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Gender-specific desensitization of group I metabotropic glutamate receptors after maternal L-glutamate intake during lactation

Antonio López-Zapata, David Agustín León-Navarro*, María Crespo, Mairena Martín

Departamento de Química Inorgánica, Orgánica y Bioquímica, Facultad de Ciencias y Tecnologías Químicas/Facultad de Medicina de Ciudad Real, Centro Regional de Investigaciones Biomédicas, Universidad de Castilla-La Mancha, Avenida Camilo José Cela, 10, 13071 Ciudad Real, Spain

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ABSTRACT

In the present work we have studied the effect of maternal intake of L-Glutamate (L-Glu) (1 g/L) during lactation on group I mGluR transduction pathway in brain plasma membrane from 15 days-old neonates. Results obtained have shown that maternal L-glutamate intake did not significantly affect neither weights of pups nor negative geotaxis reflex, an index of neurobehavioral development, but increased L-Glu plasma level in both male and female neonates. In male neonates, maternal L-Glu intake evoked a loss of $mGluR_1$ whereas no variation on $mGluR_5$ was observed as revealed by Western-blotting assay. The loss of $mGlu_1R$ was accompanied by a decrease on L-Glu-stimulated phospholipase C activity suggesting, therefore, a loss of group I mGluR functionality. Concerning female neonates, no variations were detected neither $mGluR_1$ nor $mGluR_5$ and group I mGluR functionality was also preserved.

1. Introduction

Nowadays it is estimated that human daily consumed between 30–200 mg /Kg of L-Glutamate (L-Glu) (Beyreuther et al., 2007; Fernandez-Tresguerres Hernández, 2005; He et al., 2011). An important contributor to L-Glu intake is monosodium glutamate a food additive widely used to increase the flavor of the food (Halpern, 2000). Since several researchers in the 70´s showed neuronal necrosis following L-Glu administration (Burde et al., 1971; Olney and Sharpe, 1969), the neurotoxicity of this aminoacid has been intensively studied, especially in the first stages of life. Thus, a consensus meeting on safety of monosodium glutamate on 2007 stated that placental barrier avoid the passage of glutamate from maternal plasma to fetusses (Beyreuther et al., 2007). There are, however, evidence that suggest that glutamate passes to circulation and finally may reach Central Nervous System (CNS) of fetusses following maternal oral consumption of L-Glu (Hermanussen et al., 2006). Concerning lactation period, the effect of maternal oral consumption of L-Glu on suckling babies remains elusive. It is known, however, that maternal milk contains large amounts of free aminoacids (Baldeón et al., 2014), especially glutamate and glutamine which represent nearly of 50% of total (Agostoni et al., 2000; Chuang et al., 2005; Elmastas et al., 2008; Sarwar, 2001) and some investigators have also suggested that maternal diet may change the concentration of free aminoacids in breast milk (Lindblad and Rahimtoola, 1974; Wurtman and Fernstrom, 1979). Although the role of free aminoacids in maternal milk is not completely understood, it is suggested that glutamate may play a relevant role in the growth and development of breast-fed infant by acting as neurotransmitter in the brain among others functions (Zhang et al., 2013).

In the CNS L-Glu acts as an excitatory neurotransmitter being involved in key brain processes such as development, learning and memory formation. The actions of L-Glu are mediated by a group of membrane receptors named glutamate receptors which include ionotropic (iGluRs) and metabotropic (mGluRs) glutamate receptors. iGluRs are ligand-gated ion channels responsible for fast excitatory neurotransmission and which have been further subdivided into three types (AMPA, NMDA and Kainate) according to the response to agonist. mGluRs belong to G-protein coupled receptor (GPCR) superfamily and have been subclassified into three groups: Group I ($mGluR_1$ and $mGluR_5$) which stimulate phospholipase C (PLC) activity through a $G_{q/11}$ protein, Group II ($mGluR_2$ and $mGluR_3$) and Group III ($mGluR_4$, $mGluR_6$, $mGluR_7$ and $mGluR_8$) which inhibit adenylyl cyclase (AC) activity through a Gi/o protein (Pin and Acher, 2002; Pin et al., 2003). mGluRs are distributed throughout CNS where modulate neuronal excitability and synaptic transmission (Niswender and Conn, 2010). The activity of group I-mGluR is significantly increased in developing brain

