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“Behavioural phenotyping of ubiquitous LRIG 2 knockout mice”

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Zusammenfassung

Über die Proteine aus der Familie der „leucine-rich repeat and immunoglobuline-like“ Domänen, kurz LRIG, ist noch nicht viel bekannt. Insbesondere LRIG2 wurde noch nicht umfassend erforscht. Aus diesem Grund und weil sich LRIG2 in seiner intra- und extrazellulären Struktur etwas von den anderen Proteinen dieser Familie unterscheidet, haben wir uns entschlossen, dieses spezielle Protein zu untersuchen. Wir erstellten ubiquitäre LRIG2-Knockout Mäuse, untersuchten diese auf Verhaltensunterschiede im Vergleich zu ihren Wildtyp Wurfgeschwistern und versuchten, diese mit bestimmten Gehirnregionen in Verbindung zu bringen, um damit die Rolle von LRIG2 im Gehirn, beim Lernen und anderen kognitiven Prozessen besser zu verstehen.

Die Verhaltenstests konzentrierten sich auf vier Elemente: Angst, sensorimotorisches Gating, Lernen/Gedächtnis und Geruchssinn. Ängstlichkeit wurde mit dem „Open field test“, dem „Elevated plus maze“ sowie dem „Three-chamber social interaction test“ bewertet. Das sensorimotorische Gating wurde mit dem „Startle- und prepuls-inhibition test“ untersucht. Lernen und Gedächtnis mit dem „Morris water maze“, dem „Y-maze“ und „Fear conditioning“. Darüber hinaus wurde der Geruchssinn und das Geruchsgedächtnis mit Hilfe des „Olfactory habituation/dishabituation test“ mit nicht sozialen Düften untersucht.

Abschließend wurde noch eine histopathologische Analyse des Gehirns durch ein externes Labor durchgeführt.

Die Ergebnisse unseres Verhaltensscreenings deuten darauf hin, dass vor allem Amygdala abhängige Prozesse wie emotionales Lernen/Gedächtnis und sensorischimotorisches Gating durch den Verlust von LRIG2 in Mäusen beeinträchtigt werden. Da in der histopathologischen Analyse keine strukturellen Unterschiede auffielen, gehen wir davon aus, dass diese Beeinträchtigungen durch Unterbrechungen von Signalwegen verursacht werden, in denen LRIG2 eine wichtige Rolle spielt. Außerdem nehmen wir aufgrund dieser Ergebnisse an, dass die zukünftige Erforschung von LRIG2 spezifischen Signalwegen und deren Interaktion im Bereich der Amygdala zu interessanten Erkenntnissen führen könnte.

Abstract

The study of the leucine-rich repeat and immunoglobuline-like domains (LRIG), family of proteins is in its infancy. Especially LRIG2 has not yet been extensively investigated. Considering this and due to LRIG2 differing slightly in its intra- and extracellular structure in comparison to the other proteins in this family we decided to investigate this particular protein. We generated ubiquitous LRIG2-knockout mice, screened them for behavioral differences in comparison to their wildtype littermates and tried to relate these to specific brain regions to improve the understanding of LRIG2's role in the brain and in learning processes.

Behavioral screening focused on four aspects: anxiety, sensory-motor gating, spatial memory/learning and sense of smell. Anxiety was evaluated with the open field test, the elevated plus maze as well as with the three-chamber social interaction test. Sensory-motor gating was investigated via the startle and prepuls-inhibition test. Learning and memory was assessed with the Morris water maze, the Y-maze and fear conditioning. In addition, sense and memory of smell was assessed via the olfactory habituation/dishabituation test with nonsocial odours.

Subsequently histopathological analysis of the brain was performed by an external laboratory.

Results of our behavioral screening suggests that predominantly amygdala dependent processes such as emotional learning/memory and sensory-motor gating are impeded by the ubiquitous knockout of LRIG2 in mice. Furthermore, as there were no differences in the histopathological analysis discovered, we assume that these impairments are caused by pathway interruptions in which LRIG2 plays an important role. Therefore, we assume that future research of LRIG2 pathways and their interaction with the amygdala may lead to better understanding of the role of LRIG2 in the brain.

List of abbreviations

leucine-rich and immunoglobuline like repeat domain.....	LRIG
knockout.....	KO
startlepuls.....	SP
prepuls.....	PP
startlepuls-plus-prepuls.....	SPP
short-term probe trial.....	STPT
long-term probe-trial.....	LTPT
standard deviation.....	SD

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1. Introduction

Ubiquitous leucine-rich repeat and immunoglobulin-like domain (LRIG)2-knockout (KO) mice were first reported in 2013 (1). Studying these mice will help to better understand the role of LRIG2 in the body and the effects of its absence.

LRIG2 belongs to the family of LRIG proteins which are transmembrane proteins and consist of three members: LRIG1, LRIG2 and LRIG3 (2). It is also known that the intracellular/cytoplasmic domains vary substantially between these three proteins (3). The extracellular domains of these proteins likewise differ from each other. LRIG2 however deviates more from the other proteins as its first cysteine N-flanking domain is absent and its immunoglobulin-like domains are altered (4). Therefore, it is assumed that the pathway from LRIG2 works differently than the one from LRIG1 and LRIG3. LRIG2 has been found in almost all organs and tissues (5). Thus, it is assumed to have some function in the whole body. Moreover while LRIG1 and LRIG3 have been identified as tumour suppressors (6, 7). LRIG2 has been reported to function as oncoprotein in many organs and tissues such as in glioma (1), oligodendroglioma (8), non-small cell lung cancer (9) and the cervix (10). Additionally one study showed that the absence of LRIG2 lead to better neuron regeneration after optic nerve crush (11).

Even though ubiquitous LRIG2-KO mice, have been intensively investigated in glioma (1, 8), the role of LRIG2 in cognition and learning has not yet been studied. Thus, we aimed to screen for possible behaviour abnormalities that could be associated with the absence of LRIG2 and relate these to specific brain regions, thereby better understanding the role of LRIG2 in the brain.

We created a test battery with eight behaviour tests to investigate the differences between LRIG2-KO mice and their wildtype littermate. The following is a brief summary of each test used in this study.

The open field test was first developed by Hall in 1934 to measure emotionality in rodents through locomotor activity and fecal boli deposition (12). Today it is used to study anxiety and locomotor activity in rodents by evaluating the conflict between exploration and their innate

aversion to open or exposed spaces (13–15). It is also one of the most used measurements in animal psychology as it provides well-defined behaviours while being quick to conduct and analyse (16). Animals are put in an empty square arena and are then free to explore it. Movement is tracked for a specific time depending on the research question. For quantifying locomotor ability, the animal is usually tracked for 30-60 minutes and distance travelled (cm) is evaluated. Anxiety-like behaviour is determined by the time spent in the border area in the first 10 minutes of the test.

