ABSTRACT
Connexin 30 (Cx30; also known as Gjb6 when referring to the mouse gene) is expressed in ependymal cells of the brain ventricles, in leptomeningeal cells and in astrocytes rich in connexin 43 (Cx43), leading us to question whether patients harboring GJB6 mutations exhibit any brain anomalies. Here, we used mice harboring the human disease-associated A88V Cx30 mutation to address this gap in knowledge. Brain Cx30 levels were lower in male and female Cx30A88V/A88V mice compared with Cx30A88V/+ and Cx30+/+ mice, whereas Cx43 levels were lower only in female Cx30 mutant mice. Characterization of brain morphology revealed a disrupted ependymal cell layer, significant hydrocephalus and enlarged ventricles in 3- to 6-month-old adult male and female Cx30A88V/A88V mice compared with Cx30A88V/+ or Cx30+/+ sex-matched littermate mice. To determine the functional significance of these molecular and morphological changes, we investigated a number of behavioral activities in these mice. Interestingly, only female Cx30A88V/A88V mice exhibited abnormal behavior compared with all other groups. Cx30A88V/A88V female mice demonstrated increased locomotor and exploratory activity in both the open field and the elevated plus maze. They also exhibited dramatically reduced ability to learn the location of the escape platform during Morris water maze training, although they were able to swim as well as other genotypes. Our findings suggest that the homozygous A88V mutation in Cx30 causes major morphological changes in the brain of aging mice, possibly attributable to an abnormal ependymal cell layer. Remarkably, these changes had a more pronounced consequence for cognitive function in female mice, which is likely to be linked to the dysregulation of both Cx30 and Cx43 levels in the brain.

KEY WORDS: Connexin, Connexin 30, Gap junctions, Mice, Mutant

INTRODUCTION
Understanding how connexin dysregulation and gene mutations lead to disease is complex, given that there are 21 family members in humans and that most cells express two or more of these channel-forming proteins (Sohl and Willecke, 2004). The canonical role of connexins in most cells is to assemble into hexameric channels that traffic to the cell surface and dock to connexin hexamers from an adjoining cell to create gap junction (GJ) channels (Laird, 2006). The resulting GJ channels allow for the selective passage of numerous small molecules, ranging from ions to secondary messengers to metabolites, in a process called gap junctional intercellular communication (GJIC) (Goldberg et al., 2002). In some cases, particularly in pathologies, connexin hexamers can function as hemichannels at the cell surface, allowing for highly regulated small molecule exchange between the cytoplasm and the extracellular environment (Goodenough and Paul, 2003; Leybaert et al., 2017; Srinivas et al., 2018). Often, both GJ and hemichannel function become dysregulated when connexin gene mutations produce mutant connexins that exhibit gain-of-function or loss-of-function properties, providing a molecular basis for disease (Kelly et al., 2015; Kuang et al., 2020; Laird, 2008; Laird and Lampe, 2018; Srinivas et al., 2018). At present, there are nearly 30 diseases, which present in multiple organs, linked to gene mutations in no less than half of the connexin gene family (Laird et al., 2017). Although skin disorders constitute nearly a dozen of these diseases (Srinivas et al., 2018), it remains extremely difficult to predict where and when a connexin gene mutation will present as a pathological anomaly in a tissue manifesting in disease.

