Cellular and Molecular Neurobiology

Effect of Myo-inositol treatment on hippocampal cell loss triggered by kainic acid induced status epilepticus: light and electron microscope studies

--Manuscript Draft--

Manuscript Number:	
Full Title:	Effect of Myo-inositol treatment on hippocampal cell loss triggered by kainic acid induced status epilepticus: light and electron microscope studies
Article Type:	Original Research
Keywords:	Kainic acid-induced status epilepticus. Hippocampal cell loss. Myo-Inositol pretreatment. Cytoprotective properties of Myo-inositol. Rat.
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Abstract:	Recently it was shown that myo-inositol pretreatment attenuates the seizure severity and several biochemical changes provoked by experimentally induced status epilepticus. However it remains unknown whether such properties of myo-inositol influence the structure of epileptic brain. In the present light and electron microscopic research we elucidate if pretreatment with myo-inositol has positive effect on hippocampal cell loss and cell damage provoked by kainic acid induced status epilepticus. Adult male Wistar rats were treated with (i) saline, (ii) saline + kainic acid, (iii) myo-inositol + kainic acid. The cell loss was assessed 2, 14 and 30 days after treatment in radial, oriental and pyramidal cell layers of CA1 and CA3. The ultrastructural alterations were described in CA1, 14 days after treatment. The cytoprotective effect of myo-inositol was shown. Itt was most strongly expressed in pyramidal layer of CA1 and radial and oriental layers of CA3, in less degree - in almost all other layers of both areas. Electron-microscopic studies also indicate that in comparing with rats treated with kainic acid, the ultrastructure of CA1 of animals pretreated with myo-inostiol is more preserved. Such data give us the possibility to share recent opinion that in addition to other functions, myo-inositol possesses antiepileptogenic properties.
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1. Introduction

Despite numerous anti-seizure and antiepileptic drugs that have been introduced over the last years, the recent state of epilepsy treatment has been characterized by significant limitations. Cases do not respond to drug therapy still represent the most prevailing group of epileptic patients, but even if patients who respond well to treatment, long-lasting outcomes are rarely improved (Pati and Alexopoulos 2010; Schmidt 2011; Schmidt and Loscher 2005; Walker et al. 2002; Wong 2005). It is supposed that the majority of existing antiepileptic medications are not truly antiepileptic but represent the symptomatic treatments that do not affect mechanisms of epileptogenesis (Armilio et al. 2006; Aroniadou-Anderjaska et al. 2008; Baulac et al. 2009; Wong 2005). Future studies should lead to the development of new antiepileptic treatments that could successfully cure and/or cease this disease.

Myo-inositol (MI) is physiologically important osmolyte and precursor for lipid synthesis (for review see: Fisher et al. 2002). Recently the participation of MI in the epilepsy and the changes in brain MI levels as a result of seizures have been shown. In particular, it was demonstrated that pretreatment with MI specifically reverses the pilocarpine-lithium induced limbic syndrome (e.g. for review see: Belmaker and Bersudsky 2007). In another research, as a result of kainic acid (KA) treatment, up-regulation of Na+/MI co-transporter in various levels of the hippocampus shortly after seizure induction was detected (Nonaka et al. 1999). Then it was shown that seizure focus of patients with temporal lobe epilepsy (TLE) is characterized with an increased level of MI, whereas neighboring areas have a lowered level of MI (Wellard et al. 2003). Furthermore, it was revealed that gene, coding for MI monophosphatase 2 could be febrile seizure susceptibility gene (Nakayama et al. 2004). Of particular concern is the new series of studies, where possible antiepileptic properties of physiological doses of inositols were supposed. Thus, in the first research it was shown that intraperitoneal (i.p.) pretreatment of animals with MI significantly decreases the severity of seizures induced either by KA or pentylentetrazol (PTZ) (Solomonia et al. 2007). Next series of experiments have shown that another representative of inositols, namely scyllo-Inositol (SCI), significantly reduces the severity of score and duration of PTZ-induced convulsions (Nozadze et al. 2011). The effects were specific to MI and SCI, since Mannitol – a polyol with a similar molecular weight to those of MI and SCI, had no effect on PTZ seizures (Nozadze et al. 2011). In the third series of experiments, using KA, the stratus epilepticus (SE) was induced and after 10 hours (h) the daily MI treatment was initiated. As a result it was shown that the daily MI treatment during 28 d attenuated certain biochemical changes which were associated with the process of epileptogenesis (Solomonia et al. 2010, 2012). Namely, it was demonstrated that KA-treatment decreases levels of GLUR1 subunit of AMPA-glutamate receptors α calcium-calmodulin dependent protein kinase II (CaMKII) and γ2-subunit of GABA-A receptors in the hippocampus. These alterations were almost completely reversed by daily MI treatment (Solomonia et al. 2010, 2012). Such data strongly indicate to possible antiepileptic and antiepileptogenic properties of physiological doses of MI exerting its effects on behavioral and molecular level. But the reversal of GLUR1, CaMKII or □2 subunit of GABA-A receptors amounts by MI treatment does not

necessarily mean that it also influences the structure of epileptic brain. However we suggest that studying of a unique profile of structural changes in the brain of epileptic animals pretreated with MI may provide valuable information regarding the role of MI during this disorder.

In the present research the rat model of experimental epilepsy, provoked by KA-treatment was used. It is well-known that KA, the structural analogue of glutamate, when injected i. p, intraventricularly or intravenously, activates glutamate receptors. As a result, the prolonged depolarization accompanying with chronic, spontaneous, recurrent seizures and structural abnormalities compatible to those reported in the patients with TLE, has been developed (Ben-Ari 1985; Fujikawa and Itabashi 1994; Lado et al. 2002; Rempe et al. 1997; Sater and Nadler 1987; Sloviter 1991). One of the key structural peculiarities induced by KA-treatment is the hippocampal cell loss. Our goal was to elucidate if pretreatment with MI has positive effect on temporal and spatial distribution of cell loss/damage provoked by KA-induced SE in the rat hippocampus. The following central questions were identified: (i) Does the MI pretreatment interfere with cell loss provoked by KA-induced SE in CA1 and CA3? (ii) Does the pretreatment with MI interfere with ultrastructural alterations provoked by KA-induced SE in the hippocampus?

2. Material and methods

2.1. Animals

Male adult *Wistar* rats weighting 280-300 g were used in this study. During experiments rats were housed under normal controlled environment (temperature 20-22 ^{0}C, humidity 55-60%, light on 07.30 – 19.30). Standard food pellets and tip water were available. Experimental procedures were approved by Animal Studies Committee of I. Beritashvili Center of Experimental Biomedicine.

2..2. Animal treatment

Experiments were carried out with three groups of animals and they were treated as follows:

Saline (SAL) treated rats (group: SAL)

Rats were treated i. p. singly, with SAL. These rats composed control group of animals.

SAL+KA-treated rats (group: SAL+KA)

Rats were treated i. p. with SAL and after 30 min interval, with KA dissolved in saline (10 mg/kg).

MI+KA treated rats (group: MI+KA)

Rats were treated i. p., first, with MI (Sigma) dissolved in dH2O (30 mg/kg) and after 30 min interval, with KA dissolved in SAL (10 mg/kg).

2.3. Monitoring of status epilepticus

The rats which developed repeated rearing and failing episodes during 10 h after SAL+KA or MI+KA injection were considered as animals with SE (Tuunanen et al. 1996). Only these rats were included in subsequent histological and electron-microscopic analysis.

2.4. Light microscopic (LM) study

The rat brains were studied 2, 4 and 30 days (d) after SAL, SAL+KA and MI+KA treatment (n=4 in each group).

2.4.1. Perfusion and histological procedure

Under pentobarbital injection (100mg/kg), animals underwent transcardiac perfusion with heparinized 0.9% NaCl, followed by 500 ml of 4% paraphormaldehyde in 0.1 M phosphate buffer (PB), pH-7.4 at a perfusion pressure 120 mm Hg. The brains were removed from skull; hippocampi were isolated, blocked, frozen and sectioned in the coronal plane with freezing microtome. Using the Paxinos and Watson atlas (Paxinos and Watson 2005), 30 μm thick, consecutive coronal sections were collected between -2.28 mm and -3.48 mm from bregma. Sections were kept in serial order, placed in 0.1 M PB and every 3rd section was stained with the Nissl method**.** The Nissl staining is commonly used in light microscopy for the detection of Nissl body in the cytoplasm of neurons on paraformaldehyde or formalin-fixed tissue sections. The Nissl body is stained as purple-blue. Producing the traditional multicolored cells, the Nissl staining is suitable for differentiation of diverse cytoarchitectonic fields and quantitative analysis of cells in various levels of brain. The specific morphological characteristics of hippocampal neurons give the possibility to easy distinguish pyramidal cells and interneurons from glial cells. We employed this method in order to identify the temporal and spatial distribution of cell loss in pyramidal, oriental and radial layers of CA1 and CA3 (Fig. 1). Using anatomical landmarks totally 6-10 sections/animal of similar hippocampal levels within and between experimental groups were selected.

