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A Post-ischaemic Single Administration of Galanthamine, a Cholinesterase Inhibitor, Improves Learning Ability in Rats

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Abstract

Transient forebrain ischaemia is widely observed in clinical practice. We have examined the effect of a single administration of the cholinesterase inhibitor galanthamine ($2\,mg\,kg^{-1}$, i.p.) 25 min after reperfusion in male Sprague-Dawley rats (180 ± 20 g) after a 20-min common carotid artery occlusion.

Twenty-four-hours post-ischaemia there was no difference in motor co-ordination or muscle tonus of the rats treated with or without galanthamine as assessed by the rota-rod test. Learning ability was examined using the shuttle-box test, evaluating the latency time and the number of errors for six days in succession. The performance of the ischaemic saline-injected rats was significantly impaired on days 4, 5, 6 (latency time) compared with the non-ischaemic rats and with the ischaemic animals administered galanthamine ($P < 0{\cdot}05$). Similar results were obtained when counting the number of errors (failure to cross the cage during conditioned or unconditioned stimulus). The monitoring of body temperature during the first 12-h post-ischaemia did not show any significant difference between the groups.

The data showed a beneficial effect of galanthamine on the recovery of learning ability when administered once only post-ischaemia. This suggests a direct effect on the early pathologic mechanisms of CNS damage. Cholinesterase inhibitors may prove useful in the early clinical treatment of ischaemic conditions.

Transient forebrain ischaemia is one of the most widely observed pathological findings in conditions such as cardiac arrest (Morgani-Adams-Stokes syndrome), carbon monoxide poisoning, and ischaemic stroke. Using animal models, it has been proven that cell death occurs mainly in regions with vulnerable cells such as the hippocampal CA1 region and in the cortex (Pulsinelli et al 1982; Kirino & Sano 1984). Neuronal death is accomplished within two to four days, a process that has been termed delayed neuronal cell death, and the typical characteristic is apoptosis: DNA fragmentation and caspase-3 family expression (Chen et al 1998; Guglielmo et al 1998). The delay of the neuronal degeneration together with the affected specific mediator mechanisms provides a unique opportunity for early pharmacological intervention to prevent the development of neurological symptoms. Data exist about the similarities between the pathomorphological findings in human stroke and in experimental models of transient forebrain ischaemia (Guglielmo et al 1998). Thus the rat ischaemic model could be useful for studying potentially effective drugs and regimens for clinical treatment.

Galanthamine is a cholinesterase inhibitor with a long serum half-life, a wide therapeutic index, and after prolonged administration a tolerance effect does not develop in the CNS. Also, the side effects that can occur are mild, transient and dose-dependent unlike other drugs from this group. Galanthamine is an alkaloid from the phenanthridine group, it has excellent solubility in water and easily crosses the blood–brain barrier. It can be administered orally and parenterally, it lacks hepatotoxicity (in contrast with tacrine) and has high selectivity for acetylcholinesterase (Thomsen & Kewitz 1990). Galanthamine has a well-studied clinical effectiveness, mainly in anaesthesiology, in conditions of peripheral paresis, in various forms of neuromuscular disorders and in CNS dementia of the Alzheimer's type (Paskov 1959; Cozanitis 1971; Kluzer & Lampo 1972; Thomsen et al 1990; Iliev et al 1999).

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We have examined the effect of the indirect cholinomimetic galanthamine on the restoration of learning abilities of rats after transient bicarotid ischaemia. The drug was administered once intraperitoneally immediately after reperfusion. Such a regimen of a single administration is in accordance with recent ideas on stroke treatment. We examined whether the indirect cholinomimetics influenced the pathogenic process after ischaemia or whether they had symptomatic effect on the acetylcholine deficit after repeated administration only.

Materials and Methods

Materials

The International Guiding Principles for Animal Research were strictly adhered to throughout the experiment.