Connexin 30 (Cx30; also known as Gjb6 when referring to the human gene) is best understood as one of several connexins found in the epidermis of the skin and exhibits plentiful expression in the organ of Corti in the inner ear (Di et al., 2001; Forge et al., 2003). Accordingly, mutations in the human gene (GJB6) lead to skin disease and hearing loss (Common et al., 2002; Smith et al., 2002). In the skin, GJB6 mutations result in the rare autosomal dominant disorder hidrotic ectodermal dysplasia 2 (HED2), also known as Clouston syndrome (Avshalumova et al., 2013; Fraser and Der Kaloustian, 2001; Smith et al., 2002). HED2 is characterized by thinning wiry hair, patches of alopecia, hyperkeratosis of the palms, which take on a cobblestone appearance, and nail dystrophy (Fraser and Der Kaloustian, 2001; Lamartine et al., 2000). In humans, no less than four amino acid changes in Cx30 are linked to HED2 (Baris et al., 2008; Jan et al., 2004; Smith et al., 2002). In the skin, GJB6 mutations result in the rare autosomal dominant disorder hidrotic ectodermal dysplasia 2 (HED2), also known as Clouston syndrome (Avshalumova et al., 2013; Fraser and Der Kaloustian, 2001; Smith et al., 2002). HED2 is characterized by thinning wiry hair, patches of alopecia, hyperkeratosis of the palms, which take on a cobblestone appearance, and nail dystrophy (Fraser and Der Kaloustian, 2001; Lamartine et al., 2000). In humans, no less than four amino acid changes in Cx30 are linked to HED2 (Baris et al., 2008; Jan et al., 2004; Smith et al., 2002). In the organ of Corti, Cx30 is assembled into large gap junctions in the diverse cell network that supports hair cell survival, and several GJB6 gene mutations have been documented to cause hearing loss (Berger et al., 2014; Common et al., 2002). GJB6 is also expressed in the brain, raising the possibility that patients harboring heterozygous GJB6 gene mutations might suffer from cognitive deficiencies that remain clinically unreported or become evident only during aging.

The most studied of the GJB6 disease-causing mutations has been a mutant in which alanine at position 88 is substituted with a
valine (p.A88V) (Bosen et al., 2014; Kelly et al., 2019; Lukashkina et al., 2017; Zhan et al., 2020). The autosomal dominant p.A88V mutation in Cx30 is considered to be the cause of HED2, because it has been identified in no less than seven families encompassing at least 15 individuals (Essenfelder et al., 2004; Smith et al., 2002; Zhang et al., 2003). The p.A88V mutant appears to assemble into leaky hemichannels, which disrupts Ca²⁺ and ATP homeostasis found in the skin (Essenfelder et al., 2004; Kuang et al., 2020).

In order to understand the consequences of the A88V mutation in vivo, Cx30-A88V knock-in mice were generated (Bosen et al., 2014). These mice exhibited oversized sebaceous glands and relatively mild palmoplantar hyperkeratosis (Bosen et al., 2014). In a recent elegant study, Kuang et al. (2020) developed a novel anti-Cx30 antibody that could reverse the skin pathology mediated by leaky hemichannels in homozygous Cx30-A88V mutant mice (Kuang et al., 2020), raising the profile of potential connexin-targeted therapeutics. Homozygous mice harboring the A88V mutation exhibited a second pathology of low-frequency hearing loss (Bosen et al., 2014). Intriguingly, these same mutant mice were protected from high-frequency, age-related hearing loss, suggesting that dysregulated Cx30 in the cochlea can provide a physiological advantage (Bosen et al., 2014). Further characterization of the hearing loss protection found in Cx30-A88V mice revealed that age-dependent outer hair cell loss was greatly reduced in mutant mice (Bosen et al., 2014; Kelly et al., 2019; Lukashkina et al., 2017). Notably, Cx30 is amply expressed in astrocytes, ependymal cells, leptomeningal cells and brain pericytes (Abudara et al., 2014; Dere et al., 2003; Mazare et al., 2018; Nagy et al., 2001) (see also http://mousebrain.org/genesearch.html). This raised the question as to whether Cx30-A88V mutant mice might develop additional morbidities during aging related to brain development and function.

