Genetic inhibition of Anaplastic Lymphoma Kinase rescues cognitive impairments in Neurofibromatosis 1 mutant mice.

Running title: Alk, NF1, and cognition in mice

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Abstract

Heterozygous Neurofibromatosis 1 (NF1) loss of function mutations occurs in approximately 90% of patients with neurofibromatosis. A major, disabling phenotypic consequence of reduced NF1 function is cognitive impairment; a possibly related behavioral phenotype is impaired sleep. Recent results in *Drosophila* have demonstrated a genetic interaction between Anaplastic Lymphoma Kinase (Alk) and NF1 for both associative learning and sleep. Inhibition of Alk improves associative learning and sleep in heterozygous NF1 mutant flies. The results in *Drosophila* provide a strong motivation to investigate NF1/Alk genetic interactions in mice. In *Drosophila*, activation of Alk by its ligand, Jelly belly (Jeb), is the physiologically relevant target of negative regulation by NF1. Therefore, we tested whether genetic inhibition of Alk in heterozygous NF1 mutant mice attenuates or rescues cognitive impairments in mice. Our results are consistent with the hypothesis that NF1 functions in mice biochemically to inhibit signaling from Alk through Ras. The cognitive phenotypes observed in heterozygous NF1 mutant mice are rescued or ameliorated by genetic inhibition of Alk activity. In two tests of hippocampus-dependent learning, the Morris water maze and extinction of contextual fear, mutation of one or both alleles of Alk was sufficient to improve performance to wild type or near wild type levels in NF1-/+ mice. In addition, in NF1 mice genetic inhibition of Alk improves circadian activity levels. These data are intriguing in light of the circadian alterations seen in NF1 patients and indicate that inhibition of Alk activity may cognitively benefit patients with Neurofibromatosis 1.

1. Introduction

Heterozygous Neurofibromatosis 1 (NF1) loss of function mutations occur in approximately 90% of patients with neurofibromatosis. A major, disabling phenotypic consequence of reduced NF1 function is cognitive impairment. Poor academic performance is common in children with NF1 and often requires special education [1, 2]. Mental retardation is seen in 6-7% of children, a prevalence about three times higher than that of the general population. Specific learning disabilities in reading, spelling and math occur in 20% of children without overt central nervous system pathology [3]. Impairments in visual spatial processing and more complex executive functions have also been reported [4, 5].

A possibly related behavioral phenotype is impaired sleep. Pediatric patients have problems with initiating and maintaining sleep, arousal, sleep-wake transition and hyperhidrosis [6-8]. 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 [9].

The mechanisms underlying the cognitive and sleep impairments associated with NF1 is difficult to study in humans. The phenotypes observed in mice support a specific function for Neurofibromin in the hippocampus [10]. NF1 heterozygous mutant mice show impaired hippocampus-dependent spatial learning and memory in the water maze [10-12]. This phenotype is genetically and pharmacologically susceptible to modification by manipulation of the small GTPase and proto-oncogene Ras, a regulator of the MAP kinase (MAPK) signal transduction cascade [10].

One of Neurofibromin's biochemical functions is to modulate signal transduction through the Ras-MAPK pathway. Neurofibromin is a negative regulator of this pathway

that catalyzes the conversion of activate Ras-GTP to inactive Ras-GDP [11, 13]. The Ras-MAPK signal transduction pathway is canonically responsive to receptor tyrosine kinase (RTK) activation. Studies of *Drosophila* have established that activation of Anaplastic Lymphoma Kinase (Alk) by its ligand, Jelly belly (Jeb), is the physiologically relevant target of negative regulation by NF1 with respect to regulation of body size and associative learning [14]. Alk was originally identified as a human proto-oncogene activated by chromosomal translocation in a subset of lymphomas [15]. Subsequently inappropriate Alk activation was found to cause a number of other human malignancies, including non-small cell lung cancer and neuroblastoma [16-19]. Based on the phenotypic resemblance between activation of Alk and inactivation of NF1 and genetic interactions between NF1 and Alk in *Drosophila*, Gouzi et al. hypothesized that NF1 functions as a specific negative regulator of Alk signaling to enhance learning and memory. They showed that NF1 and Alk mutations strongly interact with respect to regulation of body size and olfactory associative learning. Specifically they demonstrated in *Drosophila* that the learning impairments associated with reduced NF1 function could be corrected or rescued by genetic inhibition of Alk, and that expression of NF1 mRNA in cells that also express Alk is sufficient to rescue both body size and learning phenotypes of NF1 mutant flies [14]. Based on these results we tested whether genetic inhibition of Alk in heterozygous NF1 mutant mice attenuates or rescues cognitive impairments in mice.