2.4.2. Cell loss assessment

A systematic random sampling was employed and the neurons with distinct nucleus and nucleolus were counted with 2-dimensional counting grid (250 x 250 μm2) in 5 non-overlapping fields in CA1 and 3 fields in CA3 at the magnification 400x. Totally 30 fields per animal were analyzed and average of cell central profiles per field was used to assess anatomical damage of CA1 and CA3 in abovementioned groups of animals. The sections were analyzed with a microscope Leica MM AF.

2.4.3. Statistical analysis

To determine whether treatment type (SAL, SAL+KA, MI+KA) and time of treatment (2, 14, 30 d) or both alter the number of neurons, a two-way ANOVA was performed (factors: treatment type and time). In the case of significant effect planned comparisons were carried out using student *t*-test. The level of significance was set as $p < 0.05$. All data are presented as a mean +/-standard error of the mean (SEM).

2.5. Electron microscopic studies

2.5.1. Experimental design

EM studies were done only in the hippocampal CA1 and only at one time point, namely, on 14 d after SAL, SAL+KA and MI+KA treatment (n = 4 in each group). The choice of hippocampal area and experimental time point was based on LM data: (i) as a result of KA treatment the most prominent cell loss take place just in CA1 and 14 d after injection (Section 3.2); (ii) at this time point just in CA1 the MI - pretreatment was most effective.

2.5.2. Electron microscopic study: perfusion and material processing

Following pentobarbital injection (100 mg/kg, i.p.) rats to have EM examination of their brains underwent transcardiac perfusion with 0.9% NaCl, followed by 500 ml of 4% paraphormaldehyde and 2.5 % glutaraldehyde in 0.1 M PB, pH – 7.4 at perfusion pressure 120 mm Hg. The brains were removed from skull and placed in the same fixative overnight. The hemispheric tissue blocks containing hippocampi were cut into 400 micron-thick coronal slices. Slices were washed in 0.1 M PB and kept in 2.5 % glutaraldehyde in 0.1 M PB until processing. When processing, the slices were washed in 0.1 M PB, post-fixed in 1% osmium tetroxide in 0.1 M PB for 2 h and again washed in 0.1 M PB. The hipocampi were identified with a light microscope Leica MM AF, cut out from the coronal slices, dehydrated in graded series of ethanol and acetone and embedded in araldite. Blocks were trimmed and 70-75-nm-thick sections were cut with an ultramicrotome (Reichert), picked up on 200-mesh copper grids, double-stained with uranyl-acetate and lead-citrate and examined with JEM 100 C and Tesla BS 340 transmission electron microscopes. For each case 115 sections were observed.

3. Results

3.1. Development of SE: SAL+KA vs. MI+KA

Detailed analysis of MI pretreatment on behavioral seizure activity in rats treated with KA was performed earlier. In these studies it was shown that animals pretreated with MI develop SE significantly rare than rats treated with only KA (Nozadze et al. 2011; Solomonia et al. 2007). As expected, the present results confirmed these data. Specifically:

*SAL+KA Group***:** 30 animals were treated. 9 rats were died. From 21 survivors 18 animals developed SE. Therefore, the percent of rats with SE in the SAL+KA Group consisted 53%.

MI+KA Group: 43 animals were treated. 4 animals were died. From 39 survivors 15 animals developed SE. Therefore, the percent of rats with SE in the MI+KA Group consisted 34%.

The seizure activity wasn't observed in SAL-treated (control) rats.

As it was mentioned before only the rats that developed SE as a result of SAL+KA and MI+KA treatment were further analyzed.

3.2. LM observation

3.2.1. CA1

Oriental Layer

Two-way ANOVA revealed significant effect of type of treatment (Table 1). According to the ***t***-test (Table 2) at all time points in both, SAL+KA and MI+KA rats the cell number per counting area was significantly lower as compared with control animals (SAL+KA rats: 2 d – by 38%, 14 d – 29.4%, 30 d – 32.2%; MI+KA rats: 14 d – by 28.2%, 30 d – 29.6%, in all cases $p < 0.01$).

Significant difference between SAL+KA and MI+KA groups was observed only 2d after treatment: in MI+KA rats cell loss was less by 21% ($p < 0.05$) (Fig. 2A).

Pyramidal Cell Layer

According to the two-way ANOVA, there are significant effects of type of treatment, time and their interaction on the cell number (Table 1). According to the *t* – test (Table 2), as compared with control, SAL+KA treatment provokes significant cell loss at all experimental time points: 2 d – by 18.3%, ($p < 0.05$); 14 d – 77.1%, 30 d – 78.5%, ($p < 0.001$). In the case of MI+KA statistically significant cell loss was observed 14 d (17.6%, $p < 0.01$) and 30d (20.1%, $p < 0.05$) after treatment. Significant difference between animals of SAL+KA and MI+KA-groups was revealed 14 and 30 d after treatment: in MI+KA treated animals the cell loss was less: at 14 d - by 59.5% and at 30 d – by 58.4% ($p \leq 0.001$) (Fig. 2B). The decrease of pyramidal cells in CA1 as a result of KA-treatment and protective effect of MI pretreatment are clearly observed on the Figure 3 (A, B, C).

Radial Layer

In this layer significant effect was observed only for treatment factor (Table 1). According to the *t*-test (Table 2), in comparison with control, significant cell loss at all experimental time points provokes

SAL+KA treatment (2 d – by 32.8%, 14 d – 39.3%, $p < 0.01$; 30 d – 31 %, $p < 0,05$). In the case of MI ptretreatment significant cell loss was observed only at one time point: 14 d – 27.2%, $p < 0.01$. Significant difference between rats treated with SAL+KA and MI+KA was observed 2d after treatment: in MI+KA rats the cell loss was less by 17.5% ($p < 0.05$) (Fig. 2C).

Thus, our results reveal that the pretreatment with MI significantly decreases the cell loss produced by KA-induced SE in the CA1. This effect is most profoundly expressed in the pyramidal cell layer at 14 and 30 d after treatment, and with a less extent - in oriental and radial layers at 2 d after treatment.

3.2.2. CA3

Oriental Layer

The two-way ANOVA revealed the effect of treatment on the cell number (Table 1). According to the *t*-test (Table 3), as compared with control animals, significant cell loss was observed in both: SAL+KA (14 d – 41.4%, 30 d – 50.8%,$p < 0.01$) and MI+KA treatments (14 d – 20.4%, $p < 0.05$; 30 d – 24.8%; $p < 0.01$). The significant difference between SAL+KA and MI+KA treated groups was observed at 14 and 30 d: MI pretreatment reduces cell loss: at 14 d – by 21% and at 30 d – by 26% ($p < 0.05$) (Fig. 4A).

Pyramidal Cell Layer

The two-way ANOVA revealed the effect of treatment on neural cell number (Table 1). According to the *t*-test (Table 3), in comparing with control animals, SAL+KA and MI+KA treatments produce significant cell loss on the following time points: SAL+KA – 2 d – by 13.8%, 30 d – 27.6%, $p < 0.05$ and MI+KA treatments – 2 d – by 9.7%, 14 d – 16%, $p < 0.05$; 30 d – 15.5%, $p = 0.01$ (Table 3). Significant difference between SAL+KA and MI+KA rats wasn't observed (Fig. 4).

Radial Layer

Like pyramidal cell layer, in the radial layer the significant was only the effect of treatment (Table 1). According to the *t*-test (Table 3), in comparing with control, significant cell loss was induced by both, SAL+KA and MI+KA treatments (SAL+KA: 2 d – by 27%, $p < 0.05$; 14 d – 39.9%, $p < 0.001$; 30 d – by 52%, $p = 0.001$; MI+KA: 2 d – by 23.9%,14 d – 21.6%, 30 d – 22.5%, in all cases ($p < 0,05$). The significant difference between SAL+KA and MI+KA treatments was observed at 14 d and 30 d time points: in the case of MI pretreatment the cell loss was less: at 14 d – by 18.3% and at 30 d – by 29.5% ($p < 0.05$) (Fig. 4C).

Thus, like in the case with CA1, pretreatment with MI significantly decreases the cell loss produced by KA-induced SE in the CA3. The protective effect of MI was significant in the oriental and radial layers at 14 d and 30 d after treatment.

