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Neurotoxic effect of high dose of *L*-(+)-2-amino-3-phosphonopropionic acid in rats after intracaudatal injection[1]

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KEY WORDS *L*-(+)-2-amino-3-phosphonopropionic acid; metabotropic glutamate receptors; brain edema; *N*-methyl-*D*-aspartate receptors

ABSTRACT

AIM: To investigate neurotoxic effect of *L*-(+)-2-amino-3-phosphonopropionic acid (*L*-AP_3), a partial agonist/antagonist of metabotropic glutamate receptors (mGluRs), and explore the underlying mechanisms. **METHODS**: Consciousness and behavior of rats were evaluated after injection of *L*-AP_3, *D*-(+)-2-amino-3-phosphonopropionic acid (*D*-AP_3, an isomer of *L*-AP_3) or *L*-(+)-2-amino-4-phosphonobutyric acid (*L*-AP_4, an agonist of mGluRs) into right caudatum. Brain water, Na^+, K^+, and Ca^{2+} contents as well as the permeability of blood brain barrier (BBB) were determined 6 h after treatment of these chemicals. Histological changes at the same time point were also observed. **RESULTS**: Rats treated with *L*-AP_3 600 nmol but not 60 nmol became somnolentia. Injection of *L*-AP_3 600 nmol induced a great increase of brain water, Na^+, and Ca^{2+} contents, and a decrease of brain K^+ content ($P < 0.01$). Meanwhile, the permeability of BBB was also increased ($P < 0.01$). Electron microscopic study revealed remarkable swelling of astrocytes and degenerative changes of neurons in chemical-treated caudatum. The neurotoxic effect of *L*-AP_3 was not mimicked by *D*-AP_3 or *L*-AP_4 ($P < 0.05$). *DL*-2-Amino-5-phosphonovaleric acid, an antagonist of *N*-methyl-*D*-aspartate (NMDA) receptors, attenuated the changes induced by *L*-AP_3 ($P < 0.05$), whereas (±)-α-methyl-(4-carboxyphenyl) glycine, a non-subtype specific antagonist of mGluRs, failed to block the effect of *L*-AP_3. **CONCLUSION**: Intracaudatal injection of *L*-AP_3 induced neurotoxic effect characterized by vasogenic brain edema, neuronal degeneration, and high brain Ca^{2+} content. Neurotoxic effect of *L*-AP_3 was stereoselective and might be mediated by phospholipase C activation and partially involvement of NMDA receptors.

INTRODUCTION

Metabotropic glutamate receptors (mGluRs) are G-protein coupling receptor family, and eight subtypes have been cloned. According to their amino acid sequence similarity, signal transduction mechanisms, and agonist selectivity, mGluRs can be divided into three groups. The first group (group Ⅰ) includes $mGluR_1$ and $mGluR_5$, which are positively coupled to phospholipase C (PLC) and lead to an increase in diacylglycerol and inositol triphosphate (IP_3), and an activation of adenyl cyclases (AC) in some cases. The second and third groups (group Ⅱ and Ⅲ) include all others and lead to a decrease in forskolin-stimulated AC. During recent years, the mGluRs have been extensively studied in experimental animals and accumulative evidence indicates that mGluRs play important roles in regulating the activity of many synapses in the central nervous system and are involved in a wide range of physiological and pathological processes[1,2]. *L*-(+)-2-Amino-phosphonopropionic acid (*L*-AP_3), a structure analog of glutamate and the first compound shown to block phosphoinositide (PI) hydrolysis triggered by mGluRs, was used as the only antagonist of group Ⅰ mGluRs before 1993[3], and is still being used. However, some studies showed that *L*-AP_3 as a partial agonist could also stimulate PI hydrolysis at high doses[4]. Moreover, some reports indicated that *L*-AP_3 induced neurotoxic effect in the brain of immature rodents[5] or in cerebellar cell culture[6]. In the present work, we intended to examine the effects of *L*-AP_3 by biochemical and histological study after it was injected into caudata of rats. Some specific

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chemicals were used to analyse the underlying mechanisms.

