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Abnormal social behavior in mice with tyrosinemia type I is associated with hypermye-lination of the cerebral cortex

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Abnormal social behavior in mice with tyrosinemia type I is associated with hypermyelination of the cerebral cortex

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Abstract **-** Tyrosinemia type I (TT1) is a rare metabolic disorder that results in mutations of the enzyme FAH, which is responsible for tyrosine breakdown. Without the current treatment of 2-(2-nitro-4-trifluoromethylbenzoyl) cyclohexane-1-3-dione (NTBC), toxic metabolites build up causing severe kidney and liver dysfunction that can lead to mortality. Cognitive and social defects have recently been observed in affected individuals on NTBC, and to investigate these effects mice with tyrosinemia type I were tested utilizing the Crawley three-chambered sociability test. Social behavior is analyzed in the three chambered apparatus by observing mice interaction with either a real or dummy mouse, or between a familiar or novel mouse. The results show that mice with tyrosinemia type I spend twice as much time investigating a dummy mouse compared to a real mouse, indicating sociability deficits. Due to the importance of olfaction in mouse behavior, the olfactory abilities of the mice were also analyzed by means of the buried food test. In order to assess a biological basis for these social and cognitive impairments, mice brains were stained using Luxol fast blue to visualize myelin in the cerebral cortex. Microscopic analysis of the stained cerebral cortex showed hypermyelination in mice with tyrosinemia type I in comparison to controls. The cognitive and sociability issues observed in tyrosinemia type I mice could be attributed to malformed neuronal pathways and synapses caused by hypermyelination of the cerebral cortex.

I. Introduction

The autosomal recessive disorder tyrosinemia type I results in a deficiency of fumarlyacetoacetate hydrolase (FAH, EC 3.7.1.2), the last enzyme in the pathway of tyrosine metabolism (Fig. 1). This lack of functional FAH leads to accumulation of deleterious metabolites that can cause liver failure and fatality. Treatment with NTBC (Nitisinone) blocks the enzyme 4-hydroxyphenylpyruvate dioxygenase (HPPD, EC 1.13.11.27) upstream in the tyrosine degradation pathway, which prevents the buildup of harmful metabolites such as malylacetoacetate and fumarylacetoacetate. These metabolites can accrue to be converted to succinlyacetone, which is a diagnostic marker for tyrosinemia type I due to its production exclusively in the absence of FAH (De Jesus et. al 2014). This metabolite is identified in newborns by using tandem mass spectrometry and has detrimental effects on heme synthesis as well as neurological function. The high mortality rates in children with tyrosinemia type I before the introduction of NTBC can be attributed to these metabolites causing hepatocellular carcinoma and liver damage. The treatment with NTBC results in an alteration of the enzymatic mutation, leading to increased tyrosine concentrations and hypertyrosinemia (Bendadi et. al 2014). Although this treatment has drastically increased the survival rate in affected individuals, the exact role NTBC plays in cognitive function has yet to be elucidated.

There have been recent reports of social behavior and cognitive issues in individuals with tyrosinemia type I on long-term NTBC treatment. Lowered IQ as well as social cognition and working memory deficits have been observed in patients with TT1 compared to healthy controls (van Ginkel et. al 2016). The high mortality rate in children with tyrosinemia type I before introduction of the drug NTBC explains why any long-term cognitive effects from the disorder were not able to be examined. The treatment options for children with the disorder before NTBC included liver transplant and dietary modification of tyrosine and phenylalanine. Whether the cause of these neurological and social issues in patients with tyrosinemia type I is due to the treatment or is an effect of the disease remained undetermined. Sociability and cognition of tyrosinemia type I (FAH^{mut}) mice was investigated and compared to wildtype mice treated with NTBC (WT-NTBC) and wildtype mice drinking water (WT-water) in order to discern the cause of these issues. It has been shown that mice

with tyrosinemia type I are unable to respond effectively to change, take longer to learn and make more errors compared to WT-NTBC and WT-water mice in the Barnes maze (Hillgartner et. al 2016). This suggests that the social and cognitive problems can be attributed to the disease process and not to the treatment with NTBC. In order to further study these effects social interaction was evaluated in mice with TT1 in Crawley's three-chambered sociability test.