Abbreviations: L-Glu, L-glutamate; mGluR, metabotropic glutamate receptor; CNS, Central Nervous System; GPCR, G-protein coupled receptor; PLC, phospholipase C; A.U., arbitrary units

* Corresponding author at: Área de Bioquímica, Departamento de Química Inorgánica, Orgánica y Bioquímica, Facultad de Ciencias y Tecnologías Químicas, Avenida Camilo José Cela, 10, 13071 Ciudad Real, Spain.
E-mail address: davidagustin.leon@uclm.es (D.A. León-Navarro).

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which allowed to suggest that group I-mGluR could play a relevant role during brain development (Romano et al., 1996).

It is well known that GPCR responsiveness desensitizes after prolonged exposure to agonists through several mechanisms such as receptor phosphorylation, arrestin binding and internalization (Dhami and Ferguson, 2006). Therefore, in the present work we decided to study whether group I-mGluR signaling pathway resulted affected in neonatal brain after maternal L-Glu intake during lactation. Results obtained have shown that maternal L-Glu intake evoked a loss of $mGluR_1$ in male neonates which was also accompanied by a loss of group I mGluR functionality whereas no significant variations were observed in female neonates.

2. Materials and methods

2.1. Materials

Phosphatidylinositol-4,5-bisphosphate, [inositol-2-^{3}H(N)]- ([^{3}H]PIP_2 6.5 Ci/mmol) and [^{3}H]AMPc (27.4 Ci/mmol) were from PerkinElmer (Madrid, Spain). L-glutamate was from Tocris (London, United Kingdom). Anti-metabotropic glutamate receptor 5 ($mGlu_5$; rabbit polyclonal antibody) and anti-metabotropic glutamate receptor 1 (mGlu1; rabbit polyclonal antibody) were purchased from Upstate (Temecula, CA). β-Actin antibody (mouse monoclonal) was from Abcam (Cambridge, United Kingdom). Goat anti-rabbit IgG (H + L)-HRP conjugate and goat anti-mouse IgG (H + L)-HRP conjugate were from Bio-Rad (Hercules, CA). All other reagents were of analytical grade and obtained from commercial sources.

2.2. Animals

Eight Wistar lactating rats, kept on a 12 h light / 12 h dark cycle (lights on at 07:00 h) and with free access to food and drinking water, were treated with tap water (n = 4) and L-glutamate (1 g/L) (n = 4) in the drinking water from the day of parturition until the 15 t h day postpartum. Leakage-proof bottles were used. The drinking fluids were changed every 2 days. Rats were sexed at postnatal day 7 based on anogenital distance. At postnatal day 15, neonates were decapitated and their brains rapidly removed, frozen in liquid N_2 and stored at –70 °C until experiments were performed. Trunk blood was also collected for determination of plasma L-Glu levels. The care and use of animals were carried out accordingly with the European Directive 2010/63/EU and with Spanish laws (Real Decreto 53/2013 and Ley 32/2007) for the use of laboratory animals. All experiments were according to the Animal Experimental Committee of University of Castilla-La Mancha. Every effort was made to minimize animal suffering and to reduce the number of animals used.

2.3. Plasma membranes isolation

One male and one female brain were randomly selected from each one of the eight litters and plasma membranes were isolated following the protocol previously described (León et al., 2004). Brains were homogenized in 20 volumes of isolation buffer (50 mM Tris-HCl, pH 7.4 containing 10 mM $MgCl_2$ and protease inhibitors) in a Dounce homogenizer using pestle A (10 strokes) and B (10 strokes). After homogenization, brain preparations were centrifuged for 5 min at 1000xg in a Beckman JA 21 centrifuge (Coulter, Madrid, Spain). Supernatant was centrifuged for 20 min at 27000*g* and the pellet was finally resuspended in isolation buffer. Protein concentration was measured by the Lowry method, using bovine serum albumin as standard. Each plasma membrane isolation corresponds to one pup per litter.

