Original Research

Ctnn6 deficiency impairs allocentric navigation in mice

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Abstract
Introduction: CNTN6 is an immunoglobulin domain-containing cell adhesion molecule that belongs to the contactin family. It is involved in the development of the nervous system. We aim to determine the effect of Ctnn6 deficiency on the allocentric navigation in mice.

Methods: We recorded the travel distance and escape time of wild-type and Ctnn6 mutant male and female mice in the Morris water maze task according to the protocol.

Results: There was hardly any Ctnn6 expression in the hippocampus of postnatal day 0 (P0) mice, while obvious Ctnn6 expression was present in the hippocampal CA1 region of the P7 mice. During the acquisition period of Morris water maze task (Day 1 to 4), Ctnn6−/− male mice failed to shorten the escape time to reach platform on the third day, while the travel distance to platform was not significantly different. There was no significant difference in both escape time and travel distance to the platform among all female subjects. In the probe trial test (Day 5), spatial memory of the female mutant mice was mildly affected, while Ctnn6−/− male mice were normal. In the spatial relearning test (Day 7 to 10), Ctnn6−/− male mice showed no difference in escape time to the platform compared to the wild-type male mice, while Ctnn6 deficient female mice required shorter escape time to travel to the platform on day 7, day 8, and day 10.

Conclusions: Ctnn6 is expressed in the developing hippocampus in mice. Ctnn6 deficiency affects spatial learning and memory, indicating that Ctnn6 plays a role in the development of hippocampus and affects allocentric navigation of the animals.

Keywords
allocentric navigation, CNTN6, hippocampus, Morris water maze, spatial learning, spatial memory

1 | INTRODUCTION

Development of the central nervous system is dependent on the highly coordinated interactions between diverse cell types. Cell adhesion molecules (CAMs) are important signal molecules that mediate cell–cell and cell–extracellular matrix interactions in multiple neural developmental processes (Doving & Trotier, 1998; Schaal et al., 2003), including neuronal migration, neurite outgrowth,
Contactin-6 (CNTN6), also termed NB-3, is a member of the contactin family of immunoglobulin (Ig) domain-containing cell adhesion molecules (IgCAMs). CNTN6 contains six N-terminal Ig-like and four fibronectin type III-like (FNIII) domains and tethers to the cell membrane via a C-terminal glycosylphosphatidylinositol (GPI)-anchor (Maness & Schachner, 2007; Shimoda & Watanabe, 2009; Zuko et al., 2013). Cntn6 has been identified as a candidate risk gene of multiple psychiatric disorders including autism spectrum disorders (ASDs), schizophrenia, bipolar disorder, attention-deficit hyperactivity disorder, intellectual disability, and Tourette syndrome (Guo et al., 2012; Hu et al., 2015; Huang et al., 2017; Kashevarova et al., 2014; Kerner, Lambert, & Muthen, 2011; Nava et al., 2014; Oguro-Ando, Zuko, Kleijer, & Burbach, 2017; Okbay et al., 2016; Pinto et al., 2010; Van Daalen et al., 2011), suggesting the necessity of CNTN6 in neural development.

In mice, Cntn6 is exclusively expressed in the nervous system, such as cerebral cortex, accessory olfactory bulb, thalamus, and cerebellum (Huang, Yu, Shimoda, Watanabe, & Liu, 2012; Lee et al., 2000). However, the expression of Cntn6 displays distinct patterns in different regions in the mouse brain. The level of Cntn6 protein in the cerebrum reaches a maximum at P7 and thereafter declines to a constant low level in the adulthood (Huang et al., 2011; Lee et al., 2000). In contrast, the Cntn6 mRNA level in the cerebellum and the hippocampus increases until the adulthood (Lee et al., 2000). Plenty of studies using null mutant mice indicate that Cntn6 plays key roles in the developing and mature mouse brains (Mericati et al., 2013; Oguro-Ando et al., 2017; Shimoda & Watanabe, 2009).

