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Prenatal exposure to valproic acid induces alterations in the expression and activity of purinergic receptors in the embryonic rat brain

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Abstract

Purinergic signalling is involved in the control of several processes related to brain development, such as neurogenesis and gliogenesis, migration and differentiation of neuronal precursors, synaptogenesis and synaptic elimination to achieve a fully wired and efficient mature brain. Therefore, any deregulation of purine-dependent signalling mediated by stimulation of specific adenosine and purinergic receptor subtypes: P1, P2X, or P2Y, can lead to functional deficits and the development of neuropsychiatric disorders, including autism spectrum disorders (ASD). In this study, we investigated the changes in expression and activity of selected purinergic receptors during rat brain development in an animal model of ASD. Pregnant dams received an intraperitoneal injection of valproic acid (VPA; 450 mg/kg body weight) at embryonic day (ED) 12.5, around the time of neural tube closure. Subsequently, changes in the expression and activity of specific purinergic receptor subtypes were analysed at ED19, an important prenatal stage of brain development. Our results suggest that prenatal VPA exposure leads to a significant increase in the level and activity of adenosinergic receptors A1, A2b and A3, which are involved in the regulation of progenitor cell proliferation and nerve growth, and upregulation of purinergic P2X2/P2X3 receptors, which in turn may contribute to the postnatal neuroanatomical abnormalities and synaptic dysfunction. Conversely, the significant downregulation of P2Y1 and P2X7 receptors, together with their reduced activity in the embryonic VPA brain, may indicate disturbances in the processes of neuronal precursor migration and differentiation, dendritic and axonal formation, and glutamate/GABA imbalance, thereby altering neuronal excitability. In conclusion, defects in purinergic signalling induced by prenatal VPA exposure could have a profound impact on brain development during embryogenesis and on intellectual and behavioural functions after birth. These observations could provide clues for future implementation of potential therapeutic strategies for ASD.

Key words: *autism spectrum disorders, brain development, ATP, adenosine, P2 receptors, P1 receptors, intracellular calcium.*

Introduction

The concept of purinergic signalling, in which extracellular nucleotides and nucleosides provide the cell-to-cell communication, was first introduced by Geoffrey Burnstock in 1972 [6] and since then the properties of particular subtypes of purinergic receptors have been intensively studied, also with regard to neurodevelopmental disorders [13,35]. Nucleotides and nucleosides act as short-term signalling molecules in neurotransmission, neuromodulation, and neurosecretion. ATP has also potent, long-term roles in cell proliferation and differentiation. The various purinergic receptor subtypes are widely distributed throughout the central nervous system (CNS) where they control local signal transduction. Also the balance between the release and effects of ATP, adenosine and ectonucleotidases activities are crucial in regulating synaptic transmission neuromodulation and neurosecretion. ATP is released by “healthy” neurons and other cell types into the extracellular

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milieu under physiological conditions, as well as in the response to danger or stress signalling *via* specific or unspecific mechanisms [27]. A non-regulated nucleotide release occurs mainly from damaged or dying cells, whereas vesicular exocytosis, pore-forming channels (connexins and pannexins), or specific transporters are involved in the controlled transport [3,4,10,74].

Following the release, ATP is rapidly metabolised by extracellular ectonucleotidases to adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP), and the nucleoside adenosine (Ado) [83]. Ectonucleotidases activity is critical for controlling the balance between nucleotide- and adenosine-dependent signalling that is mediated *via* cell surface P2 and P1 receptors, respectively [1]. The P2 receptor (P2R) family includes the P2X (P2XRs) and P2Y (P2YRs) receptor subfamilies. P2XRs are ATP-gated channels allowing Ca^{2+} and Na^{+} influx and K^{+} efflux [7,35,57]. Based on the mechanism of action and pharmacology, seven subtypes (P2XR1-P2XR7) of ionotropic P2XRs have been identified so far in mammals [1]. These cation selective channels consist of at least three subunits establishing hetero- (e.g. P2XR2/3 and P2XR1/5) or homo-trimers (e.g. P2X7 receptor). Affinity of P2XRs for ATP can extensively vary, from the low micromolar to the millimolar level (e.g. P2X7 receptor). P2YRs are coupled to G proteins, triggering downstream effector signalling pathways ending with increased concentration of either intracellular Ca^{2+} or alterations in the level of cyclic adenosine monophosphate (cAMP) [42]. In mammals, eight metabotropic P2YRs (P2YR1, P2YR2, P2YR4, P2YR6, P2YR11, P2YR12, P2YR13, and P2YR14) have been so far identified and characterized. P2YR1, P2YR2, P2YR4, and P2YR6 activate Gq protein-dependent phospholipase C-β (PLC-β) that enzymatically cleaves the membrane phospholipid phosphoatidylinositol-4,5-bisphopshate (PIP_2) into diacylglycerol (DAG) and inositol trisphosphate (IP_3) which increases intracellular Ca^{2+} level *via* release from the endoplasmic reticulum. DAG remains in the membrane where it recruits and activates protein kinase C (PKC) [84]. P2YR12, P2YR13, and P2YR14 lead to inhibition of adenylyl cyclase and through it to reduction in cAMP level. P2YR11 stimulation triggers both, an increase in intracellular Ca^{2+} and cAMP level.

The P1 receptor (P1R) family includes four adenosine receptors (A1, A2A, A2B, and A3) [11]. Their activation triggers stimulation or inhibition of adenylyl cyclase, depending on the receptor subtype, thus modifying intracellular cAMP levels. The A2A and A2B receptors stimulate adenylyl cyclase and increase cAMP level, whereas A1 and A3 decrease cAMP level. Extracellular nucleotide and nucleoside signalling are present from the earliest developmental stages, fulfilling a crucial role in the control of different processes of brain development [9]. Extracellular ATP and other purines participate in the regulation of migration neurons and glia and their final allocation, as well as synaptic stabilization, maturation, and elimination to achieve a completely effective mature brain. Therefore, any deregulation of purinergic signal transduction and purinergic receptor alterations during brain development may result in functional deficits and neurodevelopmental disturbances including autism spectrum disorders (ASD) [8,13,50].

