

mgr. inż. Lidia Babiec

**Znaczenie sygnalizacji nukleotydowej
w regulacji struktury i funkcji synaps w zwierzęcym modelu autyzmu
indukowanym prenatalnym podaniem kwasu walproinowego**

Rozprawa na stopień naukowy doktora
w dziedzinie nauk medycznych i nauk o zdrowiu
w dyscyplinie nauki medyczne
w formie monotematycznego zbioru publikacji

Promotor: prof. dr hab. Agata Adamczyk

Promotor pomocniczy: dr Anna Wilkaniec

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Spis treści

1. Wykaz skrótów	- 5 -
2. Streszczenie w języku polskim	- 7 -
3. Streszczenie w języku angielskim	- 9 -
4. Innowacyjność rozprawy	- 11 -
5. Wstęp	- 12 -
5.1. Epidemiologia zaburzeń ze spektrum autyzmu	- 12 -
5.2. Obraz kliniczny zaburzeń ze spektrum autyzmu	- 13 -
5.3. Etiologia zaburzeń ze spektrum autyzmu	- 14 -
5.4. Sygnalizacja purynergiczna a zaburzenia ze spektrum autyzmu	- 18 -
6. Cel pracy	- 22 -
6.1. Cel ogólny	- 22 -
6.2. Cele szczegółowe	- 23 -
7. Metodyka	- 24 -
7.1. Zwierzęce modele zaburzeń ze spektrum autyzmu	- 24 -
7.1.1. Szczurzy model in vivo indukowany czynnikami środowiskowymi	- 24 -
7.1.2. Model stwardnienia guzowatego – myszy <i>Tsc2^{tm1Djk}/J</i>	- 26 -
7.2. Analiza zachowań o charakterze autystycznym u myszy <i>Tsc2^{tm1Djk}/J</i> i szczurów po prenatalnej ekspozycji na VPA	- 26 -
7.2.1. Test otwartego pola	- 26 -
7.2.2. Trójkomorowy test socjalizacji	- 27 -
7.2.3. Test rozpoznawania nowych obiektów	- 27 -
7.3. Metody analizy molekularnej i komórkowej	- 27 -
7.3.1. Pomiar ekspresji genów (q-PCR)	- 27 -
7.3.2. Analiza poziomu białka (Western blotting)	- 28 -
7.3.3. Analiza ultrastruktury komórek nerwowych z zastosowaniem transmisyjnej mikroskopii elektronowej	- 28 -
7.3.4. Analiza poziomu nukleotydów i adenozyiny w płynie mózgowo-rdzeniowym - (HPLC)	- 28 -
7.3.5. Analiza aktywacji receptorów purynergicznych poprzez pomiar napływu jonów wapnia (Ca^{2+}) do neuronów in vitro	- 29 -
7.3.6. Ocena poziomu wyselekcjonowanych receptorów purynergicznych w poszczególnych typach komórek OUN oraz morfologii komórek glejowych	- 29 -
7.4. Analiza statystyczna	- 29 -
9. Wnioski	- 36 -
10. Literatura	- 37 -
11. Publikacje wchodzące w skład rozprawy doktorskiej	- 50 -
11.1. Wskaźnik oddziaływania (IF) publikacji	- 50 -
11.2. Publikacja I	- 51 -
11.3. Publikacja II	- 67 -
11.4. Publikacja III	- 101 -
11.5. Publikacja IV	- 127 -
12. Oświadczenia współautorów	- 151 -

1. Wykaz skrótów

4E-BP1 – białko wiążące eukariotyczny czynnik inicjacji translacji 4E (ang. eukaryotic translation initiation factor 4E-binding protein 1)

Ada – deaminaza adenozyiny (ang. deaminase adenosine)

ADP – adenozyino-5'-difosforan (ang. adenosine diphosphate)

Akt – kinaza białkowa Akt (ang. protein kinase Akt)

AMP – adenozyino-5'-monofosforan (ang. adenosine monophosphate)

AMPK – kinaza białkowa aktywowana przez AMP (ang. 5'AMP-activated protein kinase)

AP – fosfatazy alkaliczne (ang. alkaline phosphatases)

ASD – zaburzenia ze spektrum autyzmu (ang. Autism Spectrum Disorder)

ATP – adenozyino-5'-trifosforan (ang. adenosine triphosphate)

CDC – Agencja Rządu Federalnego Stanów Zjednoczonych wchodząca w skład Departamentu Zdrowia i Opieki Społecznej (ang. Centers for Disease Control and Prevention)

DSM-5 – Kryteria Diagnostyczne Zaburzeń Psychiczych – wersja 5 (ang. Diagnostic and Statistical Manual of Mental Disorders – version 5)

eIF4E – eukariotyczny czynnik inicjujący translację 4E (ang. eukaryotic translation initiation factor 4E)

ENT – równoważny transporter nukleozydów (ang. equilibrative nucleoside transporter)

E-NPP – ekto-pirofosfatazy/fosfodiesterazy nukleotydów (ang. ecto-nucleotide pyrophosphatase/phosphodiesterase)

E-NTPD – ektofosfohydrolazy di- i trifosfonukleozydów (ang. ecto-nucleoside triphosphate diphosphohydrolase)

FXS – Zespół łamliwego chromosomu X (ang. Fragile X Syndrome)

ICD-11 – Międzynarodowa Statystyczna Klasyfikacja Chorób i Problemów Zdrowotnych – wersja 11 (ang. International Classification of Diseases – version 11)

IL-6 – interleukina 6 (ang. interleukin 6)

isoPPADS – 6-azofenylo-2',5'-disulfonowy fosforan pirydoksalu (ang. pyridoxalphosphate-6-azophenyl-2',5'-disulfonic acid)

JAK-STAT – szlak sygnalizacyjny JAK/STAT (ang. Janus kinase/signal transducer and activator of transcription)

mLST8 – cel podjednostki kompleksu rapamycyny LST8 (ang. target of rapamycin complex subunit LST8)

mRNA – matrycowy RNA (ang. messenger RNA)

mTOR – kinaza serynowo-treoninowa mTOR, ssaczy cel rapamycyny (ang. mechanistic/mammalian target of rapamycin)

mTORC1 – kompleks pierwszy kinazy mTOR (ang. mTOR complex 1)

mTORC2 – kompleks drugi kinazy mTOR (ang. mTOR complex 2)

NTPDaza – difosfohydrolaza trifosforanu nukleozydu (ang. nucleoside triphosphate diphosphohydrolase)

OUN – ośrodkowy układ nerwowy (ang. central nervous system, CNS)

P1 – receptory dla adenozyiny (ang. receptors for adenosine)

P2 – receptory dla nukleozydów (ang. receptors for nucleotides)

P2X – receptory jonotropowe P2X (ang. inotropic P2X receptors)

P2Y – receptory metabotropowe P2Y (ang. metabotropic P2Y receptors)

PI3K – kinaza 3-fosfatydylinozytolu (ang. phosphatidylinositol 3-kinase)

PPADS – 6-azofenylo-2',4'-disulfonowy fosforan pirydoksalu (ang. pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid)

PRAS40 – białko PRAS40 (ang. proline-rich Akt substrate of 40 kDa)

S6K – kinaza S6 (ang. S6 kinase)

SIN1 – białko SIN1 (ang. stress-activated protein kinase-interacting protein 1)

Tnfa – czynnik martwicy nowotworów (ang. tumor necrosis factor α)

TSC – kompleks TSC (TSC1/TSC2) (ang. Tuberous Sclerosis Complex)

UDP – urydyno-5'-difosforan (ang. uridine diphosphate)

UTP – urydyno-5'-trifosforan (ang. uridine triphosphate)

V-ATPaza – ATPaza pęcherzyków synaptycznych (ang. vesicular-type ATPase)

VNUT – pęcherzykowy transporter nukleotydów (ang. vesicular nucleotide transporter)

VPA – kwas walproinowy (ang. valproic acid)

WHO – Światowa Organizacja Zdrowia (ang. World Health Organization)

2. Streszczenie w języku polskim

Zaburzenia ze spektrum autyzmu (ASD) to grupa heterogennych zaburzeń neurorozwojowych dotyczących około 1% światowej populacji. Osoby z ASD mają trudności w komunikacji społecznej oraz wykazują powtarzalne wzorce zachowań. Etiologia ASD jest złożona i obejmuje zarówno czynniki środowiskowe, jak i genetyczne. Prenatalna ekspozycja na leki, np. walproinian sodu, jest jednym z czynników ryzyka. Podłoże genetyczne można zidentyfikować u 10-30% osób z ASD; większość związanych z nim genów wpływa na strukturę i funkcję synaps oraz szlaki sygnalizacji neuronalnej. Jednym z najczęstszych zaburzeń jednogenowych powiązanych z ASD jest stwardnienie guzowe (TSC), spowodowane mutacją genów *TSC1* lub *TSC2*, które hamują aktywność kinazy mTOR. U 26-50% pacjentów z TSC występują objawy behawioralne charakterystyczne dla ASD. Szlak mTOR reguluje translację i biosyntezę białek oraz autofagię; jego zaburzenia mogą prowadzić do nieprawidłowości w ekspresji białek synaptycznych oraz receptorów, co może powodować objawy ASD.

Sygnalizacja purynergiczna, zależna od zewnątrzkomórkowych nukleotydów, odgrywa kluczową rolę w regulacji struktury i funkcji synaps. W ośrodkowym układzie nerwowym (OUN), ATP i adenozyne są uwalniane z komórek neuronalnych i glejowych, zarówno w sposób regulowany, jak i niekontrolowany. ATP w szczelinie synaptycznej ulega degradacji przez ektonukleotydazy, kontrolując ilość biodostępnych agonistów aktywujących receptory purynergiczne (P1 dla adenozyne i P2 dla nukleotydów). Przekąźnictwo purynergiczne pośredniczy w komunikacji komórek OUN, regulując aktywację mikrogleju i uwalnianie cytokin prozapalnych. Ponadto, ATP może wzmacniać sygnalizację związaną z kinazą mTOR.

Do chwili obecnej nie ma kompleksowej analizy interakcji pomiędzy zaburzeniami sygnalizacji purynergicznej, szlakiem mTOR i poziomem białek synaptycznych w ASD. Dlatego celem niniejszej rozprawy doktorskiej było zbadanie udziału deregulacji przekąźnictwa purynergicznego w zależnych od mTOR zmianach kluczowych białek synaptycznych i rozwoju zachowań typowych dla ASD. Badania prowadzone były z wykorzystaniem dwóch modeli zaburzeń ze spektrum autyzmu: środowiskowego, gdzie ASD indukowane były prenatalną ekspozycją na kwas walproinowy (VPA) i genetycznego, u myszy z częściowym wyciszeniem genu *Tsc2*.

Otrzymane wyniki pokazują, że prenatalna ekspozycja na VPA prowadzi do deregulacji sygnalizacji purynergicznej już na wczesnym etapie rozwoju embrionalnego, w fazie

formowania się połączeń synaptycznych. W mózgu zwierząt w 19. dniu embrionalnym wykryto wzrost sygnalizacji zależnej od adenozyyny oraz zaburzenie homeostazy wapniowej wynikające z nadekspresji kanałów jonowych P2X2/P2X3 i obniżenia poziomu receptorów P2Y1 i P2Y12, co może przyczyniać się do wytworzenia nieprawidłowości neuroanatomicznych w mózgu w okresie postnatalnym. W hipokampie 52-dniowych zwierząt VPA zaobserwowano wzrost poziomu receptora P2X1 przy jednoczesnym spadku ekspresji P2X7 i P2Y1. Natomiast w korze mózgowej poziom P2X1 był znacząco obniżony. W tej strukturze zaobserwowano również znaczny wzrost poziomu receptorów P2Y1 i P2Y12. Powyższe zmiany mogą skutkować upośledzeniem zdolności poznawczych, zaburzeniami w regulacji długotrwałego wzmocnienia synaptycznego oraz aktywacją procesów zapalnych. Prenatalna ekspozycja na VPA wpływa nie tylko na ekspresję wybranych podtypów receptorów purynergicznych, ale wywołuje zmiany procesów odpowiedzialnych za regulację poziomu zewnątrzkomórkowych nukleotydów. W korze, hipokampie i mózdzku zwierząt badanych zaobserwowano wzrost ekspresji białek zaangażowanych w uwalnianie ATP oraz zwiększenie poziomu ATP, ADP i AMP w płynie mózgowo-rdzeniowym.

Obserwowane zaburzenia przekąźnictwa purynergicznego wpływają na aktywację mikrogleju w korze mózgowej i wzrost ekspresji cytokin: $Tnf\alpha$ i IL-6 oraz powodują aktywację szlaku kinazy mTOR. Badania prowadzone na modelu myszy z częściowym wyciszeniem genu *Tsc2* wykazały, że nadmierna aktywacja mTOR prowadzi do zmian anatomicznych mózgu, zaburzenia struktury synaps oraz szeregu innych nieprawidłowości ultrastrukturalnych w komórkach OUN. Ponadto, u zwierząt transgenicznych zaobserwowano zmiany poziomu kluczowych białek synaptycznych odpowiedzialnych za regulację procesu synaptogenezy oraz homeostazę pęcherzyków synaptycznych. Zastosowanie antagonistów receptorów P2 w modelu VPA normalizowało zmiany w ekspresji białek synaptycznych oraz zapobiegało zmianom behawioralnym, takim jak nadmierna lękliwość, występowanie zachowań repetytywnego oraz zmniejszenie aktywności eksploracyjnej.

3. Streszczenie w języku angielskim

Autism Spectrum Disorders (ASD) are a group of heterogeneous neurodevelopmental disorders affecting approximately 1% of the global population. Individuals with ASD have difficulties with social communication and exhibit repetitive behavior. The etiology of ASD is complex, involving both environmental and genetic factors. Prenatal exposure to drugs, such as valproic acid, is one of the risk factors for ASD. Genetic background can be identified in 10-30% of autistic individuals, with most associated genes influencing the structure and function of synapses and neuronal signaling pathways.

One of the most common monogenic disorders associated with ASD is the tuberous sclerosis complex (TSC), caused by mutations in the *TSC1* or *TSC2* genes, which inhibit the activity of the mTOR kinase. In 26-50% of TSC patients, behavioral symptoms characteristic of ASD are present. The mTOR pathway regulates translation, protein biosynthesis, and autophagy; its dysregulation can lead to abnormalities in the expression of synaptic proteins and receptors, potentially causing ASD symptoms.

Purinergic signaling, dependent on extracellular nucleotides, plays a crucial role in regulating the structure and function of synapses. In the central nervous system (CNS), ATP and adenosine are released from neuronal and glial cells both in a regulated and uncontrolled manner. Synaptic ATP is degraded by ectonucleotidases, controlling the amount of bioavailable agonists that activate purinergic receptors (P1 for adenosine and P2 for nucleotides). Purinergic signaling mediates communication between CNS cells, regulating microglial activation and the release of pro-inflammatory cytokines. Additionally, ATP can enhance mTOR pathway signaling.

There has been no comprehensive analysis of the interactions between purinergic signaling dysregulation, the mTOR pathway, and synaptic protein levels in ASD. Therefore, this doctoral dissertation aimed to investigate the involvement of purinergic signaling deregulation in mTOR-dependent alterations of key synaptic proteins and the development of ASD-like behaviors. The research was conducted using two ASD models: an environmental model, where ASD was induced by prenatal exposure to valproic acid (VPA), and a genetic model with *Tsc2* gene knockdown mice.

The results indicate that prenatal exposure to VPA leads to purinergic signaling dysregulation during synapse formation occurring at an early embryonic development. In the brains of embryos at day 19, an increase in adenosine-dependent signaling and calcium homeostasis disruption was detected due to overexpression of P2X2/P2X3 ion channels and

decreased levels of P2Y1 and P2Y12 receptors. This may contribute to neuroanatomical abnormalities in the postnatal brain. In the hippocampus of 52-day-old VPA animals, an increase in P2X1 receptor levels was observed alongside a decrease in P2X7 and P2Y1 expression. Conversely, P2X1 levels in the cortex were significantly reduced, with a significant increase in P2Y1 and P2Y12 receptors. These changes may impair cognitive abilities, disrupt long-term synaptic potentiation regulation, and activate inflammatory processes.

Prenatal VPA exposure affects not only the expression of specific purinergic receptor subtypes but also processes regulating extracellular nucleotide levels. In the cortex, hippocampus, and cerebellum of experimental animals, an increase in proteins involved in ATP release and elevated levels of ATP, ADP, and AMP in cerebrospinal fluid were observed.

Purinergic signaling disturbances affect microglial activation in the cortex and increase expression of cytokines: $Tnf\alpha$ and IL-6, leading to mTOR pathway activation. Studies in *Tsc2* knockdown mice indicated that excessive mTOR activation results in brain anatomical changes, synapse structure disruption, and other ultrastructural abnormalities in CNS cells. Additionally, transgenic animals showed altered levels of key synaptic proteins responsible for regulating synaptogenesis and synaptic vesicle homeostasis. P2 receptor antagonists normalized changes in synaptic protein expression and prevented behavioral alterations such as excessive anxiety, repetitive behavior, and reduced exploratory activity in the VPA animal model.

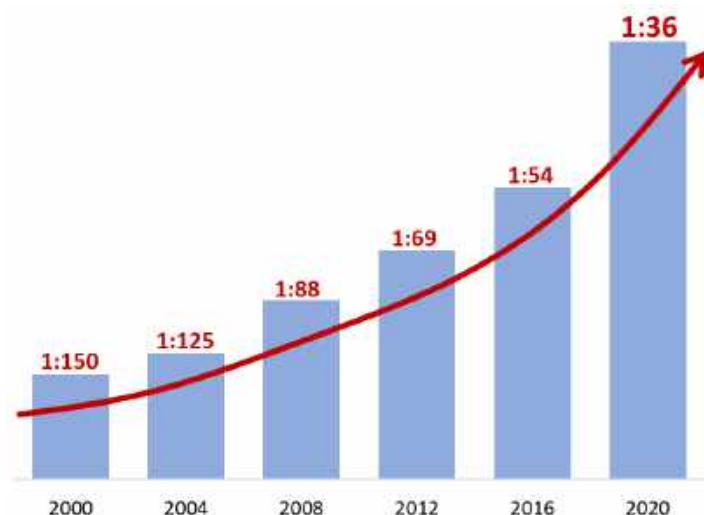
4. Innowacyjność rozprawy

- I. Deregulacja sygnalizacji purynergiczej na poziomie metabolizmu zewnątrzkomórkowych nukleotydów oraz ich receptorów w przedklinicznym modelu zaburzeń ze spektrum autyzmu (ASD).
- II. Aktywacja mikrogleju i szlaku kinazy mTOR, zależna od pobudzenia sygnalizacji purynergiczej, w zwierzęcym modelu ASD.
- III. Zmiany proteostazy i struktury synaps wywołane aktywacją sygnalizacji nukleotydowej w modelu ASD.
- IV. Zaburzenia synaptyczne spowodowane nieprawidłowym funkcjonowaniem przekąźnictwa purynergicznego, odpowiedzialne za zmiany behawioralne typowe dla autyzmu.

5. Wstęp

5.1. Epidemiologia zaburzeń ze spektrum autyzmu

Zaburzenia ze spektrum autyzmu (ang. Autism Spectrum Disorders, ASD) to grupa heterogennych nieprawidłowości neurorozwojowych, które dotyczą około 75 milionów ludzi na świecie, co stanowi blisko 1% populacji [1]. Termin „autyzm” (z gr. *autós* oznacza „sam”) został po raz pierwszy wprowadzony do psychiatrii przez szwajcarskiego lekarza Paula Eugen Bleulera w 1911 roku, aby opisać jeden z kluczowych objawów schizofrenii, jakim jest zamykanie się w sobie i brak reakcji na bodźce zewnętrzne. Austriacki psychiatra Leo Kanner wyodrębnił autyzm jako osobną kategorię diagnostyczną w 1943 roku. Przez dziesięciolecia trwały spory i kontrowersje dotyczące terminologii i klasyfikacji zaburzeń autystycznych. Brytyjska psychiatra Lorna Wing przyczyniła się do rozwiązania tych kwestii, proponując pod koniec lat 70. XX wieku termin „spektrum autystyczne”. Podkreśliła w ten sposób, że objawy autyzmu mogą mieć różny stopień nasilenia, a trudności z nimi związane można rozpatrywać jako zjawisko ciągłe [2–4]. Ostatecznie, w 2000 roku, przyjęto termin „zaburzenia ze spektrum autyzmu”, obejmujący zaburzenia autystyczne, zespół Aspergera, inne całościowe zaburzenia rozwoju oraz autyzm atypowy [5]. Z biegiem lat pojawiały się kolejne systematyzacje i kryteria diagnostyczne ASD. Obecnie na świecie obowiązują dwie klasyfikacje diagnostyczne pozwalające na rozpoznanie ASD: DSM-5 (ang. Diagnostic and Statistical Manual of Mental Disorders – version 5) opracowana przez Amerykańskie Towarzystwo Psychiatryczne [6] oraz Międzynarodowa Statystyczna Klasyfikacja Chorób i Problemów Zdrowotnych ICD-11 (ang. International Classification of Diseases – version 11) przygotowana przez Światową Organizację Zdrowia (ang. World Health Organization, WHO) [7], która weszła w życie 1 stycznia 2022 roku [8,9]. W ostatnich latach ASD diagnozowane są coraz częściej, od 2000 roku częstość występowania autyzmu wzrosła o ponad 300% (**Rycina 1**).



Rycina 1. Częstość występowania ASD w Stanach Zjednoczonych Ameryki Północnej.

Dane pochodzą z Centrum Kontroli i Prewencji Chorób (CDC) w Stanach Zjednoczonych (na podst. <https://tacanow.org/press-release/autism-prevalence-is-now-1-in-36/>; 04.06.2024).

Raport Agencji Rządu Federalnego Stanów Zjednoczonych Ameryki ds. prewencji i kontroli chorób (ang. Centers for Disease Control and Prevention, CDC) pochodzący z 2023 roku pokazuje, że autyzm występuje u 1 na 36 dzieci w wieku 8 lat, co z podziałem na płeć stanowi 4% chłopców i 1% dziewczynek w danym przedziale wiekowym [10]. W Europie autyzm diagnozuje się u około 1 na 100 dzieci. W Polsce nie prowadzono dotąd badań epidemiologicznych dotyczących ASD. Dane pochodzące z Systemu Informacji Oświatowej wskazują, że w roku szkolnym 2018/19 ponad 54 tys. dzieci z diagnozą spektrum autyzmu korzystało z kształcenia specjalnego, co oznacza 1 na 115 uczniów [11]. Jednak nie wszystkie dzieci z ASD posiadają orzeczenie o niepełnosprawności, przytoczone dane nie uwzględniają także osób dorosłych, dlatego faktyczna liczba ludzi w Polsce dotkniętych tym zaburzeniem nie jest w pełni określona i często przyjmuje się za reprezentatywne dane z badań uzyskanych w innych krajach Europy.

5.2. Obraz kliniczny zaburzeń ze spektrum autyzmu

Chociaż w ASD nie można mówić o jednym specyficznym fenotypie, istnieją charakterystyczne dla tego zaburzenia objawy, przede wszystkim trudności w interakcjach społecznych oraz w komunikacji werbalnej i niewerbalnej, a także skłonność do sztywnych, powtarzalnych wzorców zachowania [6,12]. Problemy w zakresie komunikacji i interakcji społecznych obejmują trudności w nawiązywaniu i utrzymywaniu relacji międzyludzkich, dzieci w spektrum autyzmu nie mają zdrowych, naprzemiennych kontaktów z rówieśnikami, w wielu przypadkach mają trudności ze zrozumieniem zabaw symbolicznych, naśladujących role społeczne. Cechuje je przywiązanie do codziennej rutyny i trudności z akceptowaniem

zmian w swoim otoczeniu. Zaburzenia komunikacji niewerbalnej u dzieci z diagnozą ASD często objawiają się jako nieumiejętność utrzymywania kontaktu wzrokowego oraz rozpoznawania emocji z mimiki twarzy [6,7]. Należy podkreślić, że osoby w spektrum autyzmu mogą prezentować różnorodny wzorec behawioralny oraz stopień nasilenia objawów, od niemal niezauważalnych aż do pełnej niepełnosprawności intelektualnej, która obecnie dotyka ok. 30-50% pacjentów z ASD [13].

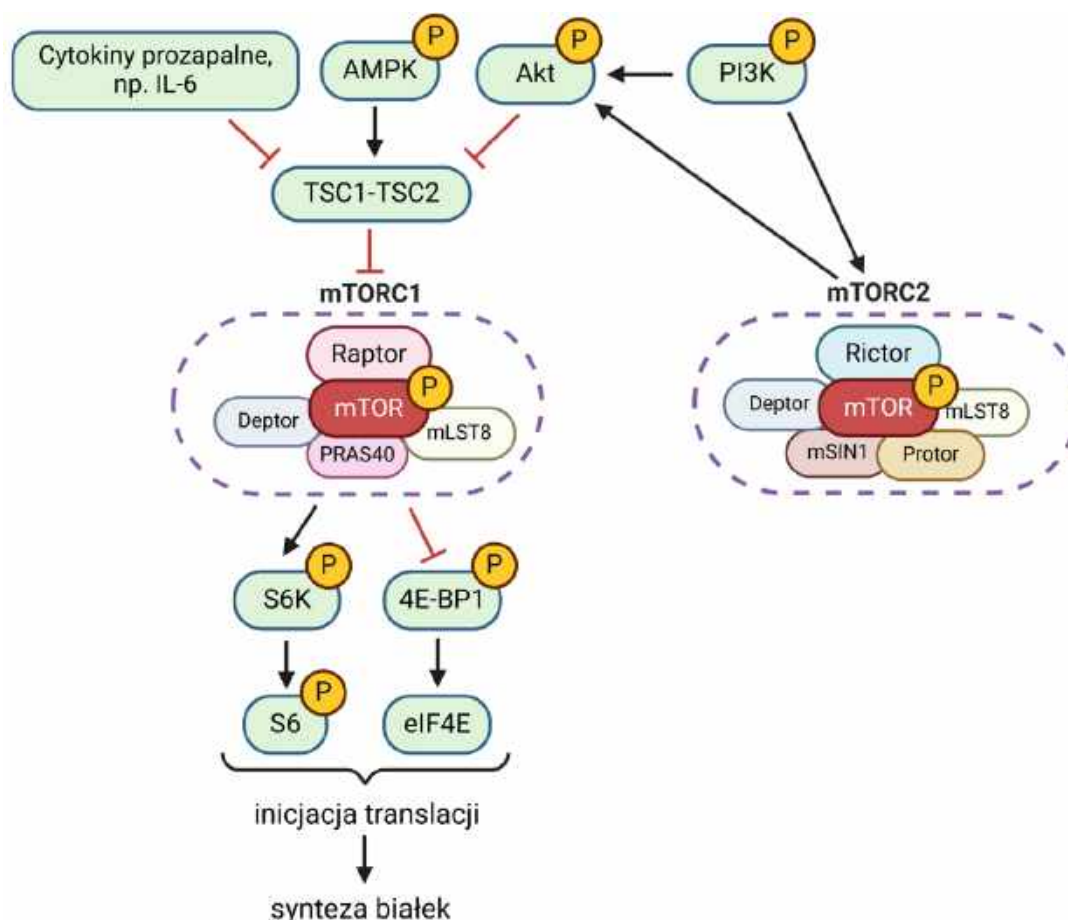
5.3. Etiologia zaburzeń ze spektrum autyzmu

Pomimo intensywnych badań prowadzonych w wielu ośrodkach naukowych na świecie, mechanizm powstawania ASD pozostaje w dużej mierze niewyjaśniony, wiadomo jednak, że w etiologii zaburzeń autystycznych istotną rolę pełnią zarówno czynniki środowiskowe, jak i genetyczne oraz interakcje pomiędzy nimi [14–17]. Najważniejsze czynniki środowiskowe to między innymi ekspozycja na zanieczyszczenie powietrza [18], wpływ metali ciężkich [19] czy plastyfikatorów i zmiękczaczy uwalnianych z powszechnie stosowanych tworzyw sztucznych [20], a także infekcje bakteryjne lub wirusowe matki na wczesnych etapach ciąży [21,22]. Jednym z najpoważniejszych zagrożeń dla rozwoju płodu są niektóre leki przyjmowane przez ciężarne, np. talidomid, mizoprostol [23,24], czy walproinian sodu, silny lek na padaczkę, który wywołuje u potomstwa zaburzenia zachowania typowe dla autyzmu [25]. Najnowsze badania retrospektywne przeprowadzone w krajach skandynawskich – Danii, Szwecji i Norwegii, wskazują na zwiększone ryzyko wystąpienia zaburzeń autystycznych u dzieci, których ojcowie byli leczeni walproinianem w ciągu trzech miesięcy przed poczęciem dziecka [26]. Z kolei udział podłoża genetycznego w rozwoju spektrum autyzmu potwierdza wysoka zgodność występowania ASD u bliźniąt jednojajowych (60–90%) w porównaniu z dwujajowymi (0–20%). I chociaż przyczynę genetyczną udaje się ustalić jedynie u 10–30% osób z ASD, a u 70–90% dzieci przyczyny ASD pozostają nieznane [27], to odkrycie genetycznych czynników zaburzeń autystycznych i badanie ścieżek, w których zaangażowane są dane geny poszerza wiedzę na temat molekularnych mechanizmów regulujących rozwój społeczny i poznawczy [28]. Znaczna większość genów, w których mutacje stanowią wysokie ryzyko wystąpienia ASD, biorą udział w regulacji struktury i funkcji synaps (*NRXN*, *NLGN*, *SHANK*) oraz szlaków sygnalizacji neuronalnej (*GluN2B*, *CACNA1C*, *SCN1A*) [29–31].

Jednym z najczęstszych zaburzeń jednogenowych, poza zespołem łamliwego chromosomu X (ang. Fragile X Syndrome, FXS), w którym wiodącym objawem jest autyzm, jest stwardnienie guzowate (ang. Tuberous Sclerosis Complex, TSC) [32,33]. Częstość

występowania zaburzeń autystycznych w TSC waha się od 26% do 50% [34,35]. Choroba ta spowodowana jest mutacją jednego z dwóch genów: *TSC1* na chromosomie 9 (9q34) lub *TSC2* na chromosomie 16 (16p13.3) [36,37]. W obu genach opisano ponad 400 różnych mutacji. Gen *TSC1* koduje białko hamartynę, a gen *TSC2* tuberynę. Białka te tworzą kompleks hamujący aktywność kinazy serynowo-treoninowej (ang. mammalian target of rapamycin, mTOR; **Rycina 2**), która poprzez swoje białka efektorowe, kinazę białkową S6 (S6K) oraz białko wiążące eukariotyczny czynnik inicjacji translacji 4E (4E-BP1) reguluje proces translacji i biosyntezy białka. Brak aktywnego kompleksu tuberyna-hamartyna powoduje aktywację szlaku mTOR. W wyniku fosforylacji 4E-BP1 przez mTOR, dochodzi do uwolnienia eukariotycznego czynnika inicjującego translację 4E (ang. eukaryotic translation initiation factor 4E, eIF4E), co uruchamia proces zależnej od kapu (koniec 5' eukariotycznego mRNA) translacji (ang. cap-dependent translation), która obejmuje około 95% komórkowego mRNA [38]. Inne czynniki inicjacji translacji, takie jak eIF3 oraz eIF4B są również kontrolowane przez ścieżkę sygnałową zależną od mTOR [39].

Kompleks TSC1-TSC2 jest regulowany potranslacyjnie przez kilka szlaków sygnałowych, m.in. przez szlak kinazy 3-fosfatydylinozytolu (PI3K) i Akt oraz szlak kinazy białkowej aktywowanej przez AMP (AMPK). Szlak PI3K/Akt negatywnie reguluje aktywność kompleksu TSC1-TSC2 przez fosforylację białka TSC2 [40,41] prowadząc do aktywacji kinazy mTOR. Natomiast podczas niekorzystnego stanu energetycznego komórki, szlak AMPK aktywuje TSC2, tym samym hamując działanie mTORC1 i aktywność mTOR [41,42].



Rycina 2. Schemat ścieżki sygnalizacyjnej kinazy mTOR.

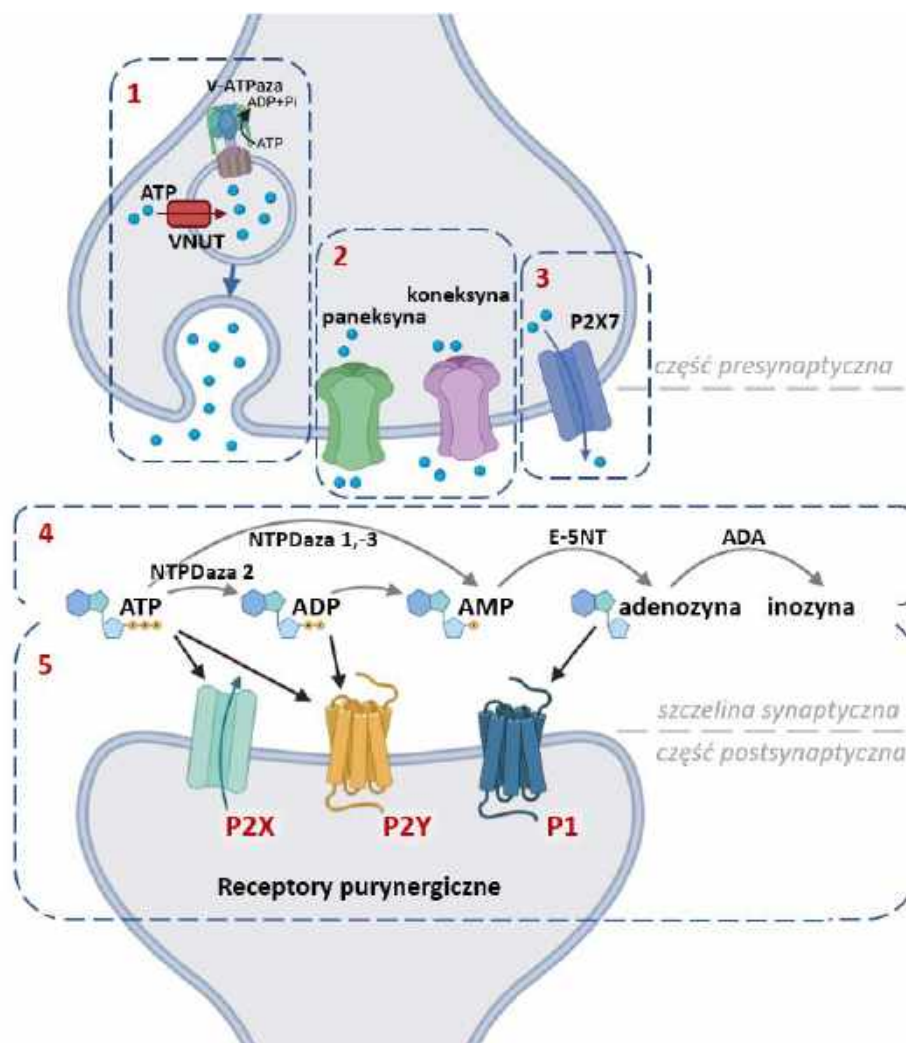
Kinaza mTOR tworzy dwa heteromeryczne, funkcjonalnie niezależne kompleksy białkowe, mTORC1 i mTORC2. Kluczowymi białkami regulatorowymi tych kompleksów są odpowiednio Raptor i Rictor. Poza kinazą mTOR, stałym składnikiem obu kompleksów jest białko stabilizujące strukturę, mLST8, oraz Deptor, który działa jako ich inhibitor. Specyficzne dla mTORC1 jest białko PRAS40, pełniące rolę inhibitora kompleksu, natomiast dla mTORC2 - czynnik SIN1, zapewniający integralność kompleksu, oraz Protor, wspierający funkcję białka Rictor. Kompleksy mTORC1 i mTORC2 pełnią odmienne role w komórce i są regulowane przez różne szlaki sygnalizacyjne. Aktywacja kompleksu mTORC1 polega na wyłączeniu jego negatywnego regulatora, heterodimeru TSC1-TSC2. Jednym z kluczowych szlaków sygnalizacyjnych zwiększających aktywność mTORC1 jest szlak PI3K/Akt. Zależna od PI3K oraz kinazy Akt fosforylacja TSC2 prowadzi do zahamowania aktywności heterodimeru TSC1-TSC2, aktywacji kompleksu mTORC1 i wzrostu aktywności kinazy mTOR. Do aktywacji kinazy mTOR poprzez inhibicję kompleksu TSC1-TSC2 mogą prowadzić również cytokiny prozapalne, takie jak IL-6. Aktywna mTOR poprzez fosforylację 4E-BP1 oraz S6K, inicjuje translację i biosyntezę białek. Przeciwną funkcję do kinaz PI3K/Akt, w regulacji aktywności mTOR pełni AMPK, która jest aktywatorem kompleksu TSC1-TSC2, co prowadzi do hamowania aktywności kompleksu mTORC1 i kinazy mTOR. W regulacji mTORC1 bierze również udział kompleks mTORC2, który jest aktywowany przez szlak PI3K i poprzez fosforylację Akt na serynie 473 (Ser473) i jej aktywację, stymuluje aktywność mTORC1 i kinazy mTOR. Na rycinie przedstawiono jedynie wybrane elementy wieloetapowej i złożonej regulacji kinazy mTOR przez kluczowe ścieżki sygnalizacyjne, które były przedmiotem badań w niniejszej rozprawie doktorskiej. Schemat został zaprojektowany z użyciem programu BioRender do tworzenia ilustracji i grafik naukowych.

W 2013 roku Santini i współpracownicy wysnuli hipotezę, iż jednym z mechanizmów leżących u podłoża zaburzeń autystycznych jest wzrost poziomu translacji zależnej od kapu, co może powodować zaburzenie struktury i funkcji synaps, a w konsekwencji nieprawidłowości w funkcjonowaniu całych obwodów neuronalnych i występowanie zachowań typowych dla autyzmu [43]. Ponadto, zależne od mTOR zmiany aktywności S6K mogą prowadzić do nieprawidłowości w poziomach synaptycznych białek strukturalnych i receptorowych [44]. Z drugiej zaś strony, nadmierna aktywacja mTOR może prowadzić do zahamowania autofagii, a w konsekwencji do akumulacji uszkodzonych białek [45,46], a jak wykazują poprzednie badania, nawet subtelne zmiany w proteomie prowadzić mogą do poważnych zaburzeń funkcji synaptycznych [47]. Zmiany zarówno w architekturze jak i funkcji synaps są cechą charakterystyczną dla ASD [48,49]; u pacjentów wykryto zaburzenie ekspresji białek odpowiedzialnych za prawidłową strukturę synaps, tj. neureksyn i neuroligin oraz białek należących do kompleksu SNARE (SNAP-25, syntaksyna, synaptobrewina) odpowiedzialnych za transport i uwalnianie pęcherzyków synaptycznych [50–59]. Wykazano również zmiany na poziomie morfologicznym, zaobserwowano istotne zwiększenie gęstości kolców dendrytycznych w porównaniu z grupą kontrolną [46,60,61]. Wysoka gęstość synaptyczna najczęściej występuje u pacjentów z ASD o niższym poziomie funkcjonowania poznawczego i powoduje zwiększenie intensywności przekazywania sygnałów, tzw. hiperłączości obserwowanej na poziomie całego mózgu, zarówno w połączeniach długozasięgowych, jak i krótkiego zasięgu, i jest związana z wyższym poziomem oscylacji sygnałów w poszczególnych regionach mózgu, co w znacznej mierze przyczynia się do występowania poważnych dysfunkcji behawioralnych [62–64]. Ponadto, w spektrum autyzmu często dochodzi do zaburzenia plastyczności synaptycznej, co może mieć swoje odzwierciedlenie w zmianach behawioralnych i skutkować upośledzeniem umiejętności komunikacji i interakcji społecznych, występowaniem zachowań repetytywnych oraz zwiększonym poziomem lęku [65,66]. Wykazano, że aktywność mTOR może regulować gęstość synaps pobudzających, co może leżeć u podstaw zakłócenia równowagi sygnałów pobudzająco-hamujących w mózgu osób z ASD [49,67]. Zatem, deregulacja sygnalizacji zależnej od kinazy mTOR może przyczyniać się do rozwoju zaburzeń ze spektrum autyzmu. Wyniki badań z zastosowaniem inhibitorów szlaku mTOR dają nadzieję na możliwość leczenia przyczynowego zaburzeń neuro-behawioralnych występujących w chorobach związanych z mutacjami genów zaburzającymi prawidłowe jego funkcjonowanie [68–71]. Do chwili obecnej niezbadaną i niewyjaśnioną

jednak kwestią pozostawało, czy deregulacja szlaku mTOR występuje w przypadkach ASD, które nie są związane z mutacjami w genach *TSC1/2* regulujących ten szlak.

5.4. Sygnalizacja purynergiczna a zaburzenia ze spektrum autyzmu

Istotną rolę w regulacji struktury i funkcji synaps, a przede wszystkim w procesach plastyczności synaptycznej pełni sygnalizacja purynergiczna zależna od zewnątrzkomórkowych nukleotydów (głównie adenozyno-5'-trifosforanu, ATP) i nukleozydów (adenozyny). Koncepcja, w której ATP jest nie tylko nośnikiem energii, ale również neuroprzekaznikiem została przedstawiona w roku 1972 przez brytyjskiego badacza Geoffreya Burnstocka [72]. W ośrodkowym układzie nerwowym (OUN) ATP i adenozyna są uwalniane zarówno z części presynaptycznej, jak i postsynaptycznej neuronów, a także z komórek glejowych. Regulowana sekrecja ATP odbywa się na drodze transportu przez pory lub kanały błonowe, w tym kanały paneksynowe (paneksyny) i koneksynowe (koneksyny), albo z udziałem swoistych przenośników błonowych, takich jak białka mające kasetę wiążącą ATP (ABC transporters), receptory P2X7 lub w procesie egzocytozy pęcherzyków synaptycznych, do których ATP jest transportowany przy udziale pęcherzykowego transportera nukleotydów (ang. vesicular nucleotide transporter, VNUT; **Rycina 3**) [73–75]. Z kolei, adenozyna uwalniana jest z komórki przez transportery nukleozydów, głównie przez równoważny transporter nukleozydów (ang. equilibrative nucleoside transporter, ENT) [76,77]. Jednak głównym źródłem adenozyiny w przestrzeni zewnątrzkomórkowej jest degradacja ATP [78]. Uwalnianie puryn może też zachodzić w sposób niekontrolowany w odpowiedzi na uszkodzenie komórki. W szczelinie synaptycznej, ATP ulega sekwencyjnej, enzymatycznej degradacji przy udziale ektonukleotydaz. Wyróżnia się 4 rodziny ektonukleotydaz: ektodifosfohydrolazy trifosfonukleozydów (E-NTPD), ekto-5'-nukleotydazy (E-5NT), pirofosfatazy/fosfodiesterazy ektonukleotydów (E-NPP) oraz fosfatazy alkaliczne (AP), ale tylko dwie pierwsze grupy są specyficzne wyłącznie dla nukleotydów [79]. ATP i ADP są hydrolizowane przy udziale E-NTPD oraz E-NPP do monofosforanu AMP, który z kolei przy udziale ekto-5'-nukleotydazy przekształcany jest do adenozyiny. Za hydrolizę adenozyiny odpowiada następnie deaminaza adenozyiny [79–83]



Rycina 3. Schemat sygnalizacji zależnej od zewnątrzkomórkowych nukleotydów i adenozyiny. Uwalnianie ATP z neuronów oraz z komórek glejowych do szczeliny synaptycznej jest kluczowym procesem w sygnalizacji nukleotydydowej i może zachodzić poprzez kilka mechanizmów: **(1)** na drodze egzocytozy zależnej od pęcherzyków synaptycznych, gdzie transport ATP do wnętrza pęcherzyka odbywa się przy udziale pęcherzykowego transportera nukleotydydów (VNUT), który wykorzystuje gradient protonowy (H^+) generowany przez aktywność pompy protonowej (V-ATPazy), następnie w odpowiedzi na potencjał czynnościowy następuje fuzja pęcherzyków z błoną synaptyczną i uwolnienie ATP; **(2)** poprzez kanały paneksynowe (paneksyny) i koneksynowe (koneksyny); **(3)** przez kanały jonowe P2X7. W szczelinie synaptycznej ATP ulega degradacji przy udziale ektonukleotydz **(4)**. ATP i ADP są hydrolizowane przez difosfohydrolazy trifosforanu nukleotydydów (NTPDaza 1, -2, -3) do AMP, który następnie jest przekształcany do adenozyiny przy udziale ekto-5'-nukleotydyazy (E-5NT). Adenozyina jest dalej hydrolizowana przez deaminazę adenozyiny (ADA). ATP i produkty jego metabolizmu aktywują receptory purynergiczne na błonie postsynaptycznej **(5)**. Receptory P2X, będące kanałami jonowymi, reagują głównie na ATP. Receptory P2Y, sprzężone z białkami G, reagują na nukleotydy purynowe (ATP, ADP) oraz pirymidynowe (UTP, UDP). Receptory P1 są specyficzne dla adenozyiny. Schemat został zaprojektowany z użyciem programu BioRender do tworzenia ilustracji i grafik naukowych.