In the visual cortex of one-month-old Cntn6−/− mice, alterations in the orientation of apical dendrites of pyramidal neurons in layer V was observed (Ye et al., 2008). Cntn6 regulates neurite outgrowth in vitro, and this property was consistent with the finding that corticospinal tract formation was delayed in the Cntn6−/− mice (Huang et al., 2011, 2012; Mercati et al., 2013). Moreover, Cntn6 contributes to glutamatergic synaptic formation between parallel fibers and Purkinje cells during postnatal cerebellar development (Sakurai et al., 2009). In addition, behavioral studies have shown that Cntn6-deficient mice display impaired motor coordination (Takeda et al., 2003).

In the hippocampus, a significant reduction in glutamatergic synapses was found in the Cntn6-deficient mice in the postnatal stage (Sakurai, Toyoshima, Takeda, Shimoda, & Watanabe, 2010; Sakurai et al., 2009). Amila Zuko et al. found that Cntn6 deficiency in the dentate gyrus (DG) may impair the fasciculation of mossy fibers that innervate pyramidal cells in the hippocampus (Cremer, Chazal, Goridis, & Represa, 1997; Heyden, Angenstein, Sallaz, Seidenbecher, & Montag, 2008; Montag-Sallaz, Schachner, & Montag, 2002; Zuko et al., 2016). Some studies showed that F3/Contactin, another member of the contactin family, promotes hippocampal neurogenesis in adult mice (Mericati et al., 2017; Puzzo et al., 2013; Sakurai et al., 2009, 2010). These studies suggest that Cntn6 may play an important role in the hippocampal development and function. However, the effect of Cntn6 deficiency on hippocampal-related behavior is still unclear.

In this study, we found that there was hardly any Cntn6 expression in the hippocampus of P0 mice, but obvious Cntn6 expression in the hippocampal CA1 region of P7 mice. Morris water maze task (MWM) was used to determine whether Cntn6 deficiency in mice would affect allocentric navigation which involves hippocampus and its related brain structures. Our results suggest that deletion of Cntn6 leads to functional deficiency of the hippocampus, especially the spatial learning ability in mice.

2 | MATERIALS AND METHODS

2.1 | Animal

Cntn6-deficient mice (Takeda et al., 2003) were maintained on a 12-hour light/dark cycle with ad libitum food and water in a specific pathogen-free (SPF) animal facility at the Capital Medical University, China. All animal procedures were approved by the university’s Committee for Animal experiments and conformed to the guidelines for the care and use of laboratory animals of the Chinese Society for Neuroscience.

Cntn6 knockout mice were generated using 129/SVJ embryonic stem cells and then were backcrossed with C57BL/6J mice for more than 20 generations. In all experiments described in this article, homozygous and heterozygous mutants were compared with their wild-type littermates.

2.2 | Colorimetric detection of LacZ expression

Cntn6+/− mice at postnatal day 0 and 7 were perfused with PBS and then with 2% paraformaldehyde dissolved in PIPES, pH 6.9, containing 2 mM MgCl₂ and 5 mM EGTA. Brains were removed and postfixed overnight at 4°C. The brains were then cryoprotected by incubation overnight in 20% sucrose containing 2 mM MgCl₂. Floating sections (50 μm) were prepared using a cryostat. Sections were washed twice in PBS containing 2 mM MgCl₂ and then incubated in PBS containing 2 mM MgCl₂, 0.005% sodium deoxycholate and 0.01% NP-40 for 10 min at 4°C. Colorimetric reaction was performed in the same solution containing 5 mM K₃[Fe(CN)₆], 5 mM K₄[Fe(CN)₆] and 0.05% 5-bromo-4-chloro-3-indolyl-D-galactoside (X-gal) at 37°C overnight. The sections were washed, mounted, air-dried and were counterstained with 0.5% neutral red to visualize the brain architecture.

