ABSTRACT

Kelly E. Baker. Targeting Kremen1 Downregulation with RVG-9R/siRNA Complexes in the Triple-Transgenic Mouse Model of Alzheimer's Disease. (Under the direction of Dr. Alexander K. Murashov). Department of Biomedical Science, July 2017.

Alzheimer's disease (AD) is a progressive disease characterized by cognitive decline and memory loss. Memory loss observed in AD results from the loss of neurons and synapses which may be caused by the disruption of the canonical Wnt signaling pathway by Dickkopf-1 (Dkk-1). Under normal conditions, the canonical Wnt signaling pathway is responsible for normal neuronal development, synaptic plasticity, and overall normal brain function. Amyloid-β (Aβ) plaques and neurofibrillary tangles are two characteristic morphological changes observed in AD. An increased level of Aβ has been associated with increased expression of Dkk-1, which may be linked to synaptic loss seen in AD. Kremen1 (Krm1) is a receptor for Dkk-1. Published and unpublished observations from our laboratory showed that silencing Krm1 with miR-431 can promote regenerative axon growth and prevent synaptic loss in a cell culture model. This study focused on downregulating Krm1 the triple-transgenic mouse model of AD (3xTg-AD). It was hypothesized that application of siRNA-431 *in vivo* would downregulate Krm1 thereby preventing synaptic loss and memory deficits in the 3xTg-AD mouse model of AD. Tail vein injections of RVG-9R/siRNA complexes and control injections were administered to 3xTg-AD mice and wild-type (WT) mice at 4, 6, or 12 months of age. Within each age cohort there were three different groups: 3xTg-AD mice injected with RVG/siRNA, 3xTg-AD mice injected with control peptide/siRNA, and WT mice injected with saline. Each group of mice was approximately half male and half female. Following the injections, the Barnes Maze was administered to each mouse in order to assess

memory function. Data gathered from the Barnes Maze shows 3xTg-AD mice have a longer primary latency in the probe phase compared to WT mice. Of mice tested, fewer 3xTg-AD mice have been successful in finding the target hole during probe phase compared to WT mice. After completion of the Barnes Maze, mice were sacrificed and brains were collected for analysis. The brains were analyzed for Krm1 downregulation at the protein and mRNA levels via Western blot and qPCR, respectively. In 4 month old mice, WT mice showed the lowest levels of Krm1 protein and mRNA expression levels and 3xTg-AD CPep/siRNA treated mice showed the highest. The 4 month old 3xTg-AD RVG-9R/siRNA treated mice had Krm1 protein and mRNA expression levels that fell between the other two groups. Immunofluorescence was performed on coronal brain sections to analyze number of synapses. Six month old 3xTg-AD CPep/siRNA treated mice had significantly fewer synapses than both the WT and 3xTg-AD RVG-9R/siRNA treated groups. In conclusion, IF, qPCR, and Western blot data reveal the potential for RVG-9R/siRNA treatment to target and downregulate Kremen1 *in vivo* and provide protection from synaptic loss. However, further studies are need to confirm the ability of RVG-9R/siRNA treatment to downregulate Kremen1.

Targeting Kremen1 Downregulation with RVG-9R/siRNA Complexes in the Triple-Transgenic Mouse Model of Alzheimer's Disease

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Kelly E. Baker

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Targeting Kremen1 Downregulation with RVG-9R/siRNA Complexes in the Triple-Transgenic Mouse Model of Alzheimer's Disease

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LIST OF ABBREVIATIONS

3xTg-AD	Triple-transgenic mouse model of Alzheimer's disease
Aβ	Amyloid-β
AD	Alzheimer's disease
CPep	Control peptide: Mr-40
DAPI	4'-6-diamidino-2-phenylindole
DKK-1	Dickkopf-1
FITC	Fluorescein isothiocyanate
IgG	Immunoglobulin G
Krm1	Kremen1
miR	micro-ribonucleic acid
PBS	Phosphate buffered saline
RVG-9R	Rabies virus glycoprotein-9R
siRNA	Short interfering ribonucleic acid
WT	Wild-type

INTRODUCTION

Alzheimer's disease

Alzheimer's disease (AD) is the most common form of dementia. More than 5 million Americans are currently living with AD (Alzheimer's Association, 2017; Taylor et al., 2017). The majority of people with AD are 65 or older (Alzheimer's Association, 2017). AD is more common in women than in men; nearly two-thirds of Americans with AD are women (Hebert et al., 2013). African-Americans are more likely to have AD than other ethnic groups (Alzheimer's Association, 2017). While some treatments for symptoms of AD are available, there is currently no known cure for AD (Götz and Ittner, 2008; Alzheimer's Association, 2017).

Memory loss and progressive cognitive decline are characteristic of AD (Wilson et al., 2012). Decline in cognitive function occurs years before the onset of dementia (Wilson et al., 2012). The most common symptom of AD is the inability to remember new information (Alzheimer's Association, 2017). Symptoms of AD can vary widely. Other commonly observed symptoms include changes in personality, poor judgement, problems with words, and problems completing familiar tasks (Alzheimer's Association, 2017).

There are two hallmark pathological changes in the AD brain: Amyloid-β (Aβ) plaques and tau neurofibrillary tangles (Alzheimer's Association, 2017). The biggest pathological changes occur in the hippocampus and subcortical regions (Götz and Ittner, 2008). Aβ plaques are comprised of a 40-42 amino acid peptide and are located outside of neurons (Götz and Ittner, 2008; Alzheimer's Association, 2017). These

plaques develop over time as $A\beta_{42}$ slowly accumulates (Selkoe, 2002). Aβ plaques are toxic to synapses (Spires-Jones and Hyman, 2014) and prevent communication between neurons at synapses (Alzheimer's Assocation, 2017). It is hypothesized that Aβ can cause synapses to fail before plaques are formed (Selkoe, 2002; Webster et al., 2014). When synaptic connections are lost memory deficits and cognitive decline can occur. Tau is a protein that, under normal conditions, helps stabilize microtubules found in axons (Götz and Ittner, 2008). However, when tau is hyper-phosphorylated it disassociates from microtubules causing tau to collapse and form tangles within the cells (Götz and Ittner, 2008). These tangles prevent essential nutrients from travelling in neurons causing them to die (Alzheimer's Association, 2017).

AD is considered to be sporadic (SAD) or familial (FAD) (Götz and Ittner, 2008). FAD is rare, accounting for 1-10% of AD cases (Bekris et al., 2011; Alzheimer's Association, 2017). Three genetic mutations have been identified in FAD: *APP*, *presenilin 1* (*PSEN1*) and *presenilin 2* (*PSEN2*) (Götz and Ittner, 2008). *APP* missense mutations account for up to 15% of FAD (Bekris et al., 2011). Mutations in the *APP* gene are associated with increased levels of Aβ (Bekris et al., 2011). Mutations in *PSEN1* result in the most severe form of AD with onset as early as the age of 30 (Bekris et al., 2011). Abnormalities in *PSEN2* do not result in as severe outcomes as those seen in *PSEN1* mutations and *PSEN2* related AD cases tend to see an onset of approximately 40 years of age (Bekris et al., 2011).These genetic mutations have been used to develop animal models, such as the triple-transgenic mouse model (Oddo et al., 2003), to study AD.

The majority (90% or more) of AD cases are sporadic and tend to be late onset AD (occurring at 65 or older) (Bekris et al., 2011). Apolipoprotein E (APOE) has been associated with AD (Götz and Ittner, 2008; Bekris et al, 2011; Alzheimer's Association, 2017). Specifically the *APOE* ε4 is linked to a higher risk of developing AD (Bekris et al., 2011; Alzheimer's Association, 2017). A recent study showed that 56% of people in the United States diagnosed with AD have at least one copy of *APOE* ε4 (Ward et al., 2012). Other than age of onset of AD, there seem to be no differences between FAD and SAD (Götz and Ittner, 2008).

Wnt Signaling

Wnt signaling is important for a multitude of cellular processes such as neuronal polarity and migration, cell proliferation, dendritogenesis, and synaptogenesis (Inestrosa and Toledo, 2008; Dickins and Salinas, 2013). Wnt signaling starts during nervous system development and is vital in the adult brain for synaptic plasticity (Chen et al., 2006; Gogolla et al., 2009; Cerpa et al., 2011; Ciani et al., 2011; Purro et al., 2012 Inestrosa et al., 2012). Wnts have been shown to play a role in nervous system development, in particular, multiple wnt ligands have been identified that influence forebrain development, which gives rise to the hippocampus (Inestrosa et al., 2012). Loss of Wnt signaling has been implicated in neurodegenerative diseases, such as Alzheimer's disease, as well as Schizophrenia (Inestrosa et al., 2012).

Wnt signaling is characterized as either canonical or non-canonical and activation of Wnt signaling can be β-catenin dependent or β-catenin independent (Inestrosa et al., 2012). The planar cell polarity (PCP) pathway (Montcouquiol et al.,

2006) and the Wnt/Ca^{2+}signaling pathway (Kohn and Moon 2005; Gorand and Nusse, 2006) are two non-canonical Wnt signaling pathways that have been elucidated. The canonical Wnt signaling pathway, or Wnt/β-catenin pathway, may be implicated in the development of AD.

Under normal conditions, the canonical Wnt signaling pathway functions to induce transcription of Wnt target genes. Extracellular Wnt ligands interact with Frizzled (Fz) receptor proteins forming complexes on the cell surface with the low density lipoprotein (LRP)-5/6 (Tamai et al. 2000; Davidson et al. 2005; Zang et al. 2005; Inestrosa et al., 2012). This complex then activates Dishevelled (Dvl), which inactivates glycogen-synthase-kinase-3β (GSK-3β). Inactivation of GSK-3β prevents the phosphorylation and degradation of β-catenin. β-catenin can then enter the nucleus and interact with T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) transcription factors, allowing for the transcription of Wnt target genes (Clevers and Nusse 2012; Nusse and Varmus 2012).

Lower levels of Wnt signaling may play a role in synaptic loss observed in AD (Puro et al., 2012). Several studies have proposed a relationship between Aβ-linked neurotoxicity and disruption of the canonical Wnt signaling pathway (De Ferrari and Inestrosa 2000; Inestrosa et al. 2000; Garrido et al. 2002; Inestrosa et al. 2002; De Ferrari et al 2003). Aβ is directly related to increased levels of Dickkopf-1 (Dkk-1) (Purro et al., 2012; Kilikck et al., 2012). Dkk-1 is a secreted antagonist of the canonical Wnt signaling pathway (Inestrosa et al., 2012; Purro et al., 2012). Dkk-1 contributes to Aβ induced synaptic loss (Purro et al., 2011) and cognitive decline seen in dementia (Dickins and Salinas, 2013). Dkk-1 is expressed at high levels in AD brains, particularly