Here, we investigated Cx30 in the brains of 3- to 6-month-old male and female Cx30-A88V mutant mice and compared them with littermate controls. We found that homozygous Cx30-A88V mice exhibited lower levels of Cx30 and sex-dependent effects on co-expressed connexin 43 (Cx43). Homozygous mutant mice presented with increased brain weight, increased ventricular size and hydrocephalus. Strikingly, despite similar structural brain changes, homozygous female mutant mice exhibited worse behavioral outcomes, including deficits in learning. These experiments illuminate how mutant Cx30 can impact the mammalian brain and contribute to pathology.

RESULTS

Female mutant mice exhibit greater reductions in connexin levels compared with males

Both Cx30 and Cx43 are expressed in the brain, most notably as the connexins forming gap junctions between astrocytes, although Cx43 is far more plentiful compared with Cx30 (Nagy et al., 2001). To investigate whether the presence of the A88V mutation in Cx30 affected either Cx30 and/or Cx43 levels in the brains of 3- to 6-month-old mutant mice, their expression and localization were assessed. In males, Cx30 mRNA levels were decreased in Cx30A88V/A88V compared with wild-type (WT) mice (Fig. 1A), whereas Cx43 mRNA levels were similar between groups (Fig. 1B). In female mice, however, Cx30 mRNA levels were lower in Cx30A88V/+ mice and further reduced in Cx30 A88V/A88V mice compared with WT mice (Fig. 1C). Furthermore, both heterozygous and homozygous mutant female mice displayed reduced Cx43 mRNA levels compared with WT mice (Fig. 1D). At the protein level, Cx30 was low in Cx30A88V/A88V mice (Fig. 2A,B), although Cx43 protein expression was unchanged between the male mouse genotypes (Fig. 2A,C). In female mice, Cx30 was also less abundant in Cx30 mutant mice compared with WT mice (Fig. 2B,D), and Cx43 was statistically less abundant only in Cx30A88V/+ mice compared with WT mice (Fig. 2D).

Homozygous mutant mice develop hydrocephaly

To determine whether the alterations in Cx30 and Cx43 affected the brain of aging mice, we examined brain weight and gross morphology in Cx30+/+, Cx30A88V/+ and Cx30A88V/A88V male and female adult mice (Fig. 3A). Analysis of coronal brain sections demonstrated
enlarged ventricular areas (indicative of volume changes) in both male and female Cx30\(^{A88V/A88V}\) mice compared with sex-matched WT and heterozygote mutant mice (Fig. 3B,C). Furthermore, both male and female Cx30\(^{A88V/A88V}\) mouse brain weights were significantly greater than those found in Cx30\(^{+/+}\) or Cx30\(^{A88V/+}\) mice, despite similar body weight (Fig. 3D,E). These findings clearly indicate that Cx30\(^{A88V/A88V}\) mice had developed hydrocephaly.

Given that Cx30 has been shown to be expressed in ependymal cells lining the brain ventricles (Kunzelmann et al., 1999), and these cells contribute to cerebral spinal fluid production, we assessed whether Cx30 and Cx43 levels and localization were changed in ependymal cells of mutant mice (Fig. 4). In both male (Fig. 4A) and female (Fig. 4B) mouse brains, Cx30 was detectible as green puncta along the ependymal cells lining the ventricles (denoted by asterisks), but to a much lesser extent than the plentiful levels of Cx43. Owing to the low level of Cx30 detected and the loss of a clearly intact ependymal cell layer of the ventricles (Fig. 4), it was difficult to determine convincingly whether homozygous mutant mice had less Cx30 compared with WT mice, although our western blot analysis might suggest that this was the case in female mutant mice. However, it is important to recognize that the level of Cx30 might also be reduced in astrocytes and leptomeningeal cells of mutant mice, but this is not possible to discern clearly in these immunofluorescence images.

To assess the localization of Cx43 and Cx30 in the whole brains of male and female WT and mutant mice, coronal sections were double immunolabeled for connexin and glial fibrillary acidic protein (GFAP) to demarcate the location of astrocytes (Fig. 5). Although Cx43 was abundant, little Cx30 immunoreactivity was detected in astrocytes from male (Fig. 5A) or female (Fig. 5B) mice. Hence, the Cx30 localization and expression patterns in brain tissue were generally concurrent with the reduced Cx30 mRNA and protein levels seen in homozygous mutant mice.

