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Pharmacological Alk Inhibition to Mitigate Behavioral Changes and Cognitive Injury in Adolescent and Adult NF1 Mutant Mice

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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 sub-chronic 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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Introduction

Neurofibromatosis type 1 (NF1) is a genetically determined neurodevelopmental disorder and tumor syndrome with an incidence of approximately 1/3000 live births. It is caused by loss of function mutations in the neurofibromin gene (*NF1)* and is estimated to effect 100,000 people in the US. Approximately 50% of the patients inherited the condition in an autosomal dominant pattern; the remaining 50% are the result of *de novo* mutations. Behavioral alterations and cognitive deficits have been found in 50-70% of children with NF1. These behavioral alterations and cognitive deficits include specific problems with attention, visual perception, language, learning, attention, and executive function. They are observed in the absence of tumors or macroscopic structural abnormalities in the central nervous system [1-6]. No effective treatments for the behavioral and cognitive disabilities of NF1 exist.

Disordered sleep is another, possibly related, phenotype associated with NF1. Pediatric patients have been documented to have problems with initiating and maintaining sleep, arousal, sleep-wake transition and hyperhidrosis [7-9]. This aspect of NF1 has not been studied extensively either in humans or in model systems. Recent results pertaining to the role of Anaplastic Lymphoma Kinase (Alk) in relation to NF1 and sleep in Drosophila provide a strong justification to investigate this in heterozygous NF1 mutant mice with a particular focus on the possible therapeutic benefit of Alk inhibition in mitigating this phenotype [10].

Positional cloning and sequence analysis of the *NF1* gene revealed it to be a negative regulator of the small GTPase, Ras, a proto-oncogene and activator of the mitogen activated protein kinase (MAP kinase) cascade [11-15]. When Ras is activated, typically by a receptor tyrosine kinase, it localizes to the plasma membrane and binds GTP. Hydrolysis of GTP to GDP converts Ras from an active to an inactive form. Neurofibromin is a Ras-GTPase activating protein that catalyzes the conversion of active GTP-Ras to inactive GDP-Ras. Based on its structure, Neurofibromin was predicted to be a direct negative regulator Ras and in turn of the MAP kinase signal transduction cascade. This biochemical function of neurofibromin is strongly supported by the tumor suppressor phenotype of *NF1* mutations [16].

Inappropriate hyperactivation of the Ras-MAP kinase cascade by mutation of *NF1* has been confirmed in humans and murine models [17-23]. Pharmacologic inhibition, even transiently, of the RAS-MAP kinase mice has ameliorated several developmental phenotypes linked to *NF1* mutation. These developmental phenotypes include abnormal cell fate determination in juvenile and adult neurogenesis and cerebellar development [24]. The cognitive impairments observed in heterozygous *NF1* mice have also been rescued by

inhibition of the Ras-MAP kinase cascade [25]. The observation that NF1 is caused by haplo-insufficiency or partial loss of function of the *NF1* gene implies that the phenotype may be amenable to interventions that modulate the signaling pathway in which it acts.

In 2011, Gouzi et al. and Walker and Bernards observed genetic interactions between Drosophila *NF1* (*dNF1)* and Drosophila Anaplastic Lymphoma Kinase (*dAlk)*, a receptor tyrosine kinase and activator of the Ras-Map kinase cascade [26, 27]. *dAlk* is the homologue of the human proto-oncogene Anaplastic Lymphoma Kinase (Alk). Alk was originally identified as an oncogene that is inappropriately activated by chromosomal translocation in anaplastic lymphomas [28]. Subsequently it was determined to cause neuroblastoma, non-small cell lung cancer and inflammatory myofibroblastic sarcoma [29-33]. It is also a likely culprit in some renal cell, breast, esophageal, colonic, glial and thyroid cancers [34]. As a frequently mutated or dis-regulated gene implicated in a variety of human tumors, Alk has been therapeutically targeted by orally active small molecule inhibitors [35, 36]. First generation Alk inhibitors have demonstrated efficacy against these tumors and are in clinical use. Crizotinib, the first FDA-approved treatment for non-small cell lung cancer, is very active against the primary tumors but has limited or no penetration of the brain. Cancer that has metastasized to the brain cannot be treated well with this compound. Newer agents such as alectinib have proven central nervous system activity and efficacy treating metastatic disease in the brain that is resistant to treatment with crizotinib [37, 38].

The identification of Alk as a physiologically relevant receptor tyrosine kinase subject to negative regulation by neurofibromin coincident with the development of Alk inhibitors with CNS activity created the opportunity to test the hypothesis that these drugs may be therapeutically beneficial in NF1. Gouzi et al observed genetic interactions between *dAlk* and *dNf1* in Drosophila both with respect to regulation of body size and associative learning [26]. Their studies were the first to identify Alk as a physiologically relevant target of negative regulation by neurofibromin. They went on to show that pharmacologic inhibition of Alk rescued or corrected associative learning defects in heterozygous *dNF1* mutant flies. They also showed that increased expression of *dNF1* in cells that also express *dAlk* was sufficient to rescue the same defect in heterozygous *dNF1* mutant flies. This argues for a specific biochemical connection based on negative regulation of Alk signaling by neurofibromin. Walker and Bernards independently found similar evidence for a genetic interaction between *dNF1* and both *dAlk* and *jelly belly (jeb)* the dALK ligand [27].

Bai and Sehgal studied the roles of dAlk and dNF1 in the regulation of sleep in Drosophila [10]. Their findings have critical implications for sleep disturbances observed in humans with NF1. dAlk is a positive regulator of sleep in flies. *dAlk* mutants have substantially increased sleep while *dNf1* mutants have decreased sleep, a phenotype similar to what has been reported in humans. As in the studies of Gouzi et al., they also observed a genetic interaction between *dAlk* and *dNf1* [26]. Reduced dAlk signaling in the central nervous system of *dNf1* mutant flies rescued or reversed the sleep phenotype just as it does the associative learning phenotype. Predicated on these studies, we have started to analyze diurnal activity of mice with reduced Alk and NF1 activity. Our preliminary data confirm similar phenotypes in mice and support the hypothesis that pharmacologic Alk inhibition will be beneficial for sleep disturbances in humans with neurofibromatosis 1.

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-9670-08af08adcffb; http://www.cancer.gov/about-cancer/treatment/drugs/alectinib; http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm476926.htm). In this project, we look 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 sub-chronic 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 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. More specifically, we are testing 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.

Key words

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

Accomplishments

In Year 3, 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 are 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 is always on a B6 background, and the non NF1 carrier is on a SVJ background

. Table 1 illustrates the mice of these breeding effort treated with jellies containing no inhibitor (control) or the Alk inhibitor at the indicated doses and behaviorally and cognitively tested. Below we describe the analyses. We are currently drafting the corresponding manuscript.

PC	0 mg/kg	3.6 mg/kg	10 mg/kg	20 mg/kg
HET F	10	10	10	10
HET M	10	10	11	10
WT F	6	6	6	6
WT M	9	11	8	10

MC	0 mg/kg	3.6 mg/kg	10 mg/kg	20 mg/kg
HET F	6	7	7	7
HET M	9	11	9	10
WT F	9	10	9	10
WT M	10	8	10	9

The results of the mice behaviorally and cognitively tested are discussed below.

Although not included in the grant proposal, we requested Alk deficient (KO) mice on the C57BL/6J background from Dr. Liliana Attisano at the University of Toronto to determine whether the Alk inhibitor might also have off target effects in Alk deficient mice. Using 4 breeding cages, we tested 58 mice with control jellies or jellies containing 20 mg/kg. The breakdown of female and male mice as part of this breeding effort is shown in Table 2. As we described these data data in the progress report from last yeatr, we do not fully discuss them here again.

Table 2. Number of offspring resulting from breeding effort involving Alk KO female and male mice on the C57BL/6J background.

	Control	**20 mg/kg**	***Total***
M	16	15	*31*
F	13	14	*27*
	29	*29*	*58*

Circadian Activity Levels

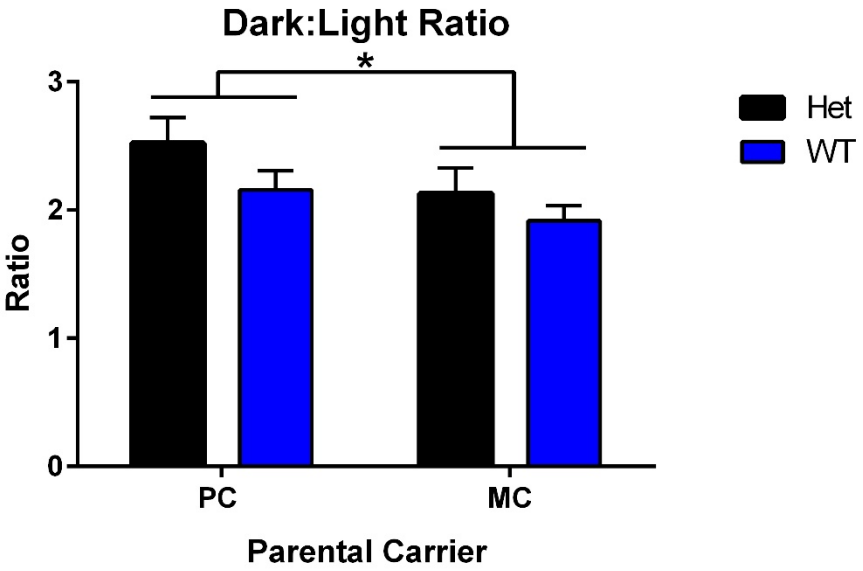


Fig. 1. Ratio activities of PC and MC NF1+/- and wild-type mice in the home cage.

