Neuropharmacology 113 (2017) 71–81

Contents lists available at ScienceDirect

Neuropharmacology

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

Agmatine rescues autistic behaviors in the valproic acid-induced animal model of autism

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ARTICLE INFO

Article history:
Received 21 March 2016
Received in revised form
9 September 2016
Accepted 12 September 2016
Available online 14 September 2016

Keywords:
Autism spectrum disorder
Therapeutics
Agmatine
E/I imbalance
Valproic acid
NMDA receptor

ABSTRACT

Autism spectrum disorder (ASD) is an immensely challenging developmental disorder characterized primarily by two core behavioral symptoms of social communication deficits and restricted/repetitive behaviors. Investigating the etiological process and identifying an appropriate therapeutic target remain as formidable challenges to overcome ASD due to numerous risk factors and complex symptoms associated with the disorder. Among the various mechanisms that contribute to ASD, the maintenance of excitation and inhibition balance emerged as a key factor to regulate proper functioning of neuronal circuitry. Interestingly, our previous study involving the valproic acid animal model of autism (VPA animal model) has demonstrated excitatory-inhibitory imbalance (E/I imbalance) due to enhanced differentiation of glutamatergic neurons and reduced GABAergic neurons. Here, we investigated the potential of agmatine, an endogenous NMDA receptor antagonist, as a novel therapeutic candidate in ameliorating ASD symptoms by modulating E/I imbalance using the VPA animal model. We observed that a single treatment of agmatine rescued the impaired social behaviors as well as hyperactive and repetitive behaviors in the VPA animal model. We also observed that agmatine treatment rescued the overly activated ERK1/2 signaling in the prefrontal cortex and hippocampus of VPA animal models, possibly, by modulating over-excitability due to enhanced excitatory neural circuit. Taken together, our results have provided experimental evidence suggesting a possible therapeutic role of agmatine in ameliorating ASD-like symptoms in the VPA animal model of ASD.

1. Introduction

Autism spectrum disorder (ASD) is a heterogeneous neurodevelopmental disorder characterized primarily by two prominent behavioral symptoms including impaired social communication and restricted or repetitive behaviors. Since ASD is a multi-gene and a multi-symptomatic disorder, it poses an immense challenge in ameliorating the impaired behaviors of affected individuals (Delorme et al., 2013). Indeed, a few approved drugs for ASD treatment fail to rescue the core ASD symptoms, especially social impairment. Prompted by these challenges, we assessed whether agmatine, an endogenous NMDA receptor antagonist, alleviates autistic behaviors using the valproic acid-induced animal model of autism.

Valproic acid (VPA) exposure during early pregnancy is known as a strong inducer of autism in offspring of exposed mothers in human as well as in experimental animals (reviewed in Ornoy, 2009). In the clinical study, a child exposed to *in utero* VPA over 1000 mg, especially during the first trimester, is the most susceptible to develop autism (Williams et al., 2001). In the previous

Abbreviations: ASD, autism spectrum disorder; ADC, arginine decarboxylase; VPA, valproic acid; NPC, neural progenitor cells; PFC, prefrontal cortex; AGM, agmatine; PZC, Piperazine-1-carboxamidine; SI, social index; SPI, social preference; CC50, convulsive current 50.

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http://dx.doi.org/10.1016/j.neuropharm.2016.09.014
0028-3908/

clinical study, the prevalence of ASD caused by *in utero* VPA exposure was 8.9%. Moreover, prenatal VPA exposure increased the risk of developing ASD up to 20 times higher than the unexposed population (Rasalam et al., 2005). In rodents, many research groups including us reported that *in utero* VPA exposure also causes autism-like symptoms in the rodent offspring, mainly social defects and repetitive behaviors (reviewed in Roullet et al., 2013). Hence, we have used the VPA-induced ASD rat model (VPA animal model or VPA-exposed rats) to test the efficacy of agmatine in ameliorating the behavioral symptoms associated with ASD.

Various studies including ours, have elucidated the pathophysiological features associated with the VPA animal model. Of particular interest, we have observed increased glutamatergic receptors expression in the cortex of rats exposed to VPA *in utero,* which was associated with an epigenetic change of Pax6, a transcription factor implicated in the induction of excitatory neuronal development (Kim et al., 2014b). Interestingly, recent reports suggested that NMDA receptor blockers can be therapeutic candidates to correct ASD symptoms caused by *in utero* VPA (Kang and Kim, 2015; Kim et al., 2014b). In addition to our studies, Rinaldi et al. reported that the NMDA-mediated long-term potentiation (LTP) is enhanced in the medial prefrontal cortex, somatosensory cortex, and amygdala of VPA-exposed offspring (Markram et al., 2008; Rinaldi et al., 2007, 2008). Based on these observations, we hypothesized that the modulation of NMDA receptor activity can potentially rescue the autism-like behavioral symptoms in VPA animal model.

