Life Sciences 232 (2019) 116605

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Opening of T-type Ca2+ channels and activation of HCN channels contribute in stress adaptation in cold water immersion stress-subjected mice

Simranjot Kaur, Nirmal Singh, Amteshwar Singh Jaggi*

Department of Pharmaceutical Sciences and Drug Research, Punjabi University, Patiala, Punjab 147002, India

ARTICLE INFO

Keywords:
Ethosuximide
Ivabradine
Stress adaptation
Behavioral changes
Corticosterone

ABSTRACT

Aim: The present study was designed to investigate the possible role of T-type Ca^{2+} channels and HCN channels in the development of stress adaptation in cold-water immersion stress-subjected mice.

Material and methods: The mice were subjected to cold-water immersion stress by placing them individually in a water tank (depth = 15.5 cm; temperature = 15 ± 2 °C) for 5 min. The mice were subjected to single episode of cold-water immersion stress for inducing acute stress; while for inducing stress adaptation, mice were subjected to repeated episodes of homotypic stressor (5 min) for 5 consecutive days. Animals were administered with ethosuximide (100 and 200 mg/kg, *i.p.*) and ivabradine (5 and 10 mg/kg, *i.p.*) before subjecting them to stress for five days. The stress-related behavioral alterations were assessed using the actophotometer, the hole board, the open field and the social interaction tests. The plasma corticosterone levels were quantified as a biochemical parameter of hypothalamic-pituitary-adrenal (HPA) axis activation.

Results: Acute stress altered the behavioral and biochemical parameters of the animals. However, repeated stress significantly restored the behavioral and biochemical alterations signifying the development of adaptation. Administration of ethosuximide and ivabradine abolished the restoration of behavioral and biochemical changes in the animals subjected to repeated stress.

Conclusion: The ethosuximide and ivabradine mediated attenuation of stress adaptation demonstrates that the opening of T-type Ca^{2+} channels and activation of HCN channels are involved in inducing stress adaptation in repeated stress-subjected animals.

1. Introduction

Stress is defined as the sum total of all the reactions in the body that leads to state of threatened homeostasis and activates a composite spectrum of adaptive physiological and behavioral responses with an aim to restore the challenged body homeostasis [4,25]. Initial exposure to a stressor results in behavioral alterations and these alterations have been attributed to the overactivation of hypothalamic-pituitary-adrenal (HPA) axis, which shows the effect by releasing glucocorticoids (corticosterone) into the blood [5]. However, repeated exposure to a homotypic stressor (same stressor) results in the blunted response in terms of restoration of behavioral and biochemical alterations in comparison to initial exposure to stressor [1,15]. This blunted response to the stress stimulus during repeated episodes of stress exposure is referred as 'stress adaptation' [36]. However, the mechanisms involved in stress adaptation during the repeated exposure to homotypic stressor are not clear yet.

The opening of T-type Ca^{2+} channels control the various physiological processes in the body including neuronal firing, pain [10,40], sleep [2] and motor coordination [31]. However, the disturbance in these channels leads to various neurological and neuropsychiatric disorders like depression [42], memory impairment [43], cognitive deficits [13], and epilepsy [9]. T-type Ca^{2+} channels are present on different stress sensitive regions including the hypothalamus, adrenal cortex, temporal lobe and dorsal region of raphe nucleus [19,33].

Studies have shown that the mice with genetic knockout of T-type Ca^{2+} channels (Cav3.2 deficient mice) exhibit anxiety-related behavior [11,29]. Accordingly, it is possible to hypothesize that the opening of T-type Ca^{2+} channels may counter the development of stress-related behavioral alterations and may also participate in the process of stress adaptation. However, there has not been any experimental study exploring the role of T-type Ca^{2+} channels in stress adaptation.

* Corresponding author at: Department of Pharmaceutical Sciences and Drug Research, Punjabi University Patiala, Patiala 147002, India.
E-mail address: amteshwarjaggi@yahoo.co.in (A.S. Jaggi).

https://doi.org/10.1016/j.lfs.2019.116605
Received 1 May 2019; Received in revised form 17 June 2019; Accepted 25 June 2019
Available online 26 June 2019

Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels were initially identified in the SA node of the heart and their role in controlling heart rate is well defined [7,27]. Later, studies identified the presence of these channels in the central nervous system including hippocampus, hypothalamus, neocortex and cerebellum [3,28]. HCN channels have been associated with a wide range of physiological processes in the brain such as regulation of pacemaker activity, synaptic transmission and membrane voltage [39]. Moreover, these channels are also associated with pathological processes related to the central nervous system such as epilepsy, depression *etc.* [18,26]. HCN channels are present on the various stress sensitive regions of the brain including hypothalamus and hippocampus. Some of the studies also suggest that blockade of HCN channels leads to development of anxiety or depression like behavior [30,31].

Based on the studies showing that the genetic knockout of T-type Ca^{2+} channels and blockade of HCN channels leads to the development of anxiety and depression-like behavior in animals, it may be hypothesized that the opening of these channels participate in alleviating anxiety. Moreover, it is also hypothesized that the process of stress adaptation (in which anxiety-related symptoms are alleviated) may also involve the opening of T-type Ca^{2+} channels and HCN channels. Accordingly, the present study was designed to investigate the possible role of T-type Ca^{2+} channels and HCN channels in the development of stress adaptation in cold-water immersion stress-subjected mice. Ethosuximide, as a selective T-type Ca^{2+} channel blocker, and ivabradine, as an HCN channel blocker, were employed as pharmacological tools to explore the role of T-type Ca^{2+} channels and HCN channels, respectively.

2. Material and methods

2.1. Experimental animals

Swiss albino mice (Lala Lajpat Rai University of Veterinary and Animal Sciences, Haryana, India) weighing 25 ± 5 g was employed in the present study. Animals were fed on standard laboratory diet (Ashirwad Industries, Kharar, Mohali, Punjab) and water *ad libitum*. They were housed in the departmental animal house and were exposed to natural cycles of light and dark. The experimental protocol was approved by Institutional Animal Ethics Committee (107/GO/ReBi/S/99/CPCSEA/2018-26) and care of the animals was carried out as per the guidelines of the Committee for the purpose of Control and Supervision of Experimental Animals (CPCSEA), Government of India (Reg. No.-107/GO/ReBi/S/99/CPCSEA).