Behavioral studies as a measure of cognitive function
Given that Cx30\(^{A88V/A88V}\) mice exhibited sex-dependent changes in Cx43 and enlarged ventricles surrounded by an apparently disrupted ependymal cell layer, it was important to determine whether heterozygous mutant mice, which model human disease, or homozygous mutant mice present with functional behavioral deficits. To assess general locomotor behavior, mice were examined using the open field test. All male mouse genotypes traveled similar distances over 30 min in an open environment (Fig. 6A,C). Interestingly, however, homozygous female mutant mice displayed more variability in locomotor activity and greater total distance traveled, suggesting increased exploratory behavior in a new environment (Fig. 6B,D).
To test the behavior of Cx30 mutant mice further, they were studied with the elevated plus maze (Fig. 7). All male mice spent the most time in the closed arm and far less time in each of the open arms (Fig. 7A). Notably, despite greater variability than WT and Cx30A88V/+ mice, female Cx30A88V/A88V mice spent significantly more time in the open arms and less time in the closed arms (Fig. 7B), supporting the notion that Cx30A88V/A88V female mice exhibit increased exploratory behavior, even in a threatening environment (open arms).

Lastly, male and female mice were subjected to the Morris water maze to assess spatial learning. For the cued test, where the position of the escape platform was clearly marked with a mounted flag, both male and female homozygous mutant mice took longer to reach the platform (Fig. 8A,B). However, in the case of the female homozygous mutant mice, statistical significance was achieved only when compared with heterozygous mutant mice, probably owing to a wide range of variance in the WT control group (Fig. 8B). During the spatial acquisition phase, on average, all male genotypes gradually reduced the time to escape over the 4 days of testing. However, Cx30A88V/A88V males exhibited longer escape times at days 2 and 3 compared with Cx30+/+ and Cx30A88V/+ mice (Fig. 8C). On day 4, however, male homozygous mutant mice demonstrated escape times similar to those of WT and heterozygous mutants (Fig. 8C). In female mice, both WT and heterozygous mutants exhibited decreasing escape time over the 4 days of testing (Fig. 8D), whereas homozygous mutants did not show any evidence of learning (Fig. 8D). During the spatial learning task, all mice demonstrated similar swimming speeds, suggesting that delayed learning or lack of learning was not attributable to poor swimming performance (Fig. 8E,F).

**DISCUSSION**

At present, no homozygous GJB6 missense mutations have been identified in the human population. However, heterozygous GJB6 missense mutations do exist in patient cohorts, with variable pathological manifestations that most often affect hearing and skin health, but it is not at all clear whether and how GJB6 mutations affect the brain, where Cx30 is widely expressed (Lamartine et al., 2000; Tan and Tay, 2000). Mutant mice lacking Cx30 demonstrate reduced exploratory activity, with evidence of increased anxiety-
like behavior (Dere et al., 2003), providing a hint that Cx30 might be linked to cognitive functions. To interrogate the nature and breadth of how Cx30 mutations cause disease, a genetically modified mouse line was generated, in which Cx30 p.A88V was expressed in both the homozygous and heterozygous context (Bosen et al., 2014). The GJB6 mutation that results in Cx30 p.A88V is best known to cause HED2, which manifests clinically as poor skin health (Lamartine et al., 2000; Smith et al., 2002). Given the potential impact of this GJB6 mutation on the brain, where Cx30 is known to be expressed in a variety of cell types, we used both heterozygous and homozygous 3- to 6-month-old Cx30-A88V mutant mice (Bosen et al., 2014) to characterize the effect of the A88V mutant on the brain and potential impact on behavioral performance. Furthermore, we investigated both male and female mouse cohorts to assess whether there were unique sex-dependent differences.

