Behavioral changes associated with loss of NSPC-derived VEGF *in vivo* after KA induced excitotoxic injury

Research Thesis

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Research Thesis

by

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Abstract

Seizures are sudden abnormal electrical activity in the brain that lead to excitotoxic tissue damage, changes in mood and behavior, and even death. Vascular endothelial growth factor (VEGF) has been shown to protect against seizure and excitotoxic injury in rats. We have recently shown that neural stem and progenitor cells (NSPCs) produce a significant amount of the VEGF in the dentate gyrus (DG). In order to study the contribution of NSPC produced VEGF in modulating seizures and their sequelae, we used $VEGF^{fl/fl}NestinCreER^{T2}$ mice in a kainic acid (KA) induced excitotoxicity model. Using $VEGF^{fl/fl}NestinCreER^{T2}$ mice following tamoxifen (TAM) injection allows for the inducible knock down (KD) of VEGF in NSPCs. After either KA or vehicle treatment, mice were given hippocampus-dependent behavioral tests consisting of a novel arm test in a Y maze, an object location test (OLT), and an elevated plus maze (EPM). Analysis of the novel arm test and the OLT confirm that the KA treatment impaired memory. Surprisingly, NSPC-specific VEGF KD seems to result in decreased memory at baseline compared to control mice and may or may not be further impacted by excitotoxic injury. Analysis of the EPM suggests that VEGF KD and KA treatment had no effects on mice's anxiety-like behavior. Elucidating the functional effects of NSPC-derived factors such as VEGF in the context of injury is a critical step to understanding how stem cells modulate brain function and will aid in the successful implementation of NSPCs as therapeutic agents.

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Introduction

Epilepsy, a neurological disorder characterized by repetitive seizures, affects more than 50 million people worldwide[1]. Seizures are a sudden, uncontrolled electrical disturbance in the brain, and they can lead to excitotoxic brain injury which is a pathological process of neuron damage caused by overactivation of glutamate receptors[2, 3]. While there are numerous antiepileptic treatments currently used to treat patients suffering from recurrent seizures, these therapies often do not completely prevent seizures and also have significant adverse effects[1]. Thus, scientists have begun exploring NSPCs as a potential new therapeutic for excitotoxic brain injury.

NSPCs are consisted of neural stem cells (NSCs) and neural progenitor cells (NPCs). NSCs are the stem cells which are self-renewing and multipotent in the nervous system and can differentiate into neural sub-types such as neurons, astrocytes and oligodendrocytes[3, 5]. NPCs are the cells directly differentiated from NSCs and have limited number of dividing times and restricted capability to differentiate into neuronal and glial cell types[6]. Collectively, these NSPCs reside in two major neurogenic niches in the brain throughout the mammalian lifespan including the subventricular zone of the forebrain and the subgranular zone of the DG of the hippocampus[7]. Previous research shows that NSPCs located in the DG of the hippocampus can mediate memory and emotions through the production of new neurons in a process called neurogenesis[8, 9]. It has been shown that seizures can acutely stimulate NSPCs to increase production of new dentate granule cells which contribute to aberrant network reorganization in the hippocampus and subsequent cognitive impairments[10, 11]. By contrast, chronic seizures

could reduce the neurogenesis possibly due to the exhaustion of NSPC pool or alterations of neurogenic niche disrupting proper functions of NSPCs[12, 13].

Incidentally, previous studies have shown that VEGF is upregulated after seizure-induced excitotoxic injury[14, 15, 16]. Interestingly, VEGF, the growth factor commonly associated with angiogenesis, has also been shown to protect against seizure and excitotoxic injury in rats[17, 18]. Most previous research assumes that neuroprotective VEGF in the brain comes from astrocytes[19]. Our lab has shown that NSPCs, in addition to their neurogenic functions, also produce a variety of growth factors and cytokines that are known to modulate inflammation[20, 21]. In particular, our lab has found that NSPCs produce almost one third of the VEGF in the relatively astrocyte-sparse DG. To date, no research has studied the effects of VEGF originating specifically from NSPCs on seizures and their sequelae.

This project focused on characterizing the effects of knocking down NSPC-derived VEGF on hippocampus-dependent behavior following excitotoxic injury as a way to investigate how NSPC-derived VEGF can modulate seizure-induced injury. Based on the functional importance of NSPCs and VEGF and the evidence of NSPCs being able to produce a significant amount of VEGF, I hypothesized that knocking down NSPC-derived VEGF would cause more severe behavioral impairments following chemical-induced excitotoxic injury.

This study used $VEGF^{fl/fl}NestinCreER^{T2}$ mouse model that enables NSPC-specific VEGF KD upon TAM injection[22, 23]. To induce excitotoxicity injury, an intraperitoneal (IP) injection of KA or vehicle (V) to KD and Con mice was administered. As an agonist of kainite receptors, KA is widely used to induce seizures in rodents and

causes strong excitotoxicity[24]. After TAM-induced recombination and KA injection, all mice began behavioral testing consisting of a novel arm test in a Y maze and an OLT for accessing short-term spatial memory, and an EPM for assessing anxiety-like behavior. Each of these tasks is a widely used assay for hippocampus-dependent behaviors[25].

