphorylation. Hyperactivation of AMPARs and NMDARs can induce an excessive Ca^{2+} entrance to the cells and activate Ca^{2+} -dependent enzymes, including protease calpain, which destroys cytoskeletal structures, leading to pathological changes in neurons [15,16]. The functional activity of calpain is closely associated with the stimulation of NMDARs [71]. On the other hand, since GluN2A subunits are believed to have a protective role in brain cells, such changes can indicate a compensatory mechanism necessary for alleviating the rate of neuron death under the influence of F^- .

It is interesting that altered expression profiles of subunits composing AMPARs and NMDARs were pronounced in the hippocampus of rats given 20 and 50 ppm F^- , whereas the decline in cognitive capacities was also evident for animals given the lowest F^- dose (5 ppm). This phenomenon suggests the existence of other cellular processes providing a negative F^- impact on brain functions. Recently, impaired neurogenesis and death of

hippocampal neurons under F^- influence was linked with activation of Notch1 (Neurogenic locus notch homolog protein 1) signaling, dysfunction of SIK2-CRTC1 (Salt inducible kinase 2-CREB-regulated transcription coactivator 1) signaling, and declined synthesis of BDNF (brain-derived neurotrophic factor) and VGF (nerve growth factor inducible) [16,72,73].

The strength of our study is the application of relatively low F^- doses, which yielded plasma F^- content comparable to that registered in the serum of humans from regions of endemic fluorosis, taking into account faster F^- clearance in rats. The long period of F^- exposure, which led to the development of stable fluorosis, also allowed us to repeat the conditions common in the areas with high environmental F^- content. Next, this study is the first to comprehensively evaluate the changes in expression of all major AMPARs and NMDARs in rat hippocampus following long-term F^- intake. However, our work has some limitations. First, as for all in vivo studies, the methods applied by us cannot distinguish between neurons and glial cells, between synaptic and extra-synaptic receptors, between their trafficking to membranes or endocytosis, so we cannot evaluate the number of receptors directly implicated in excitotoxic F^- effects, as well as cannot say which of the processes of synaptic plasticity (LTP or LTD) is disturbed. Next, the present study did not explore the possible compensatory mechanisms that can be activated in response to hyperactivation of AMPARs and NMDARs to alleviate excitotoxicity.

4. Materials and Methods

4.1. Animals

Forty male conventional healthy Wistar rats were procured from the Animal Facility of Sechenov Institute and maintained under standard conditions (temperature of 22–25 °C, 12 h light/12 h dark cycle, food and water ad libitum). Low F^- natural-ingredients diet was manufactured following recommendations of Nutrient Requirements of Laboratory Animals [74]. All procedures were in accordance with the Animal Welfare Act (2006) and approved by the Bioethics Committee of Sechenov Institute of Evolutionary Physiology and Biochemistry (Protocol # 9/2021 of 24 September 2021).

At the age of 6 weeks, the animals were randomly assigned into four groups. Control rats were given regular tap water with a natural F^- content of 0.4 ppm provided by the local water supplier. Animals from other groups received the same water supplemented with 5, 20, and 50 ppm F^- (as NaF) for 12 months. This treatment period was sufficient for rats to develop stable dental fluorosis, as was established in our previous work [16]. The doses of F^- were comparable to those existing in the water available for humans in the regions of endemic fluorosis, taking into account that the rate of F^- clearance in rats is higher than in humans. For instance, the F^- content of 1.72 μ M in the serum of rats given 22.6 ppm F^- [75] was similar to the F^- concentration in the plasma of humans consuming 2–3 ppm F^- [76,77].

The development of fluorosis was evaluated by the changes in animal teeth enamel, as described earlier [16]. While the incisors of control rats were yellowish and matte, the teeth of animals consumed 50 ppm F^- became “glass-like” with white stains, which indicates impaired enamel mineralization typical for dental fluorosis in rodents. The values of Dean’s fluorosis index calculated for the rats from control and F^- -poisoned groups are shown in Supplementary Table S1. In the group given 5 ppm F^- , less than half of the animals had normal teeth, while the majority of rats consumed 20 and 50 ppm F^- developed moderate and severe degrees of fluorosis, respectively.

4.2. F^- Determination

F^- content in the whole brain was evaluated in a separate cohort of animals exposed to background F^- content (0.4 ppm), 5 and 20 ppm F^- . One hemisphere of the brain was homogenized in phosphate-buffered solution (pH 7.4) at 4 °C. The homogenates were centrifuged for 15 min at 22,000 $\times$ g to obtain pure supernatant. F^- concentrations in the samples were determined using an F^- -specific electrode (Elit-221, Akvilon, Russia) and AgCl₃ reference electrode connected to potentiometer I-500 (Akvilon, Russia). Calibration curves were created using the standards with known F^- concentrations in water buffered

with TISAB II (pH 5.15) (AppliChem, Darmstadt, Germany). Aliquots of brain homogenates were mixed with an equal volume of TISAB II buffer. The brain samples were prepared under the same conditions as the standards. Brain F[−] content was expressed as µg of fluoride/g of tissue.

4.3. Examination of Cognitive Capacities

The efficiency of hippocampus-dependent spatial and non-spatial memory and learning was evaluated by three behavior tests widely used in rodents. The rats were subjected to behavior experiments within the last four weeks of F[−] exposure. All test series were recorded by Any-maze behavioral Video Tracking System 6.36 software and analyzed using Any-maze 4.48 program (Stoelting Co., Wood Dale, IL, USA).