Male Sprague-Dawley rats (180 ± 20 g) were caged in four groups of six animals each, on a 12-h light–dark cycle (lights on 0800 and off 2000 h). Forebrain ischaemia was accomplished using a two-vessel occlusion modification of the method of Pulsinelli et al (1982). The animals were anaesthetized with thiopental (30 mg kg^{-1}, i.p.), followed by ether inhalation. The frequency of spontaneous breathing and heart rate were monitored throughout the experiment. Both of the common carotid arteries were dissected and occluded non-traumatically for 20 min after separation from the vagal nerve. Precise measurement of body temperature was taken rectally and maintained at 37°C during ischaemia (normal biochemical functions are based on 37°C; during anaesthesia body temperature falls). All the animals underwent surgery to expose the carotid arteries, but only two groups were ischaemized. Galanthamine (Galanthamini Hydrobromidum, Sopharma, Bulgaria) was administered (2 mg kg^{-1}, i.p.) 25 min after reperfusion to one ischaemic group and to one non-ischaemic group. Behavioural tests began 24 h after surgery. Four groups of animals were tested: group 1, sham-operated group administered saline intraperitoneally; group 2, sham-operated group administered galanthamine intraperitoneally; group 3, ischaemic group administered saline intraperitoneally; group 4, ischaemic group administered galanthamine intraperitoneally.

Active avoidance test (shuttle box)

An automatic reflex conditioner (Ugo Basile, Italy) was used, allowing training and testing of the animals at the same time. The experiment was carried out using a modification of the method described by Reddy (1998). The rats were trained and tested once daily for six days in succession between 1200 and 1400 h. Twenty 12-s trials were performed each time: 4-s conditioned stimulus (light; sound 1000 Hz, 60 dB), 3-s electrical stimulus (0·5 mA), 5-s intertrial period. When an animal crossed the cage, the ongoing conditioned or unconditioned stimulus stopped. The parameters examined were the latency crossing time of the rats (integrating both conditional and unconditional stimuli responses) and the number of errors (failure to cross the cage during the conditioned or the unconditioned stimulus period). Seven days later the animals were tested again.

Rota-rod test

The rota-rod apparatus (Ugo Basile, Italy) was used to test co-ordination and muscle tonus by the method of Dunham & Miya (1957). The rat was placed on the mill for a total of 2 min and the total number of falls for each animal was counted. The test was carried out on day 1 and not repeated. This was because as a rule motor ability improves after each test and so the first test is the most informative.

Body temperature monitoring

To match the post-ischaemic data with the circadian fluctuations of body temperature, one day before ischaemia the body temperature of each animal was monitored for 24 h and recorded using an IsoThermex apparatus (Columbus Instruments, CA), connected to an IBM computer with monitoring software. The animals were fasted for 18 h before the beginning of the monitoring and the thermistors were positioned exactly at a depth of 6 cm in the rectum. After ischaemia, body temperature was measured for 12 h during which time the rats had free access to water. The differences between the post-ischaemic data and the corresponding pre-ischaemic measurement (according to the exact time of the day) were calculated and compared among the groups.

Statistical methods

Data were analysed using Graph Pad Prism Software (Ver. 2.01). The Mann–Whitney U-test and the Wilcoxon paired test were performed.

Results

The shuttle-box test is a marker for learning ability. The ischaemic plus saline group showed significant impairment in latency time in the shuttle-box test

compared with the other groups (Figure 1): sham-operated plus saline group and ischaemic plus galanthamine group (on days 4, 5, 6; $P < 0{\cdot}05$, Mann–Whitney U-test), and sham-operated plus galanthamine group (latency time on days 5, 6; $P < 0{\cdot}05$, Mann–Whitney U-test). At the same time, the ischaemic plus galanthamine group improved its performance to the level of the sham-operated plus saline and sham-operated plus galanthamine groups without displaying any significant difference with them. There was no significant difference between the groups on their first-day tests. Seven days after the last shuttle-box test the short-term memory of the rats was examined. There was still a significant impairment in the learning abilities of the ischaemic plus saline group compared with both sham-operated groups ($P < 0{\cdot}05$, Mann–Whitney U-test), but not compared with the ischaemic plus galanthamine group. Nevertheless, the results of the ischaemic plus galanthamine group were comparable with the sham-operated plus saline and the sham-operated plus galanthamine groups, supporting the favourable effect of galanthamine. None of the groups impaired its performance on day 13 compared with its own results one week before.