Fig. 1 illustrates the ratio of circadian activity levels in the active (dark) and inactive (light) periods. The ratio values were lower in the mice of the maternal than paternal breeding effort (p = 0.042). In addition, there was an effect of sex (p = 0.013).

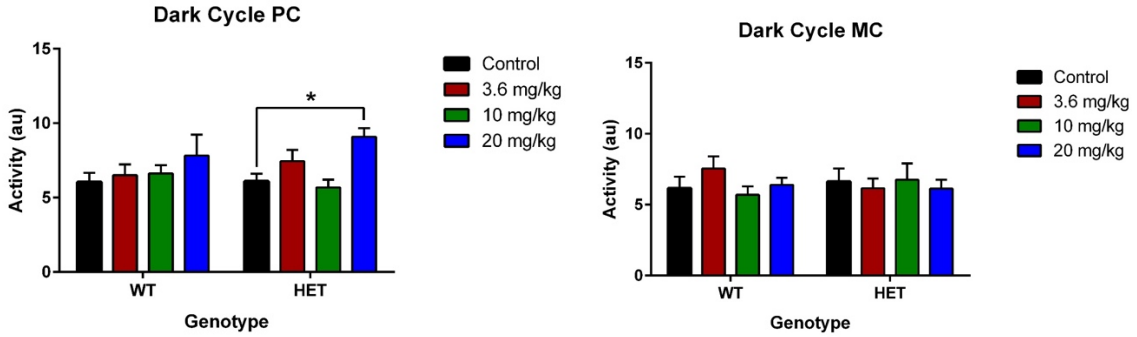


Fig. 2. Effects of pharmacological Alk inhibition on home cage activity of NF1+/- (HET) and wild-type (WT) mice part of the PC (left) and MC (right) breeding effort during the dark periods. *p = 0.002.

For the PC breeding effort, there was an effect of dose (p = 0.004) and an effect of sex (p = 0.015). When the genotypes were split up for analysis, there was an effect of dose (p = 0.001) and an effect of sex (p = 0.044) in the NF1+/- mice. Activity levels were higher in mice that received the Alk inhibitor at 20 mg/kg than in the controls (p = 0.002, Dunnett's). In contrast no treatment effects were seen in the MC breeding effort, either overall or when the genotypes were analyzed separately. An overall sex difference was seen (p = 0.039) and a sex difference was seen in the NF+/1 (p = 0.003) and WT (p =0.039) mice individually as well.

We also analyzed the ratio of activity in the dark and light periods (Fig. 3). For the MC breeding effort, there was an effect of dose (p = 0.038) and sex (p < 0.001) and a genotype x dose x sex interaction (p =0.044). When the genotypes were analyzed separately, the ratio activity was higher in WT mice that received the Alk inhibitor at 3.6 mg/kg than the control (p = 0.026, Dunnett's). There was also an effect of sex in NF1+/- (p = 0.003) and WT (p =0.045) mice. For the PC breeding effort, there was only a trend towards an effect of sex (p = 0.098) but it did not reach significance.

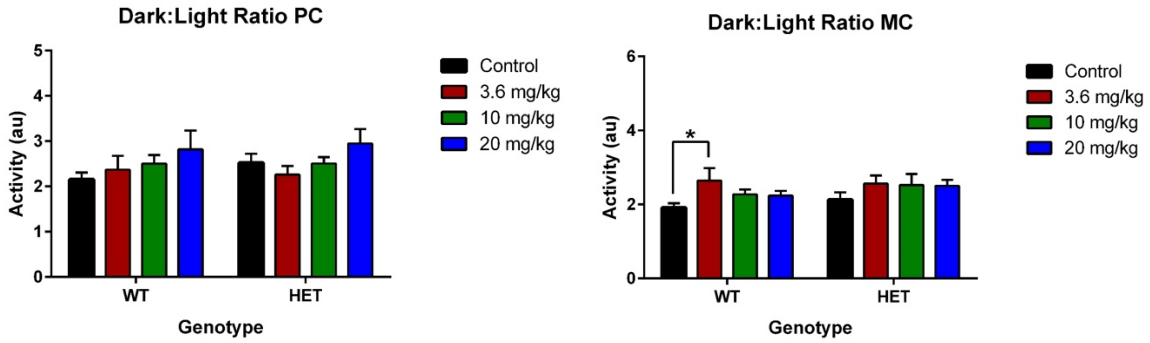


Fig. 3. Effects of pharmacological Alk inhibition on home cage ratio activity of NF1+/- (HET) and wild-type (WT) mice part of the PC (left) and MC (right) breeding effort. *p =0.026.

Overall, these data show that Alk inhibition at a dose of 20 mg/kg increases activity levels of NF1+/- mice during the active period as part of the PC, but not MC, breeding effort. In the MC, but not PC, breeding effort, the lowest dose of the Alk inhibitor used (3.6 mg/kg) increase the ratio activity in the WT but not NF1+/- mice.

In Alk KO mice, no effects of the Alk inhibitor, at 20 mg/kg, were seen in male mice during the light cycle but a trend towards decreased activity in the dark period (Fig. 4). In contrast, in female mice, there was a pattern suggesting increased activity following Alk inhibition in both the light and dark periods (Fig. 4). We next analyzed the ratio of activity in the dark and light periods (Fig. 5). While there was a trend towards a lower ratio of dark/light activity in male Alk KO mice, no alterations in the ratio activity measure were seen in female mice.

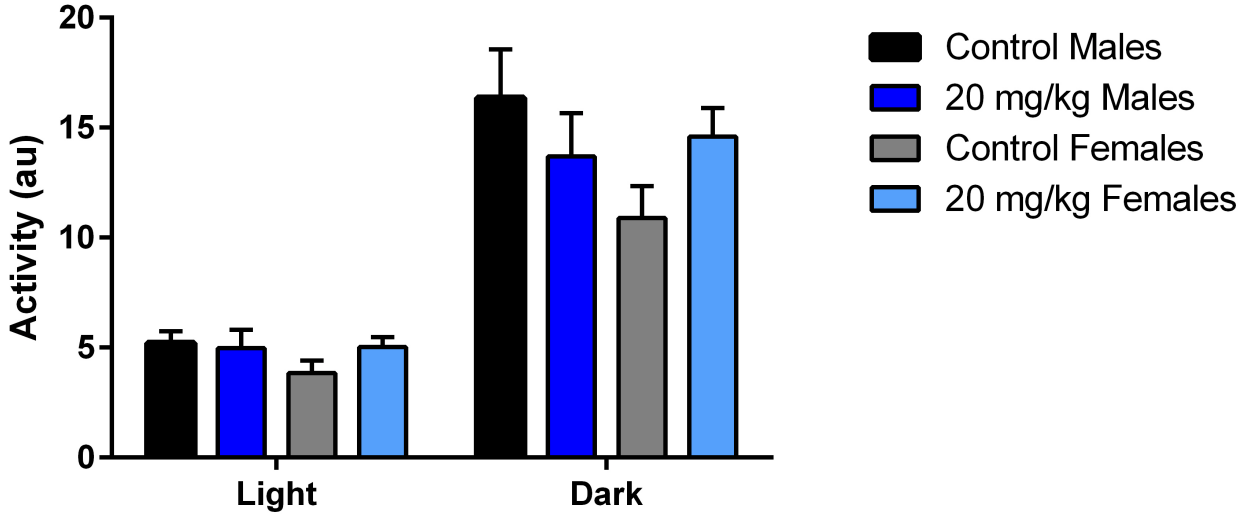


Fig. 4. Effects of pharmacological Alk inhibition on home cage activity of Alk KO female and male mice during the light (left panel) and dark (right panel) cycle. For details, see text.

