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Anaplastic Lymphoma Kinase and Leukocyte Tyrosine Kinase: Functions and genetic interactions in learning, memory and adult neurogenesis

Joseph B. Weiss [a,*], Changhui Xue [a], Ted Benice [b,c], Liquan Xue [d], Stephan W. Morris [d], Jacob Raber [b,c]

[a] Departments of Medicine and Cell and Developmental Biology, Division of Cardiovascular Medicine, Oregon Health & Science University/UHN 62, 3181 SW Sam Jackson Park Rd., Portland, OR 97239, United States
[b] Behavioral Neuroscience, Oregon Health and Science University, 3181 SW Sam Jackson Park Rd., Portland, OR 972394, United States
[c] Neurology and Division of Neuroscience, ONPRC, Health and Science University, 3181 SW Sam Jackson Park Rd., Portland, OR 97239, United States
[d] Departments of Pathology and Oncology, St. Jude Children's Research Hospital, 262 Danny Thomas Place, Memphis, TN 38105, United States

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ABSTRACT

Anaplastic Lymphoma Kinase (Alk) is a receptor tyrosine kinase expressed throughout the adult mammalian hippocampus. Recent studies in Drosophila and prior studies in *Caenorhabditis elegans* have implicated Alk signaling in learning and neurogenesis. We have studied the roles of Alk and the closely related receptor Leukocyte Tyrosine Kinase (Ltk) in learning, behavior and neurogenesis. In the hippocampus, both receptors are expressed throughout the dentate gyrus, CA1 and CA3. To assess the functional roles of Alk and Ltk in the mammalian brain, we analyzed phenotypes in Alk mutant, Ltk mutant and Alk/Ltk double-mutant mice compared to wild-type littermates. Similar to Drosophila, we found enhanced performance in spatial memory in Alk mutant mice. Also similar to Drosophila, we observed reduced neurogenesis associated with loss of Alk function. We also report genetic interactions between Alk and Ltk with respect to neurogenesis and behavioral measures such as activity, anxiety levels, and retention of spatial memory.

1. Introduction

The two closely related receptor tyrosine kinases, Anaplastic Lymphoma Kinase (Alk) and Lymphocyte Tyrosine Kinase (Ltk), are expressed in a variety of neural tissues during mammalian development and in adults (Bernards and de la Monte, 1990; Haase et al., 1991; Iwahara et al., 1997; Morris et al., 1997; Hurley et al., 2006; Degoutin et al., 2009). There are only a few functional studies of both Alk and Ltk in mammals, with just one published study addressing functions of Alk in mammalian behavior and adult neurogenesis and no publications addressing the functions of Ltk (Bilsland et al., 2008). In Drosophila, Alk and its ligand, Jelly belly (Jeb), play roles in axon targeting of photoreceptors, neuromuscular junction growth and function, as well as early mesoderm development (Rohrbough and Broadie; Weiss et al., 2001; Englund et al., 2003; Lee et al., 2003; Bazigou et al., 2007). Similar neural functions are documented for Hen1, a *C. elegans* Jeb homolog, and Scd2, a *Caenorhabditis elegans* Alk homolog (Ishihara et al., 2002; Liao et al., 2004; Reiner et al., 2008). Though neither Hen1 nor Scd2 are required for *C. elegans* development, the secreted signaling molecule Hen1 is required for integration of conflicting sensory inputs (Ishihara et al., 2002). Scd2 functions in *C. elegans* neuromuscular junction formation and in the dauer response to environmental stress (Liao et al., 2004; Reiner et al., 2008).

In three recent publications Drosophila Alk regulates 1) larval neurogenesis, 2) adult responses to ethanol and 3) body size and adult learning (Cheng et al.; Gouzi et al.; Lasek et al.). Drosophila Alk regulates the tumor suppressor Neurofibromin 1 (Nf-1) in control of body size and adult learning. In this study, we assessed whether mammalian Alk has similar functions with respect to learning and neurogenesis.

In Drosophila and *C. elegans* there is a single homologous receptor that most closely resembles Alk. In vertebrates, Alk and Ltk are found, presumably as a consequence of duplication and divergence of a common ancestral receptor. Ltk is structurally very similar to Alk and differs from it in its extracellular domain where Ltk lacks a region homologous to the amino-terminal portion of Alk (Fig. 2). In zebrafish, the development of one class of pigment cells not found in mammals, iridophores, is dependent on Ltk function (Lopes et al., 2008). Both Alk and Ltk belong to the insulin receptor superfamily of receptor tyrosine kinases, though both have diverged from other insulin receptor family members. Alk and Ltk structurally constitute a small family of receptor tyrosine kinases distinct from the insulin and insulin-related receptors (Robinson et al., 2000). The structural similarity between Alk and Ltk, along with their inferred common evolutionary origin, suggests that in mammals they may have related or even interlocking functions.

* Corresponding author. Tel.: +1 503 494 6412.
E-mail address: weissjo@ohsu.edu (J.B. Weiss).

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We explored the functional interaction between Alk and Ltk by analyzing behavioral and neurogenic phenotypes in mice harboring targeted single mutations in Alk and Ltk as well as double-mutant mice lacking both Alk and Ltk. Our objective was to determine if there is a genetic interaction between the two receptors that would support any of three possible functional relationships: 1) independent, 2) redundant or 3) antagonistic functional interactions. Our analysis focused on hippocampal neurogenesis and hippocampus-dependent behaviors because both receptors are expressed throughout the adult hippocampus.

2. Results

2.1. Alk and Ltk expression in the hippocampus

We analyzed first the expression of Alk and Ltk mRNAs in the brains of adult mice using in situ hybridization. Alk mRNA is expressed throughout the hippocampus, including the dentate gyrus (Fig. 1A–C). We also employed anti-Alk antisera and anti-doublecortin, a specific marker of committed neural progenitors, to determine whether adult born neural progenitors express Alk. Alk protein is clearly found in doublecortin-positive newborn neurons (Fig. 1D–F). Alk mRNA and protein expression is detectible throughout the hippocampus, in mature granule cells of the dentate gyrus as well as neurons in CA1 and CA3, by in situ hybridization for mRNA and immunohistochemistry using antiserum raised against Alk protein, respectively.