Con mice injected with V (ConV) were expected to show unimpaired memory and baseline anxiety-like behavior, such as avoidance of open areas, compared to other groups of mice. Con mice injected with KA (ConKA) and KD mice injected with V (KDV) were expected to show impaired memory and increased anxiety-like behavior compared to ConV. KD mice injected with KA (KDKA) were expected to exhibit the worst impaired memory and most increased anxiety-like behavior among the four groups of mice.

This study investigated the functional consequences of NSPC-specific VEGF KD on memory and anxiety. Defining the functional effects of NSPC-derived growth factors and cytokines is a critical step in the successful implementation of NSPCs as therapeutic agents for seizures.

Methods

Animals

VEGF$^{fl/fl}$ mice from Genentech, Inc were crossed with heterozygous NestinCreER^{T2} mice from The Jackson Laboratory (strain 01621). We used both male and female offspring of 8-10 weeks old of these breeders for experiments. Genotyping primers are detailed in Table 1. All mice were administered with IP injection of TAM (180mg/kg/d) dissolved in sunflower oil for 5 days. On the third day after the last TAM injection, mice were administered with IP injection of either KA (15mg/kg) or vehicle (saline). Seizure activity was observed in all mice during a period of 4 hours following KA injections. The experimental design yielded four different groups of mice: ConV (n=10), ConKA (n=15), KDV (n=11), KDKA (n=12). Two weeks following either KA or vehicle injection, mice underwent three behavioral tests consisted of a novel arm in a Y maze test, an OLT and an EPM. All animal use was in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health and approved by The Ohio State University Institutional Animal Care and Use Committee (protocol #2016A00000068).

Table 1

Gene	Primer Sequence	
	F (5' → 3')	**R** (5' → 3')
Cre-F	GCGGTCTGGCAGTAAAAACTATC	
Cre-R		GTGAAACAGCATTGCTGTCACTT
VEGF-F	TCCGTACGACGCATTTCTAG	
VEGF-R		CCTGGCCCTCAAGTACACCTT

Novel Arm Test in Y Maze

Novel arm tests were conducted in a symmetrical Y-shaped maze with three grey, opaque plastic arms (37 cm × 6 cm × 13 cm) angled 120° from each other and a camera was positioned centrally above the Y maze. Each arm was identical inside and had no intramaze cues. Spatial cues were placed on the surrounding walls outside the Y maze.

In the training trial, mice were released into one arm of the Y maze (release arm) and allowed to explore the maze with one arm blocked (novel arm) for 10 minutes. After a 2-hour intertrial interval (ITI) with the barrier to the novel arm removed, mice were released in the same arm as in the training trial and allowed to explore all arms for 10 minutes during the testing trial. This ethologically relevant test was based on rodents' inherent preference for novelty (novel arm in this experiment) and introduced no positive and negative reinforcement and very little stress to mice. The duration of time spent in each arm, frequency of visits to each arm, and latency to investigate the novel arm registered in the recorded videos were manually calculated by 2-3 observers blind to the genotype and treatment of the mice. Automated tracking software (Noldus EthoVision) was used to determine total distance traveled and average velocity of all mice to confirm that there were no inherent differences in activity or motor ability between mice.

Object Location Test (OLT)

OLTs were conducted in 4 open field areas (40 cm × 39 cm × 30 cm) made of white, opaque acrylic sheets with a camera positioned above the boxes. Spatial cues were placed on the surrounding walls outside the box. A transparent, plastic cone filled

with orange sand (6.5 cm high) and a grey, plastic, and elephant-like toy (9 cm high) were used as objects.

In preparation for the OLT that would be conducted the next day, all mice were habituated to the open field with no objects for three 10-minute trials separated by an ITI of 1 h on day 1. On the day 2 training trial, mice were allowed to investigate the two different objects in the open field for 10 minutes. After a 1-hour ITI, one of the two objects was moved to a different place compared to that in the training trial and mice were allowed to investigate the objects for 10 minutes during the testing trial. Similar to the novel arm test of Y maze, this ethologically relevant test was also based on rodents' inherent preference for novelty (the object at a different location in this experiment) and introduced no positive and negative reinforcement and very little stress to mice. Investigation time with each object during each trial registered in recorded videos were manually scored by 2-3 observers blind to the genotype and treatment of the mice. Object investigation was defined as a mouse's nose being towards and within 2 cm of the object. Climbing the object was not considered as object investigation. Fractional investigation time was calculated as (investigation time of moved object) / (investigation time of both stationary and moved objects). Automated tracking software (Noldus EthoVision) was used to determine total distance traveled and average velocity of all mice to confirm that there were no inherent differences in activity or motor ability between mice.

Elevated Plus Maze (EPM)

The EPM consisted of two open arms (6 cm × 34.5 cm) at 90° angles to two closed arms (6 cm × 34.5 cm × 21.5 cm) and all arms extend from a common central

platform (6 cm × 6 cm). The maze was elevated 70.5 cm above the ground and a camera was positioned centrally above the maze. Mice were allowed to explore all arms. Time and visits to the relatively exposed, open arms were used as a measure of anxiety, with avoidance of the open arms reflecting high anxiety. Mice's frequency of visits to and duration of time spent in closed versus open arms registered in recorded videos were manually scored by 2-3 observers blind to the genotype and treatment of the mice.