According to the number of errors (Figure 2) the ischaemic plus saline group significantly impaired its performance compared with the sham-operated plus saline group (days 3, 4 and 6; $P < 0{\cdot}05$, Mann-Whitney U-test), ischaemic plus galanthamine group (day 4; $P < 0{\cdot}01$ Mann-Whitney U-test), and the sham-operated plus galanthamine group (day 4; $P < 0{\cdot}05$, Mann-Whitney U-test). The presence of a significant difference between the ischaemic plus saline group and the sham-operated plus saline group on days 3 and 6, and the fact that the results of the ischaemic plus galanthamine group were comparable with the sham-operated plus saline group on the same days, revealed an improved performance for the ischaemic rats administered galanthamine.

The rota-rod test revealed no significant differences between the groups on day 1. From this we concluded that the behavioural tests could be carried out using the shuttle-box active avoidance apparatus, without having to take into consideration the possibility of the presence of post-ischaemic motor impairment interfering with the results.

The monitoring of rectal temperature for a period of 12-h post-ischaemia did not reveal any significant difference among the groups (Mann–Whitney U-test).

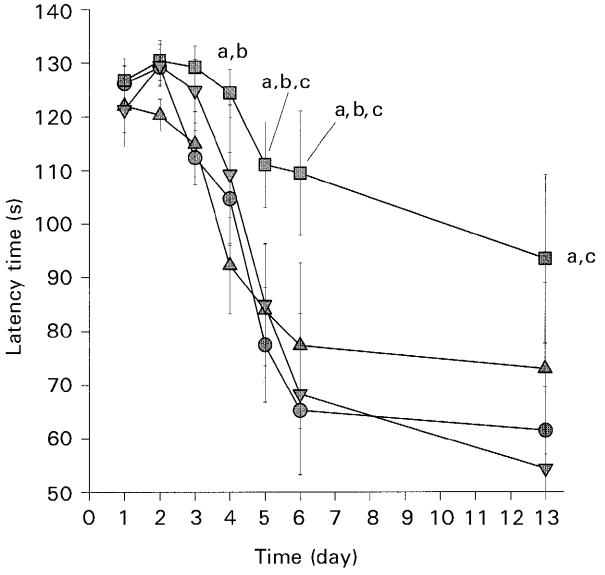

Figure 1. Learning abilities tested by the Shuttle-box test according to the parameter latency time (s). Group I showed significantly impaired learning ability compared with group S (days 4, 5, 6, 13), with group I + G (days 4, 5, 6) and with group S + G (days 5, 6, 13). Group I + G was comparable with the control groups. Each value represents the mean ± s.e.m. Mann–Whitney U-tests were performed: a, group I vs group S ($P < 0{\cdot}05$); b, group I vs group I + G ($P < 0{\cdot}05$); c, group I vs group S + G ($P < 0{\cdot}05$). ●, sham-operated group with intraperitoneal saline administration (S); ■ ischaemic group with intraperitoneal saline administration (I); ▲, ischaemic group with 2 mg kg^{-1} intraperitoneal galanthamine administration (I + G); ▼, sham-operated group with 2 mg kg^{-1}, intraperitoneal galanthamine administration (S + G).

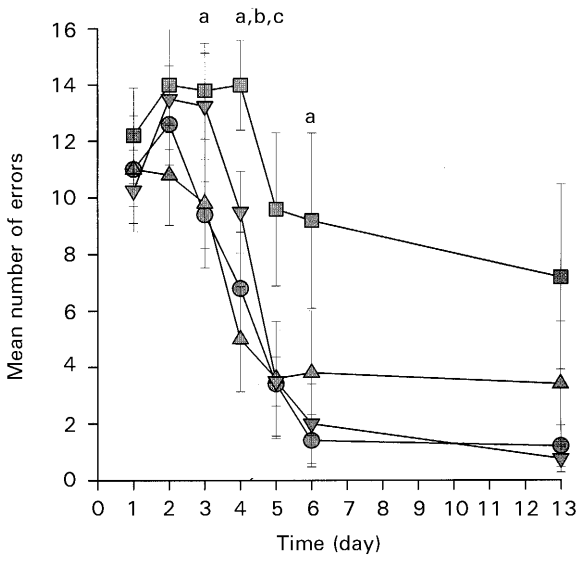

Figure 2. Learning ability tested by the Shuttle-box test according to the number of errors. Group I showed significantly impaired learning ability compared with group S (days 3, 4), with group I + G (day 4) and with group S + G (day 4). Each value represents the mean ± s.e.m. Group I + G was comparable with the control groups. Mann–Whitney U-tests were performed: a, group I vs group S ($P < 0{\cdot}05$); b, group I vs group I + G ($P < 0{\cdot}01$); c, group I vs group S + G ($P < 0{\cdot}05$). ●, sham-operated group with intraperitoneal saline administration (S); ■, ischaemic group with intraperitoneal saline administration (I); ▲, ischaemic group with 2 mg kg^{-1} intraperitoneal galanthamine administration (I + G); ▼, sham-operated group with 2 mg kg^{-1}, intraperitoneal galanthamine administration (S + G).