Ltk mRNA is also found in the adult mouse hippocampus by in situ hybridization, consistent with the Genstat database of mRNA expression in the murine central nervous system (Fig. 2). There are no available anti-sera specific for murine Ltk to confirm protein expression in the same domain as the mRNA. Ltk mRNA expression is evident throughout the hippocampus, in the dentate gyrus, CA1 and CA3. Thus, in the hippocampus, the expression of Alk and Ltk mRNAs overlap extensively, consistent with possible functional interactions between the two receptors. Lack of appropriate anti-Ltk antisera prevents definitive demonstration of co-expression of the two proteins in the same cells at the same time.

2.2. Behavioral analysis of Alk, Ltk, and Alk/Ltk double-mutant mice

First, measures of activity were assessed in the open field. There was an effect of genotype on activity in the open field ($F=7.737$, $p=0.0005$). While mice lacking either Ltk or Alk showed activity levels similar to those in wild-type mice, mice lacking both Alk and Ltk showed hypoactivity (Fig. 3).

Next, measures of anxiety were assessed in the elevated zero maze (Fig. 4). There was an effect of genotype on percentage of time spent in the open areas of the maze ($F=3.962$, $p=0.0168$). While mice lacking only Alk showed lower levels of anxiety than wild-type mice, this was not seen in mice lacking only Ltk. Mice lacking both Alk and Ltk showed intermediate anxiety levels, as compared to mice lacking only Alk or Ltk.

There was also an effect of genotype on sensorimotor function on the rotorod ($F=4.1819$, $p=0.0049$). Mice lacking only Ltk or both Ltk and Alk showed impaired rotorod performance compared to mice only lacking Alk (Fig. 5).

Finally, spatial learning and memory was assessed in the water maze. All genotypes learned to locate the visible and hidden platform locations and there were no group differences in task acquisition (Fig. 6A). However, genotype differences were seen when spatial memory retention is assessed in the water maze probe trials. In the probe trial, following the first day of hidden platform training, only mice lacking Alk alone showed spatial memory retention and spent more time searching the quadrant that contained the platform during the hidden platform training than any other quadrant (Fig. 6B). At the end of the second day of the probe trial of hidden platform training, three genotypes showed spatial memory retention, 1) mice lacking only Alk, 2) wild-type mice and 3) mice lacking only Ltk. In contrast, mice lacking both Alk and Ltk failed to show a spatial bias (Fig. 6C). After additional hidden platform training, no genotype differences were seen. All genotypes showed spatial memory retention in the third probe trial (Fig. 6D).

Next, we evaluated whether differences in measures of anxiety during the probe trial, assessed as thigmotaxis, contributed to the genotype differences in spatial memory retention in the water maze probe trials. There was no effect of genotype on thigmotaxis during

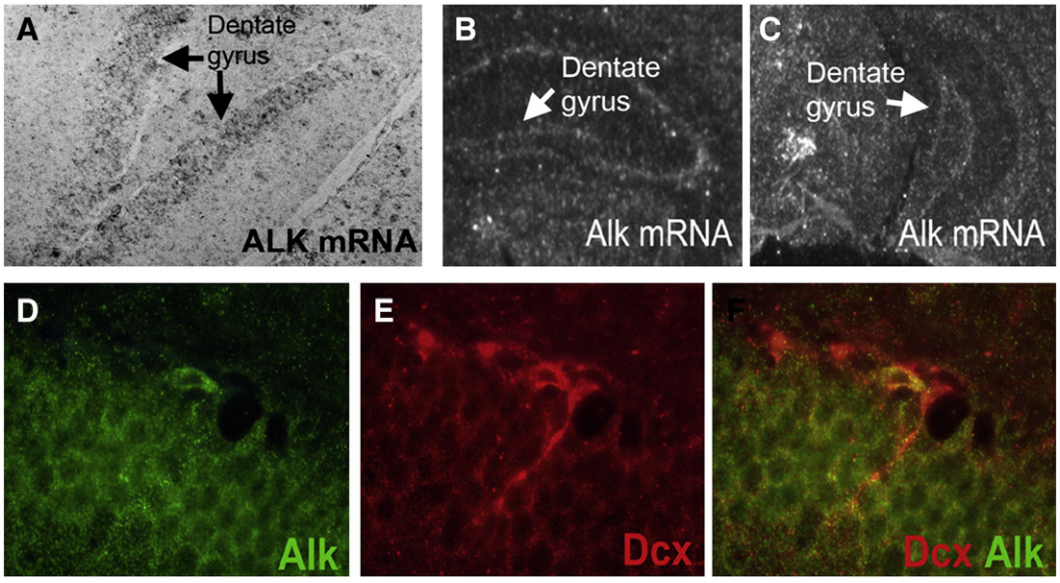

Fig. 1. Alk mRNA and protein in the dentate gyrus and newborn neurons. Alk mRNA is detected in the dentate gyrus of the hippocampus as well as CA1 and CA3 (A, B and C). Panel A. in situ hybridization performed in this study. Panels B and C, in situ hybridization from the Genstat website. Panels D–F, subgranular zone neural precursors labeled with anti-Alk antiserum (green) and anti-doublecortin antiserum (red). Note that Alk protein is found in doublecortin-positive neuroblasts.