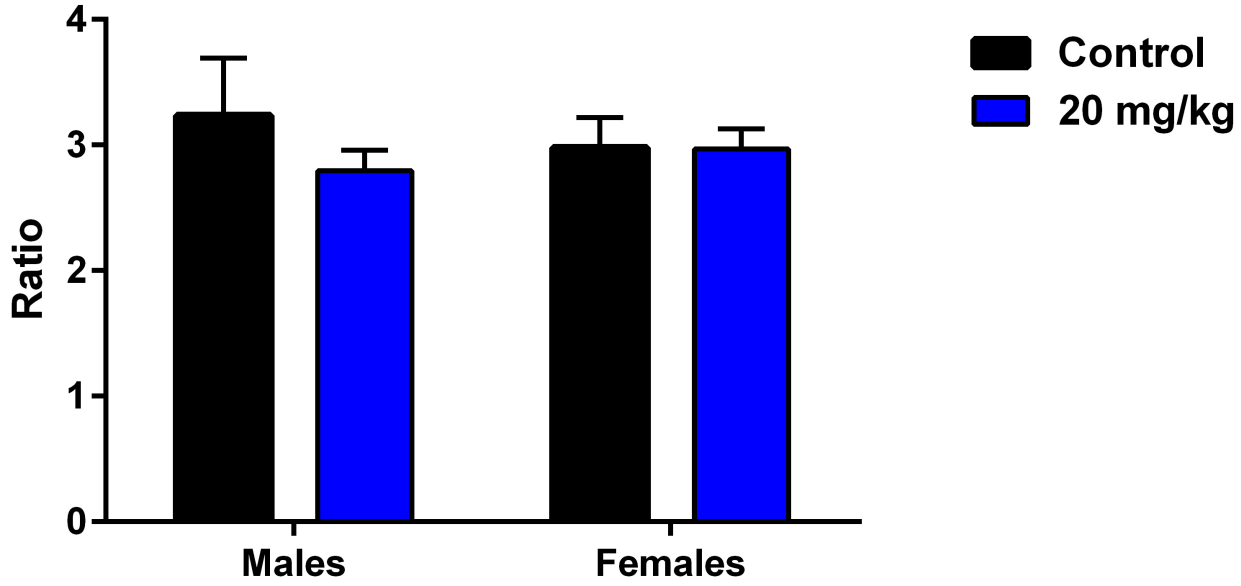


Fig. 5. Effects of pharmacological Alk inhibition on the ratio of activity in the dark and light periods in the home cage of Alk KO male (left panel) and female (right panel). For details, see text.

Spatial learning and memory in the water maze

Next spatial learning and memory was assessed in the water maze. The test was performed over 5 days. The experimental design is illustrated in Fig. 6. On day 1, the mice are trained to locate a visible platform location. There is one session, consisting of 2 trials each) in the morning and session in the afternoon. All visible and hidden platform training trials last up to 60 s. In case a mouse does not locate the platform within 60 s, it is guided to the platform by the researcher. On days 2-5, the mice are trained to locate a hidden platform. There are 2 sessions per day, one in the morning and one in the afternoon, each consisting of 2 trials each. At the beginning of each hidden platform training day, the platform is moved to a new quadrant. On days 3, 4, and 5, a probe trial (no platform) is administered in the morning prior to the 2 daily hidden platform sessions. The probe trial lasts 60 s each. In the probe trial, swimming on average closer to the platform location, indicated as a lower cumulative distance to the target, reflects better performance.

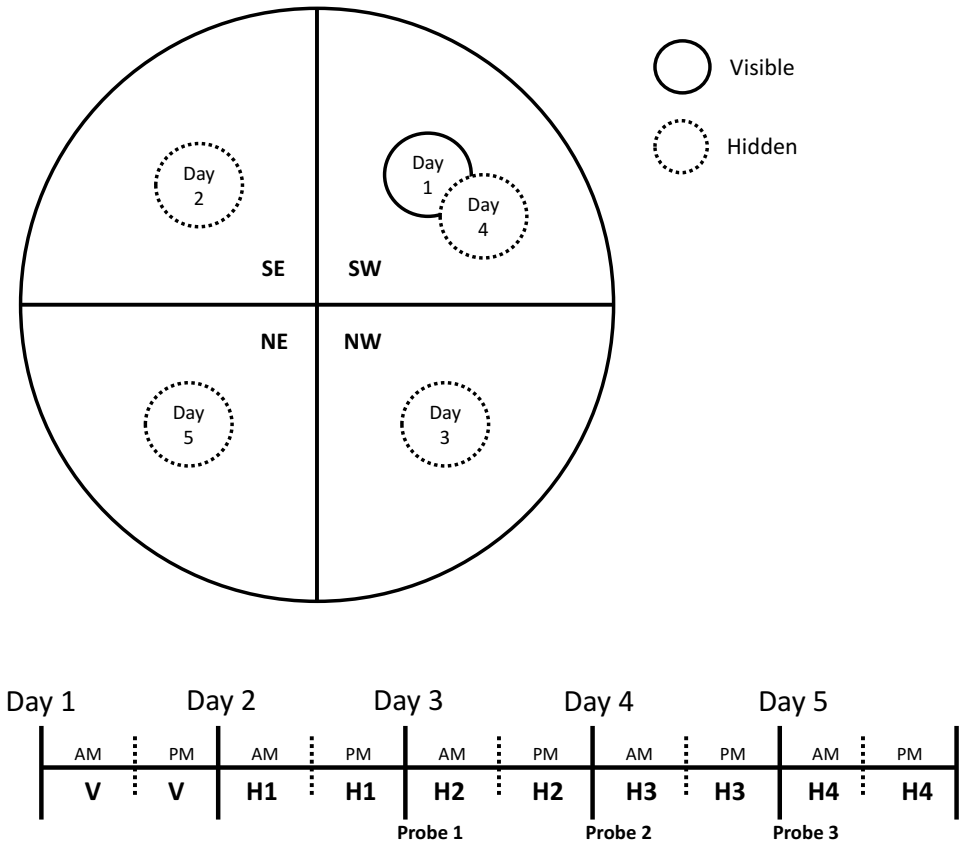


Fig. 6. Design of the spatial learning and memory test in the water maze.

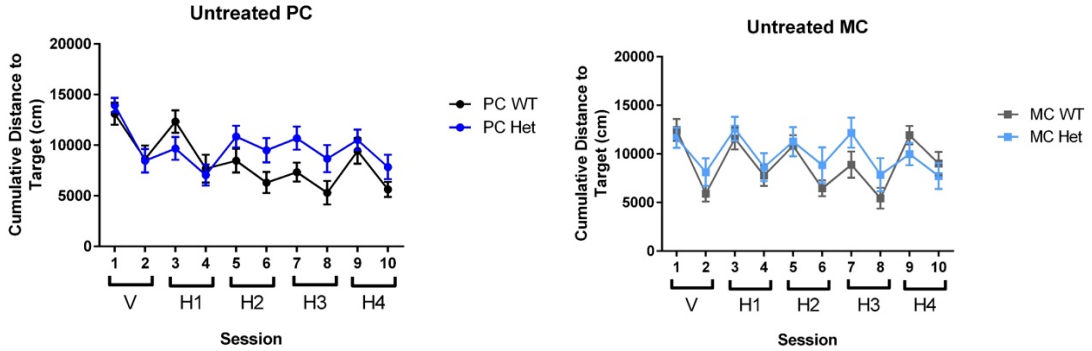


Fig. 7. Water maze performance of PC and MC NF1+/- and wild-type mice.

When performance in the water maze was analyzed (Fig. 7), using cumulative distance to the platform as performance measure, there was a genotype x session interaction (p =0.041) and an effect of session (p < 0.001). There was also a trend towards a genotype x sex x session interaction (p = 0.059). There was an effect of genotype during the training to locate the second hidden platform location (p =0.072) and an effect of genotype (p = 0.011) and a trend toward a session x breeding effort interaction (p =0.063) for the training to locate the third hidden platform location. Overall, the cognitive phenotype was clearly more pronounced in NF1+/- mice part of the PC than MC breeding effort. In addition, it is clear that the NF1+/- mice have a hard time learning a new hidden platform location, suggesting an impairment in executive function and involving the prefrontal cortex.

Next we assessed effects of Alk inhibition on performance of NF1+/- and WT mice of mice part of the PC breeding effort in the water maze (Fig. 8). For the visible platform task learning (cued training), there was a sex x dose interaction (p = 0.033). For the hidden platform training (spatial learning), there was a session x dose interaction (p = 0.026). We next analyzed the learning curves of the distinct hidden platforms separately. In NF1+/- mice, there was an effect of dose during training for the 4th hidden platform location (p = 0.034); NF1+/- mice treated with the Alk inhibitor at the 3.6 mg/kg dose performed better than the control group (p = 0.034, Dunnett's). Based on the effects of sex, we also analyzed the female and male data separately (Fig. 9). In male NF1+/- mice, there was an effect of dose (p = 0.048), and a session x dose interaction (p = 0.031).

