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Targeting ischaemic stroke with a novel opener of ATP-sensitive potassium channels in the brain

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Abstract

During cerebral ischaemia, the opening of neuronal ATP-sensitive potassium channels (K_{ATP} channels) affords intrinsic protection by regulating membrane potential. To augment this endogenous mechanism, we have synthesized a K_{ATP} opener, iptakalim. Through K_{ATP} channel activation, iptakalim affected multiple pathways of the glutamatergic system, limiting glutamate release and receptor actions, which are involved in excitotoxicity during ischaemic damage. The molecule readily penetrated the brain and showed low toxicity in animal experiments. In different animal models of stroke as well as in cell cultures, iptakalim provided significant neuroprotection, not only in promoting behavioral recovery but also in protecting neurons against necrosis and apoptosis. This compound thus has promise as a neuroprotective drug for the treatment of stroke and other forms of neuronal damage.

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Introduction

During ischaemic insult, a high rate of neuronal death always leads to permanent functional loss. Protecting against the risk of neurons dying in the ischaemic brain is thus still the main strategy for human stroke therapy. Years of animal research have resulted in a better understanding of the complex pathophysiology of ischaemic brain injury and this has led to drugs aimed at ameliorating neuronal death. Conventional neuroprotectants often target one specific receptor that participates in the neurotoxic cascade, such as the NMDA excitatory amino acid receptors. Other strategies such as the scavenging of free radicals target only one causal factor in neuronal death (Wahlgren and Ahmed, 2004). A valuable clue provided by an endogenous protective mechanism in the brain has probably been neglected. This involves positive reactions against the excess membrane depolarization that is initiated by reduced energy stores following lowered cerebral blood flow in cases of stroke. This process is often mediated by the ATP-sensitive K^+ channel (K_{ATP} channel), the only channel protein that directly couples the metabolic state of a cell to its electrical activity (Ascroft and Ascroft, 1990). This channel is usually closed in normal conditions, but is activated rapidly in response to decreases in intracellular ATP/ADP ratio under ischaemic conditions, causing a K^+ efflux. Channel opening appears to hyperpolarize the cell membrane, limiting neuronal excitability and Ca^{2+} influx, and thus blocking the subsequent neurotoxic biochemical cascade (Seino and Miki, 2003). In view of this intrinsic protective role of the K_{ATP} channel, pharmacological activation of this channel should produce neuroprotection. Some K_{ATP} openers have been reported effective in preclinical experiments (Takaba et al, 1997; Heurteaux et al, 1993; Wind et al, 1997; Lauritzen et al, 1997). However, they lack clinical effectiveness, mainly because of strong detrimental side effects or poor brain penetration.

We hypothesized that the development of K_{ATP} channel openers with low toxicity and brain-permeable characteristics would be effective for stroke therapy. We have designed and synthesized thousands of compounds and have finally screened out the most promising candidate, 2,3–dimethyl–2–butylamine, iptakalim. It is remarkably different from other known K_{ATP} channel openers, having a fatty para-amine structure (Wang et al, CN1365967A). Here we present data supporting the neuroprotective role of this compound in the amelioration of experimental stroke.

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Materials and Methods

Global ischaemic model. Mongolian gerbils (60±5g) of either sex were anesthetized, followed by bilateral carotid artery ligation for 5 min. Sham-operated gerbils were sacrificed just after exposing the carotid artery without clamping the vessels (Small et al, 1999). The animals were kept at normal body temperature before and after the operation by using heating lamps. Throughout the operation, rectal temperature was maintained at 37±0.5°C with a heat blanket with feedback control. Room temperature was regulated to 22~25°C by air-condition. Drugs were intraperitoneal administered 40 min before carotid artery ligation and every day up to day 7 following the operation. On days 1 and 5 after operation, all the gerbils were placed in an open field maze for 15 min while their locomotor activities were recorded. Locomotor activity included movements across squares and rears (rearing up on haunches), which respectively represented the exploring activities in horizontal and vertical orientation. On days 2 and 3, the gerbils were trained in the T-maze (15 pairs of trials/day). On days 4 and 6, the gerbils were given 15 pairs of trials/day and the percentages of correct choices were determined to measure the working memory of the animals (Corbett et al, 1998). For histological endpoints detected, global ischeamic gerbils treated with drugs up to day 7 were anesthetized by an overdose of sodium amylbarbitol and then were perfused transcardiac with 50 ml of saline and 50 ml of 4% buffered polyformaldehyde. Brains were removed and stored in pluformaldehyde for 24 hr. After fixation, the brains were coronally sectioned at 6μm. One set of section was stained with HE (haematoxylin and eosin) and cells in the CA1 region of hippocampus were counted. Another set of section was used for TUNEL (terminal deoxynucleotidyltransgerase-mediated dUTP nick end-labeled).

Salt load SHRsp stroke model. Stroke-prone spontaneously hypertensive rats (SHRsp, 10 weeks old, 150±30g) of either sex were given 1% NaCl solution as a substitute for drinking water every day to accelerate the onset of stroke. The trial group was orally given iptakalim or nimodipine (the positive control drug) per day for 12 weeks. The onset of stroke in each animal was recorded and succeeding neurological deficits were observed every day for 14 days. The neurological deficits were evaluated by a specially designed scoring system described previously (Zhang et al, 1996): (0), no deficit; (1), mild stress; (2), forelimb or head twitch or with severe stress; (3), hemi-paralysis, body inclined or

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disabled; (4), paralysis, tremor or convulsion. Systolic blood pressure and heart rate were measured once every 2 weeks by standard tail cuff technique (BP recorder, RBP-I, China). All animal procedures were performed in accordance with the Guide For The Care and Use of Laboratory Animals (NIH publication, 85-23, revised 1995).

Primary neuron culture and apoptosis assay. Neurons were obtained from the cortex of newborn rats, as described (Black et al, 1995). For flow cytometry, the primary culture medium was replaced on 12-day neuronal cells with low glucose DMED medium without serum. After culturing in a hypoxic container (filled with 95% N_2 + 5% CO_2) for 16 h, the cells were transferred to normal environments for 24 h. Drugs were added before the hypoxic treatment. After re-oxygenation for 24 h, cells were collected, dyed with 100 μg/ml PI and detected by flow cytometry (FACSCalibur). For electron microscopy, cells were examined using a PHILIPS TECHAI-10 transmission electron microscope.

Glutamate release and uptake assay. PC12 cells were cultured in DMEM medium containing 10% fetal cattle serum at 37 °C in a CO_2 incubator. For glutamate release, the cells were plated on to 24-well plates, high K^+ solution (80 mM) was added, and culture medium was collected after 12 h incubation. Proteins were precipitated using 0.2 M $HClO_4$ and the supernatants after centrifugation were mixed and vibrated with derived solution (OPA 20 mM, β-mercaptoethanol 2 mM, sodium tetraborate 25 mM, carbinol 50%, pH 9.6) of equal volume for 3 min. Glutamate was measured by HPLC combined with fluorescent detector analysis. For measurement of [^{3}H]-glutamate uptake, the culture medium was replaced by Hanks' balanced salt solution (HBSS) for 30 min. L-[^{3}H]-Glutamate (1 μCi/ml) was added for 7 min at 37 °C, then ice-cold HBSS was added to end the reaction. After three washes in HBSS, 200 μM NaOH was added to the cells and radioactivity was measured by liquid scintillation counting. The protein contents of the cells were determined by Lowry's assay.

Patch-clamp recording. The whole-cell configuration was conformed by sucking the neuronal membrane into the pipette when the resistance was more than 1 GΩ. The electric signals were acquired by an Axopatch-200B amplifier and analyzed using the pCLAMP 7.0 program (Axon

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Instruments). Drugs were administered by a pneumatic ejector (Picospritzer II, Parker Hannifin Co.). For potassium currents or glutamate currents recording, the extracellular bathing solution contained (in millimoles per litre): NaCl 140, $MgCl_2$ 1, KCl 5, $CaCl_2$ 3, HEPES 10, glucose 10 and TTX 0.001. For sEPSCs recording, TTX was omitted from the extracellular bathing solution. For sodium currents recording, the extracellular bathing solution contained (in millimoles per litre): NaCl 140, $MgCl_2$ 1, KCl 5, $CaCl_2$ 3, TEA 10, 4-AP 1, $CdCl_2$ 0.2, HEPES 10 and Glucose 10. For calcium currents recording, the extracellular bathing solution contained (in millimoles per litre): NaCl 140, $MgCl_2$ 1, KCl 5, $CaCl_2$ 3, TEA 10, 4-AP 1, TTX 0.001, HEPES 10 and Glucose 10. For potassium currents, glutamate currents or sEPSCs recording, the pipette solution contained (in millimoles per litre): KF 140, EGTA 10 and HEPES 10. For sodium currents recording, the pipette solution contained (in millimoles per litre): CsCl 140, TEA 10, EGTA 10 and HEPES 10. For calcium currents recording, the pipette solution contained (in millimoles per litre): CsCl 140, TEA 10, Na_2ATP 2, EGTA 10 and HEPES 10.

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Results

Profiles on brain potassium channels

Using the whole cell patch-clamp technique, we investigated the effects of iptakalim on potassium channels in cultured rat hippocampus neurons. Iptakalim enhanced the potassium currents at 1, 10 or 100 μM (Fig. 1a, left). The effect of 1μM iptakalim on potassium currents was blocked by co-application of the specific K_{ATP} blocker glibenclamide (Fig. 1a, left). Thus, iptakalim appears to open and activate the neuronal K_{ATP} channels in brain cells.