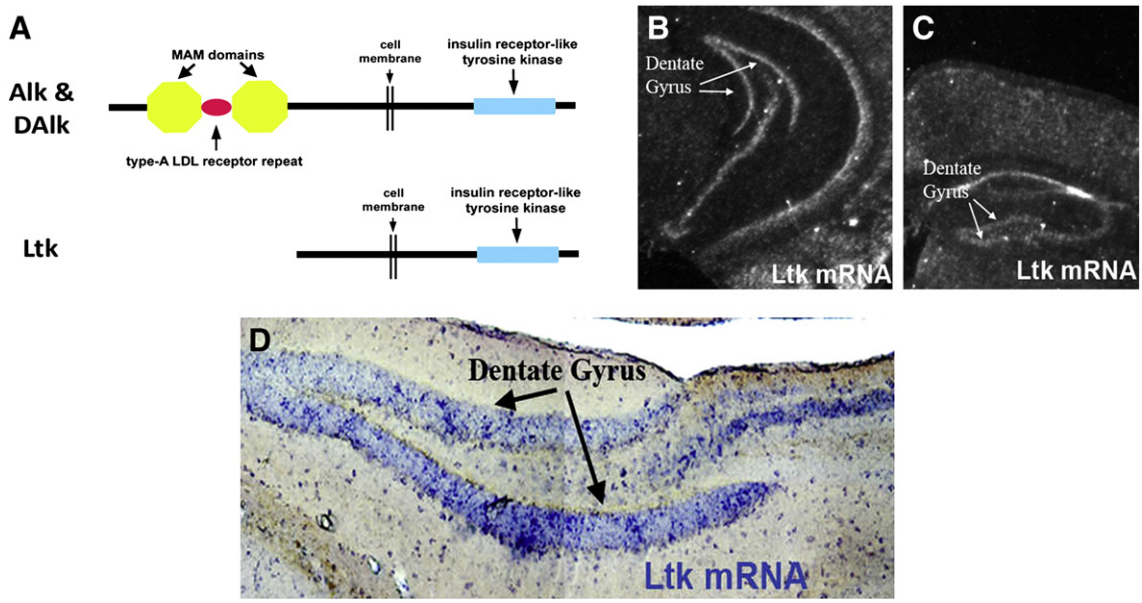

Fig. 2. Ltk, a receptor tyrosine kinase closely related to Alk, is expressed in the hippocampus in the same pattern as Alk. The homology between Alk and Ltk extends throughout both receptors, differing primarily in the amino-terminus where Ltk lacks a region found in both vertebrate and invertebrate Alk (Panel A). Panels B and C show two Genstat in situ hybridizations to detect Ltk mRNA. Note that Ltk mRNA is found throughout the hippocampus. Panel D is an in situ hybridization to detect Ltk mRNA performed in the present study to confirm Ltk expression in the dentate gyrus as well as the remainder of the hippocampus (not shown).

the first probe trial ($\chi^2_{(3)} = 2.282$, $p = 0.516$). However, there was an effect of genotype on thigmotaxis during the second probe trial ($\chi^2_{(3)} = 13.031$, $p = 0.005$). Mann–Whitney post-hoc comparisons revealed a significant difference between the Alk mutant mice (6.19 ± 1.46) and the Alk/Ltk double mutant mice (1.14 ± 0.63) ($U = 14.500$, $z = -2.881$, $p = 0.004$). In the third probe trial, there was a trend towards an effect of genotype on thigmotaxis that did not reach significance ($F(3,37) = 2.551$, $p = 0.070$). These data indicate that potential differences in thigmotaxis did not contribute to the enhanced spatial memory retention of Alk mutant mice.

2.3. Role of Alk and Ltk in adult neurogenesis

To investigate a potential functional interaction between Alk and Ltk, we compared a marker of neurogenesis in Alk and Ltk single homozygous mutant mice as well as Alk/Ltk homozygous double-mutant mice to wild-type littermates. As an indirect measure of neurogenesis, doublecortin-positive immature neurons were analyzed using immunohistochemistry and epifluoresence microscopy. With respect to doublecortin-positive cell number, a genetic interaction was observed between Alk and Ltk. The number of doublecortin-positive neurons was profoundly reduced in Alk, but not Ltk, single homozygous mutant mice compared to wild-type littermates. However, the number of doublecortin-positive neurons was much more reduced in Alk/Ltk homozygous double-mutant mice than Alk homozygous single-mutant mice (Fig. 7).

The reduction in doublecortin-positive cells observed in Alk and Alk/Ltk mutant mice led us to ask if the volume of the dentate gyrus was reduced by these mutations. We predicted that lifelong reduced production of new neurons, in the absence of effects on cell survival, would result in reduced volumes. We employed DAPI staining to define the full cellular content of the dentate gyrus and traced its boundary in each section. We them combined these two dimensional measurements from serial sections to calculate the dentate gyrus volumes. Though age correlates strongly with the dentate gyrus volumes, generally genotype does not (Fig. 8). Both in younger and older mice loss of Alk and Alk/Ltk have no effect on the volume of the dentate gyrus. One genotype, single Ltk mutants, correlates with increased dentate gyrus volume at a statistically significant level compared to age-matched wild type and Alk/Ltk mutant mice. The scientific significance of the observed correlation between genotype and dentate volume in Ltk mutant mice is unclear as we

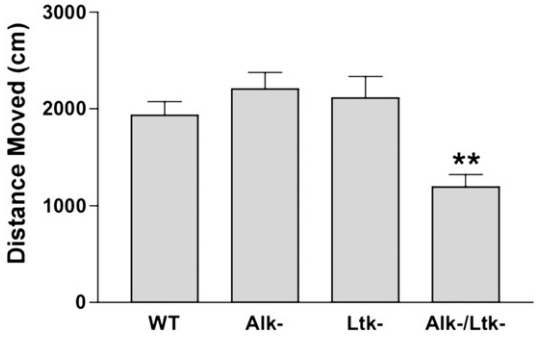

Fig. 3. Open field activity. Mice lacking both Alk and Ltk showed less activity than any other genotype. $^{**}p < 0.01$ versus any other genotype. $n = 9$–22 mice/genotype.

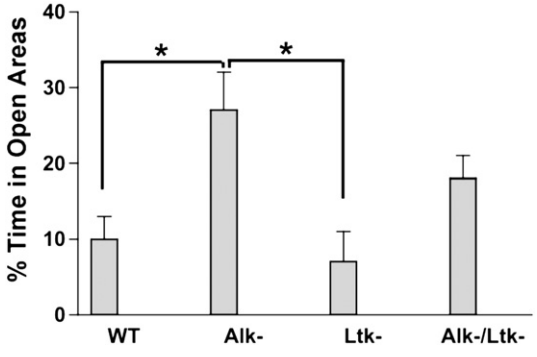

Fig. 4. Measures of anxiety in the elevated zero maze. Alk mutant mice show lower measures of anxiety than Ltk mutant and wild-type mice. $^{*}p < 0.05$. $n = 9$–22 mice/genotype.

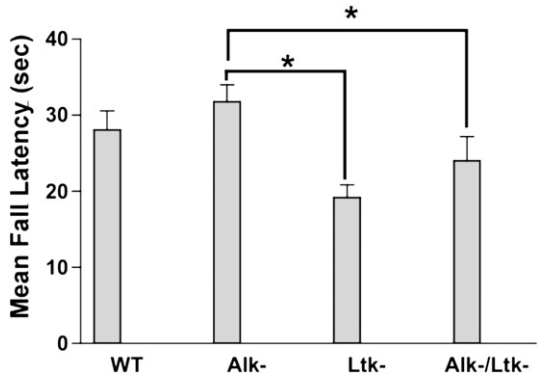

Fig. 5. Rotorod performance. Alk mutant mice stayed longer on the rotating rod than Ltk mutant and Alk/Ltk double-mutant mice. $^{*}p<0.05$. $n=9$–22 mice/genotype.

observed no hippocampal related phenotypes in these mice. The dentate gyrus of older mice, both wild type and Alk mutants, is significantly smaller than younger mice ($p=0.0001$). This result implies that the neurogenic effects of Alk and Alk/Ltk genotype are not simply attributable to reduced production of newborn neurons either during development or in adults.