3.3. Electron microscopic studies (CA1, 14 d after treatment)

3.3.1. Treatment with SAL+KA

In approximately 30% of observed cells (mostly with ultrastructural peculiarities of pyramidal neurons) several ultrastructural changes were detected. In perikaryons, large dendrites and synaptic terminals the more common was the damage of mitochondria. Thus, moderate swelling of some of them, disruption of several cristae, vacuolar degeneration or even interruption of mitochondrial membrane were present. In the cytoplasm of other cells swelled cisterns of endoplasmic reticulum or large vacuoles were observed; sometimes electron density of cytoplasm was moderately increased (Fig. 5 A,B). The nuclei were usually normal, but in some cases somewhat deformed, with small, irregular, dispersed chromatin clumps. Rarely shrunk high-osmyophilic ("dark") shapeless profiles (irreversibly degenerated presynaptic terminals or osmyophilic residues of degenerated neurons), abnormally swelled dendrites, damaged myelin sheets, enlarged glial profiles or lucent areas enveloping axonal, dendrite or somatic altered profiles were found (Fig. 5C). Among synapses relatively numerous were axo-dendritic and axo-somatic types with synaptic vesicles concentrated close to active zone.

3.3.2. Treatment with MI+KA

In comparing with SAL+KA treatment, in the case of MI pretreatment the ultrastructure of CA1 was more preserved. Only in 15% of perikaryons (mostly with the peculiarities of pyramidal cells) moderately swelled mitochondria and/or cisterns of granular and agranular reticulum were observed (Fig. 5 D,E). The majority of synapses had normal ultrastructure. In a number of spines well-developed spine-apparatus was presented. More prominent alterations, like observed as a result of SAL+KA treatment, were extremely rare.

4. Discussion

In the present research we studied, to our knowledge for first time, the efficacy of MI in preventing neuronal loss/damage provoked by excperimentally induced SE in different levels of the rat hippocampus. There were the following major findings in this research: (i) In the case of MI pretreatment, at certain time points and in some layers of CA1 and CA3 the cell loss was significantly less than after SAL+KA

treatment; (ii) In the case of MI pretreatment the ultrastructure of CA1 was more preserved than after SAL+KA treatment. The discussion of these findings is done below.

Experimental design

Earlier it was shown that i. p. pre-administration of MI or its isomer, scyllo-inositol, significantly delays the latency of seizure onset and decreases seizure scores and seizures extent induced by PTZ-injection (Nozadze et al. 2011; Solomonia et al. 2007). The MI pretreatment also diminishes the behavioral seizure activity provoked by subsequent injection of KA (Solomonia et al. 2007). Furthermore, it was revealed that 10 h MI post-treatment after KA-induced SE attenuates biochemical alterations associated with epileptogenesis (Solomonia et al. 2010, 2012). Authors proposed that such effect of MI could be the consequence of its interference with the process of epileptogenesis; therefore, MI should act as an endogenous anticonvulsant (Nozadze et al. 2011; Solomonia et al. 2010). Present results confirm abovementioned behavioral data: they indicate that pretreatment with MI significantly reduces the percentage of rats entered SE after KA injection. Based on abovementioned behavioral and biochemical responses we presupposed that MI could have positive effect on the structure of epileptic brain. But such effect could be realized simply by the decrease of seizure strength itself. For to reveal that MI pretreatment has an effect as a specific compound itself, among animals of SAL+KA and MI+KA groups we analyzed only rats which developed the same strength of convulsions: rats with SE.

The damage and loss of hippocampus cells: effect of MI+KA vs. SAL+KA

It is generally accepted that seizure activity may stem not only from inappropriate reactive synaptogenesis, but also from a continuing state of neuronal degeneration (Rempe et al. 1997; Rothman and Samaie 1985). Therefore, it is suggested that to optimize the treatment of epilepsy and SE, it is important to study not only the efficacy of different anticonvulsant/antiepileptic drugs in preventing behavioral/electrophysiological seizures and molecular changes, but also to determine how effectively they prevent seizure-induced neuronal damage (Pitkanen 1996; Pitkanen et al. 1996; Wong 2005). The highly toxic nature of KA and a high degree of specificity and complexity of its action on the hippocampus is well known: hippocampal cell death is considered as a main structural peculiarity of KA-treatment. Numerous articles and reviews have been written on the reasons, mechanisms, possible consequences and promising prevention of KA-produced hippocampal cell death (Ben-Ari 1985; Cho et al. 2008; Kotaria et al. 2010; Lee et al. 2008a; Lee et al. 2008b), but all these questions are still under investigation. In the present study we clarify possible protective effect of MI pretreatment on KA-induced hippocampal interneuron and pyramidal cell loss and damage.

The results of cell assessment clearly demonstrate that MI possesses cytoprotective properties on KA-induced neuronal cell loss/damage. These protective potencies are differentially expressed according to the

levels of the hippocampus. Otherwise: different areas and layers of the hippocampus aren't equally protected by MI. In particular, neuroprotective effect of MI was most strongly expressed in the pyramidal layer of CA1, which was the most damaged as a result of SAL+KA treatment (specifically at 14 and 30 d time points). After pyramidal cell layer of CA1, at the same time points the protective effect of MI was clearly observed in radial and oriental layers of CA3 that were also characterized with considerable loss of neurons in response to SAL+KA action. Moreover, in less degree, protecting effect of MI was observed in almost all layers of hippocampus structure.

Our electron microscopic data also point out to the cytoprotective effect of MI. Thus, in SAL+KA-treated rats we described significant mitochondrial destructions which are recognized as associated with mitochondrial dysfunctions. Such mitochondrial dysfunctions, along with increased production of reactive oxygen species and nitric oxide, are known to precede neuronal cell death and to cause subsequent epileptogenesis (Chuang 2010; Chuang et al. 2010; Kudin et al. 2002; Sobaniec-Lotowska and Lotowska 2011). However mitochondrial alterations were rare in rats pretreated with MI. Also extremely rare were other significant pathological modifications detected in the case of SAL+KA treatment: considerable vacuolar degeneration of cisterns of endoplasmic reticulum or vacuolization of parts of cytoplasm. Such modifications are also considered as leading to neuronal death. Furthermore, in this material we didn't observe the shape-less "electron-dense "residues" of cells and/or synaptic terminals that were present as a result of KA treatment.

In this study we didn't stress on the mechanisms of cell death provoked by KA-induced SE; no specific staining addressing apoptosis was used. Therefore our data do not give the possibility to discuss the nature of cell death. However, as it was mentioned above, on EM level we observed several dark shrunken perikaryons with picnotic nuclei containing small dispersed chromatin clumps and cytoplasmic vacuoles some of which could be swollen mitochondria with partly disrupted cristae or cisterns of endoplasmic reticulum. Such peculiarities remind ultrastructure of necrotic cells. Large shape-less electron-dense "residues" could also be the rests of fully destructed necrotic cells. At the same time, no "apoptotic bodies" were described.

The number of early and modern studies also indicate that different neuronal populations and brain areas are not equally protected by antiepileptic drugs. Thus, Phenobarbital treatment prevents neuronal damage to the hilar neurons but not to CA3 and CA1 pyramidal neurons in the hippocampus of rats treated with KA (Sutula et al. 1992). In another research, studying the effect of two antiepileptic drugs, vigabatrin and carbamazepine in the prevention of SE, it was demonstrated that despite similar anticonvulsant efficacy, vigabatrim treatment is more effective than carbamazepine treatment in decreasing seizure-induced neuronal damage and such neuronal protection is clearer in some regions of the hippocampus than in others and in the amygdale (Pitkanen et al. 1996). Vigabatrin treatment provokes the decrease of the glutamate levels only in the hippocampus (Halonen et al. 1991), but whether the lowered level of glutamate contribute to the neuroprotective effect of vigabatrin in the hippocampus, is unknown. As one of the factors, which could determine the antiepileptic efficacy of an anticonvulsant, the location of seizure focus was proposed

(Pitkanen, 1996). In another research, a novel compound, reported as a non-L-type voltage-sensitive calcium-channel inhibitor was described as preventing the entrance in SE and the loss of, preferentially pyramidal cells, in the hippocampal CA1 and CA3, evoked by injection of KA (Morales-Garcia et al. 2009). Partial agonist of 5-HT1A serotonin receptors with potency for the treatment of neurodegenarive diseases could also reverse oxidative stress in rat hippocampus caused by pilocarpine-induced seizures (de Freitas et al. 2010).At this step it is difficult to fully understand the selective neuroprotection of several antiepileptic treatments. It is only clear that the interference of these treatments with cell death from "epileptogenic" regions might be the consequence of complex and specific molecular processes evoked by each concrete drug on the level of various cells. It should be specially noted that there is still the need and space for novel models with unique characteristics mimicking the human conditions of pharmacoresistant epilepsy. One of such models appears to be orphenadrine induced secondarily generalized SE in rats (Rejdak et al. 2011).