2.2. Drugs and chemicals

Ethosuximide (Katwijk Chemie BV, The Netherlands) and ivabradine (Lupin Limited, India) were used in the present study and both drugs were dissolved in normal saline and they were administered through intraperitoneal (*i.p.*) route. The drugs were administered 30 min prior to each stress exposure to give a sufficient time for drug absorption and distribution in the body. The mouse corticosterone ELISA kit was used for the estimation of plasma corticosterone level (IBL International, Germany). All other chemicals and reagents used were purchased from S.D. Fine Chemicals Ltd., Mumbai, India and were of analytical grade.

2.3. Induction of cold-water immersion stress

The mice were subjected to stress by placing them individually in a tank of water at temperature of 15 °C (depth = 15.5 cm; temperature = 15 ± 2 °C) for 5 min, where they either swim or remain in upright position, keeping their heads above water level ([45]). The time period of 5 min was selected because animals were noted to sink after this time of water immersion [1].

2.4. Behavioral measurements

Before initiating the actual experimental protocol, the mice were acclimatized for 5 min on each behavioral test apparatus consecutively for three days. After acclimatization on test apparatus mice were subjected to cold-water immersion stress and after 30 min of cold-water immersion stress battery of behavioral tests was performed in mice with the sequence of the actophotometer, the hole board, the open field, the social interaction test. Each test was performed with a time gap of 5 min between the successive tests [1].

2.4.1. Actophotometer test

The locomotor activity has been considered as indicator of alertness and it was assessed by keeping the mice individually in the actophotometer. The digital actophotometer was made up of opaque metal with the square arena (30 cm × 30 cm) with six in-built photosensors and four digital counters. The movement of animals interrupts the beam of light falling on the photocell and a count is recorded digitally. Accordingly, the numbers of counts were used to indicate the locomotor activity and counts were taken for 5 min [1,23,37].

2.4.2. Hole board test

The hole board test was used to assess the explorative behavior of animals. The hole board consisted of a wooden box measuring 68 cm × 68 cm, having walls at height of 40 cm. The box was raised 28 cm above the ground on a stand. Sixteen holes each of 4 cm in diameter were cut into floor. The animals were assessed in the hole board apparatus during which the behavioral patterns (head dipping and rearing) were recorded [1,8].

2.4.3. Open field test

The open field test was used to assess the stress-related behavior in rodents on the basis of changes in the exploration, general locomotor activity, and spontaneous activity [34]. The open field apparatus consisted of a box 90 cm × 90 cm × 38 cm positioned in a dimly lighted room. The walls were painted black, while the floor was painted white and was divided by 1 cm wide black lines into 25 squares of 17 cm × 17 cm (16 peripheral squares and 9 central squares). Mice were placed in the centre of the field and number of crossing of lines and rearings were noted [1].

2.4.4. Social interaction test

The social interaction test has been employed to assess the social behavior of animals in which the behavior of an animal is observed with its social partner. Each mouse was observed with a partner mouse, which was not subjected to any type of stressor and socially housed. During the test period, the behavior such as physical contact with mutual responses and orientation toward each other was considered as social behavior of an animal and the remaining time interval was considered as non-social behavior [1,16].

2.5. Biochemical estimations

The mice were anaesthetised by the administration of sodium pentobarbital (50 mg/kg, *i.p.*) and blood was withdrawn from retro-orbital sinus after behavioral assessments. The blood was collected in to micro centrifugation tubes containing 10 µl of EDTA solution (10% *w/v*) for prevention of clotting of blood. Thereafter, tubes were centrifuged at 2000 rpm for 10 min for separation of plasma [4].

2.5.1. Plasma corticosterone estimation

Standard, control and plasma samples (20 µl each) were pipetted into discrete microtiter wells by using new disposable tips. The enzyme conjugate (200 µl) was added into each microtiter well and the contents were mixed thoroughly for 10 s. The plate was incubated for 60 min at room temperature, followed by brisk shaking of the contents. The wells

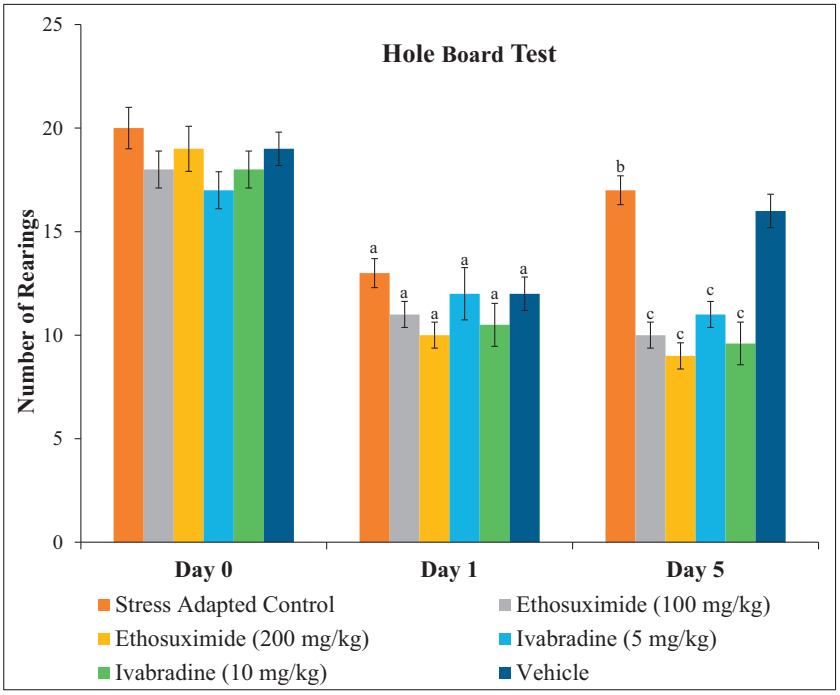

Fig. 3. Effect of cold-water immersion stress and pharmacological interventions on the exploratory behavior in terms of number of rearings using hole board test.
Values are expressed as mean ± S.E.M. with $n = 6$ in each group and statistical analysis was done by Two-way ANOVA followed by Tukey's Multiple Comparison test, [F (2,90) = 747.0] for the time factor and [F (5,90) = 72.25] for the treatment factor, a = $p < 0.0001$ as compared to respective groups on day 0; b = $p < 0.0001$ as compared to stress adapted control of day 1; c = $p < 0.0001$ as compared to stress adapted control of day 5.

were washed thrice using diluted wash solution (400 µl/well/wash). Substrate solution (100 µl) was added into each well followed by incubation at room temperature for 15 min. Stop solution (50 µl) was added to the wells of microtiter plate to stop the enzymatic reaction. The absorbance was measured at 450 nm using microtiter plate reader within 10 min of adding the stop solution.