2.4. The effect of genotype on mRNA accumulation

In several phenotypic tests, genetic interactions were found between Alk and Ltk. One possible mechanism to account for these interactions is a compensatory effect of expression levels of one receptor on the other. In particular this mechanism is suggested by the genetic interaction between Alk and Ltk in the spatial memory retention phenotype. To test this and other potential interactions at the level of mRNA accumulation, quantitative Taq-man RT-PCR was employed to measure the mRNA accumulation of both Alk and Ltk mRNA in the hippocampus of single Alk and Ltk mutants. We found no significant change in the level of Alk mRNA in Ltk mutants and no significant change in the level of Ltk mRNA in Alk mutants (Fig. 9).

3. Discussion

In this study, we analyzed hippocampal mRNA expression, behavioral phenotypes, a marker of adult neurogenesis and mRNA levels in mice with targeted mutations in the two highly related receptor tyrosine kinases, Alk and Ltk. Our results are strikingly similar to recently published papers concerning the functions of Alk in Drosophila. Specifically we found that loss of Alk function in mammals enhances retention of spatial memory, just as inhibition of Alk function in adult Drosophila enhances olfactory associative learning (Gouzi et al.). The implications of this enhanced cognitive performance are highlighted by rescue of cognitive impairment of Drosophila neurofibromin mutants as well as enhanced associative learning in wild type adult flies by administration of an orally available small molecule inhibitor of Alk. Our results support the hypothesis that inhibition of Alk in mammals, humans included, enhances cognitive performance both in normal and impaired individuals.

In Drosophila, Alk also has an essential function in protection of larval neurogenesis from nutrient stress. Alk signaling spares larval neurogenesis from growth restriction that other tissues suffer as a consequence of decreased nutrient availability (Cheng et al.). Similarly, we report decreased adult neurogenesis in mice lacking Alk and Alk/Ltk. Loss of Alk function alone resulted in about a 50% decrease in newborn neurons in both younger and older adult male mice (Fig. 7). By contrast, loss of Ltk function alone did not result in a significant reduction of newborn neurons in younger adult male mice. The

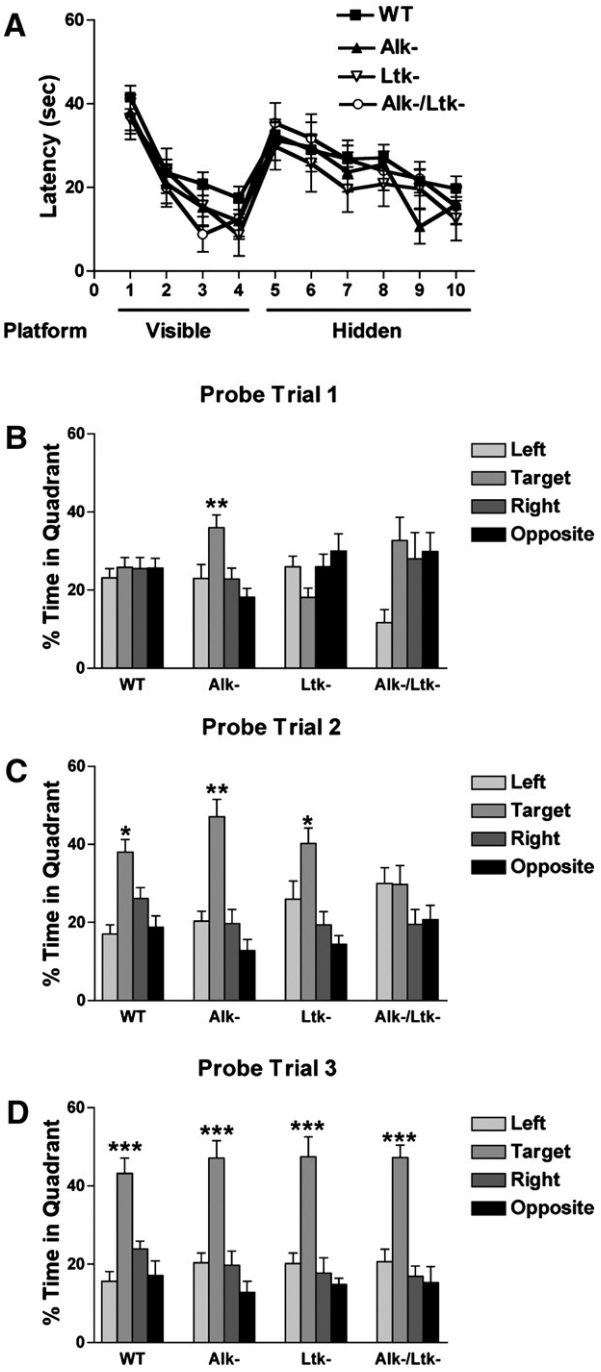

Fig. 6. Spatial learning and memory in the water maze. A. All groups learned to locate the visible and hidden platform locations and there was no genotype difference in the learning curves. Spatial memory retention was assessed in the probe trials following the first (B), second (C), and third (D) day of hidden platform training. $^{*}p<0.05$ versus any other quadrant, $^{**}p<0.01$ versus any other quadrant, $^{***}p<0.001$ versus any other quadrant. In the first probe trial, only Alk mutant mice showed spatial memory retention. In the second probe trial, only Alk/Ltk double-mutant mice failed to show spatial memory retention. Following additional training, in the third probe trial, all groups showed spatial memory retention. $n=9$–22 mice/genotype.

combined loss of both Alk and Ltk resulted in a dramatic reduction in newborn neurons to about 20% of wild-type levels in young male adult mice (Fig. 7). The simplest interpretation of these findings is that Alk and Ltk have asymmetrically redundant functions in adult neurogenesis. The enhancement of the neurogenesis phenotype of Alk mutants by removal of Ltk implies that Ltk partially compensates for loss of Alk function. Conversely, the absence of a neurogenesis

