Cognitive deficits caused by a disease-mutation in the $\alpha_3$ $\text{Na}^+/\text{K}^+$-ATPase isoform

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The Na$^+/K^+$-ATPases maintain Na$^+$ and K$^+$ electrochemical gradients across the plasma membrane, a prerequisite for electrical excitability and secondary transport in neurons. Autosomal dominant mutations in the human ATP1A3 gene encoding the neuron-specific Na$^+/K^+$-ATPase $\alpha_3$ isoform cause different neurological diseases, including rapid-onset dystonia-parkinsonism (RDP) and alternating hemiplegia of childhood (AHC) with overlapping symptoms, including hemiplegia, dystonia, ataxia, hyperactivity, epileptic seizures, and cognitive deficits. Position D801 in the $\alpha_3$ isoform is a mutational hotspot, with the D801N, D801E and D801V mutations causing AHC and the D801Y mutation causing RDP or mild AHC. Despite intensive research, mechanisms underlying these disorders remain largely unknown. To study the genotype-to-phenotype relationship, a heterozygous knock-in mouse harboring the D801Y mutation (\(\alpha_3^{+/\text{D801Y}}\)) was generated. The \(\alpha_3^{+/\text{D801Y}}\) mice displayed hyperactivity, increased sensitivity to chemically induced epileptic seizures and cognitive deficits. Interestingly, no change in the excitability of CA1 pyramidal neurons in the \(\alpha_3^{+/\text{D801Y}}\) mice was observed. The cognitive deficits were rescued by administration of the benzodiazepine, clonazepam, a GABA positive allosteric modulator. Our findings reveal the functional significance of the Na$^+/K^+$-ATPase $\alpha_3$ isoform in the control of spatial learning and memory and suggest a link to GABA transmission.

The ATP1A3 gene encodes the Na$^+/K^+$-ATPase $\alpha_3$ subunit isoform. Mutations in the ATP1A3 gene are associated with three related rare neurological disorders, rapid-onset dystonia-parkinsonism (RDP)$^3$, alternating hemiplegia of childhood (AHC)$^4$, and recently, cerebellar ataxia, areflexia, pes cavus, optic atrophy, and sensorineural hearing loss (CAPOS) syndrome$^4$. The disorders arise from autosomal dominant mutations with variable penetrance$^4$ and display overlapping symptoms that vary in severity, duration and frequency of occurrence$^4$$^8$. In the case of AHC, affected patients typically present in the context of an acute onset of paroxysmal, episodic neurological symptoms that include hemiplegia, dystonia, ataxia, or seizures. Some symptoms may persist after resolution, such as neurodevelopmental delays, attention deficits, trunk instability, dystonia or ataxia$^4$$^8$. AHC patients are easily aroused and have frequent episodes of hyperactivity and mania. These episodes can be associated with high risk of injuries for the patients (Personal communications from Jeff Wuchich, and Dr. Hendrick Rosewich). Case reports suggest that between 18 and 53% of AHC patients develop epileptic seizures$^7$.$^8$. The level of cognitive deficits is often correlated with severity of epilepsy. Consequently, developmental delay and deficits in cognitive functions are very common among patients suffering from AHC$^7$.$^9$. Interestingly, the complexity of ATP1A3-related disorders is emphasized by the fact that clinically distinct neurological diseases seem to be caused by mutations in a single gene. In fact, amino acid substitutions in the same position have been shown to cause RDP or AHC. One example of this is the disease hot spot amino acid position 801, where

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In the central nervous system (CNS), the α3 isoform is expressed in neurons whereas the α3 subunit is expressed in glia, and the α3 subunit appears to be expressed ubiquitously. Together, these Na+/K+ ATPase isoforms are responsible for maintaining the Na+ and K+ electrochemical gradients that determine cell resting membrane potentials, and support the electrical activity of excitable cells, as well as the transport of other ions and metabolites and driving neurotransmitter reuptake. Although the role of the Na+/K+ ATPases in the etiology of neurological diseases is poorly understood, reduced Na+/K+ ATPase activity has been linked to conditions such as epileptic seizures and schizophrenia. The distinguishing feature of the Na+/K+ ATPases is their several-fold lower affinity for activation by cytoplasmic Na+ compared to that of α3 Na+/K+ ATPases. In rapidly firing neurons, therefore, when action potentials increase the intracellular Na+ concentration, [Na+]i beyond levels saturating the “housekeeping” α1 Na+/K+ ATPases, activation of α3 Na+/K+ ATPases continues to increase as [Na+]i rises. As [Na+]i is linked to [Ca2+]i through the Na+/Ca2+ exchanger, the α3 isoform thus protects neurons against catastrophic elevation of [Na+]i and [Ca2+]i, and general loss of the Na+ electrochemical gradient.

Atp1a3 mouse models have provided insights into the role of the α3 isoform in neurological diseases. Currently, two knock-out and two knock-in mouse models have been reported. The heterozygous knock-out α3−/−/Atp1a3 mouse (Atp1a3tm1Klh) displayed spatial learning and memory deficits, hyperlocomotion and increased locomotor response to methamphetamine. The α3−/−/D801Y mice knock-out mouse (Atp1a3tm1Klh) showed increased sensitivity to kainate-induced dystonia and enhanced inhibitory neurotransmission of molecular-layer interneuron–Purkinje cell synapses in the cerebellar cortex. The Myshkin mouse harbors the heterozygous D801N disease mutation in the Atp1a3 gene (Myshkin, α3+/D801N, Atp1a3tm1Ling), which in humans causes AHC. The Myshkin mice are characterized by seizure activity and mania-like behavior, and showed increased response to amphetamine, similar to what has been reported for bipolar patients as well as motor dysfunction and cognitive impairments related to compromised thalamocortical functionality. The D801N mutation is found in more than one third of AHC patients. A recent study reported that heterozygous D801N knock-in mice (Mashlh1/−, Mashl, α3+/D801N) manifested several AHC-like symptoms including neuromuscular deficits, spontaneous recurrent seizures, and predispositions to kindling, to fluoroethyl seizures and to Sudden Unexpected Death in Epilepsy (SUDEP).

To further address the complex genotype-phenotype relationship particular to the D801 amino acid position (the D801N/E/V mutations are associated with AHC whereas D801Y is associated with both RDP1 and AHC), the α3−/−/D801Y mouse (Atp1a3tm1Klh) was generated and the general behavior and cognitive functions were explored. We found that α3−/−/D801Y mice display ATP1A3-related symptoms such as hyperactivity, lower threshold for PTZ-induced epileptic seizures, and reduced hippocampus-dependent cognitive performance.

