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Behavioral phenotyping of mice lacking the K_{ATP} channel subunit Kir6.2

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Abstract

ATP-sensitive potassium (K_{ATP}) channels are expressed in various tissues and cell-types where they act as so-called metabolic sensors that couple metabolic state to cellular excitability. The pore of most K_{ATP} channel types is built by Kir6.2 subunits. Analysis of a general Kir6.2 knockout (KO) mouse has identified a variety of different functional roles for central and peripheral K_{ATP} channels in situations of metabolic demand. However, the widespread distribution of these channels suggests that they might influence cellular physiology and animal behavior under metabolic control conditions. As a comprehensive behavioral description of Kir6.2 KO mice under physiological control conditions has not yet been carried out, we subjected Kir6.2 KO and corresponding wild-type (WT) mice to a test battery to assess emotional behavior, motor activity and coordination, species-typical behaviors and cognition. The results indicated that in these test situations Kir6.2 KO mice were less active, had impaired motor coordination, and appeared to differ from controls in their emotional reactivity. Differences between KO and WT mice were generally attenuated in test situations that resembled the home cage environment. Moreover, in their home cages KO mice were more active than WT mice. Thus, our results suggest that loss of Kir6.2-containing K_{ATP} channels does affect animal behavior under metabolic control conditions, especially in novel situations. These findings assign novel functional roles to K_{ATP} channels beyond those previously described. However, according to the widespread expression of K_{ATP} channels, these effects are complex, being dependent on details of test apparatus, procedure and prior experience.

Keywords: Kir6.2; K_{ATP} channels; Knockout mice; Motor activity; Species-typical behavior; Anxiety

1. Introduction

ATP-sensitive potassium (K_{ATP}) channels are widely distributed in many different tissues including pancreatic beta cells, kidney, skeletal, smooth and cardiac muscle, and the central nervous system [1]. They act as so-called metabolic sensors, coupling the metabolic state of a cell to its membrane potential and excitability: K_{ATP} channel activation hyperpolarizes cells and thus reduces their electrical activity, and in turn, activity-dependent release of hormones, mediators and neurotransmitters [2–4]. K_{ATP} channels are composed of four inwardly rectifying potassium channel subunits (either Kir6.1 or Kir6.2) and four high-affinity sulphonylurea receptor subunits (SUR1, SUR2A or SUR2B) [5]. Kir6.2 is assumed to form the pore of plasmalemma K_{ATP} channels in most tissues — excluding vascular smooth muscle or glia cells, where Kir6.1 seems to be the pore-forming subunit [3]. The central physiological role of these channels is best understood for the pancreatic beta cells, where the closure of K_{ATP} channels in response to high blood glucose levels triggers insulin secretion [6]. The generation and extensive analysis of K_{ATP} channel knockout (KO) mice has significantly advanced our understanding of channel function in other tissues and cell-types [7,8]. In particular, by analysing a general K_{ATP} Kir6.2 KO mouse, it was shown that K_{ATP} channel activation in heart, skeletal muscle and brain is beneficial in acute metabolic demand situations [9]: peripheral Kir6.2 channels are required for cardiac ischemic preconditioning [10], for the metabolic benefit of exercise training [11], for resting tension and force-

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depression of skeletal muscle during fatigue [12,13], as well as for adaptation to stress [14]. In the brain, Kir6.2 KO studies demonstrated that hypothalamic Kir6.2 channels are required for hypoglycaemia-induced glucagon secretion [15], and that K_{ATP} channel activation in the substantia nigra pars reticulata prevents seizure propagation during hypoxia [16]. In addition, activation of K_{ATP} channels in response to acute metabolic stress is neuroprotective for hippocampal neurons [17]. In contrast, under chronic metabolic stress K_{ATP} channel activation is not beneficial, but triggers selective degeneration of dopaminergic neurons [18]. In contrast to these important functional roles of peripheral and central Kir6.2-containing K_{ATP} channels in metabolic demand situations that have been clarified by analysing a general Kir6.2 KO mouse, it is less clear whether K_{ATP} channels are activated under physiological control conditions, and whether they contribute to general physiological functions and animal behaviors. To address this question, we analysed the behavioral phenotype of a general Kir6.2 KO mouse, focussing on behaviors reflecting emotionality, motor ability, species-typical behavior and cognition.

2. Materials and methods

To facilitate presentation, tests are grouped according to their primary purpose (emotionality, motor, species-typical and cognitive) although, by the multi-deterministic nature of behavior, these categories cannot always be mutually exclusive. The open field, for example, is sensitive to both basal activity and emotionality. For readers unfamiliar with these tests, a brief explanation is given at the head of each section. Testing continued for six months altogether; two tests (burrowing, static rod) being repeated at the end of the six-month long test series to confirm the persistence of the phenotype. Chronologically, the order of testing was: open field, successive alleys, static rods 1, inverted screen, horizontal bar, burrowing 1, nest construction, rotarod, weight lifting, hyponeophagia 1, hyponeophagia 2, burrow 2, spontaneous activity, burrow 3, spontaneous alternation, static rods 2. At least 24 h separated each test. Three additional tests, basal home cage activity, the plus-maze and light–dark box, were carried out on separate cohorts of mice.

2.1. Subjects

Kir6.2 knockout (KO) and Kir6.2 wild-type (WT) mice with a mixed genetic background of C57BL/6 and 129Sv [19] were back-crossed for four generations with C57BL/6 strain mice before arriving in Oxford to form a breeding stock in 1999. Wild-type mice (Kir6.2+/+) of the same genetic background were maintained in a separate colony. DNA for genotyping was extracted from ear-punches utilizing the DNA Mini kit (Qiagen). Genotyping was performed via PCR and gel-electrophoresis (2% TAE-agarosegels) using the following set of primers: Forward: TAG GCC AAG CCA GTG TAG TG, Reverse-KO: GGA GGA GTA GAA GTG GCG C, Reverse-WT: GCC CTG CTC TCG AAT GTT CT. For homozygote Kir6.2 KO mice, a PCR band of 386 bp was amplified, for Kir6.2 WT, PCR product was 222 bp, for heterozygote mice both PCR amplificates were derived from genomic DNA. PCR was carried out in 25 μl reactions in a GenAmp9600 thermocycler utilizing about 20 ng genomic DNA as template, 25 pmol each primer, 160 μM each dNTP, and Invitrogene TaqPolymerase and buffers. PCR conditions: 94 °C for 3 min, followed by 30 cycles: 94 °C for 30 s, 60 °C for 60 s, 72 °C for 60 s, final extension 72 °C for 7 min.