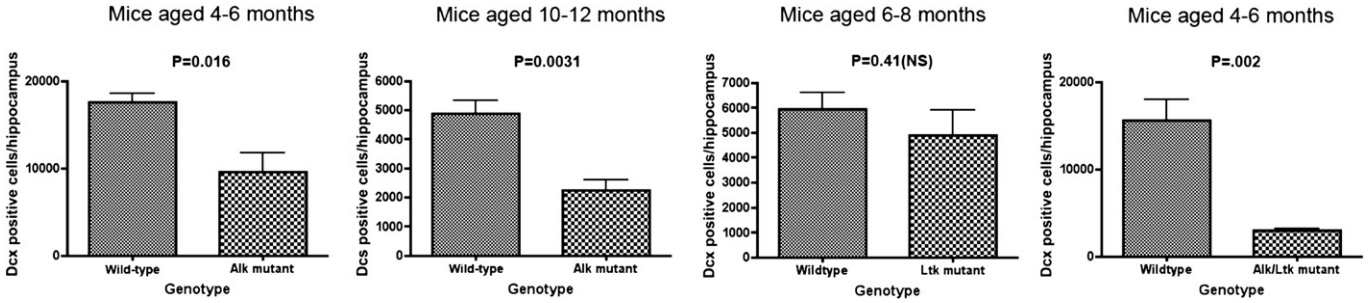

Fig. 7. Doublecortin-positive neurons in the dentate gyrus of wild type, Alk mutant, Ltk mutant and Alk/Ltk double-mutant mice. To evaluate neurogenesis the hippocampus of formaldehyde-perfused mice were serially sectioned and stained with anti-doublecortin antibodies. In a blinded fashion sections were systematically counted and the volume of dentate gyrus calculated to estimate the total numbers of doublecortin-positive cells per hippocampus. Four animals were sectioned and counted in each category. P values for two-tailed t-tests of differences between the two groups are shown above the plots.

phenotype in single Ltk mutants implies that Alk completely compensates for loss of Alk. The effect of removing Ltk function on neurogenesis can only be revealed by also removing Alk. Our experiments do not address the possible relationship between stress, Alk and neurogenesis in mammals. If the roles of Alk and Ltk in mammals are similar to those observed in Drosophila, then manipulation of Alk/Ltk signaling may be protective with respect stress-induced cognitive and emotional impairment in humans.

In contrast to our data, the one prior study of adult neurogenesis and behavioral phenotypes in mice with a targeted mutation of Alk reported enhanced neurogenesis that was age-dependent (Bilsland et al., 2008). In younger mice, 2–3 months old, no significant difference in adult neurogenesis between homozygous Alk mutant mice and wild-type littermates was reported. At six months of age, however, homozygous Alk mutant mice were reported to have enhanced hippocampal neurogenesis compared to wild-type littermates. The increased hippocampal neurogenesis correlated with increased struggle time in the tail suspension and Porsolt swim tests, assays of persistence in the face of adversity or a measure of reduced depression. The homozygous mutant Alk mice also demonstrated superior performance in novel object recognition tests. On the basis of these findings, the authors proposed Alk inhibitors as potential therapeutics for depressive psychiatric disorders.

Our results differ strikingly from this study with respect to the role of Alk in hippocampal, adult neurogenesis (Bilsland et al., 2008). There are several possible explanations for the divergent results. One derives from the different Alk alleles employed. The targeted mutation employed by Bilslund et al. is, as acknowledged in the publication, not likely to be a functional null allele. The targeted mutation we employed removes the tyrosine kinase domain of Alk and so is predicted to be a null allele. We also employed different measures of neurogenesis. Mitosis was directly measured by Bilslund et al. by incorporation of bromo-deoxyuridine, while we count specifically newborn neurons by doublecortin labeling. Decreased survival of neuroblasts lacking Alk and Ltk might account for the disparity obtained by different measures of neurogenesis.

Our results do not address at what level in the complex process of adult neurogenesis—proliferation, survival, migration, cell fate determination and differentiation, or functional integration—Alk and Ltk act, or even if they act at the same level.

We have measured dentate gyrus volume as a function of age and genotype (Fig. 8). We find that, with age, there is a statistically significant decline in the volume of the dentate gyrus that correlates with the well-established age-related decline in neurogenesis. We do not observe any significant effect of genotype on dentate gyrus volume, in spite of dramatic reduction in numbers of doublecortin-positive cells. Preservation of dentate gyrus volume, in the face of 2–5× reduction in the numbers of doublecortin-positive cells as a function of genotype, renders reduced developmental or adult neurogenesis inadequate as an explanation for the observed phenotypes. It suggests that increased survival and/or integration of mature neurons in Alk and Ltk mutants may be an important mechanism contributing to enhanced cognitive performance.

Our behavioral studies found multiple, sometimes complex, genetic interactions between Alk and Ltk. Single mutants in either Alk or Ltk demonstrate no phenotype with respect to activity as measured by distance moved in an open field, but Alk/Ltk double mutants show significantly reduced activity (Fig. 3). The simplest explanation for this phenotype is a symmetric, redundant functional interaction between Alk and Ltk in which either receptor can completely compensate for the loss of the other. The functional requirement is only revealed when both are removed.

A quite different genetic interaction is demonstrated in the elevated zero maze test of avoidance of open areas, an index of anxiety. Loss of

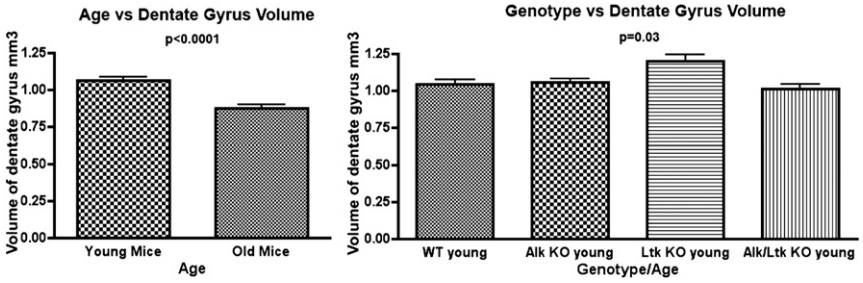

Fig. 8. Total volumes of dentate gyri as a function of age and genotype. The total volume of the dentate gyrus for each mouse used to count doublecortin-positive cells was calculated based on tracings of serial sections of DAPI stains. For comparison of young vs. old mice, 24 young mice aged 4–8 months of mixed genotypes were compared to 8 old mice aged 10–12 months of mixed genotypes. The volumes of the dentate gyrus in older mice were significantly smaller than younger mice, $p<0.001$. No significant difference was found between old mice as a function of genotype (not shown). For comparison of genotypes of young mice, 12 wild type and four of each mutant genotype were compared. Ltk KO mice had statistically significantly larger dentate gyri, $p=0.03$ after Tukey testing for multiple comparisons of one-way ANOVA.