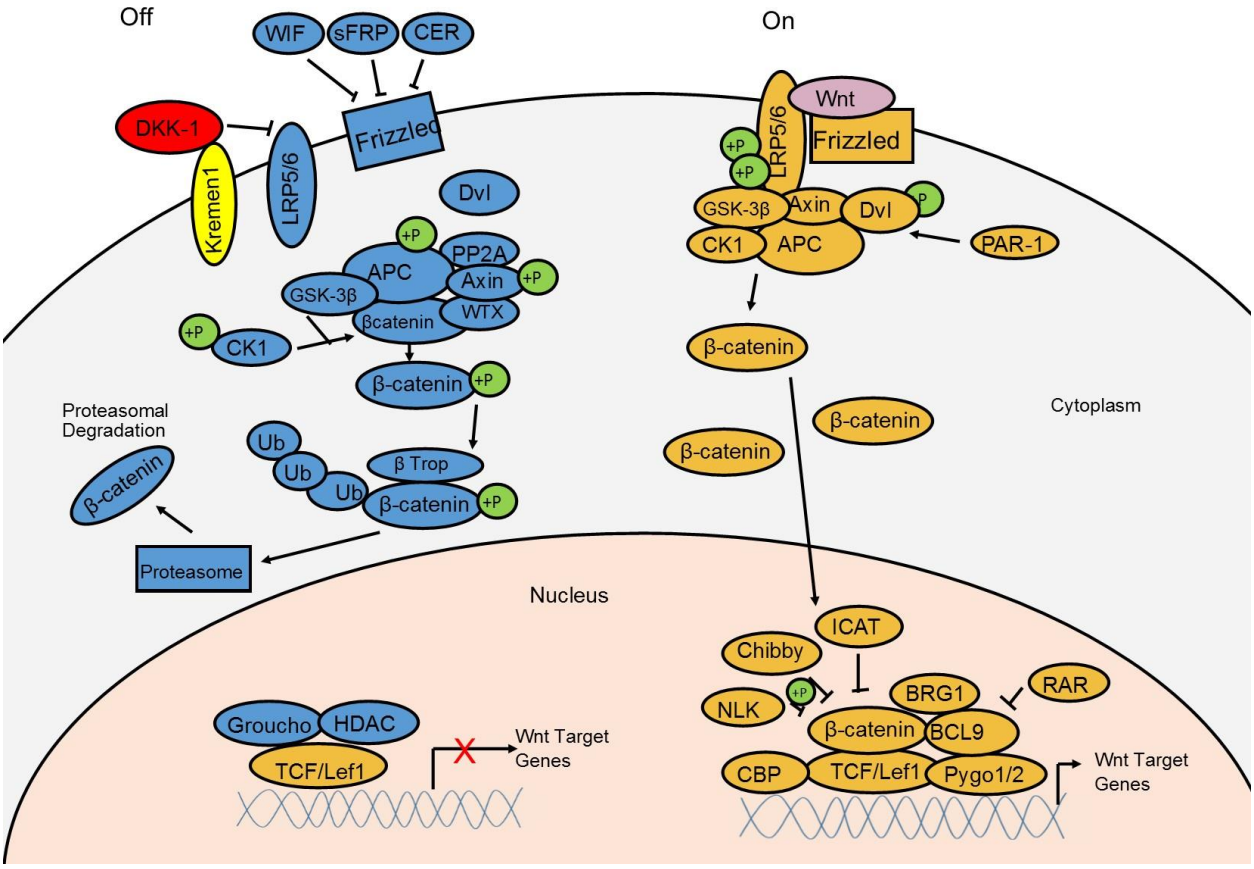
near Aβ plaques and tau neurofibrillary tangles (Caricasole et al., 2004). This secreted glycoprotein inhibits normal canonical Wnt signaling function by binding to LRP6 (Niehrs, 2006) or by inhibiting LRP6 when it binds to Kremen1, a transmembrane protein (Mao et al., 2002). When Dkk-1 interacts with LRP6, Wnt can no longer bind. This results in a cascade of events ultimately ending in phosphorylation of β-catenin by GSK-3β, ubiquitination of β-catenin, and finally proteosomal degradation of β-catenin (Fig.1). If β-catenin was not stabilized and translocated to the nucleus, but rather degraded, Wnt target genes cannot be transcribed, leading to synaptic loss and subsequent cognitive decline.

Studies have suggested that Dkk-1 is necessary for Aβ related synaptic loss (Purro et al., 2012). Exposing brain slices to Aβ rapidly increased Dkk-1 at the mRNA level (Purro et al., 2012). When Dkk-1 is knocked down, increased neurogenesis has been observed in the adult hippocampus (Seib et al., 2013). By blocking Dkk-1 with neutralizing antibodies, Aβ cannot stimulate synaptic loss as readily (Dickins and Salinas, 2013).

As previously mentioned, Dkk-1 can bind to Kremen1 to block normal function of the canonical Wnt signaling pathway. Mao et al. suggest that Kremen1 binds to Dkk-1 with high affinity (2002). Kremen1 and Dkk-1 form a complex that, in turn, inhibits LRP5/6 preventing normal function of the canonical Wnt signaling pathway. Kremen1 is a transmembrane protein that has a high affinity for Dkk-1 (Mao et al., 2002). Kremen2 also has a high affinity for Dkk-1 and forms a complex with Dkk-1 and LRP6 to induce endocytosis of the Wnt receptor (Mao et al., 2002). Knockouts of Kremen1 have been linked to disrupted thymus architecture (Osada et al., 2006) and morpholino

Figure 1. Schematic of Wnt signaling mechanisms. Disruption of the canonical Wnt signaling pathway has been associated with synaptic loss and cognitive decline observed in AD. When this pathway is functioning normally (orange), β-catenin is stabilized and Wnt target genes are transcribed. However, when Dkk-1 bind with Kremen1 and inhibits LRP5/6 (blue) the canonical Wnt signaling pathway is disrupted. β-catenin is marked for proteasomal degradation preventing its translocation into the nucleus, thereby preventing the transcription of Wnt target genes. (Adapted from Abcam Wnt pathway poster)

Canonical Wnt Signaling Pathway



oligonucleotide knockdown of Kremen1 and Kremen2 in *Xenopus* has been linked to defects in anterior neural development (Davidson et al., 2002). Triple mutant mice, *Krm1*$^{-/-}$ *Krm2*$^{-/-}$ *Dkk1*$^{+/-}$, show increased ectopic tissue growth (Ellwanger et al., 2008). Double *Krm* mutant mice develop increased bone mass and volume (Ellwanger et al., 2008).

MicroRNA-431

MicroRNAs (miRNAs) are small, noncoding RNAs that function in RNA silencing and regulating gene expression. Several miRNAs have been proposed to be involved in neurodevelopment, memory, and neurogenesis (Roshan et al., 2014). miRNA-431 (miR-431) has been shown to naturally target *Kremen1* (Wu and Murashov, 2013). *Kremen1* was shown to be downregulated by miR-431 at both the protein an mRNA levels (Wu and Murashov, 2013). miR-431 promotes regenerative axon growth by silencing *Kremen1* in a peripheral nerve injury model (Wu and Murashov, 2013). When miR-431 is overexpressed in cultured dorsal root ganglia neurons, axon length is increased (Wu and Murashov, 2013). It has been shown that miR-431 works to silence *Kremen1* by directly interacting with the 3'-untranslated region of *Kremen1* mRNA (Wu and Murashov, 2013.)

In cortico-hippocampal cell culture, miR-431 prevents synaptic loss when cells are exposed to toxic Aβ (Fig. 2). miR-431 also protects against neurite loss that occurs from Aβ exposure (Fig. 3). This illustrates the potential for using miR-431 as a therapeutic tool to prevent synaptic loss observed in AD.

Figure 2: miR-431 protects against synaptic loss. (A) A control cortico-hippocampal cell culture displaying normal synaptic puncta. (B) When cortico-hippocampal cells are exposed to Aβ, synaptic puncta are lost. (C) miR-431 prevents synapses loss induced by Aβ toxicity. White arrows indicate synaptic puncta.

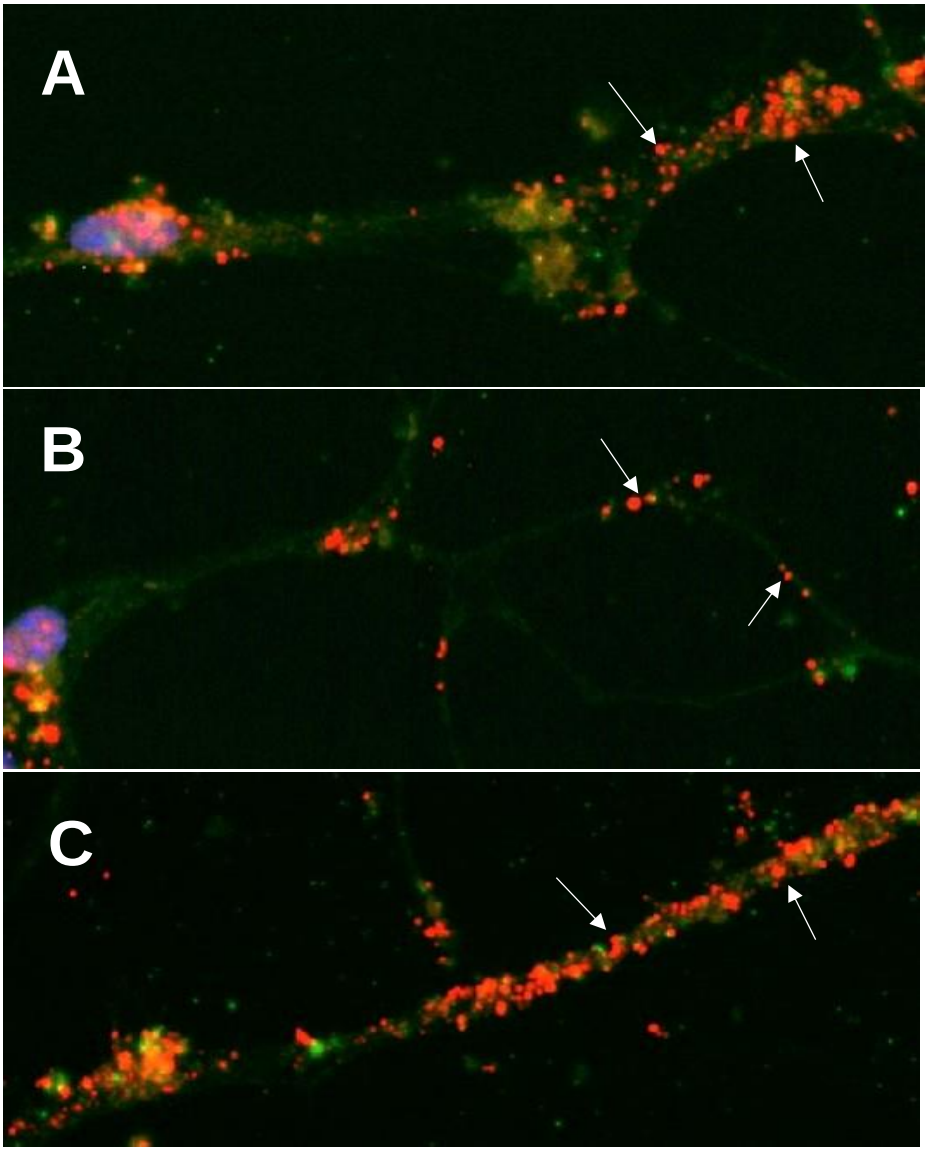
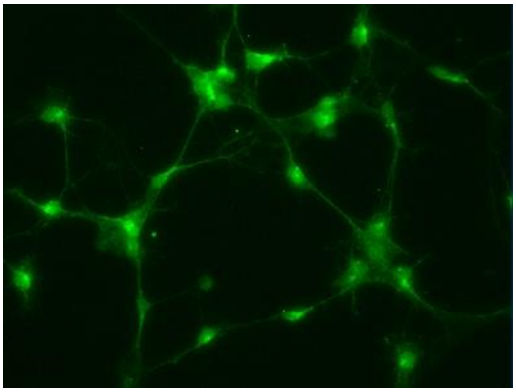

A
B
C

Figure 3: miR-431 protects against neurite loss. (A) Healthy neurites in a cortico-hippocampal cell culture. (B) Exposure to Aβ induces neurite loss in cortico-hippocampal cell culture. (C) miR-431protects neurites from Aβ toxicity.

A



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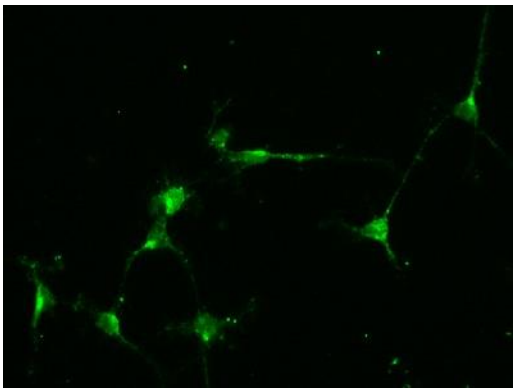

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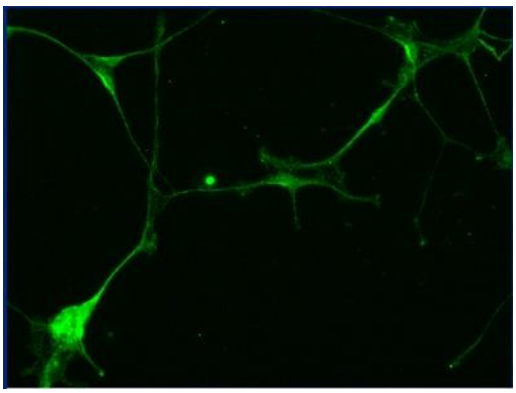


3xTg-AD Mouse Model of AD

There are at least 10 different mouse models of AD: PDAPP (Games et al., 1995), TG2576 (Hsiao et al., 1996), APP23 (Sturchler-Pierrat et al., 1997), TgCRND8 (Chishti et al, 2001), J20 (Mucke et al., 2000), App/PS1 (Jankowsky et al., 2001), TG2576 + PS1 (M146L) (Holcomb et al., 1999), APP/PS1 KI (Flood et al., 2002), 5xFAD (Oakley et al., 2006), and 3xTg-AD (Oddo et al., 2003) (Webster et al., 2014). The triple transgenic (3xTg- AD) mouse model of AD was first developed by Oddo et al. in 2003. These mice contain three transgenes (APP_{Swe}, tau_{P301L}, and $PSEN1_{M146V}$) that are associated with familial AD. This mouse line progressively develops both plaques and neurofibrillary tangles (Oddo et al., 2003), two pathological hallmarks of AD. Aβ deposits are first evident at 6 months of age and are widespread by 12 months of age (Oddo et al., 2003). Tau tangles are not apparent at 6 months of age but are apparent by 12 months of age (Oddo et al., 2003). The progressive development of Aβ plaques and tau neurofibrillary tangles mimics that of human AD pathology.