Mice used in the present experiments were about three months old when testing began. Forty-two mice in total were tested; 10 males and 11 females of both WT and Kir6.2 KO. They were housed in same-sex littermate groups of 2–6 in transparent plastic cages on aspen wood chip bedding, in a temperature and humidity controlled room on a 12 h light–dark cycle (lights on at 0600). Food and water were always available (except before tests such as hyponeophagia, see below). Each cage had a cardboard tunnel and shelter. Nesting material (a "Nestlet", a square of pressed cotton) and a small amount of forage mix (sunflower seeds, maize, dried banana, wheat, hemp, linseed) were given once a week after cage cleaning.

An additional cohort of mice (10 male WT, 11 male KO, 8 females each WT, KO) was tested on the plus-maze and the light–dark box in Marburg, Germany, because an attempted plus-maze experiment in Oxford had suffered a floor effect (virtually no mice of either genotype had ventured on to the open arms), and a light–dark box was not available in Oxford at that time. These mice (and those for home cage activity test) were from the same Oxford breeding colony, similar age, and kept under similar conditions as in Oxford. Experiments in Oxford were conducted in accordance with the UK Animals (Scientific Procedures Act), 1986, and experiments in Marburg in accordance with the regulations of the Regierungspräsidium Giessen, Germany.

2.2. Emotionality tests

All these ethologically based tests are analogues of the natural environment, where small rodents avoid areas where they would be more likely to encounter predators. The plus-maze measures the tendency of mice to remain in the closed, protected, maze arms. The successive alleys apparatus is similar, but eliminates the ambiguous central area of the plus-maze. The light–dark box analyses the conflict between a mouse's tendency to explore a novel environment versus the aversive properties of a brightly lit area. Hyponeophagia measures the reluctance of mice, depending on their anxiety level, to eat a novel food in a novel situation. A battery of tests was used, as a study on different mouse strains indicated that different test paradigms tax different aspects of anxiety, suggesting that a battery of different tests should be used in studies of anxiety-related behavior [20].

2.2.1. Elevated plus-maze

This was made of black painted wood, based on the design of Lister [21]. Two opposing arms of the + shaped maze were enclosed by 30 cm high clear acrylic walls, the other two arms were open with 0.5 cm rims. All arms were 5 cm wide. The apparatus was elevated 50 cm from the floor by a stand. A

mouse was placed on to the middle of the central square, facing the open arms. Over 5 min, the number of entries into, and the time spent, on open and closed arms were measured by a manually operated electronic timer. Time on the central square was measured indirectly by subtraction. Any faecal boli was counted.

For this and other tests that exposed successive mice to the same test apparatus, faecal boli and urine were removed after each mouse, and the apparatus was cleaned with a moist, followed by a dry tissue. Males and females were tested at separate times. Before testing began, non-experimental mice were placed on the apparatus, which was then cleaned. The aim was for the apparatus to have a weak odour of mice, to facilitate exploration, with no local odour concentrations that might provide distraction.

2.2.2. Successive alleys

This is an extended linear version of the plus-maze. It has four successively joined alleys that are designed to be increasingly anxiety-provoking: the walls become lower, the paintwork changes to grey, then white, and the alleys become narrower and more exposed (for details see Ref. [22]). The mouse was placed into the first, "safe" black alley with high walls and allowed 5 min to choose whether to explore the three further alleys. The number of entries in to, and time spent on, each alley was manually recorded by an electronic timer.

2.2.3. Light–dark box

The wooden apparatus [23] consisted of an open white-painted compartment 30 × 20 × 20 cm, with a transparent acrylic panel on one side to facilitate observation of the mouse. This was separated by a partition (with a 3 × 3 cm door) from a dark box (painted black with a lid) 15 × 20 × 20 cm. The aversiveness of the white compartment was increased by additional was placed in the middle of the dark side facing away from the door. Placing in the illumination, a 60 W anglepoise lamp placed 45 cm above the centre of the floor. The mouse dark adds an emergence component to the test, and reliably detects anxiolytic and anxiogenic effects [24,25]. The latency to cross (all four feet) to the light side, time spent on the dark side (all four feet), and the number of entries into the light side were measured for the 5-min test duration. The number of faecal boli and the presence/absence of urination were also recorded.

2.2.4. Hyponeophagia 1

Mice were restricted to 1 g food each overnight. The cage bedding was changed to reduce coprophagia and remove any uneaten forage mix. Next morning, mice were individually placed on a stainless steel sheet with a food well (12 mm diameter, 10 mm high) filled with 1 : 1 water : full cream sweetened condensed milk. The mouse was contained by a translucent 2 l plastic jug 17 cm diameter, 15 cm high, placed upside down on the sheet with the spout of the jug over the food well, thus directing the mouse's nose to the milk. The latency to start properly drinking (>2 s continuously) was measured by a stop clock. If the mouse failed to drink in 120 s it was put in a holding cage and tried again after the next mouse. Up to three 120 s tests were given per mouse. Mice that had drunk milk were put in a further separate holding cage to avoid social transmission of a food preference to untested mice.

2.2.5. Hyponeophagia 2

This was similar to hyponeophagia test 1, but the food was scattered all over the floor so that even inactive mice would instantly be aware of it; it was suspected that KO mice might have been slower to drink in hyponeophagia 1 because they were less active. Male mice (females were not used as so few drank before the cut-off time in the first test) were totally food deprived overnight (as the latencies had been long in the previous test) and placed individually in a transparent plastic box (26 × 16 × 17 cm). The floor was scattered with 25 g of chopped nuts (peanuts, almonds, walnuts) different to those in the forage mix they received as environmental enrichment. The latency until the mouse ate was noted (>3 s of continuous eating, holding a nut in the forepaws).

2.3. Motor tests

2.3.1. Basal home cage activity

Basal home cage activity of mice was monitored over 24 h by using an infrared motion detector ("mouse-E-motion") (http://www.infra-e-motion.de) attached to each home cage, with a sampling frequency of 1 Hz and a bin size of 4 min. Ten mice were monitored in parallel. Each animal was tested twice within 14 days and mean data were used for analysis.

2.3.2. Open field

The open field measures both, basal activity (squares crossed) and directed exploration (rears) both of which can be influenced by anxiety, which is more directly measured by emotional elimination scores (defaecation). The open field was a grey PVC arena, 50 × 30 × 18 cm, divided into 10 × 10 cm^2 squares. Each mouse was placed in a corner square, facing the walls, and observed for 3 min. Measures taken were the total number of squares crossed, the latency to the first rear, the total number of rears, the number of faecal boli and the presence of any urine.

