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Research report

Behavioral characterization of female zinc transporter 3 (ZnT3) knockout mice

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HIGHLIGHTS

- Female ZnT3 KO mice do not show normal improvement over time at skilled reaching.
- Female ZnT3 KO mice show decreased exploratory locomotion.
- Female ZnT3 KO mice perform normally on many tests of motor skill and cognition.
- Female ZnT3 KO mice do not exhibit the same behavioural deficits previously found in male ZnT3 KO mice.
- It is critical to conduct behavioural testing on mice of both sexes whenever possible.

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ABSTRACT

Zinc is an important element in all cells of the body, having structural, enzymatic, and regulatory functions. In some neurons, zinc is loaded into synaptic vesicles by zinc transporter 3 (ZnT3) and released into the synaptic cleft, where it can modulate neuronal function. ZnT3 knockout (KO) mice lack ZnT3 and thus lack synaptic zinc. Previous studies have examined the behavioral phenotype of ZnT3 KO mice, mostly using mixed-sex or male-only groups. In the present study we focused specifically on the behavior of female ZnT3 KO mice (2–3 months old). An extensive battery of tests was administered to assess sensorimotor and cognitive behaviours, as well as to examine for a possible schizophrenia-like phenotype. ZnT3 KO mice performed similarly to wild type controls in the majority of tests. However, they were less accurate in the skilled reach task, suggesting impaired skilled motor learning, and faster to descend a vertical pole. ZnT3 KO mice were also slower in the open field and made fewer chamber entries in the social preference test, suggesting decreased exploratory locomotion. No differences were observed in the Morris water task or fear conditioning test. This is the first study to show a behavioural phenotype specifically for female ZnT3 KO mice. Comparing our results to previous studies, it appears that there may be sex-specific effects of eliminating ZnT3. Female ZnT3 KO mice exhibit abnormalities in locomotion and at skilled motor learning, but we were unable to detect spatial or fear learning deficits previously described in male ZnT3 KO mice.

1. Introduction

Zinc is an essential metal throughout the body, having structural, enzymatic, and regulatory functions [1]. In the brain, among zinc's other functions, it is loaded into the synaptic vesicles of a subset of glutamatergic neurons by zinc transporter 3 [ZnT3; 2] and subsequently released into the synapse [3,4]. This "synaptic zinc" acts on pre- and post-synaptic neurons by binding to receptors or by entering the cell and affecting second messenger systems; these actions allow synaptic zinc to modulate neuronal function and plasticity [reviewed by 5]. Synaptic zinc is concentrated in many forebrain areas, including the neocortex (particularly layers II/III, V, and VI), hippocampus, amygdala, and striatum [6,7].

In the late 1990s, a transgenic mouse was created that lacks ZnT3 and, as a result, lacks synaptic zinc [2]. These ZnT3 knockout (KO)

Abbreviations: ASD, Autism Spectrum Disorder; KO, knockout; MWT, Morris water task; NIH, novelty-induced hypophagia; PPI, prepulse inhibition; SEM, standard error of the mean; WT, wildtype; ZnT3, zinc transporter 3.

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mice were initially tested on a battery of behavioral tests, including tests of olfaction, audition, motor coordination, nociception, and anxiety, as well as tasks requiring different types of learning and memory, such as fear memory and spatial memory. Surprisingly, ZnT3 KO mice were found to perform no differently than ZnT3 wild type (WT) mice on these tests [8], suggesting that synaptic zinc does not play an important role in the normal expression of these behaviors. This conclusion, however, has been challenged by more recent findings. Using weaker or more complex fear conditioning paradigms, ZnT3 KO mice have been shown to be mildly deficient at learning to associate an auditory tone or environmental context with a noxious foot shock [9] and are impaired at contextual discrimination [10]. They also appear to have difficulty on tasks that require behavioral flexibility, such as alternation in a T-maze [10] and the moving-platform version of the Morris water task (MWT) [11]. At an advanced age (6 months), ZnT3 KO mice perform more poorly than WT controls in the fixed-platform MWT [12], suggesting that a lack of synaptic zinc makes these mice more susceptible to the cognitive decline that occurs with age.

One shortcoming of the behavioral research that has been conducted to date on ZnT3 KO mice is a lack of focus on females. Some experiments have been conducted using only male mice [9,11]. Both male and female mice have been tested in other studies [8,12], including the initial behavioral evaluation of the ZnT3 KO mice. However, in these cases the sexes were not evaluated independently but were, instead, analyzed as mixed-sex groups, precluding the detection of any sex differences or sex-specific effects (and increasing within-group variability, to the extent that any such effects might have been present). In the case of one study, the sex of the mice used was not reported at all [10], leading one to assume that male mice were used (as this tends to be the default in rodent behavioral research) or perhaps mixed-sex groups. The importance of putting an equal focus on male and female animals has garnered increasing attention in the field of neuroscience research [e.g.,13] and is now strongly encouraged by many grant funding agencies. This is particularly important in the present context, as numerous studies have shown that male and female rodents sometimes perform differently on behavioral tests, including tests of motor behavior [reviewed by 14], anxiety-like behavior, and cognition [reviewed by 15]. Further, recent research in humans suggests that altered function of ZnT3 may have sex-specific effects, as single-nucleotide polymorphisms in *SLC30A3*, the gene that encodes ZnT3, were found to be associated with increased likelihood of having schizophrenia in females but not males [16]. Finally, estrogen is a negative modulator of ZnT3 protein levels and hippocampal synaptic zinc levels [17], providing a mechanism that might underlie sex differences in ZnT3-dependent functions.