Prepuls-inhibition is commonly used to study sensorimotor gating mechanisms in animals and humans (17–19). It is a phenomenon in which a weak stimulus (prepuls) suppresses the response to an unexpected startle-like stimulus (20). Prepuls-inhibition can be measured by using the acoustic startle reflex. In small mammals the acoustic startle reflex manifests as an involuntary whole body flinching response to a strong unexpected auditory stimulus (i.e. 80 dB and above). Neuropsychiatric disorders in humans most notably schizophrenia have been linked to deficiencies in prepuls-inhibition, animal models that reveal similar impairments play an important role in understanding these brain disorders (21, 22).

The elevated plus maze is used to measure anxiety in rodents by using their innate preference for dark and enclosed areas as opposed to open and bright areas (23–26). It is composed of a plus shaped platform with two opposing open and two opposing closed arms. The test animal is placed in the middle and is free to explore the whole platform for three minutes. During this time its movement is recorded and analysed. Animals are considered less anxious if they spend more time in the open arms and visit the open arms more frequently. Additionally, so called headdips which is a behaviour in which the mouse points its head downwards over the edge of the open arms can be counted. These are commonly used as an index for risk assessment (27, 28).

The Y-maze can be used to test spatial memory or short-term memory in rodents (29–31). It uses rodent's innate curiosity to explore previously unvisited areas. Spontaneous alternation between arms is considered working short-term memory (32).

The three-chamber social interaction test is used to test sociability and interest in social novelty. Sociability refers to the desire to seek out social contact and interaction with the

same species. Interest in social novelty is defined as the propensity of mice to spend more time with an unfamiliar novel mouse rather than a familiar one. This preference to spend more time with a novel mouse relies on the recognition of the old and new social stimulus therefore it is also considered a measurement of social memory (33, 34).

The olfactory habituation/dishabituation is used to investigate odour perception in mice. The olfactory abilities are evaluated on following factors: odour-habituation which is the progressive decrease in olfactory investigation (sniffing) towards a repeated presentation of the same odour, odour-dishabituation which is the ability to detect and the preference for novel odours and the preference for social-odours (35–37). Odour habituation is also considered an ideal model to study the neurobiology behind habituation because of the relative simplicity of the olfactory system (38).

The Morris water maze, first invented by Richard Morris (39) is one of the most regarded mazes to study spatial learning and reference memory (32, 40–42). It is not a maze in the conventional sense but an open round pool filled half-way with water or an opaque liquid in which a platform is hidden below the water-surface. Several publications showed that animals with damage to hippocampus, basal forebrain, striatum, cerebellum or neocortical areas had unique deficits in the Morris water maze proposing different roles in Morris water maze performance (42). Additionally, the Morris water maze can be used for reversal learning by training animals to escape the water to a novel platform location, usually opposite of the initial location. The reverse Morris water maze is used to test flexibility in learning.

Fear conditioning is an association learning task in which mice are presented with a neutral conditional stimulus (generally a tone) which is paired with an aversive unconditional stimulus (generally a mild electrical foot shock). After a number of pairings, often just one, mice produce robust fear learning demonstrated by a variety of fear responses/conditioned responses when exposed to the conditional stimulus alone (43, 44). To quantify fear response freezing behaviour, which is a defensive behaviour of rodents typically seen when a predator has been detected but is not in the verge of contact, is measured (45). It is defined as suppression of motion except that required for respiration (46). Brainregions such as hippocampus and the amygdala have been established as key players for fear memory

consolidation, storage and retrieval. Dysfunction in these regions may be involved in multiple forms of psychopathology such as schizophrenia and, post-traumatic stress disorder (47)

2. Material and Methods

2.1. Animals

Five female C57BL/6N (wildtype) and five female LRIG2-KO mice were used in this study. LRIG2-KO mice were generated by cloning a neomycin stop cassette between exon 1 and 2. They were established in house via stem cell injection into blastocysts on a C57BL/6N background strain. Experimental mice were littermates and originated from 4 different families. Mice were weaned at the age of three weeks and kept in same sex family groups independent of their genotype. One week prior to the start of the study, all mice were transferred to the Vienna Biocenter Core Facilities where the behavioural experiments were conducted. There, animals were kept in pairs (one LRIG2-KO and its wildtype littermate) in a standard type three individually ventilated cage equipped with aspen wood bedding, one red transparent house, aspen wood nesting material and aspen woodchips. Temperature was held at 20-24 °C and relative air humidity was between 52-78 percent throughout the whole study. Animals had a constant 14/10 day-night cycle (light: 6 am-8 pm) and free access to food (ssniff®, V1184.300, Soest, Germany) and water. Room parameters were checked daily and cages were changed at the end of each week. At the start of the study all animals were between 12-16 weeks old and weighed between 17.5 and 24.3 grams. All tests were conducted between 9 am to 6 pm. Mice were weighed at the end of each day of testing. Behavioural tests were conducted in the order they are presented in the material and methods section. At the end of the study all animals were sacrificed by cervical dislocation and organs were preserved for histopathological analysis.

The use of animals for this study was approved by the Austrian “Federal Ministry of Science and Research” with the permit numbers 2021-0.744.361 and 2022-0.498.106 and all experiments were carried out in accordance to the European Union Directive 2010/63/EU for animal experiments.

2.2. Open field test

The open field setup consisted of four 50 cm (length) x 50 cm (width) x 50 cm (height) white empty Plexiglas boxes (Fig 1). A video camera was suspended above the arena to track movement. Lights above provided 130 lux in the centre and 100 lux on the border of each

arena. Around this setup a frame with curtains was put up to separate the test environment from the experimenter. All mice were brought to the experiment-room 30 minutes before the test for habituation. At the start of each trial mice were placed in the centre of an arena and were then free to explore for 30 minutes. As we had four arenas, we tested two groups of four and one group of two mice simultaneously. Each mouse was tested once. Between groups the arenas were cleaned with water and 50 % ethanol. The video tracking software “Videomot 2” by TSE-Systems was used to record the following parameters: distance travelled, time spent in each zone (center/periphery) and rearing behaviour. The inner 25 cm x 25 cm of the open-field were considered central area. The outer 15 cm of each arena were considered peripheral.



Fig. 1 Open field maze and environment

2.3. Prepuls-inhibition of the Acoustic startle reflex

Prepuls-inhibition was assessed in the SR lab acoustic startle Box by San Diego Instruments with digitised electronic output. It consisted of a ventilated, sound- and lightproof outer chamber and an inner Plexiglas mouse restrainer resting on a platform (Fig. 2). A piezoelectric accelerometer installed in the platform converted animal movement into electrical energy (measured in mV) which was recorded and analysed by a computer and

interface assembly. The acoustic startle signals were played through a loudspeaker installed on the inside of the chamber.

