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14. ABSTRACT Neurofibromatosis type 1 (NF1) is a genetically determined neurodevelopmental disorder and tumor syndrome with an incidence of approximately 1/3000 live births. In nutritionally-stressed fly larvae Jeb and Alk protect neurogenesis from growth restriction. Concordantly, NF1 loss is associated with increased neurogenesis, an enhanced behavioral response to subchronic doses of antidepressants, and spontaneous anti-depressive-like behaviors. It is, therefore, important to assess the effects of prolonged pharmacologic Alk inhibition on sleep, hippocampal neurogenesis, depressive and anxiety-like behaviors. We test the hypothesis that the pharmacologic inhibition of Alk will have short and long-term effects on behavioral performance, including measures of anxiety, depressive like behaviors, and circadian activity, cognitive performance, and neurogenesis, in heterozygous NF1 mutant mice treated in adulthood. The Specific Aims are: 1) To determine the optimal timing and dose of pharmacologic Alk inhibition to achieve maximal cognitive performance in heterozygous NF1 mutant mice; 2) To evaluate the potential benefit of Alk inhibition on altered circadian activity levels, a non-cognitive behavioral alteration associated with NF1 that may contribute to the cognitive disabilities; and 3) To assess potential adverse behavioral and cognitive effects of Alk inhibition in wild-type and NF1 heterozygous mice.					
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1. Introduction

Neurofibromatosis type 1 (NF1), a genetically determined neurodevelopmental disorder and tumor syndrome, is caused by loss of function mutations in the neurofibromin gene (*NF1*) and associated with cognitive impairments, including problems with attention, visual perception, language, learning, attention, and executive function [1]. These cognitive impairments are observed in the absence of tumors or macroscopic structural abnormalities in the central nervous system [2-7]. Sleep-related problems, including problems with initiating and maintaining sleep, arousal, sleep-wake transition and Hyperhidrosis have been reported as well [8-10]. Consistent with the human data, NF1 heterozygous mutant mice show impaired hippocampus-dependent spatial learning and memory in the water maze [11-14] and impaired hippocampus-dependent extinction of contextual fear memory [14]. In addition, female and male flies lacking *Nf1* exhibit nocturnal hyperactivity, an increase in daytime sleep and a decrease in nighttime sleep, and impaired sleep consolidation [15, 16].

Neuronal dysfunction in NF1 patients is sex-dependent, with female patients with NF1-associated optic glioma being twice as likely to undergo brain MRI for visual symptoms and three times more likely to require treatment for visual decline than male patients [17]. In contrast, microglia in NF1 patients show sexually dimorphic cyclic AMP-dependent purinergic impairments, with microglia in males but not females being affected [18]. Glioma risk in NF1 patients is also sex-dependent, with increased risk in females and reduced risk in males associated with genetic polymorphisms in adenylate cyclase 8 [19]. While there is no parent-of-origin effect for optic gliomas in NF1 patients [20], it is not clear whether neurological phenotypes might be more pronounced in heterozygous offspring from a maternal compared to a paternal carrier. In case there would be a parent-of-origin effect in NF1 patient, that could be of clinical relevance for both diagnostic purposes and for consideration of treatment strategies.

NF1 is a negative regulator of the small GTPase, Ras, a proto-oncogene and activator of the mitogen activated protein kinase (MAP kinase) cascade [21-25] that is often activated by receptor tyrosine kinases, and binds GTP. Neurofibromin, which has been reported to function in the hippocampus [11], is a Ras-GTPase activating protein that catalyzes the conversion of active GTP-Ras to inactive GDP-Ras, lowers the activation energy of RAS's intrinsic GTPase activity, and accelerates its auto-inactivation.

Drosophila [26] [27] and subsequent mouse [14, 28] studies support a genetic interaction between NF1 and Alk, a receptor tyrosine kinase and activator of the Ras-MAP kinase cascade [26, 27, 29] and an oncogene involved in anaplastic lymphomas [30], neuroblastoma, non-small cell lung cancer, inflammatory myofibroblastic sarcoma [31-35], and likely also in some renal cell, breast, esophageal, colonic, glial and thyroid cancers [36]. Orally active Alk inhibitors developed for human tumors [37-40] have been used to demonstrate that pharmacological inhibition of Alk can rescue cognitive impairments in heterozygous *NF1* mutant flies [26] and spatial memory retention in heterozygous NF1 mice on a genetically diverse background at only one dose [41].

These pioneering studies in *Drosophila* are the predicate for a more specific therapeutic approach to the treatment of neurobehavioral NF1 phenotypes in humans. They provided the rationale for experiments we performed to test the efficacy of Alk inhibition in NF1 heterozygous mutant mice. Specifically, we started to test the hypothesis that genetic and pharmacologic inhibition of Alk in a mouse model of NF1 would ameliorate or even reverse NF1 associated neurocognitive disabilities. Confirmation of this hypothesis in mice is a necessary prelude to evaluating the therapeutic benefit of Alk inhibition to treat behavioral alterations and cognitive impairments brought on by NF1 mutations in humans. It is also critical to assess and minimize toxicity, as these studies have the ultimate objective of developing a treatment for humans with neurofibromatosis. Acceptable side effects in the context of cancer are not acceptable in the context of long-term treatment of patients with neurofibromatosis. In our pharmacological pilot studies, we used the lowest effective dose of the Alk inhibitor CH5424802 (Alectinib) (1.8 mg/kg) with activity against central nervous system tumors. This dose, which was efficacious, is more than 10 -fold lower than the maximal effective dose of (20 mg/kg). Known side effects of Alectinib used at high doses in cancer patients (600 mg taken twice daily, with dose reductions to 450 or 300 mg twice daily if patients cannot tolerate the higher dose) include constipation, diarrhea, swelling (including feet, ankles, and eyelids), weight gain, rash, dizziness, lightheadedness, fainting, shortness of breath/difficulty breathing/coughing, vision changes, muscle weakness, tiredness, rash, itchy skin, loss of appetite, dark urine, and bleeding or bruising more easily (<https://dailymed.nlm.nih.gov/dailymed/drugInfo.cfm?setid=42c49deb-713b-427a-9670-08af08adcfb>; <http://www.cancer.gov/about-cancer/treatment/drugs/alectinib>; <http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm476926.htm>). In this project, we

looked at these outcome measures for signs of toxicity for in NF1 mice treated with Alectinib in the proposed experiments. In nutritionally-stressed fly larvae Jeb and Alk protect neurogenesis from growth restriction. Concordantly, NF1 loss is associated with increased neurogenesis, an enhanced behavioral response to subchronic doses of antidepressants, and spontaneous anti-depressive-like behaviors [39]. It is, therefore, important to assess the effects of prolonged pharmacologic Alk inhibition on sleep, hippocampal neurogenesis, depressive and anxiety-like behaviors. In this project, we tested the hypothesis that the pharmacologic inhibition of Alk has short- and long-term effects on behavioral performance, including measures of anxiety, depressive like behaviors, and circadian activity, cognitive performance, and neurogenesis, in heterozygous NF1 mutant mice treated in adulthood. More specifically, we tested the following aims:

- 1) To determine the optimal timing and dose of pharmacologic Alk inhibition to achieve maximal cognitive performance in heterozygous NF1 mutant mice.
- 2) To evaluate the potential benefit of Alk inhibition on altered circadian activity levels, a non-cognitive behavioral alteration associated with NF1 that may contribute to the cognitive disabilities.
- 3) To assess potential adverse behavioral and cognitive effects of Alk inhibition in wild-type and NF1 heterozygous mice.

2. Keywords

Alk inhibitor, water maze, fear conditioning, extinction learning, circadian activity, NF1 mutant mice

3. Accomplishments

We determined the effect of the parental gene carrier on the behavioral phenotype of the offspring. While this was not an original aim, we noticed that it seems to matter whether the mother or father were the NF1 HET carrier and/or had the C57BL/6J or 129 SvJ background. Therefore, we decided to assess this in detail. There were two groups where either the father or the mother was the NF1 carrier:

- PC = B6 Paternal NF1 Carrier
- MC = B6 Maternal NF1 Carrier

The NF1 carrier was always on a B6 background, and the non NF1 carrier was on a SVJ background. We compared the behavioral and cognitive performance of NF1 heterozygous and wild-type litter mates with either the father (PC : B6 Paternal NF1 Carrier) or the mother (MC : C57BL/6J (B6) Maternal NF1 Carrier) as the NF1 carrier. The NF1 carrier was always on a B6 background, and the non-NF1 carrier was always on a 129/SvJ (SvJ) background. We assessed the ability of different doses of the the Alk inhibitor CH5424802 (Alectinib) to modulate behavioral and cognitive performance. As the results of this study were published earlier this year [42], we only summarize this part of the project and include the publication as part of the appendix.

Animals were given either vehicle or one of three doses (3.6 mg/kg, 10 mg/kg, or 20 mg/kg) of the Alk inhibitor CH5424802 (Alectinib, Chugai Pharmaceuticals, Tokyo, Japan). The vehicle for the drug was a flavored gelatin mixture in 1 cm × 1 cm squares. This route of administration was selected because voluntary oral administration would be the most relevant for human patients, and less stressful and safer for the mice than daily oral gavage. Treatment was administered daily starting 10 days prior to the first day of water maze testing. Every day when the gelatin squares were given to the mice, the researchers confirmed that the previous gelatin squares had been eaten. The mean age at start of treatment per experimental group was 6.1 ± 0.46 months.

The behavioral and cognitive phenotypes and responsiveness to Alk inhibition in heterozygous NF1 offspring depended on whether the parental carrier was maternal or paternal. Alk inhibition (20 mg/kg) increased activity levels during the dark period in NF1 Het PC, but not MC, mice. In the water maze, NF1 Het PC, but not MC, mice showed reduced cognitive flexibility and impaired ability to locate the third hidden platform location, which was improved by Alk inhibition (3.6 mg/kg). Consistent with reduced cognitive flexibility, WT, but not NF1, mice showed better performance in the third than second water maze probe trial. Finally, Alk inhibition (10 mg/kg) increased baseline activity of NF1 MC, but not PC, mice during the fear conditioning test. Thus, the effective dose depends on the behavioral test and genotype but indicates that in NF1 patients cognitive flexibility might be particularly sensitive to Alk inhibition.

These results indicate that the behavioral and cognitive phenotypes and responsiveness to Alk inhibition in heterozygous NF1 offspring depend on whether the parental carrier is maternal or paternal. These parental effects might involve genetic background differences in mitochondria function. While there was no effect of genotype or circadian activity levels, Alk inhibition (20 mg/kg) increased activity levels during the dark period in NF1 HET PC, but not MC, mice. In MC, but not PC, WT mice, Alk inhibition (3.6 mg/kg) increased the dark-light activity ratio. In the water maze, NF1 HET PC, but not MC, mice showed reduced cognitive flexibility and impaired ability to locate the third hidden platform location, suggesting impaired executive function. Alk inhibition (3.6 mg/kg) improved performance of NF1 PC mice to locate the third hidden platform location. Consistent with the hidden platform water maze data in NF1 mice, when spatial memory retention was assessed in the probe trials, WT, but not NF1, mice showed better performance in the third than second probe trial. In addition, MC WT, but not NF1, mice showed a lower cumulative distance to the third than the fourth hidden platform location. Alk inhibition (10 mg/kg) increased baseline activity of NF1 MC, but not PC, mice during the fear conditioning test. In PC, but not MC, WT mice, Alk inhibition (10 mg/kg) reduced the response to the shocks. As the NF1 carrier was always on a B6 background and the non NF1 carrier was always on a SVJ background, it is conceivable that whether the maternal or paternal is on either background might have contributed to the observed parental carrier effects. In our previous genetic and pharmacological study involving NF1 HET mice, the mice behaviorally tested were on a diverse F2 genetic background. We had been breeding male and female Alk homozygous knockout mice on the C57BL/6J back-ground with male NF1 HET mice on the 129 SvJ background. As the breeding strategy of NF1 HET mice (Alk wild-type) with Alk HET mice hardly generated NF1 HET mice (Alk wild-type), we bred F1 double HET mice (Het for both NF1 and Alk) to generate 532 F2 mice for the behavioral studies. While having NF1 mice on a F2 background is more translational relevant for humans, we expected the cognitive phenotypes in spatial learning and memory in the water maze and in extinction of contextual fear memory to be more variable and less profound on a F2 than a B6/129 SvJ F1 background. However, the cognitive phenotypes of the NF1 HET mice in the water maze and contextual fear extinction tests on a F2 background were more profound than those seen on a pure B6/129 SvJ F1 background in the current study. In the water maze, NF1 HET mice on a F2 background, already showed an impairment during training to locate the first hidden platform. No impairment was seen in NF1 mice on a pure B6/129SvJ F1 background in the current study. Part of this difference might be due to the fact that in the current study the mice were first trained to locate a visible platform and subsequently trained to locate hidden platform locations, while in our previous study NF1 HET mice on a F2 background the mice were first trained to locate the hidden platform locations and only subsequently trained to locate the visible platform location. This is unlikely to completely account for this difference, as the cognitive phenotype in extinction of contextual fear was also more profound on a F2 background than on a pure 129SvJ F1 background. It is conceivable that B6-related genes present and important for cognition might be important in protecting against the detrimental genetic effects of the NF1 HET. Nevertheless, the phenotype seen in cognitive flexibility in the water maze seen on a pure B6/129 SvJ F1 background is consistent to what has been observed for medial prefrontal cortex synaptic plasticity and working memory in the radial arm maze in the NF1 Het mice [43] [13]. The observed effects of Alk inhibition (20 mg/kg) on increasing activity levels during the dark period in NF1 HET PC mice on a pure B6/ 129 SvJ F1 background is consistent with the sleep phenotype observed in inducible NF1 homozygous knockout mice with no reproducible phenotype in NF1 HET mice on a B6/129 SvJ F1 background [44]. These data support the value of testing mutant mouse models of neurological diseases on a diverse genetic background or comparing phenotypes on distinct genetic backgrounds [45] [46]. In the current study, we used three doses of the Alk inhibitor (3.6, 10, and 20 mg/kg), all higher than the 1.8 mg/kg dose we used in our previous study [41]. The effective dose depended on the behavioral test and genotype of the mice. Alk inhibition (20 mg/kg) increased activity levels during the dark period in NF1 HET PC, while Alk inhibition (3.6 mg/kg) increased the dark-light activity ratio in MC WT mice. In contrast to home cage activity, Alk inhibition (3.6 mg/kg) improved performance to locate the third hidden platform location in NF1 PC mice. Finally, Alk inhibition (10 mg/kg) increased baseline activity of NF1 MC mice in the fear conditioning test and reduced response to the shocks in PC WT mice. These data indicate that for enhancing cognitive flexibility in the water maze a relative low dose of 1.8 or 3.6 mg/kg might be preferred over higher doses.

For enhancing activity levels during the light period in a novel environment (i.e., baseline activity in the fear conditioning test), the intermediate dose of 10 mg/kg was optimal while for enhancing activity in the dark period the highest dose of 20 mg/kg was the most effective. Interestingly, there was a trend towards increased activity of PC HET mice during the dark period treated with the lower dose of 3.6 mg/kg, a pattern not seen with treatment of the intermediate dose of 10 mg/kg. A limitation of our study design is that the mice were tested in the water maze following 10 days after the initiation of treatment and the mice were

tested for home cage activity prior to testing in the water maze. Therefore, we cannot exclude that the longer prior treatment might have contributed to the effects of the 3.6 mg/kg dose in the water maze test. Regardless, considering that side effects will likely be more carrier need to be considered in studying the behavioral and cognitive phenotypes and responsiveness to Alk inhibition in NF1 HET mice. This result might impact earlier studies as typically potential differential effects of MC versus PC NF1 HET mice were not considered or assessed. The role of the parental carrier might be important in other preclinical as well and therefore should be kept in mind for future studies. Alk inhibition improves cognitive flexibility in NF1 HET mice. NF1 HET PC mice showed reduced cognitive flexibility and impaired ability to locate the third hidden platform location, which was improved with Alk inhibition (3.6 mg/kg). Consistent with reduced cognitive flexibility in NF1 mice, they did not show better performance in the third than second probe trial as seen in WT mice. Effects of Alk inhibition on baseline activity in the fear conditioning test and on activity levels in the home cage during the dark period were seen with the 10 and 20 mg/kg dose, respectively, indicating that cognitive flexibility might be particularly sensitive to Alk inhibition. This result is consistent with the observation that NF1 is caused by haplo-insufficiency or partial loss of function of the NF1 gene and that the genetical and pharmacological susceptibility of the hippocampus-dependent functions of neurofibromin to modification by Ras [47]. A limitation of the current study is that the pharmacological treatment was initiated only 10 days prior to water maze testing. Therefore, in the study described below we determine whether following prolonged treatment there are more profound effects of Alk inhibition on behavioral and cognitive performance of NF1 HET mice. It is critical to assess and minimize toxicity, as these studies have the ultimate objective of developing a treatment for humans with neurofibromatosis. Acceptable side effects in the context of cancer are not acceptable in the context of long-term treatment of patients with neurofibromatosis. Therefore, we included additional behavioral tests as well. We just finalized the analyses and generated the related figures. We are planning to submit the related manuscript this month.

Long-term pharmacological treatment

Animals were given either vehicle or one of two doses (3.6 mg/kg or 10 mg/kg) of the Alk inhibitor CH5424802 (Alectinib, Chugai Pharmaceuticals, Tokyo, Japan). There were 10 mice/dose/sex/genotype or 120 mice in total. The vehicle for the drug was a flavored gelatin mixture in 1 cm x 1 cm squares. This route of administration was selected because voluntary oral administration would be the most relevant for human patients, and less stressful and safer for the mice than daily oral gavage. Treatment was administered daily starting 24 weeks prior to the first day of behavioral testing and 23 weeks prior to the assessment of home cage activity. Every day when the gelatin squares were given to the mice, the researchers confirmed that the previous gelatin squares had been eaten. The mean age at start of treatment was 9.28 ± 0.09 months. The mice were weighed weekly. All groups increased body weights over time but there was no effect of treatment in WT males (Fig. S1A), HET males (Fig. S1B), WT females (Fig. S1C), and HET females (Fig. S1D).

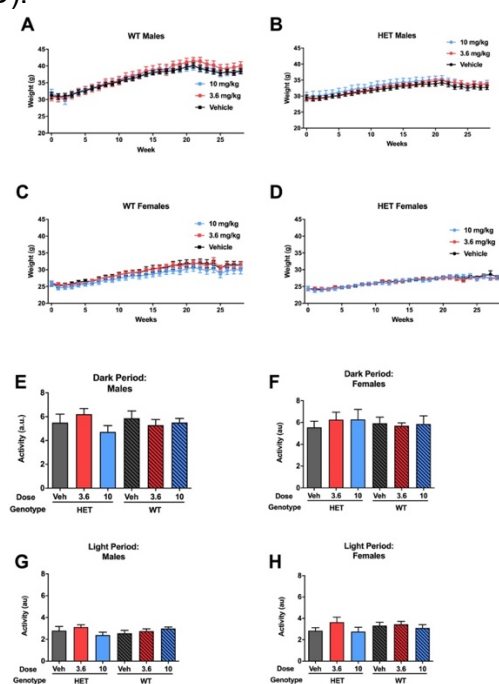


Fig. S1. No effects of long-term Alk inhibition on body weight changes and activity levels in the dark and light periods in NF1 or HET mice. For details, see text.

Mice were subsequently tested for home cage activity, performance in the rotorod, elevated zero maze, open field and novel object recognition, water maze, fear conditioning and extinction, the forced swim, and the passive avoidance tests.

Circadian activity levels were analyzed in a subset of mice using infrared home-cage activity sensors (Bioserve, St Augustin, Germany) placed above the cage. The mice were housed individually and allowed two days to acclimate to the home cage environment prior to the start of activity monitoring. Activity counts were measured every second. The ratio activity, defined as the activity in the dark period divided by the activity in the light period, was analyzed as well.

There was no effect of treatment on activity of WT (Fig. S1E) and HET (Fig. S1F) males or WT (Fig. S1G) and HET (Fig. S1H) females during the dark or light periods. However, there was an effect of treatment on the ratio dark/light activity in WT males ($F(2, 24) = 3.950$, $p = 0.034$, Fig. 1A). The ratio dark/light activity was lower in WT males treated with the Alk inhibitor at 10 mg/kg than those treated with vehicle ($p = 0.028$, Dunnett's). There was a trend towards a lower ratio dark/light activity in WT males treated with the Alk inhibitor at 3.6 mg/kg but that did not reach significance ($p = 0.069$, Dunnett's). In contrast, no effect of treatment on ratio dark/light activity was seen in HET males (Fig. 1A). There was no effect of treatment on the ratio dark/light activity in WT females (Fig. 1B). There was a trend towards an effect of treatment on the ratio dark/light activity in HET females ($F(2, 21) = 3.063$, $p = 0.070$) but neither the 3.6 nor the 10 mg/kg dose was different from the vehicle group.