Statistical Analysis

A two-way ANOVA was used to determine if there were any statistical differences between the four experimental groups, and a contingency table test was used to determine the relation between the sample values of each experiment group and two different categorical variables which are contingent on one the other. Sidak's multiple comparisons tests were performed to determine which groups were statistically different from each other. All statistical analyses were performed using GraphPad Prism software with statistical difference being $p<0.05$.

Results

NSPC specific VEGF KD and KA induced excitotoxicity

To study the effects of NSPC-derived VEGF KD, I used VEGF$^{fl/fl}$NestinCreER^{T2} mice following TAM injection to specifically knock down VEGF expression in NSPCs. This mouse model has the CreER^{T2} gene under the nestin promoter, allowing for NSPC specific expression of cre recombinase[19]. Upon TAM binding to the estrogen receptor, the cre recombinase can translocate into the nucleus, allowing for excision of exon3 leading to VEGF KD. (**Fig. 1 A**)[18]. To study the effect of excitotoxicity injury in the context of NSPC-specific VEGF KD, we administered an IP injection of KA or V to KD and Con mice. This experimental design yielded four different groups of mice: ConV, ConKA, KDV, and KDKA (**Fig. 1 B**).

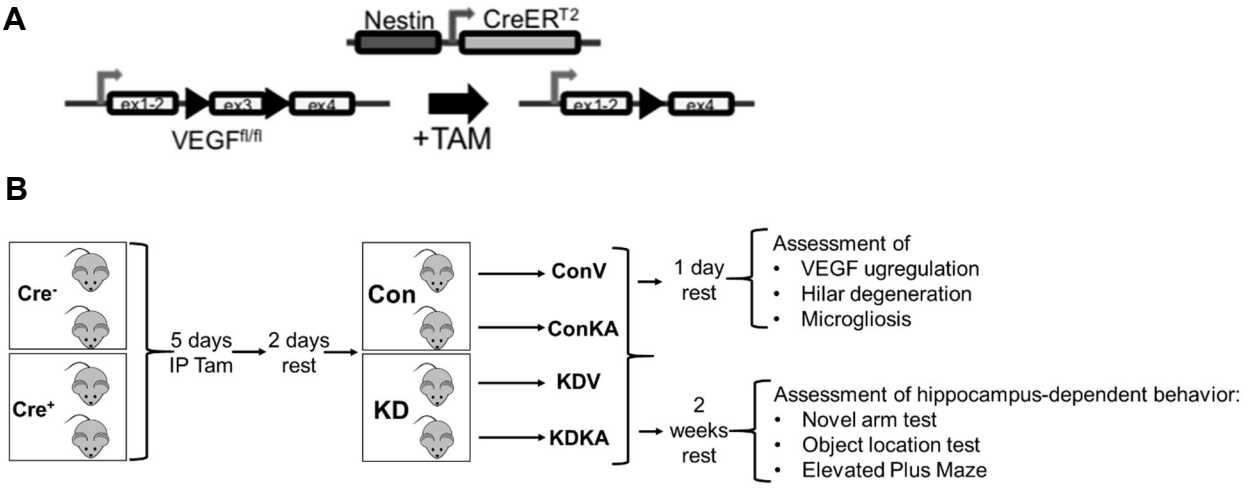

Fig. 1. Mouse model and experimental design. (**A**) VEGF$^{fl/fl}$NestinCreER^{T2} mouse model. (**B**) Cre positive (KD) and negative (Con) mice were given IP injections of TAM (180 mg/kg/d) for 5 days. Mice were given a two-day break before V or KA injection (15mg/kg). This yielded four groups: ConV, ConKA, KDV, and KDKA. After one day, mice's hippocampi were dissected for assessment of VEGF expression, hilar degeneration and microgliosis. Two weeks after either vehicle or KA inject, mice underwent hippocampus-dependent behavior tests consisted of novel arm of Y maze test, and EPM.

Preliminary data from my lab show that VEGF mRNA expression significantly decreased in KDV mice compared to ConV mice, indicating the success of VEGF KD (**Fig. 2 A**). My lab's preliminary data also show that VEGF expression increased after KA treatment, in accordance with published data, and this increase in VEGF expression was reduced in KD mice compared to Con mice (**Fig. 2 A**)[14]. Furthermore, KD mice displayed more markers of KA-induced injury than Con mice. Specifically, KDKA mice showed increased Iba1 immunofluorescence, a sign of microglial activation, compared to the other three groups (**Fig. 2 B, C**). In addition, more KDKA mice exhibited positive fluorojade C (FJC) staining than ConKA mice did, a marker of neurodegeneration **(Fig. 2 D, E)**. Cumulatively, these *in vivo* data suggest that VEGF KD in NSPCs may exacerbate KA induced injury in mice.