Discussion

The results from the shuttle-box test showed that $2\,mg\,kg^{-1}$ galanthamine administered intraperitoneally after transient 20-min ischaemia improved significantly the recovery of learning ability on days 4, 5 and 6. Similar results were observed when the number of errors was checked. There was no difference in the motor co-ordination and muscle tonus of the animals 24 h after ischaemia. According to the latency time the short-term memory tests on day 13 revealed that the ischaemic plus saline group improved its performance and became comparable with the ischaemic galanthamine-treated group. The specificity of the model could explain this recovery, because it is very close to the clinical changes that occur in patients who have suffered transient forebrain ischaemia. The favourable effect of galanthamine on the restoration of learning ability was supported by the finding that the ischaemic galanthamine-treated group on day 13, in contrast to the ischaemic saline-administered group, was not significantly different from the sham-operated plus saline group. The monitoring of rectal temperature for 12-h post-ischaemia did not reveal a significant difference among the groups. However, the behavioural effects of other dosages at more than one time point during the post-ischaemic period requires further research.

Little literature data is available about the beneficial role of indirect cholinomimetics on learning and memory in conditions of transient cerebral ischaemia: physostigmine (Yamazaki et al 1984), NIK-247 (Yamamoto et al 1993), minaprine (Yamamoto et al 1990; Karasawa et al 1992). In all of those reports the tests were held once only (mostly 24 h after ischaemia), not continuously post-ischaemia to examine the dynamics of the condition. Therefore, it was difficult to determine whether the improvement was due to the influence on the early post-ischaemic processes in the brain or to a compensation of the acetylcholine deficit, which occurred later (Ishimaru et al 1994). In this study the behavioural tests were carried out for two weeks after a single galanthamine administration 20 min after recirculation. The results led to the conclusion that it was more likely that the cholinesterase inhibitor galanthamine influenced the early post-ischaemic processes in the brain rather than the late post-ischaemic mediator deficit. The dynamics of the recovery observed with learning ability was very similar to the real clinical evolution of the ischaemic condition in man. There was an improvement in the learning and memory abilities of the ischaemic group without attaining the level of the control group.

Tanaka et al (1995) studied the neuroprotective effect of the indirect cholinomimetic ENA-713, evaluating the number of neurons surviving in the CA1 region of the hippocampus after ischaemia. Nevertheless, the increased number of neurons surviving post-ischaemia does not necessarily mean functional improvement, only behavioural tests can prove this.

Schults et al (1993) reported that after incomplete brain ischaemia in rats the outcome was made worse by physostigmine. However, those results contradict the experimental results of other researchers. It could be possible that the evaluation of the neurological outcome by Schults et al (1993) was not adequate for the model used. There could be three possible mechanisms underlying the positive pathogenic effect of galanthamine on the recovery of learning ability. Firstly, there could be an improvement in the post-ischaemic hypoperfusion. Secondly, there could be a direct effect on neurons through the cholinergic mediation they receive (especially in the hippocampus). Thirdly, hypothermia induced by the cholinomimetics could have a protective effect.

Todd et al (1986) observed that after transient ischaemia in rats, a short hyperaemic period was followed by a longer hypoperfusion mainly in hippocampus, caudoputamen and cortex. The application of a cholinesterase inhibitor could preserve the autoregulation of the cerebral blood flow (Sadoshima et al 1995), which might be explained by the enhancement of the parasympathetic innervation of the brain blood vessels (Suzuki & Hardebo 1993). The preserved cerebral blood flow could improve the restoration of homeostasis and oxygenation in the ischaemized area and facilitate the clearance of toxic metabolites, most of them participating in the induction of apoptosis and necrosis. Nevertheless, it must be noted that in higher doses the cholinomimetics can cause vasoconstriction (Miao & Lee 1991).