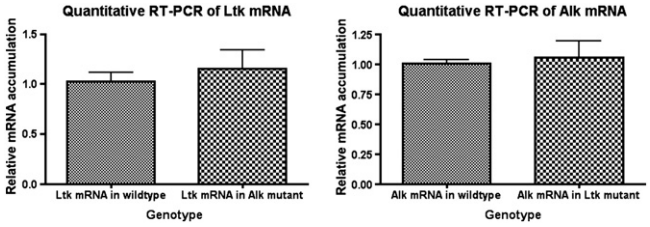

Fig. 9. Ltk and Alk mRNA in the hippocampi of wild type, Alk mutant and Ltk mutant mice. The hippocampi of wild type (n = 15 total for both experiments), Alk mutant (n = 10) and Ltk mutant (n = 7) mice were dissected out of whole brains and total RNA extracted. With Taqman RT-PCR reagents the Alk and Ltk mRNA levels were normalized to internal control Gapdh. The Ltk mRNA level in Alk mutants is not significantly different from wild type; likewise, the Alk mRNA level in Ltk mutants is not different from wild type.

Alk attenuates anxiety of being in the open, while loss of Ltk appears to enhance it, though not to a statistically significant extent, possibly because wild-type mice spend relatively little time in the open (Fig. 4). An antagonistic functional relation of the two receptors is suggested in this assay by the Alk/Ltk double mutant. In the double mutants, the loss of Ltk significantly reverses the phenotype of the Alk mutation and increases the avoidance of open areas to almost wild-type levels. In Alk mutants, the enhanced cognitive performance together with reduced measures of anxiety support Alk as an attractive therapeutic target for anxiety and cognitive disorders as well as conditions like Alzheimer's disease in which both anxiety and cognition are affected.

No genetic interaction was observed in tests of sensorimotor function on the rotorod (Fig. 5). Ltk is clearly required for normal performance on the rotorod and Alk is not. Loss of both Alk and Ltk function results in a phenotype that is not significantly different from Ltk single mutants.

With regard to the function of Alk and Ltk in the hippocampus, the retention of spatial memory as measured in the Morris water maze probe trials demonstrated the most complex genetic interaction between the two receptors. Single Alk mutants demonstrate enhanced spatial memory compared to wild type, Ltk single and Alk/Ltk double-mutants (Fig. 6). This enhanced spatial memory is evident in both the first and second probe trials where Alk single-mutants spent significantly more time searching in the appropriate quadrant for the hidden platform than any other genotype. Ltk single-mutants in this test are indistinguishable from wild type, but Alk/Ltk double mutants are impaired in spatial memory retention compared to wild type, Alk single-mutants and Ltk single-mutants. In this assay of hippocampus-dependent learning, the genetic interaction between Alk and Ltk may be redundant with Ltk over-compensating for loss of Alk.

Several mechanisms could account for the observed interaction between Alk and Ltk in hippocampus-dependent learning. Absent more mechanistic information about the physiologic basis of single mutant phenotypes, a better characterization of Ltk expression and activity in relation to Alk expression and activity is needed, along with more complete understanding of where and when Alk is functioning in hippocampus-dependent learning. We emphasize that genetic interactions constitute an entry point for more complete mechanistic studies. The scarcity of Ltk studies and attendant lack of reagents are current limitations.

Alk has been implicated in a variety of tumors and small molecule inhibitors are in clinical trials for treatment of these malignancies (Butrynski and D'Adamo; Kwak and Bang). How and to what extent these inhibitors also inhibit the normal functions of Alk and Ltk may determine the clinical tolerability of these compounds. Neuroblastoma in children is one of the most promising targets of Alk inhibitors (George et al., 2008; Janoueix-Lerosey et al., 2008; Mossâe et al., 2008). To anticipate and ameliorate the possible untoward effects of these inhibitors on the normal functions of Alk and Ltk, we will need to understand more fully how they participate in the complex activity of the mammalian central nervous system.

Both our studies and studies in Drosophila imply a potential therapeutic benefit from Alk inhibition with respect to cognitive function and measures of anxiety. The potential to enhance cognitive function and reduce measures of anxiety in mammals has numerous applications and implications. Our genetic studies provide a predicate for the investigation of this therapeutic potential, which we look forward to exploring.

4. Materials and methods

4.1. Knockout mice

Targeted mutations in Alk and Ltk were induced by homologous recombination at each locus in mouse ES cells. The targeting vectors encode neomycin resistance and their integration results in deletion of essential coding sequences. In the Alk locus exons 20 and 21 are deleted, a total of 93 amino acids. The deleted exons encode the entire intracellular juxtamembrane domain and the initial portion of the tyrosine kinase catalytic domain. In the Ltk locus exons 10 and 11 are deleted, a total of 66 amino acids. The domains deleted include the entire transmembrane domain required for anchoring the mature receptor in the plasma membrane. Targeted mutations were generated in 129 mouse strain ES cells, which were injected into C57Bl6 blastocysts to create 129/C57Bl6 chimeric mice. Subsequent to conventional breeding strategies to generate homozygous Alk and Ltk single-mutant mice, the animals were bred onto a pure C57Bl6 background for 10 generations (Stephan W. Morris, MD, personal communication). Homozygous Alk and Ltk single-null animals were crossed to create double-mutant mice. Genotyping was performed by PCR using genomic DNA and primers specific for the neomycin resistance transgene and the deleted regions as well as a novel junctional fragment created at the Alk locus.

