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Emotional disorders in adult mice heterozygous for the transcription factor *Phox2b*

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HIGHLIGHTS

- *Phox2b* is essential for the development of the autonomic nervous system.
- *Phox2b* is not expressed above the pons nor in the cerebellum.
- Mice heterozygous for *Phox2b* show mild and transient autonomic disorders at birth.
- Adult mice heterozygous for *Phox2b* also show anxiety-related behaviors.
- Mild postnatal autonomic disorders may disturb long-term emotional development.

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ABSTRACT

Phox2b is an essential transcription factor for the development of the autonomic nervous system. Mice carrying one invalidated *Phox2b* allele (*Phox2b*$^{+/-}$) show mild autonomic disorders including sleep apneas, and impairments in chemosensitivity and thermoregulation that recover within 10 days of postnatal age. Because *Phox*2b is not expressed above the pons nor in the cerebellum, this mutation is not expected to affect brain development and cognitive functioning directly. However, the transient physiological disorders in *Phox2b*$^{+/-}$ mice might impair neurodevelopment. To examine this possibility, we conducted a behavioral test battery of emotional, motor, and cognitive functioning in adult *Phox2b*$^{+/-}$ mice and their wildtype littermates (*Phox2b*$^{+/+}$). Adult *Phox2b*$^{+/-}$ mice showed altered exploratory behavior in the open field and in the elevated plus maze, both indicative of anxiety. *Phox2b*$^{+/-}$ mice did not show cognitive or motor impairments. These results suggest that also mild autonomic control deficits may disturb long-term emotional development.

1. Introduction

Phox2b is a transcription factor essential for the successful development of the autonomic nervous system [32]. Its expression predominates in the afferent and efferent pathways of respiratory, cardiovascular, and digestive reflexes [10,30–32]. In humans, *Phox2b* mutations are associated with Congenital Central Hypoventilation Syndrome (CCHS), a rare disease characterized by hypoventilation during sleep and markedly reduced ventilatory and arousal responses to hypercapnia that requires mechanical ventilation [17]. In most cases, patients with CCHS are heterozygous for a polyalanine repeat expansion mutation in the *Phox2b* gene (most frequently seven alanine expansion of the normal twenty-residue polyalanine tract). A relatively large subgroup of children with CCHS present neurocognitive disorders [17].

The role of *Phox2b* in physiological functions at organism level has mostly been investigated through genetic mouse models. Knock-in mutant mice carrying the +seven alanine heterozygous *Phox2b* mutation presented the main symptoms of CCHS [13]. These mutant mice died within a few hours following birth. Null mutant (knock-out) *Phox2b*$^{-/-}$ mice died in utero. In contrast, mice carrying one invalidated *Phox2b* allele (*Phox2b*$^{+/-}$) survived and were fertile, despite sleep apneas [14] and chemosensitivity reduction [10,36,37] and thermoregulatory impairments [35] that recovered within 10 days of postnatal age [10]. Because *Phox2b* is not expressed above the pons nor in the cerebellum, these regions were expected to develop normally in *Phox2b*$^{+/-}$ mice [20] and to ensure normal cognitive functioning. As a matter of fact, *Phox2b*$^{+/-}$ mice were not distinguishable from their wildtype littermates on gross behavioral observation.

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However, the behavioral phenotype of *Phox2b*$^{+/-}$ mice has never been systematically examined. It may be affected in *Phox2b*$^{+/-}$ mice for two reasons. Firstly, postnatal respiratory disorders such as sleep apneas, even during a short period, are considered a risk factor for neurological impairment in humans, especially in preterm infants [4,24]. In keeping with this, newborn rodents exposed to chronic intermittent hypoxia – an experimental surrogate of sleep apneas of infancy – showed cognitive impairments in adulthood [11,12,25,38]. Secondly, previous studies have shown that *Phox2b*$^{+/-}$ newborn mice displayed abnormal 5-HT metabolism compared to *Phox2b*$^{+/+}$ mice [35], a feature frequently associated with cognitive or emotional disorders [16]. In the present study, we hypothesized that adult *Phox2b*$^{+/-}$ mice would display behavioral deficits. To test this, we examined the behavioral phenotype of adult *Phox2b*$^{+/-}$ mice using motor, emotional, and cognitive tests.

2. Material and methods

2.1. Animals

Methods for generation and genotyping of *Phox2b*$^{+/-}$ mutant mice have been described in detail by Pattyn et al. [32]. Mating of seven female *Phox2b*$^{+/+}$ mice with *Phox2b*$^{+/-}$ males (all maintained in B6D2 background) yielded 24 *Phox2b*$^{+/-}$ (13 females and 11 males) and 15 *Phox2b*$^{+/+}$ (8 females and 7 males) mice. All behavioral tests were performed in the Laboratory of Biological Psychology (KU Leuven). Behavioral assessment started when animals were 8–10 weeks old. Animals were group-housed in mixed genotype groups in standard animal cages in a temperature (22 °C) and humidity controlled animal room with a 12 h light/dark cycle (lights on at 8:00 am). Water and food were available ad libitum. All behavioral tests took place during the light phase of the day. Experiments were carried out in accordance with the European Community Council Directive of 24 November 1986 (86/609/EEC) and experimental protocols were approved by the Animal Experiments Committee of KU Leuven. Tests were conducted in a specific order (Supplementary Table S1), starting with tests with a relatively low stress level building up to tests with a higher stress level [7,28]. All experiments were conducted by a single experimenter who was blind for the grouping variable (genotyping was performed only after the completion of all behavioral tests).

