国際医療福祉大学審査学位論文（博士）

大学院薬学研究科博士課程

Involvement of K_{ATP} channels including Kir6.2 in regulation of emotional behaviors and stress responses

平成 29 年度

医療・生命薬学専攻

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Abstract (Japanese)

ATP 感受性カリウムチャネルのサブユニットである Kir6.2 は脳内に多く発現しているが、情動やストレスとの関係性については不明な点が多い。

本研究では、Kir6.2 遺伝子欠損（Kir6.2$^{-/-}$）マウスの情動調節について検討した。その結果、Kir6.2$^{-/-}$マウスで一般情動行動の低下と不安様行動が認められた。また、脳内モノアミン神経上に Kir6.2 が発現していた。さらに、雌性 Kir6.2$^{-/-}$マウスで中脳におけるトリプトファン水酸化酵素タンパク質の発現量が増加していた。

次に、Kir6.2$^{-/-}$マウスのストレス応答について検討した。その結果、急性拘束ストレス刺激負荷により上昇した血中コルチコステロン濃度は、野生型より Kir6.2$^{-/-}$マウスで高値を示した。また、海馬のグルココルチコイド受容体陽性細胞上に Kir6.2 が発現していた。

以上の結果から、情動調節とストレス応答への Kir6.2 の関与が示された。

キーワード：ATP 感受性カリウムチャネル、情動行動、モノアミン神経、ストレス、HPA 系

Abstract (English)

Although Kir6.2, a pore-forming subunit of ATP-sensitive potassium (K_{ATP}) channels, is widely distributed in the brain, the mechanisms that underlie the impact of Kir6.2 on emotional behavior and stress responses are not yet fully understood. To clarify the involvement of Kir6.2 in emotional behavior, the behavioral characteristics of Kir6.2-knockout ($Kir6.2^{-/-}$) mice were investigated. $Kir6.2^{-/-}$ mice showed impaired general behavior in a locomotor activity test and open field test. In addition, anxiety-like behavior was observed in the open field test, elevated plus-maze test and light-dark test. In particular, excessive anxiety-like behavior was observed in female $Kir6.2^{-/-}$ mice. Immunohistochemical studies showed that Kir6.2 was expressed on tryptophan hydroxylase (TPH) in dorsal raphe nuclei and tyrosine hydroxylase in the ventral tegmental area and locus coeruleus. Interestingly, TPH expression in the midbrain was significantly elevated in female $Kir6.2^{-/-}$ mice. These results suggest that Kir6.2 in monoamine neurons, especially serotonergic neurons, could be involved in emotional behavior.

Furthermore, to clarify the involvement of Kir6.2 in stress responses, the changes in serum corticosterone levels induced by acute restraint stress in $Kir6.2^{-/-}$ mice were examined. In the non-stressed condition, basal corticosterone levels in $Kir6.2^{-/-}$ mice were higher than those in wild type (WT) mice. $Kir6.2^{-/-}$ mice also showed greater increases in serum corticosterone levels in response to exposure to acute restraint stress. These phenomena were more prominent in females than in males. Next, whether Kir6.2 is expressed on glucocorticoid receptor (GR)-positive cells in hippocampal CA1 and dentate gyrus (DG) was investigated. Immunohistochemical studies showed that Kir6.2 was expressed on GR-positive cells in hippocampal CA1 and DG. These results suggest that Kir6.2 in GR-positive cells could be involved in stress responses via the hypothalamus-pituitary-adrenal axis.

Keywords: K_{ATP} channel, emotional behavior, monoamine, stress response, HPA axis

Chapter 1

Involvement of K_{ATP} channels including Kir6.2 in regulation of emotional behaviors

Introduction

ATP-sensitive potassium (K_{ATP}) channels are widely expressed in many tissues or organs, including the heart, pancreas, skeletal muscle, smooth muscle and brain, and regulate cell metabolism and membrane excitability[1-6]. They are composed of inwardly rectifying potassium channel subunits (Kir6.1 and Kir6.2) and regulatory sulfonylurea receptor subunits (SUR1, SUR2A and SUR2B), and the combinations of these subunits differ in different tissues[7]. These inwardly rectifying K^+ channels are activated by Mg^{2+}-bound nucleotides and inhibited by intracellular ATP[8,9]. K_{ATP} channels play critical roles in glucose homeostasis through the release of insulin in pancreatic beta cells[7]. In the presence of high levels of glucose metabolism, and consequently increased relative levels of ATP, K_{ATP} channels close, causing the membrane potential of the cell to depolarize, activating voltage-gated calcium channels, and thus promoting the calcium-dependent release of insulin. In addition, they are important in the regulation of cardiac ischemia, adaptation to cardiac stress and skeletal muscle fatigue[10-14].

K_{ATP} channels in the brain play an important role in glucose homeostasis[15,16], and novel functions of brain K_{ATP} channels continue to be identified: they help protect against neural apoptosis following a stroke[17-19], and have recently been implicated in memory[20], the suppression of generalized seizure during hypoxia[21], and the regulation of male reproductive behavior[22]. Brain K_{ATP} channels consist of Kir6.1, Kir6.2, SUR1 and SUR2, and different combinations of these subunits in each region contribute to the diversity of K_{ATP} channels[23-26]. Kir6.2, one of the pore-forming subunits of K_{ATP} channels, is widely distributed throughout rat brain neurons and glial cells[27,28]. Especially, Kir6.2 is highly expressed in regions containing monoamine neurons such as the substantia nigra (SN), ventral tegmental area (VTA), striatum, locus coeruleus (LC), and dorsal raphe nucleus (DRN). Moreover, Kir6.2 is also observed in the synaptic neuropil in the hippocampus, amygdala, basal ganglia and cerebral cortex,

indicating that Kir6.2 is localized in dendrites and axons of monoamine neurons[28]. Therefore, it is inferred that Kir6.2 may be involved in monoamine neurotransmission. In fact, K_{ATP} channel-gated burst firing of dopamine (DA) neurons in the medial substantia nigra (m-SN) has been reported to be essential for novelty-dependent exploratory behavior in mice[29]. In addition, an *in vivo* microdialysis study showed that Kir6.2-containing K_{ATP} channels play regulatory roles in the increase in extracellular levels of DA by the perfusion of high levels of K^+ in the striatum[30].

Brain monoamine compounds, such as DA, noradrenaline (NA) and serotonin (5-HT), play critical roles in emotional behaviors and stress responses[31]. It is likely that brain Kir6.2 affects emotional behaviors and plays a role in psychiatric disorders associated with monoamine neurotransmission. Interestingly, Kir6.2-knockout ($Kir6.2^{-/-}$) mice exhibited the hypersensitive reaction to touch stimulation compared to wild type (WT) mice (data not shown). Thus, it seems that Kir6.2 deficient would affect emotional behavior. To clarify the involvement of Kir6.2 in emotional behavior, in the present study, the behavioral characteristics of $Kir6.2^{-/-}$ mice under non-stressed conditions were investigated. Furthermore, the distribution and localization of Kir6.2 in monoamine neurons of the mouse brain, and whether Kir6.2 regulates the function of monoamine neurons were examined.

Materials and Methods

The present studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the Committee on the Care and Use of Laboratory Animals of the International University of Health and Welfare.

1. Animals

In the present study, male and female Kir6.2$^{-/-}$ mice (from Drs. Miki and Seino) that were generated by targeted disruption of the KCNJ11 gene encoding the Kir6.2 subunit of K_{ATP} channels were used[32]. The Kir6.2 gene was cloned from a 129/Sv mouse genomic DNA library (Stratagene) by using its cDNA probe. A targeting vector was constructed by inserting the neomycin-resistance gene at a *Xho*I site in Kir6.2. The herpes simplex virus thymidine kinase gene was inserted downstream. The targeting vector was introduced into E14 embryonic stem (ES) cells by electroporation. Male and female C57BL/6J WT mice, the background strain of Kir6.2$^{-/-}$, were purchased from Japan SLC, Inc. (Shizuoka, Japan) and used as a WT counterpart. Both C57BL/6J and Kir6.2$^{-/-}$ mice weighing 25-30 g were housed at a room temperature of 23 ± 1°C with a 12-h light-dark cycle (light on 7:00 a.m. to 7:00 p.m.). Food and water were available *ad libitum*.

2. Experimental procedures

2.1. Locomotor activity test

To investigate the changes in circadian locomotor activity, 20- to 22-week-old mice were tested. Each mouse was placed in a cage, and circadian locomotor activity was measured by using a Supermex device (patent pending, Muromachi Kikai Co., Ltd., Tokyo, Japan). The total activity counts per hour were

automatically recorded for 72 h (starting at 19:00). The data were analyzed and stored in a personal computer using analytical software (Comp ACT HBS; Muromachi Kikai Co., Ltd., Tokyo, Japan). During this observation of circadian locomotor activity, a 12-h dark/light cycle was maintained (lights-off and -on at 19:00-7:00 and 7:00-19:00, respectively). Mice had free access to food and water for the duration of the experiment.

2.2. Open field test

To investigate changes in general emotional behavior, 15-week-old mice were tested using an apparatus based on an open field paradigm (model ST-1; Muromachi Kikai Co., Ltd., Tokyo, Japan). Each mouse was placed in the center of an open field and the behavior of the mouse in the open field was recorded for 5 min. The apparatus was made of a gray wooden box (50 x 50 x 50 cm, 170 lux). An infrared beam sensor was installed on the wall to detect the numbers and duration of rearing behaviors. Other behavioral parameters, such as the locus and distance of movement (total locomotor activity (cm)) were recorded by an overhead color CCD camera. The mice were marked by putting green seals on the tops of their heads and the color CCD camera followed the center of gravity. Data from the CCD camera were collected through a custom-designed interface (CAT-10; Muromachi Kikai Co., Ltd., Tokyo, Japan) as a reflection signal. All of the data were analyzed and stored in a personal computer using analytical software (Comp ACT HBS; Muromachi Kikai Co., Ltd., Tokyo, Japan). The results were calculated in terms of thigmotaxis (amount of time in the outer versus inner zone (17 x 17 cm) of the test enclosure). Other parameters such as rearing behavior (count and duration) and distance moved were also scored.