It is difficult to conclude which molecular event/s dictate and regulate the heterogeneous response of different hippocampal neurons to MI pretreatment described in the present study and what are biochemical mechanisms of MI action. During intense neuronal excitation, such as epileptiform bursts, massive intracellular influx of Na+, Ca2+ and Cl- takes place; it leads to water inward flow, cellular swelling and extracellular space reduction (Choi 1987; Dietzel et al. 1982; Rothman and Samaie 1985). Under such conditions various enzymes cannot function normally (Yancey et al. 1993). In addition, reduced extracellular space enhances neuronal excitability (Dudek et al. 1990; Schwartzkoin et al. 1998). For compensation of this disturbed condition neuronal and other cells increase intracellular concentration of small organic osmolytes by up-regulation of osmoprotective gene expression (e.g. taurine-transporter, tonicity-responsive enhancer binding protein, Aldol-reductase, Na+/myo-inositol co-transporter are up-regulated 2h after SE) (Nonaka et al. 1999; Rowley et al. 2011; Weise et al. 1996), which do not perturb the function of intracellular enzymes. One such important osmolyte appears to be MI (Fisher et al. 2002). Thus, one possible mechanism of MI antiepileptic action could be osmoprotection. Studying the ultrastructure of rats treated with SAL+KA, we have found swollen perikarions and dendrite shafts that were absent in the rats, pretreated with MI. It is very likely that such electron-microscopic data represent an indirect argument in favor of abovementioned suggestion. Earlier we have shown that MI inhibits ^{3}H-muscimol binding and increases ^{3}H-MK-801 (activation-dependent antagonist of NMDA receptors) binding to rat brain membranes (Solomonia et al. 2004). Based on these findings we propose that MI could exert its antiepileptic action by modulation of neuronal activity through direct influence on GABA-ergic or/and glutamatergic synaptic transmission, in particular by modulatory action on respective postsynaptic receptors**.** Generally, our histological and electron-microscopic studies indicate that antiepileptic properties of MI, in addition to molecular and behavioral levels, were reflected on the structure of hippocampus – one of the most damaged regions of epileptic brain: as a result of MI+KA treatment the pyramidal cells and interneurons of the hippocampus were much more preserved than after SAL+KA treatment. Therefore, the research confirms recent opinion that in addition to other functions, MI possesses

antiepileptic/antiepileptogenic properties and might have high potential for epilepsy treatment (Solomonia et al. 2010, 2012).

5. Conclusion

In the present study we have used Myo-inositol pretreatment in the physiological dose, which is known to attenuate the seizure severity and several molecular changes produced by status epilepticus. We show that such pretreatment significantly decreases the damage of neurons in different levels of CA1 and CA3 of the hippocampus provoked by kainic acid-induced status epilepticus We suppose that time-dependent and subfield specific changes in the hippocampal cell survival observed in Myo-inositol pretreated rats should be the consequence of Myo-inositol interference with the process of epileptogenesis at its very early stages and later.

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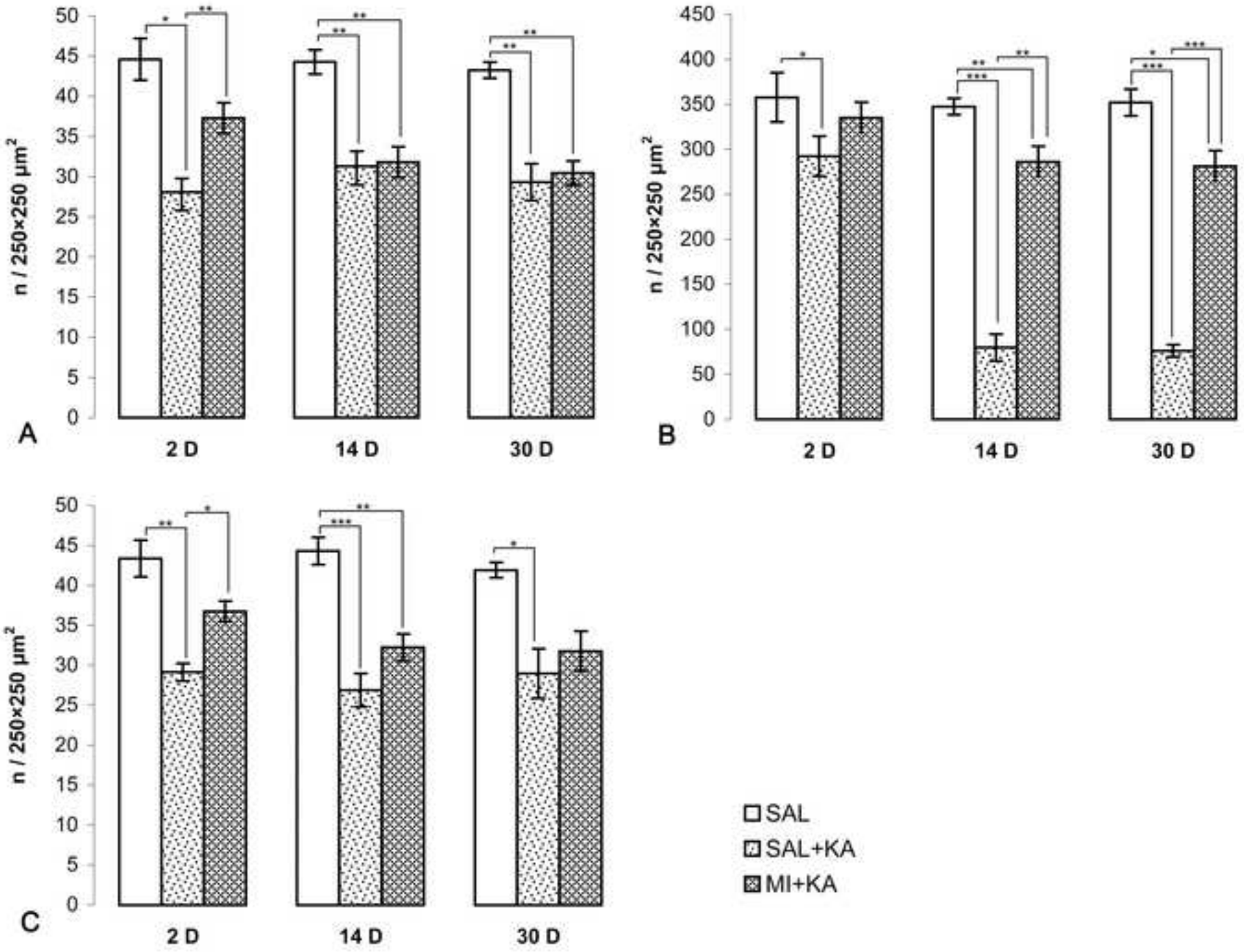

