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CENTRAL ADMINISTRATION OF A CASPASE INHIBITOR IMPAIRS SHUTTLE-BOX PERFORMANCE IN RATS

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Abstract—Recent studies suggest that caspase-3-mediated mechanisms are essential for neuronal plasticity. N-benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val- Asp(OMe)-fluoromethyl ketone (z-DEVD-fmk), a caspase inhibitor with predominant specificity toward caspase-3, has been shown to block long-term potentiation in hippocampal slices. Intrahippocampal infusion of a caspase-3 inhibitor to rats has been shown to significantly impair spatial memory in the water maze. The present work was designed to study whether i.c.v. administration of a caspase-3 inhibitor z-DEVD-fmk impairs learning in other tasks related to specific forms of memory in rats. The rats received bilateral injections of z-DEVD-fmk or N-benzyloxycarbonyl-Phe-Ala-fluoromethyl ketone (z-FA-fmk) ("control" peptide) at a dose of 3 nmol. Administration of z-DEVD-fmk significantly decreased the number of avoidance reactions in some blocks of trials in the active avoidance (shuttle box) learning, while z-FA-fmk had no effect as compared with intact rats. However, only a slight effect of the caspase inhibitor across the session was found. z-DEVD-fmk impaired development of some essential components of the two-way active avoidance performance, such as escape reaction, conditioned fear reaction, and inter-trial crossings. Measurement of caspase-3 activity in rat brain regions involved in active avoidance learning revealed most expressed z-DEVD-fmk-related inhibition of the enzyme activity (about 30%) in the fronto-parietal cortex. A similar effect was close to significant in the hippocampus, but not in the other cerebral structures studied. In primary cultures of cerebellar neurons z-DEVD-fmk (2–50 μM) inhibited caspase-3 activity by 60–87%. We suggest that moderate inhibition of caspase-3 resulting from the central administration of z-DEVD-fmk to rats may impair active avoidance learning. Taking into account previous data on the involvement of neuronal caspase-3 in neuroplasticity phenomena we assume that the enzyme may be important for selected forms of learning. © 2005 Published by Elsevier Ltd on behalf of IBRO.

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Abbreviations: CS, conditioned stimulus; DMSO, dimethyl sulfoxide; ITC, intertrial crossing; tPA, tissue-type plasminogen activator; US, unconditioned stimulus; z-DEVD-fmk, N-benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val- Asp(OMe)-fluoromethyl ketone; z-FA-fmk, N-benzyloxycarbonyl-Phe-Ala-fluoromethyl ketone.

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Caspases, cysteine-containing proteases that cleave at an aspartate residue, were first discovered a decade ago. These enzymes play distinct roles in inflammation and apoptosis (Wolf and Green, 1999) and are believed to be essential mediators of cell death (for review see Earnshaw et al., 1999; Yuan and Yankner, 2000; Troy and Salvesen, 2002). Caspases are involved in apoptosis either as upstream initiators of the proteolytic cascade (caspases-8 and -9), or as downstream effectors that cleave cellular proteins (caspases-3, -6, and -7). Among the effector caspases, caspase-3 is of particular interest in relation to programmed cell death in neurons (Troy and Salvesen, 2002). It is becoming clear, however, that certain proteases are not merely degradative enzymes but are highly regulated signaling molecules that control critical biological processes via specific limited proteolysis. The opinion that caspases are more than just killers is supported by emerging evidence from recent experiments in non-neuronal cells implicating the caspases in various, non-apoptotic aspects of cellular physiology, such as cell cycle progression, cell differentiation and proliferation, thus attesting to the pleiotropic functions of these proteases (Fadeel et al., 2000; Los et al., 2001; Fernando et al., 2002; Robertson and Zhivotovsky, 2002; Moshnikova et al., 2003; Perfettini and Kroemer, 2003; Woo et al., 2003).

There is growing evidence that in nervous tissue, so-called "apoptotic mechanisms" are not just merely apoptotic, being also involved in regulation of synaptic plasticity and growth cone motility besides programmed cell death (Gilman and Mattson, 2002). Shimohama et al. (2001a,b) suggested that differential expression of rat brain caspase family proteins during development and aging as well as differential subcellular localization of caspase family proteins in the adult rat brain indicates that caspases may contribute to regulation of synaptic plasticity (Shimohama et al., 2001a,b). A wave of active caspase-3-positive cells dividing in the proliferative zones and then migrating to the olfactory bulb as they differentiated into neurons was demonstrated by Yan et al. (2001). Recently, we have demonstrated that in early postnatal ontogenesis, a transient decline in CA1 population spike amplitude in the rat hippocampal slices coincides with caspase-3 activation during a period not related to an increase in apoptosis, suggesting involvement of caspase-3 in synaptic plasticity (Kudryashov et al., 2001, 2002). In an *in vivo* model of ischemic tolerance, McLaughlin et al. (2003) observed widespread caspase-3 cleavage, without cell death, in pre-

conditioned tissue. In an *in vitro* model of excitotoxic tolerance, they demonstrated that caspase inhibitors blocked ischemia-induced protection against *N*-methyl-D-aspartate (NMDA). These data suggest the existence of a neuroprotective pathway in which events that have been before associated with apoptotic cell death only are critical for cell survival. Thus, active caspase-3 may play a role in cellular processes such as neuronal differentiation, migration, and plasticity.

Recently, we summarized *in vitro* and *in vivo* data confirming that caspase-3-mediated mechanisms are essential for neuronal plasticity (Gulyaeva, 2003). Gulyaeva et al. (2003) demonstrated that an inhibitor of caspase-3 has blocked LTP in hippocampal slices, supporting an important function of the enzyme in neuroplasticity. A strong *in vivo* evidence is the result reported by Dash et al. (2000) who have shown that caspase-3 plays an essential role in long-term memory. They showed that intrahippocampal infusion of a caspase-3 inhibitor to rats significantly impaired spatial memory in the water maze.

