Pharmacological Study on Functions of Cannabinoid Receptors for Acute Ischemic Stroke

January 2021

Noriko SUZUKI

Pharmacological Study on Functions of Cannabinoid Receptors for Acute Ischemic Stroke

A Dissertation Submitted to
the Graduate School of Sciences and Technology,
University of Tsukuba
in Partial Fulfillment of Requirements
for the Degree of Doctor of Philosophy in Science

Doctoral Program in Biology,
Degree Programs in Life and Earth Sciences

Noriko SUZUKI

Table of Contents

Abstract 1

Abbreviations..4

General Introduction.. 7

Chapter I: Cerebroprotective Effects of TAK-937, a Cannabinoid Receptor Agonist, on Ischemic Brain Damage in Middle Cerebral Artery Occluded Rats and Non-human Primates

Abstract 15

Introduction..16

Materials and Methods..18

Results..26

Discussion..30

Tables and Figures..39

Chapter II: Contribution of Hypothermia and CB_1 Receptor Activation to Protective Effects of TAK-937, a Cannabinoid Receptor Agonist, in Rat Transient MCAO Model

Abstract 62

Introduction..63

Materials and Methods..65

Results..69

Discussion..71

Table and Figures..76

General Discussion..82

Acknowledgements..89

References..91

Abstract

The pathophysiology of brain damage after ischemic stroke involves a number of mechanisms leading to neuronal damage such as the excessive release of an excitatory amino acid glutamate and inflammatory reactions. Cannabinoid (CB) receptor agonists are expected to alleviate ischemic brain damage by modulating neurotransmission and neuroinflammatory responses via CB_1 and CB_2, respectively. TAK-937 is a selective and highly potent CB_1/CB_2 receptor agonist.

In this study, the agonistic effect of CB receptor on ischemic brain damage was examined in rat and monkey ischemic stroke models using TAK-937. Sprague-Dawley (SD) rats were subjected to 2 h transient middle cerebral artery occlusion (t-MCAO) by inserting an intraluminal suture. TAK-937 was administered intravenously for 24 h starting 2 h after MCAO. Infarct volume was determined 24 h after MCAO. Functional outcomes and brain atrophy were also evaluated 4 weeks after MCAO. Next, cynomolgus monkeys were subjected to thromboembolic MCAO. TAK-937 was administered intravenously for 24 h starting 0.5 h after MCAO. Then, infarct volume and cerebrospinal fluid (CSF) S-100β levels were determined.

In the rat t-MCAO model, TAK-937 significantly reduced infarct volume in male, female and ovariectomized rats and also improved functional outcomes and brain atrophy. In the monkey thromboembolic MCAO model, TAK-937 reduced infarct volume and S-100β levels in CSF. S-100β levels in CSF were positively correlated with infarct volume ($r = 0.81$).

As a mechanism of cerebroprotective effects of TAK-937, the relative contribution of hypothermia to the cerebroprotective effects of TAK-937 was examined because sustained hypothermia itself induces significant neuroprotective effects. In addition, the contribution of CB_1 receptor activation to the cerebroprotective effects of

TAK-937 was examined. On hypothermia, using a multi-channel brain temperature controlling system that I developed, the brain temperature of freely moving rats was telemetrically monitored and maintained between 37 and 38 ºC during the intravenous infusion of TAK-937 (100 μg/kg/h) or vehicle for 24 h after 2 h MCAO. On CB_1 receptor activation, AM251, a CB_1 receptor antagonist, was administered intraperitoneally at 30 mg/kg 30 min before starting intravenous infusion of TAK-937 (100 μg/kg/h) for 24 h. Rats were sacrificed and their brains were isolated 26 h after MCAO in both experiments.

When the hypothermic effect of TAK-937 was completely reversed by a brain temperature controlling system, the infarct-reducing effect of TAK-937 was attenuated in part, but remained significant. On the other hand, concomitant AM251 treatment with TAK-937 completely abolished the hypothermic and infarct-reducing effects of TAK-937.

I conclude that the cerebroprotective effects of TAK-937 were at least in part mediated by induction of hypothermia and mainly mediated by CB_1 receptor activation. These results suggest that cannabinoid receptor activation may be a therapeutic approach for acute ischemic stroke.

Abbreviations

ACA	anterior cerebral artery
ANOVA	analysis of variance
ATP	adenosine triphosphate
BT	body temperature
CB	cannabinoid
CBF	cerebral blood flow
CSF	cerebrospinal fluid
E2	estradiol-17β
HPBCD	hydroxypropyl-β-cyclodextrin
HR	heart rate
IL-1β	interleukin-1β
MABP	mean arterial blood pressure
MCA	middle cerebral artery
MCAO	middle cerebral artery occlusion
t-MCAO	transient middle cerebral artery occlusion
NMDA	N-methyl-D-aspartic acid
NO	nitric oxide
OVX	ovariectomized
$PaCO_2$	partial arterial blood carbon dioxide
PaO_2	partial arterial blood oxygen
PCA	posterior cerebral artery
RT	rectal temperature
SD	Sprague-Dawley
THC	tetrahydrocannabinol

TNF-α	tumor necrosis factor-α
t-PA	tissue-type plasminogen activator
TTC	2, 3, 5-triphenyltetrazolium chloride
SEM	standard error of mean

General Introduction

Stroke is the second leading cause of death worldwide. Even if one survives, it causes aftereffects such as bedridden and cerebrovascular dementia, and significantly reduces the quality of life [1]. In fact, in Japan, it is the leading cause of bedridden, accounting for 30% of the total, and medical costs are about 1.8 trillion yen, which is second next to malignant neoplasms. Stroke is mainly divided into ischemic and hemorrhagic, and ischemic stroke accounts for 70 to 80% of the whole. In other words, it is not an exaggeration to state that therapies against stroke focus almost on cures against ischemic stroke. The main causes of ischemic stroke are thrombotic and embolic vascular occlusion, which occur most frequently in the middle cerebral artery (MCA). As a current therapeutic approach for acute ischemic stroke, thrombolytic reperfusion therapy using a tissue-type plasminogen activator (t-PA) is the primary clinical treatment [2]. However, this treatment has a risk of intracranial hemorrhage, so it has a narrow therapeutic time window. In addition, there are strict usage conditions such as age restrictions and injury severity. As a result, only 3 to 5% of patients can be selected to undergo this treatment [3]. Even though the disease has a great social impact, as described above, its therapy has not been established yet. Therefore, establishment of a therapeutic method and drug development are important issues.

The pathophysiology of ischemic brain damage after stroke involves a number of mechanisms leading to neuronal damage such as the excessive release of excitatory glutamate, toxic influx of calcium into neurons and inflammatory reactions. Cerebral ischemia results in the loss of glucose and oxygen supply to neurons, resulting in rapid energy metabolic dysfunctions and ATP depletion. When ATP, an intracellular energy source, is depleted, Na^+, K^+-ATPase activity is stopped, intracellular Na^+ accumulation occurs, and the decrease in the electrochemical gradient of Na^+ causes glutamate to be

released extracellularly through the cell membrane. Excessive released glutamate binds to glutamate receptors in postsynaptic neurons and activated, resulting in an intracellular Ca^{2+} influx via voltage-gated and agonist-operated Ca^{2+} channels. In particular, excessive intracellular calcium can trigger a host of detrimental reactions, including the activation of phospholipases and proteases, the generation of free radicals, and inflammatory reactions, and leads to neuronal cell death. Thus, this neuronal injury cascade offers multiple sites for pharmacological intervention to protect the brain from ischemic damage [4, Figure 1].

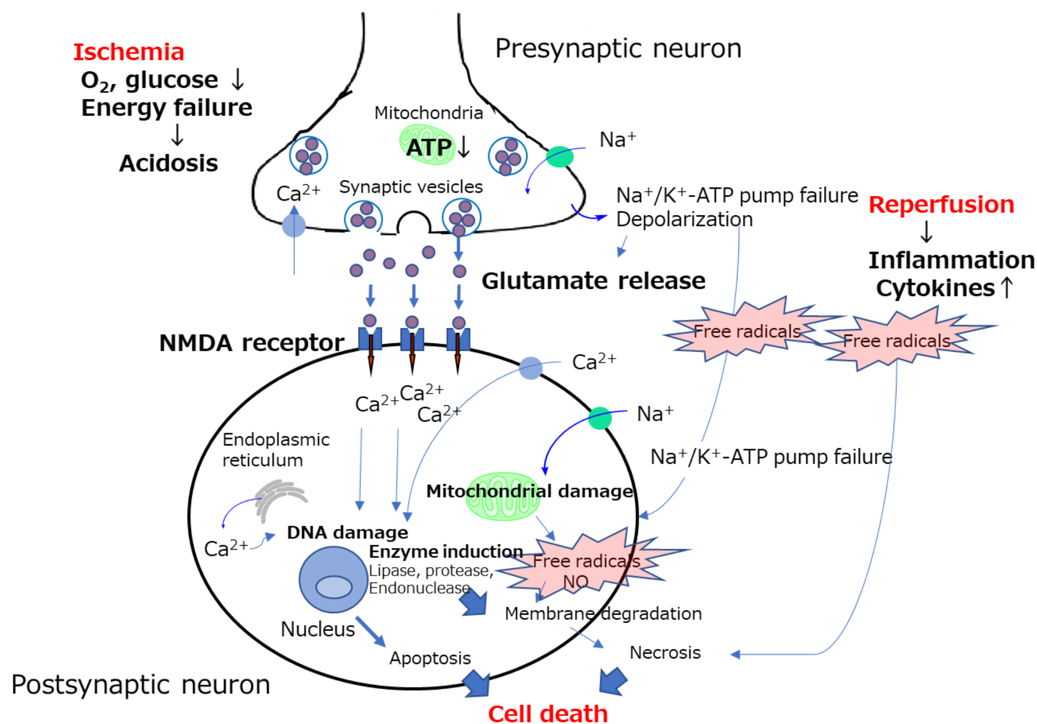


Figure 1. Pathophysiological mechanisms in the ischemic brain.

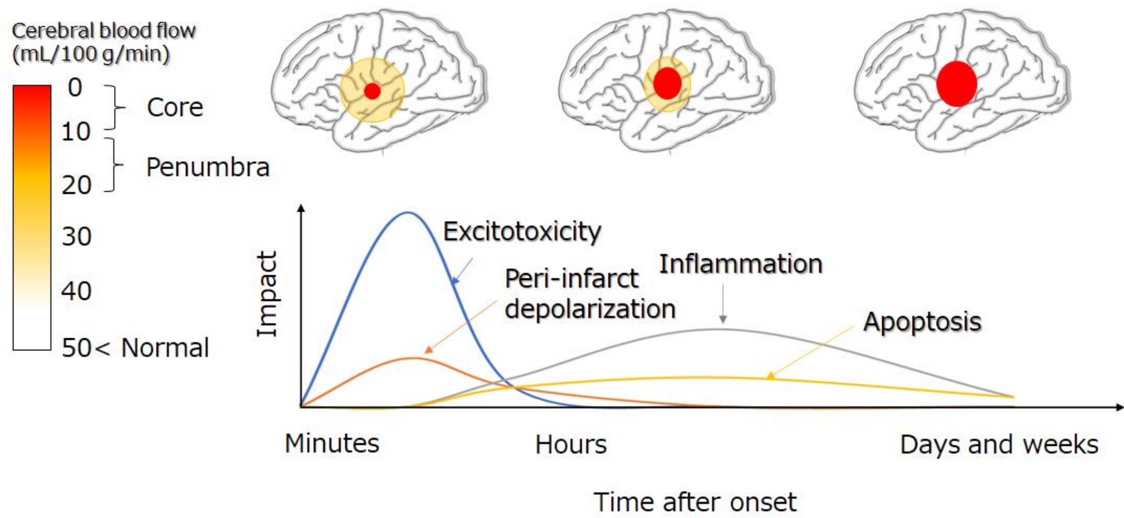
On the other hand, within 6 h of the onset of ischemic stroke, 28% of patients have a spontaneous recanalization and no vascular occlusion [5], and 70 – 80% of patients have been reperfused by intravascular thrombus removal treatment in recent years.

Reperfusion injury after resumption of blood flow has become a problem. As mechanisms of injury after reperfusion, involvement of the inflammatory reaction has been suggested, in addition to overproduction of reactive oxygen species such as superoxide, hydroxy radical and NO (Figure 1).

For these reasons, neuroprotective treatment aimed at protecting neurons against the above-mentioned ischemic cell damage-inducing substances and suppressing the progress of damage is recognized as an important therapeutic option. A radical scavenger edaravone as a neuroprotective agent has been approved only in Japan [6], but there are no neuroprotectants that can be used worldwide. Therefore, the development of other neuroprotective treatment options is highly desired.

Cerebral infarction is formed when the cerebral blood flow (CBF) is blocked or reduced, and the brain tissue controlled by the cerebral artery is damaged. At the core of cerebral ischemic infarction, cerebral metabolic dysfunctions caused by a decline of CBF immediately occur, leading to necrosis. Although it is functionally impaired, a region, the penumbra, survives for a while without cell death [7, Figure 2A]. During this period, recovery of CBF and appropriate treatment can prevent cell death and enlargement of infarct lesions in advance, which may reduce the functional disorder in prognosis. Therefore, the penumbra region is considered to be a treatable region for cerebral infarction pathology. I searched for potential therapeutic targets that suppress the depletion of high-energy phosphate compounds caused by decreased cerebral blood flow and suppressed cerebral metabolism, implying that a balance is maintained between supply and demand of energy in the brain (Figure 2B).

A



B

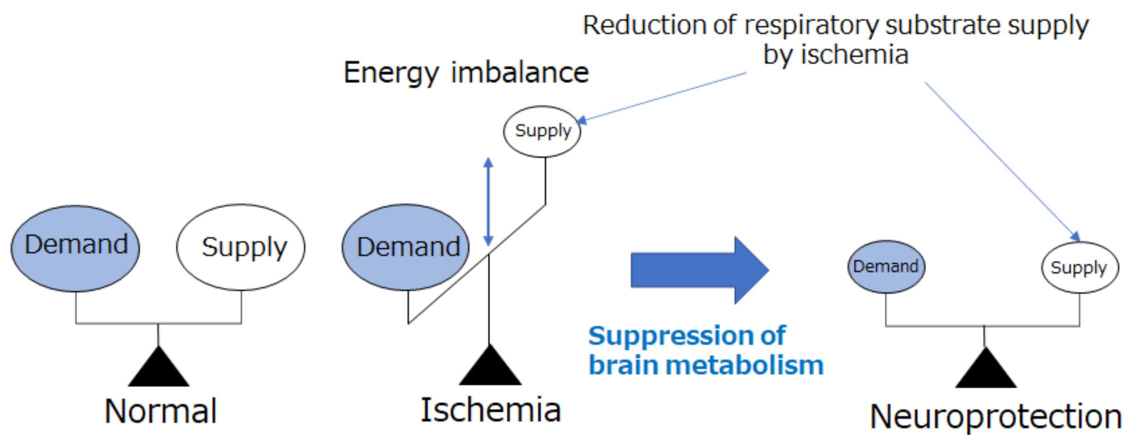


Figure 2. Image of the ischemic penumbra (A), and supply and demand balance of energy during cerebral ischemia (B).

Cannabinoid (CB) receptors were found as receptors for Δ9-tetrahydrocannabinol (THC), a major component of cannabis [8], and two subtypes have been identified so far: CB_1 and CB_2 receptors [9, 10]. CB_1 receptors are ubiquitously distributed in the brain and mainly localized in the presynaptic region [11]. CB_2 receptors

are highly expressed in organs and cells of the immune system such as the spleen and tonsils [12]. Both receptors are involved in the ability of CB to exert various pharmacological actions including inhibition of glutamate release, inhibition of intercellular calcium mobilization through inhibition of N- and Q-type voltage sensitive calcium channels, and inhibition of proinflammatory cytokine production [13]. Therefore, activation of CB receptors is expected to provide protection against the multiple cascades of ischemic brain damage, as they have anti-glutamatergic (mainly CB_1-mediated) and anti-inflammatory (which preferentially involves CB_2) properties. There are some reports on the relationship between CB and cerebral infarction. In CB_1-deficient mice, cerebral infarct lesions after cerebral ischemia-reperfusion increased compared to wild-type mice [14]. Expression of the CB_1 receptor increased in the penumbra region after cerebral ischemia [15]. A CB_2 receptor agonist also exerted neuroprotective effects in mouse focal cerebral ischemia and the effects disappeared in CB_2-deficient mice [16]. Thus, it is expected that activation of both CB receptors during cerebral ischemia suppresses the progress of cerebral infarction, as they exert anti-glutamatergic and anti-inflammatory properties.

TAK-937 [N-[(3R)-7-[(1R)-1-Hydroxyethyl]-3-(4-isopropylphenyl)-4,6-dimethyl-2,3-dihydro-1-benzofuran-5-yl]-3,3-dimethylbutanamide] synthesized by Takeda Pharmaceutical Co., Ltd. is a structurally novel CB_1/CB_2 receptor agonist *in vitro*. TAK-937 is a highly potent CB receptor ligand [IC_{50}s are 6.3 and 0.74 nmol/L in binding assays for human CB_1 and CB_2 receptors, respectively], with full agonist properties. The concentration range between specific CB receptor binding and interaction with other receptors, channels, or enzymes is at least two orders of magnitude higher, suggesting that TAK-937 is a selective CB receptor agonist.

In this thesis, I describe that the cerebroprotective effects of the cannabinoid receptor activation in acute cerebral ischemia are reliable and consistent, and that the contribution of these receptors during the progression of pathological conditions was confirmed using TAK-937. In chapter I, I examined the cerebroprotective effects of TAK-937 on infarct volume after MCAO in the rat and monkey model, and on neurological deficits after transient MCAO (t-MCAO) in rats. In chapter II, I examined the contribution of hypothermia and CB_1 receptor activation to protective effects of TAK-937 in rats.