2.3 | Morris water maze task

Learning and memory tasks of adult mice (2–4 months) were assessed using a Morris water maze task according to previous reports (Petravicz, Boyt, & McCarthy, 2014; Schenk & Morris, 1985). The stainless steel circular pool (150 cm in diameter, 51 cm in depth) was
filled with white opaque water maintained at 21 ± 1°C. The platform (10 cm in diameter) was submerged 1 cm beneath water surface. The locations of the starting points were identified using different colors and dimensions visual extra-maze cues attached to the room walls and were kept consistent during each experiment. The pool was divided into four quadrants using a computerized tracking/image analyzing system (video camcorder coupled with computational tracking system: Coulbourn Instrument). During the acquisition training trials, the platform was placed in the middle of the northwest (NW) quadrant and remained in the same position. Subjects were placed pseudorandomly with their heads facing the pool wall into each of four starting locations (northwest, northeast, southeast, and southwest) for each of four daily acquisition training trials. Trials lasted 60 s or until the subjects mounted the platform with a 30-min intertrial interval. On the first day (Day 1) of training, the subjects were manually placed on the platform and allowed to stand on it for 15–20 s if they did not find the platform after 60 s. The escape time, travel distance and mean velocity to reach the platform were recorded during the four-day training. A probe trial to test reference memory was conducted on day 5. Subjects were placed into the opposite quadrant of the platform quadrant and allowed to swim during 60 s in the absence of the platform. The number of platform crossings, the number of target quadrant crossings, and the proportion of swimming time spent in four quadrants were recorded and analyzed.

The reversal task (relearning training trial) was performed from day 7 to day 10 exactly as the acquisition training protocol, while the hidden platform was placed in the opposite quadrant (southeast). The escape time, travel distance, and mean velocity to reach the platform were recorded. The subjects were blind to the genotypes.

2.4 | Statistical analysis

A two-way ANOVA followed by the Bonferroni posttest was used to analyze escape time to platform and travel distance. The results are displayed as mean ± standard error of the mean (SEM). Multiple t test followed by the Sidak-Bonferroni method was used to analyze the time in quadrant. A one-way ANOVA followed by the Bonferroni posttest was used to analyze and obtain statistics of the entries to target quadrant.

3 | RESULTS

3.1 | Expression of Cntn6 in the developing mouse hippocampus

To assess the potential role of Cntn6 in hippocampal development, the spatiotemporal expression of Cntn6 was analyzed in the developing mouse hippocampus. The segment between initiation codon of the second exon and the Bgl I site in the second intron of the Cntn6 gene was replaced by LacZ gene, so that the generated mutant mice were expected to produce β-galactosidase instead of Cntn6 protein. The LacZ gene expression was driven by the promoter of the Cntn6 gene and accordingly reflected the expression of Cntn6 (Takeda et al., 2003). We first examine the expression of the LacZ in the whole brain (Figure 1a,b) and hippocampus (Figure 1c,d) of P0 and P7 Cntn6−/− mice via X-gal staining. The LacZ expression pattern was essentially the same as that observed in the Cntn6 in situ hybridization previously reported by Lee et al. (2000). In the hippocampus of P0 mice, there was hardly any Cntn6 expression in the CA1, CA3, and DG regions (Figure 1c). However, there was obvious Cntn6 expression in the CA1 but not in the CA3 and DG regions of P7 mice (Figure 1d). These results were indicating that Cntn6 is expressed in the developing hippocampus.

3.2 | Cntn6 deficiency affects spatial learning of male mice in the Morris water maze task

The hippocampal structure plays an important role in spatial learning and memory. It has been reported that the length and area size

![FIGURE 1](image-url)
of the suprapyramidal bundle (SPB) in the hippocampus were significantly increased in Cntn6−/− mice (Zuko et al., 2016). Here, we examined whether Cntn6 deficiency affected hippocampus-related behavior in the Morris water maze task. Over the 4-day acquisition training period, all animals improved their ability to find the submerged platform by exhibiting shorter escape time and travel distance to the platform (Figure 2a,b). There was no significant difference in performance among all female subjects (Figure 2d). However, although Cntn6−/− male mice could swim as fast as wild-type mice and willingly found a hidden platform, their escape time was significantly longer than their wild-type and Cntn6+/− littermates on the third day (Figure 2c). No significant difference in escape time was detected on the fourth day in Cntn6−/− male mice (Figure 2c). These results indicated that spatial learning is mildly compromised in the Cntn6−/− male mice.