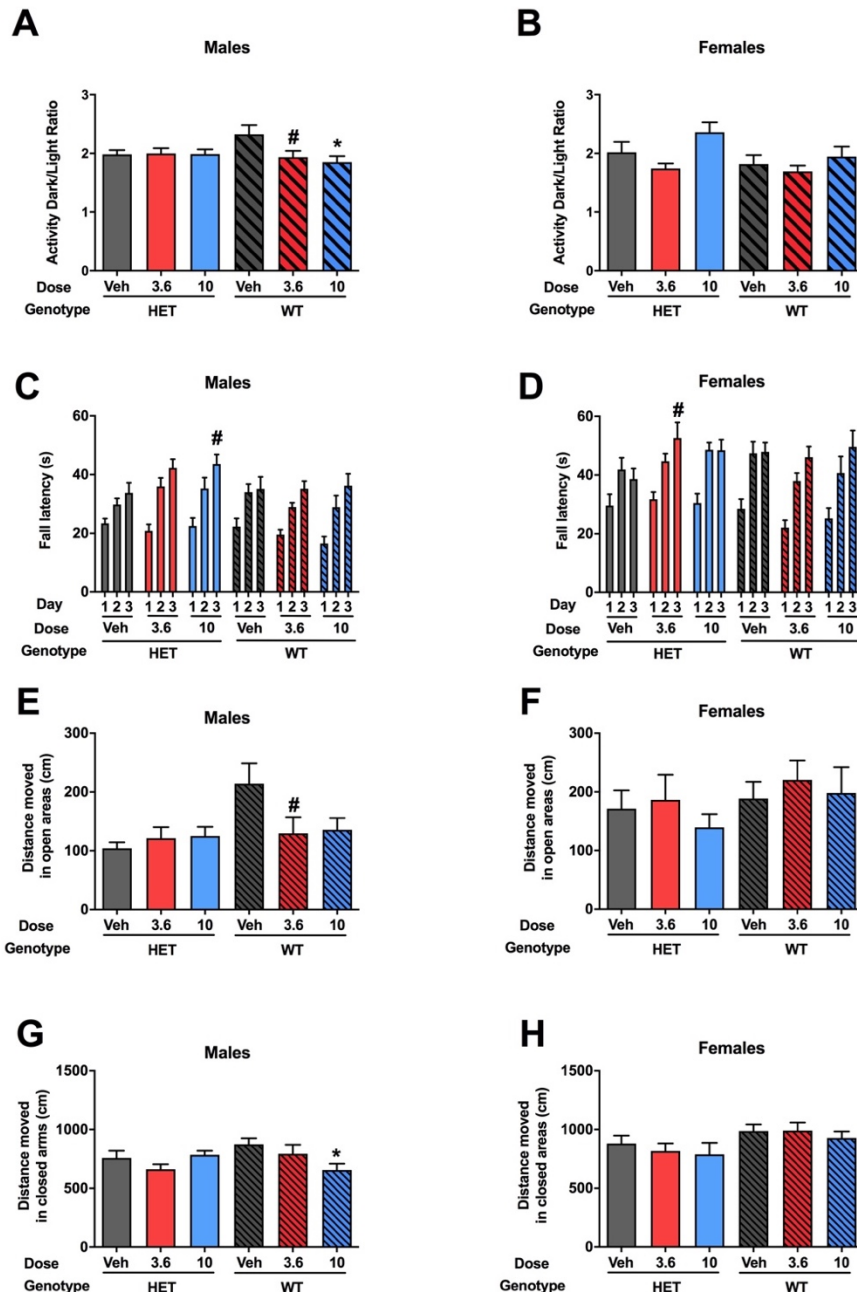


Fig. 1. Effects of long-term Alk inhibition on the dark/light ratio, and activity levels in the elevated zero maze. For details, see text.

Rotorod testing

Sensorimotor function was assessed using the rotarod. Mice were placed on a rotating rod (diameter: 3 cm, elevated: 45 cm; Rotamex-5, Columbus Instruments, Columbus, OH, USA). Rotation speed started at 5.0 rpm and accelerated 1.0 rpm every 3 s. Latency to fall (s) was recorded using photo beams located below the rod. Mice received three trials each day for 3 subsequent days. There was no effect of treatment in WT males but there was a trend towards a day x treatment interaction in HET males ($F(4, 54) = 2.307$, $p = 0.070$, Fig. 1C). There was a trend towards an effect of treatment on day 3 ($F(2, 27) = 2.705$, $p = 0.0850$), with a trend towards enhanced rotorod performance in HET males treated with the 10 mg/kg dose than those treated with vehicle ($p = 0.0749$). There was no effect of treatment in WT females but there was a day x treatment interaction in HET females ($F(4, 54) = 2.751$, $p = 0.037$, Fig. 1D). There was a trend towards an effect of treatment on day 3 ($F(2, 27) = 2.858$, $p = 0.0749$), with a trend towards enhanced rotorod performance in HET females treated with the 3.6 mg/kg dose than those treated with vehicle ($p = 0.05$).

Elevated zero maze

Measures of activity and anxiety were assessed in the elevated zero maze. The enclosure (Kinder Scientific, Poway, CA) consisted of four sections (6 cm wide), alternating between open and closed sections. Mice were placed into an open area of the maze and allowed to explore for 10 minutes. Kinder Scientific MotorMonitor was used to track the distance moved (cm) and time spent in the open and closed areas (sec). Enclosures were cleaned with 0.5% acetic acid between trials.

There was no effect of treatment on the time spent in the open areas of the elevated zero maze in WT and HET males (Fig. S2A) or WT and HET females (Fig. S2B). There was a trend towards an effect of treatment on the distance moved in the open areas of the elevated zero maze in WT males ($F(2, 29) = 2.817$, $p = 0.077$, Fig. 1E). There was a trend towards less activity of WT males treated with the 3.6 mg/kg dose than those treated with vehicle ($p = 0.077$). This trend was sex-dependent and not seen in females (Fig. 1F). There was also a trend towards an effect of treatment on the distance moved of WT males in the closed areas of the elevated zero maze ($F(2, 29) = 3.165$, $p = 0.058$, Fig. 1G). Activity levels of WT males treated with the 10 mg/kg dose was lower than those of vehicle-treated WT male ($p = 0.036$, Dunnett's).

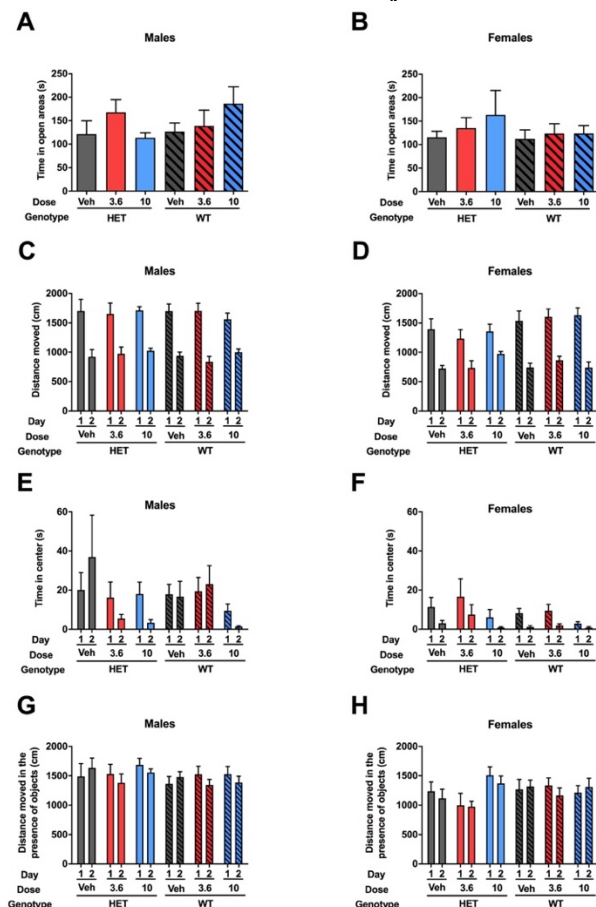


Fig. S2. No effect of long-term Alk inhibition on measures of anxiety in the elevated zero maze or activity levels in the open field containing objects. For details, see text.

Open field and novel object recognition

Activity and anxiety-like behavioral performance in an open field (41x41 cm) was assessed during two subsequent days. All tests were video recorded and total distance (as measure of activity) and time spent in the center of the open field (as measure of anxiety-like behaviour) were analysed using Noldus Ethovision video tracking. Enclosures were cleaned with 0.5% acetic acid between trials.

The novel object recognition test was used to examine object recognition by the ability of the mice to recall a familiar object after 24 hours. After a three day habituation period in the open field, mice were allowed to freely explore 2 identical objects for 15 minutes during the familiarization phase. Twenty four hours later, mice were again exposed to the open field enclosure containing one of the same objects present in the familiarization phase and one new object during the test phase for 15 minutes. The distance moved was analysed using Noldus Ethovision video tracking (Wageningen, The Netherlands) and the object exploratory behaviour of the mice (time spent exploring each subject) were analysed by manual scoring of videos. The percent time spent exploring the novel object and a discrimination index ((time spent with new object - time spent with familiar object) / total exploration time * 100%) were calculated as measure of memory retention.

When activity levels were analyzed over the two subsequent two days of open field testing, there was a trend towards a day x treatment interaction in WT males ($F(2, 27) = 2.868, p = 0.074$, Fig. S2C) but activity levels on either day were not different in the treatment groups. No effect of treatment on activity levels in the open field was seen in HET males (Fig. S2C) or WT or HET females (Fig. S2D).

Next, time spent in the more anxiety center of the open field was analyzed. There was no effect of treatment on the time WT males spent in the center of the open field (Fig. S2E). In HET males, there was a trend towards a day x treatment interaction ($F(2, 27) = 2.621, p = 0.091$, Fig. S2E) but there was no group difference in time spent in the center of the open field on either day. There was no effect of treatment on time spent in the center of the open field in WT or HET females (Fig. S2F).

Following the two days of habituation to the open field, activity levels were analyzed in the open field in the presence of objects. In males, there was no effect of treatment on activity levels in the open field containing objects in WT or HET males (Fig. S2G). In WT females, there was no effect of treatment on activity levels in the open field containing objects (Fig. S2H). There was a trend towards an effect of treatment on activity levels in the open field containing objects in HET females but it did not reach significance (Fig. S2I).

Vehicle-treated WT males showed object recognition and spent more time exploring the novel than the familiar object ($t = 2.485, p = 0.0189$, Fig. 2A). Treatment with the 3.6 mg/kg dose impaired this preference and at the 10 mg/kg dose there was only a trend towards a preference for exploring the novel object ($t = 1.575, p = 0.0797$, Fig. 2A). In contrast, object recognition was impaired in vehicle-treated HET males and those treated with the Alk inhibitor at a the 3.6 mg/kg dose (Fig. 2A). However, preference for exploring the novel object was seen in HET males treated with the Alk inhibitor at the 10 mg/kg dose ($t = 1.898, p = 0.0471$, Fig. 2A). In WT females, there was no preference for exploring the novel object in vehicle-treated mice or those treated with the Alk inhibitor at the 3.6 mg/kg dose (Fig. 2B). There was a trend towards a preference for exploring the novel object in WT females treated with the Alk inhibitor at the 10 mg/kg dose but it did not reach significance ($t = 1.465, p = 0.0905$, Fig. 2B). As in HET males, vehicle-treated HET females and those treated with the Alk inhibitor at the 3.6 mg/kg dose did not show object recognition. However, preference for exploring the novel object was seen in HET females treated with the Alk inhibitor at the 10 mg/kg dose ($t = 2.071, p = 0.0385$, Fig. 2B). The effects of the Alk inhibitor on object recognition in NF1 HET mice are very encouraging as they reveal that a clear cognitive phenotype is rescued by long-term treatment in both sexes. These data are also encouraging as they support the 10 mg/kg dose as an optimal dose for this cognitive effect.

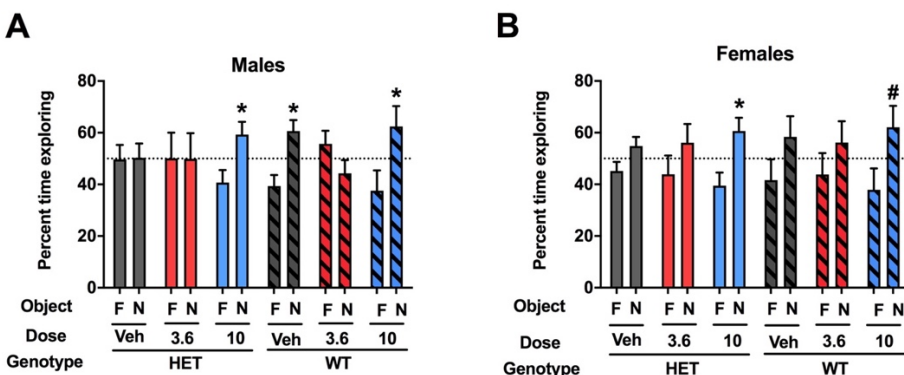


Fig. 2. Long-term Alk inhibition rescues impairments in object recognition in NF1 HET males and females. For details, see text.

Spatial learning and memory in the water maze

Mice were tested for spatial learning and memory in the water maze. A circular pool (140 cm diameter) was filled with water ($20^{\circ}\text{C} \pm 2^{\circ}\text{C}$). A platform (20 cm diameter) was located approximately 1 cm below opaque water, which contained white chalk in order to hide the platform in opaque water. A trial ended when the mouse either located and stayed on the platform for 3 s or after 60 s had elapsed. If the mouse had not located the platform within 60 s, the mouse was guided to the platform and retrieved once they had remained on it for 3 s. Mice were returned to their home cage after removal from the maze. The mice were trained to locate two locations during visible platform training (Visible Location 1: session 1; Visible Location 2: session 2) and four locations during hidden platform training (Hidden Location 1: sessions 3-4; Hidden Location 2: sessions 5-6; Hidden Location 3: sessions 7-8; Hidden Location 4: session 9-10). The sessions consisted of two trials each with a 10-minute inter-trial interval. There were two sessions per day, three hours apart. Before sessions 5, 7, and 9 mice were given a probe trial, in which the platform was removed, and they were allowed 60 s of exploration in the maze. Performance was assessed by analyzing cumulative distance to target location and latency to reach the platform. Data from the water maze was recorded and analyzed using Ethovision 7.1 XT video tracking software (Wageningen, The Netherlands) set at 6 samples/sec.

The mice were first trained to locate a visible platform in two sessions, each containing of two trials. In WT males, there was an effect of treatment on swim speeds ($F(2, 27) = 3.735$, $p = 0.037$), with lower swim speeds in mice treated with the Alk inhibitor at the 10 mg/kg dose than vehicle-treated mice ($p = 0.028$, Dunnett's, Fig. 3A). No effect of treatment on swim speeds was seen in HET males (Fig. 3B), WT females (Fig. 3C), or HET females (Fig. 3D).

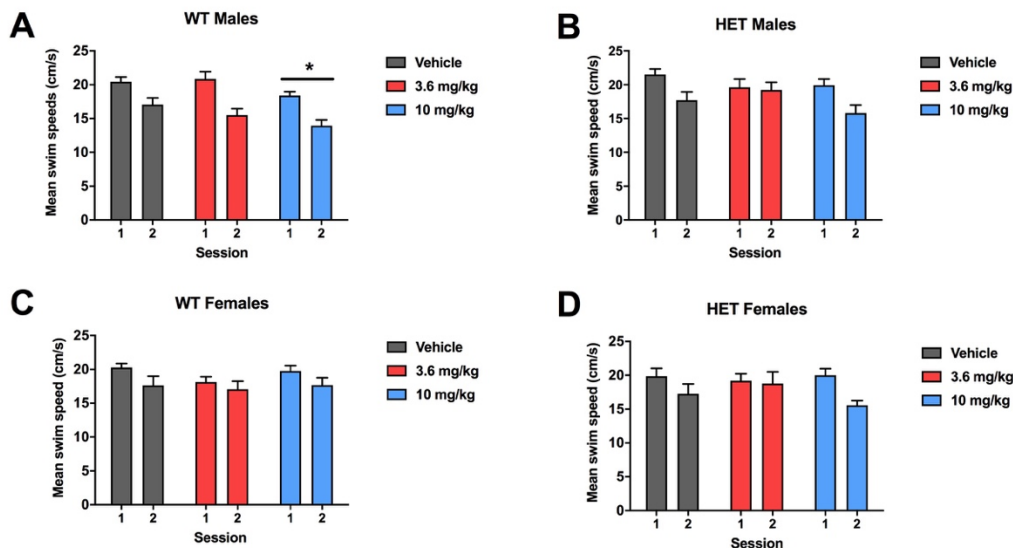


Fig. 3. Effects of long-term Alk inhibition on swim speeds of WT males.

When time to reach the platform (latency) was used as performance measure, all groups improved their performance during visible platform training but there was no effect of treatment in WT males, HET males, WT females, or HET females (Fig. S3). In WT males, there was an effect of treatment during training to locate the first hidden platform (H1) ($F(1, 27) = 6.511$, $p = 0.0167$) with worse performance in mice treated with the Alk inhibitor at the 10 mg/kg dose than in vehicle-treated mice ($p = 0.0167$, Fig. S3B). During training to locate the second hidden platform (H2), there was a trend towards a treatment x session interaction ($F(2, 27) = 3.094$, $p = 0.0617$, Fig. S3A, S3B). There was no effect of treatment during training to locate the third (H3) or fourth (H4) hidden platform. In HET males, there was no overall effect of treatment during training to locate the first, second, third, or fourth hidden platform. In session 5, there was an effect of treatment ($F(2, 27) = 4.252$, $p = 0.0248$), with better performance in HET males treated with the Alk inhibitor at the 3.6 mg/kg dose than vehicle-treated mice ($p = 0.0352$, Fig. S3C). There was no effect of treatment during training to locate any platform location in WT females. In HET females, there was no effect of treatment to locate the first, second, or fourth platform location. During training to locate the third hidden platform location, there was a trend towards better performance of HET females treated with the Alk inhibitor at 10 mg/kg than vehicle-treated mice ($p = 0.0882$, Fig. S3H).

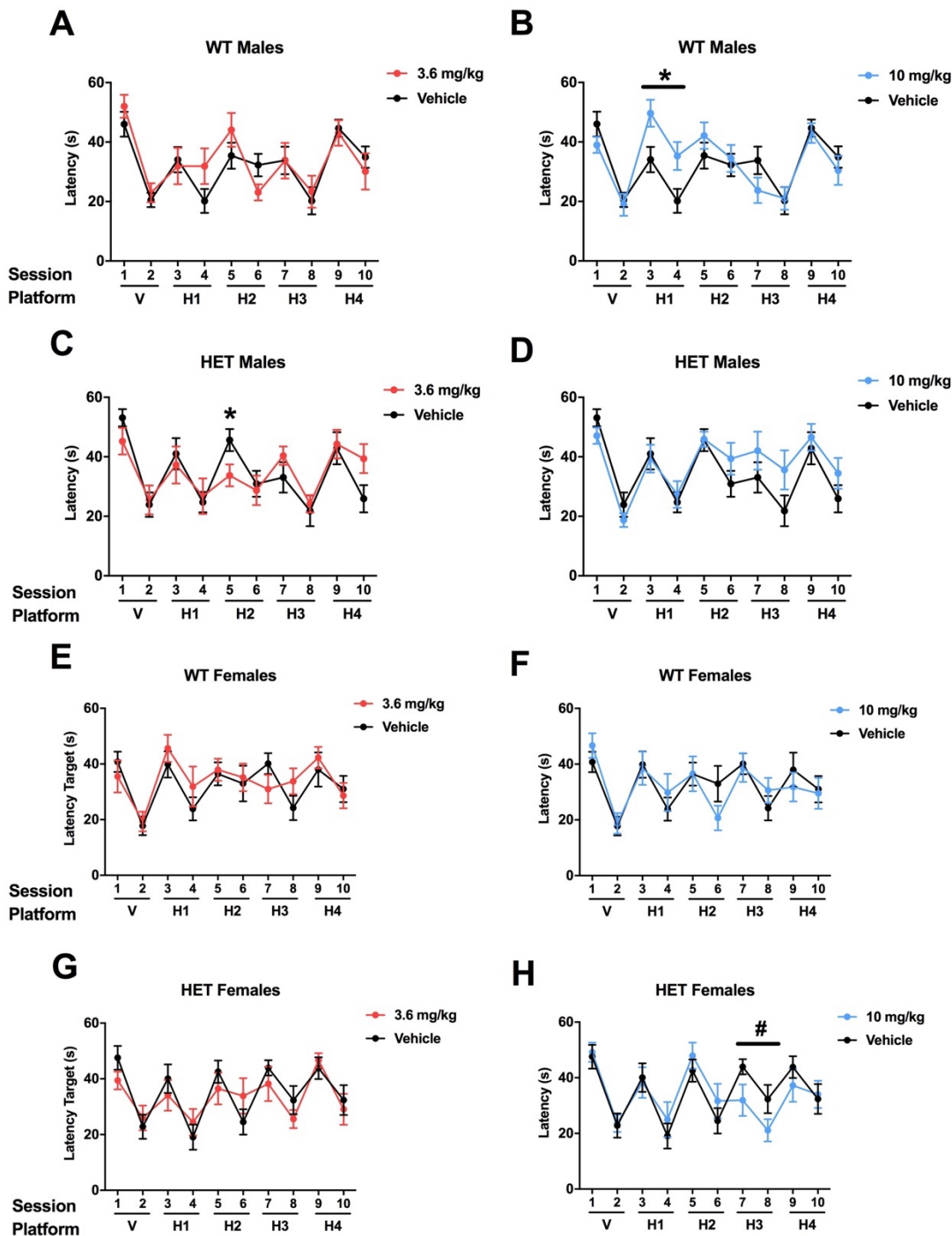


Fig. S3. Effects of long-term Alk inhibition on water maze performance latency curves of NF1 HET and WT males and females. For details, see text.