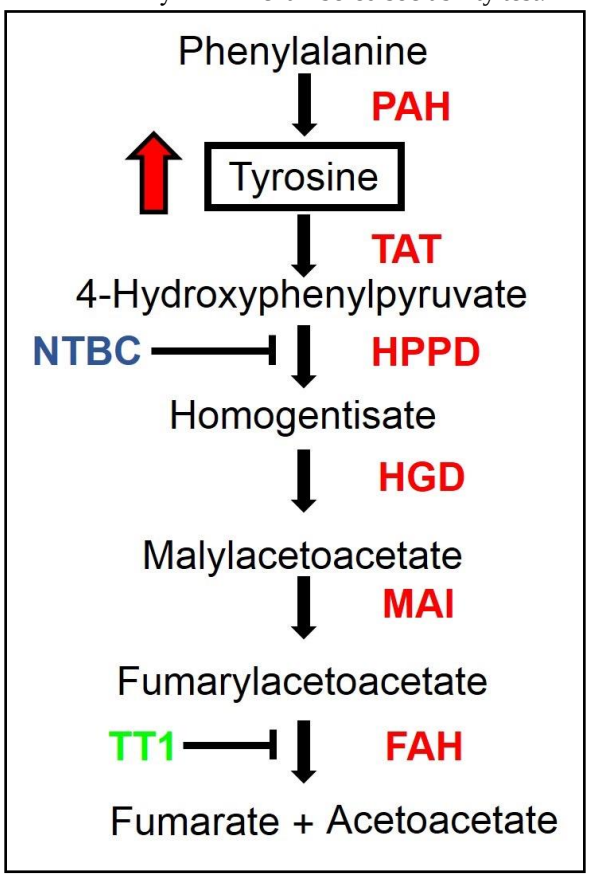

Figure 1. The tyrosine catabolism pathway. Tyrosine can be ingested from food, or synthesized by the hydroxylation of phenylalanine by phenylalanine hydroxylase (PAH). The first step in the breakdown of tyrosine is conversion to 4-hydroxyphenylpyruvate by tyrosine aminotransferase (TAT). The second step is conversion into homogentisate, by 4-hydroxyphenylpyruvate dioxygenase (HPPD) and this is the step that is inhibited by the pharmaceutical 2-[2-nitro-4-trifluoromethylbenzoyl) cyclohexane-1,3-dione (NTBC). Homogentisate is oxidized to malylacetoacetate by homogentisate dioxygenase (HGD) and isomerized by maleylacetoacetate isomerase (MAI) to form fumarylacetoacetate. The final step of tyrosine metabolism involves the breakdown of fumarylacetoacetate into fumarate and acetoacetate, and is catalyzed by fumarylacetoacetate (FAH). This is the enzyme mutated in tyrosinemia type I. In the case of tyrosinemia type I (TTI) where the enzyme FAH is compromised, toxic metabolites will accumulate and be converted to the metabolite succinylacetone which has deleterious effects on the liver, kidneys and the central and peripheral nervous system.

Sociability is examined by comparing the amount of time the mouse spends between a real mouse and a dummy (stuffed toy) mouse as well as between a familiar and novel mouse. This three-chambered apparatus is so widely used to study mouse social behavior because it provides an accurate method to analyze sociability and novel-seeking differences between various mouse strains (Pearson et. al 2010). *Mus musculus* is known for investigation of novel conspecifics as well as elevated engagement in social behaviors (Yang et. al 2011). It is therefore expected that mice with normal sociability will favor spending time with an actual mouse instead of a dummy, or no mouse, and that they will also tend to investigate a novel mouse more than a familiar mouse. Mice engage in numerous reciprocal social interactions; wherein olfactory abilities are critical for successful behavior (Moy et. al 2004). In order to ensure that the olfactory abilities of the mice were not affecting sociability, the mice were assessed with the buried food test. Mice olfaction is analyzed by how much time is taken to find a piece of palatable food buried in cage bedding. Increased latency to find the piece of food indicates gross impairments in olfaction and consequentially in social behavior. Owing to the importance of olfaction for normal mouse behavior, olfactory abilities of mice with tyrosinemia type I were analyzed to rule it out and focus on the disease process or NTBC as the cause of these neurological issues.

