Prenatal valproate treatment produces autistic-like behavior and increases metabotropic glutamate receptor 1A-immunoreactivity in the hippocampus of juvenile rats

FRANCISCO PERALTA[1], CONSTANZA FUENTEALBA[1], JENNY FIEDLER[2] and ESTEBAN ALIAGA[1]

[1]Department of Kinesiology, Faculty of Health Sciences, Universidad Católica del Maule, Talca 3460000;
[2]Department of Biochemistry and Molecular Biology, Faculty of Chemical and Pharmaceutical Sciences, Universidad de Chile, Santiago 8380000, Chile

Received May 18, 2016; Accepted July 13, 2016

DOI: 10.3892/mmr.2016.5529

Abstract. Autism spectrum disorder (ASD) is a heterogeneous neurodevelopmental disorder characterized by deficits in social communication and social interaction, and repetitive and stereotypical patterns of behavior. Previously, a common physiopathological pathway, involving the control of synaptic protein synthesis, was proposed as a convergence point in ASD. In particular, a role for local mRNA translation activated by class I metabotropic glutamate receptor type 5 (mGluR5) was suggested in genetic syndromes with autistic signs and in the prenatal exposition to the valproate model of autism. However, the role of the other members of class I metabotropic glutamate receptors, including mGluR1, has been poorly studied. The present study analyzed the immunoreactivity for mGluR1a in the hippocampus of rats prenatally treated with valproate. Pregnant dams (embryonic day 12.5) were injected with valproate (450 mg/kg) and subsequently, the behavior and mGluR1a were evaluated at postnatal day 30. Experimental rats exhibited social deficit, repetitive conduct and anxious behaviors compared with that of the control animals. Additionally, the present study observed an increased level of mGluR1a-immunoreactivity in the hilus of dentate gyrus and in the CA1 alveus region of the hippocampus. These results suggested an over-functioning of mGluR1a signaling in the hippocampus, induced in the valproate model of autism, which may serve a role in cognitive and behavioral signs of ASD.

Correspondence to: Dr Esteban Aliaga, Department of Kinesiology, Faculty of Health Sciences, Universidad Católica del Maule, Av. San Miguel 3605, Talca 3460000, Chile
E-mail: ealiaga@ucm.cl

Key words: metabotropic glutamate receptor 1A, autism, valproate, prenatal, hippocampus, alveus, hilus

Introduction

Autistic spectrum disorder (ASD) is a highly debilitating developmental neuropathology characterized by impaired social interaction and communication, and is associated with stereotypical and repetitive behaviors (1). An alteration in the genesis and maturation of neural circuits appears to produce a failed connection in several regions of the autistic brain (2). An imbalance between glutamate and GABAergic systems has been suggested and a strong bias to the excitatory neurotransmission may be characteristic of an ASD brain (3). Although ASD etiology remains elusive, it is clearly multifactorial. Genetic studies demonstrate a high degree of heterogeneity and as many as 100 genes or genomic imbalances have been implicated in this neuropathology (4). Between genetic factors, certain monogenetic types of autism are associated with mutations in genes for structural synaptic proteins; predominantly the postsynaptic cell adhesion proteins, including neuroligin 3 and 4, and their presynaptic partner neurexin 1, and the gene for postsynaptic density scaffolding protein SHANK3 (5-8). Genes for functional synaptic proteins are also mutated in ASD, including the postsynaptic ubiquitin UBE3A ligase in Angelman's patients (9,10) or calcium dependent activator protein (CAPS2/CADPS2), which is involved in neurotrophin secretion (11). Additional evidence from previous genetic disorders not included in ASD, but presenting autistic signs, involves a particular signaling pathway regulating local protein synthesis in the dendritic compartment (12,13). Tuberous sclerosis complex (TSC), a genetic type of epilepsy with autistic signs, presents with dominant mutations in TSC1 or TSC2 genes that produce over-activation of the mammalian target of rapamycin kinase (mTOR) (14). By contrast, fragile X-mental retardation is a genetic type of mental disability with autistic signs, where the FMRP gene is transcriptionally repressed, causing exaggerated protein synthesis dependent-long term depression (15,16). More recent evidence directly links autistic mutations with synaptic translation. Phosphatase and tensin homolog (PTEN) is a tumor suppressor gene that regulates TSC1/2, and individuals with germline mutations in PTEN exhibit macrocephaly and autistic behavior (17-19).

In other autistic cases, an activating mutation in the promotor region of the eukaryotic initiation factor 4E (EIF4E) gene, the rate-limiting component of eukaryotic translation, has been described (20). EIF4E is the final effector in a signaling pathway mediated by TSC/mTOR and is regulated, between others, by PTEN and FMRP that permits the generation of new proteins in a precise spatio-temporal manner for neural plasticity occurring during development and maturation of brain circuits. Therefore, alteration of dendritic protein synthesis appears to be a common defect in genetic autism (21). Glutamatergic neurotransmission is an important regulator of this pathway. In particular, glutamate metabotropic receptor type 5 (mGluR5), a member of the class I family, has a probed role in dendritic translation. For example, in fragile X-mental retardation, FMRP absence produces an excessive dendritic protein synthesis following the activation of mGluR5, resulting in synaptic pruning defects (22). However, a large number of autistic cases appear to have an environmental origin and embryonic exposition to the antiepileptic sodium valproate (VPA) in rats is a well-validated experimental model of autism (23). In this model, an altered development of neural circuits has been established. In sensorial and prefrontal cortical areas, a hyper-connectivity, hyper-reactivity and hyper-plasticity has been reported (24-26). Notably, a mis-connection implicating over-connectivity in local sort distance circuits and under-connection in the long distance circuits, is now recognized as a key characteristic in the autistic brain (2). However, the molecular mediators that cause this mis-connected brain in environmentally-induced autism are poorly investigated, even though increases in the NMDA receptor and CamKII levels have been reported in the rodent VPA model of autism (27). In addition, a role for mGluR5 has been suggested in mediating the repetitive and anxious-like behaviors in the VPA model of autism (28); however, the role of other members of class I metabotropic glutamate receptor as mGluR1 is poorly investigated. In the context of a supposed alteration in synaptic protein synthesis in autism, the present study investigated if upstream regulators of this process, including mGluRs, are located at the point of convergence between genetic and non-genetic autism. The aim of the present study was to analyze the immunoreactivity for mGluR1a and mGluR5 in the hippocampus of rats treated with VPA in the prenatal period.