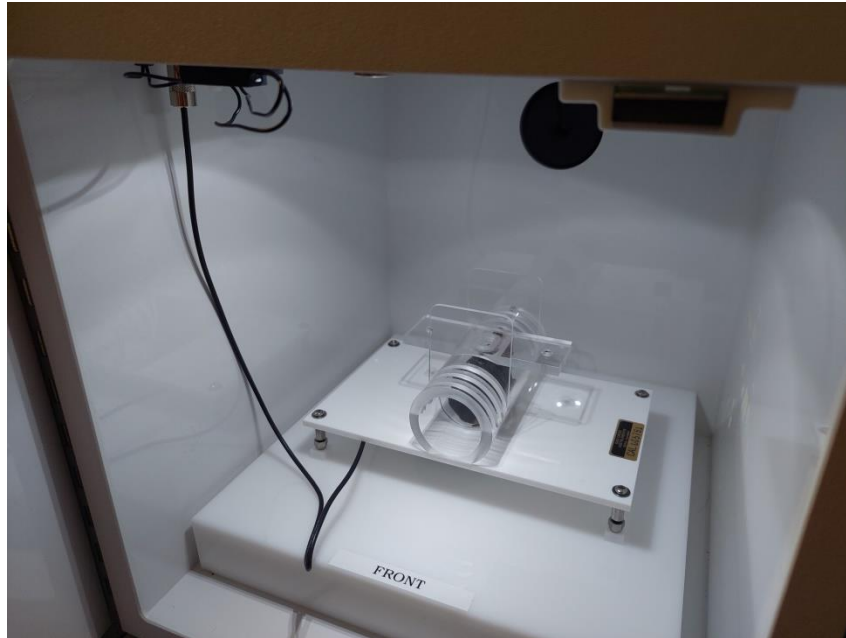


Fig. 2 Inside of the startle-box used in the startle and prepuls-inhibition test

Prepuls-inhibition testing followed a similar protocol as described in (48). Mice were placed in the restrainer and had five minutes to habituate. Subsequently they were presented with five initial startlepuls (SP) trials. Thereafter ten trial blocks were presented. Each block consisting of startlepuls trials, three prepuls (PP) trials and three prepuls-plus-startlepuls (SPP) trials. For detailed information (length, intensity, kHz) refer to Tab.1. In each block every trial type was presented at random with an inter-trial interval of an average 15 seconds. During all tests a background white-noise of 50 dB was played. 200 ms before onset of each stimulus and 200 ms after onset were recorded. The 200 ms that were recorded pre-onset were used as a baseline measurement. The maximal response for each stimulus (V_{max}) within the 200 ms after startle onset of each trial was used to determine startle reactivity and prepuls-inhibition. First startlepuls trials excluding the five initial startlepuls trials were used to determine startle reactivity. Then response to prepuls-plus-startlepuls trials was used to evaluate prepuls-inhibition. Prepuls-inhibition was calculated as a percentage of each prepuls intensity following this formula:

$$[1 - (\text{Average Vmax of SP trials} / \text{Average Vmax of PP trails})] * 100$$

As LRIG2-KO mice weighed considerably less than their wildtype littermate's outcomes from all trials were adjusted to bodyweight.

Tab. 1: Different trial types of the startle and prepuls-inhibition test

Trials	Description
Startlepuls trials	40 ms of 115 dB intensity white noise at 5k Hz
Prepuls trials	40 ms of 70, 75 or 80 dB intensity at 5 kHz
Prepuls-plus-startlepuls trials	20 ms of 70, 75 or 80 dB intensity at 5 kHz directly before Puls-alone trial

2.4. Elevated plus maze

The two opposing open arms in the elevated plus maze used in our study had a dimension of 30 cm (length) x 6 cm (width) with the two opposing closed arms additionally having a wall with a height of 30 cm. The centre zone in between the arms was 6 x 6 cm. The platform was raised on stilts 50 cm above the ground (Fig 3). The whole apparatus was made out of grey opaque plastic. A video camera above tracked all movement in the maze. Light from above ensured a light intensity of 190 lux in the open arms and 120 lux in the closed arms. Around the maze a room divider separated the test environment from the experimenter. Thirty minutes before the test mice were brought to the room for habituation. At the start of each trial the mouse was placed with its head in the middle of the platform facing an open arm and was then allowed to move freely for 5 minutes. Each mouse was tested once. Between every mouse the platform was cleaned with water and 50 % ethanol. The following parameters were recorded with the video tracking software Topscan V.3.00 by cleversinc: Time spent in open/closed arms, distance travelled and total number of headdips. Latency to visit an open arm was scored manually with a timer by re-watching the recorded video-footage. A visit was counted when the mouse was standing with both hind paws on an arm.



Fig. 3 Elevated plus maze

2.5. Y-maze

The maze consisted of a y-shaped platform made out of grey plastic 29,5 cm (length) x 6 cm (width) surrounded by 15 cm high walls (Fig 4). All mice were brought to the experimental-room 30 minutes before the start of the test for habituation. Mice were placed in one of the three arms of the maze with their head facing the dead-end. They were then free to explore the maze for five minutes. Each mouse started from a different arm and the maze was cleaned with water and 50 % ethanol between mice. Movement was captured with a video camera above the maze. Consistent lighting of 100 lux in the centre and 80 lux in the arms of the maze was provided. The video-tracking-software Topscan V.3.00 by cleversinc was used to record mouse movement. We recorded the, mean number of arm entries and the percentage of spontaneous arm alternations over total number of possible spontaneous alternations.

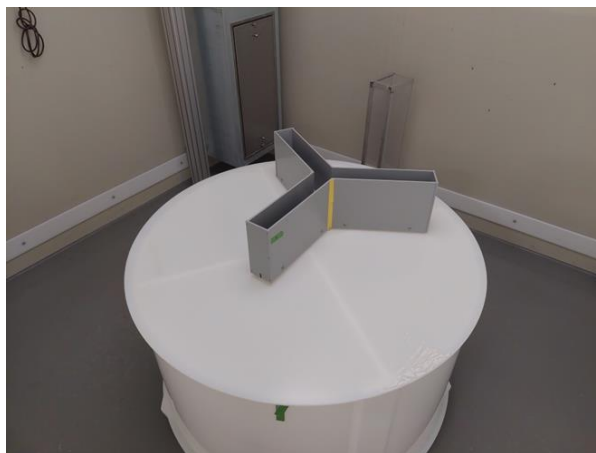


Fig. 4 Y-Maze

2.6. Three-chamber social interaction test

Mice were tested in a three chambered rectangular box made out of opaque grey plastic. The chambers could be separated by removable panels. In both outer chambers removable wire-cages with a diameter of 9 cm were placed in identical positions (Fig. 5). There was a light intensity of 90 lux inside the arena. A video camera above the apparatus recorded all trials and the program Topscan V.3.00 by cleversinc was used to track movement. A contact zone for each wire-cage was defined as the area two centimetres around the cage.

Tests were conducted on two consecutive days. On each day all mice were brought in their home-cage to the experiment room 30 minutes before the start of the experiment to habituate. There were three test trials: The first one as a baseline measurement of anxiety, the second one to test sociability and third one to test interest in social novelty.

On day one the first trial was conducted. Mice were placed in the centre chamber and were free to explore all three chambers for ten minutes. On the second day, trial two and three were performed. In trial two the chambers were separated by panels and an unfamiliar mouse was placed in one of the wire-cages in the outer chambers. Then the test-mouse was put in the centre chamber, the panels were removed and the behaviour of the test mouse was recorded. After five minutes the test-mouse was gently guided into the centre chamber and the panels were closed again. For the third trial, an additional mouse was introduced. A novel unfamiliar mouse was put in the previously empty wire-cage. Then the panels were removed and the test-mouse was observed again for five minutes.