2.3.3. Spontaneous exploration

An environment that resembled the home cage as much as possible was devised, reducing even further the minimal anxiety present in the open field. It was adapted from Ref. [26]. The spontaneous exploration test consisted of a narrow alley, which was designed to be less anxiogenic than the wider open field, and was moreover lined with bedding from the home cage to make it even less likely to provoke anxiety. The floor of a grey wooden alley 69 × 10 × 30 cm (formed from the goal arms of the T-maze used for spontaneous alternation, see below) was marked off into three 23 cm sections and covered to a depth of 1–2 cm with soiled home cage bedding from the cage of the mouse to be tested. Each mouse was placed at one end of the alley facing the wall and the number of sections crossed in 5 min was counted, also the latency to first reach a distance of one body length from the end opposite the start.

2.3.4. Static rods 1

The static rods measure motor coordination ability on a static substrate, a series of thin rods. A clamp mounted on a bench 40 cm above the floor was used to support a wooden balancing rod, 60 cm long, in a cantilevered manner, the rod projecting out from the bench. Three diameters of rods were used: 35 mm, 22 mm, and 9 mm, in a sequence starting with the widest. Soft padding was provided to cushion any falls. Each mouse was placed 2 cm from the protruding end of a rod, facing away from the bench, and the time to orientate (turn around 180°, while remaining upright, to face away from the open end) on each rod was measured, also the transit time to run the rod and reach the shelf. In this situation, mice naturally and reliably turn around and move along the rod to the supporting shelf. If the mouse failed to remain upright on the rod before orienting or transiting, or fell, it was assigned a cut-off score of 180 s. Mice failing to orientate while upright, or transit the rod before falling, were also assigned values of 180 s (the maximum test duration) on that and subsequent rods, as it was assumed they would almost certainly fall from a smaller rod.

2.3.5. Static rods 2

This was run much later (six months) than the first test, to check the persistence of the coordination deficit, especially as the rotarod results suggested that the coordination deficit seen on the first static rods test had been lost. It was run as static rods 1, but only the intermediate (22 mm diameter) rod was used.

2.3.6. Inverted screen

The inverted screen mainly measures strength, as little coordination is required to support the body by gripping the screen with all four feet. The inverted screen was a $43 \times 43\ cm^2$ wire grid, framed by a 4 cm deep wooden beading. The grid was formed of 12 mm squares of 1 mm diameter wire. The mouse was placed in the centre of the screen, which was then smoothly inverted and held 30 cm above a soft surface. The time at which the mouse fell was recorded, to a maximum of 1 min.

2.3.7. Rotarod

The rotarod measures coordination on a rod rotating about its long axis. An accelerating rotarod (Ugo Basile, Italy, model 7650) was modified to have a start speed of 2.5 r.p.m. and an acceleration rate of 20 r.p.m./min. A mouse was placed on the moving rod, which was accelerated after 10 s, and the time to a fall noted. The time from the start of acceleration until when the mouse fell was noted, up to 180 s maximum time. If a mouse fell off before the acceleration phase the time was noted and it was re-tested after a few seconds rest, for three trials maximum. The longest time the mouse stayed on was the test score.

2.3.8. Weight lifting

The weight lifting test allows better quantification of strength, as the mouse is allowed to grasp a series of increasingly heavy weights. Each weight consisted of a number of links (from one to five) cut from a light metal chain, with one end of the chain wired to a ball of thin wire. Mice held by the tail just above this ball spontaneously lifted it with their front paws. Lifting the weight off the bench for 3 s was the criterion; if this was achieved the mouse was tested on the next heaviest weight after the other mice in the cage had been tested. Testing continued until two failures to hold a weight for 3 s, the longest hold being recorded. A final total score was calculated as the product of the number of links in the heaviest chain held for the full 3 s, multiplied by the time (s) it was held. If the heaviest weight was dropped before 3 s an appropriate intermediate value was calculated. Thus a mouse holding a 5-link weight for 3 s, but unable to lift a 6-link weight, was assigned a score of $(5 \times 3) = 15$. If it held the 6-link weight for 1 s, it scored $(5 \times 3) + (1) = 16$. The five weights weighed (g) respectively: 18, 32, 47, 60 and 74.

2.3.9. Horizontal bar

The horizontal bar measures strength, particularly of the forelimbs, but performance can also be influenced by the motor coordination of the mouse. The bar was a brass rod 2 mm thick, 38 cm long, held 49 cm above the padded bench surface by supporting columns at each end. Each mouse, held by the tail, was placed at the centre of the rod and allowed to grasp the bar with its forepaws. It was left holding the bar for a maximum of 30 s or removed if it reached an end column. Either of these resulted in the maximum score of 5; falls at earlier times received a graded score from 1 (5 s or less on the bar) to 4 (21 s or less).

2.4. Species-typical behavior and cognition

2.4.1. Nest construction

Nest construction and burrowing (digging material out of a tube) are both profoundly inhibited by lesions of the hippocampus [27]. These tests, being conducted in the home cage, involve minimal stress and anxiety, so would be expected to be relatively insensitive to a genotype displaying increased emotionality. Mice were individually housed overnight, with a square of pressed cotton ("Nestlet", Datesand Ltd., Manchester, UK), which they could tear up to form a nest. Nests were rated the next morning according to the following schema, and any untorn Nestlet was also weighed:

1. Nestlet not noticeably touched (>90% intact).
2. Nestlet partially torn up (50–90% remaining intact).
3. Mostly shredded but no identifiable nest site: <50% of the Nestlet remains intact but <90% is within a quarter of the cage floor area, i.e. material is not gathered into a nest but spread around the cage.
4. An identifiable, but flat nest: >90% of the Nestlet is torn up, the material is gathered into a nest within a quarter of the cage floor area, but the nest is flat, with walls <mouse body height (curled up on its side) on >50% of its circumference.
5. A (near) perfect nest: >90% of the Nestlet is torn up, the nest is a crater, with walls >mouse body height on >50% of circumference.