MATERIALS AND METHODS

Chemicals All chemicals including *L*-AP_3, *D*-(+)-2-amino-3-phosphonopropionic acid (*D*-AP_3), *L*-(+)-2-amino-4-phosphonobutyric acid (*L*-AP_4), *DL*-2-amino-5-phosphonovaleric acid (*DL*-AP_5), and (±)-α-methyl-(4-carboxyphenyl)glycine (MCPG) were purchased from Sigma (USA).

Animal preparation Male Wistar rats (weighing 250 – 300 g) were provided by Beijing Institute of Experimental Animal Center affiliated to Chinese Academy of Medical Sciences (Certificate № 01-3008, Grade Ⅱ). After anesthetized with 10 % chloral hydrate (40 mg/kg, ip), each animal received an injection into the right caudata only once as previously described[7]. The total volume of each injection was 3 μL, which contained either normal saline (NS) or one of the following chemicals dissolved in NS at the giving dose expressed as the amount of nanogram moles in 3 μL: *L*-AP_3 60 or 600 nmol, *D*-AP_3 600 nmol, *L*-AP_4 600 nmol, *DL*-AP_5 500 nmol, MCPG 500 nmol. In two additional groups, MCPG 500 nmol plus *L*-AP_3 600 nmol, or *DL*-AP_5 500 nmol plus *L*-AP_3 600 nmol was injected with the total volume being still 3 μL. Each group consisted of 6 to 8 rats.

Measurement of brain water, Na^+, K^+, and Ca^{2+} contents Consciousness and behavior of rats were evaluated after they had recovered from anesthesia. Six hours after intracaudatal injection, rats were decapitated and bilateral caudata were taken separately to determine brain water content by the wet weight/dry weight method. Then Na^+, K^+, and Ca^{2+} contents of the samples were measured by Inductive Plasma-9000 (Jarrell-Ash, USA).

Determination of blood brain barrier (BBB) permeability In order to examine the change of BBB permeability induced by *L*-AP_3, some rats were administered with Evan's blue (EB, 50 mg/kg, iv) immediately after intracaudatal injection. The brain EB content was determined by formamide method[7].

Histology Six hours after injection of chemicals, some rats were killed by heart perfusion with 4 % paraformaldehyde. Samples from bilateral caudata were prepared for HE stain or uranyl acetate and lead citrate stain. Histological studies were performed under a Leitz light microscope and a Philips EM208S electron microscope.

Data Analysis All data were presented as $\bar{x} \pm s$. Student's *t*-test was performed with a SYSTAT software package.

RESULTS

Effects of chemicals on consciousness and behavior of rats Rats treated with *L*-AP_3 600 nmol but not 60 nmol presented somnolent and inactive after recovery from anesthesia, but this phenomenon did not occur in rats receiving NS, *D*-AP_3, or *L*-AP_4.

Effect of *L*-AP_3 on brain water, Na^+, K^+, and Ca^{2+} contents As shown in Tab 1, 6 h after treatments, *L*-AP_3 at 60 nmol did not change brain water, Na^+, K^+, and Ca^{2+} contents in injected caudatum compared ipsilaterally with NS group or contralaterally in the same group. However, *L*-AP_3 at 600 nmol did cause great increase of brain water, Na^+, and Ca^{2+} contents ($P < 0.01$) and a decrease of brain K^+ content ($P < 0.01$).

Effect of *L*-AP_3 on permeability of BBB In

Tab 1. Brain water, Na^+, K^+, and Ca^{2+} contents in right (R) or left (L) caudatum 6 h after NS or *L*-AP_3 injection into the right caudatum of rats. $n = 6$. $\bar{x} \pm s$. [a]$P > 0.05$, [c]$P < 0.01$ *vs* NS group.