Two C57BL/6J female mice which were 15 weeks old were used as stimulus mice. Mouse presentation in wire cages was randomized. The wire cages allowed for sniffing but no physical contact between stimulus and test mice. The arena and wire-cages were cleaned with water and 50 % ethanol between trials.

We assessed the duration spent in each chamber, the number of visits in respective chambers and the duration and frequency of the nose in the contact zone. Mouse behaviour was recorded by Topscan V.3.00 from cleversinc.

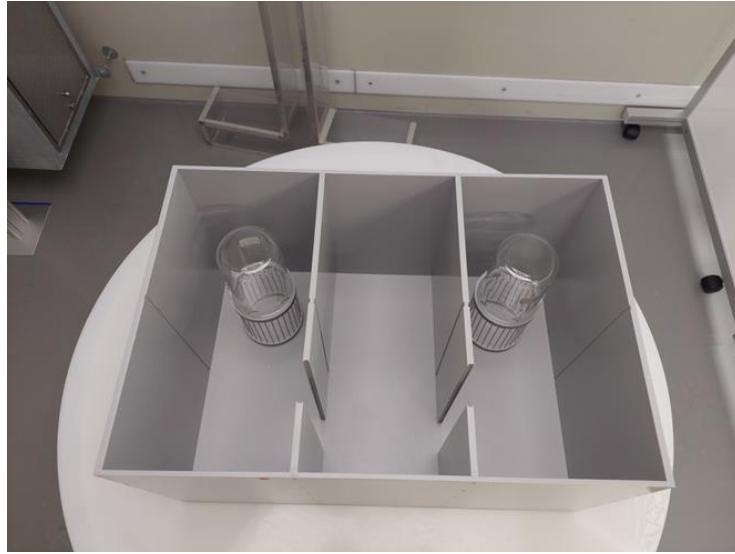


Fig. 5 Social interaction test arena

2.7. Olfactory habituation/dishabituation

We used the olfactory habituation/dishabituation test to investigate if our mice have impairments in odour perception.

The following non-social odours were prepared each day in a 10^{-5} dilution: vanilla, peanutbutter and methylic acid. Additionally, water was used as control to differentiate interest in a new object (cotton swab) with interest between the different odours. Fifty microliters of each odour were dropped on a cotton swab, before the swabs were introduced to the experimental cage through the hole in the lid normally used for drinking bottles (Fig 6). Each mouse was placed inside the experimental cage 45 minutes before the start to habituate. Subsequently the water and odour swabs were each presented three times for two minutes. Mouse behaviour was recorded via video camera and manually analysed with “Noldus observer” by Noldus. Latency to sniff the cotton swab and time sniffing at the cotton swab were analysed for each odour. Trials were always performed in a freshly bedded cage and with a light intensity of 100 lux. Tests were performed on two consecutive days, since not all mice could be tested on the same day.



Fig. 6 Odour presentation in the olfactory habituation/dishabituation test

2.8. Morris water maze

We used the Morris water maze to assess mouse learning and spatial memory. In our setup a pool with a diameter of 98 cm was used (Fig 7). The pool was divided by two imaginary lines creating a plus-shape. Each end of the line marked one cardinal point: north, east, south and west. These were not true compass directions but referred to the position relative to the experimenter. Four spatial clues were put up on the walls and room-dividers surrounding the pool for orientation. The pool was filled with room-temperature water, coloured with nontoxic white paint making it in- transparent. The platform had a diameter of 10 cm. At the surface of the water there was a light intensity of 130 lux. A camera was suspended above the maze. The video-tracking software Topscan V.3.00 by cleversinc was used to record data.

On the first training day the platform was placed in north-west position, 12 cm away from the border of the pool and 1 cm submerged under the water-surface. It was made visible by a flag placed on top of it in order for the mice to learn to search for the platform. On the following five days the flag was removed and the platform was moved to south-east position. Each day there were two training-sessions, one in the morning and one in the afternoon. In every session all mice swam four times. Each time starting from a different position in the following order: north-east, north-west, south-west, and north-east. We chose to start twice from north-east leaving out south-east due to it being very close to the platform. Between trails mice were placed in a cage under an infrared heat-lamp to warm up and rest for five minutes. This was especially important as LRIG2-KO mice have very little to no fat therefore needing longer to

warm up and recover between trials. Subsequently probe trials were performed in which the platform was removed and the time spent in the different quadrants of the maze was recorded. Time spent in the area where the platform used to be is considered successful spatial memory. A short-term probe trial (STPT) was performed directly after the last training session and a long-term probe-trial (LTPT) two days later. On training day's movement and latency to reach the platform was recorded. On probe trials movement, latency to reach the position where the platform used to be and time spent in the different quadrants of the maze were recorded. Reverse learning was initiated one day after the first LTPT. The platform was moved to north-west position and, as before five days of training, one short-term probe trial and one long-term probe trail were performed.



Fig. 7 Morris water maze setup

2.9. Fear conditioning

To perform fear conditioning, all mice were brought in their home cages to the experimental room 30 minutes before the first test for habituation. Inside the test area a ventilated soundproof chamber was placed in which the conditioning cage (Habitest Modular System, Coulbourn Instruments, United States of America) was located. The cage consisted of a grid

floor through which an electric current could be passed, walls with interchangeable panels (black/white), a lamp and an infrared capable camera which recorded and send video-data to a computer outside the test area (Fig. 8). The video data was then analysed using FreezView from Actimetrics by manually setting a threshold.

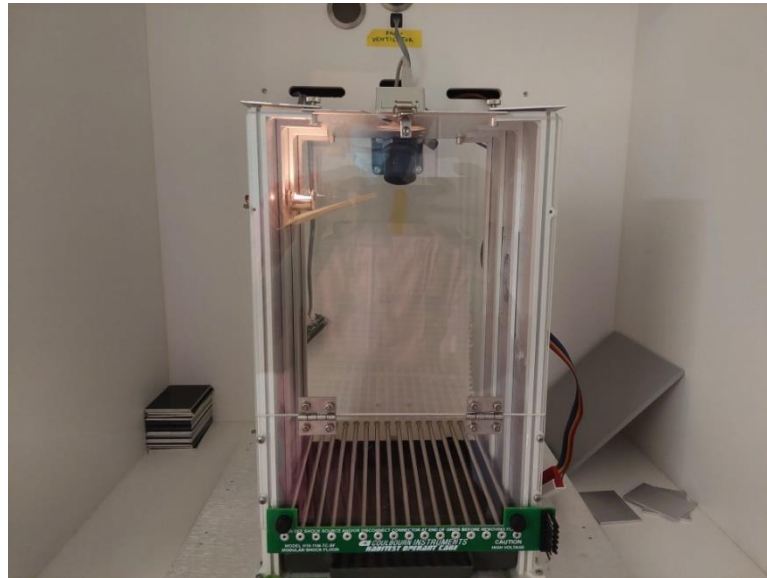


Fig. 8 Fear conditioning cage

Tests were conducted on three consecutive days. On day one fear memory acquisition was tested. The cage was fitted with all white panels, a grid floor and the cage was lit with 3 lux of white light. At the start mice had 120 seconds to explore the cage and acclimate to its conditions. Then mice were individually presented with a 10 kHz sound at 85 dB for 30 seconds. During the last two seconds of the tone a mild foot shock (0,5 mA) was delivered which co-terminated with the tone. Subsequently an inter-trial interval of 90 seconds followed before a second trial identical to the first one was conducted. Seventy seconds after the second trial mice were removed from the conditioning cage and it was cleaned with water and 50% ethanol.