4.2. Immunohistochemistry

Within 24 h of the last water maze probe trial, mice were deeply anesthetized with a cocktail of 100 mg/kg ketamine, 10 mg/kg xylazine, and 2 mg/kg acepromazine and were then perfused transcardially with PBS followed by 4% paraformaldahyde pH 7.4. Brains were removed and postfixed overnight in 4% paraformaldahyde at 4 °C, transferred to 30% sucrose solution, then embedded in cryoprotectant and stored at −80 °C until sectioning. Serial, coronal sections (50 μm) were collected through the entire dentate gyrus for each brain, and 50-μm intersection distance, comprising the entire dentate gyrus, were collected onto Superfrost microscope slides (Fisher Scientific). Sections were then stained for fluorescent visualization of DCX, a marker of immature neurons (Ming and Song, 2005). Every fifth slide was employed for anti-doublecortin immunoreactivity and DAPI staining; approximately 10 slides were stained for each side of the hippocampus to include the whole dentate gyrus. Sections were stained in 'batches', comprised of one animal from each of the experimental groups. This ensured identical labeling of brain sections between experimental groups as sections from each group in a 'batch' received equal treatment. Following an antigen retrieval step according to the manufacturer's instructions (H-3000; Vector Laboratories), sections were incubated for 2 h at room temperature in a blocking solution (5% normal donkey serum in PBS containing 0.2% TritonX-100 and 0.2% bovine serum albumen (PBT)), followed by incubation overnight at 4 °C in goat-anti-doublecortin (1:200; Santa Cruz Biotechnology) primary antibodies. The DCX antibody shows staining only in neuronal cell lines, and the antigenicity colocalizes with GFP expressed under control of the DCX promoter, indicating specificity of the antibody for DCX protein (Karl et al., 2005). Sections were incubated for 4 h at room temperature donkey-anti-

goat-IgG (diluted at 1:200) conjugated to Alexa 546 for visualization (Jackson Immuno). Following 4 washes with PBT and 1 wash with PBS (10 min each), the sections were covered with anti-fade solution containing a DAPI counterstain (Vectashield, Vector Laboratories) and were coverslipped (Fisher Scientific). Slides were stored in the dark at 4 °C until they were imaged.

Anti-Alk staining was performed in the same fashion with a primary rabbit anti-Alk antiserum diluted at 1:200 in PBS (Liquan Xue and Stephan W. Morris, St. Jude Children's Research Hospital) and secondary donkey-anti-rabbit-IgG conjugated to Alexa 488 diluted 1:200 in PBS.

4.3. In situ hybridization

The slides (50-μm sections) were collected as for DCX studies. They were post-fixed in fresh 4% paraformaldehyde for 20 min, washed with PBS 2× for 5 min, and treated with proteinase K (20 μg/ml) for 10 min at 37 °C. The sections were drained and washed with PBS for 2 min, refixed in 4% paraformaldehyde for 5 min, washed 5 min in PBS 3×, dehydrated in 70% ethanol for 5 min followed by 95% ethanol for 30 s and then air dried.

Sections were prehybridized for 1 h at 55 °C with hybridization buffer (50% formamide, 1% Denhardt's solution, 10% Dextran Sulfate, 300 mM Sodium Chloride, 20 mM Tris–HCl (pH 8.0), 5 mM EDTA (pH 8.0), 250 μg/ml tRNA) and then rinsed in 2× SSC buffer. 10 μg of Digoxigenin-labeled probe was taken up in 1 ml hybridization buffer and heat-denatured at 80 °C for 2 min, cooled and added to the sections. The sections were covered with glass coverslips, sealed and incubated in a moist chamber for at least 18 h at 55 °C. After hybridization, the slides were rinsed in prewarmed 5× SSC buffer, placed in prewarmed high stringency wash, 50% formamide 2× SSC at 65 °C for 30 min, then washed in RNase buffer (0.5 M NaCl, 10 mM Tris–HCl (pH 8), 1 mM EDTA) at 37 °C 3× for 10 min. They were then incubated with RNase A (20 μg/ml) in the same RNase buffer at 37 °C for 30 min, washed in RNase buffer at 37 °C for 15 min again and repeated high stringency wash 2× at 65 °C for 20 min each, then washed in 2× SSC, 1× SSC for 15 min each at 37 °C, washed with PBT (PBS + 0.1% Tween-20) for 15 min at room temperature. After blocking the slides by incubation with 10% heat-inactivated goat serum in PBT for 1 h at room temperature, sections were incubated with alkaline phosphatase-coupled anti-digoxigenin antibody diluted 1:1000 in PBT with 1% goat serum at 4 °C overnight. The next day, the sections were washed 4× in PBT at room temperature for 15 min each and followed by wash 2 × 10 min in NTMT buffer (100 Mm NaCl, 100 Mm Tris–HCl (pH 9.5), 50 mM MgCl2, 0.1% Tween-20, then incubated in the dark with NTMT containing 3 μl/ml NBT and 3.5 μl/ml BCIP overnight at 4 °C. The developing reaction was stopped by washing in PBT.

4.4. Stereology

The total number of DCX-positive cells in the bilateral granule cell layer (GCL) of the dentate gyrus was estimated using the optical fractionator technique (West et al., 1991). Brains from mice of various genotypes that had been through behavioral testing were selected at random. For DCX, 8–10 stacks of five images each (dissectors), with a 2-μm inter-image distance, were taken bilaterally within the GCL of each brain section. Images were collected using the 35× objective lens of a Zeiss Axiphot 2 microscope. A square counting frame (75 μm–75 μm) was laid over the computer screen with the GCL visible within, and cells were counted when they appeared within the frame in one image of the five-image stack but not in the preceding image. DCX-positive cells were counted only when the staining appeared surrounding a distinctly labeled DAPI counterstained nucleus. Using this approach, a total of 80–120 individual dissectors were counted within each brain to obtain an unbiased and accurate average density of DCX positive cells per cubic millimeter within the GCL of each mouse.

The Cavalieri principle (West et al., 1991) was used to estimate the total volume of the GCL by outlining the DAPI-counterstained GCL in the Axiovision software to obtain the GCL area in each section bilaterally. The sum of these areas was multiplied by the intersection distance and the total number of cells was then obtained by multiplying the average density measurement by the total volume estimate.

4.5. Quantitative taqman RT-PCR

For analysis of mRNA levels in various genotypes the following 5–7 month-old male mice were used; Alk mutant (n = 10), Alk wild-type siblings (n = 11) and Ltk mutant (n = 7), Ltk wild-type siblings (n = 4).

Tissue Preparation: A brain slicer (EM Corporation, Chestnut Hill, MA) was used to produce 2 mm blocks, which were placed in RNAlater (Ambion, Austin, TX). The brain was dissected with a dissecting microscope to harvest the entire hippocampus, about 100 mg of frontal cortex and cerebellum. Tissues were snap frozen and then stored at − 80 °C.

