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Cellular and behavioural effects of a new steroidal inhibitor of the *N*-methyl-D-aspartate receptor 3α5β-pregnanolone glutamate

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ABSTRACT

Preclinical studies have demonstrated a considerable role for *N*-methyl-D-aspartate (NMDA) receptors in excitotoxicity and the concurrent neuroprotective effect of NMDA receptor antagonists. Because NMDA receptors are one of the most widespread receptors in the central nervous system, application of their antagonist often leads to serious side effects ranging from motor impairment to induction of schizophrenic-like psychosis. Therefore, we have initiated development and testing of a novel synthetic NMDA receptor antagonist derived from naturally occurring neurosteroids. 20-oxo-5β-pregnan-3α-yl-L-glutamyl-1-ester (3α5βP-Glu) is a novel synthetic steroidal inhibitor of the NMDA receptor. Our results show that 3α5βP-Glu preferentially inhibits tonically activated NMDA receptors, is able to cross the blood brain barrier, does not induce psychotomimetic symptoms (such as hyperlocomotion and sensorimotor gating deficit) and reduced an excitotoxic damage of brain tissue and subsequent behavioural impairment in rats. In particular, 3α5βP-Glu significantly ameliorated neuronal damage in the dentate gyrus and subiculum, and improved behavioural performance in active allothetic place avoidance tasks (AAPA, also known as the carousel maze) after bilateral NMDA-induced lesions to the hippocampi. These findings provide a possible new therapeutic approach for the treatment of diseases induced by NMDA receptor overactivation.

1. Introduction

A number of preclinical studies have shown the remarkable ability of NMDA receptor antagonists to prevent excessive exocytosis of glutamate and neural damage (Choi et al., 1988; Choi, 1990; Rao et al., 2001; Schauwecker, 2010). However, from a clinical point of view, their therapeutic potential in stroke and traumatic brain injury treatment is rather limited (Lees et al., 2000). Since glutamate receptors are very abundant in the brain and provide important physiological functions, application of their antagonists can lead to wide variety of side effects, ranging from motor impairment to induction of psychotic symptoms (Shiigi and Casey, 1999; Manahan-Vaughan et al., 2008).

In the past decade, biomedical research has focused on the role of neurosteroids in the pathogenesis of a number of neuropsychiatric diseases and the evaluation of their therapeutic potential (Gasior et al., 1999; Melcangi et al., 2008). A number of experimental studies with animal models have shown their potential in the therapeutic treatment of several disorders of the central nervous system, including ischemia (Li et al., 2001; Pringle et al., 2003), neurodegenerative disorders (Mellon et al., 2008), traumatic brain injury (Djebaili et al., 2005) and multiple sclerosis (Morrow, 2007).

The naturally occurring compound 3α5β-pregnanolone sulfate (3α5βP-S) inhibits the activity of NMDA receptors in a use-dependent manner (Petrovic et al., 2005). Thus, sulfated neurosteroids and their analogues could be promising molecules in the therapy of central nervous system diseases. Nevertheless, enzymes constantly maintain the ratio between 3-hydroxy- neurosteroids and their sulfated esters in the brain. The enzyme steroid sulfohydrolase

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hydrolyses sulfate groups at the 3rd carbon position (Reed et al., 2005). Systemic administration of sulfated esters thus does not necessarily increase the ratio of sulfated/non-sulfated concentration in the brain (Wang et al., 1997). Furthermore 3α5βP-S do not sufficiently cross the blood-brain barrier because it is ionized molecule at physiological pH (Knapstein et al., 1968; Weaver et al., 1997; Bowen et al., 1999).

Therefore, we have started the development and testing of a novel NMDA receptor antagonist derived from the 3α5βP-S (Stastna et al., 2009). The proposed synthetic molecule should analogue of the 3α5βP-S rasistant to sulfohydrolase activity.