Materials and methods

Animals. A total of 24 pregnant Sprague-Dawley rats (Faculty of Chemical and Pharmaceutical Sciences, Universidad de Chile, Santiago, Chile) were housed in 25x45x15 cm cages under standard conditions (21˚C and 12 h light-dark cycle) with free access to food and water. To generate the animal model of autism, pregnant female rats received a single intraperitoneal injection of 450 mg/kg VPA (Sigma-Aldrich, St. Louis, MO, USA) on embryonic day 12.5 and the control group received a saline solution injection. Offspring were weaned at postnatal day 21 (P21) when males and females were separated and housed in groups of 4-5 littermates. Only male rats were used in behavioral and immunohistochemical studies. Efforts were made to minimize both the number of animals used and their suffering. All procedures were approved by the local Ethics Committee of the Science and Technology National Commission (CONICYT) in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (8th edition).

Behavioral tests. All test were performed between 9:00 and 15:00, and where recorded by a Logiteck camera located 1.5 m from the test surface. For behavioral studies, sequential tests were performed considering increased anxiogenic behavior with a 48 h inter-test period. A limit of three tests was conducted on each animal. Open-field was always the first test and Y-maze or plus-maze was the last. To reduce suffering, Y-maze and plus-maze were never performed by the same animal. Between tests the apparatus was cleaned with 5% ethanol and every test was performed with 60 db of white noise. All videos where analyzed using ANY-maze software (Stoelting, Wood Dale, IL, USA) by an operator in a blinded manner, with the exception of the Y-Maze, which was analyzed manually in a blinded manner.

Open field. Locomotor and exploratory activity was evaluated in a 60x60 cm open field arena. The animal was placed in the center of the illuminated arena and was allowed 5 min to explore the arena. Total distance, mean speed, and time and frequency of grooming behavior were then determined.

Elevated plus maze. The elevated plus maze protocol was performed with P30 rats, as described previously (29). The arms of the apparatus measured 40x10 cm and the enclosed arms where limited by walls of 30 cm high. The open arms have a 5 mm elevation in the edges to enhance the exploratory activity (30). The arms were elevated 50 cm from the floor and a camera was recording from the upside. To start the test, the animal was placed in the center of the apparatus facing to an open arm (31) and exploration was recorded during 5 min.

Three chamber social test. A three-chamber social test was performed in an apparatus consisting of three 30x22 cm chambers of transparent acrylic adjoining, each separated by 10x10 cm doors, as described previously by Moy *et al* (32). The animal was allowed to explore the central camera for 5 min and then the doors conducting to the lateral chambers were opened. One of the lateral chambers houses an animal of the same litter (familiar congener) and the other chamber was void. The social behavior of the animal was recorded for 10 min.

Y-Maze. The spatial memory-Y-Maze was performed, as described by Conrad *et al* (33), and consisted in three 46x13x32 cm arms maze disposed at angles of 120 degrees between them. The test consisted of two stages. In the first test, one animal was allowed to explore only two arms of the maze for 15 min and was subsequently returned to its home cage. The second stage was performed 4 h later, but this time the animal is allowed to explore the three arms during a 5 min duration. The initial arm was alternated between animals to avoid preference for a direction (34).

Immunohistochemistry. For immunohistochemical analysis, males were euthanized using isoflurane (Baxter,

Shanghai, China) and sacrificed by decapitation on P30. Whole brain tissue blocks were dissected briefly following decapitation and fixed overnight in 4% paraformaldehyde in phosphate buffered saline (PBS; pH 7.4). Following this, the tissue sections were cryoprotected in 30% sacarose. For free-floating immunostaining, 40 μm coronal sections were obtained in a cryostat Microm HM525 (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The tissue sections were treated for 45 min with 0.3% H_2O_2 at room temperature to block endogenous peroxidase activity and were subsequently blocked for 1 h at room temperature in blocking buffer (3% bovine serum albumin with 0.4% Triton X-100 in PBS). The tissue sections were incubated in the following primary antibodies overnight at room temperature: Rabbit anti-mGluR5 (1:100) and rabbit anti-mGluR1a (1:100), both from Thermo Fisher Scientific, Inc. The sections were subsequently rinsed and incubated for 2 h at room temperature with Biotin-SP-Conjugated anti-rabbit immunoglobulin G (H+L) (1:1,000; Jackson ImmunoResearch Labs, West Grove, PA, USA), followed by a 1 h incubation at room temperature with avidin/biotin horseradish peroxidase complex using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). Finally, immunoreactivity was detected using ImmPACT DAB peroxidase substrate (Vector Laboratories).

Image analysis. Digital microphotographs were captured using a Carl Zeiss Axiolab E microscope with a digital camera (Cannon EOS Rebel T3) and immunoreactivity quantification was performed using the public software ImageJ (NIH, Bethesda, MD, USA). In order to evaluate possible localization difference in the immunoreactivity between control and VPA groups, the present study quantified the optical density of the different hippocampal layers and corrected by the background in a no staining zone in the same slice. In addition, data from the experimental group were standardized for the control value in the same zone in order to minimize technical artefacts in immunostaining procedures performed on different days.

Statistical analysis. All data obtained from the behavioral test were analyzed by one-way analysis of variance, followed by Bonferroni post-hoc test. The statistical analysis of densitometric immunoreactivity was performed using the non-parameric Mann-Whitney test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Behavioral studies

Social behavior. The three chamber social test is a valid behavioral probe to analyze probable alterations in social approach or avoidance behavior (32). The present study evaluated the time spent in each one of the tree chambers, starting with the experimental animal in the central chamber and facing a familiar congener on one of the lateral chambers, while the other lateral chamber was empty. The expected normal behavior is a preference for spending the time in the occupied chamber even with the center and non-occupied lateral chamber. As shown in Fig. 1A, control rats exhibited a preference for the occupied chamber (398.1±10.9 sec in familiar zone vs. 116.6±9.1 sec in center; $P\leq0.001$). For the VPA-treated group, this preference is lost demonstrating no statistically significant difference between the time spent in occupied lateral chamber compared with the center or the empty zone (279.9±43.6 sec in occupied zone vs. 239.1±49.4 sec in center). This result demonstrated social deficit in the VPA group, a major characteristic of autism.