When cumulative distance to the target location was used as performance measures, all groups improved their performance during visible platform training but there was no effect of treatment in WT males, HET males, WT females, or HET females (Fig. S4). In WT males, there was an effect of treatment during training to locate the first hidden platform (H1) ($F(1, 27) = 4.196, p = 0.0259$) with worse performance in mice treated with the Alk inhibitor at the 10 mg/kg dose than in vehicle-treated mice ($p = 0.0163$, Fig. S4B). During training to locate the second platform (H2), there was a treatment x session interaction ($F(2, 27) = 4.587, p = 0.0193$, Fig. S4A, S4B). There was a trend towards an effect of treatment in session 6 ($F(2, 27) = 2.649, p = 0.0890$). There was no effect of treatment during training to locate the third (H3) or fourth (H4) hidden platform. In HET males, there was no overall effect of treatment during training to locate the first or second hidden platform. In session 5, there was an effect of treatment ($F(2, 27) = 3.765, p = 0.0361$), with better performance in HET males treated with the Alk inhibitor at the 3.6 mg/kg dose than vehicle-treated mice ($p = 0.0379$, Fig. S4C). There was a trend towards an effect of treatment during training to locate the third hidden platform ($F(2, 27) = 3.148, p = 0.0591$), with worse performance in mice treated with the 10 mg/kg dose ($p = 0.0497$, Fig.

S4D). There was no effect of treatment during training to locate the fourth hidden platform. There was no effect of treatment during training to locate any hidden platform in WT females. In HET females, there was a trend towards better performance of mice treated with the Alk inhibitor at the 10 mg/kg dose than vehicle-treated mice during the third platform location ($p = 0.0882$, Fig. S4H) but no effect of treatment during the first, second, or fourth platform location.

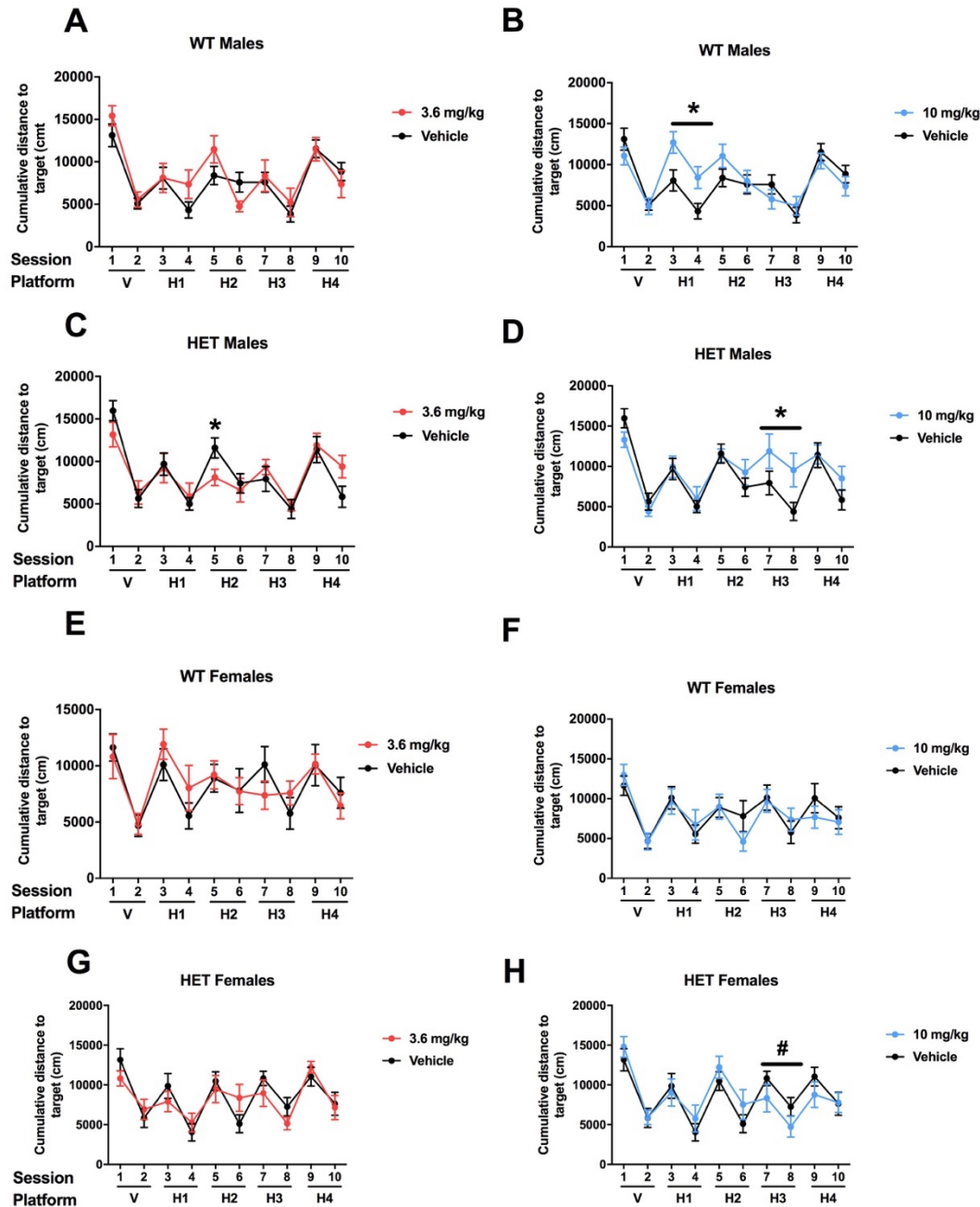


Fig. S4. Effects of long-term Alk inhibition on water maze performance cumulative distance to the target curves of NF1 HET and WT males and females. For details, see text.

Spatial memory retention was assessed in three probe trials (no platform). For the three probe trials, cumulative distance to the target platform was used as performance measure. In WT males, there was an effect of probe trial but not of treatment ($F(2, 54) = 6.660$, $p = 0.003$, Fig. S5A). In HET males, there was an effect of probe trial ($F(2, 54) = 14.522$, $p < 0.001$) and a probe trial x treatment interaction ($F(2, 54) = 3.056$, $p = 0.024$). There was no effect of treatment in the first or second probe trial. However, there was an effect of treatment in the third probe trial ($F(2, 27) = 8.726$, $p = 0.0012$, Fig. S5A), with a higher cumulative distance to the target location in mice treated with the Alk inhibitor at the 10 mg/kg dose than in vehicle-treated mice ($p = 0.0014$, Fig. S5A). There was an effect of probe trial but not of treatment in WT ($F(2, 54) = 6.106$, $p = 0.004$) and HET females ($F(2, 54) = 13.188$, $p < 0.001$) (Fig. S5B).

Because of the complexity of interpretation of probe trials with multiple hidden platform locations, we also analyzed the cumulative distance to four platform locations, one in each target quadrant, in each of the

three probe trials. In the first probe trial in vehicle-treated WT males, there was a trend towards an effect of target location ($F(3, 36) = 6.106, p = 0.08$), with a lower cumulative distance to the first than second target location ($p = 0.03$, Fig. S5C). In WT males treated with the 3.6 mg/kg dose, there was an effect of target location ($F(3, 36) = 7.561, p = 0.0005$), with a lower cumulative distance to the first than third target location ($p = 0.0077$, Fig. S5C). In WT males treated with the 10 mg/kg dose, there was also an effect of target location in WT males ($F(3, 36) = 7.740, p = 0.0004$), with a lower cumulative distance to the first than third target ($p = 0.0419$, Fig. S5c). In the first probe trial in vehicle-treated HET males, there was an effect of target location ($F(3, 36) = 7.273, p = 0.0006$), with a lower cumulative distance to the first than second ($p = 0.0002$) and third target location ($p = 0.0124$) (Fig. S5C). In HET males treated with the Alk inhibitor at the 3.6 mg/kg dose, there was also an effect of target location, ($F(3, 36) = 4.412, p = 0.0097$), with a trend towards a lower cumulative distance to the first than second ($p = 0.0540$) and third ($p = 0.0524$) target location (Fig. S5C). In HET males treated with the Alk inhibitor at the 10 mg/kg dose, there was an effect of target location, ($F(3, 36) = 6.137, p = 0.0018$), with a lower cumulative distance to the first than third ($p = 0.0392$) target location (Fig. S5C).

In the second probe trial in vehicle-treated WT males, there was an effect of target location ($F(3, 36) = 4.493, p = 0.0089$, Fig. S5E), but there was no difference between the cumulative distance to the second target location and any other location. However, the cumulative distance to the first target location was lower than that to the fourth target location ($p = 0.0154$, Fig. S5E). There was no effect of target location in WT males treated with either the 3.6 or 10 mg/kg dose of the Alk inhibitor in the second probe trial. In vehicle-treated HET males, there was an effect of target location in the second probe trial ($F(3, 36) = 7.269, p = 0.0006$), with a higher cumulative distance second than first target location ($p = 0.0002$) location and a trend towards a higher cumulative distance to the second than fourth target location ($p = 0.0587$) (Fig. S5E). The cumulative distance to the first target location was lower than that to the second ($p = 0.0002$) and fourth ($p = 0.0057$) target location. There was no effect of target location in HET males treated with the 3.6 mg/kg dose of the Alk inhibitor (Fig. S5E). There was an effect of target location in HET males treated with the 10 mg/kg dose of the Alk inhibitor in the second probe trial ($F(3, 36) = 3.552, p = 0.0238$), with a lower cumulative distance to the first than second ($p = 0.0331$) and third target location ($p = 0.0234$) (Fig. S5E).

In the third probe trial in vehicle-treated WT males, there was an effect of target location ($F(3, 36) = 5.546, p = 0.0031$, Fig. S5G). The cumulative distance to the third target location was lower than that to the second ($p = 0.0270$) and fourth ($p = 0.0010$) target location. There was no effect of target location in WT males treated with the 3.6 mg/kg dose of the Alk inhibitor. However, there was an effect of target location in HET males treated with the 10 mg/kg dose of the Alk inhibitor in the third probe trial ($F(3, 36) = 4.687, p = 0.0073$), with a higher cumulative distance to the third than fourth target location ($p = 0.0019$) (Fig. S5G).

In the first probe trial in vehicle-treated WT females, there was an effect of target location ($F(3, 36) = 6.635, p = 0.0011$), with a lower cumulative distance to the first than second ($p = 0.0012$) and third target location ($p = 0.0140$) (Fig. S5D). In WT females treated with the 3.6 mg/kg dose, there was an effect of target location during the second probe trial ($F(3, 36) = 3.254, p = 0.0327$), with a lower cumulative distance to the first than second target location ($p = 0.0355$) (Fig. S5D). In WT females treated with the 10 mg/kg dose, there was an effect of target location during the second probe trial ($F(3, 36) = 3.274, p = 0.0320$), with a lower cumulative distance to the first than second target location ($p = 0.0284$) (Fig. S5D). In the first probe trial in vehicle-treated HET females, there was no effect of target location and a trend towards a lower cumulative distance to the first than second target location ($p = 0.0861$) (Fig. S5D). There was an effect of target location in the HET females treated with the 3.6 mg/kg dose ($F(3, 36) = 8.096, p = 0.0003$), with lower cumulative distance to the first than second ($p = 0.0066$) and third ($p = 0.0016$) target location (Fig. S5D). There was also an effect of target location in HET females treated with the 10 mg/kg dose ($F(3, 36) = 6.599, p = 0.0011$), with lower cumulative distance to the first than second ($p = 0.0011$) and third ($p = 0.0086$) target location (Fig. S5D).

In the second probe trial, there was no effect of target location in vehicle-treated WT females or those treated with the 3.6 mg/kg dose. There was a trend towards an effect of target location in WT females treated with the 10 mg/kg dose ($F(3, 36) = 2.278, p = 0.0961$, Fig. S5F). There was a trend towards an effect of target location in vehicle-treated HET females ($F(3, 36) = 2.705, p = 0.0597$), with a higher cumulative distance to the first than second target location ($p = 0.0338$, Fig. S5F). There was no effect of target location in HET females treated the 10 mg/kg dose.

In the third probe trial, there was no effect of target location in WT females at any dose. In vehicle-treated HET females, there was an effect of target location ($F(3, 36) = 5.693, p = 0.0027$), with a higher cumulative distance to the third than the fourth target location ($p = 0.0007$) (Fig. S5H). There was no effect of target location in HET females treated the 3.6 or 10 mg/kg dose.

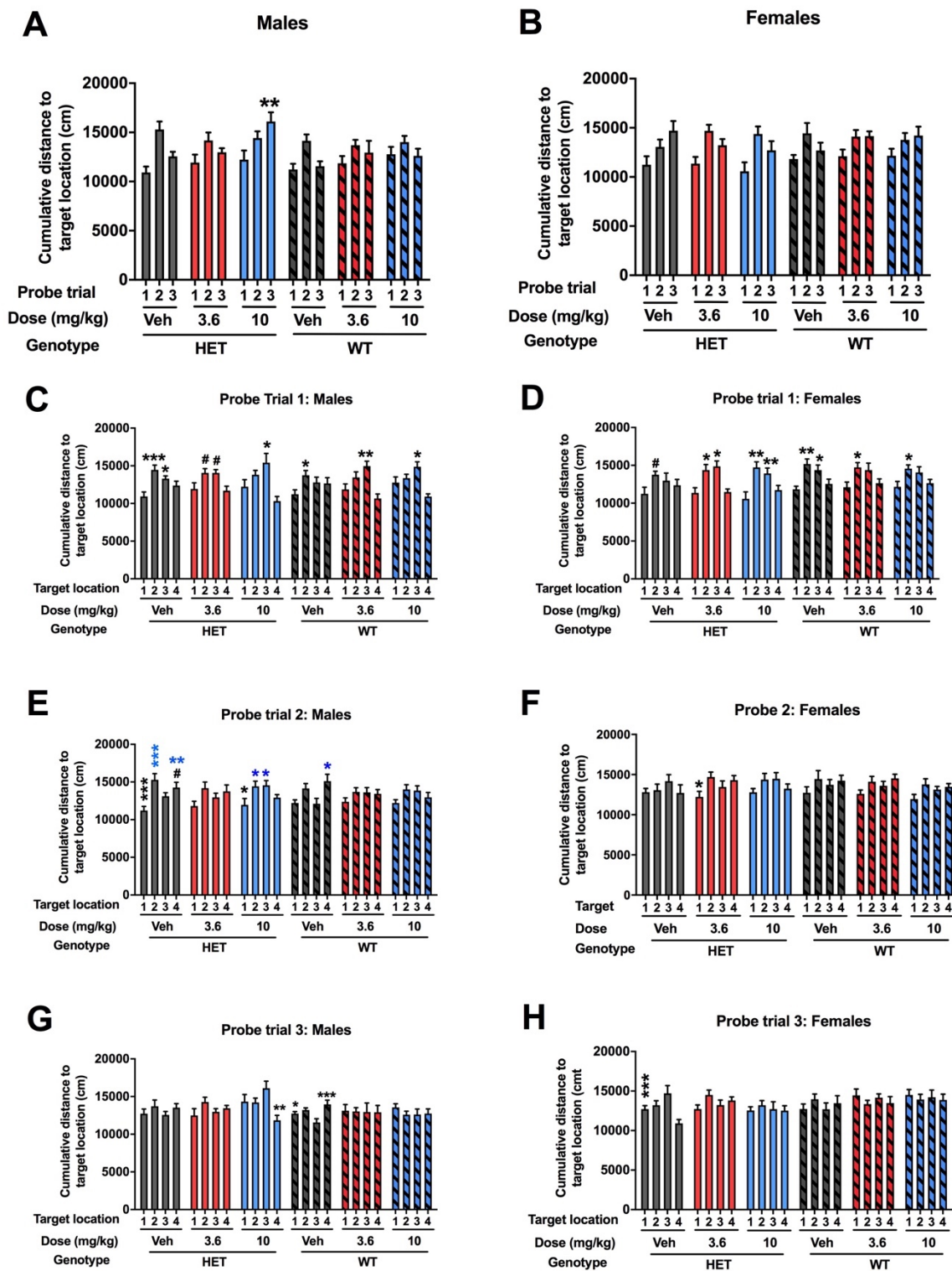


Fig. S5. Effects of long-term Alk inhibition on spatial memory retention of NF1 HETS and WT males and female in the water maze.

Contextual fear learning, memory, and extinction

Acquisition, retention, and extinction of hippocampus-dependent contextual fear conditioning was assessed using the Med Associates Video Freeze automated scoring system (Med Associates, St. Albans, VT, USA). Freezing is a recognized indicator of a conditioned fear response and is defined as the absence of motion, with the exception of respiration. On the training day, mice were placed in the chamber with a house light (100 lux) and allowed 120 s to acclimate. This was followed by two tone-shock pairings. Each tone was 80 dB at 28000 Hz and lasted for 28 s, and co-terminated with a 2 s shock at 0.6 mA, with a 120 s interval in between the two tone-shock pairings. The day after training, mice were placed back in the training environment

for 300 s during which no tone or shock were administered. This procedure was repeated for a total of 10 days during which no tone or shock were administered.

There was no effect of treatment on activity or freezing during the baseline period (prior to the first tone), movement in response to the shocks, freezing between the tone-shock pairings (ISI), or extinction of contextual fear memory in WT or HET males or females. When we analyzed freezing during the tones on the training day, in WT and HET males, freezing was higher during the second than first tone (WT: ($F(1, 27) = 62.86, p < 0.0001$; HET: ($F(1, 27) = 70.37, p < 0.0001$), but there was no effect of treatment (Fig. S6A). In WT females, freezing was higher during the second than first tone ($F(1, 27) = 82.24, p < 0.0001$) and there was a trend towards a treatment x tone interaction ($F(2, 27) = 3.108, p = 0.06$); there was a trend towards an effect of treatment on freezing during the second tone ($F(2, 27) = 2.551, p = 0.0966$), with a trend towards lower freezing levels in mice treated with the 10 mg/kg dose than vehicle treated mice ($p = 0.0958$)(Fig. S6B).

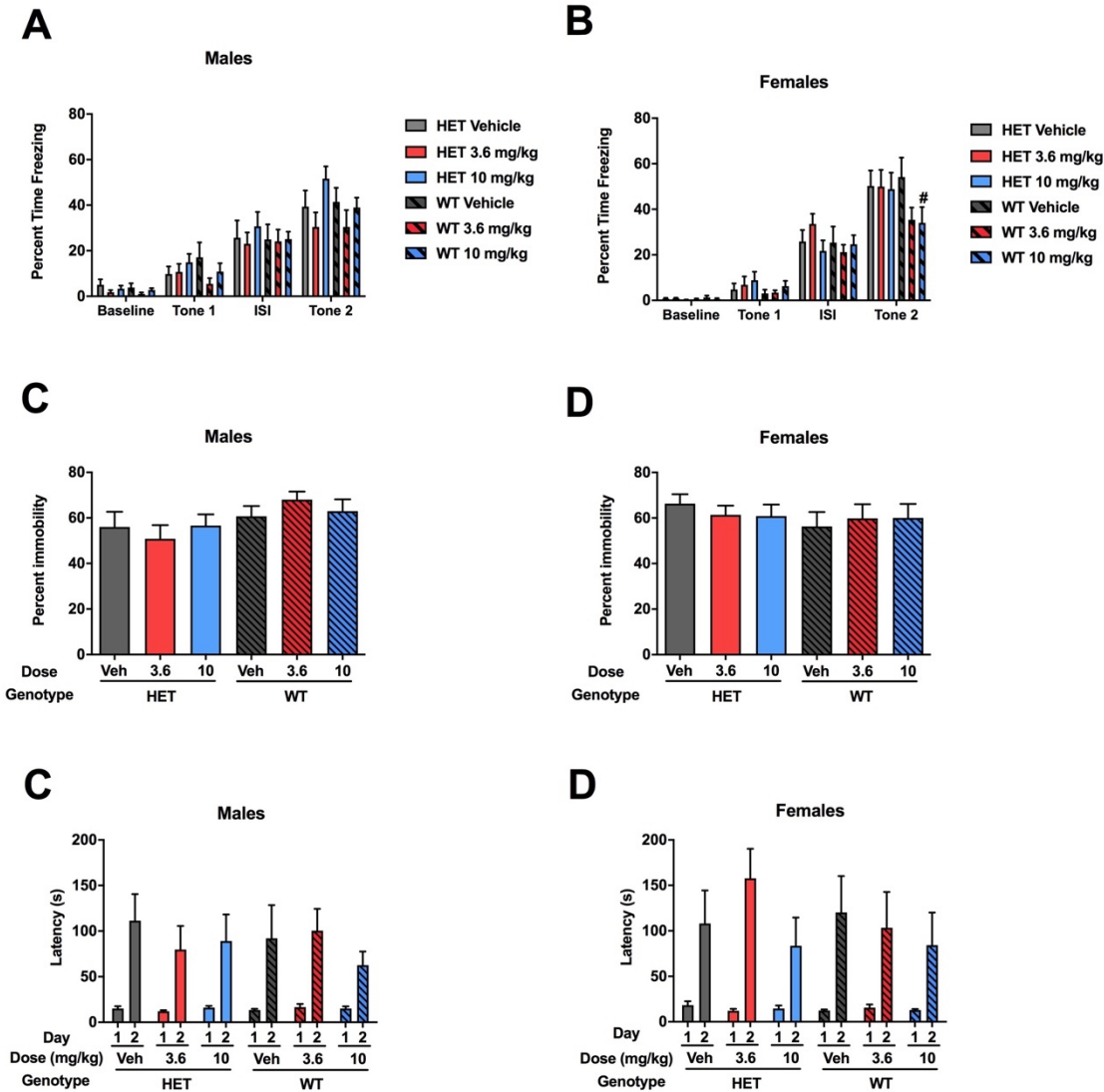


Fig. S6. Trend towards a detrimental effect of long-term Alk inhibition on freezing of WT females during the second tone during fear learning.

