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Deficits in social behavioral tests in a mouse model of alternating hemiplegia of childhood

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ABSTRACT

Social behavioral deficits have been observed in patients diagnosed with alternating hemiplegia of childhood (AHC), rapid-onset dystonia-parkinsonism and CAPOS syndrome, in which specific missense mutations in *ATP1A3*, encoding the Na^+, K^+-ATPase α3 subunit, have been identified. To test the hypothesis that social behavioral deficits represent part of the phenotype of Na^+, K^+-ATPase α3 mutations, we assessed the social behavior of the *Myshkin* mouse model of AHC, which has an I810N mutation identical to that found in an AHC patient with co-morbid autism. *Myshkin* mice displayed deficits in three tests of social behavior: nest building, pup retrieval and the three-chamber social approach test. Chronic treatment with the mood stabilizer lithium enhanced nest building in wild-type but not *Myshkin* mice. In light of previous studies revealing a broad profile of neurobehavioral deficits in the *Myshkin* model – consistent with the complex clinical profile of AHC – our results suggest that Na^+, K^+-ATPase α3 dysfunction has a deleterious, but nonspecific, effect on social behavior. By better defining the behavioral profile of *Myshkin* mice, we identify additional *ATP1A3*-related symptoms for which the *Myshkin* model could be used as a tool to advance understanding of the underlying neural mechanisms and develop novel therapeutic strategies.

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Introduction

Alternating hemiplegia of childhood (AHC; OMIM: 614820) is a rare neurodevelopmental disorder that manifests as episodic hemiplegia starting in the first 18 months of life, with a spectrum of persistent motor, movement and cognitive deficits that become progressively more apparent with age (Sweney *et al.* 2009, Panagiotakaki *et al.* 2010). Children with AHC are prone to a wide range of behavioural and psychiatric disorders, including impulsivity, lack of attention control, difficulties in acquiring speech, obsessionality and short-temperedness (Neville and Ninan 2007). Published findings of comprehensive assessments of neuropsychological functioning in children with AHC are limited to two case studies, which both report deficits in language, memory, attention and information processing, as well as difficulty with impulsivity (Shafer *et al.* 2005, Muriel *et al.* 2015).

Heterozygous missense mutations of the *ATP1A3* gene, encoding the Na^+,K^+-ATPase α3 subunit, have been identified as the primary cause of AHC (Heinzen *et al.* 2014). Na^+,K^+-ATPases are membrane-bound transporters that harness the energy of ATP hydrolysis to pump three Na^+ ions out of the cell in exchange for two K^+ ions moving inwards. Na^+,K^+-ATPase α3-expressing neurons are present throughout the nervous system (Benarroch 2011). Most *ATP1A3* mutations in AHC patients are clustered in or near transmembrane α-helix TM6, including recurrent mutations of the isoleucine at position 810: I810F, I810N and I810S (Heinzen *et al.* 2012, Rosewich *et al.* 2014a, Yang *et al.* 2014, Panagiotakaki *et al.* 2015). To date, three cases of AHC with mutation I810N have been reported, including a 22-year-old man from Belgium with autism (Yang *et al.* 2014, Panagiotakaki *et al.* 2015, Yang *et al.* 2015, Weckhuysen S 2015, Personal communication). All of the AHC mutations studied to date result in a catalytically inactive Na^+,K^+-ATPase α3 (Clapcote *et al.* 2009, Weigand *et al.* 2014).

Other missense mutations in Na^+,K^+-ATPase α3 have been identified in patients with a phenotypic continuum of *ATP1A3*-related encephalopathy: (1) rapid-onset dystonia-parkinsonism (RDP; DYT12; OMIM: 128235; Heinzen *et al.* 2014); (2) an intermediate AHC/RDP presentation (Roubergue *et al.* 2013, Boelman *et al.* 2014, Heinzen *et al.* 2014, Rosewich *et al.* 2014a, Sasaki *et al.* 2014, Termsarasab *et al.* 2015); (3) cerebellar ataxia with areflexia, pes cavus, optic atrophy and sensorineural deafness (CAPOS; OMIM: 601338; Demos *et al.* 2014, Heimer *et al.* 2015); (4) an intermediate CAPOS/AHC presentation (Rosewich *et al.* 2014b); (5) early infantile epileptic encephalopathy (EIEE; Paciorkowski *et al.* 2015); (6) relapsing encephalopathy with cerebellar ataxia (RECA; Dard *et al.* 2015). Five RDP patients with mutation T613M from an Irish family are reported to have profound difficulties socializing and maintaining relationships, resulting in a diagnosis of social anxiety disorder

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in one individual (Pittock *et al.* 2000, McKeon *et al.* 2007). Two CAPOS patients with mutation E818K from a British family have been diagnosed with autism spectrum disorder (Demos *et al.* 2014). The activity of Na^+,K^+-ATPase α3 is also impaired by its aberrant association with misfolded and aggregated proteins implicated in Alzheimer's disease (β-amyloid; Ohnishi *et al.* 2015), Parkinson's disease (α-synuclein; Shrivastava *et al.* 2015) and amyotrophic lateral sclerosis (SOD1; Ruegsegger *et al.* 2016).