On day two contextual fear conditioning was tested. Therefore, mice were individually placed in the unaltered conditioning cage for four minutes and freezing behaviour was recorded and scored.

On day three cued fear conditioning was tested. The walls of the conditioning cage were replaced with alternating black and white panels, a platform wrapped in paper towels was placed above the grid floor, the white light was turned off and the cage was lit with infrared light. As on day one mice had 120 seconds to habituate before trial start. Following the habituation, the same sound as on the first day (10 kHz; 85 dB) was presented twice for 60 seconds with an inter-trial time of 60 seconds and freezing behaviour was recorded. Mice were removed from the conditioning cage 60 seconds after the second trial.

2.10. Statistical Analysis

All data were analysed using GraphPad Prism 8.0 (GraphPad Software, United States of America). We first tested for Gaussian distribution using a Shapiro-wilk test. If the data were normally distributed parametric tests were applied, otherwise we used non parametric tests. No corrections for multiple testing were performed.

3. Results

3.1 Anxiety/Mental state

In the open field test we found that KO mice spend a significantly higher percentage of time in the centre of the open field arena compared to their wild type littermates ($t=6$, $df=10$, $p<0.001$, Fig. 9) with no difference in the distance travelled inside the maze ($U=9$, $p=0.548$, Fig. 10).

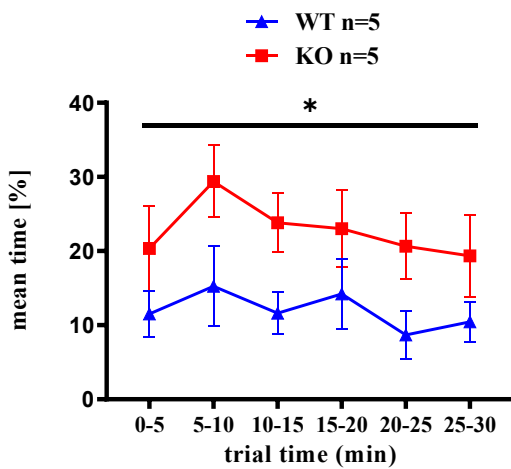


Fig. 9: Mean time and SD spent in the centre of the open field arena separated in five-minute intervals

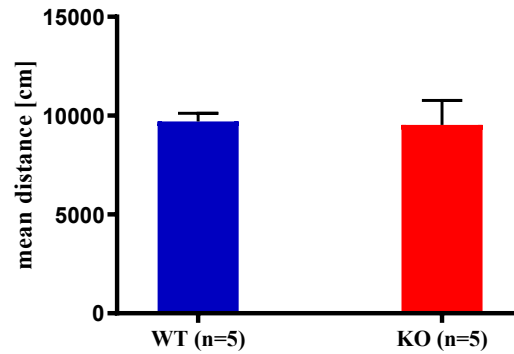


Fig. 10: Mean distance and SD travelled in the open field arena

In the elevated plus maze a significant difference in percentage of time spent in the closed arms was observed between animals. KO mice occupied the closed arms for longer than their wildtype littermates ($t=2.5$, $df=24$, $p=0.019$, Fig. 11). Furthermore KO mice covered significantly less distance inside the maze ($U=0$, $p=0.008$, Fig. 12). No significant differences between KO and wildtype mice were found in total number of head dips ($U=5$, $p=0.151$), the latency to enter ($U=7.5$, $p=0.564$), or the duration spend in the open arms ($t=0.43$, $df=24$, $p=0.672$).

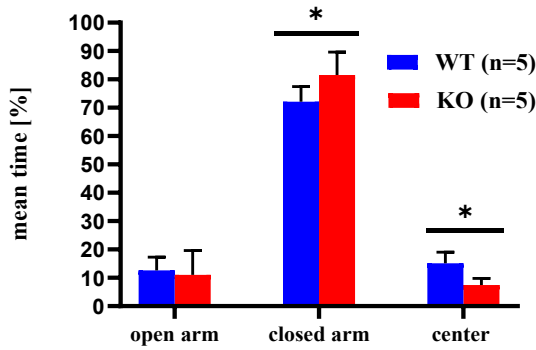


Fig. 11: Mean percentage of time and SD spent in each arm and centre of the elevated plus maze

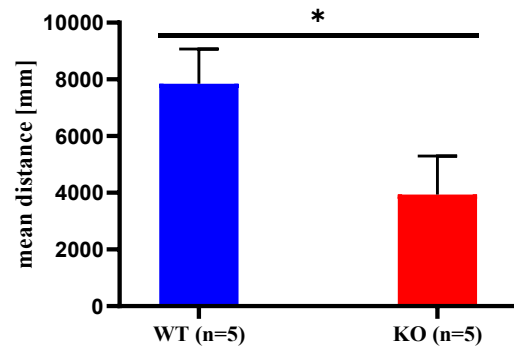


Fig. 12: Mean distance and SD covered in the elevated plus maze

In the three-chamber social interaction test, KO and wildtype mice spend a comparable amount of time in the compartment with the unfamiliar mouse ($t=0.12$, $df=8$, $p=0.906$) and the empty compartment ($t=0.18$, $df=8$, $p=0.862$, Fig. 13). Similarly, there was no difference between KO and wildtype mice in the percentage of time mice spent in the compartment with the familiar ($t=0.62$, $df=8$, $p=0.554$, Fig 14) or unfamiliar mouse ($t=0.57$, $df=8$, $p=0.586$, Fig 14)

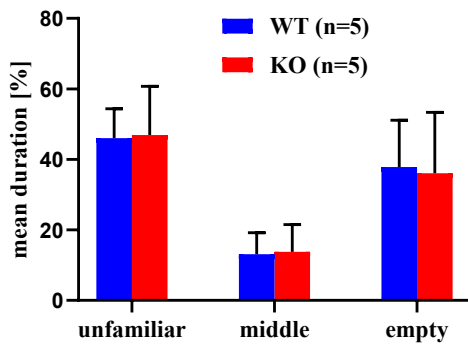


Fig. 13: Mean duration and SD spent in each chamber of the social interaction apparatus in the first trial with a caged unfamiliar mouse in one chamber and one empty cage in the other chamber

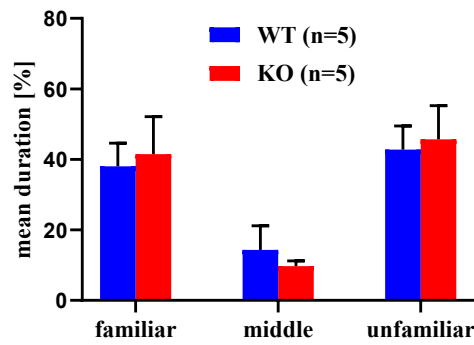


Fig. 14: Mean duration and SD spent in each chamber of the social interaction apparatus in the second trial with an unfamiliar mouse replacing the empty cage

3.2 Sensory-motor gating

In the acoustic startle and prepuls-inhibition test there was no difference in startle reactivity between SP-alone trials and SPP trials between KO and wildtype mice

($t > 1.24$, $df=8$, $p > 0.250$, Fig 15). Prepuls-inhibition showed a significant difference, as KO mice had a significantly weaker startle reaction when the prepuls was 70 dB ($t=3.95$, $df=8$, $p=0.004$) and 75 dB ($t=2.94$, $df=8$, $p=0.019$, Fig. 16) and tended to be weaker at 80dB ($t=1.78$, $df=8$, $p=0.113$).