The hippocampus has been suggested to be the primary brain structure for spatial memory acquisition, memory storage and consolidation. The hippocampal formation comprises dentate gyrus, the hippocampus proper and the adjacent parahippocampal cortices. The major excitatory input to the hippocampus arises from the entorhinal cortex via the perforant path that primarily terminates in the dentate gyrus. The dentate axons project to the CA3 region and from there the Schaffer collaterals convey the processed input to the CA1 area.

In spite of this, we did not observe any change in the excitability of CA1 pyramidal neurons in the α3−/−/D801Y mice. The cognitive deficits were rescued by administration of the GABA positive allosteric modulator, the benzodiazepine clonazepam. The α3−/−/D801Y model demonstrates a role in cognition comparable to the D801Y AHC manifestation, and will be suitable for investigations of disease mechanisms and development of therapeutic interventions.

### Results

**Non-Mendelian ratio and reduced α3 protein.** Upon generation of the α3−/−/D801Y mouse model (Fig. 1), the line was back-crossed for >7 generations before testing. Analysis of the Mendelian distribution among 200 offspring, at 3 weeks of age, showed that 35% were genotyped as α3−/−/D801Y (Fig. 2a), suggesting neontal absorption and/or perinatal death. It should be noted that homozygous α3−/−/D801Y mice died at birth. The introduction of the D801Y mutation caused a 15% reduction in total α3 protein levels, but a 33% increase of the α3 protein levels, as tested by Western blotting (WB) analysis of whole brain, cortex, hippocampus, and cerebellum lysates from adult α3−/−/D801Y mice relative to WT levels (Fig. 2b,c).

**α3−/−/D801Y caused hyperactivity, but not anxiety.** In the open field test (OF), the α3−/−/D801Y mice displayed hyperlocomotion relative to WT mice (Fig. 3a). After an initial habituation period of 8–10 minutes, WT mice showed a typical increase in horizontal rotation and meander. In contrast, α3−/−/D801Y mice showed minimal habituation and almost exclusively changed direction when reaching the walls of the enclosure (Fig. 3b,d). Track plot analysis revealed a significant increase in time spent in the OF periphery (Fig. 3c). We hypothesized this to be a consequence of the low meandering rather than an indication of anxiety. In support, when tested in the elevated plus maze (EPM), the α3−/−/D801Y mice did not discriminate between entering open and closed arms (Fig. 3e,f) and spent 240% more time in the EPM open arms compared to WT mice (Fig. 3g).

Thus, the α3−/−/D801Y mice appears to reflect hyperactivity and arousal to some degree, as they became highly agitated and displayed hyperactivity in response to handling and novel environments (described below in the Barnes Maze test), related to symptoms described for AHC patients.

**Reduced seizure threshold in the α3−/−/D801Y mice.** Corresponding to the high rate of seizures reported for AHC patients, reduced Na+/K+ ATPase activity was shown to influence seizure activity in the Myshkin mouse model and contribute to SUDEP in the Mashlh1/− mouse model. The α3−/−/D801Y mice did not develop spontaneous seizures. To determine differences in subclinical seizure thresholds, α3−/−/D801Y and WT mice were injected...
intraperitoneally with the non-competitive GABA antagonist, pentylenetetrazole (PTZ). PTZ induced a significantly stronger effect in the α\textsubscript{3}+D801Y mice as shown by the increased lethality in the α\textsubscript{3}+D801Y mice relative to WT mice (Fig. 4).

The excitability of CA1 pyramidal neurons is not changed in the α\textsubscript{3}+D801Y mice. The hippocampal CA1 region is one of the brain areas in which PTZ induces highly synchronized epileptiform burst activity. We therefore hypothesized that a reduced PTZ seizure threshold would be reflected in an increased excitability of CA1 pyramidal neurons. Using intracellular recordings in acute brain slices, no major difference in the basic membrane properties of CA1 pyramidal neurons in α\textsubscript{3}+D801Y and WT mice was found. The resting membrane potential (RMP) and input resistance (R\textsubscript{in}) were similar in α\textsubscript{3}+D801Y and WT mice (Supplementary Table 1). The threshold for induction of action potentials (APs) (Supplementary Table 1) as well as the overall composition of the APs were also similar (Fig. 5a) and typical of CA1 pyramidal neurons. The only difference found with respect to the AP was a slight, but significant, reduction of the rate of decay (rate of repolarization) in α\textsubscript{3}+D801Y mice compared to WT (Fig. 5a). Depolarizing current pulses (1 s) induced trains of APs displaying frequency accommodation in WT and α\textsubscript{3}+D801Y mice (Fig. 5b). However, the amount of accommodation was less in α\textsubscript{3}+D801Y mice compared to WT mice, primarily due to a slower initial discharge rate in α\textsubscript{3}+D801Y mice compared to WT mice (Fig. 5c). No significant difference in steady-state electroresponsive behavior, measured as the frequency vs. current (f-I) relationship, was found between α\textsubscript{3}+D801Y and WT mice (Fig. 5d).

The α\textsubscript{3}Na\textsuperscript{+}/K\textsuperscript{+}-ATPase has been suggested to serve as a “reserve” transporter activated when [Na\textsuperscript{+}]\textsubscript{i} is high, such as following prolonged high frequency discharge (reviewed in22). High frequency firing was evoked by a 20 s suprathreshold depolarizing current pulse. The assumption being that a reduced “reserve” capacity in α\textsubscript{3}+D801Y mice would lead to a higher rise in the [Na\textsuperscript{+}] concentration, resulting in a more pronounced activity-dependent

Figure 1. Generation and Targeting Strategy Screenings of the α\textsubscript{3}+D801Y knock-in mice. Diagram of the targeting strategy showing the oligonucleotide primers (grey triangles: P1, P2 and P3, P47, and P2) (a). PCR screenings verified homologous recombination in the IIBR clone, which was not observed for WT controls (b). Southern blotting analysis verified heterozygous homologous recombination in clone IIBR not observed for control (ES cells) (c). Partial Cre-excision of LoxP-NEO-LoxP cassette in the IIBR clone was verified by PCR and not observed for two controls (C1) (d). Confirmation of the third LoxP site was performed by a P\textsubscript{shai} digest (a restriction site introduced with LoxP insertion) of a PCR product (using primers 5′-gtaccccgtggatataaagt and 5′-gaagaagaaggaggttagg on genomic DNA). Treatment with the P\textsubscript{shai} enzyme gave in WT a non-digestable band of 2981 bp, and (+/-) gave 3084 bp, and 1650 bp and 1331 bp bands, whereas (-/-) gave 1650 and 1331 bp bands (e). Sequencing confirmed a heterozygous G → T base exchange in position 80 in the genomic DNA of an α\textsubscript{3}+D801Y mouse that was not present in WT (f). M: molecular DNA marker, bp: base pair, H\textsubscript{2}O: No template control where DNA was substituted with water. 