2.3. Elevated plus-maze test

To investigate changes in anxiety-like behavior, 14-week-old mice were tested using the elevated plus-maze paradigm (EPM-04M; Muromachi Kikai Co., Ltd., Tokyo, Japan). The apparatus was elevated 40 cm from the ground and the maze consisted of two opposing open arms (30 x 6 x 0.3 cm) and two opposing enclosed arms (30 x 6 x 15 cm) that were connected by a central platform (6 x 6 cm, 40 lux), thus forming the shape of a plus sign. Each mouse was placed on a central platform, and the distance that the mouse moved in the maze was recorded for 5 min by an overhead color CCD camera that tracked the center of the mouse. Moreover, the time spent in and the number of entries into open or enclosed arms were also recorded. Data from the CCD camera were collected through a custom-designed interface (CAT-10; Muromachi Kikai Co., Ltd., Tokyo, Japan) as a reflection signal. All of the data were analyzed and stored in a personal computer using analytical software (Comp ACT HBS; Muromachi Kikai Co., Ltd., Tokyo, Japan). The results were calculated as mean ratios of the time spent in the open arms to the total time spent in both the open and enclosed arms. Entries into the open arms (%), total number of entries and distance moved (cm) were also scored.

2.4. Light-dark test

To investigate the changes in anxiety-like behavior, 9-week-old mice were tested using the light-dark paradigm (LD1040; O'Hara Co., Ltd., Tokyo, Japan). The apparatus consisted of a light compartment (20 x 20 x 25 cm) and a dark compartment (20 x 20 x 25 cm). The two compartments were separated by a partition with a door, and the mouse was able to move between the two compartments. At the beginning of the observation session, the mouse was placed in the dark compartment and the distance that the mouse moved in the light and dark compartments was recorded for 10 min by an overhead CCD camera. Moreover, the time spent in each compartment, the latency to enter the light compartment and the number

of transitions between compartments were also recorded. All of the data were analyzed and stored in a personal computer using analytical software (ImageJ LD4; National Institutes of Health, MD, USA).

2.5. Rota-rod test

To investigate changes in motor coordination, 18-week-old mice were tested using the rota-rod test (MK-660B; Muromachi Kikai Co., Ltd., Tokyo, Japan). The apparatus consisted of a base platform and a rotating rod with a diameter of 3 cm and a non-slippery surface. A 30 cm-long rod was placed at a height of 15 cm from the base. The rod was divided into 5 equal sections by 6 disks. Thus, several mice were tested on the apparatus simultaneously. Each mouse was placed on the rod for 1 min and the rod was then rotated, which required the mouse to move forward. The speed of rotation was 10 rpm on days 1 and 2, and 20 rpm on days 3 and 4. Each mouse was tested on the rotating rod for a total of 5 min. If a mouse fell from the rod, it was immediately replaced. The test was performed twice a day, and at each session, the time until the first fall and the number of falls during the 5-min test were measured as indicators of motor impairment.

3. Immunohistochemistry

In the immunohistochemical analysis, mice were deeply anesthetized with sodium pentobarbital (70 mg/kg, i.p.) and perfusion-fixed with 4% paraformaldehyde (Wako Pure Chemical Industries Ltd., Osaka, Japan). The brains were quickly removed after perfusion, and post-fixed in 4% paraformaldehyde for 24 h at 4°C. Brain coronal sections (80 μm thick) were prepared on a Microslicer (DTK-1000; Ted Pella, Inc., CA, USA). The brain sections were incubated with 10% normal horse serum (NHS) in 0.01 M PBS for 1 h on ice to block nonspecific antibody binding. The primary antibody was diluted in 0.01 M PBS containing 10% NHS [1:100 Kir6.2 goat polyclonal antibody (Santa Cruz Biotechnology, Co., Ltd., CA, USA)] and

incubated for 2 days at 4°C. The samples were then rinsed with 0.01 M PBS and incubated with the appropriate secondary antibody conjugated with Alexa Fluor 488 (1:1,000) for 24 h at 4°C. The brain sections were rinsed with 0.01 M PBS, and then incubated with 10% NHS in 0.01 M PBS for 1 h on ice. The primary antibody was diluted in 0.01 M PBS containing 10% NHS [1:600 tryptophan hydroxylase (TPH) mouse monoclonal antibody (Sigma Chemical, Co., St. Louis, MO, USA) and 1:500 tyrosine hydroxylase (TH) mouse monoclonal antibody (Merck Millipore, Ltd., Darmstadt, Germany)] and incubated for 2 days at 4°C. The samples were then rinsed with 0.01 M PBS and incubated with the appropriate secondary antibody conjugated with Alexa Fluor 546 (1:1,000) for 24 h at 4°C. The brain sections were rinsed with 0.01 M PBS, and then mounted on glass slides with PermaFluor Aqueous mounting medium (Thermo Fisher Scientific, Inc., MA, USA). Fluorescence immunolabeling was detected using a confocal laser-scanning microscope (FV1000; Olympus Optical, Tokyo, Japan).

4. Western blotting

The midbrain, containing raphe, was quickly removed and homogenized in 6 volumes of ice-cold buffer. The midbrain was removed within 10 min after decapitation and stored at -70°C for future analysis. The midbrain was homogenized in 6 volumes of ice-cold buffer containing 20 mM Tris-HCl (pH7.4; Wako Pure Chemical Industries Ltd., Osaka, Japan), 2 mM ethylenediaminetetraacetic acid (EDTA; Wako Pure Chemical Industries Ltd., Osaka, Japan), 10 mM ethylene glycol-bis (2-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA; Wako Pure Chemical Industries Ltd., Osaka, Japan), 250 mM sucrose (Wako Pure Chemical Industries Ltd., Osaka, Japan), 1% Triton (Calbiochem-Novabiochem, La Jolla, CA, USA) and a protease inhibitor cocktail (Complete®; Roche Molecular Biochemicals, Mannheim, Germany), using an ultrasound homogenizer (UR-20P, TOMY SEIKO Co. Ltd., Tokyo, Japan). The homogenates were homogenized again immediately after being

centrifuged at 10,000 x *g* for 1 min at 4°C. The homogenates were centrifuged at 1,000 x *g* for 1 min at 4°C, and the supernatants were collected and stored as test samples at -70°C for future analysis.

An aliquot of test sample was diluted with an equal volume of electrophoresis sample buffer (Bio-Rad Laboratories, Co., Ltd., CA, USA). Proteins (10 μg/lane) were separated by size on 4-20% SDS-polyacrylamide gradient gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Co., Ltd., CA, USA) in 5% methanol (Wako Pure Chemical Industries Ltd., Osaka, Japan) added Tris-glycine buffer (Bio-Rad Laboratories, Co., Ltd., CA, USA) using a semi-dry electrophoretic transfer cell (Bio-Rad Laboratories, Co., Ltd., CA, USA). In addition, molecular markers (Precision plus protein dual color standards; Bio-Rad Laboratories, Co., Ltd., CA, USA) were loaded in lanes adjacent to the sample lanes before the commencement of a run. For the immunoblot detection of TPH and TH, membranes were blocked in 0.05% Tween 20-Tris-Buffered Saline (TTBS) containing 3% bovine serum albumin (BSA; Sigma-Aldrich, Co., Ltd., MO, USA) for 1 h at room temperature with agitation. The membrane was incubated with primary antibody diluted in Solution 1 (TOYOBO, Co., Ltd., Osaka, Japan) [1:1,000 TPH (Sigma Chemical, Co., St. Louis, MO, USA), and 1:1,000 TH (Merck Millipore, Ltd., Darmstadt, Germany)] overnight at 4°C. The membranes were washed in TTBS and then incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti mouse IgG (Jackson Immunoresearch Laboratories, Co., Ltd., PA, USA), which was diluted 1:2,000 or 1:10,000 in Solution 2 (TOYOBO, Co., Ltd., Osaka, Japan). After this incubation, the membranes were washed in TTBS. The antigen-antibody-peroxidase complex was then finally detected by enhanced chemiluminescence (Santa Cruz Biotechnology, Co., Ltd., CA, USA), and scanned, optimized and analyzed by Chemi Doc XRS (Bio-Rad Laboratories, Co., Ltd., CA, USA).

5. Statistical analysis

The data are presented as the mean with S.E.M. The statistical analyses were performed using two-way repeated measure ANOVA or Student's *t*-test.

Results

1. Locomotor activity test

Spontaneous locomotor activity of Kir6.2$^{-/-}$ mice as detected by the locomotor activity test is shown in Fig. 1-1. Spontaneous locomotor activity during the dark period was significantly decreased in both sexes. Such decreases were particularly apparent in female Kir6.2$^{-/-}$ mice. (Fig. 1-1A; $F_{(1,852)}$=0.168, p=0.6895 vs. male WT mice, Fig. 1-1B; $F_{(1,994)}$=4.472, p=0.0529 vs. female WT mice)

2. Open field test

General behaviors of Kir6.2$^{-/-}$ mice as detected by the open field test are shown in Fig. 1-2. Male Kir6.2$^{-/-}$ mice showed significant decreases in both the percentage of time spent in the central area (Fig. 1-2A; p<0.05) and the distance moved (Fig. 1-2B; p<0.05) compared with male WT mice. Female Kir6.2$^{-/-}$ mice showed significant decreases in rearing counts (Fig. 1-2G; p<0.01) and rearing duration (Fig. 1-2H; p<0.01) compared with female WT mice.

3. Elevated plus-maze test

Anxiety-like behaviors of Kir6.2$^{-/-}$ mice as detected by the elevated plus-maze test are shown in Fig. 1-3. The percentage of time spent in open arms was significantly decreased in Kir6.2$^{-/-}$ mice of both sexes (Fig. 1-3B and F; p<0.01). Moreover, female Kir6.2$^{-/-}$ mice showed significant decreases in distance moved (Fig. 1-3E; p<0.05) and the total number of entries (Fig. 1-3H; p<0.05) compared with female WT mice. Male Kir6.2$^{-/-}$ mice tended to show a decrease in distance moved (Fig. 1-3A; p=0.0571).

4. Light-dark test

Anxiety-like behaviors of Kir6.2$^{-/-}$ mice as detected by the light-dark test are shown in Fig. 1-4. Significant decreases in both the distance moved in the light compartment (Fig. 1-4B and H; $p<0.01$) and the number of transitions (Fig. 1-4F and L; $p<0.01$ or 0.001) and significant increases in the latency to enter the light compartment (Fig. 1-4E and K; $p<0.05$) were observed in Kir6.2$^{-/-}$ mice of both sexes. Moreover, a significant increase in the time spent in the dark compartment (Fig. 1-4I; $p<0.05$) and a decrease in the time spent in the light compartment (Fig. 1-4J; $p<0.05$) were observed in female Kir6.2$^{-/-}$ mice.