After ischaemia a reduction in the hippocampal acetylcholine level is observed. Activation of the hippocampal inhibitory interneurons by the cholinomimetic through the septal acetylcholine-mediated pathways to CA1 and CA3 could be supposed, thus inhibiting the excitotoxical damage of the pyramidal neurons. This mechanism is rather unlikely because the inhibitory interneurons in the hippocampus are the first ones to be damaged after ischaemia (Johansen 1993).

It is known that cholinesterase inhibitors cause hypothermia in mice, rats and man (Tonkopii 1975; Kokka et al 1987). It is well established that hypothermia can provide significant protection of CA1 hippocampal neurons following global ischae-

mia (Buchan & Pulsinelli 1990; Coimbra & Wieloch 1992; Colbourne & Corbett 1994). As noted by Colbourne et al (1997) durations of 12–24-h are necessary to provide lasting protection in animal models of ischaemia. However, our results did not reveal significant differences between the temperature changes of the ischaemic rats with or without galanthamine administration. It could be concluded that in our experimental model hypothermia was not the neuroprotective factor. Regarding the hypothermic effect of cholinesterase inhibitors, up to a certain level our data contradict the results of Tonkopii (1975) and Kokka et al (1987), but we should emphasize that those authors used higher dosages (Tonkopii (1975): $8\,mg\,kg^{-1}$). They also examined intact animals only, not ischaemic, operated or previously anaesthetised animals.

The model we used is very similar pathophysiologically to brain ischaemia/hypoxia in clinical conditions such as cardiac arrest (Morgani-Adams-Stokes syndrome), carbon monoxide poisoning, and ischaemic stroke. The effect of galanthamine on such conditions had not been studied previously. We conclude that the drug has a beneficial effect on the recovery of learning ability and on the speed of recovery. This could be due to influencing the early post-ischaemic processes in the brain. Its effect is observed after a single application immediately after ischaemia and so galanthamine might prove useful in the early clinical treatment of ischaemic conditions.

References

Buchan, A., Pulsinelli, W. A. (1990) Hypothermia but not the *N*-methyl-D-aspartate antagonist, MK-801 attenuates neuronal damage in gerbils subjected to transient global ischemia. J. Neurosci. 10: 311–316

Chen, J., Nagayama, T., Jin, K., Stetler, R. A., Zhu, R. L., Graham, S. H., Simon, R. P. (1998) Induction of caspase-3-like protease may mediate delayed neuronal death in the hippocampus after transient cerebral ischemia. J. Neurosci. 18: 4914–4928

Coimbra, C., Wieloch, T. (1992) Hypothermia ameliorates neuronal survival when induced 2 hours after ischaemia in the rat. Acta Physiol. Scand. 146: 543–544

Colbourne, F., Corbett, D. (1994) Delayed and prolonged post-ischemic hypothermia is neuroprotective in the gerbil. Brain Res. 654: 265–272

Colbourne, F., Sutherland, G., Corbett, D. (1997) Postischemic hypothermia: a critical appraisal with implications for clinical treatment. Mol. Neurobiol. 14: 55–85

Cozanitis, D. (1971) Experiences with galanthamine hydrobromide as curare antagonist. Anaesthetist 20: 226–229

Dunham, N. W., Miya, T. S. (1957) A note a simple apparatus for detection of neurological deficit in rats and mice. J. Am. Pharm. Ass. Sci. 46: 208–209

Guglielmo, M. A., Chan, P. T., Cortez, S., Stopa, E. G., McMillan, P., Johanson, C. E., Epstein, M., Doberstein, C. E. (1998) The temporal profile and morphologic features of neuronal death in human stroke resemble those observed in experimental forebrain ischemia: the potential role of apoptosis. Neurol. Res. 20: 283–296

Iliev, A., Traykov, V., Prodanov, D., Mantchev, G., Yakimova, K., Krushkov, I., Boyadjieva, N. (1999) Effect of the acetylcholinesterase inhibitor galanthamine on learning and memory in prolonged alcohol intake rat model of acetylcholine deficit. Methods Find. Exp. Clin. Pharmacol. 21: 297–301

Ishimaru, H., Takahashi, A., Ikarashi, Y., Maruyama, Y. (1994) Effect of transient cerebral ischemia on acetylcholine release in the gerbil hippocampus. NeuroReport 5: 601–604