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reduction of the amplitude of aPs. However, the activity-dependent reduction was similar between the two genotypes (Fig. 5e), as was the amplitude of the post-pulse slow afterhyperpolarization (Supplementary Table 1).

**Spatial learning and memory is reduced in α3+/D801Y compared to WT littermates.** More than 90% of AHC patients show signs of developmental delay or mental retardation. Similarly, a recent care report showed that 90% of RDP patients with onset at or after 18 years had trouble learning in school. The α3+/D801Y mice were tested for spatial learning and memory performance using the Barnes Maze (Hippocampus-based spatial reference memory) and passive avoidance (amygdala- and hippocampus-based memory)

In the Barnes Maze test, the mice were subjected to 4 training sessions per day for 4 days and subsequently a single test on day 5 and day 12. WT mice showed a significant reduction in latency to enter the escape tunnel (total latency). A similar learning curve was not observed for the α3+/D801Y mice (Fig. 6a). Interestingly, the WT and α3+/D801Y mice would reach the escape tunnel at the same time (primary latency) (Fig. 6b). However, once at the escape tunnel, the WT mice quickly entered, whereas the α3+/D801Y mice would walk past multiple times before entering (Fig. 6c). Further analysis of zone occupancy confirmed that both genotypes after training spent the majority of time investigating the target area. Whereas WT mice specifically occupied the target zone on testing day 5 and 12, the α3+/D801Y mice showed equal interest in the adjacent zone (−1) and remained outside for extended periods (Fig. 6d).

To assess the learning performance further, the strategy used to locate the target zone was analysed. Strategies were categorised as 1) Direct, where the mouse located the target tunnel or an adjacent hole using external cues 2) Serial, where the mouse seemingly chose a random hole and subsequently searched adjacent holes in a serial manner in a clockwise or counterclockwise direction or 3) Mixed, where the mouse displayed a more random search pattern and occasionally crosses the center of the circular platform.

Figure 2. Basic characterization of the α3+/D801Y mice. Boxplot showing skewed genotype ratio at weaning age (approximately postnatal day 21) (α3+/D801Y mice N = 70, WT mice N = 130) (a). Representative Western blots illustrating α1-3 protein expression in α3+/D801Y and WT mice in cortex (CTX), hippocampus (HC), cerebellum (CRBL) and in whole brain lysates (Brain) (full-length Western blots are shown in Supplementary Fig. 1) (b). Relative protein expression levels determined by densitometry showed a significant increase in □ throughout the brain of α3+/D801Y (N = 4). Expression of α2 was not affected (N = 3) whereas α3 expression was significantly reduced in α3+/D801Y mice (N = 3) (c).
direct strategy and had reverted to using the serial or the mixed strategy. In contrast, the WT mice still utilised the direct and serial strategies (Fig. 6e).

Fear memory is reduced in α3+/D801Y compared to WT littermates. We used passive avoidance test to assess fear memory in the mice. Compared to WT mice, the α3+/D801Y mice showed a significantly reduced latency to re-enter the dark compartment (Fig. 6f).

Alterations in inhibitory interneurons contribute to cognitive deficits associated with several psychiatric and neurological diseases. Inhibition by GABA receptors regulating neuronal activity helps to establish the appropriate network dynamics that support normal cognition 35. To investigate if GABA transmission might be involved in the cognitive deficits observed in the mice, α3+/D801Y and WT littermate mice were injected with the benzodiazepine, clonazepam and subsequently tested in the passive avoidance test. Clonazepam administrated intraperitoneally at 0.0625 mg/kg has previously been shown not to cause significant sedation or anxiolytic effect in the OF and EPM 36. At this concentration, clonazepam completely normalized the performance of α3+/D801Y mice in the passive avoidance test (Fig. 6f).

Figure 3. Increased spontaneous locomotor activity and altered exploration of the α3+/D801Y mice. The α3+/D801Y mice (N = 15) displayed hyperlocomotion (a) and traveled significantly longer in the open field test (a, insert) compared to WT mice (N = 14). Representative track plots of the first 3 minutes of open field exploration are shown in (b). The α3+/D801Y mice remained significantly longer in the open field periphery than WT mice (c). After an initial habituation period, WT mice displayed increased meander defined as horizontal rotation per distance traveled. This behavior was completely absent in the α3+/D801Y mice, here shown as the average score of 14 α3+/D801Y and 14 WT mice (d). Representative track plots of elevated plus maze exploration are shown in (g). WT mice (N = 14) preferred to enter the closed arms whereas α3+/D801Y mice (N = 15) showed no arm preference (e,f). The α3+/D801Y mice occupied the open arms significantly longer than WT mice (g). All data shown are means ± SD *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Figure 4. Reduced seizure threshold in the α3+/D801Y mice. The α3+/D801Y mice (N = 8) showed significantly reduced seizure threshold upon intraperitoneal injection with PTZ compared to WT littermates (N = 8) with no end point survival observed in the α3+/D801Y mice.
Reduced number of hippocampal dentate gyrus granule cells in the α3+/D801Y mice. Since both memory tests pointed towards hippocampal defects, we performed histological examination of this brain region (Fig. 7a). Subsequent stereological counting showed a significantly reduced number of granule layer neurons in the dentate gyrus of the α3+/D801Y mice (Fig. 7b).
Hippocampal brain sections from the $\alpha_3^{+/D801Y}$ mice revealed a large number of pyknotic nuclei within the dentate gyrus granule cell layer compared to WT littermates (Fig. 7c–f), suggesting that the reduced number of granule layer neurons in the dentate gyrus of the $\alpha_3^{+/D801Y}$ mice was partly due to this.

Discussion

$ATP1A3$ mutations have been recognized in infants and children presenting with diverse neurological symptoms. In AHC patients, some of the most devastating symptoms include bouts of hyperactivity that may cause patients to injure themselves accidentally and epileptic seizures that are associated with SUDEP and worsening of cognitive impairments. A recent case reports suggest that RDP patients also suffer from cognitive impairments, albeit to a lesser degree as many patients are able to attend and finish high school.