Forced swim test

Depressive-like behavior was assessed using the forced swim test. Groups of 4 mice were individually placed into separate 2 L glass beakers (diameter = 12.7 cm) and allowed to swim during one 6-min trial. Water in the beakers was kept at room temperature (21°C). Videos were recorded and manually scored for total time immobile for the last 5 min during the forced swim task, as described [48]. There was no effect of treatment on depressive-like behavior in the forced swim test in WT or HET males (Fig. S6C) and WT or HET females (Fig. S6D).

Passive Avoidance

In addition to fear conditioning, emotional learning and memory was also assessed with a step-through box consisting of a brightly lit compartment and an identical dark compartment connected with a sliding door (Kinder Scientific, Poway, California). Mice were given one training trial in which they were placed individually in the bright compartment and, after a habituation period of 3 s, the door was opened into the darkened compartment. Mice, being averse to bright light, were naturally inclined to enter the darkened compartment. When they did so, the door quickly shut and a slight foot-shock was delivered (0.35 mA for 3 s). After a retention interval of 24 h, each mouse received a memory retention trial. During the test trial each mouse was once again placed into the bright compartment, and the latency to re-enter the dark compartment was recorded up to 300 s. The latency to enter the dark compartment during the training trial was used as a baseline measure, and time before entering the dark chamber during the test trial was used to measure retention of passive avoidance behavior.

Lastly, we tested fear learning and memory in the passive avoidance test. All groups showed passive avoidance memory retention. WT males ($F(1, 27) = 21.887, p < 0.001$), HET males ($F(1, 27) = 24.477, p < 0.001$), WT females ($F(1, 26) = 16.233, p < 0.001$), and HET females ($F(1, 27) = 26.767, p < 0.001$) all showed higher latencies to enter the dark compartment on day 2 than day 1 (Fig. S6E, S6F). However, there was no effect of treatment on latency to enter the dark compartment during the training or testing day in WT or HET males (Fig. S6E) and WT or HET females (Fig. S6F).

4. Impact

The data described above support the therapeutic potential of pharmacological Alk inhibition in NF1^{+/-} mice and ultimately in patients with neurofibromatosis. Our data also support that it matters whether the father or the mother carries the NF1 mutation and is on the C57BL/6J background. We are not aware that there have been efforts in human NF1 patients to assess the role of a father versus a mother being carrier for NF1 but based on our data such a study seems warranted. We assessed the potential side effects of long-term treatment in NF1^{+/-} mice and confirmed the beneficial cognitive effects of Alk inhibition on object recognition in both males and females. Here is a summary of all the long-term effects:

Detrimental effects of long-term Alk inhibition in WT mice

Long-term Alk inhibition reduces the dark/light ratio in WT males; 10 mg/kg is the most effective dose.

Long-term Alk inhibition reduces activity levels of WT males in the elevated zero maze; 10 mg/kg is the most effective dose.

Trend towards reduced activity of WT males following long-term Alk inhibition in the open areas of the elevated zero maze.

Long-term Alk inhibition reduces swim speeds of WT males during visible platform training in the water maze; 10 mg/kg is the most effective dose.

Long-term Alk inhibition impairs ability of WT males to locate the first hidden platform location.

Trend towards a detrimental effect of long-term Alk inhibition on freezing of WT females during the second tone during fear learning.

Beneficial effects of long-term Alk inhibition in NF1 HET mice

Trend towards enhances performance of Alk inhibitor-treated NF1 HET males and females in the wire hang test; 10 mg/kg is the optimal dose in males, 3.6 mg/kg is the optimal dose in females.

Long-term Alk inhibition rescues impairments in object recognition in NF1 HET males and females; 10 mg/kg is the most effective dose.

Long-term Alk inhibition improves ability of NF1 HET males to locate the second hidden platform location; 3.6 mg/kg is the optimal dose.

Long-term Alk inhibition improves ability of NF1 HET females to locate the third hidden platform location; 10 mg/kg is the optimal dose.

The genotype-dependent direction of the long-term effects of Alk inhibition are consistent with a specific interaction between Alk and NF1. Although not seen under the current conditions, it is conceivable that Alk inhibition might be beneficial for cognitive performance when cognitive performance of WT mice is impaired due to age or environmental challenges. With regard to dose, in HET NF1 males, 10 mg/kg seems the optimal dose in most cases besides the water maze learning curves. In HET NF1 females, 3.6 mg/kg seems the optimal dose in the wire hang test and 10 mg/kg in the water maze. Overall, 10 mg/kg seems the optimal dose for enhancing cognitive performance.

5. Changes/Problems

We experienced problems with the performance of the wild-type littermates as part of the first breeding effort involving NF1+/- female mice and therefore we requested other mice for breeding using NF1+/- male mice instead. Based on our data, it matters whether the father or the mother is the NF1+/- carrier on a C57BL/6J background. Based on a recent study in Alk ko mice showing effects on body weight/thinness [40], we started to assess potential body weight changes as a result of NF1 genotype or Alk inhibitor treatment in mice part of the long-term study. Although initially we had planned for separate cohorts of mice for the BrdU injections and neurogenesis than the other mice on long-term treatment, we combined this effort realizing how involved the breeding is and due to the doubling of the breeding effort and number of mice as part of the parental versus maternal NF1 carrier breeding effort. We completed our ERG analyses following short-term treatment and plan to include ERG analysis following long-term treatment to determine whether the Alk inhibitor has side effects on the retina. This last part was not proposed but we decided to include it based on the generated data. Due to modified operations, we experienced additional challenges, including restrictions with regard to the number of cages of mice we were allowed to breed and with regard to behavioral testing early on when there was no vaccine yet available. Finally, we are processing the brains of our genetics study acquired during the previous pilot project and the current study to assess whether Alk deficiency in the presence and absence of NF1 heterozygosity is associated with altered neurogenesis.

6. Products

We published the results of the short-term Alk inhibition study, including the role of the parental carrier, this year in Brain Research (paper attached). The manuscript with the long-term data described above is being finalized for submission and we plan to submit this month.

7. Participants & Other Collaborating Organizations

- What individuals have worked on the project?

Name and Role	Yr 1 effort 5/15/17 to 5/14/18	Yr 2 effort 5/15/18 to 5/14/19	Yr 3 effort 5/15/19 to 5/14/20	Yr 4 effort (NCE) 5/15/20 to 5/14/21	Contribution to Project
Jacob Raber, PI	1.471 CM	1.523 CM	1.186 CM	1.0875 CM	Designed the experiments and supervised all team members for this project. Requested the Alk inhibitor via a MTA. Participated in feeding of the mice the jellies containing the treatments, analyzed all the data, generated all the figures for the published paper and reports.

Tunde Akinyeke, Postdoc	2 CM				Assisted with the daily care of the mice.
Esha Patel, RA	2.2 CM	0.8 CM			Assisted with the animal protocol approval for this project, breeding and genotyping of the mice, and daily care and treatments of the mice.
Hayley VanderJagt, RA	7.4 CM	4.3 CM			Assisted with the breeding and genotyping of the mice, and daily care and treatments of the mice.
Destine Krenik, RA		3.9 CM	4.36 CM	1.2 CM	Assisted with the breeding and genotyping of the mice, daily care and treatments of the mice, behavioral testing and related statistical analyses.
Ruby Perez, RA		2.15 CM	1.94 CM		Assisted with the breeding and genotyping of the mice, and daily care and treatments of the mice.
Sarah Holden, RA			0.65 CM		Assisted with the breeding and genotyping of the mice, daily care and treatments of the mice, maintaining of the animal protocol, and with providing detailed information on the mice for reporting requirements.

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Ended since last report:

NSCOR NNX15AK13G (Weil) 6/1/15-5/31/21 (NCE) 1.32 calendar months
NASA (Raber lab directs)

NASA Specialized Center of Research on Carcinogenesis

This Project will utilize the resources generated in the first cancer-related projects to study the neurobehavioral consequences of space irradiation and whether they are related to tumorigenesis-related outcome measures and predicted by the same or distinct biomarkers.

Role: Co-I, Subcontract PI

R21 ES027943 (Raber (contact), Kisby) 12/15/17-11/30/20 (NCE) 0.24 calendar months
NIH (Raber lab directs)

Impact of Second-Hand Smoke on Brain Metabolism and Cognition in Mice

The major goal of this project is to determine the effects of second hand smoke exposure on brain function.

New since last report:

Supplement to RF1 AG059088 (Banks, Raber) 08/01/20-07/31/21 1.98 calendar months
NIH/NIA (Raber lab directs)

Effects of apoE isoform, sex, and diet on insulin sensitivity in the brain

In this supplement, we will test the hypotheses that the ApoE phenotype will modulate the ability of SARS-CoV-2 to cross the BBB and to be sequestered by the lung and heart and that the ApoE phenotype and sex will modulate the behavioral and cognitive effects and cytokine storm induced by SARS-CoV-1.

Supplement to R21 AG065914 (Dana, Raber) 08/01/20-01/31/22 0.54 calendar months
NIH/NIA (Raber lab directs)

Neuronal activity modulation and age-related neurodegeneration

In this supplement, we will test the hypothesis that exposure to SARS-CoV VLPs will affect behavioral and cognitive performance more profoundly in htau than wild-type mice and more profoundly in males than females.

1R21AG065681 - 01A1 (Carbone) 09/15/20-08/31/2022
NIH (Raber lab directs)

0.38 calendar months

Identification of markers of early epigenetic dysregulation in Alzheimer's disease

In this proposal, a suitable human amyloid precursor protein knock in animal model will be used to characterize the exact timing of epigenetic dysregulation with respect to neuropathology in the AD brain, and to identify epigenetic marks and gene expression patterns most affected by disease onset and progression.

ADRG (Nielsen) 07/01/20-06/30/22
Stockholm Univ (Brightfocus) (Raber lab directs)

0.12 calendar months

An APOE-linked plasma profile and relevance to behavior and neurodegeneration

To determine the role of apoE from a humanized liver of E4 carriers on brain function.

Role: Collaborator

○ **What other organizations were involved as partners?**

Joseph Weiss, at Lifespan Healthcare/Brown University, served as the Other Significant Collaborator. Besides modified operations-related travel restrictions, Dr. Weiss would visit OHSU and the office of Dr. Raber twice a year to discuss this project. In addition, Drs. Weiss and Raber were continuously in contact over the period of performance of this project to discuss the latest data and adjustments needed based on modified operations or the results obtained.

8. Special Reporting Requirements

N/A

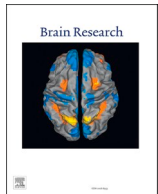
9. Appendices

Please see appended the Krenik et al. 2021 paper describing the short-term data, including the role of the parental origin.

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Role of the parental NF1 carrier in effects of pharmacological inhibition of anaplastic lymphoma kinase in Neurofibromatosis 1 mutant mice

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ABSTRACT

Neurofibromatosis type 1 (NF1), a genetically determined neurodevelopmental disorder and tumor syndrome, is associated with cognitive impairments, including in executive function and sleep-related problems. Consistent with the human data, NF1 heterozygous (Het) mice show impaired spatial learning and memory in the water maze and extinction of contextual fear memory. It is not clear whether neurological phenotypes might depend on the parental carrier. In this study, we compared the behavioral and cognitive performance of NF1 Het and wild-type litter mates with either the father (PC) or the mother (MC) as the NF1 carrier on a F1 C57BL/66/129SvJ background. The behavioral and cognitive phenotypes and responsiveness to Alk inhibition in heterozygous NF1 offspring depended on whether the parental carrier was maternal or paternal. Alk inhibition (20 mg/kg) increased activity levels during the dark period in NF1 Het PC, but not MC, mice. In the water maze, NF1 Het PC, but not MC, mice showed reduced cognitive flexibility and impaired ability to locate the third hidden platform location, which was improved by Alk inhibition (3.6 mg/kg). Consistent with reduced cognitive flexibility, WT, but not NF1, mice showed better performance in the third than second water maze probe trial. Finally, Alk inhibition (10 mg/kg) increased baseline activity of NF1 MC, but not PC, mice during the fear conditioning test. Thus, the effective dose depends on the behavioral test and genotype but indicates that in NF1 patients cognitive flexibility might be particularly sensitive to Alk inhibition.

1. Introduction

Neurofibromatosis type 1 (NF1), a genetically determined neurodevelopmental disorder and tumor syndrome, is caused by loss of function mutations in the neurofibromin gene (NF1) and associated with cognitive impairments, including problems with attention, visual perception, language, learning, attention, and executive function (Diffis-Andrews and Gutmann, 2013). These cognitive impairments are observed in the absence of tumors or macroscopic structural abnormalities in the central nervous system (Acosta et al., 2006; Hofman et al., 1994; Hyman et al., 2005; North et al., 1995; Rosser and Packer, 2003; Zoller et al., 1997). Sleep-related problems, including problems with initiating and maintaining sleep, arousal, sleep-wake transition and Hyperhidrosis have been reported as well (Johnson et al., 2005; Leschziner et al., 2013; Licit et al., 2013). Consistent with the human data, NF1 heterozygous mutant mice show impaired hippocampus-

dependent spatial learning and memory in the water maze (Costa et al., 2001; Costa and Silva, 2002; Silva et al., 1997; Weiss et al., 2017b) and impaired hippocampus-dependent extinction of contextual fear memory (Weiss et al., 2017b). In addition, female and male flies lacking Nf1 exhibit nocturnal hyperactivity, an increase in daytime sleep and a decrease in nighttime sleep, and impaired sleep consolidation (Bai and Sehgal, 2015; Williams et al., 2001).

Neuronal dysfunction in NF1 patients is sex-dependent, with female patients with NF1-associated optic glioma being twice as likely to undergo brain MRI for visual symptoms and three times more likely to require treatment for visual decline than male patients (Diggs-Andrews et al., 2014). In contrast, microglia in NF1 patients show sexually dimorphic cyclic AMP-dependent purinergic impairments, with microglia in males but not females being affected (Elmadany et al., 2020). Glioma risk in NF1 patients is also sex-dependent, with increased risk in females and reduced risk in males associated with genetic

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polymorphisms in adenylate cyclase 8 (Warrington and al., 2015). While there is no parent-of-origin effect for optic gliomas in NF1 patients (Johnson, 2012), it is not clear whether neurological phenotypes might be more pronounced in heterozygous offspring from a maternal compared to a paternal carrier. In case there would be a parent-of-origin effect in NF1 patient, that could be of clinical relevance for both diagnostic purposes and for consideration of treatment strategies.

NF1 is a negative regulator of the small GTPase, Ras, a proto-oncogene and activator of the mitogen activated protein kinase (MAP kinase) cascade (Ballester et al., 1990; Cawthon et al., 1990; Martin et al., 1990; Xu et al., 1990a; Xu et al., 1990b) that is often activated by receptor tyrosine kinases, and binds GTP. Neurofibromin, which has been reported to function in the hippocampus (Costa and Silva, 2002), is a Ras-GTPase activating protein that catalyzes the conversion of active GTP-Ras to inactive GDP-Ras, lowers the activation energy of RAS's intrinsic GTPase activity, and accelerates its auto-inactivation.

Drosophila (Gouzi et al., 2011) (Walker and Bernards, 2014) and subsequent mouse (Weiss et al., 2017b; Weiss et al., 2012) studies support a genetic interaction between NF1 and Alk, a receptor tyrosine kinase and activator of the Ras-MAP kinase cascade (Gouzi et al., 2011; Walker et al., 2013; Walker and Bernards, 2014) and an oncogene involved in anaplastic lymphomas (Morris et al., 1994), neuroblastoma, non-small cell lung cancer, inflammatory myofibroblastic sarcoma (Chen et al., 2008; George et al., 2008; Mosse et al., 2008; Sirvent et al., 2001; Soda et al., 2007), and likely also in some renal cell, breast, esophageal, colonic, glial and thyroid cancers (Palmer et al., 2009). Orally active Alk inhibitors developed for human tumors (Butrynski et al., 2010; Kodama et al., 2014; Kwak et al., 2010; Metro et al., 2016) have been used to demonstrate that pharmacological inhibition of Alk can rescue cognitive impairments in heterozygous NF1 mutant flies (Gouzi et al., 2011) and spatial memory retention in heterozygous NF1 mice on a genetically diverse background at only one dose (Weiss et al., 2017a). In this study, we compared the behavioral and cognitive performance of NF1 heterozygous and wild-type litter mates with either the father (PC: B6 Paternal NF1 Carrier) or the mother (MC: C57BL/6J (B6) Maternal NF1 Carrier) as the NF1 carrier. The NF1 carrier was always on a B6 background, and the non-NF1 carrier was always on a 129/SvJ (SvJ) background. We assessed the ability of different doses of the Alk inhibitor CH5424802 (Alectinib) to modulate behavioral and cognitive performance.

2. Results

2.1. CH5424802 increases home cage activity levels during the dark period in PC, but not MC, HET mice

Please see Table 1 for the breakdown of the behaviorally tested mice. For enhanced clarity, the statistical analyses are summarized in Table 2 and those that reached significance are indicated in bold. To assess differences in circadian activity levels between parental carriers and genotypes, we first analyzed only the mice that received vehicle treatment using a univariate ANOVA with genotype, sex, and parental carrier

Table 1

Breakdown of the behaviorally tested mice. Numbers in parenthesis indicate the subset of mice that received home cage activity monitoring.

PC	0 mg/kg	3.6 mg/kg	10 mg/kg	20 mg/kg
HET F	10 (6)	10 (7)	10 (9)	10 (10)
HET M	10 (9)	10 (7)	11 (10)	10 (8)
WT F	6 (4)	6 (6)	6 (6)	6 (6)
WT M	9 (8)	11 (9)	8 (6)	10 (9)
MC	0 mg/kg	3.6 mg/kg	10 mg/kg	20 mg/kg
HET F	6 (5)	7 (7)	7 (5)	7 (3)
HET M	9 (4)	11 (9)	9 (9)	10 (7)
WT F	9 (7)	10 (8)	9 (6)	10 (10)
WT M	10 (9)	8 (7)	10 (9)	9 (8)

as the between group factors. In the vehicle-treated animals, there was no effect of genotype on activity levels in either the dark ($F(1, 51) = 0.115$, $p = 0.737$) or the light period ($F(1, 44) = 0.200$, $p = 0.657$). However there was an effect of sex ($F(1, 51) = 6.373$, $p = 0.015$) and a sex $\times$ parental carrier interaction ($F(1, 51) = 1.950$, $p = 0.039$) in the dark cycle. When the analysis was split by parental carrier, sex remained significant only for the maternal carrier ($F(1, 24) = 8.004$, $p = 0.010$, Supplementary Fig. 1A), with the females being more active than the males. In the ratio of activity in the dark period to activity in the light cycle, parental carrier ($F(1, 51) = 2.567$, $p = 0.042$) and sex ($F(1, 51) = 6.726$, $p = 0.013$) was also significant. When split by parental carrier, there was no effect of genotype in either the paternal carriers ($F(1, 26) = 2.109$, $p = 0.160$) or the maternal carriers ($F(1, 24) = 0.577$, $p = 0.456$), but there was an effect of sex in the maternal carriers ($F(1, 24) = 11.316$, $p = 0.003$, Supplementary Fig. 1B), as was observed when only the dark cycle was analyzed.

Next we assessed the effect of the Alk inhibitor CH5424802 on circadian activity levels using sex and treatment as the between group factors. Because of the observed differences between parental carriers in the vehicle-treated mice and we a priori expected genotype differences in the treatment response, the analysis was split by parental carrier and genotype. For HET-treated PC mice, there were effects of sex ($F(1, 64) = 4.136$, $p = 0.047$, Supplementary Fig. 1C) and treatment ($F(3, 64) = 6.323$, $p = 0.001$) in the dark cycle. Post Hoc analysis revealed that mice receiving the 20 mg/kg dose were more active than the vehicle-treated PC mice ($p = 0.003$, Dunnett's post hoc test, Fig. 1A). There was no difference between vehicle-treated HET and WT PC mice. There was no effect of treatment on the ratio of dark-light activity ($F(3, 64) = 3.244$, $p = 0.234$, Fig. 1C). In PC WT littermates, there was no effect of treatment in the dark cycle ($F(3, 53) = 1.009$, $p = 0.397$, Fig. 1A), no effect of the 20 mg/kg dose on activity in the dark ($p = 0.401$), or on the ratio of dark-light activity ($F(3, 53) = 0.929$, $p = 0.435$, Fig. 1C).