Heterozygous *Myshkin* (*Atp1a3*$^{Myk/+}$; *Myk*/+) mutant mice have an I810N mutation identical to that present in AHC, which reduces total Na^+,K^+-ATPase activity (α1 + α2 + α3) in the whole brain by 42% (Clapcote *et al.* 2009). *Myk*/+ mice move with a paretic, tremulous gait that becomes transiently more severe after stress (Kirshenbaum *et al.* 2013, 2016). Other phenotypic abnormalities include neuronal hyperexcitability, and increased susceptibility to epileptic seizures in mixed 129/B6 and FVB/B6 genetic backgrounds (Clapcote *et al.* 2009). *Myk*/+ mice have, however, shown intact audioception in the acoustic startle test (Kirshenbaum *et al.* 2013), ophthalmoception in the head tracking test (Kirshenbaum *et al.* 2011a), and gustaoception in the sucrose preference test (Kirshenbaum *et al.* 2011a). To test the hypothesis that social behavioral deficits represent part of the phenotype of Na^+,K^+-ATPase α3 mutations, we assessed the social behavior of the *Myshkin* mouse model of AHC.

Materials and methods

Mice

Myk/+ males that had been backcrossed for 20 generations to the C57BL/6NCr strain were mated with C57BL/6NCrl (Charles River, Margate, UK) females to yield wild-type (+/+) and *Myk*/+ littermates. *Myk*/+ mice at N_{20} C57BL/6NCr were previously reported to be free of stress-induced seizure activity during electrocorticography (Kirshenbaum *et al.* 2011a). *Myk*/+ mice were genotyped by the presence of an *Eco*O109I (New England BioLabs, Hitchin, UK) restriction site using polymerase chain reaction primers F, 5′-CTG CCG GAA ATA CAA TAC TGA-3′ and R, 5′-ATA AAT ACC CCA CCA CTG AGC-3′. Mice were weaned at four weeks of age and grouped housed (2–5 mice/cage) with same-sex littermates. Mice were tested at 8–14 weeks of age. Males and females were included in balanced numbers, apart from the pup retrieval test. Subjects were handled daily for 5 min/day for seven days prior to testing, which was conducted during the light phase (0900–1700 h). Prior to experiments, subjects were left undisturbed in the testing environment for 30 min to allow for acclimation. All procedures involving animals were conducted in accordance with the Animals (Scientific Procedures) Act 1986, and were approved by the University of Leeds Ethical Review Committee.

Nest building and utilization

Mice were individually housed in a clean cage with nesting material comprising a 3 g 5 cm^2 square of compressed cotton ("nestlet"; Lillico, Horley, UK). During 5 min of observation, the duration and frequency of exploration and shredding of the nestlet were recorded, and then the cage was placed onto the cage rack. At 30 min, 60 min, 90 min, 3 h and 24 h, the percentage of the nestlet that was shredded was recorded, and the quality of the nest was scored as follows (Moretti *et al.* 2005): 0 = nesting material unmodified; 1 = flat nest with partially shredded nesting material; 2 = shallow nest with shredded material, but lacking fully formed walls; 3 = nest with well-developed walls; 4 = nest in a shape of a cocoon with partial or complete roof. At 3 h, utilization of the nest was assessed by recording whether the mouse was positioned inside or outside the nest. At 24 h, the height of the nest in cm was recorded.

Pup retrieval behavior

Several pairs of female littermates – one *Myk*/+ and one +/+ – were housed together from weaning to about 60 days of age, when an experienced C57BL/6NCrl stud male was introduced to each cage. Females were checked for vaginal plugs each morning to determine if mating had occurred. When both females had been mated, the male was removed and the cage was checked daily for litters. In four cages where both females had given birth within 12 h, pup retrieval behavior was assessed. On postnatal day 5, between 09:00 and 12:00 h, one of the females (counterbalanced for genotype) was temporarily removed and put into a clean cage. Next, a healthy pup with milk in its stomach was taken from the communal nest and placed at the far end of the cage. The experimenter was blind to the genotype of the pups. Latency for the remaining female to retrieve the pup was recorded. The next day (postnatal day 6), the other female was removed and the experiment was repeated.