Aktywność ektonukleotydaz jest istotnym elementem sygnalizacji purynergiczej, gdyż kontroluje ilość biodostępnych agonistów, które aktywują znajdujące się na błonie komórkowej receptory purynergiczne [79–82]. W kontekście receptorów purynergiczych należy jednak pamiętać o roli pirymidyn, wśród których znajdują się takie nukleotydy jak UTP i UDP, które również mogą oddziaływać z receptorami purynergicznymi, szczególnie receptorami P2Y. Termin "receptory purynergiczne" obejmuje zatem receptory dla puryn oraz receptory reagujące na nukleotydy pirymidynowe. Wśród purynoreceptorów wyróżnia się dwie rodziny: receptory P1 – należące do grupy receptorów metabotropowych, stymulowane przez adenozyne; oraz P2 – receptory dla nukleotydów. Wśród rodziny receptorów P2 wyodrębniono, dwie podrodziny: metabotropowe receptory P2Y oraz receptory P2X pełniące rolę kanałów jonowych, przepuszczalnych dla jonów wapnia, sodu i potasu. Receptory P2X są odpowiedzialne za szybkie przekazywanie sygnału na krótkich dystansach, i z wyjątkiem receptora P2X7, wykazują stosunkowo niskie powinowactwo do ATP (stężenie rzędu kilku μM) [84,85]. Ich czas aktywacji wynosi kilka milisekund, a związanie ATP znajdującego się w szczelinie synaptycznej jest bezpośrednio związane z otwarciem tych kanałów i napływem do wnętrza komórki jonów Na^+ i Ca^{2+} oraz z depolaryzacją błony komórkowej. Depolaryzacja aktywuje kanały wapniowe bramkowane napięciem, ułatwiając dalszy napływ jonów Ca^{2+} i wzmagając depolaryzację. Receptory P2Y są najliczniejszą grupą receptorów nukleotydowych i są zależne od białek G, które przekazują sygnał na kolejne nośniki, wyzwalając kaskadę oddziaływań we wnętrzu komórki i wyrzut jonów wapnia z wewnątrzkomórkowych magazynów oraz przekazywanie informacji aż do jądra komórkowego. Dlatego jest to przekazywanie wolniejsze, za to propagacja sygnału następuje na większych odległościach [86,87]. Do tej pory odkryto i scharakteryzowano cztery podtypy receptorów P1 (A1, A2A, A2B, A3), siedem podtypów receptorów P2X (P2X1-7) i osiem podtypów receptorów P2Y (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, P2Y14) [75,88–90]. Oprócz neuronów, receptory purynergiczne są obecne również w mikrogleju, astrocytach i oligodendrocytach. Każdy z tych typów komórek posiada unikalny wzorzec ekspresji receptorów dla nukleotydów, co jest związane z ich różną funkcją biologiczną [91]. Ze względu na uniwersalny charakter przekazywania purynergicznego zapewnia ono interakcję pomiędzy różnymi typami komórek, w tym w sygnalizacji między komórkami glejowymi a neuronami, w obrębie synaps, ale również poza nimi [75]. Wykazano, że uwalnianie ATP i adenozyne przez neurony podczas aktywacji potencjału czynnościowego wywołuje fale wapniowe w astrocytach, komórkach Schwanna i oligodendrocytach, co stanowi jeden z mechanizmów wykrywania aktywności neuronalnej

przez te komórki glejowe [92,93]. ATP reguluje również aktywność mikrogleju, prowadząc do zmian w morfologii i migracji tych komórek [94,95]. Przekaznictwo zależne od ATP i adenozyiny pełni także kluczową rolę w dwukierunkowej komunikacji między mikroglejem a neuronami, jest odpowiedzialne za regulację wydzielania cytokin [96] oraz umożliwia dalsze propagowanie sygnału pochodzącego od neuronów przez sieć komórek glejowych. Jest to niezwykle istotne, ponieważ komórki mikrogleju nie tylko są odpowiedzialne za regulację zmian zapalnych w mózgu, ale również uczestniczą w procesach synaptogenezy, plastyczności synaptycznej i neurotransmisji [97], przez co mogą wpływać na funkcje synaptyczne w miejscach odległych od sygnału inicjującego [75,98–100]. Niektóre dane kliniczne wskazują, że dysfunkcja komórek glejowych jest zaangażowana w procesy patologiczne, które mogą prowadzić do zaburzeń ze spektrum autyzmu [101], a metabolizm puryn w komórkach glejowych może być jednym z najbardziej zaburzonych szlaków w autyzmie [102]. Jednakże mimo szczegółowej wiedzy na temat funkcji komórek glejowych w ASD, nie wiadomo czy deregulacja receptorów purynergiczných bierze udział w nieprawidłowościach tych komórek towarzyszących autyzmowi. Jest to szczególnie istotne w związku z najnowszymi danymi, które wykazały, że ATP może prowadzić do aktywacji komórek mikrogleju i do wzmożenia sygnalizacji zależnej od mTOR [103]. Z kolei mTOR reguluje wrodzoną odpowiedź układu odpornościowego i przyczynia się do aktywacji makrofagów [104,105], zaś zahamowanie aktywności mTOR ma działanie przeciwzapalne [106], co wskazuje na prawdopodobieństwo istnienia pętli sprzężenia zwrotnego pomiędzy stanem zapalnym a mTOR [107]. Ponadto uwolnione przez komórki mikrogleju cytokiny, szczególnie IL-6 i Tnf α , mogą prowadzić do aktywacji mTOR w neuronach poprzez szlak PI3K/Akt, bądź też przez sygnalizację JAK/STAT [108–112].

Podsumowując, nieprawidłowości w funkcjonowaniu sygnalizacji nukleotydowej mogą stanowić kluczowy element patomechanizmu ASD, zwłaszcza w kontekście zaburzeń aktywności mTOR i powiązanych z nią syntezy/degradacji białek synaptycznych. Jednakże do chwili obecnej nie zbadano czy kinaza mTOR może pośredniczyć w przekazywaniu sygnału od receptorów purynergiczných do strukturalnych i funkcjonalnych białek synaptycznych, co stanowi lukę w naszym pojmowaniu mechanizmów współzależności zmian biochemicznych ze strukturalnymi (nieprawidłowa budowa i liczba synaps) w ASD.

6. Cel pracy

6.1. Cel ogólny

Zaburzenia ze spektrum autyzmu określane są mianem synaptopatii, czyli chorób będących wynikiem dysfunkcji synaps [49]. Istotną rolę w regulacji struktury i funkcji połączeń synaptycznych oraz w procesach plastyczności synaptycznej pełni sygnalizacja purynergiczna zależna od zewnątrzkomórkowych nukleotydów [113,114]. Istnieją przesłanki, iż przekąźnictwo nukleotydowe może mieć istotne znaczenie w tworzeniu zachowań autystycznych. Wskazują na to dotychczasowe badania przeprowadzone w mysich modelach autyzmu, gdzie niespecyficzny antagonist receptorów purynergicznych, suramina niwelowała zaburzenia behawioralne i poprawiała zdolności poznawcze [115–117]. Skuteczność terapii suraminą została również zaprezentowana w pilotażowym badaniu klinicznym z udziałem dzieci w spektrum autyzmu, u których po jednorazowej dawce zaobserwowano poprawę zdolności komunikacyjnych i interakcji społecznych oraz spadek zachowań o powtarzającym się charakterze [118]. Dotychczas nie określono, czy nieprawidłowości przekąźnictwa purynergicznego mogą wpływać na zmiany synaptyczne w zaburzeniach ze spektrum autyzmu, ani nie zbadano wpływu specyficznych modulatorów receptorów purynergicznych na zmiany związane z ASD. Nie wyjaśniono również, które ze szlaków sygnałowych są odpowiedzialne za wystąpienie korzystnych zmian behawioralnych po zastosowaniu terapii „antypurynergicznej”. Ponieważ przekąźnictwo nukleotydowe może wpływać na aktywność PI3K/Akt oraz AMPK, a poprzez te enzymy na aktywność kinazy mTOR [40,41,119–122], w warunkach patologicznych deregulacja układu purynergicznego może prowadzić do zależnych od mTOR zaburzeń syntezy/degradacji białek synaptycznych. Dlatego celem niniejszej rozprawy doktorskiej było sprawdzenie hipotezy badawczej zakładającej, że nieprawidłowości strukturalne i funkcjonalne połączeń synaptycznych w ASD wynikają z zaburzeń sygnalizacji nukleotydowej oraz, że mTOR pośredniczy w szlaku przekąźnictwa prowadzącym od receptorów nukleotydowych do dysfunkcji synaps, co rzutuje na wykształcenie objawów fenotypowych charakterystycznych dla spektrum autyzmu.

Cel ten realizowano poprzez prowadzenie badań na powszechnie uznanych modelach zwierzęcych:

- ASD indukowany czynnikami środowiskowymi: szczurzy model *in vivo* oparty na prenatalnej ekspozycji na kwas walproinowy (VPA);
- ASD indukowany czynnikami genetycznymi: myszy genetyczny model stwardnienia guzowatego, myszy B6;129S4-*Tsc2^{tm1Djk}/J*, heterozygoty *Tsc2^{+/-}*;

6.2. Cele szczegółowe

1. Zbadanie ekspresji i aktywności wybranych receptorów dla ATP i adenozyiny w krytycznej fazie embrionalnego rozwoju ośrodkowego układu nerwowego w modelu VPA (Publikacja I).
2. Analiza zmian ekspresji na poziomie genu i białka wybranych receptorów dla zewnątrzkomórkowych nukleotydów i nukleozydów w mózgu młodych 52-dniowych szczurów poddanych prenatalnej ekspozycji na VPA (Publikacja II).
3. Zbadanie wpływu prenatalnej ekspozycji na VPA na zmiany poziomu nukleotydów ATP, ADP, AMP i adenozyiny w płynie mózgowo-rdzeniowym oraz na profil ekspresji białek odpowiedzialnych za uwalnianie i metabolizm egzogennych nukleotydów w wybranych strukturach mózgu młodych zwierząt (Publikacja IV).
4. Sprawdzenie, czy zastosowanie specyficznych antagonistów receptorów P2 (PPADS i isoPPADS) reguluje aktywność komórek glejowych w korze mózgowej i hipokampie zwierząt w modelu VPA (Publikacja II).
5. Zbadanie, czy zahamowanie sygnalizacji purynergicznej moduluje aktywność kinazy mTOR oraz jej kluczowych regulatorów, AMPK i Akt w mózgu młodych, 52-dniowych zwierząt, poddanych prenatalnej ekspozycji na VPA (Publikacja IV).
6. Ocena wpływu nadmiernej aktywacji kinazy mTOR na poziom kluczowych białek synaptycznych i ultrastrukturę synaps oraz na zmiany behawioralne w genetycznym mysim modelu stwardnienia guzowatego (Publikacja III).
7. Zbadanie, czy modulacja receptorów purynergicznych wpływa na poziom białek synaptycznych oraz typowe dla autyzmu zmiany behawioralne wywołane prenatalną ekspozycją na VPA (Publikacja IV).

7. Metodyka

7.1. Zwierzęce modele zaburzeń ze spektrum autyzmu

Mnogość zaburzeń socjalnych, neurofizjologicznych oraz somatycznych obserwowanych u osób z ASD przemawia za tym, że prawdopodobnie nie mają one jednego wspólnego czynnika etiologicznego. Ze względu na różnorodność objawów ASD, nie jest możliwe zaprojektowanie jednego uniwersalnego modelu autyzmu, opracowano natomiast szereg modeli zwierzęcych odzwierciedlających poszczególne cechy tego zaburzenia. Do najpopularniejszych należą te, prowadzone na gryzoniach, ponieważ są to zwierzęta przejawiające szeroki wachlarz interakcji społecznych oraz stosują bogaty system wzajemnej komunikacji [123,124]. Dlatego mysie i szczurze modele ASD są bardzo dobrymi narzędziami do badania molekularnych podstaw choroby oraz do oceny skuteczności strategii terapeutycznych. Spośród wielu dostępnych modeli *in vivo* w niniejszym projekcie wybrano dwa, model zaburzeń ze spektrum autyzmu wywołanych działaniem czynników środowiskowych - **model VPA** oraz model ASD indukowany zmianami genetycznymi, w wyniku których dochodzi do nadmiernej aktywacji kinazy mTOR - **genetyczny model stwardnienia guzowego**. Badania prowadzono na różnych poziomach złożoności zgodnie z procedurami zaakceptowanymi przez II Lokalną Komisję Etyczną ds. Doświadczeń na Zwierzętach w Warszawie (WAW2/148/2018, WAW2/081/2019, WAW2/142/2019, WAW2/036/2019, WAW2/045/2020, WAW2/128/2021), ze szczególną dbałością o stosowanie zasady „3R”, czyli zastąpienia, ograniczenia i udoskonalenia (Replacement, Reduction, Refinement) oraz z uwzględnieniem tzw. „efektu miotu”. Zasadę tę stosuje się do badań, w których podawanie czynników wywołujących objawy choroby można zastosować tylko do całych miotów, a nie do pojedynczych osobników [125].

7.1.1. Szczurzy model *in vivo* indukowany czynnikami środowiskowymi

Badania realizowano w oparciu o przedkliniczny model autyzmu, prenatalnej ekspozycji na VPA, który jest jednym z najczęściej opisywanych zwierzęcych modeli ASD. U szczurów VPA podawany jest około 12 dnia ciąży, czyli w okresie zamknięcia się cewy nerwowej płodu [126], co prowadzi do wystąpienia charakterystycznych dla ASD zaburzeń behawioralnych u potomstwa [25,127–130]. Mechanizm działania VPA jest wielokierunkowy, związek ten jest inhibitorem deacetylazy histonowej, stanowi czynnik epigenetyczny wywołujący zmiany transkrypcji genów [131]; powoduje

zaburzenia rozwoju i dojrzewania synaps czynnościowych w sposób zależny od szlaku Wnt/ β -katenina [132]; negatywnie wpływa na łożysko i zaburza mechanizmy transportu kwasu foliowego, białek, glukozy i hormonów [133,134]. Ponadto, VPA z łatwością przenika przez łożysko, w osoczu płodu osiąga ok. 1,3 – 4,6 razy większe stężenie niż w osoczu matki [135]. Badania oparte na modelu ASD indukowanym prenatalnym podaniem VPA pokazują analogię zmian biochemicznych, strukturalnych i behawioralnych z tymi, które są obserwowane u pacjentów. Model ten spełnia podstawowe kryteria walidacji, walidację konstruktu - replikuje teoretyczne podstawy zaburzenia; walidację fasadową - symptomy ASD w modelu zwierzęcym naśladują te zaobserwowane u ludzi [136–138]; oraz walidację predykcyjną - ingerencja farmakologiczna skuteczna u ludzi, jest również efektywna w odwracaniu kluczowych objawów w modelu VPA [139].

W eksperymentach przeprowadzonych w niniejszej pracy samicom szczura szczepu Wistar w 12,5 dniu ciąży podawano dootrzewnowo VPA w dawce 450 mg/kg m.c, ponieważ ta ilość substancji czynnej powoduje umiarkowany poziom reabsorpcji ciąży, nie wpływa na liczebność płodów, nie ma istotnego wpływu na masę ciała ciężarnych samic oraz potomstwa po urodzeniu [127]. Jednocześnie jest to dawka, która wywołuje istotne zmiany behawioralne u potomstwa, takie jak upośledzona komunikacja u noworodków, czy zmniejszona aktywność eksploracyjna oraz zachowania lękowe i stereotypie u młodych dorosłych zwierząt [140]. Dokładny opis i schemat przeprowadzenia eksperymentu zamieszczono w sekcji Materiały i Metody w publikacji I (str. 392), publikacji II (str. 3-4) oraz publikacji IV (str. 2-3). Badania prowadzono na osobnikach potomnych w trzech punktach czasowych, w 19 dniu embrionalnym (Publikacja I, str. 392; Publikacja II, str. 3-4), oraz u samców w 25 i 52 dniu po urodzeniu (Publikacja II, str. 3-4; Publikacja IV, str. 2-3). Do badań wykorzystano również pierwotne hodowle neuronów z kory i hipokampa szczurów, izolowanych z 19-dniowych płodów (E19) poddanych prenatalnej ekspozycji na VPA (Publikacja I, str. 392-393). Ponadto, w celu zbadania udziału sygnalizacji purynergicznej, 52-dniowym samcom potomnym podawano dootrzewnowo antagonistów receptorów P2, PPADS (6-azofenylo-2',4'-disulfonowy fosforan pirydoksalu) oraz isoPPADS (6-azofenylo-2',5'-disulfonowy fosforan pirydoksalu) w dawce 12,5 mg/kg masy ciała (Publikacja II, str. 3-4; Publikacja IV, str. 2-3).

7.1.2. Model stwardnienia guzowego – myszy *Tsc2^{tm1Djk}/J*

Stwardnienie guzowe (ang. tuberous sclerosis complex, TSC) jest chorobą autosomalnie dominującą wywołaną mutacjami w genach *TSC1* lub *TSC2*. Około 90% pacjentów z TSC wykazuje pewien stopień zaburzeń neuropsychologicznych typowych dla ASD [141]. Częstość występowania deficytów typowych dla autyzmu u pacjentów z TSC jest wysoka [142] i charakteryzuje się wysokim zróżnicowaniem zarówno pod względem zakresu, jak i nasilenia zmian. Najistotniejsze jest jednak, że deficyty behawioralne u pacjentów z TSC w istotny sposób nakładają się na obraz zaburzeń obserwowanych u ludzi z idiopatycznym ASD [143]. Wskazuje to, że wyniki badań prowadzonych na modelach TSC mogą mieć wysokie prawdopodobieństwo walidacji również u ludzi z ASD o innej etiologii. Dlatego w niniejszym projekcie, do zbadania w jaki sposób nieprawidłowości szlaku mTOR indukują typowe dla ASD zmiany molekularne, morfologiczne i behawioralne, wybrano genetycznie modyfikowane myszy szczepu B6;129S4-*Tsc2^{tm1Djk}/J*. U tych zwierząt techniką nokautu genetycznego inaktywowany był gen *Tsc2* poprzez insercję *neo cassette* w eksonie 2. W dalszej części pracy heterozygotyczne myszy B6;129S4-*Tsc2^{tm1Djk}/J* będą określane symbolem *Tsc2^{+/-}*. Ponieważ homozygoty *Tsc2^{-/-}* obumierają w fazie zarodkowej, badania, które złożyły się na niniejszą rozprawę doktorską prowadzono na heterozygotach *Tsc2^{+/-}*, a homozygotyczne samce *Tsc2^{+/+}* z tych samych miotów stanowiły grupę kontrolną. U myszy *Tsc2^{+/-}* dochodzi do nadmiernej aktywacji kinazy mTOR, zahamowania autofagii oraz zmian behawioralnych charakterystycznych dla zaburzeń ze spektrum autyzmu [144,145], a obserwowane zaburzenia neurologiczne są znacznie poważniejsze niż w przypadku modyfikacji genu *Tsc1* [146–148]. Zwierzęta zostały komercyjnie zakupione w Jackson Laboratory (Bar Harbor, Maine, USA). Badania prowadzono na dorosłych, 50-60-dniowych samcach (Publikacja III, str. 16).

7.2. Analiza zachowań o charakterze autystycznym u myszy *Tsc2^{tm1Djk}/J* i szczurów po prenatalnej ekspozycji na VPA

7.2.1. Test otwartego pola

Test otwartego pola (ang. open field test, OF) służy do analizy zachowania ruchowego i eksploracyjnego u gryzoni, pozwala na ocenę stopnia stereotypii ruchowej zwierząt, zachowań lękowych oraz umiejętności adaptacji do nieznanego środowiska [149]. Wykorzystując ten test badano typowe dla autyzmu zachowania repetytywne, lękowe,

aktywność eksploracyjną oraz ogólną aktywność lokomotoryczną zwierząt (Publikacja III, str. 16-17; Publikacja IV, str. 4).

7.2.2. Trójkomorowy test socjalizacji

Trójkomorowy test socjalizacji (3-chamber social interaction test; Crawley's sociability and preference for social novelty test) pozwala na analizę zaburzeń interakcji społecznych w modelach ASD. Protokół ten został zaprojektowany w celu oceny preferencji między bodźcami społecznymi i niespołecznymi w warunkach równoważnej atrakcyjności i oparty jest na założeniu, iż gryzonie preferują obecność drugiego osobnika swojego gatunku („towarzyskość”, ang. sociability) oraz wykazują większe zainteresowanie nowym osobnikiem niż osobnikiem wcześniej poznanym („nowość społeczna” ang. social novelty) [150]. W badaniach zastosowano zoptymalizowany protokół, który składa się z trzech faz każdorazowo następujących po sobie w ciągu jednego dnia: fazy habituacji, fazy preferencji socjalnych oraz fazy preferencji nowości [151]. Szczegółowy opis testu znajduje się w publikacji III (Materiały i Metody, str. 17).

7.2.3. Test rozpoznawania nowych obiektów

Test rozpoznawania nowych obiektów (ang. new object recognition test, NOR) pozwala na testowanie pamięci krótko- i długotrwałej i jest stosowany do badania funkcji poznawczych i pomiaru integralności pamięci i uwagi u gryzoni [152], które często są zaburzone u pacjentów w spektrum autyzmu. Dokładny opis metody znajduje się w Publikacji III (str. 17).

7.3. Metody analizy molekularnej i komórkowej

7.3.1. Pomiar ekspresji genów (q-PCR)

Analizę ekspresji genów przeprowadzono za pomocą ilościowej reakcji łańcuchowej polimerazy (ang. quantitative polymerase chain reaction, q-PCR) z użyciem komercyjnie dostępnego zestawu TaqMan Gene Expression Assay (Thermo Fischer Scientific, USA). Pomimo tego, że metoda ta wymaga szeregu etapów prowadzących do zsyntetyzowania cDNA, jej zaletą jest wysoka specyficzność, powtarzalność wyników i wysoka czułość. Przy jej użyciu analizowano w mózgu zwierząt w modelu TSC ekspresję genów wybranych białek synaptycznych (Publikacja III, str. 18). Ponadto, zbadano wpływ prenatalnej ekspozycji na VPA na ekspresję białek zaangażowanych w uwalnianie i degradację nukleotydów (Publikacja IV, str. 3) oraz na poziom mRNA wybranych receptorów purynergicznych (Publikacje I, str. 393; Publikacja II, str. 4). Analizowano

także ekspresję cytokin prozapalnych, IL-6 i $Tnf\alpha$ (Publikacja II, str. 4; Publikacja IV, str. 3).

7.3.2. Analiza poziomu białka (Western blotting)

Identyfikację i detekcję zmian poziomu białka wybranych receptorów purynergicznym oraz wyselekcjonowanych białek synaptycznych (Publikacja I, str. 398; Publikacja II, str. 4-5; Publikacja III, str. 18, Publikacja IV, str. 3-4) przeprowadzono za pomocą ilościowej metody Western blotting. Metodą tą dokonano również oceny aktywności wybranych enzymów metabolizmu komórki: mTOR, AMPK i Akt (Publikacja IV, str. 4-5). Analiza ta pozwoliła na ogólną ocenę zmian poziomu poszczególnych białek w wybranych strukturach mózgu, bez dokładnego podziału na poszczególne typy komórek oraz przedziały komórkowe, takie jak zakończenia przed- lub postsynaptyczne.

7.3.3. Analiza ultrastruktury komórek nerwowych z zastosowaniem transmisyjnej mikroskopii elektronowej

Przy użyciu transmisyjnej mikroskopii elektronowej (ang. transmission electron microscopy, TEM) zbadano ultrastrukturę komórek neuronalnych oraz liczbę pęcherzyków synaptycznych w mózgu zwierząt w modelu TSC (Publikacja III, str. 17-18). Metoda ta pozwoliła na szczegółowe zobrazowanie i analizę zmian morfologicznych w obrębie struktur komórkowych takich jak siateczka śródplazmatyczna, aparat Golgiego, jądro komórkowe oraz osłonka mielinowa.

7.3.4. Analiza poziomu nukleotydów i adenozy w płynie mózgowo-rdzeniowym - (HPLC)

Poziom ATP, ADP, AMP i adenozy w płynie mózgowo-rdzeniowym (PMR) młodych 52-dniowych zwierząt w modelu VPA zbadano za pomocą wysokosprawnej chromatografii cieczowej (ang. high-performance liquid chromatography, HPLC). Metoda HPLC zapewnia uzyskanie stabilnych i powtarzalnych wyników badań, ponadto charakteryzuje się wysoką czułością. Szczegółowy opis metody znajduje się w publikacji IV (str. 3).

7.3.5. Analiza aktywacji receptorów purynergicznych poprzez pomiar napływu jonów wapnia (Ca^{2+}) do neuronów *in vitro*

Z wykorzystaniem sondy fluorescencyjnej Fluo-4 AM (InvitrogenTM, USA) badano wpływ prenatalnej ekspozycji na VPA na zmiany aktywności receptorów purynergicznych w hodowli pierwotnej neuronów wyizolowanych z kory mózgowej wraz z hipokampem. Zastosowanie tej metody umożliwiło detekcję napływu jonów wapnia (Ca^{2+}) do neuronów w czasie rzeczywistym w odpowiedzi na stymulację wybranymi agonistami receptorów purynergicznych (Publikacja I, str. 392-393).

7.3.6. Ocena poziomu wyselekcjonowanych receptorów purynergicznych w poszczególnych typach komórek OUN oraz morfologii komórek glejowych

Do zbadania zmian poziomu wyselekcjonowanych receptorów purynergicznych w poszczególnych typach komórek OUN u zwierząt w modelu VPA wykorzystano analizę immunohistochemiczną (ang. immunohistochemical staining, IHC). Spośród puli badanych receptorów wybrano te, których poziom znamienne zmieniał się w wybranych strukturach mózgu. Analizę zmian poziomu receptorów P2X1, P2X7, P2Y1 i P2Y12 w komórkach neuronalnych (wybarwionymi przeciwciałami anty-MAP-2), astrocytach (anty-GFAP), lub mikrogleju (anty-Iba-1) wykonano w korze przedczołowej, oraz w rejonie CA (ang. Cornu Ammonis) i zakręcie zębatym, (ang. dentate gyrus DG) hipokampa. Dokładny opis metody znajduje się w Publikacji II, str. 6-7). Metoda ta posłużyła również do analizy morfologii astrocytów i mikrogleju za pomocą rozszerzenia NeuronJ dla programu Fiji (<https://fiji.sc/>). Zmierzono pole powierzchni drzewka dendrytycznego, liczbę dendrytów pierwotnych (wychodzących z somy komórkowej) i wtórnych oraz całkowitą długość rozgałęzień przynależnych do każdej immunohistochemicznie wybarwionej komórki. Rozgałęzienie poszczególnych komórek glejowych określano metodą Sholla. Za pomocą rozszerzenia Cell Counter dla programu Fiji oznaczono też liczbę komórek mikrogleju w przeliczeniu na 1 mm². Dokładny opis metody znajduje się w Publikacji II (str. 6-7; Ryc. 8h, str. 18).

7.4. Analiza statystyczna

Do oceny, czy rozkład danych spełnia założenia rozkładu normalnego zastosowano test Shapiro-Wilka. W przypadku zestawu danych o rozkładzie normalnym, do oceny istotności statystycznej pomiędzy dwoma grupami zastosowano test *t*-Studenta, zaś

pomiędzy wieloma grupami – jednoczynnikową analizę wariancji ANOVA z testem *post-hoc* Bonferroniego. W przypadku danych o rozkładzie odbiegającym od normalnego, zastosowano nieparametryczny test Manna-Whitneya. Wyniki uznawano za istotne statystycznie, gdy $p < 0,05$. Poziom istotności statystycznej przedstawiono jako: $*p < 0,05$; $**p < 0,01$; $***p < 0,001$. Do analizy statystycznej wykorzystano program GraphPad Prism (Graph Pad Software, USA).

8. Podsumowanie

I. Prenatalna ekspozycja na kwas walproinowy powoduje deregulację receptorów purynergicznych na różnych etapach rozwoju mózgu szczura

Prenatalna ekspozycja na kwas walproinowy wywołuje znaczące zmiany w poziomie i aktywności wybranych receptorów szlaku purynergicznego w mózgu w 19. dniu rozwoju embrionalnego (E19) szczura, kiedy to zachodzą kluczowe procesy związane z dojrzewaniem OUN. Wykazano wzrost poziomu i aktywności receptorów adenozynowych A1, A2b i A3 (**publikacja I, ryc. 2A, 2A', 2A''; ryc. 3A**), które uczestniczą w regulacji różnicowania komórek progenitorowych [153] oraz procesów neurotransmisji [154,155]. Ponadto po podaniu VPA zaobserwowano zwiększenie poziomu i aktywności receptorów jonotropowych P2X2 i P2X3 (**publikacja I, ryc. 2B, 2B', 2B''; ryc. 3B, 3C, 3D, 3E**) zaangażowanych w procesy różnicowania neuronalnego oraz formowania i dojrzewania neurytów [156–163]. Z drugiej strony, obserwowany w E19 spadek poziomu i aktywności receptorów P2X7 (**publikacja I, ryc. 3F**) oraz P2Y1 (**publikacja I, ryc. 2C, 2C', 2C''; ryc. 3G**) może być związany z zaburzeniami regulowanych przez te receptory procesami migracji i różnicowania prekursorów neuronalnych, formowania dendrytów i aksonów [162,164–166] oraz homeostazy sygnalizacji pobudzającej/hamującej [167,168].

Deregulacja receptorów purynergicznych obserwowana jest również w mózgu młodych zwierząt po prenatalnej ekspozycji na VPA. W tym okresie rozwojowym zaobserwowano zmiany w poziomie receptorów P2X (**publikacja II, ryc. 5**) i P2Y (**publikacja II, ryc. 6**), natomiast ekspresja receptorów adenozynowych nie ulegała zmianie w porównaniu do kontroli (**publikacja II, ryc. 4**). Stwierdzono istotne zmiany w poziomie receptora P2X1 zarówno w korze mózgowej, jak i hipokampie młodych szczurów VPA (**publikacja II, ryc. 5 a-f**). Ponieważ receptor ten głównie znajduje się w komórkach neuronalnych (**publikacja II, ryc. 5f**), jego obniżona ekspresja w korze przedczołowej u zwierząt traktowanych VPA może sugerować zaburzenia w neuronalnej transmisji synaptycznej w tej części mózgu. W hipokampie potomstwa VPA poziom receptora P2X1 był z kolei znacząco podwyższony. Ponieważ P2X1 reguluje uwalnianie glutaminianu z zakończeń nerwowych [169] i zapewnia homeostazę pomiędzy układem pobudzającym i hamującym [169,170], jego neuronalna nadekspresja w regionie CA i zakręcie zębatym hipokampa u zwierząt VPA może sugerować zaburzenia długotrwałego wzmocnienia synaptycznego (LTP) w tej strukturze mózgu. Z kolei poziom receptora

P2X7, którego obecność potwierdzono w ciałach komórek neuronalnych oraz w mikrogleju (**publikacja II, rycina 5g**), był znacząco obniżony w hipokampie (**publikacja II, ryc. 5a, c, e, g**) 52-dniowych zwierząt po prenatalnej ekspozycji na VPA, co koresponduje z wcześniejszymi badaniami uzyskanymi na mysich modelach ASD [117]. W wyniku prenatalnej ekspozycji na VPA, u młodych zwierząt dochodzi do znaczącego wzrostu poziomu receptora P2Y1 w neuronach kory mózgu, przy jednoczesnym spadku jego poziomu w hipokampie (**publikacja II, ryc. 6 a-f**). Ponieważ receptor ten zaangażowany jest w regulację zdolności poznawczych [171], dlatego możliwe jest, że zaburzenia P2Y1 mogą przynajmniej częściowo uczestniczyć w upośledzeniu tych funkcji w ASD. W korze mózgowej zaobserwowano również indukowany prenatalnym podaniem VPA wzrost poziomu receptora P2Y12 w komórkach mikrogleju (**publikacja II, ryc. 6b, d, e, g**).

II. Prenatalna ekspozycja na kwas walproinowy skutkuje zaburzeniami metabolizmu zewnątrzkomórkowych nukleotydów w mózgu młodych zwierząt

Zwierzęta 52-dniowe, prenatalnie poddane działaniu kwasu walproinowego, wykazują nieprawidłowości nie tylko w poziomie receptorów P2, ale także w procesach uwalniania i metabolizowania nukleotydów. W płynie mózgowo-rdzeniowym (PMR) tych osobników stwierdzono znaczący wzrost poziomu ATP, ADP i AMP w porównaniu z grupą kontrolną (**publikacja IV, ryc. 1 a-c**), co potwierdza wcześniejsze doniesienia o roli zewnątrzkomórkowego ATP w wywoływaniu zmian behawioralnych charakterystycznych dla ASD [172] i koresponduje z wynikami klinicznymi, które pokazują znaczne zmiany w stężeniu puryn w PMR [173], moczu [174,175] oraz we krwi [176].

Uzyskane wyniki badań sugerują, że zwiększona ekspresja koneksyny 36 może być kluczowym mechanizmem odpowiedzialnym za podwyższone uwalnianie ATP w hipokampie i mózdzku u 52-dniowych szczurów VPA. Dodatkowo, w hipokampie wykazano zwiększoną ekspresję koneksyny 43 (**publikacja IV, ryc. 2 a-c**), co jest zgodne z wcześniejszymi badaniami pokazującymi jej podwyższony poziom w mózgach osób z autyzmem [177]. Koneksyna 36 występuje wyłącznie w neuronach [178], podczas gdy koneksyna 43 jest syntetyzowana głównie w astrocytach [178,179]. Sugeruje to, że oba typy komórek biorą udział w zaburzeniach sygnalizacji nukleotydowej w modelu VPA. Oprócz podwyższonego poziomu koneksyn, które aktywują się tylko w obecności

czynników stresowych, wykazano zwiększoną ekspresję paneksyny 1 w korze mózgowej u zwierząt po prenatalnej ekspozycji na VPA. To białko kanałowe jest głównym „niepęcherzykowym” źródłem uwalniania ATP w OUN zarówno w warunkach fizjologicznych, jak również pod wpływem stresu [180], a polimorfizmy pojedynczych nukleotydów w genie paneksyny 1 obserwowane są w ASD [181]. Z kolei poziom transkryptu VNUT, który pełni kluczową rolę w magazynowaniu ATP w pęcherzykach synaptycznych, był znacznie podwyższony we wszystkich badanych strukturach mózgu u 52-dniowych szczurów VPA. Zwiększona ekspresja białek połączeń szczelinowych oraz VNUT w mózgach zwierząt VPA może stanowić główny czynnik odpowiedzialny za podwyższone uwalnianie ATP, które wydaje się nie być kompensowane przez zwiększoną degradację ATP w szczelinie synaptycznej, ponieważ ekspresja głównych ATPaz: difosfohydrolazy trifosforanu nukleozydu 2 (*Entpd2*) i difosfohydrolazy trifosforanu nukleozydu 3 (*Entpd3*) nie uległa zmianie w mózgach zwierząt doświadczalnych (**publikacja IV, ryc. 3 a-c**). Natomiast ekspresja ekto-5'-nukleotydu (*Nt5e*), która hydrolizuje zewnątrzkomórkowy AMP do adenozyne [182] była znacznie zmniejszona (**publikacja IV, ryc. 3b, c**). Jednocześnie wykazano brak zmian w poziomie mRNA deaminazy adenozyne (*Ada*) (**publikacja IV, ryc. 3 a-c**), w związku z czym całkowity poziom adenozyne w płynie mózgowo-rdzeniowym szczurów VPA nie ulegał zmianie (**publikacja IV, ryc. 1d**).

III. Zaburzenia przekąźnictwa purynergicznego są częściowo odpowiedzialne za aktywację mikrogleju w mózgu młodych zwierząt VPA

Pobudzenie receptorów purynergiczych w przedklinicznym modelu ASD powoduje zwiększenie gęstości komórek mikrogleju oraz wzrost ekspresji cytokin: *Tnfa* i *IL-6* w korze mózgowej (**publikacja II, ryc. 9b, m, n**). Badania morfologiczne również potwierdziły wpływ prenatalnej ekspozycji na VPA na aktywację mikrogleju, zaobserwowano powiększenie ciała komórkowego oraz zmniejszenie rozgałęzienia wypustek komórkowych (**publikacja II, ryc. 9c, e-l**). W zastosowanym modelu badawczym nie stwierdzono natomiast istotnych zmian wskazujących na aktywację astrocytów (**publikacja II, ryc. 7**). Wykazano, że pobudzenie receptorów P2 odgrywa istotną rolę w zwiększeniu liczby komórek mikrogleju oraz nadekspresji cytokin w korze mózgowej zwierząt w modelu VPA. (**publikacja II, ryc. 9b, c, e-l**).

IV. Deregulacja receptorów purynergicznych oraz aktywacja szlaku mTOR są odpowiedzialne za zmiany synaptyczne obserwowane w ASD

Nadmierne pobudzenie receptorów purynergicznych w korze, hipokampie i mózdku młodych zwierząt po prenatalnej ekspozycji na VPA prowadzi do aktywacji szlaku mTOR (**publikacja IV, ryc. 4 a-f**). Przeprowadzone badania wykazały, że kanoniczna ścieżka PI3K/Akt nie leży u podstaw tego zjawiska (**publikacja IV, ryc. 6 a-f**), natomiast zahamowanie aktywności AMPK może być odpowiedzialne za aktywację mTOR w korze mózgowej i hipokampie (**publikacja IV, ryc. 5a, b, d, e**). Uzyskane wyniki wykluczają udział obu w/w ścieżek w zależnej od receptorów P2 aktywacji mTOR w mózdku (**publikacja IV, ryc. 5c,f; ryc. 6d, f**), a jako głównego potencjalnego aktywatora tego szlaku sugeruje się Il-6 (**publikacja IV, ryc. dodatkowa 1**).

Badania prowadzone na mysim genetycznym modelu TSC wykazały, że nadmierna aktywacja mTOR prowadzi do zwiększenia masy i objętości mózgu (**publikacja III, ryc. 1 d-e**) oraz wywołuje szereg zmian morfologicznych, w tym zaburzenia ultrastruktury synaps, obrzęk mitochondriów, czy rozwarstwienie osłonki mielinowej (**publikacja III, tab. 3; ryc. 3-5**). Ponadto, u zwierząt transgenicznych zaobserwowano zmiany poziomu kluczowych białek synaptycznych odpowiedzialnych za regulację procesu synaptogenezy oraz homeostazę pęcherzyków synaptycznych, takich jak synaptobrewina (VAMP1/2) i fosfo-synapsyna-1 (Ser62/67) [183–185] (**publikacja III, ryc. 2; tab. 2**). Istotną obserwacją w badaniach z wykorzystaniem modelu VPA jest to, że zmiany w ekspresji białek synaptycznych, które są modyfikowane przez zastosowanie antagonisty receptorów P2, dokładnie odpowiadają tym obserwowanym w modelu TSC (**publikacja IV, ryc. 7e, g; ryc. 8b, d**). Sugeruje to pośrednio udział szlaku mTOR w zmianach synaptycznych wywołanych przez deregulację sygnalizacji purynergicznej.

V. Antagoniści receptorów P2 zapobiegają zmianom behawioralnym obserwowanym w przedklinicznym zwierzęcym modelu ASD

W modelu VPA zaobserwowano występowanie zmian behawioralnych charakterystycznych dla ASD, zachowania repetytywne oraz zmniejszenie aktywności eksploracyjnej wskutek zwiększenia lęku (**publikacja IV, ryc. 10a, d, h**). Zastosowanie antagonistów receptorów P2, PPADS i isoPPADS zmniejszyło częstotliwość występowania zachowań stereotypowych (**publikacja IV, ryc. 10a**), obniżyło poziom lęku (**publikacja IV, ryc. 10d**) i normalizowało aktywność eksploracyjną (**publikacja IV, ryc. 10h**) zwierząt poddanych prenatalnej ekspozycji na VPA.

Podsumowując, prenatalna ekspozycja zwierząt na kwas walproinowy prowadzi do poważnych zaburzeń sygnalizacji purynergicznej, które zachodzą na różnych etapach rozwoju i obejmują zmiany ekspresji wybranych podtypów receptorów purynergicznych w neuronach i komórkach glejowych oraz reorganizację składu nukleotydów w płynie mózgowo-rdzeniowym. Deregulacja sygnalizacji purynergicznej jest w dużym stopniu odpowiedzialna za aktywację mikrogleju w korze mózgowej, zwiększenie aktywności szlaku kinazy mTOR, a w konsekwencji za zaburzenia struktury synaps, co prowadzi do pojawienia się objawów fenotypowych (behawioralnych) charakterystycznych dla ASD. Powyższe odkrycia stanowią solidną podstawę do dalszych badań mających na celu opracowanie nowoczesnych strategii terapeutycznych ukierunkowanych na blokowanie przekazywania purynergicznego w ASD.

9. Wnioski

- I. Prenatalna ekspozycja na kwas walproinowy prowadzi do deregulacji sygnalizacji purynergicznej na wczesnym etapie rozwoju embrionalnego, zarówno na poziomie receptorów adenozynowych, kanałów jonowych P2X oraz receptorów metabotropowych P2Y. Wzrost sygnalizacji zależnej od adenozyiny oraz zaburzenie homeostazy wapniowej wynikające z deregulacji receptorów dla ATP/ADP w fazie formowania się połączeń synaptycznych może przyczyniać się do wystąpienia nieprawidłowości neuroanatomicznych mózgu obserwowanych w okresie postnatalnym.
- II. U młodych zwierząt poddanych prenatalnej ekspozycji na kwas walproinowy w wybranych strukturach mózgu dochodzi do zaburzenia ekspresji receptorów P2 oraz białek zaangażowanych w metabolizm zewnątrzkomórkowych nukleotydów. Skutkuje to wzrostem poziomu ATP i jego metabolitów w płynie mózgowo-rdzeniowym.
- III. Deregulacja przekąźnictwa purynergicznego wywołana prenatalną ekspozycją na kwas walproinowy powoduje aktywację mikrogleju, wzrost ekspresji cytokin prozapalnych TNF α i IL-6 oraz aktywację ścieżki sygnalizacyjnej kinazy mTOR.
- IV. Zwiększenie aktywności szlaku mTOR wywołuje zmiany anatomiczne i morfologiczne w mózgu zwierząt w modelu TSC, a w szczególności zaburzenia proteostazy białek synaptycznych i ultrastruktury synaps.
- V. Wywołane nieprawidłowym funkcjonowaniem przekąźnictwa purynergicznego zaburzenia synaptyczne są odpowiedzialne za występowanie zmian behawioralnych typowych dla ASD, stereotypii zachowania, nadmiernej lękliwości oraz zaburzenia aktywności eksploracyjnej.

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11. Publikacje wchodzące w skład rozprawy doktorskiej

11.1. Wskaźnik oddziaływania (IF) publikacji

Publikacja I

Babiec L., Wilkaniec A., Adamczyk A. Prenatal exposure to valproic acid induces alterations in the expression and activity of purinergic receptors in the embryonic rat brain. *Folia Neuropathologica* 2022; 60 (4), 390-402. <https://doi.org/10.5114/fn.2022.123999>
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Publikacja II

Babiec L., Wilkaniec A., Matuszewska M., Pałasz E., Cieślik M., Adamczyk A. Alterations of Purinergic Receptors Levels and Their Involvement in the Glial Cell Morphology in a Pre-Clinical Model of Autism Spectrum Disorders. *Brain Sciences* 2023; 13, 1088. <https://doi.org/10.3390/brainsci13071088> (5-letni IF = 3)

Publikacja III

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Publikacja IV

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BIBLIOTEKA
Instytut Medycyny Doświadczalnej i Klinicznej
im. Mirosława Mossakowskiego
Polskiej Akademii Nauk
02-106 Warszawa, ul. A. Pawińskiego 5
tel. 22 608-66-11 NIP 525-000-81-69
e-mail: library@imdik.pan.pl

Warszawa, 21.06.2024
KIEROWNIK BIBLIOTEKI
Katarzyna Nieszporska
mgr Katarzyna Nieszporska

11.2. Publikacja I

Babiec L., Wilkaniec A., Adamczyk A. Prenatal exposure to valproic acid induces alterations in the expression and activity of purinergic receptors in the embryonic rat brain. *Folia Neuropathologica* 2022; 60 (4), 390-402.

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

Lidia Babiec, Anna Wilkaniec, Agata Adamczyk

Mossakowski Medical Research Institute, Polish Academy of Sciences, Warsaw, Poland

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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 neurotrans-

mission, 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

Communicating author:

Anna Wilkaniec, PhD, Mossakowski Medical Research Institute, Polish Academy of Sciences, Warsaw, Poland,
e-mail: awilkaniec@imdik.pan.pl

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 phosphatidylinositol-4,5-bisphosphate (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 develop-

ment [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 Fahnstock [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 $\mu\text{g}/\text{ml}$ poly-D-lysine and maintained in culture medium containing Neurobasal™ 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+} ($[\text{Ca}^{2+}]_i$) 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

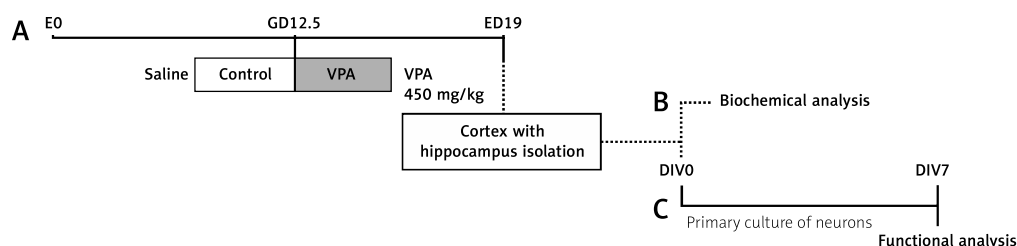


Fig. 1. Experimental design. **A**) At embryonic day 12.5 (ED12.5), either valproic acid (VPA; 450 mg/kg, *i.p.*) or saline was administered intraperitoneally to pregnant rats. Prenatal injection of VPA/saline defined the experimental groups. At embryonic day 19 (ED19), the embryos were dissected and 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 (ATP γ S), α,β -Methylene-adenosine 5'-triphosphate ($\alpha\beta$ meATP), and adenosine 5'-[β -thio]diphosphate trilithium salt (ADP β S) 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 Ca²⁺ responses, the area under the curve (AUC) was calculated as a measure for the increase in intracellular Ca²⁺.

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 Technol-

ogies, 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.

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 $\pm$ 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).