3.3 Cntn6 deficiency affects the spatial memory of female mice, but not male mice

After the 4-day successive acquisition training period, we measured the time of movement of all the experimental groups in the 60-second probe trial test on day 5 (Figure 3a). We calculated the time the mice spent in the target quadrant and the opposite quadrant after entering the pool in the last 40 s of the probe trial. Similar with the wild-type male mice, Cntn6−/+ mutant male mice spent significant shorter time in the opposite quadrant than in the target quadrant, indicating that the Cntn6 deficiency has no serious effect on male mice’s ability of recalling the previously learned spatial strategy (Figure 3b). Although Cntn6+/− and Cntn6−/− female mice also spent shorter time in the opposite quadrant, the change was not significant, (Figure 3c). We further analyzed the number of times the

![FIGURE 2](image_url)
mice crossed the target platform location. There was no significant difference in the entries to target quadrant among all experimental subjects (Figure 3d). Together, these results indicated that Cntn6 deficiency of leads to mild deficits in the spatial memory of female mice.

3.4 | Improved spatial relearning in Cntn6 deficient female mice

To investigate the effect of Cntn6 deficiency on spatial relearning, we performed a reversal task in the Morris water maze. Mice were trained for 4 additional days (day 7 to day 10) with the hidden platform placed in the opposite quadrant (Figure 4a). There was no significant difference in travel distance between wild-type and mutants mice in both sexes (Figure 4b,c). Cntn6+/− and Cntn6−/− male mice showed no difference in escape time to the platform in the reversal task (Figure 4b). Interestingly, compares with the wild-type female mice, both Cntn6+/− and Cntn6−/− female mice spent shorter time to reach the platform, and the change was significant between the wild-type and the Cntn6−/− female mice on day 7 (wild-type vs. Cntn6−/−, p = .031), day 8 (wild-type vs. Cntn6−/−, p = .0288; wild-type vs. Cntn6+/−, p = .0002), and day 10 (wild-type vs. Cntn6−/−, p = .0228) (Figure 4c). These results indicate that Cntn6 deficiency improves spatial relearning in female mice.

4 | DISCUSSION

Previous studies have shown that CNTN6 is important for the normal development and stability of the a few brain regions (Hu
et al., 2015; Kashevarova et al., 2014; Lee et al., 2000; Sakurai et al., 2009). Here, we found that the expression of Cntn6 in the hippocampal CA1 region increases during early postnatal stage, which is consistent with the data set provided by Allen Brain database (http://developingmouse.brain-map.org/gene/show/33165), suggesting that Cntn6 is necessary for hippocampal structural formation and function. In the Morris water maze task, we found Cntn6−/− male mice failed to reduce the escape time to reach the hidden platform on day 3 of the acquisition trials. Interestingly, although female Cntn6 mutant mice exhibited similar performance as the wild-type mice in the acquisition trials, their spatial memory was mildly affected in the following probe trial. Moreover, female Cntn6 mutant mice also showed a decreased escape time to reach the platform in the spatial relearning test.

The structural integrity of hippocampus is crucial for spatial learning and memory (Daugherty, Bender, Yuan, & Raz, 2016; Guderian et al., 2015; Penner & Mizumori, 2012). The so-called “trisynaptic loop” in hippocampus conducts synaptic transmission and consists of three major excitatory pathways: perforant path (from entorhinal cortex to DG), mossy fiber (from DG to CA3), and Schaffer collateral (from CA3 to CA1) (Andersen, Bliss, Lomo, Olsen, & Skrede, 1969; Inoue & Watanabe, 2014; Kesner, Lee, & Gilbert, 2004; Knierim, 2015; Lee et al., 2017; Okada & Okaichi, 2009; Piatti, Ewell, & Leutgeb, 2013; Rolls & Kesner, 2006; Rongo, 2002). The CA1 region is also thought to help encode memory into a form that can be sent back to the entorhinal cortex via the subiculum for subsequent longer-term spatial memory and consolidation, but not short-term acquisition or encoding processes (Lassalle, Bataille, & Halley, 2000; Lee & Kesner, 2004; Rolls, 2015; Rolls, Dempere-Marco, &
Deco, 2013; Rolls & Treves, 1994; Rolls & Xiang, 2006; Treves & Rolls, 1992). We found that Cntn6 is not expressed in the hippocampus of P0 mice, but is expressed in the CA1 region of P7 mice (Figure 1). Consistent with the expression pattern of Cntn6, the length and area size of mossy fiber projections in the SPB were significantly increased in the hippocampus of Cntn6−/− mice, indicating that Cntn6 deficiency may impair the fasciculation of mossy fibers (Zuko et al., 2016). We therefore used the Morris water maze task to check whether the loss of Cntn6 affects hippocampus-regulated spatial learning and memory.

