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Agmatine reduces extracellular glutamate during pentylenetetrazole-induced seizures in rat brain: A potential mechanism for the anticonvulsive effects

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Abstract

Glutamate has been implicated in the initiation and spread of seizure activity. Agmatine, an endogenous neuromodulator, is an antagonist of NMDA receptors and has anticonvulsive effects. Whether agmatine regulate glutamate release, as measured by in vivo microdialysis, is not known. In this study, we used pentylenetetrazole (PTZ)-induced seizure model to determine the effect of agmatine on extracellular glutamate in rat brain. We also determined the time course and the amount of agmatine that reached brain after peripheral injection. After i.p. injection of agmatine (50 mg/kg), increase of agmatine in rat cortex and hippocampus was observed in 15 min with levels returning to baseline in one hour. Rats, naïve and implanted with microdialysis cannula into the cortex, were administered PTZ (60 mg/kg, i.p.) with prior injection of agmatine (100 mg/kg, i.p.) or saline. Seizure grades were recorded and microdialysis samples were collected every 15 min for 75 min. Agmatine pre-treatment significantly reduced the seizure grade and increased the onset time. The levels of extracellular glutamate in frontal cortex rose two- to three-fold after PTZ injection and agmatine significantly inhibited this increase. In conclusion, the present data suggest that the anticonvulsant activity of agmatine, in part, could be related to the inhibition glutamate release.

Keywords

Agmatine; Pentylenetetrazole; Glutamate; Microdialysis; Seizures

Agmatine is an amine, synthesized by decarboxylation of L-arginine by arginine decarboxylase (ADC) [16,23]. Agmatine is widely distributed in mammalian tissue [5,22] including brain where it is unevenly distributed in various regions and enriched in subcortical regions [21]. Although the physiological functions of agmatine in the brain is still not clear, accumulating evidence indicates several pharmacological actions of agmatine. The facts that agmatine binds to NMDA receptors [25], selectively blocks NMDA receptor channels in neurons [19,30] and localized in excitatory synapses in hippocampus [24] suggest that agmatine could regulate glutamatergic neurotransmission. Moreover, agmatine inhibits the release of vasopressin in neurohypophysis [29] and norepinephrine from perivascular nerve terminals [9], suggesting a pre-synaptic action regulating the release of neuro-transmitters. Functional effects such as protection against ischemic neuronal injury [4,6,8,12,19,32,33], reduction of morphine tolerance and withdrawal symptoms [2,13,15]

and anti-seizure activity [1,3,27] all imply the inhibition of glutamatergic transmission as a possible mechanism of action. While the inhibition of NMDA receptors as one probable mechanism, the effects on pre-synaptic glutamate release may also contribute to this action. In this study, we addressed the question whether agmatine regulates the release of glutamate in rat pentylenetetrazole (PTZ)-induced seizure model using in vivo microdialysis to measure the extracellular glutamate. We also investigated the amount and time period of the accumulation of agmatine into rat brain in order to evaluate the biological half-life of injected agmatine.

Agmatine sulfate, pentylenetetrazole, glutamate and all other chemicals were purchased from Sigma (St. Louis, MO, USA). HPLC grade methanol was purchased from Fisher Scientific Company (Fair Lawn, NJ, USA). This protocol was approved by our institutional committee on animal use. Rats were cared for in accordance with National Institutes of Health guidelines. Male Sprague–Dawley rats (250–300 g, Harlan, Indianapolis, IN, USA) were purchased and maintained under conditions of 21 ± 2 °C with a 12 h/12 h light/dark cycle for 1 week before surgery.

In initial experiments, we administered agmatine to rats (50 mg/kg, i.p.) and measured the levels of agmatine in cortex and hippocampus at different time points. Rat brain regions were dissected out and processed for HPLC measurement of agmatine as described earlier [5]. A typical HPLC chromatogram of agmatine is shown in Fig. 1 for cortex samples prepared from 15 min, 1 and 2 h after agmatine injection. Agmatine values from cortex and hippocampus at all time points after agmatine injection are shown in Table 1. The levels of agmatine in cortex and hippocampus increased to two- to four-fold at 15 min, started to decrease at 30 min and reached the baseline levels at 1 h. When we injected 100 mg/kg agmatine, the levels of agmatine in frontal cortex and hippocampus doubled compared to 50 mg/kg dose although the time course was very similar in that the levels reached maximum at 15 min and returned to control levels in 60 min. Therefore, we injected agmatine 15 min prior to PTZ injection in all further experiments to determine anti-convulsive effects and glutamate release.