Chapter I

Cerebroprotective Effects of TAK-937, a Cannabinoid Receptor Agonist, on Ischemic Brain Damage in Middle Cerebral Artery Occluded Rats and Non-human Primates

Abstract

The pathophysiology of brain damage after ischemic stroke involves a number of mechanisms leading to neuronal damage such as the excessive release of an excitatory amino acid glutamate and inflammatory reactions. Cannabinoid (CB) receptor agonists are expected to alleviate ischemic brain damage by modulating neurotransmission and neuroinflammatory responses via CB_1 and CB_2, respectively. TAK-937 is a selective and highly potent CB_1/CB_2 receptor agonist. In this study, the effect of TAK-937 on ischemic brain damage was examined in rat and monkey ischemic stroke models. Sprague-Dawley (SD) rats were subjected to 2 h transient middle cerebral artery occlusion (t-MCAO) by inserting an intraluminal suture. TAK-937 was administered intravenously for 24 h starting 2 h after MCAO. Infarct volume was determined 24 h after MCAO. Functional outcomes and brain atrophy were also evaluated 4 weeks after MCAO. Next, cynomolgus monkeys were subjected to thromboembolic MCAO. TAK-937 was administered intravenously for 24 h starting 0.5 h after MCAO. Then, infarct volume and cerebrospinal fluid (CSF) S-100β levels were determined. In the rat t-MCAO model, TAK-937 significantly reduced infarct volume in male, female, and ovariectomized rats and also improved functional outcomes and brain atrophy. In the monkey thromboembolic MCAO model, TAK-937 reduced infarct volume and S-100β levels in CSF by 40%. S-100β levels in CSF were positively correlated with infarct volume. These results suggest that TAK-937 may be useful for treatment of acute ischemic stroke. Moreover, S-100β levels would be a useful surrogate biomarker for the development of TAK-937.

Introduction

Activation of CB receptors is expected to provide protection against the multiple cascades of ischemic brain damage as they have anti-glutamatergic and anti-inflammatory properties. TAK-937 is a newly synthesized selective CB receptor agonist. In this study, I examined the cerebroprotective effects of TAK-937 on ischemic brain damage.

Ischemic stroke in clinical cases includes cerebral thrombosis that is stenotic or occluded by arteriosclerotic changes in the main brain artery (i.e., atherothrombotic cerebral infarction), and cerebral embolism that is occluded by a thrombus formed in the heart or another organ. Both of them commonly occur in the middle cerebral artery (MCA). Therefore, the middle cerebral artery occlusion (MCAO) model, which causes ischemic stroke by partially occluding the cerebral artery, is widely used as an experimental model due to its similarity to clinical cases. A suture occluded model can control the ischemic duration easily by removing the suture, and is a very suitable model to evaluate the progress of cerebral infarction after reperfusion. It has been reported that most rats can survive even if cerebral infarction is formed up to 2 h of ischemic duration, but 70% of rats die within 24 h after reperfusion if the ischemic duration is extended to 3 h [17]. When the ischemic duration reached 3 h, the blood-brain barrier function was seriously impaired, vascular permeability was remarkably enhanced by reperfusion, and finally hemorrhagic infarction and cerebral edema occurred. In clinical cases, thrombolytic therapy using t-PA is limited within 4.5 h after onset due to the risk of intracranial hemorrhage [18]. In other words, it is considered that 2 h of ischemia in a rat model may correspond to the clinical limit (i.e., 4.5 h) of reperfusion therapy. Therefore, I adopted a 2-h MCAO model in rats by inserting a silicone-coated nylon embolic suture

to investigate the cerebroprotective effects of TAK-937 on ischemic stroke.

Although many neuroprotectants have demonstrated efficacy in animal ischemic stroke models, they have proved ineffective in clinical trials [19]. These failures have been attributed to a variety of factors including inadequate preclinical testing of the drugs. For example, despite the fact that a substantial number of stroke patients are women, some neuroprotectants are not effective in female rats [20, 21]. Some neuroprotectants reduce brain damage in the short-term, but do not improve neurological dysfunction in the long-term [22, 23]. Furthermore, to improve the prediction of efficacy in human stroke, the study should be replicated in higher species such as non-human primates. Although rodent stroke models are of value because of their resemblance to humans in cerebrovascular anatomy and physiology and their ease of use, it has been recommended that once a positive result is achieved in a rodent model, the study should be replicated in higher species such as non-human primates before proceeding to clinical trials [24].

In this chapter, I evaluated the cerebroprotective effects of a cannabinoid receptor agonist. At first, I examined the effect of TAK-937 on infarct volume after transient MCAO (t-MCAO) in the rat model, using both genders and ovariectomized (OVX) females, and assessed neurological deficits after transient MCAO in male rats. Moreover, I investigated whether the cerebroprotective effect of TAK-937 was replicated in a non-human primate stroke model of thromboembolic MCAO using cynomolgus monkeys.

Materials and Methods

All experiments in this study were conducted in a blind manner. Namely, experimenters who performed surgery, drug treatment, and data analysis were not informed of what had been administered to each animal until the completion of data analysis.

Chemicals

TAK-937 (N-[(3R)-7-[(1R)-1-Hydroxyethyl]-3-(4-isopropylphenyl)-4,6-dimethyl-2,3-dihydro-1-benzofuran-5-yl]-3,3-dimethylbutanamide) was synthesized by the Chemical Development Laboratories of Takeda Pharmaceutical Company Ltd. (Osaka, Japan). 2,3,5-triphenyltetrazolium chloride (TTC), saline, hydroxypropyl-β-cyclodextrin (HPBCD), halothane, heparin sodium (Hepaflush® 100 units/mL), ketamine hydrochloride (Ketalar®50 for animal use), xylazine hydrochloride (Seractal® 2% for animal use), isoflurane (Forane®) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan), Otsuka Pharmaceutical Company Ltd. (Tokushima, Japan), Sigma Aldrich (St. Louis, MO, USA), Takeda Pharmaceutical Company Ltd. (Osaka, Japan), Terumo Company Ltd. (Tokyo, Japan), Sankyo Ale Pharmaceutical Company Ltd. (Tokyo, Japan), Bayer Medical Company Japan (Tokyo, Japan), Abbott Japan (Tokyo, Japan), respectively.

Animal preparation

All animal experimental procedures were carried out in accordance with the Principles and Guidelines on Animal Experimentation of the Pharmaceutical Research

Division of Takeda Pharmaceutical Company Ltd. Sprague-Dawley (SD) rats and cynomolgus monkeys were purchased from CLEA Japan Inc. (Tokyo, Japan) and KEARI (Hashimoto, Osaka, Japan), respectively. The rats and monkeys were housed in groups of 5/cage or 1/cage, respectively. The animals were maintained under controlled conditions with temperature at 24 ± 1 °C, relative humidity of 55 ± 5% and a 12-hour lighting cycle (07:00–19:00) and were given water *ad libitum*, and fed a commercial diet.

Transient MCAO model in rats

Eight-week-old male SD rats (weighing 300 to 320 g), 10-week-old female SD rats (weighing 260 to 280 g) and OVX SD rats (weighing 340 to 370 g) were used in this study. Rats were acclimated to the experimental environment for 6 or 7 days. After acclimation, rats were weighed, and polyethylene catheters (SP-45, Natsume Seisakusyo, Japan) were implanted in the left femoral vein for continuous administration of TAK-937 or vehicle and the right femoral artery for measurement of physiological parameters. Blood gases (pH, PaO_2 and $PaCO_2$) and rectal temperature (RT) were measured using an i-STAT Portable Clinical Analyzer (Fuso Pharmaceutical Industries Ltd., Osaka, Japan) and a Physitemp (Neuroscience, Osaka, Japan), respectively. On the next day, t-MCAO was performed as previously described [25] with several modifications [26]. Briefly, under halothane anesthesia, a midline incision was made in the neck and the carotid artery bifurcation was exposed. The branches of the external carotid artery were dissected. Next, a silicon-coated nylon suture (Ø 0.3 – 0.4 mm) was inserted via the proximal external carotid artery into the internal carotid artery until its tip reached the origin of the MCA, which was detected by mild increase in resistance. The total duration of the surgery was no more than 10 min. After inserting the suture, anesthesia was withdrawn to allow the

rats to awaken. Rats were returned to their cages. Two hours after MCA occlusion, its success was judged by the appearance of hemi-paresis. Rats were then reanesthetized and MCA was reperfused by withdrawing the suture. Sham-operated rats received the same experimental surgery without ligations, arterial cut, or suture insertion. Rats that did not show hemiparesis in the sensory and motor system during MCAO were excluded from this study.

Measurement of serum estradiol-17β in rats of both genders

To determine the age of female rats in this study, changes in estradiol-17β (E2) concentration at the 4-, 10- and 16-week-old was examined. Collected serum samples were stored at -80℃ until measurement. The serum E_2 concentrations were measured using an RIA kit (Diagnostic Products Corp., CA, USA) by radioimmunoassay.

Measurement of infarct volume in t-MCAO rats

The rats were sacrificed 24 h after MCAO. The brains were quickly removed and cut into six coronal sections (2 mm thick each) using a brain matrix (RBM-4000 C, ASI instrument, MI, USA). The coronal slices were incubated in physiological saline containing 1% TTC at 37℃ for 15 min. After TTC staining, the ipsilateral hemispherical area and infarct area in each slice were measured from photographs of the slices using Adobe Photoshop®, and the ipsilateral hemispherical volume and infarct volume were determined by the integration of the area of each slice and the distances between them. The infarct size of each rat was expressed as a percentage of total ipsilateral hemispherical volume of each rat.

Assessment of neurological deficits in t-MCAO rats

The posture reflex test was performed 1 and 4 weeks after t-MCAO as previously described with a minor modification [27]. Briefly, the test consisted of the following four domains: body swing, circling behavior, paresis, and lateral push resistance (Table 1). Each domain was graded on a scale from 0 to 3 (0 = severe deficit, 1 = moderate deficit, 2 = mild deficit, and 3 = normal response). A composite score for each rat was calculated by summing up the score for each domain. The foot-fault test was performed 4 weeks after t-MCAO to assess motor impairment as previously described with a minor modification [28]. Prior to the test session, rats were habituated for 1 min on an elevated mesh wire (40 × 150 cm, 50 cm high) with a 3 × 3 cm opening. In the test session, the rats were placed onto the elevated mesh wire with a 5 × 5 cm opening. The number of ipsilateral fore- and hind-limb misplacements on the grid was counted during the first 10 steps.

Measurement of brain atrophy in t-MCAO rats

The rats were sacrificed after behavioral testing. The brains were isolated to measure the extent of atrophy. Brains were cut into six coronal sections (2 mm thick each) using a brain matrix. After TTC staining as described above, each hemispheric volume was calculated by the integration of the hemispheric area of each slice and the distances between them. The brain atrophy of each rat was calculated by the following formula: extent of atrophy (%) = 100 × (contralateral hemispherical volume − ipsilateral hemispherical volume) / (contralateral hemispherical volume).

Thromboembolic MCAO model in monkeys

Fourteen male cynomolgus monkeys (2.7 – 6.2 years old), weighing 2.5 – 7.1 kg, were used in this study. Among them, four monkeys were implanted with a telemetry transmitter (TL11M3-D70-PCT, Data Science International, St. Paul, MN, USA) in the femoral artery under ketamine hydrochloride (10 mg/kg, intramuscularly (i.m.)) and xylazine hydrochloride (2 mg/kg, i.m.) anesthesia to measure physiological parameters before and after MCAO. The monkeys were recovered from surgery, and placed in a cage mounted on a receiver to measure physiological parameters such as blood pressure, heart rate and body temperature. All parameters were continually recorded on a data receiver via a telemetry system (Dataquest IV, Data Science International, St. Paul, MN, USA). In all monkeys, an anti-coagulant-coated catheter (CNC-5H, Primetech Corporation, Tokyo, Japan) for continuous administration of TAK-937 or vehicle was inserted into the right femoral vein under ketamine hydrochloride (10 mg/kg, i.m.) and xylazine hydrochloride (2 mg/kg, i.m.) anesthesia. Autologous blood was used for thrombus. To prepare clots for embolization, each monkey was kept in a monkey chair without anesthesia, and blood was collected from the femoral vein. The collected blood was immediately transferred to a vacuum blood collection tube (Benoject II, Terumo, Tokyo, Japan) for serum separation, and filled into a polyethylene catheter (SP-45, internal diameter 0.5 mm) and kept at room temperature overnight to allow clot formation. Each clot (length 5 cm, diameter 0.38 to 0.4 mm) was aspirated into a polyethylene catheter (SP-45) filled with physiological saline. The next day, thromboembolic MCAO was induced as previously described with a minor modification [29]. Briefly, the monkeys were anesthetized with 4% isoflurane in a mixture of 50% O_2 and 50% N_2O using a facemask, followed by 1.0 to 1.5% isoflurane during the operation. The left internal carotid artery was catheterized with a polyethylene

catheter (SP-45) for clot injection. An intravascular diagnostic catheter (Prograde 2Fr, Terumo, Tokyo, Japan) was used as a guide sheath to guide to the bottom of the brain. Then, the catheter containing the clot was connected to the carotid catheter through the guide sheath, and eight clots were sequentially injected into the internal carotid artery with physiological saline. Monkeys that did not show hemiparesis in the sensory and motor system 30 min after the clot injection were excluded from this study.

Collection of cerebrospinal fluid and measurement of infarct volume in thromboembolic MCAO monkeys

Monkeys were anesthetized with ketamine hydrochloride and xylazine hydrochloride 24 h after clot injection, and cerebrospinal fluid (CSF) was collected from cisterna magna to measure S-100β. The monkeys were sacrificed and ice-cold physiological saline was inserted into the right common carotid artery to wash away the blood in the brain. After washing, brains were removed, cut into fifteen coronal sections (4 mm thick) using a brain matrix, and stained with TTC. Infarct volume was determined as described above.

Measurement of CSF S-100β

Collected CSF samples were stored at -80°C until measurement. CSF S-100β concentrations were measured using an ELISA kit (YK150; Sceti Co. Ltd., Tokyo, Japan).

Drug administration

TAK-937 was dissolved in 5% hydroxypropyl-β-cyclodextrin (HPBCD). In all rat studies, TAK-937 was intravenously administered just after reperfusion at a rate of 0.5

mL/kg/10 min (loading infusion) followed by a rate of 0.4 mL/kg/h (maintaining infusion) for 22 h after reperfusion. The concentration of TAK-937 used in all rat studies was 7.5, 25, 75 and 250 mg/mL, corresponding to the following doses applied, 3, 10, 30 and 100 μg/kg/h, respectively. In the monkey study, intravenous infusion of TAK-937 was started 30 min after clot injection at 0.2 mL/kg (bolus injection) followed by 0.5 mL/kg/h (maintaining infusion) for 23.5 h after clot injection. A solution of 5% HPBCD was administered as a vehicle control in the same manner. The dose regimen in all experiments was based on preliminary studies of the effect of TAK-937 on RT to investigate the degree of hypothermia because hypothermic effect of CB agonists was reportedly exhibited via CB_1 receptor activation in the preoptic anterior hypothalamus [30].

Statistical analysis

All data were expressed as the mean ± standard error of mean (SEM). The significance of differences between treatment groups was analyzed with the one-tailed Williams' test in the dose–response study in male rats and brain atrophy, and a probability value of $P < 0.025$ was considered to be significant. For neurological symptom scores and foot-fault tests, the Wilcoxon rank sum test was performed between the sham-operated group and the vehicle-treated group, and a probability value of $P < 0.05$ was considered to be significant. For brain atrophy, the Aspin–Welch test was performed between the sham-operated group and the vehicle-treated group, and a probability value of $P < 0.05$ was considered to be significant. Statistical differences among the vehicle-treated group and the TAK-937-treated groups were analyzed with a one-tailed Shirley–Williams' test for both the neurological symptom score and the foot-fault test, and a

probability value of $P < 0.05$ was considered to be significant. An unpaired Student's *t*-test was used when values from only two groups were compared and a probability value of $P < 0.05$ was considered to be significant. All analyses were performed using the SAS Preclinical Package Version 5.0 (SAS Institute Japan Ltd., Tokyo, Japan).

Exclusion criteria from analysis

Brain samples which had subarachnoid hemorrhage (SAH), intracerebral hemorrhage, or had no observed infarction in the striatum, which becomes the ischemic core, after TTC staining were excluded from analysis.

Results

Physiological parameters in rat t-MCAO model

Physiological parameters in male rats with t-MCAO are shown in Table 2. For all blood gas parameters in rats, there were no changes in the vehicle-treated rats throughout the experimental period, and there were no differences among groups before MCAO and during drug administration after MCAO. On the other hand, TAK-937 at 100 μg/kg/h lowered rectal temperature remarkably.

Cerebroprotective effects of TAK-937 in a dose-dependent manner in rat t-MCAO model

TAK-937 significantly ($P < 0.025$) reduced cerebral infarct volume in a dose-dependent manner with maximal reduction of infarct volume by 70% at the highest dose of 100 μg/kg/h, whereas approximately 35% of the ipsilateral hemisphere was infarcted in vehicle-treated male rats subjected to MCAO (Figure 3). The minimum effective dose of TAK-937 was 10 μg/kg/h.

Gender differences in reduction of cerebral infarct volume of TAK-937

Endogenous E2 concentrations in male and female rats were measured from 4 to 16 weeks of age. At 4 weeks of age, there was no difference between males and females. However, at 10 weeks of age, it was about 2.2 times higher in female rats than in male rats (male rats: 10.9 ± 0.6 pg/mL, female rats: 24.2 ± 3.9 pg/mL) and maintained over 16 weeks of age (Figure 4A). It was considered that 10-week-old female rats are sufficiently sexually matured. Therefore, 10-week-old female and 10-week-old OVX female rats

were used in this study.