2.4.2. Burrow 1, 2 and 3

Mice spontaneously empty a tube filled with virtually any material placed into their cage, whether these are food pellets or even soiled cage bedding [28]. Food pellets were used in

Table 1
KO mice compared with WT on the plus-maze (males and females)

Measure	Wild-type	Knockout	P
Time closed arms (s)	105.5±8.8	66.7±9.3	0.0047
Time open arms (s)	96.3±14.7	158.3±15.6	0.0068
Time central square (s)	98.2±8.41	73.0±8.92	0.048
Entries closed arms	5.0 [5.0–8.0]	5.0 [4.8–7.3]	0.87
Entries open arms	7.63±0.7	5.33±0.7	0.023
Latency open arm (s)	4.0 [1.3–17.0]	1.0 [1.0–10.5]	0.09
Defaecation (no. of boli)	0.0 [0.0–0.0]	0.0 [0.0–2.0]	0.58

Values are means±SEM, or medians and interquartile ranges [IQR] for non-parametric data.

burrowing test 1. Plastic tubes 20 cm long, 6.8 cm diameter, closed at one end and elevated 3 cm at the other end by two 50 mm machine screws, were each filled with 200 g of food pellets. At least 2 h before the end of the light phase, each mouse was placed into an individual cage with a full tube. Measurements of the amount of food pellets burrowed, i.e. displaced from the tube, were taken 2 h later just before the dark phase, and subsequently overnight.

Like the second static rods test, burrow 2 test was run towards the end of the test series to confirm that the phenotypical difference seen in burrow 1 was still present. The tubes were filled with food pellets, and the test was run exactly as the first burrowing test. For burrow 3 tests, the burrows were now filled with 90 g of fresh aspen wood chips, as used for normal cage bedding, to minimise the novelty of the substrate. The test was only run for 2 h, as by then the controls had emptied almost all the chips.

2.4.3. Spontaneous alternation

Spontaneous alternation measures working memory and cognition as mice have an innate tendency to explore their environment in a systematic way. Successful exploration depends on the ability to remember, and avoid, places recently visited. Both alternation and species-typical behaviors are sensitive to hippocampal dysfunction. Test was run in a grey painted wooden T-maze. Each arm was 30×10×29 cm. The central choice area was divided by a partition, which extended 7 cm into the start arm, allowing only one goal arm to be sampled on the first phase of a trial. A mouse was placed into the start arm; after it had entered one of the goal arms a guillotine door was shut, confining it there for 30 s. The central partition was then removed and the mouse replaced in the start arm with the goal arm doors raised. The criterion of alternation was for all of the mouse (including tail) to enter the opposite arm to that sampled. To facilitate running, the floor of the maze was covered with mixed litter from the two or three home cages of the particular sex/genotype that was being tested. The intertrial interval varied greatly depending on the overall performance of the squad, but approximated 15 min. A maximum of 90 s sample or choice time was allowed. The proportion of alternations was calculated separately from failed (>90 s without entering a goal arm) trials.

2.5. Data analysis and presentation

When the data distribution permitted (i.e. it fulfilled the criteria of normality and equality of variance) two-way ANOVAs were performed using gender and genotype as factors. Otherwise, if there were no sex differences by pairwise comparisons, the male and female data were pooled and pairwise genotype comparisons made. The *t*-test was used for comparisons of parametric data, the Mann–Whitney test for non-parametric tests (both 2-tailed). (In the Results, mean±SEM values are shown for the former, median [Interquartile Range] for the latter). Fisher exact or Chi-square tests were used for nominal data. P values <0.05 were considered as statistically significant.

3. Results

There was no difference between the body weight of KO and WT mice (at 10–11 months age). A two-way ANOVA showed an effect of sex: $F(1,38)=19.376$, $P<.0001$, but not of genotype nor an interaction (both $P>0.5$). Mean weight of males was 34.1 g and females 28.4 g.

3.1. Emotionality tests

3.1.1. Plus-maze

Many of the KO mice fell ($n=3$) or jumped off ($n=5$) the maze. Two mice that jumped off a second time were excluded from the experiment. All WT mice stayed on the maze.

KO mice spent less time than WT in the closed arms of the plus-maze, and more time on the open arms (Table 1). Two-way ANOVA showed a significant effect of genotype: $F(1,32)=9.24$, $P=0.0047$. KO mice also spent less time in the central square: $F(1,32)=4.25$, $P=0.048$. Correspondingly, they spent more time in the open arms: $F(1,32)=8.37$, $P=0.0068$. There were no gender or genotype differences in closed arm entries, but KO mice made slightly fewer open arm entries: $F(1,32)=5.67$, $P=0.023$. KO mice tended to have a lower latency to enter an open arm, but this was not significant. Neither gender nor the genotype × gender interaction was significant for any measure.

3.1.2. Successive alleys

All mice spent most time in the fully enclosed black alley 1 (see Table 2). Data for alleys 3 and 4 are not presented as so few mice ventured that far. The WT mice spent significantly more time than KO in alley 2, and this is also reflected by an increased number of entries into alleys 1 and 2.

3.1.3. Light–dark box

KO mice were slower to move to the light side, made fewer entries into the light and produced more faecal boli (see Table 3). The time KO and WT spent on the dark side was not significantly different.

Table 2
Time and entries into each alley of the successive alleys (male and female mice)

Alley	Wild-type	Knockout	P
1: Time (s)	165 [155–176]	180 [170–180]	0.017
1: Entries	2 [1–3]	0 [0–2]	0.0017
2: Time (s)	15 [4–25]	0 [0–10]	0.0048
2: Entries	2 [1–3]	0 [0–2]	0.0027

Values are medians and interquartile ranges [IQR].

Table 3
Behavior of KO and WT mice (both sexes) in the light–dark box

Measure	Wild-type	Knockout	*P*
Latency to light side (s)	11.0 [5.0–18.8]	31.0 [12.5–79.0]	0.0029
Duration in dark (s)	226.8±14.5	206.6±14.5	0.3
Entries into light	5.00 [4.0–6.0]	2.0 [1.0–4.8]	0.0079
Defaecation (no. of boli)	0.0 [0.0–0.8]	1.0 [0.0–2.8]	0.05

Values are means±SEM or medians and interquartile ranges [IQR] for non-parametric data.

3.1.4. Hyponeophagia

Hyponeophagia 1: The data were non-parametric and thus could not be analysed by two-way ANOVA, so pairwise comparisons were performed with the Mann–Whitney *U* test. There was a large sex difference; most females showed a ceiling effect, not drinking within the maximum test time of 360 s. Pooling KO and WT, the median and interquartile latency values (s) were: females, 360 [56–360]; males, 68 [12–313] ($P=0.0155$). There was no significant difference between the KO and WT females due to the ceiling effect. However, male KO mice were significantly slower than male WT to drink (see Fig. 1).