2.6. Experimental protocol

Six groups, each comprising of six Swiss albino mice were employed in the present study.

2.6.1. Group I: stress adapted control

The mice were adapted to the stressful state by subjecting them to repeated episodes of cold-water immersion stress of 5 min at 15 °C, for five days. The different behavioral tests including the actophotometer, the hole board, the open field and the social interaction tests were performed on day 0 (before stress) and after 30 min of cold-water immersion stress on day 1 and day 5. The blood was withdrawn from retro orbital sinus on the day 5 after the behavioral assessments and was used for estimation of plasma corticosterone level.

2.6.2. Group II, III: ethosuximide (100 and 200 mg/kg, i.p.) in stress adapted animal

Ethosuximide (100 and 200 mg/kg, *i.p.*) was administered, 30 min prior to each episode of cold-water immersion stress for five days and different behavioral tests were performed on day 0, day 1 and day 5. On day 5, the blood was withdrawn to estimate corticosterone as described in group I.

2.6.3. Group IV, V: ivabradine (5 and 10 mg/kg, i.p.) in stress adapted animal

Ivabradine (5 and 10 mg/kg, *i.p.*) was administered 30 min prior to each episode of cold-water immersion stress for five days and different behavioral tests were performed on day 0, day 1 and day 5. On day 5, the blood was withdrawn to estimate corticosterone as described in group I.

2.6.4. Group VI: normal saline (10 ml/kg, i.p.) as a vehicle in stress adapted animal

Normal saline (10 ml/kg, *i.p.*) as a vehicle (of ethosuximide and ivabradine) was administered 30 min for five days before subjecting animals to cold-water immersion for five days and different behavioral tests were performed on day 0, day 1 and day 5. On day 5, the blood was withdrawn to estimate corticosterone as described in group I.

2.7. Statistical analysis

The results were expressed as mean ± S.E.M. One-way ANOVA was employed to analyse the data of plasma corticosterone levels. Two-way ANOVA was employed to analyse the data of behavioral tests. ANOVA was followed by Tukey's multiple comparison *post hoc* test to compare different groups. The value of $p < 0.05$ was considered to be statistically significant.

3. Results

The data of the present study passed the normality test during the statistical analysis. Thereafter, on the basis of the results of ANOVA and *post hoc* tests, the results of different experimental groups are presented as below:

3.1. Effect of cold-water immersion stress and different pharmacological interventions on the locomotor activity in actophotometer test

A single exposure to cold-water immersion stress (acute stress) for 5 min led to significant decrease in the locomotor activity in mice on day 1 (after stress) as compared to day 0 (before stress). However, on subsequent exposures to cold-water immersion stress of 5 min each for five consecutive days (repeated homotypic stress), the locomotor activity of the animals was restored and was significantly higher on day five as compared to day one (Fig. 1). A single administration of ethosuximide (100 and 200 mg/kg, *i.p.*) and ivabradine (5 and 10 mg/kg, *i.p.*) did not modulate cold-water immersion stress induced decrease in the locomotor activity on day 1. However, administration of ethosuximide (100 and 200 mg/kg, *i.p.*) and ivabradine (5 and 10 mg/kg, *i.p.*) for five days abolished the restoration of locomotor activity on day 5.

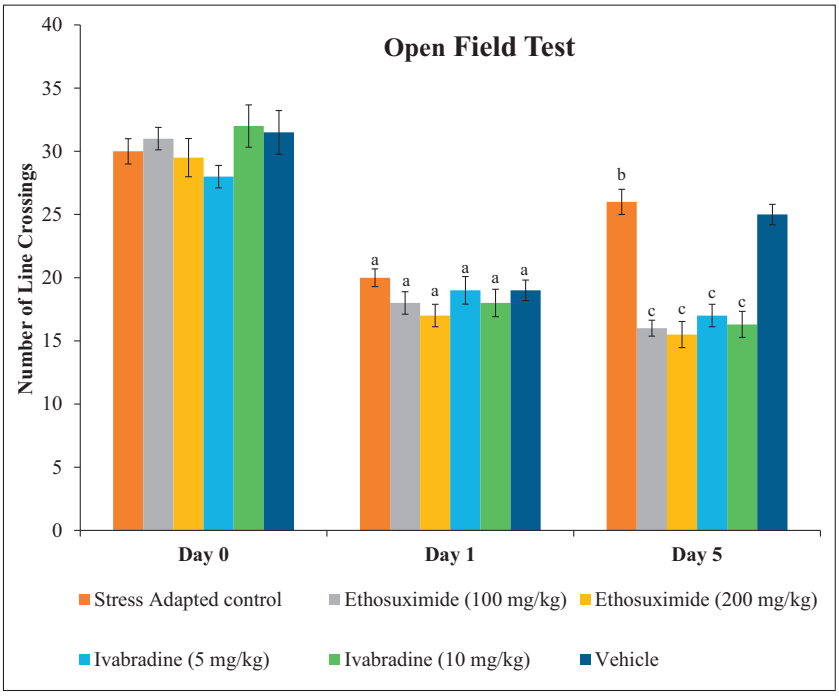

Fig. 4. Effect of cold-water immersion stress and pharmacological interventions on the motor activity of animals in terms of number of line crossings using open field test.
Values are expressed as mean ± S.E.M. with n = 6 in each group and statistical analysis was done by Two-way ANOVA followed by Tukey's Multiple Comparison test, [F (2,90) = 1366] for the time factor and [F (5,90) = 63.85] for the treatment factor, a = $p < 0.0001$ as compared to respective groups on day 0; b = $p < 0.0001$ as compared to stress adapted control of day 1; c = $p < 0.0001$ as compared to stress adapted control of day 5.