2.2. Behavioral tests

2.2.1. Open field

Mice were dark adapted for 30 min prior to testing. Mice were placed individually into a 50 × 50 cm square brightly illuminated (465 lx) arena. After a 1 min habituation period, the exploratory behavior of the animal was tracked using Ethovision video tracking equipment and software (Noldus, The Netherlands) for 10 min. The total distance traveled in the arena, velocity, frequency and duration of center visits as well as rearing behavior were registered.

2.2.2. Elevated plus maze

The apparatus consisted of a plus-shaped maze that was lifted 30 cm from the table surface. Two of the four arms (21 × 5 cm) were closed while the other two arms had no walls or roof. Animals were placed at the center of the maze and their exploratory behavior was recorded for 10 min by means of five infrared beams (four for arm entries and one for the open-arm) connected to a custom activity logger. The total number of arm visits was measured, as well as the percentage open arm visits (number of open arm visits/total number of arm visits), the ratio between the number of open and closed arm visits, and the time spent in the open arms. One animal (*Phox2b*$^{+/-}$) was excluded from the analysis because one arm of the maze was not properly closed during the recording.

2.2.3. Fear conditioning

Passive avoidance learning was evaluated in a custom apparatus consisting of a small, lit compartment and a dark compartment containing a grid floor, separated by a sliding door as previously described [5]. The experiment took place in a dark room and mice were dark-adapted 30 min prior to training and testing. In the training trial, mice were placed in the lit compartment. After 5 s, the sliding door to the dark compartment was opened, and the latency to entry in the dark compartment was measured. The sliding door was closed upon entry (four paws on the grid) and a mild foot shock (0.3 mA, 2 s) was delivered, using a constant current shocker (Med Associates Inc., Vermont, USA). Retention was measured 24 h later. The same procedure was followed and latency to enter the dark compartment was measured up to a 300 s cut-off value. Three animals (two *Phox2b*$^{+/+}$ and one *Phox2b*$^{+/-}$) were excluded from the analysis because they showed an extreme latency to entry (>90 s) during the training phase.

Context- and cue-dependent fear conditioning was assessed by studying freezing responses as previously described [5,9]. The contextual fear conditioning protocol was carried out during three consecutive days. During each phase, freezing behavior was registered by means of a sensitive Weight Transducer System (Panlab, Spain). On the first day, animals were allowed to habituate to the testing chamber (StartFear cage, Panlab, Spain) with black walls and a grid floor for 5 min. On the second day, after 2 min of exploration (baseline score), a tone (buzzer) was delivered for 30 s. This auditory stimulus served as the conditioned stimulus (CS, 30 s, 4 kHz, 80 dB) and co-terminated with the unconditioned stimulus (US), a 2 s 0.3 mA foot shock (delivered by a constant current shocker from Med Associates Inc., Vermont, USA). A second tone-shock (CS-US) pairing (shock scores) was delivered after a 1 min interval. The trial ended 1 min after the second CS-US pairing. On the third day (24 h later), mice were returned to the testing chamber (same context) for 5 min (context score). Afterwards, mice were returned to the home cage for 90 min. Then, animals were placed in a different context (white paper box, different lighting and odor) in the StartFear box for 6 min. After 3 min (preCS score), the CS was presented for 3 min (CS score). The percentage freezing was calculated for each experimental phase (baseline–shock–context–preCS–CS).

2.2.4. Spatial learning and memory in the water maze

Spatial learning and memory performance were assessed in a standard hidden-platform Morris water maze protocol. The water maze consisted of a large circular pool (150 cm diameter, 32.5 cm height) that was filled with 16 cm of water at 26 °C. A circular escape platform (15 cm diameter, height 15 cm) was hidden in a fixed position 1 cm below the water surface. Using non-toxic white paint the water was made opaque to prevent animals from seeing the platform. To locate the platform, animals thus had to rely on spatial information. Visual cues, such as posters, computer, and furniture, were available to the animals. Also, the position of the experimenter during swimming trials remained constant.

Mice were trained during 10 acquisition days (Mondays through Fridays, weekends at rest) to locate the hidden platform. Four trials were performed per acquisition day (intertrial interval: 15 min) from four fixed starting positions (the order of the starting positions was randomly altered each day). Mice that failed to reach the platform within 2 min were gently guided towards the platform where they were allowed to visually explore the surroundings for 15 s before being returned to the home cage. Ethovision video tracking equipment and software (Noldus, The Netherlands) were used to calculate escape latency, path length and swimming velocity. After five acquisition days there were two resting days. The following day (Monday morning before continuation of acquisition trials), a probe trial was conducted. During these probe trials, the platform was removed from the pool and the search pattern of the mouse was recorded for 100 s. Path length, swimming velocity, time spent in each quadrant, latency to the first entrance in the target quadrant and target position were calculated.