2.4. Neurobehavioral development: negative geotaxis reflex

During the first two weeks of postnatal period sixteen control neonates (8 male and 8 female) and sixteen neonates (8 male and 8 female) exposed to maternal L-Glu consumption during lactation were removed daily between 10:00 and 13:00 h from mothers for observation of negative geotaxis reflex which provides information about motor coordination and sensory function. During 30 s neonatal rats were placed faced down on an inclined platform (30°) and allowed to turn around and face up the ramp. Animals were returned to their home cage immediately after the observations. The mean day of appearance of this parameter was calculated.

2.5. Immunodetection of mGlu receptors

Membranes (50 μg of protein) from neonatal brain were subjected to 7.5% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) using Precision Plus Protein Standards dual colors (Bio-Rad) as molecular weight markers. Then, proteins were transferred to transfer membrane using iBlot® Dry Blotting System (Invitrogene, Barcelona, Spain) and blocked for 1 h in 5% non-fat skim milk in phosphate-buffered saline, as described earlier (Albasanz et al., 2006). Blots were then probed with anti-$mGlu_1$ (1:1000) or anti-$mGlu_5$ (1:1000) washed with 0.3% Tween-20 in PBS and incubated with second goat anti-rabbit (or anti-mouse) antiserum coupled to horseradish peroxidase (1:3000). Specific bands corresponding to the different proteins were visualized by chemiluminescent reaction using the ECL blotting detection kit from Amersham (Madrid, Spain), and images captured using Syngene G:Box (Syngene, Cambridge, UK). Quantification of the specific bands was carried out using Genetool software (Syngene). The antibody anti-β-actin (1:5000) was used as a control of protein loading.

2.6. Phospholipase C activity assay

Phospholipase C activity in plasma membranes were assayed in the presence of exogenous [^{3}H]PIP_2 as described previously (León et al., 2008). [^{3}H]PIP_2 was dried under an N_2 stream, dissolved in 2 mM sodium deoxycholate and 50 mM Tris-HCl pH 6.5, and sonicated with an Ultrasonic Processor UP 200 S (Ultrasonics Gmbh, Teltow, Germany). The phospholipase C assay was carried out for 10 min at 37 °C, incubating [^{3}H]PIP_2 (17,000 d.p.m.) with or without 20 μg plasma membrane protein in 100 μl buffer (100 mM NaCl, 1 mM sodium deoxycholate, 1 mM EGTA, 250 μM $CaCl_2$, 40 mM LiCl, and 50 mM Tris-HCl, pH 6.8). The functionality of group I mGluR transduction pathway was determined by stimulating with 1 mM L-Glu. The incubation was terminated by the addition of 360 μl chloroform/methanol/HCl (1:2:0.2 v/v) and placing the tubes on ice. After addition of 120 μl 2 M KCl and 160 μl chloroform, the tubes were centrifuged for 5 min at 3500 *g*. The upper aqueous phase (250 μl) containing [^{3}H]inositol phosphates was mixed with 3.5 ml scintillation liquid, and the radioactivity was counted in a MicroBetaJet counter (Perkin Elmer) using Betaplate Scint (Perkin Elmer).

2.7. Preparation of total RNA and cDNA and quantitative real time RT-PCR analysis

Total RNA was extracted from whole brain from male and female neonates using an ABI 6100 Nucleic Acid Prep Station according to the manufacturer´s protocol. All chemicals for the ABI 6100 were purchased from Applied Biosystems (Foster City, CA, USA). Total RNA from animals was isolated and stored individually at −80 °C. Ratio of A_{260}/A_{280} (purity of RNA) was in the range 1.9-2.1. RNA concentrations were determined from the A_{260}. One microgram of total RNA was reverse transcribed using Applied Biosystems High-Capacity cDNA Archive Kit (Applied Biosystems INC, Foster City, CA, USA). Parallel reactions for each RNA sample were run in the absence of MultiScribe Reverse Transcriptase to assess the degree of contaminating genomic DNA.

Quantitative real time RT-PCR analysis was performed as previously