Anxiety. Anxious behavior was evaluated using the elevated plus maze, where experimental animals can freely explore two protected and two unprotected elevated arms disposed perpendicularly from each other. The present study quantified accumulated time spent in open or closed arms by VPA-treated rats and have expressed the data as a percentage of the time spent for the control group in the respective arms. As shown in Fig. 1B, VPA rats spent less time, in a statistically significant manner, in the exposed open arms and more time in the protected closed arms compared with that of the control group (100±21.4% in control vs. 33.5±11.7% in VPA for open arms, $P\leq0.05$; 100±6.2% in control vs. 117.5±4.3% in VPA for closed arms, $P\leq0.05$). This result demonstrated anxious behavior in VPA-treated animals, also an important characteristic of autism.

Locomotor activity. General locomotor activity representing exploratory conduct was evaluated by quantifying distance and speed in an open field arena. As shown in Fig. 1C, a statistically significant reduction of ~20% was observed in both the total distance and the mean speed produced by VPA prenatal treatment (100±7% in control vs. 76.4±5.9% in VPA for total distance, $P\leq0.05$; 100±7.1% in control vs. 76.5±6% in VPA for mean speed, $P\leq0.05$).

Repetitive behavior. Other major characteristic of autism are repetitive and stereotypical behavior. In the same session of open field arena, the present study quantified the frequency of grooming events and accumulated time spent in that stereotypical conduct. As shown in Fig. 1D, VPA prenatal treatment increased by ~100% the occurrence and the time spent in grooming conduct during the open field session (100±14.2% in control vs. 197.8±16.7% in VPA for grooming frequency, $P\leq0.001$; 100±25.6% in control vs. 196±27.3% in VPA for time spent, $P\leq0.05$). In addition, another way to demonstrate the repetitive conduct reported in the VPA model, was the number of reentries in the same arm during the Y-maze test, a test designed to evaluate spatial memory (35). As shown in Fig. 2A, VPA prenatal treatment doubled the number of reentries during the second phase in a two-trial Y-maze (0.94±0.26 in control vs. 1.94±0.4 in VPA, $P\leq0.05$), in accord with data previously reported (36).

Spatial memory. Spatial memory was evaluated with a two trial Y-maze test. In the first trial, animals have access to only two arms in a three arms maze. At 4 h later, in the second trial, the animals have access to the three arms and the normal expected conduct is a preference for the novel arm. Memory was evaluated by the mean time permanence index, expressing the proportion of the average time spent in the novel arm in relation to the average time spent in the

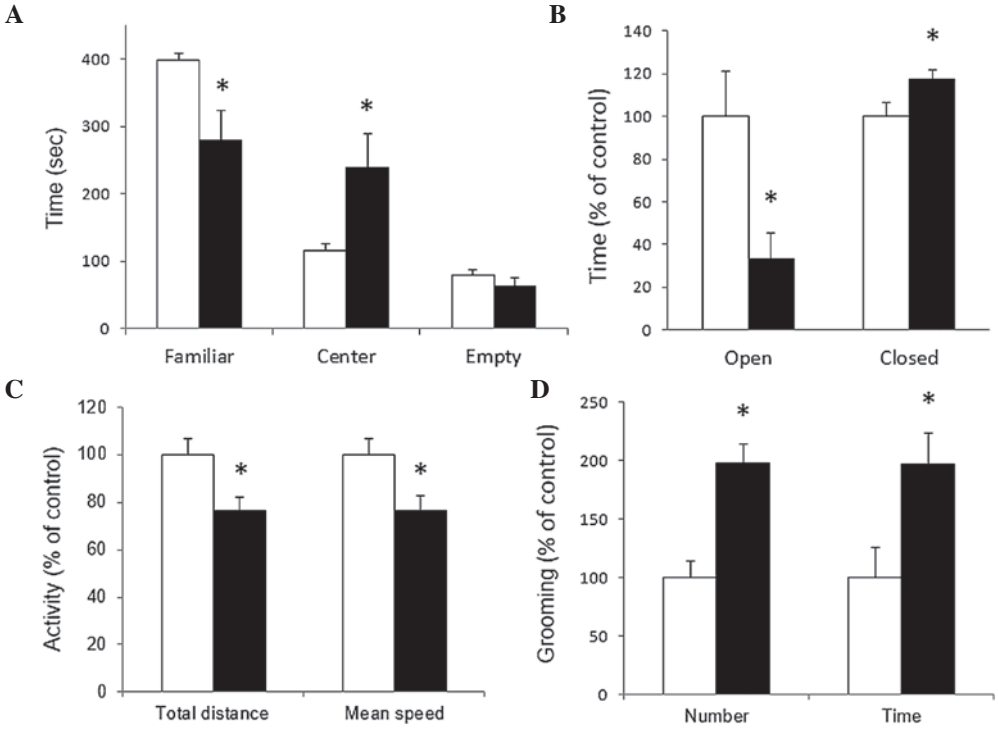

Figure 1. Behavioral and motor effects of prenatal valproate treatment. (A) Alterations in social approach were detected in the three chamber social test. The graph represents accumulated time spent in familiar, center or empty chambers during a 10 min session. A familiar animal was present in one of the lateral chambers of the test while the other lateral chamber was empty. (B) Anxious-like behavior in the elevated plus maze. The accumulated time in open or closed arms in a 5 min session is expressed as a percentage of the control. (C) Reduced locomotor activity in 5 min of 60x60 cm open field test. The total distance and mean speed are expressed as a percentage of the control animals. (D) Repetitive stereotypical behavior of grooming during open field session. The number of grooming events occurrence and the accumulated time spent in grooming are expressed as a percentage of the control. The data are expressed as the mean ± standard error of the mean (*P<0.05; n=8-11 for social test and elevated plus maze and n=13-15 for open field test). The control animals are represented by white bars and the valproate treated animals are represented by black bars. The data were analyzed using analysis of variance, followed by the Bonferroni post-hoc test.