Antipurinergic therapy has been shown to ameliorate the behavioural, ultrastructural, and biochemical symptoms in a mouse model of ASD induced by environmental factors such as maternal immune activation (MIA) [52,55] or prenatal exposure to valproic acid (VPA) [38] as well as in the genetic model, the Fmr1 knockout (Fragile X syndrome) mice [51]. The effectiveness of the compound used (suramin – non selective antagonist of purinergic receptors) was also confirmed in a small phase I/II, double-blind, placebo-controlled, translational pilot study in children with ASD, where the single intravenous administration of the drug improved scores for language and social interaction, and decreased restricted or repetitive behaviours [54]. The hypothesis that the abnormalities of purinergic signalling influence the behavioural disorders observed in ASD was also confirmed in the genetic studies on P2X4R knockout mice, where deficits in social interaction and sensory integration were shown [78]. Also, a large transcriptomic analysis of human brain tissue samples revealed that purinergic signalling abnormalities correlate with the occurrence of cardinal domains of autism: impairment of social interaction, verbal or non-verbal communication, and stereotyped or repetitive behaviours [34]. Taken together, those studies indicate that purine metabolism belongs to the most strikingly disturbed pathways in autism, but little is known about ASD-related changes in ATP-mediated signalling at the developmental brain stage, especially at the purinergic receptor level. Therefore, in the present study we aimed at analysing whether the prenatal VPA treatment induced changes in the level and activity of selected purinergic receptor subtypes in the developing brain.

Material and methods

Ethical statement

All experiments conducted with animals were approved by the Local Ethics Committee for Animal Experimentation in Warsaw, Poland (reference number WAW2/148/2018 and WAW2/036/2019), and were carried out following the EC Council Directive of 24 November 1986 (86/609/EEC) following the ARRIVE guidelines and guidelines published in the NIH Guide for the Care and Use of Laboratory Animals and the principles

presented in the "Guidelines for the Use of Animals in Neuroscience Research" by the Society for Neuroscience. The number of animals used and their suffering in this study was minimized. All manipulations were performed gently and quickly to avoid stress-induced alterations.

Animals – *in vivo* model of ASD

The Wistar female rats between 12 and 15 weeks of age weighing 210-250 g were supplied by the Animal House of the Mossakowski Medical Research Institute, Polish Academy of Sciences (Warsaw, Poland), which breeds small rodents according to the SPF standard. The animals were maintained under controlled conditions of temperature and humidity with a 12 h light/dark cycle and were allowed free access to food and water. Rats were mated, with pregnancy determined by the presence of a vaginal plug at embryonic day 0 (ED0). The single *i.p.* injection of VPA (Sigma Aldrich, MO, USA) at a dose of 450 mg/kg of body weight at ED12.5 to obtain the ASD phenotype was performed according to Nicolini and Fahnestock [56]. Control dams received a single injection of saline. Pregnant dams were sacrificed at embryonic day 19 (ED19) and foetal brain samples (cerebral cortices with hippocampi) were dissected. Collectively, for the experiments, we used 22 pregnant rats: 11 received saline (control group), and 11 received VPA (VPA group). The average yield of one pregnant dam was 10 embryos. We used half of these embryos for biochemical analysis, whereas from the rest we isolated cells to primary neuron culture. The samples were immediately used to primary culture of neurons isolation or placed in liquid nitrogen and stored at -80°C for further analysis. Experimental design was shown in Figure 1.

Primary neuronal culture

The protocol was developed following the described procedures [60] with modifications. Neurons were isolated from cortices with hippocampi of rats' embryos (ED19). Pregnant rats were euthanized, their uteri were surgically removed and embryos collected from the uteri. The embryos were decapitated and the heads were placed in a 100 mm Petri dish containing HBSS with 1% (v/v) penicillin on ice. Brains were carefully removed and placed in a fresh 100 mm dish with fresh isolation medium on ice. Under a laminar flow hood and stereotaxic microscope, embryonic cortices with hippocampi were dissected and collected into a 15 ml falcon containing fresh isolation medium. The cells were dissociated by incubation for 10 min at 37°C in digestion solution containing Trypsin (Thermo-Fisher, CA, USA) in HBSS without Ca^{2+} and Mg^{2+} (Sigma Aldrich, MO, USA). The dissociated cells containing neurons were then washed three times with foetal bovine serum (FBS), and further dissociated by gentle pipetting in culture medium. Cell viability was calculated using trypan blue staining. The number of cells added per plate was used depending on the purpose of the experiment. Neurons were plated onto plastic tissue culture dishes pre-coated with 10 ug/ml poly-D-lysine and maintained in culture medium containing Neurobasal^{TM} Plus Medium supplemented with 2% (v/v) B27 and 0.25% (v/v) Glutamate (Thermo-Fisher, CA, USA). Neuronal cultures were incubated 7 days in a culture medium in a humidified 5% CO_2 incubator at 37°C.

Fluorometric measurements of changes in the intracellular calcium concentration

Changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$) concentration in the primary neuronal culture at 7 DIV (days *in vitro*) were monitored using the fluorescent calcium-sensitive probe Fluo-4 (Thermo-Fisher, CA, USA) as described [77] with modifications. To this experiment, primary neuronal were plated onto 96-well dark plates at the density of 1.6×10^5