Social approach test

Social approach was assessed using a three-chambered apparatus (60 × 40 cm), which had two doors to allow the mouse to access left and right chambers from a central compartment (each chamber being 40 × 20 cm). Following a 15-min habituation period, two cylindrical enclosures (10 × 10.5 cm, comprising vertical metal bars 9 mm apart) were placed into the left and right chambers, into one of which an unfamiliar adult male C57BL/6 mouse (age 10 weeks; "stranger 1") was placed. Left/right placement was counterbalanced across groups. Time spent exploring each enclosure was measured for 10 min. A second unfamiliar adult male C57BL/6 mouse ("stranger 2") was then placed into the empty cylinder and time spent exploring each enclosure was measured for 10 min. The time spent exploring stranger 1 or the empty cylinder in the first phase, and time spent exploring either stranger 1 or 2 in phase two were recorded. A solution of 70% ethanol was used to clean surfaces and equipment between subjects.

Drug treatment

Lithium carbonate (Li_2CO_3) was administered in the diet at 0.4% for 28 d before the assessment of nest building, and the control group received an identical drug-free diet (CRM-P; Special Diets Services, Witham, UK). To prevent ion imbalances from lithium, all mice were provided with an additional water bottle containing 0.9% saline. Serum lithium levels were measured by a spectrophotometry kit (Roche Diagnostics, Burgess Hill, UK), and therapeutic serum lithium levels (0.75–0.95 mmol/L; Gelenberg *et al.* 1989) were reached in +/+ and *Myk*/+ mice, as previously described (Kirshenbaum *et al.* 2011a).

Data analysis

All statistics were calculated by STATISTICA (StatSoft, Tulsa, USA). Data were subjected to analysis of variance (ANOVA) with genotype, sex and drug as between-subjects factors, and time as a within-subject factor. When ANOVA detected statistically significant main effects, pairwise differences were evaluated using the Tukey–Kramer *post hoc* multiple comparison test, with significance set at $p < 0.05$. All values reported in the figures are expressed as mean ± standard error of the mean (SEM).

Results and discussion

Nest building and utilization are impaired in Myk/+ *mice*

Mice build a nest when provided with suitable material and are typically found lying in it during the daytime. A previous study (of circadian rhythms) provided anecdotal evidence of deficient nest building by *Myk*/+ mice given a ripped up paper towel (Kimberly–Clark) as nesting material (Kirshenbaum *et al.* 2011a; Figure 1(a)). As a home cage activity related to maternal care and social behavior (Peripato and Cheverud 2002, Moretti *et al.* 2005), we studied nest building in mice provided with a nestlet of compressed cotton, which requires shredding. At 30 min, 60 min, 90 min, 3 h and 24 h after mice were placed into a clean cage, the percentage of the nestlet that was shredded was recorded, and the quality of the nest was scored on a 1–4 scale. The nests of *Myk*/+ mice were of consistently lower quality than those of +/+ littermates (Figure 1(b)). The difference in nest quality was paralleled by a greater propensity of +/+ mice to shred the nesting material compared with *Myk*/+ mice, whose nestlets remained largely untouched after 24 h (Figure 1(c)). The height of nests built by +/+ mice was greater at 24 h than the nests of *Myk*/+ mice (Figure 1(d)). The difference between genotypes did not appear to be related to slower building of the nest, as it persisted even for up to one week (data not shown).

Utilization of the nest was assessed by recording the position of the mouse during rest/sleep compared with the

