ISSN 1990-519X, Cell and Tissue Biology, 2015, Vol. 9, No. 2, pp. 141–148. © Pleiades Publishing, Ltd., 2015.
Original Russian Text © R.Ya. Gordon, L.V. Shubina, M.V. Kapralova, E.V. Pershina, S.S. Khutsyan, V.I. Arkhipov, 2014, published in Tsitologiya, 2014, Vol. 56, No. 12, pp. 919–925.

Peculiarities of Neurodegeneration of Hippocampus Fields after the Action of Kainic Acid in Rats

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Received June 26, 2014

Abstract—The prolonged effect of excitotoxin (kainic acid, KA) on the morphological state of different dorsal hippocampus fields during its one-time introduction in different regions of the brain (intrahippocampal (0.2 μg/μL) and intraventricular (0.6 μg/μL)) was compared. The studies were carried out on light (cresyl violet dye) and fluorescent (fluoro-jade B) levels. The results showed that KA introduced intrahippocampally at a dose that does not result in animal epileptization caused clearly pronounced degenerative effects in hippocampus. It was found that layers of pyramidal cells in the CA3 and CA4 fields were absent 2 weeks after KA introduction and degenerative changes and cell lysis also applied to the CA1 field by the fourth week. Different level of impairments in hippocampus (from particular impairment of pyramidal neurons of the CA3 and CA4 fields to complete loss of pyramidal layers in the CA1, CA3, and CA4 fields) was observed during intraventricular introduction of KA into rats with epileptic status 4 weeks later. The layer of cells in the CA2 field was mainly maintained under both means of KA introduction, indicating the special role of this field. During one-time KA introduction, both methods of introduction resulted in long-term impairment of the nervous tissue (which probably have a common character, but different speeds of neuronal reaction from its field to the impairing factor). Activation of GluR6-containing kainate receptors on pyramidal neurons of the CA3 field is one explanation for the prolonged effect of the KA excitotoxicity; it introduces a chronic character to the single effect of KA and favors the death of the remaining neurons in a necrotic fashion while neurons die by apoptosis at the initial stage of the KA effect.

Keywords: excitotoxicity, neurodegeneration, hippocampus, CA1–CA4 fields, kainic acid, fluoro-jade B
DOI: 10.1134/S1990519X15020066

INTRODUCTION

The hippocampus is a structure that is heterogeneous in its physiological, biochemical, and morphological indices. Hippocampus fields react differently to the effects of different impairing factors (Collins, 1986; Ji, Maren, 2008; Gordon et al., 2009). Cell impairment and death caused by the effect of many damaging factors (craniocerebral injury, ischemia, hypoxia, etc.), as well as neurodegenerative diseases, is excitotoxicity. Excitotoxicity is determined as a cell death caused by the toxic effect of excitatory amino acids and their agonists (Dong et al., 2009). Neurotoxin kainic acid (KA) is an agonist of glutamate receptors. Convulsive activity is induced during its introduction to rodents or directly in the brain, and cellular impairments of the brain similar to pathological changes at human temporal lobe epilepsy are observed (Nadler, 1981; Ben-Ari, 1985; Wang et al., 2005). Experimental models on animals using KA allow its influence on the morphological state of neurons in dynamics to be studied. In spite of the fact that KA has a damaging effect on many brain structures, the hippocampus is the most sensitive to neurotoxicity induced by KA. The reasons for the special vulnerability of hippocampus neurons to the effects of excitotoxin are not clear; moreover, the mechanisms of cell impairment in separate hippocampus fields remain unclear. The study of long-term changes in physiological and morphological states of the hippocampus cells occupy a special place, since periodically occurring epileptiform activity (along with neurodegenerative changes) develops for a long time after KA introduction (Cavalheiro et al., 1982; Ben-Ari, 1985; Tokuhara et al., 2007; Vincent, Mulle, 2009). In order to study late irreversible degenerative changes in the hippocampus neurons, fluorescent fluoro-jade B dye is successfully used; along with classical dyes that detect the state of cells (cresyl violet), it allows a more complete picture of neuron degeneration in dynamics to be seen (Schmued, Hopkins, 2000; Anderson et al., 2005).

[1] *Abbreviations*: KA—kainic acid.