Administration of TAK-937 at 30 μg/kg/h showed a significant reduction of cerebral infarct volume in both genders compared with the vehicle-treated group (males: $P < 0.001$, females: $P < 0.05$, Figure 4B). There was no significant difference in the rate of reduction on cerebral infarction ($P = 0.29$).

Regarding the influence of OVX on infarct volume, the mean infarct volume of female and OVX female rats were 29.5 ± 2.6% and 36.4 ± 3.1%, respectively, which tended to increase in OVX female rats (Figure 5A).

Regarding the cerebroprotective effects of TAK-937 in the OVX female rat t-MCAO model, TAK-937 significantly reduced cerebral infarct volume ($P < 0.001$, Figure 5B). TAK-937 exerted cerebroprotective effects regardless of the presence of endogenous E2.

Effect of TAK-937 on neurological deficits in t-MCAO rats

Vehicle-treated rats exhibited significant ($P < 0.001$) neurological deficits as indicated by a reduction of the posture reflex score 4 weeks after t-MCAO compared with sham-operated rats whose score was almost 12, meaning “completely normal”, throughout the experimental period. TAK-937 significantly improved the neurological deficits in a dose-dependent manner ($P < 0.025$, Figure 6B).

In the foot-fault test, vehicle-treated rats showed a significant ($P < 0.001$) increase in the number of foot-faults 4 weeks after t-MCAO compared sham-operated rats. TAK-937 decreased the number of foot-faults in a dose-dependent manner with a significant decrease at 100 μg/kg/h ($P < 0.025$, Figure 6C).

In the histological analysis, vehicle-treated rats showed significant ($P < 0.025$)

atrophy of the ipsilateral hemisphere at 4 weeks after t-MCAO compared with sham-operated rats. TAK-937 significantly reduced hemispherical atrophy in a dose-dependent manner ($P < 0.025$, Figure 6D).

Furthermore, a negative correlation (r = 0.74) between hemispherical atrophy and neurological score, and a positive correlation (r = 0.68) between hemispherical atrophy and foot-fault rate, were observed (Figure 7A and 7B).

Physiological parameters in monkey thromboembolic MCAO model

Physiological parameters in monkeys with thromboembolic MCAO are shown in Table 3. Cardiovascular parameters such as mean arterial blood pressure (MABP) or heart rate (HR) did not change following the administration of TAK-937. On the other hand, TAK-937 lowed body temperature.

Protective effects of TAK-937 on infarct volume after thromboembolic MCAO in monkeys

Typical TTC-stained brain slices for vehicle- and TAK-937-treated groups are shown in Figure 8A. In the vehicle-treated group, infarct areas extended from the parietal to temporal cortex, but not to basal ganglia. TAK-937 reduced the area of infarcts between 20 and 60 mm from the frontal pole compared with vehicle, with a maximal reduction of 32 to 60 mm (Figure 8B). These areas of infarct between 4 and 60 mm of the stereotaxic levels were integrated to give a volume of the infarct (Figure 8C). TAK-937 tended to reduce infarct volume by 40% compared with vehicle-treatment.

Correlation between infarct volume and CSF S-100β concentration

S-100β concentration in CSF taken 24 h after clot injection is shown in Figure 9A. TAK-937 tended to reduce S-100β levels in CSF by 40% compared with vehicle-treatment, although statistical significance was not observed. Moreover, a positive correlation between infarct volume and CSF S-100β concentration was observed ($r = 0.81$, Figure 9B).

Discussion

In this study in rats, a 2 h t-MCAO model was adopted by inserting an intraluminal suture to investigate the effect of TAK-937, a selective and highly potent CB_1/CB_2 receptor agonist, on ischemic brain damage. In conducting these experiments, I cared about the identified problems in past *in vivo* studies [31] to ensure the use of methods with rigorous evaluation. Specifically, animals showing "zero infarction", which indicates no infarction in the striatum, were excluded. In this 2 h t-MCAO model in rats, the striatum is recognized as the ischemic core region, which cannot be rescued under the 2 h ischemic condition of MCA. Therefore, it is considered that the animals showing "zero infarction" had insufficient reduction in cerebral blood flow (CBF) in the area controlled by MCA. For this reason, clear exclusion criteria from analysis were set as described here. In addition, all experiments in this study were conducted in a blind manner so as not to overestimate the effects of TAK-937.

In this study, effects of TAK-937 were evaluated in two animal models of ischemic stroke: t-MCAO in rats and thromboembolic MCAO in monkeys. In rats, the protective effect of TAK-937 administered from 2 h after MCAO was dose-dependent and associated with an improvement in neurological deficits and motor impairment for at least 4 weeks. TAK-937 tended to be effective in the monkey thromboembolic MCAO model. Furthermore, the reduction in infarct volume was also observed in female and OVX female rats. These findings suggest that CB receptor activation reduces ischemic brain damage regardless of gender and that the effect is sustained over the long-term, including functional improvements.

TAK-937 did not affect either blood gas parameters ($PaCO_2$, PaO_2 and pH) or cardiovascular parameters at cerebroprotective doses. $PaCO_2$ is an index of respiratory

acidosis and is the largest factor affecting CBF by vasoconstrict or relax controlled pH in the extracellular fluid of the brain [32]. When CO_2 passes the blood-brain barrier (BBB), extracellular H^+ concentration increases, pathways mediated by NO-cGMP and prostaglandin-cAMP, as well as hyperpolarization by activation of ATP-sensitive K^+ channels, are relaxed brain artery by suppressing electrical-dependent Ca^{2+} channels [33]. Relaxation of the cerebral artery further reduces CBF in the ischemic region. There is a general agreement that hypotension during acute ischemic stroke is detrimental to perfusion of the ischemic brain because CBF passively follows changes in blood pressure, as cerebral autoregulation is impaired under these conditions. These conditions exacerbate the pathology of cerebral ischemia. Thus, therapeutic agents for acute ischemic stroke should not decrease blood pressure. Although the effect of TAK-937 on CBF was not examined in the present study, no effect was anticipated because HU-210, a synthetic CB receptor agonist, did not influence the CBF after cerebral ischemia [34]. On the other hand, TAK-937 reduced body temperature in a dose-dependent manner. Hypothermia has been suggested as one of the mechanisms for the cerebroprotective effects of TAK-937 because physical hypothermia offered protection from cerebral injury in pre-clinical and clinical settings of cerebral ischemia [35, 36]. An additional study on the contribution of hypothermia to the protective effects of TAK-937 is described in chapter II.

The areas suffering from cerebral ischemia are divided into the ischemic core and the ischemic penumbra that is ischemic but still viable cerebral tissue. In the core region, brain tissue has already died due to a severe reduction of CBF. In the penumbra region, although functional impairment is observed, cell death has not yet occurred by residual blood flow via collateral blood flow. Thus, it is considered that if early recovery

of blood flow and a subsequent suppressing secondary cytotoxic mechanism are achieved, then infarct expansion can be reduced.

In the t-MCAO model used in this study, since the origin of MCA was occluded, the lateral striatum and lateral frontoparietal cortex supplied by MCA became the core region. On the other hand, the frontoparietal cortex, medial segment of striatum, and thalamus, which are supplied by the anterior cerebral artery (ACA) and posterior cerebral artery (PCA), are located around the core region. ACA and PCA form an arterial anastomosis called the Willis annulus at the fundus of the brain with MCA, which becomes a form of collateral circulation when MCA is occluded. Therefore, the frontoparietal cortex, medial segment of striatum, and thalamus are considered to be the penumbra regions. In the rat coronal section stained with TTC 22 h after reperfusion, the infarct area in the vehicle-treated group had an extensive range, including the lateral striatum and lateral frontoparietal cortex supplied by MCA, the frontoparietal cortex supplied by ACA, and the medial segment of striatum and thalamus supplied by PCA. It was considered that the penumbra region in the vehicle-treated group could not be rescued only by the subsequent reperfusion of blood flow. Administration of TAK-937 reduced cerebral infarct volume in a dose-dependent manner. Although the lateral striatum and lateral frontoparietal cortex could not be rescued as a core, a reduction in infarct volume was observed in the penumbra region: frontoparietal cortex, medial segment of striatum and thalamus in a dose-dependent manner. These results suggest that the CB receptor agonist TAK-937 rescued the penumbra, which is a therapeutic area of the 2 h t-MCAO model in rats.

Moreover, the cerebroprotective effects of TAK-937 were examined in female rats and OVX female rats in addition to male rats to examine gender differences.

According to epidemiological studies, there are gender differences on the number of strokes and on the prevalence of stroke. In recent years, it has been reported that the incidence is higher [37], the mortality is higher, and the functional outcome is worse in elderly women than in men [38, 39]. The causes are considered to be not only a long average life span, but also the depletion of estrogen, a female hormone. There are many reports that estrogen has neuroprotective effects by itself, and its mechanism of action has also been reported [40, 41, 42]. Furthermore, there are gender differences in excitotoxic signal pathways after cerebral ischemia treatment [43], and there may be a gender difference in drug efficacy or infarct formation in the rodent cerebral infarct model [44, 45]. In this study, 10-week-old female rats and 10-week-old OVX female rats were used. The blood level of E2, the most active estrogen, increased gradually in 6-week-old female rats, and almost reached a plateau when they were 8 weeks old [46]. In my experiments, the E2 content of female and male rats was almost the same when they were 4 weeks old, whereas the E2 content in 10-week-old female rats increased to almost the same level as that in 16-week-old female rats. Thus, it was considered that 10-week-old female rats used for my experiments have a high endogenous E2 content and are sufficiently sexually matured. On the other hand, the ovariectomy of 10-week-old OVX female rats was performed on 5-week-old female rats before the E2 production cycled when endogenous E2 content was considered to be low, although E2 content was not measured. First, a comparison of cerebral infarct volume between male and female rats showed that female rats tended to be smaller than male rats (male rats: 34.7 ± 3.4%, female rats: 27.8 ± 3.6%). This result may be due to estrogen having cerebroprotective effects. This hypothesis was supported by the result of a comparison of cerebral infarct volume between naïve female and OVX female rats, where the latter tended to be larger than the former (naïve female

rats: 29.5 ± 2.6%, OVX female rats: 36.4 ± 3.1%). Exacerbation of infarct formation by OVX is consistent with a clinically higher prevalence of cerebral infarction which is more severe in postmenopausal women than in premenopausal women. Next, the cerebroprotective effect of TAK-937 in the female and OVX female rat t-MCAO models was investigated. Administration of TAK-937 was 30 μg/kg/h based on the results of a dose-dependent study in male rats. As a result, TAK-937 significantly reduced infarct volume in both male and female rats, and there was no gender difference in terms of efficacy. In OVX female rats, TAK-937 exerted a significant cerebral infarct-reducing effect, regardless of the presence of an endogenous hormone.

Treatment with TAK-937 not only reduced infarct volume but also improved functional outcomes in the posture reflex score for neurological dysfunction and the foot-fault test for motor impairment, which are more sensitive ways to assess chronic functional deficits in the rat t-MCAO model [27, 28, 47]. TAK-937 also attenuated brain atrophy 4 weeks after t-MCAO in rats, indicating that TAK-937 improved ischemic brain damage both functionally and histologically over the long term. Moreover, high correlations were observed in neurological score and motor impairment, against brain atrophy. These results are the first findings to show the long-term efficacy of the CB receptor agonist exclusively by administration at an early stage of onset in a preclinical study, although endogenous or synthetic CB receptor agonists have been reported to be effective *in vitro* and *in vivo* in the short term. In the posture reflex test, Bederson's methods were used with a minor modification [27]. Neurological deficits, including hemiplegia, are known to occur as a sequela of injury to the frontal cortex and striatum, and are closely related to the severity of neurological deficits and the extent of brain injury. This test is widely used because it has an excellent ability to detect injuries and can be

evaluated within 3 to 5 min per rat. The foot-fault test is a useful method to easily evaluate the long-term motor function of the fore- and hind-limbs on the paralyzed side after t-MCAO, and does not require training before MCAO. On the other hand, regarding the brain injury in the rat t-MCAO model used in this study, the cerebral cortex and striatum in the MCA perfusion area became the ischemic core, leading to infarction. It has been reported that the MCA perfusion area controls sensory and motor functions, and that if this area becomes infarcted and injured, long-term motor functional deficits are observed [48]. Thus, although there are many reports on the relationship between cerebral infarct formation and neurological deficits or motor functional deterioration in an untreated time-course, the long-term functional improvement and attenuation of brain atrophy by drug administration of TAK-937 also showed a high correlation between them. These results suggested that the posture reflex test and foot-fault test are useful evaluation methods that reflect the severity of brain injury.

Furthermore, I observed a cerebroprotective trend of TAK-937 in the monkey thromboembolic MCAO model. Given the limited supply of nonhuman primates, 2 μg/kg/h was selected as the tested dose based on a preliminary study with normal monkeys where an approximately 1 °C decrease in body temperature was observed at that dose. As a sufficient cerebroprotective effect was observed at such a hypothermic dose of TAK-937 in rats, the dose was considered to be appropriate. Consequently, there were no statistically significant effects. However, sample size could be estimated for obtaining statistically significant effects from this result. A further study with appropriate sample size and additional doses is worth exploring to try and confirm the protective effect of TAK-937 in a monkey stroke model.

Thus far, the involvement of CB receptors in cerebroprotection or

neuroprotection and their mechanisms have been investigated using various research tools such as CB receptor agonists and genetically modified mice. In models of excitotoxicity, two CB receptor agonists (WIN 55,212-2 and CP 55,940) attenuated calcium overload [49, 50] and inhibited glutamate release [50]. CB_1 receptor knockout mice showed increased infarct volume in a model of cerebral ischemia [14]. Moreover, some reports suggested that CB receptor agonists reduced the production of pro-inflammatory cytokines such as IL-1β and TNF-α after brain disease [51, 52]. These results suggested that activation of both CB_1 and CB_2 receptors exerted neuroprotective effects against cerebral ischemic damage. However, it has not been well characterized which receptor contributes most to the cerebroprotective effects [53]. Thus, the contribution of each CB receptor to cerebroprotective effects of TAK-937 was assessed using selective CB_1 and CB_2 receptor antagonists.

In contrast, it was reported that selective CB_1 receptor antagonists, such as SR141716, decreased cerebral infarction [54]. That contradiction could be explained by the diverse cellular distribution of CB_1 receptors and difference in experimental conditions. It is suggested that activation of CB_1 receptors in neurons would bring neuroprotection via suppression of excitotoxicity. On the other hand, activation of CB_1 receptors in cerebral blood vessels may cause a loss of autoregulation of CBF resulting in deterioration during cerebral ischemia. It was reported that the CB_1 receptor agonist induced more profound hypotension and bradycardia in an anesthetic condition but did not much affect cardiovascular parameters in conscious rats [55]. As anesthesia in my protocol was minimized, it is considered that the effect of the CB_1 receptor agonist on the cardiovascular system was not profound, as shown in Tables 2 and 3. Moreover, the effect on excitotoxicity was dominant because TAK-937 was sustainably administered

throughout early onset to progression of cerebral damage, differing from the design for CB_1 receptor antagonists, where anesthetic time was prolonged and the compounds were administered just before the onset of ischemia when hemodynamic changes would be large.

In addition to those findings, I found for the first time that the CB receptor agonist achieved cerebroprotection and reduced CSF S-100β in a monkey stroke model. S-100β, which is produced and released by activated astrocytes, it leaks from damaged brain cells into the CSF during brain injury such as cerebral ischemia. S-100β levels have been correlated with infarct size and functional outcome in ischemic stroke patients [56], suggesting that it would be a surrogate marker for the degree of brain damage and would be useful in establishing a prognosis after acute ischemic stroke. The present findings that CSF S-100β correlated with infarct volume in the monkey stroke model used in this study and that TAK-937 simultaneously reduced infarct volume and CSF S-100β suggest that S-100β could also be utilized as a surrogate endpoint in future clinical studies for TAK-937. However, from the practical viewpoints of clinical settings of acute ischemic stroke, measuring S-100β levels in plasma/serum is desired as it would not be easy to collect CSF samples from patients. Actually, S-100β was measured in plasma simultaneously with CSF collection because animals had to be sacrificed to determine cerebral infarct volume, but S-100β was not detected in plasma 24 h after MCAO. Several studies reported that the peak serum S-100β value was delayed 2 days from the CSF peak [57]. Thus, further studies, for example the measurement of plasma S-100β at appropriate timing, are required to apply S-100β as a surrogate marker to serve as a prognosis of the efficacy of TAK-937. As the release of S-100β is accompanied by the production of inflammatory cytokines (IL-1β and TNF-α) which contribute to delayed brain damage

[58, 59], it will be important to investigate the anti-inflammatory properties of TAK-937.

In conclusion, the experiment in this chapter provides experimental evidence for the first time that a CB receptor agonist exerts cerebroprotection and improvement of long-term neurologic outcome in rats and monkeys subjected to MCAO-inducing brain ischemia. In addition, the cerebroprotective effect of the CB receptor agonist is not sexually dimorphic, unlike many other neuroprotectants. Furthermore, the correlation between infarct volume and CSF S-100β levels suggests that S-100β would be a good indicator of the efficacy of candidates in preventing brain damage. The data provide preclinical evidence that CB receptor agonists would be beneficial therapeutic agents for the treatment of ischemic stroke.

Tables and Figures

Table 1. Neurological scoring

The test consisted of the following four domains: body swing, circling behavior, paresis, and lateral push resistance. Each domain was graded on a scale from 0 to 3 (0 = severe deficit, 1 = moderate deficit, 2 = mild deficit, and 3 = normal response). A composite score for each rat was calculated by summing up the score for each domain.