Injected substance	Caudatum	Water content/%	Cation content/μmol·g^{-1} dry weight		
			Na^+	K^+	Ca^{2+}
NS	R	78.0 ± 0.7	195 ± 7	439 ± 21	7.7 ± 2.2
	L	77.7 ± 0.6	186 ± 18	442 ± 13	6.9 ± 1.8
L-AP_3	R	78.2 ± 0.7[a]	209 ± 31[a]	427 ± 10[a]	8.1 ± 2.8[a]
(60 nmol)	L	77.3 ± 0.8[a]	181 ± 7[a]	425 ± 17[a]	6.4 ± 1.5[a]
L-AP_3	R	82.2 ± 2.3[c]	443 ± 146[c]	297 ± 64[c]	19 ± 7[c]
(600 nmol)	L	77.6 ± 1.0[a]	203 ± 34[a]	402 ± 61[a]	7.1 ± 1.4[a]

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rats receiving 600 nmol L-AP_3, there were blue stain in injected caudatum 6 h and 24 h but not 1 h after injection. Six hours after treatment, EB content in injected caudatum was (12.6 ± 4.5) μg/g wet weight, compared with (5.2 ± 1.7) μg/g wet weight in NS group ($P < 0.01$) and (3.9 ± 3.0) μg/g wet weight ($P < 0.01$) in contralateral caudatum.

Morphological changes after L-AP_3 treatment In rats treated with L-AP_3 600 nmol, there was conspicuous swelling in injected caudatum 6 h after injection. Remarkable astrocyte swelling with loss of organellae and cytoplasmic protein was observed with electron microscope (Fig 1A). Capillaries were surround by edematous astrocytic endfeet which were filled with swelling fluid (Fig 1B). Some neurons underwent degenerative changes and nuclear membranes became folded. There were also some shrunk neurons present (Fig 1C). Large amount of astrocytic processes around neurons, axons, and dendrites were swelling. Myelin sheathes also swelled.

Effects of other agents on brain water, Na^+, K^+, and Ca^{2+} contents As shown in Tab 2, although increases of brain water, Na^+, and Ca^{2+} contents were induced ($P < 0.01$) with no change of brain K^+ content, D-AP_3 caused changes less than those induced by L-AP_3 ($P < 0.05$). Unlike L-AP_3, L-AP_4, an agonist of group III mGluRs, only induced increases of brain water and Na^+ contents ($P < 0.01$) with no change of brain K^+ and Ca^{2+} contents, but these changes were much less than those induced by L-AP_3 ($P < 0.05$).

Having no effects on brain water, Na^+, K^+, and Ca^{2+} contents, MCPG, a non-subtype specific antagonist of mGluRs, did not block the changes induced by L-AP_3 but balanced the K^+ decrease ($P < 0.05$) when co-injected with L-AP_3. DL-AP_5, an antagonist of N-methyl-D-aspartate (NMDA) receptors, which slightly increased brain Na^+ and Ca^{2+} contents by itself ($P < 0.01$), could attenuated the changes of brain water and K^+ content induced by L-AP_3 ($P < 0.05$) when co-injected with L-AP_3 (Tab 2).

DISCUSSION

The present study has clearly shown that high dose of L-AP_3 (600 nmol) injected intracaudatally caused increases of brain water, Na^+, and Ca^{2+} contents, and decrease of brain K^+ content accompanied by breakdown of BBB, suggesting that L-AP_3 induced vasogenic brain edema. Given to the neuronal degeneration revealed by electron microscope and somnolent status of rats after L-AP_3 treatment, all these data indicated that high dose of L-AP_3 produced neurotoxic actions in adult rats.

In our previous work, L-AP_3 could attenuate astrocytic swelling induced by *trans*-1-aminocyclopentance-1, 3-diacarboxylic acid (ACPD) in culture[8], but in the present work L-AP_3 *per se* induced astrocytic swelling and neuronal degeneration *in vivo*. Reasons for the discrepancy may lie in the difference of the animal age[9], the concentration of L-AP_3[10], and the cell types involved[11].