The aim of the present study was investigation of the prolonged effect of KA excitotoxin on the morphological state of different hippocampus fields during its single introduction into different brain departments (intrahippocampal and intraventricular). Comparison of the different methods of KA introduction in the brain will allow one to detect the speed and degree of impairment of different fields of dorsal hippocampus, as well as (subsequently) the most efficient approaches to decreasing excitotoxic brain impairments.

MATERIALS AND METHODS

The experiments were carried out on male rats of the Wistar line (with a weight of 180–200 g). Animals were kept and used in accordance with the rules of the Council of European Society (directive from 1986).

Intrahippocampal KA introduction. Stereotaxic double-sided KA (Sigma, United States) was introduced to rats of the experimental group ($n = 14$) under the effect of pentobarbital anesthesia (30 mg/kg). Microinjection was conducted by a 1-μL (0.2 μg) Hamilton syringe to the left and right dorsal hippocampus (coordinates AP: –3.0, ML: ±3.0, V:—3.0) (Paxinos, Watson, 1986). Transient convulsive phenomena of limbic nature (twitching of the head and forelimbs, shaking, et al.) arose in rats for 2–4 h after KA introduction, but no convulsion was observed subsequently. Isotonic NaCl solution was introduced to the control animals ($n = 6$) in the same amounts and at the same time as to the experimental rats.

Intraventricular KA introduction. KA (0.6 μg in 1 μL) was introduced unilaterally into right lateral ventricle of the animal brain (AP = –0.9, L = 1.5, H = 3.5). For this, neurosurgical operation for implantation of directing cannula (using which KA was once introduced) was carried out a week before the beginning of experiments. Behavioral epileptic status was observed in all animals ($n = 7$) after intraventricular KA introduction. Isotonic NaCl solution was introduced to the control animals ($n = 4$) in the same amounts and at the same time as to the experimental rats.

Morphological studies. Two and four weeks after KA microinjection in hippocampus and four weeks after KA microinjection into the brain ventricle, animals were decapitated, the brain was extracted and fixed in the Carnoy mixture (ethanol–chloroform–acetic acid in a ratio of 6 : 3 : 1, respectively), and put into paraffin. Sections of 10-μm thickness were prepared from blocks.

Light microscopy. The state of pyramidal hippocampus neurons (CA1, CA2, CA3, and CA4 fields) were detected by staining of the brain sections by cresyl violet (Fluka, United States) (according to Nissl). Histological preparations were analyzed using an AxioImager M1 optic microscope (Carl Zeiss, Germany). The length of the impaired region of hippocampus fields was determined on the section photos by means of the ImageJ program (http://imagej.en.softonic.com).

Fluorescent microscopy. Late features of neuronal degeneration were histochemically detected by fluorescent fluoro-jade B dye (EMD Millipore, United States). Staining by fluoro-jade B was performed according to the method described by Schmued and Hopkins (2000) with modifications that we introduced. Dehydrated preparations were placed into 0.06% potassium permanganate solution for 30 min at room temperature and then placed into freshly prepared 0.0001% fluoro-jade B solution in 0.1% acetic acid for 25 min. Sections were then washed with distilled water, and the stained preparations were put into balsam (Sigma, United States) and covered by a cover glass. The state of neurons on sections was determined by means of a fluorescent DM 6000 microscope (Leica, Germany).

Data are presented as a mean and standard error of the mean.

RESULTS

KA introduction into dorsal hippocampus. The results showed that KA applied in a dose that did not result in animal epileptization caused clearly pronounced degenerative changes in the left and right sides of the dorsal hippocampus. When staining by cresyl violet, neurons in the control animals had an evenly stained cytoplasm and nucleoplasm, pronounced plasmatic and nuclear membranes, and clearly pronounced nucleolus (Fig. 1a). Degradation of pyramidal cell layers in CA3 and CA4 hippocampus fields occurred 2 weeks after the microinjection as a result of neuron lysis throughout 0.7 ± 0.2 mm (Fig. 2a). Swelling of cytoplasm, its vacuolization, and loss of membrane integrity (which are usually morphological traits of necrosis) are typical for neurons bordering on damaged areas (Kroemer et al., 2009). Pyramidal neurons of the CA1 field remained almost intact (Fig. 2a). Partial loss of the layer of pyramidal neurons in CA1 field (0.77 ± 0.36 mm) was observed in 4 weeks (in addition to an increase in the neuron impairment in CA3 and CA4 fields (1.2 ± 0.24 mm)). Traits of necrosis were registered in most remaining neurons (Fig. 2d).