Examination of brain morphology revealed hydrocephalus in Cx30A88V/A88V mice, in which the brain weight and ventricles were enlarged in male and female mice compared with heterozygous and WT groups. In male mouse brains, Cx30 levels were lowest in Cx30A88V/A88V mice compared with the other genotypes. In female brains, however, both Cx30A88V+/+ and Cx30A88V/A88V mice demonstrated lower brain levels of Cx30 compared with WT mice, although it was not possible to assign this decrease to any one specific cell type. Interestingly, only female mutant mouse brain tissue exhibited lower levels of Cx43, suggesting that females might have a more severe phenotype, which received support from our behavioral studies. Only female Cx30A88V/A88V mice were also the only group to spend a greater proportion of time in the open arm of the elevated plus maze. Although the elevated plus maze can report on anxiety-like behavior, it is likely that the phenotype in this task is more related to deficits in innate behavior to recognize the threat of the open arm. Both male and female Cx30A88V/A88V mice performed less efficiently in the cued learning task compared with heterozygous and WT groups. These results might suggest that their vision or ability to detect visual cues could also be impaired. Nonetheless, homozygous mutant mice were still able to find the platform, albeit taking a longer time. Remarkably, in the spatial learning test, female Cx30A88V/A88V mice were unable to learn the location of the hidden platform during the acquisition phase. Overall, our findings indicate that the Cx30-A88V mutant impacts the development and health of the male and female mouse brain, but female mice exhibit far greater cognitive deficits.

In the brain, Cx30 is found in astrocytes, but to a much lesser extent than Cx43 (Charvériat et al., 2017). Cx30 is also expressed in the ependymal cell layer separating the brain parenchyma from the ventricle space. Scale bars: 50 μm (20 μm for insets).
cerebral spinal fluid (CSF) space of the ventricles and in leptomeningeal cells surrounding blood vessels in the subarachnoid space (De Bock et al., 2014). Interestingly, these tissues engage in CSF production and circulation (Brinker et al., 2014), which might explain, in part, the hydrocephalus phenotype seen in aging Cx30A88V/A88V mice. Using cellular expression models, our group and others have previously shown that the Cx30-A88V mutant is cytotoxic when ectopically expressed in HeLa cells and keratinocytes (Berger et al., 2014; Essenfelder et al., 2004; Lu et al., 2018), most probably owing to its assembly into leaky hemichannels (Essenfelder et al., 2004; Kuang et al., 2020). This raises the possibility that the homozygous expression of the A88V mutant is causing ependymal cells to malfunction. Ependymal cells are typically coupled via gap junctions with one another and with the surrounding glial cells (Brinker et al., 2014; De Bock et al., 2014). Although the ependymal cell layer of homozygous mutant mice appeared unorganized, Cx43 was still readily observed, suggesting that this cell layer was still somewhat intact, although Cx30 levels were low. We suspect that low Cx30 function might have compounding disruptive effects on normal CSF production and flow in the brain, resulting in hydrocephalus. Astrocytes have also been implicated in governing CSF fluid exchange, in addition to choroid plexus CSF production (Brinker et al., 2014; De Bock et al., 2014), because their processes are interspersed in the ependymal layer and tethered to brain capillaries. In related studies, Cx30/Cx43 astroglial knockout mice also demonstrate reduced aquaporin 4 levels in the brain (Ezan et al., 2012), altering transmembrane water flux (Simard et al., 2003). Although morphological brain anomalies have not been reported in HED2 patients, on occasion patients with connexin 26 (Cx26; also known as GJB2 when referring to the gene)-linked keratitis-ichthiosis-deafness (KID) syndrome present with Dandy-Walker malformation resulting in cystic dilatation of the fourth ventricle and hydrocephalus (Todt et al., 2009), linking connexin status to CSF homeostasis.