At first acquisition of spatial learning was evaluated via repetitive training during which the mice use distinct spatial cues to swim from the starting position to the submerged platform. On day 3 of the acquisition training trials, the Cntn6−/− male mice took longer time to find the hidden platform than the wild-type male mice, indicating that Cntn6−/− male mice learn more slowly but catch up at a later stage of the acquisition training trials (Figure 2). This increase in escape time on day 3 in Cntn6−/− male mice is not due to impaired motor coordination as their swimming speed was comparable with the wild-type male mice, and they performed equally well on day 1, 2, and 4 of the acquisition trials (Figure 2). After the acquisition training, a single probe trial was performed on day 5 with the platform withdrawn from the water tank to assess their spatial memory. The Cntn6−/− male mice performed similar as the wild-type mice, while the spatial memory in female mutant mice was mildly compromised (Figure 3). Interestingly, in the relearning/reversal phase (day 7 to 10) when mice were forced to find the submerged platform at a different location, Cntn6−/− and Cntn6−/− female mice performed better than their wild-type littermates (Figure 4), while no difference was detected in the male mice, suggesting that female Cntn6 mutant mice are less perseverative for the previous acquisition platform location and are more readily to adapt to the changed contingencies.

Contactin family belongs to immunoglobulin (Ig) domain-containing cell adhesion molecules (IgCAMs) and contains six members, CNTN1 (Contactin), CNTN2 (TAG-1), CNTN3 (BIG-1), CNTN4 (BIG-2), CNTN5 (NB-2), and CNTN6 (NB-3) (Shimoda & Watanabe, 2009). CNTN6 is structurally and functionally similar to the other five family members. CNTN4 and CNTN6 followed by the close homologue of L1 (CHL1) are located on chromosome 3p25pter in the human genome (Kamei, Tsutsumi, Taketani, & Watanabe, 1998; Wei et al., 1998; Zeng et al., 2002). The deletion of this locus will cause 3p deletion syndrome with symptoms of microcephaly, growth retardation, intellectual disability, and distinctive facial features (Dijkhuizen et al., 2006; Fernandez et al., 2004, 2008). These three genes are closely located on chromosome 6p− in the mouse genome and exhibit similar expression pattern. Thus, we speculate that the mild effect of Cntn6 deficiency on learning and memory may be due to the compensational effects of other contactin family members for the in the Cntn6−/− brain.

Our results show that Cntn6 mutant mice exhibit sexual difference in spatial learning and memory impairments. The selection of female mice was random and did not exclude the factors of the menstrual cycle. Cntn6−/− male show slower spatial learning, while female mutant mice may be compromised in long-term memory retention. No sexual difference in hippocampus morphology or architecture has been discovered in the Cntn6 mutant mice. Sex hormones are involved in the cognitive differences between men and women, and sex-selective effects were also detected with regard to spatial learning and memory (Piber, Nowacki, Mueller, Wingenfeld, & Otte, 2018). Young males rodents also have an advantage in spatial learning in Morris water maze tasks (Brandes, Brandys, & Yehuda, 1989). Male and female mice perform the same when they are 6 months old, suggesting that the sex difference in young animals may reflect a difference in maturation rate (Bucci, Chiha, & Gallagher, 1995). We also found that Cntn6−/− female mice have an advantage in spatial relearning during the reversal task compared with the wild-type female mice. Reversal learning is a form of cognitive flexibility, an executive process that allows the adaptive modification of behavior in response to changes (Rygula, Walker, Clarke, Robbins, & Roberts, 2010). It has been reported that abnormal hippocampal structure leads to inflexible behaviors in women (Vilà-Balló et al., 2017). We therefore speculate that the Cntn6 deficiency may specifically increase cognitive flexibility in female mice.

In conclusion, Cntn6 is expressed during postnatal hippocampal development. The absence of Cntn6 affects hippocampal spatial learning and memory. However, its cellular and molecular mechanism need further study.

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CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

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