4.6. RNA extraction and reverse transcription

Total RNA was extracted from hippocampus, frontal cortex and cerebellum nuclei under RNase-free conditions using TriZol reagent (Invitrogen) according to the TriZol RNA isolation protocol of the W. M. Keck foundation biotechnology microarray resource laboratory at Yale University. Total RNA (5 μg) was used to generate cDNA with the SuperScript III First-Strand Synthesis System (Invitrogen).

4.7. Real-time PCR quantification assays

Quantification real-time PCR analysis was performed with the Stratagene Mxpro3500p System using Taqman universal PCR master mix according to manufacturer's specifications. Taqman gene expression assays containing primers and probes for mouse Alk (assay ID Mm00431770_ml), mouse Ltk (assay ID Mm01324624_gl) and mouse Gapdh (assay ID Mm99999915_gl) was prepared by ABI. The target gene-specific probe and the Gapdh internal control probe were labeled using the reporter dye FAM. Multiplex PCR was performed with Alk, Ltk and Gapdh primers and probes using PCR protocol: 50 °C for 2 min, and then 95 °C for 10 min (initial denaturing) followed by 40 cycles of amplification at 95 °C for 15 s (denaturing), 60 °C for 1 min (annealing), and completed with 1 cycle of 80 °C for 1 min. All samples were run in triplicate. The qPCR reaction contained 5 μl 2× master mix, 0.5 μl 20× of forward and reverse primers and probe, 1 μl cDNA and nuclease-free water to 10 μl final volume.

4.8. Real-time PCR analysis

Relative quantification analysis was done using the comparative $\Delta\Delta C_T$ method. The data were expressed as an n-fold change in gene expression normalized to a reference gene and relative to calibrator sample. The reference gene Gapdh was used to normalize the target genes, the mean ΔC_T of Alk wild-type samples or Ltk wild-type samples was used as an internal calibrator when comparing Alk mutant or Ltk mutant versus wild type. $\Delta\Delta C_T = \Delta C_T$ (C_T target gene − C_T Gapdh) − calibrator (mean of ΔC_T wild-type (C_T wild-type − C_T Gapdh)), fold change = $2^{-\Delta\Delta CT}$.

The mean ± SEM of the n-fold change was determined for relative expression levels of Alk gene or Ltk gene in Alk mutant or Ltk mutant mice. A two-tailed *t*-test was employed to detect statistically significant changes ($p < 0.05$ was considered significant).

5. Behavioral tests

Mice were first tested for exploratory behavior and measures of anxiety in the open field and the elevated zero maze. Subsequently, mice were tested for sensorimotor function using the rotorod. Finally, spatial learning and memory was assessed using in the water maze.

5.1. Open field

To assess exploratory behavior, open field activity was assessed individually for 10 min in brightly lit enclosures (16 in. × 16 in. square) equipped with a 16 × 16 array of infrared photocells for measuring horizontal movements and computer-quantified automatically (Kinder Scientific, Poway, CA). The total distance moved during the test measured activity levels. After each assessment of open field activity, the equipment was cleaned with 5% acetic acid to remove residual odors.

5.2. Elevated zero maze

Measures of anxiety were also assessed using the elevated zero maze. The custom built elevated zero maze consisted of two enclosed areas (safe environment) and two open areas (anxiety-provoking environment), identical in length (35.5 cm; Kinder Scientific, Poway, CA). Mice were placed in the closed part of the maze and allowed free access for 10 min. A video tracking system (Noldus Information Technology, Sterling, VA) set at 6 samples/s was used to calculate the distance moved, and percent time spent in the open areas of the maze.

5.3. Rotorod

To assess sensorimotor function, the mice were tested on the rotorod (Rotamex-5, Columbus Instruments, Columbus, OH). Mice were placed on an elevated rod (3 cm × 9.5 cm spindle 44.5 cm elevated) initially rotating at 5 rpm. The speed of the rotating rod was increased by 1 rpm every 3 s to a maximum of 24 rpm. Each trial ended when a fall was recorded by photo beams aligned with each individual mouse or if a mouse did not fall from the rod within 300 s. Mice received 3 trials, 30 min apart, for 3 consecutive days, and the mean fall latency was calculated.

5.4. Water maze

The mice were first trained to locate a platform clearly marked by a beacon (non-spatial training, days 1 and 2), and then to locate a platform beneath opaque (white non-toxic chalk) water (spatial training, days 3–5) using the available spatial cues in the room. The time to locate the platform, distance moved, swim speed, and cumulative distance to the target was calculated. To assess spatial memory retention, a probe trial (platform removed) was performed 1 h after the last hidden platform training trial on each of the three hidden platform training days. The time spent swimming in the target quadrant (where the platform was located during hidden platform training), and in the three non-target quadrants (right, left, and opposite quadrants) was measured. Thigmotaxis was measured as the amount of time mice spent in the outer zone of the maze. All performance measures were calculated using Noldus Ethvision video tracking set at 6 samples/s.

5.5. Statistical analyses

All statistical analyses were performed using SPSS software (SPSS Inc., Chicago, Il, USA), SigmaStat (Systat Software Inc., San Jose, CA) or GraphPad Prism software (San Diego, CA, USA). A p value of <0.05 was considered statistically significant. ANOVAs were used with genotype as between-subject factor to assess effects of genotype on exploratory behavior and measures of anxiety in the open field and elevated zero maze. Repeated measures ANOVA were used to compare water maze learning curves. Thigmotaxis data for each probe trial were analyzed using one-way ANOVA with genotype as a between subjects variable. As these data were not normally distributed, Kruskal–Wallis (KW) one-way ANOVA was conducted, with Mann–Whitney comparisons post-hoc using a Bonferroni corrected alpha (α/n) of 0.008 to adjust for the six post-hoc comparisons, in the instance of a significant effect of group as determined by the KW test. Data were expressed as means ± SEM. $p<0.05$ was considered significant for all tests.

Statistical analysis of doublecortin-positive cell numbers, quantitative RT-PCR and Dentate Gyrus Volumes all employed the two-tailed, unpaired T-test for comparisons of two groups with presumed Gaussian variation. Statistical analysis of Dentate Gyrus volumes of various genotypes employed one-way ANOVA for unpaired measurements with presumed Gaussian variation and Tukey's post-test analysis for multiple comparisons. $P<0.05$ was considered significant for all tests.

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