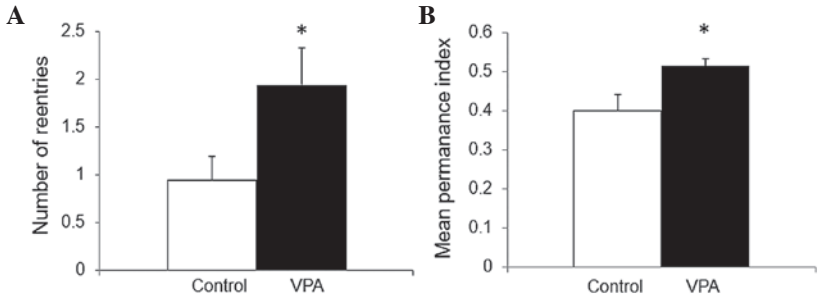

Figure 2. Effect of prenatal valproate treatment in stereotypical behavior and memory in Y-maze. (A) Repetitive behavior was evaluated by the number of reentries in a same arm during the Y-maze test. (B) Moderate increases of spatial memory were observed during the Y-maze test. Memory was evaluated by the mean permanence index, expressing the proportion of the average time spent in the novel arm in relation to the average time spent in the novel plus the known one. The data are presented as the mean ± standard error of the mean (*P<0.05; n=13-15). The control is represented by white bars while the valproate treated animals are represented by black bars. The data were analyzed using analysis of variance, followed by the Bonferroni post-hoc test.

novel plus the known arm. As shown in Fig. 2B, a moderate increase of spatial memory was produced by VPA prenatal treatment (0.4±0.04 in control vs. 0.5±0.02 in VPA, P≤0.05).

Immunohistochemical study

mGluR5 expression. Immunohistochemical analysis for mGluR5 in hippocampal formation showed intense labeling in all dendritic fields, while cellular layers displayed less staining in control and VPA-treated rats (Fig. 3A and B). Semi-quantitative analysis of mGluR5 immunoreactivity labeling intensity demonstrated no effect of treatment in any subfields and layers of the hippocampus analyzed (Fig. 3C).

mGluR1a expression. Immunoreactive staining for mGluR1a exhibited a distinctive pattern in the hippocampal subfields, staining a number of cellular processes in all dendritic stratus of the hippocampus (hilus of dentate gyrus, stratum radiatum and stratum oriens of CA1 and CA3). A less intense staining was evident in the cellular layers (stratum pyramidale and stratum granulosum) (Fig. 4). The most intense label intensity was found in the stratum alveus of CA1 and in its limits with stratum oriens, as shown in Fig. 4A. In this layer, somatic and dendritic elements were stained, which most probably corresponded to the alveus interneurons and their profuse dendritic network, as previously reported (37,38). The

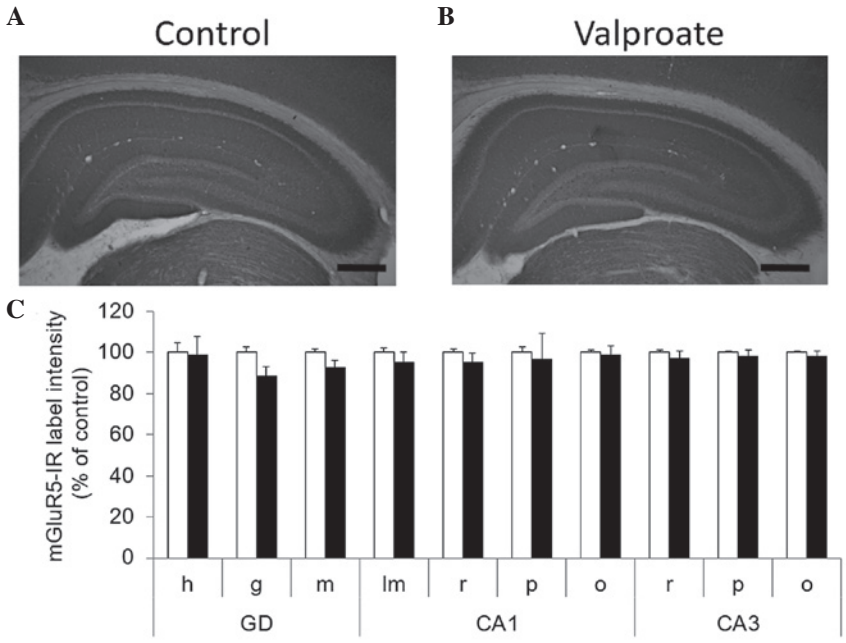

Figure 3. No effect was observed in hippocampal mGluR5 immunoreactivity following prenatal valproate treatment. Digital microphotographs showing immunohistochemical staining for mGluR5 in dorsal hippocampal formation from postnatal day 30 (A) control and (B) valproate treated animals (scale bar, 500 μm). (C) The label intensity of digital microphotographs was analyzed in the different layers of hippocampal subfields and were expressed as a percentage of the control. The data are presented as the mean ± standard error of the mean (n=6). The control is represented by white bars and the valproate treated animals are represented by black bars. The data were analyzed using Mann-Withney test. h, hilus; g, granular; m, molecular; lm, lacunosum moleculare; r, radiatum; p, pyramidal; o, oriens; a, alveus; GD, dentate gyrus; CA, Ammon's horn.