To verify the doses of PTZ and the behavioral effects of agmatine, we initially observed the seizure activity after injecting PTZ with and without prior injection of agmatine. Following each PTZ injection, each rat was placed in acrylic observation chamber, one at a time, and behavioral seizures observed for 30 min as described earlier [1]. The seizure was rated according to the following scale: (0) no change in behavior, (I) myoclonic jerks, (II) minimum seizures with convulsions through out the body (III) fully developed minimal seizures, clonus of the head muscles and forelimbs, (IV) major seizures (generalized without the tonic phase), (V) generalized tonic-clonic seizures began with running, followed by the loss of righting ability, then short tonic phase progressed to clonus of all four limbs. The time between the injection of PTZ and the first myoclonic jerk was defined as the onset time. After testing doses ranging from 50 to 80 mg/kg of PTZ, we settled for 60 mg/kg as this dose caused reproducible grade V seizure in rats with animals returning to normal state in 1 h.

The effect of pre-injection of agmatine (100 mg/kg, i.p.) on PTZ-induced seizure grades and onset time is shown in Table 2. The dose of agmatine was chosen as the most promising in previous experiment [1]. In saline injected rats, 8 out of 11 rats showed grade V seizures whereas only one rat showed grade V seizure in agmatine pre-treated rats. In contrast, most rats in agmatine-treated group showed only grades II and III seizure (9 rats out of 11). Moreover, the onset time was significantly higher in agmatine-treated compared to saline group. Based on these behavior studies, we designed experiments to determine the effect of

agmatine on extracellular glutamate levels in PTZ-treated rat cortex using in vivo microdialysis.

The surgical procedures and microdialysis sampling were performed using modifications of techniques detailed in our earlier studies [7]. The rats were anesthetized with halothane inhalation and a CMA/11 microdialysis guide cannula (CMA Microdialysis, N. Chelmsford, MA) was implanted with the tip directed toward the frontal cortex. The coordinates for these surgeries were +3.2 mm anterior to bregma, -3.5 mm from the midline and -1.5 mm from the surface of the skull. Four stainless steel screws were secured to the skull, an aluminum protective cap was placed around guide cannula and dental acrylic will be applied to anchor the assembly to the skull surface. Rats were allowed 3–4 days of post-operative recovery time. The microdialysis probes (CMA/11, outside diameter 0.24 mm, membrane length 2 mm, molecular weight cut off 6000 Da) were purchased from CMA Microdialysis (N. Chelmsford, MA) and were equilibrated according to manufacturer's specifications and used within 3 months. Initially, microdialysis probes were perfused with artificial cerebrospinal fluid (aCSF; 140 mM NaCl, 2.7 mM KCl, 1.2 mM CaCl₂, 1 mM MgCl₂, 0.3 mM NaH₂PO₄, 2.7 mM Na₂HPO₄, pH 7.4). For collecting microdialysis samples, each rat was placed in an individual CMA/120 System (CMA Microdialysis, MA) and microdialysis probe was placed into the frontal cortex guide cannula. The probe was perfused with aCSF at a flow rate of 1 µl/min for an equilibration period of 3–4 h. Thereafter, a series of four sequential 15 min samples for each rat was taken prior to drug injection to demonstrate a steady baseline. These four samples were averaged and all subsequent values were expressed as a percentage of this preinjection value (% of baseline). The in vitro recovery of glutamate was determined by immersion of probes in saline containing 10 µM of glutamate which ranged from 20 to 25% for glutamate.