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

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

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 purinergic receptors, 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 down-regulated 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 μ M) (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 μ M) 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 μ M) ($p < 0.0001$) (Fig. 3C). In addition, the results showed that treatment with ATP γ S (100 μ M), the ATP analogue with increased metabolic stability, also caused a significant increase in the $[\text{Ca}^{2+}]_i$ by about 118% ($p = 0.0033$) in VPA cells (Fig. 3D). In order to determine whether the increase in $[\text{Ca}^{2+}]_i$ 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 μ M 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 μ M), a potent agonist of P2X7 receptor (and weak agonist of P2X4). However, a significant decrease in $[\text{Ca}^{2+}]_i$ 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 (ADP β S, 10 μ M, stable ADP analogue) also caused a significant decrease in $[\text{Ca}^{2+}]_i$ 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

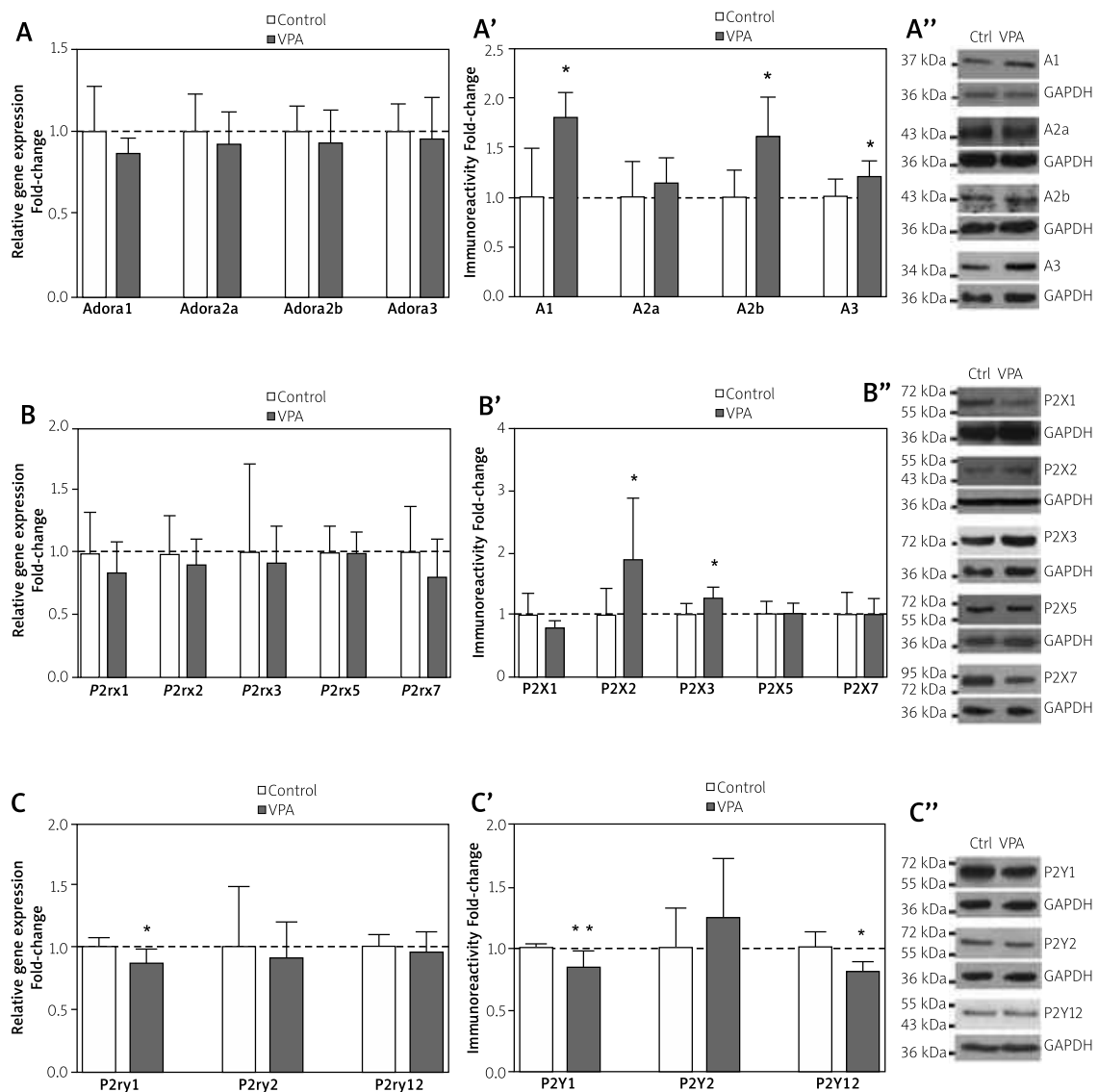


Fig. 2. The impact of prenatal valproic acid (VPA) exposure on the purinergic receptors' expression in the brain of 19-day-old rat embryos. The mRNA level of selected adenosine (A), ionotropic (B), and metabotropic (C) receptors in the cerebral cortex with the hippocampus of rat embryos, which were prenatally exposed to VPA treatment, was measured by real-time PCR, and normalized to Actb (β -actin). Data represent the mean value $\pm$ 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 $\pm$ 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.

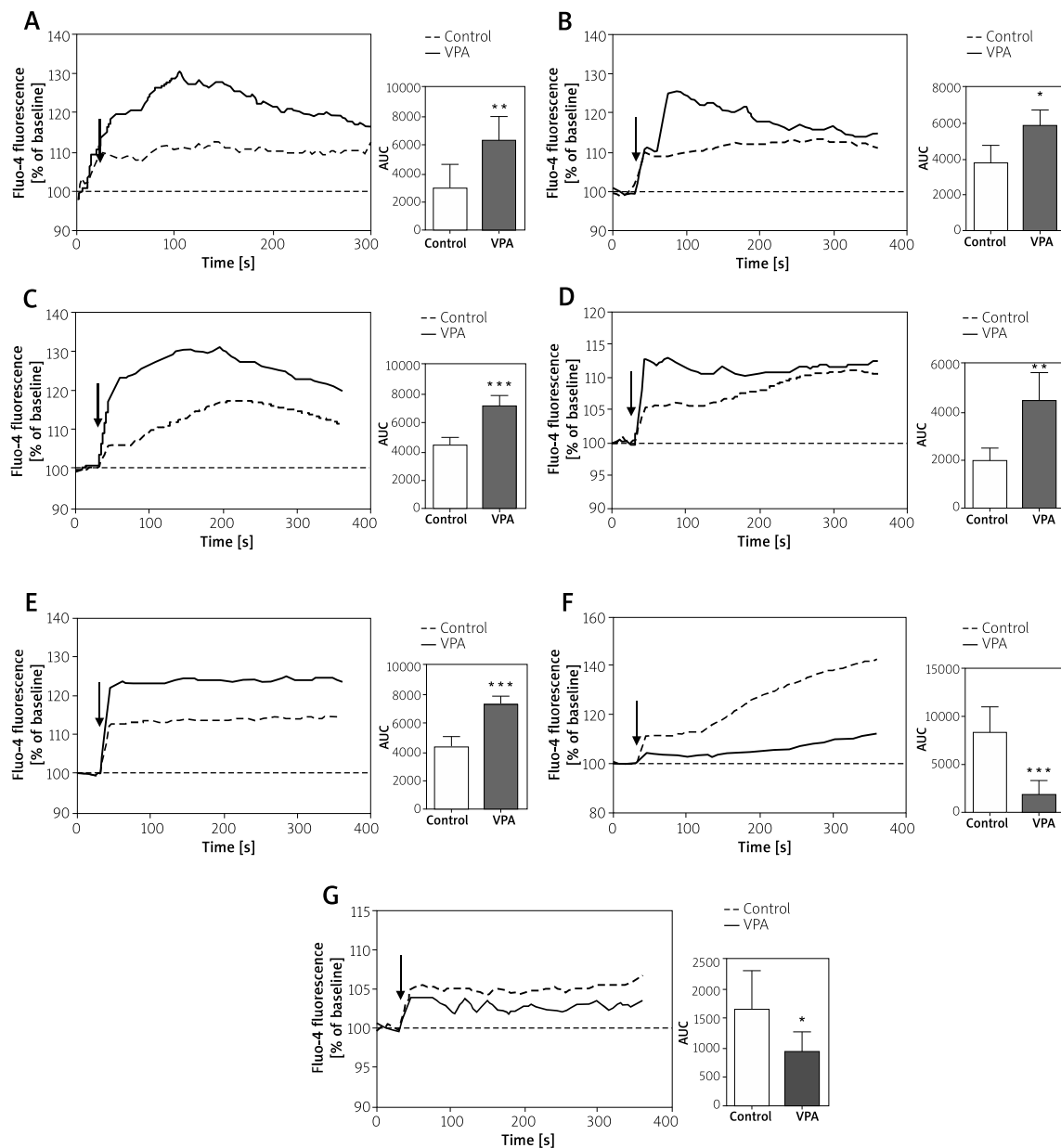


Fig. 3. The influence of selected purinergic receptor activation on intracellular calcium concentrations in the primary neuronal culture. The intracellular calcium concentrations in the culturing primary neurons isolated from the cerebral cortex with the hippocampus of 19-day-old embryos prenatally exposed 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 μ M Adenosine (A), 1 μ M ATP (B), 100 μ M ATP (C), 100 μ M ATP γ S (D), 30 μ M $\alpha\beta$ meATP (E), 300 μ M BzATP (F) or 10 μ M ADP β S (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 $\pm$ 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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11.3. Publikacja II

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Article

Alterations of Purinergic Receptors Levels and Their Involvement in the Glial Cell Morphology in a Pre-Clinical Model of Autism Spectrum Disorders

Lidia Babiec, Anna Wilkaniec ^{*}, Marta Matuszewska , Ewelina Pałasz , Magdalena Cieřlik 
and Agata Adamczyk 

Department of Cellular Signalling, Mossakowski Medical Research Institute, Polish Academy of Sciences, Pawińskiego 5, 02-106 Warsaw, Poland; lbabiec@imdik.pan.pl (L.B.); mmatuszewska@imdik.pan.pl (M.M.); epalasz@imdik.pan.pl (E.P.); mcieslik@imdik.pan.pl (M.C.); aadamczyk@imdik.pan.pl (A.A.)

* Correspondence: awilkaniec@imdik.pan.pl; Tel.: +48-226080600

Abstract: Recent data suggest that defects in purinergic signalling are a common denominator of autism spectrum disorders (ASDs), though nothing is known about whether the disorder-related imbalance occurs at the receptor level. In this study, we investigated whether prenatal exposure to valproic acid (VPA) induces changes in purinergic receptor expression in adolescence and whether it corresponds to glial cell activation. Pregnant dams were subjected to an intraperitoneal injection of VPA at embryonic day 12.5. In the hippocampi of adolescent male VPA offspring, we observed an increase in the level of P2X1, with concomitant decreases in P2X7 and P2Y1 receptors. In contrast, in the cortex, the level of P2X1 was significantly reduced. Also, significant increases in cortical P2Y1 and P2Y12 receptors were detected. Additionally, we observed profound alterations in microglial cell numbers and morphology in the cortex of VPA animals, leading to the elevation of pro-inflammatory cytokine expression. The changes in glial cells were partially reduced via a single administration of a non-selective P2 receptor antagonist. These studies show the involvement of purinergic signalling imbalance in the modulation of brain inflammatory response induced via prenatal VPA exposure and may indicate that purinergic receptors are a novel target for pharmacological intervention in ASDs.

Keywords: purinergic signalling; microglia; autism spectrum disorders; valproic acid



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1. Introduction

Growing evidence demonstrates the involvement of purinergic signalling in brain development and neurotransmission [1,2]. Unlike other neurotransmitter systems, which are characterised by high specificity, the major hallmarks of purinergic signalling are its omnipresence and versatility [3,4]. Not only is ATP secreted from pre- or post-synaptic terminals, as well as from other cells in the central nervous system (CNS) in response to neurotransmitter stimulation, but it is also secreted in response to a variety of other stimuli, like inflammation or cellular damage [5,6]. Moreover, the release of ATP is mediated via various mechanisms, including vesicle exocytosis, depending on the vesicular nucleotide transporter (VNUT) and SNARE complex [7]; the activation of membrane channels (such as connexins, pannexins, P2X7 receptor); or specific transporters [8–11], like ATP-binding cassette (ABC) transporter [8–10]. Unlike classical neurotransmitters, which are inactivated via their reuptake from the synaptic cleft, ATP undergoes rapid enzymatic degradation by cell membrane ectonucleotidases [12]. Each of the products (ATP, ADP, AMP, adenosine) involved in the multistep enzymatic conversion of ATP to adenosine can activate different types of purinergic receptors localized on pre- and post-synaptic membranes [13]. Currently, four receptors of adenosine (P1 type), seven subtypes of ionotropic ATP receptors (P2X), and eight subtypes of metabotropic (P2Y) ATP receptors are recognized, many of which can form heteromultimers [14]. ATP and its end product, adenosine, are important

signalling molecules in both pre-natal and post-natal development [15], regulating the short-term neurotransmission/neuromodulation in the CNS, as well as longer-term effects, including neuronal development and neuroregeneration [14,16–18]. Apart from neurons, purinergic receptors are also present in glial cells, as well as in each of the following cell subtypes: microglia, astrocytes, and oligodendrocytes. These subtypes express a unique pattern of purinergic receptors, which are responsible for orchestrating specific intracellular downstream signalling that leads to specific responses [19]. Moreover, purinergic receptors are indispensable in regulating the specific interaction between glia and neurons that largely extend beyond synapses [3]. Neuronal release of ATP and adenosine during action potential firing was previously shown to evoke calcium waves in astrocytes, Schwann cells, and oligodendrocytes, as well as state the novel mechanism of sensing neuronal activity by glial cells [20,21]. ATP also regulates the activity of microglia, which are the immune cells of the nervous system, leading to rapid changes in their morphology and migration [22,23], as well as triggering cytokine secretion [24]. Glial cells not only form “the scaffold” for neurons but also participate in the synaptogenesis and synaptic plasticity, homeostasis, elimination of used transmitters, facilitation of signal transduction; and in immune system responses [25]. Importantly, astrocytes and microglia strictly cooperate, and purinergic signalling is the main mode of communication between them [3,26–28]. Thus, ATP and adenosine may be indispensable in bidirectional microglia–neuron communication, as they enable glia to detect synaptic function, further propagate the signal through the glial cell network, and influence synaptic function at distant sites. These unique properties greatly expand the significance of purinergic signalling in brain function, especially under pathological conditions accompanying neurodegenerative or neurodevelopmental disorders [29,30]. Many previous data have found defects in nucleoside/nucleotide signalling to be a common denominator of autism spectrum disorders (ASDs). Studies of ASD animal models have indicated that the deregulation of the enzyme activities involved in the control of ATP and adenosine levels may be involved in the impairment of social interaction [31]. Some clinical reports point out that glial cell deregulation is also engaged in pathological processes that may lead to autism since their critical role in enabling brain functions, astrocytes, and microglia are increasingly linked to ASD features [32]. To date, although astrocytes and microglia actively respond to brain homeostasis aberration via activation, which is reflected in morphology change [33,34], most studies on ASD have focused on changes in the number of astroglia markers or the number of glial cells, rather than a thorough analysis of morphological changes in these cells [35]. Recent studies indicate that purine metabolism in glial cells seems to be the most strikingly disturbed pathway in autism [36], though, currently, nothing is known about whether the ASD-related imbalance also occurs at the receptor level. Moreover, despite the detailed role of glial cell function in ASD, little is known about the involvement of particular purinergic receptor subtypes in abnormalities of these cells. Given that different purinergic receptor subtypes may participate in various processes across CNS development, in the present study, we first aimed to investigate the changes in the expression pattern of the selected purinergic receptors in the cortex and hippocampus of rats at three developmental stages: the late prenatal stage (gestational day 19), weanling (postnatal day 25), and adolescence (postnatal day 52). Next, we analysed whether, in the brain of adolescent male autistic-like rats, structure-dependent alterations in particular purinergic receptors’ expression in the brain occur. In addition, we explore whether changes in purinergic signalling may be associated with the activation of glial cells and neuroinflammatory responses.

2. Materials and Methods

2.1. Ethical Statement

All experiments conducted with animals were approved by the Local Ethics Committee for Animal Experimentation in Warsaw, Poland (reference numbers: WAW2/148/2018, WAW2/036/2019, WAW2/128/2021) and were carried out following the EC Council Directive of 24 November 1986 (86/609/EEC), the ARRIVE guidelines, and guidelines published

in the NIH Guide for the Care and Use of Laboratory Animals. Additionally, the principles presented in the “Guidelines for the Use of Animals in Neuroscience Research” by the Society for Neuroscience were followed. We made an effort to use the minimal number of animals necessary to obtain results and proceeded with the animals gently to reduce stress and suffering.

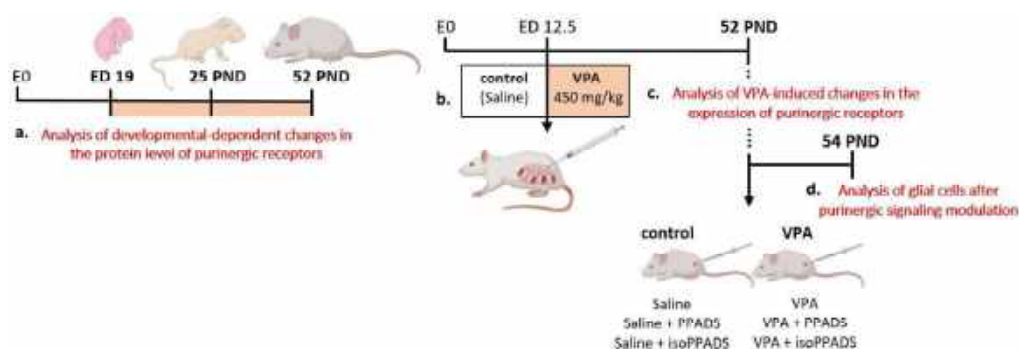
2.2. Animals—In Vivo Model of ASD

We obtained Wistar female rats from the Animal House of the Mossakowski Medical Research Institute, Polish Academy of Sciences (Warsaw, Poland). The rats were aged between 12 and 15 weeks and had a standard weight range of 210–250 g. They were bred according to the SPF standard for small rodents and kept under a 12-h light/dark cycle with access to food and water ad libitum, and the temperature and humidity were strictly controlled.

To determine pregnancy, the presence of a vaginal plug was observed and recorded as embryonic day 0. On gestational day 12.5, pregnant rats received a single intraperitoneal injection of valproic acid (VPA) at a dose of 450 mg/kg of body weight to induce the ASD phenotype [37]. Control dams were given a single injection of saline. All pregnant dams were injected at the same time of the day (noon). To minimize the risk of litter effect, randomly selected animals from at least 2 or 3 litters in each experimental group.

For purinergic receptors analysis, several of the control and VPA dams were sacrificed on embryonic day 19, and foetal brain samples (cerebral cortices with hippocampi) were microdissected. The remaining pregnant rats were allowed to give birth and raise offspring under normal conditions. The day of birth was considered postnatal day (PND) 1. At 22–23 days of age, the rat pups were separated from their mothers and housed in groups of 3–4 individuals in open polycarbonate cages with an enriched environment. Juvenile and adolescent rats (25 PND and 52 PND, respectively) were sacrificed by decapitation. The brains were quickly removed, and two brain parts, the hippocampus and cerebral cortex, were collected and placed in liquid nitrogen.

To analyse the modulation of purinergic signalling, male offspring from the control or VPA groups were divided into three groups at 52 PND. The rats received a single intraperitoneal injection of either PPADS or isoPPADS at a dose of 12.5 mg/kg of body weight. Control rats received the appropriate volume of the vehicle. After 48 h, the rats were sacrificed. Samples for biochemical analysis were collected as mentioned earlier, while for IHC staining, rats were perfused with 4% PFA before decapitation and brain removal. The samples were stored at -80°C until use. Experimental design is shown on the Scheme 1.



Scheme 1. Experimental design. (a) The analysis of changes in the levels of specific purinergic receptors during rat brain development was conducted at three stages: ED 19, 25 PND, and 52 PND.

(b) A Valproic acid-induced model of ASD was performed by a single intraperitoneal injection of VPA (450 mg/kg, i.p.) or saline into pregnant rats. (c) The investigation of VPA-induced alteration on the expression of selected purinergic receptors was performed on the brains of 52-day-old animals. (d) To analyse the modulation of purinergic signalling, rats at 52 PND were divided into three groups and subsequently injected with PPADS (P2X receptor antagonist), isoPPADS (12.5 mg/kg, i.p.) (P2 receptor antagonist), or saline. Analysis of glial cells and inflammatory cytokine expression was performed on the brains of 54-day-old animals. The figure was created using BioRender.com.

2.3. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

To isolate RNA from the brain samples, we used TRI-reagent (Sigma-Aldrich, St. Louis, MO, USA). The extraction procedure was carried out according to the manual provided by the manufacturer. RNA quantity and quality were analysed with spectrophotometer measurements using NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). Subsequently, we digested the remaining trace amounts of DNA with DNase I (Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer's instructions.

For reverse transcription, we utilized the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer's instructions. The mRNA level of the selected purinergic receptors was measured using real-time PCR, employing the TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) listed in Table 1. The analysis was performed on the ABI PRISM 7500 apparatus. *Actb* was used as the reference gene, and each sample was analysed in three technical replicates. The $\Delta\Delta C_t$ method was employed to calculate the relative changes in mRNA levels, and the results were expressed as RQ.

Table 1. A detailed list of used TaqMan Gene Expression Assays for qRT-PCR.

Receptor Gene Symbol	Assay ID	Cat. No.
<i>Adora1</i>	Rn00567668_m1	4331182
<i>Adora2a</i>	Rn00583935_m1	4331182
<i>Adora2b</i>	Rn00567697_m1	4331182
<i>Adora3</i>	Rn00563680_m1	4331182
<i>P2rx1</i>	Rn00564454_m1	4331182
<i>P2rx2</i>	Rn00586491_m1	4331182
<i>P2rx3</i>	Rn00579301_m1	4331182
<i>P2rx4</i>	Rn00580949_m1	4331182
<i>P2rx5</i>	Rn00589966_m1	4331182
<i>P2rx7</i>	Rn00570451_m1	4331182
<i>P2ry1</i>	Rn00562996_m1	4331182
<i>P2ry2</i>	Rn02070661_s1	4331182
<i>P2ry12</i>	Rn02133262_s1	4331182
<i>Il6</i>	Rn01410330_m1	4331182
<i>Tnf</i>	Rn99999017_m1	4331182
<i>Actb</i>	Rn00667869_m1	4331182

2.4. Western Blot Analysis

To determine the protein level of selected purinergic receptors, we employed the Western blotting method. After homogenising the tissue samples, we mixed them with a Laemmli buffer. Denaturation was carried out at 95 °C for 5 min. Proteins were separated using standard 10% or 15% SDS-PAGE gels. Subsequently, the proteins were transferred to nitrocellulose membranes using the “wet” transfer method at 50 V for 2 h.

Immunodetection of specific antibodies began with a 5-min wash in TBS-T (Tris-buffered saline with Tween 20 buffer: 100 mM Tris, 140 mM NaCl, and 0.1% Tween 20, pH 7.6). To block non-specific binding, we incubated the membranes with a 5% BSA solution in TBS-T or a 5% non-fat milk solution in TBS-T for 1 h at room temperature (RT). The membranes were then incubated with the primary antibodies, and detailed protocols can be found in Table 2. After three washes with TBS-T, the membranes were incubated with appropriate secondary antibodies for 1 h at RT, followed by another 3 washes with TBS-T.

Table 2. A detailed list of used antibodies and employed protocols for Western blotting.

Receptor	Company	Cat. No.	Conditions
A1	Thermo Fischer, Carlsbad, CA, USA	PA1041A	Blocking: 5% milk in TBS-T, 1 h at RT I-ab 1:500 in 5% milk in TBS-T, 2 h at RT II-ab anti-rabbit 1:5000 in 5% milk in TBS-T, 1 h at RT
A2a	Sigma-Aldrich, St. Louis, MO, USA	AB1559P	Blocking: 2% BSA in TBS-T, 1 h at RT I-ab 1:200 in 2% BSA in TBS-T, overnight at 4 °C II-ab anti-rabbit 1:4000 in 5% milk in TBS-T, 1 h at RT
A2b	Sigma-Aldrich, St. Louis, MO, USA	AB1589P	Blocking: 2% BSA in TBS-T, 1 h at RT I-ab 1:250 in 2% BSA in TBS-T, overnight at 4 °C II-ab 1:4000 in 5% milk in TBS-T, 1 h at RT
A3	Sigma-Aldrich, St. Louis, MO, USA	AB1590P	Blocking: 5% BSA in TBS-T, 1 h at RT I-ab 1:500 in 2% BSA in TBS-T, overnight at 4 °C II-ab 1:4000 in 5% milk in TBS-T, 1 h at RT
P2X1	Thermo Fischer, Carlsbad, CA, USA	APR-001	Blocking: 5% BSA in TBS-T, 1 h at RT I-ab 1:500 in 5% BSA in TBS-T, overnight at 4 °C II-ab 1:4000 in 5% milk in TBS-T, 1 h at RT
P2X2	Thermo Fischer, Carlsbad, CA, USA	PA5-119683	Blocking: 5% BSA in PBS, 1 h at RT I-ab 1:500 in 5% BSA in PBS, 2 h at RT II-ab 1:5000 in 5% milk in TBS-T, 1 h at RT
P2X3	Sigma-Aldrich, St. Louis, MO, USA	P0121	Blocking: 2% BSA in TBS-T, 1 h at RT I-ab 1:200 in 2% BSA in TBS-T, overnight at 4 °C II-ab 1:4000 in 5% milk in TBS-T, 1 h at RT
P2X4	Alomone Labs, Jerusalem, Israel	APR-024	Blocking: 5% milk in TBS-T, 1 h at RT I-ab 1:400 in 5% BSA in TBS-T, overnight at 4 °C II-ab 1:4000 in 5% milk in TBS-T, 1 h at RT
P2X5	Alomone Labs, Jerusalem, Israel	APR-027	Blocking: 5% milk in TBS-T, 1 h at RT I-ab 1:400 in 5% BSA in TBS-T, overnight at 4 °C II-ab 1:4000 in 5% milk in TBS-T, 1 h at RT
P2X7	Alomone Labs, Jerusalem, Israel	APR-008	Blocking: 5% BSA in TBS-T, 1 h at RT I-ab 1:500 in 5% BSA in TBS-T, overnight at 4 °C II-ab 1:4000 in 5% milk in TBS-T, 1 h at RT
P2Y1	Sigma-Aldrich, St. Louis, MO, USA	P-6487	Blocking: 5% milk in TBS-T, 1 h at RT I-ab 1:100 in TBS-T, overnight at 4 °C II-ab 1:4000 in 5% milk in TBS-T, 1 h at RT
P2Y2	Alomone Labs, Jerusalem, Israel	APR-010	Blocking: 5% milk in TBS-T, 1 h at RT I-ab 1:100 in 2% BSA, overnight at 4 °C II-ab 1:4000 in 5% milk in TBS-T, 1 h at RT
P2Y12	Thermo Fischer, Carlsbad, CA, USA	702516	Blocking: 5% BSA in TBS-T, 1 h at RT I-ab 1:1000 in 5% milk in TBS-T, overnight at 4 °C II-ab 1:1000 in 5% milk in TBS-T, 1 h at RT
GFAP	Abcam, Cambridge, UK	ab53554	Blocking: 5% milk in TBS-T, 1 h at RT I-ab 1:750 in TBS-T, overnight at 4 °C II-ab 1:4000 in 5% milk in TBS-T, 1 h at RT
Iba-1	Cell Signaling, USA	17198	Blocking: 5% milk in TBS-T, 1 h at RT I-ab 1:1000 in TBS-T, overnight at 4 °C II-ab 1:4000 in 5% milk in TBS-T, 1 h at RT
HRP-GAPDH	Proteintech, Rosemont, IL, USA	HRP-60004	Blocking: 5% milk in TBS-T, 1 h at RT I-ab 1:50,000 in 5% milk in TBS-T, 1 h at 4 °C
Goat anti-rabbit	Proteintech, Rosemont, IL, USA	SA00001-2	1:4000 or 1:1000 in 5% milk in TBS-T, 1 h at RT

The detection of antibodies was performed using a chemiluminescent reaction and the Clarity Western ECL Substrate (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. After stripping, the immunolabelling of GAPDH as a loading control was performed. Densitometric analysis was conducted using TotalLab v1.11 software.

2.5. Immunohistochemistry Staining

The animals were deeply anaesthetized with ketamine and transcardially perfused with cold (4 °C) phosphate-buffered saline (PBS), followed by 4% paraformaldehyde (PFA) in PBS. The brains were then removed and placed in 4% PFA for 24 h, followed by immersion in 30% sucrose for cryoprotection. The brains were frozen on dry ice and stored at −80 °C.

For double immunohistochemical staining, the animal brains from the control and VPA groups at 52 PND were used, while for single staining, the animal brains from the control, control + PPADS, control + isoPPADS, VPA, VPA + PPADS, and VPA + isoPPADS groups at 54 PND were used. Staining was performed on 40 µm coronal sections of the hippocampus and cerebral cortex.

For double staining, the sections were first incubated with primary antibodies against MAP-2, GFAP, or Iba-1 for 1 h at RT and overnight at 4 °C. On the following day, the sections were washed in PBS (3 × 5 min) and incubated with the following primary antibodies: P2X1 and P2Y1. Then, they were directly labelled with an ATTO-488 fluorescent dye, or P2X7 and P2Y12. For single staining, primary antibodies against Iba-1 and GFAP were used.

The next day, the sections were washed in PBS (3 × 5 min), and incubated for 1 hour at RT with the appropriate secondary antibody conjugated with Alexa Fluor 594. The secondary antibody was diluted to working concentrations in PBS with 1% BSA, and 0.3% Triton X100. To prevent photobleaching, incubation with fluorescent antibodies was conducted in the dark. The concentration and conditions of the antibodies are indicated in Table 3. Finally, after washing (3 × PBS), the sections were transferred onto microslides. The slides were coated with Vectashield Vibrance mounting medium with DAPI, which counterstained cell nuclei, and covered with a coverslip.

Table 3. Detailed list of used antibodies and employed protocols for immunohistochemistry.

Receptor	Company	Cat. No.	Conditions
P2X1	Alomone Labs, Jerusalem, Israel	APR-022-AG	I-ab 1:50 in PBS containing 1% BSA, 0.3% Triton X; incubation for 1 h at RT, and overnight at 4 °C
P2X7	Alomone Labs, Jerusalem, Israel	APR-004	I-ab 1:100 in PBS containing 1% BSA, 0.3% Triton X; incubation for 1 h at RT, and overnight at 4 °C
P2Y1	Alomone Labs, Jerusalem, Israel	APR-021-AG	I-ab 1:50 in PBS containing 1% BSA, 0.3% Triton X; incubation for 1 h at RT, and overnight at 4 °C
P2Y12	Thermo Fischer, Carlsbad, CA, USA	702516	I-ab 1:200 in PBS containing 1% BSA, 0.3% Triton X; incubation for 1 h at RT, and overnight at 4 °C
MAP-2	Invitrogen, Carlsbad, CA, USA	AP18	I-ab 1:1000 in PBS containing 1% BSA, 0.3% Triton X; incubation for 1 h at RT, and overnight at 4 °C
GFAP	Abcam, Cambridge, UK	ab53554	I-ab 1:500 in PBS containing 1% BSA, 0.3% Triton X; incubation for 1 h at RT, and overnight at 4 °C
Iba-1	Abcam, Cambridge, UK	ab5076	I-ab 1:500 in PBS containing 1% BSA, 0.3% Triton X; incubation for 1 h at RT, and overnight at 4 °C

Images were obtained at the Laboratory of Advanced Microscopy Techniques, Mossakowski Medical Research Institute, Polish Academy of Sciences, using a confocal laser scanning microscope LSM780 (Zeiss, Jena, Germany) with z-stack acquisition using a 10× objective. For the analysis of glial cell morphology, a 40× objective was utilized, as described in [38].

Quantitative analysis of astrocytic and microglial processes, cell bodies, and Sholl analysis were performed following the method outlined by Sanagi et al. [39] with modifications. Five microglia or astrocytes were randomly selected per section, and their values were averaged, except for astrocytes in the hippocampus, for which we averaged five cells derived from each of the three hippocampal layers.

To analyse the images of astrocytes or microglia, the images were converted to an 8-bit format, and the dendrites of individual cells were traced using the NeuronJ plugin for Fiji [40]. The surface area of the cell body, the surface area of the dendritic tree, the number of primary dendrites (those originating from the cell soma), the number of secondary dendrites, and the total length of processes belonging to each glial cell were analysed.

The branches of individual glial cells were determined using the Sholl method [41]. Each cell was analysed by selecting the centre of its body, and then the number of intersections at circles of increasing diameter from the centre was counted using the SNT plugin for Fiji. The diameters of all processes were measured at intervals of 2.5 µm.

2.6. Statistical Analysis

All results are presented as the mean values ± standard deviation (SD). To analyse differences between the average values, we used a Student's *t*-test (between two groups) or one-way analysis of variance (ANOVA) with a Bonferroni comparison post hoc test (between multiple groups). Statistical significance was considered at $p < 0.05$. The analysis was conducted using Graph Pad Prism version 6.0 (Graph Pad Software, San Diego, CA, USA).

3. Results

3.1. The Expression of Selected Purinergic Receptors during Brain Development

To gain insight into the changes in purinergic receptor expression at different stages of central nervous system (CNS) development, the protein levels of specific receptors were evaluated in the cortex with hippocampus isolated from the foetal brain (ED19), as well as in the cerebral cortex and hippocampus isolated from 25-day-old (weanling, PND 25), and 52-day-old (young adult, PND 52) rats. An average of four animals from two litters were used for each data point.

It was observed that all subtypes of adenosine receptors were present in the embryonic brain, albeit to varying extents, and their expression continued into adulthood (Figure 1). The A1 receptor showed strong expression in the embryonic brain, with a subsequent decline in expression (Figure 1a). The expression of the A2a receptor was lowest at ED19 and significantly increased in the cortex and hippocampus of rats at PND 25, as well as in the hippocampus at PND 52 (Figure 1b). In adulthood, the level of the A2a receptor significantly decreased in the brain cortex compared to the hippocampus (Figure 1b). Furthermore, the A2a receptor was significantly upregulated in the brain cortex of weanling animals compared to young adult rats. This may be related to the important role of A2a receptors in controlling glutamatergic long-term potentiation (LTP) [42] during the enhanced synaptic plasticity observed during the weanling period in rats [43]. The level of the A2b receptor for adenosine remained constant thorough the investigated developmental stages, with a slight increase observed in the hippocampus of 25-day-old animals (Figure 1c). The A3 receptor level was lowest in the embryonic brain but significantly increased at PND 25 and remained constant until adulthood (Figure 1d).

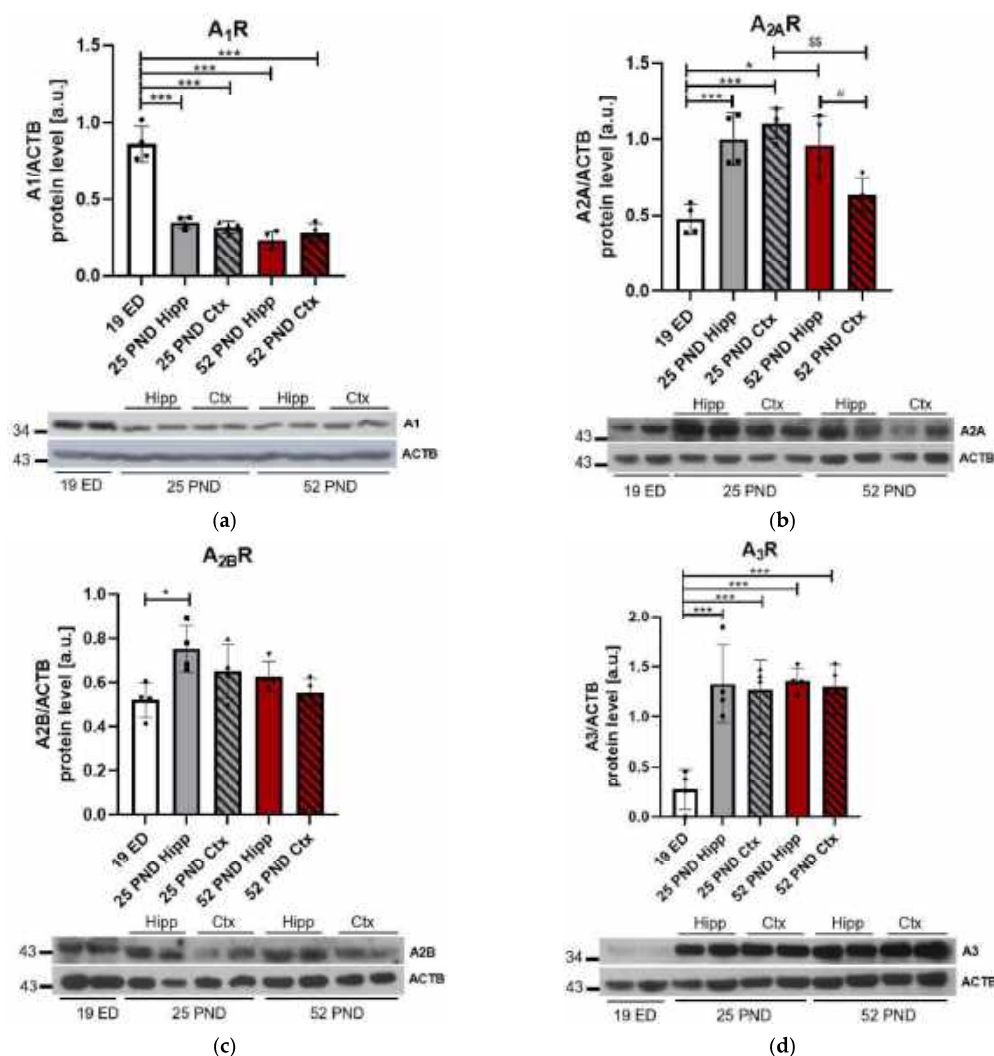


Figure 1. Protein expression of adenosine receptors during rat brain development. The immunoreactivity of A1 (a), A2a (b), A2b (c), and A3 (d) receptors in the brain of 19-day-old rat embryos, as well as in the hippocampus (Hipp) and cortex (Ctx) of 25-day-old and 52-day-old rats, was determined using Western blotting. Actin (ACTB) was used as a loading control. The data represent the mean value $\pm$ SD from four independent experiments. * $p < 0.05$, ** $p < 0.01$ vs. ED19, \$\$\$ $p < 0.001$ vs. cortex PND 25, # $p < 0.05$ vs. hippocampus PND 52, as determined using one-way ANOVA followed by the Bonferroni post hoc test.

From the investigated ionotropic receptors for ATP, the level of the P2X1 receptor remained constant throughout rat brain development, with a minor increase observed in the cortex of 52-day-old animals (Figure 2a). The P2X2 receptor exhibited the lowest expression in the embryonic brain, and its immunoreactivity significantly increased in the hippocampus of 25-day-old and young adult animals. P2X2 receptor was also significantly upregulated in the hippocampi of both weanling and young adult animals when compared to cortices at their respective ages (Figure 2b). A marked predominance of the P2X3 receptor

was observed in the foetal brain, followed by a substantial decline during postnatal periods (Figure 2c). In contrast, the P2X4 receptor was not detected at ED19, but its expression was observed in the brains of weanling rats, with the highest level observed in the cortex of 52-day-old animals (Figure 2d).

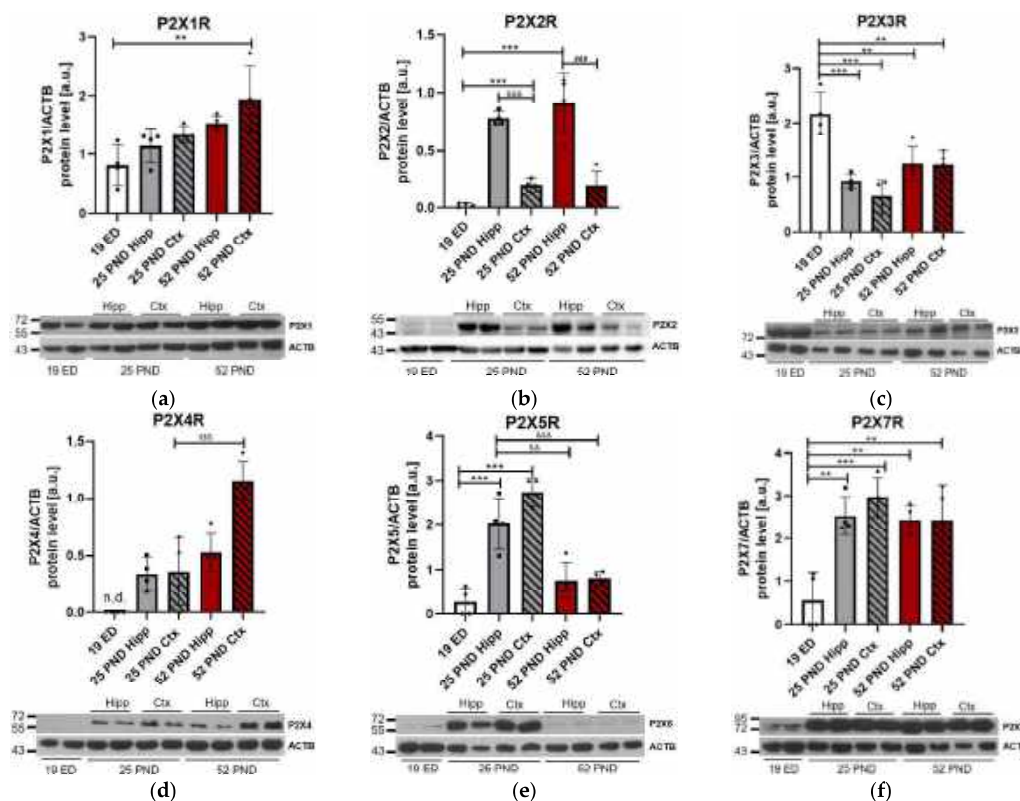


Figure 2. Ionotropic purinergic receptor protein expression during rat brain development. The immunoreactivity of P2X1 (a), P2X2 (b), P2X3 (c), P2X4 (d), P2X5 (e), and P2X7 (f) receptors in the brain of 19-day-old rat embryos, as well as in the hippocampus (Hipp) and cortex (Ctx) of 25- and 52-day-old rats, was determined using Western blotting. Actin (ACTB) was used as a loading control. The data represent the mean value $\pm$ SD from four independent experiments. ** $p < 0.01$, *** $p < 0.001$ vs. ED19, && $p < 0.01$, &&& $p < 0.001$ vs. hippocampus PND 25, \$\$\$ $p < 0.001$ vs. cortex PND 25, ### $p < 0.001$ vs. hippocampus PND 52, as determined using one-way ANOVA followed by the Bonferroni post hoc test; n.d.—not detected.

The P2X5 and P2X7 proteins exhibited the lowest expression at ED19. While a significant elevation in the P2X5 receptor expression level was observed in both the hippocampus and cortex of weanling animals, the protein expression of this receptor significantly declined at PND 52 (Figure 2e). The functional role of the P2X5 receptor in the CNS has been previously demonstrated to be widely distributed in adult rodents [44], in contrast to humans, where it occurs as a nonfunctional splice variant [45]. Therefore, the peak expression of this receptor during weaning may suggest its important role during brain maturation. Interestingly, several reports have linked P2X5 receptor expression with the differentiation and turnover of various non-neuronal cells, as well as in different cancer

tissues [46]. However, to date, no data are available on the functional role of this receptor in the CNS.

On the other hand, the P2X7 receptor was found to be abundantly present in the brain tissue of both 25-day-old and young adult animals (Figure 2f).

From the large group of metabotropic purinergic receptors, we selected P2Y1, P2Y2, and P2Y12 for our investigations based on their role in synaptic transmission, regulation of voltage-gated ion channels, modulation of neuronal circuits, gliotransmission, regulation of inflammatory response, and synaptic plasticity [47]. Moreover, those receptors have also been shown to regulate the release of neurotransmitters, including glutamate, in the synapses of the hippocampus and cerebral cortex [48], as well as to inhibit the excitatory transmission mediated by postsynaptic NMDA receptors and to increase the inhibitory action of the GABA receptors, promoting LTP [47,49]. We observed that all investigated P2Y receptor subtypes were expressed in the embryonic tissue. The levels of both P2Y1 and P2Y12 receptors remained constant in all investigated developmental periods (Figure 3a,c). In contrast, the level of the P2Y2 receptor slightly increased in the hippocampus of 25-day-old animals, as well as in the cortex of 52-day-old rats (Figure 3b).

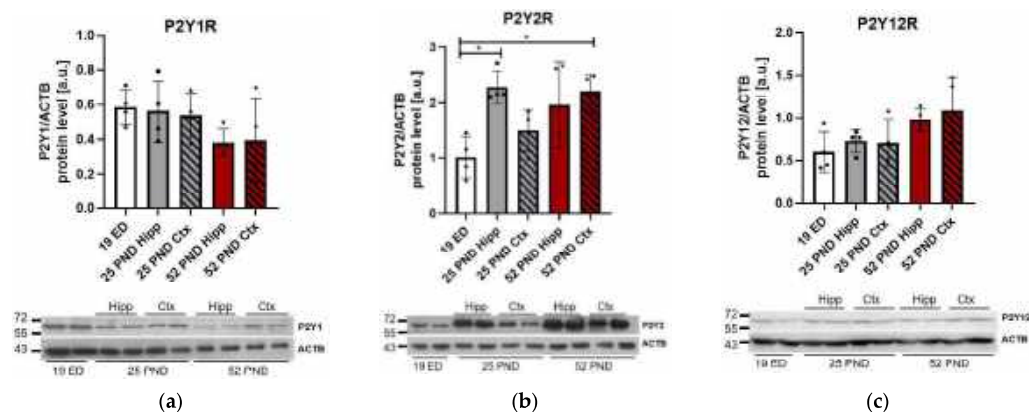


Figure 3. Metabotropic purinergic receptor protein expression during rat brain development. The immunoreactivity of P2Y1 (a), P2Y2 (b), and P2Y12 (c) receptors in the brain of 19-day-old rat embryos, as well as in the hippocampus (Hipp) and cortex (Ctx) of 25- and 52-day-old rats, was determined using Western blotting. Actin (ACTB) was used as a loading control. The data represent the mean value $\pm$ SD from four independent experiments. * $p < 0.05$ vs. ED19, as determined using one-way ANOVA followed by the Bonferroni post hoc test.

3.2. The Impact of Prenatal Exposure to Valproic Acid (VPA) on the mRNA, Protein Expression Patterns, and Immunolocalization of Purinergic Receptors Subtypes in the Brain of 52-Day-Old Animals

Prenatal exposure to VPA produces relevant autistic-like phenotypes in rat offspring, and the specific implementation of this model was recently validated in our study to induce pathological alterations in synapse ultrastructure and function, as well as to cause aberrant behavioural outcomes in offspring [50]. Using this model, we have previously demonstrated the disturbances in purinoreceptors levels occurring at the prenatal stage in animals subjected to VPA treatment [51]. Additionally, the activity of selected purinergic receptor subtypes was significantly altered in neuronal cells isolated from embryonic tissue, suggesting their strong involvement in improper brain development during the early stage of brain formation. In this study, we investigated whether significant alteration in purinergic receptor levels also occurs during the developmental period of late adolescence (PND 52), a critical phase for the exacerbation of autism-related symptoms.

Pregnant rats were injected with VPA or saline (control) during midgestation (ED12.5), and cortices and hippocampi from the offspring at PND 52 were collected. The mRNA levels and protein expression of selected P1, P2X, and P2Y receptors were measured as described above and compared to the matched control samples. Five to six animals from at least three litters were used. Average values were compared between saline and VPA offspring for each receptor in the selected brain regions. For immunohistochemistry staining, we investigated the abundance of only those receptors in which we observed the most prominent changes in the brains of animals prenatally exposed to VPA. Three animals from at least two litters were included for analysis. Images from the brains of VPA offspring were analysed and compared to control samples for each selected receptor in the prefrontal cortex and hippocampus of 52-day-old animals.