Cx30-deficient mice have been characterized in behavioral studies to avoid open field areas (Dere et al., 2003), whereas mice harboring Cx43-deficient astrocytes exhibit greater exploratory behavior (Frisch et al., 2003; Theis et al., 2003). We found that only female Cx30A88V/A88V mice demonstrated greater locomotive activity in the open field test, with a preference for exploring the open arm of the elevated plus maze to the point of even peering over the end of the arm. The reason for this same behavior not occurring in male homozygous mutant mice is not clear, but might be related to the observed reduction in Cx43 expression levels in females. This alteration might affect connection to astrocytes that are extensively coupled by Cx43 gap junctions. The conserved expression of Cx43 in all male genotypes might be enough to sustain astrocyte-dependent communication mechanisms affecting behavioral patterns, regardless of reduced functional Cx30 in the brain of Cx30A88V/A88V mice. The reason why any A88V mutant mice have
Sex differences were also evident, because female homozygous mutant mice presented with a range of behavioral alterations. Whether these alterations occur because female mice are less resilient to the ventricular dysfunction or owing to the compound effect of Cx43 reduction observed in females remains to be determined. Importantly, both male and female heterozygous mutant mice, which model autosomal dominant HED2, did not present with major brain structural or behavioral changes. These results suggest that heterozygous GJB6 mutations might not cause enough deficits to affect cognitive function in humans. By contrast, the major brain phenotypes and the sex-specific connexin differences in mutant mouse brains impacting behavioral outcomes might contribute to the lack of homozygous GJB6 missense mutations in the human population. Nevertheless, our study firmly adds to the growing body of evidence that connexins found in the brain might play crucial roles in cognitive function.

**MATERIALS AND METHODS**

**Mice**  
Cx30ΔASV/ΔASV and Cx30ΔASV/ΔASV mice were generated as previously described (Bosen et al., 2014) and kindly provided by Dr Klaus Willecke (Life and Medical Sciences Institute, Bonn, Germany), bred in a CD-1 background, and genotyped as previously described (Bosen et al., 2014; Kelly et al., 2019; Lukashkina et al., 2017). Three- to 6-month-old male and female Cx30+/− (noted as WT in figures), Cx30ΔASV/− and Cx30ΔASV/ΔASV mice were used in littermate-controlled experiments and housed in the animal care facilities at the University of Western Ontario. Mice were provided with unlimited food and water and maintained in the dark for 12 h each day. Mice were euthanized via CO2 asphyxiation. In keeping with the Canadian Council of Animal Care, all studies were reviewed and approved by the Animal Care Committee at the University of Western Ontario (Protocol 2015-030; 2019-009).

**Histology**  
Brains fixed in 4% paraformaldehyde (PFA) were embedded in paraffin, and 5- to 10-μm-thick sections were collected at 100 μm intervals. After staining with Hematoxylin and Eosin, brain sections were imaged. For all mouse groups (n=3 cohorts), brain ventricle areas were measured using ImageJ and the data expressed relative to the total cross-sectional area of the brain.

**qPCR analysis**  
RNA was collected using Qiagen RNeasy kits (Qiagen) from dissected and flash-frozen whole brains of 3- to 6-month-old male and female mice. Complementary DNA (cDNA) was produced using the first-strand cDNA synthesis kit (SuperScript VILO; Thermo Fisher Scientific). Transcript levels were analyzed using mouse-specific primers (Cx43, 5′-AAATGTC-TGCTATGACAAGTCCTTC-3′ and 5′-CTTTGAGCTCCTCTCTTTC-TTGTT-3′; Cx30, 5′-GGCGGATGTTGTACCCCTG-3′ and 5′-TTCTTTCAGGGCATGTTG-3′; and Gapdh, 5′-CGACTTCACA-GCAACTCCCACCTCTCC-3′, 5′-TGGGTGGTTCAGGTTTCTTACTCCT-3′ and the PowerUp SYBR Green Mastermix (Thermo Fisher Scientific) in a Bio-Rad CFX96 real-time system. Transcripts were normalized to GAPDH mRNA. Normalized mRNA expression levels were analyzed using the ΔΔCt method, which was calculated using Bio-Rad software. A WT mouse sample was set as the control for all calculations, and data were expressed relative to this sample (n=5-7 per group for males, n=4-8 per group for females).