5. Rota-rod test

Motor coordination of Kir6.2$^{-/-}$ mice as detected by the rota-rod test is shown in Fig. 1-5. In male Kir6.2$^{-/-}$ mice, an apparent reduction in the latency to fall (Fig. 1-5A; $F_{(1,77)}=3.893$, $p=0.0741$ vs. male WT mice) and a tendency for an increase in the number of falls (Fig. 1-5B; $F_{(1,77)}=2.839$, $p=0.1201$ vs. male WT mice) from the rotating rod were observed on day 3. In female Kir6.2$^{-/-}$ mice, a significant reduction in the latency to fall (Fig. 1-5C; $F_{(1,91)}=10.536$, $p=0.0064$ vs. female WT mice) and a significant increase in the number of falls (Fig. 1-5D; $F_{(1,91)}=5.792$, $p=0.0317$ vs. female WT mice) from the rotating rod were observed on day 3.

6. Immunohistochemistry

The distribution and localization of Kir6.2 in monoamine neurons of mouse brain are shown in Fig. 1-6. Kir6.2 was expressed in TPH-positive cells in the DRN of male WT mice (Fig. 1-6A, B and C-i, ii). Kir6.2 was also expressed in TH-positive cells in the VTA (Fig. 1-6D, E and F-i, ii) and the LC (Fig. 1-6G, H and I-i, ii) of male WT mice.

7. Western blotting

The changes in the protein levels of TPH and TH in the Kir6.2$^{-/-}$ mouse midbrain are shown in Fig. 1-7. Western blotting showed that whole-cell protein levels of TPH in the midbrain were significantly increased in female Kir6.2$^{-/-}$ mice (Fig. 1-7F; $p<0.05$).

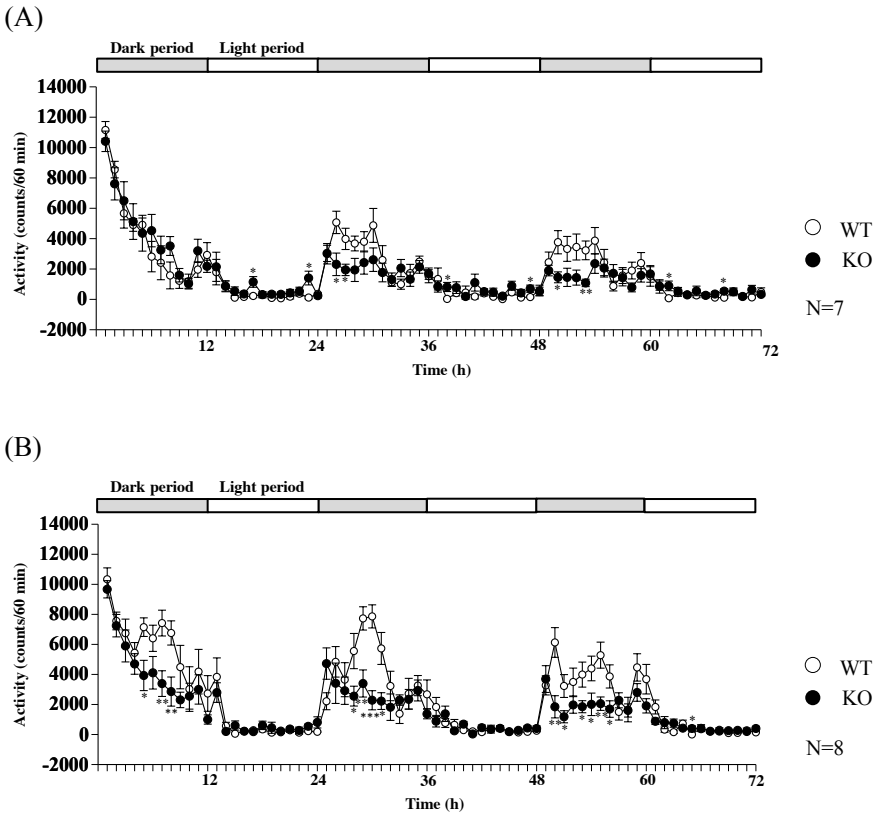


Fig. 1-1. Spontaneous locomotor activity in Kir6.2$^{-/-}$ mice as detected by the locomotor activity test. Each mouse was placed independently in a cage with the same shape as its home cage, and its locomotor activity was recorded for 72 h. (A) Analysis of male mice. (B) Analysis of female mice. Each point represents the mean with SEM of 7-8 mice. Open and closed circles represent wild type and Kir6.2$^{-/-}$ mice, respectively. *p<0.05, **p<0.01, ***p<0.001 vs. wild type mice. WT: wild type mice, KO: Kir6.2$^{-/-}$ mice.

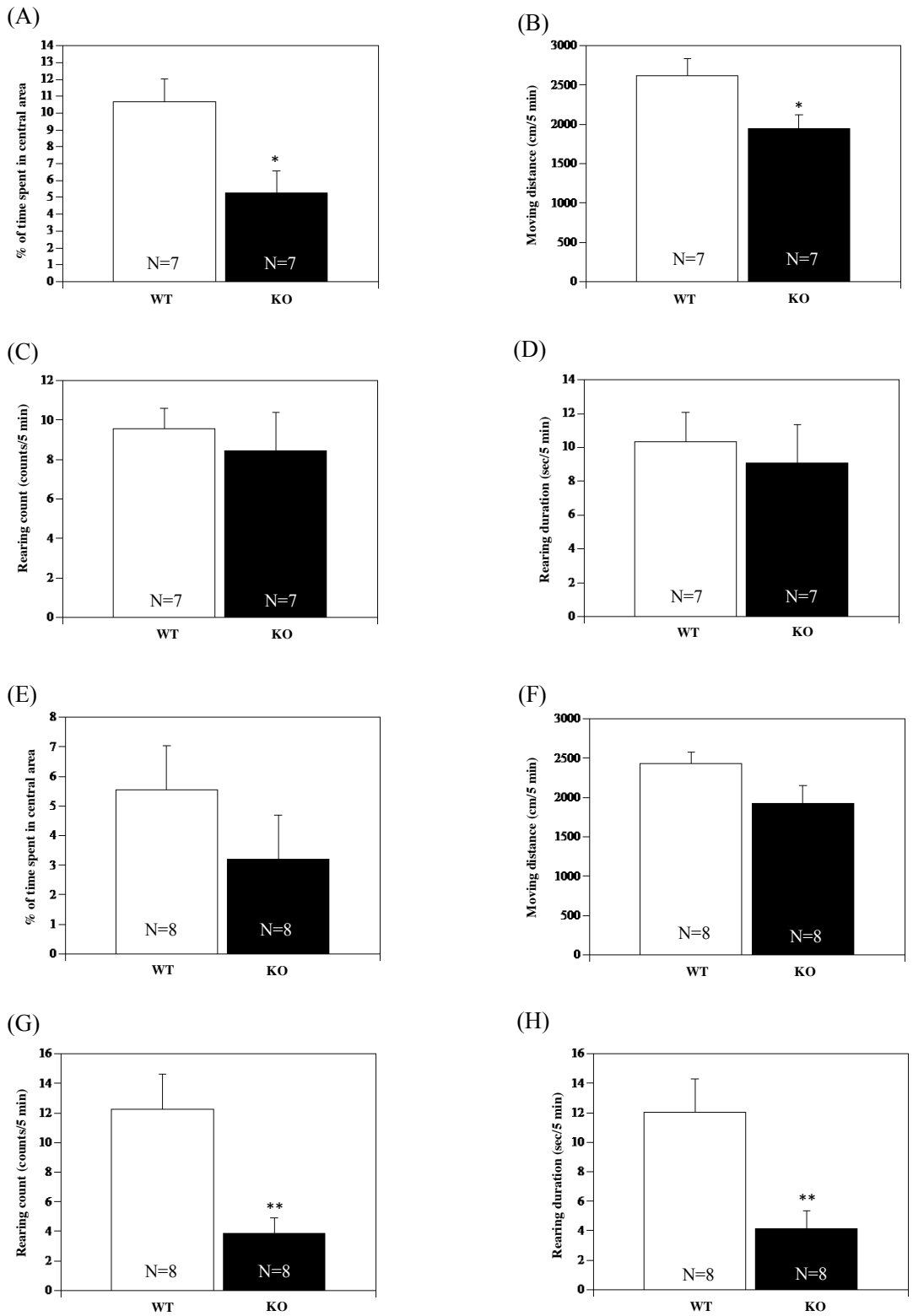


Fig. 1-2. General behaviors of Kir6.2$^{-/-}$ mice as detected by the open field test. Each mouse was placed in the center of an open field and the behavior of the mouse in the open field was recorded for 5 min. (A-D) Analysis of male mice. (E-H) Analysis of female mice. The percentage of time spent in the central area (A, E), the distance moved (B, F), the rearing counts (C, G) and the rearing duration (D, H) were scored. Each column represents the mean with SEM of 7-8 mice. *p<0.05, **p<0.01 vs. wild type mice. WT: wild type mice, KO: Kir6.2$^{-/-}$ mice.