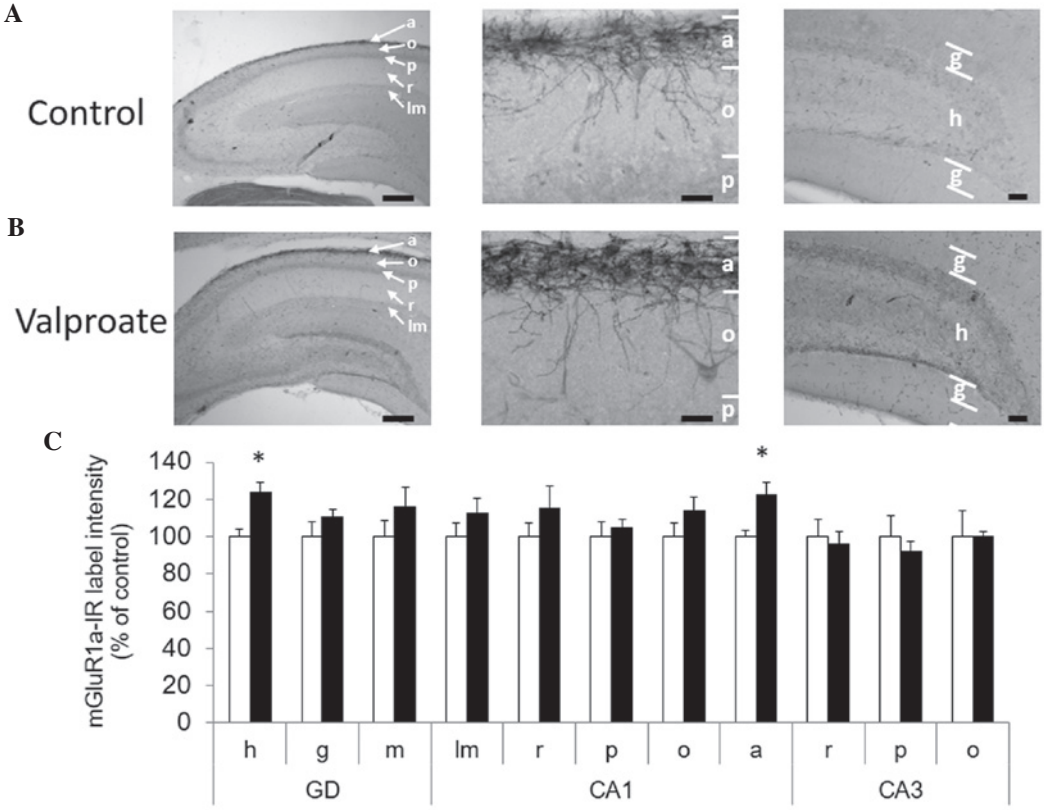

Figure 4. Increase of mGluR1a-immunoreactivity in gyrus dentate hilus and CA1 alveus subfields of hippocampus induced by prenatal valproate treatment. Digital microphotographs showing immunohistochemical staining for mGluR1a in dorsal hippocampal formation from postnatal day 30 (A) control and (B) valproate treated animals (scale bar, 500 μm). Detail of CA1 oriens/alveus limit from control and valproate treated animals (left panels). Detail of dentate gyrus from control and VPA-treated animals (middle panels). The right panels are the left panel images at a higher magnification (scale bar, 50 μm). (C) Label intensity of digital microphotographs was analyzed in the different layers of hippocampal subfields and expressed as a percentage of the control. The data are presented as the mean ± standard error of the mean (*P<0.05). The control is represented by white bars and the valproate treated animals are represented by black bars. The data were analyzed using the Mann-Withney test. h, hilus; g, granular; m, molecular; lm, lacunosum moleculare; r, radiatum; p, pyramidal; o, oriens; a, alveus; GD, dentate gyrus; CA, Ammon's horn.

second most intense staining was found in the hilus of dentate gyrus, immediately adjacent to granular cell layer (Fig. 4C). VPA-treated rats exhibited clearly more intense staining in these two later regions, CA1 alveus and hilus (Fig. 4A and B). Semi-quantitative analyses revealed a statistically significant increase of mGluR1a immunoreactivity labeling intensity in CA1 stratum alveus (100±6.8 5% in control vs. 130.9±7.5% in VPA, P≤0.05) and hilus of dentate gyrus (100±6.3% in control vs. 131.6±8.8% in VPA, P≤0.05). These results indicate that treatment with VPA increased mGluR1a immunoreactivity in selected areas of the hippocampus without altering mGluR5.

Discussion

Animal models of autism are characterized by social deficit, anxiety, and repetitive and stereotypical behaviors (39). In the present study, treatment of the rat with a unique dose of 450 mg/kg VPA on gestational day 12.5 produced the expected triad of signs. At P30, VPA-exposed rats exhibited social deficit in the three chamber social test, an anxious profile in the elevated plus maze and repetitive behavior, quantified as self-grooming conducts or re-entries during the Y-maze performance. Additionally, all VPA-treated rats exhibited a crooked tail phenotype from the earliest postnatal stages of development, a mild teratogenic effect, usually observed in the VPA model of autism (40). All characteristics in the experimental group confirmed this as an autistic-like model of study. The immunoreactivity of mGluR5 and mGluR1a in the hippocampal region of P30 male control and VPA-treated rats were assessed. The most important finding reported in the present study was the increased mGluR1a immunoreactivity in the stratum alveus of CA1 and in the hilus of dentate gyrus subfields of hippocampus produced by prenatal treatment with VPA, without changes in mGluR5 immunostaining.

In the open field arena, a hypokinetic condition that is consistent with a reduction in exploratory activity was observed, as has been previously reported by others in VPA-treated rats using the same test (41). This was also demonstrated previously with a motor alteration, and a balance and coordination deficit was reported in the rotarod and vertical pole test (42). Motor alteration reinforces the idea that biological mechanisms underlying autism affect synaptic development in diverse brain circuits. Based on genetic evidence, synaptic protein synthesis regulated by the mTOR pathway appears to be a point of convergence between several types of autism and affecting different brain regions, where an apparent increase in mTOR signaling may be the common pathophysiological marker. In effect, in the TSC+/-rodent, a hyperactive mTOR causes altered autophagy that most likely mediates a synaptic pruning deficit, parallel to these observed in an autistic child (43). However, a recent report demonstrated decreased mTOR signaling in the temporal cortex of prenatally treated VPA rats and in human postmortem fusiform gyrus of idiopathic autism (44). These authors suggest that a bidirectional alteration in the mTOR signaling pathway can be prejudicial to synaptic development and not only its exacerbation. Another interpretation is that in VPA-induced autism exists with an increased synaptic protein synthesis independent of mTOR, and that decreased mTOR signaling may be a compensatory change to excessive activation of a different pathway. Thus, it is fundamental in the VPA model to evaluate other possible regulators of synaptic protein synthesis.

Upstream regulators of synaptic protein synthesis initiated by the mTOR pathway or in parallel to it are metabotropic glutamate receptors. Their relevance to autistic behavior has been clearly highlighted in fragile X-mental disability, a neurological disorder with autistic signs (45). In the mouse model for fragile X-syndrome, a hypersensitivity to mGluR5 stimulation and increased Erk activation, causing increased synaptic protein synthesis in the hippocampus, was demonstrated (46). In agreement with that previous study, the mGluR5 receptor antagonist, 2-methyl-6-phenylethyl-pyrididine, reduces increased self-grooming and marble burying, however, no anxiety in VPA-treated mice (28). However, in that previous report, anxiety was evaluated by open field test and no social interaction was assessed. Nonetheless, the role of the other member of Class I mGluR receptor, mGluR1, has rarely been studied in environment-induced autism. In this context, it is important that mGluR5 and mGlu1 receptors have no redundant roles in the hippocampus as they differentially regulate CA1 pyramidal cell function (47).