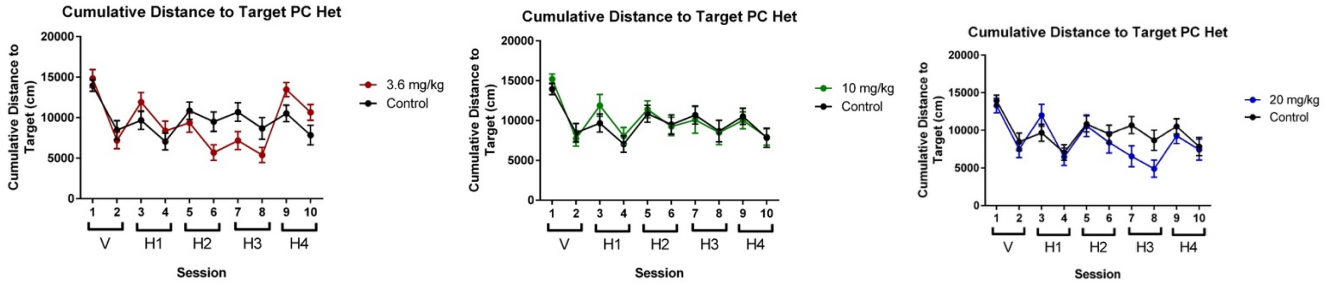


Fig. 8. Effect of pharmacological Alk inhibition on spatial learning of NF1+/- mice part of PC breeding effort.

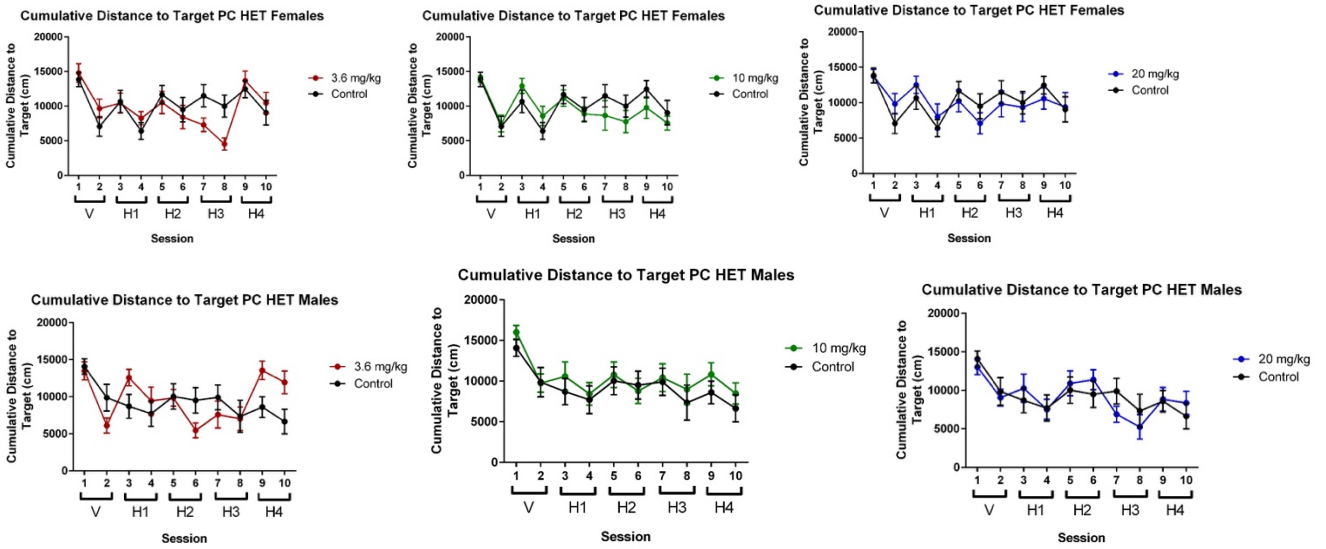


Fig. 9. Effect of pharmacological Alk inhibition on spatial learning of NF1+/- female (top panels) and male (bottom panels) mice part of MC breeding effort.

When we assessed effects of Alk inhibition on performance of NF1+/- and WT mice of mice part of the MC breeding effort in the water maze (Fig. 10), there was a session x sex x dose interaction for visible platform training (p = 0.016) and a trend towards a session x dose interaction (p = 0.095). For NF1+/- mice, there was a trend towards a session x dose interaction for *training* to locate the first hidden platform location (p = 0.057) and fourth hidden platform location (p = 0.078).

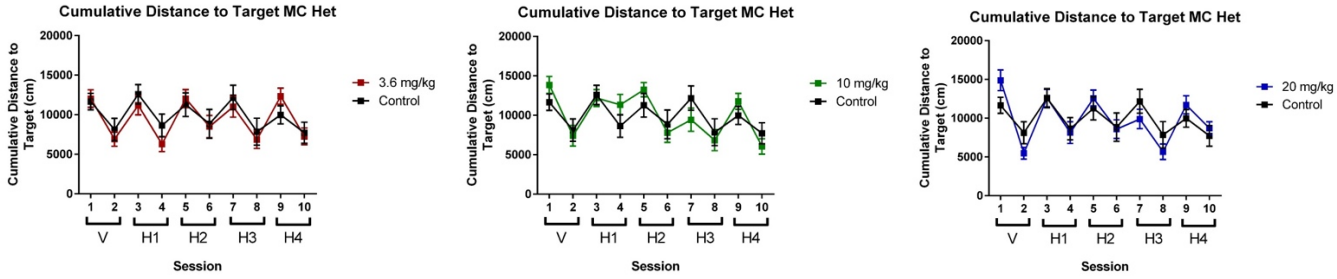


Fig. 10. Effect of pharmacological Alk inhibition on spatial learning of NF1+/- mice part of MC breeding effort.

When we assessed the female and male NF1+/- mice, separately there was a session x dose interaction (p = 0.006); mice treated with the 20 mg/kg dose of the Alk inhibitor performed more poorly than the control in the first visible platform session (not shown). No treatment effects were seen in NF1+/- mice as part of the MC breeding effort.

In WT mice part of the PC breeding effort, a trend towards a session x dose interaction was observed for visible platform training. The mice treated with the Alk inhibitor at the 3.6 mg/kg dose performed better than control mice in the first visible platform session (not shown). During hidden platform training, there was a trend towards an effect of sex (p = 0.064). When the learning curves of the distinct hidden platform locations were analyzed separately, there was a trend towards an effect of sex during the first hidden platform training (p = 0.052) and a trend towards an effect of dose during the second hidden platform training (p = 0.091)(not shown).

In WT mice part of the MC breeding effort, there was a session x sex x dose interaction for visible platform training (p = 0.020)(not shown). There was also a trend towards an effect of sex for the first hidden platform training (p = 0.075). When the female and male data were analyzed separately, there was a trend towards a session x dose interaction in the females (p = 0.057). This was not seen in the male mice.

When time to reach the platform (latency) was analyzed as performance measure, there was a session x sex x genotype interaction for visible platform training (p = 0.023). In NF1+/- mice bred as part of the PC breeding effort, there was a sex x dose interaction for visible platform training (p = 0.033) and a trend towards a session x dose interaction for hidden platform training (p = 0.096) (Fig. 11). Similar for what was seen for the cumulative distance analysis in Fig. 9, there was a beneficial effect of the Alk inhibitor at the lowest dose used (3.6 mg/kg) during the third hidden platform location and for the latency outcome measure also during the second hidden platform location. When we analyzed performance of NF1+/- mice part of the MC breeding effort, there was a session x sex x dose interaction for visible platform training (p = 0.041).The beneficial effect of Alk inhibition during the third hidden platform training of NF1+/- mice part of the PC breeding effort was not seen in NF1+/- mice part of the MC breeding effort (Fig. 12) but a slight beneficial effect was seen during the first hidden platform training.

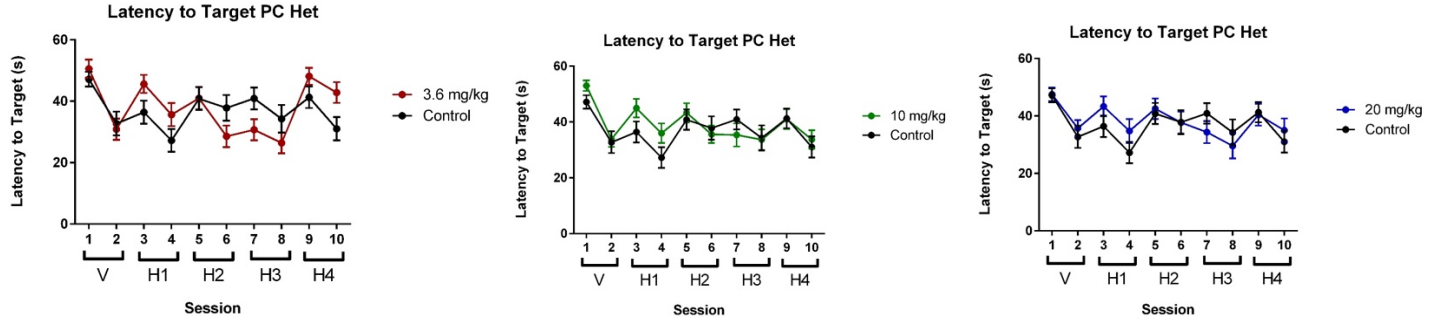


Fig. 11. Effect of pharmacological Alk inhibition on spatial learning of NF1+/- mice part of PC breeding effort.