For HET MC mice, there was an effect of sex in both the dark cycle ($F(1, 48) = 10.027$, $p = 0.003$, Supplementary Fig. 1D) and on the ratio of dark to light ($F(1, 48) = 9.710$, $p = 0.003$, Supplementary Fig. 1F), with the females having higher scores than the males. In contrast to the HET PC mice, there was no effect of treatment in either the dark cycle ($F(3, 48) = 0.486$, $p = 0.694$, Fig. 1B) or on the dark-light activity ratio ($F(3, 48) = 1.444$, $p = 0.244$, Fig. 1D) in HET MC mice. Thus, the effect of Alk inhibition on activity levels in HET mice during the dark period was also parental carrier-dependent.

Much like in their HET littermates, in WT MC mice there was an effect of sex in both the dark cycle ($F(1, 63) = 4.460$, $p = 0.039$, Supplementary Fig. 1E) and on the dark-light activity ratio ($F(1, 63) = 4.225$, $p = 0.045$, Supplementary Fig. 1G). Females were more active than their male littermates. In WT MC mice, there was no effect of treatment on activity in the dark cycle ($F(3, 63) = 1.191$, $p = 0.321$, Fig. 1B). However, while there was no overall effect of treatment on the dark-light activity ratio ($F(3, 63) = 2.008$, $p = 0.123$), WT MC mice treated with the 3.6 mg/kg treatment showed a higher dark-light activity ratio than vehicle-treated WT MC mice (Fig. 1D).

2.2. HET mice are impaired in locating the second and third hidden platform location in the water maze

Next, we assessed spatial learning and memory in the water maze (see Fig. 2A and 3A for distinct hidden platform locations). First, we analyzed the vehicle-treated mice (Fig. 2B and C). The data for visible and hidden platform sessions were analyzed separately using a repeated measure ANOVA using sex, genotype, and parental carrier as between group factors. When cumulative distance to the target was used as the measure of performance, there was an effect of session in both the visible ($F(1, 55) = 46.447$, $p < 0.001$) and hidden ($F(5.403, 297.148) = 9.173$, $p < 0.001$) platform sessions, indicating that the mice improved their performance with training. During the visible platform sessions, there

Table 2
Summary of statistical results.

Analyses	Measures	Effects	Direction of effects
Vehicle-treated mice	Dark cycle	Genotype: $F(1, 51) = 0.115$, $p = 0.737$	
	Light cycle	Genotype: $F(1, 44) = 0.200$, $p = 0.657$	
	Dark cycle	Sex: $F(1, 51) = 6.373$, $p = 0.015$	
Analysis split by parental carrier	Dark cycle	Sex $\times$ Parental carrier interaction: $F(1, 51) = 1.950$, $p = 0.039$ MC; Sex: $F(1, 24) = 8.004$, $p = 0.010$	F > M
	Ratio dark period/light cycle	PC: $F(1, 51) = 2.567$, $p = 0.042$; sex $F(1, 51) = 6.726$, $p = 0.013$	
Analysis split by parental carrier	Ratio dark period/light cycle	Genotype: $F(1, 26) = 2.109$, $p = 0.160$; MC: $F(1, 24) = 0.577$, $p = 0.456$	
	Ratio dark period/light cycle	Sex in MC: $F(1, 24) = 11.316$, $p = 0.003$	
Alk inhibitor	Dark cycle	HET-treated PC mice; Sex: $F(1, 64) = 4.136$, $p = 0.047$; Treatment: $F(3, 64) = 6.323$, $p = 0.001$; WT-treated PC mice; Treatment: $F(3, 53) = 1.009$, $p = 0.397$ Treatment: $F(3, 64) = 3.244$, $p = 0.234$; WT-treated PC mice; Treatment: $F(3, 53) = 0.929$, $p = 0.435$	HET PC mice receiving the 20 mg/kg dose > vehicle-treated PC mice
	Ratio dark period/light cycle	Treatment: $F(3, 64) = 3.244$, $p = 0.234$; WT-treated PC mice; Treatment: $F(3, 53) = 0.929$, $p = 0.435$	
	Dark cycle	HET-treated MC mice; Sex: $F(1, 48) = 10.027$, $p = 0.003$; Treatment: $F(3, 48) = 0.486$, $p = 0.694$	F > M
	Ratio dark period/light cycle	HET-treated MC mice; Sex: $F(1, 48) = 9.710$, $p = 0.003$; Treatment: $F(3, 48) = 1.444$, $p = 0.244$	F > M
	Dark period	WT MC mice; Sex: $F(1, 63) = 4.460$, $p = 0.039$	F > M
	Ratio dark period/light cycle	WT MC mice; Sex: $F(1, 63) = 4.225$, $p = 0.045$	F > M
	Dark period	WT MC Mice; Treatment: $F(3, 63) = 1.191$, $p = 0.321$	
	Ratio dark period/light cycle	WT MC Mice; Overall treatment: $F(3, 63) = 2.008$, $p = 0.123$	WT MC mice treated with the 3.6 mg/kg > vehicle-treated WT MC mice
	Cumulative distance to the visible target platform	Vehicle-treated mice; effect of session: $F(1, 55) = 46.447$, $p < 0.001$; trend toward a session $\times$ sex $\times$ genotype interaction ($F(1, 55) = 3.957$, $p = 0.052$; no main effect of sex ($F(1, 55) = 0.082$, $p = 0.776$), genotype ($F(1, 55) = 0.466$, $p = 0.498$), or parental carrier ($F(1, 55) = 2.016$, $p = 0.161$; when the sexes were analyzed separately: no effect of genotype in females: $F(1, 27) = 0.615$, $p = 0.440$ or males $F(1, 33) = 0.079$, $p = 0.781$; no interaction with session and genotype in females: $F(1, 27) = 1.034$, $p = 0.318$; a trend toward a session $\times$ genotype interaction in males $F(1, 33) = 3.024$, $p = 0.091$ Vehicle-treated mice; effect of session: $F(5.403, 297.148) = 9.173$, $p < 0.001$; session $\times$ genotype interaction: $F(5.403, 297.148) = 0.641$; trend towards an effect of genotype during H2: $F(1, 60) = 3.347$, $p = 0.072$; effect of genotype during H3: $p = 0.011$; trend toward a session $\times$ parental carrier interaction during H3: $F(1,60) = 6.934$, $p = 0.063$	
Analysis split by parental carrier	Cumulative distance to the hidden target platform	Vehicle-treated mice; trend toward an effect of genotype during H2 in PC mice: $F(1, 31) = 3.958$, $p = 0.056$, but not in MC mice: ($F(1, 29) = 0.508$, $p = 0.482$; in H3, effect of genotype in PC mice: $F(1, 31) = 4.753$, $p = 0.037$, but not in MC mice: $F(1, 29) = 2.537$, $p = 0.112$	Phenotype in the water maze more pronounced in PC than MC mice
Analysis split by genotype		effect of session in HET mice: $F(4.234, 131.256) = 4.036$, $p = 0.003$; effect of session in WT mice: $F(5.088, 147.544) = 8.853$, $p < 0.001$ effect of session: $F(1, 60) = 36.578$, $p < 0.001$; session $\times$ sex $\times$ genotype interaction ($F(1, 60) = 5.409$, $p = 0.023$; effects of sex: $F(1, 60) = 0.011$, $p = 0.916$; effect of genotype: $F(1, 60) = 0.432$; effect of parental carrier: $F(1, 60) = 2.510$, $p = 0.118$; trend for a sex $\times$ genotype interaction during the first session: ($F(1, 60) = 3.512$, $p = 0.066$; no effect of genotype in females: $F(1, 30) = 1.944$, $p = 0.175$; no effect of genotype in males: $F(1, 36) = 1.506$, $p = 0.228$; sexes analyzed separately; males: session $\times$ genotype interaction: $F(1, 33) = 4.280$, $p = 0.046$; effect of genotype: $F(1, 33) = 0.225$, $p = 0.639$; females; effect of genotype: ($F(1, 27) = 0.440$, $p = 0.513$ effect of session: $F(5.698, 341.882) = 8.774$, $p < 0.001$; effects of sex: $F(1, 60) = 0.054$, $p = 0.817$; effect of genotype: $F(1, 60) = 1.676$, $p = 0.200$; effect of parental carrier: $F(1, 60) = 1.062$, $p = 0.307$ HET PC mice; effect of session: $F(1, 73) = 86.38$, $p < 0.001$; sex $\times$ treatment interaction: $F(3,73) = 3.073$, $p = 0.033$; effect of sex: $F(1,73) = 0.037$, $p = 0.849$; effect of treatment: $F(3,73) = 0.383$, $p = 0.766$; sexes analyzed separately; males; effect of treatment: $F(3, 37) = 2.889$, $p = 0.048$; females; effect of treatment: $F(3, 36) = 0.851$, $p = 0.475$	
Alk inhibitor	Cumulative distance to the visible target platform		Males that received the 3.6 mg/kg treatment > males that received the 10 mg/kg treatment

(continued on next page)

Table 2 (continued)

Analyses	Measures	Effects	Direction of effects
	Cumulative distance to the hidden target platform	HET PC mice; effect of session: F(5.177, 377.900) = 7.902, $p < 0.001$; session $\times$ treatment interaction: F(15.530, 377.900) = 1.841, $p = 0.026$; effect of sex: F(1,73) = 0.561, $p = 0.456$; effect of treatment: F(3,73) = 0.081, $p = 0.970$; each day analyzed separately; effect of treatment on day 4: F(1,73) = 3.037, $p = 0.034$	Mice that received the 3.6 mg/kg treatment > mice that received the vehicle treatment during training to locate the third hidden platform location
	Cumulative distance to the visible target platform	WT PC mice; effect of session: F(1,54) = 52.318, $p < 0.001$; trend toward a session $\times$ treatment interaction: F(3,54) = 2.633, $p = 0.059$; effect of sex: F(1, 54) = 0.242, $p = 0.624$; effect of treatment: F(3,54) = 1.498, $p = 0.226$; sessions analyzed separately; trend toward an effect of treatment during session 1: F(3, 61) = 2.333, $p = 0.084$	
	Cumulative distance to the hidden target platform	WT PC mice; effect of session: F(4.712, 254.451) = 7.619, $p < 0.001$	
	Cumulative distance to the visible target platform	HET MC mice; effect of session: F(1,58) = 73.176, $p < 0.001$; effect of sex: F(1,58) = 0.611, $p = 0.438$; effect of treatment: F(3,58) = 0.536, $p = 0.660$; session $\times$ sex $\times$ treatment interaction: F(3,58) = 3.735, $p = 0.016$; trend towards a session $\times$ treatment interaction: F(3,58) = 2.220, $p = 0.095$; sexes analyzed separately; males; session $\times$ treatment interaction: F(3, 35) = 4.988, $p = 0.006$; effect of treatment: F(3, 35) = 0.442, $p = 0.724$; each session analyzed separately; effect of dose in session 1: F(3, 38) = 3.660, $p = 0.021$; females; effect of treatment: F(3, 23) = 2.096, $p = 0.129$	Males that received the 20 mg/kg dose < vehicle treated littermates
	Cumulative distance to the hidden target platform	HET MC mice; effect of session: F(4.976, 288.615) = 15.563, $p < 0.001$; effect of sex: F(1,58) = 0.322, $p = 0.573$; effects of treatment: F(3,58) = 0.120, $p = 0.948$	
	Cumulative distance to the visible target platform	WT MC mice; effect of session: F(1, 66) = 71.579, $p < 0.001$; session $\times$ sex $\times$ treatment interaction: F(3, 66) = 3.327, $p = 0.025$; effect of sex: F(1, 66) = 0.126, $p = 0.724$; effect of treatment: F(3, 66) = 0.212, $p = 0.888$; sexes analyzed separately; females: trend toward a session $\times$ treatment interaction: F(3, 34) = 2.768, $p = 0.057$	
	Cumulative distance to the hidden target platform	WT MC mice; effect of session: F(5.508, 357.996) = 15.627, $p < 0.001$; effect of sex: F(1, 65) = 0.072, $p = 0.789$; effect of treatment: F(3, 65) = 0.122, $p = 0.947$	
	Latency to the visible target platform	HET PC mice; effect of session: F(1, 73) = 61.740, $p < 0.001$; sex $\times$ treatment interaction: F(3, 73) = 3.084, $p = 0.033$; effect of sex: F(1, 73) = 0.081, $p = 0.777$; effect of treatment: F(3, 73) = 0.545, $p = 0.653$; analysis split by sex; males; effect of treatment: F(3, 37) = 2.893, $p = 0.048$; females; effect of treatment: F(3, 36) = 1.015, $p = 0.397$	Males that received the 3.6 mg/kg treatment > males that received the 10 mg/kg treatment
	Latency to the hidden target platform	HET PC mice; effect of session: F(5.639, 411.650) = 7.161, $p < 0.001$; trend toward a session $\times$ treatment interaction: F(16.917, 411.650) = 1.487, $p = 0.096$; effect of sex: F(1,73) = 1.203, $p = 0.276$; effect of treatment: F(3, 73) = 0.160, $p = 0.923$	
	Latency to the visible target platform	WT PC mice; effect of session: F(1, 54) = 35.029, $p < 0.001$; effect of sex: F(1,54) = 0.376, $p = 0.542$; effect of treatment: F(3,54) = 1.126, $p = 0.347$	
	Latency to the hidden target platform	WT PC mice; effect of session: F(5.480, 295.895) = 6.998, $p < 0.001$; trend towards an effect of sex: F(1, 54) = 2.870, $p = 0.096$; effect of treatment: F(3, 54) = 1.887, $p = 0.143$	
	Latency to the visible target platform	HET MC mice; effect of session: F(1, 58) = 65.749, $p < 0.001$; session $\times$ sex $\times$ treatment interaction: F(3, 58) = 2.927, $p = 0.041$; effect of sex: F(1, 58) = 0.706, $p = 0.404$; effect of treatment: F(3, 58) = 0.413, $p = 0.744$; analysis split by sex; males: session $\times$ treatment interaction: F(3, 35) = 3.618, $p = 0.022$; effect of treatment: F(3, 35) = 0.239, $p = 0.869$; sessions analyzed separately; trend toward an effect of treatment in session 1: F(3, 38) = 2.560, $p = 0.071$	
	Latency to the hidden target platform	HET MC mice; effect of session: F(5.353, 310.454) = 14.562, $p < 0.001$; effect of sex: F(1, 65) = 1.093, $p = 0.300$; effect of treatment: F(3, 65) = 0.361, $p = 0.781$	
	Latency to the visible target platform	WT MC mice; effect of session: F(1, 65) = 68.491, $p < 0.001$; session $\times$ sex $\times$ treatment interaction: F(3, 65) = 3.699, $p = 0.016$; effect of sex: F(1, 65) = 1.093, $p = 0.300$; effect of treatment: F(3, 65) = 0.361, $p = 0.781$; analysis split by sex; females; trend towards a session $\times$ treatment interaction: F(3, 34) = 2.771, $p = 0.056$	
	Latency to the hidden target platform	WT MC mice; effect of session: F(5.759, 374.315) = 11.789, $p < 0.001$	
	Cumulative distance to the target location in the probe trials	Vehicle-treated mice; effect of probe trial: F(2, 116) = 3.119, $p = 0.048$; probe $\times$ genotype interaction: F(2, 116) = 4.602, $p = 0.012$; effect of sex: F(1, 58) = 0.001, $p = 0.973$; effect of genotype: F(1, 58) = 2.272, $p = 0.137$; effect of parental carrier: F(1, 58) = 0.950, $p = 0.0334$ WT mice; effect of probe trial: F(2, 56) = 6.080, $p = 0.004$	

(continued on next page)

Table 2 (continued)

Analyses	Measures	Effects	Direction of effects
Analysis split by genotype	Cumulative distance to the target location in the probe trials		Better performance of WT mice in the third than the second probe trial
Analysis split by parental carrier	Cumulative distance to the target location in the probe trials	PC HET mice; effect of probe trial: $F(2, 138) = 3.773$, $p = 0.025$; effect of treatment: $F(1, 69) = 1.452$, $p = 0.235$	
Analysis split by parental carrier	Cumulative distance to the target location in the probe trials	PC WT mice; trend towards an effect of probe trial: $F(2, 104) = 3.068$, $p = 0.051$; trend towards a probe trial $\times$ treatment interaction: $F(6, 104) = 1.853$, $p = 0.096$; effect of treatment: $F(3, 52) = 0.488$, $p = 0.692$	
Analysis split by parental carrier	Cumulative distance to the target location in the probe trials	MC HET; effect of probe trial: $F(2, 116) = 0.993$, $p = 0.373$; effect of treatment: $F(3, 58) = 0.791$, $p = 0.504$	
Analysis split by parental carrier	Cumulative distance to the target location in the probe trials	MC WT mice; probe trial $\times$ treatment interaction ($F(6, 130) = 2.680$, $p = 0.017$; effect of probe trial: $F(2, 130) = 2.972$, $p = 0.055$; effect of treatment: $F(3, 65) = 2.099$, $p = 0.109$	
	Baseline motion during fear conditioning test	Effect of genotype: $F(1, 68) = 4.524$, $p = 0.037$	
	Baseline freezing during fear conditioning test	Effect of genotype: $F(1, 68) = 4.027$, $p = 0.049$	
	Motion during shock in fear conditioning test	Shock $\times$ sex $\times$ genotype interaction: $F(1, 61) = 5.036$, $p = 0.028$; effect of sex: $F(1, 61) = 8.946$, $p = 0.004$; effect of parental carrier: $F(1, 61) = 18.739$, $p < 0.001$; sex $\times$ genotype interaction ($F(1, 61) = 4.940$, $p = 0.030$; analysis split by sex; males; males effect of parental carrier: $F(1, 34) = 13.797$, $p = 0.001$; females; shock $\times$ parental carrier interaction: $F(1, 27) = 2.945$, $p = 0.098$; effect of genotype: $F(1, 27) = 8.395$, $p = 0.007$; effect of parental carrier: $F(1, 27) = 6.348$, $p = 0.018$; trend towards a shock $\times$ genotype interaction: $F(1, 27) = 3.065$, $p = 0.091$; analyzing each shock separately; shock 1; effect of sex: $F(1, 68) = 4.724$, $p = 0.034$; effect of parental carrier: $F(1, 68) = 22.203$, $p < 0.001$; sex $\times$ genotype interaction: $F(1, 68) = 10.131$, $p = 0.002$; trend towards an effect of genotype: $F(1, 68) = 3.235$, $p = 0.077$.	
	Freezing during the tones in the fear conditioning test	Effect of sex: $F(1, 61) = 2.486$, $p = 0.120$; effect of genotype: $F(1, 61) = 2.076$, $p = 0.155$; effect of parental carrier: $F(1, 61) = 2.069$, $p = 0.155$	
	Baseline motion during fear conditioning test	PC HET mice; effect of treatment: $F(3, 80) = 0.230$, $p = 0.875$	
	Baseline motion during fear conditioning test	PC WT mice; effect of treatment: $F(3, 61) = 1.278$, $p = 0.291$	
	Baseline motion during fear conditioning test	MC HET mice; effect of treatment: $F(3, 65) = 4.016$, $p = 0.012$	MC HET mice treated with the 10 mg/kg dose > than those treated with vehicle
	Motion during shock in fear conditioning test	PC HET mice; effects of treatment: $F(3, 73) = 0.060$, $p = 0.981$; trend toward a sex effect: $F(1, 73) = 3.694$, $p = 0.059$	
	Motion during shock in fear conditioning test	PC WT mice; effect of treatment: $F(3, 54) =$, $p = 0.008$; trend towards an effect of sex: $F(1, 54) = 3.510$, $p = 0.066$	PC WT mice that received the 10 mg/kg dose < than the 3.6 mg/kg and vehicle-treated PC WT mice
	Motion during shock in fear conditioning test	MC HET mice; effect of sex: $F(1, 58) = 8.441$, $p = 0.005$; treatment $\times$ shock interaction: $F(3, 58) = 3.181$, $p = 0.031$; effect of treatment: $F(3, 58) = 1.646$, $p = 0.189$; analysis of each shock separately; effect of treatment during shock 2: $F(3, 65) = 2.794$, $p = 0.048$	
	Extinction of contextual fear	Vehicle-treated mice; effect of day: $F(5.348, 326.216) = 25.297$, $p < 0.001$; a day $\times$ parental carrier interaction: $F(5.348, 326.216) = 6.749$, $p < 0.001$; trend towards a day $\times$ parental carrier $\times$ sex interaction: $F(5.348, 326.216) = 2.030$, $p = 0.069$; effect of sex: $F(1, 61) = 7.139$, $p = 0.010$; effect of genotype: $F(1, 61) = 0.500$, $p = 0.482$; effect of parental carrier: $F(1, 61) = 1.454$, $p = 0.233$	
	Extinction of contextual fear	PC HET mice; effect of day: $F(6.009, 438.638) = 15.833$, $p < 0.001$; effect of sex: $F(1, 73) = 16.716$, $p < 0.001$; effect of treatment: $F(1, 73) = 1.480$, $p = 0.227$	
	Extinction of contextual fear	PC WT mice; effect of day: $F(5.426, 292.982) = 16.815$, $p < 0.001$	
	Extinction of contextual fear	MC HET mice; MC HET mice; effect of day: $F(4.791, 277.870) = 35.641$, $p < 0.001$; trend towards a day $\times$ sex interaction: $F(4.791, 277.870) = 2.197$, $p = 0.058$; effect of sex: $F(1, 58) = 6.058$, $p = 0.017$; effect of treatment: $F(3, 58) = 1.686$, $p = 0.180$	
	Extinction of contextual fear	MC WT mice; effect of day: $F(5.230, 345.173) = 55.312$, $p < 0.001$; trend toward a day $\times$ treatment interaction: $F(5.230, 345.173) = 1.623$, $p = 0.062$; trend towards an effect of sex: $F(1, 66) = 3.192$, $p = 0.079$	

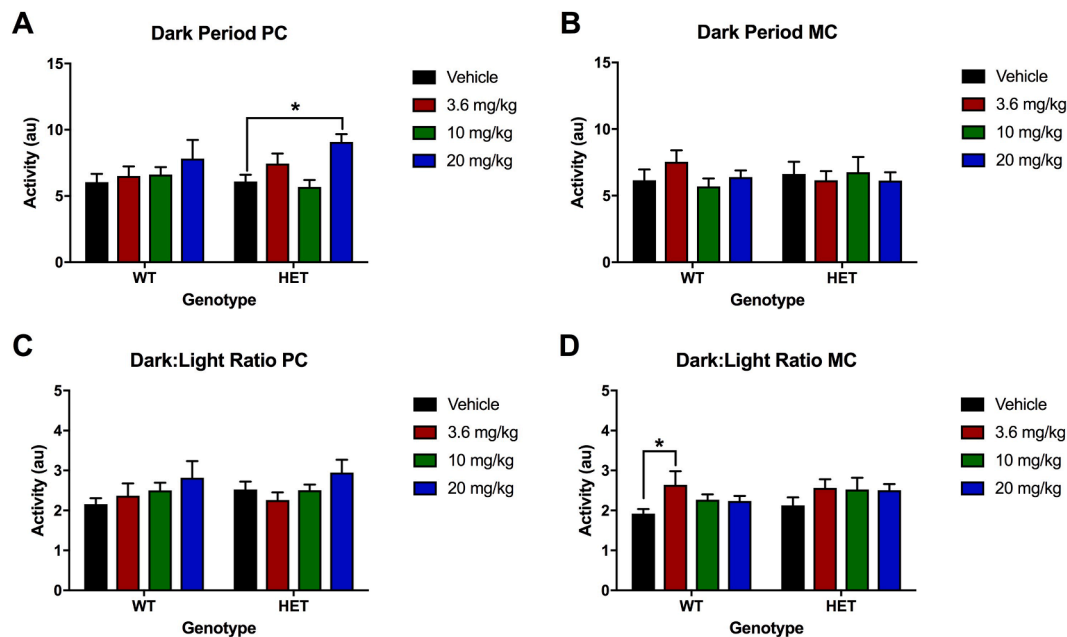


Fig. 1. Circadian activity levels. **A.** Activity levels of PC WT and HET mice during the dark period. PC HET mice that received the 20 mg/kg dose were more active than those that received vehicle ($p = 0.003$, Dunnett's post hoc). **B.** Similar activity levels of MC WT and HET mice during the dark period. **C.** There was no significant difference in the ratio of activity during the dark period compared to the light period for PC mice. **D.** In MC WT mice, the dark-light ratio was larger in mice that received the 3.6 mg/kg dose than those that received vehicle ($p = 0.045$, Dunnett's).