The present study have analyzed mGluR5 and mGluR1a immunoreactivity in the hippocampal region of P30 male rats treated with VPA on embryonic day 12.5. The most important finding was the increased mGluR1a immunoreactivity in the stratum alveus, including the limit oriens/alveus, of the CA1 field of the hippocampus and in the hilus of dentate gyrus produced by prenatal treatment with VPA. These class I mGluR immunoreactive cells in CA1 oriens/alveus certainly correspond to inhibitory GABAergic interneurons (38). The hippocampus has a high diversity of inhibitory interneurons, which form complex feedback and feedforward circuits. These are fundamental for hippocampal functions and, at least four different types of interneurons have been described in the oriens/alveus region, according to their electrical responses to class I mGluRs agonist, trans-(1S, 3R)-1-aminocyclopentane-1, 3-dicarboxylic acid (ACPD) (48). Oriens/alveus interneurons type I express mGluR1, mGluR5 and somatostatin, while type II express mGluR1 and calbindin, and type III express only mGluR5. Type IV also express class I mGluRs, but they do not respond to ACPD (48). Judging by its profuse mGluR1a-immunoreactive dendritic arborization, the cells with increased labeling in the CA1 oriens/alveus of VPA-treated animals may correspond to type I or II. Notably, some of the oriens/alveus interneurons I, II and IV project to the CA1 stratum lacunosum moleculare and are termed O-LM interneurons (38,49). In functional terms, O-LM interneurons type I and type II have an interesting function, collaborating with the septal area (50) in the hippocampal theta rhythmic oscillations, which are involved in several neurobiological processes, including locomotion, defense, affect, learning and memory (51). In the context of autistic signs, the function for O-LM cells in the exclusion of aversive stimuli during hippocampal contextual representation in the fear-learning paradigm is interesting (52). The increased mGluR1a immunoreactivity in the alveus/oriens region can represents mGluR1a-expressing O-LM interneurons innervating the last portion of pyramidal dendrites laying in the lacunosum moleculare region. Therefore, in this way, increased mGluR1a-mediated activity of O-LM interneurons can produce altered inhibition, most

likely increased, in the distal portion of apical dendritic tree of CA1 pyramidal neurons. Those distal ramifications, also termed tufts, lie in the stratum lacunosum moleculare and modulates proximal synapsis of the same neurons lying in stratum radiatum (53). Following this line of reasoning, and knowing that the stratum radiatum and lacunosum moleculare represents distinct CA1 input pathways, it was expected that an altered activity of O-LM interneurons can exert long-lasting heterosynaptic effects altering the interplay between two functionally and spatially distinct pathways, affecting finally the network properties. This alteration can affect the role of O-LM interneurons in theta oscillations affecting the functions in which CA1 O-LM interneurons-mediated feedback circuits are involved. Therefore, that simple change in the mGluR1a immunoreactivity may have important consequences for the hippocampal integration and diverse neurobiological functions. In this context, the role of O-LM interneurons and hippocampal theta oscillations in defense, affect and, in particular, contextual fear learning deserves special attention. The suggested function of O-LM interneurons in the exclusion of aversive stimuli of hippocampal dependent fear learning (52) and the probed role of mGluR1 in long term plasticity of the same cells (37) suggests that the increases in mGluR1a immunoreactivity reported in the present study may be relevant to explain certain specific signs of autism.

Increased immunoreactivity for mGluR1a in the hilus may corresponds to hilar interneurons and it may have important consequences for hippocampal functioning. Granular cells of the dentate gyrus receive the major hippocampal inputs coming from the entorhinal cortex and subsequently, mossy fibers from granular cells innervate proximal dendrites of pyramidal neurons in CA3. In addition, mossy fibers also innervate profusely interneurons in the hilus of dentate gyrus and in the CA3. Therefore, granular layer activation by entorhinal inputs produces a rather general reduction of CA3 pyramidal cell excitability, a phenomena that likely aids to filter memory information (54). The majority of hilar GABAergic neurons are mGluR1a- and substance P-immunoreactive, and small type terminals of mossy fibers innervate almost all of these (55). In addition, a part of hilar mGluR1 immunoreactive interneurons are somatostatin-positive and form a feedback loop from the hilar region to the outer molecular layer of dentate gyrus (55,56). Thus, increased mGluR1a immunoreactivity in the hilus can affect forward and feedback inhibitory loops, modulating the hippocampal information processing.

By contrast, increased mGluR1a immunoreactivity may be a result of increased transcription or altered mRNA processing, translation or stability. In base to these results, the present study failed to determine the origin of increased mGluR1a immunoreactivity, but suggested an over-functioning of that specific type of class I mGluR, in line with the previous evidence for the involvement of this family of receptors in autism. Analysis of mRNA is required and it is interesting to determinate mGluR1a and mGluR1b levels since an interesting cooperative function between 1a and 1b isoforms has been previously demonstrated (57). In addition, the present study cannot discard the role of mGluR5 in the VPA model of autism and the possible cooperative signaling between homodimers of metabotropic glutamate receptors 1 and 5 is highly interesting (58).

In conclusion, the results of the present study suggested that prenatal treatment with VPA produces mGluR1a over-functioning in hippocampal interneurons of juvenile rats. This likely alters distinct regulatory loops affecting hippocampal processing that may be associated with autistic signs. The functional involvement of mGluR1a in autism models require further research as a possible novel pharmacological target in autism.

Acknowledgements

The present study was supported by the National Found for Scientific and Technological Research (nos. 1110855 and 1120528).