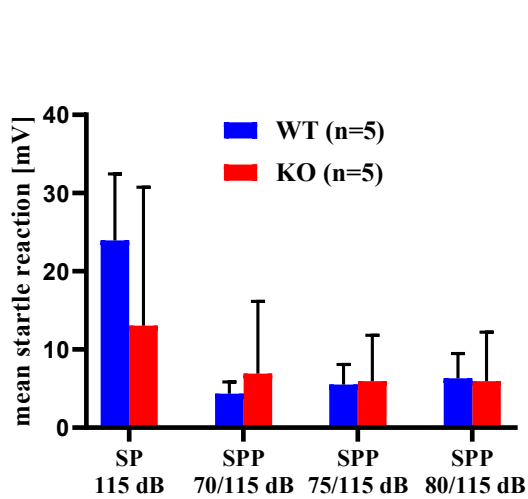


Fig. 15: Mean startle reaction and SD in SP-alone and SPP-trials normalized to bodyweight in the startle and prepulsinhibition test

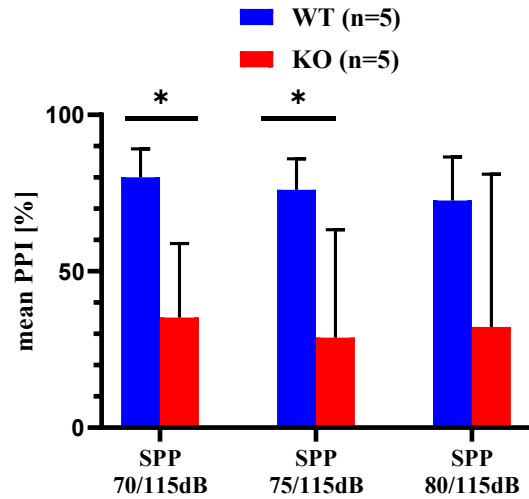


Fig. 16: Mean PPI and SD in all prepuls intensities normalized to bodyweight in the startle and prepulsinhibition test

3.3 Memory and learning

In the Morris water maze LRIG2-KO mice required on average significantly more time to reach the hidden platform on the first ($t=2.4$, $df=8$, $p=0.04$) and fifth ($t=3.7$, $df=8$, $p=0.006$) training day than their wildtype littermates. Additionally, as seen in Fig. 17 wildtype mice showed on average continuous improvement in finding the platform while KO mice did not.

In the short-term probe trial KO mice required on average significantly more time to reach the area where the platform used to be during training than their wildtype littermates ($U=2$, $p=0.03$, Fig. 18). Furthermore, KO mice spend on average significantly less time in the target quadrant where the platform used to be during training than their wildtype littermates ($t=3.3$, $df=8$, $p=0.012$, Fig. 19).

In the long-term probe trial, there was no differences in the average time to reach the target area where the platform used to be during training between KO and wildtype mice

($U=10$, $p=0.691$, Fig. 18). Additionally, there was no difference in average time spent in the target quadrant where the platform used to be during training ($t=1.3$, $df=8$, $p=0.245$, Fig. 20).

In the reverse Morris water maze, there was no difference in the average time to reach the hidden platform in all training days ($t<1.49$, $df=8$, $p>0.176$). Additionally, there was no difference in average time to reach the target area where the platform used to be during training as well as in the average time spent in the target quadrant in the short-term probe trial (latency: $U=7$, $p=0.31$; time: $t=1.7$, $df=8$, $p=0.12$) and long-term probe trial (latency: $U=9$, $p=0.55$; time: $t=0.27$, $df=8$, $p=0.27$)

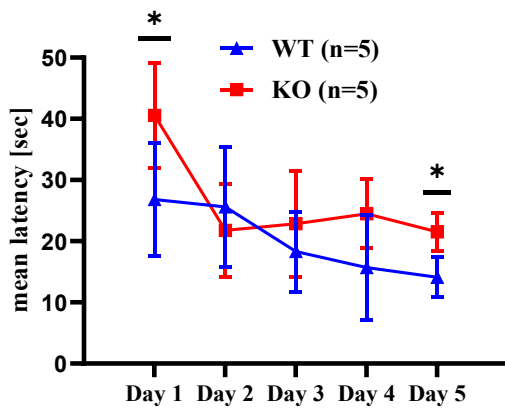


Fig. 17 Mean latency and SD to reach the hidden platform on each training day in the MWM

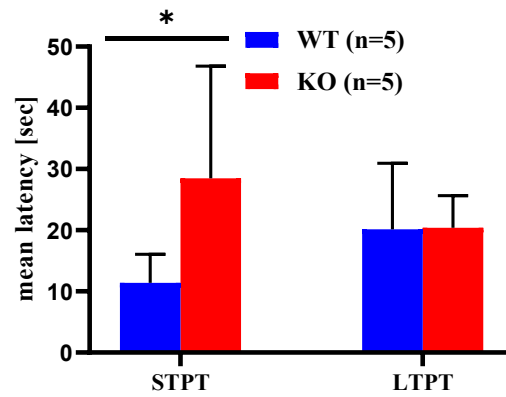


Fig. 18 Mean latency and SD to reach the hidden platform in the short- and long-term probe trial of the MWM

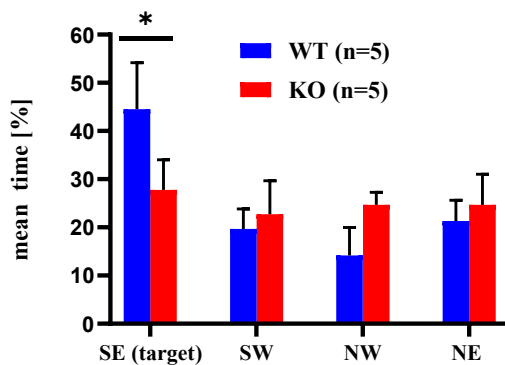


Fig. 19 Mean time and SD spent in each quadrant of the MWM in the short-term probetrial

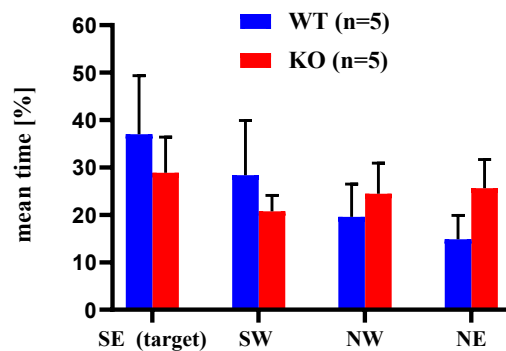


Fig. 20 Mean time and SD spent in each quadrant of the MWM in the long-term probetrial

In the Y-maze there was no difference in the latency to leave the starting arm ($U=11.5$, $p=0.881$) and no difference in the mean number of arm entries between KO and wildtype mice ($U=3.5$, $p=0.064$, Fig. 21). Additionally, there was no difference in the percentage of spontaneous arm alternations ($U=8$, $p=0.413$, Fig. 22).