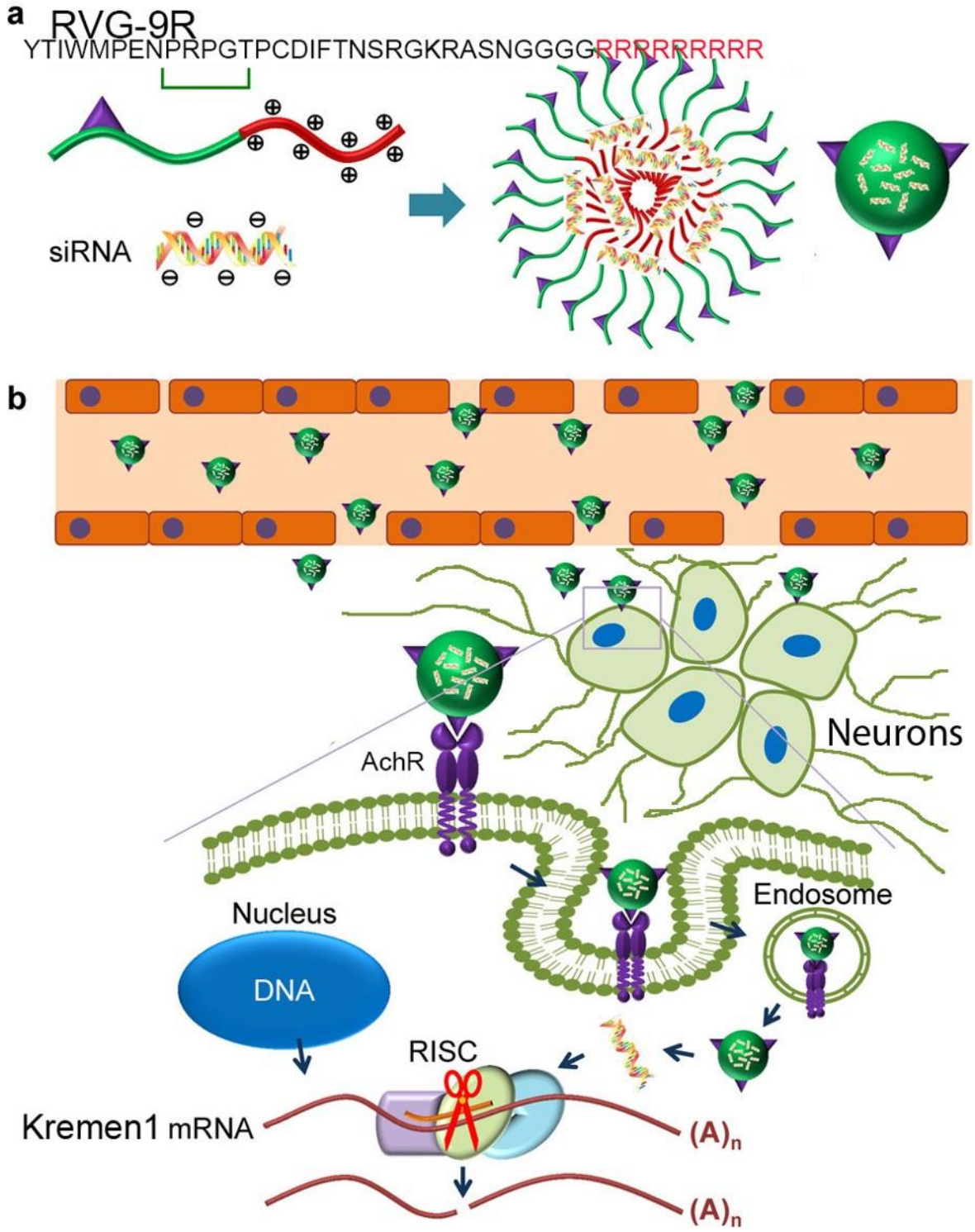
Synaptic transmission and long-term potentiation (LTP) are both impaired in 3xTg-AD mice by 6 months of age (Oddo et al., 2003). Both male and female 3xTg-AD mice appear to be equally affected by their transgenes, unlike other mouse models of AD (Oddo et al., 2003). The age-dependent progression of pathology and behavioral changes in the 3xTg-AD mouse model allows for more opportunities to study different aspects and mechanisms of AD.

Rabies Virus Glycoprotein-9R

The blood-brain barrier (BBB) is an obstacle for designing therapeutics aimed at treating diseases and disorders of the central nervous system (CNS). The BBB acts as a protective barrier for the brain, preventing molecules circulating in the blood stream from passing into the brain. Neurotrophic viruses, such as the rabies virus, are able to cross the BBB. Harnessing the ability of these viruses to cross the BBB could provide a potential mechanism for delivery of therapeutics to the brain (Huey et al., 2016).

A small peptide derived from the rabies virus, rabies virus glycoprotein (RVG), has been shown to successfully deliver short-interfering RNA (siRNA) across the blood brain barrier in a mouse model (Kumar et al., 2007). RVG specifically binds with nicotinic acetylcholine receptors on neuronal cells (Lentz et al., 1981; Kumar et al., 2007). A positively charged arginine nonamer at the end of RVG (RVG-9R) allows for negatively charged siRNA to bind with the peptide (Fig.4). Application of RVG-9R/siRNA *in vivo* does not produce inflammatory cytokines or anti-peptide antibodies (Kumar et al., 2007).

Figure 4: Brain targeted siRNA delivery across BBB involving RVG/Ach receptor interactions. The positively charged arginine nonamer at the end of the RVG peptide binds negatively charged siRNA forming RVG/siRNA complexes. The complexes pass through the blood-brain barrier. The RVG peptide specifically binds to nicotinic acetylcholine receptors on neurons and the complex is taken into the cell via receptor-mediated endocytosis. Once inside the cell, the siRNA will induce RNA interference thereby blocking expression of Kremen1 (Modified from: Huang et al., 2015)


a
RVG-9R
YTIWMPENPRPGTPCDIFTNSRGKRASNGGGGRRRRRRRRR
siRNA
b
AchR
Neurons
Endosome
Nucleus
DNA
RISC
Kremen1 mRNA
(A)n
(A)n

MATERIALS AND METHODS

Animals

Experiments were conducted on 3xTg-AD mice (B6;129-Psen1tm1Mpm Tg(APPSwe,tauP301L)1Lfa/Mmjax) and wild-type (WT) control mice (101045 B6129SF2/J). Animals were group housed and kept on a twelve-hour light/dark cycle with unlimited access to food and water. All animal experiments were approved by ECU IACUC.

Cortico-hippocampal Cultures

Primary cortico-hippocampal cultures were used to determine effective molar ratios for RVG-9R and siRNA specific to Kremen1 (Sigma, St. Louis, MO). The hippocampus and cortical tissue was isolated from adult mice and snipped in Hibernate A with B27 Supplement and GlutaMax (HABG; Hybernate A, 1xB27, 0.5mM Glutamax, Thermo Fisher Scientific, Waltham, MA). Cortico-hippocampal tissue was transferred to 5mL HABG and incubated at 30°C for 8min. Tissue was then dissociated in papain in Hibernate A–Ca^{2+} for 30min at 30°C. The tissue was then re-suspended in HABG. Cortico-hippocampal tissue was triturated. Next, neurons were separated by a density gradient made with OptiPrep Density Gradient Solution (Sigma) according to Brewer et al. (1997). The neuronal portion was then re-suspended in Neurobasal A with B27 and 10ng/ml mouse PDGF and 5ng/ml mouse FGF (Thermo Fisher Scientific). Neurons were then plated on poly-D-lysine coated coverslips (Sigma). Neurons were grown for 14-21 days. Approximately 1/3 of the media was removed and replaced 4 days following plating and then once a week from then on.

For three days prior to transfection, approximately 1/3 of the media was removed and replaced with antibiotic-free media. Complexes of RVG-9R with FAMsiRNA (Ambion, Cambridge, MA, USA) and complexes of a control peptide with FAMsiRNA were prepared in a range of molar ratios (peptide:siRNA): 1:10, 1:15, 1:30 and 1:50. Peptides and FAM siRNA were combined and incubated for 20min at RT. Neurons were transfected for 48hrs before they were fixed with ice cold 4% paraformaldehyde in PBS.

List of Peptides:

RVG-9R (Yr-41): YTIWMPENPRPGTPCDIFTNSRGKRASNGGGGrrrrrrrrr (PepMic, Suzhou, China)

Mr-40: MNLLRKIVKNRRDEDTQKSSPASAPLDGGGGrrrrrrrrr (PepMic)

Coverslips were washed three times with PBST (phosphate buffered saline with 0.3% triton) and then blocked with 10% goat serum in PBST for 1hr at RT. Blocking serum was then removed and cells were incubated in primary antibody solution (Kremen1 1:200, rabbit polyclonal IgG) overnight at 4°C. The next day primary antibodies were removed and cells were washed three times in PBST. Cells were incubated in secondary antibodies (Alexa Fluor™ 488 goat anti-mouse IgG (H+L) and Alexa Fluor™ 594 goat anti-rabbit IgG (H+L), Invitrogen) for 1hr. Coverslips were then washed another three times in PBST prior to being incubated in DAPI (Sigma) for 5min. Coverslips were then mounted on slides with anti-fading medium Fluoro-Gel (Electron Microscopy Sciences, Hatfield, PA).

Slides were imaged using an Olympus IX81 Inverted fluorescent microscope and CellSens Dimension software (Olympus America, Inc.).

Organ Specificity Testing

To ensure brain specific delivery of siRNA by RVG-9R, two wild-type mice (one male and one female) received tail vein injections of RVG-9R/FAMsiRNA (FAMsiRNA, Sigma) or the control peptide (Mr-40)/FAMsiRNA. Peptide/siRNA complexes were formed in a 1:15 molar ratio and 5% glucose was used as the vehicle. Two tail vein injections were administered to each mouse 6 hours apart. Mice were then sacrificed 10 hours after the second injection.

Mice were perfused with 60 mL phosphate buffered saline (1x PBS, pH 7.4) followed by 60 mL 4% paraformaldehyde in PBS (pH 7.4). The brain, kidney, liver, heart, spleen, and lungs were harvested from both mice. Organs were fixed in 4% paraformaldehyde in PBS (pH 7.4) overnight at 4°C. The organs were then transferred to 30% sucrose in d. H_2O where they were stored at 4°C until they sunk to the bottom of the container. Organs were then frozen in OCT (Tissue-Tek, Sakura Finetek, Torrance, CA). Once frozen, organs were sliced in 50µm sections on a cryostat and store in PBS with sodium azide until they were stained.

Slides were imaged with consistent gain and exposure time using an Olympus IX81 Inverted fluorescent microscope and CellSens Dimension software (Olympus America, Inc., Center Valley, PA).

Tail Vein Injections

Three different types of injections were administered during this experiment: saline, RVG-9R/siRNA, and Control Peptide/siRNA. Wild-type mice received a saline injection. 3xTg-AD mice were randomly assigned to receive an injection of RVG-

9R/siRNA or Control Peptide/siRNA. siRNA specifically targeted mouse Kremen1 (Sigma). Male and Female mice were injected at one of three ages: 4 months, 6 months, or 12 months. Refer to Table 1 for information regarding exact sample sizes and breakdown of treatment groups by sex.

Peptides and siRNA were prepared in a 1:15 molar ratio in 5% glucose. Peptides and siRNA were combined with the vehicle and incubated at RT for 20min. 3xTg-AD mice received 50µg of siRNA. Total injection volume was 175µL.

Mice were removed from their home cage and placed into a restrainer. Injections were made into the lateral tail vein. Mice were then returned to their home cage. Injections were administered 48 hours prior to the start of behavioral testing (Fig. 5).

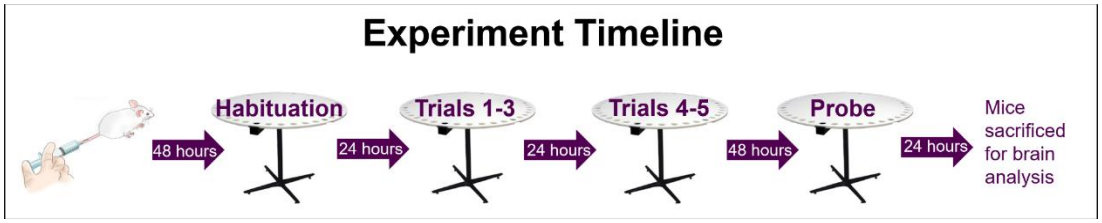
The Barnes Maze

The Barnes Maze was designed to test spatial learning and memory in rats and has been modified for use with mice (Barnes, 1979). The elevated circular maze has 40 holes around its circumference. Beneath one of these holes, the escape hole, is an escape box. The location of the escape box is static throughout the course of the protocol. Extra-maze cues on the walls around the maze (orange triangle, blue circle, and yellow square of approximately the same size) and repeated training trials (five) are used for the mouse to learn the location of the escape box. On the last day of the protocol the escape box is removed and the mouse is tested to determine if it learned the location of the escape box. There are several versions of the Barnes maze operating protocol varying in number of training trials, training days, among other

Table 1: Experimental groups. Breakdown of mice used in this study by treatment, age and sex.