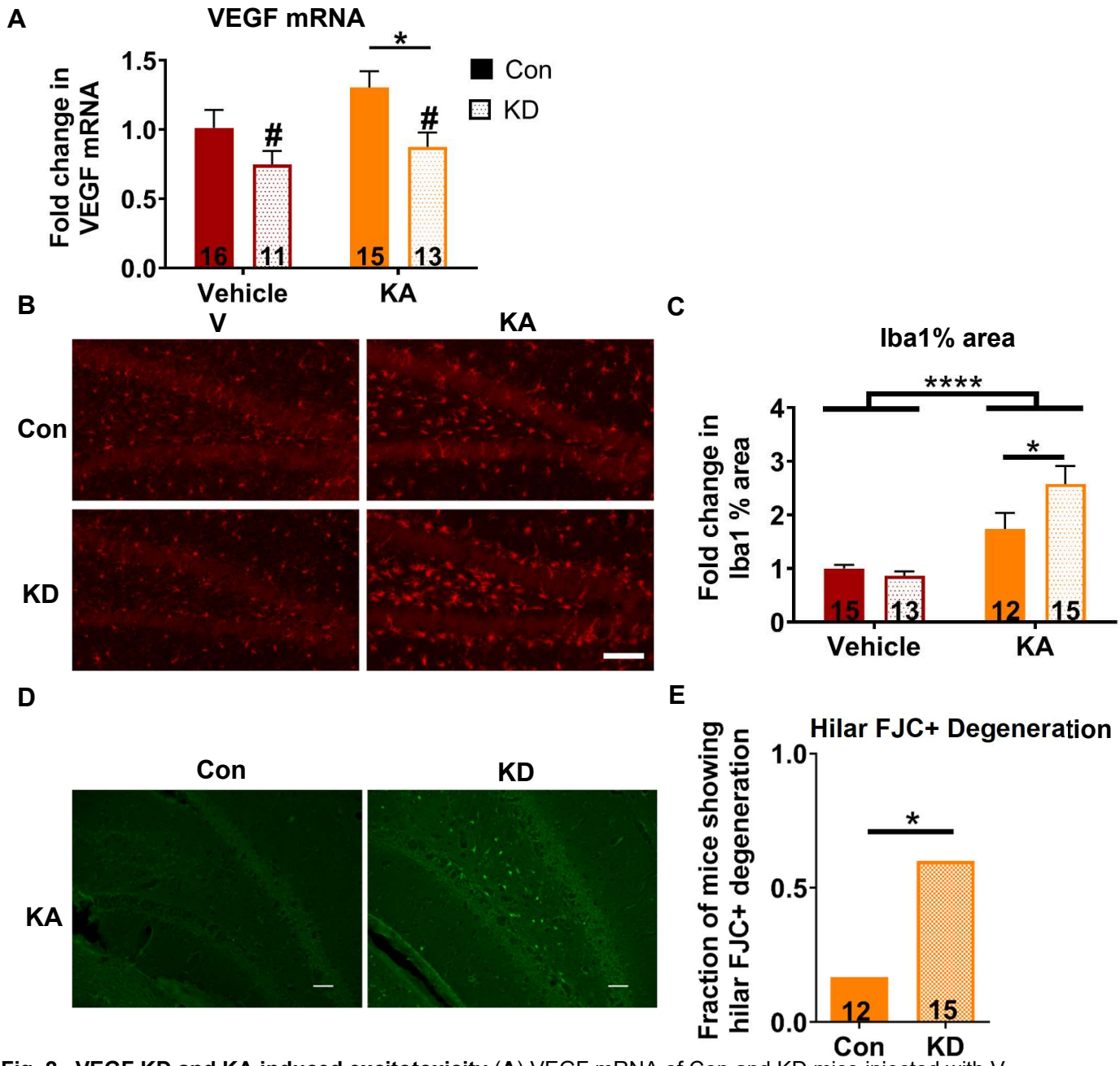

Fig. 2. VEGF KD and KA induced excitotoxicity (**A**) VEGF mRNA of Con and KD mice injected with V or KA was quantified by rt-qPCR. KD mice showed less VEGF expression compared to Con mice (two-way ANOVA, genotype, #p=0.0048, F (1,51) =8.685; KA, p=0.0816, F (1,51) =8.685), but KA treatment decreased more VEGF expression in KD mice than in Con mice (Sidak's multiple comparisons test, *p=0.0048). (**B**) Representative images of Iba1% staining in the DG in the four groups of mice. (Scale bars: 100 µm.) (**C**) KA treated mice showed increased Iba1% staining compared to V treated mice (two-way ANOVA, genotype, p=0.1330 F (1,51) =2.331, KA, ****p<0.0001, F (1,51) =27.77). There was no difference in fold change in Iba1% staining between V injected Con and KD mice, but KA injected KD mice showed increased Iba1% staining compared to Con mice (Sidak's multiple comparisons test, *p=0.0283). (D) Representative images of FJC+ staining in ConKA and KDKA mice. (Scale bars: 100 µm.) (**E**) More KD mice showed hilar FJC+ degeneration than Con mice (Contingency test, Chi-square=5.185, *p=0.0228). Data represents mean ± SEM

KA and VEGF KD impaired hippocampus-dependent short-term spatial memory in mice

The novel arm test was conducted in a Y maze to assess mice's hippocampus-dependent short term spatial memory (**Fig. 3 A, B**). Mice have an inherent preference for novelty and if they have accurate memory for the training trial, they should show preference for visiting the previously blocked arm. This preference for the novel arm was therefore used as a measure of short-term memory. While more ConKA, KDV, KDKA mice visited either the familiar or release arms instead of novel arm, the difference compared to ConV was not significant (**Fig. 3 C**). Additionally, there was a similar trend in KD mice showing an increased latency to investigate the novel arm (**Fig. 3 D**). Collectively, even though it did not reach significance, the data seem to indicate a potential impact on short term spatial memory by knocking down VEGF in NSPCs.