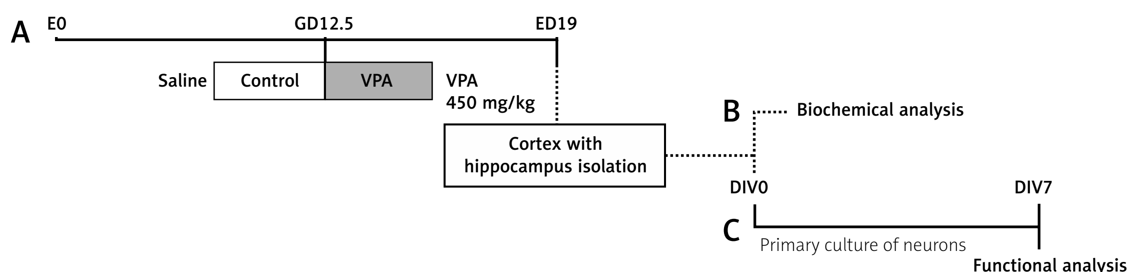

Fig. 1. Experimental design. **A**) At embryonic day 12.5 (ED12.5), either valproic acid (VPA; 450 mg/kg, *i.p.*) or saline was administered intraperitoneally to pregnant rats. Prenatal injection of VPA/saline defined the experimental groups. At embryonic day 19 (ED19), the embryos were dissected and their cortex with hippocampus was isolated. **B**) Gene expression and protein level were determined by biochemical analysis (qPCR, and Western blot analysis, respectively). **C**) *In vitro* studies involved the establishment of primary neuronal cultures. The functional analysis (intracellular calcium level) was performed at day *in vitro* (DIV) 7.

cells/ml. Neurons were loaded with 1 mM Fluo-4 AM supplemented with 0.02% Pluronic® F-68 for 60 min at 37°C in a Neurobasal Plus. Neurons were washed two times with colourless HBSS. After a second washing, the fluorescence was measured using a microplate reader FLUOstar Omega (Ortenberg, Germany) set at 485 nm excitation and 538 nm emission wavelengths. After determining the baseline fluorescence of the neurons, the changes in fluorescence after the addition of the test compounds were recorded every 15 s for 300 s. We used following test compounds:

Adenosine, adenosine-5'-triphosphate tetralithium salt (ATP) and 2'(3')-O-(4-Benzoylbenzoyl)adenosine 5'-triphosphate triethylammonium salt (BzATP) from Sigma Aldrich (MO, USA); adenosine-5'-(γ-thio)-triphosphate tetralithium salt (ATPγS), α,β-Methyleneadenosine 5'-triphosphate (αβmeATP), and adenosine 5'-[β-thio]diphosphate trilithium salt (ADPβS) from Bio-Techne (MN, USA). This treatment did not have any significant impact on cell viability. The results of fluorescence measurements are presented as percent changes in fluorescence intensity relative to the basal level vs. duration of measurement (%F/F0). To quantify the change in the dynamics of the Ca^{2+} responses, the area under the curve (AUC) was calculated as a measure for the increase in intracellular Ca^{2+}.

Quantitative real-time polymerase chain reaction (qRT-PCR)

RNA from samples was isolated with TRI-reagent according to the manufacturer's protocol (Sigma Aldrich, Saint Louis, MO, USA). RNA quantity and quality were controlled by spectrophotometric analysis using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Digestion of DNA contamination was performed with DNase I according to the manufacturer's protocol (Sigma Aldrich, Saint Louis, MO, USA). Reverse transcription was performed with a High Capacity cDNA Reverse Transcription Kit according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). Expression levels of mRNA for selected purinergic receptors were measured with real-time PCR, using the TaqMan Gene Expression Assays indicated in Table I (Applied Biosystems, Foster City, CA, USA) with *Actb* as the reference gene, on an ABI PRISM 7500 apparatus. Each sample was analysed in three technical replicates, and the mean Ct values were used for further analysis. The relative changes in mRNA levels were calculated using the $^{\Delta\Delta}$Ct method and expressed as RQ.

Table I. The detailed information about TaqMan Gene Expression Assays used for qRT-PCR

Receptor gene symbol	Assay ID	Cat. No.
Adora1	Rn00567668_m1	4331182
Adora2a	Rn00583935_m1	4331182
Adora2b	Rn00567697_m1	4331182
Adora3	Rn00563680_m1	4331182
P2rx1	Rn00564454_m1	4331182
P2rx2	Rn00586491_m1	4331182
P2rx3	Rn00579301_m1	4331182
P2rx4	Rn00580949_m1	4331182
P2rx5	Rn00589966_m1	4331182
P2rx7	Rn00570451_m1	4331182
P2ry1	Rn00562996_m1	4331182
P2ry2	Rn02070661_s1	4331182
P2ry12	Rn02133262_s1	4331182
Actb	Rn00667869_m1	4331182

Western blot analysis

The immunochemical analysis of the protein level of selected purinergic receptors was performed by the Western blotting method under standard conditions. Tissue samples were homogenized, mixed with a Laemmli buffer, and denatured at 95°C for 5 min. After standard 10% SDS-PAGE separation, the proteins were "wet"-transferred to nitrocellulose membranes under standard conditions, and the proteins were detected by immunodetection with specific antibodies. The membranes were washed for 5 min in TBS-T (Tris-buffered saline with Tween 20 buffer: 100 mM Tris, 140 mM NaCl and 0.1% Tween 20, pH 7.6), and non-specific binding was blocked for 1 h at room temperature (RT) with 5% BSA in TBST or with 5% non-fat milk solution in TBST. Membranes were probed with the primary antibodies with the appropriate concentration and conditions indicated in Table II. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the loading controls. Primary antibodies were purchased from Alomone Labs (Jerusalem, Israel), Sigma Aldrich (Saint Louis, MO, USA), and Thermo Fisher (Carlsbad, CA, USA). The membranes were washed three times in TBS-T, incubated for 60 min at RT with appropriate secondary antibodies, and washed again 3 times in TBS-T. Antibodies were detected using the chemiluminescent reaction and a Clarity Western ECL Substrate (Bio-Rad Laboratories, Hercules, CA, USA) under standard conditions. After stripping, the immunolabelling of GAPDH was performed as a loading control. Densitometric analysis was performed with the TotalLab software.

Statistical analysis

The results are expressed as mean values ±SD. Differences between the means were analysed using a Student's *t*-test. Statistical significance was accepted at $p < 0.05$. The statistical analyses were performed using Graph Pad Prism version 8.0 (Graph Pad Software, San Diego, CA).