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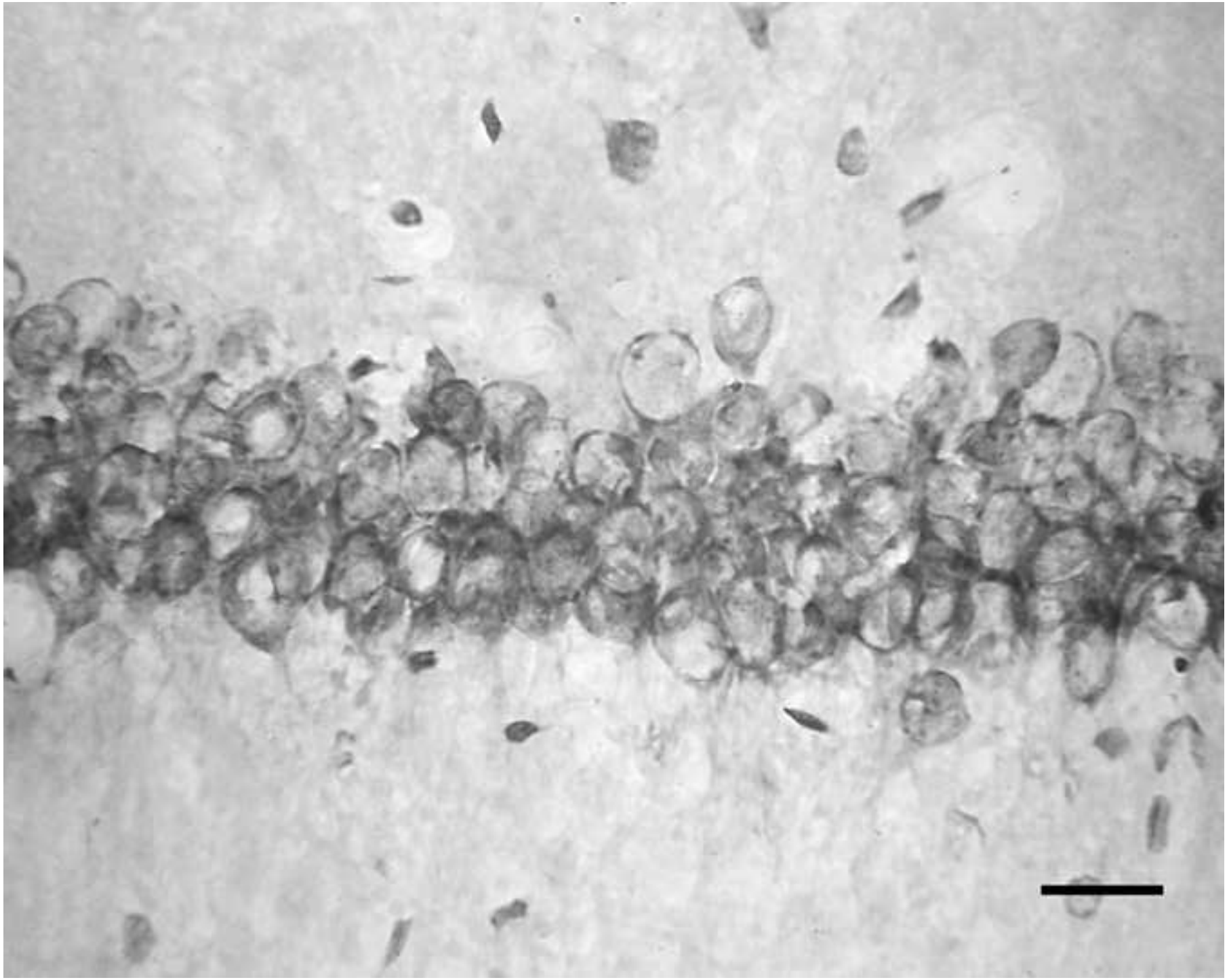

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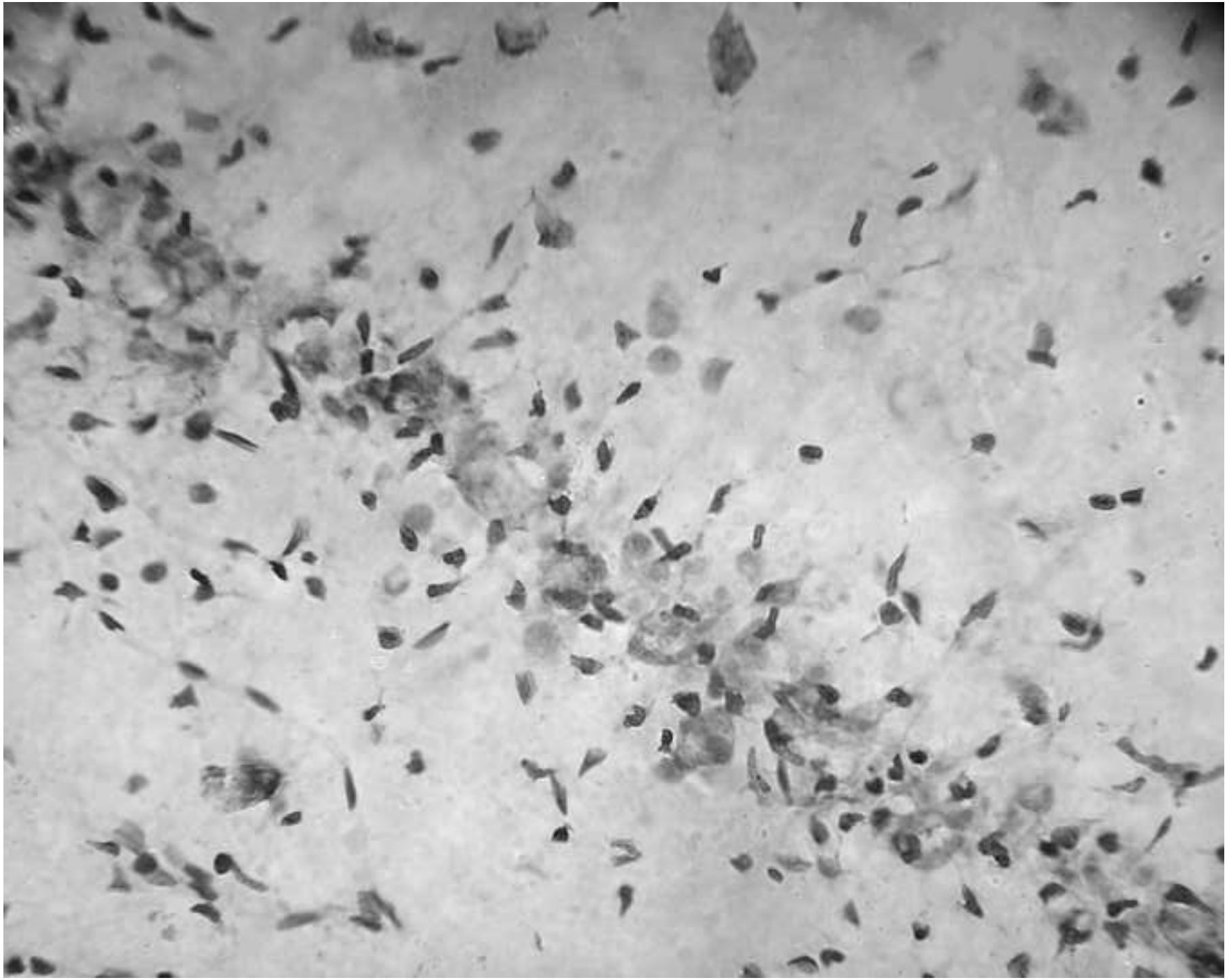

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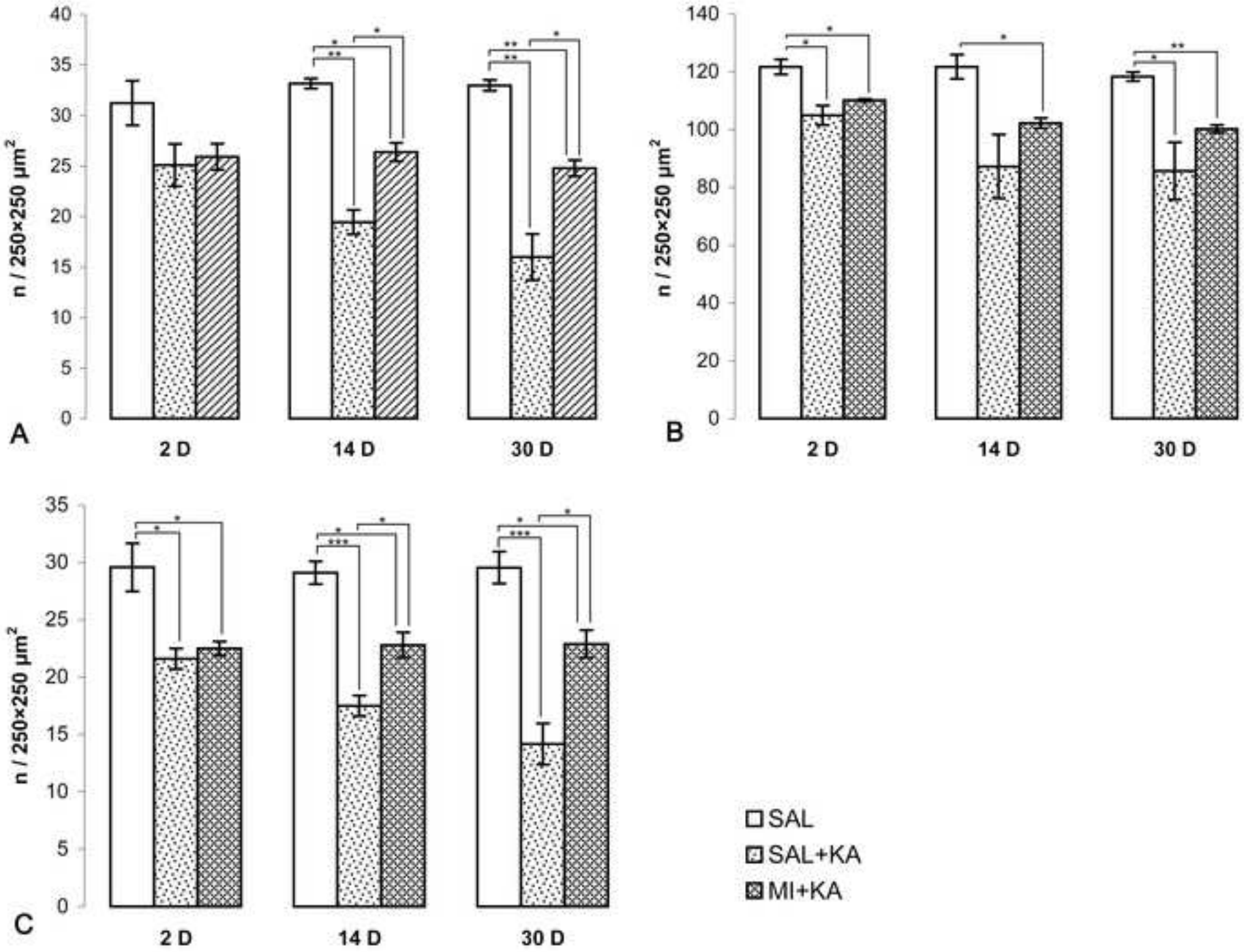