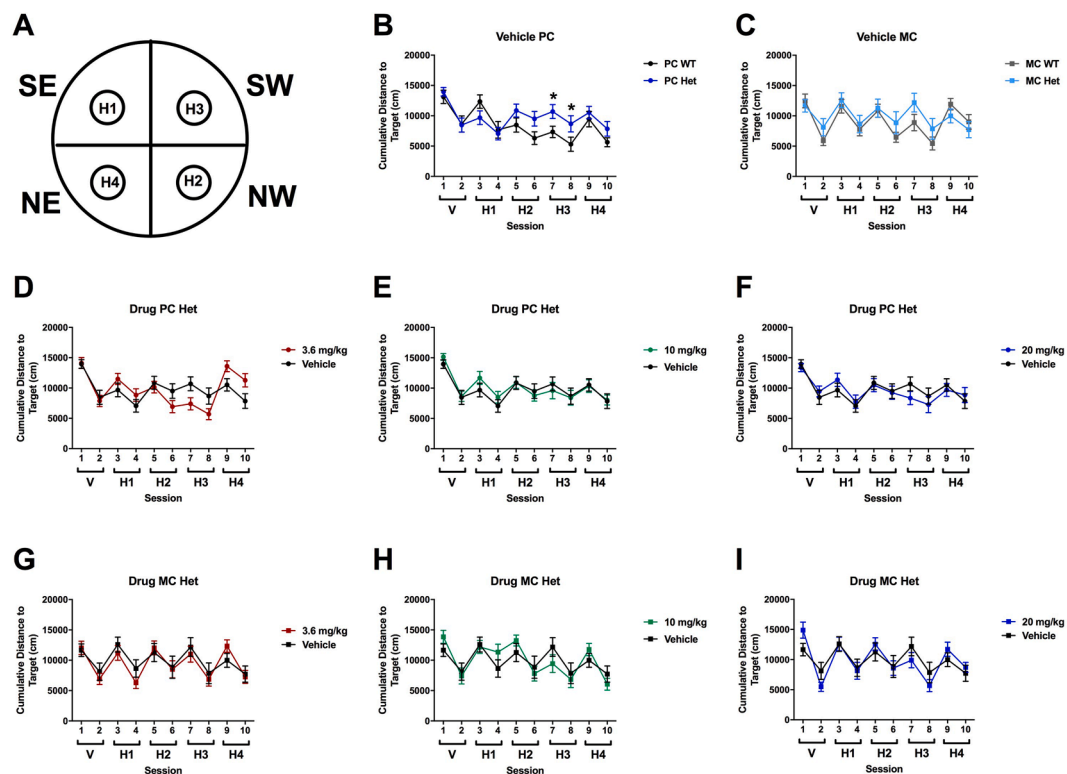


Fig. 2. Spatial learning and memory in the water maze using cumulative distance to the platform as performance measure. **A.** Mice were trained to locate two visible platform locations (Visible Location 1: session 1; Visible Platform 2: session 2) and four hidden platform locations (Hidden Location 1: sessions 3–4; Hidden Location 2: sessions 5–6; Hidden Location 3: sessions 7–8; Hidden Location 4: session 9–10). The four hidden platform locations are illustrated. **B–C** Performance in the water maze was not significantly different between genotypes or parental carriers. **D–F.** In PC HET mice, there was a sex $\times$ treatment interaction. **G–I** In MC HET mice, there was no significant effect of treatment on performance in the water maze.

was a trend towards a session $\times$ sex $\times$ genotype interaction ($F(1, 55) = 3.957$, $p = 0.052$), but there was no main effect of sex ($F(1, 55) = 0.082$, $p = 0.776$), genotype ($F(1, 55) = 0.466$, $p = 0.498$), or parental carrier ($F(1, 55) = 2.016$, $p = 0.161$). When the sexes were analyzed separately, there was still no effect of genotype in the females ($F(1, 27) = 0.615$, $p = 0.440$) or the males ($F(1, 33) = 0.079$, $p = 0.781$), nor was there an interaction between session and genotype in the females ($F(1, 27) = 1.034$, $p = 0.318$). In the males, there was a trend towards a session $\times$ genotype interaction ($F(1, 33) = 3.024$, $p = 0.091$) but that did not reach significance.

During the hidden platform sessions, there was a session $\times$ genotype interaction ($F(5.403, 297.148) = 0.641$, $p = 0.017$, Fig. 2B, C). During the training to locate the second hidden platform location (H2), there was a trend towards an effect of genotype ($F(1, 60) = 3.347$, $p = 0.072$). During the training to locate the third hidden platform location (H3), there was an effect of genotype ($p = 0.011$) and a trend toward a session $\times$ parental carrier interaction ($F(1, 60) = 6.934$, $p = 0.063$). When the performance of the PC and MC mice was analyzed separately, there was still a trend toward an effect of genotype during the training to locate the second platform location in the PC ($F(1, 31) = 3.958$, $p = 0.056$), but not in the MC ($F(1, 29) = 0.508$, $p = 0.482$), mice. During the training to locate the third hidden platform location, there was an effect of genotype in the PC ($F(1, 31) = 4.753$, $p = 0.037$), but not the MC ($F(1, 29) = 2.537$, $p = 0.112$), mice. Overall, the cognitive phenotype seemed more pronounced in PC than MC mice. The HET mice had a hard time learning a new hidden platform location, suggesting an impairment in executive function. There were no main effects of sex ($F(1, 55) = 0.145$, $p = 0.705$), genotype ($F(1, 55) = 3.312$, $p = 0.704$), or parental carrier ($F(1, 55) = 1.394$, $p = 0.243$). When the genotypes were analyzed separately, there was an effect of session in both the HET ($F(4.234, 131.256) = 4.036$, $p = 0.003$) and WT mice ($F(5.088, 147.544) = 8.853$, $p < 0.001$), indicating that they both improved their performance with training.

Latency to reach the target platform was also used as a measure of performance (Fig. 3B and C). Similar to the cumulative distance analysis, there was an effect of session during both the visible ($F(1, 60) = 36.578$, $p < 0.001$) and hidden ($F(5.698, 341.882) = 8.774$, $p < 0.001$) platform sessions. During the visible platform sessions, there was a session $\times$ sex $\times$ genotype interaction ($F(1, 60) = 5.409$, $p = 0.023$). There were no main effects of sex ($F(1, 60) = 0.011$, $p = 0.916$), genotype ($F(1, 60) = 0.432$), or parental carrier ($F(1, 60) = 2.510$, $p = 0.118$). When sessions were analyzed separately, there was a trend towards a sex $\times$ genotype interaction during the first session only ($F(1, 60) = 3.512$, $p = 0.066$). The analysis was then split by sex, but there was no effect of genotype in either the females ($F(1, 30) = 1.944$, $p = 0.175$) or the males ($F(1, 36) = 1.506$, $p = 0.228$). Based on the sex $\times$ genotype interaction, the sexes were analyzed separately. In the males, there was a session $\times$ genotype interaction ($F(1, 33) = 4.280$, $p = 0.046$), but there was no main effect of genotype ($F(1, 33) = 0.225$, $p = 0.639$) during the visible platform sessions. In the females, there was no effect of genotype on performance during the visible platform sessions ($F(1, 27) = 0.440$, $p = 0.513$). There were also no main effects of sex ($F(1, 60) = 0.054$, $p = 0.817$), genotype ($F(1, 60) = 1.676$, $p = 0.200$), or parental carrier ($F(1, 60) = 1.062$, $p = 0.307$) during the hidden platform sessions.

2.3. CH5424802 improves performance of HET PC mice in locating the second and third hidden platform location in the water maze

We next assessed the effect of CH5424802 on water maze performance using cumulative distance to the platform as performance measure (Fig. 2). In HET PC mice (Fig. 2D–F), there was an effect of session during both the visible ($F(1, 73) = 86.38$, $p < 0.001$) and hidden ($F(5.177, 377.900) = 7.902$, $p < 0.001$) platform sessions. For the visible platform sessions, there was no main effect of sex ($F(1, 73) = 0.037$, $p = 0.849$) or treatment ($F(3, 73) = 0.383$, $p = 0.766$) but there was a

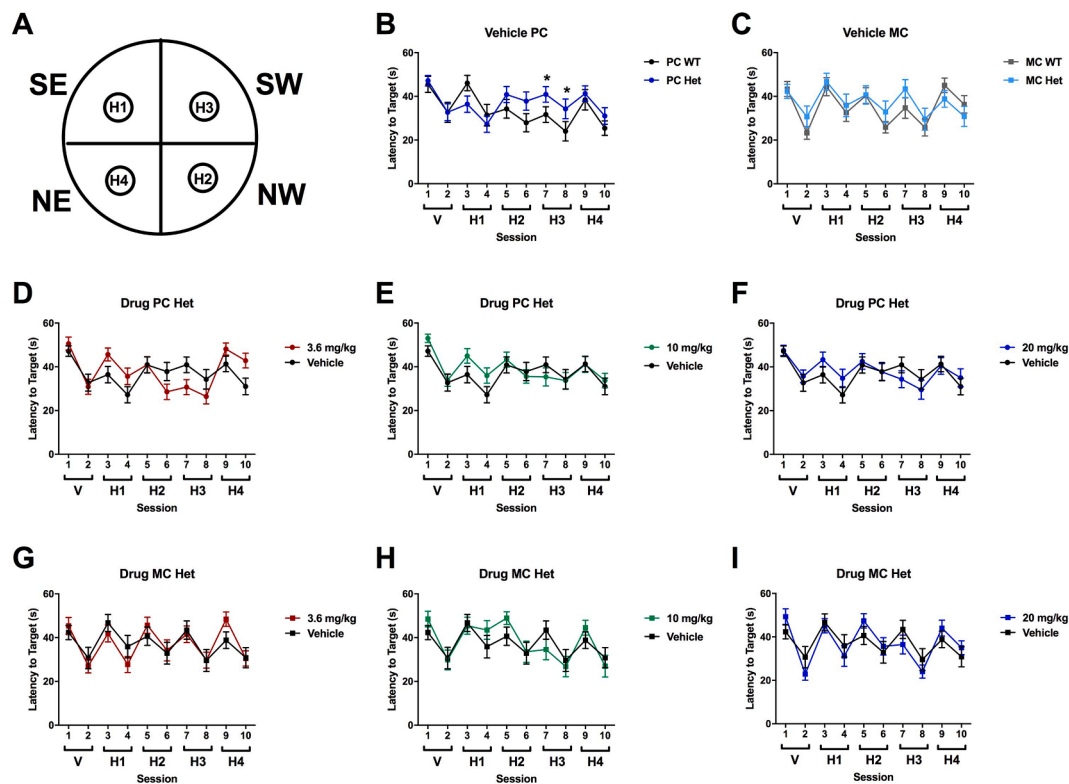


Fig. 3. Spatial learning and memory in the water maze using latency as performance measure. **A.** The four hidden platform locations are illustrated. **B–C.** In vehicle-treated mice, the performance in the water maze was not significantly affected by sex, genotype, or parental carrier. **D–F.** In PC HET mice, there was no significant effect of treatment on performance. **G–I.** In MC HET mice, there was also no significant effect of treatment on performance. **G–I.**

sex $\times$ treatment interaction ($F(3,73) = 3.073$, $p = 0.033$). The sexes were then analyzed separately. There was no effect of treatment in the females ($F(3, 36) = 0.851$, $p = 0.475$, [Supplementary Fig. 2A](#)), but there was an effect of treatment in the males ($F(3, 37) = 2.889$, $p = 0.048$, [Supplementary Fig. 2B](#)). Males that received the 3.6 mg/kg treatment performed better than those that received the 10 mg/kg treatment ($p = 0.037$, Tukey post hoc test).

For the hidden sessions, there was a session $\times$ treatment interaction ($F(15.530, 377.900) = 1.841$, $p = 0.026$), but no main effects of sex ($F(1,73) = 0.561$, $p = 0.456$) or treatment ($F(3,73) = 0.081$, $p = 0.970$). A beneficial effect of treatment was seen in PC Het mice at the 3.6 mg/kg dose during training to locate the third hidden platform location; mice treated with the Alk inhibitor at the 3.6 mg/kg dose swam closer to the platform location than genotype-matched vehicle-treated mice (repeated-measures ANOVA: $F(1,36) = 4.620$, $p = 0.038$, [Fig. 2D](#)). This effect was parental carrier-dependent and not seen in MC Het mice ([Fig. 2G](#)).

When each day of hidden platform training was then analyzed separately, an effect of treatment was revealed on the fourth day of water maze training ($F(1,73) = 3.037$, $p = 0.034$). There was a trend towards mice that received the 3.6 mg/kg treatment performing more poorly compared to the vehicle-treated mice ($p = 0.060$, Tukey post hoc) and mice that received the 10 mg/kg ($p = 0.075$, Tukey post hoc), and the 20 mg/kg treatments ($p = 0.094$, Tukey post hoc).

In WT PC mice, there was an effect of session during the visible ($F(1,54) = 52.318$, $p < 0.001$) and the hidden platform sessions ($F(4.712, 254.451) = 7.619$, $p < 0.001$). During the visible platform sessions, there was a trend toward a session $\times$ treatment interaction ($F(3,54) = 2.633$, $p = 0.059$), but there was no effect of sex ($F(1, 54) = 0.242$, $p = 0.624$) or treatment ($F(3,54) = 1.498$, $p = 0.226$). When sessions were analyzed separately, there was a trend toward an effect of treatment during the first visible platform session ($F(3, 61) = 2.333$, $p = 0.084$). There was no effect of treatment during the second visible platform session ($F(3, 61) = 1.588$, $p = 0.203$). During the hidden platform sessions, there was a trend toward an effect sex ($F(1, 54) = 3.571$, $p = 0.064$), but not of treatment ($F(3,54) = 0.144$, $p = 0.144$).

In HET MC mice ([Fig. 2G–I](#)), there was also an effect of session during the visible ($F(1,58) = 73.176$, $p < 0.001$) and hidden ($F(4.976, 288.615) = 15.563$, $p < 0.001$) platform sessions. During the visible platform sessions, there was no effect of sex ($F(1,58) = 0.611$, $p = 0.438$) or treatment ($F(3,58) = 0.536$, $p = 0.660$). There was a session $\times$ sex $\times$ treatment interaction ($F(3,58) = 3.735$, $p = 0.016$) and a trend towards a session $\times$ treatment interaction ($F(3,58) = 2.220$, $p = 0.095$). When the sexes were analyzed separately, there was a session $\times$ treatment interaction ($F(3, 35) = 4.988$, $p = 0.006$), but no main effect of treatment ($F(3, 35) = 0.442$, $p = 0.724$), in males. Each visible session was then analyzed separately. There was an effect of dose during the first visible platform session ($F(3, 38) = 3.660$, $p = 0.021$); the males that received the 20 mg/kg dose had a higher cumulative distance to the target location (i.e., poorer performance) than sex-matched vehicle treated littermates ($p = 0.019$, Tukey's post hoc test). In the females, there was no effect of treatment ($F(3, 23) = 2.096$, $p = 0.129$) or treatment $\times$ session interaction ($F(3, 23) = 1.444$, $p = 0.256$). During the hidden platform sessions, there was no main effect of sex ($F(1,58) = 0.322$, $p = 0.573$) or treatment ($F(3,58) = 0.120$, $p = 0.948$).

In WT MC mice, there was an effect of session during the visible ($F(1, 66) = 71.579$, $p < 0.001$) and hidden platform sessions ($F(5.508, 357.996) = 15.627$, $p < 0.001$). During the visible platform sessions, there was a session $\times$ sex $\times$ treatment interaction ($F(3, 66) = 3.327$, $p = 0.025$), but there was no effect of sex ($F(1, 66) = 0.126$, $p = 0.724$) or treatment ($F(3, 66) = 0.212$, $p = 0.888$). When the sexes were analyzed separately, there was a trend towards a session $\times$ treatment interaction in the females ($F(3, 34) = 2.768$, $p = 0.057$) but not in the males ($F(3, 31) = 1.401$, $p = 0.261$). During the hidden platform

sessions, there was no effect of sex ($F(1, 65) = 0.072$, $p = 0.789$) or treatment ($F(3, 65) = 0.122$, $p = 0.947$) on performance.

Next, latency to the target platform was analyzed as performance measure ([Fig. 3](#)). In HET PC mice ([Fig. 3D–F](#)), there was an effect of session during the visible ($F(1, 73) = 61.740$, $p < 0.001$) and hidden ($F(5.639, 411.650) = 7.161$, $p < 0.001$) platform sessions. During the visible platform sessions, there was no main effect of sex ($F(1, 73) = 0.081$, $p = 0.777$) or treatment ($F(3, 73) = 0.545$, $p = 0.653$) but there was a sex $\times$ treatment interaction ($F(3, 73) = 3.084$, $p = 0.033$). The analysis was then split by sex, and revealed that there was no effect of treatment in the females ($F(3, 36) = 1.015$, $p = 0.397$, [Supplementary Fig. 2C](#)), but there was an effect of treatment in the males ($F(3, 37) = 2.893$, $p = 0.048$, [Supplementary Fig. 2D](#)). Consistent with the cumulative distance to the target location data described above, males that received the 3.6 mg/kg treatment had lower latency times (better performance) compared to mice that received the 10 mg/kg treatment ($p = 0.031$, Tukey post hoc). During the hidden platform sessions, there was a trend toward a session $\times$ treatment interaction ($F(16.917, 411.650) = 1.487$, $p = 0.096$) but no main effects of sex ($F(1, 73) = 1.203$, $p = 0.276$) or treatment ($F(3, 73) = 0.160$, $p = 0.923$). A trend towards a beneficial effect of treatment to locate the third hidden platform location was seen in PC Het mice treated with the 3.6 mg/kg dose; there was a trend towards mice treated with this dose of the Alk inhibitor reaching the platform location quicker than genotype-matched vehicle-treated mice (repeated-measures ANOVA: $F(1,36) = 3.754$, $p = 0.061$, [Fig. 3D](#)). This trend was parental carrier-dependent and not seen in MC Het mice ([Fig. 3G](#)).

In WT PC mice, there was an effect of session during the visible ($F(1, 54) = 35.029$, $p < 0.001$) and the hidden platform sessions ($F(5.480, 295.895) = 6.998$, $p < 0.001$). During the visible platform sessions, there was no effect of sex ($F(1,54) = 0.376$, $p = 0.542$) or treatment ($F(3,54) = 1.126$, $p = 0.347$). During the hidden platform sessions, there was a trend towards an effect of sex ($F(1, 54) = 2.870$, $p = 0.096$), but not of treatment ($F(3, 54) = 1.887$, $p = 0.143$).