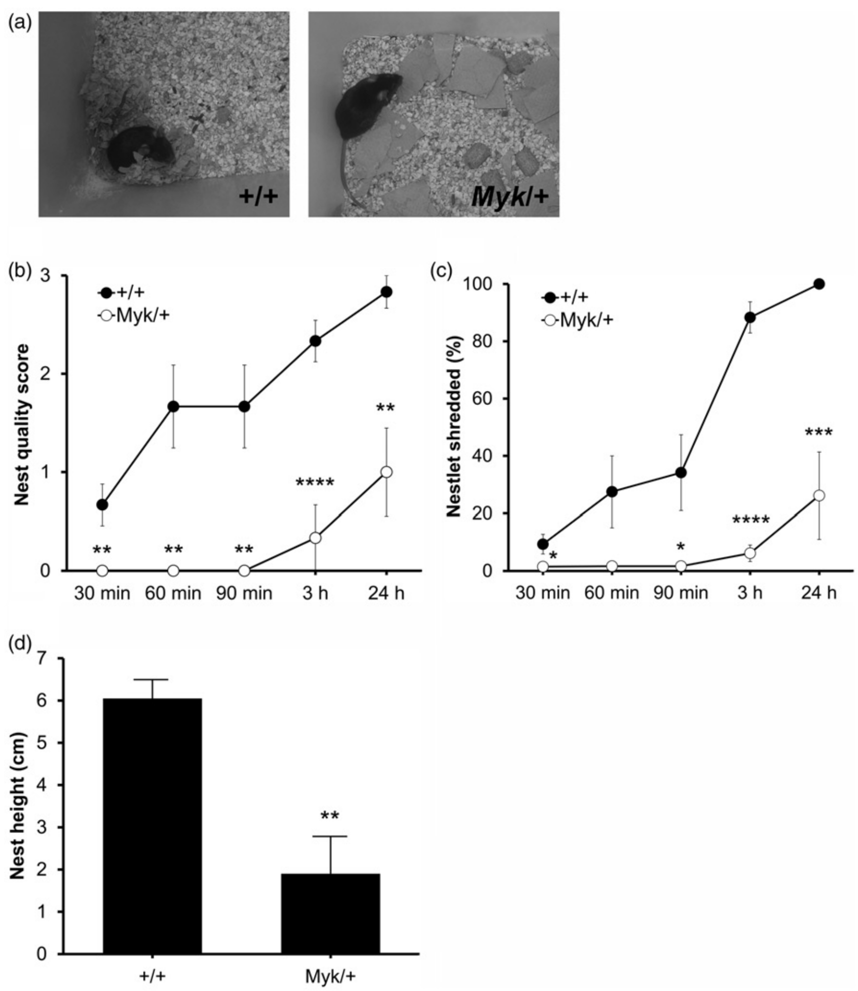

Figure 1. Nest building behavior. (a) Representative examples of nest building by singly-housed *Myk*/+ (right) and +/+ (left) mice provided with a ripped up paper towel in a previous circadian rhythm study. (b) Nest quality score (0–4 scale) at 30 min, 60 min, 90 min, 3 h and 24 h after placement into a clean cage. Main effects of genotype ($F(1, 44) = 73.86$, $p < 0.0001$) and time ($F(4, 44) = 8.66$, $p < 0.0001$) were observed. (c) Percentage of nestlet shredded at 30 min, 60 min, 90 min, 3 h and 24 h after placement into a clean cage. Main effects of genotype ($F(1, 44) = 74.12$, $p < 0.0001$), time ($F(4, 44) = 17.87$, $p < 0.0001$) and genotype × time interaction ($F(4, 44) = 7.73$, $p < 0.0001$) were observed. (d) Height of nests at 24 h after placement into a clean cage. A main effect of genotype ($F(1, 8) = 15.82$, $p < 0.01$) was observed. *Myk*/+ mice ($n = 6$); +/+ mice ($n = 6$). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ versus +/+ mice.

location of the nest in the light phase of the 12:12 light:dark cycle. Upon observation at 24 h, 100% (6 of 6) of +/+ mice versus 16.7% (1 of 6) of *Myk*/+ mice were found to be resting. *Myk*/+ mice are known to exhibit less REM and non-REM sleep (Kirshenbaum *et al.* 2011a, 2014). All of the +/+ mice were resting inside the nest, whereas the single resting *Myk*/+ mouse was found outside the nest.

Most behavioral tests in experimental animals depend on the measurement of motor output and may be influenced by underlying motor dysfunction. *Myk*/+ mice have a tremor (Kirshenbaum *et al.* 2013), which could conceivably disrupt fine motor skills, so we cannot formally exclude the possibility that tremor may have impaired physical manipulation of the nestlets. However, our finding that nest utilization and building time were both reduced in *Myk*/+ mice suggests that the difference in nesting behavior is more likely due to decreased interest in building the nest than simple impairment in execution of a motor task. Indeed, deficient nest building was first observed in *Myk*/+ mice given nesting material – a ripped up paper towel – that does not require extensive shredding. Moreover, similar abnormalities in nest building and utilization have been exhibited by two mouse models of Rett syndrome, an autistic spectrum disorder, independent of whether gross body tremor was present ($Mecp2^{tm1Hzo}$; Moretti *et al.* 2005) or absent ($Mecp2^{tm1.1Bird}$; Samaco *et al.* 2008) in the mice.

Chronic treatment with the mood stabilizer lithium is reported to promote sociability in the *Fmr1* knock-out mouse model of Fragile X syndrome (Mines *et al.* 2010), a developmental disorder that often includes symptoms of autism (Kaufmann *et al.* 2004). Lithium has also been shown to reduce the hyperambulation and risk-taking behavior of *Myk*/+ mice (Kirshenbaum *et al.* 2011a), so we subjected a separate cohort of mice to chronic dietary treatment with Li_2CO_3 and observed their nest building behavior in a clean cage. Over 5 min of observation, *Myk*/+ mice engaged in fewer bouts of shredding, and spent less time exploring and shredding the nesting material than +/+ littermates, but there was no genotypic difference in the number of exploratory approaches (Figure 2(a,b)). Wild-type mice spent 30.9 ± 3.8% of the time building the nest, whereas *Myk*/+ mice spent only 1.2 ± 0.7% of the time actively manipulating the same material. We found that Li_2CO_3 treatment had no effect on any nest building and utilization parameter in *Myk*/+ mice (Figure 2(a–e)), but it did enhance the nest building behavior of +/+ mice at 90 min and 3 h after placement into a clean cage (Figure 2(c,d)).