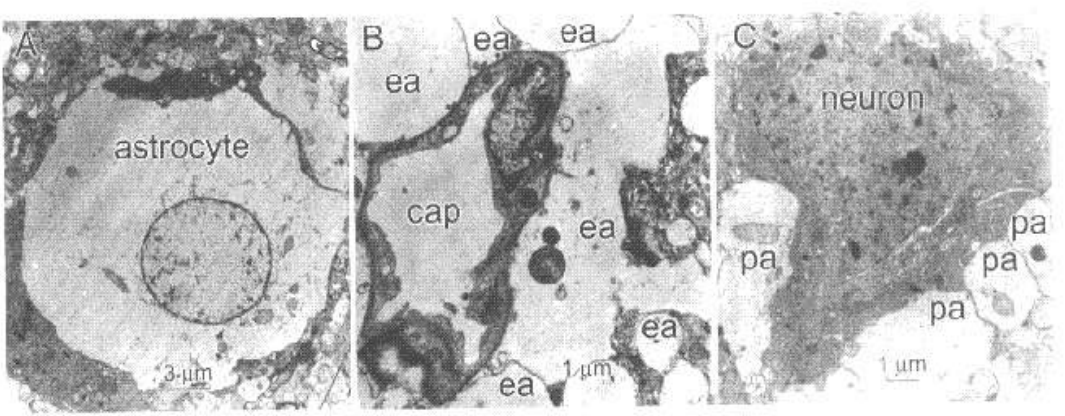

Fig 1. Electron microscopy showing remarkable swelling of astrocytes and degenerative changes of neurons in rat caudatum injected with L-AP_3 for 6 h. A) a swelling astrocyte with loss of organellae and cytoplamic protein, ×3150; B) a capillary (cap) surrounded by swelling endfeet of astrocytes (ea), ×6300; C) a shrunk neuron surrounded by swelling processes of astrocytes (pa), ×6300.

Tab 2. Effects of *L*-AP_3, *D*-AP_3, *L*-AP_4, *DL*-AP_5, and MCPG on brain water, Na^+, K^+, and Ca^{2+} contents in right caudatum 6 h after intracaudatal injection in rats. $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* NS group. $^dP > 0.05$, $^eP < 0.05$ *vs* *L*-AP_3 group.

Group	*n*	Water content/%	Cation content/$\mu mol \cdot g^{-1}$ dry weight		
			Na^+	K^+	Ca^{2+}
NS	6	78.0 ± 0.7	195 ± 7	439 ± 21	7.7 ± 2.2
L-AP_3	6	82.2 ± 2.3^c	443 ± 146^c	297 ± 64^c	19 ± 7^c
D-AP_3	6	79.6 ± 0.7^{ce}	297 ± 22^c	443 ± 12^e	17.6 ± 2.0^c
L-AP_4	8	79.3 ± 0.4^{ce}	248 ± 24^{ce}	442 ± 28^e	8.7 ± 2.2^e
MCPG	8	78.3 ± 0.6^a	206 ± 22^a	447 ± 17^a	10 ± 6^a
MCPG + *L*-AP_3	6	81.3 ± 1.5^d	361 ± 77^d	390 ± 48^e	14 ± 5^d
DL-AP_5	8	78.6 ± 0.7^a	278 ± 49^e	432 ± 18^a	12.4 ± 2.7^e
DL-AP_5 + *L*-AP_3	6	79.8 ± 1.0^e	306 ± 76^d	403 ± 31^e	15 ± 6^e