The amount of glutamate in the dialytic samples were measured by reverse phase HPLC using a Supelco Hypersil ODS column (150 mm × 4.6 mm I.D., 5 µm, 100 Å) and fluorescence detection (excitation wavelength of 350 nm and emission wavelength of 450 nm; Waters 402, Marlborough, MA). The mobile phase consisted of 0.1 M sodium phosphate (mono and dibasic)/methanol buffer (63:37, v/v; pH 5.2). The derivatizing agent was consisted of *o*-phthalaldehyde (50 mg), 2-mercaptoethanol (40 µl), absolute ethanol (0.9 ml) and 0.1 M borate buffer to make a final volume of 10 ml. Fifteen microliters of a standard solution or microdialysis sample was mixed with 15 µl of the derivatizing agent solution for 1 min and immediately injected into the HPLC system.

After the recovery period of 3 or 4 days after surgery, each rat was randomly assigned and marked to a control group ($n = 9$, 4 animals for saline + saline and 5 animals for saline + PTZ) or for treatment with agmatine ($n = 6$, agmatine + PTZ). Following baseline sample collection, rats were injected with saline or agmatine (100 mg/kg, i.p.). After 15 min sample collection, the rats then were injected with PTZ (60 mg/kg, i.p.) or saline. Throughout the experiment, dialysis samples were collected every 15 min into tubes for 120 min and were analyzed by HPLC. The baseline extracellular glutamate level was 2.85 ± 0.76 µM calculated from in vitro recovery experiments. As shown in Fig. 2, PTZ injection induced a large increase in extracellular glutamate in 0–15 min sample and levels returned to baseline in 15–30 min sample. When agmatine was injected 15 min before PTZ injection, the increase in extracellular glutamate levels was largely reduced. The levels of glutamate during PTZ-induced seizure were significantly lower at 0–15 min samples in agmatine-injected rats compared to saline-injected rats. Agmatine injection in normal rats slightly, but not significantly, reduced baseline extracellular glutamate levels (data not shown).

We report in this study, for the first time, the inhibition of in vivo glutamate release by agmatine as measured by extracellular glutamate. Agmatine has been shown to exert several

actions that are related to its ability to inhibit NMDA receptors [4,19,30]. The protective effects of agmatine on excitotoxic injury in vitro and ischemic injury in vivo are well documented [4,6,11,12,32,33]. While the centrally administered agmatine potentiates morphine analgesia, peripheral agmatine reduces morphine tolerance and withdrawal symptoms [2,13,14,31]. Although the exact mechanism for these actions is not clear, the effect on morphine tolerance is similar to NMDA receptor antagonists [28] and may be mediated by the inhibition of NMDA receptors. More recently, several reports have indicated that agmatine reduces convulsive seizures induced by chemical or electrical stimulation [1,3,27]. Since all these conditions involve higher glutamate release and excessive NMDA receptor activation, agmatine could be acting by reducing glutamatergic transmission. While the blockade of NMDA receptors by agmatine have been reported in neuronal cells [30], effects on in vivo glutamate release have not been investigated.

Findings from the central effects of agmatine on functional/behavioral parameters in vivo have not been consistent and vary with the route of administration and doses used (see review [18]). The major unanswered issues are the penetration of agmatine into brain and its biological half-life after peripheral injection. We have shown in this study that agmatine levels increased in brain within 15 min after peripheral injection and last only for about 30 min. A similar pattern was also observed with plasma agmatine levels after i.p. injection of agmatine (data not shown). From the total brain agmatine levels at all time points, we calculated that only about 1% of injected agmatine reached the brain. The reason for such low levels of agmatine reaching brain could be due to the rapid metabolism of agmatine in peripheral tissues like kidney and liver where agmatine could be oxidized by diamine oxidase [10] or hydrolyzed by agmatinase [26]. While our previous report in newborn rat pups indicated a large increase in total brain agmatine levels after peripheral injection [6], we observed only smaller increase in adult rat brain suggesting that the maturation of the blood-brain barrier could restrict the penetration of agmatine into brain. These findings provide the basis for the use of relatively higher doses of agmatine and repeated administration in adult rats for in vivo functional studies. In previous studies, agmatine, at doses ranging from 5 to 160 mg/kg given peripherally or centrally, showed significant anti-seizure activity [1,3,27]. In this study, since our objective is to determine the effect on glutamate release, we used a relatively higher dose of 100 mg/kg, although lower doses may also be effective. As agmatine can be administered even up to 300 mg/kg without any cardiovascular or locomotor effects in normal rats [20], this dose was considered reasonable.