2. Material and Methods

2.1. Animals

Four male and 3 female Alk^{-/-} mice on the C57BL/6J background were received from Dr. Lilia Attisano at the University of Toronto, Canada, and 2 male NF1^{-/+} mice, on the 129 Svj genetic background, were received from Dr. Nancy Ratner at the Cincinnati Children's Hospital, OH. Alk^{+/+}; NF1^{+/-} mice were first bred with Alk^{-/+} mice. As this breeding strategy hardly generated Alk^{+/+}; NF1^{+/-} mice, we subsequently bred F1 double heterozygous Alk^{-/+}/NF1^{-/+} mice to generate 532 F2 mice of the different genotypes on a B6/129Svj background. The number of mice and nomenclature of the genotypes used for cognitive testing in this genetic study are indicated in Table 1. DNA extracted from tail biopsies were used for NF1 and Alk genotyping. The polymerase chain reaction (PCR) protocols from the Attisano and Ratner laboratories were used for genotyping. The following primers were used for the NF1 genotyping: NF1 31: 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. NF1^{+/+} runs as a 200 bp product and NF1^{-/+} as a 400 bp product. The initial denaturing was at 95⁰ C for 5 min. The denature, anneal, and extend conditions were respectively: 94⁰ C for 30 sec, 63⁰ C for 30 sec, and 72⁰ C for 45 sec. Thirty five cycles were used with a finale extension cycle at 72⁰ C for 5 min. The primer sequences used for the Alk genotyping were: ALK WT 5': ACCCCCTCACAGCGGACACCTATC; ALK WT 3': TGGGGACAGGGGCAGATGATTGAC; ALK neo 5': ATCTCCTGTCATCTCACCTTGCTC; and ALK neo 3': GTAAAGCACGAGGAAGCGGTCAGC. Alk^{+/+} runs as a 600 bp fragment and Alk neo as a 490 bp fragment. The initial denaturing was at 93⁰ C for 5 min. The denature, anneal, and extend conditions were respectively: 93⁰ C for 30 sec, 54⁰ C-56⁰ C for 30

sec, and 72⁰ C for 45 sec. Thirty cycles were used with a finale extension cycle at 72⁰ C for 7 min. All mice were tested at three to four months of age. The mice were housed under a constant 12 hr light: 12 hr dark cycle. Food (PicoLab Rodent Diet 20, no. 5053; PMI Nutrition International, St. Louis, MO), and water were provided *ad libitum*. All procedures were approved by Oregon Health & Science University (OHSU) Institutional Animal Care and Use Committee (IACUC).

2.2. Spatial learning and memory in the water maze

Mice were tested for spatial learning and memory in the water maze [20]. A circular pool (140 cm diameter) was filled with water (22°C ± 2°C). The water was made opaque with white chalk in order to hide the platform. The platform (20 cm wide) was located approximately 1 cm below the water level. Trials ended when the mice reached the platform and remained on it for 3 seconds or when 60 seconds had elapsed. In trials in which the mice did not find the platform, they were guided to the platform and allowed to remain on it for 3 seconds. Upon removal from the maze, the mice were dried with absorbent towels and returned to their home cages. The mice were trained to locate a platform in three distinct locations during hidden platform water maze training (Hidden Location 1: sessions 1-4; Hidden Location 2: sessions 5-6; Hidden Location 3: sessions 7-8). Each session consisted of two trials, with a 10 min inter-trial interval. There were two sessions per day, three hours apart. Subsequently, the mice were trained to locate a visible platform (Visible Location: sessions 9-10). Performance measures included time to reach the platform (latency), **distance moved**, and swim speeds. The swimming patterns of the mice were analyzed using the Ethovision video tracking system (Wageningen, The Netherlands) set at 6 samples/sec.