4.3.1. Novel Object Recognition Test (NOR)

The formation of short-term and long-term non-spatial memory was evaluated using the NOR test. Before testing, the rats were habituated to an experimental cage by being allowed to explore it for 5 min in the absence of objects. The next day, a training session was performed first. The rats were placed at the center of the cage in front of two identical objects and allowed to examine them for 5 min. The formation of short-term memory was examined 1 h after training and the efficiency of long-term memory 24 h after. During both testing sessions, one of the familiar objects was replaced with a new one. All objects were odorless glasses of different shapes and colors but with similar volumes and heights. The recognition was considered successful if the rats touched the objects with their noses or hands. The objects were cleaned with 70% ethanol after each rat to remove the smell of the previous animal. The results of both testing sessions are presented as discrimination index: $DI = (\text{novel object}) / (\text{novel object} + \text{familiar object}) \times 100\%$ [78].

4.3.2. Y-Maze Test

The Y-maze test was performed a week after the NOR test and used to evaluate spatial working memory. The maze was made of black plastic and contained three arms (50 × 10 × 30 cm) positioned at a constant angle. Each rat was placed at the center of the maze and allowed to freely explore it for 8 min. The number of individual arm entries and their sequence were scored by an observer in a triplet set, with successful entry defined as all four animal legs inside the arm. The percentage of spontaneous alternation behavior was calculated as the ratio of actual to possible alternations: $C_{alt} = N_{alt} / (N_t - 2) \times 100\%$, where N_{alt} —number of alternations, N_t —number of total arms entries [79].

4.3.3. Morris Water Maze Test

Spatial learning and formation of memory were evaluated by the Morris water maze test [80] a week after the Y-maze test. The experiments were performed in a circular plastic pool (TS1004-M2G, Water maze test, OpenScience, Moscow, Russia) filled with opaque water (temperature of 22–24 °C), which was changed every day. The pool was divided into four quadrants (north, south, east, and west) used as the starting points. Before the test series, the animals were habituated to water by swimming for 60 s. The next day, the escape platform was fixed in the geometric center of the northwest quadrant, 1 cm above the water, and four objects of various shapes were placed on the walls of the pool to help animals learn about the environment. The rats were allowed to find the platform and stay on it for 15 s. During the next four consecutive days, the animals were subjected to hidden-platform acquisition tests from different sides. The rats were allowed to find the platform submerged 1 cm below water for 60 s. The time required to locate the platform (escape latency) was established. If the rat failed to find the platform within testing time, it was guided there by the researcher, and escape latency was fixed at 60 s. After finding the platform, the rats were allowed to rest on it for 15 s. Each day, four consecutive swimming attempts from different starting points with a rest interval of 15 s were performed. The swimming time, pathway, and speed were analyzed. The second stage of the test (spatial

probe) was performed on day 5. The platform was removed, and the rats were placed into the pool facing the wall in the quadrant opposite the original position of the platform. The swimming pathway was recorded for 60 s. The time of the first entry to the target quadrant and a few other parameters have been analyzed in the Any-maze program.

4.4. mRNA Extraction and cDNA Synthesis

In two weeks after the end of behavior tests, the rats were anesthetized with Zoletil-100 by intraperitoneal injection (50 mg/kg BW) and sacrificed by dissecting the abdominal aorta. The brains were removed and placed in ice-cold conditions. The hippocampi from one hemisphere were isolated and incubated in an Intact RNA reagent (Eurogen, Moscow, Russia) for 24 h at 4 °C to stabilize RNA. Then, the samples were homogenized in the appropriate volume of monophasic aqueous ExtractRNA reagent (Eurogen, Moscow, Russia) according to the manufacturer's instructions. Total RNA concentration (absorption at 260 nm) and purity (260/280 nm absorption ratio) were evaluated using a CLARIOstar Plus multimodal reader (BMG LABTECH, Ortenberg, Germany). The absorption ratio at 260/280 nm exceeded 1.8 in all samples. Total RNA (1 µg) was reverse transcribed into cDNA using an MMLV RT kit (Eurogen, Moscow, Russia). The reaction mixture was incubated for 5 min at 25 °C, 60 min at 42 °C, and 5 min at 70 °C. cDNA was diluted 10-fold before the PCR step. The primers were provided by Eurogen (Moscow, Russia) (Supplementary Table S2). The melting temperature was optimized using the Primer Blast tool (<http://www.ncbi.nlm.nih.gov/tools/primer-blast>, accessed on 1 October 2024). The product size was 70–250 base pairs, and GC content was 40–60%.

4.5. Quantitative Real-Time PCR

RT-qPCR was performed on a C1000 Touch Thermal Cycler equipped with the CFX96 detection system (Bio-Rad, Hercules, CA, USA). The reactions were carried out using qPCRmix-HS SYBR Master Mix (Eurogen, Moscow, Russia). The mixture contained 17 µL of dH₂O, 2 µL of direct and reverse primer (500 nM), 1 µL of cDNA, and 5 µL of qPCRmix-HS SYBR. The cycling protocol included initial denaturation at 95 °C for 5 min followed by 40 amplification programs, each consisting of a denaturation step at 95 °C for 5 s, an annealing step at 57–63 °C for 10 s, and an extension step at 72 °C for 30 s. The specificity of amplification was verified by analysis of melting curves.

qPCR curves were analyzed using CFX Manager version 3.1 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The expression of target genes was normalized to that of the *Eef1a1/Ppia* pair of reference genes, showing stable expression in our previous work [81]. The threshold cycle (Ct) values for target and reference genes were less than 40 for all reactions. The relative expression of genes was estimated by the comparative $2^{-\Delta\Delta C_t}$ method, where ΔC_t is the difference between studied and reference genes [82]. The average Ct and standard deviation (SD) values for each qPCR product were calculated from three individual repeats.