We analysed the expression of adenosine receptors in the brains of animals prenatally exposed to VPA. As shown in Figure 4, the mRNA levels for adenosine receptors in both the hippocampus (Figure 4a) and cortex (Figure 4b) did not change after VPA prenatal exposure compared to the control. Additionally, the analysis of protein levels in the investigated brain structures showed no alterations (Figure 4c,d).

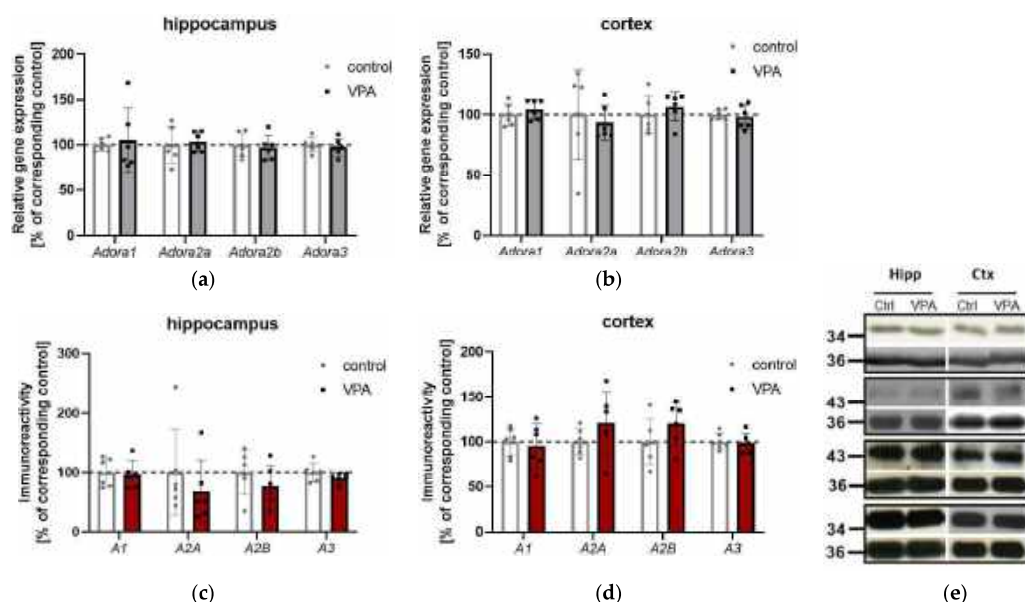


Figure 4. The effect of prenatal treatment with VPA on the gene expression and protein levels of adenosine receptors in the hippocampus and cortex of 52-day-old rats. The mRNA levels of adenosine receptors in the hippocampus (a) and cortex (b) of young adult animals prenatally exposed to VPA treatment were measured by real-time PCR and normalized to Actb (β -actin). Data are shown as a percentage of corresponding control and represent the mean value $\pm$ SD for 5–6 independent experiments. The immunoreactivity of adenosine receptors in the hippocampus (c) and cortex (d) of young adult animals prenatally exposed to VPA treatment was analysed by Western blotting. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. Representative pictures are shown (e). Data are shown as a percentage of the corresponding control and represent the mean value $\pm$ SD for 5–6 independent experiments. Statistical analysis was determined using the Student's *t*-test.

The expression of ionotropic receptors in the brain, caused by prenatal VPA treatment, was distinct and region-specific (Figure 5). In the hippocampus, the P2rx1 mRNA significantly increased, whereas the mRNA level of P2rx7 was lower in this brain structure

at PND 52 (Figure 5a). These results are consistent with the analysis of protein levels, where we detected an increase in P2X1 and a significant decrease in P2X7 (Figure 5c). In contrast, in the cortex of animals prenatally exposed to VPA, the mRNA level of the ionotropic P2rx1 decreased, and the P2rx7 remained unchanged, but there was an increase in the expression of P2rx3 (Figure 5b). The significant decrease in P2X1 and the elevation of the P2X3 receptor in the cortex of young adult VPA rats were also confirmed at the protein level (Figure 5d). Prenatal exposure to VPA treatment did not influence the expression and protein levels of P2X2, P2X4 and P2X5 receptors in the analysed brain structures (Figure 5a–d). In the following studies, based on the previous data concerning the relevance of particular ionotropic purinergic receptors to brain function [52,53], we also analysed the co-localization of P2X1 and P2X7 receptors on neurons and microglial cells (the nonoverlapping images for the particular photograph are included in Supplementary Figures S1–S6). As shown in Figure 5f, the P2X1 (green immunostaining) co-localized predominantly with neuronal MAP-2-positive cells of either the prefrontal cortex or DG and CA regions of the hippocampus. Basal P2X1 expression in the GFAP-stained astrocytes in the prefrontal cortex and hippocampal areas was low, whereas positive co-expression of the P2X1 with the glial marker Iba-1 was not visible. Prenatal treatment with VPA produced a noticeable increase in P2X1 immunoreactivity in the neuronal cells in the hippocampal areas, while P2X1 neuronal staining was visually decreased in the prefrontal cortex. We did not observe any VPA-induced changes in P2X1 expression in GFAP-stained astrocytes.

The immunohistochemical staining of P2X7 revealed that this receptor is abundant in the perikarya of granule cells in the CA and DG regions of the hippocampus, as well as in the neurons of the prefrontal cortex. The immunostaining of this receptor was also observed in the microglial processes, but it was not visualized on the astrocytes. In both regions of the hippocampus examined, the immunostaining of this receptor visually decreased in the microglial cells of animals treated with VPA, but its abundance on neuronal cells appeared unchanged in the investigated brain areas (Figure 5g).

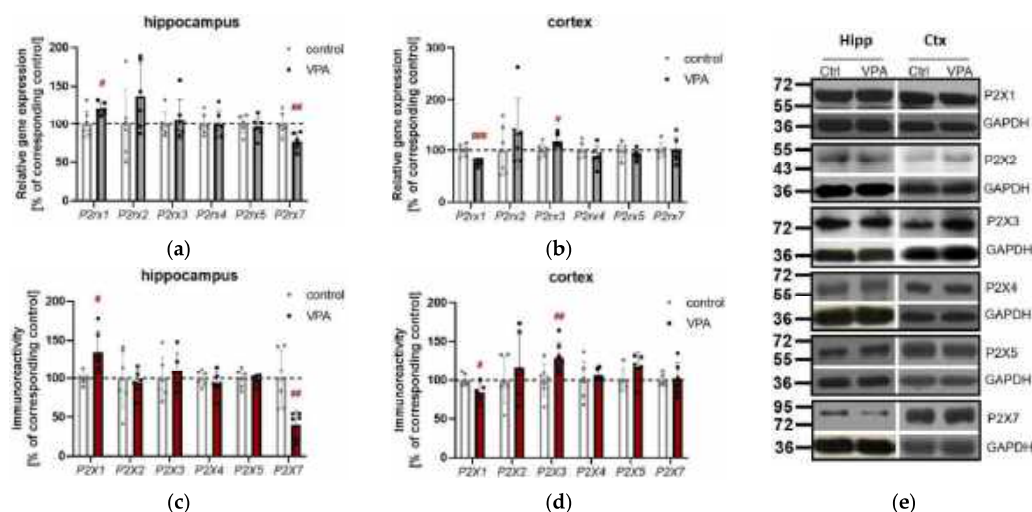


Figure 5. Cont.

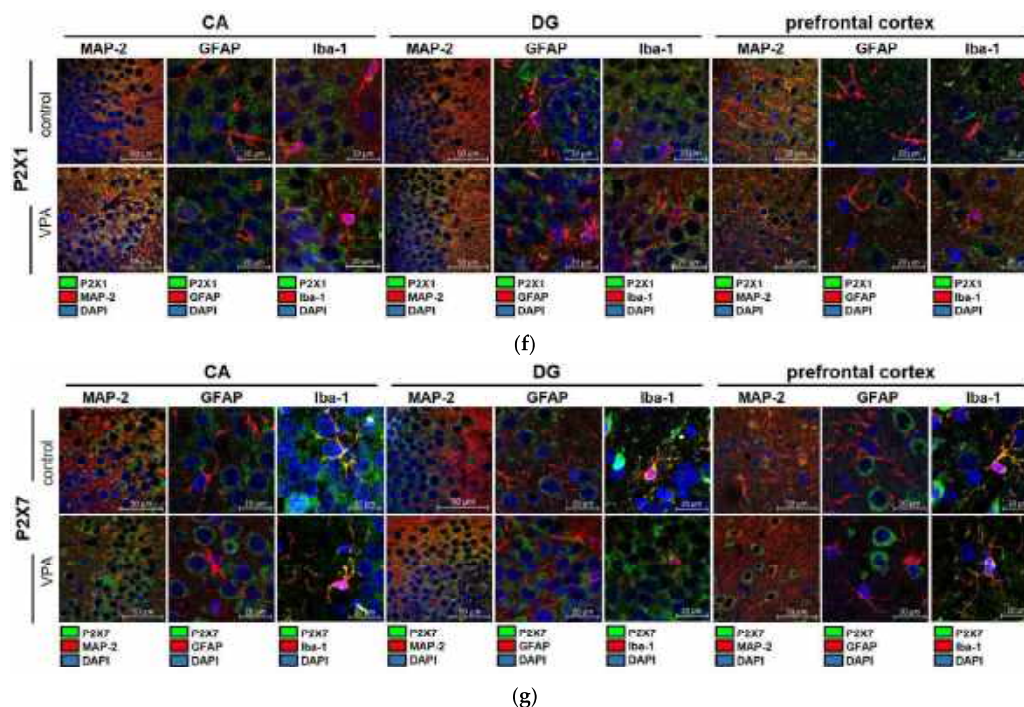


Figure 5. The effect of prenatal treatment with VPA on the gene expression, protein level, and immunolocalization of selected ionotropic receptors in the hippocampus and cortex of 52-day-old rats. The mRNA levels of adenosine receptors in the hippocampus (a) and cortex (b) of young adult animals prenatally exposed to VPA treatment were measured by real-time PCR and normalized to Actb (β -actin). Data are shown as a percentage of the corresponding control and represent the mean value $\pm$ SD for 5–6 independent experiments; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. corresponding control, as determined using the Student's *t*-test. The immunoreactivity of ionotropic receptors in the hippocampus (c) and cortex (d) of young adult animals prenatally exposed to VPA treatment was analysed by Western blotting. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. Representative pictures are shown (e). Data are presented as a percentage of corresponding control and represent the mean value $\pm$ SD for 5–6 independent experiments; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. corresponding control, as determined using the Student's *t*-test. The immunohistochemical analysis of co-localisation of the P2X1 receptor (green) (f) in the neurons (MAP-2, red), astrocytes (GFAP, red) and the glial cells (Iba-1, red) (f), as well as the analysis of co-localisation of the P2X7 receptor (green) (g) in the neurons (MAP-2, red), astrocytes (GFAP, red), and the glial cells (Iba-1, red) (g), was determined in the cortex, dentate gyrus (DG), and cornu ammonis (CA) of the hippocampus. The nuclei were counterstained with DAPI (blue). Scale bar = 50 μ m for MAP-2 and 20 μ m for GFAP and Iba-1.

Regarding the metabotropic receptors, we observed a significant decrease in the mRNA level of P2ry1 in the hippocampus of young adult rats following VPA exposure (Figure 6a), which was confirmed by the lower protein level for this receptor (Figure 6c). In the cerebral cortex, however, we observed opposite alterations: a substantial increase in the mRNA of P2ry1. Moreover, we observed an increased mRNA level of the P2ry12 receptor (Figure 6b). These abnormalities in the cortex were confirmed by the protein level for those receptors (Figure 6d).

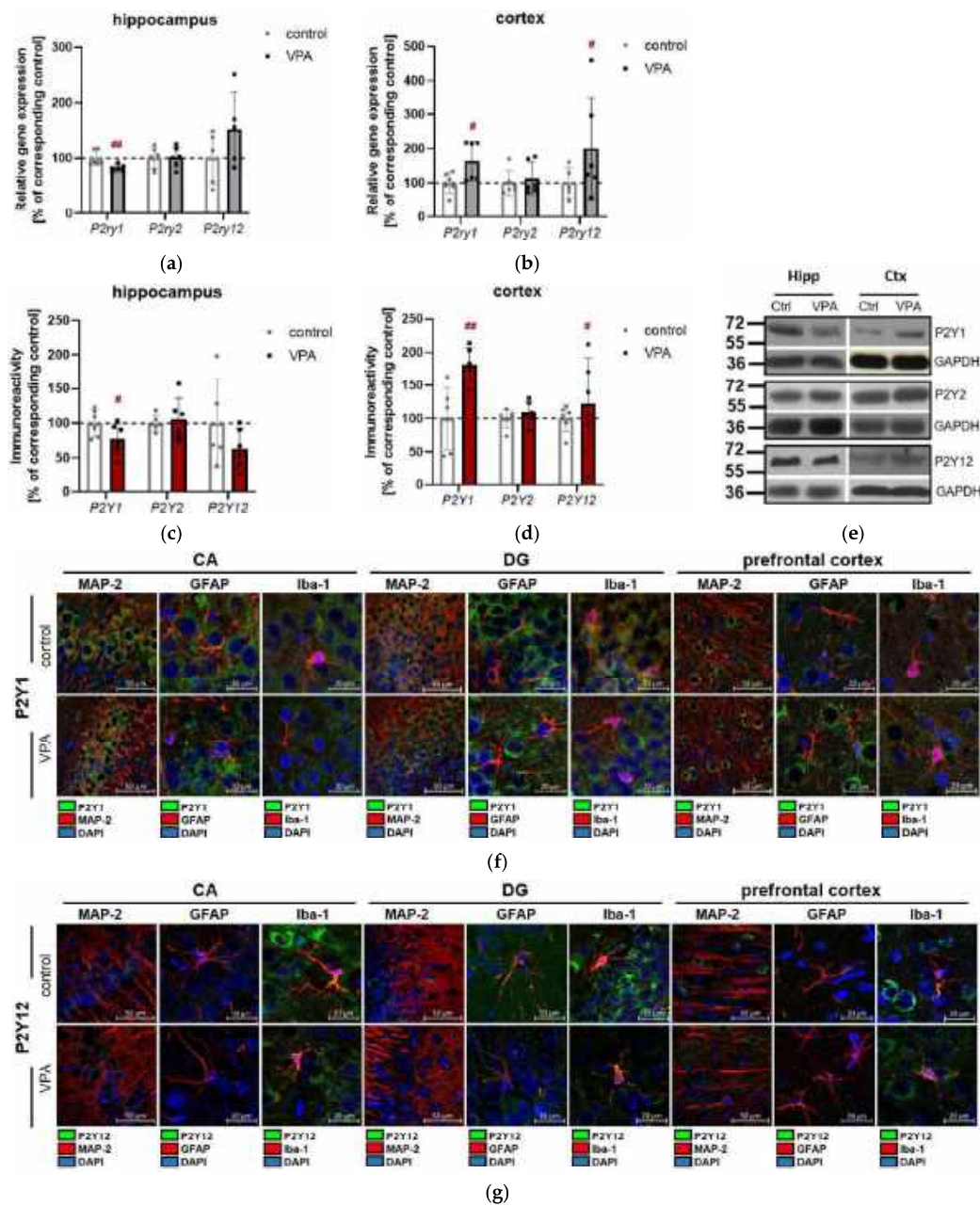


Figure 6. The effect of prenatal treatment with VPA on the gene expression, protein level, and immunolocalization of selected metabotropic receptors in the hippocampus and cortex of 52-day-old

rats. The mRNA levels of metabotropic receptors in the hippocampus (a) and cortex (b) of young adult animals prenatally exposed to VPA treatment were measured by real-time PCR and normalized to Actb (β -actin). Data are shown as a percentage of the corresponding control and represent the mean value $\pm$ SD for 5–6 independent experiments; # $p < 0.05$, ## $p < 0.01$ vs. corresponding control as determined using the Student's *t*-test. The immunoreactivity of metabotropic receptors in the hippocampus (c) and cortex (d) of young adult animals prenatally exposed to VPA treatment was analysed by Western blotting. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. Representative pictures are shown (e). Data are presented as a percentage of the corresponding control and represent the mean value $\pm$ SD for 5–6 independent experiments; # $p < 0.05$, ## $p < 0.01$ vs. corresponding control as determined using the Student's *t*-test. The immunohistochemical analysis of co-localisation of the P2Y1 receptor (green) (f) in the neurons (MAP-2, red), astrocytes (GFAP, red) and the glial cells (Iba-1, red) (f), as well as the analysis of co-localisation of the P2Y12 receptor (green) (g) in the neurons (MAP-2, red), astrocytes (GFAP, red), and the glial cells (Iba-1, red) (g), was determined in the cortex, dentate gyrus (DG), and cornu ammonis (CA) of the hippocampus. The nuclei were counterstained with DAPI (blue). Scale bar = 50 μ m for MAP-2 and 20 μ m for GFAP and Iba-1.

The analysis of P2Y1 immunostaining in the investigated brain areas showed its co-expression with MAP-positive neurons, whereas the co-expression of P2Y1 with glial marker Iba-1 was not visible. Surprisingly, weak colocalization was observed with the astrocyte marker GFAP, both under control conditions and after treatment with VPA (Figure 6f, Supplementary Figure S2). The specificity of the antibody was confirmed by the absence of immunoreactivity in tissue slices pretreated with the blocking peptide (ALOMONE) (Supplementary Figure S13).

The analysis of P2Y12 co-localisation revealed its predominant presence in the rat hippocampus and prefrontal cortex microglial cells. The immunoreactivity of this receptor was not observed in GFAP-stained astrocytes and MAP-2-stained neuronal processes in each investigated brain area (Figure 6g).

3.3. The Impact of Prenatal Exposure to Valproic Acid (VPA) on the Morphology of Astrocytes and Microglia, and the Expression of Pro-Inflammatory Cytokines in the Brain of 52-Day-Old Animals

Our previous data showed that prenatal exposure to VPA resulted in an increase in proinflammatory signalling in the brain of adolescent rats in a region-specific manner [50]. Thus, in this study, we addressed the question of whether the effect of VPA on the changes in purinergic receptor levels may be associated with the activation of glial cells and neuroinflammatory response. Since astrocytes, the most abundant cells within the CNS involved in the regulation of neuroinflammatory response, have been shown to express a variety of purinergic receptor subtypes, we decided to investigate the activity and morphological changes occurring in astrocytes in animals prenatally exposed to VPA.

The characteristic feature of reactive astrocytes is the mRNA and protein overexpression of glial fibrillary acidic protein (GFAP) [54]. We found that the reactive protein levels of this astrocyte marker were not changed in the hippocampus or cortex of the VPA group when compared with the controls (Figure 7a,b). Next, we performed morphometric analysis of GFAP immunolabeled astrocytes in adolescent control and VPA-exposed male rats. Since astrocytes display large heterogeneity in the hippocampus, which appears to be layer-specific [55], we conducted morphological analyses in different layers of the hippocampus. The reconstruction of skeletonized GFAP-positive structures was performed in confocal z-stacks obtained from the CA1 and DG subfields, including the CA1 stratum oriens, stratum lacunosum moleculare, stratum radiatum, DG stratum moleculare, and hilus. The results obtained from each subregion were then averaged. A similar analysis was performed on z-stacks acquired from the prefrontal cortex. We did not observe changes in the cell bodies' surface area in the investigated brain structures (Figure 7d,e). Using Sholl analysis, it is possible to represent the complexity of the 3D astrocyte structure of the rat brain on a one-dimensional scale (Figure 7c). The analysis revealed that the astrocyte

backbone in terms of arbour complexity (Figure 7f,g), total process length (Figure 7h,i), and number of primary (Figure 7j,k) and secondary processes (Figure 7l,m) was not significantly altered in animals prenatally exposed to VPA. We also superimposed spheres of increasing radius (2.5 μm increase in radius per step, Figure 7c) beginning at the centre of the cell body and measured the number of process intersections that each sphere encountered. We found that in both the hippocampus (Figure 7n) and prefrontal cortex (Figure 7o), astrocytes of VPA-exposed animals did not show a significant loss of complexity (the number of intersections at each radius remained unchanged).

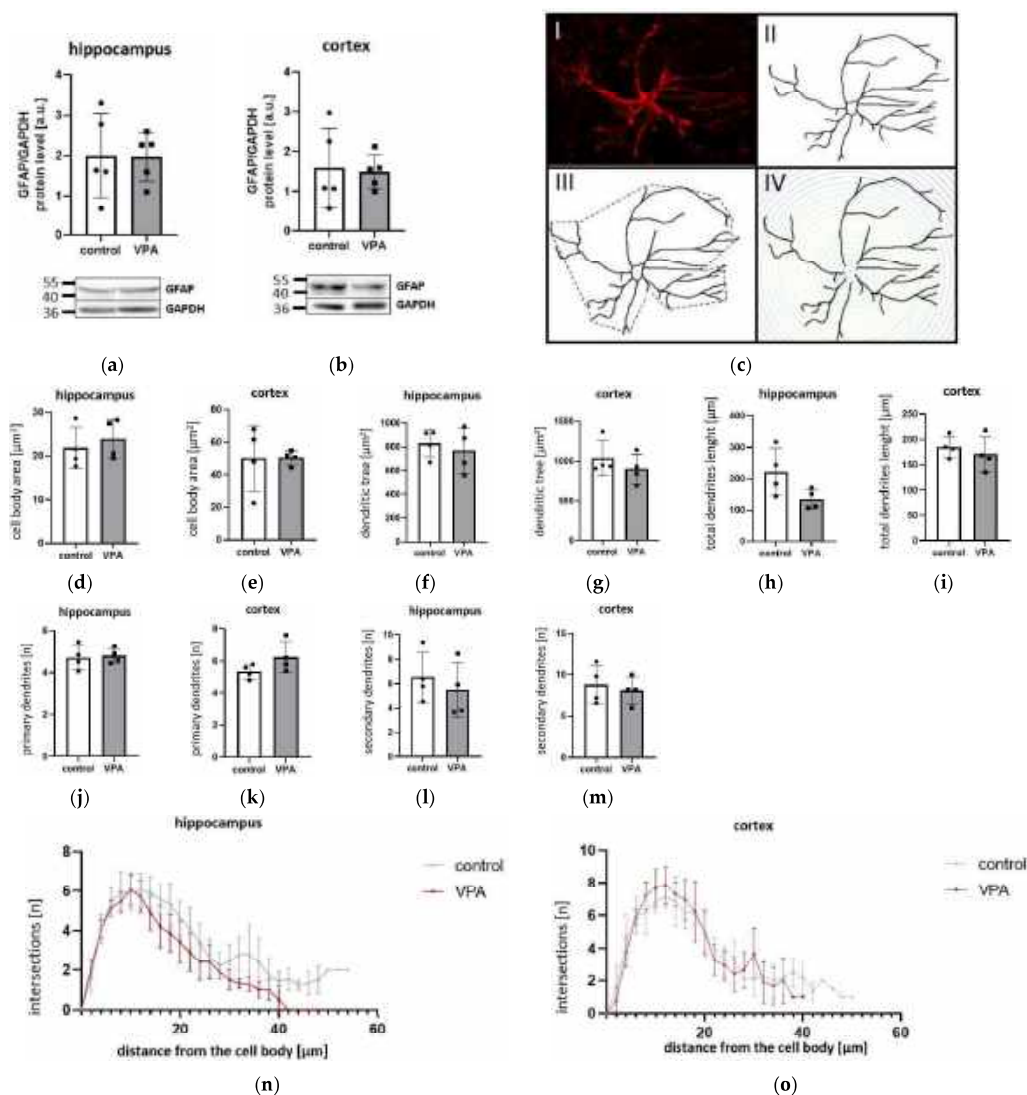


Figure 7. The effect of prenatal treatment with VPA on the morphology of astrocytes in the hippocampus and cortex of 52-day-old rats. The immunoreactivity of GFAP in the hippocampus (a) and cortex (b) of young adult animals prenatally exposed to VPA treatment was analysed by Western

blotting, and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. Representative pictures are shown. Data are presented as the mean value $\pm$ SD for 5 independent experiments. The subsequent steps in the morphology analysis of astrocytes are shown (c). The image of an GFAP-labelled astrocyte (I) was converted to an 8-bit format. Processes were traced by the NeuronJ plugin for Fiji (II). The surface area of the binary profile of the cell was marked with a dotted line around the astrocyte (III). Digitally applied concentric circles were applied every 2.5 μ m, centred on the soma centre (IV), and used to count the number of intersections for the Sholl analysis. The surface of the cell body of astrocytes in the hippocampus (d) and cortex (e) of animals prenatally exposed to VPA was measured. Data are presented as the mean value $\pm$ SD for four independent experiments. The dendritic tree of cells in the hippocampus (f) and cortex (g) of 52-day-old animals prenatally exposed to VPA was analysed as the surface area of cells together with processes. Data are presented as the mean value $\pm$ SD for four independent experiments. The total length of processes in the hippocampus (h) and cortex (i) was measured with the NeuronJ plugin. Data are presented as the mean value $\pm$ SD for four independent experiments. The primary dendrites in the hippocampus (j) and cortex (k), as well as the secondary processes in the hippocampus (l) and cortex (m) of young adult animals prenatally exposed to VPA treatment, were measured. Data are presented as the mean value $\pm$ SD for four independent experiments. Sholl analysis of complexity branching of astrocytes in the hippocampus (n) and cortex (o) of 52-day-old animals prenatally exposed to VPA treatment was performed with the SNT plugin for Fiji. Data are presented as the mean value $\pm$ SD for four independent experiments. Statistical analysis for each data set was determined using Student's *t*-test.

To assess the involvement of microglial pathology in the hippocampus of adolescent VPA animals, we first analysed the protein level of Iba-1, but we detected no differences between groups (Figure 8a). Next, we quantified the number and morphological complexity of microglia in the CA1 and DG subfields using confocal microscopy on Iba1-stained sections (Figure 8b). We observed that neither the number of Iba1+ microglial cells per mm² of tissue in VPA-exposed animals nor their morphology, in terms of a cell body area (Figure 8c), arbour complexity (Figure 8d), and total process length (Figure 8e), displayed significant differences compared to controls. The analysis revealed no differences in the number of primary branches in Iba-1+ cells from VPA-exposed animals compared to control animals (Figure 8f). However, the number of secondary branches significantly decreased in the hippocampi of animals treated with VPA (Figure 8h). This was further evidenced by the decreased arborization of microglia in VPA-exposed rats, especially at 15–20 μ m distances from the soma (Figure 8i,k).

Next, we evaluated the mRNA expression levels of interleukin-6 (IL-6) and tumour necrosis factor- α (TNF α), proinflammatory cytokines often linked with microglia activation in ASD. These results demonstrate that in the hippocampus, Il6 and Tnf α mRNA levels were unchanged in VPA-treated animals (Figure 8m,n). We also analysed the involvement of the selective blockade of P2X and overall purinergic P2 receptors with PPADS and isoPPADS, respectively, and observed that the treatment with both drugs had a negligible effect on microglial number, morphology, and activation in control and VPA-treated animals (Figure 8). Additionally, in the hippocampus, these antagonists did not influence the morphological changes of microglial cells evoked by prenatal VPA treatment (Figure 8g,k).

In contrast to the hippocampus, in the prefrontal cortex, we observed that the protein level for Iba-1 increased in the VPA group (Figure 9a). Moreover, the number of Iba1+ cells per mm² of tissue in VPA animals was significantly higher compared to controls, while treatment with non-selective P2 receptor antagonist isoPPADS resulted in a reduction in the number of microglial cells in VPA-exposed animals to the control level (Figure 9b). Microglial cells in the prefrontal cortex of VPA-treated animals exhibited a typically activated morphology, characterised by a significant increase in the size of microglial cell soma (Figure 9c), a decrease in arbour complexity (Figure 9d), and a decrease in total process length (Figure 9e) compared to the control. We also observed a decrease in the number of primary and secondary branches in Iba-1+ cells (Figure 9f,g) and a reduction in the number of proximal (Figure 9i) and distal (Figure 9j–k) intersections per radius in the mi-

croglia of VPA-exposed animals when compared to controls. Interestingly, treatment with the selective P2X purinergic receptor antagonist did not influence the above-mentioned changes in microglial cells morphology, whereas the non-selective P2 antagonist isoPPADS prevented the enlargement of cell bodies of Iba+ cells but had a negligible effect on changes in microglial cell arborisation. However, this compound significantly inhibited the increase in the expression of Il6 and Tnf α induced by prenatal exposure to VPA (Figure 8m,n).

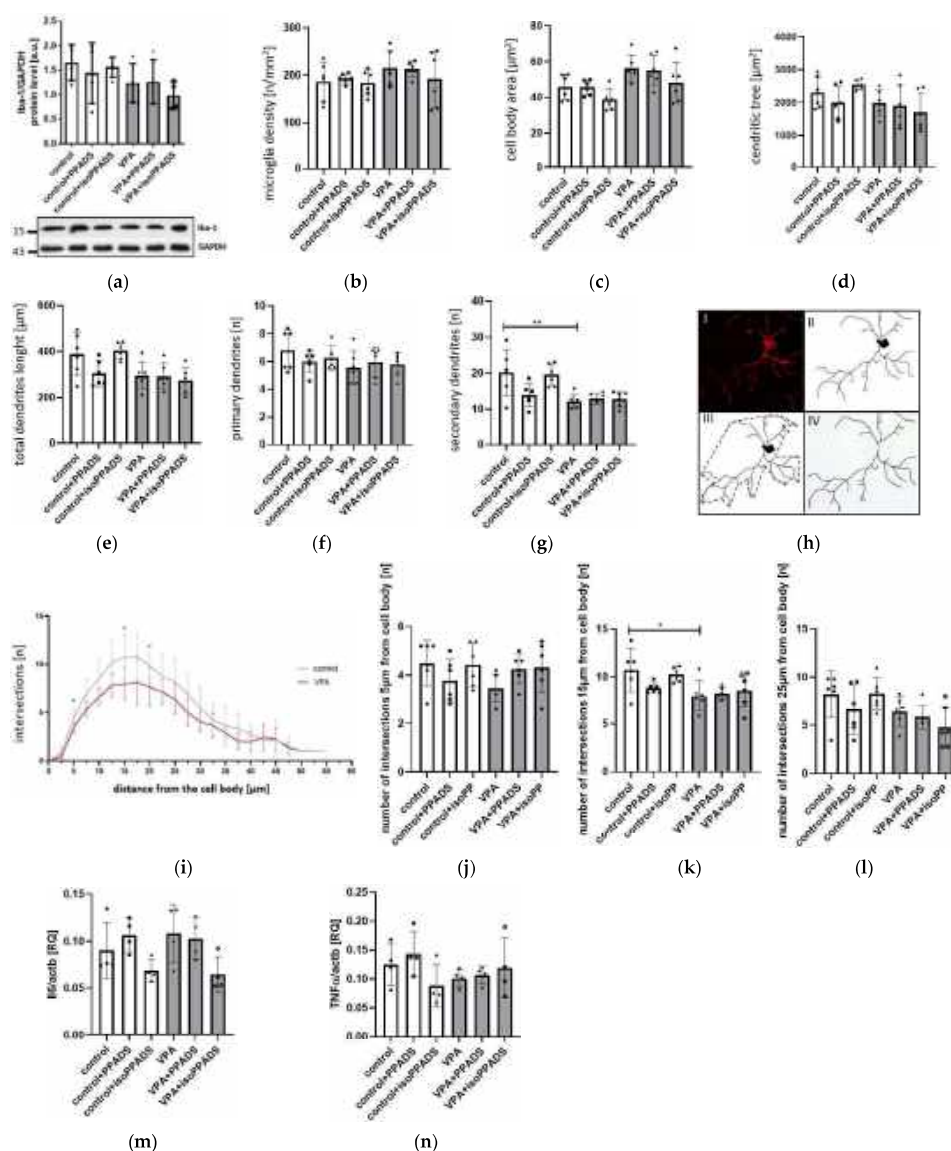


Figure 8. The effect of the inhibition of purinergic signalling on the morphology of microglia and cytokine expression in the hippocampus of 52-day-old rats prenatally treated with VPA. The effect of

PPADS/isoPPADS treatment on the immunoreactivity of Iba-1 in the hippocampus (a) of young adult animals prenatally exposed to VPA treatment was analysed by Western blotting. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. Representative pictures are shown. Data are presented as the mean value $\pm$ SD for four independent experiments. The effect of PPADS/isoPPADS treatment on the density of microglia (b) as the number of cells per $1 \mu\text{m}^2$ in the hippocampus of young adult animals prenatally exposed to VPA treatment was measured with the Cell Counter plugin for Fiji. Data are presented as the mean value $\pm$ SD for six independent experiments. The effect of PPADS/iso-PPADS treatment on the surface of the cell body of microglia (c) and dendritic tree (d) in the hippocampus of animals prenatally exposed to VPA was measured. Data are presented as the mean value $\pm$ SD for 6 independent experiments. The effect of PPADS/iso-PPADS treatment on the total length of microglial processes (e) in the hippocampus of 52-day-old animals prenatally exposed to VPA was measured with the NeuronJ plugin. Data are presented as the mean value $\pm$ SD for six independent experiments. The effect of PPADS/iso-PPADS treatment on the number of primary (f) and secondary (g) dendrites in the hippocampus of young adult animals prenatally exposed to VPA treatment was counted. Data are presented as the mean value $\pm$ SD for six independent experiments. * $p < 0.05$, ** $p < 0.01$, vs. corresponding control, as determined using one-way ANOVA followed by the Bonferroni test. The subsequent steps in the morphology analysis of microglia are shown (h). The image of the Iba1-labeled microglia cell (I) was converted to an 8-bit format. Processes were traced by the NeuronJ plugin for Fiji (II). The surface area of the binary profile of the cell was marked with a broken line around the microglia. (III) Digitally applied concentric circles were applied every $2.5 \mu\text{m}$, centred on the soma centre (IV) and were used to count the number of intersections for Sholl analysis. Sholl analysis of complexity branching of microglia (i) in the hippocampus of 52-day-old animals prenatally exposed to VPA treatment was performed with the SNT plugin for Fiji. Data are presented as the mean value $\pm$ SD for six independent experiments. * $p < 0.05$ vs. corresponding control as determined using Student's *t*-test. The effect of PPADS/iso-PPADS treatment on the number of intersections $5 \mu\text{m}$ (j), $15 \mu\text{m}$ (k), as well as $25 \mu\text{m}$ (l) from the centre of the cell body, was determined. * $p < 0.05$, vs. corresponding control, as determined using one-way ANOVA followed by the Bonferroni test. The effect of PPADS/iso-PPADS treatment on the mRNA level of Il6 (m) and TNF α (n) in the hippocampus of young adult animals prenatally exposed to VPA treatment was measured by real-time PCR and normalized to Actb (β -actin). Data are presented as the mean value $\pm$ SD for four independent experiments.

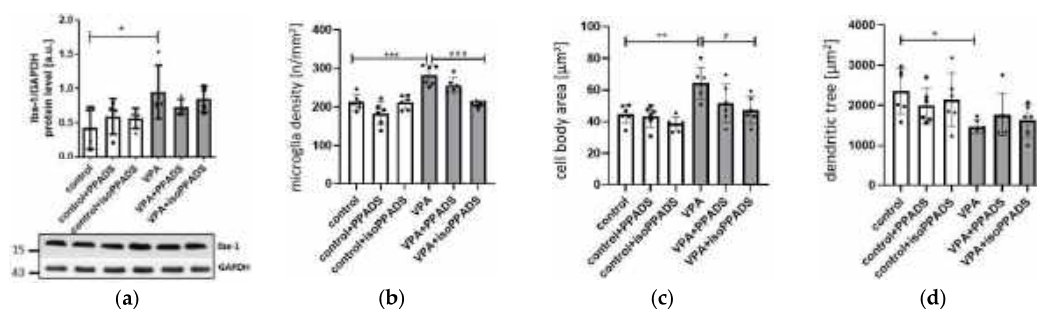


Figure 9. Cont.

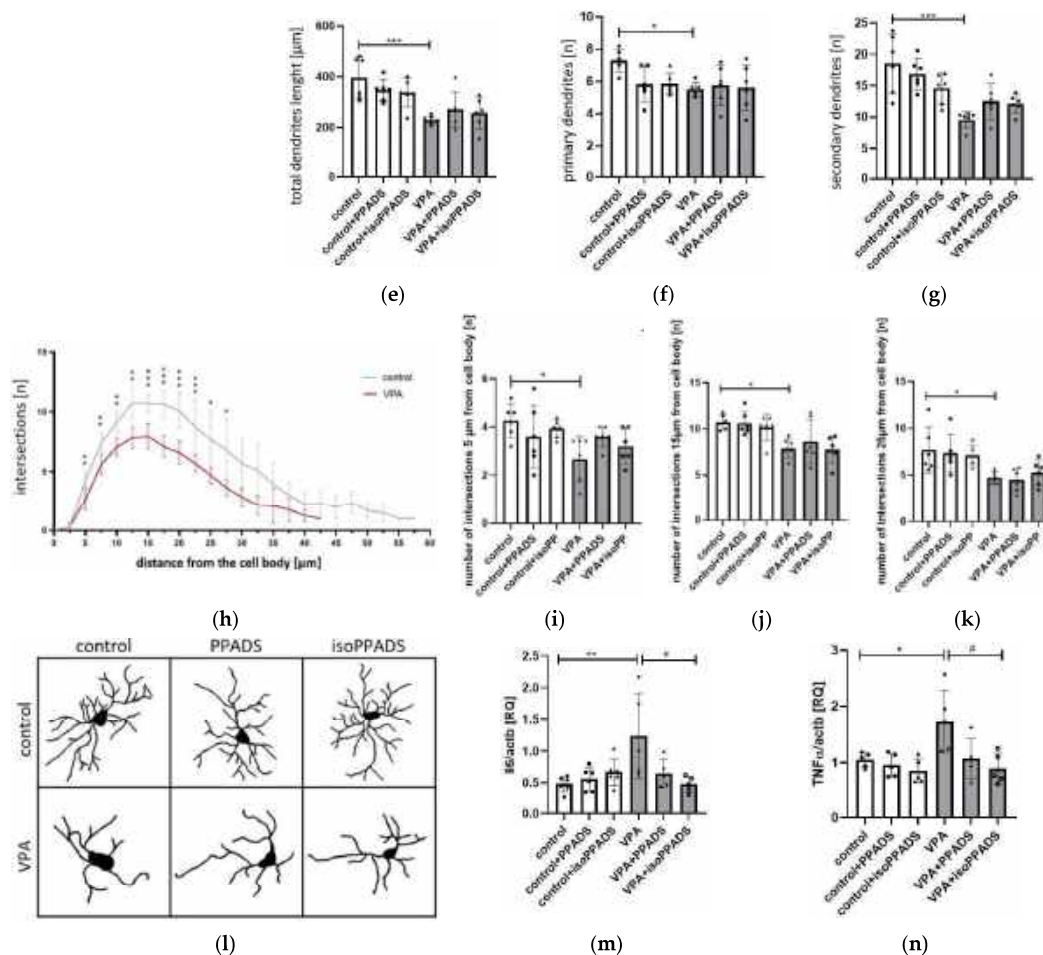


Figure 9. The effect of the inhibition of purinergic signalling on the morphology of microglia and cytokine expression in the cortex of 52-day-old rats prenatally treated with VPA. The effect of PPADS/isoPPADS treatment on the immunoreactivity of Iba-1 in the cortex (a) of young adult animals prenatally exposed to VPA treatment was analysed by Western blotting. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. Representative pictures were shown. Data are presented as the mean value $\pm$ SD for four independent experiments. The effect of PPADS/iso-PPADS treatment on the density of microglia (b) as the number of cells per $1 \mu\text{m}^2$ in the cortex of young adult animals prenatally exposed to VPA treatment was measured with the Cell Counter plugin for Fiji. Data are presented as the mean value $\pm$ SD for six independent experiments. * $p < 0.05$, *** $p < 0.001$ vs. corresponding control, # $p < 0.05$, ### $p < 0.001$ vs. VPA, as determined using one-way ANOVA followed by the Bonferroni test. The effect of PPADS/isoPPADS treatment on the surface of the cell body of microglia (c) and dendritic tree (d) in the cortex of animals prenatally exposed to VPA was measured. Data are presented as the mean value $\pm$ SD for six independent experiments. * $p < 0.05$, ** $p < 0.01$ vs. corresponding control, # $p < 0.05$ vs. VPA, as determined using one-way ANOVA followed by the Bonferroni test. The effect of PPADS/isoPPADS treatment on the total length of microglial processes (e) in the cortex of 52-day-old animals prenatally exposed to was

measured with the NeuronJ plugin. Data are presented as the mean value $\pm$ SD for six independent experiments. *** $p < 0.001$ vs. corresponding control, as determined using one-way ANOVA followed by the Bonferroni test. The effect of PPADS/iso-PPADS treatment on the number of primary (f) and secondary (g) dendrites in the cortex of young adult animals prenatally exposed to VPA treatment was measured. Data are presented as the mean value $\pm$ SD for six independent experiments. *** $p < 0.001$, vs. corresponding control as determined using one-way ANOVA followed by the Bonferroni test. Sholl analysis of complexity branching of microglia (h) in the cortex of 52-day-old animals prenatally exposed to VPA treatment was performed with the SNT plugin for Fiji. Data are presented as the mean value $\pm$ SD for six independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. corresponding control, as determined using Student's *t*-test. The effect of PPADS/iso-PPADS treatment on the number of intersections at 5 μ m (i), 15 μ m (j), as well as at 25 μ m (k) from the centre of the cell body, was determined. * $p < 0.05$ vs. corresponding control, as determined using one-way ANOVA followed by the Bonferroni test. The representative data of microglial cells in the investigated groups are presented (l). The effect of PPADS/iso-PPADS treatment on the mRNA level of Il6 (m) and TNF α (n) in the cortex of young adult animals prenatally exposed to VPA treatment was measured by real-time PCR and normalized to Actb (β -actin). Data are presented as the mean value $\pm$ SD for six independent experiments. * $p < 0.05$, # $p < 0.05$ vs. VPA as determined using one-way ANOVA followed by the Bonferroni test.

4. Discussion

In recent years, it has been shown that purinergic signalling disruption might be involved in the pathomechanisms related to ASDs. The P2X and P2Y receptors are known to control a wide range of molecular processes that are disturbed in autism, such as synaptogenesis and brain development [13], the inflammatory response and microglial activation [56], regulation of intestinal motility and permeability [57,58], chemosensory transduction of taste [59] and hearing [60], and sensitivity to food allergens [61]. It was previously demonstrated that the non-selective blockade of purinergic receptors corrects aberrant behaviour and prevents morphological and biochemical changes in ASD animal models [62–65]. Following those observations, the present study provides experimental evidence suggesting for the first time that ASD-related disturbances in purinergic signalling might be directly related to changes in the expression of particular subtypes of purinergic receptors occurring in adolescence. Moreover, these alterations might, at least in part, be responsible for microglial activation and neuroinflammatory response induction in the brains of young adult animals.

In this study, we used an environmentally triggered rat models of ASD based on prenatal exposure to valproic acid (VPA), a commonly used antiepileptic drug [66,67]. Several large retrospective studies have shown that VPA intake during pregnancy increases the risk of ASD prevalence almost eightfold when compared to the general population [68–70]. Administering VPA to pregnant rodent females at a critical time during gestation results in the development of many structural and behavioural features in the adult offspring that can be observed in ASD patients [50,71]. Because it meets the criteria of construct, face, and predictive validity, the animal model of VPA exposure is considered relevant to a psychiatric condition described in humans and is still one of the most frequently used ASD models [72–74]. Numerous studies have documented that the teratogenicity of VPA is connected to the deregulation of epigenetic processes that control the coordinated execution of protein expression involved in neural network maturation [75]. In utero VPA exposure increases histone acetylation and methylation in mouse foetal tissue [76], which can lead to increased synapses and robust facilitation of excitatory synapse maturation [77]. We have previously observed that prenatal exposure to VPA alters the levels of presynaptic and postsynaptic density proteins, as well as synaptic cell adhesion molecules, in adult offspring [50]. Additionally, in that study, VPA exposure induced regional changes in the expression of proinflammatory genes [50]. The current study extends these observations by showing the deregulation of the protein level of the particular subtypes of purinergic receptors in the cortex and hippocampus of the rat offspring as one of the molecular targets of the

VPA action. Moreover, our data suggest that the impairment of the purinergic system might partially contribute to the activation of microglia in the cortex of VPA-treated animals.

We observed prominent ASD-related changes in purinergic receptors in early adulthood. These changes in receptor expression may directly or indirectly influence the behavioural or cognitive phenotype of ASD. Overall, alterations in the expression of each purinergic receptor subtype result in the deregulation of cellular Ca^{2+} level, which largely affects social interaction and induces stereotypic behaviour [78]. Moreover, specific receptors belonging to the P2 subfamily regulate unique cellular pathways related to neurotransmission, synaptic plasticity, or the inflammatory response. Therefore, any change in their expression may trigger specific molecular changes related to ASD pathology.

In our study, we found that the purinergic P2X1 receptor was significantly impacted in both the cortex and hippocampus of adult VPA offspring. The level of this receptor was the highest in the adult rat cortex, where P2X1 mediates an appreciable fraction of excitatory synaptic current in ~85% of layer V neocortical pyramidal neurons [79]. Consistent with an earlier study showing that microglial cells of adult rat brains do not express P2X1 [80], our study demonstrated that this receptor is predominantly expressed in neuronal cells in the prefrontal cortex of adult rats. The down-regulation of this receptor in cortical regions of VPA-treated animals may suggest disturbances in neuronal synaptic transmission in this brain area. Interestingly, in the hippocampus, where the level of P2X1 is significantly increased in VPA offspring, this receptor was shown to stimulate the evoked release of glutamate from purified nerve terminals [81]. Therefore, the overexpression of this receptor in neurons in the hippocampal cornu ammonis or dentate gyrus regions may suggest its indirect role in the elevation of hippocampal LTP. This study greatly extends previous findings, showing that prenatal VPA exposure disrupts neurogenesis and alters the expression levels of multiple proteins associated with glutamatergic and GABAergic neurotransmission in nonhuman primates [82].