2.3. Contextual fear learning, memory, and extinction

The mice were tested for learning, memory, and extinction of hippocampus-dependent contextual fear conditioning using Med Associates NIR Video and automated analysis (Med Associates, St. Albans, VT, USA) utilizing Med Associates Video Freeze automated scoring system. Freezing is defined as the absence of motion with the exception of respiration. The freezing response is a widely used indicator of a conditioned fear response [21, 22]. On day 1 (training), each mouse was placed inside the enclosure with a house light (100 Lux) on during the training trial. The test involved a 60 second baseline period in which activity was recorded, followed by four tone-shock pairings. The tones were 80 dB at 2,800 Hz for 30 sec, co-terminating with 2 sec shocks (0.7 mA). Activity in the novel environment was defined as movement during the 60-second baseline period. The inter-trial interval (ITI) (time interval between the subsequent foot shocks) was 90 seconds. Motion during shock (arbitrary units from proprietary index) was measured to evaluate potential treatment-induced differences in response to the aversive stimulus. Percent time freezing during each subsequent ITI and tone presentation was measured to assess acquisition of the fear response. On days 2 (day 1 of extinction) through 8 (day 7 of extinction), mice were exposed to the training environment for 300 seconds and motion and freezing levels were analyzed.

2.4. Home cage activity

In a subset of mice, circadian activity levels were assessed using infrared home-cage activity sensors (Biobserve, St Augustin, Germany). Mice were individually housed and acclimated to the home-cage environment for three days prior to activity monitoring.

Activity counts were measured every second. The subset of mice included Alk^{+/+}; NF1^{-/+} mice ($n = 6$ mice) and Alk^{-/+}; NF1^{-/+} ($n = 3$ mice), Alk^{-/-}; NF1^{+/+} ($n = 10$ mice) and Alk^{-/+}; NF1^{+/+} ($n = 10$ mice).

As we did not have the equipment for assessment of circadian home cage activity at the beginning of this project and the involved breeding scheme took three years, we did not have wild-type litter mates we could include as statistical comparison for the circadian data. For illustrative purposes, we did include circadian data of 13 C57Bl6/J age-matched wild-type male mice.

2.5. Statistical analyses

All data are shown as mean $\pm$ standard error of the mean (SEM). Statistical analyses were performed using SPSS[™] software (Chicago, IL). The data were analyzed using Analysis of Variance (ANOVA)s with genotype and sex as between group factors. 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 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). ANOVA was used with genotype and sex as the between factors, followed up by post-hoc tests when appropriate, as indicated. We considered $p < 0.05$ as statistically significant.

3. Results

3.1. Spatial learning and memory in the water maze

The time to reach the hidden target location (latency) is shown in Fig. 1A and Fig. 1B. The data were analyzed, for each platform location separately, using repeated-measures ANOVAs with genotype and sex as between group factors. For training to locate the first platform location, there was a significant NF1 x Alk genetic interaction ($F(1,111) = 7.490$, $p = 0.007$) and a significant effect of Alk ($F(2,111) = 7.456$, $p = 0.001$). Compared to Alk+/+; NF1+/+ mice, Alk+/+; NF1-/+ mice showed impairments in their ability to reach the platform (Fig. 1B, $p = 0.048$, Tukey-Kramer; $p = 0.022$, Dunnett's multiple comparisons). Reducing Alk activity either by removing one copy of the gene in the Alk-/+; NF1-/+ group or completely removing functional Alk in the Alk-/-; NF1-/+ group mitigated this impairment.

A similar pattern was seen when distance moved was analyzed as performance measure. There were significant effects of NF1 ($F(1,111) = 5.293$, $p = 0.023$) and Alk ($F(2,111) = 8.426$, $p < 0.001$). Compared to Alk+/+; NF1+/+ mice, Alk+/+; NF1-/+ mice swam more. In addition, compared to Alk+/+; NF1+/+ mice, Alk-/+; NF1+/+ mice ($p = 0.005$) and Alk-/+; NF1-/+ ($p = 0.008$) swam less.

For training to locate the second platform location, there was a significant effect of Alk ($F(2,111) = 6.096$, $p = 0.003$), and a trend towards a NF1 x Alk genetic interaction ($F(1,111) = 3.629$, $p = 0.059$) **when latency was used as performance measure.** There was also a sex x session interaction ($p = 0.004$). When the sexes were analyzed separately, there was NF1 x session interaction in the females ($p = 0.045$) but not males.