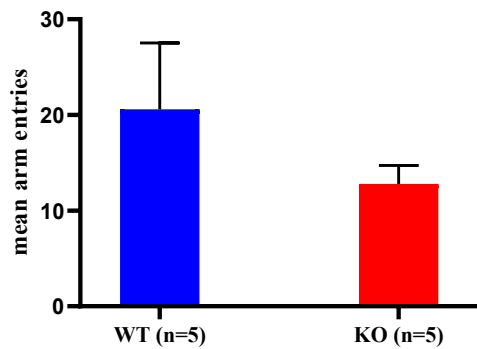


Fig. 21 Mean number of arm entries and SD in the Y-maze

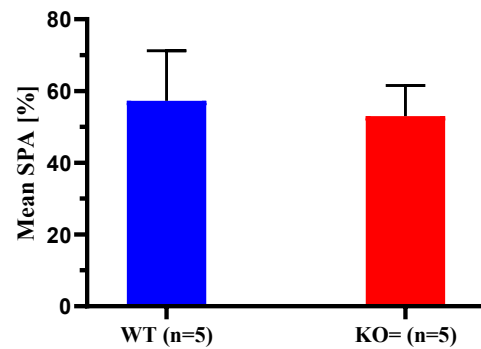


Fig. 22 Mean spontaneous arm alternations in percent and SD in the Y-maze

In the fear conditioning test fear acquisition was successful in both groups. We found no difference in the contextual fear test between groups, as KO and wildtype mice showed an equal amount of freezing behaviour ($U=11$, $p=0.841$, Fig. 23). However, when cued fear was tested, KO mice showed significantly more freezing behaviour at the first ($t= 3.2$, $df= 8$, $p=0.013$, Fig. 24) and the second presentation ($t= 4.5$, $df= 8$, $p=0.002$, Fig 24) of the conditioning tone.

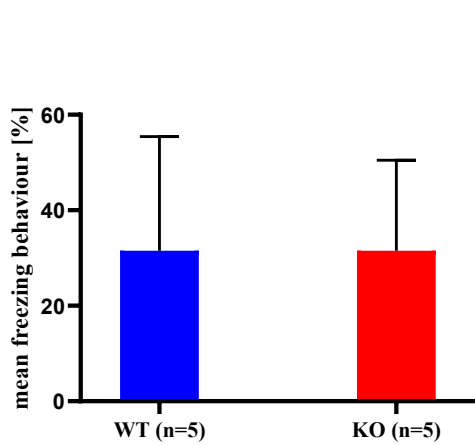


Fig. 23 Mean percentage of time and SD of freezing behaviour on the second day of the fear conditioning test measuring contextual fear

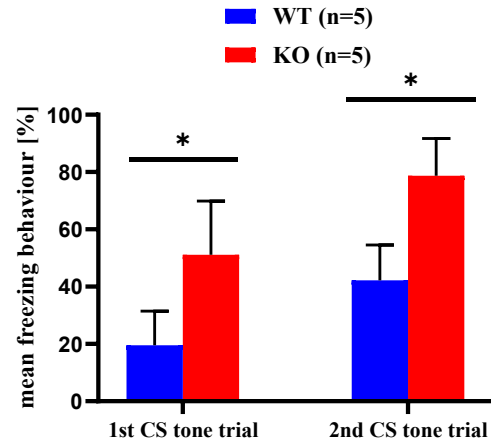


Fig. 24 Mean percentage of time and SD of freezing behaviour in each conditioning tone trial on the third day of the fear conditioning test measuring cued fear conditioning

3.4 Olfactory sense and memory

In the olfactory habituation/dishabituation test no habituation occurred on consecutive presentations of the same odour as we found no decline between the first and third odour presentation (not considering water) in both groups (all $U < 5$, $p > 0.167$, Fig 25). Similarly, mice did not show the expected odour dishabituation when novel odours were introduced. Only between the third presentation of peanut butter and the first presentation of methylbutyric acid ($t = 2.39$, $df = 8$, $p = 0.044$, Fig 26) we observed a reduced latency to sniff. All other latencies between odours did not differ in both groups (all $t < 1.108$, $df = 8$, $p > 0.3$, Fig. 26).

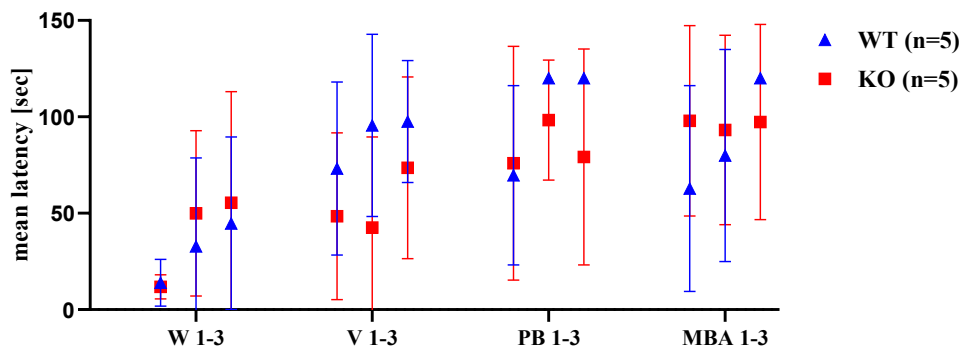


Fig. 25: Mean latency and SD to sniff at presented odours in the odour habituation/dishabituation test. Each odour was presented three times (W=water, V=vanilla, PB=peanutbutter and MBA=methylbutyric acid)

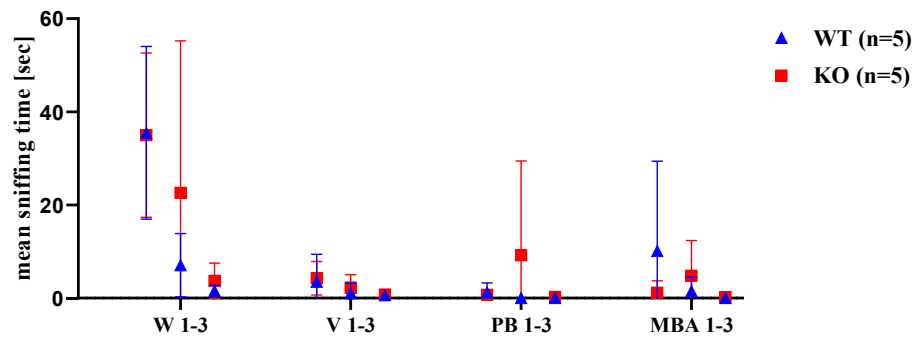


Fig. 26: Mean sniffing time and SD at presented odours in the odour habituation/dishabituation test. Each odour was presented three times (W=water, V=vanilla, PB=peanutbutter and MBA=methylbutyric acid)

4. Discussion

The goal of this study was to screen for possible behaviour and learning abnormalities that could be associated with the absence of LRIG2 and relate these to specific brain regions, thereby better understanding the role of LRIG2 in the brain. As we performed our study with a small sample size, the statistical power to detect differences was low. In addition, as we only investigated females the general applicability of our results is limited and future research with bigger sample sizes are required. Nevertheless, the differences we found between LRIG2-KO mice and their wildtype littermates could point future research to brain areas which might be affected by the absence of LRIG2.