The experiments using fluoro-jade B, which allows one to detect irreversible neurodegenerative changes, showed that positive labeling is registered in the hippocampus CA3 and CA4 fields 2 weeks after intrahippocampal KA introduction (Fig. 2b). Moreover, in addition to fluorescence in pyramidal neurons, a fluorescent label was also observed in adjacent to them section regions (Fig. 3b). Most likely, reactive astroglia was an object of such fluorescence, since fluoro-jade B (in addition to degenerating neurons) is able to stain reactive microglia and astroglia in the process of neuronal degeneration (Damjanas et al., 2007). In this period of time, astrogliosis (being a final component of

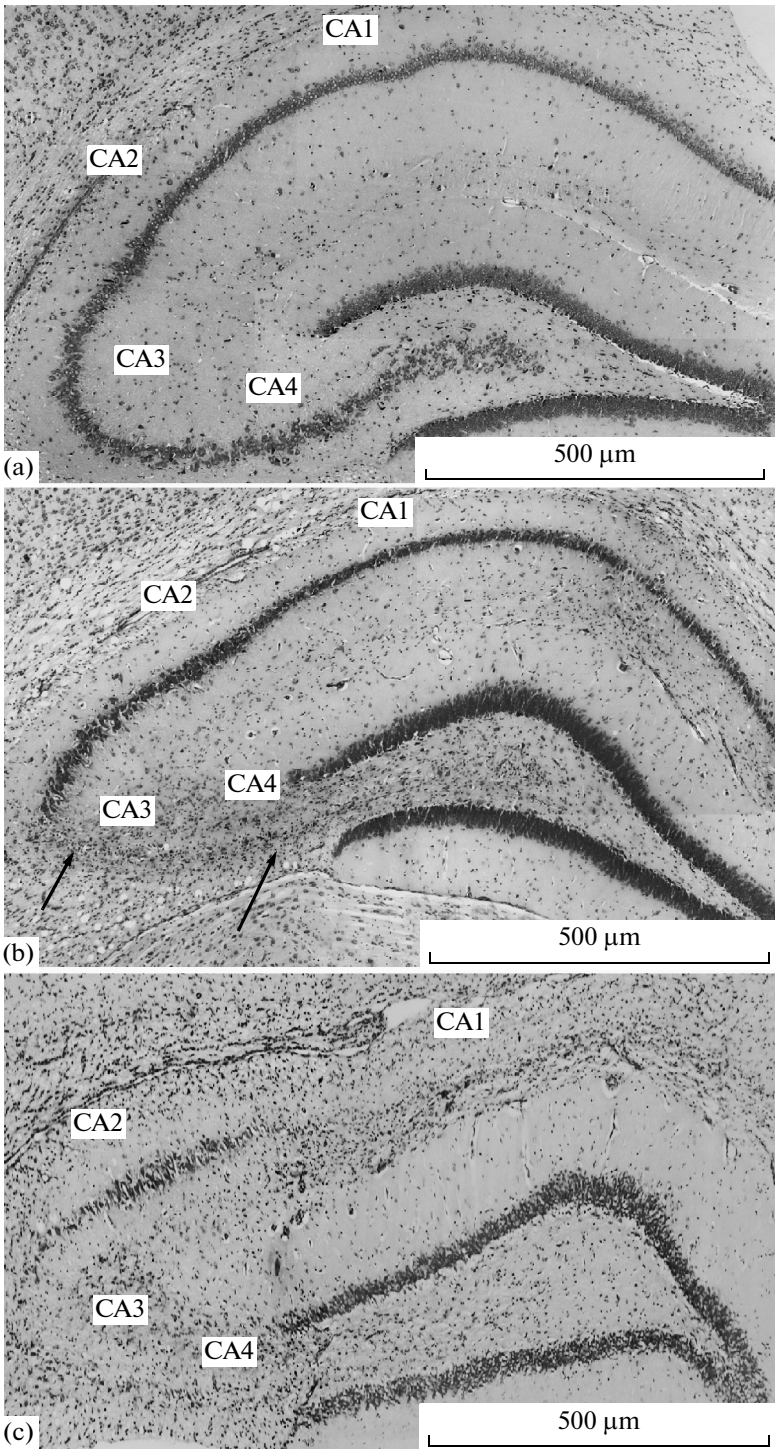

Fig. 1. Degenerative changes in rat hippocampus 4 weeks after intraventricular introduction of KA (0.6 μg/1 μL). Sections of rat brain stained by cresyl violet. (a) Control animals; (b) degradation of pyramidal neuron layer of CA3 and CA4 hippocampus fields (designated by *arrows*); and (c) loss of cells layer in CA1, CA3, and CA4 fields and particular loss of neurons of CA2 field. Ob. 10×.

gliosis) is manifested in activation of astrocytes that surround neurons. As opposed to the results obtained in 2 weeks (Fig. 2c), the appearance of fluorescence in CA1 field was registered 4 weeks after KA microinjection (Fig. 2f) with preserved positive labeling of CA3 and CA4 fields (Fig. 2e).

KA introduction into the brain ventricle. Epileptic status developed in almost all animals 4 weeks after