When distance moved was used as performance measures, there was an Alk x session interaction ($F(2,111) = 3.691, p = 0.028$) and a NF1 x session x sex interaction ($F(1,111) = 5.015, p = 0.027$) but no significant main effects of NF1, Alk, or sex.

For training to locate the third platform location, there was a significant NF1 x Alk x session interaction ($F(1,111) = 10.218, p = 0.002$), and a significant effect of Alk ($F(1,111) = 10.218, p = 0.002$) **when latency was used as performance measure**. Remarkably, the protective effect of Alk against the detrimental effects of NF1 on spatial learning and memory in the water maze was more pronounced in Alk-/+; NF1-/+ than Alk-/-; NF1-/+ mice. There was also an Alk x session x sex interaction ($p = 0.029$). When the sexes were analyzed separately, there were no significant effects.

When distance moved was used as performance measure, there was only a significant effect of session ($F(1,111) = 36.013, p < 0.001$).

These significant effects in the water maze seen **when latency was used as performance measure** could not be attributed to the pattern of genotype differences in swim speeds seen in the water maze (Fig. 1C). Compared to Alk+/+; NF1+/+ mice, Alk-/-; NF1+/+ mice showed reduced swim speeds ($p < 0.001$).

3.2. Learning, memory, and extinction of contextual fear

Next the mice were tested for acquisition and extinction of hippocampus-dependent contextual fear. During training (day 1), there was an effect of genotype on motion prior to the first tone (baseline motion, Fig. 2A). There were significant effects of NF1 ($F(1,111) = 5.169, p = 0.025$), an Alk x NF1 interaction, and an NF1 x Alk x sex

interaction ($F(1,111) = 4.508, p = 0.036$) for baseline motion ($F(1,111) = 5.816, p = 0.018$). Thus prior to the first training event, Alk+/+; NF1+/+ mice moved more than Alk-/+; NF1-/+ ($p = 0.012$) and Alk-/-;NF1-/+ ($p = 0.014$) mice. These data indicate that in NF1-/+ mice, reducing Alk expression is associated with reduced general activity levels in a novel environment.

Next the response to the shock was analyzed (Fig. 2B). There were significant effects of NF1 ($F(1,111) = 15.398, p < 0.001$), sex ($F(1,111) = 10.523, p = 0.002$), an Alk x sex interaction ($F(2,111) = 4.533, p = 0.013$), and an NF1 x Alk x sex interaction ($F(1,111) = 7.166, p = 0.009$). Alk+/+; NF1-/+ mice showed a lower response to shock than Alk+/+; NF1+/+ ($p = 0.002$, Tukey's post hoc test), 2xHet ($p = 0.031$), and Alk-/-; NF1+/+ mice ($p = 0.007$). There were no effects of genotype or interactions with genotype for response to the tone (not shown). There was no effect of genotype on freezing between the tone-shock pairings during training but freezing increased following additional training ($p < 0.001$, Fig. 2C), indicating that all genotypes acquired the fear learning task equally well.

Next, we determined the ability of the mice to suppress the memory when placed daily in the same environment as that used for learning the tone-shock association. The data are shown in Fig. 3A and Fig. 3B. There were a significant Alk x NF1 x sex x day interaction ($F(3.455,383.522) = 3.6984, p = 0.009$) and a significant Alk x day interaction ($F(6.910,383.522)=2.172, p = 0.037$). Freezing levels decreased more in Alk+/+; NF1+/+ than Alk+/+; NF1-/+ mice (Fig. 3B). This effect was mitigated in Alk-/-; NF1-/+ and Alk-/-; NF1+/+ mice. Consistent with this pattern, when the difference in freezing levels between days 1 and 7 were compared as measure of extinction of a contextual fear memory, the freezing levels on day 1 were significantly higher than those on day 7 in Alk+/+; NF1+/+, Alk+/+; NF1-/+, Alk-/-; NF1-/+, and Alk-/-; NF1+/+ mice ($p < 0.0001$, Sidak's multiple

comparisons). However, freezing levels on days 1 and 7 were not different in Alk^{+/+}; NF1^{-/+} mice, indicating a lack of extinction of contextual fear in these mice that was rescued by reducing Alk activity (Fig. 3B).