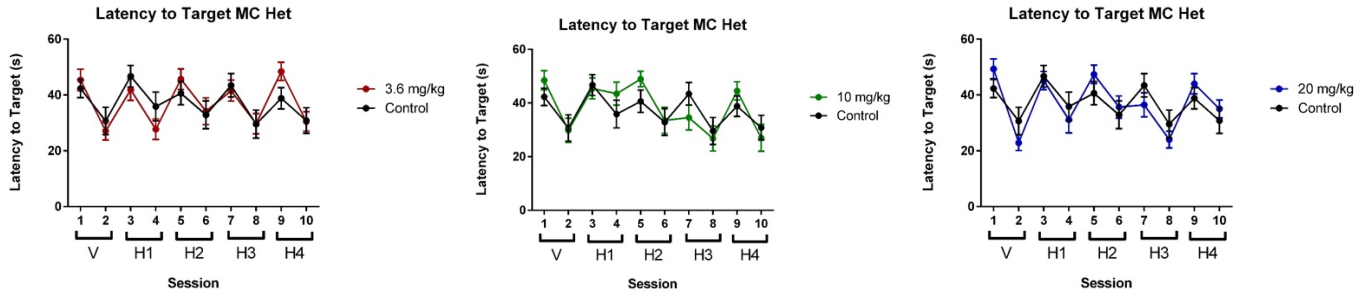


Fig. 12. Effect of pharmacological Alk inhibition on spatial learning of NF1+/- mice part of the MC breeding effort.

In WT mice part of the PC breeding effort, there was no effect of Alk inhibition during visible or hidden platform training (not shown). There was a trend towards an effect of sex during hidden platform training but that did not reach significance (p = 0.096). In WT mice part of the MC breeding effort, there was a session x sex x dose interaction for visible platform training (p = 0.016).

Finally, as swim speeds can affect the latency and cumulative distance performance measures, we analyzed swim speeds during visible platform training. There was a genotype x breeding effort interaction (p = 0.029), an effect of breeding effort (p = 0.020), and an effect of genotype (p < 0.001). In NF1+/- mice bred as part of the PC breeding effort, there was no effect of Alk inhibition on swim speeds. There was a trend toward an effect of dose on swim speeds of WT mice bred as part of the PC effort but it did not reach significance (p = 0.095). In NF1+/- mice bred as part of the MC breeding effort, there was a session x sex x dose interaction (p = 0.001). There was a sex x dose interaction for swim speeds of WT mice bred as part of the MC effort (p = 0.043).

Next, spatial memory retention was assessed in the probe trials (no platform). Using cumulative distance to the platform location pertinent to the last hidden platform location prior to the probe trial as performance measure, there was a genotype x probe trial interaction (p = 0.012) (Fig. 13). When the genotypes were analyzed separately, there was an effect of probe trial in WT (p = 0.004) but not NF1+/- mice (p = 0.1). Interestingly, there was a step-wise decrease in performance across the three probe trials in NF1+/- mice, while in WT mice, performance in the first and third probe trials seemed similar.

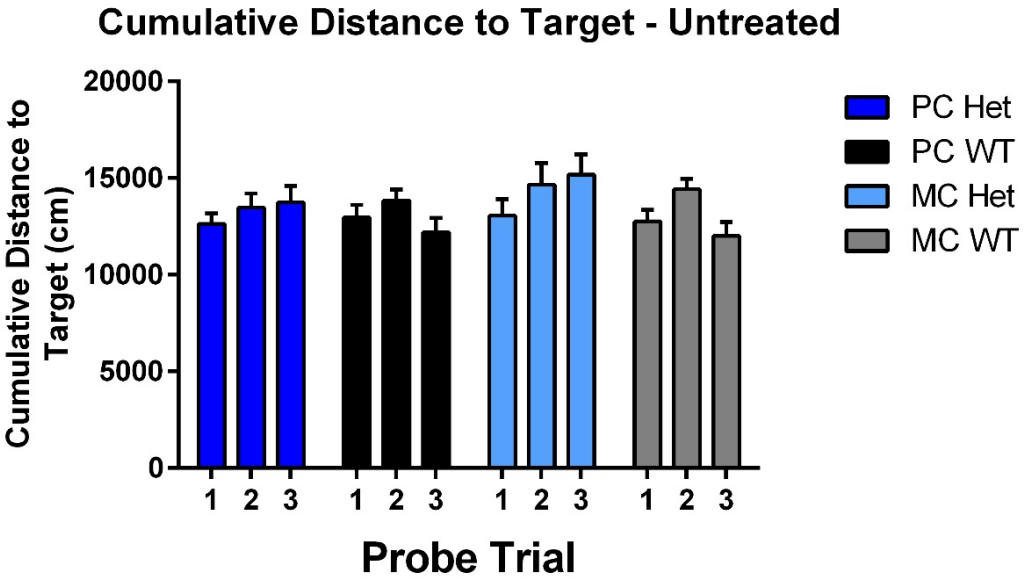


Fig. 13. Spatial memory retention of NF1+/- and WT mice part of the HC and MC breeding efforts.

Recognizing that with distinct hidden platform locations, the interpretation of the second and third probe trials are more complicated as the mice might search for the platform in an earlier hidden platform location than the most recently trained one. Therefore, we also analyzed the cumulative distance to the four distinct platform locations (Fig. 14). Interestingly, there seemed an overall genotype difference; the pattern seen in NF1+/- and WT mice in the three probe trials seemed distinct. In general, as lower cumulative distance values reflect better performance, the performance seems better in WT than NF1+/- mice.

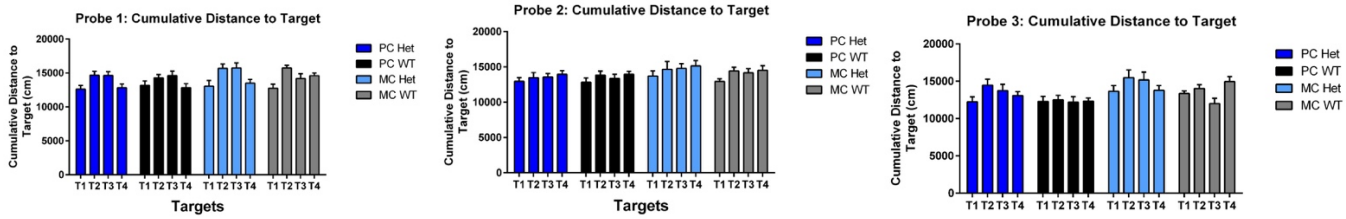


Fig. 14. Spatial memory retention of NF1+/- and WT mice part of the HC and MC breeding efforts. T1-T4 indicate the four distinct hidden platform locations.

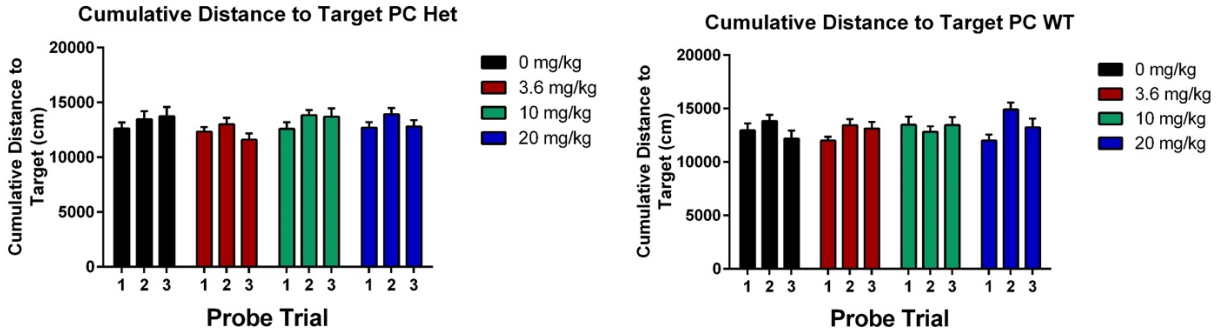


Fig. 14. Spatial memory retention of NF1+/- and WT mice part of the HC breeding effort in the water maze.