The present work was designed to study whether inhibition of caspase-3 activity by i.c.v. administration of N-benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val- Asp(OMe)-fluoromethyl ketone (z-DEVD-fmk), a caspase inhibitor with predominant specificity toward caspase-3, impairs learning in other tasks related to specific forms of memory.

EXPERIMENTAL PROCEDURES

Subjects and experimental groups

Forty-nine adult male Wistar rats were supplied by Stolbovaya Breeding Center (Moscow Region, Russia). Animals, weighing 250–350 g at the beginning of the experiment, were housed five per a cage at 12-h light/dark cycle (8:00 a.m.–8:00 p.m.) and fed *ad libitum*. All experiments were made in accordance with the European Communities Council Directive (86/609/EEC) for the care and use of animals for experimental procedures; the protocol was approved by the institutional Animal Care and Ethics Committee. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Experiments were performed on two separate groups of animals. The effects of z-DEVD-fmk and N-benzyloxycarbonyl-Phe-Ala-fluoromethyl ketone (z-FA-fmk) on learning were studied using the first group of rats subjected to a two-way active avoidance procedure. Three sub-groups of animals were tested in the task: intact, z-DEVD-fmk- and z-FA-fmk-treated. The second group of animals which were not subjected to the learning procedure was used to study caspase-3 activity in brain regions after central administration of the peptides.

Surgical procedure

Animals under chloral hydrate anesthesia (350 mg/kg) were positioned in a stereotaxic frame and a midline sagittal incision was made in the scalp. Holes were drilled in the skull over the lateral ventricles using the following co-ordinates (Paxinos and Watson, 1982): 0.8 mm posterior to bregma; 1.5 mm lateral to the sagittal suture. A stainless steel guide cannula (0.9 mm external diameter, 9 mm length) was placed sub-durally through holes drilled in the scull and secured to the scull with dental acrylic. Stainless steel stylets were inserted into the guide cannulas and kept in place prior to injections. After surgery rats were housed individually and kept under these conditions until the end of the experiment.

Two-way active avoidance

Active avoidance testing was conducted in a 50×25×25 cm two-way automated shuttle-box (Multiscreen-4, Multiscreen Moscow, Russia) constructed of gray painted steel. The front panel of the box was made of clear Plexiglas. The shuttle-box was equipped with a grid electrifiable floor made of stainless steel wire (2 mm in diameter) and was divided on two equal compartments by black Plexiglas wall. A square hole (7×10 cm) in the center of the wall was made to allow a communication between compartments of the experimental chamber. The shuttle-box was situated in the sound-protected deeply lighted room. The conditioned stimulus (CS) was a flashlight, producing by two 6 W bulbs situated on a faceplate of each compartment. The unconditioned stimulus (US) was intermittent electric footshock (pulses of 50 Hz, 1.5 mA, 400 ms duration electric stimulation). Each training trial consisted of 5 s presentation of CS followed by simultaneous US presented for 40 s at maximum. The trials were given with 1 min intertrial interval. The shuttle-box was connected to a computer that controlled the training schedule and scored avoidances, escapes and non-responses, latencies and the number of crossings that the animals made.

The rats were trained in a massed session (60 trials) of a two-way active avoidance task. Immediately before the first trial of acquisition session, the rats received a 5 min adaptation period consisting of free ambulation in the shuttle-box in order to be familiarized with the learning environment. During the learning training each trial consisted of 5 s CS presentation followed by US, so as the US coincided with CS up to 40 s. The animals could avoid the shock by crossing to the adjacent dark compartment during the first 5 s of CS presentation. The following parameters of rat performance were registered: 1) number of avoidance responses; 2) number of reactions on CS (rearing, turning, freezing, flinching, moving across compartment, vocalization etc.); 3) latencies of reaction on CS or US; 4) intertrial crossings (ITC). The number of avoidances, reaction latencies and ITC was checked automatically by computer. The type and the number of reactions to CS were controlled visually and subsequently divided onto three groups according to the definition of Savonenko et al. (2003): 1) freezing reactions, defined as the lack of any movement except that related to respiration; 2) preparatory response during CS presentation, i.e. turning of the body and orienting of the head toward the opening during CS presentation, excluding the cases when preparatory response was followed by avoidance reaction; 3) attention reaction to the CS: any change in ongoing behavior, which was observed during first seconds of CS presentation, as initiation of preparatory response, dissipation of freezing, or break of any previous activity.

Drug preparation and treatment

Inhibitor of caspase-3, z-DEVD-fmk, or the "control" peptide recommended by the producing company, z-FA-fmk (both Enzyme Systems Products, Livermore, USA), were diluted in dimethyl sulfoxide (DMSO), aliquoted and stored under −40 °C. Immediately before the injection stock solutions were diluted by sterile saline 1:100 (v/v). The final concentration of z-DEVD-fmk or z-FA-fmk was 750 μM.

Ten to 14 days after surgery rats were assigned into two groups and subjected to i.c.v. injection either of two peptides. All injections were made using 10 μl Hamilton syringe equipped with a 26S-gauge beveled needle. The rats were gently restrained, cannulas' caps and stylets were removed and syringe needle was inserted into guide cannula. The needle was directed vertically down to 4.0 mm beneath the scull. The rats received bilateral injections of z-DEVD-fmk or z-FA-fmk at a dose of 3 nmol in 4 μl of 1% DMSO (2 μl into each ventricle). After this procedure rats were returned into their home cages. The administration was performed 20–22 h before behavioral testing.