Age	Strain + Treatment		
4 Months	**WT + Saline** Males n=10 Females n=10 Total n=20	**3xTg + RVG/siRNA** Males n=10 Females n=10 Total n=20	**3xTg + CPep/siRNA** Males n=7 Females n=13 Total n=20
6 Months	**WT + Saline** Males n=10 Females n=10 Total n=20	**3xTg + RVG/siRNA** Males n=13 Females n=7 Total n=20	**3xTg + CPep/siRNA** Males n=10 Females n=10 Total n=20
12 Months	**WT + Saline** Males n=10 Females n=11 Total n=21	**3xTg + RVG/siRNA** Males n=3 Females n=8 Total n=11	**3xTg + CPep/siRNA** Males n=2 Females n=8 Total n=10

Figure 5: Experimental timeline. From the time a mouse is injected to the time of sacrifice is 7 days. Mice received a tail vein injection 48 hours prior to starting the Barnes maze protocol. Twenty-four hours following the habituation phase of the Barnes maze training trials begin. The first day of training consists of 3 training trials. The following day mice receive two more training trials. Forty-eight hours after completing the last training trial, mice are subjected to the probe phase of the Barnes maze. During the probe phase of the Barnes maze the escape box is no longer present. Twenty-four hours following completion of the probe phase mice were sacrificed and brains were taken for molecular analysis.


Experiment Timeline
Habituation
Trials 1-3
Trials 4-5
Probe
48 hours
24 hours
24 hours
48 hours
24 hours
Mice sacrificed for brain analysis

changes. The protocol used in this study was adapted from the short training protocol described by Attar et al. (2013).

Prior to the start of each Barnes maze habituation, training or probe phase, mice were removed from their home cages and placed in individual holding cages. Five cages were placed in the testing room regardless of the number of mice being tested on a particular day.

The first day of the Barnes Maze protocol is referred to as the habituation day. On this day the mouse is placed in the center of the maze under an opaque start box for 10 seconds. This ensures a random orientation of each mouse upon the start of the trial. The start box is removed and the mouse is guided to the escape box via the experimenter's hands. Once the mouse enters the escape box it remains inside for 2 minutes. The mouse is then returned to its holding cage.

Twenty-four hours following the habituation phase the training phase begins. The training phase consists of five training trials over the course of two days: 3 training trials on the first day and 2 training trials 24 hours later. For each training trial the mouse is placed on the center of the maze under the opaque start box for 10 seconds. Once the start box is removed the mouse is allowed to explore the maze for 180 seconds. During this 180 seconds, primary errors, primary latency, total errors, and trial end time are recorded. Primary errors are defined as any nose poke made in a hole that is not the escape hole that occurs prior to the initial finding of the escape hole. Primary latency is the amount of time that passes from the start of the training trial until the mouse initially finds the escape hole. Total errors are the nose pokes made in a hole that is not the escape hole. The trial end time is the time at which the mouse enters the escape box or

180 seconds. If the mouse does not enter the escape box during the 180 second exploratory period the mouse is guided via the experimenter's hands to enter the box. The mouse remains inside the escape box for 60 seconds. The mouse is then removed and returned to its holding cage. Each mouse is allowed a minimum of 15 minutes between training trials.

Forty-eight hours following the last training trial is the probe phase. During this 120 second phase the escape box is removed from the maze. Primary errors, primary latency, and total errors are recorded. Additionally, the number of nose pokes made into the escape hole are also recorded.

Several measures were collected for each mouse during the Barnes maze including: primary errors, primary latency, total errors, and number of nose pokes in the escape hole. Each of these measures is used to indicate if a mouse is successfully learning the location of the escape hole over the course of the Barnes maze protocol.

Primary errors are defined as the number of nose pokes a mouse makes into a hole that is not the escape hole, prior to initially finding the escape hole. The fewer the primary errors a mouse makes indicates better learning. The number of primary errors is counted for each mouse during all five training trials and the probe phase. Primary errors were analyzed by treatment group for each age cohort. In addition, primary errors were then analyzed by sex and treatment for each age group.

Twenty-four hours after completing the Barnes Maze protocol mice were sacrificed for brain analysis.

Immunofluorescence

Twenty-four hours following completion of the probe phase of the Barnes maze mice were euthanized via an overdose of isoflurane. A maximum of 3 mice of each sex from each age cohort and treatment group were randomly selected for immunofluorescence. Mice were perfused with 60 mL phosphate buffered saline (1x PBS, pH 7.4) followed by 60 mL 4% paraformaldehyde in PBS (pH 7.4). The brain was removed and fixed in 4% paraformaldehyde in PBS (pH 7.4) overnight at 4° C. The following day brains were transferred to 30% sucrose and remained in the solution until they sank. Brains were then frozen in OCT (Tissue-Tek).

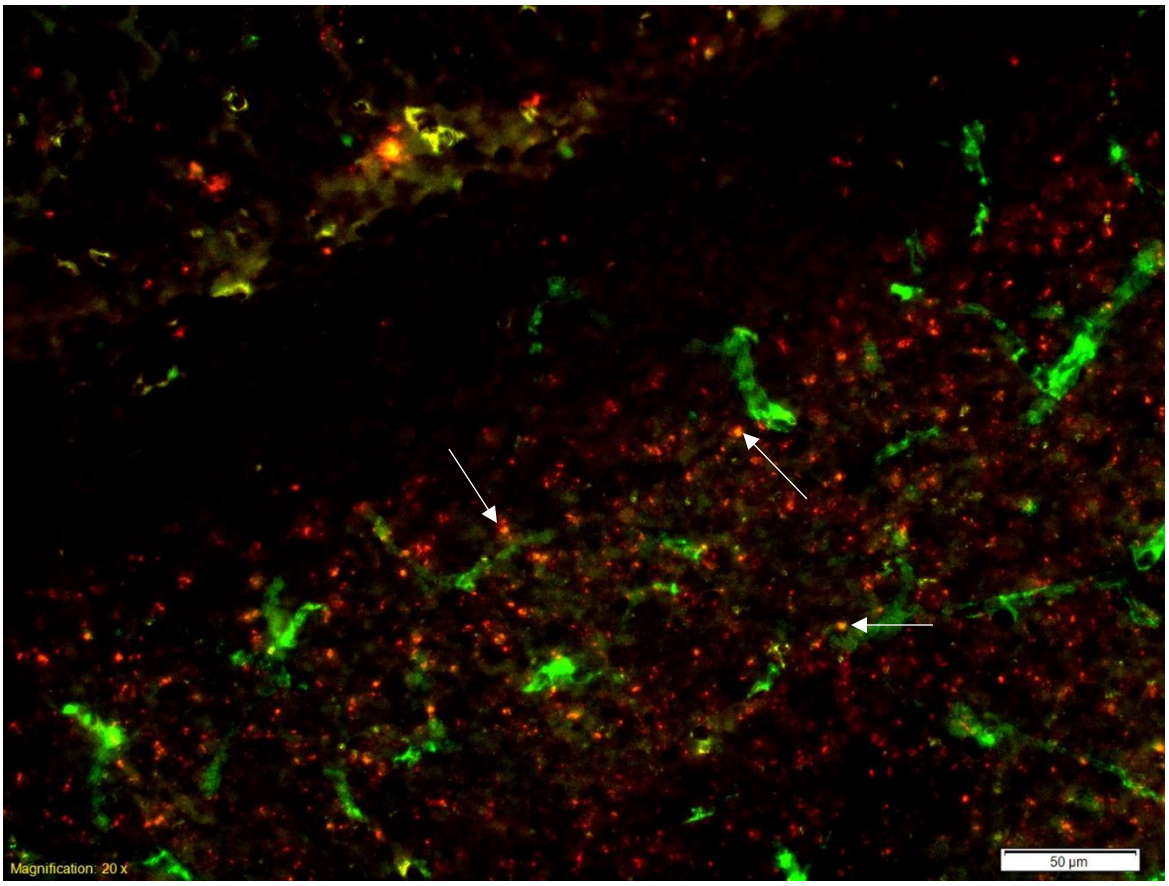
50 µm thick coronal sections were sliced on a cryostat (Cryotome series 77210166, Shandon) and stored in PBS with sodium azide (10%). Free floating sections were stained for post-synaptic marker PSD95 (1:200, mouse monoclonal IgG_{2a}, Upstate), and pre-synaptic marker synapsin (1:1000, rabbit affinity isolated antibody, Sigma). Sections were washed with approximately 1mL of PBST (phosphate buffered saline with 0.3% triton) on a rocker for 10 minutes. The PBST was then removed and replaced and sections were washed for 10 minutes on a rocker. Following the second wash of PBST, PBST was removed and approximately 1mL of cold 80% methanol was added to the wells. Sections were washed on a rocker for an additional 10 minutes. Methanol was then removed and sections were washed two times in PBST for 5 minutes each on a rocker. The PBST was then removed. Sections were incubated in 0.5mL of blocking solution (10% goat serum in PBST) on a rocker for 1 hour. Following this incubation period, the blocking solution was removed and 200µL of the primary antibody solution was added to the sections. Sections incubated in primary antibodies overnight on a rocker in a cold room (4°C).

The next day the primary antibodies were removed and sections were washed 3 times in PBST for 10 minutes each on a rocker. Following the third wash, sections were incubated in 200µL of secondary antibodies (Alexa Fluor™ 488 goat anti-mouse IgG (H+L) and Alexa Fluor™ 594 goat anti-rabbit IgG (H+L), Invitrogen) for 1 hour on a rocker. The secondary antibodies were then removed and sections were washed 3 times in PBST for 10 minutes each on a rocker. Following the third wash PBST was removed and sections were incubated in DAPI for 5 minutes. DAPI was then removed and distilled water was added to the sections. Sections were then mounted on gelatinized slides and allowed to air dry overnight.

The next day slides were immersed in 100% ethanol for 10 minutes. Slides were then transferred to Safeclear (Fisher Scientific, Kalamazoo, MI) and immersed for 10 minutes. DPX mounting media (Fluka BioChemika) was then used to place coverslips over tissue. Slides then air dried overnight.

Slides were imaged using an Olympus IX81 Inverted fluorescent microscope and CellSens Dimension software (Olympus America, Inc., Center Valley, PA). Merged images of PSD95 and synapsin staining from the cortex and hippocampus were then used to count synapses (Fig. 6). The number of synapses were compiled from each treatment group at each age. Synapse numbers from 3xTg-AD group were normalized to those found in age-matched WT groups. Number of synapses from males and females were analyzed both together and separately.

Figure 6: Merged image of PSD95 and synapsin staining. Thirty images from the cortex and hippocampus regions were taken from coronal brain slices of three mice of both sexes, all treatment groups, and all ages and used to count synapses. Arrows indicate areas where PSD95 and synapsin staining overlap. These areas represent synapses. All synapses in an image were counted and recorded.


Magnification: 20 x
50 µm

Western Blot

Twenty-four hours following completion of the probe phase of the Barnes maze mice were sacrificed for brain analysis. Mice were euthanized by an overdose of isoflurane. Brains were then removed and immediately frozen in liquid nitrogen.

Whole brain tissue samples from 3 mice per sex per treatment group per age were ground using mortar and pestle technique. Brain samples were then homogenized in RIPA buffer (150mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris, pH8.0) with Halt Protease and Phosphatase Inhibitor Cocktail, EDTA-free (Thermo Scientific, Rockford, IL) at 4°C. Brain lysate was then incubated on ice for 30min and subsequently centrifuged at 10,000rpm for 30min at 4°C. The supernatant, containing the protein, was then removed and protein concentration was analyzed via BCA assay using the Pierce™ BCA Protein Assay Kit (Thermo Scientific). Six BSA standards were prepared in d. H_2O ranging from 0µg/µL to 1µg/µL for a final volume of 50µL. Samples were prepared as 1:50 dilutions in d. H_2O for a final volume of 50µL. 200µL of working solution (1:50 preparation of BCA Reagent A:BCA Reagent B) was added to all standards and samples. Standards and samples were then incubated at 37°C for 30 min. Standards and Samples were then allowed to cool at RT for 30 min. 200µL of each standard and sample was then loaded in to a 96 well plate. The 96 well plate was analyzed by KC4™ Data Analysis Software (BioTek, Winooski, VT) to determine sample protein concentrations.