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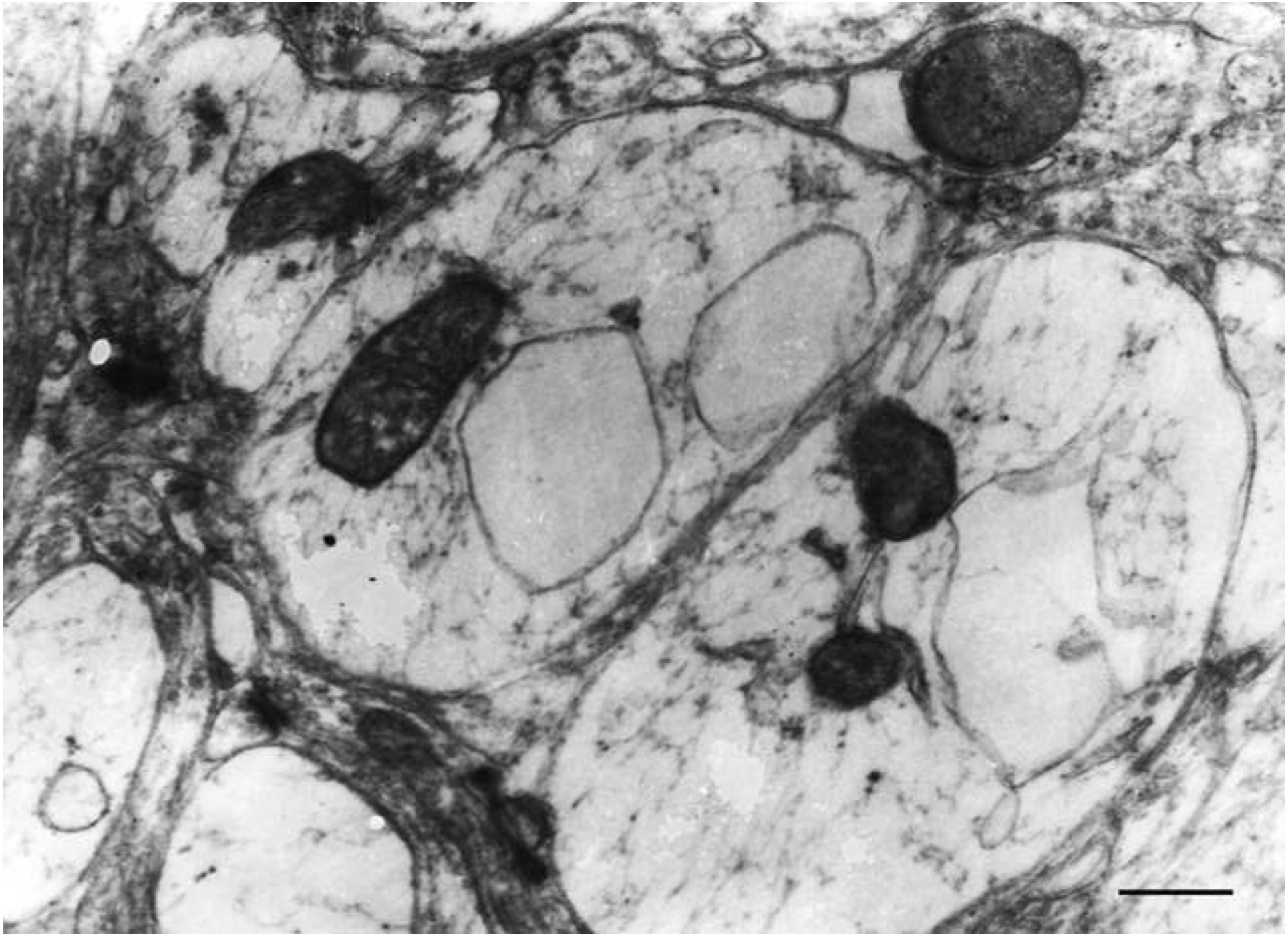

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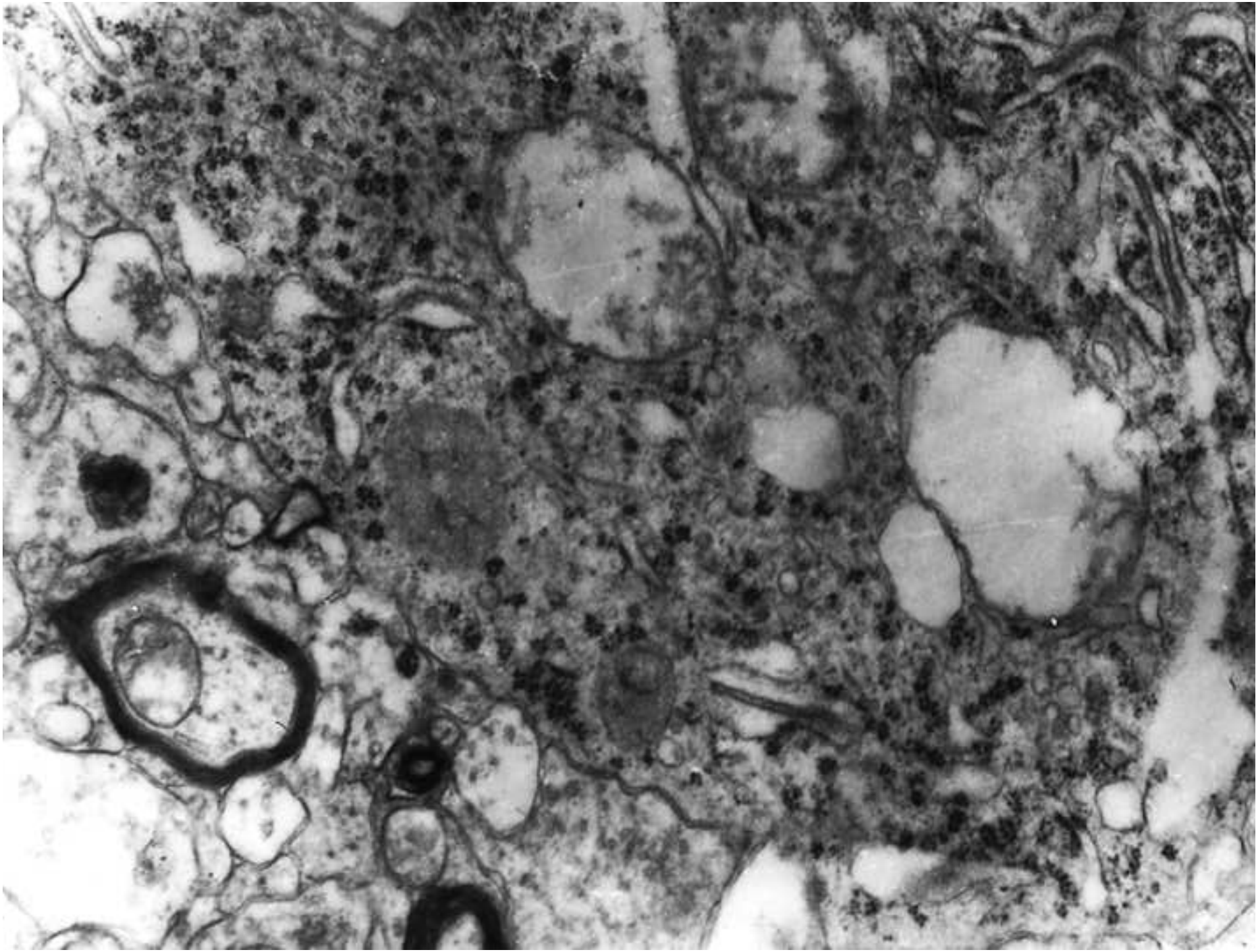