Myk/+ dams display deficit in pup retrieval

Ultrasonic vocalizations (USV) emitted by pups removed from the nest will elicit search-and-retrieval behavior in lactating dams (Young *et al.* 2010). As a measure of maternal care, we compared the pup retrieval latencies of *Myk*/+ and +/+ dams. *Myk*/+ dams exhibited deficient maternal behavior by taking longer than +/+ dams to initiate pup retrieval (Figure 3). CAPOS patients with *ATP1A3* mutations exhibit optic atrophy and sensorineural deafness (Demos *et al.* 2014), but the intact audioception of *Myk*/+ mice (Kirshenbaum *et al.* 2013) suggests that the reduced maternal retrieval behavior of *Myk*/+ dams is unlikely to be a simple consequence of hearing loss. While it is possible that *Myk*/+ pups may have emitted fewer USV than +/+ pups, this is unlikely to be responsible for the reduced pup retrieval of *Myk*/+ dams because all pups were removed by the experimenter from communal nests containing on average 75% +/+ pups and 25% *Myk*/+ pups.

Myk/+ mice show reduced social interaction

Laboratory mice are naturally social animals (Bailey and Crawley 2009). To assess the social interaction of *Myk*/+ mice, we utilized the three-chamber social approach test, which has been extensively used in studies of sociality in a variety of mouse lines (Yang *et al.* 2011). The sociability phase of the test measures the preference of the subject to explore either a novel adult male conspecific enclosed in a ventilated container (Stranger 1) or an identical but otherwise empty container. This task has face validity to the tendency of autistic children to spend more time playing with an inanimate toy than engaged in social interactions with other children (Ryan *et al.* 2008). By contrast with +/+ mice, *Myk*/+ mice spent less time exploring the novel mouse, and did not show a preference for exploring the novel mouse versus the empty container (Figure 4(a)).

In the social novelty phase of the test, the subject encountered a second novel adult male mouse in the previously empty container (Stranger 2), as well as the now familiar conspecific. By contrast with +/+ mice, *Myk*/+ mice spent less time exploring the novel mouse, and did not demonstrate a preference for exploring the novel mouse versus the previously introduced mouse (Figure 4(b)). This apparent inability to discriminate between Stranger 1 and Stranger 2 is consistent with the reported social recognition deficiency of *Myk*/+ mice; mutants showed reduced recognition of a juvenile male C57BL/6NCrl mouse 24 h after being exposed to it for 2 min, but not for 10 min (Kirshenbaum *et al.* 2015). The reduced social interaction of *Myk*/+ mice was not due to deficient ambulation within the arena, as there were no genotypic differences in distance travelled during either phase of the test (Figure 4(c)).

Impairments in social approach and nest building have previously been exhibited by mice deficient in the CNTNAP2 and SHANK2 genes implicated in autism (Peñagarikano *et al.* 2011, Won *et al.* 2012); SHANK2-deficient mice also show a deficit in pup retrieval (Won *et al.* 2012). Mice rely heavily upon olfaction during typical social interactions (Otmakhova *et al.* 1992). Social approach by male C57BL/6J mice in the three-chamber test is driven primarily by social olfactory cues (Ryan *et al.* 2008). CNTNAP2 and SHANK2 deficient mice were shown to have intact olfactory function (Peñagarikano *et al.* 2011, Won *et al.* 2012), so their abnormal social behavior cannot be attributed to olfactory deficits. Olfaction usually implies detection of compounds in gaseous or airborne form (remote chemoreception), whereas gustation involves direct contact with a substrate (contact chemoreception). The

