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Effects of NAAG peptidase inhibitor 2-PMPA in model chronic pain – relation to brain concentration

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Abstract

N-acetylated-alpha-linked-acidic peptidase (NAAG peptidase) converts *N*-acetyl-aspartyl-glutamate (NAAG, mGluR3 agonist) into *N*-acetyl-aspartate and glutamate. The NAAG peptidase inhibitor 2-PMPA (2-(phosphonomethyl)pentanedioic acid) had neuroprotective activity in an animal model of stroke and anti-allodynic activity in CCI model despite its uncertain ability to penetrate the blood–brain barrier. The NAAG concentration in brain ECF under basal conditions and its alteration in relation to the brain ECF concentration of 2-PMPA is unclear. We therefore assessed those brain concentrations after i.p. administration of 2-PMPA, using *in vivo* microdialysis combined with LC/MS/MS analysis. Administration of 2-PMPA (50 mg/kg) produced a mean peak concentration of 2-PMPA of 29.66 ± 8.1 μM. This concentration is about 100,000 fold more than is needed for inhibition of NAAG peptidase, and indicates very good penetration to the brain. Application of 2-PMPA was followed by a linear increase of NAAG-concentration reaching a maximum of 2.89 ± 0.42 μM at the end of microdialysis. However, during the time the anti-allodynic effects of 2-PMPA were observed, the NAAG concentration in the ECF did not reach levels which are likely to have an impact on any known target. It appears therefore that the observed behavioural effects of 2-PMPA may not be mediated by NAAG nor, in turn, by mGluR3 receptors.

Keywords: Anxiety; NAAG; NAAG peptidase; 2-PMPA; Brain microdialysis; Brain concentration; HPLCMSMS; Pain; CCl; Carboxypeptidase II (GCPII)

1. Introduction

Glutamate receptors and glutamatergic system in general are attractive targets for new drugs, due to their widespread involvement in pathomechanisms or symptomatological expression of many diseases (Danysz et al., 1995; Kew and Kemp, 2005). However, such a widespread distribution and large array of functions means that certain physiological processes might be indiscriminately affected by glutamatergic drugs, resulting in undesirable side-effects. Indeed, only a few drugs in clinical use target the glutamatergic system, such as memantine, tampanel, riluzole, and acamprosate (Parsons et al., 1998). More discrete and selective interference may result in an improved therapeutic profile, and it has been suggested that inhibition of metallopeptidase *N*-acetylated-alpha-linked-acidic dipeptidase (Blakely et al., 1988) (also known as NAAG peptidase, glutamate carboxypeptidase II, GCP II, NAALADase) could fulfil these expectations (Tsai and Coyle, 1995; Neale et al., 2000). The existence of another NAAG peptidase (called glutamate carboxypeptidase III) was shown recently (Bzdega et al., 2004). The differences between GC

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II and GC III are poorly understood yet. Hence, it will not be distinguished between the NAAG peptidases in the presented study.

NAAG peptidase, which is expressed in the outer cell membrane of astrocytes (Berger et al., 1993) cleaves NAAG (*N*-acetyl-aspartylglutamate) (Westbrook et al., 1986; Robinson et al., 1987) into NAA (*N*-acetylaspartate) and glutamate (reviewed in Neale et al., 2000). NAAG seems to be released and taken up by nerve terminals (Cassidy and Neale, 1993). Manipulation of NAAG metabolism has been discussed as a promising therapeutic concept for a number of CNS disorders (Bederson et al., 1986; Neale et al., 2005). These include amyotrophic lateral sclerosis (Tsai et al., 1991), epilepsy (Meyerhoff et al., 1992), schizophrenia, motor neuron diseases (Tsai et al., 1993), ischemia (Sager et al., 1995; Tortella et al., 2000), Alzheimer's disease and Huntington's disease (Passani et al., 1997), see for review (Zhou et al., 2005). Inhibition of NAAG peptidase could result in neuroprotective activity due to decreased levels of pro-toxic glutamate (Slusher et al., 1999). However, even in the early 1990's there were indications that NAAG also interferes with metabotropic glutamate receptors, since it was shown to inhibit forskolin-stimulated cAMP increase (Wroblewska et al., 1993), later characterized as being mGluR3-mediated (Bischofberger and Schild, 1996; Wroblewska et al., 1997; Ghose et al., 1997). Several groups confirmed later that NAAG is neuroprotective through the activation of mGluR3 (Orlando et al., 1997; Bruno et al., 1998). Such actions at mGluR3 have been therefore proposed as mechanisms underlying neuroprotective effects of NAAG peptidase inhibitors *in vivo*, e.g. shown in models of ischemia (Lu et al., 2000; Cai et al., 2002).