A conventional membrane ligand-receptor saturation and competitive binding assay was used to explore the possibility that specific iptakalim binding sites may be present in the rat brain. We labeled binding sites in the rat hippocampus, striatum and cortex membranes with [^{3}H]-iptakalim. The Kd values were 2.30, 2.45 and 2.16 nM respectively, and the corresponding Bmax values were 1073, 858 and 715 fmol/mg protein (Fig. 1*b-d*.). Unlabeled iptakalim, or the specific K_{ATP} opener pinacidil, was incubated with rat cortex membrane for 75 min at 25°C. Both iptakalim and pinacidil inhibited the binding of [^{3}H]-iptakalim in dose-dependent manners, with IC_{50} values of 7.08 nM and 6.19 nM, and Ki values of 3.18 nM and 2.72 nM, respectively (Fig. 1*e*). Thus, iptakalim has a high affinity to neuronal K_{ATP} channels in brain.

Effects against the brain glutamatergic system

Glutamate has been implicated as a mediator of neuronal injury in many neurological disorders, so interrupting the glutamatergic system in the brain, for example by inhibition of glutamate release or blockade of glutamate receptors, has been widely evaluated for stroke therapy. We therefore investigated the effects of iptakalim on glutamate release and uptake process. iptakalim dose-dependently inhibited the glutamate release stimulated with 80 mM KCl solution that could be prevented by glibenclamide. Iptakalim at concentrations of 0.1, 1.0, 10, 100 μM inhibited glutamate release by 19.8%, 36.8%, 50.5%, and 60.3%, respectively, and they were decreased to 3.6%, 20.0%, 36.4%, and 48.9% (n = 6) corresponding in the presence of glibenclamide at 1 μM. In the glutamate uptake experiment, 5 and 10 μM iptakalim significantly increased the uptake of [^{3}H]-glutamate into PC12 cells from 2.12 ± 0.16 to 2.72 ± 0.24 ($P < 0.05$, n = 6) and 2.82 ± 0.24 pM/mg protein/min ($P < 0.05$, n = 6) respectively. Simultaneous treatment with 10 μM iptakalim and 20 μM glibenclamide resulted in no change in [^{3}H]-glutamate uptake compared with controls ($P > 0.05$, n = 6). Thus,

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iptakalim inhibits glutamate release and enhances glutamate uptake in brain through activated K_{ATP} channels.

To determine whether iptakalim influences glutamate receptor function, we examined its effects on glutamate-evoked currents in cultured rat hippocampus neurons. Using voltage-clamp at a holding potential of –70 mV, flow pipette application of glutamate (100 μM) for 1 s evoked an inward current. Co-application of 1, 10 and 100 μM iptakalim decreased the current amplitudes significantly. When 30 μM glibenclamide was applied simultaneously, the inhibition of 100 μM iptakalim was attenuated significantly (Fig. 2*a*–*d*). However, application of glibenclamide alone had no significant effect on glutamate-induced currents ($P > 0.05$). We also tested the effect of iptakalim on currents evoked by 100 μM NMDA, an agonist for the NMDA glutamate receptor. Iptakalim reduced the NMDA-currents and glibenclamide also antagonized this effect of iptakalim (Fig. 2*a*–*d*). Thus, iptakalim might suppress the responsiveness of hippocampal neurons to glutamate.

Last, we tested the effects of iptakalim on spontaneous synaptic activity in cultured rat hippocampus neurons. Spontaneous events were recorded at a holding potential of –70 mV. The events detected under these conditions were probably spontaneous excitatory postsynaptic currents (sEPSCs) mediated by spontaneous glutamate release, because the glutamate receptor antagonists MK-801 (10 μM) and NBQX (10 μM) abolished them. Iptakalim dose-dependently inhibited the sEPSCs by decreasing both current frequency and amplitude, reversibly (Figs 2*e*, *f*). Because these two indications respectively reflect transmitter release from presynaptic terminals and drug action to postsynaptic sites, these results supported the idea that iptakalim down-regulates the glutamatergic system. In separate studies, we also noted that iptakalim had no significant effects on sodium and calcium channels, the two other fundamental ion channels in neurons, which are also important for glutamate release and receptor activity (Fig. 1*a*).

Protective effects against neuronal ischaemic injury

To test the neuroprotective effects of iptakalim *in vitro*, we used high concentrations of glutamate to mimic ischaemic injury in PC12 cells. After treatment with 5 mM glutamate for 24 h, cell viability was assessed by an MTT assay. With increased doses, iptakalim significantly attenuated glutamate-induced cytotoxity and this effect was reversed by co-application of glibenclamide (Fig.

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3*a*).

We also explored the effects of iptakalim on neuronal apoptosis using cultured cortical neurons subjected to hypoxia-hypoglycaemia. Flow cytometry was used to measure the DNA content of the neurons and to assess the percentage that was apoptotic. Neurons exposed to hypoxia appeared in the 'G1 sub-peak' and the proportion of apoptosis was 23.8 ± 7.4% after 16 h hypoxia, higher than in the control (2.3 ± 1.2%). Iptakalim at concentrations of 0.1, 1 and 10 μM decreased the proportion of apoptosis dose-dependently, and this effect was blocked by simultaneous application of glibenclamide (Fig. 3*b*). Transmission electron microscopy showed that the neurons in the model group displayed typical apoptotic features such as shrinking of the cell body, chromatin condensation and a crescent structure. Iptakalim thus significantly attenuated apoptotic cell death following hypoxia (Fig. 3*c*).

Protective effects against brain ischaemic injury

Initially, we used the gerbil bilateral carotid artery occlusion model of global ischaemia to investigate the potential efficacy of iptakalim. In behavioral measurements, treatment with iptakalim dose-dependently reduced the increases of squares crossed and rears counted that were evoked by global cerebral ischaemia. Ketamine, the positive control drug, significantly decreased only the rears counted (Figs 4*a*, *b*). The T-maze task revealed significant ischaemic working-memory impairments that were not improved by ketamine, whereas iptakalim markedly increased the percentage of correct choices made by the ischaemic gerbils to obtain a food reward (Fig. 4*c*). Thus, intraperitoneally administered iptakalim reduces the increase of locomotor activity evoked by ischaemia and alleviates ischaemia-induced working-memory impairments. We also tested the effects of iptakalim histologically. HE staining showed extensive losses (overall counted averaged ~15% of sham group) of hippocampus CA1 zone pyramidal neurons in gerbils were induced by global ischemia for 5 min. Treatment with iptakalim dose-dependently suppressed ischemia-evoked hippocampus damage and increased the viable CA1 neurons counted of hippocampus. Treatment with ketamine significantly decreased the number of necrotic neurons and increased the remaining number of healthy neuron in hippocampus CA1 zone (~79% of sham group) (Fig. 4*e*). A TUNEL test showed that ischaemia-evoked apoptotic neuron death in the CA1 zone of the hippocampus was attenuated by ketamine and by an increased dose of iptakalim (Fig. 4*f*). Once ischaemic damage occurs, various

transmitters in brain often change accordingly. To investigate the effects of iptakalim on hippocampal amino acid transmitters during global cerebral ischaemia, the levels of amino acids were assayed by HPLC analysis 60 min after operation. Iptakalim had no effects on the concentrations of these transmitters under non-ischaemic conditions (data not shows), but it reversed the ischaemia-evoked increases in glutamate, aspartic acid, glutamine and glycine. However, it did not reverse the ischaemia-induced increases in γ-aminobutyric acid or taurine (representative results are shown in Fig. 4*d*.).

We also evaluated the therapeutic effects of iptakalim on the model of stroke induced by salt load in stroke-prone spontaneously hypertensive rats (SHRsp). Iptakalim dose-dependently decreased the incidence and mortality of stroke, delayed the onset and prolonged survival time after onset, and nimodipine had similar effects (Figs 5*a*, *b*). The neurological deficit scores within 14 days after the onset of stroke showed that iptakalim and nimodipine alleviated these deficits significantly compared to vehicles (Fig. 5*c*). The systolic blood pressure of SHRsp in vehicle group increased continuously during the experimental period. Under the same experimental conditions, iptakalim 0.25, 1.0 and 4.0mg/kg/d decreased SBP significantly by around 20, 32 and 41 mmHg respectively ($p<0.01$). The heart rates remained unchanged by iptakalim at dose of 0.25, and 1.0 mg/kg/d ($p>0.05$), but were decreased markedly by around 40 beat per minute when treated with iptakalim 4.0 mg/kg/d ($p<0.01$).

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Discussion

Potassium channels, including K_{ATP} channels, mediate the actions of some classical drug targets, such as antidiabetic drugs (Kazic and Gojkovic-Bukarica, 1999). According to the development of CNS therapeutics, the specific targeting of potassium channels is relatively new. Here we suggest a new anti-stroke approach by the augmentation of an endogenous regulator, the K_{ATP} channel. Iptakalim has specific binding sites in the brain and is believed to open this channel moderately. Iptakalim also regulates the mRNA expression of Kir6.x and SUR, two major subunits of the K_{ATP} channels (Liu et al, 2003). Here we found that iptakalim suppressed neuronal necrosis and apoptosis induced by hypoxia and by high glutamate concentrations in cell culture; moreover, in an animal model, iptakalim not only inhibited neuronal necrosis and apoptosis, but also definitely protected brain function. These *in vivo* and *in vitro* results suggest that iptakalim may be neuroprotective in ischaemic stroke and in other acute or chronic neurodegenerative diseases.

Besides nerve tissues, K_{ATP} channels are also found in other tissues, such as pancreatic islets, cardiac muscles, and vascular smooth muscles (Fujita and Kurachi, 2000). Iptakalim shows no significant or detectable effects on heart rates, insulin and glucagon excretion (unpublished observation). However, it demonstrated selective effects on reducing blood pressure in hypertensive animals. (Wang H, 2003) Because hypertension is the major risk factor of stroke, this aspect of iptakalim is likely to expand its therapeutic range.