Body swing		
	0	Immediate and maximum twisting
	1	Maximum twisting after short delay
	2	Slight twisting
	3	Extension of both forelimbs toward the floor
Circling behavior		
	0	Spontaneous and continuous circling toward the paretic side
	1	Consistent circling toward the paretic side
	2	Inability to walk straight
	3	Normal walk
Paresis		
	0	Complete paresis of the forelimb contralateral to the ischemic side
	1	Moderate paresis
	2	Slight paresis
	3	Normal
Lateral push resistance		
	0	Falling with minimal resistance toward the paretic side
	1	Moderately reduced resistance toward the paretic side
	2	Slightly reduced resistance toward the paretic side
	3	Resistance equally in both directions

Table 2. Effect of TAK-937 on physiological parameters in rats with t-MCAO

Blood gases (pH, PaO_2 and $PaCO_2$) and rectal temperature (RT) were measured using an i-STAT Portable Clinical Analyzer (Fuso Pharmaceutical Industries Ltd., Osaka, Japan) and a Physitemp (Neuroscience, Osaka, Japan), respectively. Data are indicated as the mean ± SEM.

Parameter	Dose	No. of		Time after drug administration (h)		
	(µg/kg/h)	animals	Before	0	5	24
$PaCO_2$	Vehicle	6	39.9 ± 1.5	40.6 ± 1.6	40.7 ± 1.3	39.9 ± 0.5
(mmHg)	3	6	39.2 ± 1.1	41.0 ± 1.7	42.6 ± 1.3	42.2 ± 1.4
	10	6	39.9 ± 1.3	40.6 ± 1.8	40.0 ± 0.9	39.2 ± 0.5
	30	6	41.9 ± 1.0	39.4 ± 1.1	41.0 ± 1.1	40.7 ± 0.8
	100	6	39.2 ± 0.6	42.5 ± 1.4	43.2 ± 1.5	43.5 ± 1.5
PaO_2	Vehicle	6	81 ± 2	84 ± 4	89 ± 2	90 ± 1
(mmHg)	3	6	82 ± 2	82 ± 2	85 ± 2	85 ± 2
	10	6	81 ± 4	85 ± 2	83 ± 2	89 ± 2
	30	6	82 ± 3	89 ± 4	84 ± 1	86 ± 1
	100	6	82 ± 3	81 ± 2	79 ± 2	83 ± 1
pH	Vehicle	6	7.45 ± 0.01	7.44 ± 0.01	7.46 ± 0.01	7.47 ± 0.00
	3	6	7.46 ± 0.01	7.42 ± 0.01	7.43 ± 0.01	7.49 ± 0.01
	10	6	7.46 ± 0.01	7.44 ± 0.01	7.45 ± 0.01	7.48 ± 0.01
	30	6	7.45 ± 0.01	7.45 ± 0.01	7.43 ± 0.01	7.47 ± 0.01
	100	6	7.46 ± 0.01	7.42 ± 0.01	7.41 ± 0.01	7.47 ± 0.01
RT	Vehicle	6	38.5 ± 0.1	39.5 ± 0.1	38.9 ± 0.3	37.8 ± 0.3
(˚C)	3	6	38.7 ± 0.1	39.3 ± 0.1	37.7 ± 0.3	38.1 ± 0.2
	10	6	38.5 ± 0.1	39.2 ± 0.2	38.6 ± 0.4	38.0 ± 0.1
	30	6	38.6 ± 0.1	39.4 ± 0.1	37.3 ± 0.7	38.0 ± 0.2
	100	6	38.3 ± 0.2	39.4 ± 0.1	34.1 ± 0.7	37.3 ± 0.2

Table 3. Effect of TAK-937 on physiological parameters in monkeys with thromboembolic MCAO

All parameters were continually recorded on a data receiver via a telemetry system (Dataquest IV, Data Science International, St. Paul, MN, USA). Data are indicated as mean. MABP: mean arterial blood pressure, HR: heart rate, BT: body temperature.

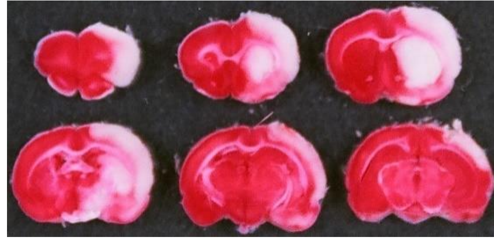
Parameter	Dose	No. of		Time after drug administration (h)		
	(μg/kg/h)	animals	Before	0	5	24
MABP	Vehicle	2	116	117	116	114
(mmHg)	2.0	2	114	117	116	114
HR	Vehicle	2	160	151	158	150
(beats/min)	2.0	2	158	150	157	149
BT	Vehicle	2	37.1	37.4	37.8	38.1
(˚C)	2.0	2	37.0	37.1	35.8	36.4

Figure 3. Cerebroprotective effects of TAK-937 on cerebral infarction after t-MCAO in male rats.

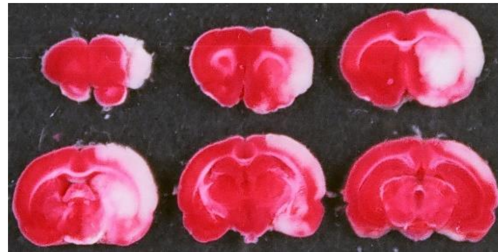
(A) Typical images of cerebral infarction after TTC staining in each group, and (B) protective effects of TAK-937 on cerebral infarct volume. Infusion of vehicle or TAK-937 started just after reperfusion. Brains were obtained after 2 h MCAO and 22 h reperfusion. Infarct volume was determined by staining with TTC. Data are indicated as the mean ± SEM. Significant differences from the vehicle-treated group are indicated by $^{*}P < 0.025$ (one-tailed Williams' test). Numbers in parentheses show the number of rats used.

A

Vehicle-treated group

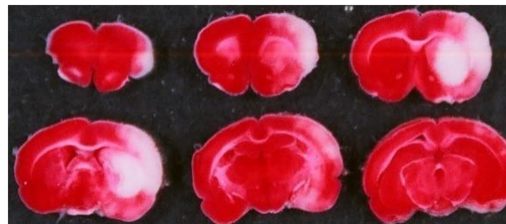


TAK-937 at 3 μg/kg/h-treated group



TAK-937 at 10 μg/kg/h-treated group

TAK-937 at 30 μg/kg/h-treated group



TAK-937 at 100 μg/kg/h-treated group

B

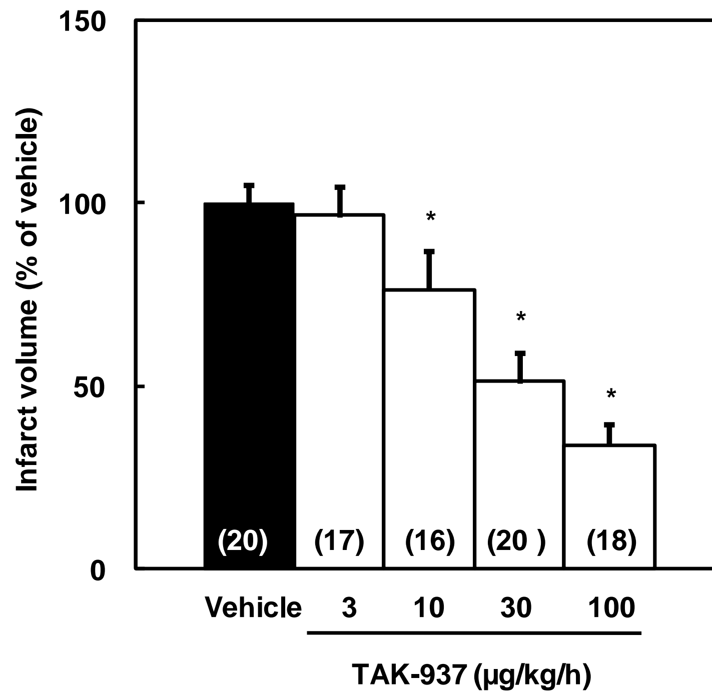
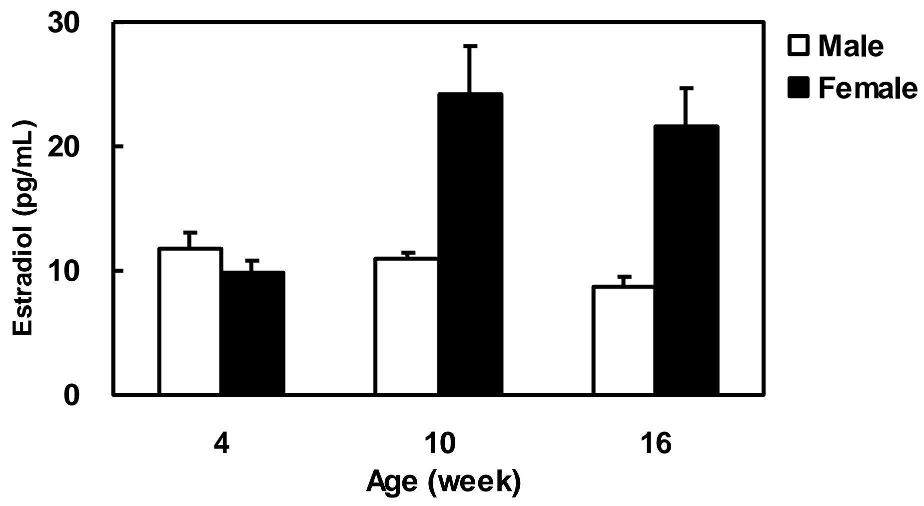


Figure 4. Gender differences in reduction of cerebral infarct volume of TAK-937.

(A) Endogenous estradiol-17β (E2) concentrations in rats of both genders. Serum E2 concentrations were measured using an RIA kit (Diagnostic Products Corp., CA, USA) by radioimmunoassay. Data are indicated as the mean ± SEM. N=6-8. (B) Cerebroprotective effects of TAK-937 on cerebral infarction after t-MCAO in rats of both genders. Eight-week-old male and 10-week-old female SD rats were used. Infusion of vehicle or TAK-937 at 30 μg/kg/h started just after reperfusion. Brains were obtained after 2 h MCAO and 22 h reperfusion. Infarct volume was determined by staining with TTC. Data are indicated as the mean ± SEM. Mean infarct volume of vehicle in male and female rats was 283.3 ± 15.6 and 204.5 ± 28.1 mm^3, respectively. Significant differences from the vehicle-treated group are indicated by $^{***}P < 0.001$ and $^{*}P < 0.05$ (Student's *t*-test). Numbers in parentheses show the number of rats used.

A



B

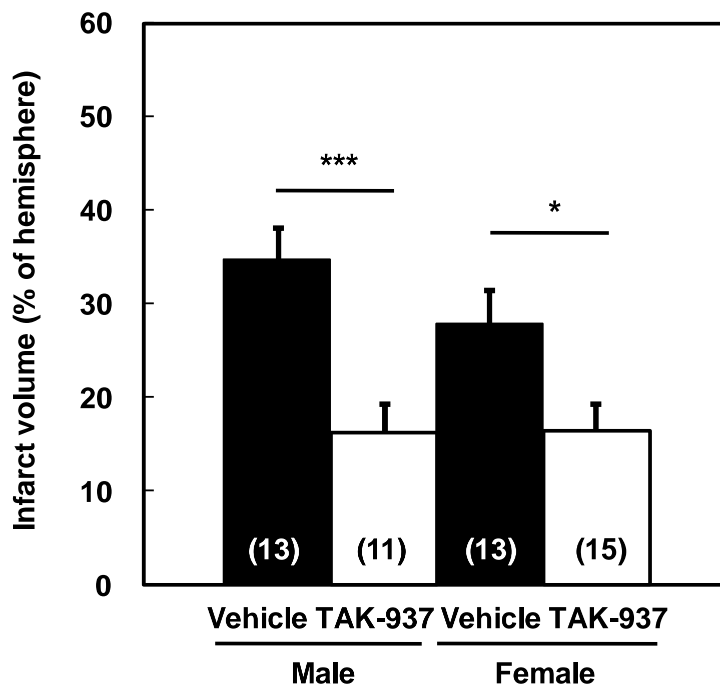
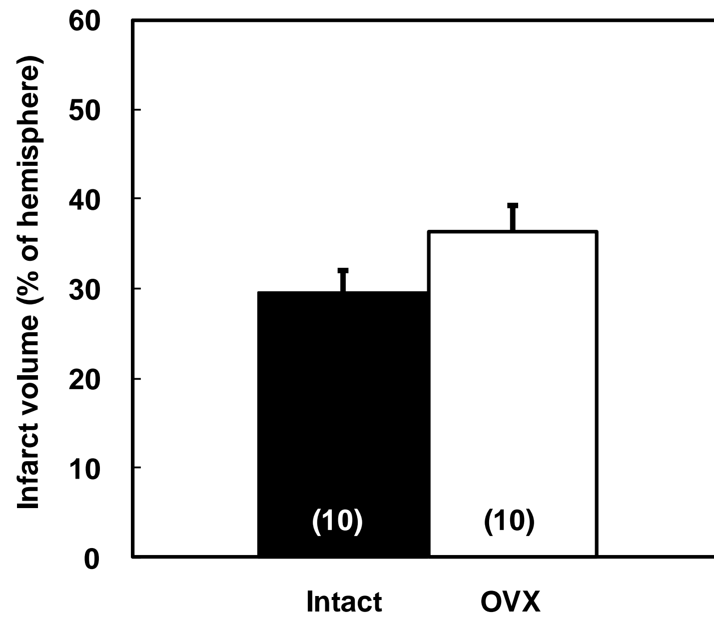


Figure 5. Cerebroprotective effects of TAK-937 on cerebral infarction after t-MCAO in female rats and OVX female rats.

(A) Comparison of cerebral infarction after t-MCAO between intact and OVX female rats. Brains were obtained after 2 h MCAO and 22 h reperfusion. (B) Protective effects of TAK-937 on cerebral infarction after t-MCAO in female and OVX rats. Infusion of vehicle or TAK-937 (30 μg/kg/h) started just after reperfusion. Infarct volume was determined by staining with TTC. Data are indicated as the mean ± SEM. Mean infarct volume of the vehicle in OVX female rats was 299.4 ± 27.2 mm^3. Significant difference from the vehicle-treated group is indicated by $^{***}P < 0.001$ (Student's *t*-test). Numbers in parentheses show the number of rats used.

A



B

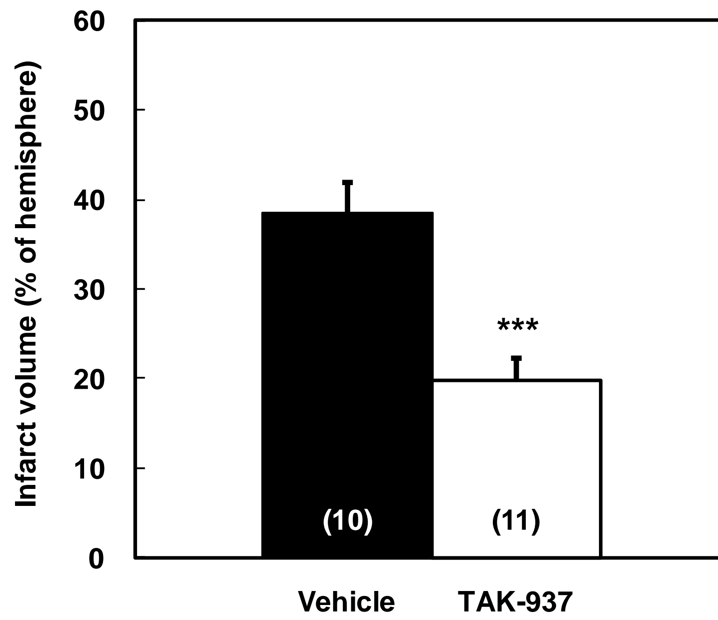
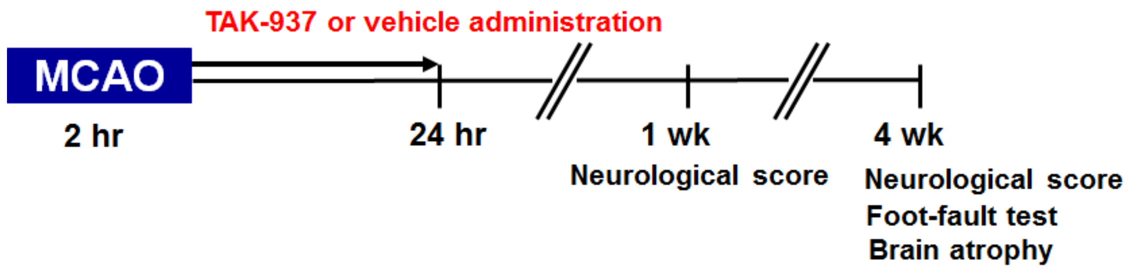


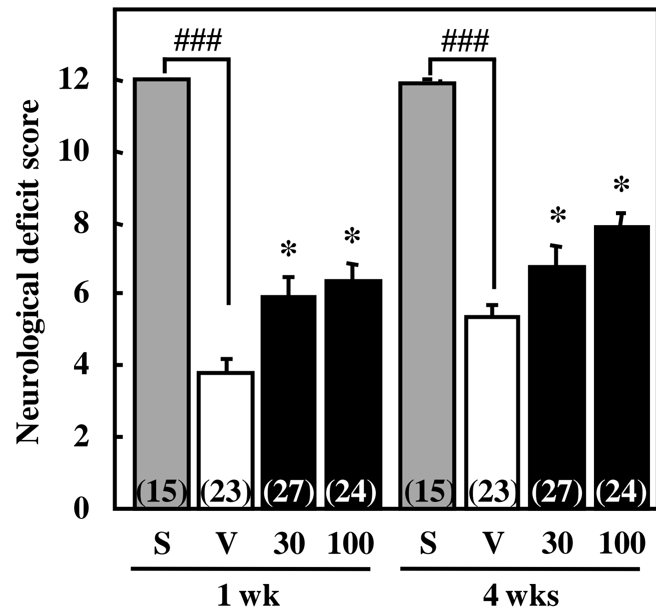
Figure 6. Ameliorative effects of TAK-937 on neurological deficit score, foot-faults and brain atrophy after t-MCAO in rats.