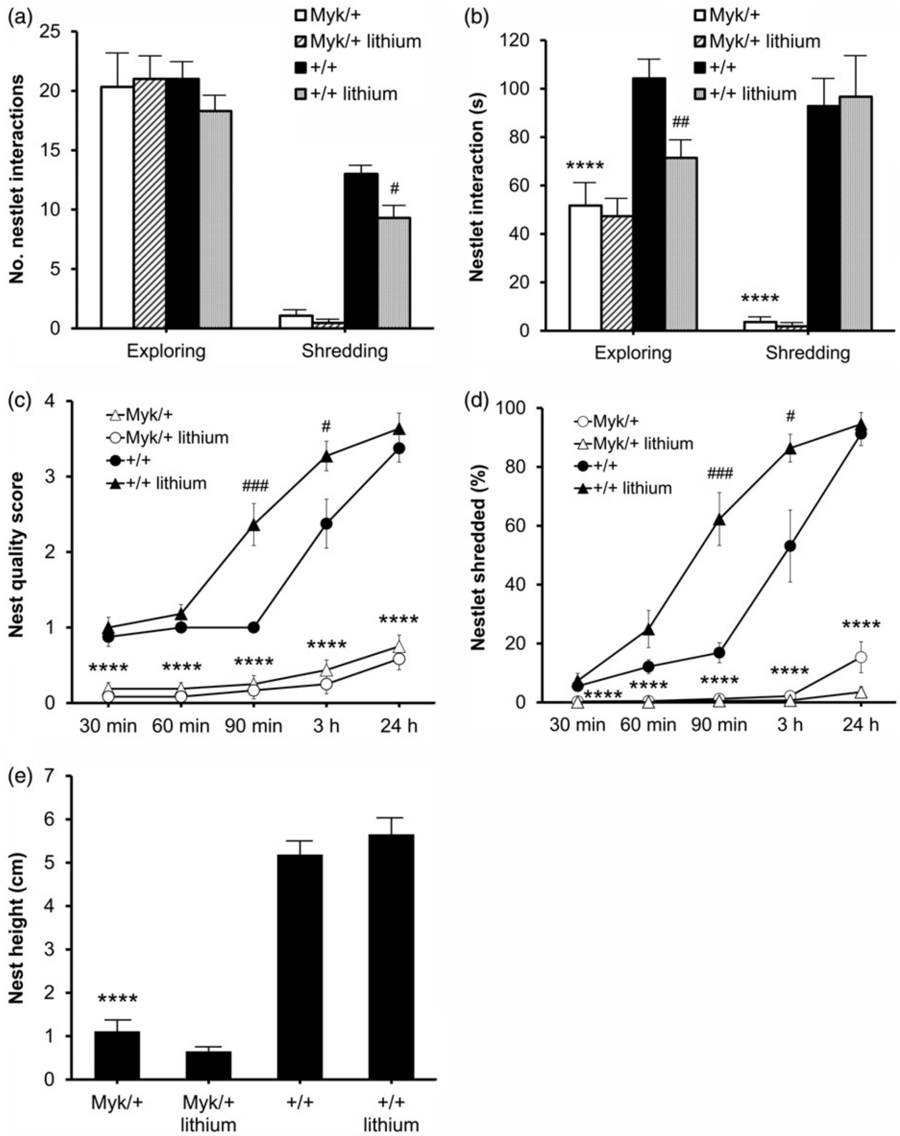

Figure 2. Effects of chronic lithium treatment on nesting building. (a) Bouts of exploration and shredding of the nesting material. Main effects of genotype (F(1, 37) = 233.31, $p < 0.0001$), drug (F(1, 37) = 9.80, $p < 0.01$) and genotype × drug interaction (F(1, 37) = 5.39, $p < 0.05$) on bouts of shredding were observed. (b) Time spent exploring and shredding the nesting material over 5 min of observation in a clean cage. Main effects of genotype (F(1, 37) = 18.06, $p < 0.0001$) and drug (F(1, 37) = 4.39, $p < 0.05$) on time spent exploring, and a main effect of genotype (F(1, 37) = 101.27, $p < 0.0001$) on time spent shredding, were observed. (c) Nest quality score (0–4 scale) at 30 min, 60 min, 90 min, 3 h and 24 h after placement into a clean cage. Main effects of genotype (F(1, 212) = 595.26, $p < 0.0001$), drug (F(1, 212) = 9.87, $p < 0.0001$), time (F(4, 212) = 74.68, $p < 0.0001$), genotype × drug interaction (F(1, 212) = 24.82, $p < 0.0001$) and genotype × time interaction (F(4, 212) = 33.99, $p < 0.0001$) were observed. (d) Percentage of nestlet shredded at 30 min, 60 min, 90 min, 3 h and 24 h after placement into a clean cage. Main effects of genotype (F(1, 212) = 579.50, $p < 0.0001$), drug (F(1, 212) = 21.25, $p < 0.0001$), time (F(4, 212) = 100.05, $p < 0.0001$), genotype × drug interaction (F(1, 212) = 38.89, $p < 0.0001$), genotype × time interaction (F(4, 212) = 67.39, $p < 0.0001$), sex × drug interaction (F(1, 212) = 5.39, $p < 0.05$) and drug × time interaction (F(4, 212) = 5.69, $p < 0.0001$) were observed. (e) Height of nests at 24 h after placement into a clean cage. A main effect of genotype (F(1, 40) = 238.88, $p < 0.0001$) was observed. *Myk*/+ mice on standard diet (n = 15); *Myk*/+ mice on lithium diet (n = 11); +/+ mice on standard diet (n = 8); +/+ mice on lithium diet (n = 10). *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$ *Myk*/+ mice on standard diet versus +/+ mice on standard diet. #$p < 0.05$; ###$p < 0.001$ +/+ mice on lithium diet versus +/+ mice on standard diet.

demonstration of sucrose preference and conditioned taste aversion by *Myk*/+ mice (Kirshenbaum *et al.* 2011a, 2013) suggests that their gustatory perception is intact, but we cannot formally exclude the presence of a specific olfaction defect in *Myk*/+ mice.