We selected a lead structure (Fig. 1), 20-oxo-5β-pregnan-3α-yl-L-glutamyl-1-ester (3α5β-pregnanolone glutamate, 3α5βP-Glu). In the present study we show the distribution of 3α5βP-Glu and its penetration into the brain, its binding and action on native NMDA receptors in cultured hippocampal neurons, the effect of 3α5βP-Glu on spontaneous behaviours related to schizophrenia-like behaviour (prepulse inhibition of the acoustic startle response and locomotor activity in an open field test) and its effect against neuronal damage and behavioural impairment induced by intrahippocampal application of NMDA.

2. Materials and methods

2.1. Animals

Adult male Long-Evans rats (weighting from 250 to 350 g) from a breeding colony of the Institute of Physiology AS CR, Prague were used in the study. Rats were kept in transparent Plexiglas cages measuring 25 × 25 × 50 cm located in an air-conditioned animal room with a 12:12 hour light-dark cycle with the lights turned on beginning at 7:00 a.m. Animals had free access to water and food. All experiments were done in accordance with European Union regulations on animal care and protection, the Animal Protection Code of the Czech Republic and NIH guidelines.

2.2. 3α5βP-Glu synthesis

3α5βP-Glu was prepared by esterification of 3α-hydroxy-5β-pregnan-20-one (Steraloids INC, USA) with a protected glutamate (Boc-L-Glu(OBzl)-OH, Bachem AG, Germany) catalysed by DCC and DMAP. Esterification was followed by deprotection of the carboxy and amino groups. The structure was confirmed by IR, NMR, MS, and HR-MS spectra. Other reagents used were purchased from Sigma Aldrich (Germany).

2.3. 20-oxo-5β-pregnan-3α-yl-L-glutamyl-1-ester [9,12,12-3H] (3α5βP-Glu- d_3) synthesis

A deuterated analogue was prepared from 11α-hydroxy-progesterone (ACROS Organics, USA). This was converted by stereoselective hydrogenation to yield 5β-steroid, followed by protection of the 3- and 20- oxo groups and mild oxidation of the 11α-OH group. Deuterium exchange at enolisable positions was then conducted under basic conditions using MeOD as an economical and convenient source of deuterium. Deuterated 11-ketone was reduced with $LiAlH_4$ to fix deuterium atoms. Oxygen functionality was removed using the Barton-McCombie reaction. The sequence was then finished using acid catalysed deprotection followed by stereo- and regioselective reduction of the 3-keto group into a tri-deuterated analogue of the parent pregnanolone. Coupling with protected glutamic acid followed by deprotection gave the desired [9,12,12-3H] pregnanolone glutamate at >89% isotopical purity. The overall yield of this sequence was 13%. Ratios and positions of deuterium labels were determined by MS and NMR analyses. All other reagents used were purchased from Sigma Aldrich (Germany).

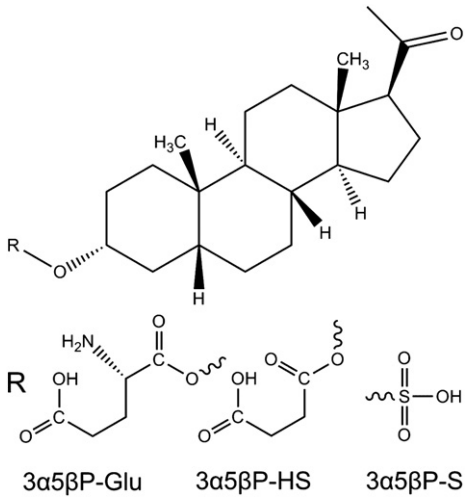

Fig. 1. Chemical structure of 3α5βP-Glu (An L-glutamic acid residue replaces the sulfate group at position 3 on the steroidal ring A), 3α5βP-HS and 3α5βP-S.

2.4. Hippocampal cultures

Primary dissociated hippocampal cultures were prepared from 1- to 2-day-old postnatal rats. Animals were decapitated, and hippocampi were dissected. Trypsin digestion followed by mechanical dissociation was used to prepare cell suspensions. Neuronal cultures were maintained in Neurobasal™-A (Invitrogen, USA) medium supplemented with glutamine (0.5 mM) and B-27 Serum-Free Supplement (Invitrogen, USA).