We also observed a significant increase in the level of P2X3 receptors in the brain cortex of adult VPA offspring. Among other purinergic receptors, the expression of P2X3 is known to rapidly decrease after birth [83], and in our study, we demonstrated that it is relatively low in the brain of weanling and adult rats. Moreover, its expression and function are predominantly attributed to the primary sensory neurons of the spinal cord [84] and are implicated in the modulation of pain sensitivity [85]. Some data suggest that P2X3 receptors can form stable multimers with P2X1 [86] or P2X2 [87] receptors, and antagonists of P2X2/3 and P2X3 have been shown to inhibit glutamate release in the hippocampus, suggesting their role in the modulation of excitatory synaptic transmission [81]. However, little is known about the relevance of the P2X3 receptor in the brain cortex function, and the conditions under which this alteration becomes functionally important remain to be determined. Interestingly, upregulation of P2X3 receptors in a murine model has previously been associated with increased scratching behaviour [88], which is a common problem in autistic patients [89], suggesting that alterations in P2X3 may be connected with sensory hypersensitivity observed in some individuals with ASD.

Previously, the purinergic P2X7 receptor was indicated as a key factor involved in maternal immune-activation-induced autism-like behavioural and biochemical features [90]. Activation of this ligand-gated ion channel, which is sensitive to high extracellular ATP levels, is mainly associated with inflammatory conditions. Its stimulation acts as a cellular signal for inflammasome activation and the post-translational processing of proinflammatory cytokines, including IL-1 β , IL-18, TNF- α and IL-6 [91]. Initially, it was believed that the functional expression of the P2X7 receptor was exclusive to oligodendrocytes and microglia in the CNS [92]. However, recent evidence has shown that P2X7 receptors are widely present in neuronal cells [93,94]. Their activation mediates transmembrane ion fluxes that mediate fast excitatory transmission in the hippocampus and primary cerebrocortical neurons. P2X7 is also involved in the release of glutamate, GABA, and ATP from neuronal terminals [11,95,96]. Excessive stimulation of P2X7 receptors leads to increased production of nitric oxide and induces mitochondrial dysfunction in neuronal cells [97].

In this study, we demonstrated the presence of P2X7 receptors in the neuronal perikarya of the prefrontal cortex and pyramidal cell layers of the CA and DG in the hippocampus. The level of this receptor was significantly decreased in the hippocampus of VPA offspring. Interestingly, we did not observe significant activation of microglial cells in the hippocampus of VPA offspring, suggesting that the down-regulation of this receptor may also occur in microglia, leading to a lack of activation in these cells. This unexpected effect was also observed in the study of the Naviaux group, where prenatal exposure to poly-I:C resulted in a marked decrease in P2X7 receptor levels [62]. The decrease was normalized after the administration of non-selective purinergic receptor antagonists, such as suramin. The authors of that study suggested that the observed decrease in receptor expression may be a result of chronically elevated levels of ATP and other nucleotides. The idea that the neuronal P2X7 receptor may be overstimulated in ASDs is supported by a study showing that a single dose of a highly selective and brain-permeable P2X7 antagonist, JNJ47965567, alleviated behavioural alterations in the offspring [90].

Recent data have suggested the mitochondrial localization of the P2X7 receptor and its involvement in the control of mitochondrial respiration, mitochondrial matrix Ca^{2+} levels, and regulation of the expression pattern of oxidative phosphorylation enzymes [98]. Genetic deletion (P2X7 knockout mice) or subchronic pharmacological inhibition has been shown to increase carbohydrate oxidation [99]. Disturbances in energetic metabolism have been implicated in cell deregulation and the progression of ASDs [100]. The use of the ketogenic diet in ASD patients has also been shown to significantly improve behaviour and intellect [101,102]. Therefore, we hypothesize that the decline in the P2X7 receptor levels after prenatal VPA administration may be involved in the deregulation of energy metabolism in neuronal cells. However, this interesting hypothesis requires further examination using sophisticated quantitative methods.

It has previously been demonstrated that action potentials trigger the axonal release of ATP and glutamate, and the elevated synaptic ATP acts predominantly on astrocytic P2Y1 metabotropic receptors [103]. Surprisingly, our findings showed that under physiological conditions, P2Y1 is predominantly expressed in neuronal cells in the hippocampus or prefrontal cortex. This is in agreement with other researchers showing that the P2Y1 receptor was mainly localized to neuronal cells of the cerebral cortex, cerebellum, hippocampus, caudate nucleus, putamen, globus pallidus, subthalamic nucleus, red nucleus, and midbrain [29,104,105]. Moreover, it has been demonstrated that P2Y1 is expressed in the soma and mossy fibre terminals of the CA3 subfield of the hippocampus [103], and in physiological conditions, it is involved in reducing neurotransmitter release [105]. However, the expression of these receptors significantly increases in astrocytes during reactive astrogliosis, where they play the predominant role in the regulation of single-cell transients and calcium waves [106,107]. P2Y1 expression in astrocytes has been demonstrated to be increased under different pathological conditions, including oxidative stress [108,109] and ischemia [110]. In contrast to those studies, we did not observe a significant increase in P2Y1 levels in the astrocytes in the cortex and hippocampus of VPA animals. What we did notice was a significant elevation of this receptor's level exclusively in the neuronal cells in the brain cortex with a simultaneous decrease in its level in the hippocampus. It has previously been demonstrated that P2Y1 overactivation in the medial prefrontal cortex significantly alters cognitive abilities in rats, manifesting in a delay in completing tasks and reduced learning abilities [111]. Executive dysfunction and social cognition deficits are often associated with autism in humans [112], therefore it is possible that P2Y1 disturbances might, at least partly, participate in neurocognitive impairment in individuals with ASD.

It is unclear why we did not detect an elevation of P2Y1 expression in astrocytes, but this is partially in agreement with previous studies showing that P2Y1 was significantly upregulated in neurons and microglia, but not in the astrocytes, in status epilepticus [105]. Other studies have also demonstrated the upregulation of P2Y1 in microglial cells following TGF- β treatment [113]. Unlike these reports, we did not observe any increase in the expression of P2Y1 in microglial cells after VPA exposure. However, our study largely extends

the previous findings by defining an interesting aspect of P2Y1, which is its overexpression in different cell subtypes depending on the type of insult or pathological state.

Interestingly, the overexpression of P2Y1 in astrocytes has been predominantly linked to protective effects, such as the prevention of astrocyte damage caused by oxidative stress [108] or the inhibition of neuronal damage induced by IL-6 release [109]. Treatment with the P2Y1 agonist also had beneficial effects in reducing ischemic lesions through an increase in intracellular Ca^{2+} mobilization and enhanced mitochondrial metabolism in astrocytes [110]. In our study, we did not observe significant changes in astrocytic morphology and number, parameters that have been previously shown to be closely associated with the response of astrocytes to different pathological cues, including alterations in the metabolic state of neighbouring cells [114,115]. Therefore, we speculate that in VPA animals, the overexpression of P2Y1 in astrocytes might be somehow blocked, thereby preventing the protective activity of these cells. The lack of proper support from astrocytes may be the cause of neuronal or synaptic alterations, which may lead to the incidence of neuropsychiatric disorders, including autism. This hypothesis is especially interesting in light of the data showing that impaired calcium signalling in astrocytes resulted in atypical social interaction and repetitive behaviour in mice [78]. Astrocyte abnormalities have also been identified post-mortem in the cortex of brains of individuals with autism [35,116,117], and recently, the transplantation of iPSC-derived human ASD astrocytes to the healthy mouse brain was sufficient to induce repetitive behaviour as well as impaired memory and long-term potentiation [118].

Finally, we demonstrated that the expression of the metabotropic P2Y12 is significantly enhanced in the cortex of animals prenatally exposed to VPA. These receptors are generally considered to be a signature of microglia since they are universally and specifically expressed in these cells in all regions of rodent [119] and human brains [120]. The presence of P2Y12 distinguishes CNS resident microglia from peripheral macrophages [22,119]. Our study also showed the specific abundance of this purinergic receptor subtype in the microglial cells in the brains of control and VPA-treated rats. Moreover, we observed that, similar to humans [120], the expression of these receptors is stable across different stages of rat brain development.

The activation of P2Y12 receptors on microglia has been associated with various pathological conditions in the CNS [22,121,122]. Compared to physiological extracellular ATP levels in the brain, high concentrations of this signalling molecule (1 mM) have been shown to induce P2Y12-receptor-dependent prompt chemotaxis of microglia towards the ATP-releasing source [23]. Moreover, stimulation of the P2Y12 receptor in microglia rapidly induces lamellipodia formation, process extension, and morphological conversion [22] into the ramified state. Microglial process convergence towards hyperactivated neurons has been specifically mediated by P2Y12 [31,123], and this receptor also participates in the formation of neuron–microglia junctions, thus regulating mitochondrial function in neurons, neuronal calcium load, and functional connectivity [124]. Therefore, the activation of P2Y12 on microglial cells has been previously considered neuroprotective. Furthermore, robust expression of P2Y12 was demonstrated in the ‘resting’ state of microglia but is dramatically reduced after microglial activation [22]. Activation of microglial TLR-4 receptors by lipopolysaccharide (LPS) has been associated with almost a total loss of P2Y12 receptors [125,126]. Also, gradual and almost complete disappearance of P2Y12 receptors on microglia has been observed in stroke [127].

Considering these findings, we expected that P2Y12 overexpression in the cortex of animals prenatally exposed to VPA would result in increased microglial chemotaxis and intensification of microglial process extension. However, while we observed an increase in the number of microglial cells in the cortex of VPA rats mediated by purinergic receptors, the morphology of microglial cells underwent a transition that is characteristic of reactive microgliosis, which has previously been associated with the down-regulation of microglial P2Y12 receptor expression [128], as demonstrated in various neurological disorders [128,129]. However, recent studies have indicated the presence of P2Y12 in activated

microglia in post-mortem tissues of patients suffering from major depressive disorder [130] or Alzheimer's disease [131]. Moreover, it has recently been demonstrated that microglial P2Y₁₂ is a critical mediator of stress-induced neuronal remodelling in the prefrontal cortex and contributes to associated behavioural consequences [132].

Also, in animal models of neuropathic pain, upregulation of the P2Y₁₂ receptor in microglial cells resulted in the activation of the GTP-RhoA/ROCK2 signalling pathway, elevation of phosphorylated p38-mitogen-activated protein kinase (MAPK) levels and upregulation of excitatory synaptic transmission in the dorsal horn [121,133]. Taking into consideration the recent data obtained from the high-resolution single-cell technologies, which reveal the large diversity of microglial cells populations both under homeostatic and pathological conditions [134], it is therefore plausible that epigenetic changes induced by prenatal VPA exposure might result in the formation of P2Y₁₂-positive microglial cells that adopt an activation-specific phenotype. Consistent with this suggestion, it was demonstrated that P2Y₁₂-positive microglia can overexpress pro-inflammatory mediators, such as iNOS, IL-1 β , and TNF- α , upon LPS stimulation, and this effect was significantly reduced by P2Y₁₂-inhibitor treatment [135]. In line with that study, we demonstrated that the purinergic receptor antagonist prevented cytokine overexpression in the cortex of animals prenatally exposed to VPA. Previous evidence has shown that aberrant release of cytokines, especially IL-6, alters neural cell adhesion and migration, and also disrupts the balance of excitatory and inhibitory circuits in ASD patients [136]. Our study additionally shows the direct involvement of purinergic receptors in the brain cytokine imbalance, which is closely related to the pathogenesis of autism. Despite the apparent involvement of purinergic signalling in the elevation of cytokine expression in cortical microglial cells, our study revealed that those receptors are not involved in the regulation of morphological changes observed in microglia in the cortex of VPA animals. In light of previous observations showing that microglial process surveillance could also be regulated by other factors, such as β 2-adrenergic receptors [137] or changes in extracellular adenosine levels [138], it is possible that the observed changes in microglial morphology could be induced by one of those mechanisms. This is particularly interesting since both β 2-adrenergic receptors [139,140] and imbalances in adenosine signalling [141] have previously been shown to be involved in conditions related to ASD. Another noteworthy observation that explains our results is that mutations or epigenetic changes can induce abnormalities in P2Y₁₂ function in platelets, leading to clopidogrel resistance [142,143]. Therefore, prenatal exposure to histone acetylase inhibitor VPA might induce epigenetic changes that result in the expression of dysfunctional P2Y₁₂ receptors. In that case, the overexpression of this receptor in microglial cells might be ineffective in preventing microglial cell de-ramification. This interesting hypothesis appears to be quite probable in light of the data showing valproate-mediated disturbances of haemostasis and platelet function [144] and will be further tested in our future research.

We are aware of the limitations of this study. First, Western blotting analysis of the brain tissue homogenate only allows for the net assessment of changes in particular protein levels without providing information about their alteration in specific cell subtypes and/or cellular compartments, such as pre- or post-synaptic terminals. To partially overcome these limitations, we performed IHC staining on selected purinergic receptors to observe their presence in particular cells within the CNS. Furthermore, we focused on the investigation of the expression of particular receptor subtypes only in a selected stage of CNS development, while purinergic signalling might also be significantly altered in other stages of neuronal development when neurogenesis, neuronal migration, axon growth, and functional axonal maturation occur. Finally, a general limitation of using the VPA rodent model is that the results obtained might not easily translate to the broader human ASD population. Even though prenatal exposure to VPA recapitulates many features characteristic of idiopathic autism, the aetiology of this disorder in the majority of people is not directly connected to prenatal exposure to HDAC inhibitors. Thus, there is a risk that the observed phenomenon may not appear in a certain population of patients. However, the results obtained in this study indicate an urgent need to investigate the changes in purinergic receptors

expression and activity in humans suffering from ASD and the relation of these changes to disorder outcomes. This is especially pertinent since a few previous studies showed a certain effectiveness of purinergic receptor inhibition on motor coordination and social impairments in animal ASD models [62,63,65], the genetic Fragile X model [64], as well as in autistic children [145]. Therefore, studying the changes in purinergic neurotransmission in ASD seems to be a promising target for future investigations, especially when various centrally penetrant P2 receptors antagonists are now undergoing clinical trials for the treatment of CNS disorders, such as neuropathic pain, multiple sclerosis, stroke, epilepsy, or Alzheimer's disease [146,147].

5. Conclusions

In summary, our report is the first to show that prenatal exposure to VPA induces significant structure-dependent changes in the expression of purinergic receptors in early adulthood. Moreover, we demonstrated the partial involvement of purinergic signalling imbalance in modulating the brain inflammatory response induced by prenatal VPA exposure. Thus, this study establishes a solid foundation for future investigations into the involvement of purinergic receptor deregulation in ASD, and the development of therapeutic strategies for treatment of this neurodevelopmental disorder.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/brainsci13071088/s1>. The impact of prenatal exposure to VPA on P2X1 receptor level in the neurons (Figure S1), astrocytes (Figure S2), and microglia (Figure S3) of the hippocampus and the prefrontal cortex at 52 PND; the impact of prenatal exposure to VPA on P2X7 receptor level in the neurons (Figure S4), astrocytes (Figure S5), and microglia (Figure S6) of the hippocampus and the prefrontal cortex at 52 PND; the impact of prenatal exposure to VPA on P2Y1 receptor level in the neurons (Figure S7), astrocytes (Figure S8), and microglia (Figure S9) of the hippocampus and the prefrontal cortex at 52 PND; the impact of prenatal exposure to VPA on P2Y12 receptor level in the neurons (Figure S10), astrocytes (Figure S11), and microglia (Figure S12) of the hippocampus and the prefrontal cortex at 52 PND; antibody specificity (Figure S13).

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11.4. Publikacja III

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*autorzy równorzędni



Article

Synaptic Alterations in a Transgenic Model of Tuberous Sclerosis Complex: Relevance to Autism Spectrum Disorders

Grzegorz A. Czapski ^{1,†}, Lidia Babiec ^{1,†}, Henryk Jeśko ^{1,‡}, Magdalena Gąssowska-Dobrowolska ¹,
Magdalena Cieślak ¹ , Marta Matuszewska ¹, Małgorzata Frontczak-Baniewicz ², Karolina Zajdel ²
and Agata Adamczyk ^{1,*}

¹ Department of Cellular Signalling, Mossakowski Medical Research Institute, Polish Academy of Sciences, Pawińskiego 5, 02-106 Warsaw, Poland; gczapski@imdik.pan.pl (G.A.C.); lbabiec@imdik.pan.pl (L.B.); hjesko@imdik.pan.pl (H.J.); mgassowska@imdik.pan.pl (M.G.-D.); mcieslik@imdik.pan.pl (M.C.); mmatuszewska@imdik.pan.pl (M.M.)

² Electron Microscopy Platform, Mossakowski Medical Research Institute, Polish Academy of Sciences, Pawińskiego 5, 02-106 Warsaw, Poland; mbaniewicz@imdik.pan.pl (M.F.-B.); kzajdel@imdik.pan.pl (K.Z.)

* Correspondence: aadamczyk@imdik.pan.pl

† These authors contributed equally to this work.

‡ Henryk Jeśko passed away while this paper was being written.



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Abstract: Tuberous sclerosis complex (TSC) is a rare, multi-system genetic disease with serious neurological and mental symptoms, including autism. Mutations in the *TSC1/TSC2* genes lead to the overactivation of mTOR signalling, which is also linked to nonsyndromic autism. Our aim was to analyse synaptic pathology in a transgenic model of TSC: two-month-old male B6;129S4-Tsc2^{tm1Djk}/J mice with Tsc2 haploinsufficiency. Significant brain-region-dependent alterations in the expression of several synaptic proteins were identified. The most prominent changes were observed in the immunoreactivity of presynaptic VAMP1/2 (ca. 50% increase) and phospho-synapsin-1 (Ser62/67) (ca. 80% increase). Transmission electron microscopy demonstrated serious ultrastructural abnormalities in synapses such as a blurred structure of synaptic density and a significantly increased number of synaptic vesicles. The impairment of synaptic mitochondrial ultrastructure was represented by excessive elongation, swelling, and blurred crista contours. Polyribosomes in the cytoplasm and swollen Golgi apparatus suggest possible impairment of protein metabolism. Moreover, the delamination of myelin and the presence of vacuolar structures in the cell nucleus were observed. We also report that Tsc2^{+/-} mice displayed increased brain weights and sizes. The behavioural analysis demonstrated the impairment of memory function, as established in the novel object recognition test. To summarise, our data indicate serious synaptic impairment in the brains of male Tsc2^{+/-} mice.

Keywords: tuberous sclerosis complex; synaptic dysfunction; autism; animal model

1. Introduction

Tuberous sclerosis complex (TSC) is an autosomal, dominant genetic disease evoked by the mutation of either the *TSC1* or *TSC2* gene; these genes encode the proteins Tsc1 (hamartin) and Tsc2 (tuberin), respectively. The disorder affects approximately 1 in 5800 people and is typically characterised by widespread hamartomas in multiple organ systems, including the brain [1]. In the brain, hamartin and tuberin have been implicated in the control of cortical lamination, the regulation of dendritic arborization, neuronal migration, polarization, axonal outgrowth, and spine formation [2,3]. Pathological mutations in these genes disrupt the interaction between hamartin and tuberin and affect cell function via the improper regulation of the mechanistic/mammalian target of rapamycin (mTOR) signalling cascade and multiple downstream effectors responsible for the modulation of key cellular processes, including protein synthesis, autophagy, cell growth, and proliferation [4]. Specifically, in the brain, the mTOR signalling pathway is involved in the regulation of

synaptogenesis, corticogenesis, the connectivity of neuronal circuits, the proliferation of cerebral cells, and dendritic spine density [5]. In particular, the hamartin-tuberin complex inhibits the activity of Ras-family GTPase, the small G-protein Rheb (Ras homolog enriched in the brain), which plays a critical role in the activation of mTOR kinase. Therefore, the dysfunction of hamartin and/or tuberin leads to the overactivation of the mTOR kinase-dependent pathway. The serine/threonine protein kinase mTOR exists in two diverse complexes: mTORC1 (mTOR, Raptor, and LST8) and mTORC2 (mTOR, Rictor, LST8, and SIN1). Upon stimulation by growth factors, signals related to nutrient availability, or hypoxia, mTOR phosphorylates a variety of substrates, affecting numerous essential cell processes, including the regulation of several components involved in protein synthesis, such as initiation and elongation factors, and the biogenesis of ribosomes themselves. mTOR-dependent translation is regulated by the control of two critical core components of the translation-initiation machinery: ribosomal protein S6 kinase beta-1 (S6K1 or p70S6K) and the eIF-4E-binding protein 1 (4E-BP1). S6K1 is phosphorylated by mTOR at Ser389. After activation, S6K1 phosphorylates the ribosomal subunit protein S6, leading to an increase in protein translation. 4E-BP1 is a repressor of the translation-initiation complex (eIF-4E), which is required for the 5'-cap-dependent translation of mRNA. When phosphorylated by mTOR, 4E-BP1 dissociates from eIF-4E, allowing for the initiation of 5'-cap-dependent RNA translation. A growing body of evidence indicates that the mTOR pathway is the central regulator of cap-dependent local translation at the synapse [6]. Disturbances in local-translation control and changes in the quantities of proteins at synapses could affect synaptic structure and function and might be responsible for a shift in the balance of synaptic plasticity and associated processes of learning and memory as well as behaviour. Therefore, the dysregulation of synaptic protein synthesis at synapses has been implicated in the pathogenesis of several diseases, including autism, epilepsy, and neurodegenerative disorders [7,8].

In addition, the overactivation of the mTOR pathway leads to the impairment of autophagy [9]. Overactivated mTORC1 phosphorylates ULK1 (Unc-51-like autophagy-activating kinase) at Ser757, leading to its inhibition and, consequently, a decrease in autophagic activity [10,11]. However, in *Tsc2*-knockdown neurons, the accumulation of the autophagic marker LC3-II and of the autophagy substrate p62 has been observed [9]. The data suggest that, in neurons, despite the mTOR-dependent inhibitory phosphorylation of ULK1 at Ser757, increased AMPK-dependent activating phosphorylation of ULK1 at Ser555 occurs, leading, in effect, to increased autophagic activity through the AMPK-dependent activation of ULK1.

Nevertheless, abnormalities in the mTOR-dependent signalling pathway have been suggested in the pathology of several disorders, from neurodevelopmental syndromes in which autism is highly prevalent, to neurodegenerative disorders such as Alzheimer's disease (AD) and Parkinson's disease (PD) [12–14]. There is convincing evidence that mutations in the *TSC2* gene evoke more severe neurological manifestations, including intellectual disability, than mutations in the *TSC1* gene [15–18].

Therefore, the aim of our study was to determine the effect of haploinsufficiency of *Tsc2* on the expression of synaptic proteins responsible for the regulation of neurotransmitter release and neuronal plasticity. Structural changes in synapses were analysed using transmission electron microscopy (TEM), and appropriate behavioural tests were performed. We identified alterations in the expression of key synaptic proteins and serious ultrastructural changes at synapses in the brains of male *Tsc2*^{+/-} mice, as compared to *Tsc2*^{+/+} animals. Moreover, memory function was impaired, as demonstrated in the novel object recognition test; however, exploratory and social behaviours were not affected in *Tsc2*^{+/-} mice.

2. Results

In our study, we used strain B6;129S4-*Tsc2*^{tm1Djk/J}, which is a murine model of TSC. As shown in Figure 1a,b, the level of the *Tsc2* protein was significantly reduced in the

TSC murine model compared to the control wild-type (wt) $Tsc2^{+/+}$ animals. Consequently, processes downstream of Tsc2 and the mTOR pathway were affected, as demonstrated by alterations of the brain weights in $Tsc2^{+/-}$ mice, suggesting impaired development of the central nervous system and megalencephaly. The average weight of the animal's brain was increased by 3.42 percent in $Tsc2^{+/-}$ mice, whereas the weight of the body was not changed (Figure 1c–e).

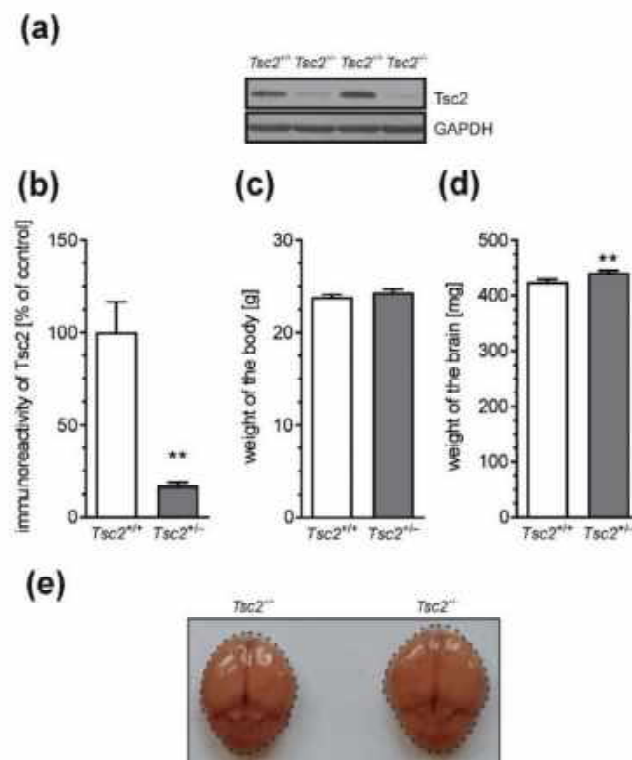


Figure 1. The effect of Tsc2 haploinsufficiency on body weight in mice. (a) The protein level of Tsc2 in the brain cortex of $Tsc2^{+/+}$ and $Tsc2^{+/-}$ two-month-old male mice was analysed by Western blotting. Typical blots are presented. (b) Densitometric analysis of immunoreactivity of Tsc2 in the brain cortex. Data were normalised to GAPDH. $n = 3$. (c,d) The effect of Tsc2 insufficiency on the body weight and brain weight. (e) Example photographs of the brains of $Tsc2^{+/+}$ and $Tsc2^{+/-}$ two-month-old male mice. $n = 30$. Data represent the mean values $\pm$ SEMs. ** $p < 0.01$ vs. wild-type animals, as determined by Student's *t*-test.

To reveal the impact of Tsc2 haploinsufficiency on the expression of synaptic neurotransmission-related proteins, the mRNA and protein levels were analysed by using qPCR and Western blotting, respectively. Calcium-binding synaptic vesicle protein synaptotagmin-1 (Syt1); phosphorylated synapsin (p-Syn), which is responsible for synaptic vesicle docking, fusion, and recycling; the SNARE proteins VAMP1/2, syntaxin 1 (Stx1), and SNAP25; and neuroligins (Nlgn), cell-adhesion molecules that maintain trans-synaptic connections, were investigated in the cerebral cortex, hippocampus (regions CA1/CA2 and CA2/CA3), and cerebellum. It was observed that the expression of specific synaptic proteins was significantly changed in $Tsc2^{+/-}$ mice (Table 1). The mRNA levels for *Vamp1*, *Vamp2*, *Syt1* and *Nlgn1* were modestly increased in the brain cortex of $Tsc2^{+/-}$

mice, whereas the expression of these genes in the hippocampus and cerebellum was not changed. The expression of *Stx1a*, *Stx1b*, *Snap25* and *Nlgn3* was not altered in any of the studied brain structures.

Table 1. The effect of Tsc2 haploinsufficiency on the mRNA levels for synaptic proteins.

mRNA	Hippocampus		Cortex		Cerebellum	
	Tsc2 ^{+/+}	Tsc2 ^{+/-}	Tsc2 ^{+/+}	Tsc2 ^{+/-}	Tsc2 ^{+/+}	Tsc2 ^{+/-}
<i>Vamp1</i>	1.00 ± 0.05	0.99 ± 0.06	1.00 ± 0.05	1.15 ± 0.03 *	1.00 ± 0.07	0.94 ± 0.08
<i>Vamp2</i>	1.00 ± 0.04	0.96 ± 0.03	1.00 ± 0.03	1.22 ± 0.04 **	1.00 ± 0.03	0.95 ± 0.08
<i>Syt1</i>	1.00 ± 0.03	0.96 ± 0.04	1.00 ± 0.03	1.13 ± 0.04 *	1.00 ± 0.05	0.99 ± 0.08
<i>Stx1a</i>	1.00 ± 0.07	0.78 ± 0.04	1.00 ± 0.05	1.11 ± 0.04	1.00 ± 0.24	0.87 ± 0.06
<i>Stx1b</i>	1.00 ± 0.04	0.94 ± 0.02	1.00 ± 0.02	1.00 ± 0.02	1.00 ± 0.03	0.88 ± 0.06
<i>Snap25</i>	1.00 ± 0.08	0.95 ± 0.04	1.00 ± 0.05	1.13 ± 0.03	1.00 ± 0.04	0.84 ± 0.09
<i>Syn1</i>	1.00 ± 0.04	1.11 ± 0.02	1.00 ± 0.06	1.07 ± 0.07	1.00 ± 0.08	1.00 ± 0.07
<i>Nlgn1</i>	1.00 ± 0.09	0.95 ± 0.08	1.00 ± 0.03	1.30 ± 0.03 ***	1.00 ± 0.09	1.04 ± 0.07
<i>Nlgn3</i>	1.00 ± 0.05	1.06 ± 0.02	1.00 ± 0.05	0.98 ± 0.02	1.00 ± 0.02	0.96 ± 0.04

The levels of mRNA in the hippocampus, cortex, and cerebellum were determined by using real-time PCR and calculated using the $\Delta\Delta C_t$ method, with *Actb* as a reference gene. Data presented in the table are relative quantity (RQ) ± SEM ($n = 3-6$). Each sample was from a separate animal. Samples were isolated from at least three different litters. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.01$, compared with the control group, as determined using Student's *t*-test.

Concomitantly, significant changes in the levels of key synaptic proteins were observed (Figure 2, Table 2). The level of VAMP1/2 was significantly ($p < 0.05$) increased in the hippocampus, cerebral cortex, and cerebellum, whereas the levels of Stx1 and Syt1 were reduced in the cerebral cortex but were unchanged in the hippocampus and cerebellum. Interestingly, the immunoreactivity of phospho-synapsin (Ser62/67) was significantly ($p < 0.01$) increased in the hippocampus and cerebral cortex. The levels of the other tested synaptic proteins were not changed in any of the analysed brain structures. Immunoreactivity of neuroligins 1 and 3 was not detected in the cerebellum.

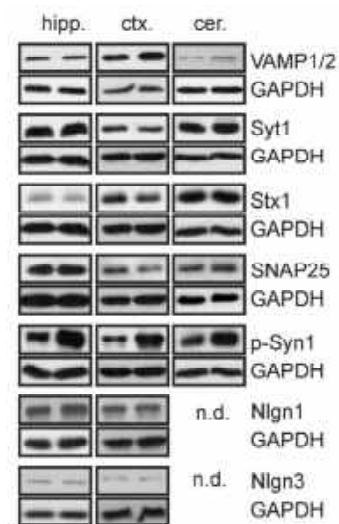


Figure 2. The effect of Tsc2 haploinsufficiency on levels of synaptic proteins. The immunoreactivity of synaptic proteins in the hippocampus (hipp.), cortex (ctx.), and cerebellum (cer.) was determined by using Western blotting. Representative pictures are presented. GAPDH was used as a loading control. n.d.—not detected; densitometric analysis is presented in Table 2.

Table 2. The effect of Tsc2 haploinsufficiency on levels of synaptic proteins—densitometric analysis.

Protein	Hippocampus		Cortex		Cerebellum	
	Tsc2 ^{+/+}	Tsc2 ^{+/-}	Tsc2 ^{+/+}	Tsc2 ^{+/-}	Tsc2 ^{+/+}	Tsc2 ^{+/-}
Vamp1/2	1.00 ± 0.14	1.56 ± 0.08 *	1.00 ± 0.08	1.46 ± 0.19 *	1.00 ± 0.09	1.59 ± 0.17 *
Syt1	1.00 ± 0.09	1.04 ± 0.05	1.00 ± 0.04	0.86 ± 0.04 *	1.00 ± 0.21	1.03 ± 0.19
Stx1	1.00 ± 0.10	0.95 ± 0.12	1.00 ± 0.05	0.80 ± 0.03 **	1.00 ± 0.13	1.10 ± 0.14
Snap25	1.00 ± 0.19	0.82 ± 0.09	1.00 ± 0.18	0.73 ± 0.08	1.00 ± 0.30	1.07 ± 0.33
p-Syn	1.00 ± 0.11	1.77 ± 0.11 **	1.00 ± 0.23	1.94 ± 0.24 *	1.00 ± 0.11	1.20 ± 0.18
Nlgn1	1.00 ± 0.08	1.09 ± 0.02	1.00 ± 0.07	1.08 ± 0.15	not detected	not detected
Nlgn3	1.00 ± 0.07	1.13 ± 0.08	1.00 ± 0.11	1.15 ± 0.23	not detected	not detected

The immunoreactivity of synaptic proteins in the hippocampus, cortex, and cerebellum was determined by using Western blotting. GAPDH was used as a loading control. Results of the densitometric analysis were normalised to GAPDH and calculated as relative changes and are presented as the mean value ± SEM. (*n* = 3–6). Data were analysed using Student's *t*-test. * *p* < 0.05 and ** *p* < 0.01. Each sample was from a separate animal. Samples were isolated from at least three different litters.

Since the amount and availability of synaptic proteins affect not only synaptic signalling but also the structure and plasticity of neurons, and the fact that changes in synaptic morphology and number are linked to the majority of psychiatric and neurological disorders, from autism to neurodegenerative disorders, a comprehensive ultrastructural analysis of the brains of Tsc2^{+/-} mice using transmission electron microscopy (TEM) was performed.

We analysed neurons in the cerebral cortex, cerebellum, and two regions of the hippocampus, CA1/CA2 and CA2/CA3. A full list of the pathological alterations in all the analysed brain structures of Tsc2^{+/-} mice is presented in Table 3. Example microphotographs showing control tissue from Tsc2^{+/+} animals and typical alterations in Tsc2^{+/-} animals are presented in Figures 3–5. The analysis demonstrated that Tsc2 haploinsufficiency evoked prominent pathological changes in the morphology of neurons. We observed a blurred structure of synaptic density (Figure 4a,b) and a significantly increased number of synaptic vesicles (Figure 4c,d). Some synapses were extremely overloaded with synaptic vesicles in the brains of Tsc2^{+/-} animals. Numerous overloaded synaptic endings were observed exclusively in the hippocampus; very few were observed in the cortex and cerebellum. Semi-quantitative analysis of the average number of synaptic vesicles was performed. As demonstrated in Figure 6, a statistically significant difference in the number of synaptic vesicles in the synaptic ending was confirmed only in the CA2/CA3 region of the hippocampus. We also detected impairment of mitochondrial ultrastructure, represented by swelling and blurred crista contours. The mitochondrial pathology was typically observed in synaptic endings. Additionally, significantly elongated mitochondria with blurred constrictions were observed, which was distinctively different from those in the control tissue from Tsc2^{+/+} animals, where the mitochondria were predominantly of ball-like structure or sausage-shaped (Figure 4e,f). These morphological differences may be due to the different patterns in the expression of fission/fusion proteins as well as defects in mitophagy since mTOR kinase is involved in the regulation of both phenomena [19]. We found that some of the endoplasmic reticulum networks were fragmented, with polyribosomes freed in the cytosol (Figure 5a,b). Moreover, the swelling of the Golgi apparatus in Tsc2^{+/-} animals was evident (Figure 5c,d). Untypical vacuolar structures were observed in the neuronal nuclei, which may indicate certain cytoskeleton disorders (Figure 5e,f). Additionally, we observed the delamination of myelin in Tsc2^{+/-} mice (Figure 5g,h).

Table 3. Pathological alterations of neuronal ultrastructure found in the brains of $Tsc2^{+/-}$ mice.

	CA1/CA2	CA2/CA3	Cortex	Cerebellum
blurred structure of synaptic density	+	+	+	+
overloaded synaptic endings	+	+	+/-	+/-
swollen mitochondria	+	+	+	+
excessively elongated mitochondria	+	+	+	+
polyribosomes in the cytoplasm	+	+	+	+
swollen Golgi apparatus	+	+	+	+
vacuolar structures in the cell nucleus	+	+	+	+
delamination of myelin	+	+	+	+

+ alteration frequently observed in the tissue of $Tsc2^{+/-}$ animals; +/- alteration exceptionally observed in the tissue of $Tsc2^{+/-}$ animals.

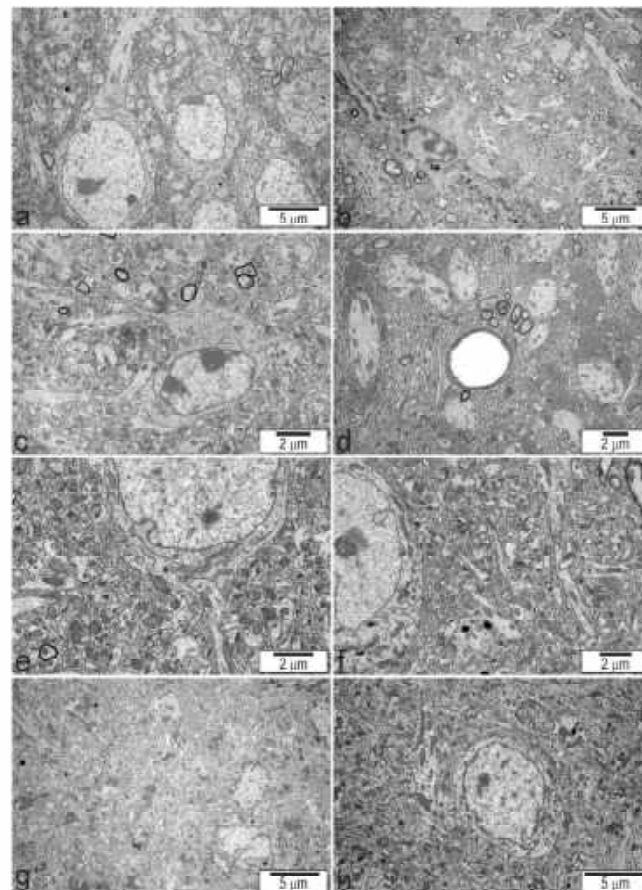


Figure 3. The effect of $Tsc2$ haploinsufficiency on the ultrastructure of neuronal cells in the CA1/CA2 (a,b). and CA2/CA3 (c,d) regions of the hippocampus, brain cortex (e,f), and cerebellum (g,h). (a,c,e,g)—control group $Tsc2^{+/+}$; (b,d,f,h)— $Tsc2^{+/-}$ group. Typical electronograms at low magnification are presented.

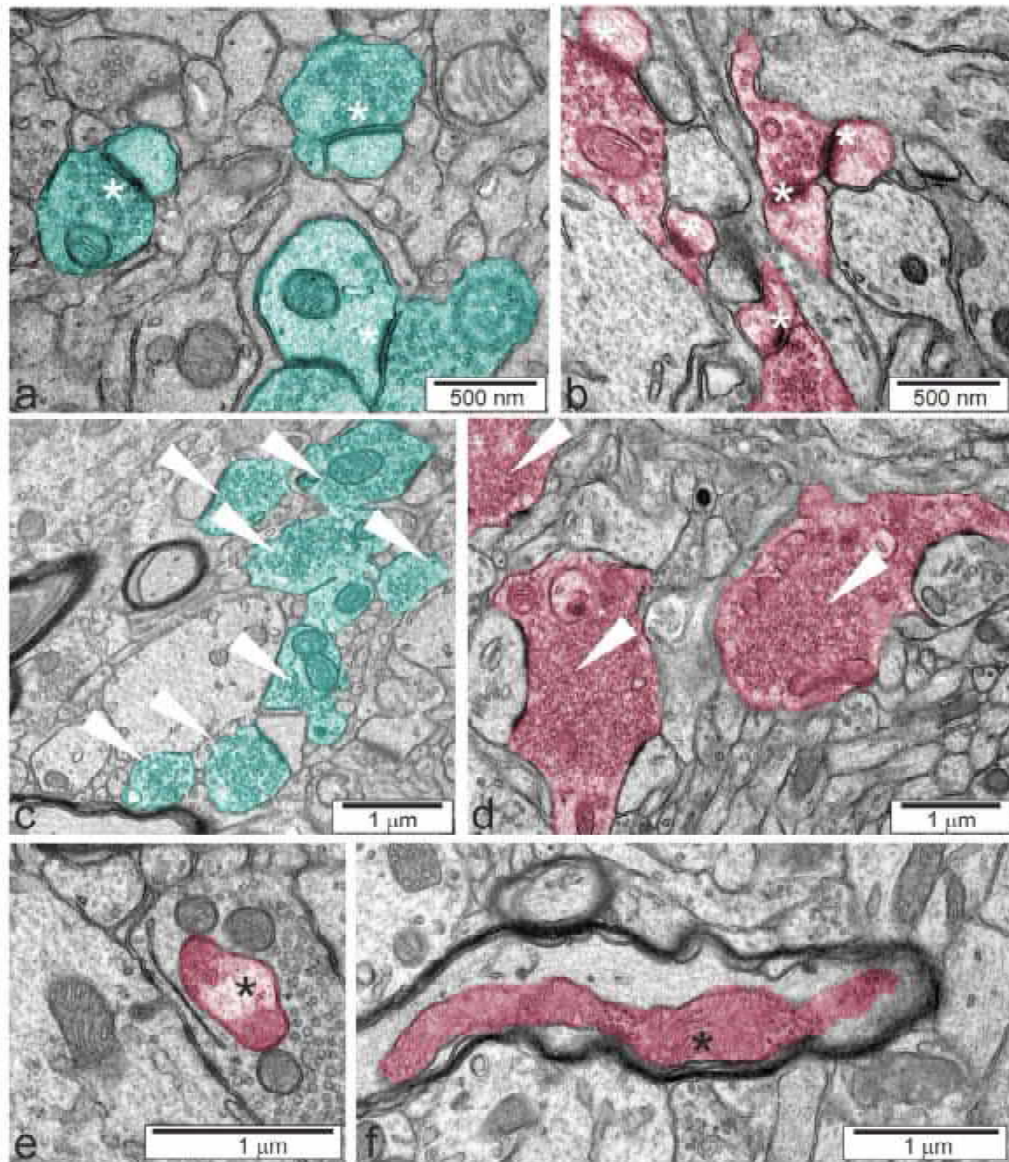


Figure 4. Typical electronograms presenting morphological alterations of neuronal cells in the hippocampus. (a) Correct synaptic density (white asterisk), (b) blurred structure for synaptic density (white asterisk), (c) correct synapses (arrowheads), (d) overloaded synaptic endings (arrowheads), (e) swollen mitochondrion (black asterisk), and (f) excessively elongated mitochondrion (black asterisk). The colour was used to label control (turquoise) and altered (reddish) structures.

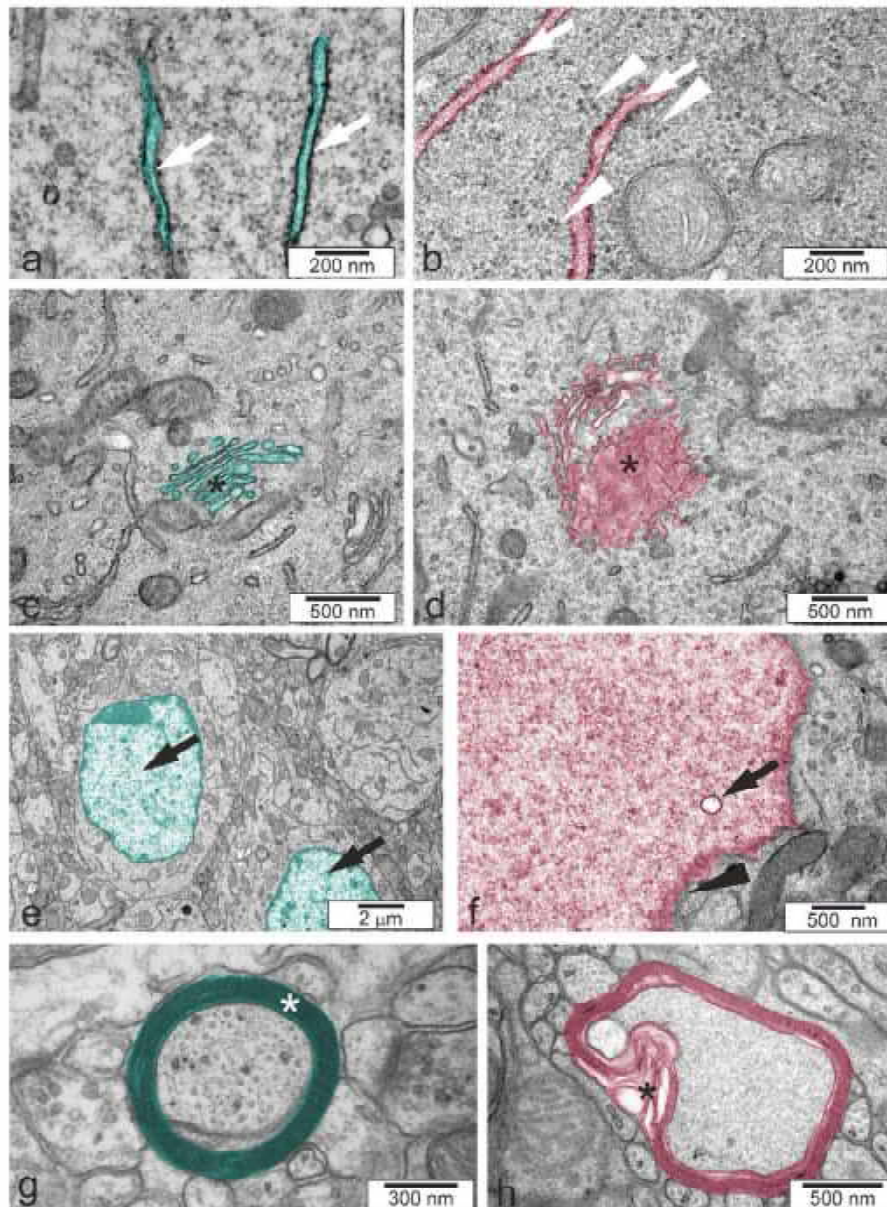


Figure 5. Typical electronograms presenting morphological alterations of neuronal cells in the hippocampus. (a) Correct cytoplasm and endoplasmic reticulum (white arrow), (b) swollen endoplasmic reticulum (white arrows) and polyribosomes in the cytoplasm (white arrowheads), (c) correct Golgi apparatus (black asterisk), (d) swollen Golgi apparatus (black asterisk), (e) correct nuclei (black arrows), (f) vacuolar structures in the cell nucleus (black arrow) and diffused nuclear envelope (black arrowhead), (g) correct structure of myelin (white asterisk), and (h) delamination of myelin (black asterisk). The colour was used to label control (turquoise) and altered (reddish) structures.