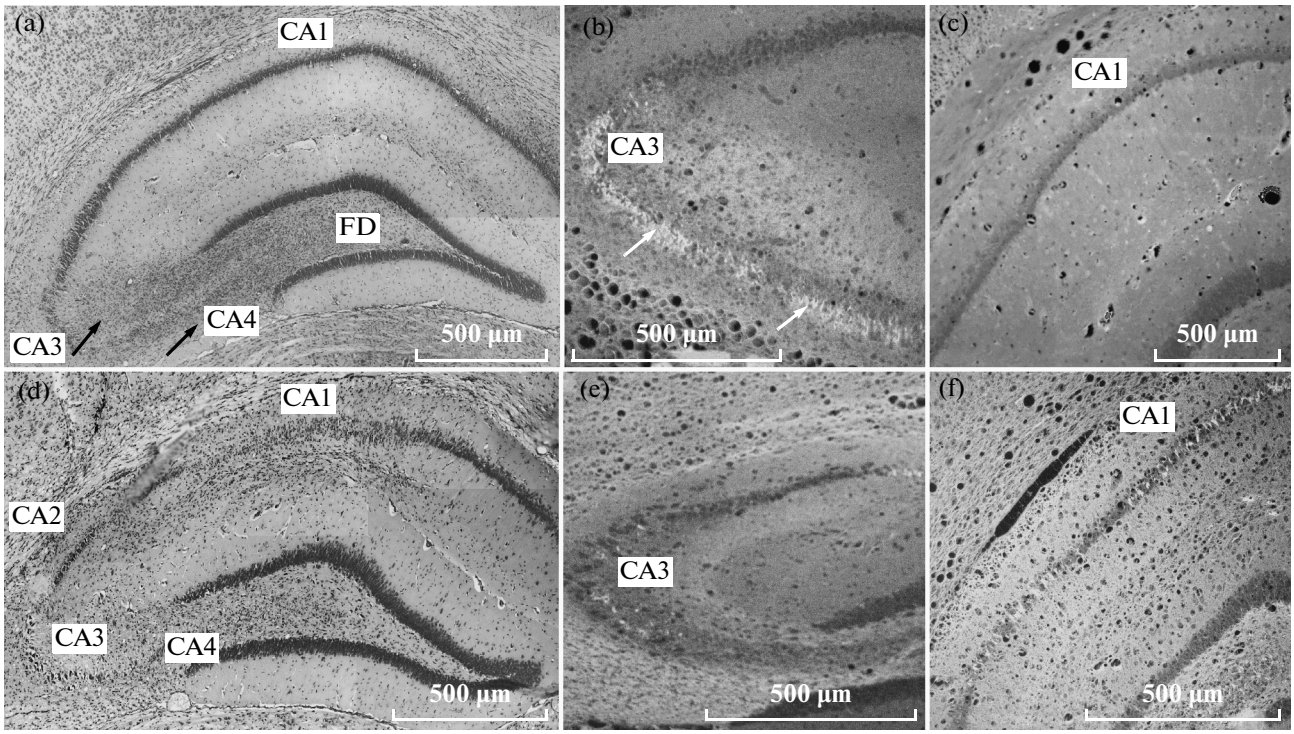

Fig. 2. Histological picture of the brain sections. The hippocampus impairment during intrahippocampal introduction of KA (0.2 μg/1 μL) in 2 (a–c) and 4 (d–f) weeks. Staining of sections by (a, d) cresyl violet (according to Nissl) and (b, c, e, f) fluoro-jade B. (a) Degradation of pyramidal cell layers in CA3 and CA4 fields (designated by *arrows*), CA1 and CA2 remain almost intact; (b) labeling of impaired neurons in CA3 and CA4 fields (designated by *arrows*); (c) absence of labeling in CA1 field; and (d) loss of neuron layer in CA3–CA4 fields, significant loss of neuron layer in CA1 field, partial impairment of neurons of CA2 field. Labeling of impaired neurons of (e) CA3–CA4 fields and (f) and CA1 field. Ob. 10×.

intraventricular KA introduction. It should be noted that different levels of impairments (from partial impairment of pyramidal neurons of CA3 and CA4 fields (Fig. 1b) to the loss of pyramid layers in the CA1, CA3, and CA4 fields (Fig. 1c)) were observed in all rats in the injured right half of the dorsal hippocampus. When determining the level of cellular degeneration by means of fluoro-jade B in these animals, respectively, fragmentary positive labeling both exclusively in hippocampus CA3 and CA4 fields and including CA1 field was observed. In addition, similar changes in the hippocampus took place both 2 and 4 weeks after intrahippocampal KA introduction. The state of hippocampus neurons almost did not differ from the control in the intact left side of the brain.