Application of iptakalim regulated the pathology of multiple neurotransmitter release, inhibiting the excess release of excitotoxic amino acids and glycine from sensitive brain regions induced by acute global ischaemia in gerbils. Nevertheless, it never affected these in normal animals. This selectivity implies that the action of iptakalim may be selective for ischaemic cells, with little influence on normal ones.

In addition to neuroprotective virtues, effective neuroprotectants should enter the brain easily. Following oral (p.o.) administration, iptakalim quickly enters the mouse brain at stable levels (T_{max} = 30 min; C_{max} = 2.25 μg/g in brain; 3 mg/kg, p.o.) with a brain: plasma ratio of 3.2. The drug concentration in brain decreased to 18% of C_{max} within 3 h, and reduced to a very low level at 6 h after administration (unpublished observation). The blood–brain barrier is usually the rate-limiting step in the translation of many neuroprotective molecules, such as neurotrophin, into clinically effective

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neurotherapeutics (Pardridge, 2002). iptakalim penetrates the brain quickly and freely, even when taken orally. This profile may be attributed to its unusual chemical structure and low molecular weight.

Glutamate, the major neurotransmitter in the central nervous system (CNS), is the critical cause of excitotoxicity in CNS pathology (Choi, 1988). We have shown that iptakalim, via its action on neuronal K_{ATP}, decreases the toxicity of glutamate and exerts protective effects. The evidence comes from three sets of results: (a) inhibition of glutamate release by opening the presynaptic K_{ATP} channels both *in vivo* and *in vitro*; (b) inhibition of glutamate receptor activity by opening the postsynaptic K_{ATP} in patch-clamp experiments and (c) enhancement of uptake by the glutamate transporter, and decrease in glutamate concentration in the synaptic cleft by opening the K_{ATP}. Because glutamate transporter functional deficiency is found in many neurodegenerative diseases (Ferrarese et al, 2000; Vandenberg, 1998), researchers are looking for drugs that effectively stimulate this transporter. That glutamate uptake can be enhanced through K_{ATP} channel opening suggests a clue for developing novel drugs.

In the binding test, pinacidil competed with [^{3}H] iptakalim with regular inhibition curves extrapolating to complete inhibition at saturation, this compatible with a direct competition for a homogeneous class of binding sites of iptakalim. The fact that glibenclamide did not inhibit [^{3}H] iptakalim binding indicated it binding to a class of site which is different from iptakalim. Glibenclamide is a specific blocker of K_{ATP} channels and has a specific binding site for SUR subunits of K_{ATP}. The opening of K_{ATP} by KCO or other modulators could be significant inhibited by glibenclamide, but the blocker may do not occupy the same binding site of some kind of KCO directly just like iptakalim. The possible explanation may be that glibenclamide bind to a different binding site negatively allosterically coupled to the iptakalim site. It will change the conformation of SUR and thus regulate the specific binding of iptakalim in the negative allosteric manner in brain membranes. This hypothesis could be supported by our results in cardiac membranes (Cui et al, 2004). In that study, the specific bindings of [^{3}H] glibenclamide could not be displaced by iptakalim or pinacidil, and iptakalim could accelerate the dissociation kinetic process of [^{3}H] glibenclamide binding with SUR without affecting the association kinetic process. That indicated the allosterical modulation of iptakalim on the binding sites of K_{ATP} blocker glibenclamide.

Iptakalim is relatively safer with few side effects (unpublished observation). In acute toxicity

studies, iptakalim has shown low toxicity in mice, rats and dogs. The LD_{50} values are 63 mg/kg (i.v.) and 338 mg/kg (p.o.) in mice, and 413 mg/kg (p.o.) in rats. In dogs, the highest non-fatal dose is 205 mg/kg (p.o.). In long-term toxicity studies, no iptakalim-related cytotoxic effects were observed following oral administration for 180 days at doses up to 64 mg/kg/day in rats. Iptakalim was not genotoxic when evaluated in a battery of *in vitro* and *in vivo* assays, and was not teratogenic in mice. In another series of experiments, at the therapeutic doses, iptakalim exhibits no side effects on the central nervous, respiratory, digestive and endocrine systems. Therefore, this compound has wide potential safe dose range if used for anti-stroke therapy.

In conclusion, iptakalim influences the multiple-pathway glutamatergic system via K_{ATP} channel activation, and produces definite neuroprotection *in vivo* and *in vitro*. In addition, this compound passes the blood-brain barrier readily and shows less adverse effects in animals. Iptakalim can be expected to develop into a potential candidate compound against stroke.

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Footnotes

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Legends for Figures

Figure 1. Effects of iptakalim (Ipt) on potassium channels. a: Effects of iptakalim alone or in combination with glibenclamide (Gli) on potassium currents (left) in cultured rat hippocampus neurons. Data are expressed as means ± SDs (n = 9). **, $P < 0.01$ versus control. ##, $P < 0.01$ versus iptakalim 1 μM alone. Iptakalim had no significant effects on sodium currents (middle, n = 5) or calcium currents (right, n = 6) in cultured rat hippocampus neurons. b–d: Saturation curves of binding of [^{3}H]-Ipt to sulfonylurea receptors of K_{ATP} channels from rat cerebral cortex (b), hippocampus (c), and striatum (d) membrane preparations. Inset shows a Scatchard plot for saturation binding. e: Competitive inhibition of iptakalim, pinacidil (Pin) and glibenclamide on the specific binding of [^{3}H]-Ipt in rat cerebral cortex membrane preparations. Data are expressed as means ± SDs (n = 4).

Figure 2. Effects of iptakalim (Ipt) on glutamate (Glu) receptors and transmission. a: Iptakalim (100 μM) inhibited glutamate and NMDA-evoked currents in cultured rat hippocampus neurons. Glutamate or NMDA were used at 100 μM. b: Different doses of iptakalim on the relative amplitudes of glutamate and NMDA-evoked currents (n = 6). ** $P < 0.01$ versus control. c and d: Antagonism of glibenclamide (Gli) against the inhibitory effects of iptakalim in glutamate and NMDA-evoked currents. Iptakalim or glibenclamide were used at 100 μM and 30 μM, respectively. Data were recorded from six neurons. ** $P < 0.01$ versus controls; #, $P < 0.05$, ##, $P < 0.01$ versus iptakalim treatment. e and f: spontaneous excitatory postsynaptic currents from six neurons. * $P < 0.05$; ** $P < 0.01$ versus controls. All data are expressed as means ± SDs.

Figure 3. Neuroprotective properties of iptakalim (Ipt) in cell culture. a: Effects of iptakalim and glibenclamide (Gli) against glutamate (Glu)-induced cytotoxicity in PC12 cells (n = 6). **, $P < 0.01$ versus normal; ##, $P < 0.01$ versus glutamate treatment; △△, $P < 0.01$ versus treatment with glutamate and iptakalim. b: Effects on apoptosis induced by hypoxia in rat cortical neurons. Data are expressed as means ± SDs. 10,000 neurons were counted in each group. * $P < 0.05$; ** $P < 0.01$ versus the hypoxic vehicle group. # $P < 0.05$ versus the 10 μM Ipt group. c: Apoptosis shown by transmission electron microscopy. I: Normal neurons; II, III: Hypoxic neurons; IV: The neurons were treated with Ipt at 10 μM under hypoxic conditions. Bar = 1 μm

Figure 4. Neuroprotective properties of iptakalim (Ipt) in globally ischaemic gerbils. a, b and c: Open field locomotion scores (a, b) and working memory activity in T-maze (c) (n = 8). d, Contents of glutamate (Glu) and GABA in hippocampus (n = 7). Data are expressed as means ± SDs. * $P < 0.05$; ** $P < 0.01$ versus sham-operated group; # $P < 0.05$; ## $P < 0.01$ versus ischemia group. e, Normal cell counts (percentage of sham) in medial, middle, and lateral sector of CA1 zone in dorsal hippocampus of gerbils with global cerebral ischaemia. Data are expressed as mean±SD, n= 6. $^{\#}P< 0.05$, $^{\#\#}P< 0.01$ versus ischaemia group. f: TUNEL staining of CA1 pyramidal neurons in dorsal hippocampus of sham-operated; ischaemic; ketamine (Ket)-treated; gerbils treated with iptakalim at 0.5, 1.0, 2.0, and 4.0 mg/kg (I–VII). Short arrowheads indicate TUNEL-positive neurons. Bar = 15 μm.

Figure 5. Neuroprotective properties of iptakalim (Ipt) in stroke-prone spontaneously hypertensive rats (SHRsp). a: Incidence and mortality raters caused by stroke. b: Time of stroke appearance and survival. There were 12 animals in each group. Data are expressed as means ± SDs. * $P < 0.05$; ** $P < 0.01$ versus vehicle-treated group. c: Scores of neurological deficits after the onset of stroke.