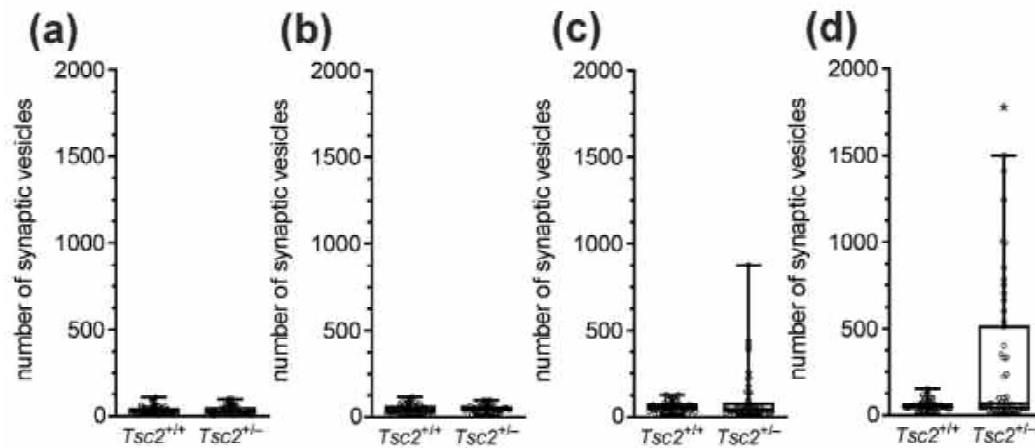


Figure 6. The effect of *Tsc2* haploinsufficiency on the number of synaptic vesicles in (a) cerebral cortex, (b) cerebellum, (c) CA1/CA2 region of the hippocampus, and (d) CA2/CA3 region of the hippocampus. The quantitative analysis of microphotographs was performed for 5 animals in each group; 10 random synapses from each animal were taken for analysis ($n = 50$). Data were not normally distributed and are presented as medians with interquartile ranges, minimums, and maximums. * $p < 0.05$ vs. wild-type animals, as determined using the Mann–Whitney test.

To examine whether *Tsc2*^{+/-} haploinsufficiency was associated with behavioural deficits, several aspects of animals' behaviour were analysed. As shown in Figure 7, exploratory activity and anxiety-related behaviours were not changed in *Tsc2*^{+/-} mice, as determined in the open-field test. The distance travelled during the test (Figure 7a) and the exploration of the central zone (Figure 7b,c) were the same in *Tsc2*^{+/+} and *Tsc2*^{+/-} animals. Although the numbers of episodes of defecation (Figure 7d), rearings (Figure 7e), climbing (Figure 7f), and grooming (Figure 7g,h) behaviour did not show statistically significant differences between *Tsc2*^{+/+} and *Tsc2*^{+/-} animals ($p > 0.05$), a weak but tenacious drift toward increased anxiety in *Tsc2*^{+/-} mice was visible. Because TSC haploinsufficiency is known to induce autistic behaviours in patients, a three-chamber Crowley's test was also performed. In this test, the animal is allowed to freely investigate two chambers connected by a third one. The first chamber contains a cage with another animal, and the second chamber contains an empty cage. Wild-type animals prefer staying in the chamber with another animal. When social behaviour is impaired, the preference toward an animal's cage is significantly reduced. As shown in Figure 8a, both *Tsc2*^{+/+} and *Tsc2*^{+/-} mice spend significantly more time on the exploration of the chamber with the unknown animal than on the exploration of the chamber containing an empty cage; however, no difference between *Tsc2*^{+/+} and *Tsc2*^{+/-} was observed. The analysis of the time spent on the direct investigation of the cages provides more specific results. As shown in Figure 8b, the cage with an animal was investigated for significantly longer by the tested animal than the empty cage; however, no differences between *Tsc2*^{+/+} and *Tsc2*^{+/-} were detected. Finally, we performed a novel object recognition test. As shown in Figure 9, the index of discrimination (ID) in *Tsc2*^{+/-} mice is reduced compared to that in *Tsc2*^{+/+} mice.

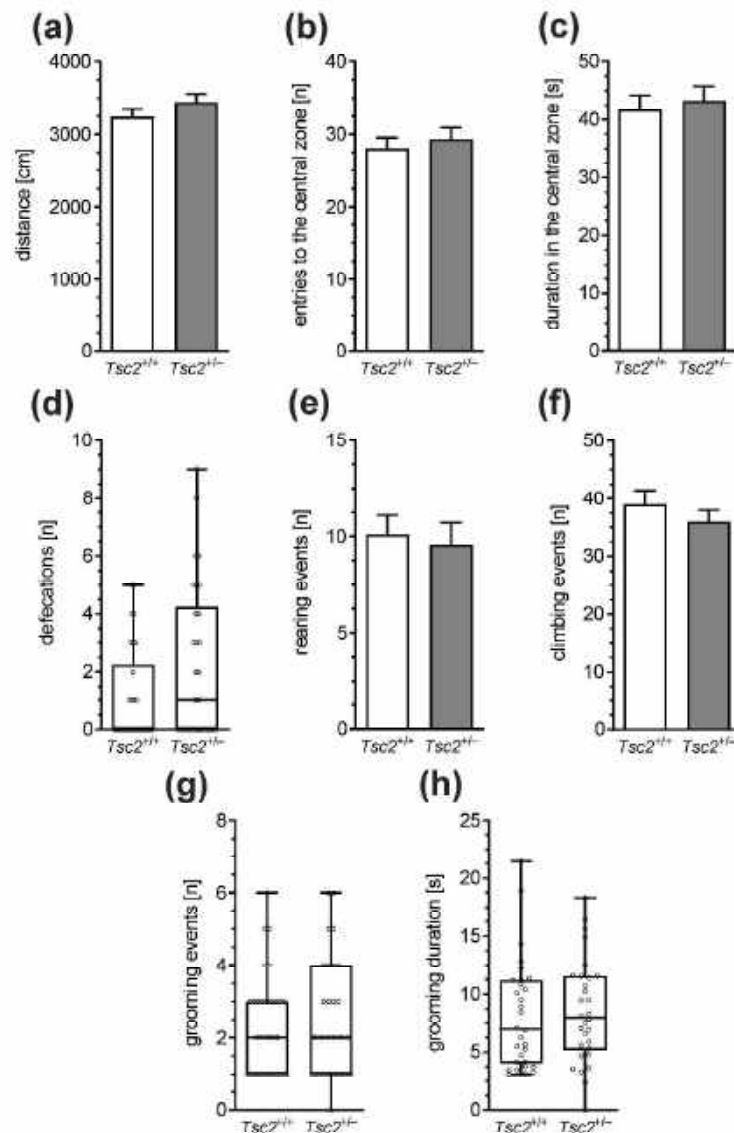


Figure 7. The effect of *Tsc2* haploinsufficiency in mice on behaviour (exploratory activity). The exploratory activity of male mice was analysed in an open-field test. (a) The total distance travelled by animals, (b) the number of entries to the central zone, (c) the time spent in the central zone, (d) the number of defecation events, (e) the number of rearing events, (f) the number of climbing events, (g) the number of grooming events, and (h) the total time spent on self-grooming. Data (a–c,e,f) represent the mean values $\pm$ SEMs from $n = 30$ independent experiments. Data (d,g,h) not normally distributed are presented as medians with interquartile ranges, minimums, and maximums ($n = 30$). Each data point is from a separate animal.

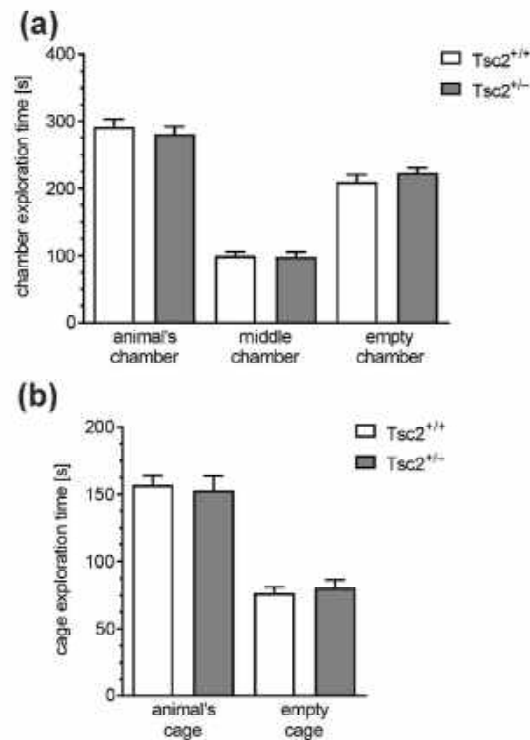


Figure 8. The effect of *Tsc2* haploinsufficiency in mice on behaviour (sociability). (a) The time spent by the tested animal in the chamber; (b) the time spent by the tested animal on the exploration of cages. Data represent the mean values $\pm$ SEMs from $n = 20$ independent experiments. Each data point is from a separate animal.

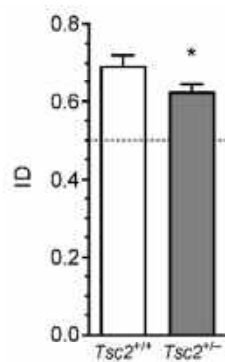


Figure 9. The effect of *Tsc2* haploinsufficiency in mice on behaviour (cognitive function). The memory of male mice was analysed in a novel object recognition test. Index of discrimination (ID) was calculated, as described in the Methods section. Presented results are means $\pm$ SEMs from $n = 10$ animals in each group. * $p < 0.05$, compared to control, as determined using Student's *t*-test. Each data point is from a separate animal.

3. Discussion

In our study, we used a commercially available murine transgenic model of TSC. In the strain B6;129S4-*Tsc2*^{tm1Djk/J}, the gene *Tsc2* was inactivated by the insertion of the *neo* cassette into exon 2 [20]. *Tsc2*^{-/-} homozygosity is lethal in the embryonic stage. *Tsc2*^{+/-} mice with *Tsc2* immunodeficiency develop symptoms resembling human TSC. Because of the deficiency of *Tsc2*, the activity of the *Tsc1/Tsc2* complex is decreased, leading to the overactivation of the mTOR pathway, which is the primary cause of TSC-related symptoms, including tumours of the brain, skin, heart, lungs, and kidney [1,21]. In the brain, cortical tubers are typical abnormalities [22]. The presence of tuber-like lesions has been reported in several rodent models of TSC [23–25]. However, in other experimental models, despite some typical TSC symptoms being mimicked, the presence of cortical tubers has not been observed, suggesting that the neurocognitive symptoms may, rather, be caused by the impairment of neuronal connectivity or signalling [26,27]. These pathological tuber-like alterations were also not observed in our study in two-month-old animals.

TSC patients have many neurological and mental symptoms, including autism [1]. A growing body of evidence indicates that the neurobiological basis of autism in TSC may be abnormal synaptic plasticity and alterations in neural connectivity [28,29]. Hamartin (*Tsc1*) and tuberin (*Tsc2*) regulate synaptic growth and function in neurons, and their loss causes enlarged spines [30]. A hyperactive mTOR pathway, as a result of *Tsc2* insufficiency, is probably responsible for the autism-related symptoms in TSC. The mTOR pathway plays essential roles in the translational machinery, protein-synthesis-dependent long-term potentiation (LTP), synaptic plasticity, and neural network and connectivity formation. The mTOR-kinase-dependent regulation of the activity of the mRNA cap-binding factor eIF4E and S6K is a major effector mechanism through which synaptic protein synthesis is modulated [31]. Kelleher and Bear hypothesised that an increase in the activity of the mTOR pathway could lead to excessive plasticity-related synaptic protein synthesis that may result in changes in synaptic connectivity and cognitive impairment and be one of several pathogenic mechanisms leading to autism [31]. The impairment of synaptic plasticity was also reported in a rat model of TSC (*Tsc2*^{+/-} rats) [32].

In the current research, we identified alterations in synaptic proteostasis and synaptic ultrastructure impairments, as we previously observed in animal models of autism evoked by environmental factors [33,34]. Concomitantly, a higher synaptic protein level was observed in fragile X syndrome (FXS), a single-gene disorder associated with autism [31]. In the present study, we showed a significantly higher level of key presynaptic proteins involved in the kinetics of synaptic vesicles (SVs), fusion, and neuroexocytosis, such as p-synapsin and synaptobrevin (VAMP1/2). Both p-synapsin and VAMP1/2 were increased in the cerebral cortex and hippocampus; however, in the cerebellum, only VAMP1/2 was upregulated. Changes in synaptic protein expression are dependent on the brain structure, and the greatest disturbance of synaptic protein homeostasis has been observed in the cerebral cortex, where, additionally, a deficit of synaptotagmin and syntaxin-1 has been demonstrated.

Synapsins are a family of phosphoproteins that are the most abundant on SVs. They are involved in the regulation of various synaptic functions such as the formation of presynaptic terminals, the regulation of the vesicle reserve pool at presynaptic terminals, synaptogenesis, and synaptic vesicle docking. Synapsin-1, the major isoform in neurons, plays a key role in the regulation of the SV life cycle and maintains a reserve pool of SVs in the vicinity of the active zone. The binding of synapsin-1 to SV depends on the degree of its phosphorylation. In the dephosphorylated form, synapsin-1 binds to SV, whereas the phosphorylated form dissociates from SVs and enables their translocation to the plasma membrane, their entry to the path of exocytosis, and the release of neurotransmitters. Since synapsin-1 in its phosphorylated form plays a key role in mediating the trafficking of SVs to the active zone, an increase in its level could be responsible for increased neurotransmitter release. Interestingly, this neuron-specific phosphoprotein is expressed with divergent contents and plays diverse roles in excitatory glutamatergic and inhibitory

GABAergic neurons [35,36]. Synapsins maintain the reserve pool of glutamatergic vesicles, while regulating the size of the readily releasable pool of GABAergic vesicles. Therefore, we suggest that the mTOR-dependent upregulation of p-synapsin may underlie the E/I imbalance observed in autism spectrum disorders (ASD). Like synapsin-1, VAMP1/2 is localised to SVs and plays an important role in the regulation of neuroexocytosis and the kinetics of neurotransmitter release. Together with syntaxin-1 and SNAP-25, VAMP1/2 forms the SNARE complex that provides the energy to drive fusion. Changes in the expression and activity of any of the SNARE proteins could affect SV fusion, neurotransmitter release, and plasticity. Higher expression of VAMP1/2 could promote SNARE complex formation and exocytosis. In our study, we observed a significant increase in both the mRNA and protein levels of VAMP1/2 in the cerebral cortex and higher protein levels in the hippocampus and cerebellum. Changes in VAMP1/2 levels were also observed in the experimental models of ASD. Prenatal exposure to polyinosinic-polycytidylic acid (PIC) evoked an increase in VAMP1/2 levels in offspring [37]. Similarly, prenatal exposure to valproic acid (VPA) induced an increase in the VAMP1/2 level in the hippocampus but not in the cerebral cortex [33]. In the present work, despite the increase in the level of VAMP1/2, we observed a significantly lower level of syntaxin-1 in the cerebral cortex, which can lead to the dysfunction of the SNARE complex and impaired neurotransmission. Syntaxin-1 is a membrane protein localised to the plasma membrane of the presynaptic active zone and involved in vesicle fusion. In addition to participating in SNARE complex formation, syntaxin-1 binds synaptotagmin on SVs, allowing SV fusion with the plasma membrane. The level of the latter protein is also lowered in the cerebral cortex, despite increased expression of the *Syt1* gene, indicating disturbances of SV cycling in this brain structure. We also noted a higher expression of neuroligin-1 in the cerebral cortex, which, however, is not reflected in the protein level. Therefore, this phenomenon has no biological effect. All these results indicate that specific presynaptic molecules involved in the SV recycling events, the regulation of synaptic plasticity, and brain development are affected by mTOR-kinase-dependent signalling. The activation of this molecular machinery regulating synaptic protein synthesis could be responsible for pathological changes in synaptic structure, function, and plasticity. mTOR is also a key regulator of gene transcription, so its overactivation significantly affects the activity of several transcription factors (e.g., STAT3, PPAR- γ , HIF-1 α , TFEB, and YY1) and the expression of many genes [38]. Moreover, the mTOR pathway also regulates epigenetic mechanisms [39]. A recent study demonstrated a general decrease in the hippocampal histone H3 acetylation level in a mouse model of TSC, suggesting that gene expression may be an important factor in TSC pathology [40]. This also raises the possibility of wide alterations of the expression profile in TSC, including for genes encoding synaptic proteins.

Along with the dysfunction of synaptic proteins, dramatic changes in the ultrastructure of the synapses were identified. Since we observed significant dysregulation of specific synaptic proteins in both the cerebral cortex and hippocampus, but statistically significant differences in the number of synaptic vesicles exclusively in the CA2/CA3 region of the hippocampus, it is possible that other proteins not investigated here could be responsible for the observed synaptic overload in the hippocampus. Therefore, the molecular mechanisms responsible for overloaded synaptic hippocampal endings remain unclear and further research is needed to fully understand the link between the activation of mTOR signalling and synapse deregulation.

The alterations of synaptic density in *Tsc2*^{+/-} mice may be directly related to fluctuations in synaptic proteins. The blurred structure of the synaptic cleft without clearly marked pre- and postsynaptic membranes and a disturbed synaptic membrane were previously observed in experimental models of ASD, in adolescent rat offspring after prenatal exposure to LPS or VPA [33,41]. The synaptic imbalance in TSC was recently suggested to be the result of astrocytes' impairment [42]. Dooves and co-workers suggested that factors released by affected astrocytes alter the synaptic balance. The presence of giant synapses in the hippocampi of *Tsc2*^{+/-} animals may also be related to impaired synthesis of

synaptic proteins. Interestingly, the impairment of the endoplasmic reticulum, an increased fraction of free polyribosomes, and the impairment of the Golgi apparatus may indicate that local protein synthesis, modification, and transport in $Tsc2^{+/-}$ animals may be the crucial pathological event triggering further perturbations. Abnormal translation at the synapse was suggested to contribute to the pathological mechanisms of ASD [43].

The local synthesis of synaptic proteins requires energy delivered by synaptic mitochondria [44,45]. Our electron microscopic analysis of mitochondria revealed morphological alterations in $Tsc2^{+/-}$ mice, including common swelling and occasionally excessive elongation. Mitochondrial and bioenergetic anomalies have been observed in patients with ASD and in TSC models [46,47]. Along with these pathological changes in the ultrastructure, our TEM studies indicated the delamination of the myelin sheath in $Tsc2^{+/-}$ animals. This abnormal myelination could contribute to disturbances in processing speed and in neuronal signal transduction. In a recent study by Mühlebner and co-workers, myelin pathology in cortical tubers resected from TSC patients was demonstrated [48]. The level of myelin-associated oligodendrocyte basic protein was decreased and was associated with the presence of autism-related symptoms. Overactivation of the mTOR pathway is probably responsible for the TSC-related impairment of the maturation of oligodendroglia and production of the myelin sheath [49].

In addition, the present results indicate that defects in the mTOR signalling pathway lead to the abnormal enlargement of the brain, megalencephaly. The data are in line with the theory that the dysregulation of mTOR is central to multiple forms of developmental megalencephaly and macrocephaly [50]. The presence of brain enlargement is consistent with the increased brain weight that has also been observed by others [51]. Numerous studies show that mutations in different genes acting as regulators of mTOR signalling and, consequently, the activation of the mTOR pathway lead to megalencephaly or hemimegalencephaly [52–56]. Our results are also in line with previous studies that used a mouse model of TSC, $Tsc2^{lox/ko};hGFAP-Cre$. In this model, the *Tsc2* gene has been removed from most neurons and glia of the cortex and hippocampus by targeted Cre-mediated deletion in radial glial neuroprogenitor cells. The $Tsc2^{lox/ko};hGFAP-Cre$ mice demonstrated significant brain pathology including brain overgrowth, postnatal megalencephaly, cortical and hippocampal lamination defects, enlarged dysplastic neurons and glia, and astrogliosis [57,58]. Likewise, other studies showed that $Tsc1^{GFAP}$ KO mice developed dramatic, diffuse megalencephaly and higher brain weights compared to control mice [59]. It should also be emphasised that hemimegalencephaly has been demonstrated in some patients with TSC along with abnormal myelination patterns and gyral abnormalities in the enlarged hemisphere [53,60–62]. Although megalencephaly and hemimegalencephaly are not typical symptoms of TSC, the case reports confirm the involvement of the activation of the mTOR pathway in processes leading to brain overgrowth, similar to our studies with $Tsc2^{+/-}$ mice. In addition, macrocephaly was observed in children with TSC, but to a lesser extent than in autistic patients [63]. In addition, it was suggested that the overactivation of the mTOR pathway that regulates cell growth, survival, and differentiation could contribute to neuronal hypertrophy and hyperconnectivity leading to network dysfunction and megalencephaly, which, in turn, contributes to the development of autism symptoms [64]. Macrocephaly is present in ca. 15% of patients with idiopathic autism, and head and brain growth is disproportionate to body growth in early childhood [65–67]. Therefore, our TSC model effectively recapitulates the brain structure pathology observed in ASD patients with higher mTOR signalling and suggests that disturbances in the mTOR pathway might be responsible for this phenomenon in some autistic patients.

As reported previously, the vast majority of TSC patients have neurological and mental symptoms, including autism, epilepsy, mental retardation, cognitive impairment, attention-deficit hyperactivity disorder, sleep disruption, and anxiety [1]. Recent studies demonstrated that the prevalence of autism in patients with TSC is over 40–50% [68–71]. The inhibition of mTOR was shown to be an effective strategy for preventing epileptic seizures and behavioural alterations [72]. Treatment with mTOR inhibitors was also demon-

strated to be effective in experimental models of autism [73]. Moreover, approximately 50% of TSC patients are developmentally delayed or have an intellectual disability [74].

Experimental animal models successfully replicate only selected typical TSC-related changes. In $Tsc2^{+/-}$ rats, impairment of synaptic plasticity, enhanced episodic-like memory, reduced exploration behaviour, and no differences in anxiety-related behaviour and hippocampus-dependent learning were observed [32,75–77]. Additionally, autism-like social deficits were present, and the exploration of novel objects was reduced [76,77]. In $Tsc2^{+/-}$ mice, synaptic plasticity was also affected [78]. Correspondingly, cognitive deficits were observed [79]. Our behavioural analysis demonstrated that, despite the significant impairment of synaptic structure, the animal's behaviour was almost intact. The exploratory activity, as determined using the open field test, was not changed in $Tsc2^{+/-}$ mice. Additionally, we analysed the numbers of climbings and rearings, and the grooming behaviour, which may indicate changes in anxiety levels in animals. We observed a weak but persistent drift toward increased anxiety in $Tsc2^{+/-}$ mice; however, the difference was without statistical significance. Possibly, the strength of anxiety-like behaviours is very low in $Tsc2^{+/-}$ mice, therefore, much more numerous experimental groups should be tested to confirm this phenomenon.

Because TSC haploinsufficiency is related to autistic behaviours in a high fraction of patients, we analysed the sociability of $Tsc2^{+/-}$ mice in a three-chamber test. The impairment of social behaviour was demonstrated in several experimental models of TSC [27,76,80]. However, in our analysis, $Tsc2^{+/+}$ and $Tsc2^{+/-}$ mice presented the same levels of sociability which is consistent with previously published results of the analysis of the same mouse strain [79,81]. The origin of this inconsistency in experimental models is elusive. It may be caused by the variability in experimental conditions. Another question is the discrepancy between the high frequency of autistic symptoms in TSC patients and the absence of autistic symptoms in our experimental model. We suppose that additional environmental factors may be necessary to induce social behaviour impairment in patients (and animals) with mutations in the *TSC1/2* genes. For example, prenatal viral infection, which is a risk factor for neuropsychiatric disorders, could be such an interfering factor. The data indicate that maternal immune activation (poly(I:C) model) and *Tsc2* haploinsufficiency cooperate to disrupt adult social behaviour in mice [81]. Another important factor interacting with *TSC1/2* haploinsufficiency could be age. The dynamics of the development of autistic behaviours are not fully understood. It was demonstrated that autistic behaviours may be acquired with age [82].

The most significant behavioural alteration we observed was in the novel object recognition test, which tests hippocampus-dependent memory function [83]. This result may indicate that the learning and memory processes in *Tsc2*-haploinsufficient mice are slightly impaired compared to those in wild-type mice. However, the result of this test may also have been caused by motor stereotypies. It was recently suggested that the novel object exploration test (not to be confused with the novel object recognition test) may be a potential assay for higher-order repetitive behaviours in mice [84,85]. Repetitive behaviours are a part of autism but may also occur in other conditions such as attention deficit hyperactivity disorder, obsessive-compulsive disorder, and schizophrenia, or even during typical development. Additional studies would be necessary to fully understand the meaning of the reduced ID in the novel object recognition test in $Tsc2^{+/-}$ mice.

Our study supports and extends evidence for the synaptic alterations in TSC. We demonstrate in a rodent model of TSC that inactivation of *Tsc2* gene triggers pathological alterations in synaptic ultrastructure as well as brain overgrowth. In parallel, we indicate changes in the expression of several synaptic proteins that could be responsible for the impairment of synaptic plasticity and memory function.

4. Materials and Methods

4.1. Chemicals

TRI Reagent, DNase I, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rabbit monoclonal anti-Tsc2 antibody was purchased from Novus Biologicals (Centennial, CO, USA). Mouse monoclonal anti-VAMP1/2 antibody, rabbit polyclonal anti-phospho-synapsin (Ser62/67) antibody, and mouse monoclonal anti-syntaxin-1 were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Rabbit polyclonal anti-synaptotagmin-1 antibody and rabbit monoclonal anti-SNAP25 antibody were obtained from Cell Signalling (Beverly, MA, USA). Rabbit polyclonal anti-GAPDH antibody and goat anti-rabbit IgG antibody were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sheep anti-mouse IgG antibody was purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). The chemiluminescent reagent Clarity Western ECL Substrate was purchased from Bio-Rad Laboratories (Hercules, CA, USA). The cOmplete protease inhibitor cocktail was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Reagents for reverse transcription (High Capacity cDNA Reverse Transcription Kit) and PCR (TaqMan Fast Advanced Master Mix) were obtained from Thermo Fisher Scientific (Paisley, UK). DMSO and all the other common reagents were from Sigma-Aldrich (St. Louis, MO, USA).

4.2. Animals

Young male B6;129S4-Tsc2^{tm1Djk/J} mice (*Mus musculus*) were provided by The Jackson Laboratory and bred in The Laboratory For Genetically Modified Animals of the Mossakowski Medical Research Institute PAS (Warsaw, Poland), which breeds small rodents according to the SPF standard. Animals were analysed at postnatal days 50–60 (PND50–60). The animals were maintained under controlled temperature and humidity conditions with a 12 h light/dark cycle. All of the experiments conducted on animals were approved by the Local Ethics Committee for Animal Experimentation in Warsaw (reference numbers WAW2/081/2019, WAW2/142/2019, and WAW2/045/2020) and were carried out following the EU Directive 2010/63/EU for animal experiments. Every effort was made to minimise the number of animals used and reduce the amount of pain and distress. In the whole study, a group of 60 animals was tested. To avoid the litter effect [86], for every test/assay, data from at least three different litters were analysed.

To determine the genotype, DNA was isolated from the tail as previously described [87]. The PCR was performed in accordance with the protocol recommended by the Jackson Laboratory (<https://www.jax.org/strain/004686>; accessed on 16 October 2019) in the presence of EvaGreen dye (Biotium, Inc., Fremont, CA, USA). The melt curve analysis was performed using the ABI PRISM 7500 apparatus to distinguish between the different genotypes.

4.3. Behavioural Analysis

One week before the first behavioural test, animals were transported to the Behavioural facility anteroom with an inverted day–night phase. Starting from postnatal day (PND) 43, all the animals were subjected, every day, to handling procedures and to the sound-isolated analysis room (overhead diffused light, ca. 12 lux). The behavioural tests were performed by a blinded operator who was not present in the analysis room during the test. The animals' behaviour was recorded using a Basler acA1300-60 GigE camera (Basler AG, Germany), and the behaviour was analysed by the blinded operator or calculated using Ethovision XT 10 (Noldus Information Technology, Wageningen, The Netherlands). All the tests were performed in the morning (the beginning of the night phase).

4.3.1. Open-Field Test

The open-field test was performed as previously described [33]. In this test, general locomotor activity, novel environment exploration, and anxiety-related behaviour may be measured. Sixty animals (30 in each experimental group) at 50–51 PND were individually placed in the corner of an open-field chamber (dark grey PCV box, 55 × 55 × 30 cm), and

the total distance and the exploration of the central zone (diameter, 36.5 cm) and the border zone were analysed. The numbers of episodes of grooming, rearings, and climbings were counted by the blinded operator.

4.3.2. Novel Object Recognition Test

The test was performed on twenty mice (10 in each group) at PND 54–56, as previously described with modifications [88]. This test exploits the natural tendency of rodents to explore novel objects, for testing non-spatial memory. A day before testing, mice were individually subjected to a habituation session; they were allowed to freely explore the test chamber (a dark grey PCV box, 30 cm × 20 cm × 30 cm) for 5 min. The experimental session consisted of two trials. In the first trial (T1), two identical objects (O1) were placed in the chamber. During the second trial (T2), one object, O1, was replaced with the alternative object, O2. As testing objects, we used a set of plastic bricks and a cell-culture bottle. The objects presented during the sessions were free of olfactory traits, and their positions in the chamber were randomised to eliminate spatial bias in the task. At the beginning of each trial, mice were placed at the centre of the box, with their heads oriented in the opposite direction to the object. The respective durations of T1 and T2 were 5 and 2 min. T2 started 2 h after T1. The basic measurement was the total time spent by mice exploring objects during the T1 and T2 trials. The exploration of an object was defined as follows: directing the nose at a distance of 2 cm to the object and/or touching it with the nose. The climbing time was excluded from the analysis. The index of discrimination (ID) was calculated for each animal and expressed as a ratio: the time spent exploring the novel object/the added time exploring novel and known objects.

4.3.3. Three-Chamber Social Interaction Test (Crawley's Sociability and Preference for Social Novelty Test)

The three-chamber social interaction test was performed on forty mice (20 in each experimental group), as described previously with modifications [33]. This test assesses general sociability in rodents. The mouse normally prefers to spend more time with another mouse. At PND 58–59, mice were introduced to a three-chamber social interaction apparatus (100 × 55 × 30 cm). Openings between the compartments (10 cm-wide doors) allowed the animals to access all three chambers. In phase I, each tested animal was allowed to explore the environment freely for 10 min for habituation. After the habituation phase (phase I), the subject was gently guided by the operator to the central chamber, and the two entrances were blocked. Two metal wire cages, the first one containing a sex-, age-, and weight-matched mouse and the second one empty, were placed in the left and right chambers (the order was randomised). Then, the two entrances were opened to allow the tested animal to explore the new environment freely for 10 min, and the social preference was measured (social stimulus vs. non-social stimulus)—phase II. Individual movement tracks were recorded by using a video system and analysed using the BehaView 0.0.16 software <http://www.pmbogusz.net/?a=behaview> (accessed on 24 August 2021). The time spent in each chamber and the time spent on direct interaction with the animal were measured by a blinded operator.

4.4. Transmission Electron Microscopy (TEM) Analysis

TEM analysis was performed as described previously [41]. Ten animals (5 in each experimental group) at PND 60 were anaesthetised with ketamine and xylazine (100 and 10 mg/kg, respectively, i.p.) and perfused through the ascending aorta initially with 0.9% NaCl in 0.01 M sodium-potassium phosphate buffer, pH 7.4, and afterward with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, at 4 °C. Small (ca. 1–2 mm³) pieces of brain tissue were dissected: (i) from the brain cortex—primary somatosensory area, layer 1–4; (ii) from the hippocampus—cornu ammonis (CA) subregions CA1/CA2 and subregions CA2/CA3 (both pyramidal layer and stratum radiatum were collected); (iii) from the cerebellum—Crus2 (both molecular and granular layers).

All the structures were identified according to the Allen Brain Atlas for the adult mouse (<https://atlas.brain-map.org/>; accessed on 24 August 2021) [89].

Tissue specimens were post-fixed in the ice-cold fixative solution for 24 h and placed in a mixture of 1% OsO₄ and 0.8% K₄[Fe(CN)₆]. After dehydration in a series of ethanol gradients, the tissue specimens were embedded in epoxy resin (Epon 812) at 60 °C for 24 h. Ultra-thin sections (60 nm) from the somatosensory cortex were stained with uranyl acetate and lead citrate and then examined by transmission electron microscopy (JEM 1011, Jeol, Japan) using a MORADA camera and iTEM 1233 software, operated at 80 kV.

To assess the numbers of synaptic vesicles in nerve terminals, electronograms under a magnification of 50,000 × were taken. The numbers of vesicles in 10 random nerve endings in each animal were counted; in total, 50 nerve endings from 5 animals were analysed. The data were not normally distributed and, therefore, are presented as medians with interquartile ranges, minimums, and maximums.

4.5. Analysis of the mRNA Level

The tissue samples for mRNA analysis were collected at PND 60 and stored at −80 °C until analysis. Total RNA was isolated using TRI Reagent (Sigma Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol, and the digestion of contaminating DNA was performed using DNase I according to the manufacturer's protocol (Sigma Aldrich, St. Louis, MO, USA). The RNA quantity and quality were checked by spectrophotometric analysis. A reverse transcription was performed using the High Capacity cDNA Reverse Transcription Kit according to the manufacturer's protocol (Thermo Fisher Scientific, Paisley, UK). Quantitative PCR was performed on an ABI PRISM 7500 apparatus using commercially available TaqMan Gene Expression Assays: Mm01185107_g1 (*Vamp1*), Mm01325243_m1 (*Vamp2*), Mm00436858_m1 (*Syt1*), Mm00444008_m1 (*Stx1a*), Mm02394624_s1 (*Stx1b*), Mm01276449_m1 (*Snap25*), Mm00449772_m1 (*Syn1*), Mm02344307_m1 (*Nlgn1*), Mm01225951_m1 (*Nlgn3*), and Mm00607939_s1 (*Actb*). The relative levels of mRNA were calculated using the $\Delta\Delta C_t$ method.

4.6. Western Blotting

The tissue samples for WB were collected at PND 60 and stored at −80 °C until analysis. The tissue samples were homogenised, mixed with Laemmli buffer, and denatured at 95 °C for 5 min. After SDS-PAGE, the proteins were transferred to a nitrocellulose membrane under standard conditions and used for immunochemical analysis, followed by chemiluminescent detection. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) level was analysed as a loading control. In all the experiments, densitometric analysis of the immunoblots was performed using normalization to the immunoreactivity for GAPDH. Densitometric analysis and size-marker-based verification were performed with the TotalLab4 software (NonLinear Dynamics Ltd., Newcastle upon Tyne, UK).

4.7. Statistical Analysis

The results are expressed as mean values $\pm$ SEMs. In all the analyses, each datapoint is from a separate animal. The normality and equality of the group variances were tested using Shapiro–Wilk tests. Differences between means were analysed using (i) unpaired Student's *t*-tests or two-way ANOVAs with Bonferroni post-hoc tests for data with normal distributions, or (ii) Mann–Whitney tests for data with non-normal distributions. The level of statistical significance was set at $p < 0.05$. The statistical analyses were performed using Graph Pad Prism version 8.0 (Graph Pad Software, San Diego, CA, USA).

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11.5. Publikacja IV

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Inhibition of purinergic P2 receptors prevents synaptic and behavioral alterations in a rodent model of autism spectrum disorders.

Lidia Babiec^a, Anna Wilkaniec^{a,*}, Elżbieta Gawinek^a, Wojciech Hilgier^b,
Agata Adamczyk^a

^a Department of Cellular Signaling, Mossakowski Medical Research Institute, Polish Academy of Sciences, Pawińskiego 5, 02-106 Warsaw, Poland

^b Department of Neurotoxicology, Mossakowski Medical Research Institute, Polish Academy of Sciences, Pawińskiego 5, 02-106 Warsaw, Poland

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ABSTRACT

Background: Extracellular nucleotides acting through specific purinergic receptors play a key role in synaptic transmission and neuromodulation. Therefore, any deregulation of purine-dependent signaling may lead to synaptic dysfunction and the development of autism-like behaviors.

Method: Here, we used a well-established autism spectrum disorders (ASD) model of a single prenatal exposure to valproic acid (VPA). Wistar female rats received an *i.p.* injection of VPA (450 mg/kg body weight) at gestational day 12.5. To study the involvement of purinergic signaling in the alterations of the key synaptic proteins and the development of ASD-like behaviors in VPA offspring at 52. post-natal day, we performed a single *i.p.* injection of the non-selective P2-purinoreceptor antagonists PPADS and isoPPADS (12.5 mg/kg body weight) 24 h before behavioral tests.

Results: The results showed an increased level of ATP and its metabolites, ADP, and AMP in the cerebrospinal fluid of VPA offspring indicating significant dysregulation in extracellular nucleotides- signaling, that may result in synaptic aberrations. Treatment with isoPPADS significantly prevented alterations of the selected synaptic proteins evoked by prenatal VPA exposure along with prevention of the overactivation of mTOR kinase, while both purinergic antagonists reversed autism-like behavior. This suggests that apart from the activation of the mTOR signaling cascade, purinergic deregulation may influence molecular pathways other than synaptic protein imbalance.

Conclusions: Disturbances in the ATP turnover and overstimulation of purinergic receptors are responsible for synaptic proteins' dyshomeostasis and the appearance of ASD-like behaviors. mTOR kinase might be partially responsible for the development of autistic symptoms evoked by hyperpurinergia.

1. Introduction

Autism spectrum disorders (ASD) are complex, multisystem diverse group of neurodevelopmental conditions characterized by abnormalities in social communications and restricted repetitive behaviors that manifest in early childhood (Lord et al., 2020).

* Corresponding author.

E-mail address: awilkaniec@imdik.pan.pl (A. Wilkaniec).

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Environmental factors in the early stage of development together with genetic and epigenetic factors are involved in the pathogenesis of ASD, though the exact causes that predispose an individual to develop autism are not fully understood (Masini et al., 2020; Persico & Bourgeron, 2006; Yoon et al., 2020). Several studies indicated that mutations in genes that encode proteins involved in the regulation of synapse formation and dendritic spine maturation such as cell adhesion molecules *NRXN* and *NLGN*, scaffolding proteins *SHANK*, or proteins involved in synaptic transcription and translation *TSC1/2* and *FMR1* play a causal role in ASD pathogenesis (Stühf, 2017; Trobiani et al., 2020). Thus, any variations in molecular pathways affecting various aspects of synaptic function might represent mechanisms underlying the risk of ASD. Purinergic signaling plays a critical role in the control of synapses, such as stability and elimination, trans-synaptic signaling as well as synaptogenesis (Rodrigues et al., 2019). The role of hyperpurinergia in the pathogenesis of autism and related spectrum disorders has been suggested since the discovery that suramin, a broad-range modulator of purinergic signaling improves core symptoms of ASD in a small human clinical trial (J. C. Naviaux et al., 2014, 2015; R. K. Naviaux, 2018; R. K. Naviaux et al., 2013). However, the molecular pathways that could be involved in synaptic dysfunction and autism-like features evoked by defects in purinergic signaling have not been explored so far. Purinergic signaling is a complex and dynamic communication system consisting of receptors, and extracellular enzymes that metabolize/hydrolyze ATP and its derivatives ADP, AMP, and adenosine as well as nucleotide-releasing channels and transporters (Burnstock et al., 2010). The release of endogenous ATP is a critical step in initiating a purinergic signaling cascade and could be triggered by various stimuli. Apart from the massive leakage of nucleotides upon cell damage under stress conditions by nonspecific mechanisms (Dosch et al., 2018; Yegutkin, 2008; H. Zimmermann, 1994), ATP might be released into extracellular space via multiple different specific pathways, such as electrodiffusional movement through membrane ion channels, including connexin (Cx) hemichannels and pannexin (Panx) channels (Dahl, 2015; Dosch et al., 2018; Taruno, 2018), diffusion by nucleotide-specific ATP-binding cassette (ABC) transporters (Dunn et al., 2020), and vesicular exocytosis facilitated by vesicular nucleotide transporter (VNUT) that actively transport nucleotides into synaptic vesicles and is responsible for the vesicular storage of ATP (Hasuzawa et al., 2020).

Following synaptic release, ATP is hydrolyzed to ADP, AMP, adenosine, and inorganic phosphate by several ectonucleotidases including ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) family, ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP) family, ecto-5'-nucleotidase/CD73, and alkaline phosphatases (Langer et al., 2008; Robson et al., 2006; H. Zimmermann, 2006, 2021). Thus, by controlling the duration of activity of purinergic receptor agonists, the activity of ectonucleotidases is a key factor for purinergic neurotransmission (Langer et al., 2008; Robson et al., 2006; H. Zimmermann, 2006).

Extracellular nucleotides activate fifteen different isoforms of cell surface receptors, seven subtypes of membrane ion channels termed P2X receptors (P2X₁-P2X₇) permeable to sodium, potassium, and calcium that open within milliseconds of the binding of ATP and eight metabotropic G-protein coupled P2Y receptors (subfamily principally Gq protein-coupled: P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁ and principally Gi protein-coupled: P2Y₁₂-P2Y₁₄) (Kennedy, 2021; Sheng & Hattori, 2022). Purinoceptor activation could control the PI3K-AKT/mTOR signaling pathway that consists of two parts: phosphatidylinositol 3 (PI3K) and its downstream protein kinase B (PKB), also known as Akt, and mammalian/mechanistic target of rapamycin (mTOR) kinase. Deregulation of PI3K-AKT/mTOR signaling pathway might be associated with alterations in protein synthesis and neuronal circuit connectivity (M. Yang et al., 2022). Thus, this signaling pathway has been suggested to play an important role in the regulation of certain features of autism. Inhibition of mTOR activity resulted in augmented anxiety (Koehl et al., 2021; A. Wang et al., 2020), while its enhanced activity was connected with repetitive behavior (Hoeffer et al., 2008) and impaired social communication occurrence (Xing et al., 2019). Together, abnormalities in mTOR and PI3K/Akt function, have recently been identified as a significant risk factor for ASD (Windén et al., 2018). However, whether the PI3K-AKT/mTOR signaling pathway deregulation leads to changes in synaptic structure and function evoked by hyperpurinergia in ASD is unknown.

In the following study we tested the hypothesis that purinergic signaling hyperactivation is involved in the alterations of the key synaptic protein level and leads to the development of autism-like behaviors in the pre-clinical epigenetic valproic acid-induced rodent model of ASD. To test the role of P2 receptor signaling in the modulation of the PI3K-AKT/mTOR pathway and improving autistic features we used two non-selective purinergic P2 receptor antagonists PPADS and isoPPADS. Our results indicated significant correction of the core behavioral symptoms in the VPA-induced model of ASD using non-selective purinergic P2 receptor antagonists that inhibit purinergic signaling. We directed for the first time that treatment with isoPPADS partially mitigated alterations of the selected synaptic proteins evoked by prenatal VPA exposure along with prevention of the overactivation of mTOR kinase, suggesting that this enzyme is at least partially responsible for the development of autistic symptoms evoked by purinergic signaling activation.

2. Methods

2.1. Animals – in vivo model of ASD

We obtained Wistar female rats from the Animal House of the Mossakowski Medical Research Institute, PAS (Warsaw, Poland). The rats were aged between 12 and 15 weeks and had a standard weight range of 210–250 g. They were bred according to the SPF standard for small rodents and kept under a 12-h light/dark cycle with access to food and water ad libitum, and the temperature and humidity were strictly controlled.

To determine pregnancy, the presence of a vaginal plug was observed and recorded as embryonic day 0. On gestational day 12.5, pregnant rats received a single intraperitoneal injection of valproic acid (VPA) at a dose of 450 mg/kg of body weight to induce the ASD phenotype (Nicolini & Fahnestock, 2018). Control dams were given a single injection of saline. All pregnant dams were injected at the same time of the day (noon). The pregnant rats were allowed to give birth and raise offspring under normal conditions. The day of birth was considered post-natal day (PND) 1. At 22–23 days of age, the rat pups were separated from their mothers and housed in

Table 1
Detailed information about TaqMan Gene Expression Assays used for qRT-PCR.

Gene Symbol	Assay ID
<i>Gja1</i>	Rn01433957
<i>Gjd2</i>	Rn00439121
<i>Panx1</i>	Rn01447976
<i>Panx2</i>	Rn01308054
<i>Slc17a9</i>	Rn01215082
<i>Entpd2</i>	Rn01438929
<i>Entpd3</i>	Rn00710251
<i>Nt5e</i>	Rn00665212
<i>Ada</i>	Rn00590523
<i>IL-6</i>	Rn01410339
<i>Tnfr</i>	Rn01525859
<i>Actb</i>	Rn00667869

groups of 3–4 individuals in open polycarbonate cages with an enriched environment.

Young adult male rats at 52 PND were divided into two groups: animals from the first one were sacrificed by decapitation at 52 PND and the materials (CSF and brain samples) were collected to analyze the purinergic signaling orchestration, while the rest of the animals were employed to analyze the involvement of purinergic receptors modulation. Part of either control or VPA male offspring from the control or VPA groups were divided into three groups at 52 PND. The rats received a single intraperitoneal injection of either PPADS or isoPPADS (Tocris Bioscience, UK) at a dose of 12.5 mg/kg of body weight (according to (Pereira et al., 2013)). Control rats received the appropriate volume of the vehicle. After 24 h the behavioral test was performed, and after another 24 h, rats were sacrificed. After the dissection, they were placed in liquid nitrogen. All the samples were stored at -80°C for further analysis. Animals from at least 3 litters in each experimental group (randomly selected) were tested to reduce the risk of litter effect. Moreover, to avoid time differences between groups, tissue isolation was performed in pairs of animals: one animal from control group and another one from the VPA.

2.2. Cerebrospinal fluid collection and HPLC analysis of nucleotides (ATP, ADP, AMP) and nucleoside (adenosine)

The concentration of ATP, ADP, AMP, and adenosine was determined in the cerebrospinal fluid (CSF) by HPLC with a fluorescence detector according to the method of Kawamoto et al. (Kawamoto et al., 1998) with some modifications. Cerebrospinal fluid collection was performed by direct CC puncture using a collection apparatus (consists of a 1 ml syringe, a disposable intravenous infusion needle and a clip) with negative pressure as described (Li et al., 2016). Cerebrospinal fluid collection made between 11 a.m. and 1 p.m. Derivatization of cerebrospinal fluid (plasma) was made with 2-chloroacetaldehyde solution and acetate buffer (pH 4.5) through heating at 60°C for 60 min. The reaction was terminated by placing the samples on ice. Then 25 ml of the reaction solution was injected into the HPLC apparatus. The concentrations of ATP, ADP, AMP and adenosine were measured by reverse-phase HPLC with Hypersil Gold column (250 X 4.6 mm, Thermo Fischer Scientific, USA). The mobile phase buffer consisted 100 mM KH_2PO_4 , 5 mM tetrabutylammonium bromide and 2% CH_3CN (pH 3.3). The flow rate was 1.0 ml/min, and the wavelengths for excitation and emission were 270/420 nm respectively.