The highly variable nature of $ATP1A3$ diseases even for patients carrying the same mutation, has poised the theory that other factors such as genetic background, epigenetic modifications and environmental triggers influence the disease course.

Several studies show that $\alpha_3$ expression is influenced by changes in $[\text{Na}^+]_3$ and $[\text{K}^+]_3$, both are likely affected by reduced $\alpha_3\text{Na}^+/\text{K}^+\text{ATPase}$ activity. The compensatory upregulation of $\alpha_3$ protein in response to the reduced $\alpha_3$ protein expression in whole $\alpha_3^{+/K014}$ brain lysates is therefore to be expected. Similar observations have previously been reported for the $\alpha_3^{+/K014}$ mice.

Figure 6. Reduced spatial memory and learning abilities in the $\alpha_3^{+/D801Y}$ mice. In Barnes maze analysis, $\alpha_3^{+/D801Y}$ (N = 6) and WT (N = 8) mice were monitored for (a) the time to enter the hidden tunnel i.e. total latency, (b) the time to first visiting the hidden tunnel i.e. the primary latency and (c) the number of target visits. (d) Zone occupancy and (e) target hole strategies (Direct, serial and mixed). The passive avoidance test, (f) the $\alpha_3^{+/D801Y}$ (N = 4) and WT (N = 6) mice differed in their response to clonazepam (Clz) compared to the vehicle treatment. All data shown are means ± SD. *P < 0.05.
Homozygous \( \alpha_3^{+/D801Y} \) mice died at birth, suggesting that \( \alpha_1 \) upregulation could not compensate for \( \alpha_3 \) loss at this stage. This is in accordance with suggestions that \( \alpha_1 \) and \( \alpha_3 \) differ not only in substrate affinity but also in localisation\(^39\) and that the CNS shifts from predominantly using the \( \alpha_1 \) and \( \alpha_2 \) isoforms during early development to \( \alpha_1 \) and \( \alpha_3 \) during post-natal development\(^26\).

In exploration-based tests for anxiety-like behavior, such as the OF and EPM, it can be difficult to dissociate symptoms of hyperactivity and attention deficits from anxiety-like behavior, as they may interfere with spontaneous exploratory locomotion. Although the \( \alpha_3^{+/D801Y} \) mice spent significantly more time in the OF periphery, we propose this to be a direct consequence of the lack of meander rather than a trait of anxiety. In support, the \( \alpha_3^{+/D801Y} \) did not discriminate between entering open and closed arms in the EPM and spent significantly more time than WT mice exploring the EPM open arms. In further support of this hypothesis, similar behavior was described for an attention deficit mouse model\(^40,41\).

**Figure 7.** Number of neurons in hippocampus dentate gyrus granular layer is reduced in the \( \alpha_3^{+/D801Y} \) mice. The number of neurons in the dentate gyrus granular layer (a) was significantly reduced in the \( \alpha_3^{+/D801Y} \) mice \((N = 10, n = 408,217)\) compared to WT littermates \((N = 10, n = 477,124)\) (b). All data shown are means ± SD. \(*P < 0.05\). Hippocampal brain sections from the WT (c,d) and \( \alpha_3^{+/D801Y} \) (e,f) mice revealed a large number of pyknotic nuclei within the dentate gyrus granule cell layer in the \( \alpha_3^{+/D801Y} \) compared to WT littermates.
The hyperactive phenotype observed in the OF was induced response to handling and novelty in general. This was particularly evident during the memory tests, where repeated handling and the stressful environment caused some \( \alpha_3^{+/D801Y} \) mice to become so agitated that they would jump off the testing platforms repeatedly and hurt themselves. These mice were omitted from the study. We believe this behavior could reflect in some degree the hyperactive and manic episodes of AHC patients.

Reduced \( \text{Na}^+/K^+\)-ATPase activity has previously been described in human epilepsy patients\(^{39}\). There is a growing appreciation that genetic factors contribute to the etiology of seizures\(^{42}\). With the recent case report of D801Y patient diagnosed with late onset mild AHC, it is possible that other symptoms of \( ATPIA3 \)-related disorders are further affected by the genetic background.

The \textit{Myskkin} and \textit{Mash}\(^{+/-} \) mice were originally maintained in the 129S1/SvImJ and 129Sv background, respectively, and developed spontaneous tonic-clonic seizures and epileptic discharges as well as a SUDEP-like phenotype\(^{26,29}\).

Despite a reduced PTZ seizure threshold, we did not observe spontaneous seizures in the \( \alpha_3^{+/D801Y} \) mice. Spontaneous seizures have also not been reported for the \( \alpha_3^{+/KOH} \) and \( \alpha_3^{+/\Delta E2-6} \) mice\(^{23,24}\). Supporting a strong contribution from genetic background, the latter two \textit{Atp1a3} mouse models were maintained in the seizure resistant C57BL/6J strain\(^{45}\). Given the close relationship to the C57BL/6J strain, it is likely that some of the same genetic modifiers play a role in the seizure phenotype of the \( \alpha_3^{+/D801Y} \) mice. In further support of this theory, the \textit{Myskkin} mice became resistant to stress-induced seizures once maintained in the seizure-resistant C57BL/6J strain for 20 generations\(^{27}\).

Electrophysiological measurements in acute brain slices from naïve animals showed only minor differences between the genotypes. This cannot account for the decreased seizure threshold. It therefore seems unlikely that the increased excitability of the \( \alpha_3^{+/D801Y} \) mice can be explained by changes in the basic membrane properties of the CA1 pyramidal neurons.

The hippocampus regulates the generation of long term memory\(^{44}\) and spatial learning\(^{45}\) and has been shown to be critical for spatial memory in human subjects with hippocampal damage (reviewed in\(^{46}\)). Together, the presented memory tests suggest an essential role of hippocampal \( \alpha_3 \text{Na}^+/K^+\)-ATPase in consolidating particularly long-term spatial memory and fear-dependent memory, whereas short-term memory seemed less affected.