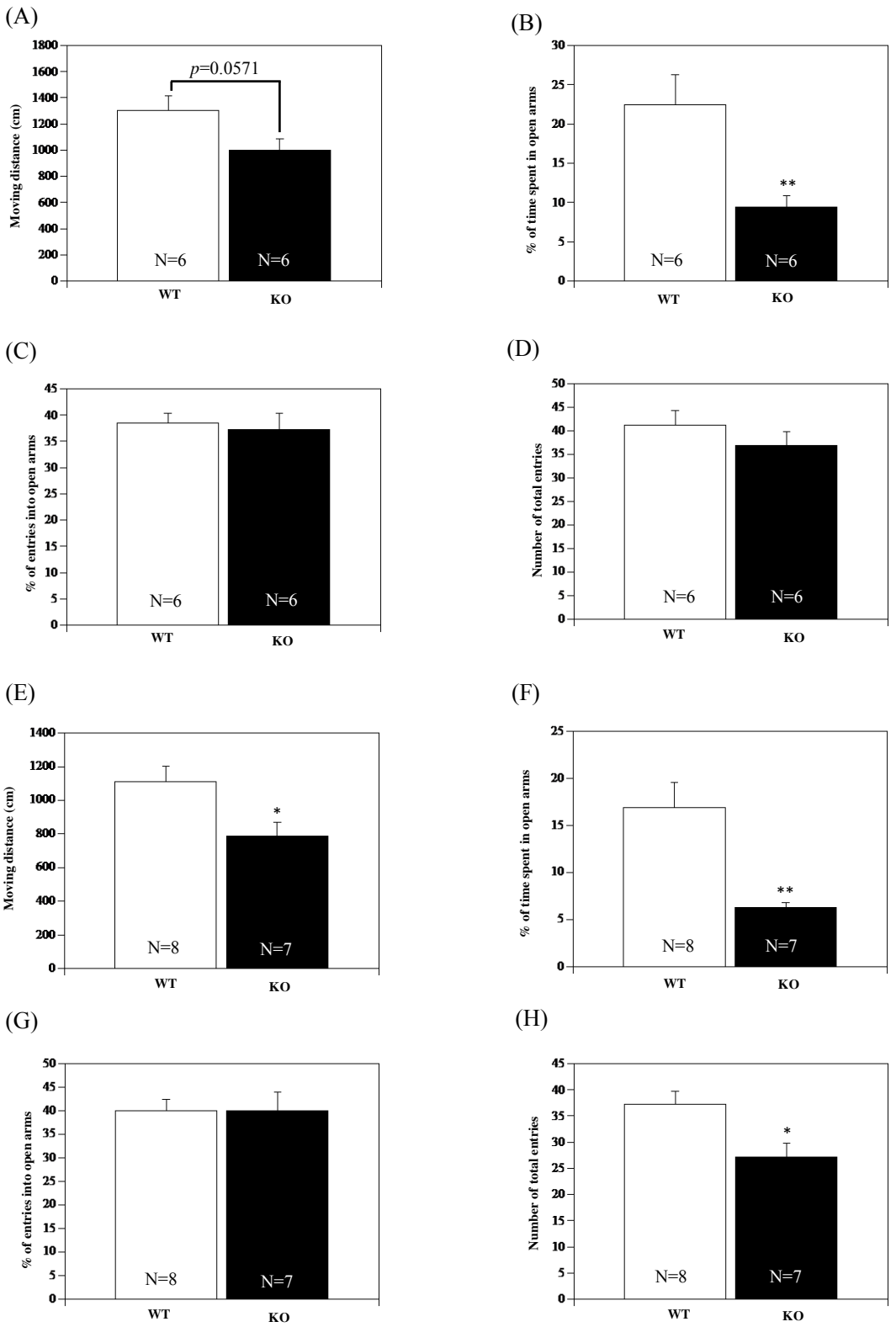


Fig. 1-3. Anxiety-like behaviors of Kir6.2$^{-/-}$ mice as detected by the elevated plus-maze test. Each mouse was placed on a central platform, and the distance that the mouse moved in the maze was recorded for 5 min. (A-D) Analysis of male mice. (E-H) Analysis of female mice. The distance moved (A, E), the percentage of time spent in open arms (B, F), the percentage of entries into open arms (C, G) and the total number of entries into both open and enclosed arms (D, H) were scored. Each column represents the mean with SEM of 6-8 mice. *p<0.05, **p<0.01 vs. wild type mice. WT: wild type mice, KO: Kir6.2$^{-/-}$ mice.

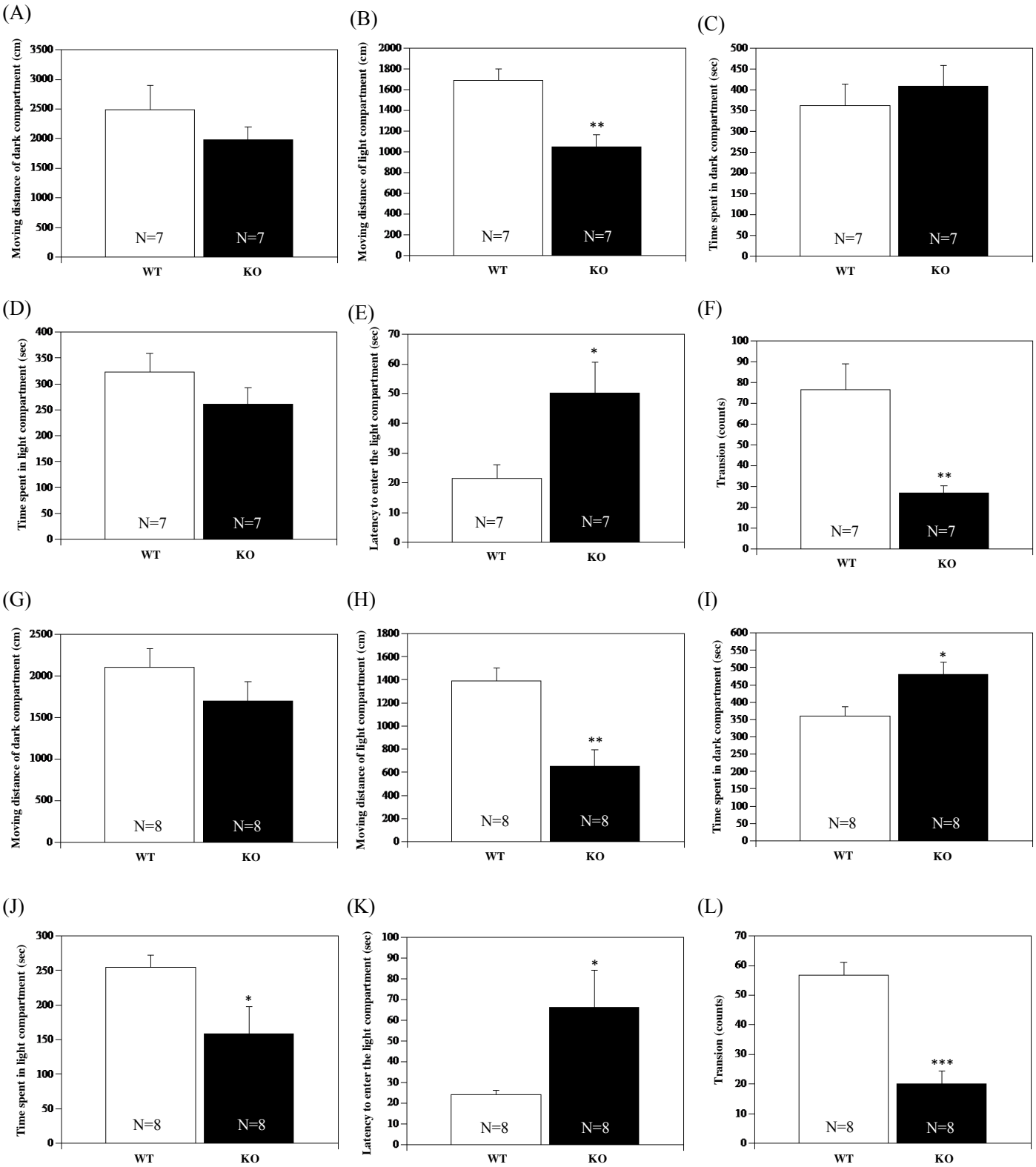


Fig. 1-4. Anxiety-like behaviors of Kir6.2$^{-/-}$ mice as detected by the light-dark test. Each mouse was placed in the dark compartment, and the distance that the mouse moved in the box for 10 min was recorded. (A-F) Analysis of male mice. (G-L) Analysis of female mice. The distances moved in the dark compartment (A, G) and the light compartment (B, H), the times spent in the dark (C, I) and light compartments (D, J), the latency to enter the light compartment (E, K) and the number of transitions between compartments (F, L) were scored. Each column represents the mean with SEM of 7-8 mice. *p<0.05, **p<0.01, ***p<0.001 vs wild type mice. WT: wild type mice, KO: Kir6.2$^{-/-}$ mice.