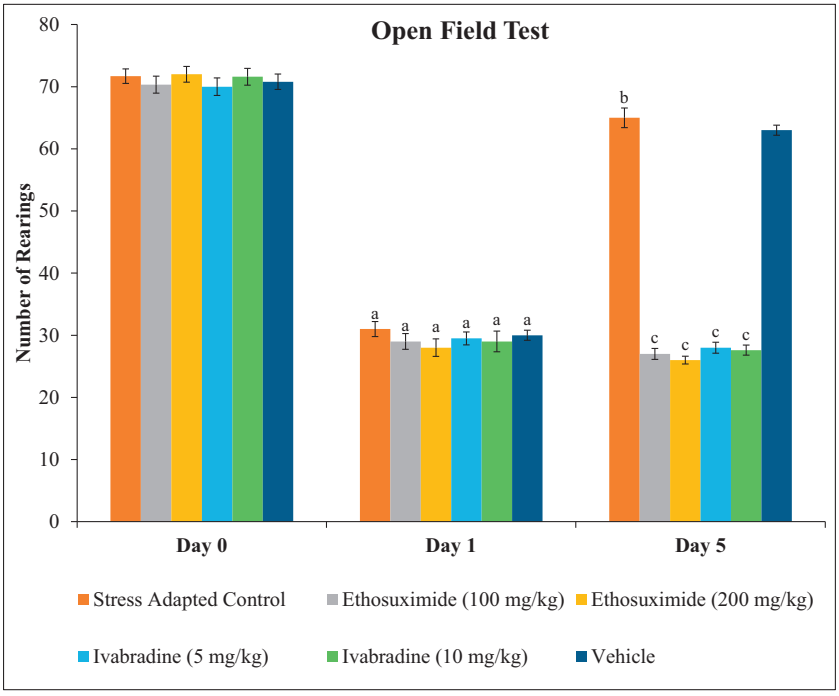

Fig. 5. Effect of cold-water immersion stress and pharmacological interventions on the exploratory behavior of animals in terms of number of rearings using open field test.
Values are expressed as mean ± S.E.M. with n = 6 in each group and statistical analysis was done by Two-way ANOVA followed by Tukey's Multiple Comparison test, [F (2,90) = 11,772] for the time factor and [F (5,90) = 550.1] for the treatment factor, a = $p < 0.0001$ as compared to respective groups on day 0; b = $p < 0.0001$ as compared to stress adapted control of day 1; c = $p < 0.0001$ as compared to stress adapted control of day 5.

Vehicle administration did not modulate the locomotor activity either day 1 or day 5 (Fig. 1).

3.2. *Effect of cold-water immersion stress and different pharmacological interventions on head dips and rearings (exploratory behavior) in hole board test*

The number of rearings and head dips in the hole board apparatus was significantly decreased after exposing the animals to cold-water immersion stress for 5 min on day one as compared to day 0 (before stress). However, there was restoration of number of head dips and rearings after repeated exposure to cold-water immersion stress of 5 min each for five days. The administration of ethosuximide (100 and 200 mg/kg, *i.p.*) and ivabradine (5 and 10 mg/kg, *i.p.*) for five days significantly abolished the restoration of number of rearings and head dips on day five, without any significant effect on day 1. Administration of vehicle did not abolish the restoration of exploratory behavior of the animals on day 5 (Figs. 2 and 3).

3.3. *Effect of cold-water immersion stress and different pharmacological interventions on line crossings (motor activity) and rearings (exploratory behavior) in open field test*

A single exposure to cold-water immersion stress for 5 min (acute

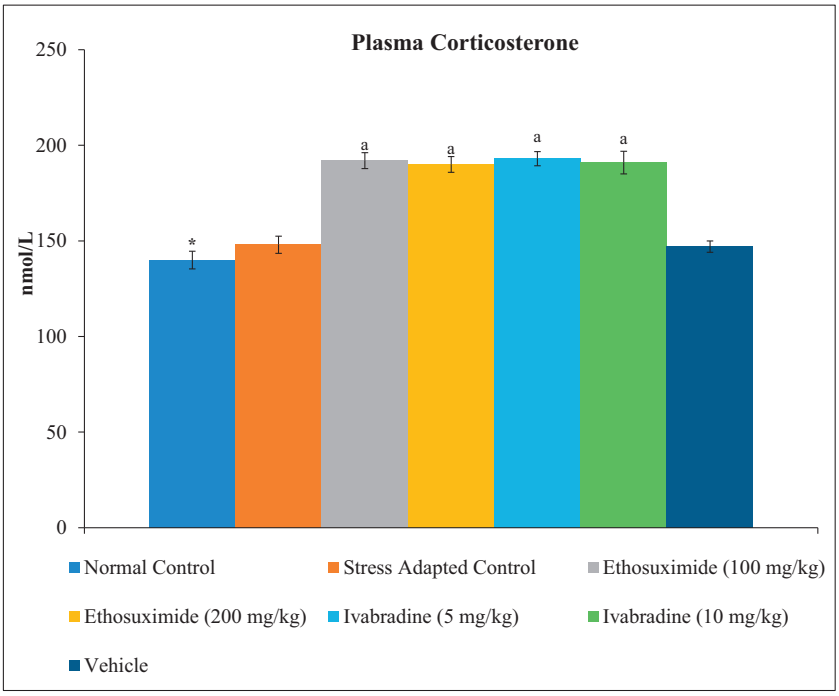

Fig. 6. Effect of cold-water immersion stress and pharmacological interventions on plasma corticosterone levels in animals.
Values are expressed as mean ± S.E.M. with n = 6 in each group and statistical analysis was done by One-way ANOVA followed by Tukey's Multiple Comparison test, [F (5,30) = 208.3] for the treatment factor.* = The value is taken from the our previous studies [6,22]; a = $p < 0.0001$ as compared to stress adapted control.