The \textit{Barnes maze} test showed that the \( \alpha_3^{+/D801Y} \) mice took significantly longer time to enter the target tunnel. This difference was not caused by an inability of the \( \alpha_3^{+/D801Y} \) mice to locate the tunnel, but rather the fact that the \( \alpha_3^{+/D801Y} \) mice spent upwards of four times longer at the entrance before entering. The \textit{Barnes maze} test relies on the instinct of mice to escape a brightly lit area and to seek protection in a tunnel. The mouse is guided towards the tunnel via external cues mounted on the surrounding walls. The lack of discrimination between open and closed arms in the EPM would suggest that the instinct to seek cover is suppressed in the \( \alpha_3^{+/D801Y} \) mice. Similar behavior has previously been described in the dopamine transporter knockout mouse, a mouse model for attention-deficit/hyperactivity disorder (ADHD) and schizophrenia-like behavior\(^{40,41}\).

We propose that the increased total latency is a consequence of failure to process the stressful surroundings of the \textit{Barnes maze}, originally created to drive the mice into the tunnel.

\textit{Barnes maze} track plot analysis revealed that the mice irrespective of genotype employed similar search strategies during the first 4 days of training: At day 5, both genotypes used the serial and direct strategies to an equal extent, suggesting that the \( \alpha_3^{+/D801Y} \) mice have functional short-term spatial memory. The direct approach requires that the mice navigate using external cues, confirming that the vision of the \( \alpha_3^{+/D801Y} \) mice was intact.

At day 12, the \( \alpha_3^{+/D801Y} \) mice no longer used the direct strategy and reverted to predominantly using the serial and to a lesser extent the mixed approach. This suggests that their long-term spatial memory was affected. Primary latencies were not affected by this change in tactics. We initially expected a compensatory effect of the hyperlocomotion, previously observed in OF, but average speed for both genotypes was similar (not shown).

The \( \alpha_3^{+/D801Y} \) showed reduced fear memory in the passive avoidance test. It was clear that the right environment, the mice were fully capable of entering a dark compartment. A likely explanation for poor performance of the \( \alpha_3^{+/D801Y} \) in the \textit{Barnes maze} is therefore that the stressful environment interfered with the decision-making of the \( \alpha_3^{+/D801Y} \) mice.

The reduced spatial learning and memory abilities in the \( \alpha_3^{+/D801Y} \) mice suggested dysfunction of the amygdala and hippocampal brain regions. Histological examination revealed a large number of pyknotic nuclei within the granule layer of the dentate gyrus. Similar nuclear morphology has previously been described in ouabain-treated cultured cortical neurons undergoing hybrid cell death\(^{45}\). Furthermore, similar hippocampal pathologies has previously been reported in rats injected with ouabain into the hippocampus\(^{48}\) and in a rat model of pilocarpine-induced chronic epilepsy\(^{49}\), thus strengthening the link between \( \text{Na}^+/K^+\)-ATPases perturbations and seizure.

The \( \alpha_3 \) isoform is highly expressed in the GABAergic basket cells in the subgranular zone\(^{17}\) that are responsible for proliferation of granule cells during early development. It is therefore likely that the reduced number of granule cells in the dentate gyrus is the result of skewed apoptosis/proliferation in this region and that this is directly affected by the reduced \( \alpha_3 \) activity.

Memory deficits were noted in the heterozygous knock-in mouse models \textit{Myskkin}\(^{30}\) and \textit{Mash}\(^{1+/-} \; 29\) as well as the heterozygous knock-out model \( \alpha_3^{+/KOH} \) mice\(^{23}\), strongly supporting the role of the \( \alpha_3 \) in hippocampus-dependent cognition. The \( \alpha_3^{+/KOH} \) mice showed reduced expression of the N-methyl-D-aspartic acid receptor (NMDR)\(^{31}\). The NMDR has a well-documented role in the formation of several memories, including spatial, olfactory and contextual memory\(^{52}\). NMDA receptor expression was reduced by applying ouabain to cerebellar neurons\(^{33}\). Reduced NMDA receptor NR1 expression was described in homozygous E18 \textit{Myskkin} mice, but not in heterozygous E18 and adult \textit{Myskkin} mice\(^{26}\).

The co-expression of the \( \alpha_3 \) isoform in GABAergic neurons suggests an association between the \( \text{Na}^+/K^+\)-ATPase and GABA transmission. The role of GABA\(_A\) receptors in learning and memory and neurological...
disorders is well documented (recently reviewed\textsuperscript{34}). In particular, GABA regulates oscillations implicated in learning and memory, by generating synchronized inhibitory post synaptic potentials. Dysfunctions caused by the \(\alpha_3\) isoform has previously been linked to GABA transmissions in the \(M\_Myosin\) mouse and aberrant cerebellar function in the \(\alpha_3\) mice\textsuperscript{24}. To test if increasing GABAergic transmission could improve learning, the \(\alpha_3^{+/D801Y}\) mice was treated with the benzodiazepine, clonazepam. A single injection rescued passive avoidance performance and thus fear-dependent memory. Interestingly, similar effects were recently reported for the \(Scn1a^{+/D801Y}\) mouse model for Dravet's syndrome\textsuperscript{56}, a disease where GABAergic neurotransmission is specifically impaired by a mutation in the \(SCN1A\) gene encoding voltage-gated sodium channel \(Na_v1.1\). Interestingly, the \(Scn1a^{+/D801Y}\) mice also exhibited hyperactivity, and impaired context-dependent spatial memory. Supporting the hypothesis that GABA is a major contributor towards \(ATP1A3\)-related diseases is the fact that GABA\(_{\alpha_3}\) receptors are implicated in childhood epilepsy\textsuperscript{55}, and patients with temporal lobe epilepsy exhibit altered expression of the mRNA encoding the GABA\(_{\alpha_3}\) receptor in several hippocampal sub-regions\textsuperscript{56-58}. It was clear that PTZ, as a temporal lobe epileptic inducer, lowered the seizure threshold in the \(\alpha_3^{+/D801Y}\) mice compared to WT mice, supporting the role of GABA.

The effects of clonazepam are associated with allosteric activation of the ligand-gated GABA\(_{\alpha_3}\) receptor\textsuperscript{59}. The current note of GABA\(_{\alpha_3}\) receptor complex subunits is that the GABA\(_{\alpha_3}\) subunit might be implicated in memory and learning, however, the GABA\(_{\alpha_3}\) subtype alteration in the \(\alpha_3^{+/D801Y}\) mice remains to be elucidated.