In NF1+/- mice part of the PC breeding effort, there was an effect of probe trial (p = 0.025) (Fig. 14, left panel). In WT mice as part of the PC breeding effort, there was a trend towards an effect of probe trial (p = 0.051) and a trend towards a probe trial x dose interaction (p = 0.096) (Fig. 14, right panel). In NF1+/- mice part of the MC breeding effort, there was an effect of probe trial (p = 0.025) (Fig. 15, left panel). In WT mice as part of the PC breeding effort, there was a probe trial x dose interaction (p = 0.017) and a trend towards an effect of probe trial (p = 0.055) (Fig. 15, right panel).

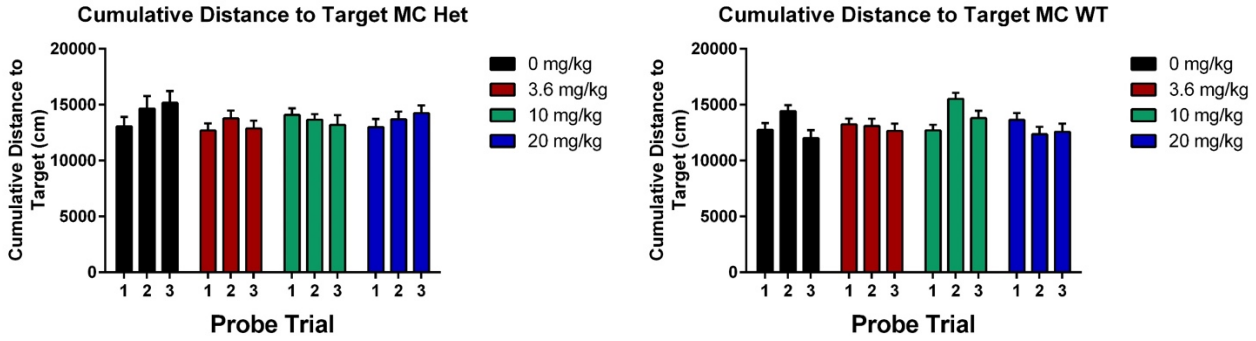


Fig. 15. Spatial memory retention of NF1+/- and WT mice part of the MC breeding effort in the water maze.

In NF1+/- mice of both the PC and MC breeding efforts, mice treated with the Alk inhibitor ar the 3.6 mg/kg dose showed better performance than the controls. This was not seen in WT mice.

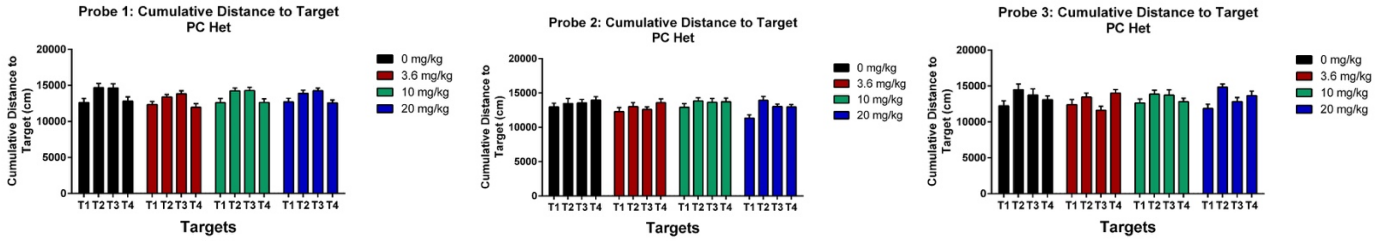


Fig. 16. Spatial memory retention of NF1+/- mice part of the PC breeding effort. T1-T4 indicate the four distinct hidden platform locations.

Next, we analyzed the cumulative distance to the four distinct platform locations (Fig. 16). In general, NF1+/- mice part of the PC breeding effort treated with the 3.6 mg/kg dose of the Alk inhibitor show somewhat lower cumulative distance to the target values. This was seen to a lesser extent in NF1+/- mice part of the MC breeding effort (Fig. 17) or WT mice part of either breeding effort (not shown).

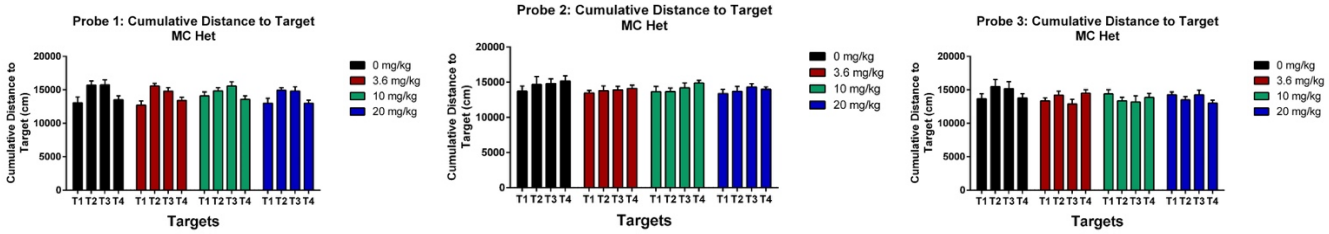


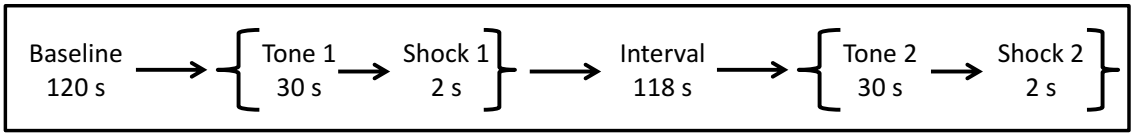
Fig. 17. Spatial memory retention of NF1+/- mice part of the MC breeding effort. T1-T4 indicate the four distinct hidden platform locations.

In Alk KO mice, there were no clear effects of the Alk inhibitor, at 20 mg/kg, seen on spatial learning and memory or spatial memory retention in the water maze in our preliminary analysis (not shown).

Finally, the mice were tested for contextual fear learning, contextual fear memory, and extinction of contextual fear memory. The experimental paradigm is illustrated in Fig. 18.

Fear Conditioning Paradigm

- 11 days total, 1 session per day per mouse. Each session lasts a total of 5 minutes.
  - Day 1: Training
    - 0:00-2:00 = Baseline, followed by 30-second Tone 1, followed by 2-second Shock 1.
    - 2:33-4:31 = Interval, followed by 30-second Tone 2, followed by 2-second Shock 2.



  - Days 2-11: Extinction
    - Mice are placed in the chamber for a total of 5 minutes (no tones or shocks).

Fig. 18. Experimental paradigm of acquisition, memory, and extinction of contextual fear.

There was an effect of genotype on the activity of the mice prior to the tone during fear learning ($p = 0.037$) (Fig. 19, left panel). In addition, during the two shocks there was a shock x sex x genotype interaction (p = 0.028), a sex x genotype interaction ($p = 0.030$), an effect of sex ($p = 0.004$), and an effect of breeding effort ($p < 0.001$). When the percent freezing was analyzed, there was an effect of genotype for freezing during the baseline ($p = 0.049$) (Fig. 19, right panel).

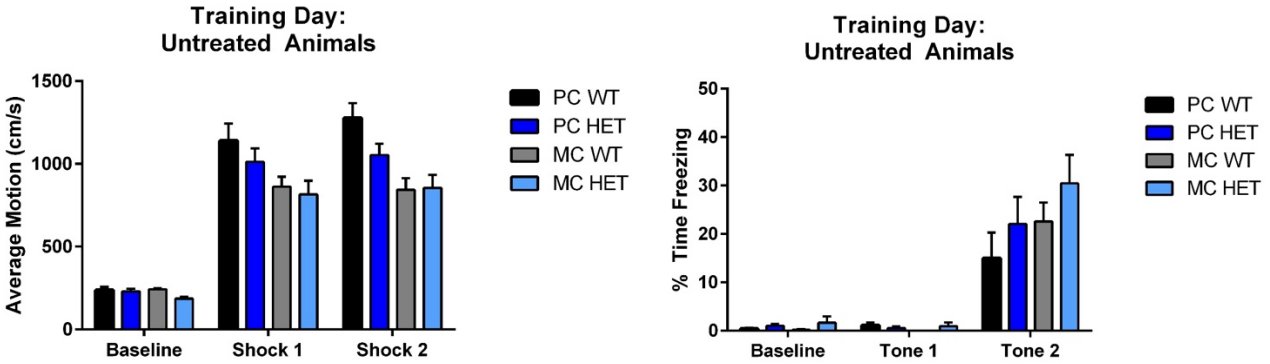


Fig. 19. Activity levels (left panel) and percent freezing (right panel) of NF1+/- and WT mice of the PC and MC breeding efforts.