Johansen, F. F. (1993) Interneurons in rat hippocampus after cerebral ischemia. Acta Neurol. Scandinav. 88 (Suppl.): 150

Karasawa, Y., Araki, H., Otomo, S. (1992) Cholinomimetic activity of minaprine is related to the amelioration of delayed neuronal death in gerbils. Physiol. Behav. 52: 141–147

Kirino, T., Sano, K. (1984) Selective vulnerability in the gerbil hippocampus following transient ischemia. Acta Neuropathol. 62: 201–208

Kluzer, G., Lampo, B. (1972) Importance of the association of Nivalin and vitamins of the B group in the treatment of neuropathies. Minerva Med. 63: 1686–1697

Kokka, N., Clemons, G. K., Lomax, P. (1987) Relationship between the temperature and endocrine changes induced by cholinesterase inhibitors. Pharmacology 34: 74–79

Miao, F. J., Lee, T. J. (1991) VIP-ergic and cholinergic innervations in internal carotid arteries of the cat and rat. J. Cardiovasc. Pharmacol. 18: 369–378

Paskov, D. S. (1959) Nivalin: pharmacological characteristics. Med. Fizkult. (Sofia) 3: 272

Pulsinelli, W. A., Brierly, J. B., Plum, F. (1982) Temporal profile of neuronal damage in a model of transient forebrain ischemia. Ann. Neurol. 11: 491–499

Reddy, D. S. (1998) Preclinical and clinical behavioral paradigms for testing drugs that affect learning and memory processes. Methods Find. Exp. Clin. Pharmacol. 20: 249–277

Sadoshima, S., Ibayashi, S., Fujii, K., Nagao, T., Sugimori, H., Fujishima, M. (1995) Inhibition of acetylcholinesterase modulates the autoregulation of cerebral blood flow and attenuates ischemic brain metabolism in hypertensive rats. J. Cereb. Blood. Flow. Metab. 15: 845–851

Schults, J. A., Hoffman, W. E., Albrecht, R. F. (1993) Sympathetic stimulation with physostigmine worsens outcome from incomplete brain ischemia in rats. Anesthesiology 79: 114–121

Suzuki, N., Hardebo, J. E. (1993) The cerebrovascular parasympathetic innervation. Cerebrovasc. Brain Metab. Rev. 5: 33–46

Tanaka, K., Mizukawa, K., Ogawa, N., Mori, A. (1995) Post-ischemic administration of the acetylcholinesterase inhibitor ENA-713 prevents delayed neuronal death in the gerbil hippocampus. Neurochem. Res. 20: 663–667

Thomsen, T., Kewitz, H. (1990) Selective inhibition of human acetylcholinesterase by galanthamine in vitro and in vivo. Life Sci. 46: 1553–1558

Thomsen, T., Bickel, U., Fischer, J. P., Kewitz, H. (1990) Galanthamine hydrobromide in a long-term treatment of Alzheimer's disease. Dementia 1: 46–51

Todd, N. V., Picozzi, P., Crockard, H. A., Ross Russell, R. W. (1986) Recirculation after cerebral ischemia. Simultaneous measurement of cerebral blood flow, brain edema, cerebrovascular permeability and cortical EEG in the rat. Acta Neurol. Scand. 74: 269–278

Tonkopii, V. D. (1975) Mechanism of the hypothermic action of reversible cholinesterase inhibitors. Farmakol. Toksikol. 38: 141–144

Yamamoto, T., Yatsugi, S., Ohno, M., Furuya, Y., Kitajima, I., Ueki, S. (1990) Minaprine improves impairment of working memory induced by scopolamine and cerebral ischemia in rats. Psychopharmacology 100: 316–322

Yamamoto, T., Ohno, M., Kitajima, I., Yatsugi, S., Ueki, S. (1993) Ameliorative effect of the centrally active cholinesterase inhibitor, NIK-247, on impairment of the working memory in rats. Physiol. Behav. 53: 5–10

Yamazaki, N., Take, Y., Nagaoka, A., Nagawa, Y. (1984) Beneficial effect of idebenone (CV-2619) on cerebral ischemia-induced amnesia in rats. Jpn. J. Pharmacol. 36: 349–356