The results presented here strengthen the ongoing debate of the complexity of the \(ATP1A3\)-related diseases: Why some mutations are specific to RDP, AHC or CAPOS, and why the same mutations may produce intermediate symptoms could give rise to so very different disease courses. Given that the D801Y mutation has been shown to cause RDP and AHC in human patients, the \(\alpha_3^{+/D801Y}\) mice may present as a unique platform to investigate this further. \(ATP1A3\)-related diseases have no effective treatments\textsuperscript{4}. It is therefore interesting that the cognitive deficits in the \(\alpha_3^{+/D801Y}\) mice could be reverted by a single low dose of clonazepam. This novel mouse model could be helpful for future developments of targeted treatments in neuropsychology and memory functions\textsuperscript{60}.

**Methods**

**Generation of the \(\alpha_3^{+/D801Y}\) KI mouse model.** Vector preparation. Preparation of LoxP-NEO-LoxP (PCG sing LPN_Bcl_F: 5’-cggtgatca-ataacttcgtatagca and LPN_Bcl_R: 5’-cggtgatca-gcctgctattgtcttc) and 2XTK cassette were performed as described\textsuperscript{61}. The 2XTK cassette (3652 bp) was cloned into Strataclone\textsuperscript{TM} blunt PCR cloning vector, pSC-B (3.5 kb) using the SacI restriction enzyme site (generating the pSC-B-2XTK). The 5’ end 5921 bp PCG product (A3.1交错 sac 2R: 5’-GCAACCAAAATGGAGACTCC and I1F not: 5’-GAAGGCTTCCTCTCCCTGACAT) was cloned into the pSC-B-2XTK (generating the pSC-B-2XTK-5HOM), and a LoxP site-containing PCR product (LP_BstX1_F: 5’-cgccgannnnttgg-ataacttcgtataatgt and LP_BstX1_R: 5’-cgccgannnnttgg-ataacttcgtataatgt) was inserted using a BstX1 restriction enzyme (pSC-B-2XTK-5HOM-LP). The LoxP-NEO-LoxP vector was cloned into the pSC-B-2XTK-LP vector using the BclI restriction enzyme (generating the pSC-B-2XTK-5HOM-LP-LPNLP). The 1660bp 3’ end PCR product (I21 R sac2: 5’-GCCACGGCTAACCCTCAACT and A3.1 intersect saca 2F: 5’-GCAACCAAAATGGAGACTCC) was cloned into Strataclone\textsuperscript{TM} blunt PCR cloning vector, pSC-B (3.5 kb) (generating the p-SC-B-3HOM construct).

Site-directed mutagenesis was performed to introduce the D801Y mutation using the following primers to introduce this mutation, atp1a3-blpmut-F 5’-cggtgatca-gcctgctattgtcttc and atp1a3-blpmut-R 5’-gctgctattgtcttc. The pSC-B-2XTK-5HOM-LP-LPNLP and p-SC-B-3HOM was combined using a Clal site, generating the Atp1a3 D801Y gene targeting vector (15.642 bp).

C7 ES cells (from 129 S1/Sv)\textsuperscript{62} were electrooporated with the linear targeting vector and double selected with G418 (350 \(\mu\)g active substance/ml) and FIAU (0.5 \(\mu\)g/ml) (Fig. 1a). Targeting efficiency was 3/360 double resistant ES cell colony TM144 II B12 was confirmed by 5’ end and 3’ end PCR's using primers located to the NEO cassette sequence (P47) and Atp1a3 sequence specific primers (A3.1交错_F4 and A3.1交错_R4) located to Atp1a3 sequences not included in the gene targeting vector, as follows; 5’-PshaI, a site that was deleted introducing the D801/Y mutation was confirmed by DNA sequencing (Fig. 1f). Transgenic \(\alpha_3^{+/D801Y}\) knock-in (K1) mice \((\text{Atp1a3}^{+/\text{D801Y}})\) were crossed to C57/BL6J (Janvier) background, and heterozygous \(\alpha_3^{+/D801Y}\) mice were identified using the following primers; A3.1交错_F4 5’-cggtgatca-ataacttcgtatagca and A3.3.Rarm: 5’-cgccgannnnttgg-ataacttcgtataatgt) was inserted using a BstX1 restriction enzyme (pSC-B-2XTK-5HOM-LP). The LoxP-NEO-LoxP insert was cloned into the pSC-B-2XTK-LP vector using the BclI restriction enzyme (generating the pSC-B-2XTK-5HOM-LP-LPNLP). The 1660bp 3’ end PCR product (I21 R sac2: 5’-GCCACGGCTAACCCTCAACT and A3.1 intersect saca 2F: 5’-GCAACCAAAATGGAGACTCC) was cloned into Strataclone\textsuperscript{TM} blunt PCR cloning vector, pSC-B (3.5 kb) (generating the p-SC-B-3HOM construct).

Partial cre of transfected ES cells. The NEO cassette was removed by partial Cre-enzyme treatment leaving a single Loxp site in intron 16 obtained by transfecting TM144 II B12 ES cells with linearized Cre-enzyme encoding plasmid, as described\textsuperscript{61}. Successful partial Cre-enzyme treatment was confirmed for the TM144 II B12 cre 2 and 15 clone by PCR (A3.1交错_C1 and A3.Rev.RC) (data not shown) and PshaI digestions of the PCR product generated specific band patterns (Fig. 1e) (No cre: 1650 bp and 1331 bp, full cre: 1902 bp, 1650 bp and 1331 bp, partial cre; 3084 bp, 1650 bp and 1331 bp). The TM144 II B12 cre 8 revealed at non-cre event. The final introduction of the D801/Y mutation was confirmed by DNA sequencing (Fig. 1f). Transgenic \(\alpha_3^{+/D801Y}\) knock-in (K1) mice \((\text{Atp1a3}^{+/\text{D801Y}})\) were crossed to C57/BL6J (Janvier) background, and heterozygous \(\alpha_3^{+/D801Y}\) mice were identified by qPCR genotyping (described below).
Genotyping. Heterozygous $\alpha_3^{+/D801Y}$ mice were genotyped by High Resolution Melt analysis (Roche, Basel, Switzerland) on a lightcycler 96 (Roche, Basel, Switzerland). using forward primer tcatgctaatctccacctg and reverse primer agtagcagccaggcttacca (Sigma-Aldrich; Schnelldorf, Germany).