Results

Our previous study indicated that prenatal exposure to VPA induced neuroinflammation and reactive oxygen species (ROS) generation in the cerebral cortex and hippocampus of young-adult offspring along with pathological changes in the ultrastructure of synapses as well as behavioural alterations typical for autism spectrum disorders (ASD) [31]. Since disturbed functioning of extracellular nucleotide-dependent signal transduction has been also suggested to be associated with autism pathology and purinergic signalling control key processes of brain development, such as embryonic neurogenesis, synaptogenesis and synaptic stability [64,86] we

Table II. The concentration of the antibodies and the experimental conditions for Western blotting

Receptor	Company	Cat. No.	Conditions
A1	Thermo Fisher, USA	PA1041A	Blocking: 5% milk in TBS-T, 1 hr, RT Primary antibody diluted in 5% milk in TBS-T (1 : 500), incubation 2 h, RT Secondary antibody anti-rabbit diluted in 5% milk in TBS-T (1 : 5000), 1 hr in RT
A2a	Sigma Aldrich, USA	AB1559P	Blocking: 2% BSA in TBS-T, 1 hr, RT Primary antibody diluted in 2% BSA in TBS-T (1 : 200), incubation overnight, 4°C Secondary antibody anti-rabbit diluted in 5% milk in TBS-T (1 : 4000), 1 hr, RT
A2b	Sigma Aldrich, USA	AB1589P	Blocking: 2% BSA in TBS-T, 1 hr, RT Primary antibody diluted in 2% BSA in TBS-T (1 : 250), incubation overnight, 4°C Secondary antibody anti-rabbit diluted in 5% milk in TBS-T (1 : 4000), 1 hr, RT
A3	Sigma Aldrich, USA	AB1590P	Blocking: 5% BSA in TBS-T, 1 hr, RT Primary antibody diluted in 2% BSA in TBS-T (1 : 500), incubation overnight, 4°C Secondary antibody anti-rabbit diluted in 5% milk in TBS-T (1 : 4000), 1 hr, RT
P2X1	Thermo Fisher, USA	APR-001	Blocking: 5% BSA in TBS-T, 1 hr, RT Primary antibody diluted in 5% BSA in TBS-T (1 : 500), incubation overnight, 4°C Secondary antibody anti-rabbit diluted in 5% milk in TBS-T (1 : 4000), 1 hr, RT
P2X2	Thermo Fisher, USA	PA5-119683	Blocking: 5% BSA in PBS, 1 hr, RT Primary antibody diluted in 5% BSA in PBS (1 : 500), incubation 2 hr, RT Secondary antibody anti-rabbit diluted in 5% BSA in PBS (1 : 5000), 1 hr, RT
P2X3	Sigma Aldrich, USA	P0121	Blocking: 2% BSA in TBS-T, 1 hr, RT Primary antibody diluted in 2% BSA in TBS-T (1 : 200), incubation overnight, 4°C Secondary antibody anti-rabbit diluted in 5% milk in TBS-T (1 : 4000), 1 hr, RT
P2X4	Alomone Labs, Israel	APR-024	Blocking: 5% milk in TBS-T, 1 hr, RT Primary antibody diluted in 5% BSA in TBS-T (1 : 400), incubation overnight in 4°C Secondary antibody anti-rabbit diluted in 5% milk in TBS-T (1 : 4000), 1 hr, RT
P2X5	Alomone Labs, Israel	APR-027	Blocking: 5% milk in TBS-T, 1 hr, RT Primary antibody diluted in 5% BSA in TBS-T (1 : 400), incubation overnight in 4°C Secondary antibody anti-rabbit diluted in 5% milk in TBS-T (1 : 4000), 1 hr, RT
P2X7	Alomone Labs, Israel	APR-008	Blocking: 5% BSA in TBS-T, 1 hr, RT Primary antibody diluted in 5% BSA in TBS-T (1 : 500), incubation overnight in 4°C Secondary antibody anti-rabbit diluted in 5% milk in TBS-T (1 : 4000), 1 hr in RT
P2Y1	Sigma Aldrich, USA	P-6487	Blocking: 5% milk in TBS-T, 1 hr, RT Primary antibody diluted in TBS-T (1 : 100), incubation overnight, 4°C Secondary antibody anti-rabbit diluted in 5% milk in TBS-T (1 : 4000), 1 hr in RT
P2Y2	Alomone Labs, Israel	APR-010	Blocking: 5% milk in TBS-T, 1 hr, RT Primary antibody diluted in 2% BSA (1 : 100), incubation overnight, 4°C Secondary antibody anti-rabbit diluted in 5% milk in TBS-T (1 : 4000), 1 hr in RT
P2Y12	Thermo Fisher, USA	702516	Blocking: 5% BSA in TBS-T, 1 hr, RT Primary antibody diluted in 5% milk in TBS-T (1 : 1000), incubation overnight, 4°C Secondary antibody anti-rabbit diluted in 5% milk in TBS-T (1 : 1000), 1 hr in RT
HRP-GAPDH mouse monoclonal	Proteintech, UK	HRP-60004	Blocking: 5% milk in TBS-T, 1 hr, RT Primary antibody diluted in 5% milk in TBS-T (1 : 50,000), incubation 1 hr, 4°C
Goat anti-rabbit IgG (H+L)	Proteintech, UK	SA00001-2	Antibody diluted in 5% milk in TBS-T (1 : 4000 or 1 : 1000), 1 hr, RT

have investigated the VPA-induced changes in the gene expression and protein level of particular subtypes of purinergic receptors at the early prenatal stage (ED19) along with activity analysis in primary culture neurons.