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Fig1a

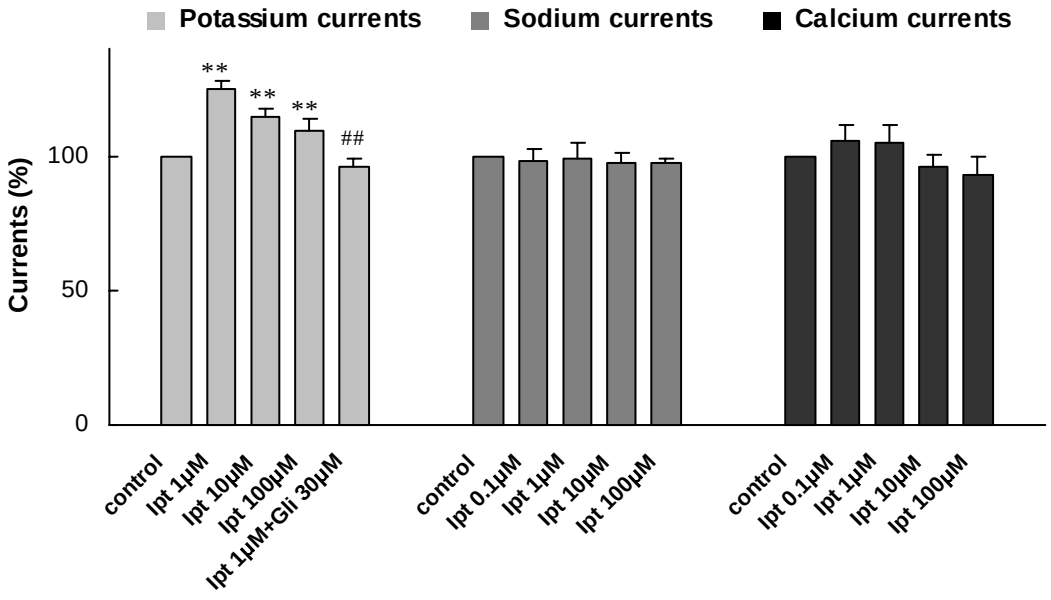

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Fig1b

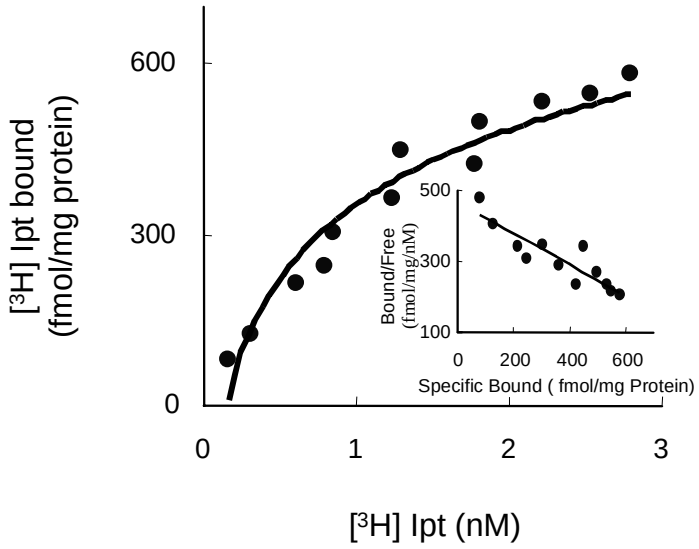

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Fig1c

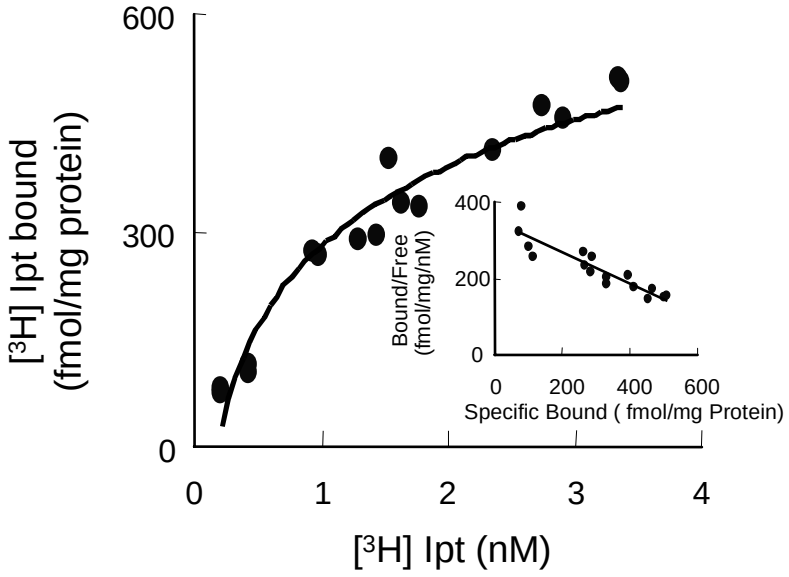

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Fig1d

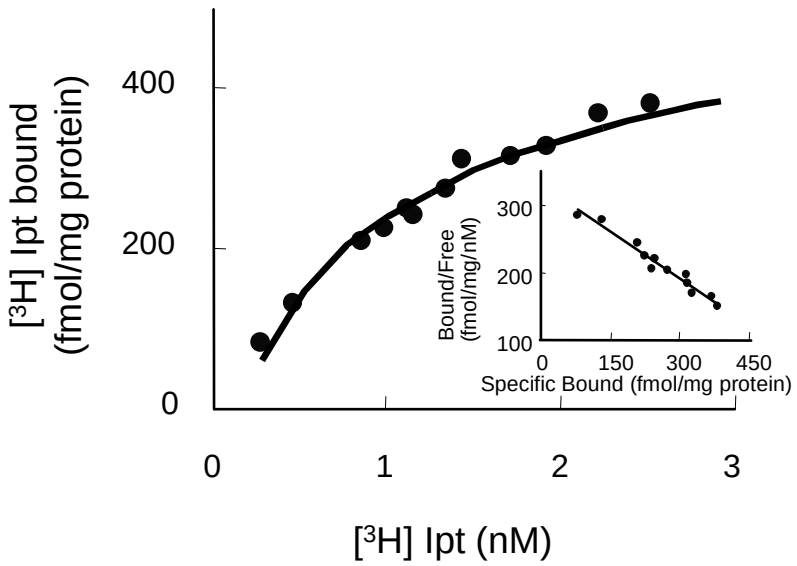

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Fig1e

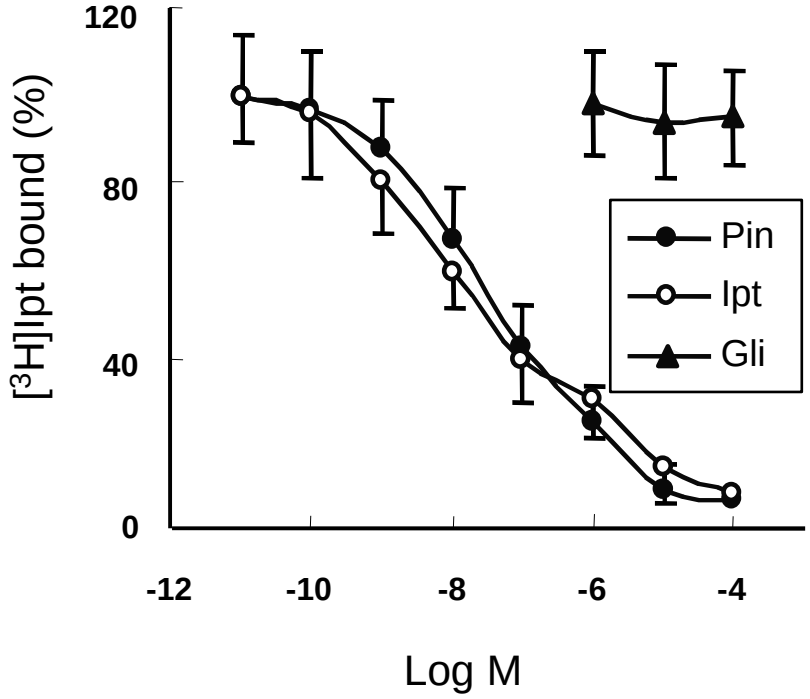