4.6. Western Blotting Analysis

The hippocampi from the second hemisphere were frozen at −80 °C until processing. The subcellular fractions enriched with cytosolic and membrane proteins (further referred to as cytosol and membranes, respectively) were obtained using a slightly modified method of detergent extraction recommended for Mem-PER Plus Membrane Protein Extraction kit (#89842; ThermoFisher Scientific, Waltham, MA, USA). The hippocampi were minced with permeabilization buffer supplemented with inhibitors of proteases (Sigma-Aldrich P8340, Merck, Darmstadt, Germany) and phosphatases (1 mM Na₃VO₄ 2 H₂O and 1 mM Na₂-EDTA). Non-homogenized material was sedimented by centrifugation at 1000 × g for 15 min and 4 °C. The supernatant was centrifuged at 11,000 × g for 15 min and 4 °C, then the aliquots of the second supernatant were centrifuged at 25,000 × g for 15 min and 4 °C to obtain the fraction enriched with cytosolic proteins, and the final pellet was solubilized to obtain the fraction containing membrane and membrane-associated proteins. The purity

of obtained fractions was examined by immunoblotting with membrane proteins Na-K-ATPase, postsynaptic density protein PDS95, mitochondrial membrane protein VDAC (voltage-dependent anion channel), cytosolic protein GAPDH and cytoskeletal protein β -actin. For this purpose, an equal amount of total protein (30 μ g) from all fractions was loaded into wells. As seen in Supplementary Figure S4, the fractions enriched with cytosolic and membrane proteins were reasonably homogenous and contained only small amounts of the proteins from other subcellular fractions.

The samples were frozen at -80°C until use. The total protein content in the probes was determined by the Lowry method. The samples were solubilized in Laemmli buffer, then 60 or 100 μ g/well of cytosolic and 30 μ g/well of membrane proteins were separated by SDS-PAGE in 10% polyacrylamide gels and transferred to nitrocellulose membranes. Non-specific membrane binding was blocked for 1 h at room temperature with a 5% skim milk solution. Then the membranes were incubated overnight with primary antibodies against GluA1 (1:750), phospho-GluA1 (1:750), GluA2 (1:750), phospho-GluA2 (1:750), GluA3 (1:750), GluN1 (1:500), phospho-GluN1 (1:500), (1:200), GluN2A (1:500), phospho-GluN2A (1:200), GluN2B (1:500) and phospho-GluN2B (1:200), followed by 1-h treatment with secondary HRP-conjugated antibodies (1:1000). Immunostained proteins were visualized by exposure with ECL detection system. To control protein loading, the membranes were stripped by two rounds of 30-min incubation with an acidic solution containing 25 mM glycine and 1% SDS (pH 2.0), followed by 10-min TBS washing and re-probed with antibodies against GAPDH for cytosol or β -actin for membranes (1:1000) followed by secondary HRP-conjugated antibodies. Relative protein expression was evaluated by densitometric analysis using the ImageJ program (NIH, Bethesda, MD, USA). The optical densities of target proteins were normalized to that of reference proteins.

Rabbit antibodies to GluA1 (#13185), phospho-GluA1 (#8084), GluA2 (#5306), phospho-GluA2 (#3921), GluA3 (#4676), GluN1 (#5704), phospho-GluN1 (#3381), GluN2A (#4205), phospho-GluN2A (#4206), GluN2B (#4207) and phospho-GluN2B (#4208) were purchased from Cell Signaling Technology (Danvers, MA, USA). Mouse monoclonal antibodies against GAPDH (sc-32233) and β -actin (sc-517582) were obtained from Santa Cruz Biotechnology. Secondary antibodies and chemiluminescent ECL systems were purchased from Cytiva (Marlborough, MA, USA), and X-ray film, developer, and fixer solutions were purchased from Agfa CEA (Mortsel, Belgium).

4.7. Statistics

The statistical analysis was performed using GraphPad Prism 6 software (San Diego, CA, USA). Shapiro-Wilk normality test was applied to check for the Gaussian distribution of the samples. The results of the Y-maze test were evaluated by the Mann-Whitney U-test. The parameters obtained in the MWN acquisition test, such as mean latencies and distance, were analyzed by two-way repeated measures (RM) ANOVA. All other data were evaluated by one-way ANOVA followed by Bonferroni multiple comparison test. The data are presented as mean $\pm$ SEM for parametric results and boxplots for non-parametric data. The differences were considered statistically significant at $p < 0.05$.

5. Conclusions

The results of our study revealed that the cognitive decline of F-poisoned rats is accompanied by the changes in expression of a few subunits composing AMPA and NMDA receptors in hippocampal cells. The major findings are the prevalence of Ca^{2+} -permeable GluA1- and GluA3-containing AMPA receptors in membranes, internalization of Ca^{2+} -impermeable GluA2-containing AMPARs, upregulation of *Grin2-3* genes, and shift in the ratio between GluN2A and GluN2B subunits composing NMDA receptors. All these phenomena might indicate disturbed synaptic processes and are typical for neurological disorders.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms252111796/s1>.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Bioethics Committee of Sechenov Institute of Evolutionary Physiology and Biochemistry (Protocol # 9/2021 of 24 September 2021).