Although this study has demonstrated *L*-AP_3 can elicit a neurotoxic effect, it is unclear whether this is mediated via an agonist action at mGluRs. *D*-AP_3 is an isomer of *L*-AP_3 but has no stimulative effect on PLC[9]. In our present study, *D*-AP_3 induced less changes of brain water contents than *L*-AP_3 and different electrolyte changes from *L*-AP_3, indicating the neurotoxic effect of *L*-AP_3 was stereoslective and might be mediated by PLC activation. Furthermore, since *D*-AP_3 is a selective inhibitor of phosphoserine phosphatase, more pontent than *L*-AP_3[11], the possibility that neurotoxicity of *L*-AP_3 may be due to its inhibition of phosphoserine phosphatase appears not to be sound. In addition, the fact that an agonist of group Ⅲ mGluRs *L*-AP_4[12] induced less brain edema than *L*-AP_3 after injected intracaudatally may exclude the involvement of group Ⅲ mGluRs in the neurotoxic effects of *L*-AP_3. The data that a non-subtype specific antagonist of mGluRs MCPG did not block brain edema induced by *L*-AP_3 made it difficult to distinct which subtypes of mGluRs were involved in the neurotoxic effect of *L*-AP_3. The possible explain may be that the receptor subtype mediating effects of *L*-AP_3 is different from MCPG-sensitive receptors[13].

According to our data, neurotoxicity of *L*-AP_3 was characterized by the presence of remarkable increase of brain Ca^{2+} content and neuron degeneration, which was similar to the effects of NMDA receptor activation. In addition, *DL*-AP_5 attenuated brain edema induced by *L*-AP_3 in the present study. Thus these results suggest that NMDA receptors might be involved in the neurotoxic effect of *L*-AP_3. An involvement of NMDA receptors in *L*-AP_3 neurotoxicity is also reported in cultured cerebellar neurons[6].

In conclusion, our results demonstrated that *L*-AP_3 injected into rat caudatum at a high dose induced neurotoxic effects characterized by vasogenic brain edema, neuronal degeneration, and high Ca^{2+} content. These effects of *L*-AP_3 were stereo-selective and might be mediated by PLC activation and partially involvement of NMDA receptors.

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大鼠尾状核内注射高剂量 *L*-(+)-2-氨基-3-磷酸基丙酸的神经毒性[1]

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关键词 *L*-(+)-2-氨基-3-磷酸基丙酸; 代谢型谷氨酸受体; 脑水肿; *N*-甲基-*D*-天门冬氨酸受体

目的: 观察代谢型谷氨酸受体部分激动剂/拮抗剂 *L*-(+)-2-氨基-3-磷酸基丙酸(*L*-AP_3)脑内注射引起的神经毒性作用, 并探讨其机制. **方法**: 大鼠尾状核内微量注射药物后, 观察动物意识状态和活动情况, 测定脑组织含水量、Na^+、K^+、Ca^{2+}含量及血脑屏障(BBB)通透性变化, 并进行组织学研究. **结果**: *L*-AP_3 600 nmol 脑内注射后动物出现嗜睡, 并引起脑组织含水量、Na^+和Ca^{2+}含量增加, K^+含量减少, 同时 BBB 通透性增加 $P < 0.01$, *L*-AP_3 60 nmol 未产生上述变化. 电镜检查发现 *L*-AP_3 引起星形胶质细胞高度肿胀, 神经元变性坏死. *D*-(+)-2-氨基-3-磷酸基丙酸和 *L*-(+)2-氨基-4-磷酸丁酸不能模拟 *L*-AP_3 引起的变化, *DL*-2-氨基-5-磷酸基戊酸可以减轻 *L*-AP_3 的作用, (±)-α-甲基-(4-羧基苯基)甘氨酸不能减轻 *L*-AP_3 的作用. **结论**: 脑内注射高剂量的 *L*-AP_3 引起神经毒性作用, 以血管源性脑水肿、神经元损伤及脑组织高Ca^{2+}含量为特征, 此作用是立体构型特异的, 可能与磷脂酶 C 激活有关, 部分通过 NMDA 受体介导.

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