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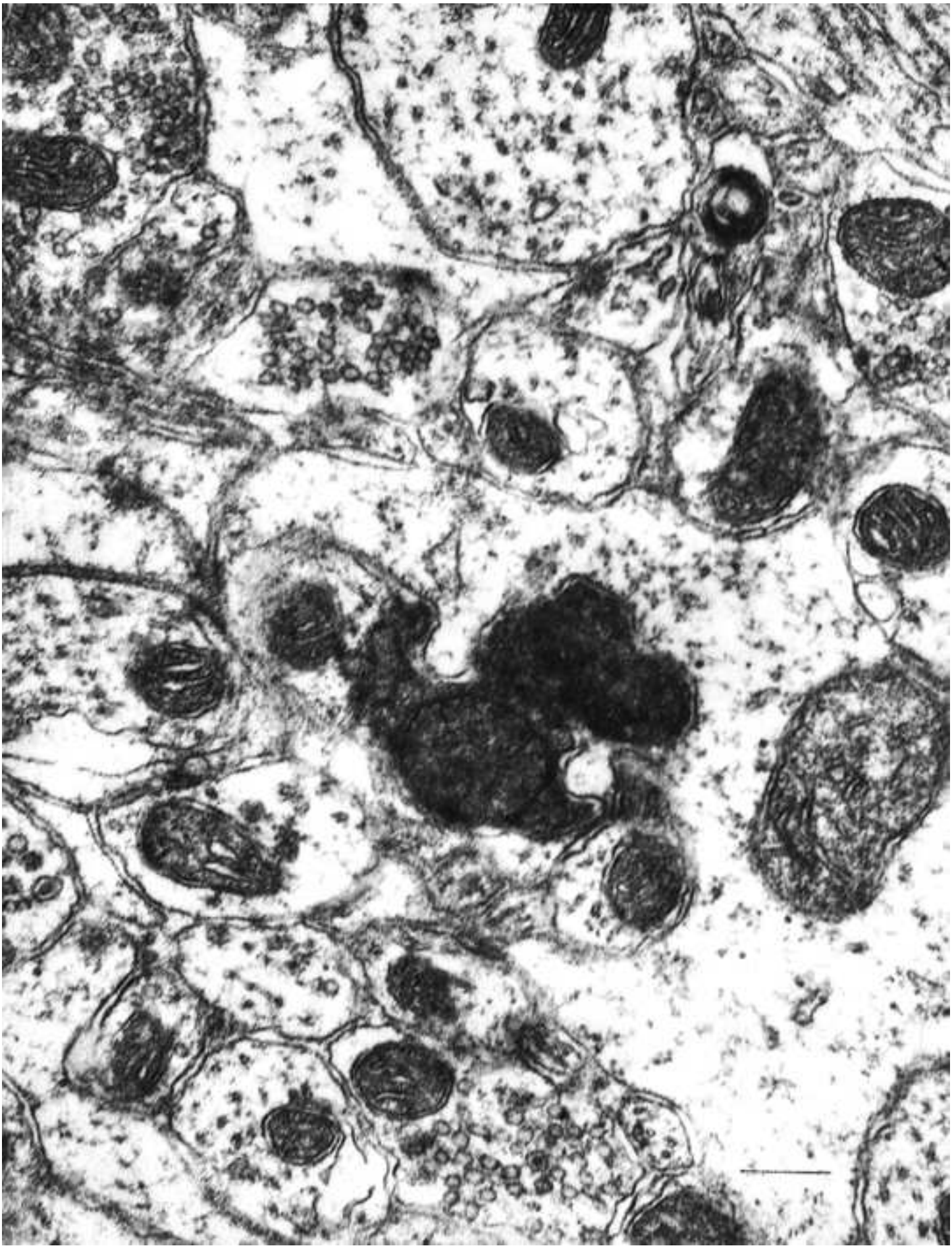

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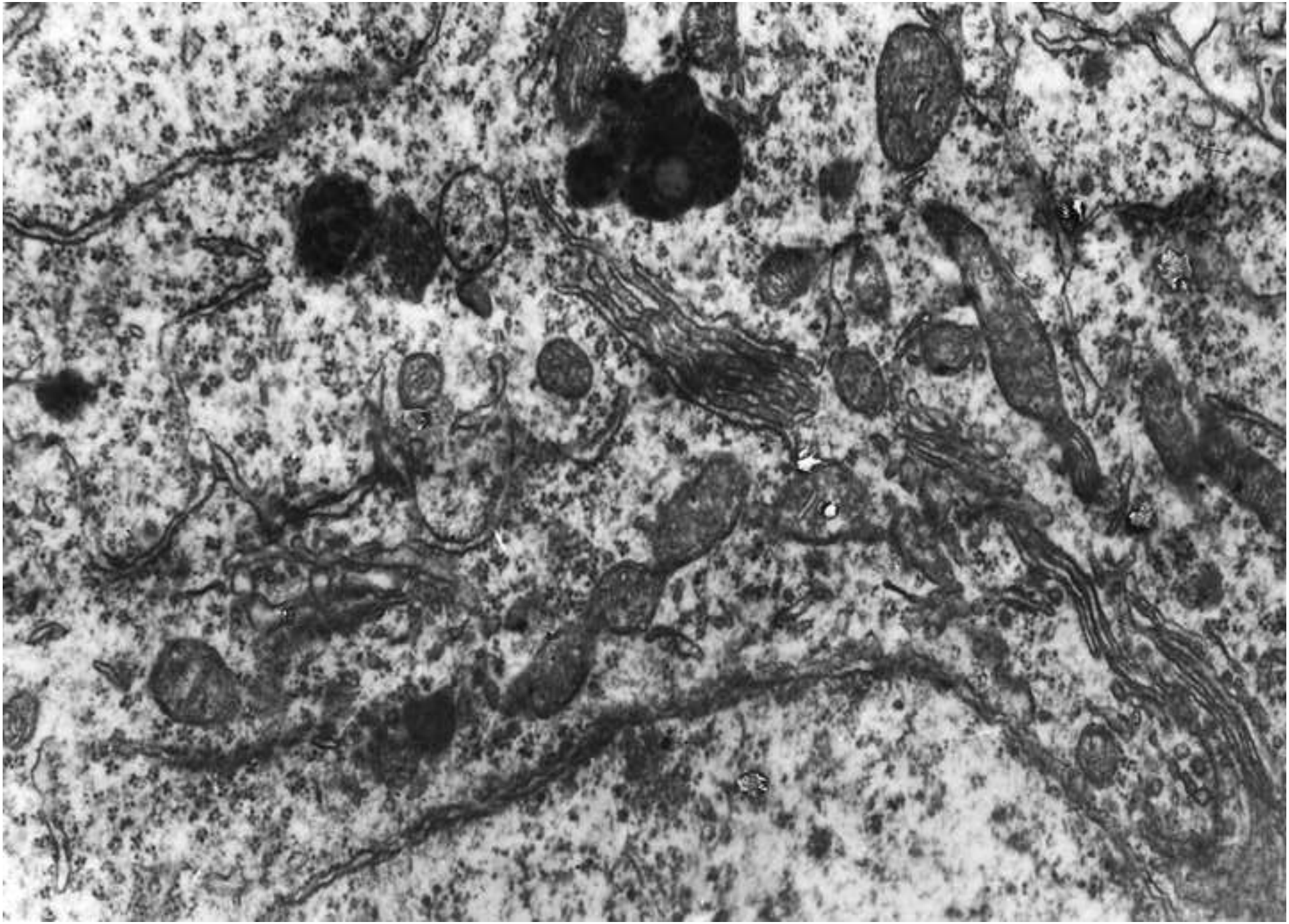