As there were genotype differences in motion prior to the first tone on the training day, we also analyzed the percent time freezing during the extinction days using motion at baseline during training as a covariate. As sphericity was violated, we used a Greenhouse-Geisser correction. There was an effect of day ($F(3.452, 376.713) = 8.770, p < 0.001$), an Alk x NF1 x sex x day interaction ($F(3.452, 376.713) = 3.295, p = 0.016$), and a trend towards a NF1 x day interaction ($F(3.452, 376.713) = 2.207, p = 0.078$). These data indicate that genotype differences in baseline motion cannot account for the genotype differences seen in the percent freezing during the extinction days.

Based on the genotype differences in activity levels during the baseline on the training day, we also analyzed the average motion during the extinction days (Figs 3D-F). As the sphericity was violated, a Greenhouse Geisser correction was used. There was an effect of day ($F(1.996, 221.527) = 16.318, p < 0.001$) and an Alk x NF1 x sex interaction ($F(1, 111) = 4.865, p = 0.029$) but there were no main effects (Figs 3D, E). When the activity levels on days 1 and 7 were compared (Fig. 3F), there was an effect of day ($F(1, 111) = 86.325, p < 0.001$), a NF1 x Alk x day x sex interaction ($F(1, 111) = 11.143, p = 0.001$), a NF1 x Alk x day interaction ($F(1, 111) = 4.945, p = 0.028$), an NF1 x Alk x sex interaction ($F(1, 111) = 8.094, p = 0.005$), and a NF1 x day interaction ($F(1, 111) = 6.627, p = 0.011$).

Similar to the pattern seen for percent freezing, when the difference in motion between days 1 and 7 were compared, the motion levels on day 1 were

significantly lower than those on day 7 in Alk+/+; NF1+/+, Alk-/-; NF1-/+, and Alk-/-; NF1+/+ mice ($p < 0.0001$, Sidak's multiple comparisons) and Alk-/+; NF1-/+ ($p < 0.01$, Sidak's multiple comparisons). However, motion levels on days 1 and 7 were not different in Alk+/+; NF1-/+ mice, and this was rescued by reducing Alk activity (Fig. 3F).

3.3. Circadian activity levels

In aged female Rhesus macaques, reduced activity levels during the active period are associated with cognitive impairments [23]. Therefore, we determined whether in Alk+/+; NF1-/+ mice, the cognitive impairments were also associated with reduced activity levels (Fig. 4A). Activity levels during the dark phase, when the mice are active, seemed lower Alk+/+; NF1-/+ mice than the other genotypes, but it did not reach significance. The difference between activity levels during the dark and light phase was smaller in Alk+/+; NF1-/+ mice than Alk-/+; NF1-/+ mice (Fig. 4B).

In Fig. 4C, circadian data are shown for age-matched C57Bl6/J mice. Although we could not directly statistically compare the genotypes as the wild-type mice were not littermates of the mutant mice, these data suggest that NF1-/- mice have lower activity levels than wild-type mice.

These data suggest that in NF1 mice genetic inhibition of Alk improves circadian activity levels. These data are especially intriguing in light of the sleep disturbances reported in NF1 patients and studies in *Drosophila* demonstrating genetic effects of Alk, Nf1 and genetic interactions between them in regulation of sleep.

4. Discussion

Alk has been characterized primarily as an oncogene. Based on its biochemical function as a receptor tyrosine kinase it has been successfully targeted for pharmacologic inhibition to treat malignancies. Orally active small molecule Alk inhibitors have shown notable effectiveness in the treatment of lung cancer and are being tested for the treatment of neuroblastomas that are caused by uncontrolled or inappropriate activation of Alk [24-26]. Outside of the context of oncogenesis, the normal function of Alk in mammals is poorly understood. In *C. elegans* and *Drosophila* specific functions in smooth muscle development, axon targeting, neurogenesis and regulation of synaptic structure and function have all been demonstrated [27-35]. Alk and its ligands Jeb, in *Drosophila*, and Hen-1, in *C. elegans*, have also been found to regulate learning, sleep, body size and the developmental response to environmental stress [[9][14, 36] . Expression studies in mammals have demonstrated Alk mRNA in both the developing and adult nervous system [37, 38]. This expression pattern as well as the findings in invertebrate species has focused recent investigations on behavioral phenotypes in mammals [38,39].