To examine the neurological source for these intellectual and social problems, myelin from the cerebral cortex of mice was stained using Luxol fast blue for microscopic visualization. The cerebral cortex is responsible for many aspects of brain function such as learning, attention and memory. Oligodendrocytes are responsible for myelination within the central nervous system, while Schwann cells produce myelin for the peripheral nervous system. Myelin is a fatty substance made of lipids and proteins that works to insulate neurons to help propagate action potentials and increase neuronal activity. It stems as an outgrowth of glial cells and acts to increase electrical resistance and decrease capacitance across

the membrane (Jahn et. al 2009). Signals are propagated in the central nervous system through salutatory conduction from one node of Ranvier to another, resulting in rapid depolarization of the membrane. This efficiency is due to multiple layers of myelin surrounding the nerve cells, acting as an insulator to maintain quick and accurate electrical impulses. Any defect in myelination can result in altered synapses as well as impaired neuronal pathways that subsequently alter cognition and behavior. Myelin was therefore assessed in mice with tyrosinemia type I to investigate any morphological differences that could explain this impaired cognition seen in TT1 mice.

II. Materials and Methods

Tyrosinemia type I mice

All mice were housed with 2-5 mice in each cage with corn cob bedding (Harlan), given water ad libitum and fed with mouse chow (Teklad Global 18 % Protein Rodent Diet with 0.1 % phenylalanine and 0.6 % tyrosine). All cages were supplied with cotton for nesting as well as wheels for lifestyle enrichment. All mice care and experimental protocols were approved by the UAH IACUC committee.

Social Behavior

The Crawley three-chambered sociability test utilizes the natural tendency of mice to investigate novel conspecifics as well as engage in high levels of sociability in order to compare different mouse strains. The testing apparatus is separated into three chambers, with a central chamber in which the mouse is habituated to before testing sociability. The mouse is placed into the apparatus after a dummy (stuffed toy) mouse and a stranger mouse familiarized with the cage are placed into small wire cages in either the left or right chamber. The doors blocking the entry into the side chambers were taken off, and the number of entries as well as time spent within each chamber was recorded for 10 minutes. Screen dividers were placed around the apparatus to ensure that the mice were not affected by the experimenters and the chambers were cleaned in between each trial. The side of the real and toy mouse as well as the side of the familiar and novel mouse was alternated for each trial. The mice were also tested for novel preference by habituating the mouse to the central chamber for 5 minutes followed by placing an unfamiliar mouse into one of the wire cages in either of the side chambers. The mouse is allowed to explore for 10 minutes and a novel (unfamiliar) mouse is then placed into the other wire cage. Time spent in and entries into each chamber for 10 minutes is measured in order to compare the novelty-seeking behavior of the mice (Moy et al. 2004).

Olfactory test

To analyze olfactory abilities in the mice the buried food test was conducted using Teddy Graham's as the palatable food to be buried in the cage bedding. A couple of days before the experiment, a teddy graham was placed into the mice cages and consumption was monitored to ensure its palatability to the mice. All food was taken away from the mice the night before testing. The test evaluates impairments in olfaction by observing how long it takes in a 15-minute trial for overnight-fasted mice to find the buried food. Mice who have been fasted overnight with normal olfactory abilities should therefore be able to find the buried food relatively quickly (Yang et. al 2009). The cage was filled with 3cm of corn cob (Harlan) bedding and the mice were allowed to explore the cage for 5 minutes. A separate cage was used for each trial and medical dividers were placed around the cage to eliminate any outside effects. After acclimating to the cage, the mouse was taken out while a teddy graham was buried and was then returned to the cage for 15 minutes to search for it. The latency to find the teddy graham was measured as well as if the cookie was consumed.

Mouse brain histochemistry

In order to examine the myelin in the cerebral cortex of the mice, brains were extracted and fixed with 10 percent formalin. The brain was placed into a brain block in order to section it for analyzation of the cerebral cortex. The tissue was then sequentially dehydrated, cleared, paraffin embedded and sectioned onto microscopic slides for staining to visualize the myelin. A microtome was used to slice the tissue into 20-micrometer sections for staining. The cerebral cortex was stained with Luxol fast blue and counterstained with cresyl violet, with the myelin staining blue and the cell nucleus staining purple. The cresyl violet acts to clearly distinguish the myelin sheath that is stained blue from the purple Nissl-stained cells (Kluver and Barrera, 1953).