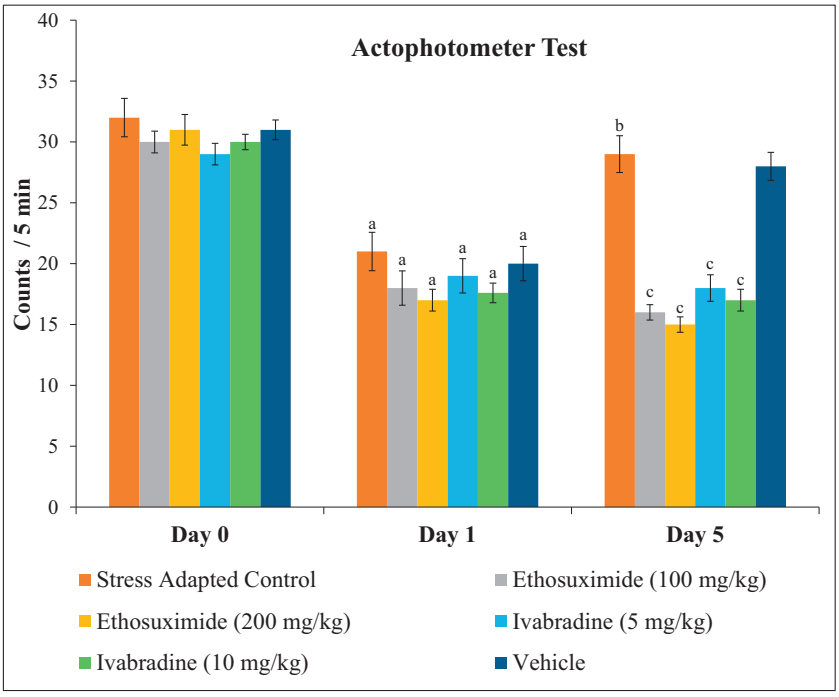

Fig. 1. Effect of cold-water immersion stress and pharmacological interventions on the locomotor activity of animals in terms of counts using actophotometer test.
Values are expressed as mean ± S.E.M. with $n = 6$ in each group and statistical analysis was done by Two-way ANOVA followed by Tukey's Multiple Comparison test, [F (2,90) = 1103] for the time factor and [F (5,90) = 119.9] for the treatment factor, a = $p < 0.0001$ as compared to respective groups on day 0; b = $p < 0.0001$ as compared to stress adapted control of day 1; c = $p < 0.0001$ as compared to stress adapted control of day 5.

stress) led to significant decrease in the exploratory behavior in terms of line crossings and rearings on day one, as compared to mice on day 0 (before stress). However, on subsequent exposure to cold-water immersion stress of 5 min each for five consecutive days (repeated stress), the motor activity and exploratory behavior of the animals was restored and was significantly higher on day five as compared to day one. The administration of ethosuximide (100 and 200 mg/kg, *i.p.*) and ivabradine (5 and 10 mg/kg, *i.p.*) for five days significantly abolished the restoration of the motor activity and exploratory behavior of the animals on day five. Administration of vehicle in stress subjected mice did not significantly modulate restoration of number of line crossings and rearings on day 5 (Figs. 4 and 5).

3.4. Effect of cold-water immersion stress and different pharmacological interventions on the social (following) and non-social (avoidance) behavior of animals in social interaction test

The single exposure to cold-water immersion stress for 5 min led to development of non-social (avoiding the partner) behavior in mice on day one, when compared to animals on day 0 (before stress). However, on subsequent exposures to cold-water immersion stress (5 min each) for five consecutive days, the social (following the partner) behavior improved as compared to behavior of mice on day one. Administration of ethosuximide (100 and 200 mg/kg, *i.p.*) and ivabradine (5 and 10 mg/kg, *i.p.*) for 5 days abolished the improvement of social behavior

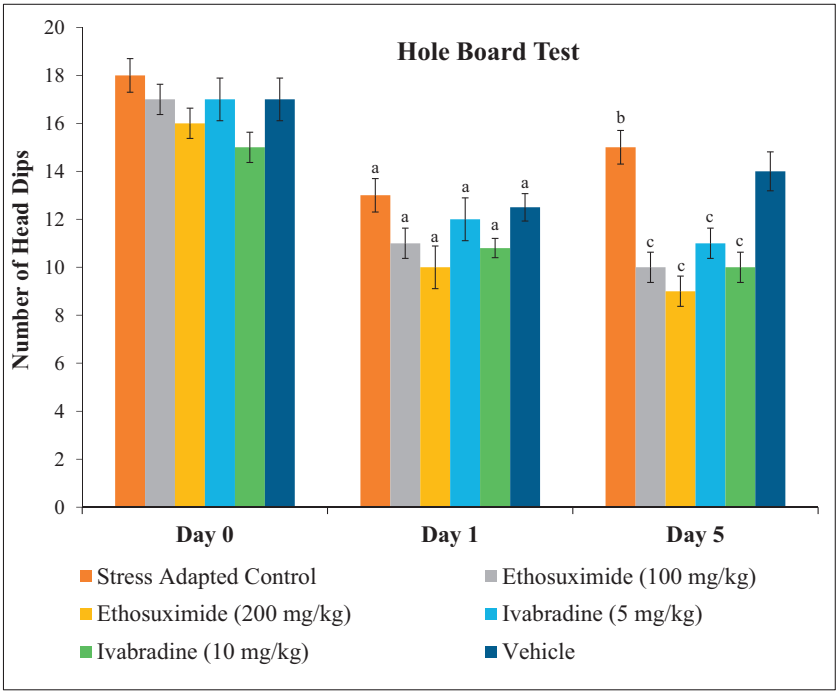

Fig. 2. Effect of cold-water immersion stress and pharmacological interventions on the exploratory behavior of animals in terms of number of head dips using hole board test. Values are expressed as mean ± S.E.M. with $n = 6$ in each group and statistical analysis was done by Two-way ANOVA followed by Tukey's Multiple Comparison test.
[F (2,90) = 676.9] for the time factor and [F (5,90) = 69.90] for the treatment factor, a = $p < 0.0001$ as compared to respective groups on day 0; b = $p < 0.0001$ as compared to stress adapted control of day 1; c = $p < 0.0001$ as compared to stress adapted control of day 5.

Table 1
Effect of cold-water immersion stress and pharmacological interventions on social and non-social behavior using open field test. Values are expressed as mean ± S.E.M. with n = 6 in each group and statistical analysis was done by two-way ANOVA followed by Tukey's Multiple Comparison test. For following [F (2,90) = 41,226] for the time factor and [F (5,90) = 2351] for the treatment factor; For avoidance, [F (2,90) = 35,226] for the time factor and [F (5,90) = 2009] for the treatment factor. a = $p < 0.0001$ as compared to respective groups on day 0; b = $p < 0.0001$ as compared to stress adapted control of day 1; c = $p < 0.0001$ as compared to stress adapted control of day 5.