20µg of each protein sample was prepared with NuPAGE® LDS Sample Buffer (4X) (Invitrogen, Carlsbad, CA), NuPAGE® Sample Reducing Agent (10X) (Invitrogen),

and d. H_20 and incubated at 70°C for 10 minutes. Samples were then loaded in Bolt™ 4-12% Bis-Tris Plus gels (Invitrogen). Samples were run in MES SDS Running Buffer (Bolt™, Novex by Life Technologies, Carlsbad, CA) in the Bolt Mini Gel Tank (Novex by Life Technologies) at 165V for 35min. SeeBlue® Plus2 Prestained (Invitrogen) standard was used according to manufacturer's suggestions.

Gels were transferred on to iBlot®2 NC Regular Stacks (Invitrogen) via the iBlot (Invitrogen). Membranes were stained for Kremen1 (primary antibody 1:1000 rabbit polyclonal, Invitrogen; secondary antibody 1:4000 IRDye®800CW goat anti-rabbit, Li-Cor, Lincoln, NE) and β-actin (primary antibody 1:5000 mouse monoclonal, Abcam; secondary antibody 1:4000, IRDye®680RD goat anti-mouse, Li-Cor) with the iBind Western Device (Life Technologies). Membranes were imaged with the Odyssey CLX (Li-Cor). Images were then analyzed for band intensity using ImageJ. Quantifications for Kremen1 were relative to the loading control, β-actin.

q-PCR

Twenty-four hours following completion of the probe phase of the Barnes maze mice were sacrificed for brain analysis. Mice were euthanized by an overdose of isoflurane. Brains were then removed and immediately frozen in liquid nitrogen. Whole brain tissue samples from 4 mice of both sexes from each treatment group and all age groups were ground using mortar and pestle technique. Approximately 250mg tissue was used for RNA isolation.

RNA was isolated from tissue samples using mirVana miRNA Isolation kit (Invitrogen by Thermofisher Scientific). RNA concentrations were measured by

NanoDrop. cDNA were then Δprepared using SuperScript IV VILO kit (Thermo Scientific). PCRs were run using PowerUp SYBR Green Master Mix (Applied Biosystems, Life Technologies) in triplicate for each cDNA sample on the QuantStudio 6 (Life Technologies) system.

List of Primers

Kremen1: 5'-ACAGCCAACGTGCAGATTAC-3' and

3'-TGTTGTACGGATGCTGGAAG-5' (Invitrogen)

S12: 5'-TGGCCCGGCCTTCTTTATG-3' and

3'-CCTAAGCGGTGCATCTGGTT-5' (Invitrogen)

q-PCR results were quantified using the $2^{-\Delta\Delta CT}$ method. Quantification of gene expression was calculated against S12.

Statistical Analysis

Barnes maze data was analyzed by multi-factor analysis of variance (ANOVA) with Statistical Analysis Software (SAS, Cary, NC, USA). Data gathered from immunofluorescence, western blots, and PCR were analyzed by one-way ANOVA followed by student's t-test with GraphPad Prism version 6 for Windows (GraphPad Software, San Diego, CA, USA). Results were displayed as mean ± standard error of the mean

RESULTS

Transfection Efficiency

Transfection efficiency was calculated using cortico-hippocampal cultures that were transfected for 48 hours. Cultures were either transfected with Lipofectamine and FAMsiRNA or with RVG-9R and FAMsiRNA. The Lipofectamine transfected cells served as a positive control. 48 hours following the transfection cells were fixed and stained.

Lipofectamine successfully transfected cells with FAMsiRNA at a rate of 62.29%. When RVG-9R was used as the vehicle for transfection, cells were successfully transfected with FAMsiRNA at a 47.35% rate (Fig.7).

Organ Specificity Testing

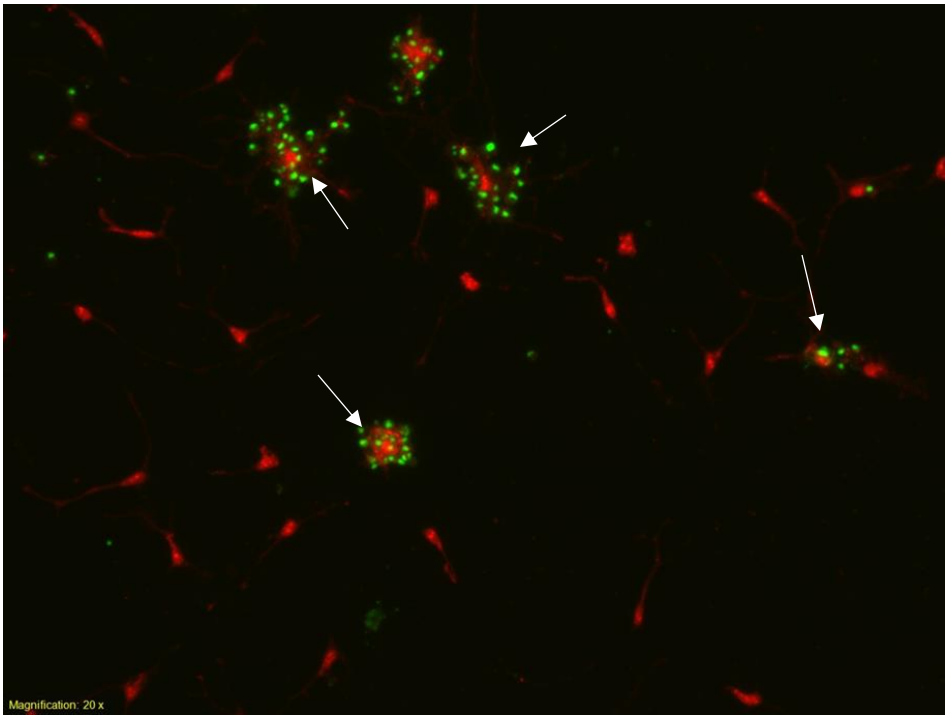
Upon examining the organs under the microscope, FITC fluorescence was only present in brain tissue. No FITC fluorescence was seen in the heart, kidney, liver, lung, or spleen tissue (Fig 8). These results confirm that siRNA complexed with RVG-9R can be delivered to the brain via intravenous injection and that the siRNA will only be delivered to the brain and no other organ systems. This ensures the siRNA specific to Kremen1 will be delivered to the brain.

Barnes Maze

Four month old mice generally saw a decrease in mean primary errors over the course of the Barnes maze protocol for all three treatment groups (Fig. 9A). Four month on WT mice decrease from 14.57 ± 3.43 mean primary errors at trial 1 to 6.55 ± 1.48

Figure 7: Transfection of cortico-hippocampal cell culture with RVG-9R/FAMsiRNA and Lipofectamine/FAMsiRNA. White arrows indicate successfully transfected cells. (A) Cortico-hippocampal cells transfected with RVG-9R/FAMsiRAN were successfully transfected at a rate of 47.35%. (B) Cortico-hippocampal cells transfected with Lipofectamine/FAMsiRNA were successfully transfected at a rate of 62.29%.

A



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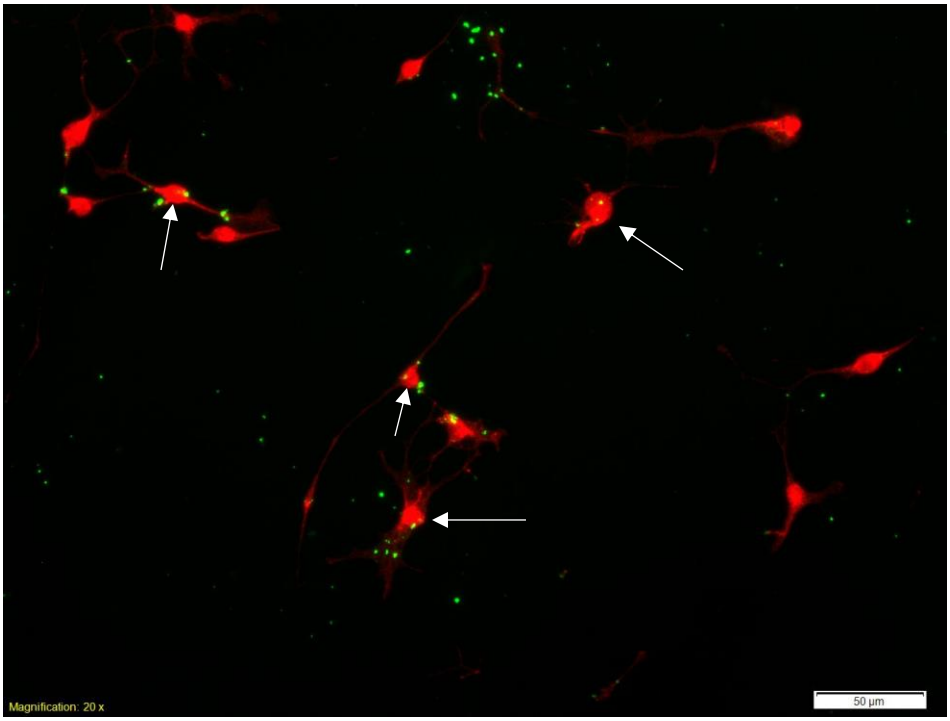
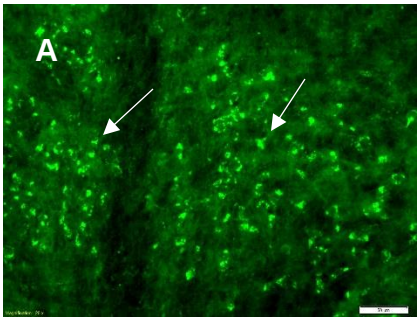


Figure 8: Organ specificity testing results. RVG-9R/FAMsiRNA injections delivered the FAMsiRNA only to the brain. White arrows indicate FAMsiRNA. (A) Brain of mouse injected with RVG-9R/FAMsiRNA. (B) Brain of mouse injected with CPep/siRNA. (C) Heart tissue of mouse injected with RVG-9R/FAMsiRNA. (D) Kidney tissue from mouse injected with RVG-9R/FAMsiRNA. (E) Liver tissue from mouse injected with RVG-9R/FAMsiRNA. (F) Lung tissue from mouse injected with RVG-9R/siRNA. (G) Spleen tissue from mouse injected with RVG-9R/siRNA.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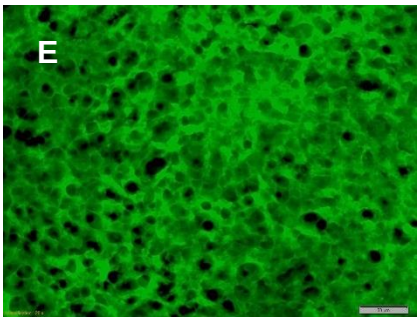
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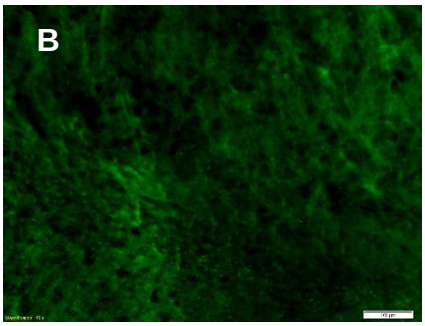
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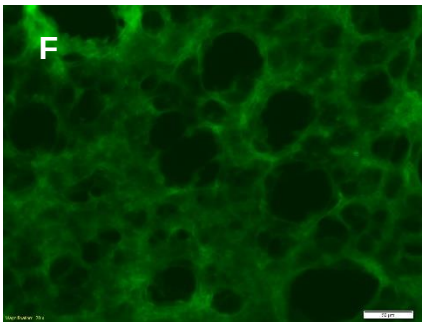
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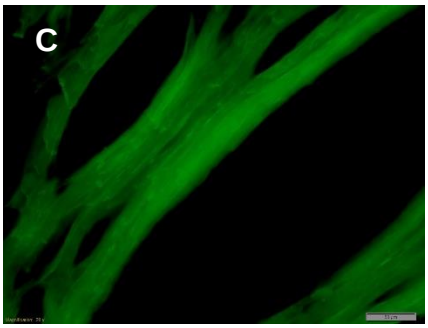
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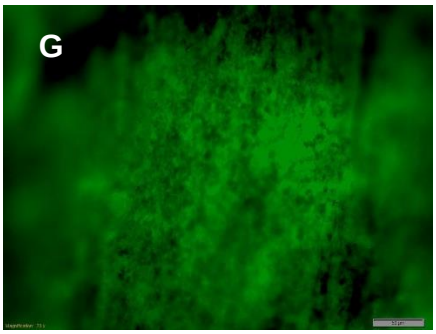
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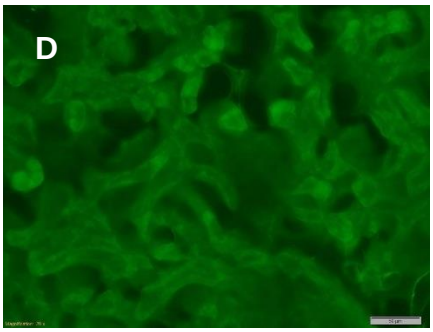
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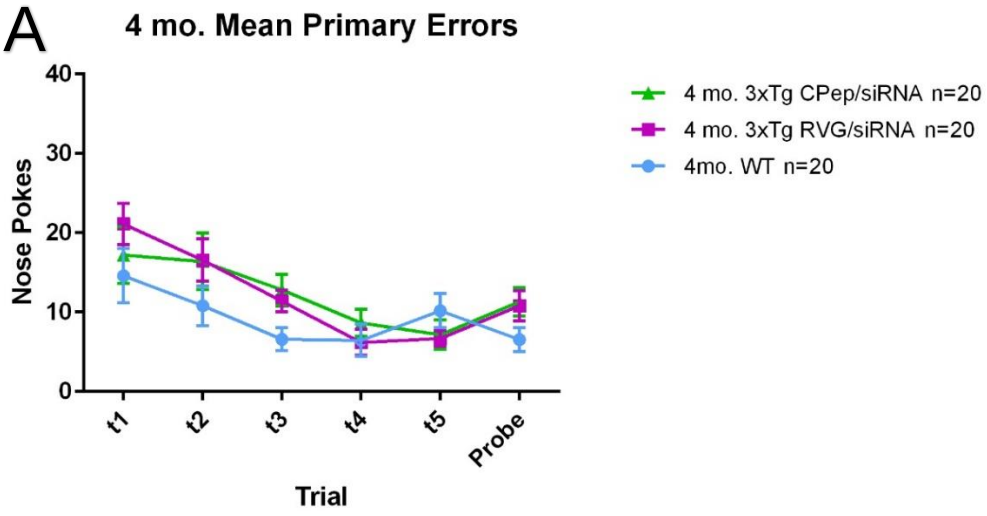
Figure 9: Primary errors for 4 month old mice. (A) Mean primary errors of 4 month old mice. Primary errors decrease over the course of the training trials. (B) Primary errors tend to decrease over the course of the training trials. Once tested in the probe phase primary errors for both groups of 3xTg-AD mice. However, as we hypothesized the 3xTg-AD group treated with RVG/siRNA had fewer primary errors than the 3xTg-AD mice treated with the control peptide/siRNA, although these differences were not statistically significant. (C) Mean primary errors generally decreased over the course of the training trials for all three groups and increased during the probe phase, as is expected. No statistically significant differences were found. All data displayed as mean ± standard error of the mean.