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Fig2a

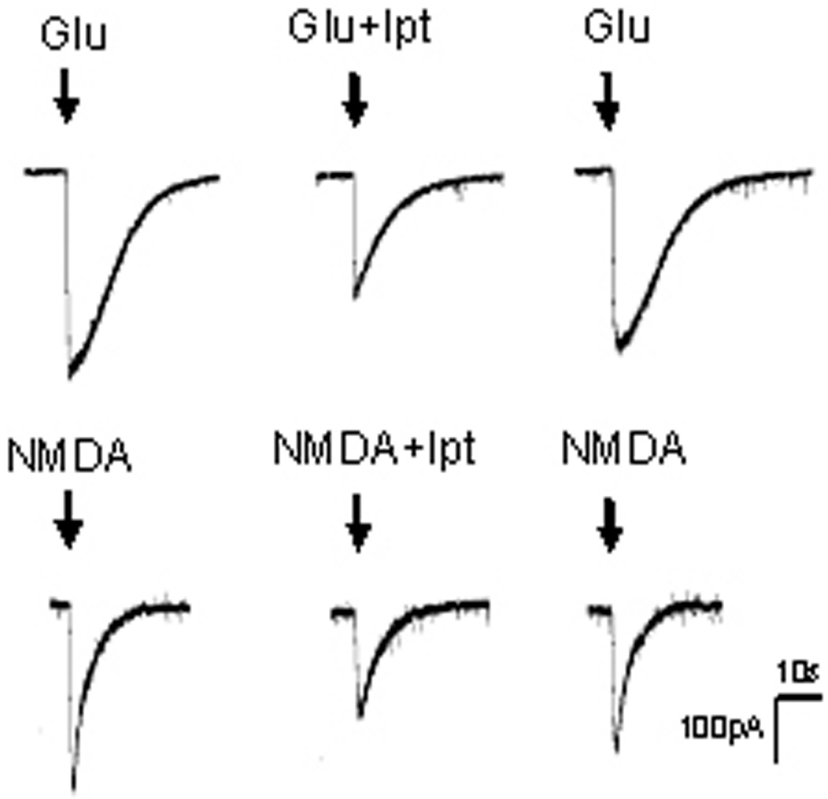

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Fig2b

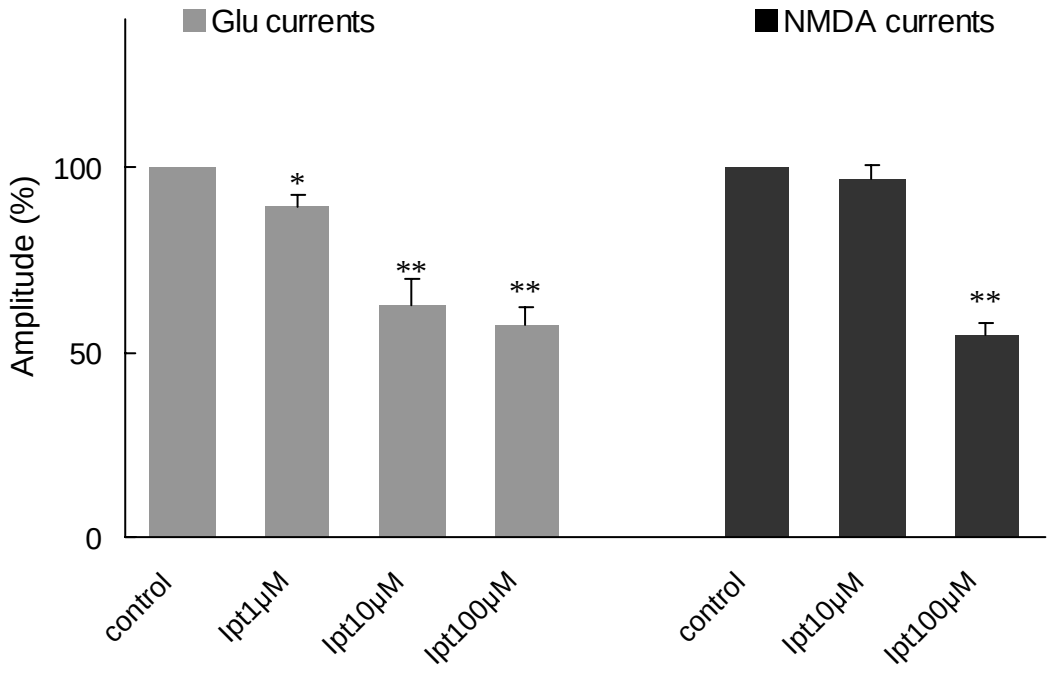

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Fig2c

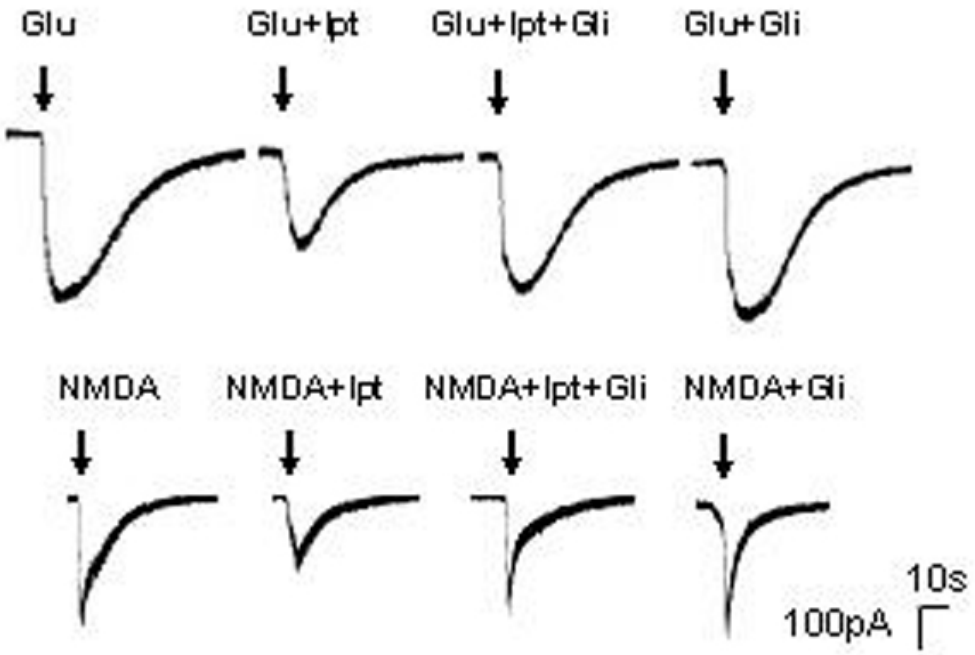

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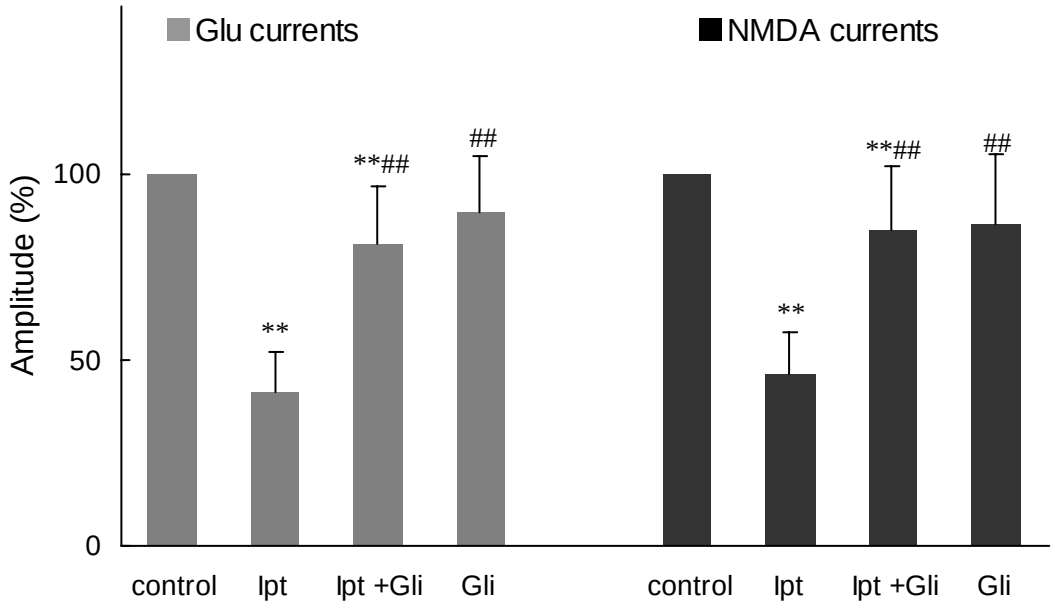
Fig2d



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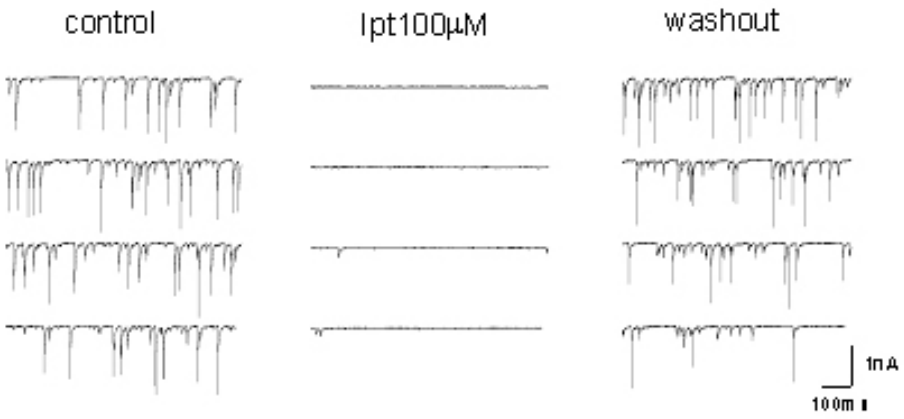