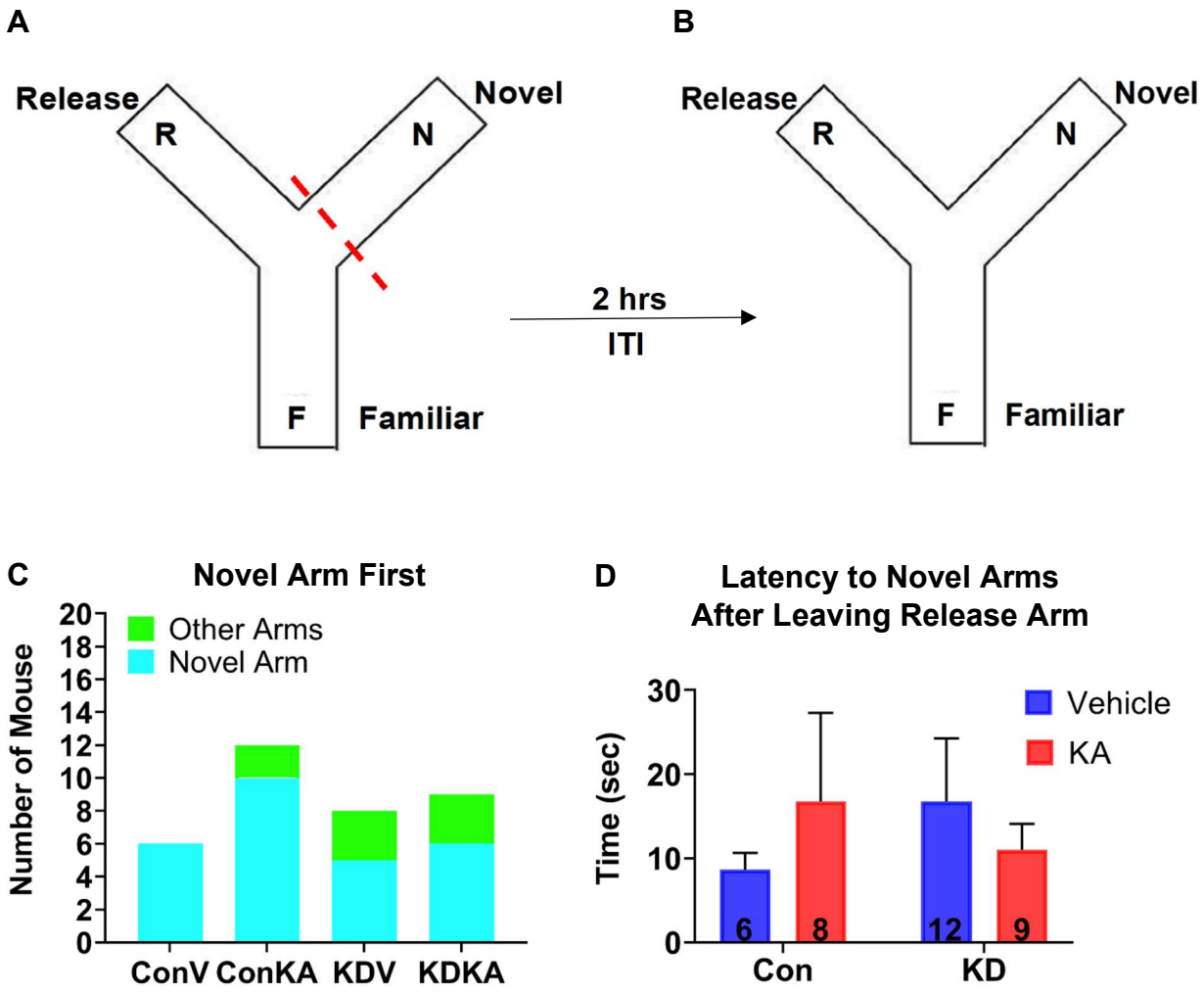

Fig. 3. Short term spatial memory performance of mice based on novel arm test. (**A**, **B**) Schematic drawing of novel arm of Y maze test. (**C**) There was no significant difference of visiting novel arm first instead of other arms among four groups of mice (Contingency table test, Chi-square= 5.810, *p*= 0.1212) (**D**) There was no significant difference of latency to visit novel arm after leaving releasing arm among four groups of mice (two-way ANOVA, genotype, *p*=0.6835, F (1,44) =0.1684; KA, *p*=0.8907, F (1,44) =0.01912). Data represents mean ± SEM.