A


4 mo. Mean Primary Errors
Nose Pokes
40
30
20
10
0
t1
t2
t3
t4
t5
Probe
Trial
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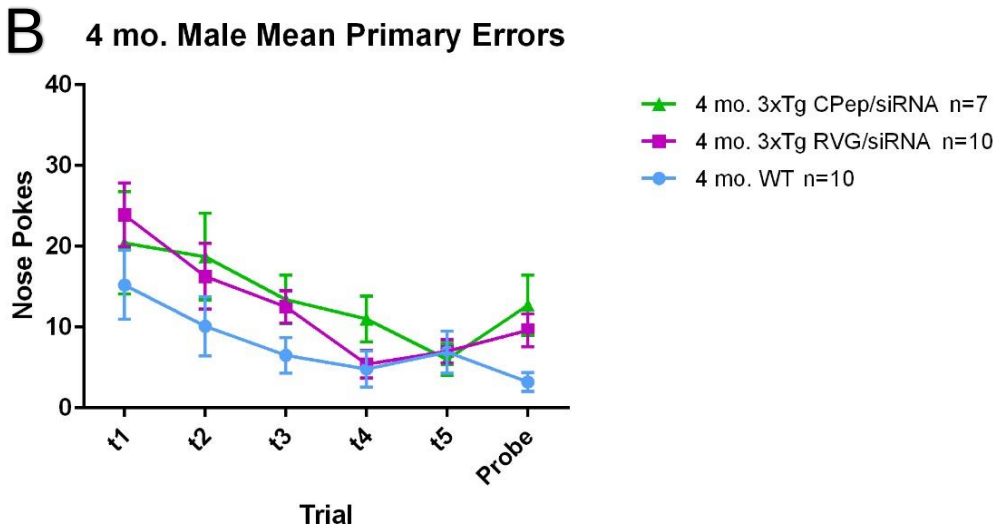
4 mo. 3xTg RVG/siRNA n=20
4mo. WT n=20

B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Nose Pokes
40
30
20
10
0
t1
t2
t3
t4
t5
Probe
Trial
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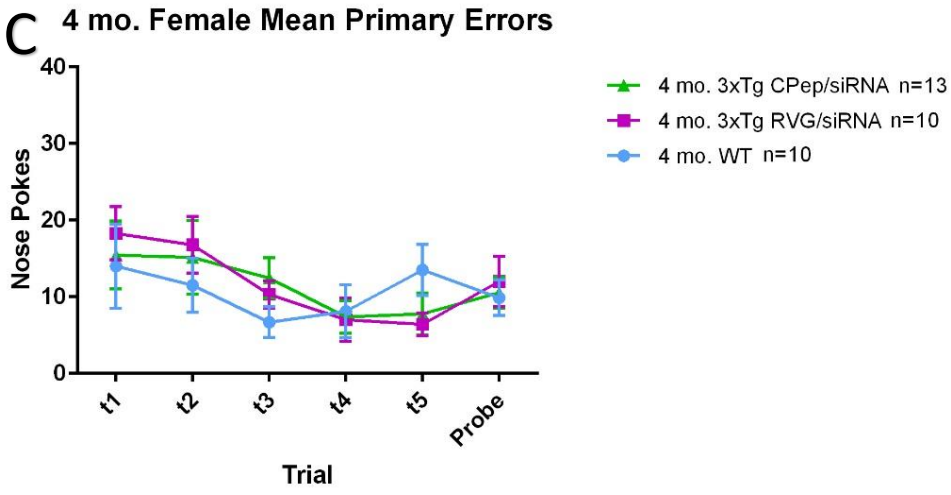
4 mo. 3xTg RVG/siRNA n=10
4 mo. WT n=10

C


4 mo. Female Mean Primary Errors
Nose Pokes
40
30
20
10
0
t1
t2
t3
t4
t5
Probe
Trial
4 mo. 3xTg CPep/siRNA n=13
4 mo. 3xTg RVG/siRNA n=10
4 mo. WT n=10

mean primary errors at the probe phase. Four month old 3xTg-AD RVG-9R/siRNA treated mice had 21.1 ± 2.63mean primary errors in trial 1 and only 10.8 ± 1.92 mean primary errors in the probe phase. Four month old 3xTg-AD CPep/siRNA treated mice had 17.2 ± 3.59 mean primary errors in trial 1 versus 10.8 ± 1.82 mean primary errors in the probe phase.

When mice are separated by sex and treatment group more pronounced differences can be observed. For example, when examining only male mice, at the probe phase 4 month old WT males performed the best with 6.2 ± 1.18 mean primary errors (Fig. 9B). The 4 month old male 3xTg-AD RVG-9R/siRNA treated mice performed worse (23.9 ± 2.06) than the WT but better than the 3xTg-AD CPep/siRNA (20.43 ± 3.72) treated mice as was hypothesized, although, these differences are not statistically significant.

The mean primary errors for the 4 month old female groups also tended to decrease over the course of the Barnes maze protocol (Fig. 9C). Four month old WT females had 14.0 ± 5.50 mean primary errors in trial 1 and slightly decrease their mean primary errors in the probe phase to 9.9 ± 2.33. Four month old female 3xTg-AD RVG-9R/siRNA treated mice saw a slight decrease from 18.3 ± 3.47 to 12.0 ± 3.31 mean primary errors from trial 1 to the probe phase. The four month old female 3xTg-AD CPep/siRNA treated mice performed approximately the same as the four month old WT mice with 15.5 ± 4.46 mean primary errors in trial 1 and 10.5 ± 2.05 mean primary errors in the probe phase.

Six month old mice WT also show a decrease in mean primary errors from training trial 1 (21.6 ± 2.55) to the probe phase (5.90 ± 1.51), indicating successful

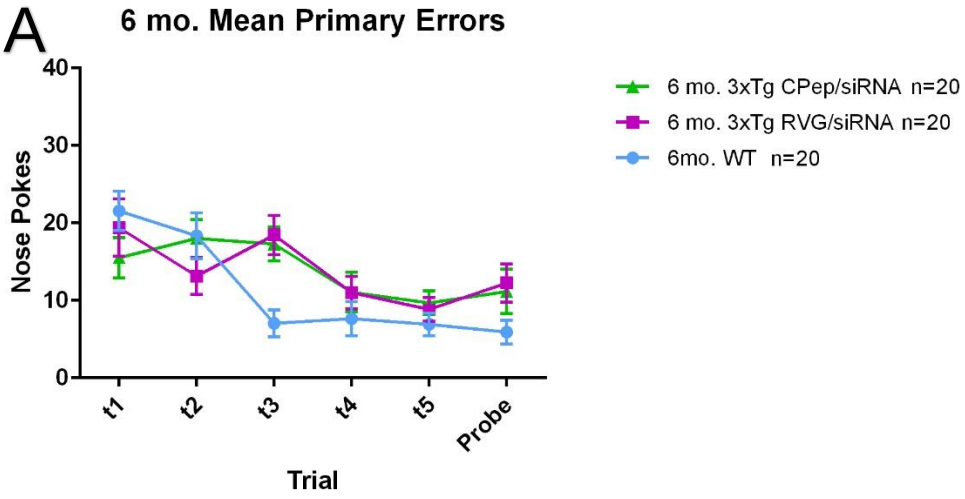
learning. Both 6 month old 3xTg-AD groups had higher mean primary errors compared to 6 month old WT mice for most of the Barnes maze protocol. (Fig. 10A). 6 month old 3xTg-AD RVG-9R/siRNA mice started off similarly to their WT counterparts with 19.4 ± 3.69 mean primary errors in trial 1. This group saw a slightly less robust decrease in mean primary errors in the probe phase with 12.3 ± 2.47. Six month old 3xTg-AD CPep/siRNA treated mice had a similar pattern to the other two treatment groups. In trial 1 3xTg-AD CPep/siRNA had 15.5 ± 2.60 mean primary errors and this decreased to 11.2 ± 2.86 in the probe phase.

6 month old male mice show similar patterns in mean primary errors as the 6 month old group as a whole. 3xTg-AD RVG-9R/siRNA treated and CPep/siRNA treated 6 month old male mice tended to have higher mean primary errors compared to the 6 month old male WT mice (Fig. 10B). In trial 1, WT males had 19.7 ± 2.65 mean primary errors and this decreased to 6.2 ± 2.73 in the probe phase. Male 3xTg-AD RVG-9R/siRNA treated mice had 12.7 ± 3.52 mean primary errors in the probe phase, down from 21.3 ± 5.41 in trial 1. The male 3xTg-AD CPep/siRNA treated mice began with 15.2 ± 3.83 mean primary errors in trial 1, which decrease to 9.0 ± 4.75 in the probe phase.

Six month old female mean primary errors decreased over the course of the Barnes maze protocol for all treatment groups (Fig. 10C). At the probe phase, both 3xTg-AD female groups had mean primary errors higher than the 6 month old female WT group (WT: 5.6 ± 1.46; 3xTg-AD RVG-9R/siRNA: 11.4 ± 3.00; 3xTg-AD CPep/siRNA: 13.3 ± 3.31).

Figure 10: Primary errors for 6 month old mice. (A) Mean primary errors for 6 month old mice followed a general trend of decreasing over the course of the Barnes maze protocol. At the probe phase, mean primary errors for 3xTg mice treated with RVG/siRNA was approximately the same as the mean primary errors for 3xTg mice treated with the control peptide/siRNA. (B) Mean primary errors generally decreased for all three treatment groups over the course of the five training trials. At the probe phase, both 3xTg groups had more mean primary errors than the wild type group. (C) Mean primary errors for all three treatment groups decreased over the course of the Barnes maze protocol. At the probe phase, both 3xTg treatment groups had higher mean primary errors compared to the wild type group, however, these differences are not statistically significant. All data displayed as mean ± standard error of the mean.