(A) Scheme of experimental protocol. Infusion of vehicle or TAK-937 started just after reperfusion. (B) Ameliorative effects of TAK-937 on neurological deficit score. Neurological symptoms were assessed 1 week and 4 weeks after MCAO. (C) Ameliorative effects of TAK-937 on foot-faults. The foot-fault test was performed 4 weeks after MCAO. (D) Ameliorative effects of TAK-937 on brain atrophy. After behavioral testing, rats were sacrificed and brains were isolated to measure the extent of atrophy. S: sham-operated group, V: vehicle-treated group, 30: TAK-937 30 μg/kg/h-treated group, 100: TAK-937 100 μg/kg/h-treated group. Data are indicated as the mean ± SEM. Significant difference from the sham-operated group is indicated by $^{###}P < 0.001$ (Student's *t*-test) and significant differences from the vehicle-treated group are indicated by $^{*}P < 0.025$ (one-tailed Williams' test). Numbers in parentheses show the number of rats used.

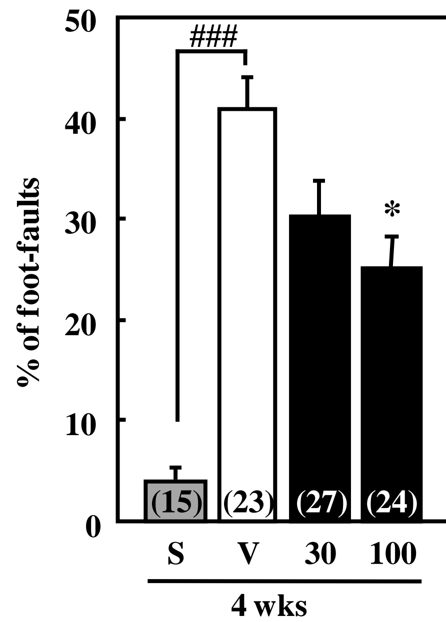
A



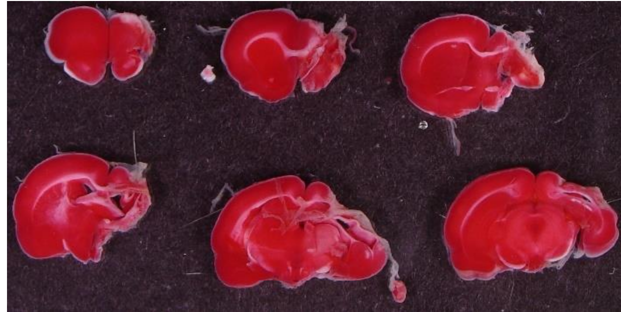
B



C



D



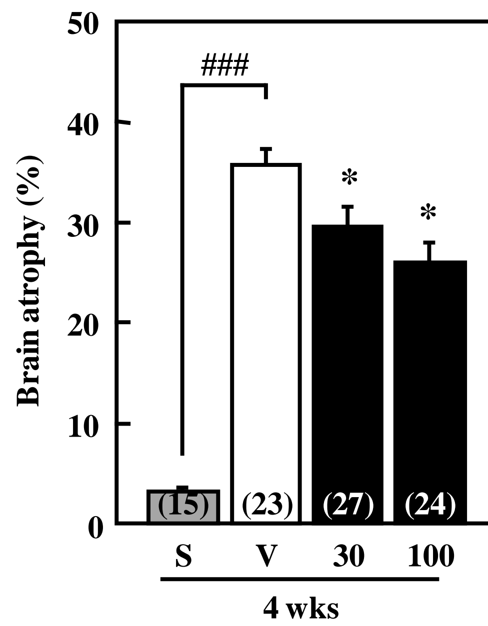


Figure 7. Correlation between brain atrophy and neurological score (A) or foot-fault rate (B) 4 weeks after t-MCAO in rats.

A negative correlation between neurological score (y-axis) and brain atrophy (x-axis) ($r = 0.74$, A) and a positive correlation between foot-fault rate (y-axis) and brain atrophy ($r = 0.68$, B) were shown in a rat t-MCAO model. Vehicle: vehicle-treated group (N=23), 30: TAK-937 30 μg/kg/h-treated group (N=27), 100: TAK-937 100 μg/kg/h-treated group (N=24).

A

y = -0.1908x + 12.506
r = 0.7376

Neurological score

Vehicle
30
100

Brain atrophy (%)

B

y = 1.1756x - 3.8349
r = 0.6798

Foot fault rate (%)

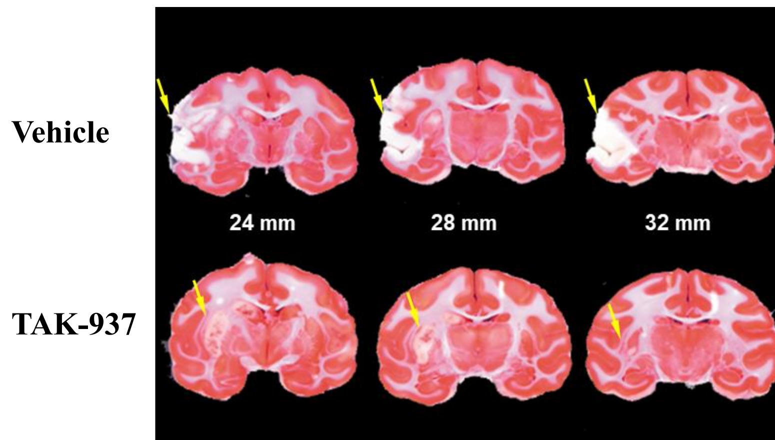
Vehicle
30
100

Brain atrophy (%)

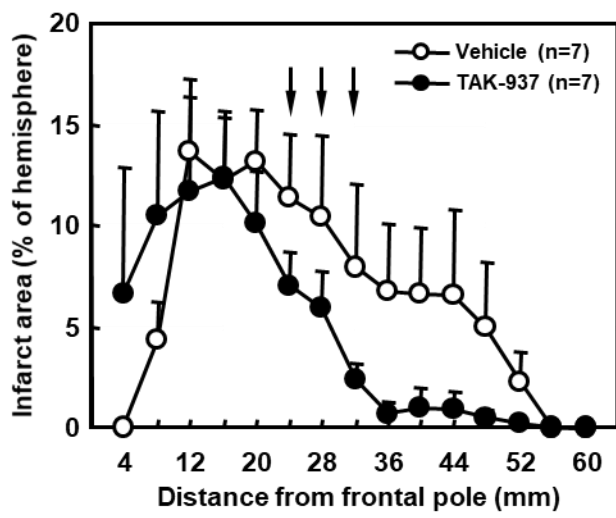
Figure 8. Typical images of cerebral infarction from treated monkeys (A), and protective effects of TAK-937 on cerebral infarct area (B) and volume (C) after thromboembolic MCAO in monkeys.

Infusion of vehicle or TAK-937 (2 μg/kg/h) started 30 min after clot injection for 23.5 h. Brains were obtained 24 h after clot injection. Infarct area was determined by staining with TTC. Data are indicated as the mean ± SEM. Mean infarct volume with vehicle was 2.7 ± 1.7 cm^3. Numbers in parentheses show the number of monkeys used. Black arrows indicate the position of brain sections shown in the above images.

A



B



C

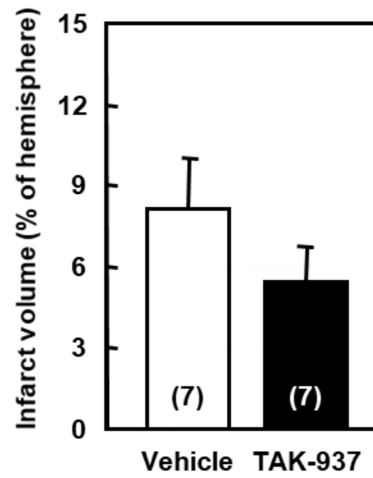
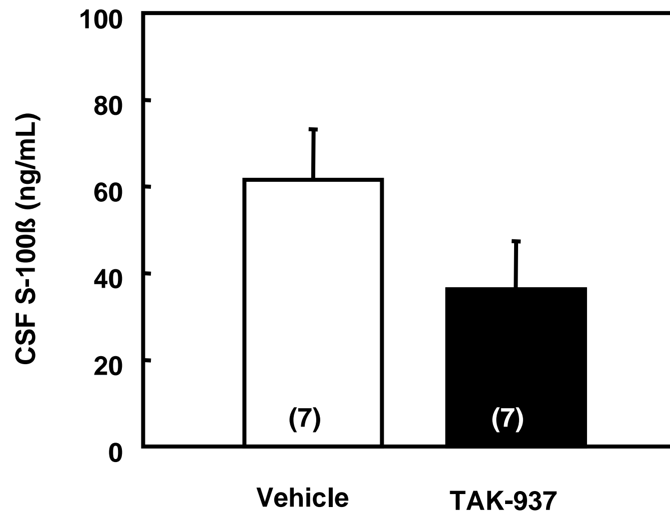


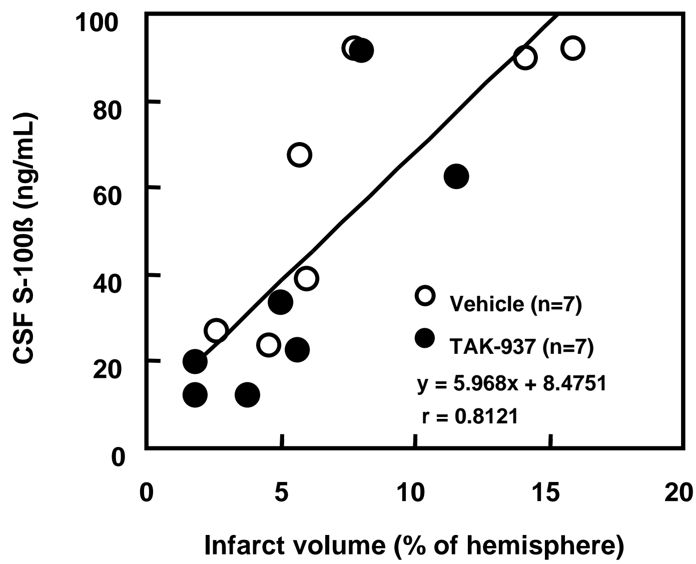
Figure 9. Effect of TAK-937 on CSF S-100β concentration (A) and correlation between CSF S-100β concentration and cerebral infarction (B) 24 h after thromboembolic MCAO in monkeys.

CSF was collected 24 h after clot injection. Each point with its vertical bar indicates the mean ± SEM. Numbers in parentheses show the number of monkeys used.

A



B



Chapter II

Contribution of Hypothermia and CB_1 Receptor Activation to Protective Effects of TAK-937, a Cannabinoid Receptor Agonist, in Rat Transient MCAO Model

Abstract

Cannabinoid (CB) receptor agonists are expected to alleviate ischemic brain damage by modulating neurotransmission and neuroinflammatory responses via CB_1 and CB_2 receptors, respectively. In Chapter I, TAK-937, a novel potent and selective CB_1 and CB_2 receptor agonist, was shown to exert significant cerebroprotective effects accompanied by hypothermia after transient middle cerebral artery occlusion (t-MCAO) in rats. Sustained hypothermia itself induces significant neuroprotective effects. In the present studies, the relative contribution of hypothermia and CB_1 receptor activation to the cerebroprotective effects of TAK-937 were examined. Using a multichannel brain temperature controlling system I developed, the brain temperature of freely moving rats was telemetrically monitored and maintained between 37 and 38°C during intravenous infusion of TAK-937 (100 μg/kg/h) or vehicle for 24 h after 2 h MCAO. AM251, a selective CB_1 receptor antagonist, was administered intraperitoneally at 30 mg/kg 30 min before starting intravenous infusion of TAK-937 (100 μg/kg/h) for 24 h. Rats were sacrificed, and their brains were isolated 26 h after MCAO in both experiments. When the hypothermic effect of TAK-937 was completely reversed by a brain temperature controlling system, the infarct-reducing effect of TAK-937 was attenuated in part, but remained significant. On the other hand, concomitant AM251 treatment with TAK-937 completely abolished the hypothermic and infarct-reducing effects of TAK-937. It can be concluded that the cerebroprotective effects of TAK-937 were, at least in part, mediated by the induction of hypothermia, and mainly mediated by CB_1 receptor activation.

Introduction

In chapter I, it was confirmed that TAK-937, a selective CB_1/CB_2 receptor agonist, has dose-dependent cerebroprotective effects in a rat t-MCAO model. TAK-937 improved not only histological damage in the short term, but also long term (4 weeks) neurological dysfunction and impairment of motor function in the rat t-MCAO model. Furthermore, TAK-937 showed protective effects after embolic MCAO in cynomolgus monkeys. Cerebroprotection by TAK-937 at its optimal doses was accompanied by a decrease in body temperature, which is one of the various actions of cannabinoids.

Body temperature is controlled by the hypothalamus, a central locus for thermoregulation. Ischemia of the hypothalamus causes pyrexia. In humans, body temperature in acute stroke is correlated with stroke severity, infarct size, mortality, and functional outcome [60]. Hyperthermia worsens brain injury. In contrast, hypothermia, even a 1°C decrease, reduces ischemic brain injury [61]. Hypothermia has been shown to be neuroprotective as exemplified by the reduced mortality and improved recovery of cardiac arrest survivors [62, 63] and in neonates after hypoxia/ischemia [64], although the utility of induced hypothermia for the treatment of ischemic stroke patients has not yet been established [65]. In my laboratory's previous study, post-ischemic mild hypothermia that was induced within 4 h after reperfusion showed significant cerebroprotective effects in a rat t-MCAO model [66]. Taken together, it is considered that the hypothermic component of cannabinoid treatment could be a part of their mechanisms for cerebroprotection. In this chapter II, I determined to what extent hypothermia contributes to cerebroprotection by TAK-937 in a rat t-MCAO model. I also investigated the involvement of CB_1 receptor in the cerebroprotective and hypothermic

effects of TAK-937.

Materials and Methods

Chemicals

AM251, N-(Piperidine-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide, a selective CB_1 receptor antagonist, was synthesized at the Medical Chemistry Research Laboratories of Takeda Pharmaceutical Company Ltd. Methylcellulose was purchased from Shin-Etsu Chemical Co., Ltd. (Tokyo, Japan). The other materials that were used are the same as those in Chapter I.

Animal preparation

Animal preparation was described in Chapter I.

Transient MCAO model in rats

The MCAO model was prepared in the same manner as in Chapter I. Eight-week-old male Sprague-Dawley (SD) rats (weighing 300 to 330 g) were used in this study.

Contribution of hypothermia to cerebroprotective effects

To assess the contribution of hypothermia to the neuroprotective effects of TAK-937, body temperature was controlled using a multichannel brain temperature controlling system (HEM software, Notocord Systems, Croissy, France, Figure 10A) according to previous methods, with modifications [66, 67]. One day before MCAO, the left femoral vein of rats was catheterized with a polyethylene tube for continuous administration of TAK-937 or vehicle. Subsequently, rats were implanted with a brain telemetry probe (Model XM-FHBP; Mini-Mitter Co., Bend, OR, USA) as follows. Under halothane

anesthesia, the rats were moved to a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) and a midline scalp incision was made. In the skull, a small burr hole was drilled 2 mm left of the bregma. The brain telemetry probe covered with a protector was lowered 4 mm below the skull surface and placed into the hole using dental cement. This method allowed the temperature in the contralateral cortex or corpus callosum to be measured. Anesthesia was discontinued and rats were housed individually in cages resting upon the telemetry receivers (RPC-1, DataSciences Int., St. Paul, MN, USA) that continuously sampled brain temperature. On the next day, t-MCAO was produced as described in Chapter I. Rats were returned to their cages and once again placed upon the telemetry receivers during MCAO. The intravenous administration of TAK-937 or vehicle was started just after reperfusion. Using a multichannel brain temperature controlling system, the brain temperature of freely moving rats was telemetrically monitored and maintained between 37 and 38°C for 24 h after reperfusion of the vehicle-treated and TAK-937-treated and warmed groups as follows. For the vehicle-treated group, two cooling fans equipped on the cage lid were turned on through an analogue feedback circuit when brain temperature became higher than 38°C, and the fans were stopped when brain temperature reached below 37°C. For the TAK-937-treated and warmed group, an infrared heat lamp was turned on through an analogue feedback circuit when brain temperature became lower than 37°C, and the lamp was stopped when brain temperature reached over 38°C. For the TAK-937-treated group, rats were kept at room temperature without controlling brain temperature. Brain temperature was recorded every 2 min for 2 sec on a receiver connected to a computer with a Dataquest A.R.T. System (DataSciences Int., St. Paul, MN, USA). Rectal temperature was measured before MCAO, just before drug administration, and 3, 5 and 24 h after drug administration with an electronic digital

thermometer (BAT-12, Physitemp Instruments, Clifton, NJ, USA). Infarct volumes were determined 24 h after administration as described in Chapter I.