Data Availability Statement: The data generated and analyzed during the current study will be available from the corresponding authors upon reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Johnston, N.R.; Strobel, S.A. Principles of fluoride toxicity and the cellular response: A review. *Arch. Toxicol.* **2020**, *94*, 1051–1069. [[CrossRef](#)] [[PubMed](#)]
2. Lubojanski, A.; Piesiak-Panczyszyn, D.; Zakrzewski, W.; Dobrzynski, W.; Szymonowicz, M.; Rybak, Z.; Mielan, B.; Wiglusz, R.J.; Watras, A.; Dobrzynski, M. The Safety of Fluoride Compounds and Their Effect on the Human Body—A Narrative Review. *Materials* **2023**, *16*, 1242. [[CrossRef](#)]
3. Bailey, K.; Chilton, J.; Dahi, E.; Lennon, M.; Jackson, P.; Fawell, J. (Eds.) *Fluoride in Drinking Water*; WHO (World Health Organization), WHO Press: Geneva, Switzerland, 2006; pp. 37–39.
4. Till, C.; Green, R. Controversy: The evolving science of fluoride: When new evidence doesn't conform with existing beliefs. *Pediatr. Res.* **2020**, *90*, 1093–1095. [[CrossRef](#)] [[PubMed](#)]
5. Agalakova, N.I.; Nadei, O.V. Inorganic fluoride and functions of brain. *Crit. Rev. Toxicol.* **2020**, *50*, 28–46. [[CrossRef](#)] [[PubMed](#)]
6. Adkins, E.A.; Brunst, K.J. Impacts of Fluoride Neurotoxicity and Mitochondrial Dysfunction on Cognition and Mental Health: A Literature Review. *Int. J. Environ. Res. Public Health* **2021**, *18*, 12884. [[CrossRef](#)] [[PubMed](#)]
7. Ottapillakkil, H.; Babu, S.; Balasubramanian, S.; Manoharan, S.; Perumal, E. Fluoride Induced Neurobehavioral Impairments in Experimental Animals: A Brief Review. *Biol. Trace Element Res.* **2022**, *201*, 1214–1236. [[CrossRef](#)]
8. Duan, Q.; Jiao, J.; Chen, X.; Wang, X. Association between water fluoride and the level of children's intelligence: A dose-response meta-analysis. *Public Health* **2017**, *154*, 87–97. [[CrossRef](#)]
9. Strunecka, A.; Strunecky, O. Chronic Fluoride Exposure and the Risk of Autism Spectrum Disorder. *Int. J. Environ. Res. Public Health* **2019**, *16*, 3431. [[CrossRef](#)]
10. Grandjean, P. Developmental fluoride neurotoxicity: An updated review. *Environ. Health* **2019**, *18*, 110. [[CrossRef](#)]
11. Veneri, F.; Vinceti, M.; Generali, L.; Giannone, M.E.; Mazzoleni, E.; Birnbaum, L.S.; Consolo, U.; Filippini, T. Fluoride exposure and cognitive neurodevelopment: Systematic review and dose-response meta-analysis. *Environ. Res.* **2023**, *221*, 115239. [[CrossRef](#)]
12. Żwierzeło, W.; Maruszewska, A.; Skórka-Majewicz, M.; Gutowska, I. Fluoride in the Central Nervous System and Its Potential Influence on the Development and Invasiveness of Brain Tumours—A Research Hypothesis. *Int. J. Mol. Sci.* **2023**, *24*, 1558. [[CrossRef](#)] [[PubMed](#)]
13. Guth, S.; Hüser, S.; Roth, A.; Degen, G.; Diel, P.; Edlund, K.; Eisenbrand, G.; Engel, K.-H.; Epe, B.; Grune, T.; et al. Toxicity of fluoride: Critical evaluation of evidence for human developmental neurotoxicity in epidemiological studies, animal experiments and in vitro analyses. *Arch. Toxicol.* **2020**, *94*, 1375–1415. [[CrossRef](#)] [[PubMed](#)]
14. Miranda, G.H.N.; Alvarenga, M.O.P.; Ferreira, M.K.M.; Puty, B.; Bittencourt, L.O.; Fagundes, N.C.F.; Pessan, J.P.; Buzalaf, M.A.R.; Lima, R.R. A systematic review and meta-analysis of the association between fluoride exposure and neurological disorders. *Sci. Rep.* **2021**, *11*, 22659. [[CrossRef](#)]
15. Nadei, O.V.; Ivanova, T.I.; Sufieva, D.A.; Agalakova, N.I. Morphological Changes of the Rat Hippocampal Neurons Following Excessive Fluoride Consumption. *J. Anat. Histopathol.* **2020**, *9*, 53–60. [[CrossRef](#)]
16. Nadei, O.V.; Khvorova, I.A.; Agalakova, N.I. Cognitive Decline of Rats with Chronic Fluorosis Is Associated with Alterations in Hippocampal Calpain Signaling. *Biol. Trace Element Res.* **2019**, *197*, 495–506. [[CrossRef](#)]
17. Vani, M.L.; Reddy, K.P. Effects of fluoride accumulation on some enzymes of brain and gastrocnemius muscle of mice. *Fluoride* **2000**, *33*, 17–26.
18. Jiang, S.; Su, J.; Yao, S.; Zhang, Y.; Cao, F.; Wang, F.; Wang, H.; Li, J.; Xi, S. Fluoride and Arsenic Exposure Impairs Learning and Memory and Decreases mGluR5 Expression in the Hippocampus and Cortex in Rats. *PLoS ONE* **2014**, *9*, e96041. [[CrossRef](#)]