Fluorescence was absent in experiments with fluoro-jade B in all studied methods of KA introduction in places of complete absence of cells in impaired hippocampus fields. No fluorescent label was observed in the control preparations in animals after introduction of physiological solution.

It should be noted that the cell layer remained in the CA2 field under both methods of KA introduction (Figs. 1c, 2d), although some neurons of this layer were impaired.

DISCUSSION

In the present work, the effect of KA on the dorsal hippocampus during one-time introduction was studied. The dorsal hippocampus is more vulnerable than other parts of the hippocampus to such effects (Gordon et al., 2013). During intraventricular introduction, KA has an effect on many brain structures that are adjacent to ventricles, while neurotoxin acts more locally (first of all, impairing neighboring neurons and their associations) during intrahippocampal injections. In addition, neurons of the CA3 field are initially impaired, and then those of the CA1 field. Similar results were also obtained by a number of authors during intraventricular KA introduction (Nadler, Cuthbertson, 1980; Franck, 1984). According to our studies, if the above-described dynamics of hippocampus field impairment is observed at intrahippocampal KA microinjections, then different levels of impairments for the same period (4 weeks after KA introduction) are observed during intraventricular introduction.

We note that the observed excitotoxic impairment of hippocampus is considered by many authors as typical for temporal lobe epilepsy in human (Nadler et al., 1978; Cavalheiro et al., 1982; Babb et al., 1995; Cavazos et al., 2004). Indeed, in the present work we