Hyponeophagia 2: Only male mice were tested due to the ceiling effect observed for females in hyponeophagia 1 test. KO mice were now very much quicker than WT to begin eating (see Fig. 1).

3.2. Motor tests

3.2.1. Basal home cage activity

A two-way analysis of variance on the 24 h motor activity counts showed an effect of genotype: $F(1,41)=50.29$, $P<0.0001$, and of gender: $F(1,41)=12.97$, $P=0.0008$, but no genotype–gender interaction. Wild-type males gave significantly lower mean counts (8361±637 SEM) than wild-type females (11,213±1244 SEM). KO mice were significantly more active than wild-type (about 1.7-fold), giving mean scores of 14,498±486 (males) and 18,424±1169 (females). Thus, the loss of Kir6.2 channels increased activity proportionately to gender.

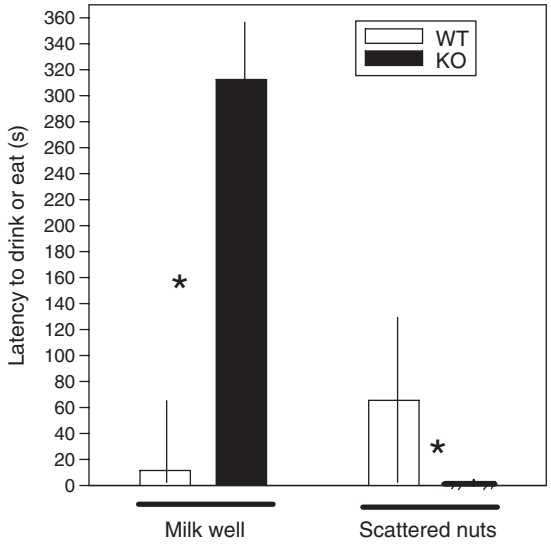

Fig. 1. (Male) KO mice were slower to drink milk from a food well, but quicker to eat nuts from the cage floor. Values are medians and interquartile ranges (s). *$P<0.05$, WT versus KO.

3.2.2. Open field

The KO mice were significantly less active than WT, crossing fewer squares, making fewer rears and being slower to make their first rear (see Fig. 2A). WT emotional elimination (defaecation and urination) scores were low, those of the KO mice being slightly (non-significantly) higher. Two-way ANOVA showed significant effects of genotype on squares crossed: $F(1,38)=8.69$, $P=0.0054$, and on rears: $F(1,38)=13.91$, $P=0.0006$. There were no significant effects of sex or sex×genotype on either measure. A pairwise comparison showed that KO mice were slower to make their first rear than WT mice.

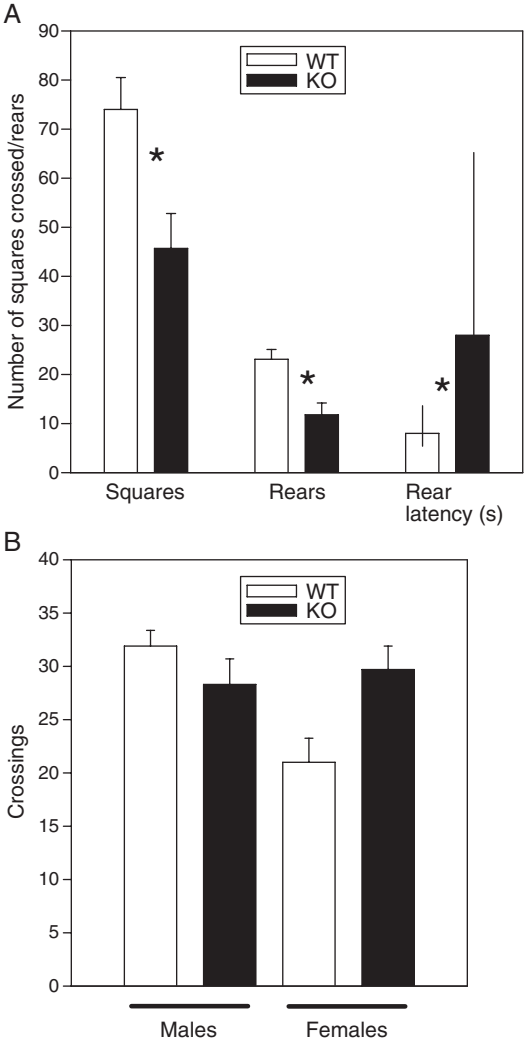

Fig. 2. A: Kir6.2 KO mice (both sexes) were much less active than WT controls on all measures of activity in a novel open field. Values are mean±SEM for squares crossed and rears; rear latencies are medians and interquartile ranges. *$P<0.05$, WT versus KO. B: Activity (crossings) when mice were tested in a novel alley but on familiar home cage bedding. KO females made more crossings of designated areas than their same-sex controls. Values are mean±SEM. There was also a group×sex interaction: female KO mice were more active.

Table 4
Median [IQR] values for orientation and transit times for rods 1–3

Measure	Wild-type	Knockout	P
Rod 1 orientation (s)	7 [5–16]	24 [8–180]	0.007
Rod 1 transit (s)	30 [16–78]	180 [41–180]	0.007
Rod 1 fall	0/21	3/21	
Rod 2 orientation (s)	12 [9–17]	180 [23–180]	0.005
Rod 2 transit (s)	16 [12–21]	180 [37–180]	0.001
Rod 2 fall	3/21	11/21	0.020
Rod 3 orientation (s)	180 [12–180]	180 [180–180]	0.019
Rod 3 transit (s)	180 [21–180]	180 [180–180]	0.008
Rod 3 fall	10/21	19/21	0.006

Mice (both sexes) were successively tested from rod 1 (the largest) to rod 3 (the smallest). The proportion of mice falling from each rod is also shown.

3.2.3. Spontaneous exploration

There was no significant difference between the WT and KO mice in the latency to initially run the length of the alley; values were (s) 8 [7–11] and 9 [7–12], respectively. In terms of total line crossings, however, females were less active, especially WT (see Fig. 2B). In contrast to other tests, within females at least, KO were more active than WT. A two-way ANOVA revealed no significant effect of group, but there was an effect of sex, $F(1,38)=4.95$, $P=0.0321$, and a group × sex interaction, $F(1,38)=8.39$, $P=0.0062$.

3.2.4. Static rods 1

KO mice were markedly impaired on this task (see Table 4); they were slower to orientate away from the open end of each rod and to run (transit) along them, and more fell off.