Support for the involvement of Na^+,K^+-ATPase α3 in the regulation of social behavior is provided by heterozygous *Atp1a3*$^{tm1Ling/+}$ mice, which have a point mutation in *Atp1a3* intron 4 that reduces hippocampal α3 protein expression by around 60% and whole brain Na^+,K^+-ATPase activity by around 16%, without visible neurological defects (Moseley *et al.* 2007, Kirshenbaum *et al.* 2011b). *Atp1a3*$^{tm1Ling/+}$ mice show robust gustaoception in the sucrose preference test (Kirshenbaum *et al.* 2011b) and exhibited deficits in motor coordination and balance only in females and only after exposure to restraint stress for five days (DeAndrade *et al.* 2011). Under standard husbandry conditions, *Atp1a3*$^{tm1Ling/+}$ mice show normal social approach in the three-chamber test, but both sexes showed deficient sociability and preference for social novelty, and a reduction in whole brain Na^+,K^+-ATPase activity of around 33%, after exposure to chronic variable stress, comprising single housing and one to two unpredictable mild stressors per day for six weeks (Kirshenbaum *et al.* 2011b).

Conclusion

The observation of social behavioral deficits in patients diagnosed with AHC (I810N; Panagiotakaki *et al.* 2015), RDP (T613M; Pittock *et al.* 2000, McKeon *et al.* 2007), and CAPOS syndrome (E818K; Demos *et al.* 2014) raised the hypothesis that social deficits represent part of the complex phenotype of Na^+,K^+-ATPase α3 mutations. Consequently, we assessed the social behavior of the *Myshkin* mouse model of AHC, which has an I810N mutation identical to that found in an AHC patient with co-morbid autism. *Myk*/+ mice displayed deficits in nest building, pup retrieval and the three-chamber social approach test, suggesting that Na^+,K^+-ATPase α3 dysfunction has a deleterious effect on social behavior. This finding supports the notion that social deficits are part of the complex phenotype of Na^+,K^+-ATPase α3 mutations.

Previous behavioral analyses have revealed novelty-induced hyperambulation, increased risk-taking behavior, motor dysfunction and cognitive impairment in *Myk*/+ mice (Kirshenbaum *et al.* 2011a, 2013, 2015). This broad profile of neurobehavioral deficits, to which the present study has added social behavioral deficits, is consistent with the complex clinical profile of AHC patients, which includes a wide range of behavioral and psychiatric disorders (Neville and Ninan 2007). These multiple behavioral deficits are likely to be interrelated, such that a deficit in attention, for example, could be manifested as impairments in several behavioral tests that require sustained attention.

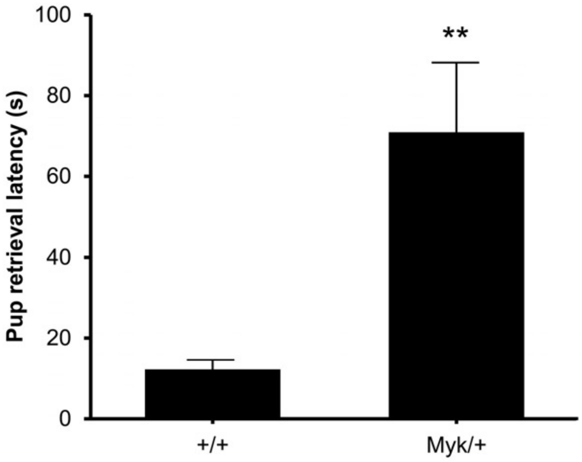

Figure 3. Pup retrieval. Latency of *Myk*/+ ($n = 8$) and +/+ ($n = 8$) dams to return pup to the nest location. A main effect of genotype ($F(1, 14) = 11.20$, $p < 0.01$) was observed. **$p < 0.01$ *Myk*/+ mice versus +/+ mice.