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Fig2f

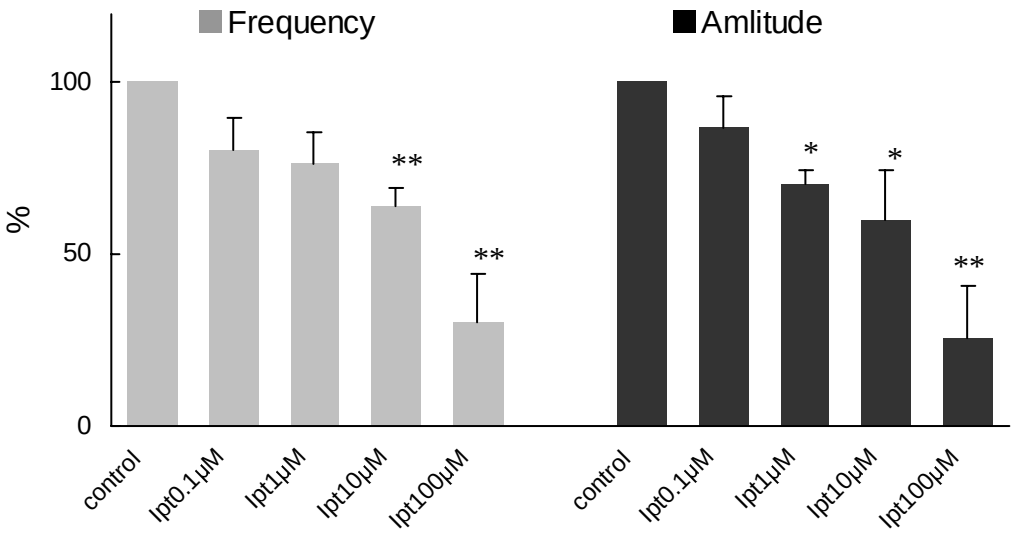

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Fig3a

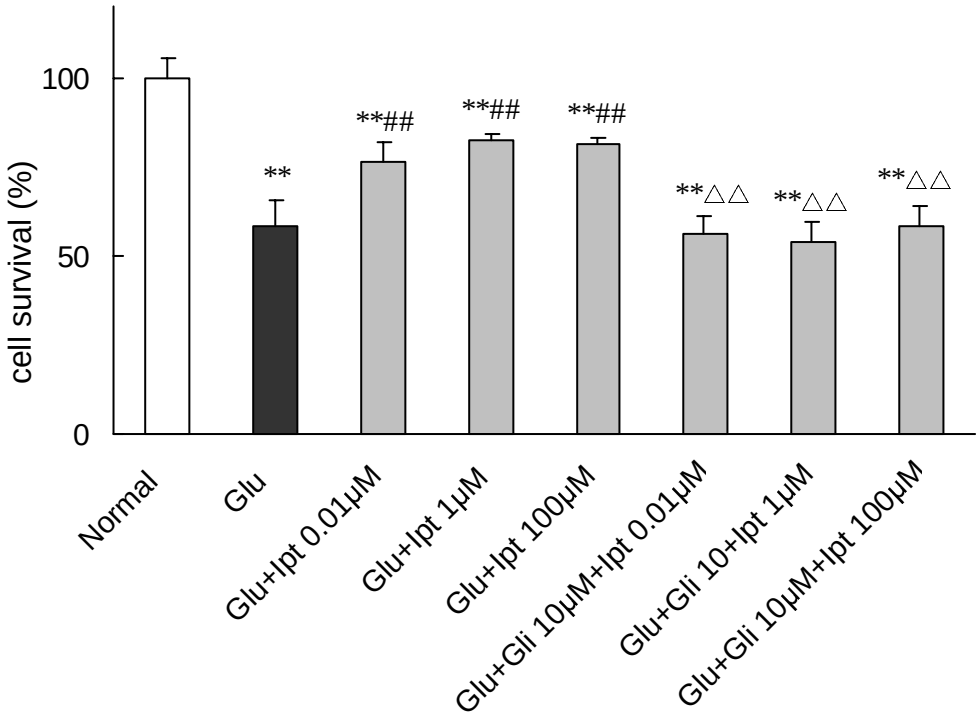

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Fig3b

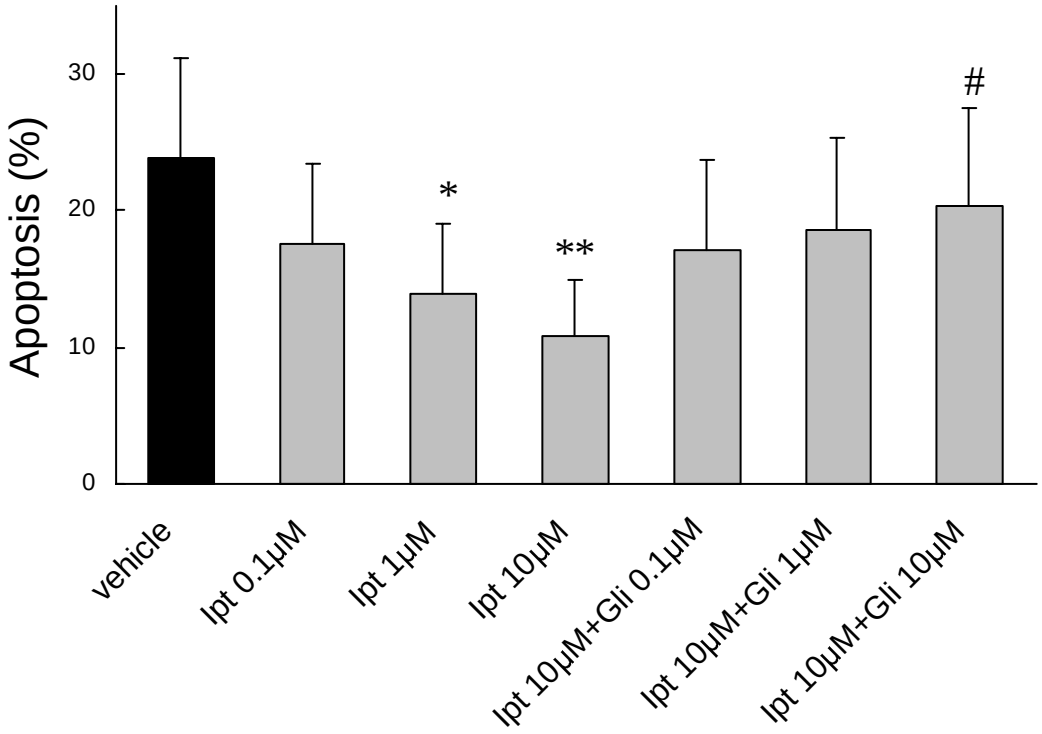

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Fig3c

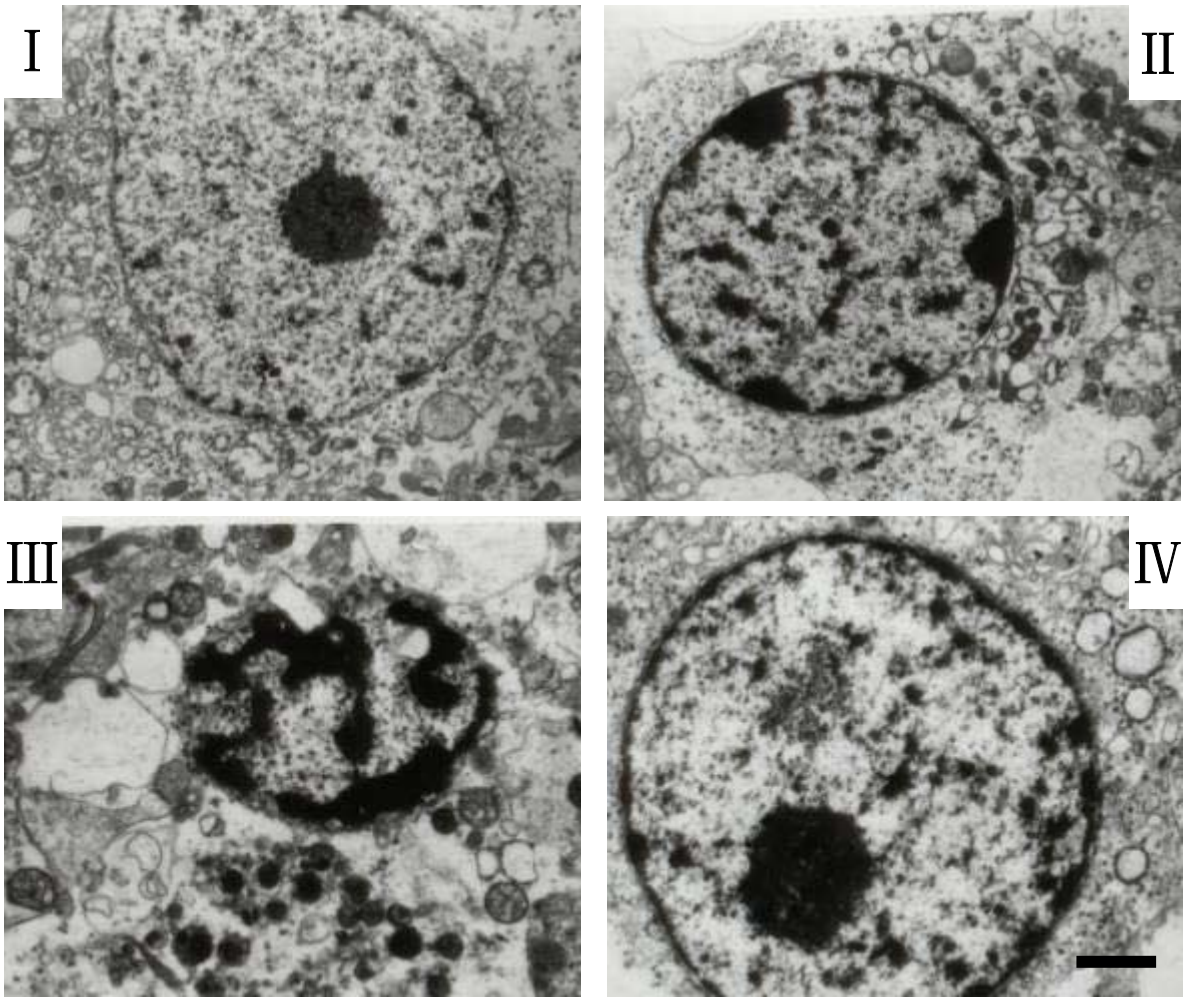