3.2.5. Static rod 2

The KO mice were once again, six months later, less competent than the WT animals. Although not significantly impaired on initial orientation on rod 2, their transit time was significantly higher; median values (s) were 33 [18–59] for WT and 180 [123–180] for KO ($P<0.0001$). Also more KO mice fell off the rod than did WT (13/21 and 1/21, respectively, $P<0.0001$).

3.2.6. Inverted screen

There were no group differences on this relatively undemanding task; all mice held on for 60 s.

3.2.7. Rotarod

There was no impairment in the KO mice. The group medians for time (s) on the rod ranged from 72 to 97 s, well within the cut-off of 180 s. A two-way ANOVA revealed no effect of genotype, sex or interaction (all P values >0.1).

Table 5
Median [IQR] values for the weight (g) burrowed in test 1 (male and female mice)

Burrow duration	Wild-type	Knockout	P
2 h	94 [59–137]	6 [2–34]	<0.0001
Overnight	200 [194–200]	124 [34–164]	<0.0001

3.2.8. Weight lifting

Unusually, the females scored higher than males. ANOVA revealed a significant effect of sex, $F(1,38)=21.18$, $P<.0001$, but no significant effect of genotype nor an interaction. Mean ± SEM scores were: females 10.3 ± 0.4 and males 7.9 ± 0.4.

3.2.9. Horizontal bar

The KO mice were impaired. The difference between the performance scores of the groups just failed to attain statistical significance (median ± IQR: WT 5 [5]; KO 5 [3–5]. However, the proportion of mice that fell from the bar was significantly greater in the KO group (8/21) than in the WT (1/21) (Fisher exact test $P=0.021$).

3.3. Species-typical behavior and cognition

3.3.1. Nest construction

There were no significant differences between groups. Both made good nests (median score WT 4.5, KO 4.0) and left very little of the Nestlets unshredded (median weights (g) = 0 for both WT and KO).

3.3.2. Burrow 1

Most WT mice emptied approximately half the tube contents in 2 h and the rest overnight (see Table 5 and Fig. 3). KO mice burrowed very little in 2 h but emptied over half of the tube overnight.

3.3.3. Burrow 2 and 3

The second burrowing test, again using food pellets (200 g), showed that the deficit in the KO mice seen in the first burrowing test at the start of behavioral testing was still present, if indeed not greater (Fig. 3). However, when wood chips (90 g) were presented in the burrows in test 3 this difference was much smaller, although still significantly different.

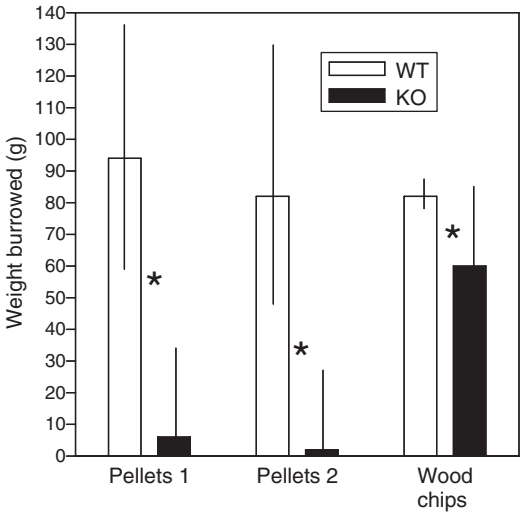

Fig. 3. Kir6.2 KO mice (of both sexes) burrowed many fewer food pellets from a tube than WT controls, but when wood chips were substituted for pellets, this difference was considerably reduced. Values are medians and interquartile ranges (s). *$P<0.05$, WT versus KO.

3.3.4. Spontaneous alternation

WT mice alternated at a high rate (90.9±2.2%) and KO mice at a lower rate (77.1±3.7%) (means±SEM), a statistically significant difference ($P=0.0027$, *t*-test). However, KO mice were also slower to run. The median [IQR] time to run on the sample phase of the trials (choice times are not reported because of the problem of missing data due to failure to run on some samples) was 22.4 [16.4–32.9] s (KO) versus 12.9 [10.0–14.4] s (control) ($P<0.0001$, *U* test). There were also more failures to complete trials (i.e. sample or choice>90 s). WT mice failed to complete 6/110 trials and KO 44/110 (Chi-square=35.4, $df=1$, $P<0.0001$).

4. Discussion

We have analysed the behavioral phenotype of a general Kir6.2 KO mouse in comparison to WT mice of the same genetic background. KO mice were significantly more active than WT in their home cages, but less active in the open field, impaired on the static rods and horizontal bar, as well as burrowing tests 1 and 2. The results of the open field test, light–dark box, plus-maze, successive alleys, hyponeophagia, rotarod static rods and horizontal bar suggested that Kir6.2 KO mice can differ from WT in both emotionality and motor coordination, but these changes are situationally dependent, i.e. their behavior does not suggest less anxiety or motor coordination in all situations. This is to be expected, given that test situations often differ in the type of anxiety elicited, and treatments can simultaneously increase or decrease different measures of anxiety [29]. The rank order of anxiety in different mouse strains has been found to vary as a function of test type in a test battery [20,26].

One possible explanation might be that KO mice differ most from WT when the situation is unfamiliar. Thus the WT–KO differences were reduced when familiar wood chips were used in the third burrowing test (food pellets would be familiar, but only in the food hopper, not underfoot). The reduced activity seen in the open field was alleviated in the spontaneous exploration alley test (Fig. 2B), when the mice were run on bedding from their own home cage, indeed female KO mice were even more active than WT.

Although males and females gave similar results in many tests (plus-maze, successive alleys, light–dark box, open field, static rods, and rotarod) there were gender differences on the hyponeophagia 1 test. Paradoxically, in two minimally anxiogenic activity tests, namely spontaneous exploration in an alley and home cage activity, females were less active in the former and more active in the latter. At present we have no specific explanation for these differences.

Some aspects of the behavior of the KO mice seemed superficially similar to those of 129 strain mice. For example they were generally slower to act and sometimes seemed more emotional. The KO mice as well as the WT mice were backcrossed to C57BL/6 mice for four generations, thus they should possess the same genetic background. Yet it was possible that 129-related genes might still be influencing behavior. A closer comparison between the present mice and 129 mice (129S2/SvHsd), however, shows a number of differences in behavioral phenotype [22]. Specifically, 129 mice performed worse on the rotarod than C57BL/6 in the latter study, whereas the Kir6.2 KO in the present work did not differ from WT. Transit times on the multiple static rods were almost always less than 60 s in 129 mice, whereas the median KO value was the cut-off, 180 s. Two tests in the present experiment measured "relatively pure" locomotor activity; closed arm entries in the plus-maze [30] and spontaneous exploration in the alley. Both tests are indeed very similar in apparatus and procedure. KO and WT mice did not differ on either measure; in contrast, Contet et al. reported that C57BL/6 mice were almost three times more active than 129 mice in the closed arms of the plus-maze. It therefore seems difficult to attribute all the WT–KO differences to 129-related gene effects [22].