References

1. Frith U and Happé F: Autism spectrum disorder. Curr Biol 15: R786-R790, 2005.
2. Wass S: Distortions and disconnections: Disrupted brain connectivity in autism. Brain Cogn 75: 18-28, 2011.
3. Blatt GJ: The neuropathology of autism. Scientifica (Cairo) 2012: 703675, 2012.
4. Betancur C: Etiological heterogeneity in autism spectrum disorders: More than 100 genetic and genomic disorders and still counting. Brain Res 1380: 42-77, 2011.
5. Jamain S, Quach H, Betancur C, Råstam M, Colineaux C, Gillberg IC, Soderstrom H, Giros B, Leboyer M, Gillberg C, *et al*: Mutations of the X-linked genes encoding neuroligins NLGN3 and NLGN4 are associated with autism. Nat Genet 34: 27-29, 2003.
6. Moessner R, Marshall CR, Sutcliffe JS, Skaug J, Pinto D, Vincent J, Zwaigenbaum L, Fernandez B, Roberts W, Szatmari P and Scherer SW: Contribution of SHANK3 mutations to autism spectrum disorder. Am J Hum Genet 81: 1289-1297, 2007.
7. Durand CM, Betancur C, Boeckers TM, Bockmann J, Chaste P, Fauchereau F, Nygren G, Rastam M, Gillberg IC, Anckarsäter H, *et al*: Mutations in the gene encoding the synaptic scaffolding protein SHANK3 are associated with autism spectrum disorders. Nat Genet 39: 25-27, 2007.
8. Hung AY, Futai K, Sala C, Valtschanoff JG, Ryu J, Woodworth MA, Kidd FL, Sung CC, Miyakawa T, Bear MF, *et al*: Smaller dendritic spines, weaker synaptic transmission, but enhanced spatial learning in mice lacking Shank1. J Neurosci 28: 1697-1708, 2008.
9. Dindot SV, Antalffy BA, Bhattacharjee MB and Beaud: The Angelman syndrome ubiquitin ligase localizes to the synapse and nucleus and maternal deficiency results in abnormal dendritic spine morphology. Hum Mol Genet 17: 111-118, 2008.
10. Kishino T, Lalande M and Wagstaff J: UBE3A/E6-AP mutations cause Angelman syndrome. Nat Genet 15: 70-73, 1997.
11. Sadakata T, Washida M, Iwayama Y, Shoji S, Sato Y, Ohkura T, Katoh-Semba R, Nakajima M, Sekine Y, Tanaka M, *et al*: Autistic-like phenotypes in Cadps2-knockout mice and aberrant CADPS2 splicing in autistic patients. J Clin Invest 117: 931-943, 2007.
12. Costa-Mattioli M, Sossin WS, Klann E and Sonenberg N: Translational control of long-lasting synaptic plasticity and memory. Neuron 61: 10-26, 2009.
13. Hoeffer CA and Klann E: mTOR signaling: At the crossroads of plasticity, memory and disease. Trends Neurosci 33: 67-75, 2010.
14. de Vries PJ and Howe CJ: The tuberous sclerosis complex proteins-a GRIPP on cognition and neurodevelopment. Trends Mol Med 13: 319-326, 2007.
15. Feng Y, Zhang F, Lokey LK, Chastain JL, Lakkis L, Eberhart D and Warren ST: Translational suppression by trinucleotide repeat expansion at FMR1. Science 268: 731-734, 1995.
16. Koekkoek SK, Yamaguchi K, Milojkovic BA, Dortland BR, Ruigrok TJ, Maex R, De Graaf W, Smit AE, VanderWerf F, Bakker CE, *et al*: Deletion of FMR1 in Purkinje cells enhances parallel fiber LTD, enlarges spines, and attenuates cerebellar eyelid conditioning in Fragile X syndrome. Neuron 47: 339-352, 2005.