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Fig4a

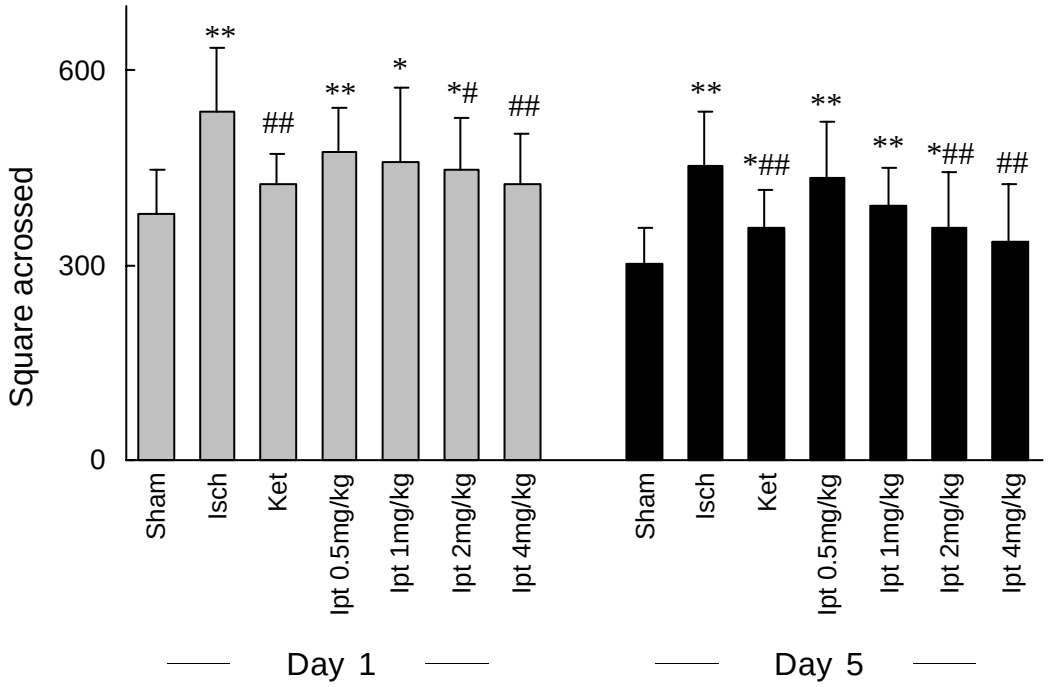

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Fig4b

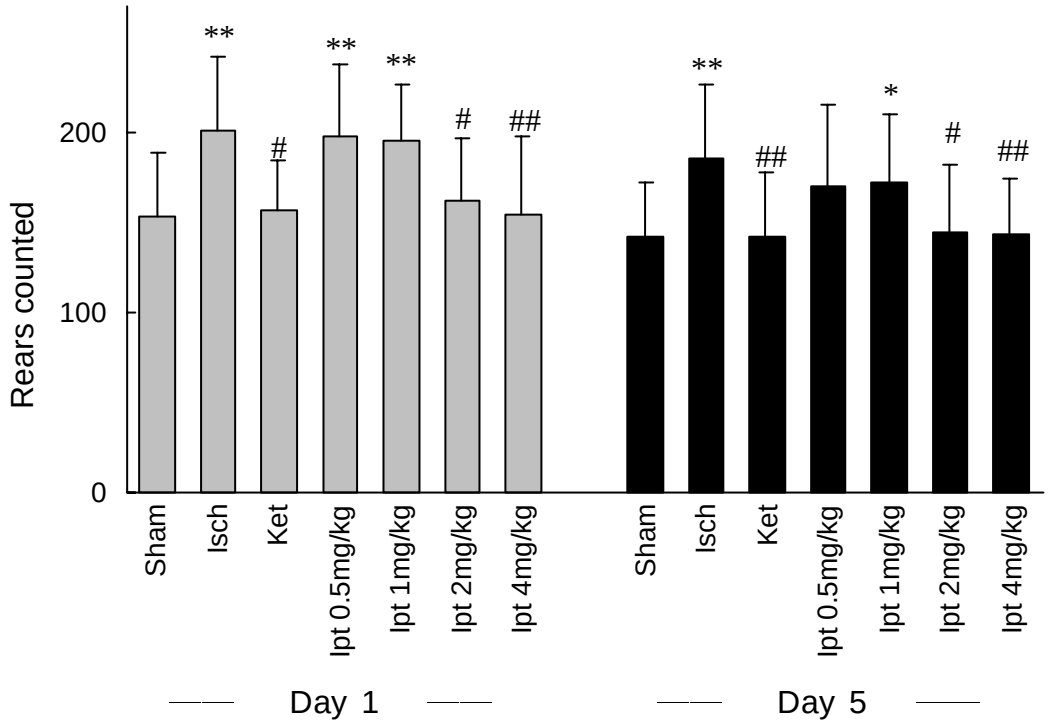

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Fig4c

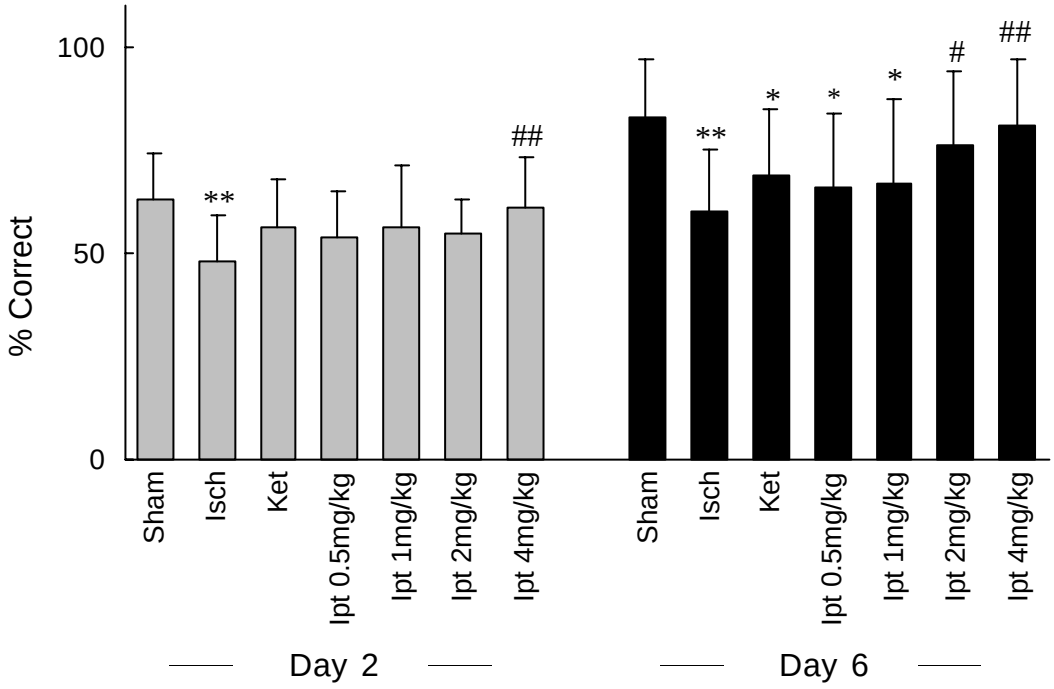

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Fig4d

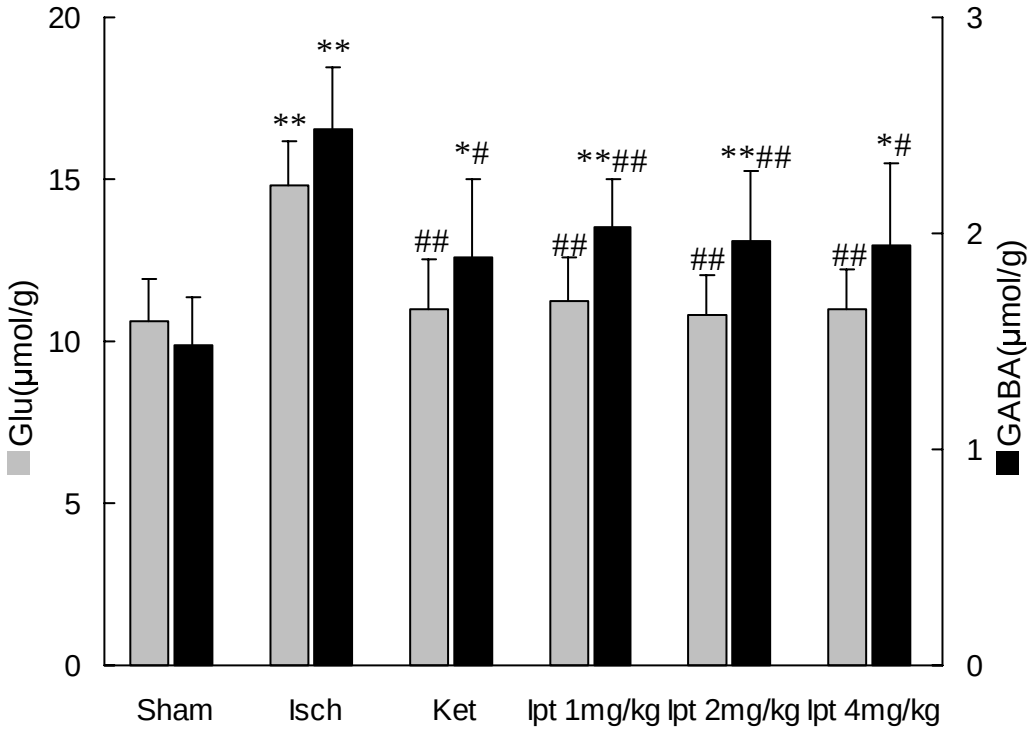

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Fig4e