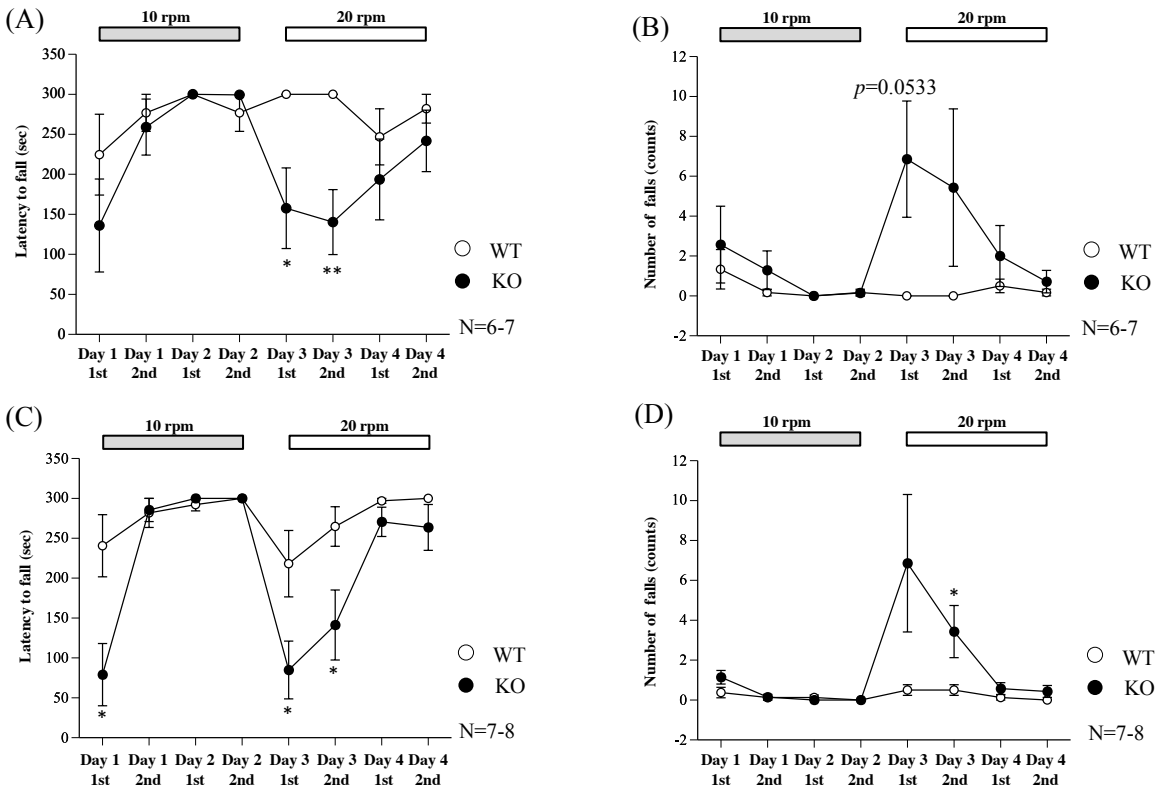


Fig. 1-5. Motor coordination of Kir6.2$^{-/-}$ mice as detected by the rota-rod test. Each mouse was placed on the rod for 1 min and the rod was then rotated, which required the mouse to move forward. The speed of rotation was 10 rpm on days 1 and 2, and 20 rpm on days 3 and 4. Each mouse was tested on the rotating rod for a total of 5 min. If a mouse fell from the rod, it was immediately replaced. Twice a day, the time until the first fall and the number of falls during the 5 min test period were measured as indicators of motor impairment. (A, B) Analysis of male mice. (C, D) Analysis of female mice. The latency to the first fall (A, C) and the number of falls (B, D) from a rotating rod were scored. Each point represents the mean with SEM of 6-8 mice. Open and closed circles represent wild type and Kir6.2$^{-/-}$ mice, respectively. *p<0.05 , **p<0.01 vs wild type mice. WT: wild type mice, KO: Kir6.2$^{-/-}$ mice.

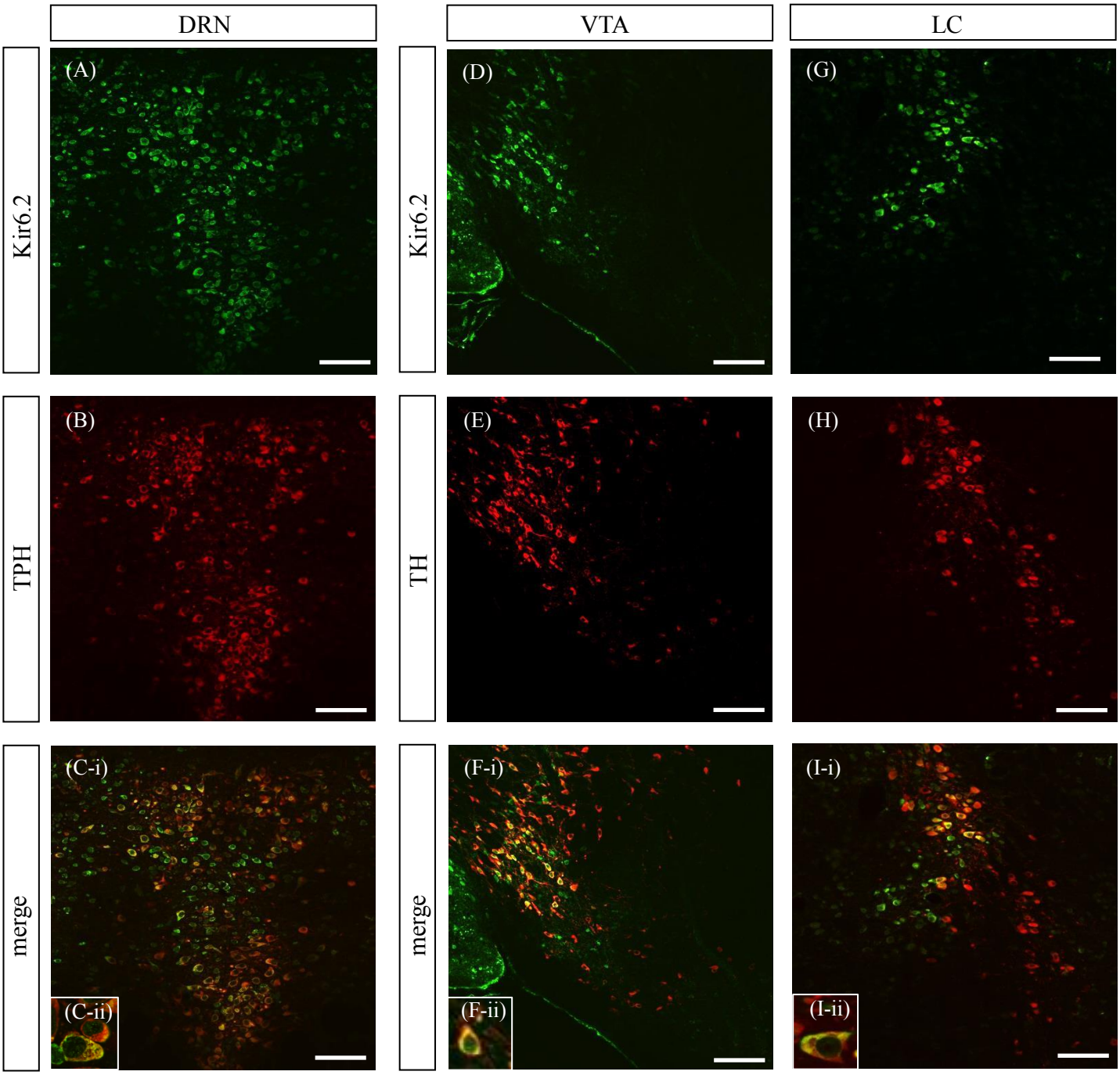


Fig. 1-6. Distribution and localization of Kir6.2 in the midbrain of wild type mouse. *Left*: Kir6.2 labeled in green (A) and tryptophan hydroxylase (TPH) labeled in red (B) were expressed in the dorsal raphe nuclei (DRN) of male wild type mice (C-i, ii). *Middle*: Kir6.2 labeled in green (D) and tyrosine hydroxylase (TH) labeled in red (E) were expressed in the ventral tegmental area (VTA) of male wild type mice (F-i, ii). *Right*: Kir6.2 labeled in green (G) and tyrosine hydroxylase (TH) labeled in red (H) were expressed in the locus coeruleus (LC) of male wild type mice (I-i, ii). Scale bar = 100 μm.

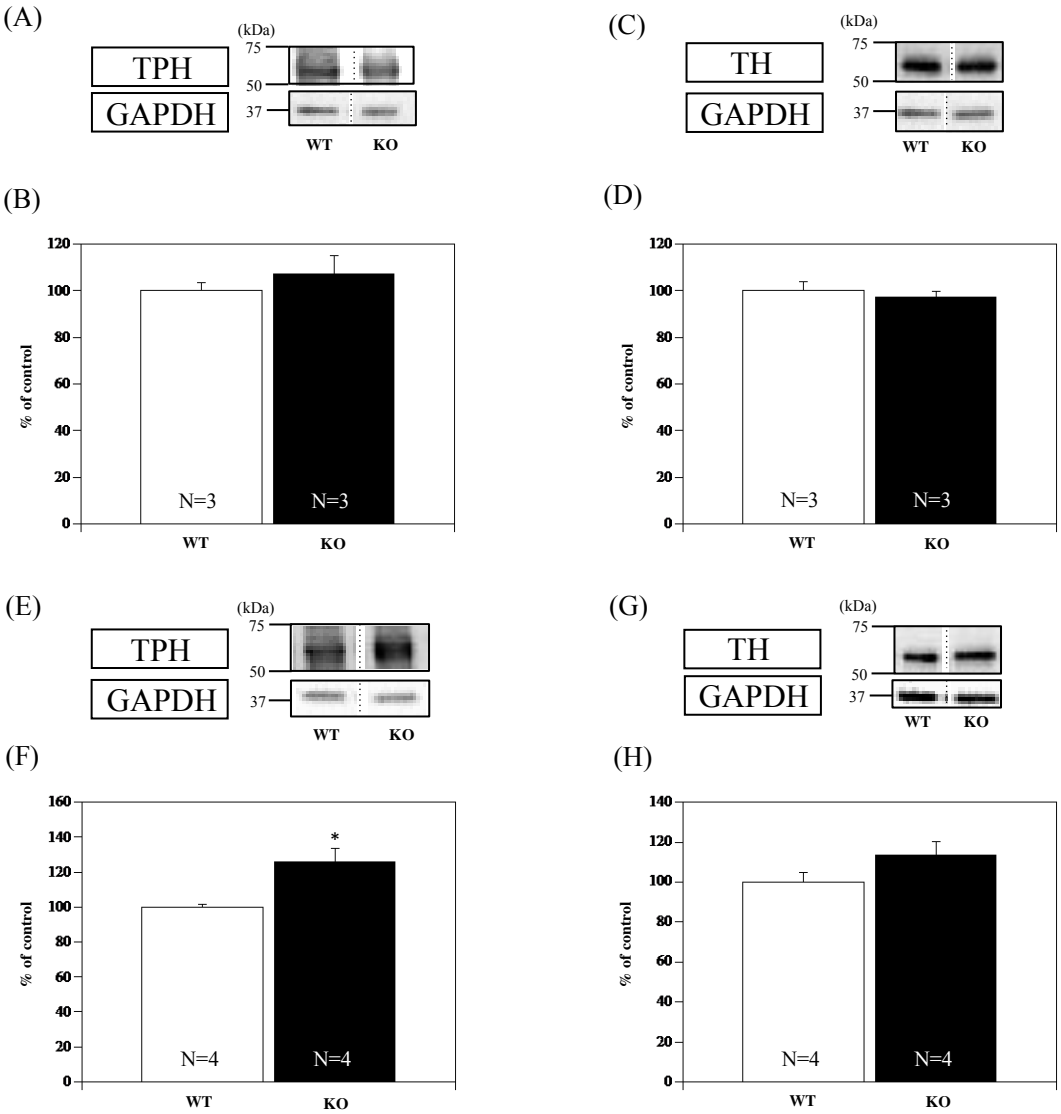


Fig. 1-7. Changes in the protein levels of tryptophan hydroxylase (TPH) and tyrosine hydroxylase (TH) in the midbrain of Kir6.2$^{-/-}$ mice. (A-D) Analysis of male mice. (E-H) Analysis of female mice. (A, C, E, G) Representative Western blotting of TPH (A, E) and TH (C, G) in the midbrain of wild type and Kir6.2$^{-/-}$ mice. (B, D, F, H) Quantitative analysis of immunoreactivities for TPH (B, F) and TH (D, H). Each column represents the mean with SEM of 3-4 mice. *p<0.05 vs wild type mice. WT: wild type mice, KO: Kir6.2$^{-/-}$ mice.