Animal ethics and conditions. All in vivo studies were performed using $\alpha_3^{+/D801Y}$ mice and WT obtained by crossing $\alpha_3^{+/D801Y}$ mice (generation N ≥ 8) with C57BL/6Jrj mice (Janvier). Mice were kept at a daily 12 h light/dark cycle. Tests were performed during the light phase. The mice were maintained as heterozygotes ($\alpha_3^{+/D801Y}$) since homozygotes were neonatally lethal. Mice used for all experimental procedures are between 10–20 weeks of age. Experimental protocols involving mice, performed at Aarhus University were performed according to the Danish national and Institutional regulations and approved by the Animal Experiments Inspectorate under the Danish Ministry of Justice (permit numbers 2012-15-2934-00621, 2013-15-2934-00815 and 2014-15-2934-01029) to KLH.

Basic Characterization. Western blot. The protocol for Western blot was performed as previously described. Primary antibodies (anti $\alpha_1$ 1:2000 (a6f-c, DevelopmentalStudies Hybridoma Bank), anti $\alpha_2$ 1:1000 (07674, EMD Millipore, US), anti $\alpha_3$ 1:1000 (06172, EMD Millipore, US), anti DAT 1:500 (MAB369, EMD Millipore, US), anti TH 1:2000 (AB152, EMD Millipore, US) and GAPDH 1:1000 (ab9485, Abcam, Cambridge, UK)) were incubated overnight at 4 °C. Next day, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (swine anti-rabbit 1:2000 (Dako, Glostrup, Denmark), rabbit anti-mouse 1:2000 (Dako, Glostrup, Denmark)) for 1 hour at room temperature. Visualization was done with a LAS 3000 imager (Fujifilm, Tokyo, Japan) using Amersham ECL Western Blotting Detection Kit (GE Healthcare, Buckinghamshire, UK) as the detection reagent. ImageJ version 1.48 v was used for densitometric analysis of the Western blots.

In vitro electrophysiology. Preparation of brain slices. Male mice were anesthetized with isoflurane and decapitated. The brain was removed and quickly placed in dissection medium (in mM; 120 NaCl, 2 KCl, 1.25 KH$_2$PO$_4$, 6.6 HEPES acid, 2.6 NaHEPES, 20 NaHCO$_3$, 2 CaCl$_2$, 2 MgSO$_4$ and 10 D-glucose, bubbled with 95% O$_2$ and 5% CO$_2$) at 4 °C. The hippocampus was dissected free, and 400 μm slices were cut using a McIlwain tissue chopper. One slice was immediately transferred to the recording chamber, where it was placed on a nylon-mesh grid at the interface between warm (31–32 °C) aCSF (in mM; 124 NaCl, 3.25 KCl, 1.25 NaH$_2$PO$_4$, 20 NaHCO$_3$, 2 CaCl$_2$, 2 MgSO$_4$ and 10 D-glucose, bubbled with 95% O$_2$ and 5% CO$_2$, pH 7.3) and warm humidified gas (95% O$_2$, 5% CO$_2$). Perfusion flow rate was 1 ml/min. The slice rested for at least one hour before electrophysiological recordings were started. The remaining slices were stored in dissection medium bubbled with 95% O$_2$ and 5% CO$_2$ at room temperature.

Electrophysiological recordings. Intracellular recordings were obtained using borosilicate glass electrodes (1.2 mm OD; Clark Electromedical, Pangbourne, UK) filled with 4M K$^+$ acetate and placed in stratum pyramidale in area CA1. Conventional recording techniques were employed, using a high-input impedance amplifier (Axoclamp 2A, Molecular Devices, USA) with bridge balance and current injection facilities. Signals were digitized online using a Digidata 1440 interface and transferred to a computer for analysis employing pCLAMP (version 10, Molecular Devices). Inclusion criteria were a stable resting membrane potential (RMP) ≥ 70 mV. The time-dependent decay in AP amplitude during 20 s repetitive firing was estimated using the following formula: (1st AP – last AP)/1st AP.

Analysis. $R_n$ was evaluated from the current-voltage relationship close to RMP; the AP threshold was measured using short (4 ms) depolarizing current pulses of increasing intensity; the rates of rise and decay of the AP were taken as the maximal slopes; the frequency vs. current (f-I) relationship was estimated with 500 ms depolarizing current pulses of increasing intensity. Frequency accommodation was estimated as the variance of interspike duration during repetitive ﬁring of 16–19 APs evoked by a 1 s depolarizing current pulse from a baseline potential of −65 mV. The time-dependent decay in AP amplitude during 20 s repetitive firing was estimated using the following formula: (1st AP – last AP)/1st AP. Unless otherwise indicated, values are given as mean ± S.E.M, and the unpaired Student’s t-test or Mann-Whitney rank sum test were used for statistical evaluation. For multiple comparisons the two-way ANOVA was used. The level of significance was set at 5%.

Behavioral paradigms. Experiments were conducted blinded using $\alpha_3^{+/D801Y}$ mice and age-matched WT littermates. Mice were transferred to the test room one hour prior to testing for acclimation and tests were performed 1–2 days after last cage change. Behavioral apparatuses were cleaned between tests in 70% EtOH and only one gender was tested per experiment.

Open field. Mice were placed in a 50 × 50 cm open field (Stoelting Europe; Dublin, Ireland) and monitored for 20 minutes using the ANY-maze software V4.99 (Stoelting, USA). Three zones were defined: A peripheral zone measuring approximately 8 cm from the walls, an intermediate zone extending another 8 cm into the apparatus and a center zone.
Elevated plus maze. Entries into the open and closed arms of the elevated-plus maze (Stoelting Europe; Dublin, Ireland), time spent in these arms, as well as distance traveled was recorded for 10 minutes using the ANY-maze software (Stoelting Company).

Barnes Maze and passive avoidance were performed as recently described. Trials were recorded by using computerised tracking/image analyser system and analysed using the ANY-maze tracking system (Stoelting Company). The following parameters were recorded: errors, distance from tunnel, search strategy and time that the mouse took to escape into the tunnel i.e. total latency. Errors were defined as nose pokes and head deflections over any hole that did not have the tunnel. The search strategies were determined by examining each mouse’s daily session and defined in to three categories: (1) Direct (spatial): Moving directly to target hole or to an adjacent hole before visiting the target. (2) Mixed: Hole searches separated by crossing through the center of the maze or unorganised search. (3) Serial: The first visit to the target hole was preceded by visit at least two adjacent holes in serial manner, clockwise or counter clockwise direction.

The passive avoidance test was initiated on the acquisition day (A). The mouse was placed in a brightly lit compartment with an electronically controlled door leading into a dark compartment. The latency (s) was recorded for the mouse to enter the dark compartment. Once in the dark compartment, the door closed and the mouse received an electric shock (0.42 mA for 1 s). Twenty-four hours later (retention day, R), the mouse was reintroduced to the same brightly lit compartment and the latency to enter the dark compartment was recorded as an indicator of memory of the shock.