19. Reddy, Y.P.; Tiwari, S.; Tomar, L.K.; Desai, N.; Sharma, V.K. Fluoride-Induced Expression of Neuroinflammatory Markers and Neurophysiological Regulation in the Brain of Wistar Rat Model. *Biol. Trace Element Res.* **2020**, *199*, 2621–2626. [\[CrossRef\]](#)
20. Niu, R.; Sun, Z.; Cheng, Z.; Li, Z.; Wang, J. Decreased learning ability and low hippocampus glutamate in offspring rats exposed to fluoride and lead. *Environ. Toxicol. Pharmacol.* **2009**, *28*, 254–258. [\[CrossRef\]](#)
21. González-Alfonso, W.L.; Pavel, P.; Karina, H.-M.; Del Razo, L.M.; Sanchez-Peña, L.C.; Zepeda, A.; Gensebatt, M.E. Chronic exposure to inorganic arsenic and fluoride induces redox imbalance, inhibits the transsulfuration pathway, and alters glutamate receptor expression in the brain, resulting in memory impairment in adult male mouse offspring. *Arch. Toxicol.* **2023**, *97*, 2371–2383. [\[CrossRef\]](#)
22. Kim, J.-H.; Marton, J.; Ametamey, S.M.; Cumming, P. A Review of Molecular Imaging of Glutamate Receptors. *Molecules* **2020**, *25*, 4749. [\[CrossRef\]](#) [\[PubMed\]](#)
23. Chakraborty, P.; Dey, A.; Gopalakrishnan, A.V.; Swati, K.; Ojha, S.; Prakash, A.; Kumar, D.; Ambasta, R.K.; Jha, N.K.; Jha, S.K.; et al. Glutamatergic neurotransmission: A potential pharmacotherapeutic target for the treatment of cognitive disorders. *Ageing Res. Rev.* **2023**, *85*, 101838. [\[CrossRef\]](#) [\[PubMed\]](#)
24. Diering, G.H.; Huganir, R.L. The AMPA Receptor Code of Synaptic Plasticity. *Neuron* **2018**, *100*, 314–329. [\[CrossRef\]](#) [\[PubMed\]](#)
25. Purkey, A.M.; Dell'acqua, M.L. Phosphorylation-Dependent Regulation of Ca²⁺-Permeable AMPA Receptors During Hippocampal Synaptic Plasticity. *Front. Synaptic Neurosci.* **2020**, *12*, 8. [\[CrossRef\]](#) [\[PubMed\]](#)
26. Italia, M.; Ferrari, E.; Di Luca, M.; Gardoni, F. GluA3-containing AMPA receptors: From physiology to synaptic dysfunction in brain disorders. *Neurobiol. Dis.* **2021**, *161*, 105539. [\[CrossRef\]](#)
27. Chater, T.E.; Goda, Y. The Shaping of AMPA Receptor Surface Distribution by Neuronal Activity. *Front. Synaptic Neurosci.* **2022**, *14*, 833782. [\[CrossRef\]](#)
28. Hanley, J.G. Subunit-specific trafficking mechanisms regulating the synaptic expression of Ca²⁺-permeable AMPA receptors. *Semin. Cell Dev. Biol.* **2014**, *27*, 14–22. [\[CrossRef\]](#)
29. Hardt, O.; Nader, K.; Wang, Y.-T. GluA2-dependent AMPA receptor endocytosis and the decay of early and late long-term potentiation: Possible mechanisms for forgetting of short- and long-term memories. *Philos. Trans. R. Soc. B Biol. Sci.* **2014**, *369*, 20130141. [\[CrossRef\]](#)
30. Wu, Q.-L.; Gao, Y.; Li, J.-T.; Ma, W.-Y.; Chen, N.-H. The Role of AMPARs Composition and Trafficking in Synaptic Plasticity and Diseases. *Cell. Mol. Neurobiol.* **2022**, *42*, 2489–2504. [\[CrossRef\]](#)
31. Chen, S.-R.; Zhou, H.-Y.; Byun, H.S.; Pan, H.-L. Nerve Injury Increases GluA2-Lacking AMPA Receptor Prevalence in Spinal Cords: Functional Significance and Signaling Mechanisms. *J. Pharmacol. Exp. Ther.* **2013**, *347*, 765–772. [\[CrossRef\]](#)
32. Chen, S.-R.; Zhang, J.; Chen, H.; Pan, H.-L. Streptozotocin-Induced Diabetic Neuropathic Pain Is Associated with Potentiated Calcium-Permeable AMPA Receptor Activity in the Spinal Cord. *J. Pharmacol. Exp. Ther.* **2019**, *371*, 242–249. [\[CrossRef\]](#)