In HET MC mice ([Fig. 3G–I](#)), there was also an effect of session during the visible ($F(1, 58) = 65.749$, $p < 0.001$) and hidden ($F(5.353, 310.454) = 14.562$, $p < 0.001$) platform location sessions. During the visible platform sessions, there was no main effect of sex ($F(1, 58) = 0.706$, $p = 0.404$) or treatment ($F(3, 58) = 0.413$, $p = 0.744$). However, there was a session $\times$ sex $\times$ treatment interaction ($F(3, 58) = 2.927$, $p = 0.041$). When the analyses were split by sex, there was a session $\times$ treatment interaction ($F(3, 35) = 3.618$, $p = 0.022$), but no main effect of treatment ($F(3, 35) = 0.239$, $p = 0.869$) in males. When each session was analyzed separately, there was a trend towards an effect of treatment on the first session ($F(3, 38) = 2.560$, $p = 0.071$). Post hoc analysis revealed that the mice treated with the 20 mg/kg dose performed worse than vehicle-treated during the first visible platform session, though it did not reach significance ($p = 0.060$, Tukey's post hoc test). There was no effect of treatment on performance during the second visible platform session ($F(3, 38) = 0.813$, $p = 0.496$). In the females, there was no session $\times$ treatment interaction ($F(3, 23) = 0.876$, $p = 0.468$) or main effect of treatment ($F(3, 23) = 1.666$, $p = 0.202$). During the hidden platform sessions, there was no main effect of sex ($F(1, 58) = 0.066$, $p = 0.798$) or treatment ($F(3, 58) = 0.064$, $p = 0.978$).

In WT MC mice, there was an effect of session during the visible ($F(1, 65) = 68.491$, $p < 0.001$) and hidden platform sessions ($F(5.759, 374.315) = 11.789$, $p < 0.001$). During the visible platform sessions, there was no effect of sex ($F(1, 65) = 1.093$, $p = 0.300$) or treatment ($F(3, 65) = 0.361$, $p = 0.781$). However, there was a session $\times$ sex $\times$ treatment interaction ($F(3, 65) = 3.699$, $p = 0.016$). Analyses was then split by sex. In the females, consistent with the cumulative distance to the target location data described above, there was a trend towards a session $\times$ treatment interaction ($F(3, 34) = 2.771$, $p = 0.056$), but no main effect of treatment ($F(3, 34) = 0.469$, $p = 0.706$). During the hidden platform sessions, there was no main effect of sex ($F(1, 65) = 0.000$, $p = 0.990$) or treatment ($F(3,$

65) = 0.004, $p = 1.000$).

Spatial memory retention was assessed in three probe trials (no platform). For the three probe trials, cumulative distance to the target platform was used as performance measure. The vehicle-treated mice were analyzed first (Fig. 4), using repeated measures ANOVA. There was an effect of probe trial ($F(2, 116) = 3.119$, $p = 0.048$), and a probe $\times$ genotype interaction ($F(2, 116) = 4.602$, $p = 0.012$). There were no main effects of sex ($F(1, 58) = 0.001$, $p = 0.973$), genotype ($F(1, 58) = 2.272$, $p = 0.137$), or parental carrier ($F(1, 58) = 0.950$, $p = 0.0334$). When the analyses were split by genotype, the effect of probe trial remained significant only in the WT mice ($F(2, 56) = 6.080$, $p = 0.004$), which seemed driven by better performance in the third than the second probe trial. This pattern was genotype-dependent and not seen in HET mice (Fig. 4).

The probe trial analyses were then split by parental carrier and genotype as described previously, and the effect of treatment was assessed. In the PC HET mice (Fig. 5A), there was an effect of probe trial ($F(2, 138) = 3.773$, $p = 0.025$), but no effect of treatment ($F(1, 69) = 1.452$, $p = 0.235$). In the PC WT littermates (Fig. 5B), there was a trend towards an effect of probe trial ($F(2, 104) = 3.068$, $p = 0.051$) and a probe trial $\times$ treatment interaction ($F(6, 104) = 1.853$, $p = 0.096$), but there was no main effect of treatment ($F(3, 52) = 0.488$, $p = 0.692$). In the HET MC mice (Fig. 5C), there was no effect of probe trial ($F(2, 116) = 0.993$, $p = 0.373$), or treatment ($F(3, 58) = 0.791$, $p = 0.504$). In the MC WT mice (Fig. 5D), there was a probe trial $\times$ treatment interaction ($F(6, 130) = 2.680$, $p = 0.017$), but the effect of probe trial did not reach significance ($F(2, 130) = 2.972$, $p = 0.055$). There was no effect of treatment ($F(3, 65) = 2.099$, $p = 0.109$).

Recognizing the complexity of interpretation of probe trials with multiple hidden platform locations, we also analyzed the cumulative distance to four platform locations, one in each target quadrant, in each of the three probe trials (Fig. 6). In the first probe trial (Fig. 6A), MC and PC HET mice had a smaller cumulative distance to the target location T1 than to the untrained target locations T2 and T3, but not T4. In contrast, MC WT mice had only a lower cumulative distance to T1 than T2 and PC WT mice showed no significantly lower cumulative distance to T1 than any of the other target locations. These data suggest a reduced spatial bias in PC WT mice. During the second probe trial (Fig. 6B), the cumulative distance to T2 was not lower than to any other target location. In the third probe trial (Fig. 6C), the cumulative distance of MC WT mice to T3 was lower than that to T4. This pattern was genotype-dependent and parental carrier-dependent and not seen in PC WT or MC or PC HET mice.

2.4. Effects of CH5424802 on contextual fear learning, memory and extinction in PC and MC HET and WT mice

First, the vehicle-treated mice were analyzed for fear learning during the training day (Fig. 7). A univariate ANOVA revealed an effect of genotype on baseline motion ($F(1, 68) = 4.524$, $p = 0.037$, Fig. 7A) and

baseline freezing ($F(1, 68) = 4.027$, $p = 0.049$, Fig. 7B). A repeated measures ANOVA for motion during the shock revealed a shock $\times$ sex $\times$ genotype interaction ($F(1, 61) = 5.036$, $p = 0.028$). There were main effects of sex ($F(1, 61) = 8.946$, $p = 0.004$), parental carrier ($F(1, 61) = 18.739$, $p < 0.001$), and a sex $\times$ genotype interaction ($F(1, 61) = 4.940$, $p = 0.030$). The analysis was then split by sex. In the females (Supplementary Fig. 3A), there was trend towards a shock $\times$ genotype interaction ($F(1, 27) = 3.065$, $p = 0.091$) and a shock $\times$ parental carrier interaction ($F(1, 27) = 2.945$, $p = 0.098$). There were main effects of genotype ($F(1, 27) = 8.395$, $p = 0.007$) and parental carrier ($F(1, 27) = 6.348$, $p = 0.018$). In the males (Supplementary Fig. 3B), there was only an effect of parental carrier ($F(1, 34) = 13.797$, $p = 0.001$).

Due to the interactions with shock, each shock was analyzed separately with a univariate ANOVA. During the first shock, there were effects of sex ($F(1, 68) = 9.198$, $p = 0.004$) and parental carrier ($F(1, 68) = 8.789$, $p = 0.004$). During the second shock, there was an effect of sex ($F(1, 68) = 4.724$, $p = 0.034$), parental carrier ($F(1, 68) = 22.203$, $p < 0.001$), and a sex $\times$ genotype interaction ($F(1, 68) = 10.131$, $p = 0.002$). There was a trend towards an effect of genotype as well ($F(1, 68) = 3.235$, $p = 0.077$).

A repeated measures ANOVA for freezing during the tones revealed a significant increase in freezing from the first tone to the second tone ($F(1, 61) = 69.664$, $p < 0.001$), consistent with fear learning in all the groups. There were no main effects of sex ($F(1, 61) = 2.486$, $p = 0.120$), genotype ($F(1, 61) = 2.076$, $p = 0.155$), or parental carrier ($F(1, 61) = 2.069$, $p = 0.155$).

Next, the effect of the treatment was assessed in PC mice (Fig. 8A,B). There was no effect of treatment on baseline motion in either the HET ($F(3, 80) = 0.230$, $p = 0.875$, Fig. 9A) or the WT ($F(3, 61) = 1.278$, $p = 0.291$, Fig. 9B) PC mice. A repeated measures ANOVA for the motion during the shocks revealed no effects of treatment in the PC HET mice ($F(3, 73) = 0.060$, $p = 0.981$), but there was a trend towards an effect of sex ($F(1, 73) = 3.694$, $p = 0.059$) (Fig. 8A). In the PC WT mice, there was also a trend towards an effect of sex ($F(1, 54) = 3.510$, $p = 0.066$) and an effect of treatment ($F(3, 54) = 0.008$, $p = 0.008$) (Fig. 8B). PC WT mice that received the 10 mg/kg dose were less active during the shock than the vehicle-treated ($p = 0.005$, Tukey's post hoc test) and the 3.6 mg/kg-treated PC WT mice ($p = 0.011$, Tukey's post hoc test).

In contrast to the PC HET mice, there was an effect of treatment on baseline motion in MC HET mice ($F(3, 65) = 4.016$, $p = 0.012$, Fig. 9C). In MC HET mice, those treated with the 10 mg/kg dose were more active during the baseline period than those treated with vehicle ($p = 0.024$, Tukey post hoc test). Similar to the PC WT mice, there was no effect of treatment on baseline motion in MC WT mice ($F(3, 73) = 0.077$, $p = 0.972$, Fig. 9D).

A repeated measures ANOVA for the motion during the shocks revealed no effect of treatment in the MC HET mice ($F(3, 58) = 1.646$, $p = 0.189$), but there was an effect of sex ($F(1, 58) = 8.441$, $p = 0.005$, Supplementary Fig. 3C-D) and a treatment $\times$ shock interaction ($F(3,$

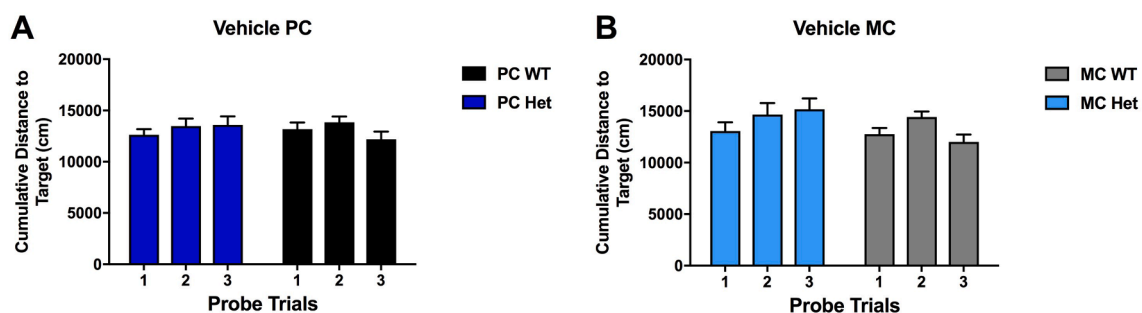


Fig. 4. Performance of vehicle-treated mice in the water maze probe trials. During the probe trials, the target platform was removed and memory retention was tested 24 h after training for one of three hidden platform locations. There was no effect of parental carrier, genotype, or sex. There was an effect of probe trial and a probe trial $\times$ genotype interaction. When the genotypes were split up for analysis, there remained an effect of probe trial in WT, but not HET, mice.

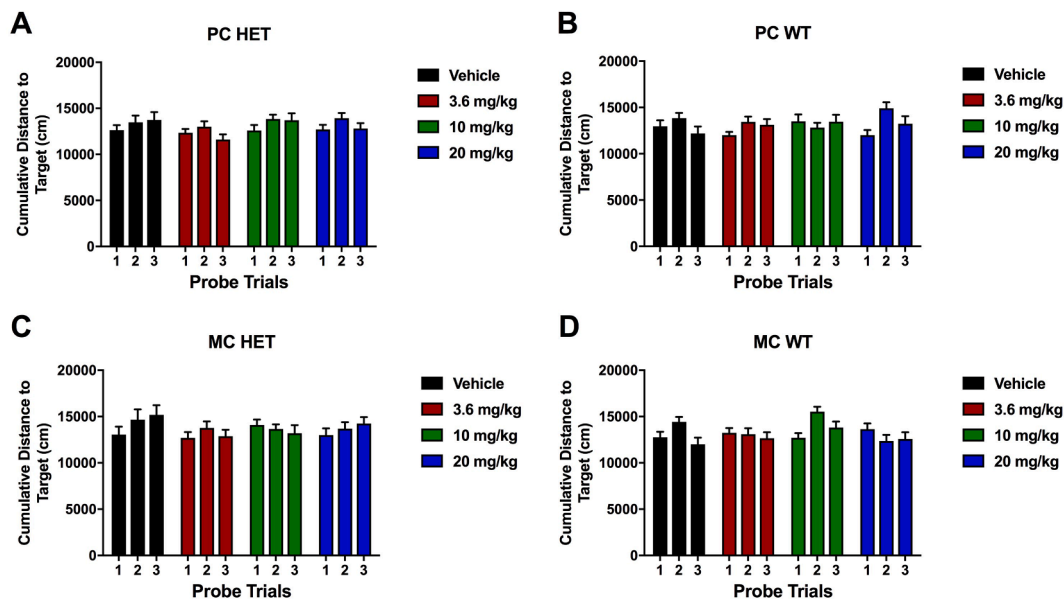


Fig. 5. Performance of drug-treated mice in the water maze probe trials. **A.** In PC HET mice, there was an effect of probe trial, but no effect of treatment. **B.** In PC WT mice, there was no effect of probe trial or treatment. **C.** In MC HET mice, there was no effect of probe trial or treatment. **D.** In MC WT mice, there was a probe trial $\times$ treatment interaction.

58) = 3.181, $p = 0.031$). When each shock was analyzed with a univariate ANOVA, there was no effect of treatment during the first shock ($F(3, 65) = 1.137$, $p = 0.342$), but there was an effect of treatment during the second shock ($F(3, 65) = 2.794$, $p = 0.048$). Post hoc analysis revealed a trend toward a larger response to the shock in MC HET mice that received the 3.6 mg/kg than that seen in vehicle-treated mice ($p = 0.094$, Tukey post hoc test, Fig. 9C). This was genotype-dependent and not seen for motion during the shocks in WT mice ($F(3, 66) = 0.087$, $p = 0.967$).

Finally, we assessed extinction of contextual fear (Fig. 9). During extinction of contextual fear, there was an effect of day ($F(5.348, 326.216) = 25.297$, $p < 0.001$), consistent with extinction of contextual fear, a day $\times$ parental carrier interaction ($F(5.348, 326.216) = 6.749$, $p < 0.001$), and trend towards a day $\times$ parental carrier $\times$ sex interaction ($F(5.348, 326.216) = 2.030$, $p = 0.069$) in the vehicle-treated mice. There was a main effect of sex ($F(1, 61) = 7.139$, $p = 0.010$, Supplementary Fig. 4A–D), but no effect of genotype ($F(1, 61) = 0.500$, $p = 0.482$) or parental carrier ($F(1, 61) = 1.454$, $p = 0.233$).

While in the PC HET mice there was an effect of day ($F(6.009, 438.638) = 15.833$, $p < 0.001$) and sex ($F(1, 73) = 16.716$, $p < 0.001$, Supplementary Fig. 4A,B), there was no effect of treatment ($F(1, 73) = 1.480$, $p = 0.227$). In the PC WT mice, there was only an effect of day ($F(5.426, 292.982) = 16.815$, $p < 0.001$). A repeated measures ANOVA for all 10 days of extinction revealed an effect of day for the MC HET mice ($F(4.791, 277.870) = 35.641$, $p < 0.001$), and a trend towards a day $\times$ sex interaction ($F(4.791, 277.870) = 2.197$, $p = 0.058$). Sex itself was significant ($F(1, 58) = 6.058$, $p = 0.017$, Supplementary Fig. 4C,D) but treatment was not ($F(3, 58) = 1.686$, $p = 0.180$). In MC WT mice, there was an effect of day ($F(5.230, 345.173) = 55.312$, $p < 0.001$) and a trend towards a day $\times$ treatment interaction ($F(5.230, 345.173) = 1.623$, $p = 0.062$) and a trend towards an effect of sex ($F(1, 66) = 3.192$, $p = 0.079$).

3. Discussion

The results of this study indicate that the behavioral and cognitive phenotypes and responsiveness to Alk inhibition in heterozygous NF1 offspring depend on whether the parental carrier is maternal or paternal. These parental effects might involve genetic background differences in mitochondria function. While there was no effect of genotype or

circadian activity levels, Alk inhibition (20 mg/kg) increased activity levels during the dark period in NF1 HET PC, but not MC, mice. In MC, but not PC, WT mice, Alk inhibition (3.6 mg/kg) increased the dark-light activity ratio. In the water maze, NF1 HET PC, but not MC, mice showed reduced cognitive flexibility and impaired ability to locate the third hidden platform location, suggesting impaired executive function. Alk inhibition (3.6 mg/kg) improved performance of NF1 PC mice to locate the third hidden platform location. Consistent with the hidden platform water maze data in NF1 mice, when spatial memory retention was assessed in the probe trials, WT, but not NF1, mice showed better performance in the third than second probe trial. In addition, MC WT, but not NF1, mice showed a lower cumulative distance to the third than the fourth hidden platform location. Alk inhibition (10 mg/kg) increased baseline activity of NF1 MC, but not PC, mice during the fear conditioning test. In PC, but not MC, WT mice, Alk inhibition (10 mg/kg) reduced the response to the shocks. As the NF1 carrier was always on a B6 background and the non NF1 carrier was always on a SVJ background, it is conceivable that whether the maternal or paternal is on either background might have contributed to the observed parental carrier effects.

In our previous genetic and pharmacological study involving NF1 HET mice, the mice behaviorally tested were on a diverse F2 genetic background (Weiss et al., 2017a; Weiss et al., 2017b). We had been breeding male and female Alk homozygous knockout mice on the C57BL/6J background with male NF1 HET mice on the 129 SvJ background. As the breeding strategy of NF1 HET mice (Alk wild-type) with Alk HET mice hardly generated NF1 HET mice (Alk wild-type), we bred F1 double HET mice (Het for both NF1 and Alk) to generate 532 F2 mice for the behavioral studies. While having NF1 mice on a F2 background is more translational relevant for humans, we expected the cognitive phenotypes in spatial learning and memory in the water maze and in extinction of contextual fear memory to be more variable and less profound on a F2 than a B6/129 SvJ F1 background. However, the cognitive phenotypes of the NF1 HET mice in the water maze and contextual fear extinction tests on a F2 background were more profound than those seen on a pure B6/129 SvJ F1 background in the current study. In the water maze, NF1 HET mice on a F2 background, already showed an impairment during training to locate the first hidden platform (Weiss et al., 2017b). No impairment was seen in NF1 mice on a pure B6/129 SvJ F1 background in the current study. Part of this difference might be due to

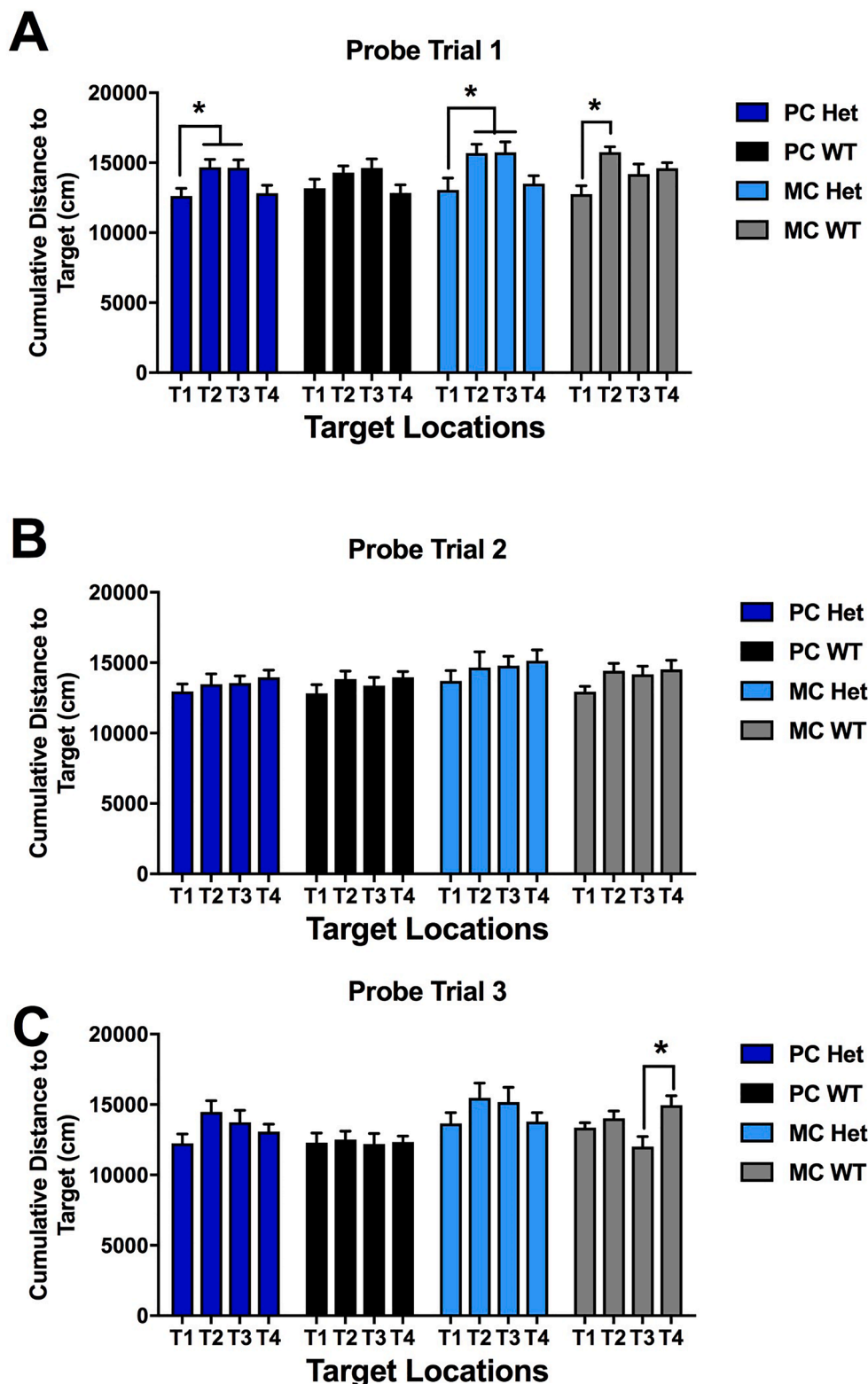


Fig. 6. Performance of vehicle-treated mice in the water maze probe trials as cumulative distance to four platform locations in each probe trial. Platform locations are listed in the order in which the mice were trained to locate a platform in the quadrant. So the T1 location is the first hidden platform location the mice were trained to locate, the T2 location is the second hidden platform location the mice were trained to locate, and the T3 location is the third hidden platform location the mice were trained to locate. **A.** MC and PC HET mice had a smaller cumulative distance to the T1 than the untrained T2 or T3 locations. MC WT mice has a smaller cumulative distance to the T1 location than the T2 location. No significant preference was seen in PC WT mice. **B.** In the second probe trial, no groups had a significant lower cumulative distance to T2 location than any other target location. **C.** In the third probe trial, MC WT mice had a lower cumulative distance to the T3 than the T4 location. * $p < 0.05$ (Dunnett's).