A


6 mo. Mean Primary Errors
Nose Pokes
40
30
20
10
0
t1
t2
t3
t4
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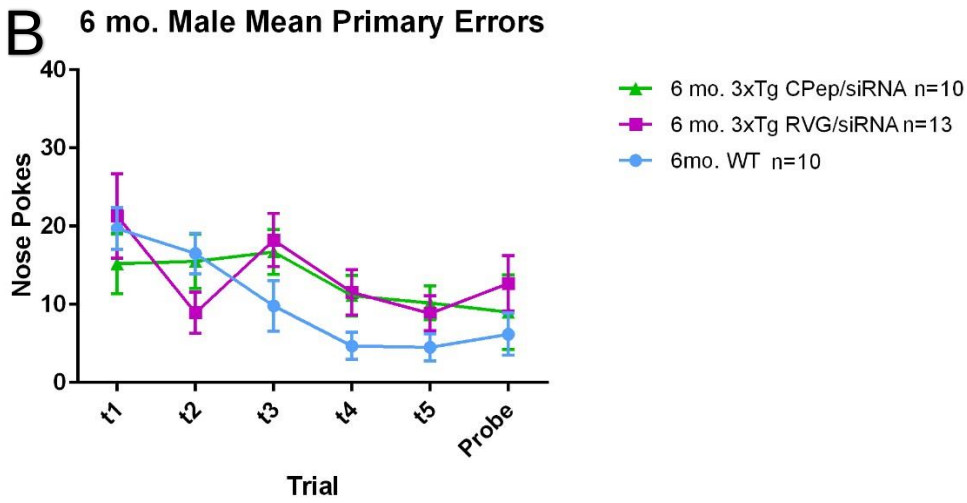
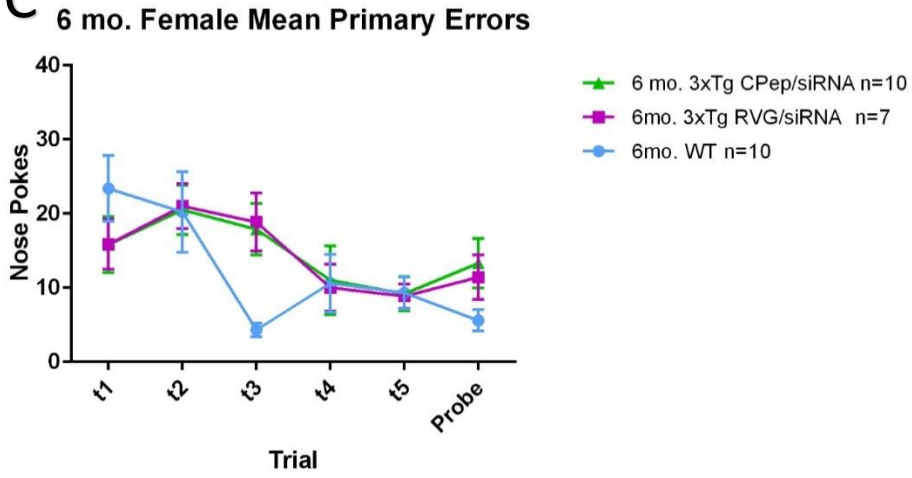
Probe
Trial
6 mo. 3xTg CPep/siRNA n=20
6 mo. 3xTg RVG/siRNA n=20
6mo. WT n=20

B


6 mo. Male Mean Primary Errors
Nose Pokes
40
30
20
10
0
t1
t2
t3
t4
t5
Probe
Trial
6 mo. 3xTg CPep/siRNA n=10
6 mo. 3xTg RVG/siRNA n=13
6mo. WT n=10

C


6 mo. Female Mean Primary Errors
Nose Pokes
40
30
20
10
0
t1
t2
t3
t4
t5
Probe
Trial
6 mo. 3xTg CPep/siRNA n=10
6mo. 3xTg RVG/siRNA n=7
6mo. WT n=10

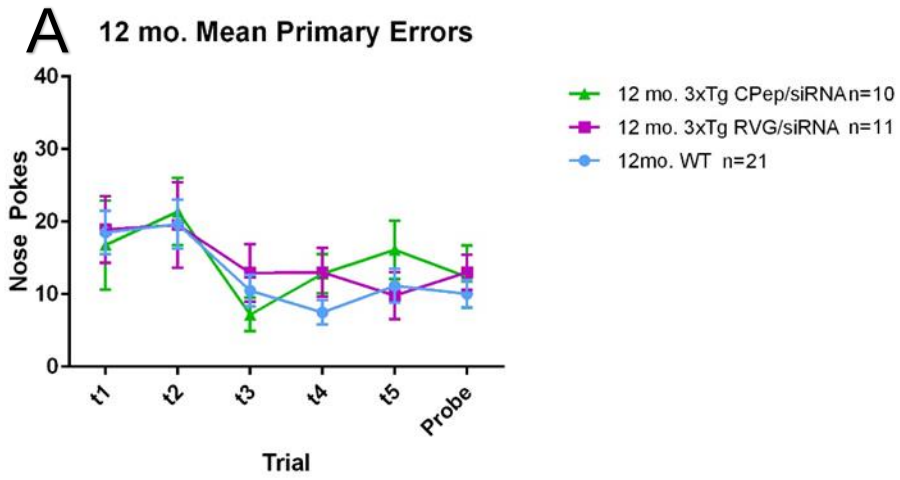
Like the 4 month old and 6 month old groups, the 12 month old groups showed a decline in mean primary errors during the Barnes maze protocol (Fig. 11A). This is most clearly observed in the 12 month females (Fig. 11B). The small sample size for the 3xTg-AD 12 month old males (3xTg-AD RVG-9R/siRNA n=3; 3xTg-AD CPep/siRNA n=2) makes it difficult to interpret any trends in the data. The 12 month old WT males saw a decline in mean primary errors from 21.8 ± 5.34 in trial 1 to 10.7 ± 2.42 in the probe phase. Interestingly, the 12 month old 3xTg-AD RVG-9R/siRNA males saw an increase in primary errors over the course of the training trials. At trial 1 this group had 11.3 ± 5.55 mean primary errors at the probe phase 19.7 ± 4.18. This is likely due to the high variability in behavior and small sample size. The 3xTg-AD CPep/siRNA males displayed the lowest mean primary errors in trial 1 and the probe phase compared to the other two treatment groups (t1=4.5 ± 4.5; probe=9.0 ± 3.0). This is again, likely due to the small sample size (n=2).

Overall, mean primary errors in the probe phase tended to increase with age as was expected (Fig. 12). Twelve month old 3xTg-AD mice treated with RVG-9R/siRNA displayed the highest mean primary errors in the probe phase, 13.0 ± 2.43, compared to all other groups. The 6 month old WT group had the lowest mean primary errors in the probe phase with 5.9 ± 1.51.

Primary latency is defined as the amount of time that passes from the start of the trial until a mouse first pokes its nose into the escape hole. Shorter primary latencies indicate better learning and memory of the location of the escape box. If a mouse did not find the escape hole during a trial, primary latency for that trial was recorded as the full length of time for that trial (180 sec for t1-t5 and 120 sec for probe phase).

Figure 11: Primary errors for 12 month old mice. (A) Mean primary errors for all three 12 month old treatment groups slightly decreased over the course of the Barnes maze protocol. At the probe phase, all three treatment groups had approximately the same mean primary errors. (B) 12 month old male wild type mice showed a decrease in mean primary errors over the course of the training trials and probe phase as was expected. Trends for the 3xTg RVG/siRNA treated and 3xTg control peptide/siRNA treated groups are difficult to interpret due to the small sample sizes (n=3 and n=2, respectively). (C) Mean primary errors tended to decrease for all three treatment groups over the course of the five training trials and probe phase. Wild type mice tended to have fewer mean primary errors compared to both 3xTg treatment groups. At the probe phase, wild type mice had the lowest mean primary errors, followed by the 3xTg RVG/siRNA treatment group. The 3xTg control peptide/siRNA treated group had the highest mean primary errors at the probe phase. All data displayed as mean ± standard error of the mean.

A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Nose Pokes
40
30
20
10
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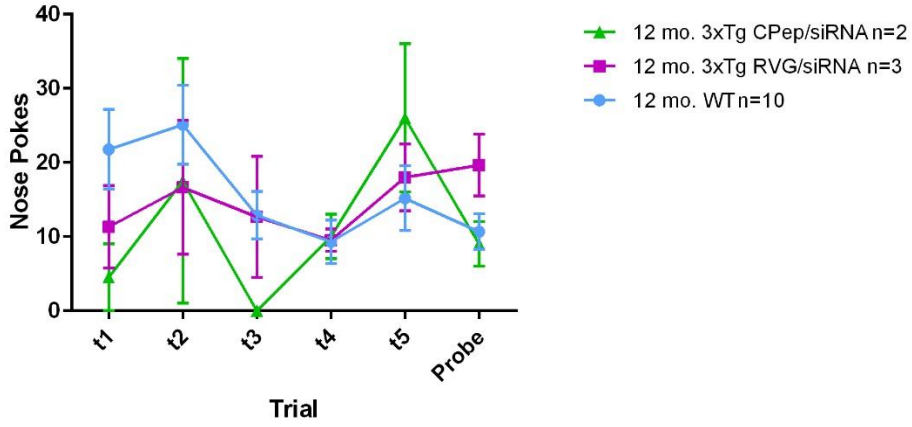
t1
t2
t3
t4
t5
Probe
Trial
12 mo. 3xTg CPep/siRNA n=10
12 mo. 3xTg RVG/siRNA n=11
12mo. WT n=21

B


12 mo. Male Mean Primary Errors
Nose Pokes
40
30
20
10
0
t1
t2
t3
t4
t5
Probe
Trial
12 mo. 3xTg CPep/siRNA n=2
12 mo. 3xTg RVG/siRNA n=3
12 mo. WT n=10

C

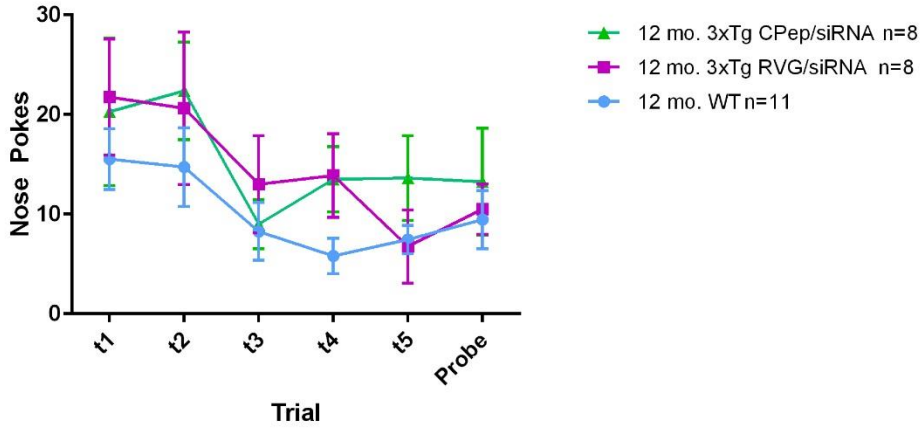
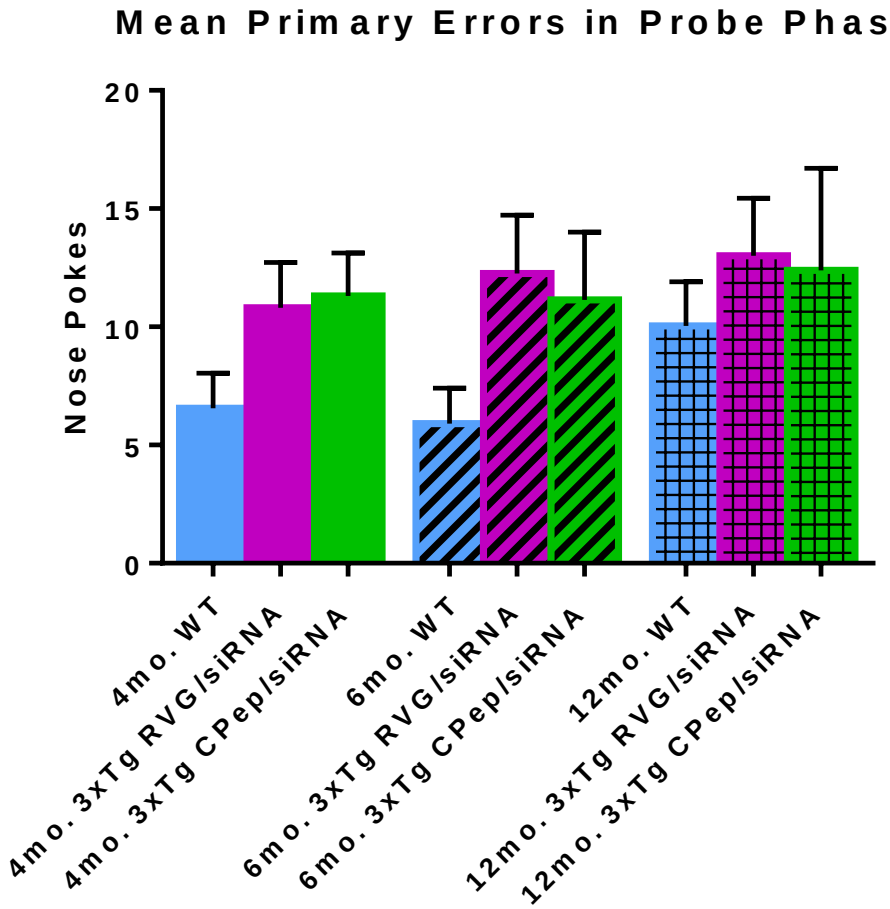

12 mo. Female Mean Primary Errors
Nose Pokes
30
20
10
0
t1
t2
t3
t4
t5
Probe
Trial
12 mo. 3xTg CPep/siRNA n=8
12 mo. 3xTg RVG/siRNA n=8
12 mo. WT n=11

Figure 12: Mean primary errors in the probe phase for all treatment groups across all ages. Mean primary errors tends to increase with age, regardless of treatment or strain. All 3xTg groups had higher mean primary errors compared to the age-matched wild type controls. All data displayed as mean ± standard error of the mean.