While in NF1+/- mice bred as part of the PC breeding effort there was no effect of Alk inhibition on activity levels at baseline or during the shock, in WT mice there was an effect of dose ($p = 0.008$), and trend towards an effect of sex ($p = 0.066$) and shock x sex interaction ($p = 0.093$) (Fig. 20). In NF1+/- mice bred as part of the MC breeding effort, there was an effect of Alk inhibition on activity levels during the baseline period ($p = 0.012$). Activity levels were higher in mice that received the Alk inhibitor at 10 mg/kg than those in the control mice.

While in NF1+/- mice bred as part of the PC breeding effort there was no effect of Alk inhibition on freezing levels at baseline, in WT mice there was an effect of dose ($p = 0.045$), sex ($p = 0.016$), and a sex x dose interaction ($p = 0.045$) (not shown). Freezing levels were higher in WT mice that received the Alk inhibitor at 10 mg/kg than control mice ($p = 0.020$, Dunnett's). In NF1+/- mice bred as part of the MC breeding effort, there was an effect of dose ($p = 0.010$), and a

trend towards a dose x tone interaction (p = 0.088) for freezing during the tones. In WT mice bred as part of the MC breeding effort, there was only an effect of sex (p = 0.018).

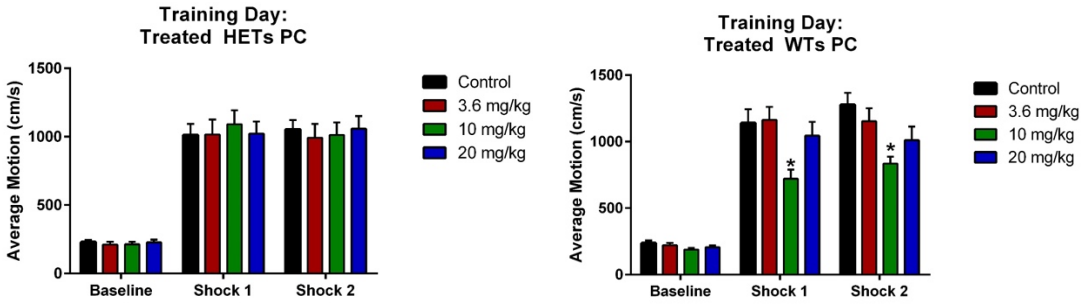


Fig. 20. Activity levels of NF1+/- (left panel) and WT (right panel) mice of the PC breeding efforts during fear learning.

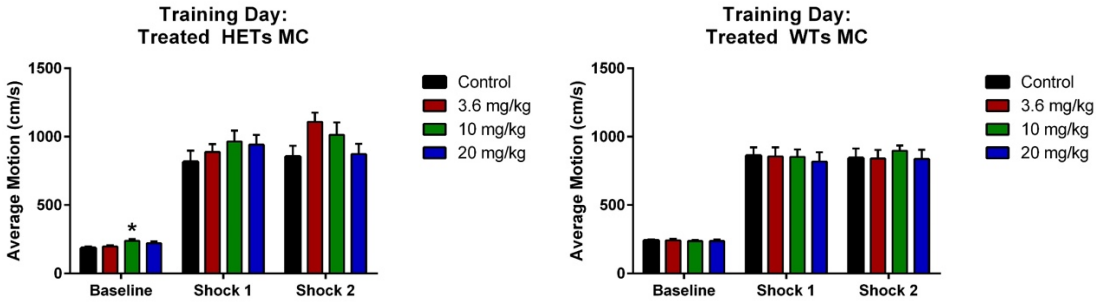


Fig. 21. Activity levels of NF1+/- (left panel) and WT (right panel) mice of the MC breeding efforts during fear learning.

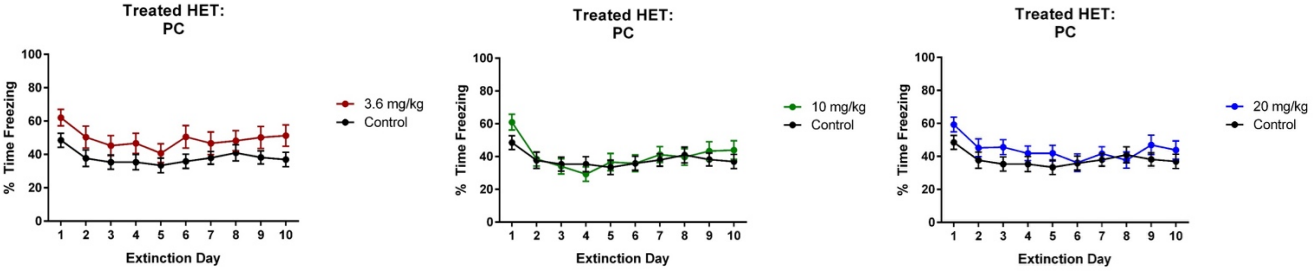


Fig. 22. Extinction learning of NF1+/- mice of the PC breeding effort.

When extinction of fear conditioning was assessed in NF1+/- mice part of the PC breeding, there was a trend towards a day x sex x dose interaction (p = 0.089), day x sex interaction (p = 0.089), and effects of sex and day (both, p < 0.001) (Fig. 22). In NF1+/- mice part of the MC breeding, there was a trend towards a day x sex interaction (p = 0.058), and effects of sex (p = 0.017) and day (p < 0.001) (Fig. 23). The fear extinction curves of the WT mice part of the PC and MC breeding efforts are shown in Figs. 24 and 25, respectively. In general, more profound treatment effects were seen in WT part of the PC than the MC breeding effort. We noted that most extinction occurred between days 1 and 5 and are in the process to only analyze the extinction curves for those days.

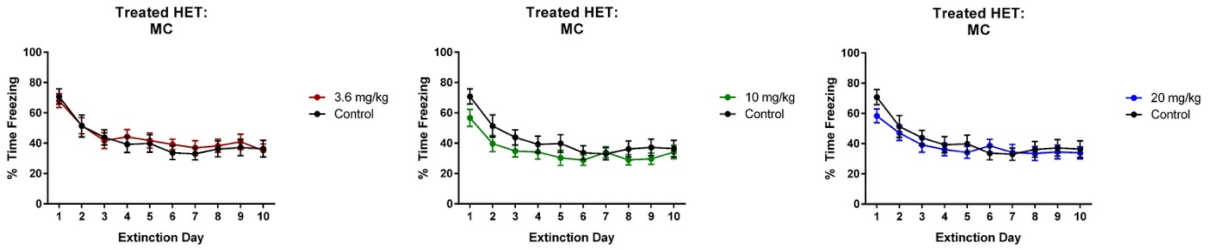


Fig. 23. Extinction learning of NF1+/- mice of the MC breeding effort.

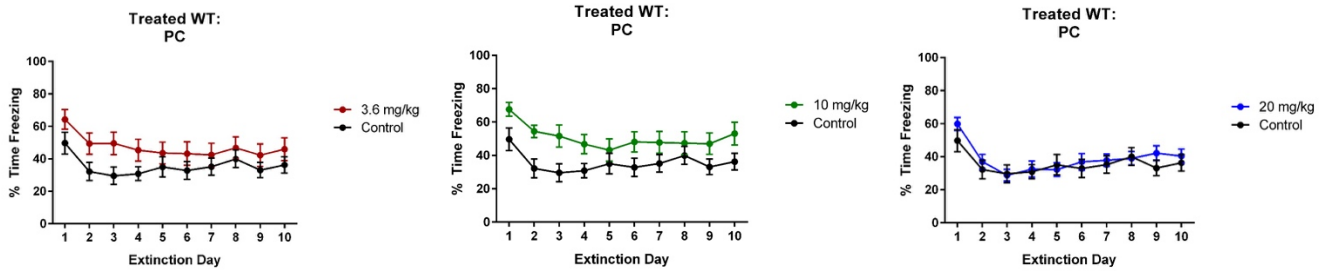


Fig. 24. Extinction learning of WT mice of the PC breeding effort.

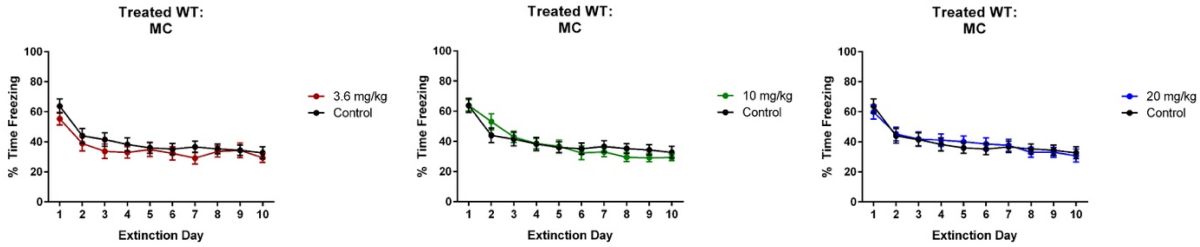


Fig. 25. Extinction learning of WT mice of the MC breeding effort.