2.5. Electrophysiological recording from cultured cells

Experiments were performed on cultured hippocampal neurons prepared as described above. Whole-cell voltage-clamp recordings were made with a patch-clamp amplifier (Axopatch 1D; Axon Instruments, Inc., Foster City, CA, USA) after a capacitance and series resistance (<10 MΩ) compensation of 80–90%. Agonist-induced responses were low-pass filtered at 1 kHz with an 8-pole Bessel filter (Frequency Devices, Haverhill, MA, USA), digitally sampled at 5 kHz and analysed using pClamp software version 9 (Axon Instruments, USA). Patch pipettes (3–4 MΩ) pulled from borosilicate glass were filled with a Cs^+-based intracellular solution containing 125 mM gluconic acid, 15 mM CsCl, 5 mM EGTA, 10 mM HEPES, 3 mM $MgCl_2$, 0.5 mM $CaCl_2$, and 2 mM ATP-Mg salt (pH-adjusted to 7.2 with CsOH). The extracellular solution (ECS) contained 160 mM NaCl, 2.5 mM KCl, 10 mM HEPES, 10 mM glucose, 0.2 mM EDTA and 0.7 mM $CaCl_2$ (pH-adjusted to 7.3 with NaOH). Glycine (10 μM), an NMDA receptor co-agonist, was present in the control and test solutions. The drugs were purchased from Sigma Aldrich (Germany) or Tocris Cookson Ltd. (Avonmouth, UK). 3α5βP-Glu solutions were made from a freshly prepared 20 mM stock in DMSO. Control experiments were performed in extracellular solution containing DMSO at the same concentration present in steroid containing solutions. A microprocessor-controlled fast-perfusion system, with a time constant of solution exchange around cells of 10 ms, was used to apply test and control solutions (Vyklicky et al., 1990). Results are presented as mean ± S.E.M. with n equal to the number of cells studied.

2.6. Determination of 3α5βP-Glu plasma and tissue levels

3α5βP-Glu levels were determined in plasma and tissue samples. On the day of the experiment, rats were divided into groups based on their scheduled sacrification time (15, 30, 45, 60, 90, 120, 180 min and 24 or 48 h post-injection) and administered with a single dose 1 mg/kg, i.p. of 3α5βP-Glu dissolved in hydroxypropyl-β-cyclodextrine (β-CD, 72 mM saline solution, Sigma–Aldrich, Germany) adjusted pH7.4 by 1 M NaOH. Each group consisted of four rats. Plasma and tissue (brain, lung, liver, heart and kidney) were isolated.

2.6.1. Plasma

We added 10 ng of the internal standard (3α5βP-Glu-d_3), 0.5 mL of acetonitrile and 0.25 mL of methanol to a 0.5 mL aliquot of rat plasma, followed by ultrasonication for 20 min and centrifugation at 6500 g for 10 min. The supernatant was filtered using a 0.2 μm PTFE filter.

2.6.2. Tissue

The tissue was weighted and the 3α5βP-Glu- d_3 internal standard (10 ng/1 g tissue) was added to the whole tissue and the sample was homogenised. The tissue was then extracted with methanol:acetonitrile (1:1), sonicated for 20 min and centrifuged at 6500 g for 10 min. The supernatant was filtered using a 0.2 μm PTFE filter, evaporated to dryness and reconstituted in 1 mL of methanol and 9 mL of acetonitrile. The sample was then centrifuged at 6500 g for 5 min and the supernatant was filtered. The final solution was evaporated and reconstituted in 1 mL of methanol.

2.6.3. Analysis

An HPLC-MS system equipped with a Luna C18 column (5 μm × 20 mm × 2 mm; Phenomenex, USA), using a methanol/water mixture (70:30) as the eluent at a flow rate of 150 mL/min was used. The column was heated to 25 °C. The injection volume was 20 μL. The HPLC system was directly coupled to a mass spectrometer Agilent 6320 Ion Trap (Agilent, USA) equipped with an electrospray ion source operated in ionization mode ESI+ for 3α5βP-Glu and 3α5βP-Glu-d_3. Selective reaction