Contribution of CB_1 receptor activation to cerebroprotective effects

One day before MCAO, the left femoral vein was catheterized with a polyethylene tube for continuous administration of TAK-937 or vehicle. On the next day, t-MCAO was produced as described in Chapter I. AM251 at 30 mg/kg or vehicle was intraperitoneally injected at 2 mL/kg 30 min before reperfusion. The intravenous administration of TAK-937 or vehicle was started just after reperfusion. Rectal temperature was measured before MCAO, just before drug administration, and 1, 3, 5, 8, and 24 h after drug administration with an electronic digital thermometer (BAT-12, Physitemp Instruments, Clifton, NJ, USA). Infarct volumes were determined 24 h after administration, as described in Chapter I.

Measurement of infarct volume in t-MCAO rats

Infarct volume was measured in the same manner as in Chapter I.

Drug administration

TAK-937 administration was examined in the same manner as in Chapter I. The concentration of TAK-937 was 250 μg/mL, which means that the applied dose of TAK-937 was 100 μg/kg/h. TAK-937 was administered at a dose of 125 μg/kg/10 min, followed by 100 μg/kg/h for 24 h. A solution of 5% hydroxypropyl-β-cyclodextrin (HPBCD) was administered as vehicle control at the same administration rate as TAK-937. AM-251 was suspended in 0.5% methylcellulose.

Statistical analysis

All data were expressed as the mean ± SEM. Statistical analysis between two groups was assessed using a two-tailed, unpaired Student's *t*-test. Differences among three or four groups were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's test. $P < 0.05$ was considered to be significant. All statistical analyses were performed using SAS Preclinical Package Version 5.0 (SAS Institute Japan Ltd., Tokyo, Japan).

Results

Contribution of hypothermia to cerebroprotective effects of TAK-937

The time course of brain temperature changes in TAK-937, TAK-937 plus warming, and vehicle-treated groups are shown in Figure 10B. Rectal temperatures at some time points were simultaneously measured (Table 4). TAK-937 at 100 μg/kg/h lowered brain temperature to about 35°C, but the rats in the vehicle-treated group showed no significant change. The hypothermic effect of TAK-937 was completely reversed to the level of brain temperature in the vehicle-treated group by warming rats with the multichannel brain temperature controlling system. The infarct volumes measured 1 day after MCAO in TAK-937, TAK-937 plus warming, and vehicle-treated groups were 105.7 ± 20.5, 200.8 ± 16.3, and 272.8 ± 25.6 mm^3, respectively (Figure 10C), indicating that TAK-937 significantly ($P < 0.001$) reduced cerebral infarct volume and that the infarct-reducing effect of TAK-937 was attenuated in part by warming, although a significant ($P < 0.05$) reduction in cerebral infarct volume was still observed.

Contribution of CB_1 receptor activation to cerebroprotective effects of TAK-937

The time course of changes in rectal temperature in TAK-937, AM251, TAK-937 plus AM251, and vehicle-treated groups are shown in Figure 11A. TAK-937 gradually lowered rectal temperature, and the maximum point of decrease was 34.4 ± 0.5°C 5 h after administration, whereas the vehicle-treated group rats showed no significant change. The hypothermic effect of TAK-937 was completely reversed by AM251, a selective CB_1 receptor antagonist. The infarct volumes measured 1 day after MCAO in TAK-937, AM251, TAK-937 plus AM251, and vehicle-treated groups were 114.3 ± 18.4, 294.9 ±

36.8, 309.8 ± 27.3, and 317.0 ± 28.9 mm^3, respectively (Figure 11B), indicating that TAK-937 significantly ($P < 0.001$) reduced infarct volume and that the infarct-reducing effect of TAK-937 was completely inhibited by AM251.

Discussion

In chapter I, TAK-937 was shown to reduce infarct volumes after t-MCAO at doses where hypothermia was induced. However, hypothermia itself exerted strong neuroprotection. Hypothermia is considered to suppress the depletion of high-energy phosphoric acid caused by the suppression of cerebral blood flow and cerebral metabolism and maintains a balance between supply and demand in the brain, which leads to neuroprotection. In the present study, I sought to examine the contribution of hypothermia and CB_1 receptor activation on the protective effects of TAK-937 on cerebral infarction after transient MCAO in rats, and to investigate the mechanism of the cerebroprotective effects of TAK-937.

To maintain a constant body temperature in animals, conventional apparatus such as a conductive water mattress, convective air warmer, and heating lamp are used in anesthetic conditions, where the body temperature generally drops. However, it is difficult and impractical for that type of apparatus to be used with conscious, freely moving animals. Therefore, I developed a multichannel brain temperature controlling system to maintain a constant brain temperature of conscious rats strictly in a range between 37 and 38°C by modification of our original brain temperature controlling system used to induce mild hypothermia [66], referring to the method of DeBow and Colbourne [67]. As shown in Figure 10B, brain temperature of control rats was kept constant throughout the drug administration period. Using this system, we revealed that the cerebroprotective effect of TAK-937 was partially attenuated by reversing hypothermia to normothermia. However, significant protection by TAK-937 was still observed in this condition. This finding suggests that the cerebroprotective effects of TAK-937 are mediated in part by the

induction of hypothermia. The present finding that the cerebroprotective effect of TAK-937 was seen without hypothermia is consistent with the finding in Chapter I that TAK-937, at 10 mg/kg/h where hypothermia was not seen, significantly reduced infarct volume after t-MCAO. Thus, it was suggested that mechanisms other than hypothermia would be involved in cerebroprotective effects of TAK-937. In this experiment, I simultaneously measured brain temperature telemetrically and rectal temperature manually at some time points (Table 4), because it was pointed that rectal temperature might not accurately reflect brain temperature, especially during global ischemia. As a result, I confirmed that there was little difference (about 0.5°C) between brain temperature and rectal temperature in the experiment, and both temperatures changed to parallel. This observation is supported by a report showing a positive correlation ($r = 0.91$) between brain temperature and rectal temperature in the same model as mine [68]. Based on this finding, I only measured rectal temperature in the experiment of the contribution of CB_1 receptor activation.

To pharmacologically reverse the hypothermic effect of TAK-937, a CB_1 receptor antagonist was applied because it has been reported that the hypothermic effect of cannabinoids is mediated by CB_1 receptors and not CB_2 receptors [69]. As shown in Figure 11A, the hypothermic effect of TAK-937 was completely reversed by the concomitant administration of AM251, a selective CB_1 receptor antagonist. Moreover, the cerebroprotective effect of TAK-937 was also completely reversed, in contrast to the physical reversal of hypothermia. This result suggests that the cerebroprotective effect of TAK-937 is almost mediated by CB_1 receptor agonist activity, and that mechanisms of cerebroprotection by TAK-937 other than hypothermia may also be mediated by the CB_1 receptor.

Thus far, several studies have investigated the contribution of hypothermia to neuroprotection by several known CB receptor agonists. HU-210, a synthetic CB agonist, demonstrated a dose-dependent reduction in infarct volume after focal cerebral ischemia, and cerebroprotection was associated with indirect protective effects of hypothermia [70]. Another synthetic CB agonist WIN 55,212-2-induced hypothermia (to approximately 34°C) significantly reduced infarct volume after focal and global cerebral ischemia, and this cerebroprotection was still observed after warming the animals to reverse their body temperature to the same level as that of control animals [71]. Thus, my study further confirmed the involvement of hypothermia in the cerebroprotective effect of CB receptor agonists and clearly indicated that other mechanisms, mainly mediated by CB_1 receptors, exist.

Mechanisms underlying the protective effects of CB receptor agonists unrelated to hypothermia have been shown in several *in vitro* studies. HU-210 and WIN 55,212-2 significantly reduced excitatory postsynaptic currents in a dose-dependent manner and inhibited the synaptic release of glutamate in rat dorsolateral striatal brain slices [72]. Synthetic CB agonists, CP 55,940 and WIN 55,212-2 remarkably reduced hippocampal cell death in cultured neurons subjected to high levels of N-methyl-D-aspartic acid (NMDA), and WIN 55,212-2 also inhibited the NMDA-induced increase in intracellular calcium concentration by initiating CB_1-mediated inhibition of adenylate cyclase [73]. Furthermore, CP 55,940 inhibited the release of nitric oxide (NO) from rat microglial cells via CB_1 receptor, which may play an important role in the central nervous system to elicit immune-mediated neurodegenerative inflammatory processes and cause brain injury [74]. These mechanisms may act additively or synergistically with hypothermia. It seems prudent to investigate whether TAK-937 also exerts those actions to fully

understand the mechanisms of its cerebroprotective effects.

In addition, the potential contribution of CB_2 receptor agonist activity to the cerebroprotective effects of TAK-937 cannot be neglected because the contribution of CB_2 receptor activation could be masked by strong CB_1 receptor activation. In order to examine the involvement of CB_2 receptor activation, I tried to determine the appropriate doses and dosing schedule for CB_2 receptor antagonist to block CB_2 receptor activation certainly utilizing some biomarkers, similar to the use of body temperature in the case of CB_1 receptor activation. The anti-inflammatory effects of CB_2 receptor activation by examining the changes in pro-inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor α (TNF-α) are considered as pharmacodynamic (PD) markers [75]. I explored the potential for utility of those pro-inflammatory cytokines as PD markers using AM630, a CB_2 receptor antagonist [76, 77], but the appropriate experimental conditions to examine the involvement of CB_2 receptor activation in our model could not be identified. In other words, substantial doses of AM630 did not block the suppressive effects of TAK-937 on cytokine elevation. Several studies reported that CB_2 receptor involved in microglial/macrophage activation, and influenced on alternative mediators/markers such as IL-10 and transforming growth factor β (TGF-β) [78]. Further studies are required to determine the appropriate dosing using PD markers, such as IL-10 or TGF-β, to clarify the involvement of CB_2 receptor activation.

Therapeutic hypothermia is well established in experimental animals [66, 68] and is applied clinically to patients with cardiac arrest [62, 63]. On the other hand, therapeutic hypothermia is not widely applied in acute ischemic stroke [65]. Several clinical trials to assess the efficacy of hypothermia on acute ischemic stroke are currently underway [79, 80]. One of the reasons why therapeutic hypothermia is not so widespread

is considered to be its inconvenience. Hypothermia with mechanical cooling provokes strong physiological counter responses such as vasoconstriction, shivering, hypertension, and tachycardia. Those responses should be cautiously monitored in well-organized facilities that provide specialist instruments and expert clinical support, such as in intensive care units, resulting in expensive treatment. In contrast, TAK-937 did not change blood gas parameters (PaO_2, $PaCO_2$, or pH) in a rat MCAO model, nor did it change mean arterial pressure or heart rate at doses effective in a cynomolgus monkey embolic MCAO model, although TAK-937 induced hypothermia in both models as described in chapter I. Thus, drug-induced hypothermia using the CB receptor agonist appears to be superior to mechanical cooling.

In conclusion, I found that the cerebroprotective effects of the highly potent and selective CB_1 and CB_2 receptor agonists were mediated partially by the induction of hypothermia and mainly by CB_1 receptors. CB activation promises to be a beneficial therapeutic target for the treatment of acute ischemic stroke patients.

Table and Figures

Table 4. Effect of TAK-937 on rectal and brain temperature in rats with t-MCAO.

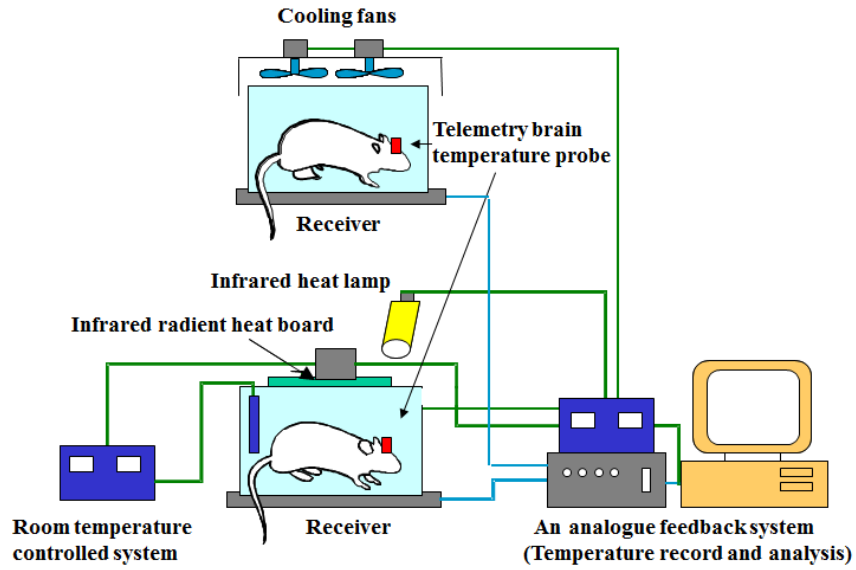
Brain temperature was recorded every 2 min for 2 sec on a receiver connected to a computer with Dataquest A.R.T. System (DataSciences Int., St. Paul, MN, USA) using a brain telemetry probe. Rectal temperature was measured using an electronic digital thermometer (BAT-12, Physitemp Instruments, Clifton, NJ, USA) before MCAO, just before drug administration, and 1, 3, 5, 8, and 24 h after drug administration. Data are indicated as the mean ± SEM.

Parameter	Groups	No. of animals	Before	Time after drug administration (h)			
				0	3	5	24
Brain Temp. (˚C)	Vehicle	10	36.7 ± 0.2	38.7 ± 0.2	37.7 ± 0.2	37.6 ± 0.1	37.4 ± 0.1
	TAK-937	10	36.9 ± 0.2	38.7 ± 0.2	35.6 ± 0.4	35.2 ± 0.4	36.9 ± 0.5
	TAK-937 + warming	11	36.9 ± 0.2	38.9 ± 0.1	37.5 ± 0.1	37.5 ± 0.1	37.6 ± 0.1
Rectal Temp. (˚C)	Vehicle	10	37.3 ± 0.1	39.4 ± 0.1	38.2 ± 0.1	38.2 ± 0.1	37.9 ± 0.1
	TAK-937	10	37.3 ± 0.1	39.4 ± 0.1	35.5 ± 0.4	35.0 ± 0.4	36.9 ± 0.4
	TAK-937 + warming	11	37.4 ± 0.1	39.3 ± 0.1	37.7 ± 0.1	37.8 ± 0.1	37.8 ± 0.1

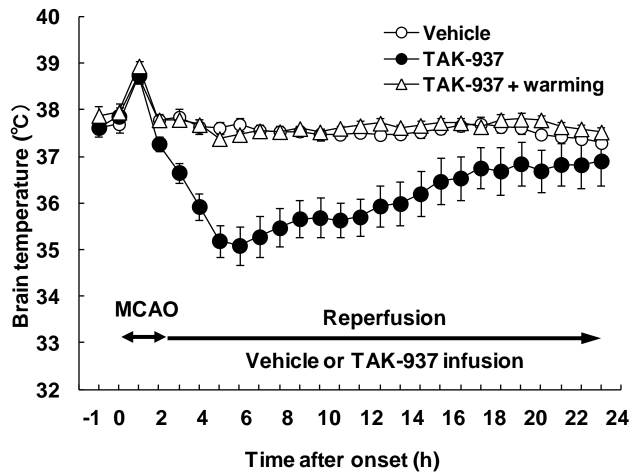
Figure 10. Contribution of hypothermia to cerebroprotective effects of TAK-937 after t-MCAO in rats.

Scheme of multichannel brain temperature controlling system (A), brain temperature (B), and infarct volume (C). Infusion of vehicle or TAK-937 at 100 μg/kg/h was started just after reperfusion. For the vehicle-treated group, two cooling fans were turned on through an analogue feedback circuit when brain temperature became higher than 38°C (upper figure). For the TAK-937 plus warming group, an infrared heat lamp was turned on through an analogue feedback circuit when brain temperature became lower than 37°C (lower figure). The brain temperature of freely moving rats was telemetrically monitored and maintained between 37 and 38°C for 24 h after reperfusion. Brains were obtained 24 h after reperfusion. Infarct volume was determined by staining with 2, 3, 5-triphenyltetrazolium chloride (TTC). Data are indicated as the mean ± SEM. Significant differences from the corresponding vehicle-treated group are indicated by $^{***}P < 0.001$ and $^{*}P < 0.05$ (Dunnett's test), and from the corresponding TAK-937-treated group is indicated by $^{\#\#}P < 0.01$ (Student's *t*-test). The number of rats used is shown in parentheses.

A.



B



C

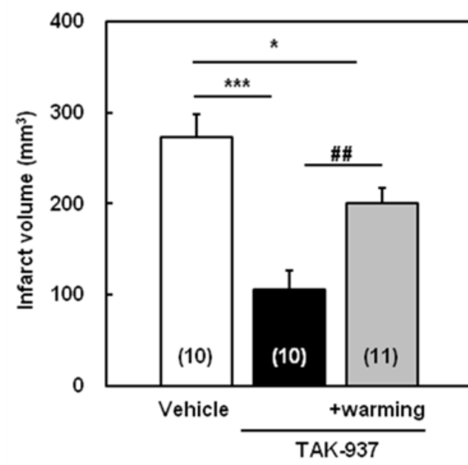
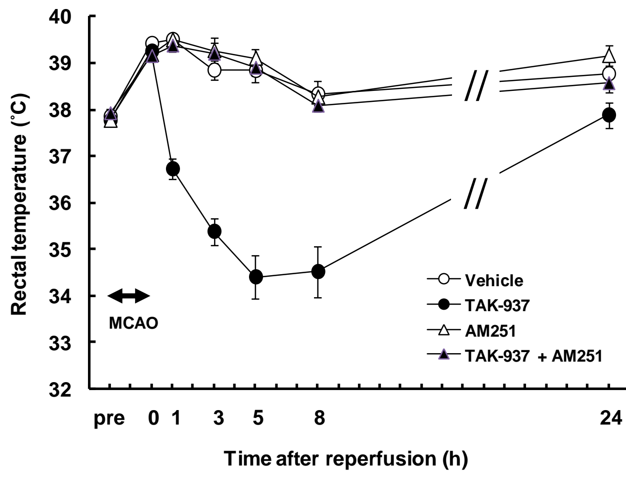


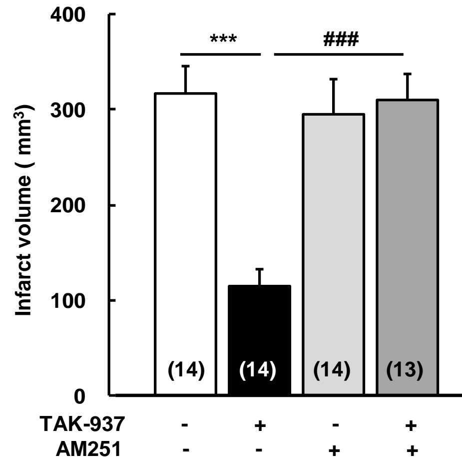
Figure 11. Reversal of cerebroprotection of TAK-937 by AM251, a CB_1 antagonist, after t-MCAO in rats.