Experimental groups	Social interaction test					
	Following (*sec*)			Avoidance (sec)		
	Day 0	Day 1	Day 5	Day 0	Day 1	Day 5
Stress adapted control	250.6 ± 1.37	100.6 ± 0.55[a]	244.0 ± 1.01[b]	49.4 ± 1.11	199.4 ± 1.25[a]	56.0 ± 1.06[b]
Ethosuximide (100 mg/kg)	244.0 ± 0.85	99.5 ± 1.02[a]	99.0 ± 0.36[c]	56.0 ± 1.18	200.5 ± 0.99[a]	201.0 ± 0.36[c]
Ethosuximide (200 mg/kg)	249.0 ± 0.93	98.7 ± 0.66[a]	96.0 ± 0.25[c]	51.0 ± 1.00	201.3 ± 1.33[a]	204.0 ± 0.36[c]
Ivabradine (5 mg/kg)	253.0 ± 1.06	100.0 ± 0.96[a]	98.0 ± 0.36[c]	47.0 ± 1.06	200.0 ± 1.46[a]	202.0 ± 0.25[c]
Ivabradine (10 mg/kg)	243.3 ± 1.40	99.0 ± 1.12[a]	97.0 ± 0.36[c]	56.7 ± 0.71	201.0 ± 1.52[a]	203.0 ± 0.68[c]
Vehicle	246.5 ± 1.17	100.3 ± 0.33[a]	243.0 ± 0.40	53.5 ± 0.54	199.7 ± 0.30[a]	57.0 ± 0.36

after five consecutive exposures to cold-water immersion stress. However, administration of vehicle did not modulate restoration of social behavior on day 5 (Table 1).

3.5. Effect of cold-water immersion stress and different pharmacological interventions on the plasma corticosterone levels

After five consecutive exposures to cold-water immersion stress, the plasma corticosterone levels were comparable to normal plasma corticosterone levels and much lower in comparison to acute stress subjected animals (Based on previous studies, [6,22]. Administration of ethosuximide (100 and 200 mg/kg, *i.p.*) and ivabradine (5 and 10 mg/kg, *i.p.*) for 5 days abolished the restoration of plasma corticosterone levels. However, administration of vehicle did not alter the levels of plasma corticosterone in a significant manner (Fig. 6).

4. Discussion

In the present study, a single episode of cold-water immersion stress for 5 min produced significant changes in the behavior of mice including attenuation of locomotor activity, a decrease in the exploratory behavior and social behavior in the actophotometer, the hole-board, the open field and the social interaction tests respectively. Several studies have reported that induction of acute stress in experimental animals decreases the locomotor activity, the exploratory behavior and alters the social behavior [1,8,16,22]. Cold-water immersion stress is one of the most frequently employed methods to induce stress in mice for studying different aspects of stress [1,38] including exploring the new pharmacological agents for stress management or to explore the mechanisms involved in stress adaptation [14,22,38]. On repeated exposure to homotypic stressor of 5 min duration for five days, acute stress induced behavioral changes were restored on the 5th day, which reflects the development of stress adaptation. Moreover, the repeated application of the same stressor for 5 consecutive days also attenuated acute stress induced increase in plasma corticosterone levels. In line with this, studies from our laboratory have shown that acute exposure to stressor for 5 min produces significant increase in the plasma corticosterone levels and alters behavioral parameters on the first day as compared to non-stress control mice. However, on repeated exposure to the same stressor for 5 days, there is normalization of plasma corticosterone levels and behavioral parameters [1,4].

To explore the mechanisms involved in the development of stress

adaptation in mice, ethosuximide, as a T-type Ca^{2+} channel blocker, was employed as a pharmacological tool. Ethosuximide is clinically used in the treatment of absence seizures [32,44]. A single administration of ethosuximide (100 and 200 mg/kg, *i.p.*), 30 min before the induction of acute stress (5 min), did not show any effect on behavioral and biochemical changes in mice on day 1. However, repeated administration of ethosuximide (100 and 200 mg/kg, *i.p.*) for five days, 30 min before subjecting the animals to repeated episodes of stress, abolished the restoration of behavioral and biochemical changes assessed on day 5 as a part of adaptive process in mice. The inhibition of stress adaptation in the presence of ethosuximide signifies the important role of T-type Ca^{2+} channels in the process of stress adaptation. Studies have shown that blockade of T-type Ca^{2+} channels lead to development of anxiety [12]. Moreover, previous studies also suggest that T-type Ca^{2+} channel deficient mice exhibit symptoms of anxiety, depression, and insomnia *etc.* [11,29]. However, it is the first study documenting that opening of T-type Ca^{2+} channels in the brain may be involved in the process of stress adaptation. Ethosuximide-mediated increase in the plasma corticosterone levels in repeated stress subjected mice indicates that the activation of T-type Ca^{2+} channels may inhibit the over activation of HPA axis to normalize the corticosterone levels. Since normalization of HPA axis is central to stress adaptation process [24, 41, 36] and T-type Ca^{2+}channels are expressed on the different components of HPA axis [33,35]; therefore, it is possible that during the repeated exposure to stress, the activation of T-type Ca^{2+} channels in the brain play a key role in normalizing the HPA axis and inducing stress adaptation.

To further explore the mechanisms involved in the process of adaptation, ivabradine (HCN channel blocker) was administered for five days in repeated stress subjected mice. Ivabradine is clinically used for the management of stable angina and symptomatic chronic heart failure [20]. A single dose administration of ivabradine (5 and 10 mg/kg, *i.p.*) in acute stress (5 min) subjected mice did not show any effect on the behavioral and biochemical changes. Nevertheless, repeated administration of ivabradine (5 and 10 mg/kg, *i.p.*) abolished the restoration of the behavioral and biochemical changes as a part of adaptive process in repeated stress subjected mice. Ivabradine mediated abolishment of adaptation process in repeated stress subjected mice demonstrates that HCN channels in the brain may also be involved in process of adaptation in response to repeated episodes of stress. HCN channels are present on various stress sensitive regions of the brain such as hypothalamus and hippocampus. Studies have suggested that blockade of HCN channels by the administration of various pharmacological agents including ZD7288 leads to induction of anxiety or depression like behavior [17,30,31]. It suggests that HCN channels may function in the brain to alleviate anxiety and related behavioral changes. Accordingly, it is possible that repeated exposure to stress may trigger the activation of the brain localized HCN channels to overcome stress-associated behavioral alterations. Like T-type Ca^{2+}channels, HCN channels are also present on the HPA axis [3,21,28] and ivabradine-mediated increase in corticosterone levels suggests the role of HCN channels in preventing the over activation of HPA axis. Based on the results of this study, it is not possible to delineate the precise molecular events triggered following HCN channels activation during the process of stress adaptation. Nevertheless, it is the first study describing the key role of HCN channel activation in the process of stress adaptation.