III. Results

Mice were tested for social behavior in a three-chambered Crawley sociability test and the time the three populations of mice spent interacting in the zones with the real mouse and dummy mouse were measured (Fig. 2). WT-water mice spent equal amounts of time in the zone containing the mouse 222 ± 16.67 s (n = 8), as in the zone with the dummy mouse, 201.1 ± 20.07 s (n = 8, ns, $P = 0.4356$). The mice drinking NTBC behaved similarly also not discriminating between the real mouse and the dummy mouse, spending 250.7 ± 35.51 s, (n = 9) with the real mouse and 186.7 ± 22.46 s (n = 9) with the dummy mouse (ns, $P = 0.1468$). However, the mouse with tyrosinemia type 1 (FAH^{mut}) spend about twice as much time with the dummy mouse than the real mouse, spending 283.1 ± 24.75 s (n = 10) in the zone with the dummy mouse and only 148.1 ± 23.25 s (n = 10, $P = 0.0009$), in the zone with the real mouse (Fig. 2A). An example of the exact path an FAH^{mut} mouse took in the 10 minutes long experiment is shown in Figure 2C. The dummy mouse was then replaced with a novel mouse, and a test of social novelty performed. The WT-water mouse showed a preference for the novel mouse and spent 223.4 ± 16.58 s, (n = 7) in the zone with the novel mouse but only 157 ± 16.34 s (n = 7) with the familiar mouse ($P = 0.0147$). The WT-NTBC mouse showed similar behavior, spending about a minute and a half more time interacting with the novel mouse than the familiar mouse (259 ± 20.58 s, n = 9 vs 153.7 ± 19.34 s, n = 9, $P = 0.0018$). However, the mice with tyrosinemia type I (FAH^{mut}), did not discriminate between the two mice and spent similar time in the chambers housing the novel mouse (204 ± 12.51 s (n = 10) and the familiar mouse (195.3 ± 19.55 s, n = 10, $P = 0.7126$). An example of a WT-NTBC mouse in the social novelty test is shown in Figure 2D.

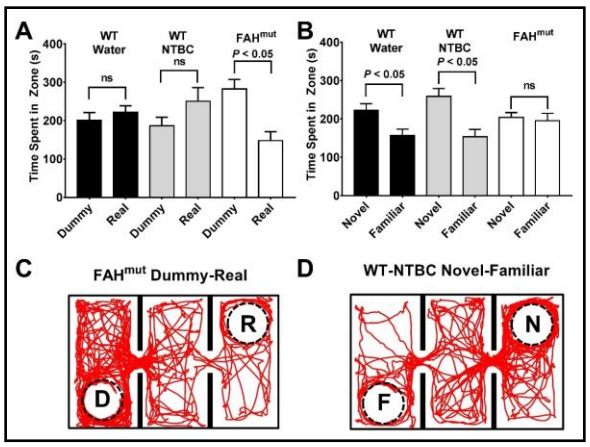

Figure 2. Mice with tyrosinemia type I show abnormal social behavior. A. Time spent in zone containing the dummy mouse or the real mouse. B. Time spent in zone containing the novel mouse or the familiar mouse. C. Example of mouse trail in the social experiment. The letter D represents the dummy mouse and R represents the real mouse. D. Example of mouse trail in the social novelty experiment. The letter F represents the familiar mouse and N represents the Novel mouse.

Mice were tested in a simple experimental test of their olfactory skills by timing how long it took them to find a buried Teddy Graham biscuit (Fig. 3). There was no difference in the abilities of the three groups of mice to find the Teddy Graham, $F(2, 16) = 0.3127$, $P = 0.7359$, with WT-water taking 24.4 ± 11.74 s (n = 5), WT-NTBC taking 36.77 ± 10.79 s (n = 6) and FAH^{mut} taking a total of 33.19 ± 9.367 s (n = 8).