the fact that in the current study the mice were first trained to locate a visible platform and subsequently trained to locate hidden platform locations, while in our previous study NF1 HET mice on a F2 background the mice were first trained to locate the hidden platform locations and only subsequently trained to locate the visible platform location. This is unlikely to completely account for this difference, as the cognitive phenotype in extinction of contextual fear was also more profound on a

F2 background (Weiss et al, 2017b) than on a pure 129SvJ F1 background. It is conceivable that B6-related genes present and important for cognition might be important in protecting against the detrimental genetic effects of the NF1 HET. Nevertheless, the phenotype seen in cognitive flexibility in the water maze seen on a pure B6/129 SvJ F1 background is consistent to what has been observed for medial pre-frontal cortex synaptic plasticity and working memory in the radial arm

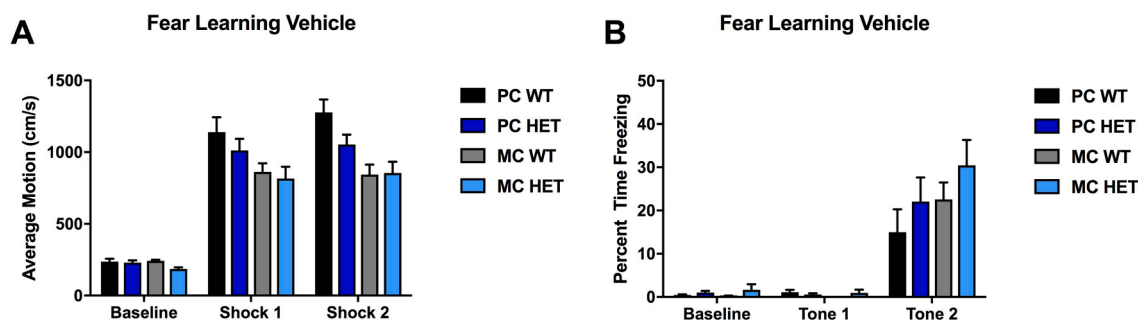


Fig. 7. Fear learning in vehicle-treated mice. **A.** At baseline, there was an effect of genotype on average motion, but no effect of parental carrier. During the shocks, there was an effect of parental carrier. **B.** Baseline freezing was different between genotypes and there was an effect of tone in all groups, indicating that all groups learned.

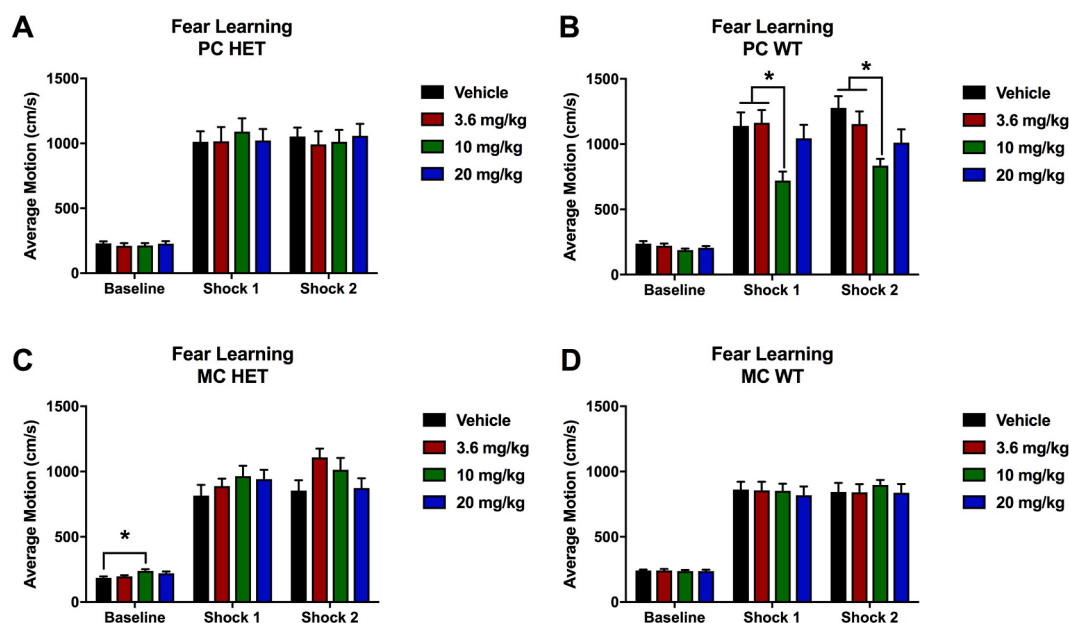


Fig. 8. Fear learning in drug-treated mice. **A.** In PC HET mice, there was no significant effect of treatment on either baseline motion or motion during the shocks. **B.** In PC WT mice, there was no effect of treatment on baseline motion but during the shocks the mice that received the 10 mg/kg dose were less active than the vehicle-treated mice ($p = 0.005$, Tukey post hoc) and those that received the 3.6 mg/kg dose ($p = 0.011$, Tukey). **C.** In MC HET mice, those that received the 10 mg/kg dose were more active at baseline than those that received vehicle ($p = 0.024$, Tukey). There was an effect of treatment during the second, but not first, shock. There was a trend towards increased activity during the shock of mice that received the 3.6 mg/kg dose than those that received vehicle ($p = 0.094$, Tukey). **D.** In MC WT mice, there were no effects of treatment on the baseline motion or motion during the shocks.

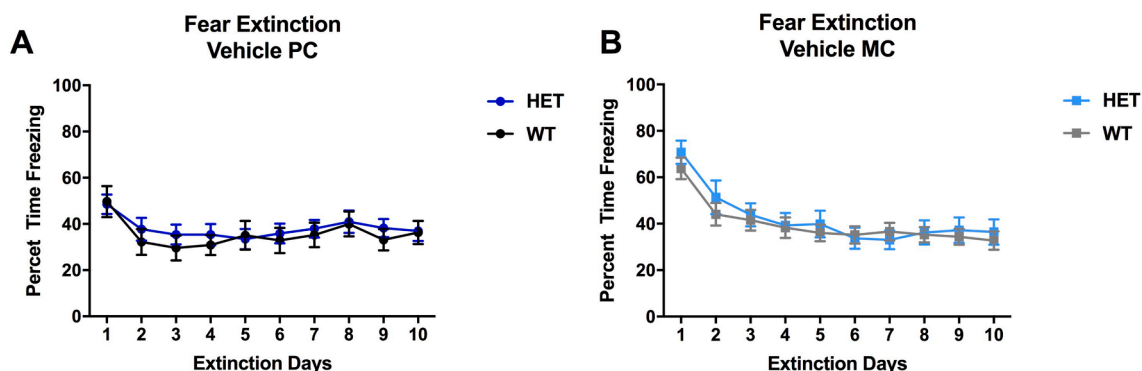


Fig. 9. Extinction of contextual fear memory in the vehicle-treated mice. There was a day $\times$ parental carrier interaction but no effect of genotype in PC (A) or MC (B) mice.

maze in the NF1 Het mice (Shilyansky et al., 2010; Silva et al., 1997).

The observed effects of Alk inhibition (20 mg/kg) on increasing activity levels during the dark period in NF1 HET PC mice on a pure B6/129 SvJ F1 background is consistent with the sleep phenotype observed in inducible NF1 homozygous knockout mice with no reproducible phenotype in NF1 HET mice on a B6/129 SvJ F1 background (Anastasakis et al., 2019). These data support the value of testing mutant mouse models of neurological diseases on a diverse genetic background or comparing phenotypes on distinct genetic backgrounds (Onos et al., 2019; Sittig et al., 2016).

In the fear conditioning test, MC HET mice treated with the Alk inhibitor at the 10 mg/kg dose were more active during the baseline period than those treated with vehicle. In contrast, there was no effect of Alk inhibition at this dose on home cage activity of MC HET mice, suggesting that this effect might only be revealed in a novel but not a familiar environment.

In the current study, we used three doses of the Alk inhibitor (3.6, 10, and 20 mg/kg), all higher than the 1.8 mg/kg dose we used in our previous study (Weiss et al., 2017a). The effective dose depended on the behavioral test and genotype of the mice. Alk inhibition (20 mg/kg) increased activity levels during the dark period in NF1 HET PC, while Alk inhibition (3.6 mg/kg) increased the dark-light activity ratio in MC WT mice. In contrast to home cage activity, Alk inhibition (3.6 mg/kg) improved performance to locate the third hidden platform location in NF1 PC mice. Finally, Alk inhibition (10 mg/kg) increased baseline activity of NF1 MC mice in the fear conditioning test and reduced response to the shocks in PC WT mice. These data indicate that for enhancing cognitive flexibility in the water maze a relative low dose of 1.8 or 3.6 mg/kg might be preferred over higher doses. For enhancing activity levels during the light period in a novel environment (i.e., baseline activity in the fear conditioning test), the intermediate dose of 10 mg/kg was optimal while for enhancing activity in the dark period the highest dose of 20 mg/kg was the most effective. Interestingly, there was a trend towards increased activity of PC HET mice during the dark period treated with the lower dose of 3.6 mg/kg, a pattern not seen with treatment of the intermediate dose of 10 mg/kg. A limitation of our study design is that the mice were tested in the water maze following 10 days after the initiation of treatment and the mice were tested for home cage activity prior to testing in the water maze. Therefore, we cannot exclude that the longer prior treatment might have contributed to the effects of the 3.6 mg/kg dose in the water maze test. Regardless, considering that side effects will likely be more pronounced with higher doses, the 3.6 mg/kg dose seems preferred.

There was no effect of genotype or circadian activity levels in NF1 HET mice. In contrast, daytime and nighttime sleep were highly fragmented in NF1 knockout male flies, with a reduced average sleep bout duration and an increased number of sleep bouts increased. A sleep phenotype was less clear in NF1 knockout female flies, which might relate to sexual dimorphic regulation of sleep (Bai and Sehgal, 2015). In contrast to these studies in flies, conditional NF1 knockout mice, showed increased non-REM sleep (NREM)-to-wake and wake-to-NREM transitions, decreased bout durations during awake and NREM sleep states under both light and dark conditions, and a higher percentage of delta power during awake and NREM sleep states under all light conditions (Anastasakis et al., 2019). Although our earlier data suggested lower circadian activity levels in NF1 heterozygous mice, we could not directly statistically compare them to wild-type mice as they were not littermates (Weiss et al., 2017b). The current study indicates that within the limitation of the pure B6/129 SvJ F1 genetic background used that NF1 heterozygous mice do not have a circadian phenotype.

In summary, the results of this study indicate that effects of parental carrier need to be considered in studying the behavioral and cognitive phenotypes and responsiveness to Alk inhibition in NF1 HET mice. This result might impact earlier studies as typically potential differential effects of MC versus PC NF1 HET mice were not considered or assessed. The role of the parental carrier might be important in other preclinical

models as well and therefore should be kept in mind for future studies. Alk inhibition improves cognitive flexibility in NF1 HET mice. NF1 HET PC mice showed reduced cognitive flexibility and impaired ability to locate the third hidden platform location, which was improved with Alk inhibition (3.6 mg/kg). Consistent with reduced cognitive flexibility in NF1 mice, they did not show better performance in the third than second probe trial as seen in WT mice. Effects of Alk inhibition on baseline activity in the fear conditioning test and on activity levels in the home cage during the dark period were seen with the 10 and 20 mg/kg dose, respectively, indicating that cognitive flexibility might be particularly sensitive to Alk inhibition. This result is consistent with the observation that NF1 is caused by haplo-insufficiency or partial loss of function of the NF1 gene and that the genetical and pharmacological susceptibility of the hippocampus-dependent functions of neurofibromin to modification by Ras (Costa and Silva, 2002). A limitation of the current study is that the pharmacological treatment was initiated only 10 days prior to water maze testing. Therefore, in future studies, we will determine whether following prolonged treatment there are more profound effects of Alk inhibition on behavioral and cognitive performance of NF1 HET mice.

4. Experimental procedure

4.1. Animals

Three male and three female heterozygous (HET) NF1 mice on a C57BL/6J (B6) background were received from Dr. David H. Gutmann at Washington University, Saint Louis, MO, and bred with wild type (WT) mice on a 129 SvJ (SvJ) background from JAX (Bar Harbor, Main, MA). As it might matter whether the mother or father is the NF1 HET carrier and/or had the C57BL/6J or 129 SvJ background, we set up breeding pairs with either the father (PC : B6 Paternal NF1 Carrier) or the mother (MC : B6 Maternal NF1 Carrier) as the NF1 carrier. The NF1 carrier (F1 as part of the original three NF1 mice or F2 based on a subsequent generation) was always on a B6 background, and the non NF1 carrier was always on a SVJ background. All 143 PC mice were F1, whereas 68 of the MC mice were F1 and 72 were F2. For genotyping of the offspring, DNA was extracted from tail biopsies collected at weaning and processed for polymerase chain reaction (PCR). The following primer sequences were used to produce a 400 base pair (bp) product for HET mice and a 200 bp product for WT mice: NF1 31A: AGTA TTG AAT TGA AGC ACC TTT GTT TG; NF1 31B: CTG CCC AAG GCT CCC CCA G; and NF1 neoTK: GCG TGT TCG AAT TCG CCA ATG. The initial denaturing was at 95 °C for 2 min. The denature, anneal, and extend conditions were as follows: 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 45 s. This was run for 37 cycles with a final extension at 72 °C for 5 min. Animals were housed under a 12-hour light dark cycle. Food and water were provided ad libitum. Testing was conducted and data analyzed by an experimenter blind to the genotype of the mice. The group code was broken once the data was analyzed. All procedures complied with the ARRIVE guidelines, NIH Guide for the Care and Use of Laboratory Animals, and were approved by Oregon Health and Science University (OHSU) institutional Animal Care and Use Committee (IACUC).

4.2. Pharmacological treatment

Animals were given either vehicle or one of three doses (3.6 mg/kg, 10 mg/kg, or 20 mg/kg) of the Alk inhibitor CH5424802 (Alectinib, Chugai Pharmaceuticals, Tokyo, Japan). The vehicle for the drug was a flavored gelatin mixture in 1 cm × 1 cm squares. This route of administration was selected because voluntary oral administration would be the most relevant for human patients, and less stressful and safer for the mice than daily oral gavage. Treatment was administered daily starting 10 days prior to the first day of water maze testing. Every day when the gelatin squares were given to the mice, the researchers confirmed that the previous gelatin squares had been eaten. The mean age at start of treatment per experimental group was 6.1 ± 0.46 months.

4.3. Home cage activity

Circadian activity levels were analyzed in a subset of mice using infrared home-cage activity sensors (Bioserve, St Augustin, Germany) placed above the cage. The mice were housed individually and allowed two days to acclimate to the home cage environment prior to the start of activity monitoring. Activity counts were measured every second. The ratio activity, defined as the activity in the dark period divided by the activity in the light period, was analyzed as well.

4.4. Spatial learning and memory in the water maze

Mice were tested for spatial learning and memory in the water maze. A circular pool (140 cm diameter) was filled with water ($20^{\circ}\text{C} \pm 2^{\circ}\text{C}$). A platform (20 cm diameter) was located approximately 1 cm below opaque water, which contained white chalk in order to hide the platform in opaque water. A trial ended when the mouse either located and stayed on the platform for 3 s or after 60 s had elapsed. If the mouse had not located the platform within 60 s, the mouse was guided to the platform and retrieved once they had remained on it for 3 s. Mice were returned to their home cage after removal from the maze. The mice were trained to locate two locations during visible platform training (Visible Location 1: session 1; Visible Location 2: session 2) and four locations during hidden platform training (Hidden Location 1: sessions 3–4; Hidden Location 2: sessions 5–6; Hidden Location 3: sessions 7–8; Hidden Location 4: session 9–10). The sessions consisted of two trials each with a 10-minute inter-trial interval. There were two sessions per day, three hours apart. Before sessions 5, 7, and 9 mice were given a probe trial, in which the platform was removed, and they were allowed 60 s of exploration in the maze. Performance was assessed by analyzing cumulative distance to target location and latency to reach the platform. Data from the water maze was recorded and analyzed using Ethovision 7.1 XT video tracking software (Wageningen, The Netherlands) set at 6 samples/sec.

4.5. Contextual fear learning, memory, and extinction

Acquisition, retention, and extinction of hippocampus-dependent contextual fear conditioning was assessed using the Med Associates Video Freeze automated scoring system (Med Associates, St. Albans, VT, USA). Freezing is a recognized indicator of a conditioned fear response and is defined as the absence of motion, with the exception of respiration. On the training day, mice were placed in the chamber with a house light (100 lx) and allowed 120 s to acclimate. This was followed by two tone-shock pairings. Each tone was 80 dB at 28000 Hz and lasted for 28 s, and co-terminated with a 2 s shock at 0.6 mA, with a 120 s interval in between the two tone-shock pairings. The day after training, mice were placed back in the training environment for 300 s during which no tone or shock were administered. This procedure was repeated for a total of 10 days during which no tone or shock were administered.

4.6. Statistical analysis

All data are shown as mean $\pm$ standard error of the mean (SEM). Statistical analyses were performed using SPSSTM software (Chicago, IL). The data were analyzed using Analysis of Variance (ANOVA) with genotype, sex, and parental carrier as the between group factors, followed up by post-hoc tests when appropriate, as indicated. Our initial analysis revealed a parental carrier $\times$ sex interaction ($F = 5.579$, $p = 0.019$), a parental carrier $\times$ genotype $\times$ sex $\times$ treatment interaction ($F = 4.157$, $p = 0.043$) and a trend towards a parental carrier $\times$ treatment interaction ($F = 2.237$, $p = 0.084$) for freezing during the baseline period in the fear conditioning test, a parental carrier $\times$ platform location interaction ($F = 3.341$, $p = 0.002$), effect of parental carrier ($F = 6.006$, $p = 0.015$) and a trend towards a parental carrier $\times$ genotype $\times$ sex $\times$ treatment interaction ($F = 2.325$, $p = 0.075$) for cumulative distance to the target location during the visible platform trials in the water maze, and a trend

towards a parental carrier $\times$ genotype $\times$ sex interaction ($F = 2.952$, $p = 0.087$) for cumulative distance to the target location during the hidden platform trials in the water maze, and a trend towards a parental carrier $\times$ dose interaction for home cage activity during the dark period ($F = 2.459$, $p = 0.064$) and for the ratio activity during the dark and light period ($F = 2.264$, $p = 0.082$). Based on these results, treatment effects in each MC and PC genotype were analyzed separately. Performance during training to locate a particular platform location in the water maze and extinction of contextual fear memory were analyzed using repeated-measures ANOVAs. If a violation of sphericity occurred indicating that the variances of the differences between all combinations of the groups were not equal, Greenhouse-Geisser corrections were used. Tukey's and Dunnett's multiple comparison post-hoc tests and were used, as indicated. All figures were generated using GraphPad Prism software (San Diego, CA). We considered $p < 0.05$ as statistically significant.

CRedit authorship contribution statement

Destine Krenik: Data curation, Formal analysis, Methodology, Project administration, Validation, Visualization, Writing – original draft. **Joseph B. Weiss:** Conceptualization. **Jacob Raber:** Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Supervision, Validation, Visualization, Writing – original draft.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.brainres.2021.147594>.

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