**Western blotting**  
Mouse brain tissue lysates were prepared on ice via homogenization of the whole brain in lysis buffer (150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100 and 10 mM Tris-HCl) containing protease and phosphatase inhibitors (Roche-Applied Sciences; 100 mM NaF and 100 mM Na3VO4). Thirty microgram samples of protein from tissues lysates were resolved on a 10% SDS-PAGE gel and transferred nitrocellulose membranes using an
iBlot Dry Blotting system (Invitrogen). Membranes were blocked in 3% bovine serum albumin-PBS (blocking solution) for 30 min at room temperature. Membranes were immunolabeled using the following primary antibodies: rabbit anti-Cx43 (1:5000; Sigma-Aldrich; C6219); rabbit anti-Cx30 (1:300; Thermo Fisher Scientific; 71-2200) and mouse anti-GAPDH (1:10,000; Santa Cruz Biotechnology; sc-365062), diluted in blocking solution at 4°C overnight. Membranes were washed three times, for 5 min each time, with PBS containing 0.5% Tween 20 and incubated with fluorescence-tagged secondary anti-rabbit Alexa Fluor 680 (1:10,000; LI-COR Biosciences; ab175772) or anti-mouse IRdye 800 (1:10,000; Rockland Immunochemicals; 610-132-003). Quantification of protein expression was performed using an Odyssey Infrared Imaging System and accompanying software for densitometry analysis (LI-COR Biosciences). Samples were quantified after normalization to GAPDH loading controls (n=5-7 per group for males, n=5-8 per group for females).

Immunofluorescence microscopy

Male and female mouse brains were dissected and immersed in 10% formalin at 4°C for ~48 h, washed in PBS, immersed in 30% sucrose and maintained at 4°C for cryopreservation. Brains were washed three times, for 5 min each time, with PBS containing 0.5% Tween 20 and incubated with fluorescence-tagged secondary anti-rabbit Alexa Fluor 680 (1:10,000; LI-COR Biosciences; ab175772) or anti-mouse IRdye 800 (1:10,000; Rockland Immunochemicals; 610-132-003). Quantification of protein expression was performed using an Odyssey Infrared Imaging System and accompanying software for densitometry analysis (LI-COR Biosciences). Samples were quantified after normalization to GAPDH loading controls (n=5-7 per group for males, n=5-8 per group for females).

Experimental design of behavioral studies

Cognitive and sensorimotor function was assessed in two cohorts of mice (57 total, minimum of eight mice from each genotype/sex grouping). Experiments were performed in the following order: open field test, elevated plus maze and spatial and cued Morris water maze. Mice aged 3-6 months were housed with littermates when possible in the rodent neurobehavioral core facility at Robarts Research Institute for at least 1 week before testing. Each day, mice were brought to the laboratory and left unattended in their home cages for at least 20 min before testing. All experiments were performed in the following order: open field test, elevated plus maze and spatial learning phase of the Morris water maze (WT versus A88V; P<0.05, **P<0.01,§§P<0.001).
performed by the same experimenter between 09.00 and 17.00, during the light phase of the light-dark cycle.

Open field test
General locomotor activity was assessed as previously described by Zubrycki et al. (1990). Briefly, mice were placed in diagonally opposite 20×20 cm quadrants of a square arena and left to explore the enclosure freely in the absence of the experimenter. For 30 min, locomotor activity in the x-, y- and z-axes was recorded by the infrared beam detectors of open field locomotor boxes (Omnitech Electronics, Columbus, OH, USA), and data were acquired using VersaMax software (Omnitech Electronics). Enclosure surfaces were thoroughly cleaned with 70% ethanol before each trial to remove debris and scent cues. For each box, two mice were recorded simultaneously, and genotype/sex group pairings were balanced to minimize potential group interactions.