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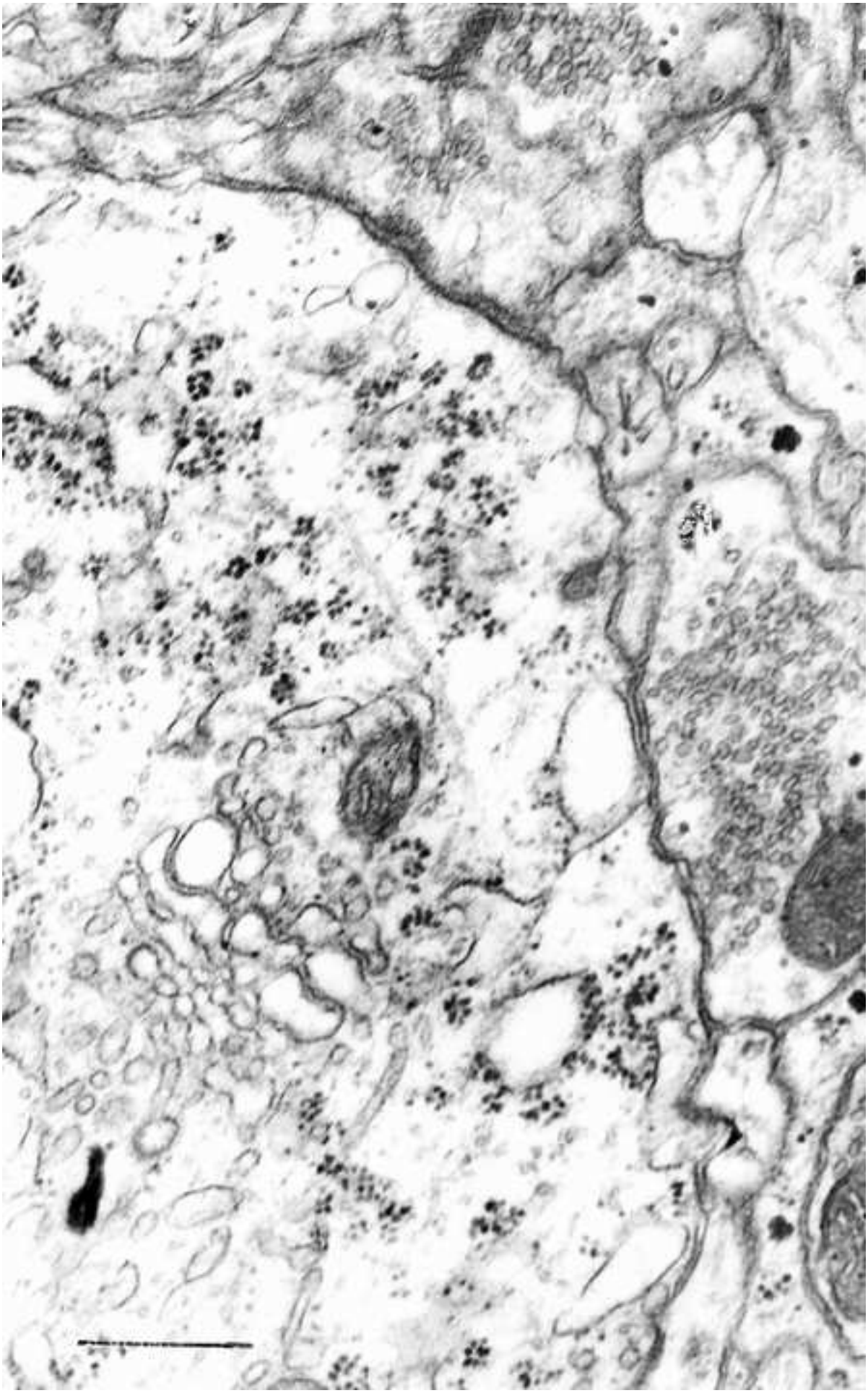

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Fig. 1. Sections of the rat brain hemisphere (A) and hippocampal subregion (B) demonstrating one of the levels used for cell counting. (A) Scale = 1.4 μm; (B)OL – Oriental Layer, PL – Pyramidal Layer, RL – Radial Layer; Scale = 60 μm.

Fig. 2. Mean number of neurons +/- SEM in the Oriental (A), Pyramidal (B) and Radial (C) layers of CA1 in rat treated with KA and MI+KA. 2D, 14D, 30D – 2, 14, 30 days – timeframe of KA and MI+KA exposure; n / 250 x 250 μm2 - number of cell counts per counting frame area (250 x 250 μm2); • -$p<0.05$, •• - $p<0.01$, ••• - $p<0.001$.

Fig. 3. The photomicrographs demonstrating the obvious decrease of principle cells in pyramidal layer of CA 1 and protective effect of MI. A. – SAL, B. – SAL+KA, C. – MI+KA. Magnification x 200; Scale = 25 μm.

Fig. 4. Mean number of neurons +/- SEM in the Oriental (A), Pyramidal (B) and Radial (C) layers of CA3 in rat treated with KA and MI+KA. 2D, 14D, 30D – 2, 14, 30 days – timeframe of KA and MI+KA exposure; n / 250 x 250 μm2 - number of cell counts per counting frame area; • - $p<0.05$, •• -$p<0.01$, ••• - $p<0.001$.

Fig. 5A: **Two dendritic shafts with big vacuoles in the CA1 area of the hippocampus;** 14 d after SAL+KA treatment. X 25 000 (Bar – 0.4 μm).

Fig. 5B: **Destructed mitochondria and swelled cisterns of endoplasmic reticulum in the CA1 area of the hippocampus;** 14 d after SAL+KA treatment. X 25 000 (Bar - 0.4 μm).

Fig. 5C: Degenerated synaptic terminal partly engulfed with astrocyte processes in the CA1 area of the hippocampus; 14 d after SAL+KA treatment. X 25 000 (Bar - 0.4 μm).

Fig. 5D: Fragment of perikaryon in the CA1 area of the hippocampus; 14 d after MI+KA treatment. X 25 000 (Bar - 0.4 μm).

Fig. 5E: Fragment of perikaryon in the CA1 area of the hippocampus; 14 d after MI+KA treatment. X 25 000 (Bar – 0.4 μm).

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Treatment factors and their interaction						
	Treatments		Time		Interaction	
	$F_{3, 32}$	P	$F_{3, 32}$	P	$F_{3, 32F}$	P
CA1 Area						
Oriental layer (OL)	48.66	<.0001	1.52	0.2399	1.15	0.3583
Pyramidal layer (PL)	322.9	<.0001	75.38	<.0001	22.26	<.0001
Radial layer (RL)	43.6	<.0001	1.48	0.2485	0.4	0.8066
CA3 Area						
Oriental layer (OL)	55.5	<.0001	3.04	0.0674	2.89	0.0448
Pyramidal layer (PL)	19.25	<.0001	3.13	0.0628	0.3	0.8749
Radial layer (RL)	60.23	<.0001	3.3	0.055	1.8	0.1633

Table. 1. Table 1. Summary of two - way ANOVAs results. F- variance ratio from two-way ANOVA; P-probability.

CA 1 Area										
Timeframe of treatment		2 Days			14 Days			30 Days		
Type of treatment		SAL	SAL+KA	MI+KA	SAL	SAL+KA	MI+KA	SAL	SAL+KA	MI+KA
Layers	Statistics									
OL	Mean	44.64±2.6	28.10±1.7	37.30±1.9	44.30±1.5	31.30±1.9	31.83±1.9	43.28±1.0	29.33±2.3	30.47±1.5
	P-value		0.006†	0.087† 0.037‡		0.003†	0.007† 0.855‡		0.005†	0.006† 0.696‡
PL	Mean	357.8±27.4	292.4±22.2	335.3±4.7	347.7±9.0	79.7±15	286.43±4.0	352.2±15	76±6.8	281.3±2.0
	P-value		0.025†	0.218† 0.088‡		0.000†	0.003† 0.001‡		0.000†	0.017† 0.000‡
RL	Mean	43.35±2.3	29.13±1.1	36.73±13	44.3±1.7	26.88±2.1	32.23±1.7	41.90±0.97	28.98±3.1	31.77±2.5
	P-value		0.005†	0.060† 0.015‡		0.001†	0.008† 0.119‡		0.028†	0.063† 0.521‡

Table 2. Effect of KA and MI administration on the number of neurons in the oriental (OL), pyramidal (PL) and

radial layers (RL) of CA1. Data are given as mean ± SEM; † - indicates vs. SAL; ‡ - indicates vs. SAL+KA.

CA 3 Area										
Timeframe of treatment		2 Days			14 Days			30 Days		
Type of treatment		SAL	SAL+KA	MI+KA	SAL	SAL+KA	MI+KA	SAL	SAL+KA	MI+KA
Layers	Statistics									
OL	Mean	31.25±2.2	25.10±2.1	25.93±1.3	33.18±0.5	19.45±1.2	26.4±0.9	33.0±0.54	16±2.3	24.8±0.81
	P-value		0.111†	0.104† 0.753‡		0.002†	0.023† 0.010‡		0.006†	0.004† 0.042‡
PL	Mean	121.8±2.6	105±3.4	110.2±0.49	121.8±4.2	87.3±11	102.3±1.7	118.4±1.5	85.7±9.9	100.3±1.3
	P-value		0.017†	0.022† 0.269‡		0.056†	0.024† 0.256‡		0.046†	0.01† 0.236‡
RL	Mean	29.58±2.1	21.6±0.9	22.5±0.6	29.10±1.0	17.5±0.9	22.8±1.1	29.55±1.4	14.18±1.8	22.9±1.2
	P-value			0.024†	0.046† 0.472‡		0.000†	0.013† 0.018‡		0.001†

0.02