In Yr 3, we also started the treatment and assessment of the long-term effects of Alk inhibition. Although initially we had planned for a shorter period of treatment prior to starting the behavioral testing, due to COVID-19 and modified operations, we requested permission to continue treating the mice as we were not able to work on campus to test the mice yet. In addition, Dr. Raber organized for shipment of NF1+/- and WT littermates to Legacy so that mice would be available to continue the project in case of reaching an emergency situation at OHSU. Fortunately, this scenario did not happen and recently these mice were shipped back to OHSU. For the first cohort of 33 mice on long-term treatment, the mice were treated for 24-25 weeks prior to the first behavioral test. This testing is currently ongoing. We are now planning for a second long-term treatment testing cohort.

Impact

The updated preliminary 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 carrier 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 are in the process of assessing the potential side effects of long-term treatment of the lowest dose(s) showing beneficial cognitive effects in NF1+/- mice.

Changes/problems

As described above, 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 have 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 included based on the generated data. 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.

Products

We are finalizing the pharmacological dose-response analyzes described above for a manuscript with these data we plan to submit in the near future.

References

1. North, K., et al., *Cognitive function and academic performance in children with neurofibromatosis type 1.* Dev Med Child Neurol, 1995. **37**: p. 427-36.
2. Acosta, M.T., G.A. Gioia, and A.J. Silva, *Neurofibromatosis type 1: new insights into neurocognitive issues.* Curr Neurol Neurosci Rep, 2006. **6**: p. 136-43.

3. Rosser, T.L. and R.J. Packer, *Neurocognitive dysfunction in children with neurofibromatosis type 1.* Curr Neurol Neurosci Rep, 2003. **3**: p. 129-36.
4. Hofman, K.J., et al., *Neurofibromatosis type 1: the cognitive phenotype.* J Pediatr, 1994. **124**(4): p. S1-8.
5. Hyman, S.L., A. Shores, and K.N. North, *The nature and frequency of cognitive deficits in children with neurofibromatosis type 1.* Neurology, 2005. **65**: p. 1037-44.
6. Zoller, M.E., B. Rembeck, and L. Backman, *Neuropsychological deficits in adults with neurofibromatosis type 1.* Acta Neurol Scand, 1997. **95**: p. 225-32.
7. Licis, A.K., et al., *Prevalence of Sleep Disturbances in Children With Neurofibromatosis Type 1.* J Child Neurol, 2013. **28**: p. 1400-1405.
8. Leschziner, G.D., J.F. Golding, and R.E. Ferner, *Sleep disturbance as part of the neurofibromatosis type 1 phenotype in adults.* Am J Med Genet A, 2013. **161A**: p. 1319-22.
9. Johnson, H., et al., *Psychological disturbance and sleep disorders in children with neurofibromatosis type 1.* Dev Med Child Neurol, 2005. **47**: p. 237-42.
10. Bai, L. and A. Sehgal, *Anaplastic Lymphoma Kinase Acts in the Drosophila Mushroom Body to Negatively Regulate Sleep.* PLoS Genet, 2015. **11**: p. e1005611.
11. Cawthon, R.M., et al., *A major segment of the neurofibromatosis type 1 gene: cDNA sequence, genomic structure, and point mutations.* Cell, 1990. **62**: p. 193-201.
12. Xu, G.F., et al., *The neurofibromatosis type 1 gene encodes a protein related to GAP.* Cell, 1990. **62**: p. 599-608.
13. Ballester, R., et al., *The NF1 locus encodes a protein functionally related to mammalian GAP and yeast IRA proteins.* Cell, 1990. **63**: p. 851-9.
14. Martin, G.A., et al., *The GAP-related domain of the neurofibromatosis type 1 gene product interacts with ras p21.* Cell, 1990. **63**: p. 843-9.
15. Xu, G.F., et al., *The catalytic domain of the neurofibromatosis type 1 gene product stimulates ras GTPase and complements ira mutants of S. cerevisiae.* Cell, 1990. **63**: p. 835-41.
16. Parada, L.F., *Neurofibromatosis type 1.* Biochim Biophys Acta, 2000. **1471**: p. M13-9.
17. Oliveira, A.F. and R. Yasuda, *Neurofibromin is the major ras inactivator in dendritic spines.* J Neurosci, 2014. **34**: p. 776-83.
18. Yunoue, S., et al., *Neurofibromatosis type I tumor suppressor neurofibromin regulates neuronal differentiation via its GTPase-activating protein function toward Ras.* J Biol Chem, 2003. **278**: p. 26958-69.
19. Bollag, G., et al., *Loss of NF1 results in activation of the Ras signaling pathway and leads to aberrant growth in haematopoietic cells.* Nat Genet, 1996. **12**: p. 144-8.
20. Chen, Y.H., S.M. Gianino, and D.H. Gutmann, *Neurofibromatosis-1 regulation of neural stem cell proliferation and multilineage differentiation operates through distinct RAS effector pathways.* Genes Dev, 2015. **29**: p. 1677-82.
21. Hegedus, B., et al., *Neurofibromatosis-1 regulates neuronal and glial cell differentiation from neuroglial progenitors in vivo by both cAMP- and Ras-dependent mechanisms.* Cell Stem Cell, 2007. **1**: p. 443-57.
22. Lau, N., et al., *Loss of neurofibromin is associated with activation of RAS/MAPK and PI3-K/AKT signaling in a neurofibromatosis 1 astrocytoma.* J Neuropathol Exp Neurol, 2000. **59**: p. 759-67.
23. Sharma, R., et al., *Hyperactive Ras/MAPK signaling is critical for tibial nonunion fracture in neurofibromin-deficient mice.* Hum Mol Genet, 2013. **22**: p. 4818-28.
24. Kim, B.S., et al., *Differential regulation of observational fear and neural oscillations by serotonin and dopamine in the mouse anterior cingulate cortex.* Psychopharmacology, 2014. **231**: p. 4371-4381.
25. Wang, Z., et al., *Selective ablation of Ctip2/Bcl11b in epidermal keratinocytes triggers atopic dermatitis-like skin inflammatory responses in adult mice.* PLoS One, 2012. **7**: p. e51262.
26. Gouzi, J.Y., et al., *The receptor tyrosine kinase alk controls neurofibromin functions in Drosophila growth and learning.* PLoS Genet, 2011. **7**: p. e1002281.
27. Walker, J.A. and A. Bernards, *A Drosophila screen identifies neurofibromatosis-1 genetic modifiers involved in systemic and synaptic growth.* Rare Dis, 2014. **2**: p. e28341.
28. Morris, S.W., et al., *Fusion of a kinase gene, ALK, to a nucleolar protein gene, NPM, in non-Hodgkin's lymphoma.* Science, 1994. **263**: p. 1281-4.
29. Chen, Y., et al., *Oncogenic mutations of ALK kinase in neuroblastoma.* Nature, 2008. **455**: p. 971-4.
30. George, R.E., et al., *Activating mutations in ALK provide a therapeutic target in neuroblastoma.* Nature, 2008. **455**: p. 975-8.
31. Mosse, Y.P., et al., *Identification of ALK as a major familial neuroblastoma predisposition gene.* Nature, 2008. **455**: p. 930-5.
32. Soda, M., et al., *Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer.* Nature, 2007. **448**: p. 561-6.
33. Sirvent, N., et al., *ALK probe rearrangement in a t(2;11;2)(p23;p15;q31) translocation found in a prenatal myofibroblastic fibrous lesion: toward a molecular definition of an inflammatory myofibroblastic tumor family?* Genes Chromosomes Cancer, 2001. **31**: p. 85-90.
34. Palmer, R.H., et al., *Anaplastic lymphoma kinase: signalling in development and disease.* Biochem J, 2009. **420**: p. 345-61.
35. Kwak, E.L., et al., *Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer.* N Engl J Med, 2010. **363**: p. 1693-703.

36. Butrynski, J.E., et al., *Crizotinib in ALK-rearranged inflammatory myofibroblastic tumor.* N Engl J Med, 2010. **363**: p. 1727-33.
37. Kodama, T., et al., *Antitumor activity of the selective ALK inhibitor alectinib in models of intracranial metastases.* Cancer Chemother Pharmacol, 2014. **74**: p. 1023-8.
38. Metro, G., et al., *Alectinib's activity against CNS metastases from ALK-positive non-small cell lung cancer: a single institution case series.* J Neurooncol, 2016 **129**: 355-361.
39. Li, Y., et al., *Neurofibromin modulates adult hippocampal neurogenesis and behavioral effects of antidepressants.* J Neurosci, 2012. **32**: p. 3529-3539.
40. Orthofer, M. and e. al, *Identification of ALK in thinness.* Cell, 2020. **181**: p. 1246-1262.