Elevated plus maze
Exploratory behaviors were assessed as previously described by Martins-Silva et al. (2011). Briefly, mice were placed on an elevated white '+-'-shaped platform with opposing open arms perpendicular to opposing closed arms. Closed arms were shaded by ~20-cm-high black plastic walls. Mice were placed individually in the middle of the maze facing a closed arm and left to explore freely for 5 min. Video monitoring recorded the position of the animal to ANY-maze software (Wood Dale, IL, USA), and the percentages of time mice spent in the center, open and closed arms were recorded and averaged over four trials. The target platform position of the hidden platform (15 cm-tall orange and black flag). The time to escape was recorded and averaged over four trials. If the Shapiro-Wilk test was used to compare genotype means for time spent in the open and closed arms.

Morris water maze
The spatial and cued water maze tests were performed as previously described by Vorhees and Williams (2006). Briefly, mice were placed initially in individual test cages for 5 min before testing. The spatial learning phase consisted of lowering mice into a 1.2-m-diameter water bath at one of four positions and leaving them to explore the bath for up to 90 s. Mice that failed to find the clear target platform submerged 1.5 cm below the water surface were guided towards it and left on the platform for 15 s before returning to their test cage. Four up-lights placed near the bath lit the testing room. The water temperature was maintained at 25°C, and prominent visual cues (black and white patterns) were located on the walls around the water bath. Each animal performed four trials per day, in which they were lowered into the bath at a different initial position. Mice had a minimum of 30 min to rest between trials and were tested on four consecutive days. Video monitoring tracked the position of the animal in the water bath, and the time to escape the maze (finding and climbing onto the platform) was recorded for each trial. An average escape time was calculated for each mouse on each day of spatial acquisition. After the spatial trial, a cued learning trial was performed, wherein mice were tested in an identical manner to the spatial acquisition task, with the addition of a prominent visual cue indicating the position of the hidden platform (15 cm-tall orange and black flag). The time to escape was recorded and averaged over four trials. The target platform position, order of initial positions and order of animal testing were balanced by genotype/sex groupings.

Statistics
Results are provided as means±s.e.m. and compared between genotypes within male and female cohorts to evaluate sex as a biological variable. One-way ANOVA with Tukey’s post hoc test was used to determine changes between genotype means for mRNAs and protein expression, body and brain weight, total distance traveled in the open field test, and cued learning escape time in the Morris water maze. If the Shapiro–Wilk test for normality deemed genotype data non-normally distributed, a non-parametric Kruskal–Wallis test with Dunn’s multiple comparisons was performed on genotype means. Two-way ANOVA with Tukey’s post hoc test was used to compare genotype means for time spent in the open and closed arms of the elevated plus maze. Two-way repeated-measures ANOVA with Tukey’s post hoc test was used to compare escape time and swimming speed means for the spatial learning phase of the Morris water maze. All statistical analyses were achieved using Prism v.8 (GraphPad, La Jolla, CA, USA). Means were considered statistically significant when P<0.05.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: N.M.N.-K., E.R.P., D.W.L.; Methodology: N.M.N.-K., E.R.P., K.B., M.A.M.P.; Formal analysis: N.M.N.-K., E.R.P., M.A.M.P.; Investigation: N.M.N.-K., E.R.P., K.B.; Resources: M.A.M.P., D.W.L.; Data curation: N.M.N.-K., E.R.P., K.B.; Writing - review & editing: N.M.N.-K., E.R.P., K.B., M.A.M.P., D.W.L.; Visualization: D.W.L.; Supervision: M.A.M.P., D.W.L.; Project administration: D.W.L.; Funding acquisition: D.W.L.

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