33. Liu, B.; Liao, M.; Mielke, J.G.; Ning, K.; Chen, Y.; Li, L.; El-Hayek, Y.H.; Gomez, E.; Zukin, R.S.; Fehlings, M.G.; et al. Ischemic Insults Direct Glutamate Receptor Subunit 2-Lacking AMPA Receptors to Synaptic Sites. *J. Neurosci.* **2006**, *26*, 5309–5319. [\[CrossRef\]](#)
34. Babaei, P. NMDA and AMPA receptors dysregulation in Alzheimer's disease. *Eur. J. Pharmacol.* **2021**, *908*, 174310. [\[CrossRef\]](#) [\[PubMed\]](#)
35. Martín-Belmonte, A.; Aguado, C.; Alfaro-Ruiz, R.; Itakura, M.; Moreno-Martínez, A.E.; de la Ossa, L.; Molnár, E.; Fukazawa, Y.; Luján, R. Age-Dependent Shift of AMPA Receptors From Synapses to Intracellular Compartments in Alzheimer's Disease: Immunocytochemical Analysis of the CA1 Hippocampal Region in APP/PS1 Transgenic Mouse Model. *Front. Aging Neurosci.* **2020**, *12*, 577996. [\[CrossRef\]](#)
36. Alfaro-Ruiz, R.; Aguado, C.; Martín-Belmonte, A.; Moreno-Martínez, A.E.; Merchán-Rubira, J.; Hernández, F.; Ávila, J.; Fukazawa, Y.; Luján, R. Alteration in the Synaptic and Extrasynaptic Organization of AMPA Receptors in the Hippocampus of P301S Tau Transgenic Mice. *Int. J. Mol. Sci.* **2022**, *23*, 13527. [\[CrossRef\]](#) [\[PubMed\]](#)
37. Kuniishi, H.; Yamada, D.; Wada, K.; Yamada, M.; Sekiguchi, M. Stress induces insertion of calcium-permeable AMPA receptors in the OFC–BLA synapse and modulates emotional behaviours in mice. *Transl. Psychiatry* **2020**, *10*, 154. [\[CrossRef\]](#) [\[PubMed\]](#)
38. Yonezawa, K.; Tani, H.; Nakajima, S.; Nagai, N.; Koizumi, T.; Miyazaki, T.; Mimura, M.; Takahashi, T.; Uchida, H. AMPA receptors in schizophrenia: A systematic review of postmortem studies on receptor subunit expression and binding. *Schizophr. Res.* **2022**, *243*, 98–109. [\[CrossRef\]](#) [\[PubMed\]](#)
39. Reinders, N.R.; Pao, Y.; Renner, M.C.; da Silva-Matos, C.M.; Lodder, T.R.; Malinow, R.; Kessels, H.W. Amyloid- β effects on synapses and memory require AMPA receptor subunit GluA. *Proc. Natl. Acad. Sci. USA* **2016**, *113*, E6526–E6534. [\[CrossRef\]](#)
40. Berchtold, N.C.; Sabbagh, M.N.; Beach, T.G.; Kim, R.C.; Cribbs, D.H.; Cotman, C.W. Brain gene expression patterns differentiate mild cognitive impairment from normal aged and Alzheimer's disease. *Neurobiol. Aging* **2014**, *35*, 1961–1972. [\[CrossRef\]](#)
41. Peng, S.-X.; Pei, J.; Rinaldi, B.; Chen, J.; Ge, Y.-H.; Jia, M.; Wang, J.; Delahaye-Duriez, A.; Sun, J.-H.; Zang, Y.-Y.; et al. Dysfunction of AMPA receptor GluA3 is associated with aggressive behavior in human. *Mol. Psychiatry* **2022**, *27*, 4092–4102. [\[CrossRef\]](#)
42. Yang, L.; Jin, P.; Wang, X.; Zhou, Q.; Lin, X.; Xi, S. Fluoride activates microglia, secretes inflammatory factors and influences synaptic neuron plasticity in the hippocampus of rats. *NeuroToxicology* **2018**, *69*, 108–120. [\[CrossRef\]](#) [\[PubMed\]](#)
43. Sun, Z.; Zhang, Y.; Xue, X.; Niu, R.; Wang, J. Maternal fluoride exposure during gestation and lactation decreased learning and memory ability, and glutamate receptor mRNA expressions of mouse pups. *Hum. Exp. Toxicol.* **2017**, *37*, 87–93. [\[CrossRef\]](#) [\[PubMed\]](#)