The OLT was also conducted to assess mice's hippocampus-dependent short term spatial memory (**Fig. 4 A, B, C**). Similar to the novel arm of in a Y maze test, mice with intact hippocampal memory should show a preference for novel stimulus, which in this case was the object in the novel location (i.e., the moved object). During the training trial, mice in four groups investigated both objects for similar amounts of time, which indicates that mice had no inherent preference for either object (**Fig. 4 D**). During the testing trial, KA treated mice spent less time investigating moved object compared to V treated mice, indicating impaired memory caused by KA treatment (**Fig. 4 E**). KD mice also spent less time investigating moved object compared to Con mice in the V treated groups (**Fig. 4 E**), which implies VEGF KD alone impaired mice's short-term spatial memory. During both training and testing trials, VEGF KD and KA treatment had no effects on total investigation time of both moved and stationary objects among four groups of mice (**Fig. 4 F, G**). However, in the testing trial KDKA mice spent more time investigating both objects compared to KDV and ConKA mice (**Fig. 4 F, G**).

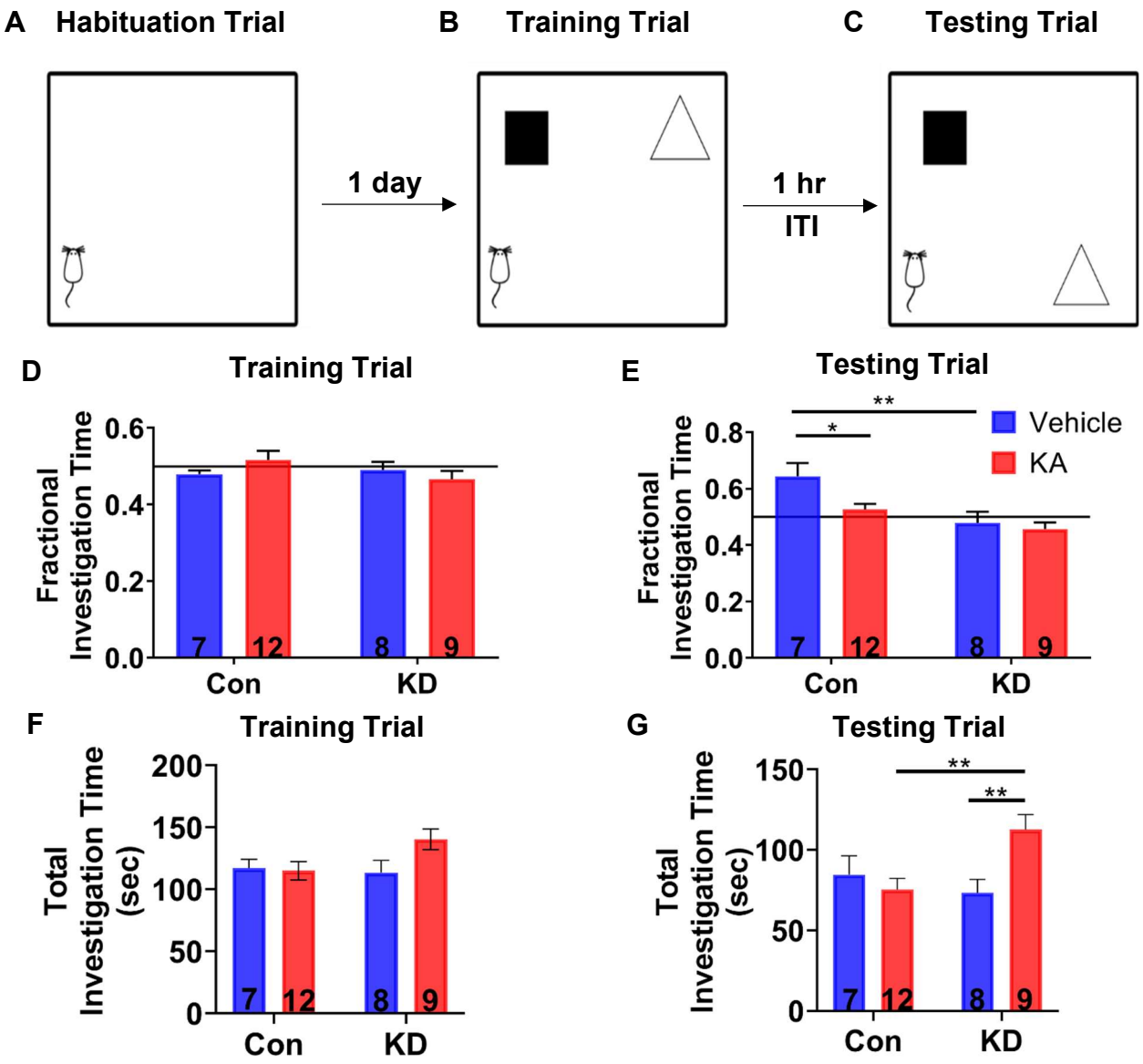

Fig. 4. Short term spatial memory performance of mice based on OLT. (**A**, **B**, **C**) Schematic drawing of OLT. (**D**) In training trial, there was no significant difference of preferring visiting either moved or stationary object among four group of mice (two-way ANOVA, KD, p=0.3829, F (1,32) =0.7827; KA, p=0.7839, F (1,32) =0.7647). (**E**) In testing trial, KA treated mice spent less time investigating moved object compared to V treated mice (two-way ANOVA: *p=0.032); KD mice spent less time investigating moved object compared to Con mice (two-way ANOVA: genotype, **p=0.0007, F (1,32) =14.16; KA, *p=0.0320, F (1,32) =5.027); ConKA mice spent less time investigating moved object compared to ConV (Sidak's multiple comparisons test, *p=0.0207). (**F**) There was no significant difference of investigation time of moved and stationary objects among four groups of mice in training trial ((two-way ANOVA, genotype, p=0.2032, F (1,32) =1.688; KA, p=0.1535, F (1,32) =2.138). (**G**)VEGF KD and KA treatment had no main effects on investigation time of moved and stationary objects among four groups of mice in testing trial. KDKA ((two-way ANOVA, genotype, p=0.1628, F (1,32) =2.051; KA, p=0.1535, F (1,32) =2.768); KDKA mice spent more time investigating both objects compared to KDV mice (Sidak's multiple comparisons test, **p=0.0089); KDKA mice spent more time investigating both objects compared to ConKA mice (Sidak's multiple comparisons test, **p=0.0063). Data represents mean ± SEM.

KA and VEGF KD had no effect on anxiety-like behavior

The EPM, a common test for anxiety, was conducted. The EPM consisted of two open arms and two closed arms (**Fig. 5 A**). Time and visits to the relatively exposed, open arms were used as measures of anxiety-like behavior, with avoidance of the open arms reflecting high anxiety. Time spent and number of entries in open arms did not differ among the four groups of mice (**Fig. 5 B**, **C**). The lack of differences indicates KA and VEGF KD might not have impacts on mice's anxiety. Number of entries in both closed and open arms did not differ statistically among the hour groups of mice (**Fig. 5 D**), suggesting no difference in locomotor activity.