17. Butler MG, Dasouki MJ, Zhou XP, Talebizadeh Z, Brown M, Takahashi TN, Miles JH, Wang CH, Stratton R, Pilarski R and Eng C: Subset of individuals with autism spectrum disorders and extreme macrocephaly associated with germline PTEN tumour suppressor gene mutations. J Med Genet 42: 318-332, 2005.
18. Goffin A, Hoefsloot LH, Bosgoed E, Swillen A and Fryns JP: PTEN mutation in a family with Cowden syndrome and autism. Am J Med Genet 105: 521-524, 2001.
19. Zori RT, Marsh DJ, Graham GE, Marliss EB and Eng C: Germline PTEN mutation in a family with Cowden syndrome and Bannayan-Riley-Ruvalcaba syndrome. Am J Med Genet 80: 399-402, 1998.
20. Neves-Pereira M, Müller B, Massie D, Williams JH, O'Brien PC, Hughes A, Shen SB, Clair DS and Miedzybrodzka Z: Deregulation of EIF4E: A novel mechanism for autism. J Med Genet 46: 759-765, 2009.
21. Kelleher RJ IIIrd and Bear MF: The autistic neuron: Troubled translation? Cell 135: 401-406, 2008.
22. Kao DI, Aldridge GM and Greenough WT: Altered mRNA transport, docking and protein translation in neurons lacking fragile X mental retardation protein. Proc Natl Acad Sci USA 107: 15601-15606, 2010.
23. Markram K, Rinaldi T, La Mendola D, Sandi C and Markram H: Abnormal fear conditioning and amygdala processing in an animal model of autism. Neuropsychopharmacology 33: 901-912, 2008.
24. Silva GT, Le Bé JV, Riachi I, Rinaldi T, Markram K and Markram H: Enhanced long-term microcircuit plasticity in the valproic acid animal model of autism. Front Synaptic Neurosci 1: 1, 2009.
25. Rinaldi T, Perrodin C and Markram H: Hyper-connectivity and hyper-plasticity in the medial prefrontal cortex in the valproic acid animal model of autism. Front Neural Circuits 2: 4, 2008.
26. Rinaldi T, Silberberg G and Markram H: Hyperconnectivity of local neocortical microcircuitry induced by prenatal exposure to valproic acid. Cereb Cortex 18: 763-770, 2008.
27. Rinaldi T, Kulangara K, Antoniello K and Markram H: Elevated NMDA receptor levels and enhanced postsynaptic long-term potentiation induced by prenatal exposure to valproic acid. Proc Natl Acad Sci USA 104: 13501-13506, 2007.
28. Mehta MV, Gandal MJ and Siegel SJ: mGluR5-antagonist mediated reversal of elevated stereotyped, repetitive behaviors in the VPA model of autism. PLoS One 6: e26077, 2011.
29. WalfA AA and Frye CA: The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. Nat Protoc 2: 322-328, 2007.
30. Fernandes C and File SE: The influence of open arm ledges and maze experience in the elevated plus-maze. Pharmacol Biochem Behav 54: 31-40, 1996.
31. Pellow S, Chopin P, File SE and Briley M: Validation of open:Closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J Neurosci Methods 14: 149-167, 1985.
32. Moy SS, Nadler JJ, Perez A, Barbaro RP, Johns JM, Magnuson TR, Piven J and Crawley JN: Sociability and preference for social novelty in five inbred strains: An approach to assess autistic-like behavior in mice. Genes Brain Behav 3: 287-302, 2004.
33. Conrad CD, Grote KA, Hobbs RJ and Ferayorni A: Sex differences in spatial and non-spatial Y-maze performance after chronic stress. Neurobiol Learn Mem 79: 32-40, 2003.
34. Conrad CD, Galea LA, Kuroda Y and McEwen BS: Chronic stress impairs rat spatial memory on the Y maze, and this effect is blocked by tianeptine pretreatment. Behav Neurosci 110: 1321-1334, 1996.
35. Dellu F, Mayo W, Cherkaoui J, Le Moal M and Simon H: Two-trial memory task with automated recording: Study in young and aged rats. Brain Res 588: 132-139, 1992.
36. Edalatmanesh MA, Nikfarjam H, Vafaee F and Moghadas M: Increased hippocampal cell density and enhanced spatial memory in the valproic acid rat model of autism. Brain Res 1526: 15-25, 2013.
37. Lapointe V, Morin F, Ratté, S , Croce A, Conquet F and Lacaille JC: Synapse-specific mGluR1-dependent long-term potentiation in interneurones regulates mouse hippocampal inhibition. J Physiol 15: 125-135, 2004.
38. Shigemoto R, Kinoshita A, Wada E, Nomura S, Ohishi H, Takada M, Flor PJ, Neki A, Abe T, Nakanishi S and Mizuno N: Differential presynaptic localization of metabotropic glutamate receptor subtypes in the rat hippocampus. J Neurosci 17: 7503-7522, 1997.
39. Patterson PH: Modeling autistic features in animals. Pediatr Res 69: 34R-40R, 2011.
40. Kim KC, Kim P, Go HS, Choi CS, Yang SI, Cheong JH, Shin CY and Ko KH: The critical period of valproate exposure to induce autistic symptoms in Sprague-Dawley rats. Toxicol Lett 201: 137-142, 2010.
41. Schneider T and Przewłocki R: Behavioral alterations in rats prenatally exposed to valproic acid: Animal model of autism. Neuropsychopharmacology 30: 80-89, 2005.
42. Kim JE, Shin MS, Seo TB, Ji ES, Baek SS, Lee SJ, Park JK and Kim CJ: Treadmill exercise ameliorates motor disturbance through inhibition of apoptosis in the cerebellum of valproic acid-induced autistic rat pups. Mol Med Rep 8: 327-334, 2013.
43. Tang G, Gudsnuk K, Kuo SH, Cotrina ML, Rosoklija G, Sosunov A, Sonders MS, Kanter E, Castagna C, Yamamoto A, *et al*: Loss of mTOR-dependent macroautophagy causes autistic-like synaptic pruning deficits. Neuron 83: 1131-1143, 2014.
44. Nicolini C, Ahn Y, Michalski B, Rho JM and Fahnestock M: Decreased mTOR signaling pathway in human idiopathic autism and in rats exposed to valproic acid. Acta Neuropathol Commun 3: 3, 2015.
45. Bear MF, Huber KM and Warren ST: The mGluR theory of fragile X mental retardation. Trends Neurosci 27: 370-377, 2004.
46. Osterweil EK, Krueger DD, Reinhold K and Bear MF: Hypersensitivity to mGluR5 and ERK1/2 leads to excessive protein synthesis in the hippocampus of a mouse model of fragile X syndrome. J Neurosci 30: 15616-15627, 2010.
47. Mannaioni G, Marino MJ, Valenti O, Traynelis SF and Conn PJ: Metabotropic glutamate receptors 1 and 5 differentially regulate CA1 pyramidal cell function. J Neurosci 21: 5925-5934, 2001.
48. van Hooft JA, Giuffrida R, Blatow M and Monyer H: Differential expression of group I metabotropic glutamate receptors in functionally distinct hippocampal interneurons. J Neurosci 20: 3544-3551, 2000.
49. Kullmann DM: Interneuron networks in the hippocampus. Curr Opin Neurobiol 21: 709-716, 2011.
50. Stewart M and Fox SE: Do septal neurons pace the hippocampal theta rhythm? Trends Neurosci 13: 163-168, 1990.
51. Chee SS, Menard JL and Dringenberg HC: The lateral septum as a regulator of hippocampal theta oscillations and defensive behavior in rats. J Neurophysiol 113: 1831-1841, 2015.
52. Lovett-Barron M, Kaifosh P, Kheirbek MA, Danielson N, Zaremba JD, Reardon TR, Turi GF, Hen R, Zemelman BV and Losonczy A: Dendritic inhibition in the hippocampus supports fear learning. Science 343: 857-863, 2014.
53. Han E and Heinemann S: Distal dendritic inputs control neuronal activity by heterosynaptic potentiation of proximal inputs. J Neurosci 33: 1314-1325, 2013.
54. Scharfman HE and Myers CE: Hilar mossy cells of the dentate gyrus: A historical perspective. Front Neural Circuits 6: 106, 2013.
55. Acsády L, Kamondi A, Sík A, Freund T and Buzsáki G: GABAergic cells are the major postsynaptic targets of mossy fibers in the rat hippocampus. J Neurosci 18: 3386-3403, 1998.
56. Baude A, Nusser Z, Roberts JD, Mulvihill E, McIlhinney RA and Somogyi P: The metabotropic glutamate receptor (mGluR1 alpha) is concentrated at perisynaptic membrane of neuronal subpopulations as detected by immunogold reaction. Neuron 11: 771-787, 1993.
57. Techlovská S, Chambers JN, Dvořáková M, Petralia RS, Wang YX, Hájková A, Nová A, Franková D, Prezeau L and Blahos J: Metabotropic glutamate receptor 1 splice variants mGluR1a and mGluR1b combine in mGluR1a/b dimers in vivo. Neuropharmacology 86: 329-336, 2014.
58. Sevastyanova TN and Kammermeier PJ: Cooperative signaling between homodimers of metabotropic glutamate receptors 1 and 5. Mol Pharmacol 86: 492-504, 2014.