The major limitation of the present study is that apart from blocking the T-type calcium channels and HCN channels in the brain, ethosuximide and ivabradine may also exert systemic effects due to wide spread presence of these channels in the peripheral tissues. Accordingly, it may not be ruled out that the peripheral effects produced by these pharmacological agents may also affect the behavioral parameters measured in this study. Therefore, the precise role of these channels in stress adaptation may be elucidated in the future studies by employing the transgenic animals with specific knock out of genes encoding these channels in the brain. Since stress activates mainly two pathways in the body *i.e.* the HPA axis and the sympatho-adrenal medullary system; therefore measurement of both plasma corticosterone and plasma epinephrine levels may give better representative picture of stress induction and stress adaptation in comparison to plasma corticosterone levels alone.

5. Conclusion

Ethosuximide and ivabradine attenuate stress adaptation in mice demonstrating that the stress response depends on the T-type Ca^{2+} channels and HCN ion channels. Moreover, the attenuation of stress adaptation in the presence of ethosuximide and ivabradine suggests that the activation of T-type Ca^{2+} channels and HCN channels is critical in inducing stress adaptation in repeated stress subjected mice.

Declaration of Competing Interest

There authors declare no conflict of interest which interferes with the integrity of the manuscript.

Acknowledgement

The authors are grateful to the Department of Pharmaceutical Sciences and Drug Research, Punjabi University, Patiala, Punjab, India for providing the technical facilities for this work. The authors are also thankful to the Katwijk Chemie BV, The Netherlands for giving us the gift sample of Ethosuximide to do this work.

References

[1] A. Agrawal, A.S. Jaggi, N. Singh, Pharmacological investigations on adaptation in rats subjected to cold water immersion stress, Physiol. Behav. 103 (2011) 321–329.
[2] M.P. Anderson, T. Mochizuki, J. Xie, W. Fischler, J.P. Manger, E.M. Talley, et al., Thalamic Cav3.1 T-type Ca2+ channel plays a crucial role in stabilizing sleep, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 1743–1748.
[3] A. Arroyo, B. Kim, R.L. Rasmusson, G. Bett, J. Yeh, Hyperpolarization-activated cation channels are expressed in rat hypothalamic gonadotropin-releasing hormone (GnRH) neurons and immortalized GnRH neurons, J. Soc. Gynecol. Investig. 13 (2006) 442–450.
[4] A. Bali, A.S. Jaggi, Investigations in foot shock stress of variable intensity in mice: adaptation and role of angiotensin II, Eur. J. Pharmacol. 761 (2015) 86–94.
[5] A. Bali, N. Singh, A.S. Jaggi, Investigations into mild electric foot shock stress-induced cognitive enhancement: possible role of angiotensin neuropeptides, J. Renin-Angiotensin-Aldosterone Syst. 14 (2013) 197–203.
[6] N. Bhatia, A.S. Jaggi, N. Singh, P. Anand, R. Dhawan, Adaptogenic potential of curcumin in experimental chronic stress and chronic unpredictable stress induced memory deficits and alterations in functional homeostasis, J. Nat. Med. 65 (2011) 532–543.
[7] M. Biel, A. Schneider, C. Wahl, Cardiac HCN channels: structure, function, and modulation, Trends Cardiovasc Med 12 (2002) 206–212.
[8] G.R. Brown, C. Nemes, The exploratory behavior of rats in the hole-board apparatus: is head-dipping a valid measure of neophilia? Behav. Process. 78 (2008) 442–448.
[9] S.M. Cain, T.P. Snutch, T-type calcium channels in burst-firing, network synchrony, and epilepsy, Biochim. Biophys. Acta 1828 (2013) 1572–1578.
[10] A. Francois, N. Kerckhove, M. Meleine, A. Alloui, C. Barrere, A. Gelot, V.N. Uebele, J.J. Renger, A. Eschalier, D. Ardid, E. Bourinet, State-dependent properties of a new T-type calcium channel blocker enhance Ca(V)3.2 selectivity and support analgesic effects, Pain 154 (2013) 283–293.
[11] G. Gangarossa, S. Laffray, E. Bourinet, E. Valjent, T-type calcium channel $Ca_v3.2$ deficient mice show elevated anxiety, impaired memory and reduced sensitivity to psychostimulants, Front. Behav. Neurosci. 8 (2014) 92.
[12] A. Gironell, J. Marin-Lahoz, Ethosuximide for essential tremor: an open-label trial, Tremor Other Hyperkinet Mov 6 (2016) 378.
[13] N. Husain, Y. Yabuki, Y. Shinoda, K. Fukunaga, Acute treatment with T-type calcium channel enhancer SAK3 reduces cognitive impairments caused by methimazole-induced hypothyroidism via activation of cholinergic signaling, Pharmacology 101 (2018) 309–321.
[14] A.S. Jaggi, N. Bhatia, N. Kumar, N. Singh, P. Anand, R. Dhawan, A review on animal models for screening potential anti-stress agents, Neurol. Sci. 32 (2011) 993–1005.
[15] G.J. Kant, T. Eggleston, L. Landman-Roberts, C.C. Kenion, G.C. Driver, J.L. Meyerhoff, Habituation to repeated stress is stressor specific, Pharmacol. Biochem. Behav. 22 (1985) 631–634.
[16] R. Kaur, A.S. Jaggi, N. Singh, Studies on effect of stress preconditioning in restrain stress-induced behavioral alterations, Yakugaku Zasshi 130 (2010) 215–221.
[17] B. Kelmendi, M. Holsbach-Beltrame, A.M. McIntosh, L. Hilt, E.D. George,