Behavioral functions for an Alk ligand were first described in *C. elegans*. An unbiased, forward genetic screen for mutations that effect integration of conflicting sensory inputs identified Hen-1, the *C. elegans* homologue of Jeb, as a signal that participated in behavioral response to simultaneous, conflicting attractive and aversive stimuli [29]. One of the most striking findings was that the Hen-1 requirement was not developmental. The behavioral phenotype could be rescued by a temporally controlled transgene only if it was expressed in adults. The behavioral phenotype of Hen-1 mutants included an effect on associative learning.

In *Drosophila*, Jeb and Alk were first characterized for their roles in early muscle development [27, 30, 39]. Investigation of their functions in the nervous system has

required conditional genetic techniques to circumvent embryonic lethality. Several functions for Jeb and Alk have been established in the developing and adult *Drosophila* nervous system: 1) Jeb and Alk are essential for correct axon targeting of a subset of photoreceptors [32]; 2) Late embryonic maturation of the larval neuromuscular junction requires Jeb and Alk, though they are not required for axon targeting or synapse formation in this context [40]; 3) Jeb and Alk protect neurogenesis when developing larvae are nutritionally challenged [35]; and 4) Jeb and Alk have been implicated in associative learning and regulation of body size upstream of Nf1 [14]; 5) in the context of transynaptic signaling Jeb activation of Alk in the larval neuromuscular junction, a glutamatergic synapse, was shown to be a potent negative regulator of synaptic transmission [33]; and 6) most recently Jeb and Alk in *Drosophila* have been found to regulate sleep and once again to interact functionally with NF1 [9].

Based on the two studies that have reported genetic interactions between Jeb/Alk and NF1 in *Drosophila* we sought to evaluate whether this pathway is evolutionarily conserved in mammals. If the hypothesis is correct then pharmacologic inhibition of Alk may be a potent pharmacologic approach to treating cognitive and other behavioral limitations in patients with neurofibromatosis, a strategy that has already been applied in *Drosophila* [14].

Our results are consistent with the hypothesis that, as in *Drosophila*, NF1 functions biochemically to inhibit signaling from Alk through Ras. The cognitive phenotypes observed in heterozygous NF1 mutant mice are rescued or ameliorated by genetic inhibition of Alk activity. In two tests of hippocampus-dependent learning, the Morris water maze and extinction of contextual fear, mutation of one or both alleles of Alk was sufficient to improve performance to wild type or near wild type levels in NF1-/+ mice. These findings argue strongly that pharmacologic modulation of Alk activity with orally available small molecule Alk inhibitors may cognitively benefit patients with

Neurofibromatosis 1. **We recognize that it would be important to also test the effects of NF1 and Alk on spatial memory retention in probe trials (no platform) following training for the individual platform location and will plan to include them as part of future studies.**

Jeb/Alk signaling in the brain of *Drosophila* also regulates sleep [9]. Conditional, temperature sensitive *Drosophila alk* mutants have a long sleep phenotype at non-permissive temperatures. As in assays of associative learning and regulation of body size this long sleep phenotype is sensitive to the gene dosage of *Nf1*. We observed a shift in activity from typical nocturnal predominance to more non-circadian pattern in NF1-/+ mice. This phenotype, potentially related to short sleep, was normalized by genetic reduction in Alk activity. This again supports the potential therapeutic utility of pharmacologic Alk inhibition to address disordered sleep behavior in NF1 patients.

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Figure legends

Fig. 1A. Latency to reach the platform in the water maze. The mice were trained to locate a platform in three distinct locations during hidden platform water maze training (Hidden Location 1: sessions 1-4; Hidden Location 2: sessions 5-6; Hidden Location 3: sessions 7-8). Subsequently, the mice were trained to locate a visible platform (Visible Location: sessions 9-10). **1B.** Compared to Alk+/+; NF1+/+ mice, Alk+/+; NF1-/+ mice showed impairments in their ability to reach the platform ($p = 0.048$, Tukey-Kramer; $p = 0.022$, Dunnett's multiple comparisons). Reducing Alk in the Alk-/+; NF1-/+ and Alk-/-; NF1-/+ mice mitigated this impairment. **1C.** Swim speeds in the water maze. Compared to Alk+/+; NF1+/+ mice, Alk-/-; NF1+/+ mice showed reduced swim speeds ($p < 0.001$). For training to locate the first platform location, there was an effect of Alk ($F(2,111) = 7.456$, $p = 0.001$) and a NF1 x Alk interaction ($F(1,111) = 7.490$, $p = 0.007$). For training to locate the second platform location, there was an effect of Alk ($F(2,111) = 6.096$, $p = 0.003$). For training to locate the third platform location, there was an effect of Alk ($F(2,111) = 4.088$, $p = 0.019$). See Table 1 for the number of mice tested in the water maze.