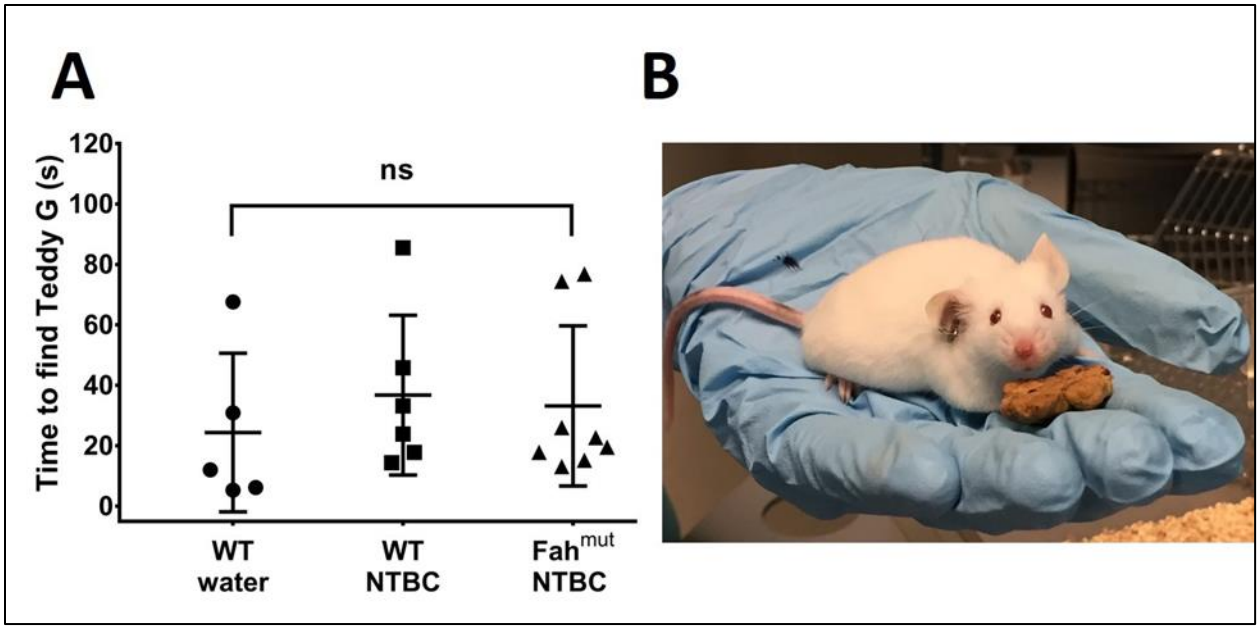

Figure 3. Mice with tyrosinemia type I are efficient in finding a Teddy Graham. A. There was no difference between the three groups of mice in finding a Teddy Graham buried in their cage. B. A picture of a mouse and a Teddy Graham.

To investigate any alterations in brain myelin, we isolated fixed and stained mouse brains from WT-water, WT-NTBC and FAH^{mut} mice. Brains were stained with the specific myelin dye Luxol fast blue and counterstained with cresyl violet. Brain slices from FAH^{mut} mice showed increased myelination (Fig. 4, Left Panel) as indicated by deep blue staining. Brains from the treatment control group of NTBC-WT mice showed less intense staining (Fig 4, Right Panel). Upon magnification of the cerebral cortex up to 10x, a diffuse hypermyelination was seen in the FAH^{mut} mice (Fig. 4, Bottom Panels).

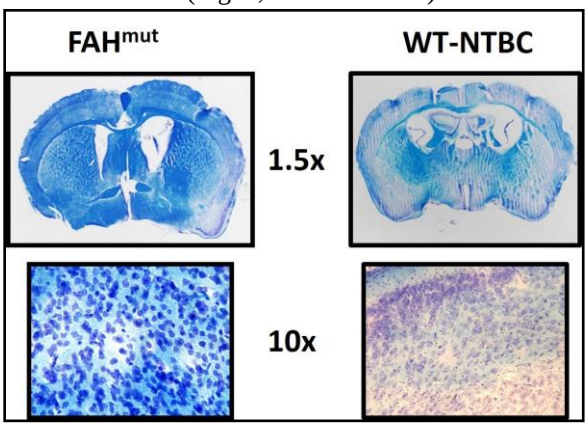

Figure 4. Mice with tyrosinemia type 1 show hypermyelination of the cerebral cortex. Left Panel. Here we show a brain slice from a FAH^{mut} mouse where myelin is stained blue. Right Panel. A brain slice from a WT-NTBC mouse shows much lighter staining for myelin. The cell nuclei are stained purple in both panels but are more visible in the WT-NTBC mouse.

IV. Discussion

The exact cause of the intellectual and social impairments recently reported in individuals with tyrosinemia type I has yet to be illuminated. The findings of altered sociability and hypermyelination in mice with tyrosinemia type I indicate possible neuronal pathways for impaired cognitive function and sociability (Fig 5.). Whether the cause of these social and cognitive problems was from the disease process or from treatment with NTBC remained unknown. Although it has been shown that NTBC increases the amount of blood tyrosine levels and transport to the brain, wildtype mice on NTBC show no altered learning or behavior. Tyrosinemia type I mice display hypermyelination of the cerebral cortex as well as behavioral and cognitive impairments in testing compared to WT-NTBC and WT-water mice. This indicates that these neurological problems can be accredited to the pathophysiology of the disease, and not to the treatment with NTBC.