Agmatine clearly reduced the convulsive effect of PTZ both in terms of severity and onset time. Although we cannot predict the exact mechanism of action, the decrease in glutamate release could be contributing factor. Increased extracellular glutamate during PTZ-induced seizures has been widely reported in brain regions (review [17]). Agmatine almost completely inhibited the increase in extracellular glutamate during PTZ-induced seizure. While the inhibition of NMDA receptors was proposed as the mechanism of action of agmatine in reducing convulsive seizures, our data suggest that the inhibition of glutamate release may also contribute to this action. Agmatine has been shown to inhibit the release of norepinephrine from vascular nerve terminals [29] and vasopressin from neurohypophysis, probably by inhibiting pre-synaptic voltage-dependent calcium channels [9]. It is conceivable that similar action by agmatine in glutamatergic terminals could inhibit the release of glutamate during convulsive seizure. Such an action of agmatine could also provide molecular basis for the protective effects of agmatine during morphine withdrawal or ischemic injury, conditions associated with higher glutamate release.

In conclusion, our findings clearly indicate that exogenous agmatine reduces PTZ-induced seizures and inhibits increased glutamate release. Whether endogenous agmatine inhibits glutamate release and blocks NMDA receptor activation are not known. Since agmatine

immunoreactivity has been detected in vesicles of asymmetric synapses (excitatory) in hippocampus presumably co-localized with glutamate [24], it is conceivable that endogenous agmatine may regulate glutamatergic neurotransmission in brain. Thus, increasing brain agmatine either by exogenous administration or by augmenting endogenous levels could be a novel way to reduce epileptic seizures.

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Fig. 1.

An example HPLC chromatogram of cortex samples prepared from rats injected with agmatine (50 mg/kg, i.p.). The agmatine peak (22 min) is shown in relation to an internal standard peak (25 min) in samples prepared at different time points after agmatine injection.

Fig. 2.

Effect of agmatine on cortical extracellular glutamate during PTZ-induced seizure. The amount of glutamate in dialysis samples was measured by HPLC and expressed as percent of basal level ($2.85 \pm 0.76 \mu\text{M}$). Values are mean $\pm$ S.E. from five animals for saline + PTZ and six animals for agmatine + PTZ groups and three animals for saline group. * $p < 0.01$ compared to saline + PTZ group; # $p < 0.01$ compared to saline group.

Table 1

Agmatine levels in brain after peripheral injection of agmatine in rats

	Time after injection (min)					
	0	15	30	60	120	240
Frontal cortex	336 ± 52	688 ± 125*	482 ± 112*	296 ± 98	342 ± 105	318 ± 118
Hippocampus	105 ± 38	481 ± 86*	232 ± 87*	135 ± 56	148 ± 67	143 ± 98

Agmatine (50 mg/kg) was injected by i.p. and brain regions dissected out at specified time. Samples, prepared from these regions, were injected in to the HPLC system, the amount agmatine was determined and expressed as ng/g wet weight. Values are mean ± S.E. from four rats in each group.

* $p < 0.01$ compared to 0 time point.

Table 2

Effect of agmatine on PTZ-induced seizure grade and onset time

	Seizure grade					Onset time (s)
	I	II	III	IV	V	
Saline + PTZ	0	0	1	2	8	70 ± 5
Agmatine + PTZ	1	5*	4	0	1**	120 ± 10**

Agmatine (100 mg/kg, i.p.) was injected 15 min prior to PTZ (60mg/kg, i.p.) injection and convulsive seizures were observed and graded for 30 min. The time lapsed between PTZ injection and the first myoclonic jerk was measured as the onset time.

* $p < 0.05$.

** $p < 0.01$ compared saline group.