Fig. 2. Acquisition of hippocampus-dependent contextual fear. **A.** When activity levels were analyzed prior to the first tone-shock pairing, Alk+/+; NF1+/+ mice moved more than Alk-/+; NF1-/+ ($p = 0.012$) and Alk-/-; NF1-/+ ($p = 0.014$) mice. **B.** Response to the shock. Alk+/+; NF1-/+ mice showed a lower response to shock than Alk+/+; NF1+/+ ($p = 0.002$, Tukey's post hoc test), 2xHet ($p = 0.031$) and Alk-/-; NF1+/+ mice ($p = 0.007$). **C.** The freezing levels between the tone-shock pairings during training were similar in all

the genotypes. All genotypes learned the task; the freezing levels increased with training ($p < 0.001$). See Table 1 for the number of mice tested in the water maze.

Fig. 3. Extinction of contextual fear memory. **A.** The ability of the mice to suppress the contextual fear memory. **B.** During extinction trials, freezing levels decreased more in Alk+/+; NF1+/+ than Alk+/+; NF1-/+ mice. This effect was mitigated in Alk-/-; NF1-/+ and Alk-/-; NF1+/+ mice. **C.** The difference in freezing levels between days 1 and 7. The freezing levels on day 1 were significantly higher than those on day 7 in Alk+/+; NF1+/+, Alk-/-; NF1-/+, Alk-/-; NF1-/+, and Alk-/-; NF1+/+, but not in Alk+/+; NF1-/+ mice ($^{\#}p < 0.0001$, Sidak's multiple comparisons). These data indicate a lack of extinction of contextual fear in NF1 mice that is mitigated by reducing Alk expression. See Table 1 for the number of mice tested in the water maze. **D. Average motion of all genotypes during the extinction days.** **E. Average motion of Alk+/+; NF1+/+ and Alk+/+; NF1-/+ mice during the extinction days.** **F. The difference in motion levels between days 1 and 7. The motion levels on day 1 were significantly lower than those on day 7 in Alk+/+; NF1+/+, Alk-/-; NF1-/+, and Alk-/-; NF1+/+ mice ($^{\#}p < 0.0001$, Sidak's multiple comparisons) and Alk-/-; NF1-/+ ($^{**}p < 0.01$, Sidak's multiple comparisons). However, motion levels on days 1 and 7 were not different in Alk+/+; NF1-/+ mice, and this was rescued by reducing Alk activity (Fig. 3F).**

Fig. 4. Circadian activity levels. **A.** Activity levels during the dark phase seemed lower Alk+/+; NF1-/+ mice than the other genotypes. **B.** The difference between activity levels during the dark and light phase seemed especially much smaller in Alk+/+; NF1-/+ mice than Alk-/-; NF1-/+ mice. Alk+/+; NF1-/+: $n = 6$ mice; Alk-/-; NF1-/+: $n = 3$ mice; Alk-/-;

NF1^{+/+}, $n = 10$ mice; and Alk^{-/+}; NF1^{-/+}: $n = 10$ mice. **C. Activity of age-matched C57BL6/J wild-type male mice. $n = 13$ mice. These data were acquired over 6 days. For the panel on the right, the data is from 72 hours at the end of the activity monitoring period.**

Table 1. Number of mice and genotype nomenclature of the genetic study.

Nomenclature	Alk Genotype	NF1Genotype	Females	Males	Total
Alk+/+; NF1+/+	WT	WT	11	13	24
Alk+/+; NF1-/+	WT	NF1-/+	11	10	21
Alk-/-; NF1+/+	Alk-/-	WT	11	12	23
Alk-/-; NF1-/_	Alk-/-	NF1-/+	12	16	28
Alk-/+; NF1-/+	Alk-/+	NF1-/+	10	15	25

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Figure 1

Figure 2

Figure 3

Figure 4