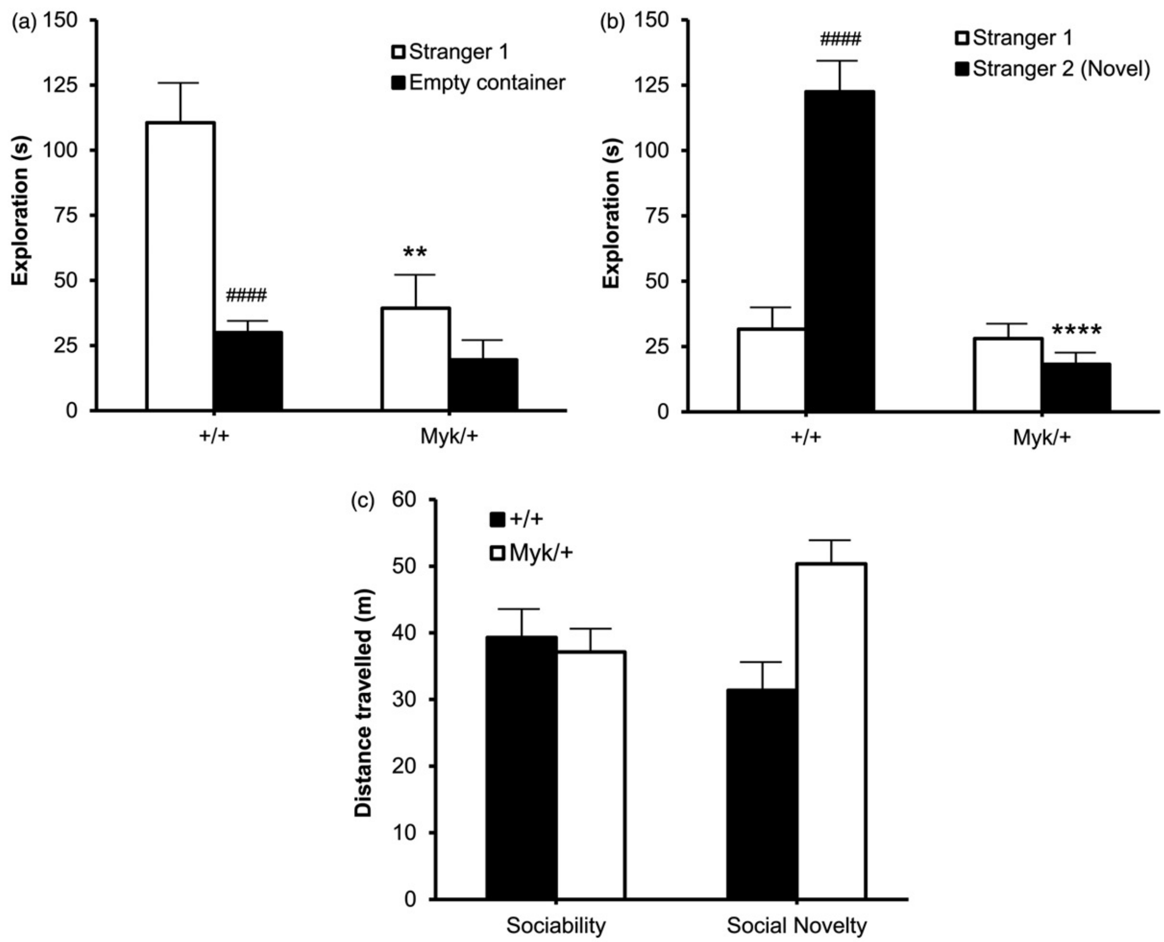

Figure 4. Three-chamber social approach test. (a) Sociability phase: time spent by the subject exploring a novel adult male mouse (Stranger 1) or an empty container. A main effect of genotype ($F(1, 14) = 5.98$, $p < 0.05$) on time in contact with the novel mouse was observed. (b) Social Novelty phase: time spent by the subject exploring the mouse previously explored (Stranger 1) and a second novel adult male mouse (Stranger 2). A main effect of genotype ($F(1, 14) = 26.71$, $p < 0.0001$) on time in contact with Stranger 2 was observed. (c) Distance travelled (m) in the Sociability and Social Novelty phases of the test. There was no main effect of genotype or sex. *Myk*/+ mice ($n = 8$); +/+ mice ($n = 10$). **$p < 0.01$; ****$p < 0.0001$ versus +/+ mice. $^{####}p < 0.0001$ versus Stranger 1.

Na^+,K^+-ATPase α3 is highly expressed in the hippocampal CA2 and amygdala (Grillo *et al.* 1997), brain regions that are important in the regulation of social behavior (Hitti and Siegelbaum 2014, Stevenson and Caldwell 2014, Mineur *et al.* 2016, Radke *et al.* 2015), but the phenotypic abnormalities of *Myk*/+ mice are not restricted to social behavioral deficits. It is, therefore, conceivable that the reduced outcomes in the three social behavioral tests reported herein may be the consequence of an underlying general behavioral abnormality, resulting from Na^+,K^+-ATPase α3 dysfunction; this could also be a possible explanation for the wide range of neurobehavioral deficits exhibited by AHC patients.

By better defining the behavioral profile of *Myk*/+ mice, the present study has identified additional *ATP1A3*-related symptoms for which the *Myk*/+ model could be used as a tool to advance understanding of the underlying neural mechanisms and develop novel therapeutic strategies. Future work could apply exploratory factor analysis to a balanced selection of variables that best characterize the behavioral variability of *Myk*/+ and +/+ mice (Valenti *et al.* 2001), to statistically dissect the involvement of factors that underlie deficits in various tests.

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Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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