Given the lack of focus to date on female ZnT3 KO mice, the purpose of the present study was to examine the behavior, specifically, of these animals, using tests that have so far only been applied to male mice or mixed-sex groups, as well as tests that have not previously been applied to ZnT3 KO mice at all. The tests were divided into three batteries, each focused on a different objective. The first battery of tests was designed to assess general and skilled motor function. In particular, we were interested in the function of the striatum, which plays a role in motor learning [18], among other things. Notably, the striatum contains a large amount of synaptic zinc [19], though the function of synaptic zinc in the striatum is not yet known. The second battery was intended primarily to assess whether female ZnT3 KO mice exhibit a schizophrenia-like behavioral phenotype, given the finding of Perez-Becerril et al. [16] that polymorphisms of *SLC30A3* increase the risk for schizophrenia specifically in females. This battery included tests of exploratory behavior, emotionality, sociability, and sensorimotor gating. Altered locomotor activity, decreased preference for social interaction, and impaired sensorimotor gating are all phenotypes in mice that have potential relevance to human schizophrenia [20]. The aim of the third test battery was to assess female ZnT3 KO mice on tests that have previously been used to demonstrate minor behavioural abnormalities in male mice or mixed-sex groups. These included tests of spatial cognition and fear memory [8,9,11].

2. Methods

2.1. Animals

All procedures were approved by the Life and Environmental Sciences Animal Care Committee of the University of Calgary and conformed to the guidelines set out by the Canadian Council on Animal Care. Mice were housed in plastic cages ($28 \times 17 \times 12$ cm) with woodchip bedding, paper nesting material, and one enrichment object. The cages were kept in a temperature- and humidity-controlled room (22 °C; 25–30% humidity) under a 12:12 h light/dark cycle (lights on during the day), and the mice had ad libitum access to food (LabDiet Mouse Diet 9F, #5020) and tap water except as noted below. The female ZnT3 WT and KO mice used for this study were on a mixed C57BL/6 × 129/SvEv genetic background and were bred from heterozygous pairs. The mice were weaned on postnatal day 21 and group-housed (2–5 mice per cage) with same-sex littermates. The mice were 2–3 months old at the time of behavioral testing.

2.2. Behavioral testing

All behavioral testing was conducted during the mice's light phase. The mice were tested in three cohorts, each of which was subjected to a different battery of tests. This was done in order to limit the length of time over which behavioural testing was conducted and the number of tests each mouse was subjected to, and to decrease order effects and possible effects of extensive handling. The first cohort ($n = 14$ WT, 12 KO) was subjected primarily to motor tests. The second cohort ($n = 10$ WT, 12 KO) was subjected to a more diverse battery that included tests of locomotion, anxiety-like behavior, sociability, and sensorimotor gating. The third cohort ($n = 13$ WT, 11 KO) was subjected to cognitive tests for spatial and fear memory. For each test battery, the tests were conducted in the order presented below. Fig. 1 shows where each test fits in terms of what the test is assessing (sensorimotor function, emotionality, or learning, memory, and cognition).

2.2.1. Test battery 1

All tasks, except for footprint analysis, were recorded for offline scoring using a digital video camera (Sony HDR-SR8; 60 fps). Mice were acclimatized to the testing room for 1 h per day over the 3 days prior to the start of the battery.

2.2.1.1. Skilled reach task. Mice were food-restricted for the skilled reach task. During food restriction, the mice were given enough food to maintain 85–90% of their body weight (2 g of food/animal/day). The food was provided after the testing session for the day was completed. Sugar pellets (0.2 g/animal/day; Noyes Precision Formula F sucrose pellets, Lancaster, NH) were given alongside the food.

The skilled reach task is a test of motor learning and fine motor skills. There are also motivation and reward aspects to the test. Mice were placed in a modified Plexiglas reaching box (13 cm wide × 15 cm long × 40 cm high) and trained to reach through a slit (1 cm wide) in the box to retrieve a sugar pellet on a ledge (1.5 cm above ground) in front of the slit. Mice were habituated to the sugar pellets by placing them in their home cages for 5 days prior to training. Mice were habituated to the reaching box over 3 days. The first day mice were placed in littermate pairs in the reaching box for