Discussion

Brain monoamine neurons play a critical role in several emotional behaviors, and Kir6.2 is highly expressed in brain regions containing monoamine neurons[28]. It is therefore possible that brain Kir6.2 affects emotional behaviors associated with monoamine neurotransmission. In the present study, the involvement of Kir6.2, pore-forming subunits of K_{ATP} channels, in emotional behaviors under non-stressed conditions were investigated.

In the spontaneous locomotor activity test, both male and female Kir6.2$^{-/-}$ mice exhibited hypolocomotion during the dark period. In the open field test, male Kir6.2$^{-/-}$ mice showed a significant decrease in the time spent in the central area. A decrease in the distance moved was also observed. Moreover, female Kir6.2$^{-/-}$ mice showed significant decreases in both rearing counts and rearing duration. These results indicate that Kir6.2 could be involved in general emotional behaviors. These findings strongly agree with a previous report that Kir6.2$^{-/-}$ mice differ from WT mice with respect to their behavior in novel situations[33]. Furthermore, mice carrying the human Kir6.2 mutation V59M, valine-to-methionine at position 59, which results in an increased expression of Kir6.2 in the brain, are more active and show an increase in exploratory behavior[34,35]. Taken together, these results suggest that Kir6.2 influences the regulation of general emotional behaviors under non-stressed conditions.

To clarify the effect of Kir6.2 mutation on anxiety-like behavior, the behavior of Kir6.2$^{-/-}$ mice was checked in the elevated plus-maze test and the light-dark test. In the elevated plus-maze test, both male and female Kir6.2$^{-/-}$ mice showed a significant decrease the percentage of time spent in open arms. In the light-dark test, both male and female Kir6.2$^{-/-}$ mice showed a significant decrease in the distance moved in the light compartment and an increase in the latency to enter the light compartment. Moreover, female Kir6.2$^{-/-}$ mice showed a significant increase in the time spent in the dark compartment. These results

strongly agree with previous reports that Kir6.2$^{-/-}$ mice exhibit increased anxiety-like behavior and Kir6.2-increased mice (Kir6.2-V59M) showed reduced anxiety-like behavior in the elevated plus-maze, the successive alleys test, and the light-dark test[33,35]. In addition, the novel finding of the present study is that excessive anxiety-like behavior was observed in female Kir6.2$^{-/-}$ mice. Namely, increase in time spent in dark compartment in the light-dark test, a indicator of anxiety, was more pronounced in female than male Kir6.2$^{-/-}$ mice. Although the reason for this result is not clear, it may be due to a gender difference in monoamine neurons.

Kir6.2 is also expressed in basal ganglia and the cerebellum, which controls motor coordination[23,28]. Therefore, it is possible that both the decrease in general emotional behaviors and the increase in anxiety-like behaviors observed in Kir6.2$^{-/-}$ mice were caused by the impairment of motor coordination. To investigate possible motor impairment under a Kir6.2 deficiency, Kir6.2$^{-/-}$ mice were tested using the rota-rod test. At a rod-rotating speed of 10 rpm, a Kir6.2 deficiency did not significantly affect either the latency to fall or the number of falls from the rotating rod in male. In contrast, under these conditions, significant shortening of the latency to fall was observed in female Kir6.2$^{-/-}$ mice. At a rod-rotating speed of 20 rpm, Kir6.2$^{-/-}$ mice showed both a decrease in the latency to fall and an increase in the number of falls from the rotating rod over 5 min, and these impairments were more prominent in females. These results indicate that although Kir6.2$^{-/-}$ mice may be impaired with regard to advanced motor coordination, they are capable of simple exercise. In addition, Kir6.2$^{-/-}$ mice showed the hypersensitive reaction to touch stimulation and the movements of Kir6.2$^{-/-}$ mice were very quick compared with WT mice (data not shown). Moreover, in forced swim test, Kir6.2 deficient failed to change in the immobility time in both sexes (data not shown). These results would be the evidence that Kir6.2 deficient failed to induce sever impairment of motor coordination and affect on general malaise. However, it was a fact that partial movement

disorder-like behavior was observed in Kir6.2$^{-/-}$ mice. Therefore, the phenotype of emotional behavior obtained in the present study needs to consider a possible impairment of mild motor coordination.

In the next set of experiments, the expression pattern of Kir6.2 in 5-HT, DA or NA neurons was focused. The immunohistochemical analysis showed that Kir6.2 was expressed in TPH-positive cells in the DRN, and in TH-positive cells in the VTA and LC of WT mice. Therefore, Kir6.2 might regulate monoamine neurons in the brain. K_{ATP} channel-gated burst firing in DA neurons of the m-SN is essential for novelty-dependent exploratory behaviors in mice[29]. In addition, an *in vivo* microdialysis study showed that Kir6.2-containing K_{ATP} channels regulate the increase in extracellular levels of DA with the perfusion of high levels of K^+ in the striatum[30]. Although these reports indicate that Kir6.2 plays a functional role in monoamine neurons in the brain, they focused on DA neurons in the SN. The novel finding in the present study is that Kir6.2 may play a functional role in DA neurons in the VTA as well as other monoamine neurons. Further studies are needed to determine whether Kir6.2 in monoamine neurons regulates the release of 5-HT, DA and NA, and influences the behavioral changes in Kir6.2$^{-/-}$ mice.

Another question to consider is whether a deficiency of Kir6.2 affects the expression of functional enzymes in brain monoamine neurons. The expression of TPH and TH in the midbrain of WT mice was checked. Western blotting analysis suggested that protein levels of TPH, the rate-limiting enzyme in 5-HT synthesis, in whole-cell lysate obtained from the midbrain were significantly increased in female, but not male, Kir6.2$^{-/-}$ mice. On the other hand, there were no differences in the expression of TH in either male or female Kir6.2$^{-/-}$ mice. These results indicate that the synthesis of 5-HT may be increased in female Kir6.2$^{-/-}$ mice. It has been reported that 5-HT_{2A} and 5-HT_{2C} receptors modulate anxiety-like behaviors[36-39]. One explanation for why excessive anxiety-like behavior was observed in Kir6.2$^{-/-}$ mice may be that 5-HT synthesis was increased and 5-HT_{2A} and 5-HT_{2C} receptors were stimulated. On the other hand, previous studies in stress-adaptive and -maladaptive animals have provided evidence that an increase in brain 5-HT

signaling may be a key factor in the adaptation to stress[40-43]. In addition, stimulation of 5-HT_{1A} receptor produced emotional resistance to stress stimuli in mice[44-46]. TPH expression was also increased and decreased in the midbrain in stress-adaptive and -maladaptive mice, respectively[47]. These reports indicate that the constitutive activation of brain 5-HT neurons could be necessary for the development of stress adaptation. Thus, another possibility is that an increase in TPH in the midbrain of female Kir6.2$^{-/-}$ mice might be compensatory mechanisms to excessive anxiety.

At least in my understanding, there is no report on the gender differences on the role of Kir6.2 in emotional regulation. To clarify this point, the present study has carried out the two sets of simple experiments. First, the possibility that there are gender differences on the expression of Kir6.2 was considered. Although the expression of Kir6.2 protein in the midbrain and hippocampus of WT mice has been checked, there were no changes in protein levels among sexes in these brain regions (data not shown). Furthermore, to clarify the involvement of Kir6.2 in stress responses, the changes in serum corticosterone levels induced by acute restraint stress in Kir6.2$^{-/-}$ mice were investigated. Surprisingly, gender differences of stress response were observed in Kir6.2$^{-/-}$ mice. Detail of them was described in chapter 2.

K_{ATP} channels play a critical role in glucose metabolism through the release of insulin. Another concern in the present study is the possibility that a deficiency of Kir6.2 induced abnormal glucose metabolism, and hyperglycemia caused emotional abnormality. This possibility may be excluded by unpublished observation that Kir6.2$^{-/-}$ mice developed diabetes when fed a high-fat diet, but showed a well-controlled blood glucose level under normal feeding.

In summary, the present study demonstrated that K_{ATP} channels including Kir6.2 regulate emotional behaviors such as anxiety-like behavior. A possible mechanism for such regulation may involve the influence of Kir6.2 on monoamine neurons in the brain. In particular, the observation of excessive anxiety-like behavior in female Kir6.2$^{-/-}$ mice might be due to the activation of brain 5-HT neurons.

Although there are still many questions regarding the mechanisms of the relationship between Kir6.2 and emotional regulation, the present study provides important evidence suggesting that K_{ATP} channels may be a novel target for the treatment of psychiatric disorders.

Chapter 2

Involvement of K_{ATP} channels including Kir6.2 in stress responses

Introduction

As mentioned in a previous chapter, K_{ATP} channels including Kir6.2 can regulate emotional behaviors. A possible mechanism for such regulation may involve the influence of Kir6.2 on monoamine neurons in the brain. Moreover, Kir6.2 is also highly expressed in brain regions such as the hippocampus, hypothalamus and pituitary, which play important roles in stress responses[28,48]. The hypothalamic-pituitary-adrenal (HPA) axis is a complex set of direct influences and feedback interactions among three endocrine glands (the hypothalamus, the pituitary gland, and the adrenal glands), which is activated by exposure to stress[49]. The paraventricular nucleus (PVN) of the hypothalamus contains neuroendocrine neurons that synthesize and secrete corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP). These hormones sequentially lead to secretion of the adrenocorticotropic hormone (ACTH) from the anterior lobe of the pituitary. ACTH releases glucocorticoids (cortisol in humans and corticosterone in mice and rats) from the adrenal cortex into plasma. Regulatory control over the HPA axis is mediated via negative feedback by the glucocorticoid receptor (GR) in the hippocampus, hypothalamus and pituitary[50]. Kir6.2 is expressed on corticotrophs (ACTH cells) in the anterior lobe of the pituitary gland in rats[48], and plays possible roles in the control of ACTH secretion. Moreover, iptakalim, a K_{ATP} channel opener, inhibits the increase in serum corticosterone induced by chronic mild stress[51]. Therefore, brain Kir6.2 could affect stress responses and play a role in psychiatric disorders associated with the HPA axis. To clarify the involvement of Kir6.2 in stress responses, in the present study, the serum concentrations of corticosterone in Kir6.2$^{-/-}$ mice under stressed conditions were investigated. Furthermore, the distribution and localization of Kir6.2 in the hippocampus were examined.