The impact of prenatal exposure to valproic acid on the mRNA and protein level of purinergic receptors subtypes in the foetal brain

The relative expression of mRNA and protein level was performed in the cerebral cortex with hippocampus isolated from foetal brain (ED19). Prenatal exposure to VPA causes changes in the expression of selected purinoceptors, especially adenosine receptors. Although we did not observe any significant alterations in the mRNA expression (Fig. 2A), we showed a distinct increase in the protein level for these receptors as compared to control (Fig. 2A'). The protein level of A1 ($p = 0.0202$), A2b ($p = 0.024$), and A3 receptor ($p = 0.0135$) was significantly increased by about 80%, 60%, and 11%, respectively. Similarly, ionotropic P2X receptors were modulated on the protein level without changes in the mRNA expression (Fig. 2B). We have observed the significant elevation of the protein level of P2X2 ($p = 0.0397$) and P2X3 ($p = 0.0201$) receptor by about 87% and 25%, respectively (Fig. 2B'), while the expression of P2X4 receptor was undetectable (data not shown). Among the investigated P2Y receptors, exclusively P2Y1 was significantly downregulated since mRNA level decreased by 13% (Fig. 2C, $p = 0.0467$) and protein level by 15% (Fig. 2C', $p = 0.0076$). In addition, immunoreactivity of P2Y12 receptor decreased by about 18% (Fig. 2C', $p = 0.0157$).

The impact of prenatal exposure to valproic acid on the selected purinergic receptor activation in the primary cultured neurons

As described in Material and methods section, pregnant rats were injected with VPA or saline (control) at ED12.5 and the next at ED19 cerebral cortex with the hippocampus was dissected from rat embryos to establish primary neuronal cultures. At day *in vitro* (DIV) 7, neurons in culture were loaded with fluorescence probe Fluo-4 and treated with subsequent purinergic receptor agonists.

In our study we reported that treatment with adenosine (100 μM) (Fig. 3A) results in increased intracellular Ca^{2+} concentration (about 115%, $p < 0.0053$) in the neurons isolated from rats after prenatal VPA exposure. Moreover, as shown in Figure 3B, treatment with ATP (1 μM) leads to significantly higher, by about 55% ($p = 0.0138$), intracellular Ca^{2+} mobilization in neurons isolated from the brain of foetuses prenatally exposed to VPA. The increase in intracellular Ca^{2+} level was more prominent (about 99% increase) when cells were treated with ATP at a higher concentration (100 μM) ($p < 0.0001$) (Fig. 3C). In addition, the results showed that treatment with ATPγS (100 μM), the ATP analogue with increased metabolic stability, also caused a significant increase in the $[Ca^{2+}]_i$ by about 118% ($p = 0.0033$) in VPA cells (Fig. 3D). In order to determine whether the increase in $[Ca^{2+}]_i$ is related to P2X ionotropic or P2Y metabotropic receptor, we treated culturing primary neurons with appropriate selective agonists. After activation of P2X2/3 receptors with 30 μM α,β-methylene (α,β-me) ATP, which is a specific agonist of P2X2/3 receptors, we observed a significant increase in the intracellular calcium concentration by about 64% ($p < 0.0001$) (Fig. 3E) suggesting activation of these ATP-gated calcium entry channels. To study the involvement of P2X7 receptor that has been suggested to be activated in autism-like pathology [73], primary neurons were treated with BzATP (300 μM), a potent agonist of P2X7 receptor (and weak agonist of P2X4). However, a significant decrease in $[Ca^{2+}]_i$ was observed in primary neurons from VPA embryos comparing to controls by about 77% ($p = 0.0003$) (Fig. 3F). What is important, activation of P2Y metabotropic receptors (P2Y1, P2Y12, and P2Y13) by the agonist adenosine 5′-O-2-thiodiphosphate (ADPβS, 10 μM, stable ADP analogue) also caused a significant decrease in $[Ca^{2+}]_i$ in primary neurons from VPA embryonic brain by about 44% ($p = 0.0444$) (Fig. 3G).

Discussion

Several clinical and preclinical studies on animal models of ASD have suggested disturbed functioning of the purinergic signalling system in autism and related spectrum disorders [53,82,86]. The metabolomic studies have revealed significant alterations in the purine and pyrimidine metabolite profile in the urine and plasma of ASD individuals [33]. Also, a non-selective blockade of purinergic receptors reversed abnormal behaviour and prevented morphological and biochemical changes in ASD animal models [38,51,52,55]. Moreover, the inhibition of P2X7R reduced inflammatory response in poly I:C subjected pregnant animals, thus preventing autism-like phenotype in the offspring. Our recent study extends the previous findings by showing that in the ASD animal model triggered by prenatal exposure to VPA the expression and activity of the specific subtypes of purinergic receptors are significantly altered in the embryonic rat brain, thus partially defining the molecular aspect of the earlier observations.

The animal model chosen for this study recapitulates the characteristics of idiopathic autism with an epigenetic or environmental background and is the