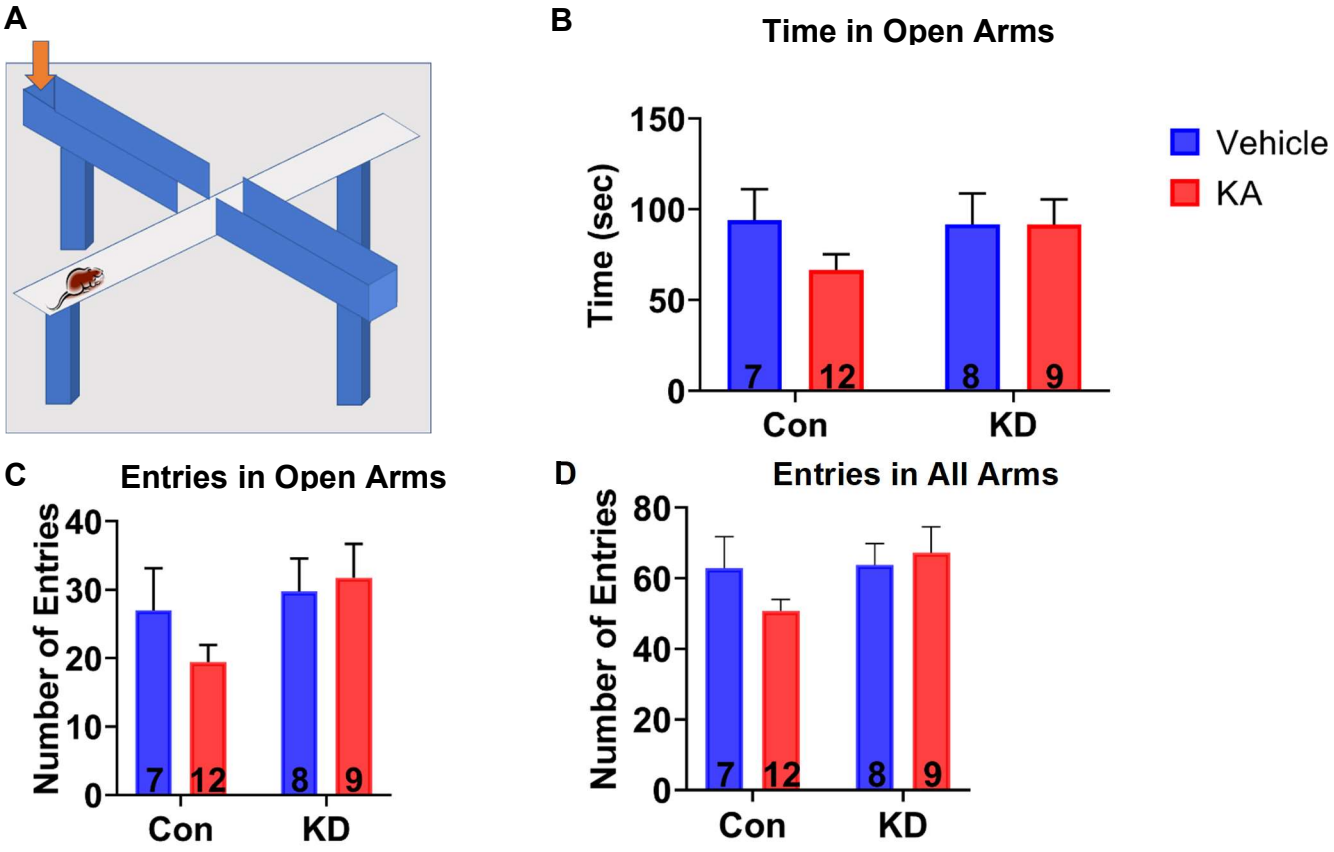

Fig. 5. Anxiety-like behavior performance of mice based on EPM. (**A**) Schematic drawing of EPM. The red arrow indicated the releasing place of every mouse. (**B**) There was no significant difference of time spent in open arms among four group of mice (two-way ANOVA, genotype, p=0.4152, F (1,32) =0.6815; KA, p=0.3236, F (1,32) =1.005). (**C**) There was no significant difference of entries in open arms among four group of mice (two-way ANOVA, genotype, p=0.0999, F (1,32) =2.872; KA, p=0.5377, F (1,32) =0.3882). (**D**) There was no significant difference of number of entries in closed and open arms among four groups of mice (two-way ANOVA, genotype, p=0.1754, F (1,32) =1.920; KA, p=0.4958, F (1,32) =0.4748). Data represents mean ± SEM.

Discussion

This thesis investigated the behavioral changes associated with loss of NSPC-derived VEGF after KA induced excitotoxic injury. Excitotoxic tissue damage could be caused by seizures, and changes in mood and behavior are also observed after seizures[2, 22, 23]. Previous studies have shown that VEGF is upregulated after seizure induced excitotoxicity and can protect against seizure and excitotoxic injury in rats [14, 15]. Incidentally, our lab has shown that NSPCs in the DG of the hippocampus produce a significant amount of VEGF. Importantly, the DG of the hippocampus is a crucial brain region for modulating memory and emotions by producing new neurons[8, 9]. So, we hypothesized that knocking down NSPC-derived VEGF would cause more severe behavioral impairments following excitotoxic injury.

Similar to previous study, our preliminary data also show that VEGF expression was increased after KA treatment compared to that after V treatment. Additionally, we found that VEGF expression was decreased in the KD mice compared to the Con mice after KA treatment. Importantly, the preliminary data also show that KA induced excitotoxic injury was exacerbated in the absence of NSPC-derived VEGF, including increased neuroinflammation and neuron degeneration. The exacerbated excitotoxicity might indicate the later impairments in behavioral functions.

To investigate specific behavioral changes, including hippocampus-dependent short-term spatial memory and anxiety-like behavior, associated with loss of NSPC-derived VEGF after KA induced excitotoxic injury, we utilized the novel arm test in a Y maze test, the OLT, and the EPM. Results from the novel arm test seem to suggest a