Materials and Methods

The present studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the Committee on the Care and Use of Laboratory Animals of the International University of Health and Welfare.

1. Animals

In the present study, male and female Kir6.2$^{-/-}$ mice (from Drs. Miki and Seino) that were generated by targeted disruption of the KCNJ11 gene encoding the Kir6.2 subunit of K_{ATP} channels were used[32]. The Kir6.2 gene was cloned from a 129/Sv mouse genomic DNA library (Stratagene) by using its cDNA probe. A targeting vector was constructed by inserting the neomycin-resistance gene at a *Xho*I site in Kir6.2. The herpes simplex virus thymidine kinase gene was inserted downstream. The targeting vector was introduced into E14 embryonic stem (ES) cells by electroporation. Male and female C57BL/6J WT mice, the background strain of Kir6.2$^{-/-}$, were purchased from Japan SLC, Inc. (Shizuoka, Japan) and used as a WT counterpart. Both C57BL/6J and Kir6.2$^{-/-}$ mice weighing 25–30 g were housed at a room temperature of 23 ± 1°C with a 12-h light-dark cycle (light on 7:00 a.m. to 7:00 p.m.). Food and water were available *ad libitum*.

2. Measurement of serum corticosterone concentrations

Seven- to nine-week-old mice were exposed to single restraint stress for 60 min by insertion into a syringe (50 mL). Just after this exposure to restraint stress, mice were sacrificed by decapitation and their blood was collected from 14:00 to 18:00. Blood samples were centrifuged at 2,380 x *g* for 15 min, and serum was stored at -20^{o}C for future analysis. The corticosterone concentrations were determined by

competitive enzyme immunoassay (AssayPro, St. Charles, MO, USA) according to the manufacturer's instructions.

3. Immunohistochemistry

In the immunohistochemical analysis, mice were deeply anesthetized with sodium pentobarbital (70 mg/kg, i.p.) and perfusion-fixed with 4% paraformaldehyde (Wako Pure Chemical Industries Ltd., Osaka, Japan). The brains were quickly removed after perfusion, and post-fixed in 4% paraformaldehyde for 24 h at 4°C. Brain coronal sections (80 μm thick) were prepared on a Microslicer (DTK-1000; Ted Pella, Inc., CA, USA). The brain sections were incubated with 10% normal horse serum (NHS) in 0.01 M PBS for 1 h on ice to block nonspecific antibody binding. The primary antibody was diluted in 0.01 M PBS containing 10% NHS [1:100 Kir6.2 goat polyclonal antibody (Santa Cruz Biotechnology, Co., Ltd., CA, USA)] and incubated for 2 days at 4°C. The samples were then rinsed with 0.01 M PBS and incubated with the appropriate secondary antibody conjugated with Alexa Fluor 488 (1:1,000) for 24 h at 4°C. The brain sections were rinsed with 0.01 M PBS, and then incubated with 10% NHS in 0.01 M PBS for 1 h on ice. The primary antibody was diluted in 0.01 M PBS containing 10% NHS [1:100 glucocorticoid receptor (GR) rabbit polyclonal antibody (Santa Cruz Biotechnology, Co., Ltd., CA, USA)] and incubated for 2 days at 4°C. The samples were then rinsed with 0.01 M PBS and incubated with the appropriate secondary antibody conjugated with Alexa Fluor 546 (1:1,000) for 24 h at 4°C. The brain sections were rinsed with 0.01 M PBS, and then mounted on glass slides with PermaFluor Aqueous mounting medium (Thermo Fisher Scientific, Inc., MA, USA). Fluorescence immunolabeling was detected using a confocal laser-scanning microscope (FV1000; Olympus Optical, Tokyo, Japan).

4. Statistical analysis

The data are presented as the mean with S.E.M. The statistical analyses were performed using one-way ANOVA with the Bonferroni/Dunnett multiple comparison test.

Results

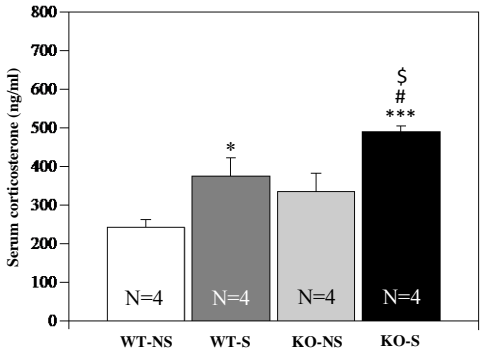
1. Serum corticosterone concentrations

The effects of exposure to acute restraint stress on serum corticosterone concentrations in mice are shown in Fig. 2-1. In the non-stressed condition, the deficiency of Kir6.2 tended to increase corticosterone concentrations in males and significantly increased these concentrations in females (Fig. 2-1B; $p<0.01$ vs. wild type non-stressed mice). Exposure to acute restraint stress for 60 min significantly increased corticosterone concentrations in WT mice and Kir6.2$^{-/-}$ mice of both sexes (Fig. 2-1A, B; $p<0.05$ or 0.01 vs. wild type non-stressed mice, $p<0.05$ or 0.001 vs. Kir6.2$^{-/-}$ non-stressed mice). In the stressed condition, corticosterone concentrations in Kir6.2$^{-/-}$ mice of both sexes were higher than those in WT mice (Fig. 2-1A, B; $p<0.05$ or 0.001 vs. wild type stressed mice).

2. Immunohistochemistry

The distribution and localization of Kir6.2 in the mouse hippocampus are shown in Fig. 2-2. Kir6.2 was expressed in GR-positive cells in CA1 (Fig. 2-2A-F) and dentate gyrus (DG) (Fig. 2-2G-L) of female WT mice.

(A)



(B)

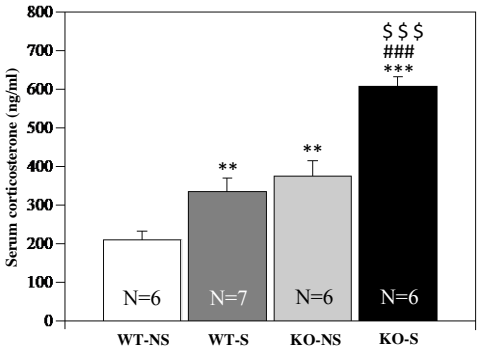


Fig. 2-1. Influences of acute restraint stress on serum corticosterone concentration. (A) Analysis of male mice. (B) Analysis of female mice. Each column represents the mean with SEM of 4-7 mice. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ vs. wild type non-stressed mice. $^{\#}p<0.05$, $^{\#\#\#}p<0.001$ vs. wild type stressed mice. $^{\$}p<0.05$, $^{\$\$\$}p<0.001$ vs. Kir6.2$^{-/-}$ non-stressed mice. WT-NS: wild type non-stressed mice, WT-S: wild type stressed mice, KO-NS: Kir6.2$^{-/-}$ non-stressed mice, KO-S: Kir6.2$^{-/-}$ stressed mice.

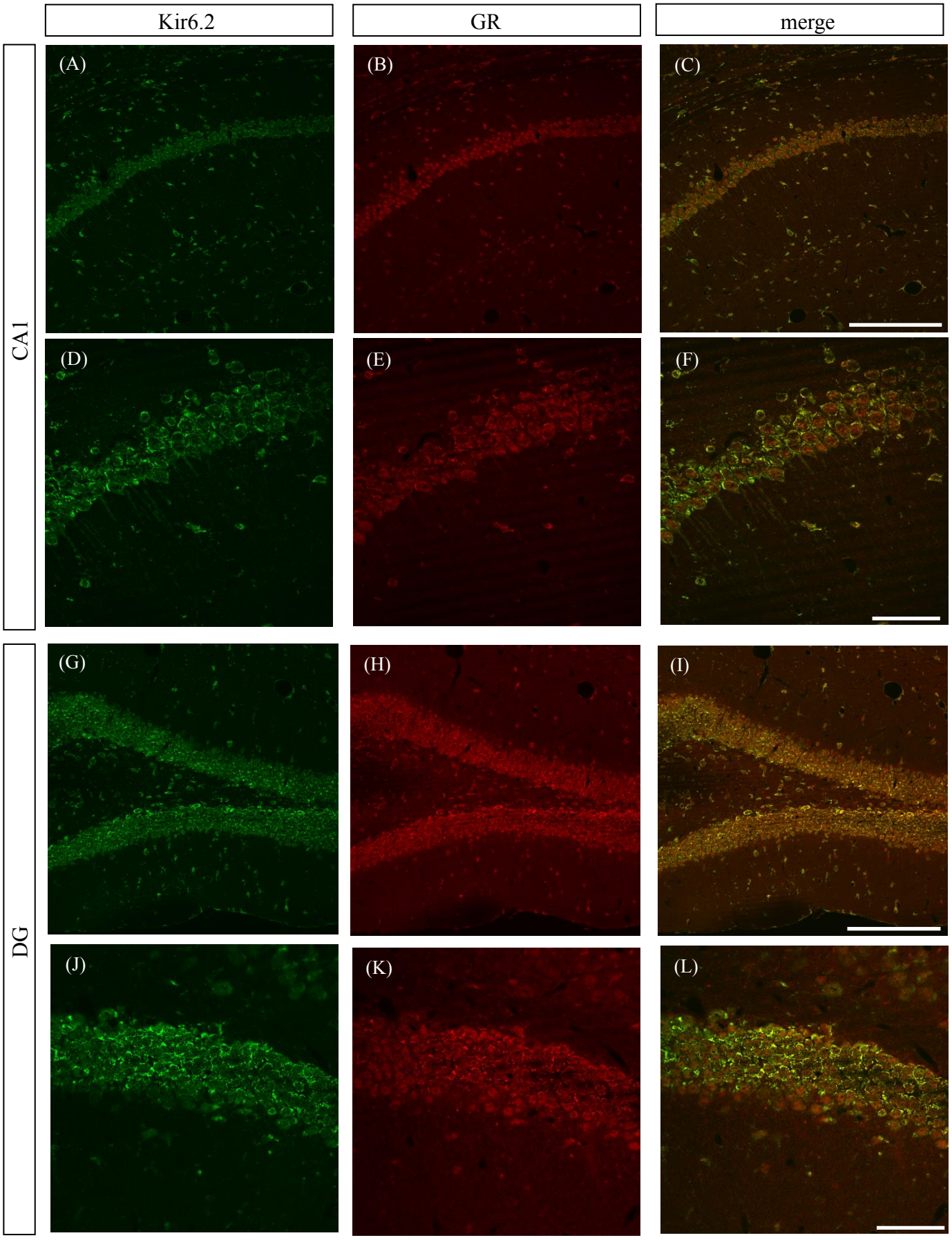


Fig. 2-2. Distribution and localization of Kir6.2 in the hippocampus of mice. Kir6.2 labeled in green (A, D, G, J) and GR labeled in red (B, E, H, K) were expressed in CA1 (C, F) and DG (I, L) of the hippocampus in female wild type mice. (A, B, C, G, H, I) Scale bar = 200 μm. (D, E, F, J, K, L) Scale bar = 50 μm.