The result that tyrosinemia type I mice spend twice as much time with a dummy mouse than with a real mouse compared to WT-water and WT-

NTBC mice suggests impaired sociability caused by the disorder. TT1 mice also show no preference for social novelty in comparison to WT-water and WT-NTBC mice who spent more time with a novel mouse. This lack of social preference for a real mouse and a novel mouse in TT1 mice is indicative of diminished sociability and altered exploratory mouse behavior. These results help to demonstrate the significantly different behavioral traits and sociability impairments in mice with tyrosinemia type I compared to the WT-NTBC and WT-water mice and further demonstrates the effects produced by the disorder.

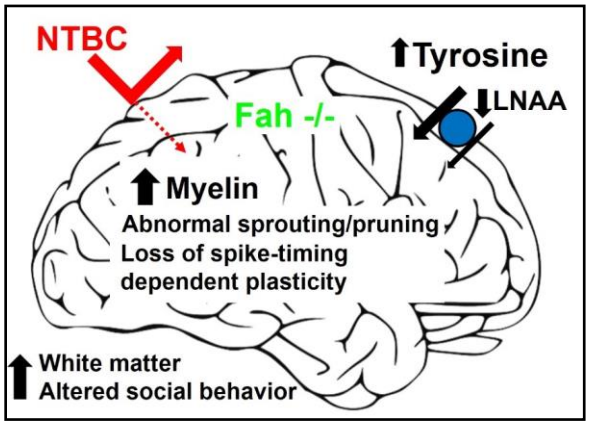

Figure 5. A schematic hypothesis to explain the social behavior changes observed in tyrosinemia type I mice. We propose that NTBC has limited permeability across the blood-brain barrier, providing insufficient inhibition of HPPD. Hence, NTBC is less efficient at the treatment of tyrosinemia type I of the CNS. The enzyme fumarylacetoacetate (FAH) is not expressed in the tyrosinemia type I mice, and results in the increased accumulation of myelin. Myelin can alter neuron sprouting and pruning and can cause the loss of spike-timing dependent plasticity. This altered neuronal architecture or the increased myelination itself is contributing to the altered social behavior in mice.

The lack of difference in time to find the teddy graham in the buried food test between all three genotypes indicates normal and equivocal olfactory abilities. This further demonstrates the suggestion that the disease process of tyrosinemia type I is responsible for the cognitive and sociability problems presenting in TT1 mice. Microscopic analysis of the mice brains revealed dramatic differences in the myelination of TT1 mice in comparison to WT-water and WT-NTBC mice. TT1 mice brains all expressed hypermyelination of the cerebral cortex after staining with Luxol fast blue, indicating different neuronal circuits that could aid in explaining the altered behavior. Myelin undergoes continuous maintenance from glial cells as well as developmental pruning of connections in order to form efficient and accurate neural connections (Lui et. al 2013). In order for proper cognitive and social abilities, neuronal circuits must have normal myelination or else the electrical impulses will be impaired. It is therefore suggested that these neurological deficits are associated with the observed hypermyelination of the cerebral cortex of mice with tyrosinemia type I.

V. Conclusion

The hypermyelination in mice with tyrosinemia type I may be associated with the impaired social behavior we observed. Behavioral testing of mice with tyrosinemia type I have shown altered memory and cognitive function as well as impairments in social behavior. Hypermyelination could lead to malformed neuronal connections as well as altered synapse formation and cause neurological problems. Any defect in white matter myelination of the central nervous system can lead to impaired action potential propagation and further cause deficits in cognition and social behavior. The altered memory, learning, and sociability observed in mice with tyrosinemia type I in comparison to WT-NTBC and WT-water mice indicates that these issues are a product of the disease and not with treatment of NTBC. This indicates that the social and cognitive impairments observed in individuals with tyrosinemia type I could be accounted for by deficits in neuropsychological development.

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Author contributions

MM and GM planned, designed and carried out experiments. MM and GM analyzed the data and wrote the manuscript.MM and GM have no conflicts of interest. MM and GM agree to the conditions of publication including the availability of data and materials.

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