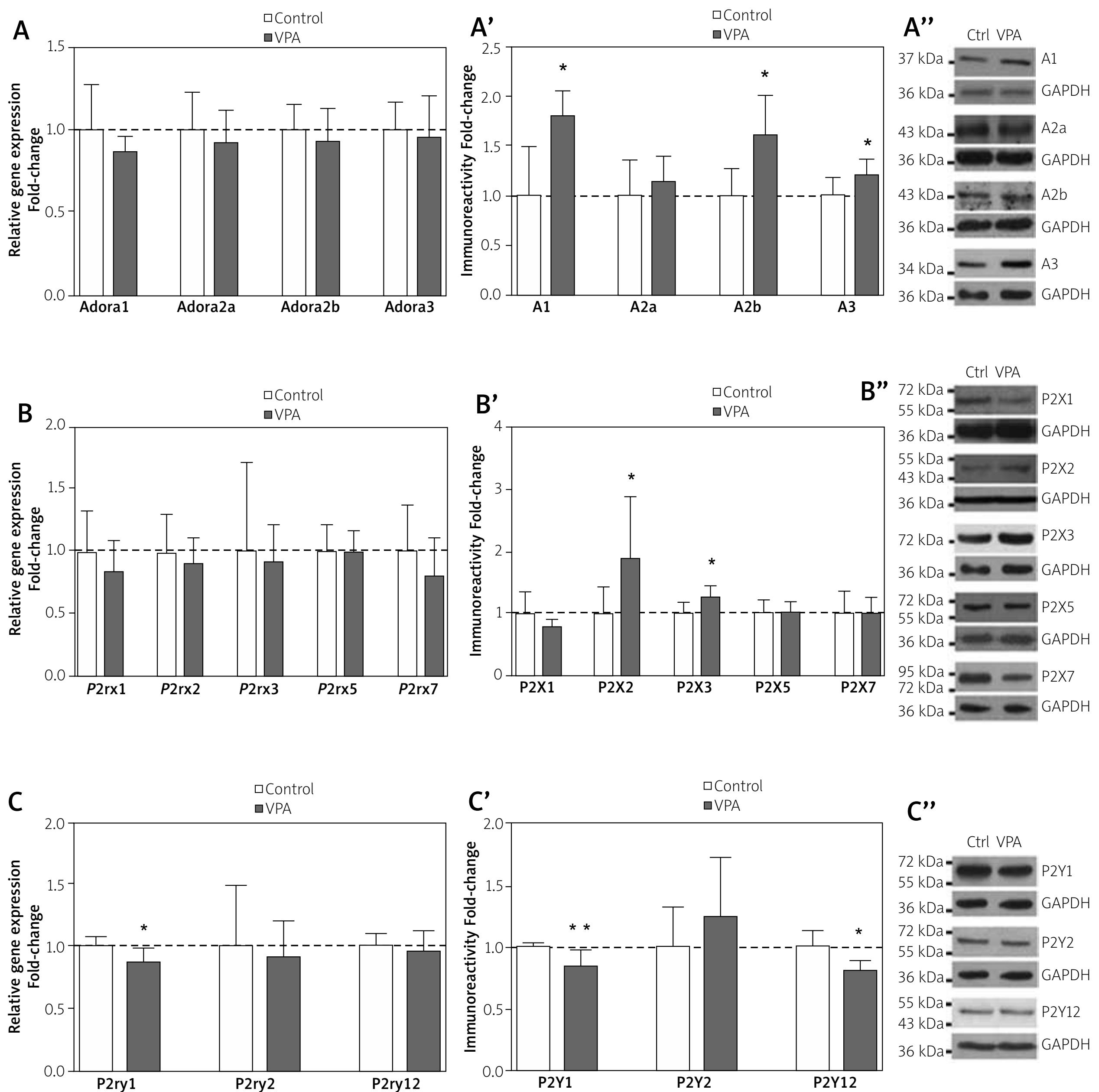

Fig. 2. The impact of prenatal valproic acid (VPA) exposure on the purinergic receptors' expression in the brain of 19-day-old rat embryos. The mRNA level of selected adenosine (**A**), ionotropic (**B**), and metabotropic (**C**) receptors in the cerebral cortex with the hippocampus of rat embryos, which were prenatally exposed to VPA treatment, was measured by real-time PCR, and normalized to Actb (β-actin). Data represent the mean value ±SD from 4-9 independent experiments; $^{*}p < 0.05$ vs. the corresponding control as determined using Student's *t*-test. The immunoreactivity of selected adenosine (**A'**), ionotropic (**B'**), and metabotropic (**C'**) receptors was measured by Western blot method. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. Data represent the mean value ±SD from 3-7 independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. the corresponding control determined using the Student's *t*-test. The representative pictures of adenosine (**A''**), ionotropic (**B''**), and metabotropic (**C''**) receptors were shown.