Inhibition of NAAG peptidase may provide synergistic neuroprotection through both an increase in levels of mGluR3 agonist NAAG and an subsequent decrease in glutamate levels, as suggested in several publications (Cai et al., 2002; Neale et al., 2005). Hence, NAAG peptidase may be indeed a very attractive therapeutic target, particularly with regard to chronic or acute neurodegeneration. The first inhibitors of NAAG peptidase were *N*-acetylated glutamate analogs, but had only moderate affinity for their target and were not likely to penetrate to the CNS (Serval et al., 1992). However, very potent (of affinity below 1–2 nM) agents have subsequently been discovered, among them 2-PMPA (2-(phosphonomethyl)pentanedioic acid) (Jackson et al., 1996). This agent has been shown to be neuroprotective in an animal model of ischemia, in spite of the fact that the structure is very hydrophilic – not normally a good indication of favourable CNS penetration (Slusher et al., 1999). This concern stimulated the search for more lipophylic agents with better predicted blood-brain barrier penetration, and in turn, several groups of such compounds have been designed (Kozikowski et al., 2001; Jackson et al., 2001; Williams et al., 2001; Jackson and Slusher, 2001).

However, one intriguing question remains: how does 2-PMPA exerts CNS activity e.g. in ischemia (Slusher et al., 1999) if it does not penetrate to the CNS. One explanation would be that the functional effects result from trace levels of this compound reaching the brain, given its high affinity (<1 nM). Another explanation could be that BBB breakdown occurs in ischemia models, resulting in 'leakage' to the CNS (Preston et al., 1993; Nishino et al., 1994). The aim of the present study was therefore to evaluate the effects of 2-PMPA in a model not connected with BBB disruption, i.e. the chronic constriction injury (CCI) model of chronic pain (Bennett et al., 2003). The concentrations of 2-PMPA in the brain and changes in CNS NAAG levels were measured using brain microdialysis. Currently, data regarding basal extracellular NAAG levels are very scarce and diverse (Lin et al., 1995; Sager et al., 1995; Slusher et al., 1999). However, this information is crucial in order to determine whether NAAG reaches levels in the brain capable of affecting known or suggested targets such as mGluR3 or NMDA receptors (Neale et al., 2000).

2. Materials and methods

2.1. Subjects

The experiments are approved by the relevant local authorities and were performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

For the CCI model, adult male experimentally naive Wistar rats (230–270 g, Rappolovo, St. Petersburg, Russia) were used. Animals were kept individually in plastic cages (T3) with water and standard rodent chow (Volosovo, St. Petersburg, Russia) available *ad libitum* in a colony room maintained at 21 ± 1 °C temperature and 50–70% humidity. All experiments were conducted during the light period of a 12 h/12 h day/night cycle (lights on from 08:00 to 20:00 h).

For microdialysis, experimentally naive adult male Sprague–Dawley rats (200–250 g; *Janvier*, France) were housed in groups of up to five per cage (type IV). Colony room temperature and humidity were maintained respectively at 20 ± 1 °C and 60 ± 3%. Food and water was available *ad libitum* and the animals were kept under an alternating 12 h/12 h day-night cycle (lights on at 07:00 h).

2.2. CCI model of chronic pain – procedure

2.2.1. General procedure

At the beginning of the experiment, all animals were screened for baseline tactile reactivity for both paws on two consecutive days. Based on these data, rats with no initial difference in withdrawal thresholds between paws were subjected to surgical manipulations. Three additional baseline tactile tests were held on Day 4, Day 7 and Day 11 post-surgery to monitor the development of tactile allodynia. Based upon previous experience, it was expected that no further decrements in paw withdrawal thresholds (on ligated side) would be observed after Day 11. Rats that were used for cannulation of the right jugular vein had individual tactile paw withdrawal threshold difference of at least 10 g. The rats were used for behavioural testing after a recovery period of at least 3 days after surgery, and drugs were therefore administered starting from Day 14.

During the 'drug test' days, animals were subjected to behavioural testing procedures (see below) which were repeated nine times – once before the drug or its vehicle was administered and 15, 30, 45, 60, 90, 120, 150 and 180 min after the drug/vehicle administration. Each trial was repeated twice at intervals of approximately 2 min, as described previously (Chen et al., 2002).

The procedure followed for sciatic nerve injury was originally described by Bennett and Xie (1988).

2.2.2. Behavioural testing

Tactile sensitivity. Rats were placed in a plastic cage with a metal grid bottom, which allows full access to the paws. A short habituation period (10 min) preceded the test period. The paw withdrawal thresholds were determined using a procedure described in Chaplan et al. (1994). Paws were touched with