44. Lu, W.; Roche, K.W. Posttranslational regulation of AMPA receptor trafficking and function. *Curr. Opin. Neurobiol.* **2012**, *22*, 470–479. [[CrossRef](#)] [[PubMed](#)]
45. Wang, J.Q.; Guo, M.-L.; Jin, D.-Z.; Xue, B.; Fibuch, E.E.; Mao, L.-M. Roles of subunit phosphorylation in regulating glutamate receptor function. *Eur. J. Pharmacol.* **2013**, *728*, 183–187. [[CrossRef](#)] [[PubMed](#)]
46. Corti, E.; Duarte, C.B. The role of post-translational modifications in synaptic AMPA receptor activity. *Biochem. Soc. Trans.* **2023**, *51*, 315–330. [[CrossRef](#)]
47. Crombag, H.S.; Sutton, J.M.; Takamiya, K.; Holland, P.C.; Gallagher, M.; Haganir, R.L. A role for alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid GluR1 phosphorylation in the modulatory effects of appetitive reward cues on goal-directed behavior. *Eur. J. Neurosci.* **2008**, *27*, 3284–3291. [[CrossRef](#)]
48. Caudal, D.; Godsil, B.P.; Mailliet, F.; Bergerot, D.; Jay, T.M. Acute Stress Induces Contrasting Changes in AMPA Receptor Subunit Phosphorylation within the Prefrontal Cortex, Amygdala and Hippocampus. *PLoS ONE* **2010**, *5*, e15282. [[CrossRef](#)]
49. Caudal, D.; Rame, M.; Jay, T.M.; Godsil, B.P. Dynamic Regulation of AMPAR Phosphorylation In Vivo Following Acute Behavioral Stress. *Cell. Mol. Neurobiol.* **2016**, *36*, 1331–1342. [[CrossRef](#)]
50. Lee, M.T.; Peng, W.-H.; Kan, H.-W.; Wu, C.-C.; Wang, D.-W.; Ho, Y.-C. Neurobiology of Depression: Chronic Stress Alters the Glutamatergic System in the Brain—Focusing on AMPA Receptor. *Biomedicines* **2022**, *10*, 1005. [[CrossRef](#)]
51. Zhao, Y.-B.; Hou, X.-F.; Li, X.; Zhu, L.-S.; Zhu, J.; Ma, G.-R.; Liu, Y.-X.; Miao, Y.-C.; Zhou, Q.-Y.; Xu, L.; et al. Early Memory Impairment is Accompanied by Changes in GluA1/p-GluA1 in APP/PS1 Mice. *Curr. Alzheimer Res.* **2022**, *19*, 667–673. [[CrossRef](#)]
52. Paoletti, P.; Bellone, C.; Zhou, Q. NMDA receptor subunit diversity: Impact on receptor properties, synaptic plasticity and disease. *Nat. Rev. Neurosci.* **2013**, *14*, 383–400. [[CrossRef](#)] [[PubMed](#)]
53. Gardoni, F.; Di Luca, M. Protein-protein interactions at the NMDA receptor complex: From synaptic retention to synaptonuclear protein messengers. *Neuropharmacology* **2021**, *190*, 108551. [[CrossRef](#)]
54. Cercato, M.C.; Vázquez, C.A.; Kornisiuk, E.; Aguirre, A.I.; Coletti, N.; Snitcowsky, M.; Jerusalinsky, D.A.; Baez, M.V. GluN1 and GluN2A NMDA Receptor Subunits Increase in the Hippocampus during Memory Consolidation in the Rat. *Front. Behav. Neurosci.* **2017**, *10*, 242. [[CrossRef](#)] [[PubMed](#)]
55. Franchini, L.; Carrano, N.; Di Luca, M.; Gardoni, F. Synaptic GluN2A-Containing NMDA Receptors: From Physiology to Pathological Synaptic Plasticity. *Int. J. Mol. Sci.* **2020**, *21*, 1538. [[CrossRef](#)] [[PubMed](#)]
56. Holehonnur, R.; Phensy, A.J.; Kim, L.J.; Milivojevic, M.; Vuong, D.; Daison, D.K.; Alex, S.; Tiner, M.; Jones, L.E.; Kroener, S.; et al. Increasing the GluN2A/GluN2B Ratio in Neurons of the Mouse Basal and Lateral Amygdala Inhibits the Modification of an Existing Fear Memory Trace. *J. Neurosci.* **2016**, *36*, 9490–9504. [[CrossRef](#)] [[PubMed](#)]
57. Acutain, M.F.; Luft, J.G.; Vazquez, C.A.; Popik, B.; Cercato, M.C.; Epstein, A.; Salvetti, A.; Jerusalinsky, D.A.; Alvares, L.d.O.; Baez, M.V. Reduced Expression of Hippocampal GluN2A-NMDAR Increases Seizure Susceptibility and Causes Deficits in Contextual Memory. *Front. Neurosci.* **2021**, *15*, 644100. [[CrossRef](#)] [[PubMed](#)]
58. Oka, M.; Ito, K.; Koga, M.; Kusumi, I. Changes in subunit composition of NMDA receptors in animal models of schizophrenia by repeated administration of methamphetamine. *Prog. Neuro-Psychopharmacol. Biol. Psychiatry* **2020**, *103*, 109984. [[CrossRef](#)]
59. Sabo, S.L.; Lahr, J.M.; Offer, M.; LA Weekes, A.; Sceniak, M.P. GRIN2B-related neurodevelopmental disorder: Current understanding of pathophysiological mechanisms. *Front. Synaptic Neurosci.* **2023**, *14*, 1090865. [[CrossRef](#)]
60. Sivakumar, S.; Ghasemi, M.; Schachter, S.C. Targeting NMDA Receptor Complex in Management of Epilepsy. *Pharmaceuticals* **2022**, *15*, 1297. [[CrossRef](#)]
61. Zubareva, O.E.; Kovalenko, A.A.; Kalemenev, S.V.; Schwarz, A.P.; Karyakin, V.B.; Zaitsev, A.V. Alterations in mRNA expression of glutamate receptor subunits and excitatory amino acid transporters following pilocarpine-induced seizures in rats. *Neurosci. Lett.* **2018**, *686*, 94–100. [[CrossRef](#)]
62. Postnikova, T.Y.; Zubareva, O.E.; Kovalenko, A.A.; Kim, K.K.; Magazanik, L.G.; Zaitsev, A.V. Status epilepticus impairs synaptic plasticity in rat hippocampus and is followed by changes in expression of NMDA receptors. *Biochemistry* **2017**, *82*, 282–290. [[CrossRef](#)] [[PubMed](#)]
63. Li, Y.; Chang, L.; Song, Y.; Gao, X.; Roselli, F.; Liu, J.; Zhou, W.; Fang, Y.; Ling, W.; Li, H.; et al. Astrocytic GluN2A and GluN2B Oppose the Synaptotoxic Effects of Amyloid- β 1-40 in Hippocampal Cells. *J. Alzheimer's Dis.* **2016**, *54*, 135–148. [[CrossRef](#)] [[PubMed](#)]
64. Du, Z.; Song, Y.; Chen, X.; Zhang, W.; Zhang, G.; Li, H.; Chang, L.; Wu, Y. Knockdown of astrocytic Grin2a aggravates β -amyloid-induced memory and cognitive deficits through regulating nerve growth factor. *Aging Cell* **2021**, *20*, e13437. [[CrossRef](#)] [[PubMed](#)]
65. Zhang, W.; Chen, X.; Du, Z.; Mao, X.; Gao, R.; Chen, Z.; Wang, H.; Zhang, G.; Zhang, N.; Li, H.; et al. Knockdown of astrocytic Grin2a exacerbated sleep deprivation-induced cognitive impairments and elevation of amyloid-beta. *Sleep Med.* **2022**, *100*, 280–290. [[CrossRef](#)]
66. Wei, N.; Dong, Y.-T.; Deng, J.; Wang, Y.; Qi, X.-L.; Yu, W.-F.; Xiao, Y.; Zhou, J.-J.; Guan, Z.-Z. Changed expressions of N-methyl-d-aspartate receptors in the brains of rats and primary neurons exposed to high level of fluoride. *J. Trace Elements Med. Biol.* **2017**, *45*, 31–40. [[CrossRef](#)]
67. Ge, Y.; Chen, L.; Yin, Z.; Song, X.; Ruan, T.; Hua, L.; Liu, J.; Wang, J.; Ning, H.; Ge, Y.; et al. Fluoride-induced alterations of synapse-related proteins in the cerebral cortex of ICR offspring mouse brain. *Chemosphere* **2018**, *201*, 874–883. [[CrossRef](#)]