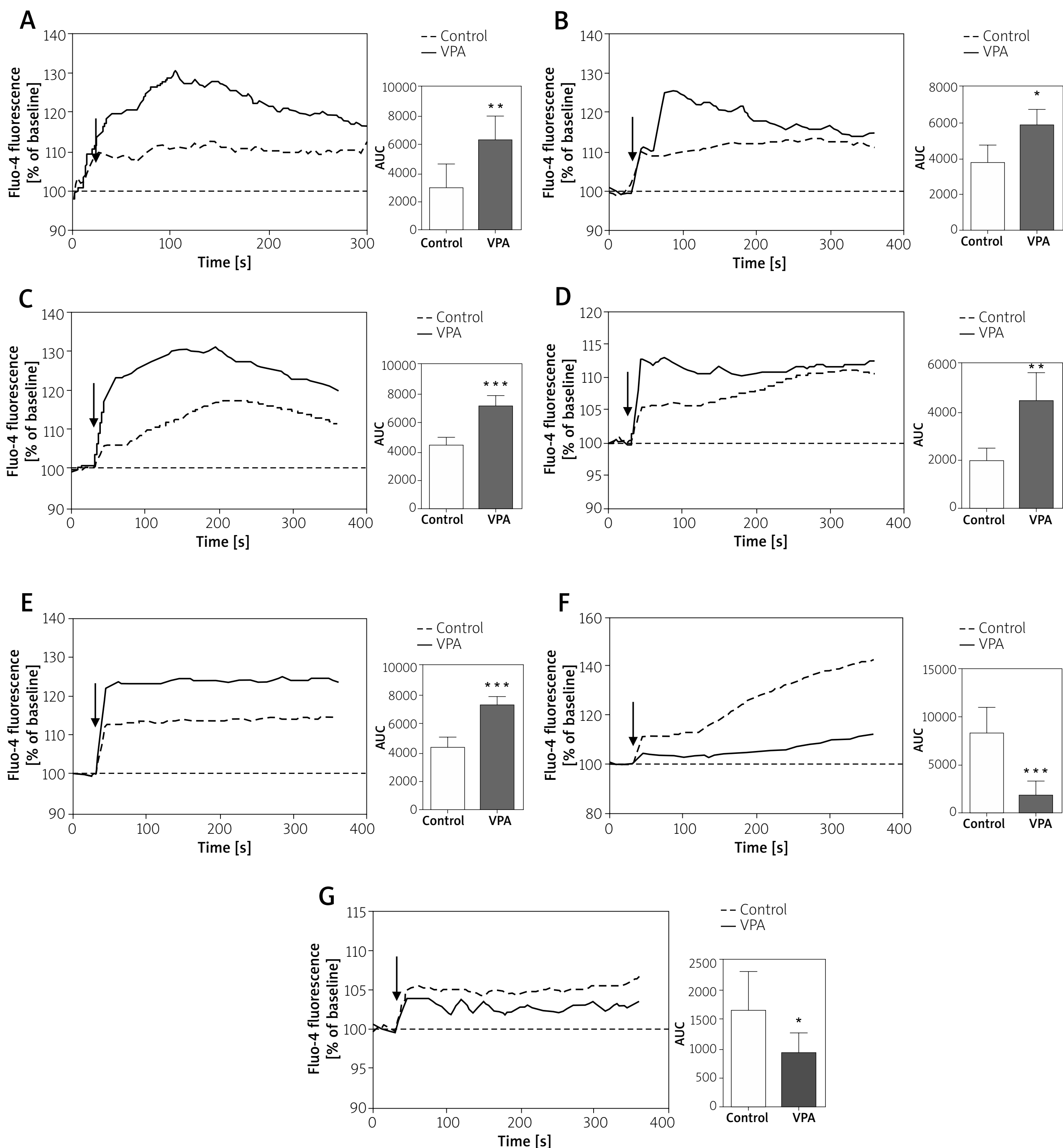

Fig. 3. The influence of selected purinergic receptor activation on intracellular calcium concentrations in the primary neuronal culture. The intracellular calcium concentrations in the culturing primary neurons isolated from the cerebral cortex with the hippocampus of 19-day-old embryos prenatally exposed to valproic acid (VPA) was measured by the fluorescent calcium-sensitive probe Fluo-4. Baseline Fluo-4 was monitored for 30 s prior to the addition of selected purinergic receptor agonists. Culturing primary neurons were treated with: 100 μM Adenosine (**A**), 1 μM ATP (**B**), 100 μM ATP (**C**), 100 μM ATPγS (**D**), 30 μM αβmeATP (**E**), 300 μM BzATP (**F**) or 10 μM ADPβS (**G**). Fluorescence was monitored for another 300 s, and values were converted to %F/F0, where F0 is the fluorescence value of the first record (0 s). The responses were quantitated by measuring the AUC value. The results are presented as the mean value ±SD from 4-11 independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to control using the Student's *t*-test.

most frequently used in the studies of ASD [49,56]. Valproic acid is a first-generation anti-epileptic drug (AED), which has been shown highly effective in the treatment of AED due to a variety of different neural mechanisms: inhibition of the voltage-dependent sodium channels, enhanced GABAergic signalling, reduced NMDA-receptor-mediated glutamate excitation and increased serotonergic inhibition [47,59]. Such a broad spectrum is highly valuable in AED treatment, suggesting improved effectiveness with reduced side effects. However VPA is also an inhibitor of histone deacetylases (HDAC), therefore it indirectly influences the execution of gene expression that contributes to the high teratogenicity of this drug. VPA has been associated with a variety of major and minor foetal malformations, including neural tube defects, cardiovascular abnormalities, genitourinary defects, developmental delay, endocrinological disorders, or limb defects [2]. It was also demonstrated that VPA intake during pregnancy, especially in the first trimester, significantly increases the risk of ASD prevalence in offspring [17]. Our earlier studies showed that prenatal exposure to VPA leads to significant impairment of communication in neonatal rats, followed by the decrease in exploratory activity in adolescence, and the occurrence of anxiety-like and repetitive behaviours, which are characteristic features of ASD [31,32]. In addition, in young adult VPA offspring, the significant synaptic impairment accompanied by deregulation of key pre- and postsynaptic proteins and significant alterations in the level of microtubule-associated proteins (MAPs) was observed [31,32]. Also, the exposure of cortical neurons isolated from rat embryos to VPA in concentrations modelled on dose applicable in human patients, revealed the marked functional excitatory/inhibitory imbalance in neuronal networks *in vitro*, which is largely caused by the epigenetic effect of VPA [30]. Since the results of VPA treatment are observed very early during development, thus we have decided to analyse changes in purinergic receptors level and activity in the foetal brain, where the impact of purines function is much greater than in the mature brain, especially in the context of regulation of neuro- and gliogenesis [80,85].

In the prenatal stage, when ASD-related abnormalities in cell proliferation, neurogenesis, migration, and differentiation occur [19], we have observed the profound elevation of the protein level of the A1, A2b, and A3 subtypes of adenosine receptors. In the rat brain, the expression of adenosine receptors begins during mid-late embryogenesis [76]. While A1 and A3 receptors induce adenylyl cyclase (AC) inhibition through G proteins of the Gi and Go family [28,44], A2A and A2B mediated Gs protein activation leads to the stimulation of AC. However, beyond these canonical signalling mechanisms, the stimulation of A1 and A3 receptors can also elicit Gq-mediated and IP3 receptor-regulated release of calcium ions from intracellular stores [40]. In accordance with these studies our results also indicated that in the VPA animals, where the profound increase in A1, A2b and A3 subtypes of adenosine receptors occurs, the significant increase in cytoplasmic calcium level upon adenosine treatment was observed. It was previously demonstrated that the pharmacological inhibition of adenosine metabolism disrupts foetal development [46]. Moreover, gene polymorphisms of the adenosine deaminase (ADA), which catalyses the irreversible adenosine conversion into inosine may be a risk factor for the development of autism [5]. Conversely, the exposure of pregnant mice to the subtype-selective adenosine type 2A receptor (A2AR) antagonist resulted in the delayed migration and insertion of γ-aminobutyric acid (GABA) neurons into the hippocampal circuitry during the first postnatal week in offspring, which was associated with increased neuronal network excitability and increased susceptibility to seizures in response to a seizure-inducing agent [69]. Altogether those results suggest that the either up- or down-regulation of adenosine signalling in the brain under various pathophysiological conditions might be involved in pathology related to ASD. Our study extends the previous findings by showing that exposure to potent inducer of the ASD phenotype resulted in the deregulation of the adenosine receptors level and activity. It was previously demonstrated that acting essentially through A1 receptors, adenosine modulates immature synaptic and network activity in different regions such as the rat hippocampus or developing cortex [9,43]. Given that the first synapses in the rat foetal brain are found at a rather early stage of cortical development: at ED16, and the presence of axo-somatic contacts on neurons situated above and below cortical plate could be found at ED18 and 19 [45], it is thus possible that VPA-induced elevation of A1 receptor could be responsible for the deregulation of signal transduction in those newly formed neuronal connections, leading to the abnormalities observed in the postnatal period. Opposite to A1 receptor, the data about the physiological or pathological functions of A2b and A3 receptor subtypes in neurogenesis are scarce. Also when compared to the high level of A1 and A2a receptor expression in the brain, the level of A2B and A3 receptors were found to be relatively low [71], therefore it is difficult to speculate about the significance of these changes in the context of ASD aetiology. Some data suggested the expression of A2b is significantly elevated in response to adverse conditions, including necrosis and inflammation [79], and lack of this receptor increases the tissue susceptibility to ischemic and