4.1 Anxiety, mental state and locomotor ability

We used three tests to assess anxiety like behaviour in our animals. The elevated plus maze, the three-chamber social interaction test and the open field test. The open field test indirectly assesses anxiety like behaviours in recording exploration. In addition, we used this test to investigate locomotor ability.

We found that KO mice spent more time in the closed arms of the elevated plus maze and moved less overall which could imply higher levels of anxiety in KO mice than in their wildtype littermates. Paradoxically, KO mice also spent more time in the centre of the open field test compared to their wildtype littermates, which could be interpreted as being less anxious.

Even though these results might seem contradictory at first glance, there might be a functional explanation. The aversive stimulus in the elevated plus maze is much greater than the one in the open field test. Therefore, we expect the results of the elevated plus maze to be a better indicator for anxiety. The aversive stimulus in the open field might have been too weak to elicit anxiety like behaviour in the mice, and spending more time in the centre of the open field might rather indicate poor vigilance behaviour in KO mice. This interpretation would be in line with our observation of the general behaviour of the KO mice, which seem to be duller and less alert than their wildtype littermates. As we found no difference in the distance covered in the open field test, we assume that KO mice have comparable locomotor abilities to their wildtype littermates and no obvious motoric impairments.

The results of the three-chamber social interaction test were surprising, as KO mice and their wildtype littermates behaved differently than expected. Neither group spend more time with the novel subject as predicted by the test. However, as both groups showed the same response, we assume that there is no difference between them and further studies would be required to investigate differences in social anxiety between mice.

4.2 Sensory-motor gating

Sensory-motor gating was evaluated with the startle and prepuls-inhibition test.

KO and wildtype mice were equally responsive in the initial startlepuls trials, revealing that they do not differ in their ability to startle. However, prepuls-inhibition was clearly reduced in KO mice, suggesting that LRIG2 plays an important role in prepuls-inhibition. Prepuls-inhibition is commonly associated with the following forebrain areas: thalamus, striatum, globus pallidus, hippocampus, insula, and the temporal, parietal and frontal cortices as well as the Amygdala (49, 50). Thus, LRIG2 might be important in some or all these brain regions for proper function and future studies are required to assess how LRIG2 affects sensory-motor gating.

4.3 Memory and learning

We performed the Morris water maze, Y-maze and Fear conditioning test to investigate learning and memory in LRIG2-KO mice.

In the Morris water maze LRIG2-KO mice did not show consistent improvement in finding the hidden platform over the five training days leading up to the probe trials other than their wildtype littermates. In the short-term probe trial (platform was removed and mice were tested immediately), KO mice took longer to reach the target area where the platform used to be and spend less time in the correct quadrant compared to their wildtype littermates. In the long-term probe trial (platform was removed and mice were tested two days later), as well as in both reverse water maze probe trials, no more differences in the performance between LRIG2-KO and their wildtype controls were found.

Our results could indicate cognitive impairments in LRIG2-KO mice. Though alternatively, the poorer performance of the KO mice in the short-term probe trial could be explained by

their reduced body size as this might have impacted their swimming abilities. Given that we found no difference in the performance of KO and wildtype mice in the long-term probe trial and in the reversed learning trials, it is unlikely that the difference found in the short-term probe trial would solely be explained by impaired swimming abilities. Additionally, our finding could be related to the fact that the task is physiologically more challenging for KO mice due to their relatively bigger body surface. This would be in line with our observation that KO mice took longer to recover under the heating lamp after each trial than their wildtype controls. Additional tests are required to clarify whether KO mice show reduced spatial learning abilities.

In the Y-maze there was no difference in the total number of arm entries. Therefore, we were able to compare spontaneous arm alternations between KO and wildtype mice which is used as an indicator for working memory. As there was no difference in spontaneous arm alternations between groups, we assume that LRIG2-KO mice have no saliences in working memory. Brain regions usually associated with working memory are the fronto-parietal brain regions, including the prefrontal, cingulate, and parietal cortices. Additionally, recent studies suggest a role of subcortical regions (such as the midbrain and cerebellum) in working memory(51). Therefore, our results could imply that these regions are not be affected by the absence of LRIG2.

Finally, in the fear conditioning test, we found no differences in fear acquisition and contextual fear between LRIG2-KO mice and their wildtype littermates. However, when cued fear was tested LRIG2-KO mice responded stronger, i.e. with more freezing behaviour than their wildtype controls without any habituation between conditioned stimulus presentations.

As contextual fear is dependent on the hippocampus these results suggest that the hippocampus function in LRIG2-KO mice might not be affected by the absence of LRIG2. Cued fear is independent of the hypothalamus and mostly dependent on the amygdala(44), therefore the difference in the cued fear test suggests that LRIG2-KO mice might have alternations in the amygdala affecting emotional learning.

4.4 Olfactory sense and memory

Unfortunately, the olfactory habituation and dishabituation test did not work as expected. Mice spent most time at the cotton swab when water was presented and almost no time at other odour presentations. The high interest in the water cotton swabs can most likely be explained by the interest in the novel object rather than the associated odour. Additionally, the sniffing duration did not decline with consecutive odour presentations or increase when new odours were presented. Therefore, no habituation and dishabituation occurred in either group.

4.5 Histopathology

Histopathological analysis of various brain regions revealed no structural differences between LRIG2-KO mice and their wildtype littermates suggesting that behavioural differences are likely explained by differences in the signaling pathways, which are not yet fully studied.

4.6 Closing remarks

We aimed to investigate the effect of LRIG2 in learning processes and in the brain. We used ubiquitous LRIG2-KO mice for our study and given that LRIG2 is present in multiple tissues in the body, we cannot exclude that any effects in the brain are enforced or overwritten by the fact that LRIG2 was ubiquitously absent in the body. Furthermore we only investigated one sex and a small sample size, thus it is possible that we missed differences between LRIG2-KO and their wildtype littermates. Still, our findings suggest that the ubiquitous KO of LRIG2 in mice leads primarily to impairment in amygdala dependent processes and that hippocampus dependent learning and memory is not affected, at least in females. Furthermore, as no differences were found in histopathological analysis our data suggest that these impairments are not of structural nature.

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