4.1. Kir6.2 KO mice often – but not always– show altered motor behavior

Assessment of motor behavior is important in interpreting any behavioral task [31]. Motor coordination and muscle strength were tested in several different ways to fully evaluate apparent deficits in KO mice. Motor tests that primarily measured muscle strength (the weight lifting and inverted screen) showed no deficits for KO mice. This is in accordance with previous studies of this KO mouse, showing that the force–frequency curve, the twitch, and the tetanic force of skeletal muscle, as well as basal metabolic aerobic capacity, were not significantly different between Kir6.2 KO and WT mice [11,12]. However, during fatigue, K_{ATP} channels (made up by Kir6.2 and SUR2A) are normally activated, and in consequence muscle-force is depressed due to reduction of action potential amplitude — in particular in older mice (12 month) [13]. In addition, under the treadmill exercise-stress test, with stepwise escalating velocity and incline, Kir6.2 KO tolerated lower workload and endured shorter times of exercise stress, due to impaired cardiac responses and depleted functional reserve of heart muscle [14].

Since the rotarod is similar to a treadmill (but we did not vary workload), one might expect that KO mice would also perform less well on the rotarod. But in contrast, they performed as well as WT mice (similar as for weight lifting), arguing against fatigue as a cause for the observed motor deficits in other tests. Moreover, basal home cage activity of KO mice was not reduced but significantly higher than those of WT. Possibly, the moving substrate of the rotarod provided sufficient stimulation to overcome a deficit in coordinated activity. Another explanation might be that the rotarod-situation was not entirely unfamiliar to the mice, as each housing cage was provided with a cardboard tube as environmental enrichment that was unstable when the mice jumped on it.

On the horizontal bar, and particularly the static rods, the Kir6.2 KO mice were impaired. Motor coordination is controlled by specific brain regions, in particular by basal ganglia and cerebellar circuits, where Kir6.2 K_{ATP} channels are expressed [4,32]. The static rods – where mice were placed on a high, exposed, cantilevered rod – may also have provoked more anxiety than the rotarod where mice were placed on a rod with a large flange to each side of them. Likewise, although the inverted screen test may be easier than the horizontal bar test, it could be that the KO mice

were unaffected on the screen as it closely resembled the multiple bars of their home cage lids. All considered, the complex motor–behavioral phenotype of Kir6.2 KO mice is more likely to be caused by loss of central K_{ATP} channels in the brain, than by peripheral deficits due to loss of cardiac or skeletal muscle K_{ATP} channels. If the mice were suffering from peripherally induced muscle weakness their behavior would have been depressed across the whole spectrum of tests, not on the restricted number that was observed. A case in point is the rotarod versus the static rods; the former required the mice to run continuously for around 1 min, the latter to walk 0.5 m. Yet the KO mice were impaired on the static rods but not the rotarod which would have required much more energy expenditure.

4.2. Altered species-typical behavior of Kir6.2 KO mice in response to novelty

Species-typical behaviors such as nesting and burrowing were assessed, as these are sensitive to strain differences and lesions [22,27]. Kir6.2 KO mice, like hippocampal lesioned mice [27] showed a strong impairment of food pellet burrowing, both early on in the series of tests (burrowing 1) and towards the end (burrowing 2). But unlike lesioned mice they made good nests. One explanation for this was that Nestlets were provided as part of the enriched housing, and were therefore familiar to the mice, whereas digging in food pellets had not hitherto been possible as these were always in the food hopper. Thus, KO mice may be more susceptible to novelty per se suppressing behavior. This also accords with the burrowing test 1 and 2 where the greatest difference from WT mice was on the initial 2 h test rather than the overnight test, when they would have had time to adapt to the test conditions — although a ceiling effect on the WT overnight scores could also have been a factor. This familiarity hypothesis was strengthened by burrowing test 3, where the WT–KO burrowing difference was considerably reduced compared with burrow tests 1 or 2. In burrow test 3 the burrowing tube was filled with the wood chips that were routinely used for cage bedding, rather than food pellets, and the KO mice burrowed large amounts of them. Alternatively, burrowing may simply be a more sensitive test than nesting. Thus, altered species-typical behavior of Kir6.2 KO mice might indicate that they are differentially affected by novelty, perhaps due to a dysfunctional emotional response, or their deficit may rather be cognitive, due to difficulties in using or initiating new motor skills under novel conditions. Altered response to novelty depends on various neurotransmitter systems including dopamine [33], thus deficits in motor plan switching could arise from basal ganglia dysfunction. However, the Kir6.2 KO mice did not show a clear hyperdopaminergic phenotype that is characterised by hyperactivity and impaired response habituation [34], and striatal dopamine levels in the KO mouse were not different from WT [18].

4.3. Kir6.2 KO mice are generally less active and show altered anxiety behavior

KO mice were less active in the open field test. This could have been due to either lower motility per se, or a secondary effect resulting from increased anxiety. This test was designed not to be unduly anxiety-provoking, as it was conducted in a grey apparatus of moderate size without supplementary lighting. Its primary purpose was to provide a general screen for any gross behavioral abnormalities, as recommended by other laboratories engaged in phenotyping [31,35]. Normal animals would be expected to show considerable locomotion, with relatively low levels of emotionally induced defaecation and urination, as indeed the WT mice did. Nevertheless, the novelty of the situation would be expected to be mildly anxiogenic, and in anxious mice this would inhibit activity. The KO mice were markedly less active on every measure of activity (latency to first rear, total rears, squares crossed), yet their defaecation and urination scores were not significantly higher than those of WT mice. This suggests that either the KO mice are fundamentally slower, rather than more anxious, than WT, or alternatively that the activity measures were more sensitive to anxiety than the defaecation/urination scores. Indeed, in the light–dark box, the defaecation scores in the KO mice were significantly higher. However, we cannot rule out the possibility that higher levels of emotional elimination of faeces or urine in KO mice were caused directly by altered autonomous control or organ function of the intestinal or urinary systems, both of which possess functional K_{ATP} channels [36,37].