2.3. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

To isolate RNA from the brain samples, we used TRI-reagent (Sigma-Aldrich, St. Louis, MO, USA). The extraction procedure was carried out according to the manual provided by the manufacturer. RNA quantity and quality were analysed with spectrophotometer measurements using NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). Subsequently, we digested the remaining trace amounts of DNA with DNase I (Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer's instructions.

For reverse transcription, we utilized the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer's instructions. The mRNA level of the selected purinergic receptors was measured using real-time PCR, employing the TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) listed in Table 1. The analysis was performed on the ABI PRISM 7500 apparatus. *Actb* was used as the reference gene, and each sample was analysed in three technical replicates. The mean cycle threshold (Ct) values were used for further analysis, and relative quantification was performed by the $\Delta\Delta\text{Ct}$ method and expressed the ratio between control and VPA samples as a percentage of the control (set as 100%).

2.4. Western blot analysis

To determine the protein level of mTOR pathway activity we employed the Western blotting method. After homogenizing the tissue samples, we mixed them with a Laemmli buffer. Denaturation was carried out at 95°C for 5 min. Proteins were separated using standard 6%, 10% or 15% SDS-PAGE gels. Subsequently, the proteins were transferred to nitrocellulose membranes using the "wet" transfer method at 50 V for 2 h.

Immunodetection of specific antibodies began with a 5-minute wash in TBS-T (Tris-buffered saline with Tween 20 buffer: 100 mM

Table 2
The concentration of the antibodies and the experimental conditions for Western blotting:.

Antibody name	Company/Cat #	Dilution
mTOR	Cell Signaling 2983	1:1000 5% milk in TBS-T 0.1%
p-mTOR (Ser2448)	Cell Signaling 5536	1:1000 5% milk in TBS-T 0.1%
AMPK	Cell Signaling 5831	1:500 TBS-T 0.1%
p-AMPK(Thr172)	Cell Signaling 50081	1:500 TBS-T 0.1%
anti-p-4E-BP1(Thr37/46)	Cell Signalling 2855	1:800 TBS-T 0.1%
Akt	Cell Signaling 4691	1:750 2% BSA in TBS-T 0.1%
p-Akt(Ser473)	Cell Signaling 4691	1:500 2% BSA in TBS-T 0.1%
SYP	Santa Cruz Biotechnology sc-55507	1:1000 TBS-T 0.1%
p-Syn1(Ser62/Ser67)	Santa Cruz Biotechnology sc-135709	1:500 TBS-T 0.1%
SNAP25	Cell Signalling #5309	1:1000 5% milk in TBS-T 0.1%
VAMP1/2	Santa Cruz Biotechnology sc-20039	1:300 5% milk in TBS-T 0.1%
PSD95	Santa Cruz Biotechnology sc-32290	1:400 TBS-T 0.1%
Vinculin	Cell Signaling 13901	1:1000 5% milk in TBS-T 0.1%
HRP-GAPDH	Proteintech HRP-60004	1:8000 5% milk in TBS-T 0.1%
Goat-anti-mouse	Thermo Fischer Scientific 31430	1:4000 5% milk in TBS-T 0.1%
Goat anti-rabbit	Proteintech, SA00001-2	1:4000 5% milk in TBS-T 0.1%

Tris, 140 mM NaCl, and 0.1% Tween 20, pH 7.6). To block non-specific binding, we incubated the membranes with a 5% BSA solution in TBS-T or a 5% non-fat milk solution in TBS-T for 1 h at room temperature (RT). The membranes were then incubated with the primary antibodies indicated in Table 2. After three washes with TBS-T, the membranes were incubated with appropriate secondary antibodies for 1 h at RT, followed by another 3 washes with TBS-T.

The detection of antibodies was performed using a chemiluminescent reaction and the Clarity Western ECL Substrate (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions and visualized using the Fusion FX imaging system (Vilber Lourmat, France). After stripping, the immunolabelling of GAPDH as a loading control was performed. Densitometric analysis was conducted using TotalLab v1.11 software.

2.5. Behavioral analysis

We performed an Open Field test according our previously published methodology (Czapski et al., 2021; Gąssowska-Dobrowolska et al., 2020a) with modifications. This test enables to measure the general locomotor activities, anxiety-related behavior, exploratory activity, and the typical for autism repetitive behavior. Animals were habituated both to the experimenter and to the testing room every day for one week before experiment. At 52 PND rats were injected with a single i.p. injection of PPADS or isoPPADS at a dose of 12.5 mg/kg of body weight (controls received the appropriate volume of the vehicle). After 24 h the behavioral test was performed. All tests were performed between 8 a.m. and 12 p.m. in a sound-isolated room with a constant illumination of ca. 25 lx, while a blinded operator was not present in the analysis room during the test. Rats were individually placed in the center of the arena (grey PCV box 55 × 55 × 50 cm), and the activity was recorded for 10 min with a video camera. The total distance moved and the rat's velocity was measured using Ethovision XT 10 (Noldus Information Technology, The Netherlands). The duration of grooming, the number of climbs and rearings, and the number of defecation events were scored manually with BehaView 0.0.22. After each trial, the test chamber was cleaned with 10% ethanol.

2.6. Statistical analysis

All results are presented as the mean values ± standard deviation (SD). To analyze differences between the average values, we used a Student's t-test (between two groups) or one-way analysis of variance (ANOVA) with a Bonferroni comparison post hoc test (between multiple groups). Statistical significance was considered at $p < 0.05$. The analysis was conducted using Graph Pad Prism version 6.0 (Graph Pad Software, San Diego, CA, USA).

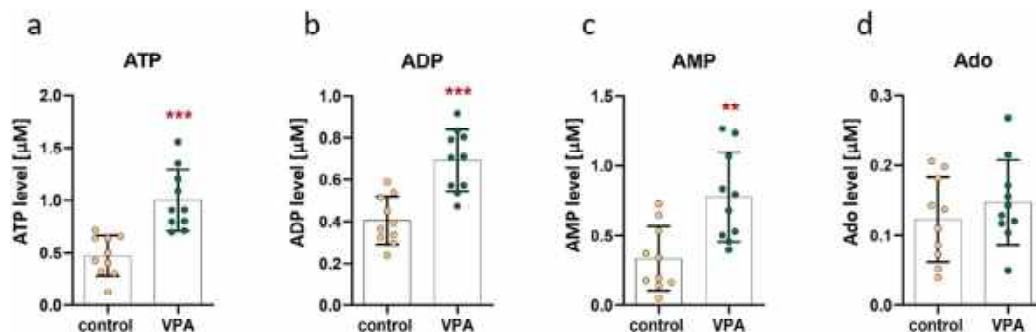


Fig. 1. The effect of prenatal valproic acid exposure on the levels of (a) ATP, (b) ADP, (c) AMP, and (d) adenosine (Ado) in the cerebrospinal fluid determined by HPLC analysis. Data are expressed as a mean value $\pm$ SD for 10 independent experiments (N = 10). * $p < 0.01$, *** $p < 0.001$ versus corresponding control as determined using the Student's t-test.

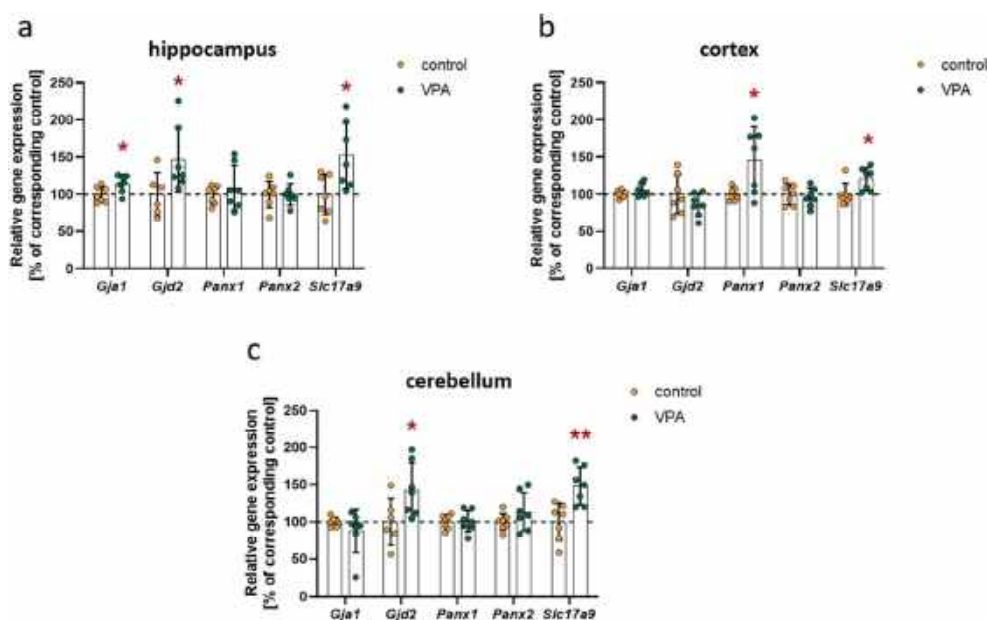


Fig. 2. The effect of prenatal valproic acid exposure on the expression of proteins involved in ATP release in the (a) hippocampus, (b) brain cortex, and (c) cerebellum. Data are expressed as a mean value $\pm$ SD for 5–7 independent experiments (N = 5–7). * $p < 0.05$, ** $p < 0.01$ versus corresponding control as determined using the Student's t-test and presented as a percentage of the control (set as 100%). *Gja1* - connexin 43; *Gjd2* - connexin 36; *Panx1* - pannexin 1; *Panx2* - pannexin 2; *Slc17a9* - vesicular nucleotide transporter (VNUT).

3. Results

To investigate changes in purine metabolism we first measured the level of nucleotides (ATP, ADP, AMP) and adenosine in the CSF of the adolescent rats prenatally exposed to VPA. As shown in Fig. 1, the level of ATP ($t_{18} = 4.797$, $p = 0.0001$), ADP ($t_{18} = 4.871$, $p = 0.0001$) and AMP ($t_{18} = 3.525$, $p = 0.0024$) was significantly elevated in the CSF of VPA animals, while the concentration of adenosine was similar as in the control group (Fig. 1d).

Next, we investigated the expression of proteins involved in the release and hydrolysis of nucleotides in the selected brain areas of VPA offspring. We observed the elevation in the mRNA level of connexin 46 (*Gja1*, $t_{12} = 2.450$, $p = 0.0306$) and connexin 36 (*Gjd2*, $t_{11} = 2.227$, $p = 0.0478$) in the hippocampus (Fig. 2a), while in the brain cortex of VPA rats, the significant increase in *Panx1* expression ($t_{12} = 2.712$, $p = 0.0189$) was detected (Fig. 2b). Moreover, we observed in the cerebellum increased mRNA level for *Gjd2* ($t_{11} = 2.261$,

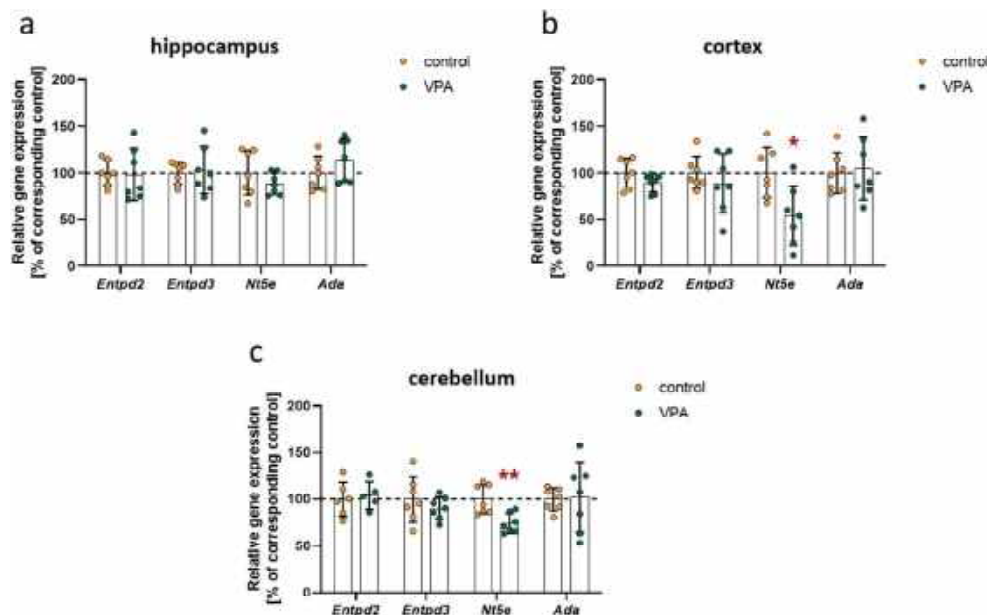


Fig. 3. The effect of prenatal valproic acid exposure on the expression of ectonucleotidases in the (a) hippocampus, (b) brain cortex, and (c) cerebellum. Data are expressed as a mean value $\pm$ SD for 6–7 independent experiments ($N = 6-7$). * $p < 0.05$, ** $p < 0.01$ versus corresponding control as determined using the Student's *t*-test and presented as a percentage of the control (set as 100%). *Entpd2* - Ectonucleoside triphosphate diphosphohydrolase 2; *Entpd3* - Ectonucleoside triphosphate diphosphohydrolase 3; *Nt5e* - ecto- 5'-nucleotidase; *Ada* - deaminase adenosine.

$p = 0.0450$, Fig. 2c) in VPA animals. In those animals also the increased expression of *Slc17a9*, encoding vesicular nucleotide transporter (VNUT) was observed in the hippocampus ($t_{12} = 2.657$, $p = 0.0209$), brain cortex ($t_{12} = 2.556$, $p = 0.0252$), and cerebellum ($t_{12} = 3.607$, $p = 0.0036$) (Fig. 2a-c). We did not observe any alterations in the expression of *Panx2* in the investigated brain structures (Fig. 2a-c).

From the set of ectonucleotidases that hydrolyze extracellular nucleotides, we observed the significant decrease in the expression of AMP-converting enzyme, ecto-5'-Nucleotidase (*Nt5e*) in the brain cortex ($t_{12} = 2.878$, $p = 0.0139$) and cerebellum ($t_{12} = 3.630$, $p = 0.0034$) of VPA offspring (Fig. 3b,c). No significant alteration in the mRNA level of ATP and ADP hydrolyzing ectonucleoside triphosphate phosphohydrolase: 2 (*Entpd2*) and 3 (*Entpd3*), as well as adenosine deaminase (*Ada*), was observed (Fig. 3a-c).

To reveal the direct impact of the purinergic signaling on the mammalian target of rapamycin (mTOR), which is the catalytic subunit of the mTORC1 complex contributing to synaptic proteins homeostasis, we analyzed mTOR level and phosphorylation on Ser2448 as a marker of mTORC1 activation. As shown in Fig. 4a-c, prenatal exposure to VPA increased the phosphorylation of mTOR in the hippocampus, brain cortex, and cerebellum ($F_{5,30} = 4.864$, $p = 0.0013$; $F_{5,30} = 4.173$, $p = 0.0053$; $F_{5,30} = 6.640$, $p = 0.0006$, respectively), while the level of total mTOR remained unchanged (Fig. 4d-f). The enhanced mTOR phosphorylation was not observed in the hippocampus ($F_{5,30} = 4.864$, $p = 0.0149$), brain cortex ($F_{5,30} = 4.173$, $p = 0.0415$), and cerebellum ($F_{5,30} = 6.640$, $p = 0.0023$) of animals treated with the single dose of isoPPADS (Fig. 4a-c).

To confirm mTOR activation in the brain after prenatal VPA treatment, we have examined the phosphorylation of its substrate: eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1). In each of the investigated brain structures, the level of p-4E-BP1 was increased in VPA animals ($F_{5,30} = 1.698$, $p < 0.0001$ in the hippocampus, $F_{5,30} = 1.295$, $p = 0.0024$ in the brain cortex, and $F_{5,30} = 1.297$, $p = 0.0006$ in the cerebellum) (Fig. 4g-i). Also, isoPPADS treatment significantly prevented 4E-BP1 phosphorylation after VPA treatment in the investigated brain areas ($F_{5,30} = 7.871$, $p = 0.0010$ in the hippocampus, $F_{5,30} = 1.295$, $p = 0.0010$ in the brain cortex, and $F_{5,30} = 1.297$, $p = 0.0155$ in the cerebellum) (Fig. 4g-i).

To gain insight into the mechanism leading to mTOR phosphorylation, we analyzed two upstream kinases, 5'-adenosine monophosphate-activated protein kinase (AMPK) and serine/threonine protein kinase, Akt. We have observed, that in the cortex and hippocampus of VPA-treated rats, the phosphorylation of AMPK, which is responsible for the activation of this kinase, is significantly decreased ($F_{5,30} = 8.828$, $p < 0.0001$ and $F_{5,30} = 3.221$, $p = 0.0009$, respectively) (Fig. 5a,b), whereas in the cerebellum, the activity of AMPK significantly increased ($F_{5,30} = 5.051$, $p = 0.0012$) (Fig. 5c). Treatment with isoPPADS increased phosphorylation of AMPK in the hippocampus ($F_{5,30} = 8.828$, $p = 0.0301$) and cortex ($F_{5,30} = 3.221$, $p = 0.0442$) (Fig. 5a,b).

We have observed that the phosphorylation of Akt kinase, a positive modulator of mTOR activity (Hay, 2005), was significantly increased in the cerebellum ($F_{5,29} = 5.012$, $p = 0.0349$) (Fig. 6c), but not in the hippocampus or brain cortex of VPA treated rats

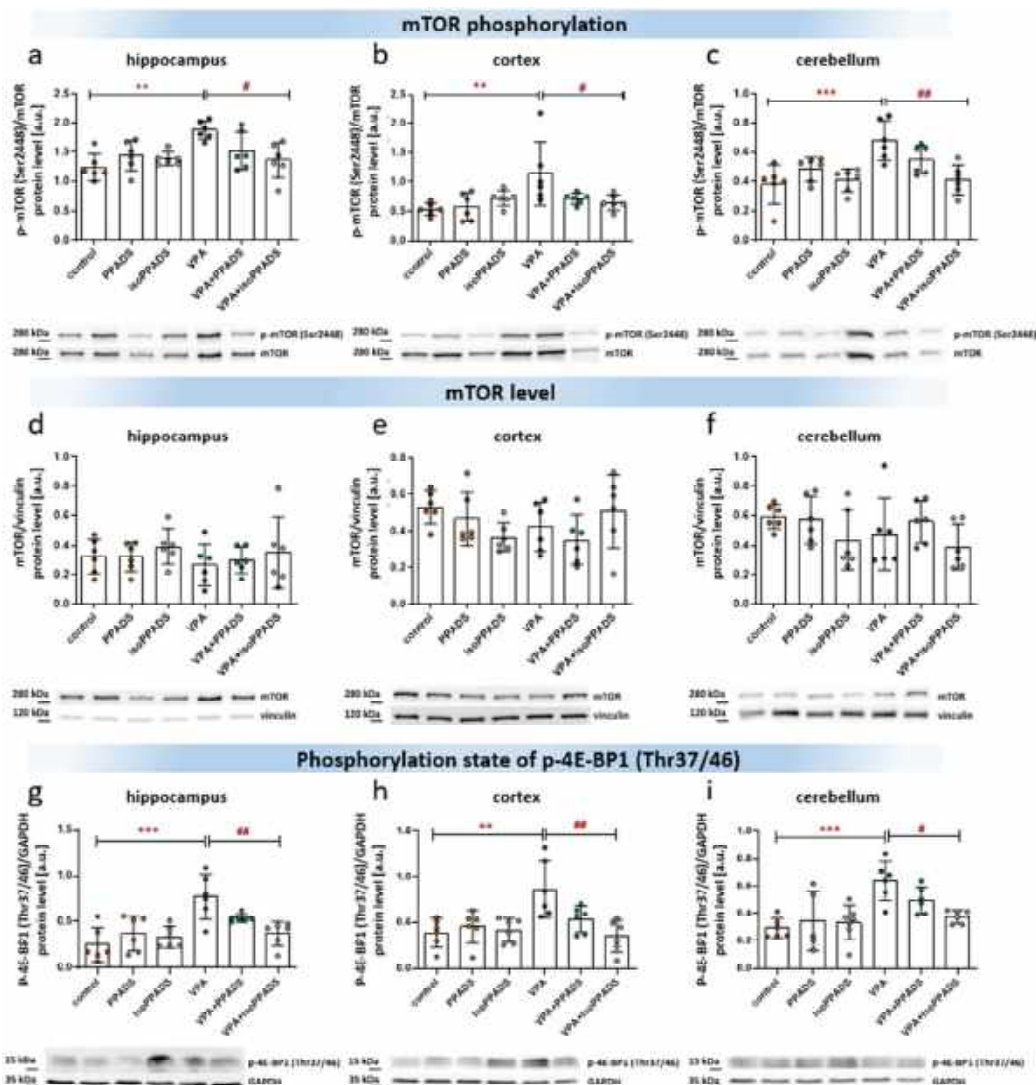


Fig. 4. The effect of the purinergic receptors inhibition on the mTOR activity in selected brain regions of animals prenatally exposed to VPA. Data are expressed as a mean value $\pm$ SD for 6 independent experiments ($N = 6$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus control, # $p < 0.05$, ## $p < 0.05$ versus VPA as determined by one-way ANOVA followed by Bonferroni's post-hoc comparison test.

(Fig. 6a,b). Purinergic receptor inhibitors did not influence the activity of Akt (Fig. 6a-c). Also, the level of the total Akt was not influenced by prenatal exposure to VPA in the investigated brain areas (Fig. 6d-f).

To study the involvement of purinergic signaling dysfunction in the alterations of the key pre- and postsynaptic proteins, we non-selectively block P2 receptors using pyridoxal phosphate derivatives PPADS and its positional isomer isoPPADS. These are relatively non-subtype-selective P2X antagonists, which also block several P2Y subtypes (Y.-C. Kim et al., 1998, 2001). We performed a single *i.p.* injection of PPADS and isoPPADS (12.5 mg/kg) 48 h prior of tissue collection. We have observed the structure-dependent changes in the level of synaptic vesicles' marker proteins: synapsin-1 (Syn-1), which regulates the synaptic vesicles docking and trafficking, and synaptophysin (SYP), the integral membrane protein of synaptic vesicles (Fig. 7a). While the Syn-1 level was upregulated in the

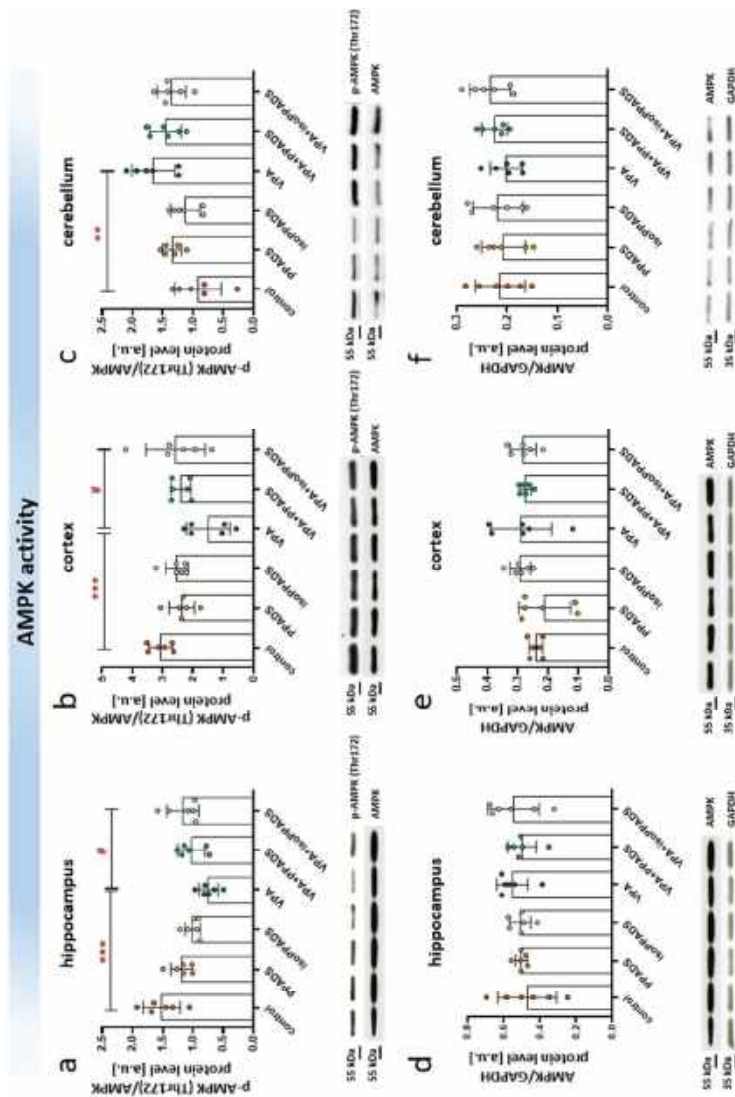


Fig. 5. The effect of the purinergic receptors inhibition on AMPK activity in selected brain regions of animals prenatally exposed to VPA. Data are expressed as a mean value $\pm$ SD for 6 independent experiments (N = 6). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus control, # $p < 0.05$ versus VPA by one-way ANOVA followed by Bonferroni's post-hoc comparison test.

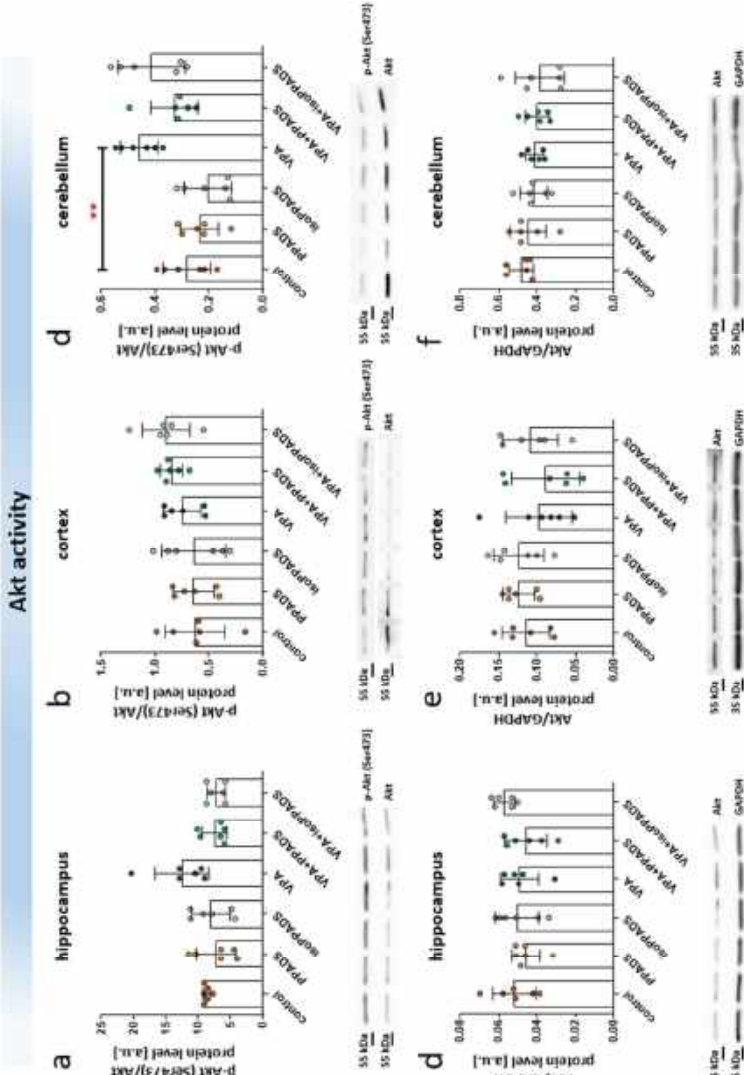


Fig. 6. The effect of the purinergic receptors inhibition on the Akt kinase activity in selected brain regions of animals prenatally exposed to VPA. Data are expressed as a mean value $\pm$ SD for 6 independent experiments ($N = 6$). * $p < 0.05$ versus control as determined by one-way ANOVA followed by Bonferroni's post-hoc comparison test.

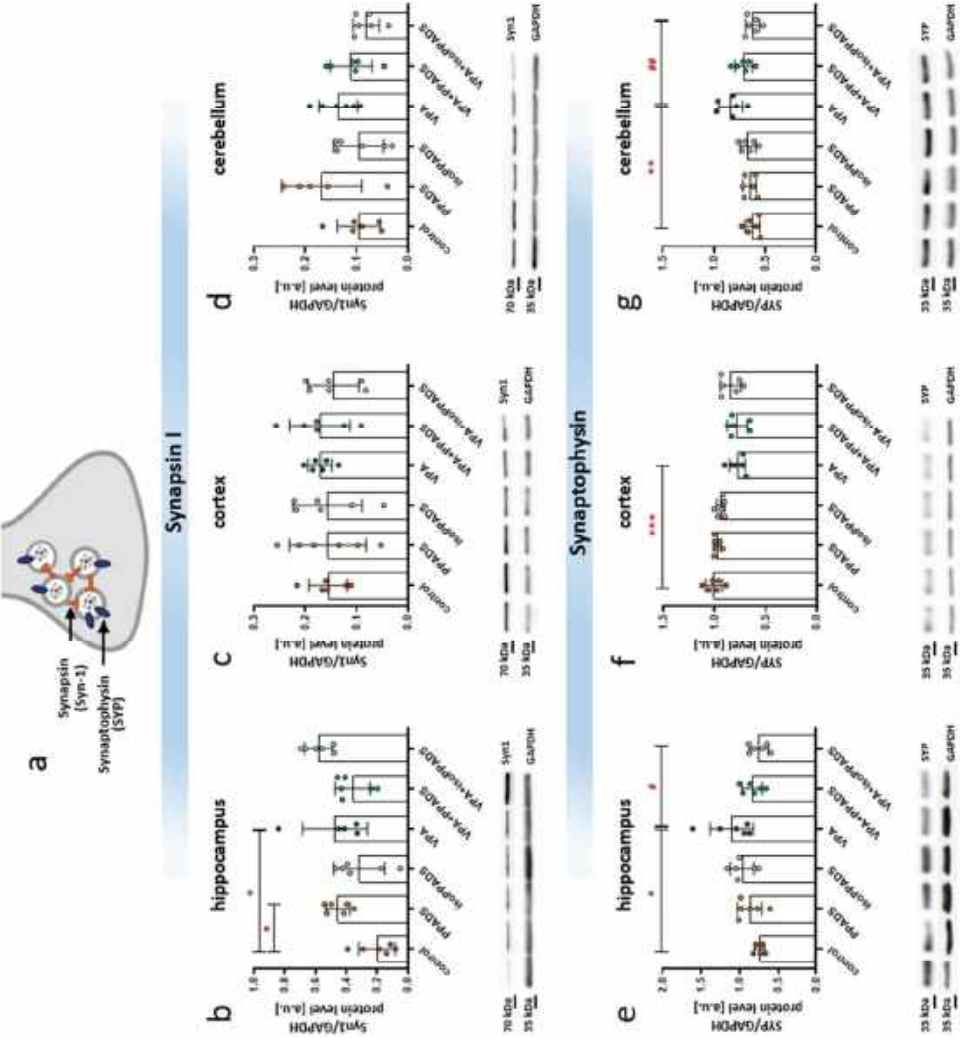


Fig. 7. The effect of the purinergic receptors inhibition on the immunoreactivity of pre-synaptic protein level in selected brain regions of animals prenatally exposed to VPA. Data are expressed as a mean value $\pm$ SD for 5–6 independent experiments ($N = 5-6$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus control; # $p < 0.05$, ## $p < 0.01$ versus VPA as determined by one-way ANOVA followed by Bonferroni's post-hoc comparison test.

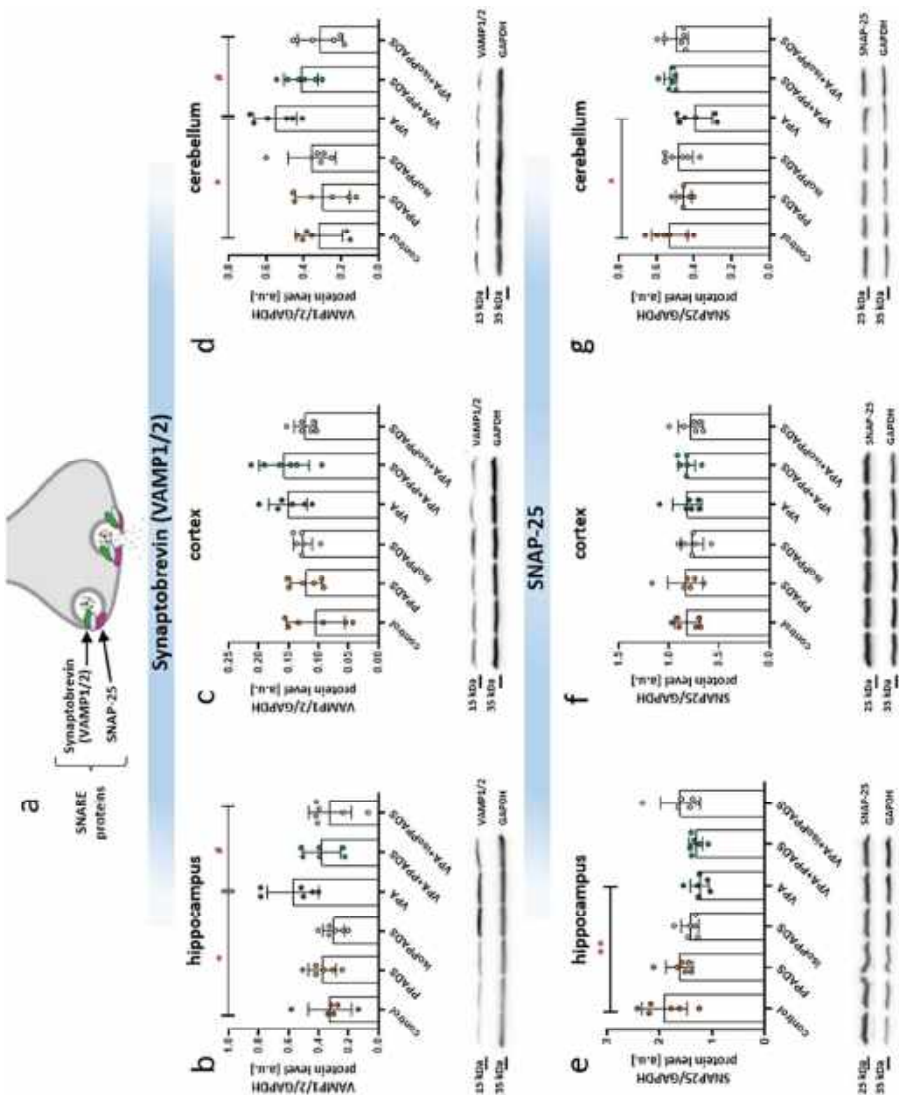


Fig. 8. The effect of the purinergic receptors inhibition on the level of synaptobrevin (VAMP1/2) and SNAP-25 in selected brain regions of animals prenatally exposed to VPA. Data are expressed as a mean value $\pm$ SD for 6 independent experiments (N = 6). * $p < 0.05$, ** $p < 0.01$ versus control, # $p < 0.05$ versus VPA as determined by one-way ANOVA followed by Bonferroni's post-hoc comparison test.

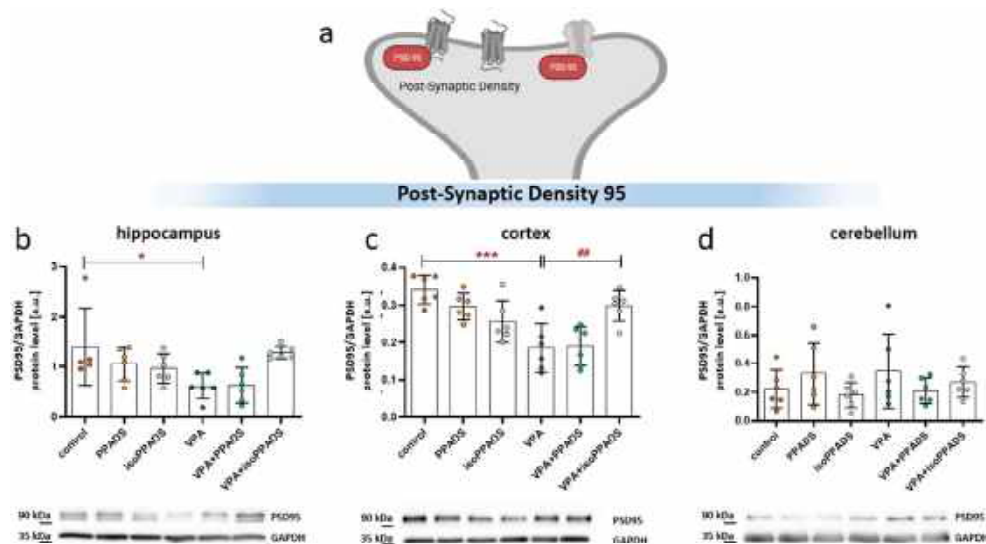


Fig. 9. The effect of the purinergic receptors inhibition on the level of post-synaptic density protein PSD95 in selected brain regions of animals prenatally exposed to VPA. Data are expressed as a mean value $\pm$ SD for 5–6 independent experiments ($N = 5-6$). * $p < 0.05$, ** $p < 0.001$ versus control, ## $p < 0.01$ versus VPA as determined by one-way ANOVA followed by Bonferroni's post-hoc comparison test.

hippocampus of VPA animals ($F_{5,29} = 5.871$, $p = 0.0335$, Fig. 7b), no significant alterations of this protein in the brain cortex and cerebellum were detected (Fig. 7c,d). Additionally, in the hippocampus and cerebellum, we observed a substantial increase in SYP ($F_{5,29} = 3.845$, $p = 0.0210$ and $F_{5,30} = 5.995$, $p = 0.0012$, respectively, Fig. 7e,g), while in the brain cortex of animals prenatally exposed to VPA a significant decrease ($F_{5,30} = 10.32$, $p = 0.0002$) in the expression of this protein was noted (Fig. 7e). The purinergic signaling modulation had a negligible effect on elevated level of Syn-1 in the hippocampus, however we observed in the control group significant increase after PPADS treatment ($F_{5,29} = 5.871$, $p = 0.0401$) (Fig. 7b). Treatment with non-selective P2 receptor antagonist (isoPPADS) normalized the elevated level of SYP in the hippocampus ($F_{5,29} = 3.845$, $p = 0.0178$) and cerebellum ($F_{5,29} = 5.995$, $p = 0.0011$) in VPA rats (Fig. 7e,g).

In our animal model, we have also analyzed the effect of the purinergic receptors blockade on the alterations in SNARE complex proteins [Fig. 8a, (Czapski et al., 2021; Gąsowska-Dobrowolska et al., 2020a)]. As shown in Figs. 8b and 8d, treatment with isoPPADS prevented VAMP1/2 elevation in the hippocampus and cerebellum ($F_{5,30} = 3.537$, $p = 0.0364$ in hippocampus and $F_{5,30} = 3.764$, $p = 0.0274$ in the cerebellum). However purinergic receptors' antagonists did not affect the down-regulation of SNAP-25 in the hippocampus ($F_{5,30} = 4.543$, $p = 0.0043$) and cerebellum ($F_{5,30} = 2.961$, $p = 0.0408$) of juvenile VPA rats (Fig. 8e,g). In the brain cortex, we did not observe alterations in both investigated proteins (Fig. 8c,f).

Postsynaptic density protein-95 (PSD95), the major scaffolding protein that regulates synaptic function (Fig. 6a), was significantly decreased in the hippocampus ($F_{5,29} = 4.000$, $p = 0.0351$) (Fig. 9b) and brain cortex ($F_{5,30} = 9.875$, $p < 0.0001$) (Fig. 9c) of VPA animals, while no significant change was observed in the cerebellum (Fig. 9d). Treatment with isoPPADS normalized PSD95 level in the cortex of juvenile VPA rats ($F_{5,30} = 9.875$, $p = 0.0062$) (Fig. 9c), but had no effect on decreased level of this protein in hippocampus (Fig. 9b).

Finally, we have examined whether purinergic signaling disturbances contribute to the occurrence of autistic behavior in animals prenatally exposed to VPA. We have observed that in the juvenile VPA rats, the total duration of self-grooming was increased ($F_{5,53} = 3.760$, $p = 0.0112$), indicating an enhancement of their autism-like repetitive behavior (Fig. 10a). This abnormal activity was significantly attenuated by either PPADS ($F_{5,53} = 3.760$, $p = 0.0164$) or isoPPADS ($F_{5,53} = 3.760$, $p = 0.0426$) treatment.

The analysis of the locomotor function of VPA animals showed no differences. While the velocity between rats in the investigated group did not alter (Fig. 10b), we observed a significant decrease in the total length of distance traveled ($F_{5,48} = 3.565$, $p = 0.0275$, Fig. 10c). The anxiety level was indicated by the measurement of defecation events as well as a number of central zone entries and time spent in the central zone (Fig. 10d-f.). We have observed that the number of defecations increased substantially in VPA animals ($F_{5,54} = 3.760$, $p < 0.0001$), and this number decreased in the rats' group after purinergic receptors inhibition by PPADS ($F_{5,54} = 3.760$, $p = 0.0002$), or isoPPADS ($F_{5,54} = 3.760$, $p = 0.0138$, Fig. 10d). We did not observe any significant change in the number of central entries or time spent in central zone in VPA animals when compared to control (Fig. 10e,f). Although we did not observe the changes in the number of climbs in VPA animals (Fig. 10g), we observed reduction in the number of rearing events suggesting diminished exploratory activity ($F_{5,50} = 5.939$, $p = 0.0366$), rescued after either PPADS ($F_{5,50} = 5.939$, $p = 0.0011$) or

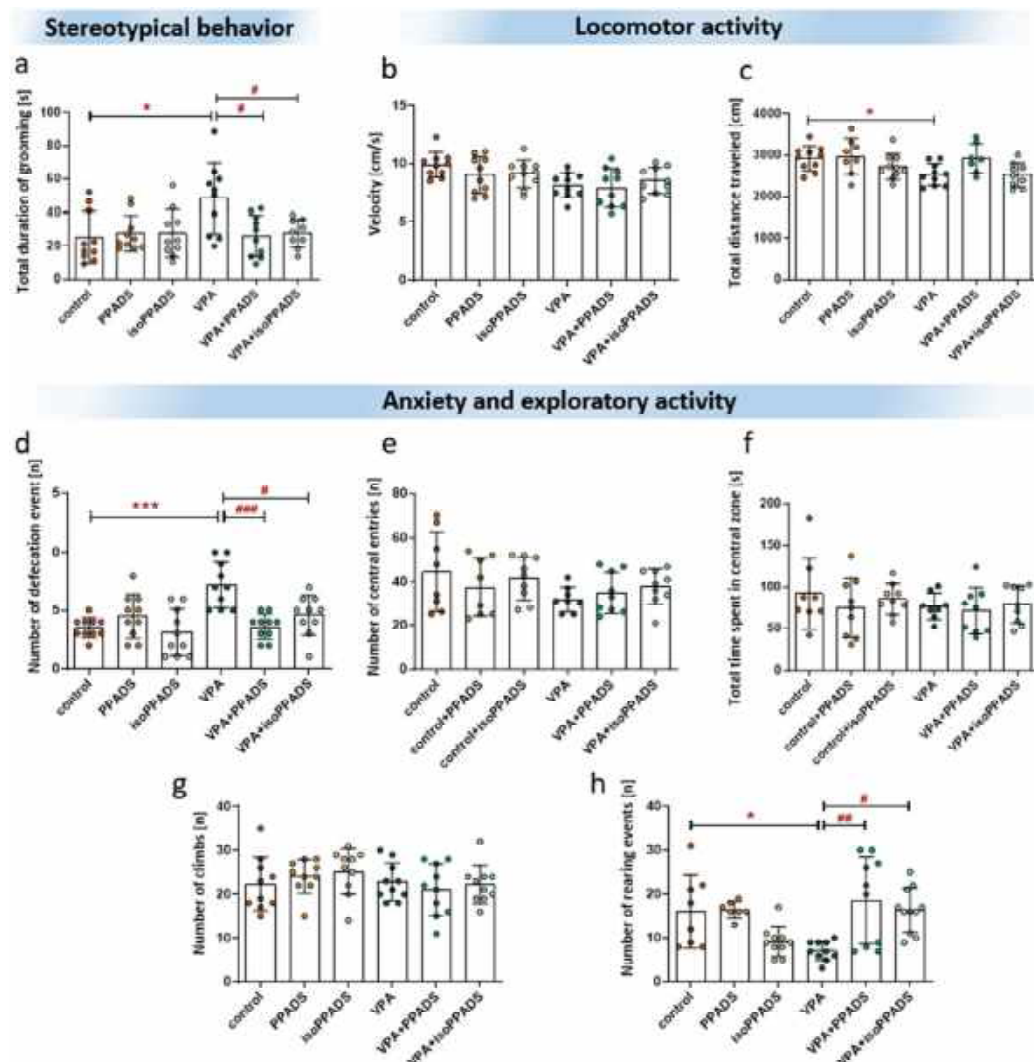


Fig. 10. The effect of the purinergic receptors inhibition on the stereotypical behavior, locomotor activity and anxiety of animals prenatally exposed to VPA. Data are expressed as a mean value $\pm$ SD for 7–10 independent experiments ($N = 7–10$). * $p < 0.05$, *** $p < 0.001$ versus control, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ versus VPA as determined by one-way ANOVA followed by Bonferroni's post-hoc comparison test.

isoPPADS treatment ($F_{5,50} = 5.939$, $p = 0.0125$, Fig. 10h).