R.R. Kitchen, B.C. Carlyle, C. Pittenger, V. Coric, S. Nolen-Hoeksema, G. Sanacora, A.A. Simen, Association of polymorphisms in HCN4 with mood disorders and obsessive-compulsive disorder, Neurosci. Lett. 496 (2011) 195–199.
[18] C.S. Kim, D. Johnston, A possible link between HCN channels and depression, Chronic Stress (Thousand Oaks) 2 (2018), https://doi.org/10.1177/2470547018787781 Jan-Dec.
[19] K.M. Knutson, S.T. Rakowsky, J. Solomon, F. Krueger, V. Raymont, M.C. Tierney, E.M. Wassermann, J. Grafman, Injured brain regions associated with anxiety in Vietnam veterans, Neuropsychologia 51 (2013) 686–694.
[20] J.S. Koruth, A. Lala, S. Pinney, V.Y. Reddy, S.R. Dukkipati, The clinical use of ivabradine, J. Am. Coll. Cardiol. 70 (2017) 1777–1784.
[21] K. Kretschmannova, M. Kucka, A.E. Gonzalez-Iglesias, S.S. Stojilkovic, The expression and role of hyperpolarization-activated and cyclic nucleotide-gated channels in endocrine anterior pituitary cells, Mol. Endocrinol. 26 (2012) 153–164.
[22] N. Kumar, N. Singh, A.S. Jaggi, Anti-stress effects of cilnidipine and nimodipine in immobilization subjected mice, Physiol. Behav. 105 (2012) 1148–1155.
[23] B. Kumari, A. Kumar, A. Dhir, Protective effect of non-selective and selective COX-2 inhibitors in acute immobilization stress induced behavioral and biochemical alterations, Pharmacol. Rep. 59 (2007) 699–707.
[24] R. Kvetnansky, L. Mikulai, Adrenal and urinary catecholamines in rats during adaptation to repeated immobilization stress, Endocrinology 81 (1970) 738–743.
[25] I. Kyrou, C. Tsigos, Stress hormones: physiological stress and regulation of metabolism, Curr. Opin. Pharmacol. 9 (2009) 787–793.
[26] Y. Noam, C. Bernard, T.Z. Baram, Towards an integrated view of HCN channel role in epilepsy, Curr. Opin. Neurobiol. 21 (2011) 873–879.
[27] A. Noma, H. Irisawa, Membrane currents in the rabbit sinoatrial node cell as studied by the double microelectrode method, Pflugers Arch. 364 (1976) 45–52.
[28] T. Notomi, R. Shigemoto, Immunohistochemical localization of Ih channel subunits, HCN1-4, in the rat brain, J. Comp. Neurol. 471 (2004) 241–276.
[29] A. Papazoglou, C. Henseler, A. Lundt, C. Wormuth, J. Soos, K. Broich, D. Ehninger, M. Weiergräber, Gender specific hippocampal whole genome transcriptome data from mice lacking the $Ca_v2.3$ R-type or $Ca_v3.2$ T-type voltage-gated calcium channel, Data Brief 12 (2017) 81–86.
[30] K. Park, S. Lee, S.J. Kang, S. Choi, K.S. Shin, Hyperpolarization-activated currents control the excitability of principal neurons in the basolateral amygdala, Biochem. Biophys. Res. Commun. 361 (2007) 718–724.
[31] Y.G. Park, H.Y. Park, C.J. Lee, S. Choi, S. Jo, H. Choi, Y.H. Kim, H.S. Shin, R.R. Llinas, D. Kim, Ca(V)3.1 is a tremor rhythm pacemaker in the inferior olive, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 10731–10736.
[32] P.N. Patsalos, Properties of antiepileptic drugs in the treatment of idiopathic generalized epilepsies, Epilepsia 46 (2005) 140–148.
[33] K.L. Powell, H. Tang, C. Ng, I. Guillemain, G. Dieuset, G. Dezsi, N. Çarçak, F. Onat, B. Martin, T.J. O'Brien, A. Depaulis, N.C. Jones, Seizure expression, behavior, and brain morphology differences in colonies of genetic absence epilepsy rats from Strasbourg, Epilepsia 55 (2014) 1959–1968.
[34] L. Prut, C. Belzung, The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review, Eur. J. Pharmacol. 463 (2003) 3–33.
[35] J. Qiu, M.A. Bosch, K. Jamali, C. Xue, M.J. Kelly, O.K. Rønnekleiv, Estrogen upregulates T-type calcium channels in the hypothalamus and pituitary, J. Neurosci. 26 (2006) 11072–11082.
[36] C. Rabasa, C. Munoz-Abellan, N. Daviu, R. Nadal, A. Armario, Repeated exposure to immobilization or two different foot shock intensities reveals differential adaptation of the hypothalamic-pituitary-adrenal axis, Physiol. Behav. 103 (2011) 125–133.
[37] D.S. Reddy, S.K. Kulkarni, Role of GABA-A and mitochondrial diazepam binding inhibitor receptors in the anti-stress activity of neurosteroids in mice, Psychopharmacology 128 (1996) 280–292.
[38] S. Retana-Marquez, H. Bonilla-Jaime, G. Vazquez-Palacios, R. Martinez-Garcia, J. Velazquez-Moctezuma, Changes in masculine sexual behavior, corticosterone and testosterone in response to acute and chronic stress in male rats, Horm. Behav. 44 (2003) 327–337.
[39] R.B. Robinson, S.A. Siegelbaum, Hyperpolarization-activated cation currents: from molecules to physiological function, Annu. Rev. Physiol. 65 (2003) 453–480.
[40] S.M. Todorovic, V. Jevtovic-Todorovic, T-type voltage-gated calcium channels as targets for the development of novel pain therapies, Br. J. Pharmacol. 163 (2011) 484–495.
[41] L.D. Van de Kar, M.L. Blair, Forebrain pathways mediating stress induced hormone secretion, Front Neuro endo crinol 20 (1999) 1–48.
[42] J. Xu, Y. Yabuki, M. Yu, K. Fukunaga, T-type calcium channel enhancer SAK3 produces anti-depressant-like effects by promoting adult hippocampal neurogenesis in olfactory bulbectomized mice, J. Pharmacol. Sci. 137 (2018) 333–341.
[43] Y. Yabuki, X. Jing, K. Fukunaga, The T-type calcium channel enhancer SAK3 inhibits neuronal death following transient brain ischemia via nicotinic acetylcholine receptor stimulation, Neurochem. Int. 108 (2017) 272–281.
[44] G.W. Zamponi, J. Striessnig, A. Koschak, A.C. Dolphin, The physiology, pathology, and pharmacology of voltage-gated calcium channels and their future therapeutic potential, Pharmacol. Rev. 67 (2015) 821–870.
[45] K.S. Lee, B.V. Lim, M.H. Jang, M.C. Shin, T.H. Lee, Y.P. Kim, H.S. Shin, S.Y. Cho, H. Kim, M.S. Shin, E.H. Kim, C.J. Kim, Hypothermia inhibits cell proliferation and nitric oxide synthase expression in rats, Neurosci Lett. (2002) 53–56.