The spontaneous activity test in the alley assessed behavior in a novel environment, but anxiety was lowered to the absolute minimum by the alley being narrow, with home cage bedding on the floor. This was therefore a minimally anxiogenic "experimental crucis" for the interpretation of the other tests involving motor activity. KO and WT mice behaved similarly under such conditions, suggesting that the lower activity of KO mice in the other testing conditions was indeed not a primary motor deficit, but secondary to an altered response. The higher basal home cage activity of KO animals confirmed this interpretation. Decreased exploratory drive, or delayed decision-making, in novel or semi-novel situations (such as the spontaneous alternation testing) is an alternative interpretation.

To further assess a possible anxiety phenotype of Kir6.2 KO mice, more specific tests of anxiety were used. In the light–dark box, the KO mice were slower to enter the brightly lit area than WT, and also produced more faecal boli. Both results suggest KO had higher anxiety levels. However, the time spent in the dark was not significantly higher for KO mice. Also the number of entries into the light was lower for KO mice, so their increased latency to enter the light area could be interpreted as reflecting decreased activity, rather than increased anxiety. This ambiguity between decreased activity or increased anxiety also applies to transitions between alleys 1 and 2, which were lower for KO mice in the successive alleys test. KO mice spent less time on the more anxiogenic second alley, but again this could be interpreted as reflecting either lower basal activity or increased anxiety. Also, few WT mice ventured on to alley 2, so the test suffered from a partial floor effect.

On the elevated plus-maze, a mouse exhibiting a decrease in open arm entries or time would be considered to have an increased level of anxiety. However, the Kir6.2 KO mice spent a significantly longer time on the open arms than WT (see Table 1).

Entries into closed arms of the plus-maze have been shown to reflect general locomotor activity rather than anxiety [30]. Kir6.2 KO mice did not differ from WT mice on this measure, suggesting their basal activity was not reduced (in accord with the spontaneous exploration alley test and home cage activity measurements). Observation of the KO mice revealed that unlike WT mice, which made stretch-attend movements towards the open arms, the KO mice simply stopped on the central square or the open arms, or even moved backwards. This might be due to anxiety-induced freezing, perhaps coupled with perceptual difficulties that inhibited their evaluation of the central area or open arms as aversive until they were completely on them. The plus-maze made in Marburg deliberately used clear acrylic walls to the closed arms, to decrease their attractiveness by increasing the light levels, as a previous test in Oxford using black opaque walls had encountered a floor effect, with few mice venturing out. Several mice either jumped or fell off the maze. This behavior might be interpreted as a panic reaction, a variant of anxiety [38]. The observed non-anxious behavioral 'baseline' was far from optimal and may explain why KO mice did not show consistent changes in emotionality in this test. Baselines on this ethologically based test are notoriously difficult to control.

The two hyponeophagia test results were notably different, with KO mice showing apparently increased neophobia for milk in a discrete food well, and decreased neophobia with scattered food. One possible reason is lower levels of exploratory behavior in the KO mice. However, the behavior of the mice towards the food was very different in the two tests. In the first test, when KO mice did find the food well they would generally wait a longer time than WT mice before properly drinking, and showed clear signs of anxiety such as approach–retreat behavior towards the well. The subjective impression was that these mice were genuinely anxious about drinking, not just slow to find the milk. It is also possible that any delay in finding food in a small, inescapable and unfamiliar environment would in itself lead to increased anxiety. By contrast, the behavior of KO mice in the cage with food scattered all over the floor was totally different; they almost instantly started to eat the food, and their behavior was remarkably similar to that of hippocampal lesioned animals [27]. The hyponeophagia tests epitomised the crucial nature of apparently small differences in test environment and procedure. One possible explanation might again relate to familiarity: the mice were normally fed small amounts of forage mix in their home cages, which was very similar to the chopped nuts which were offered, spread on the floor, in hyponeophagia 2. In hyponeophagia 1 on the other hand, the milk, and the discrete metal well it was presented in, were totally unfamiliar.

Spontaneous alternation was also slightly impaired in KO mice, but was still considerably greater than chance levels. Intra-septal injections of the K_{ATP} channel blocker glibenclamide has in contrast been shown to significantly enhance spontaneous alternation performance in the rat [39]. The number of failed trials was also greater in Kir6.2 KO than WT mice, consistent with the lower activity seen in other tests like the open field, so slowness on the trials that were successfully completed may at least partly explain the alternation deficit. Further work would be necessary to test if the slight impairment of KO mice in this task was indeed due to a cognitive deficit.

4.4. How could K_{ATP} channel activity affect anxiety and motor behavior?

Taken together, in addition to altered anxiety behaviors, KO mice showed increased home cage activity but decreased activity in certain test situations, altered responses to novelty, and reduced motor coordination. These characteristics might be secondary to a primary change in emotionality. A number of neuronal circuits and neurotransmitter systems throughout the brain are involved in anxiety behavior, and a variety of transgenic animals have been demonstrated to display anxiety phenotypes — predominantly with manipulations affecting neurotransmitter receptors and transporters of the norepinephrine, serotonin, GABA, or CRH/HPA systems [38]. Due to their ubiquitous expression throughout the brain, Kir6.2-containing K_{ATP} channels are present in many of the key systems involved in anxiety. In these neurons, the lack of functional K_{ATP} channels could lead to more depolarized membrane potentials and increased electrical activity, which in turn would alter presynaptic transmitter release [4].

5. Conclusion

The general functional roles of central and peripheral K_{ATP} channels under physiological conditions are still unclear. K_{ATP} channels are assumed to be mainly in the closed state under physiological control conditions and to become active only in response to metabolic stress, e.g. ischemia [9]. Accordingly, the here described general Kir6.2 KO mouse has been analysed predominantly under metabolic stress situations, but the altered basal behavioral phenotype was not described. Our findings suggest that K_{ATP} channels are partly activated under physiological conditions, at least at some point during development. However, since we analysed a general Kir6.2 KO mouse, with Kir6.2-containing K_{ATP} channels absent throughout all brain and peripheral tissues, it is not possible to identify the cellular substrates of the changed behavioral phenotype. This common problem of analysing general KO mice can ultimately only be overcome by developing tissue-specific models. Future studies and generation of tissue-specific KO mice might address a possible functional role of K_{ATP} channels in specific neuronal populations that influence anxiety and motor behavior. However, we do not exclude the possibility that peripheral K_{ATP} channels influenced behavior. Both, loss of central and peripheral K_{ATP} channels may contribute in concert to the observed phenotype.

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