Rectal temperature (**A**) and infarct volume (**B**). Infusion of vehicle or TAK-937 at 100 µg/kg/h started just after reperfusion. CB_1 receptor antagonist AM251 was intraperitoneally injected 30 min before reperfusion. Brains were obtained 24 h after reperfusion. Infarct volume was determined by staining with TTC. Data are indicated as the mean ± SEM. Significant differences from the corresponding vehicle-treated group are indicated by $^{***}P < 0.001$ (Dunnett's test), and from the corresponding TAK-937-treated group is indicated by $^{\#\#\#}P < 0.001$ (Student's *t*-test). The number of rats used is shown in parentheses.

A



B



General Discussion

As described in the general introduction, ischemic stroke is the top leading cause of long-term disability. In Japan, intravenous therapy with thrombolytic agent recombinant tissue-type plasminogen activator (rt-PA) was approved for acute ischemic stroke within 3 h of onset in 2005, and the time allowance for the use of the therapy was extended to 4.5 h of onset in 2012. With the progress of treatment in acute ischemic stroke, such as endovascular treatment as well as thrombolytic therapy, the recanalization rate of an occluded artery has become as high as 70 to 80% in recent years. The combined therapies with recanalization treatments and neuroprotectants are expected to become a mainstream treatment for ischemic stroke. In fact, several clinical trials of such combined therapies have been conducted in the United States. Furthermore, it is expected that neuroprotectant therapy can be applied to acute ischemic stroke patients at first to prolong the therapeutic time window and to allow thrombolytic therapy at later time points. Under such backgrounds, the demand for effective neuroprotective agents against cell damage after thromboembolic and/or reperfusion has increased more than when I investigated to generate them in 2000's. To develop anti-stroke therapies, many neuroprotective agents have been tested so far, but none of them have been proved effective in clinical trials, even now. One of the reasons for such failures would be that preclinical studies for those neuroprotective agents lacked long-term functional assessment, which is a major endpoint in clinical trials. In fact, a non-competitive MK-801, an NMDA receptor antagonist, reduced brain damage in the short-term, but did not improve neurological dysfunction in the long-term, and was clinically ineffective [23].

In chapter I, the selective CB receptor agonist reduced infarct volume in male, female and OVX rats, and also improved long-term functional outcomes and brain atrophy in the rat t-MCAO model. Moreover, I found for the first time that CB receptor

agonists have observable cerebroprotective effects in higher species. These studies are also important when considering the outcomes in future clinical trials that are related to functional outcomes and safety. Moreover, it has been observed that coadministration of CB receptor agonist with thrombolytic agent, t-PA, provided greater therapeutic efficacy than t-PA treatment alone, and also showed efficacy in aged rats [81]. Thus, I have covered almost all of the studies that should be conducted in non-clinical studies prior to clinical studies.

Actually, a phase I clinical trial of TAK-937 was conducted in 2009 to characterize the safety, tolerability profile, and pharmacokinetic parameters, to identify a well-tolerated TAK-937 loading dose, and to assess the pharmacodynamic effects of TAK-937. As a result, due to the cardiovascular side effects observed at doses below the target plasma concentration (1-1.5 ng/mL), the current safety margin for TAK-937 did not support further clinical development for the indication of acute ischemic stroke.

In chapter II, I found that the cerebroprotective effects of TAK-937 were totally mediated by CB_1 receptor activation and at least in part mediated by the induction of hypothermia in a rat t-MCAO model. Activation of CB_1 receptor suppresses cAMP production via G protein-mediated inhibition of adenylate cyclase. The decrease of cAMP suppresses intracellular Ca^{2+} influx by voltage-gated Ca^{2+} channel, and as a result, the release of various transmitters such as glutamate-caused neuronal excitation, is suppressed. One of the mechanisms of action of TAK-937 is considered to be the inhibition of excitotoxicity of an excessive glutamate signal, which is a common pathway with MK-801, the NMDA receptor antagonist that failed in a clinical study, as described above. Differing from MK-801, which acts exclusively on the NMDA receptor, which is a glutamate receptor subtype, TAK-937 acts on various cascades including upstream of

MK-801. In addition, hypothermia caused by CB_1 activation is considered to contribute to additive and/or synergistic cerebroprotective effects (Figure 12).

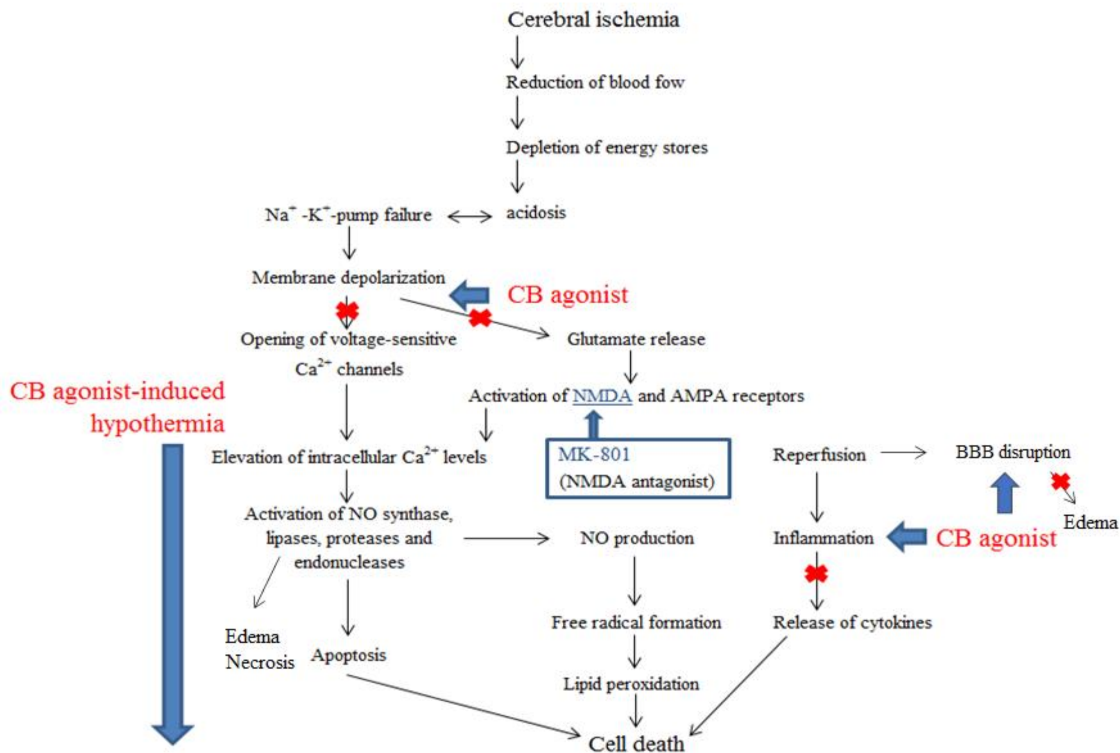


Figure 12. Putative mechanisms of actions of a CB agonist on ischemic cell death pathway.

In my rat t-MCAO model, brain injury was not apparent until 10 h after ischemia. On the other hand, inflammatory responses, including cytokine production, were induced about 6 h after occlusion. Therefore, it was suggested that the production of inflammatory cytokines occurred preceding ischemic brain injury and might be promoting factors of brain injury. TAK-937 has more potent CB_2 receptor agonistic activity than CB_1. The CB_2 receptor is mainly expressed in peripheral immune cells such as the spleen and tonsils, and is also expressed in astrocytes and microglia in the brain [82]. It was reported that

CB_2 receptor was relevant to the regulation of inflammatory responses in immune cells and mediated the anti-inflammatory effect against activated astrocytes and microglia [83, 84]. However, in this study, CB_2 receptors appear to have contributed little to the cerebroprotective effect of TAK-937. In fact, TNF-α, an inflammatory cytokine expressed within 1-6 h after cerebral ischemia, is localized in axons of neurons but not in astrocytes [85, 86]. Hence, it is thought that inflammatory cytokines such as TNF-α are produced by neurons, and then these cytokines induce activation of glial cells such as oligodendrocytes and astrocytes to produce more inflammatory cytokines, causing inflammation and gliosis. TNF-α activates NF-κB by activating IκB kinase (IKK) that phosphorylates inhibitory-κB (IκB) [87]. It has been reported that an endogenous cannabinoid 2-arachidonoylglycerol (2-AG) reduces cerebral edema and cerebral injury in a model of head injury, and its mechanism of action is that the CB_1 receptor is involved in the suppression of NF-κB, suggesting that the CB_1 receptor may be involved in the intracellular inflammatory signaling pathway [88]. Furthermore, in the same model as this study, it was reported that CB_2-positive macrophages derived from microglia accumulated at the site of brain injury 3 days after the onset of cerebral ischemia [89], suggesting that there is little involvement of CB_2 receptor activation at an early stage, i.e., within 24 h after the onset of cerebral ischemia. Therefore, it may be difficult to recognize the involvement of the CB_2 receptor. As described above, it was reconfirmed that the cerebroprotective effect of TAK-937 in this rat t-MCAO model is mediated by CB_1 receptor activation.

After my studies, a large amount of research on mechanisms of ischemic injury have been conducted, especially studies from the viewpoint of glial cells have been increasing. In recent years, it was demonstrated that the ratio of glial cells to neurons in

the human brain is 1:1 with a range of 40-130 billion glial cells, although the human brain is believed to contain about 100 billion neurons and one trillion glial cells, with a neuron:glia ratio of 1:10 over the past five decades [90]. Glial cells are thought to have roles in structurally and metabolically supporting neurons, but recently, they have been thought to be involved in neural function by exchanging neurotransmitters with neurons. Thus, recent studies have focused on not only neurons but also glial cells. Astrocytes of the glial cell population are the first responders to ischemic stress [91]. When aerobic metabolism shuts down because of oxygen and glucose deprivation, lactate production from glycogen stored predominantly in glial cells continues, which leads to severe acidosis. Beppu *et al.* demonstrated, using optogenetic tools, that acidosis in astrocytes triggers glutamate release from astrocytes followed by neuronal cell death via glutamate excitotoxicity [92]. Ischemia activates not only cell death pathways but also endogenous protective mechanism pathways. There is increasing interest in understanding the mechanisms of endogenous neuronal protection and repair as potential strategies because hundreds of clinical trials failed to show therapeutic effects against ischemic stroke. It has been reported that endogenous cannabinoids are involved in triggering this protective mechanism [93]. An acute elevation of endogenous CB stimulates the CB_1 receptor in astrocytes, suppresses the GLT1 receptor, increases ambient glutamate, activates postsynaptic NR2B, and induces postsynaptic AMPA receptor endocytosis, which elicits long-term depression (LTD) at glutamatergic synapses to trigger neuroprotection. In addition, endocannabinoid signaling regulates neural progenitor cells in the adult brain [94], which means that CB activation is involved in post-developmental neurogenesis in the brain. CB activation induces PI3K/Akt and MEK/MAPK/CREB signaling pathways that influence cell proliferation, differentiation and survival, and also promote the

integration of immature neurons into existing circuitry. Interestingly, the mammalian target of rapamycin complex1 (mTORC1) signaling is a target of the PI3K/Akt pathway and mTORC signaling also contributes to CB_2-regulated neural progenitor cell proliferation, although there are considerable reports that indicate that the involvement of the CB_1 receptor is crucial [95]. As described above, CB activation is considered to exert cerebroprotective effects by suppressing excitotoxic mechanisms in the super acute phase, anti-inflammation and anti-apoptosis in the acute phase, and neurogenesis in the chronic phase of ischemic stroke. Since the involvement of CB_2 activation might still be involved in these actions, I would like to construct an experimental model that can further explore the involvement of CB_2 activation, and to propose a long-lasting neuroprotective effect at lower doses without CB_1-mediated adverse effects.

In conclusion, my first study provides clear evidence, for the first time, that a CB receptor agonist provides cerebroprotection and improvement of long-term neurologic outcome in rats and monkeys subjected to MCAO-induced brain ischemia. In addition, my second study provides evidence that the cerebroprotective effects of the CB receptor agonist are at least in part mediated by the induction of hypothermia, and mainly mediated by CB_1 receptor activation. My studies support that the activation of CB receptors is effective for acute ischemic stroke, and would add valuable findings to the pathophysiology of, and provide potential therapeutic approaches for, human cerebral ischemic stroke.

Acknowledgements

I am deeply grateful to Professors Chikafumi Chiba and Tomoki Chiba, and Associate Professors Kyoichi Sawamura and Hidekazu Kuwayama, University of Tsukuba, for guiding my work and valuable discussions through my doctoral program.

I am very thankful to Drs. Masato Shimojo and Motohisa Suzuki, Takeda Pharmaceutical Company Limited, for guiding my work and valuable discussions through my work.

I appreciate Dr. Koji Murakami and Tetsuya Tsukamoto, Takeda Pharmaceutical Company Limited, for their contributions and helpful support.

I also thank Kazuhiro Hamajo, Takeda Pharmaceutical Company Limited, for his technical support.

Finally, I would like to offer appreciation to my family for supporting my life in University of Tsukuba.

References

1. World Health Organization, http://www.who.int/mediacentre/factsheets/fs310/en/ (2018).
2. Martínez-Sánchez P, Díez-Tejedor E, Fuentes B, Ortega-Casarrubios MA, Hacke W. Systemic reperfusion therapy in acute ischemic stroke. *Cerebrovasc Dis.* 2007; 24 Suppl 1: 143-52.
3. Wardlaw JM, Warlow CP, Counsell C. Systematic review of evidence on thrombolytic therapy for acute ischaemic stroke. *Lancet*. 1997; 350: 607-614.
4. Dirnagl U, Iadecola C, Moskowitz M.A. Pathobiology of ischemic stroke; an integrated view. *Trends Neurosci.* 1999; 22: 391-397.
5. Moussa HK, Graffagnino C. Nonocclusion and spontaneous recanalization rates in acute ischemic stroke. *Arch Neurol.* 2002; 59: 1870-3.
6. Abe K. Edaravone. *Nippon Rinsyo.* 2006; 64 Suppl 7: 548-53.
7. Astrup J, Siesjo BK, Symon L. Thresholds in cerebral ischemia – the ischemic penumbra. *Stroke.* 1981; 12: 723-5.
8. Gaoini Y, Mechoulam R. Isolation, structure and partial synthesis of an active constituent of hashish. *J Am Chem Soc.* 1964; 86: 1646-1647.
9. Matsuda LA, Lolait SJ, Brownstein MJ, Young AC, Bonner TI. Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature.* 1990; 346: 561-564.
10. Munro S, Thomas KL, Abu-Shaar M. Molecular characterization of a peripheral receptor for cannabinoids. *Nature.* 1993; 365: 61-5.
11. Herkenham M, Lynn AB, Little MD, Johnson MR, Melvin LS, de Costa BR, *et al*. Cannabinoid receptor localization in brain. *Proc Natl Acad Sci USA*. 1990: 87, 1932-1936.

12. Galiègue S, Mary S, Marchand J, Dussossoy D, Carrière D, Carayon P, *et al.* Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. *Eur J Biochem.* 1995; 232: 54-61.

13. Pertwee RG. Pharmacology of cannabinoid CB_1 and CB_2 receptors. *Pharmacol Ther.* 1997; 74: 129-180.

14. Parmentier BS, Jin K, Mao XO, Xie L, Greenberg DA. Increased severity of stroke in CB_1 cannabinoid receptor knock-out mice. *J Neurosci.* 2002; 22: 9771-9775.

15. Jin KL, Mao XO, Goldsmith PC, Greenberg DA. CB_1 cannabinoid receptor induction in experimental stroke. *Ann Neurol.* 2000; 48: 257-261.

16. Murikinati S, Jüttler E, Keinert T, Ridder DA, Muhammad S, Waibler Z, *et al.* Activation of cannabinoid 2 receptors against cerebral ischemia by inhibiting neutrophil recruitment. *FASEB J.* 2009; 2: 1-11.

17. Koizumi J, Yoshida Y, Nakazawa T, Ooneda G. Experimental studies of ischemic brain edema. 1. A new experimental model of cerebral embolism in rats in which recirculation can be introduced in the ischemic area. *Jpn J Stroke.* 1986; 8: 1-8.

18. Powers WJ, Rabinstein AA, Ackerson T, Adeoye OM, Bambakidis NC, Becker K, *et al*. Guidelines for the early management of patients with acute ischemic stroke: 2019 update to the 2018 guidelines for the early management of acute ischemic stroke: a guideline for healthcare professionals from the American Heart Association/ American Stroke Association. *Stroke.* 2019; 50: e344-e418.