inflammatory injury [16,24,29]. Thus, the observed elevation of this receptor's level might be a non-specific protective response to VPA-induced damage. It was previously demonstrated that the activation of the A2b receptor induces a reversible increase in the distance between the centrosome and nucleus, thus reducing cell migration [58]. Moreover, adenosine acting through A2b was shown to increase the number of progenitor cells and stimulate nerve growth regeneration in adult zebrafish [63]. Those observations might provide cues to the potential role of A2b adenosinergic receptors in the deregulation of neuronal migration and neurogenesis after VPA exposure, but this interesting feature requires further extensive studies.

Among the purinergic P2 receptors or ATP, P2X2, P2X3, and P2X7 are the first expressed in neuronal precursors [14], suggesting their important role in the regulation of proliferation, migration, and differentiation of those progenitor cells. In the present study, we reported up-regulation of purinergic P2X2 and P2X3 ion channels in the foetal brain of VPA offspring along with the increased activity of these receptors. Previously, the elevated expression of P2X2 receptor in differentiating neuronal precursors was observed [68], and activation of this receptor was found crucial for the inhibition of progenitor cell proliferation and activation of their differentiation into neurons [80]. Apart from the direct role of P2X2 receptor in neuronal precursor specification, the possible indirect function of this receptor acting via intracellular calcium signalling in the regulation of neuritogenesis and outgrowth is possible [39,65,70,81]. P2X3 receptors were also found on the motor neurons and their outgrowing neurites suggesting P2X3 role in axon outgrowth and elongation [14,20]. Underscoring these crucial developmental roles, the present studies reveal that VPA-dependent up-regulation of P2X2/P2X3 receptors might affect calcium signalling in the foetal brain, which in turn might contribute to neuroanatomic abnormalities and synaptic dysfunction observed in the mature brain in adolescent offspring. P2X3 expression also appears early at embryonic development, but in contrast to other P2 receptors the peak level of those receptors in the brain is observed at ED11 and afterwards, their expression gradually declines in the stages that follow and reach the significant down-regulation in the neonatal rat brain [14,15]. This purinergic receptor abundance is mainly observed in the peripheral sensory neurons, which are considered as the ATP sensors since they are activated by nanomolar ATP concentrations [15,25]. Recent evidence suggests that P2X3 receptor function not only induces neuron depolarisation and neurotransmitter release, but it also triggers intracellular molecular changes like the regulation of neuronal plasticity and the release of neuromodulators [26]. Since P2X3 receptor is largely involved in the formation of proper sensory innervation, it is thus possible that the changes in this receptor level and activity observed upon VPA exposure in the foetal brain might have a substantial impact on deficits in somatosensory neurons functions that are indeed observed at the majority of ASD individuals, appearing as both hyper- and hypo-reactivity, with abnormal responses to tactile stimulation [75].

In contrast to P2X2 up-regulation with differentiation, the decreased expression of P2X7 and P2Y1 in embryonic rat telencephalon neural progenitor cells was observed upon their differentiation into neurons [72]. P2X7 and P2Y1 receptors are both expressed in the embryonic ventricular and subventricular zones, where P2Y1 activation mediates Ca^{2+} waves in radial glial cells, which are critically required to entrain ventricular zone cell proliferation [66] and P2X7 receptors are involved in the regulation of neural progenitor level via induction of the necrotic cell death [21]. P2Y1 receptor-mediated calcium signalling is also critical for cortex development, especially progenitor cell migration and their further differentiation into neurons and astrocytes [61]. P2Y1 also participates in neurite outgrowth [37], and also promotes axonal elongation [62], while P2X7-mediated Ca^{2+}-influx arrests growth cone motility and inhibits axonal extension [18,22,36], and this inhibitory effect of P2X7 in axonal outgrowth prevails over P2Y1 action. Accordingly, while P2Y1 receptor promotes neuroblast migration [12], the activation of P2X7 in neuroblasts induces the phagocytosis of surrounding apoptotic cells [48]. Since we have observed the significant down-regulation of P2Y1 and P2X7 along with the reduced activity of those receptors in the VPA embryonic brain, this might suggest the elevation in the neural progenitors level, together with the disturbances of the processes of migration and differentiation of neuronal precursors and dendritic and axonal formation. Furthermore, the observed downregulation of P2Y1 receptors in the VPA embryonic brain could suggest the deregulation of glutamate and GABA release, thereby altering neuronal excitability [23,41]. Since P2Y12 receptors are mainly localized on the microglia and macrophages that are not mature at the stage of E19, thus the expression of P2Y12 receptor is very low at this developmental period [67], it is difficult to speculate about the significance of this receptor down-regulation induced by VPA exposure. Undoubtedly, further studies regarding the unrevealed roles of P2Y12 receptor in ASD-related pathology are necessary to understand the meaning of this observation.

Summarizing, defects in the purinergic signalling pathway evoked by prenatal exposure to VPA might have a profound impact on brain development during

embryogenesis and on intellectual and behavioural functions after birth. Although our promising results showed activation of specific purinoceptors in the rat ASD model, we are only at the beginning of understanding the role of purinergic signalling in ASD pathology to ultimately establish P1 and/or P2 receptors as a possible therapeutic target in ASD.

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Disclosure

The authors report no conflict of interest.

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