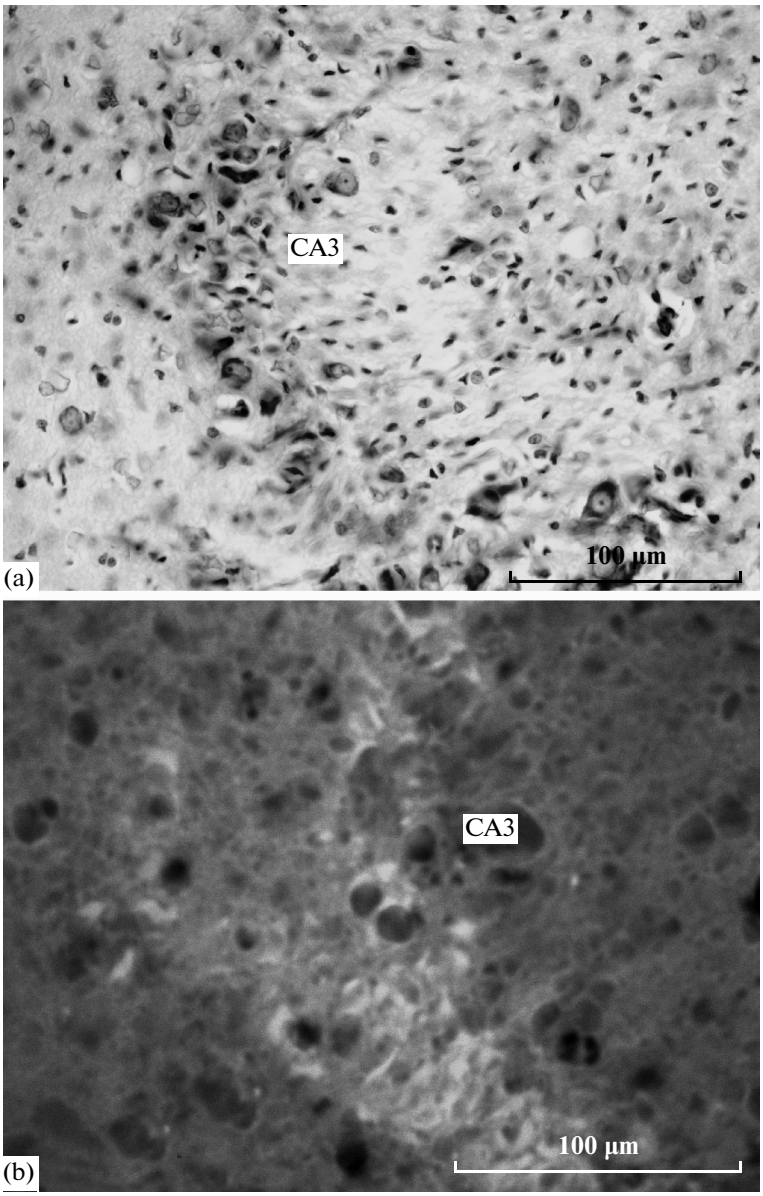

Fig. 3. State of pyramidal neurons of hippocampus CA3 field in rats 2 weeks after intrahippocampal introduction of KA. (a) Staining by cresyl violet; (b) labeling by fluoro-jade B of impaired neurons and astroglia. Ob. 40×.

observed loss of neuronal layers in fields 1, 3, 4, and irreversible damages of the main bulk of remained neurons, revealed by labeling with Fluoro-Jade B (in both cases of administration), similar to sclerosis signs at a temporal epilepsy. In addition, it was possible to observe gliosis pronounced in fluorescence of reactive microglia and astroglia (in the case of intrahippocampal KA introduction) (Meldrum, Corsellis, 1984; Blümcke et al., 2002). Some authors suggest that loss of neurons and gliosis are the main indices of hippocampus sclerosis (Sofroniew, 2009; Zhang et al., 2010).

It is typical that, if excitotoxicity caused by the introduction of ischemia, hypoxia, and amyloid (Aβ) in the model of Alzheimer's disease first of all impairs neurons of the CA1 field (O'Mara et al., 2000; Nie et al., 2010; Gordon et al., 2012), while pyramidal CA3 cells and morphologically diverse neurons of the CA4 region become the most vulnerable during temporal lobe epilepsy (Margerison, Corsellis, 1966; Nadler, 1981).

In temporal lobe epilepsy, the loss of neurons in the CA1 and CA3 hippocampus fields is accompanied by insignificant impairment of neurons of dentate fascia, the CA2 field, and subiculum (Sloviter, 1983; Loup et al., 2000; Andrioli et al., 2007; Mercer et al., 2007; Wittner et al., 2009), which was also observed in our work with both routes of KA introduction. Since partial or complete loss of the neurons of the hippocam-

pus CA1, CA3, and CA4 fields occurs in patients with epilepsy or in experimental models, it has been suggested that the remaining neurons of the CA2 field and parahippocampal regions may be an epileptogenic substrate under a prolonged KA effect (Jefferys, Traub, 1998). Other authors have detected that the CA2 field, dentate fascia, and subiculum in the human hippocampal formation are resistant to the loss of cells during temporal lobe epilepsy (Sloviter, 1983; Mercer et al., 2007; Wittner et al., 2009).