19. O'Collins VE, Macleod MR, Donnan GA, Horky LL, van der Worp BH, *et al*. 1,026 experimental treatments in acute stroke. *Ann Neurol.* 2006; 59: 467-477.

20. Bramlett HM, Dietrich WD. Neuropathological protection after traumatic brain injury in intact female rats versus males or ovariectomized females. *J Neurotrauma.* 2001;

18: 891-900.

21. Hurn PD, Vannucci SJ, Hagberg H. Adult or perinatal brain injury; does sex matter? *Stroke.* 2005; 36: 193-195.
22. Tan Z, Li X, Kelly KA, Rosen CL, Huber JD. Plasminogen activator inhibitor type 1 derived peptide, EEIIMD, diminishes cortical infarct but fails to improve neurological function in aged rats following middle cerebral artery occlusion. *Brain Res.* 2009; 1281: 84-90.
23. Valtysson J, Hillered L, Andiné P, Hagberg H, Persson L. Neuropathological endpoints in experimental stroke. *Acta Neurochir.* 1994; 129: 58-63.
24. Stroke Therapy Academic Industry Roundtable. Recommendation for standards regarding preclinical neuroprotective and restorative drugs. *Stroke.* 1999; 30: 2752-2758.
25. Longa EZ, Weinstein PR, Carlson S, Cummins R. Reversible middle cerebral artery occlusion without craniotomy in rats. *Stroke.* 1989; 20: 84-91.
26. Kiyota Y, Pahlmark K, Memezawa H, Smith ML, Siesjo BK. Free radicals and brain damage due to transient middle cerebral artery occlusion: the effect of dimethylthiourea. *Exp. Brain Res.* 1993; 95: 388–396.
27. Bederson JB, Pitts LH, Tsuji M, Nishimura MC, Davis RL, *et al*. Rat middle cerebral artery occlusion; evaluation of the model and development of a neurologic examination. *Stroke.* 1986; 17: 472-476.
28. Hernandez TD, Schallert T. Seizures and recovery from experimental brain damage. *Exp Neurol.* 1988; 102: 318-324.
29. Kito G, Nishimura A, Susumu T, Nagata R, Kuge Y, *et al*. Experimental thromboembolic stroke in cynomolgus monkey. *J Neurosci Methods.* 2001; 105: 45-

53.

30. Rawls SM, Cabassa J, Geller EB, Adler MW. CB_1 receptors in the preoptic anterior hypothalamus regulate WIN 55212-2 [(4,5-dihydro-2-methyl-4(4-morpholinylmethyl)-1-(1- naphthalenyl-carbonyl)-6H-pyrrolo[3,2,1ij]quinolin-6-one) - induced hypothermia. *J Pharmacol Exp Ther.* 2002; 301: 963-968.
31. Macleod MR, Worp HB, Sena ES, Howells DW, Dirnagl U, *et al*. Evidence for the efficacy of NXY-059 in experimental focal cerebral ischemia is confounded by study quality. *Stroke.* 2008; 39: 2824-9.
32. Koehler RC, Traystman RJ. Bicarbonate ion modulation of cerebral blood flow during hypoxia and hypercapnia. *Am J Physiol.* 1982; 243: 33-40.
33. Brian JE Jr. Carbon dioxide and the cerebral circulation. *Anesthesiology.* 1998; 88: 1365-86.
34. Leker RR, Gai N, Mechoulam R, Ovadia H. Drug-induced hypothermia reduces ischemic damage; effects of the cannabinoid HU-210. *Stroke.* 2003; 34: 2000-2006.
35. Lazzaro MA, Prabhakaran S. Induced hypothermia in acute ischemic stroke. *Expert Opin Investig Drugs.* 2008; 17: 1161-1174.
36. Miyazawa T, Tamura A, Fukui S, Hossmann KA. Effect of mild hypothermia on focal cerebral ischemia; Review of experimental studies. *Neurol Res.* 2003; 25: 457-464.
37. Seshadri S, Beiser A, Hayes MK, Kase CS, Au R, Kannel WB, *et al*. The lifetime risk of stroke. Estimates from the Framingham study. *Stroke.* 2006; 37: 345-50.
38. Bousser MG. Stroke in women. The 1997 Paul Dudley white international lecture. *Circulation.* 1999; 99: 463-7.
39. Kapral MK, Fang J, Hill MD, Silver F, Richards J, Jaigobin C, *et al*. Sex differences in stroke care and outcomes. Results from the registry of the Canadian stroke network.

Stroke. 2005; 36: 809-14.

40. Wise PM, Dubal DB, Wilson ME, Rau SW, Bottner M, Rosewell KL. Estradiol is a protective factor in the adult and aging brain: understanding of mechanisms derived from *in vivo* and *in vitro* studies. *Brain Res Rev.* 2001; 37: 313-9.
41. Hurn PD, Brass LM. Estrogen and stroke. A balanced analysis. *Stroke*. 2003; 34: 338-41.
42. Miller VM, Duckles SP. Vascular actions of estrogens: functional implications. *Pharmacol Rev*. 2008; 60: 210-41.
43. Lang JT, McCullough LD. Pathways to ischemic neuronal cell death: are sex differences relevant? *J Translational Medicine.* 2008; 6: 33-42.
44. Chen CH, Toung TJK, Hurn PD, Koehler RC, Bhardwaj A. Ischemic neuroprotection with selective κ-opioid receptor agonist is gender specific. *Stroke.* 2005; 36: 1557-61.
45. Liu F, Li Z, Li J, Siegel C, Yuan R, McCullough LD. Sex differences in caspase activation after stroke. *Stroke.* 2009; 40: 1842-8.
46. Ojeda NB, Grigore D, Robertson EB, Alexander BT. Estrogen protects against increased blood pressure in postpubertal female growth restricted offspring. *Hypertension.* 2007; 50: 679-85.
47. Zhang L, Schallert T, Zhang ZG, Jiang Q, Arniego P, Li Q, *et al*. A test for detecting long-term sensorimotor dysfunction in the mouse after focal cerebral ischemia. *J Neurosci Methods.* 2002; 117: 207-214.
48. Freret T, Chazalviel L, Roussel S, Bernaudin M, Schumann-Bard P, Boulouard M. Long-term functional outcome following transient middle cerebral artery occlusion in the rat: correlation between brain damage and behavioral impairment. *Behav Neurosci.* 2006; 120: 1285-98.

49. Grundy RI. The therapeutic potential of the cannabinoids in neuroprotection. *Expert Opin. Investig. Drugs.* 2002; 11: 1365-1374.

50. Huang SY, Bridges D, Grigorenko E, McCloud S, Boon A, Hampson RE, *et al*. Cannabinoids produce neuroprotection by reducing intracellular calcium release from ryanodine-sensitive stores. *Neuropharmacology.* 2005; 48: 1086-1096.

51. Croxford JL, Miller SD. Immunoregulation of a viral model of multiple sclerosis using the synthetic cannabinoid R+WIN55,212. *J Clin Invest.* 2003; 111: 1231-1240.

52. Panikashvili D, Shein NA, Mechoulam R, Trembovler V, Kohen R, Alexandrovich A, *et al*. The endocannabinoid 2-AG protects the blood-brain barrier after closed head injury and inhibits mRNA expression of proinflammatory cytokines. *Neurobiol Dis.* 2006; 22: 257-264.

53. Nagayama T, Sinor AD, Simon RP, Chen J, Graham SH, Jin K, *et al*. Cannabinoids and neuroprotection in global and focal cerebral ischemia and in neuronal cultures. *J Neurosci.* 1999; 19: 2987-2995.

54. Muthian S, Rademacher DJ, Roelke CT, Gross GJ, Hillard CJ. Anandamide content is increased and CB_1 cannabinoid receptor blockade is protective during transient, focal cerebral ischemia. *Neuroscience.* 2004; 129: 743-750.

55. Lake KD, Martin BR, Kunos G, Varga K. Cardiovascular effects of anandamide in anesthetized and conscious normotensive and hypertensive rats. *Hypertension.* 1997; 29: 1204-1210.

56. Missler U, Wiesmann MT, de Bruijn CH, Lamers KJ. Release of glial tissue-specific proteins after acute stroke; a comparative analysis of serum concentration of protein S-100β and glial fibrillary acidic protein. *Stroke.* 2000; 31: 2670-2677.

57. Petzold A, Keir G, Lim D, Smith M, Thompson EJ. Cerebrospinal fluid (CSF) and

serum S100β: release and wash-out pattern. *Brain Res Bull*. 2003; 61: 281-285.

58. Matsui T, Mori T, Tateishi N, Kagamiishi Y, Satoh S, Katsube N, *et al*. Astrocytic activation and delayed infarct expansion after permanent focal ischemia in rats. Part I; Enhanced astrocytic synthesis of S-100β in the periinfarct area precedes delayed infarct expansion. *J Cereb blood flow Metab.* 2002; 22: 711-722.
59. Swanson RA, Ying W, Kauppinen TM. Astrocyte influences on ischemic neuronal death. *Curr Mol Med.* 2004; 4: 193-205.
60. Reith J, Jørgensen HS, Pedersen PM, Nakayama H, Raaschou HO, *et al*. Body temperature in acute stroke: relationship to stroke severity, infarct size, mortality, and outcome. *Lancet* 1996; 347: 422–425.
61. Colbourne F, Sutherland G, Corbett D. Postischemic hypothermia: a critical appraisal with implications for clinical treatment. *Mol. Neurobiol.* 1997; 14: 171–201.
62. The Hypothermia after Cardiac Arrest Study Group. Mild therapeutic hypothermia to improve the neurological outcome after cardiac arrest. *N. Engl. J. Med.* 2002; 346: 549–556.
63. Bernard SA, Gray TW, Buist MD, Jones BM, Silvester W, *et al*. Treatment of comatose survivors of out-of-hospital cardiac arrest with induced hypothermia. *N. Engl. J. Med.* 2002; 346: 557–563.
64. Shankaran S, Laptook AR, Ehrenkranz RA, Tyson JE, McDonald SA, *et al*.; National Institute of Child Health and Human Development Neonatal Research Network. Whole-body hypothermia for neonates with hypoxic-ischemic encephalopathy. *N. Engl. J. Med.* 2005; 353: 1574–1584.
65. Adams HP, Zoppo G, Alberts MJ, Bhatt DL, Brass L, *et al*. Guidelines for the early management of adults with ischemic stroke. *Stroke* 2007; 38: 1655–1711.

66. Ohta H, Terao Y, Shintani Y, Kiyota Y. Therapeutic time window of post-ischemic mild hypothermia and the gene expression associated with the neuroprotection in rat focal cerebral ischemia. *Neurosci. Res.* 2007; 57: 424–433.

67. DeBow S, Colbourne F. Brain temperature measurement and regulation in awake and freely moving rodents. *Methods* 2003; 30: 167–171.

68. Maier CM, Ahern K, Cheng ML, Lee JE, Yenari MA, *et al*. Optimal depth and duration of mild hypothermia in a focal model of transient cerebral ischemia: effects on neurologic outcome, infarct size, apoptosis, and inflammation. *Stroke* 1998; 29: 2171-2180.

69. Rawls SM, Cabassa J, Geller EB, Adler MW. CB_1 receptors in the preoptic anterior hypothalamus regulate WIN 55,212-2 [(4,5-dihydro-2-methyl)-4-(4-morpholinylmethyl)-1-(1-naphthalenyl-carbonyl)-6H-pyrro-lo[3,2,1ij]quinolin-6-one)]-induced hypothermia. *J. Pharmacol. Exp. Ther.* 2002; 301: 963–968.

70. Leker RR, Gai N, Mechoulam R, Ovadia H. Drug-induced hypothermia reduces ischemic damage: effects of the cannabinoid HU-210. *Stroke* 2003; 34: 2000–2006.

71. Bonfils PK, Reith J, Hasseldam H, Johansen FF. Estimation of the hypothermic component in neuroprotection provided by cannabinoids following cerebral ischemia. *Neurochem. Int.* 2006; 49: 508–518.

72. Gerdeman G, Lovinger DM. Cannabinoid receptor inhibits synaptic release of glutamate in rat dorsolateral striatum. *J. Neurophysiol.* 2001; 85: 468–471.

73. Zhuang SY, Bridges D, Grigorenko E, McCloud S, Boon A, *et al*. Cannabinoids produce neuroprotection by reducing intracellular calcium release from ryanodine-sensitive stores. *Neuropharmacol.* 2005; 48: 1086–1096.

74. Waksman Y, Olson JM, Carlisle SJ, Cabral GA. The central cannabinoid receptor

(CB_1) mediates inhibition of nitric oxide production by rat microglial cells. *J. Pharm. Exp. Ther.* 1999; 288: 1357–1366.

75. Gallily R, Breuer A, Mechoulam R. 2-Arachidonylglycerol, an endogenous cannabinoid, inhibits tumor necrosis factor-α production in murine macropharges, and in mice. *Eur J Pharmacol.* 2000; 406: R5-R7
76. Hosohata K, Quock RM, Hosohata Y, Burkey TH, Makriyannis A, *et al*. AM630 is a competitive cannabinoid receptor antagonist in the guinea pig brain. *Life Sci.* 1997; 61: PL115-PL118.
77. Ross AR, Brockie HC, Stevenson LA, Murphy VL, Templeton F, *et al*. Agonist-inverse agonist characterization at CB_1 and CB_2 cannabinoid receptors of L759633, L759656 and AM630. *Br J Pharmacol.* 1999; 126: 665-672.
78. Zarruk JG, Fernández-López D, García-Yébenes I, García-Gutiérrez MS, Vivancos J, *et al*. Cannabinoid type 2 receptor activation downregulates stroke-induced classic and alternative brain macropharge/microglial activation concomitant to neuroprotection. *Stroke* 2012; 43: 211-219.
79. Kammersgaard LP, Rasmussen BH, Jørgensen HS, Reith J, Weber U, *et al*. Feasibility and safety of inducing modest hypothermia in awake patients with acute stroke through surface cooling: a case-control study. The Copenhagen stroke study. *Stroke* 2000; 31: 2251–2256.
80. Krieger DW, Georgia MA, Abou-Chebl A, Andrefsky JC, Sila CA, *et al*. Cooling for acute ischemic brain damage (COOL AID). An open pilot study of induced hypothermia in acute ischemic stroke. *Stroke* 2001; 32: 1847–1854.
81. Murakami K, Suzuki M, Suzuki N, Hamajo K, Tsukamoto T, Shimojo M. Cerebroprotective effects of TAK-937, a novel cannabinoid receptor agonist, in

permanent and thrombotic focal cerebral ischemia in rats: therapeutic time window, combination with t-PA and efficacy in aged rats. *Brain Res.* 2013; 1526: 84-93.

82. Galiegue S, Mary S, Marchand J, Dussossoy D, Carriere D, Carayon P, *et al*. Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. *Eur J Biochem.* 1995; 232: 54-61.
83. Stella N. Cannabinoid and cannabinoid-like receptors in microglia, astrocytes, and astrocytomas. *Glia.* 2010; 58: 1017-1030.
84. Zarruk JG, Fernández-López D, García-Yébenes I, García-Gutiérrez MS, Vivancos J, Nombela F, *et al*. Cannabinoid type 2 receptor activation downregulates stroke-induced classic and alternative brain macrophage/microglial activation concomitant to neuroprotection. *Stroke.* 2012; 43: 211-219.
85. Liu T, Clark RK, McDonnell PC, Young PR, White RF, Barone FC, *et al*. Tumor necrosis factor-alpha expression in ischemic neurons. *Stroke.* 1994; 25: 1481-1488.
86. Wang X, Yue TL, Barone FC, White RF, Gagnon RC, Feuerstein GZ. Concomitant cortical expression of TNF-alpha and IL-1 beta mRNAs follows early response gene expression in transient focal ischemia. *Mol Chem Neuropathol.* 1994; 23: 103-114.
87. Schreck R, Rieber P, Baeuerle PA. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1. *EMBO J.* 1991; 10: 2247-2258.
88. Panikashvili D, Mechoulam R, Beni SM, Alexandrovich A, Shohami E. CB_1 cannabinoid receptors are involved in neuroprotection via NF-κB inhibition. *J Cereb Blood Flow Metab.* 2005; 25: 477-484.
89. Ashton JC, Rahman RMA, Nair SM, Sutherland BA, Glass M, Appleton I. Cerebral hypoxia-ischemia and middle cerebral artery occlusion induce expression of the

cannabinoid CB_2 receptor in the brain. *Neurosci. Lett.* 2007; 412: 114-117.

90. von Bartheled CS, Bahney J, Herculano-Houzel S. The search for true numbers of neurons and glial cells in the human brain: a review of 150 years of cell counting. *J Comp Neurol.* 2016; 524: 3865-3895.
91. Rossi DJ, Brady JD, Mohr C. Astrocyte metabolism and signaling during brain ischemia. *Nat. Neurosci.* 2007; 10: 1377-1386.
92. Beppu K, Sasaki T, Tanaka KF, Yamanaka A, Fukazawa Y, *et al*. Optogenetic countering of glial acidosis suppresses glial glutamate release and ischemic brain damage. *Neuron.* 2014; 81: 314-320.
93. Wang F, Han J, Higashimori H, Wang J, Liu J, *et al*. Long-term depression induced by endogenous cannabinoids produces neuroprotection via astroglial CB_1R after stroke in rodents. *J Cereb Blood Flow Metab.* 2019; 39: 1122-1137.
94. Prenderville JA, Kelly AM, Downer EJ. The role of cannabinoids in adult neurogenesis. *Br J Pharmacol.* 2015; 172: 3950-3963.
95. Palazuelos J, Ortega Z, Diaz-Alonso J, Guzman M, Galve-Roperh I. CB_2 cannabinoid receptors promote neural progenitor cell proliferation via mTORC1 signaling. *J Biol Chem.* 2012; 287: 1198-1209.