4. Discussion and implications

In recent years there has been an increasing number of animal and human studies indicating purinergic signaling disturbances as a key factor involved in the prevalence of neurodevelopmental disorders (Boccazzi et al., 2023; Dai et al., 2023; Reynolds et al., 2021). Several studies on ASD individuals reported elevated levels of purine metabolism components in the urine (Gevi et al., 2016; A. Liu et al., 2019), as well as increased levels of uric acid in the blood (Vanwong et al., 2017). Those observations were followed by a series of genetic, metabolomic, and clinical evidence pointing to an imbalance of purine metabolism in autism (Likhitweerawatong et al., 2021; Naviaux et al., 2017). In this study, we have shown that the disturbances in the extracellular ATP turnover result in the overstimulation of purinergic receptors that are responsible for the dysfunction in synaptic protein homeostasis and behavioral alterations. We have

performed those studies using a well-established ASD model of a single prenatal exposure to valproic acid (Mabunga et al., 2015), which is suitable for the investigation of a broad range of brain pathology associated with ASD including aberrant neurodevelopment, altered neurotransmission, oxidative stress, inflammation, and behavioral changes (Banerjee et al., 2013; Chaliha et al., 2020; Cui et al., 2020; Maisterrena et al., 2022; Naviaux, 2018; Pangrazzi et al., 2020; Wang et al., 2023; Zhong et al., 2023). Because this model meets the criteria of construct, face, and predictive validity, it is widely used to examine the potential therapeutic interventions for ASD (Chaliha et al., 2020). It was observed that in prenatal exposure to VPA, the major behavioral aberrations appear before puberty, which is the unique feature of this model when compared with other animal models of neurodevelopmental disorders, especially schizophrenia (Schneider & Przewlocki, 2005, pp. 1300518). Therefore we have decided to perform the study in the developmental period of late rodent adolescence (PND 52), also because it is one of the critical phases of exacerbation of autism-related symptoms. Our previous research showed significant alterations in rat brains of VPA animals at this stage, including inflammation and altered synaptic protein expression (Gassowska-Dobrowolska et al., 2020b) along with purinergic receptors dyshomeostasis (Babiec et al., 2023). This period of rodent development corresponds to the phase of late adolescence in humans, where ASD youth show early deficits in social motivation that during development lead to socio-cognitive deficits (Burnside et al., 2017; Chevallier et al., 2012). Moreover, adolescence is the critical period for the development of high-order cognitive functions (Yurgelun-Todd, 2007).

There are a few reports considering the changes in extracellular nucleotide levels as a potential marker of ASD (Essa et al., 2013). Significant differences in the composition of the purine metabolites were detected in the CSF of autistic patients when compared with healthy individuals (Brister et al., 2022). Following those observations, we have shown a significant increase in the level of ATP and its derivatives in CSF in the pre-clinical model of ASD. Since the CSF composition is critical for the maintenance of brain function from prenatal to postnatal stages (Xu et al., 2021), any irregularities in its components might be linked with the occurrence of clinical ASD manifestations (Shen, 2018). The critical involvement of exogenous ATP in inducing behavioral changes was documented by showing that only a single injection of extracellular ATP is able to induce rapid and short-lasting changes in the animal behavior characteristics for ASD (Zolkipli-Cunningham et al., 2021). The question remains about the origin of the ATP in the CSF of ASD animals and whether those changes could directly reflect the changes in ATP level in the synaptic cleft. In the CNS, ATP is released not only from neurons but also from microglia and astrocytes (Dale et al., 2023). ATP could be released from damaged cells but it is also present in the secretory vesicles and is co-released to the extracellular space with classical neurotransmitters (Svensson et al., 2019). ATP liberation occurs also in a controlled manner, via connexin or pannexin channels. Importantly, our results suggest increased gap junctional activity as a key mechanism responsible for the elevated ATP release in the hippocampus and cerebellum of autistic rats. This conclusion is supported by the observation of the increased expression of connexin 36 in those brain structures. Interestingly, this gap junctional protein is exclusively expressed in neurons (Vicario et al., 2017) and displays a gradual decrease during post-natal brain maturation (Belousov & Fontes, 2013). This age-dependent change is known to be dependent on NMDA receptor (NMDAR) activity (Arumugam et al., 2005). Previous results demonstrated that NMDAR subunit NR1 was decreased in hippocampal neurons isolated from animals prenatally exposed to VPA (Traetta et al., 2021). Also, deficits in social behavior were demonstrated to be common for either NMDA receptor hypofunction (Cao et al., 2022) or increased gap-junctional activity (E. Lee et al., 2021). It is therefore tempting to speculate that the limited NMDAR neuronal function in young adult VPA animals may fail to promote age-dependent connexin 36 downregulation. Interestingly, we also found the increased expression of connexins 43 exclusively in the hippocampus of the ASD rat model. In the adult brain, this gap junctional protein is mostly found in the astrocytes (Sánchez et al., 2020; Vicario et al., 2017), and was demonstrated to be up-regulated in the brains of autistic individuals (Fatemi et al., 2008). In addition to identifying elevation in connexin proteins in VPA animals, our results demonstrated the abnormal expression of pannexin 1 in the brain cortex. In contrast to connexins, which are able to open only when stress factors are present, ATP release by pannexins also occurs under physiological conditions (Scemes et al., 2009). Also, single nucleotide polymorphisms in the pannexin 1 gene have been linked to autism spectrum disorders (Davis et al., 2012). In turn, the transcript level for vesicular nucleotide transporter (VNUT), which plays an essential role in vesicular ATP storage, was significantly enhanced in all investigated structures of VPA animals. VNUT is localized in the secretory vesicles of neuronal, endocrine, and immune cells, where it regulates vesicular ATP release and purinergic chemical transmission (Miyaji et al., 2011). Since VNUT is a key molecule in initiating purinergic signaling for neurological, metabolic, and immunological functions, the observed deregulation of this protein leading to abnormal ATP release might significantly impair brain activity in autistic rats. The increased gap junctional and vesicular activity in the brains of VPA animals may represent a major factor responsible for elevated ATP release that seems not to be compensated by raised ATP degradation in the synaptic cleft, since the expression of major ATPases: ectonucleoside triphosphate diphosphohydrolase 2 and ectonucleoside triphosphate diphosphohydrolase 3 was not changed in the experimental animals' brains. On the other hand, the expression of ecto-5'-nucleotidase, which hydrolyses extracellular AMP into adenosine (Dwyer et al., 2020) was significantly decreased, thus the total level of adenosine in CSF of VPA rats was unchanged. This observation corresponds with previous data showing the elevated catabolism of ATP and ADP, with the concomitant decrease in AMP and adenosine (ADO) in the brains of zebrafish prenatally exposed to VPA (Zimmermann et al., 2017). Therefore, the mechanisms involved in the control of ATP and adenosine levels may be at least partially involved in the pathophysiological mechanisms of autism, especially since it was demonstrated that adenosine-modulating activities like swimming, deep pressure massage, or a ketogenic diet may significantly decrease core disease symptoms in a broad range of individuals with ASD (Masino et al., 2011).

The increased purine concentration in the extracellular matrix in specific brain areas may produce more robust activation of P2 receptors, which are present on each type of cell within CNS, that can directly influence the several molecular pathways in the particular brain cell subtypes. Among them, we have focused on analyzing the mTOR activity, which is known to be an important downstream effector protein in purinergic signaling. The disturbance of the mTOR pathway was previously suggested to be the major factor contributing to the occurrence of autistic phenotype in VPA animal models (Gassowska-Dobrowolska et al., 2021, 2022; Jezzi et al., 2022; Lieberman et al., 2020). An abnormal mTOR signaling has been identified in around 10% of autistic individuals (Bhandari

et al., 2020) and has been associated with macrocephaly, seizures, and learning deficits (Bhandari et al., 2020; Czapski et al., 2021; Yeung et al., 2017). Interestingly, more than half of the autism susceptibility genes are directly or indirectly associated with disturbances in mTOR activity (Trifonova et al., 2019). The extracellular ATP can directly influence the activation of mTOR in the human lung microvascular endothelial cells (HLMVEC) (Ahmad et al., 2004), liver cells (Sun et al., 2013), skeletal muscle (Hornberger et al., 2006; Ito et al., 2018), regulatory and helper T cells (Cobbold, 2013; Roy & Awasthi, 2019). mTOR phosphorylation is a required step in ATP-induced cell proliferation, which can be inhibited by rapamycin (Gerasimovskaya et al., 2005; Rosborough et al., 2014). Importantly, extracellular ATP triggers mTOR activation in a way similar to LPS (Hu et al., 2020), and mTOR is largely involved in the regulation of gene expression of inflammatory cytokines and chemokines in ASD patients (Temiz-Resitoglu et al., 2017; B. Wang et al., 2022). Additionally to those studies, we have observed that abnormal P2 receptor stimulation after prenatal VPA exposure leads to increased mTOR phosphorylation in the brain. In our study, we have employed two non-selective antagonists of P2 receptors, PPADS, and isoPPADS to modulate purinergic signaling in animals prenatally exposed to VPA treatment. Those compounds initially were believed to predominantly block P2X receptors. However, there are several reports, showing their considerable affinity for P2Y receptors (Boyer et al., 1994; Brown et al., 1995; Schachter et al., 1996). Interestingly, we have observed that only isoPPADS was effective in preventing mTOR phosphorylation on S2448. Concerning the differences in the way our two antagonists work, we know that isoPPADS activity is stronger than PPADS in relation to purinergic signaling inhibition (Felix et al., 2012) and it inhibits both P2X and P2Y receptors without displaying clear selectivity for any particular subtype (Bültmann et al., 1996, 1998; Hansmann et al., 1997). Moreover, isoPPADS is more potent in blocking P2X2, P2Y1 (Y.-C. Kim et al., 1998), as well as P2X1 receptors (Jacobson et al., 1998). Taking into consideration the observed distinct effects of non-selective purinergic receptor antagonists, it is thus possible that the stimulation of the purinergic receptors for which isoPPADS is more effective is responsible for changes in mTOR phosphorylation. However, the involvement of the particular P2 receptor subtype in the observed phenomena should be confirmed in future studies.

The molecular mechanisms underlying the P2 receptor-dependent regulation of mTOR activity are multifaceted. It was previously demonstrated that stimulation of P2Y1, P2Y2, and P2Y12 receptors resulted in mTOR activation in a manner largely independent of the canonical pathway of PI3 kinase/Akt (Hassanian et al., 2015; Ito et al., 2018; Moore et al., 2014). In our study, the changes in Akt phosphorylation were observed only in the cerebellum of VPA-exposed rats, moreover, this alteration was not influenced by isoPPADS treatment. Therefore we could speculate that the canonical pathway of the PI3K/Akt is not responsible for mTOR phosphorylation. However, mTOR receives input from multiple other signaling pathways, like mitogenic signals, or energy metabolism sensors (Memmott & Dennis, 2009). One of those mechanisms of mTOR regulation is its inhibition by the master metabolic sensor, AMPK. This kinase can inhibit mTOR both directly, through the phosphorylation of Raptor (Gwinn et al., 2008), as well as indirectly through the phosphorylation and activation of TSC2 (Inoki et al., 2003). It was previously demonstrated that extracellular ATP induced the inhibition of AMPK activity (Chi et al., 2014). P2 receptors are known to modulate AMPK activity, mainly via intracellular Ca^{2+} concentration and by CaMKK (da Silva et al., 2006; Piwkowska et al., 2011). Increasing evidence indicates that AMPK/mTOR pathway has a pivotal role in neuronal morphogenesis and synaptic plasticity. For example, AMPK acting through the mTOR pathway influences long-term potentiation (Potter et al., 2010), regulates dendrite growth and arborization in cultured hippocampal neurons (Ramamurthy et al., 2014) as well as dendrite retraction and synapse elimination in retinal ganglion cells (Belforte et al., 2021). It was previously demonstrated that prenatal exposure to VPA induces changes in the AMPK activity in the brain of adult animals (Gąssowska-Dobrowolska et al., 2022), followed by enhanced synaptic plasticity (Sui & Chen, 2012), impairment in long-term potentiation (Mohammadkhani et al., 2022), changes in synapse ultrastructure (Gąssowska-Dobrowolska et al., 2020b) and parvalbumin interneurons dysfunction in amygdala (Zahedi et al., 2023). Thus, in the cortex and hippocampus of VPA animals, a P2-mediated decrease in AMPK activity seems to be responsible for mTOR deregulation and synaptic dysfunction. The question arises about the mechanisms underlying mTOR changes in the cerebellum since the AMPK pathway is not changed within this structure. Moreover, the activation of the master regulator of mTOR, Akt is increased in this structure but seems to be P2-receptor independent. Aside from the Akt or AMPK pathways, there are many other mTOR regulators that are the sensors of amino acid and nutrient status and are connected with P2 receptor activity, like receptor tyrosine kinase-dependent Ras, GSK3 β , REDD1, p53 or MAPK p38 (Bao et al., 2015; Evangelisti et al., 2020; D.-F. Lee et al., 2007; Saxton & Sabatini, 2017). The possible involvement of those factors in regulating mTOR in the cerebellum could be taken into consideration in future studies. Interestingly, it was previously demonstrated that inflammation induces the activation of mTOR in the brain (X. Wang et al., 2016). In our previous paper, we observed that purinergic antagonists significantly prevented the up-regulation of IL-6 and TNF α in the brain cortex of VPA animals (Babiec et al., 2023). Those cytokines were previously shown to regulate mTOR independently from Akt or AMPK pathways; while TNF α induces direct mTOR phosphorylation (Balato et al., 2019), IL-6 activates the Stat3 signaling thus promotes the mTOR phosphorylation and activation (Pinno et al., 2016). Here, we have also observed that in the cerebellum of VPA-exposed animals the mRNA level of IL-6, is significantly increased, and isoPPADS exclusively prevented this up-regulation, while the expression of TNF α was not significantly changed (Fig. S1). Since extracellular ATP is an important pro-inflammatory signal transducer (Cauwels et al., 2014), the P2-receptor-dependent mTOR activation may be at least partly mediated by the pro-inflammatory cytokines.

Additionally to the effect of P2 receptors on mTOR activity, the feedback mechanism of regulation of purine synthesis by mTOR was also observed. It was demonstrated that de novo biosynthesis of purines requires the assembly of biosynthetic enzymes into dynamic cellular bodies named purinosome, and this assembly is dependent on mitochondrial function and tightly regulated by mTOR (French et al., 2016). Our study is thus consistent with the previous findings that exogenous ATP triggers mTOR activation (Hu et al., 2020; Roy & Awasthi, 2019), and also provides the first compelling evidence for the direct association of P2 receptor deregulation with the impairment of some of the proteins related to synaptic structure and function in ASD. Similarly, as in our previous paper (Gąssowska-Dobrowolska et al., 2020a), we have observed that treatment with VPA evoked structure-dependent changes in the whole panel of synaptic proteins that regulate the maintenance of the synaptic vesicles reserve pool (Zhang & Augustine, 2021), vesicle trafficking and

regulation of SNARE complex formation (White & Stowell, 2021), as well as post-synaptic scaffolding proteins. The abnormalities in some of those proteins could be directly associated with the ASD behavioral phenotype as previously shown in animal and human studies (Liu et al., 2016), whereas changes in others are associated with the occurrence of some specific features of the disease like the gene polymorphism of SNAP-25 was shown to be related with the degree of hyperactivity in ASD children (Guerini et al., 2011).

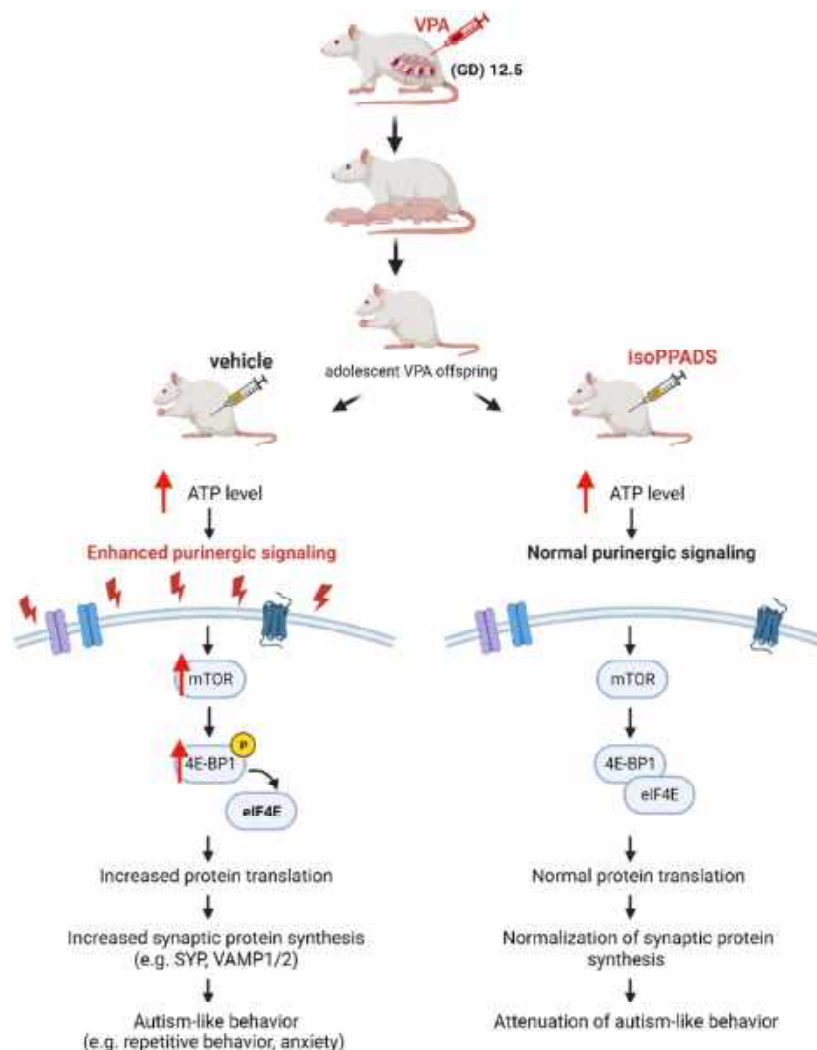
Thus, in addition to previous studies, showing that P2X and P2Y receptors can modulate synaptic transmission and plasticity (Guzman & Gerevich, 2016; Pankratov et al., 2009), this report demonstrates the crucial role of P2 receptors in the deregulation of synaptic function and neurotransmitter release in the pathology related to ASD. The other compelling observation from our experiments is that the blockade of P2 receptors normalized the level of selected synaptic proteins changed in autistic animals, like synaptophysin and synaptobrevin (VAMP1/2) in the hippocampus and cerebellum, or PSD95 in the cortex, while the other VPA-induced changes were not influenced by this treatment. This clearly indicates that the deleterious effect of prenatal VPA exposure on the changes in synaptic function is not only mediated by aberrant purinergic signaling stimulation. Moreover, we have observed that similarly to mTOR activation, only isoPPADS, was able to normalize the level of some synaptic proteins changed in VPA animals, suggesting the possible involvement of mTOR in the synaptic protein disturbances in the VPA model. While the regulation of synaptic protein levels by mTOR is complex and tightly linked to synaptic plasticity and neuronal activity (Gao et al., 2021), the role of mTOR in direct regulation of VAMP1/2 synaptophysin and PSD95 was clearly indicated (Czapski et al., 2021; A. Wang et al., 2020; Xiao et al., 2017). Interestingly, in our previous studies using the transgenic model of tuberous sclerosis complex 2 (Tsc2) haploinsufficiency, where the improper regulation of the mTOR signaling cascade occurs, we have observed that the brain-region-dependent alterations in the expression of synaptic proteins exactly follow the pattern of the VPA-induced changes that are influenced by isoPPADS treatment (Czapski et al., 2021), based on this observation we could speculate that our study provides the first compelling evidence for the direct association of P2 receptor-mediated mTOR depletion to impairment of some of the proteins related to synaptic structure and function in ASD. It is not clear, which molecular mechanism is responsible for the decrease in PSD95 in the brain cortex and hippocampus of VPA animals since it was previously shown that p-mTOR positively correlates with PSD95 expression in ASD individuals (Tang et al., 2014; Y. Yang et al., 2019). Some studies indicated that PSD-95 colocalizes with the P2Y1 (Siow et al., 2010) and P2X4 receptors (Lalo et al., 2016) in postsynaptic scaffold and this interaction is crucial in the modulation of NMDA receptors activity and efficacy of

Table 3
Summary of antipurinergic therapy results in the pre-clinical epigenetic VPA-induced rodent model of Autism Spectrum Disorders (ASD).

		Effect after prenatal VPA exposure	Response to antipurinergic therapy
Pre-Synaptic proteins			
Synapsin-1 (Syn-1)	Hippocampus	↑	No
	Cortex	Unchanged	Unchanged
	Cerebellum	Unchanged	Unchanged
Synaptophysin (SYP)	Hippocampus	↑	Normalized
	Cortex	↓	No
SNARE complex proteins	Cerebellum	↑	Normalized
	Hippocampus	↑	Normalized
VAMP1/2	Cortex	Unchanged	Unchanged
	Cerebellum	↑	Normalized
	Hippocampus	↓	No
SNAP-25	Cortex	Unchanged	Unchanged
	Cerebellum	↓	No
Post-Synaptic Density protein			
PSD-95	Hippocampus	↓	No
	Cortex	↓	Normalized
	Cerebellum	Unchanged	Unchanged
mTOR kinase activity			
p-mTOR kinase	Hippocampus	↑	Normalized
	Cortex	↑	Normalized
	Cerebellum	↑	Normalized
p-4E-BP1	Hippocampus	↑	Normalized
	Cortex	↑	Normalized
Up-stream modulators of mTOR kinase	Cerebellum	↑	Normalized
	Hippocampus	↓	Normalized
AMPK activity	Cortex	↓	No
	Cerebellum	↑	No
	Hippocampus	Unchanged	Unchanged
Akt kinase activity	Cortex	Unchanged	Unchanged
	Cerebellum	↑	No
Behavioral abnormalities			
Stereotypies		↑	Normalized
Velocity		Unchanged	Unchanged
Total distance travelled		↓	No
Anxiety		↑	Normalized
Exploratory activity		↓	Normalized

excitatory synapses. Therefore it is probable that persistent elevation of exogenous ATP in the brain of VPA animals induces P2 receptor hyperactivity, an increase in Ca^{2+} influx, and PSD95-mediated deregulation of the NMDA receptors. Thus, the decrease in PSD95 could be the negative feedback mechanism that normalizes the increased net magnitude of LTP. In agreement with this hypothesis, it was recently shown that following Ca^{2+} influx, binding of Ca^{2+} + calmodulin (CaM) to PSD-95 triggers loss of synaptic PSD-95 (Chowdhury & Hell, 2019).

Finally, the increased purinergic activity may also represent a major factor responsible for the altered social behavior of VPA animals. Our results are consistent with the previous studies from poly(IC) (Naviaux et al., 2014; Naviaux et al., 2013) or Fragile X



Rycine 1. Suggested molecular link between the disturbances in extracellular ATP-dependent signaling and the synaptic, and behavioral alterations in an animal pre-clinical model of ASD. Maternal exposure to valproic acid (VPA; 450 mg/kg body weight) at gestational day (GD) 12.5 induced significant increase in the level of ATP and its hydrolysis products (ADP and AMP) in the cerebrospinal fluid (CSF) of adolescent offspring leading to enhanced purinergic signaling. Overstimulation of ATP-dependent signaling induced mTOR kinase activation and enhanced 4E-BP1 phosphorylation. Phosphorylation of 4E-BP1 leads to dissociation of eIF-4E (eukaryotic translation initiation factor 4E), followed by increased protein translation. Those changes resulted in the increased synaptic protein level (e.g. SYP, VAMP1/2) and development of autism-like behaviors. These pathological changes were attenuated after VPA offspring treatment with isoPPADS (P2 receptors inhibitor). Purinergic receptors inhibition normalized mTOR activation and synaptic proteins level along with attenuation of autism-like behavior such as repetitive behavior and anxiety.

(Fmr1 knockout) mouse model of ASD Naviaux et al. (2015)) showing that the non-selective purinergic receptors blockade results in the improvement of deficits in behavior. Also in the animal model of prenatal exposure to VPA, treatment with the non-selective P2 receptors blocker, Suramin, resulted in the modification of behavioral and inflammatory processes (Hirsch et al., 2020). Interestingly, in our studies, some of the behavioral manifestations like repetitive behavior were reversed by both employed non-selective purinergic blockade. This clearly indicates that apart from changing the mTOR signaling cascade in ASD, purinergic deregulation may influence other features related to brain dysfunction and connected to P2 receptors malfunction, like increased inflammatory response or metabolic dysfunction (Babiec et al., 2022; Naviaux et al., 2014; Naviaux et al., 2013).

We are aware of the limitations of this study. First, we have decided to conduct our studies in one particular stage of CNS development, while purinergic signaling might also be significantly altered in earlier stages when neurogenesis, neuronal migration, axon growth, and functional axonal maturation occur. Also, our experiments were performed exclusively on male offspring, because large reports are showing the gender differences in a broad spectrum of aberrations in rats exposed to VPA on day 12.5 of gestation: while males presented a broad range of behavioral, neuroanatomical, and immunological changes, female offspring showed only minor deficits (Kazlauskas et al., 2019; K. C. Kim et al., 2013; Mowery et al., 2015; Schneider et al., 2008). Additionally, the synaptic alterations are greater in males (K. C. Kim et al., 2013). mTOR activity also significantly varies by sex in rodents (Baar et al., 2016) and the purine metabolism and the expression of selected purinergic receptors are largely affected by estrogens (Bereiter et al., 2022; Hebanowska et al., 2021; Wei et al., 2023). There is also a suggestion of the potential cross-talk between P2 and estrogen receptor systems (Crain & Watters, 2010). Due to the possible involvement of female sex hormones in the observed parameters, we have decided to conduct our experiments using only male offspring, being aware that there is a risk that the observed phenomenon may not appear in a certain, female population. Therefore, the findings derived from this research could suggest a novel avenue for upcoming studies concerning gender differences. Finally, a general limitation of the studies using non-selective inhibitors is that we are not able to select the specific receptor(s) that are responsible for the occurrence of the observed phenomena. However, the results obtained in this study may indicate a new pathway in future investigations and indicate an urgent need to investigate the changes in purinergic signaling in humans suffering from ASD and the relation of these changes to disorder outcomes.

In summary, we have shown that the disturbances in the extracellular ATP turnover and overstimulation of purinergic receptors triggers mTOR kinase activation. mTOR-dependent signaling pathway could be at least partially responsible for the alterations of the synaptic proteins level and homeostasis and appearance of behaviors associated with ASD (Table 3, Rycine 1). Therefore, purinergic signaling-targeting interventions may have therapeutic relevance to ASD treatment.

Ethical statement

All experiments conducted with animals were approved by the Local Ethics Committee for Animal Experimentation in Warsaw, Poland (reference numbers: WAW2/148/2018, WAW2/036/2019, WAW2/128/2021) and were carried out following the EC Council Directive of 24 November 1986 (86/609/EEC), the ARRIVE guidelines, and guidelines published in the NIH Guide for the Care and Use of Laboratory Animals. Additionally, the principles presented in the "Guidelines for the Use of Animals in Neuroscience Research" by the Society for Neuroscience were followed. We made an effort to use the minimal number of animals necessary to obtain results and proceeded with the animals gently to reduce stress and suffering.

CRediT authorship contribution statement

Adamczyk Agata: Conceptualization, Data curation, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. **Gawinek Elżbieta:** Investigation. **Hilgier Wojciech:** Investigation. **Babiec Lidia:** Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Visualization, Writing – original draft. **Wilkaniec Anna:** Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Supervision, Validation, Visualization, Writing – review & editing.

Declaration of Competing Interest

The authors report no conflict of interest.

Data availability

Data will be made available on request.

Acknowledgements

Investigation of ATP, ADP, AMP, and adenosine level in the cerebrospinal fluid of VPA animals (Fig. 1), as well as determination of purinergic signaling modulation on the mTOR pathway activity (Figs. 4–6) and synaptic proteins dysregulation (Fig. 7–9) after valproic acid prenatal exposure was funded by The National Science Centre Grant: 2017/25/B/NZ4/01969. Analysis of the expression of proteins related to ATP release and hydrolysis in the brain of animals prenatally treated with VPA (Figs. 2,3), as well as the impact of purinergic signaling modulation on the autism-like behavior (Fig. 10) after valproic acid prenatal exposure was funded by The National Science Centre Grant: 2021/41/N/NZ4/02350.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.rasd.2024.102353](https://doi.org/10.1016/j.rasd.2024.102353).

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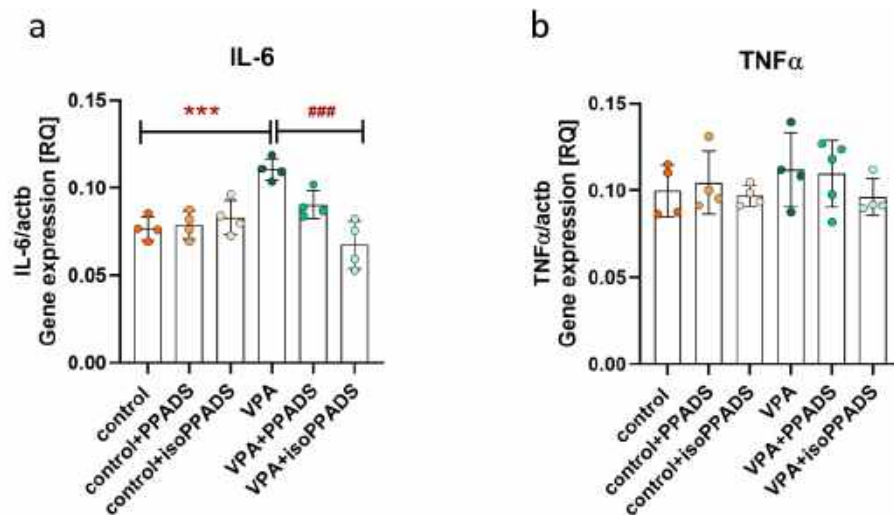
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Supplementary Figure 1. The effect of the purinergic receptors inhibition on the IL-6 and TNF α expression in the cerebellum of animals prenatally exposed to VPA. Data are expressed as a mean value $\pm$ SD for 4-5 independent experiments (N=4-5). * $p < 0.05$ versus control; # $p < 0.05$ versus VPA as determined by one-way ANOVA followed by Bonferroni's post-hoc comparison test.

12. Oświadczenia współautorów



Instytut Medycyny Doświadczalnej i Klinicznej
im. Mirosława Moczakowskiego
Polskiej Akademii Nauk

Zakład Komórkowej Transdukcji Sygnału
mgr. inż. Lidia Babiec
e-mail: lbabiec@imdik.pan.pl

Warszawa, 13.06.2024 r.

Oświadczenie kandydata

Niniejszym poświadczam swój wkład w powstanie następujących publikacji:

- Babiec L., Wilkaniec A., Adamczyk A. Prenatal exposure to valproic acid induces alterations in the expression and activity of purinergic receptors in the embryonic rat brain. *Folia Neuropathol* 2022; 60 (4), 390-402. <https://doi.org/10.5114/fn.2022.123999>
- Babiec L., Wilkaniec A., Matuszewska M., Pałasz E., Cieślik M., Adamczyk A. Alterations of Purinergic Receptors Levels and Their Involvement in the Glial Cell Morphology in a Pre-Clinical Model of Autism Spectrum Disorders. *Brain Sciences* 13, 1088. <https://doi.org/10.3390/brainsci13071088>

Wkład w powyższe publikacje obejmuje:

Wykonywanie doświadczeń (zakładanie hodowli neuronów *in vitro*, analiza aktywności, qPCR i Western Blotting receptorów purynerycznych, barwienie IHC; analiza ilości i morfologii komórek glejowych) oraz iniekcje zwierząt; analiza i interpretacja wyników; udział w pisaniu manuskryptu.

- Czapski G.A., Babiec L., Jęsko H., Gąssowska-Dobrowolska M., Cieślik M., Matuszewska M., Frontczak-Baniewicz M., Zajdel K., Adamczyk A. Synaptic Alterations in a Transgenic Model of Tuberous Sclerosis Complex: Relevance to Autism Spectrum Disorders. *International Journal of Molecular Sciences* 2021, 22, 10058. <https://doi.org/10.3390/ijms221810058>

Wkład w powyższą publikację obejmuje:

Adaptacja oraz przeprowadzenie genotypowania myszy TSC, wykonanie analizy Western blotting białek synaptycznych oraz przeprowadzenie badań behawioralnych w badanym modelu; udział w pisaniu manuskryptu i odpowiedzi na recenzje.

- Babiec L., Wilkaniec A., Gawinek E., Hilgier W., Adamczyk A. Inhibition of purinergic P2 receptors prevents synaptic and behavioral alterations in a rodent model of autism spectrum disorders. *Research in Autism Spectrum Disorders* 2024, 112, 102353. <https://doi.org/10.1016/j.rasd.2024.102353>

Wkład w powyższą publikację obejmuje:

Adaptacja metod oraz przeprowadzenie doświadczeń (qPCR, Western Blotting), iniekcje zwierząt oraz przeprowadzenie badań behawioralnych; analiza i interpretacja wyników, udział w pisaniu manuskryptu i odpowiedzi na recenzje; pozyskanie środków oraz zarządzanie projektem.

Lidia Babiec
Lidia Babiec



Instytut Medycyny Doświadczalnej i Klinicznej
Im. Mirosława Mossakowskiego
Polskiej Akademii Nauk

Zakład Komórkowej Transdukcji Sygnału
prof. dr hab. n. med. Agata Adamczyk

e-mail: aadamczyk@imdik.pan.pl

Warszawa, 13.06.2024 r.

Oświadczenie współautora

Niniejszym poświadczam swój wkład w powstanie następujących publikacji:

- Czapski G.A., Babiec L., Jęśko H., Gąssowska-Dobrowolska M., Cieślik M., Matuszewska M., Frontczak-Baniewicz M., Zajdel K., Adamczyk A. Synaptic Alterations in a Transgenic Model of Tuberous Sclerosis Complex: Relevance to Autism Spectrum Disorders. *International Journal of Molecular Sciences* 2021, 22, 10058.
<https://doi.org/10.3390/ijms221810058>

Wkład w powyższe publikacje obejmuje:

Udział w sformułowaniu koncepcji pracy i nadzór merytoryczny; analiza i interpretacja wyników; udział w pisaniu manuskryptu i odpowiedzi na recenzje; pozyskanie środków oraz zarządzanie projektem.

- Babiec L., Wilkaniec A., Adamczyk A. Prenatal exposure to valproic acid induces alterations in the expression and activity of purinergic receptors in the embryonic rat brain. *Folia Neuropathol* 2022; 60 (4), 390-402.
<https://doi.org/10.5114/fn.2022.123999>
- Babiec L., Wilkaniec A., Matuszewska M., Pałasz E., Cieślik M., Adamczyk A. Alterations of Purinergic Receptors Levels and Their Involvement in the Glial Cell Morphology in a Pre-Clinical Model of Autism Spectrum Disorders. *Brain Sciences* 13, 1088.
<https://doi.org/10.3390/brainsci13071088>
- Babiec L., Wilkaniec A., Gawinek E., Hilgier W., Adamczyk A. Inhibition of purinergic P2 receptors prevents synaptic and behavioral alterations in a rodent model of autism spectrum disorders. *Research in Autism Spectrum Disorders* 2024, 112, 102353.
<https://doi.org/10.1016/j.rasd.2024.102353>

Wkład w powyższe publikacje obejmuje:

Udział w sformułowaniu koncepcji pracy i nadzór merytoryczny; adaptacja metod badawczych do warunków eksperymentalnych; analiza i interpretacja wyników; korekta manuskryptu i udział w odpowiedzi na recenzje; pozyskanie środków oraz zarządzanie projektem.


Agata Adamczyk



Instytut Medycyny Doświadczalnej i Klinicznej
im. Mirosława Mossakowskiego
Polskiej Akademii Nauk

Zakład Komórkowej Transdukcji Sygnału
dr Anna Wilkaniec

e-mail: awilkaniec@imik.pan.pl

Warszawa, 13.06.2024 r.

Oświadczenie współautora

Niniejszym poświadczam swój wkład w powstanie następujących publikacji:

- Babiec L., Wilkaniec A., Adamczyk A. Prenatal exposure to valproic acid induces alterations in the expression and activity of purinergic receptors in the embryonic rat brain. *Folia Neuropathol* 2022; 60 (4), 390-402.
<https://doi.org/10.5114/fn.2022.123999>
- Babiec L., Wilkaniec A., Matuszewska M., Pałasz E., Cieślik M., Adamczyk A. Alterations of Purinergic Receptors Levels and Their Involvement in the Glial Cell Morphology in a Pre-Clinical Model of Autism Spectrum Disorders. *Brain Sciences* 13, 1088.
<https://doi.org/10.3390/brainsci13071088>

Wkład w powyższe dwie publikacje obejmuje:

Udział w sformułowaniu koncepcji pracy i nadzór merytoryczny; adaptacja metod badawczych i przygotowanie materiału do badań; analiza i interpretacja wyników; przygotowanie tekstu manuskryptu oraz udział w odpowiedzi na recenzje.

- Babiec L., Wilkaniec A., Gawinek E., Hilgier W., Adamczyk A. Inhibition of purinergic P2 receptors prevents synaptic and behavioral alterations in a rodent model of autism spectrum disorders. *Research in Autism Spectrum Disorders* 2024, 112, 102353.
<https://doi.org/10.1016/j.rasd.2024.102353>

Wkład obejmuje:

Udział w sformułowaniu koncepcji pracy i nadzór merytoryczny; adaptacja metod badawczych do warunków eksperymentalnych; analiza i interpretacja wyników; korekta manuskryptu i udział w odpowiedzi na recenzje.

A. Wilkaniec
Anna Wilkaniec



Instytut Medycyny Doświadczalnej i Klinicznej
im. Mirosława Mossakowskiego
Polskiej Akademii Nauk

Zakład Komórkowej Transdukcji Sygnału
dr Magdalena Cieślik

e-mail: mcieslik@imdik.pan.pl

Warszawa, 15.05.2024 r.

Oświadczenie współautora

Niniejszym poświadczam swój wkład w powstanie następujących publikacji:

- Czapski G.A., Babiec L., Jęśko H., Gąssowska-Dobrowolska M., Cieślik M., Matuszewska M., Frontczak-Baniewicz M., Zajdel K., Adamczyk A. Synaptic Alterations in a Transgenic Model of Tuberous Sclerosis Complex: Relevance to Autism Spectrum Disorders. *International Journal of Molecular Sciences* 2021, 22, 10058.
<https://doi.org/10.3390/ijms221810058>

Wkład obejmuje:

Przygotowanie materiału do badań oraz udział w odpowiedzi na recenzje.

- Babiec L., Wilkaniec A., Matuszewska M., Pałasz E., Cieślik M., Adamczyk A. Alterations of Purinergic Receptors Levels and Their Involvement in the Glial Cell Morphology in a Pre-Clinical Model of Autism Spectrum Disorders. *Brain Sciences* 13, 1088.
<https://doi.org/10.3390/brainsci13071088>

Wkład obejmuje:

Udział w iniekcji zwierząt oraz przygotowanie materiału do badań.


Magdalena Cieślik



Instytut Medycyny Doświadczalnej i Klinicznej
im. Mirosława Mossakowskiego
Polskiej Akademii Nauk

Zakład Komórkowej Transdukcji Sygnału
dr hab. Grzegorz Czapski, prof. IMDiK

e-mail: gczapski@imdik.pan.pl

Warszawa, 15.05.2024 r.

Oświadczenie współautora

Niniejszym poświadczam swój wkład w powstanie następującej publikacji:

Czapski G.A., Babiec L., Jęsko H., Gąssowska-Dobrowolska M., Cieślik M., Matuszewska M.,
Frontczak-Baniewicz M., Zajdel K., Adamczyk A. Synaptic Alterations in a Transgenic Model of
Tuberous Sclerosis Complex: Relevance to Autism Spectrum Disorders.

International Journal of Molecular Sciences 2021, 22, 10058.

<https://doi.org/10.3390/ijms221810058>

Wkład obejmuje:

Adaptacja metod i przeprowadzenie badań behawioralnych oraz analizy Western blotting;
wykonanie analizy statystycznej; przygotowanie rycin do publikacji, tekstu manuskryptu oraz
udział w odpowiedzi na recenzje.



Grzegorz Czapski



Instytut Medycyny Doświadczalnej i Klinicznej
im. Mirosława Mossakowskiego
Polskiej Akademii Nauk

Zakład Komórkowej Transdukcji Sygnału
dr hab. Magdalena Gąssowska-Dobrowolska

e-mail: mgassowska@imdik.pan.pl

Warszawa, 15.05.2024 r.

Oświadczenie współautora

Niniejszym poświadczam swój wkład w powstanie następujących publikacji:

Czapski G.A., Babiec L., Jęsko H., Gąssowska-Dobrowolska M., Cieślik M., Matuszewska M.,
Frontczak-Baniewicz M., Zajdel K., Adamczyk A. Synaptic Alterations in a Transgenic Model of
Tuberous Sclerosis Complex: Relevance to Autism Spectrum Disorders.

International Journal of Molecular Sciences 2021, 22, 10058.

<https://doi.org/10.3390/ijms221810058>

Wkład obejmuje:

Adaptacja oraz wykonanie analizy Western blotting białek synaptycznych w badanym modelu;
udział w odpowiedzi na recenzje.

Magdalena Gąssowska-Dobrowolska



Instytut Medycyny Doświadczalnej i Klinicznej
im. Mirosława Mossakowskiego
Polskiej Akademii Nauk

Zakład Komórkowej Transdukcji Sygnału
mgr inż. Marta Matuszewska

e-mail: mmatuszewska@imdik.pan.pl

Warszawa, 15.05.2024 r.

Oświadczenie współautora

Niniejszym poświadczam swój wkład w powstanie następujących publikacji:

- Czapski G.A., Babiec L., Jęsko H., Gąssowska-Dobrowolska M., Cieślik M., Matuszewska M., Frontczak-Baniewicz M., Zajdel K., Adamczyk A. Synaptic Alterations in a Transgenic Model of Tuberous Sclerosis Complex: Relevance to Autism Spectrum Disorders. *International Journal of Molecular Sciences* 2021, 22, 10058.
<https://doi.org/10.3390/ijms221810058>

Wkład obejmuje:

Udział w prowadzeniu badań behawioralnych oraz odpowiedzi na recenzje.

- Babiec L., Wilkaniec A., Matuszewska M., Pałasz E., Cieślik M., Adamczyk A. Alterations of Purinergic Receptors Levels and Their Involvement in the Glial Cell Morphology in a Pre-Clinical Model of Autism Spectrum Disorders. *Brain Sciences* 13, 1088.
<https://doi.org/10.3390/brainsci13071088>

Wkład obejmuje:

Udział w iniekcji zwierząt oraz analizie qPCR i Western Blotting receptorów purynergicznym.

Marta Matuszewska



Instytut Medycyny Doświadczalnej i Klinicznej
Im. Mirosława Mossakowskiego
Polskiej Akademii Nauk

Zakład Komórkowej Transdukcji Sygnału
mgr Elżbieta Gawinek

e-mail: egawinek@imdik.pan.pl

Warszawa, 15.05.2024 r.

Oświadczenie współautora

Niniejszym poświadczam swój wkład w powstanie następującej publikacji:

Babiec L., Wilkaniec A., Gawinek E., Hilgier W., Adamczyk A. Inhibition of purinergic P2 receptors prevents synaptic and behavioral alterations in a rodent model of autism spectrum disorders. Research in Autism Spectrum Disorders 2024, 112, 102353.
<https://doi.org/10.1016/j.rasd.2024.102353>

Wkład obejmuje:

Udział w iniekcji zwierząt oraz przeprowadzaniu badań behawioralnych.

Elżbieta Gawinek



Instytut Medycyny Doświadczalnej i Klinicznej
im. Mirosława Mossakowskiego
Polskiej Akademii Nauk

Zakład Neurotoksykologii
dr n. przyr. Wojciech Hilgier

e-mail: whilgier@gmail.com

Warszawa, 30.04.2024 r.

Oświadczenie współautora

Niniejszym poświadczam swój wkład w powstanie następującej publikacji:

Babiec L., Wilkaniec A., Gawinek E., Hilgier W., Adamczyk A. Inhibition of purinergic P2 receptors prevents synaptic and behavioral alterations in a rodent model of autism spectrum disorders. Research in Autism Spectrum Disorders 2024, 112, 102353.

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Wkład obejmuje:

Adaptacja metody analitycznej oznaczania poziomu nukleotydów (ATP, ADP i AMP) oraz adenozyiny w płynie mózgowo-rdzeniowym zwierząt do warunków doświadczalnych. Opracowanie wyników oraz opis metody.

Wojciech Hilgier



Instytut Medycyny Doświadczalnej i Klinicznej
im. Mirosława Mossakowskiego
Polskiej Akademii Nauk

Zakład Komórkowej Transdukcji Sygnału
dr Ewelina Pałasz

e-mail: epalas@imdik.pan.pl

Warszawa, 15.05.2024 r.

Oświadczenie współautora

Niniejszym poświadczam swój wkład w powstanie następującej publikacji:

Babiec L., Wilkaniec A., Matuszewska M., Pałasz E., Cieślik M., Adamczyk A. Alterations of Purinergic Receptors Levels and Their Involvement in the Glial Cell Morphology in a Pre-Clinical Model of Autism Spectrum Disorders. Brain Sciences 13, 1088.
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Wkład obejmuje:

Przygotowanie materiału oraz adaptacja metody barwienia IHC receptorów purynergicznym do warunków doświadczalnych.

Ewelina Pałasz



Instytut Medycyny Doświadczalnej i Klinicznej
im. Mirosława Mossakowskiego
Polskiej Akademii Nauk

Pracownia Badań Mikroskopowa – Elektronowych
prof. dr hab. n. med. Małgorzata Frontczak-Baniewicz

e-mail: mbaniewicz@imdik.pan.pl

Warszawa, 06.06.2024 r.

Oświadczenie współautora

Niniejszym poświadczam swój wkład w powstanie następującej publikacji:

Czapski G.A., Babiec L., Jęsko H., Gąssowska-Dobrowolska M., Cieślik M., Matuszewska M.,
Frontczak-Baniewicz M., Zajdel K., Adamczyk A. Synaptic Alterations in a Transgenic Model of
Tuberous Sclerosis Complex: Relevance to Autism Spectrum Disorders.

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Analiza zdjęć TEM; udział w odpowiedzi na recenzje.

Małgorzata Frontczak-Baniewicz



Instytut Medycyny Doświadczalnej i Klinicznej
im. Mirosława Mossakowskiego
Polskiej Akademii Nauk

Pracownia Badań Mikroskopowo – Elektronowych
dr n. med. Karolina Zajdel

e-mail: karolina.zajdel@ncbj.gov.pl

Warszawa, 14.06.2024 r.

Oświadczenie współautora

Niniejszym poświadczam swój wkład w powstanie następującej publikacji:

Czapski G.A., Babiec L., Jęsko H., Gąssowska-Dobrowolska M., Cieślik M., Matuszewska M.,
Frontczak-Baniewicz M., Zajdel K., Adamczyk A. Synaptic Alterations in a Transgenic Model of
Tuberous Sclerosis Complex: Relevance to Autism Spectrum Disorders.

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Adaptacja metody i wykonanie zdjęć TEM; udział w odpowiedzi na recenzje.

Karolina Zajdel

Karolina Zajdel