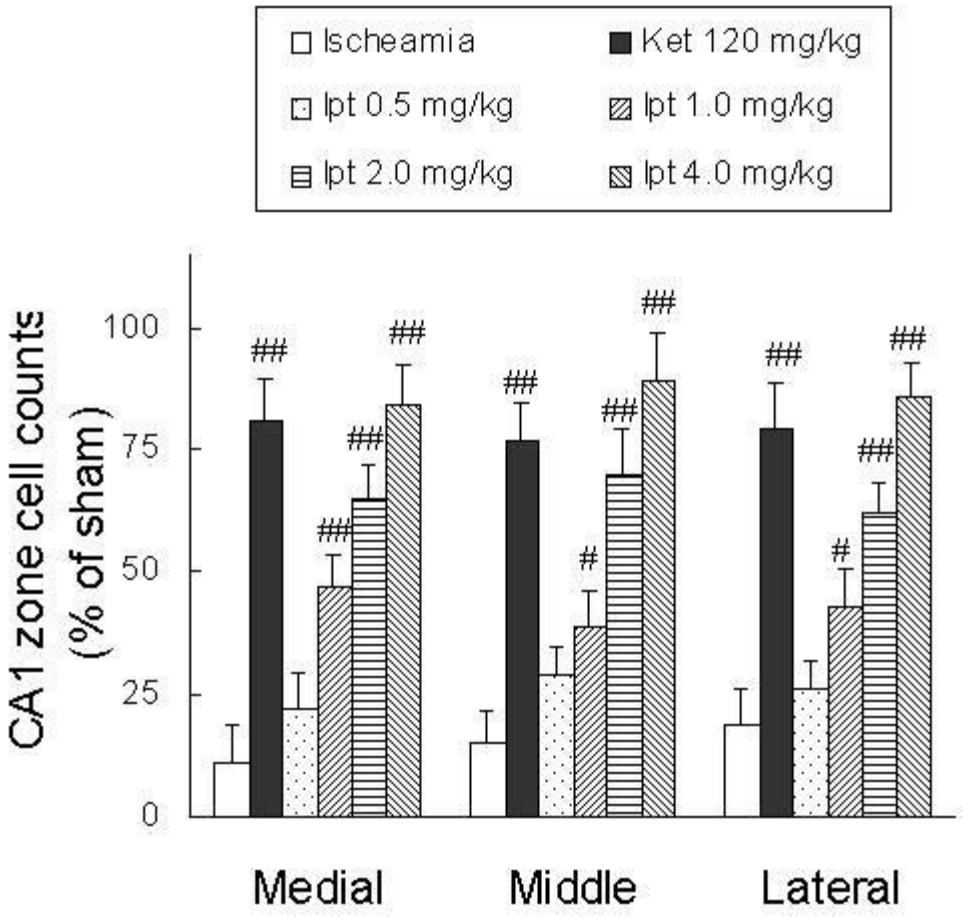

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Fig4f

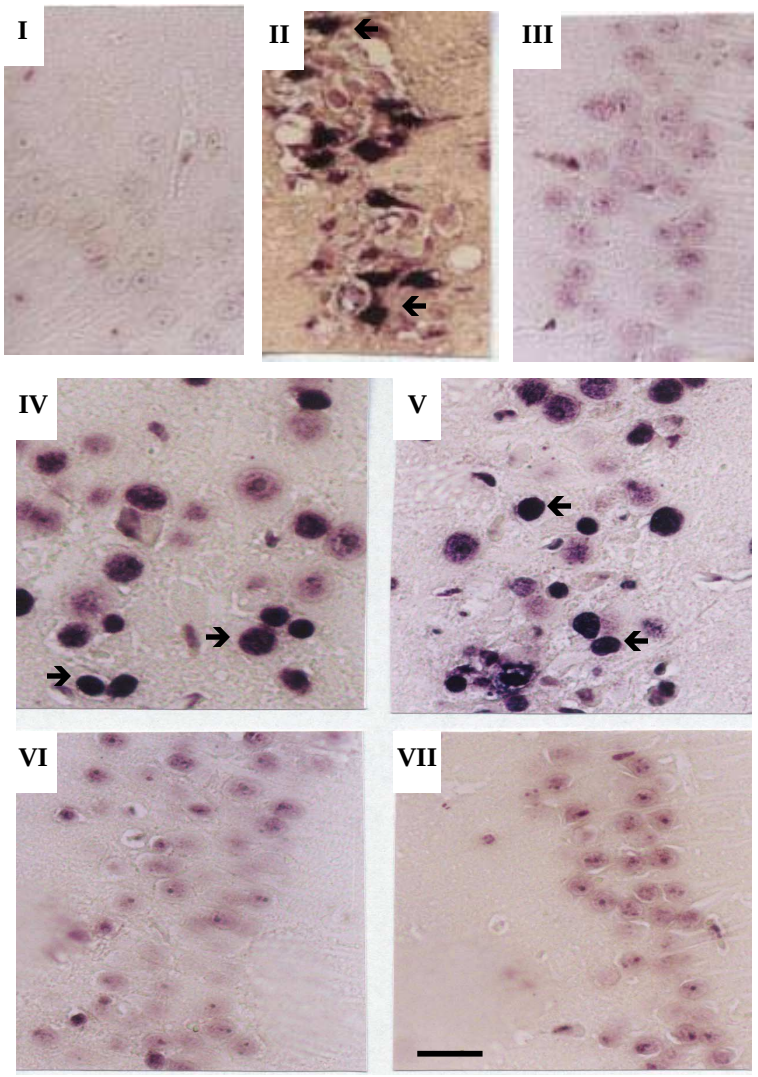

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Fig5a

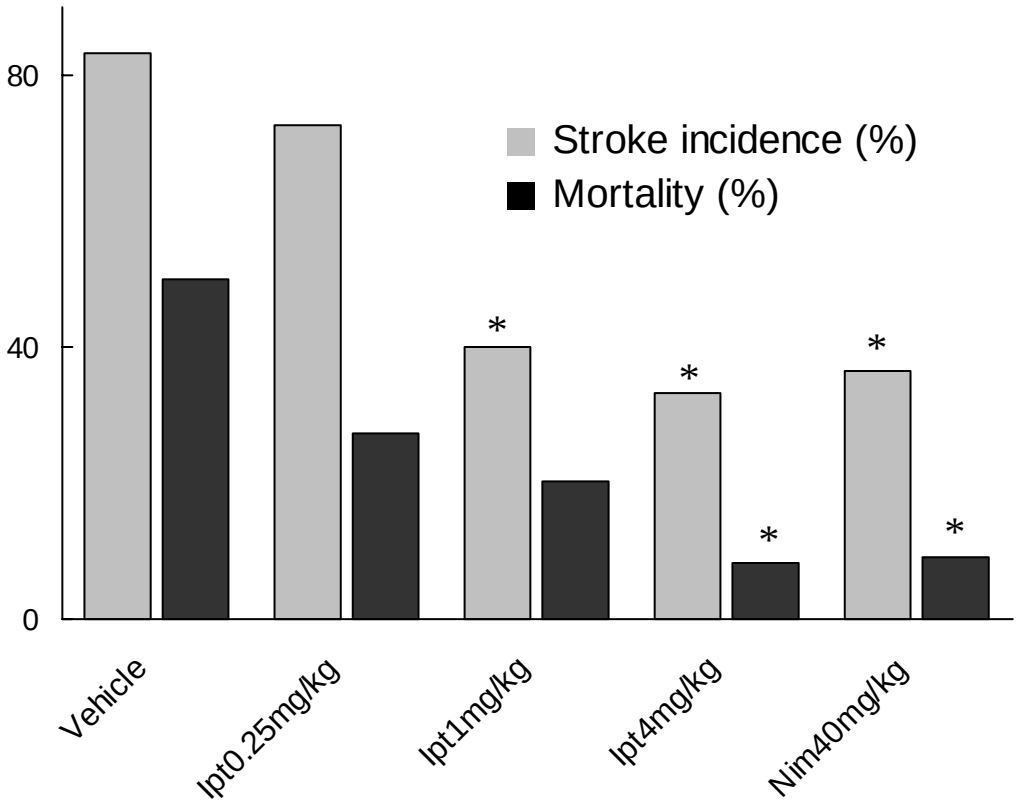

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Fig5b

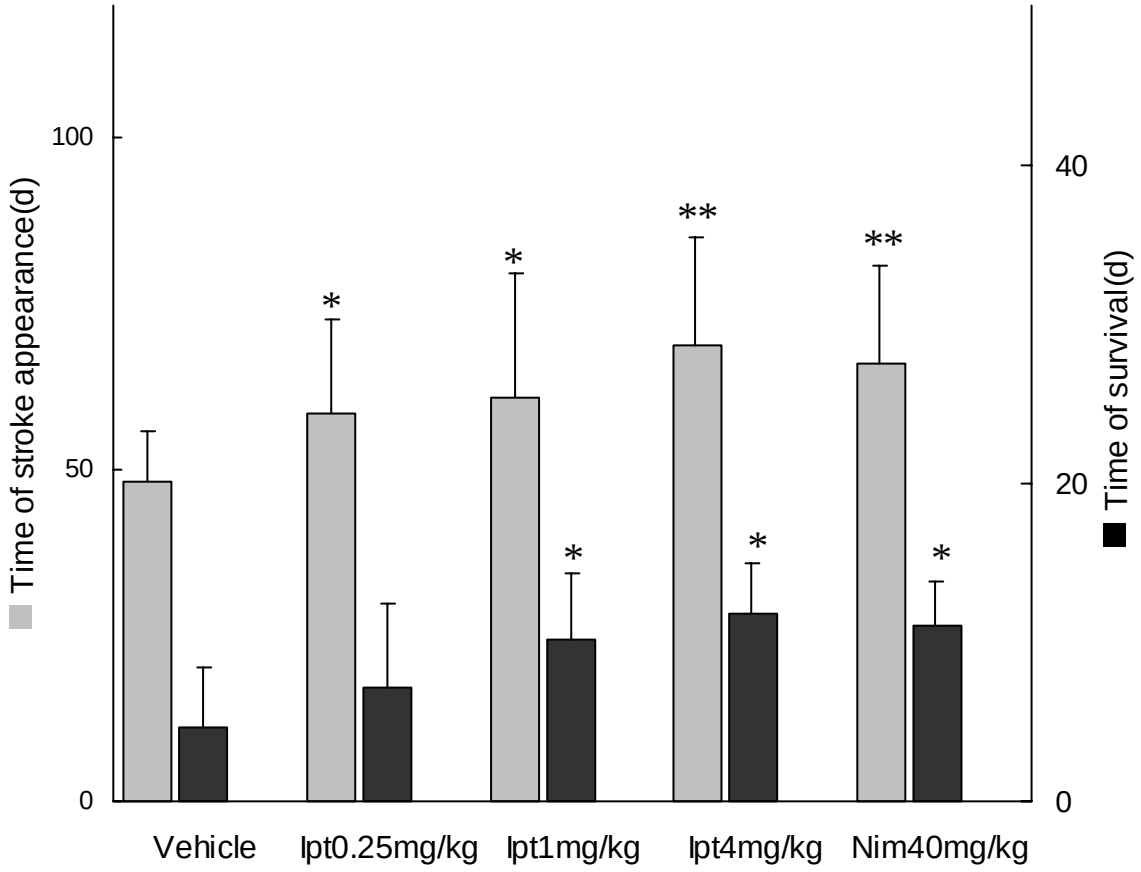

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Fig5c