Agmatine has been recently known as a source of polyamine. Agmatine is also believed to act as a neurotransmitter stored in the synaptic vesicle and released when neurons are depolarized (Goracke-Postle et al., 2006; Halaris and Plietz, 2007; Reis et al., 1998). Agmatine is known as a ligand for various targets such as imidazoline, α2 adrenergic receptors, voltage-gated ion channels, nitric oxide synthase (iNOS/nNOS), and act as an NMDA receptor blocker (reviewed in Piletz et al., 2013a). Agmatine can reduce the NMDA currents by the interaction between its guanidine group of agmatine and pores of NMDA receptor channels (Yang and Reis, 1999). This function of agmatine demonstrates its potential to reduce the neuronal over-excitability in autism. Importantly, agmatine has been proposed as a candidate drug for other psychiatric disorders such as depression, ischemia, and epilepsy (reviewed in Piletz et al., 2013a). Based on these properties, we assessed the therapeutic potential of agmatine to rescue the behavioral deficits associated with ASD.

Here, we have shown that agmatine can rescue the social impairment, hyperactivity, and repetitive behaviors observed in the VPA animal model. Agmatine also increased the threshold of seizure activity caused by electric shock stimulation. Moreover, we reasoned that neuronal over-excitability might be induced by the increased glutamatergic neurons which enhance sensitivity to glutamates. To elucidate the molecular target mediating the over-excitability of cellular signaling, we investigated the ERK1/2 signaling based on several reports. First, the Fragile X Syndrome-causing knockdown or loss of FMRP (a syndromic ASD) in neural progenitor cells enhanced glutamatergic neuronal differentiation and, possibly, increased the glutamatergic neuronal number (Jeon et al., 2014; Tervonen et al., 2009). Interestingly, the correction of increased ERK1/2 phosphorylation in $Fmr1^{-/-}$ mice using the ERK1/2 inhibitor, lovastatin, reduced exaggerated neuronal activity (Osterweil et al., 2013). Second, ERK1/2 is the downstream signaling of NMDA receptors and ERK1/2 activity can be used as a marker of the NMDA receptor blocking potential (Thomas and Huganir, 2004). Hence, we measured the phosphorylation of ERK1/2 in the prefrontal cortex (PFC) and hippocampus of VPA-exposed rats. Fascinatingly, we found the ERK1/2 phosphorylation was overly active in the VPA-exposed rats and agmatine treatment relieved the active ERK1/2 signaling in their prefrontal cortex and hippocampus.

Our study clearly demonstrates the therapeutic potential of agmatine to ameliorate autism-like behavioral symptoms and shed an insight into the mechanistic rationale involved in the impairment of neural circuit function in ASD.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium/F12 (DMEM/F12) was obtained from Gibco BRL (U.S.) and the B-27 supplement was purchased from Invitrogen (U.S.). Poly-L-ornithine, sodium valproate (valproic acid, VPA), agmatine sulfate (agmatine, A7127), and memantine hydrochloride (memantine, M9292) were obtained from Sigma-Aldrich (U.S.). Piperazine-1-carboxamidine was purchased from Frontier Scientific Services, Inc. (U.S.). Antibodies for ERK1/2 (9102) and phosphor-ERK1/2 (Thr202/Tyr204, 9101S) were purchased from cell signaling technology (U.S.). β-actin (A5316) was purchased from Sigma-Aldrich (U.S.).

2.2. Cell cultures

Rat primary neural progenitor cells (NPCs) were isolated from cerebral cortex of embryonic day 14 (E14) SD rats as described previously (Kim et al., 2014b). For differentiation, NPCs were subcultured into poly-L-ornithine (0.1 mg/ml) pre-coated multi-well plates (5×10^5/ml) using B27 supplement-containing DMEM/F12 medium without growth factors. Valproic acid (0.5 mM) was treated 3 h after subculture. Half the volume of the media was changed every two days. After 10 days in vitro (DIV), agmatine (200 μM) or memantine (10 μM) was treated to the cells and after 30min, cells were lysed for Western blot analysis to measure the phosphorylation of ERK.

2.3. In vivo brain preparation

At postnatal day 28, rats were sacrificed by cervical dislocation to obtain the prefrontal cortices and hippocampi separately, which were rapidly frozen in liquid nitrogen. For the treatment group, agmatine (50 mg/kg, Fig. 6) was injected i.p. 30 min prior to brain preparation. Brain samples were stored at −80 °C for further analysis. Subjects were randomly selected from four litters for control and agmatine-treated groups, and another four litters for VPA and VPA + agmatine treated groups (N = 5–7 per group). Animals for brain preparation were not used in other behavior studies.

2.4. Western blot analysis

Cells were washed twice with PBS and lysed with 2× SDS-PAGE sample buffer (120 mM Tris-HCl (pH 6.8), 20% glycerol, 4% SDS, 28.8 mM 2-mercaptoethanol, and 0.01% bromophenol blue). Brain tissues were homogenized using RIPA buffer (150 mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) with protease and phosphatase inhibitors (protease inhibitor cocktail (Roche, No.11873580001), 30 mM NaF, 1 mM PMSF and 1 mM Na_2VO_4). Protein levels were quantified using BCA analysis and the samples were equalized based on the amount of proteins before being diluted with 5× SDS-PAGE sample buffer. Equal amounts of proteins (10 μg) for each condition were resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes. Following transfer, membranes were incubated with