Clonazepam passive avoidance rescue. Thirty minutes prior to passive avoidance training, the mice received 0.0625 mg/kg clonazepam intraperitonially (Roche, Hvidovre, Denmark) dissolved in 0.9% sterile saline (vehicle) or vehicle alone.

PTZ seizure threshold. Mice were given 75 mg/kg pentlenetetrazole (Sigma-Aldrich; Schnelldorf, Germany) or 0.9% NaCl vehicle IP and monitored and video-recorded for 30 minutes after which they were euthanized.

Brain sampling and immunohistochemistry. Mice were deeply anesthetized with an overdose of pentobarbital. Approximately 0.05 mL per 10 g body weight pentobarbital was given intraperitonially (50 mg/mL pentobarbital, Aarhus University Hospital). When sedated, the mice were fixed upon a polystyrene board, and their chests were cut open with a blunt pair of scissors. The mice were perfused with 20 mL ice cold phosphate buffered solution (PBS) transcardially and subsequently with 20 mL ice cold 4% paraformaldehyde (PFA) in PBS. The brains were carefully dissected out and post-fixed in 4% PFA, PBS 4 °C over night (ON). The brains were cut in half following the midline and the olfactory bulb and cerebellum were dissected from the right hemispheres, and these halves were used for sampling. The left hemispheres were saved for later studies.

The tissues were infiltrated in paraffin using a Shandon CitadelTM Tissue Processor (Thermo Scientific). The right hemispheres were coronally sliced on a microtome at a thickness of 30 μm. A stereotactic atlas of the mouse brain (Paxinos and Franklin, 2003, second edition) was used to identify a region juxtaposed to the hippocampus in order to have a visual guideline for initiating the collection of the sections. The point of reference chosen was the dorsal third ventricle at approximately Bregma −0.22 mm that was approximately situated 0.70 mm frontally to the hippocampus. The hippocampus stretches from Bregma −0.94 mm to −3.88 mm according to the atlas. Every second section was collected on Superfrost ® Plus Microscope Slides (Thermo Scientific) spanning 4 series. One of the series was used for stereological analysis. Microscopic examination ensured that the hippocampus had been fully sectioned. After collection of the sections, the slides were put in the oven at 65 °C for 30 min. Sections were then deparaffinised Xylene (2 × 15 min), 99% EtOH (3 × 5 min), 96% EtOH (3 × 5 min) and 70% EtOH (2 × 5 min). Sections for stereological analysis were stained with toluidine blue, subsequently dehydrated and cover slipped. Sections for histological assessment were stained with Hoechst and cover slipped.

Stereological analysis. The optical fractionator was used as counting methodology, and quantitative stereological analysis was performed by the same person who was blinded to the phenotype of the mice.

Equipment. Counting was done on a computerized optical microscope (Olympus BX50) equipped with a motorized stage and focus control system (Prior Scientific, ProScan™ III). A highly specialized software program (Visiopharm integrator system version 4.5.1.324) was used for counting.

Sampling. Every second section was collected spanning 4 series in order to have at least six sections of the hippocampus in every series. Counting was only done on one of them giving a section sampling fraction,ssf, of 1/8. The final tissue thickness after shrinkage was determined to be approximately 25 μm, which allowed the height of the disector to be 15 μm with safeguard zones of 5 μm at the top and 5 μm at the bottom. The top of the tissue is excluded from the counting as sectioning can extract parts of cells close the sectioning plane. The height of the disector is used to calculate the height sampling fraction, \( h_{s}f = h/\bar{r}_{Q} \), where \( \bar{r}_{Q} \) is the \( Q \)-weighted mean section thickness which can be calculated as:

\[
\bar{r}_{Q} = \frac{\sum (t_{i} \cdot Q_{i})}{\sum Q_{i}}
\]

(1)

\( t_{i} \) is the local section thickness placed in the \( i \)th counting frame with a disector count of \( Q_{i} \).
An unbiased counting frame with an area, \( a = 76 \mu m^2 \), was superimposed on each field of view within the area of the granular layer of dentate gyrus. The step length was 100 \( \mu m \) in both the x and y plane and hence the sampling area fraction was:

\[
\text{asf} = \frac{a(\text{frame})}{(dx \times dy)} = \frac{76 \mu m^2}{100 \mu m \times 100 \mu m} = 0.0076
\]

In order to achieve a sample estimate, \( N \), with a CE less than 0.1, the number of sections, the step-length and the area of the counting frame were dimensioned so that ~200 cells were counted per series.

**Counting.** The GrDG of each hippocampus present on a slide was delineated using a 4 \( \times \) objective at a final magnification of 135 \( \times \). Subsequently, meander counting was performed using the 100 \( \times \) objective at a final magnification of 3366 \( \times \). At each field of view, the microscope was slowly focused down from top to bottom of the section. All non-pyknotic neurons sampled by the 2D unbiased counting frame and located within the height of the optical disector were counted. Pyknotic cells were counted as a separate population of cells.

**Statistical analysis.** All data were shown as mean ± s.d. (or SEM) and statistical analyses were done using Graphpad Prism version 5.01 or 6.03 (GraphPad Software Inc, La Jolla, CA, USA) or the R software (R Foundation for Statistical Computing, Vienna, Austria)\(^{48} \) (Supplementary Table 1). The obtained male and female mice data were only pooled when this was statistically validated (\( P < 0.05 \)), and all statistical tests and the \( P \)-values obtained are presented in Supplementary Table 1.

**Data availability.** The \( \alpha_3^{+/-D801Y} \) mouse model is available through a Material Transfer Agreement (MTA).

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Author Contributions
PN. and K.L.-H. conceived the study. K.L.-H. outlined the overall cloning strategy and cloning of the targeting construct. PB. devised screening procedures for the $\alpha_3^{+/D801Y}$ mouse model, and managed initial backcrossing. E.-M.F. directed ES cell work. T.H.H. directed all main experimental outlines and breeding protocols, and performed most of the behavioral experiments as well as IHC. T.J.I. performed Western blot analysis, and contributed to PTZ experiments. A.H. performed mice genotypings. J.R.N. contributed towards IHC. The hippocampal electrophysiology was performed by SN. and MA. T.H.H. and K.L.-H. interpreted the data and wrote the manuscript. All authors co-wrote the manuscript, read and approved the final manuscript.

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