Special attention should be paid to the fact that KA does not induce convulsions in animals during intrahippocampal introduction and results in approximately the same cellular impairments in the dorsal hippocampus in almost all animals, while morphological impairments differ significantly during intraventricular introduction (in the presence of epileptic status in animals). Such a difference in different methods of KA introduction may be due to the following reasons. First, excitotoxic death of neurons causes reorganization of neuronal networks of the whole temporal region. Neurons of the CA3 field are a primary target during intrahippocampal KA introduction; they are the most sensitive to its action due to the presence of a large amount of kainate receptors on them (Vincent, Mulle, 2009). The pyramidal cells of the CA3 field are 10–30 times more sensitive to KA application than are CA1 neurons (Vincent, Mulle, 2009). Second, neurons of the CA3 field have physiological abilities to generate and increase the effect of the impairing action of KA (Jefferys, Traub, 1998). An increase in the excitability of neurons of the CA3 and CA4 fields during synaptic release of glutamate stimulates Schaffer collaterals directed toward neurons of the CA1 field. The death of neurons of the CA3 field as a result of the impairing action of KA results in morphological changes also in neurons of the CA1 field, which are based on biochemical changes (Yi et al., 2013). Therefore, degradation of neurons of the CA1 field is observed after degradation of neurons of the CA3/CA4 field (Lancaster et al., 2009). The large role played by neurons of the CA1 field in maintenance of permanent hyperexcitability of hippocampus cells, amplification, and synchronization of epileptic discharges in efferents of hippocampal formation (because the CA1 field is its final projection on subiculum) should be noted (Greene, Totterdell, 1997; Menendez de la Prida et al., 2003; Cavazos et al., 2004).

During intraventricular introduction of KA, its action is apparently performed in accordance with the lamellar hypothesis (Andersen et al., 2000). The entorhinal cortex is projected through the perforating pathway onto the dentate fascia, which in turn is projected through the mossy fiber pathway on the region of CA3 field pyramids. The CA3 field is projected through Schaffer collaterals to the region of pyramidal neurons of the CA1 field, which is projected to the subiculum (which sends efferents to the entorhinal cortex, generating a loop within the hippocampal system).

In order to understand the mechanisms of neurodegenerative diseases associated with excitotoxicity, the question of why a single action of KA on the brain (at any method of its introduction) results in long-term increasing impairments of the nervous tissue (which, however, begin to appear almost immediately after its introduction) is important (Tokuhara et al., 2007). The answer is to some extent clarified by the peculiarities of kainate receptors, which are the main targets for KA in its epileptogenic and excitotoxic effects (Vincent, Mulle, 2009; Melyan et al., 2011). Kainate receptors of glutamate perform postsynaptic response in pyramidal neurons of the CA3 field and interneurons of the hippocampus CA1 field (Melyan et al., 2011). Being ionotropic, they contain a GluR6 subunit, which is typical for metabotropic receptors (Lancaster et al., 2009). Activation of receptors containing GluR6 favors a prolonged increase in the excitability of pyramidal neurons of the hippocampus (Rodríguez-Moreno, Sihra, 2007, 2011; Melyan, Wheal, 2011).

Thus, the data obtained allow it to be thought that degeneration of hippocampus neurons after two different types of central introduction of KA has common mechanisms, but neurons of hippocampus fields have different speeds of reaction to the excitotoxin effect.

It should be noted that neurons usually die by apoptosis under one effect of an impairing factor. A chronic effect results in necrotic neuronal death, although a combination of properties of different types of death is possible (Artal-Sanz, Tavernarakis, 2005). As for KA, the type of cell death and time of their death depend on its dose (Tokuhara et al., 2007).

According to our data, typical traits of necrosis are already observed 2 and 4 weeks after introduction of KA in the remaining neurons, which occurs during the chronic effect of an impairing factor. Moreover, in addition to the already lyzed neurons, the remaining impaired cells (detected by fluoro-jade B) cannot be restored (Schmued, Hopkins, 2000), which is probably one of the main reasons for the prolonged action of KA.

Thus, activation of a Glu6R-containing kainate receptor (which brings about a prolonged effect of KA excitotoxicity) probably results in a chronic character in a single action of KA and favors the death of the remaining neurons in a necrotic fashion while at the initial stage of the effect of KA they die by apoptotic way.

ACKNOWLEDGMENTS

We are grateful to the Laboratory of Optical Spectral Analysis Methods team headed by V.A. Yashin.

This work was supported by the Russian Foundation for Basic Research (projects nos. 12-04-00470 and 12-04-00776-a), as well as by Leading Scientific Schools (project no. 850.2012.4).

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Translated by A. Barkhash