68. Pupo, G.; Gouverneur, V. Hydrogen Bonding Phase-Transfer Catalysis with Alkali Metal Fluorides and Beyond. *J. Am. Chem. Soc.* **2022**, *144*, 5200–5213. [[CrossRef](#)]
69. Qin, J.; Chai, G.; Brewer, J.M.; Lovelace, L.L.; Lebioda, L. Fluoride Inhibition of Enolase: Crystal Structure and Thermodynamics. *Biochemistry* **2005**, *45*, 793–800. [[CrossRef](#)]
70. Agalakova, N.I.; Gusev, G.P. Molecular Mechanisms of Cytotoxicity and Apoptosis Induced by Inorganic Fluoride. *ISRN Cell Biol.* **2012**, *2012*, 403835. [[CrossRef](#)]
71. Yildiz-Unal, A.; Korulu, S.; Karabay, A. Neuroprotective strategies against calpain-mediated neurodegeneration. *Neuropsychiatr. Dis. Treat.* **2015**, *11*, 297–310. [[CrossRef](#)]
72. Zhou, G.; Hu, Y.; Wang, A.; Guo, M.; Du, Y.; Gong, Y.; Ding, L.; Feng, Z.; Hou, X.; Xu, K.; et al. Fluoride Stimulates Anxiety- and Depression-like Behaviors Associated with SIK2-CRTC1 Signaling Dysfunction. *J. Agric. Food Chem.* **2021**, *69*, 13618–13627. [[CrossRef](#)] [[PubMed](#)]
73. Du, Y.; Feng, Z.; Gao, M.; Wang, A.; Yan, X.; Chen, R.; Liu, B.; Yu, F.; Ba, Y.; Zhou, G. Impaired neurogenesis induced by fluoride via the Notch1 signaling and effects of carvacrol intervention. *Environ. Pollut.* **2024**, *356*, 124371. [[CrossRef](#)] [[PubMed](#)]
74. National Research Council. *Nutrient Requirements of Laboratory Animals*; The National Academies Press: Washington, DC, USA, 1995; pp. 12–79. [[CrossRef](#)]
75. Lyaruu, D.M.; Bronckers, A.L.J.J.; Santos, F.; Mathias, R.; DenBesten, P. The effect of fluoride on enamel and dentin formation in the uremic rat incisor. *Pediatr. Nephrol.* **2008**, *23*, 1973–1979. [[CrossRef](#)] [[PubMed](#)]
76. Ekstrand, J.J. Relationship between Fluoride in the Drinking Water and the Plasma Fluoride Concentration in Man. *Caries Res.* **1978**, *12*, 123–127. [[CrossRef](#)] [[PubMed](#)]
77. Spencer, A.; Do, L.; Mueller, U.; Baines, J.; Foley, M.; Peres, M. Understanding Optimum Fluoride Intake from Population-Level Evidence. *Adv. Dent. Res.* **2018**, *29*, 144–156. [[CrossRef](#)]
78. Antunes, M.; Biala, G. The novel object recognition memory: Neurobiology, test procedure, and its modifications. *Cogn. Process.* **2011**, *13*, 93–110. [[CrossRef](#)]
79. Ukkirapandian, K.; Elumalai, K.; Udaykumar, K.P.; Vp, S.; Rangasmy, M. Behavioral and Biochemical Assays for Autism Models of Wistar Rats. *Cureus* **2024**, *16*, e52066. [[CrossRef](#)]
80. Hernández-Mercado, K.; Zepeda, A. Morris Water Maze and Contextual Fear Conditioning Tasks to Evaluate Cognitive Functions Associated with Adult Hippocampal Neurogenesis. *Front. Neurosci.* **2022**, *15*, 782947. [[CrossRef](#)]
81. Nadei, O.V.; Agalakova, N.I. Optimal Reference Genes for RT-qPCR Experiments in Hippocampus and Cortex of Rats Chronically Exposed to Excessive Fluoride. *Biol. Trace Element Res.* **2023**, *202*, 199–209. [[CrossRef](#)]
82. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. *Methods* **2001**, *25*, 402–408. [[CrossRef](#)]

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