potential impact on short-term spatial memory by knocking down VEGF in NSPCs, even though they did not reach significance. And results from the OLT show that KA treatment impaired mice's short-term spatial memory. Surprisingly, results from the OLT have also suggested that VEGF KD alone impair mice's short-term spatial memory which may or may not be further impacted by KA induced excitotoxicity. This particular finding indicates that NSPC-derived VEGF might be essential for the proper memory function. Noticeably, in the testing trial KDKA mice spent more time investigating both stationary and moved objects compared to KDV and ConKA mice, suggesting that there might be potential interaction impact of VEGF KD and KA treatment on locomotor abilities and motivation to investigate. However, in both training and testing trials there were no main effects of VEGF KD and KA treatment on total investigation time of both objects.

Even though our data suggest that VEGF KD and KA treatment impacted the short-term spatial memory which is modulated by the DG of the hippocampus, the results from the EPM indicate that they had no effects on mice's anxiety-like behavior which the DG also plays an important role in[8, 9]. By contrast, one previous study has shown that VEGF treatment helped preserve normal anxiety functioning in mice completely after acute seizures[17]. So, I think my experiment might possibly fail to capture the impact of KA treatment and VEGF KD on mice's anxiety-like behavior.

For future studies, it will be helpful to analyze the data from the open field habituation trials during the first day of OLT as another way to assess mice's anxiety-like behavior. Mice's time spent in the center of the open field could be used to

measure the anxiety-like behavior, with more time spent in the center reflecting less anxiety[24]. The data from the open field habituation trials could be used to confirm whether the lack of effect of KA treatment and VEGF KD on mice's anxiety-like behavior as shown in the EPM is valid or not. In order to determine the potential memory exacerbation of KA effect by KD of NSPC-derived VEGF, future studies could elongate the training trial's duration of the OLT to provide mice more time to form spatial memory. Because by doing that decreases the difficulty of OLT, it would avoid the ceiling effect of assessing mice's short-term spatial memory and could highlight the exacerbation effect of KA effect by KD of NSPC-derived VEGF on memory.

Conclusion

Here we investigated behavioral changes associated with loss of NSPC-derived VEGF after KA induced excitotoxic injury, because of the importance of VEGF on protecting against seizures caused excitotoxic injuries, and also the crucial role of NSPCs, which produce a significant amount of VEGF, on mediating memory and emotions. Overall, our results suggest that KA treatment and VEGF KD could potentially impair mice's short-term spatial memory based on the novel arm test in a Y maze and the OLT but had no effects on mice's anxiety-like behavior based on the EPM. This thesis aimed to contribute to the further research of NSPCs' therapeutic functions by enhancing our understanding of NSPCs' impacts on modulating behavior changes after brain injuries.

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