Discussion

The HPA axis plays a critical role in several stress responses[49,50]. Kir6.2 is widely distributed throughout rat brain neurons and glial cells[27,28]. Kir6.2 is highly expressed in brain regions that control the HPA axis and corticosterone secretion[28,48]. Because brain Kir6.2 is likely to affect stress responses associated with the HPA axis, the present study investigated the involvement of Kir6.2 in stress responses.

Glucocorticoid hormones are the final step in the activation of the HPA axis and are known to function in the biological response to stress[49,52]. In addition, acute restraint stress increases the plasma corticosterone concentration in rodent[53]. To clarify the stress response in Kir6.2$^{-/-}$ mice, this study examined the change in the corticosterone concentration induced by exposure to acute restraint stress. In the non-stressed condition, basal corticosterone concentrations tended to be increased in male Kir6.2$^{-/-}$ mice and were significantly increased in female Kir6.2$^{-/-}$ mice compared to those in WT mice. Furthermore, exposure to acute restraint stress induced significant increases in corticosterone concentrations in Kir6.2$^{-/-}$ mice of both sexes compared to those in Kir6.2$^{-/-}$ non-stressed mice. Interestingly, these corticosterone concentrations were significantly higher than those in stressed WT mice in both sexes. These results suggest that Kir6.2 could be involved in the regulation of serum corticosterone levels. Kir6.2 is expressed on cells containing ACTH in the anterior lobe of the pituitary gland in rats[48]. On the other hand, Kir6.2 was not expressed on cells containing prolactin, follicular stimulating hormone, or growth hormone[48]. These findings suggest that Kir6.2 may play important roles in ACTH cells in the pituitary gland. Moreover, acute restraint stress elevated levels of ACTH and corticosterone, and activated cAMP-PKA-CREB signaling pathway which is involved in CRH gene expression in the PVN. These changes were normalized by iptakalim, a K_{ATP} channel opener[54]. In addition, the expression of Kir6.2 mRNA and protein has been

demonstrated in an adrenal chromaffin cell line (MAH cells)[55]. These reports suggest that Kir6.2 may regulate the secretion of corticosterones.

Another finding of the present study is the gender difference in serum corticosterone concentrations in Kir6.2$^{-/-}$ mice. Basal corticosterone concentrations in the non-stressed condition tended to be increased in male Kir6.2$^{-/-}$ mice and were significantly increased in female Kir6.2$^{-/-}$ mice compared to those in WT mice. In addition, although there was no difference in the percentage corticosterone increase under exposure to acute restraint stress, the concentrations were higher in female Kir6.2$^{-/-}$ mice. Therefore, it is considered that the change in the stress response due to Kir6.2 deficiency is more prominent in females. Since activation of the HPA axis is influenced by other endocrine systems such as sex hormones, the higher corticosterone levels in females has been thought to reflect activation of the HPA axis by ovarian estrogen[56,57]. Thus, the gender difference in the increase in serum corticosterone levels due to exposure to acute restraint stress in Kir6.2$^{-/-}$ mice might involve activation of the HPA axis by ovarian estrogen.

Moreover, as described in chapter 1, an increase in TPH level was observed in female Kir6.2$^{-/-}$ mice. The projection of 5-HT neuron from the midbrain raphe nuclei to the PVN is important for the activation of HPA axis[58,59]. Pharmacological stimulation of this pathway by acute administration of a selective serotonin reuptake inhibitor or 5-HT agonist increases plasma ACTH and corticosterone[60–65]. Gender differences in the central 5-HT system, such as decreased serotonin transporter binding in female rodents and humans or a greater stress-induced increase in amygdala 5-HT levels in females[61,66,67], may influence the HPA axis response and/or feedback mechanisms essential for homeostatic maintenance. Considering these reports, central 5-HT nervous systems might also be involved in the gender difference of the stress response caused by deficiency of Kir6.2.

On the other hand, it is necessary to examine whether Kir6.2 directly controls the HPA axis. The hippocampus regulates the endocrine stress system by modulating hypothalamic paraventricular nucleus

activity. Chronic dysregulation of the HPA axis in response to stress is associated with impaired glucocorticoid function and inhibition of negative feedback via the hippocampal GR[50]. Kir6.2 is highly expressed in regions associated with negative feedback such as the hippocampus, hypothalamus and pituitary[28,48]. Therefore, the next set of experiments focused on whether Kir6.2 is expressed in GR-positive cells in the CA1 and DG. The immunohistochemical analysis showed that Kir6.2 was expressed in GR-positive cells in the CA1 and DG of WT mice. Taken together, these results suggest that Kir6.2 could influence the HPA axis via GR regulation. One possible explanation for why excessive corticosterone secretion was observed in Kir6.2$^{-/-}$ mice after restraint stress is the dysregulation of negative feedback mechanisms via GR.

In summary, the present study demonstrated that K_{ATP} channels including Kir6.2 can regulate stress responses such as corticosterone secretion via the HPA axis. In addition, because higher corticosterone concentrations were observed in female Kir6.2$^{-/-}$ mice exposed to acute restraint stress, there is a gender difference in this regulation. A possible mechanism for this regulation may involve the influence of Kir6.2 on GR-positive cells in the hippocampus. Dysregulation of HPA axis is associated with mood disorders[50,68,69]. Although there are still many unanswered questions regarding the mechanisms of the relationship between Kir6.2 and the response to stress, the present study provides important evidence suggesting that K_{ATP} channels may be a novel target for the treatment of psychiatric disorders.

Conclusion

In chapter 1, the present study demonstrated that K_{ATP} channels including Kir6.2 regulate emotional behaviors such as anxiety-like behavior, and a possible mechanism for such regulation may involve the influence of Kir6.2 on monoamine neurons in the brain. In particular, the observation of excessive anxiety-like behavior in female Kir6.2$^{-/-}$ mice might be due to the activation of brain 5-HT neurons.

In chapter 2, the present study demonstrated that K_{ATP} channels including Kir6.2 can regulate stress responses such as corticosterone secretion via the HPA axis, and this regulation may involve the influence of Kir6.2 on GR-positive cells in the hippocampus. In addition, because higher corticosterone concentrations were observed in female Kir6.2$^{-/-}$ mice exposed to acute restraint stress, there is a gender difference in this regulation.

The present study provides evidence that Kir6.2 on monoamine neurons, in particular 5-HT neurons, may be involved, at least in part, in the regulation of emotional behaviors and stress responses. Moreover, excessive anxiety-like behavior and stress responses in female Kir6.2$^{-/-}$ mice may be caused by the changes in the HPA axis activity induced by the 5-HT neurons activation. Rodent and human studies have shown that females exhibit higher levels of glucocorticoids in response to various stressors[70-74]. Furthermore, the prevalence of affective disorders is 2 times greater in females than males[75], which may relate to gender differences in stress sensitivity and 5-HT neurocircuitry[66,76-78].

Although further studies are necessary in order to conclude a causal association between Kir6.2 and emotional regulation, the present findings provide new insights into the elucidation of why psychiatric disorders are greater in females.

List of Publications

This dissertation is based on the following original publications:

1, Atsumi Saito, Kazuya Miyagawa, Hiroko Miyagishi, Kazuhiro Kurokawa, Akira Umeda, Yasumasa Okada, Minoru Tsuji, Hiroshi Takeda: Possible involvement of monoamine neurons in the emotional abnormality in Kir6.2-deficient mice. Physiology and Behavior (*in press*): Chapter 1

2, 齋藤淳美，宮川和也，宮岸寛子，黒川和宏，梅田 啓，岡田泰昌，辻 稔，武田弘志：ATP 感受性カリウムチャネル Kir6.2 のストレス応答における役割．国際医療福祉大学学会誌 (印刷中): Chapter 2

Acknowledgements

This research will never be materialized without the help of the following people and organizations.

First, I would like to express my gratitude and appreciation to Professor Hiroshi Takeda (Department of Pharmacology, School of Pharmacy, International University of Health and Welfare) and Professor Minoru Tsuji (Department of Pharmacology, School of Pharmacy, International University of Health and Welfare) for their encouragement, helpful guidance in my research work and preparing this dissertation, and for giving a chance of this research work.

I would like to thank Dr. Kazuya Miyagawa (Department of Pharmacology, School of Pharmacy, International University of Health and Welfare) for a great technical assistance and helpful suggestions. Also I would like to thank Dr. Kazuhiro Kurokawa (Department of Pharmacology, School of Pharmacy, International University of Health and Welfare) and Dr. Hiroko Miyagishi (Department of Pharmacology, School of Pharmacy, International University of Health and Welfare) for helpful suggestion in my research work.

I wish to thank all of graduate and undergraduate students of Department of Pharmacology, School of Pharmacy, International University of Health and Welfare for helpful guidance in my research work.

I am grateful to Professor Akira Umeda (Department of Internal Medicine, International University of Health and Welfare Shioya Hospital) and Dr. Yasumasa Okada (Department of Internal Medicine, National Hospital Organization Murayama Medical Center) for providing the Kir6.2$^{-/-}$ mice.

I would like to express my deepest condolences to animals that were sacrificed for the present study.

Finally, I would like to express my gratitude to my parents, grandmother, sister and friends for their assistance in my life.

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