4mo. WT n=20; 4mo. 3xTg RVG/siRNA n=20; 4mo. 3xTg CPep/siRNA n=20

6mo. WT n=20; 6mo. 3xTg RVG/siRNA n=20; 6mo. 3xTg CPep/siRNA n=20

12mo. WT n=21; 12mo. 3xTg RVG/siRNA n=11; 12mo. 3xTg CPep/siRNA n=10

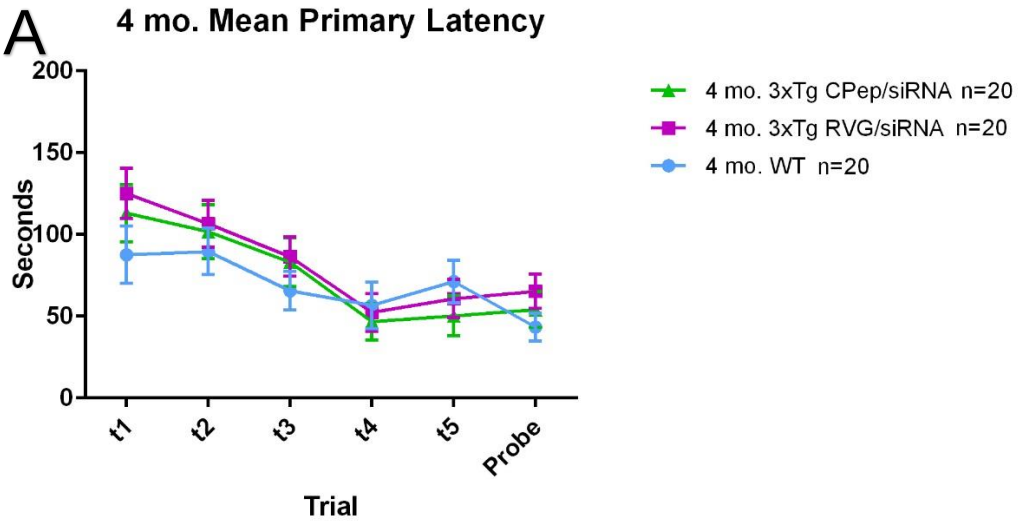
Mean primary latency for 4 month old mice steadily decreased over the course of the Barnes maze training trials (Fig. 13A). Four month old WT mice tended to have the shortest mean primary latency compared to both 3xTg-AD groups, as was expected. Mean primary latency for the 4 month WT group decreased from 87.6 ± 18.66 sec in trial 1 to 43.4 ± 9.42 sec in the probe phase. Both 3xTg-AD 4 month old groups followed the same general trend. Four month old 3xTg-AD mice treated with RVG-9R/siRNA began with a mean primary latency of 125.2 ± 16.71 sec in trial 1. Their mean primary latency decreased by nearly half to 65.3 ± 11.62 sec in the probe phase. The 4 month old 3xTg-AD CPep/siRNA mice followed suit with a decrease from 113.1 ± 18.83 sec in trial 1 to 54.1 ± 11.68 sec in the probe phase.

Four month old male mice display a trend in mean primary latency that supports the hypothesis. The WT group had the shortest mean primary latency, followed by the 3xTg-AD RVG-9R/siRNA treated group, and the 3xTg-AD CPep/siRNA treated group had the longest mean primary latency over the course of the training trials (Fig. 13B). At trial 1, 4 month old male WT mice had a mean primary latency of 98.7 ± 26.11 sec, 3xTg-AD RVG-9R/siRNA had a mean primary latency of 120.3 ± 21.63 sec, and 3xTg-AD CPep/siRNA had a mean primary latency of 169.6 ± 10.43 sec. By the probe phase, 4 month old WT mice had a mean primary latency of 29.4 ± 9.78 sec, 3xTg-AD RVG-9R/siRNA had a mean primary latency of 47.0 ± 12.90 sec, and 3xTg-AD CPep/siRNA had a mean primary latency of 72.1 ± 23.78 sec.

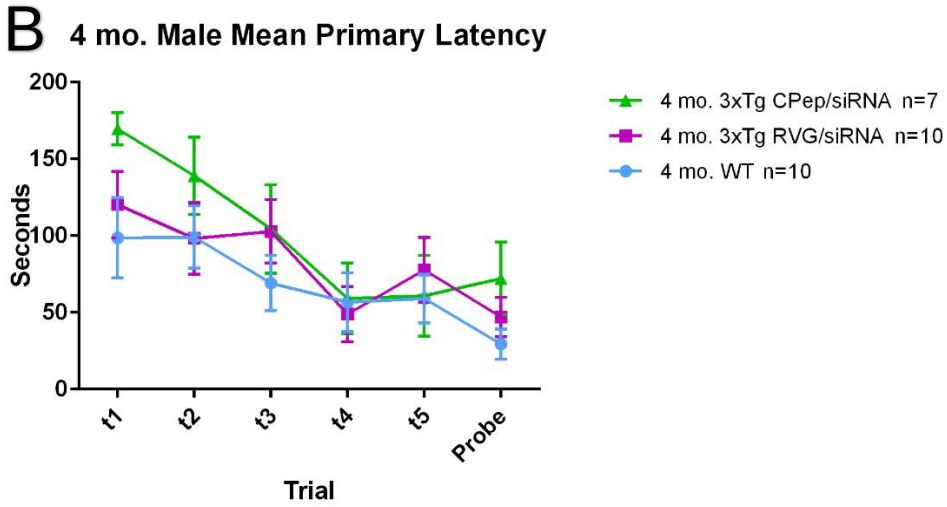
Four month old female mice also displayed decreased in mean primary latency in all treatment groups across the Barnes maze training trials, though the decreases seem to be more modest than the 4 month old males (Fig. 13C). Four month old female WT

Figure 13: Primary latencies for 4 month old mice. (A) Primary latency tends to decrease over the course of the training trials. (B) Primary latency for these mice tended to decrease over the course of the training. The 3xTg mice treated with RVG/siRNA had a lower mean primary latency during the probe phase compared to the 3xTg mice treated with the control peptide/siRNA. (C) Primary latency generally decreased over the five training trials for all three groups. During the probe phase, mean primary latency for the 3xTg RVG/siRNA treated group was the highest among the three groups. All data displayed as mean ± standard error of the mean.

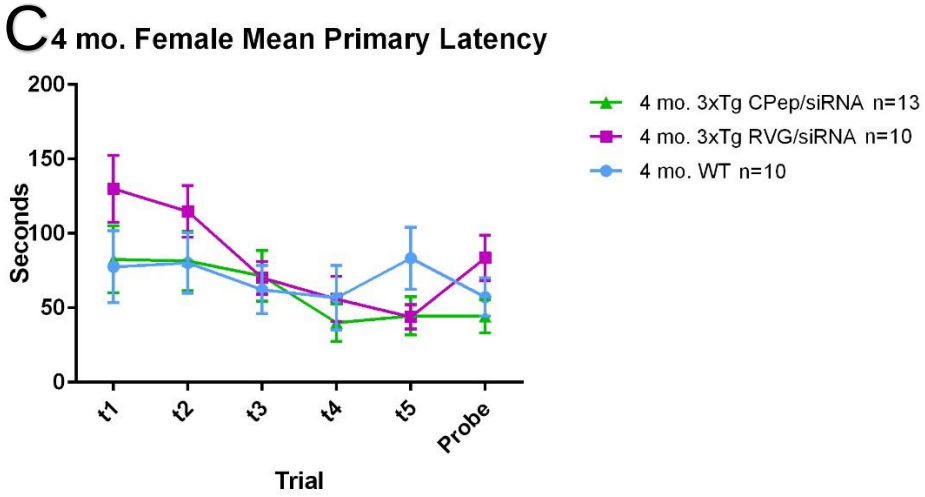
A


4 mo. Mean Primary Latency
200
150
100
50
0
Seconds
t1
t2
t3
t4
t5
Probe
Trial
4 mo. 3xTg CPep/siRNA n=20
4 mo. 3xTg RVG/siRNA n=20
4 mo. WT n=20

B


4 mo. Male Mean Primary Latency
200
150
100
50
0
Seconds
t1
t2
t3
t4
t5
Probe
Trial
4 mo. 3xTg CPep/siRNA n=7
4 mo. 3xTg RVG/siRNA n=10
4 mo. WT n=10

C


4 mo. Female Mean Primary Latency
200
150
100
50
0
Seconds
t1
t2
t3
t4
t5
Probe
Trial
4 mo. 3xTg CPep/siRNA n=13
4 mo. 3xTg RVG/siRNA n=10
4 mo. WT n=10

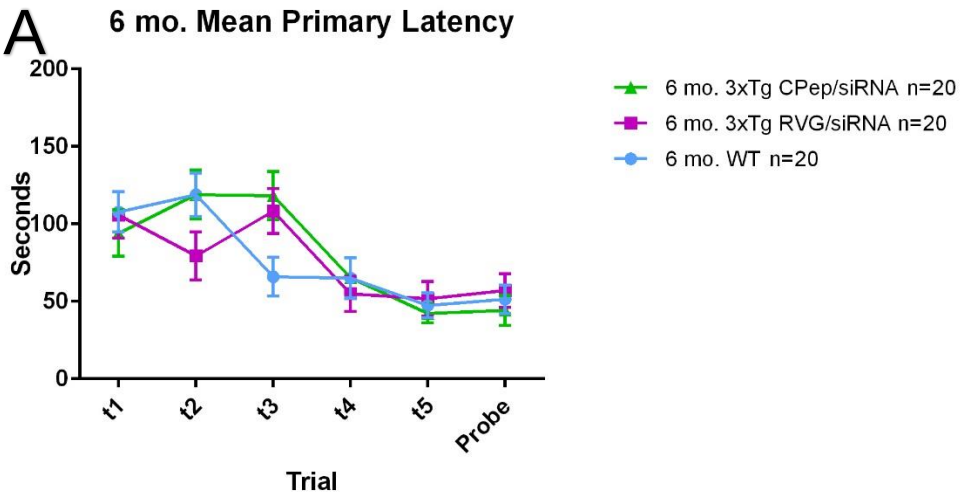
mice had a mean primary latency of 77.6 ± 24.20 sec in trial 1 and saw this decrease to 57.3 ± 12.94 sec in the probe phase. Four month old 3xTg-AD RVG-9R/siRNA females had a mean primary latency of 130.0 ± 22.62 sec in trial 1, which consistently decreased until trial 5 before increasing to 83.5 ± 15.20 sec in the probe phase. Four month old 3xTg-AD CPep/siRNA treated females had a decrease in mean primary latency from 82.6 ± 22.48 sec in trial 1 to 44.4 ± 11.05 in the probe phase. Among the three 4 month old female groups the 3xTg-AD CPep/siRNA treated mice performed the best in the probe phase with regards to primary latency.

Six month old mice also displayed decreases in mean primary latency over the course of the five training trials and probe phase (Fig. 14A). WT mice and 3xTg-AD RVG-9R/siRNA treated mice had approximately the same mean primary latency in trial 1 (107.8 ± 12.92 sec and105.8 ± 14.87 sec, respectively). The 6 month old 3xTg-AD CPep/siRNA treated mice had a slightly shorter mean primary latency at trial 1 at 94.1 ± 15.02 sec. All three treatment groups saw decreases in mean primary latency by the probe phase. At the probe phase, all three 6 month old treatment groups performed almost equally: WT= 51.4 ± 9.22 sec; 3xTg-AD RVG-9R/siRNA=57.0 ± 10.99 sec; and 3xTg-AD CPep/siRNA=44.2 ± 9.46sec.

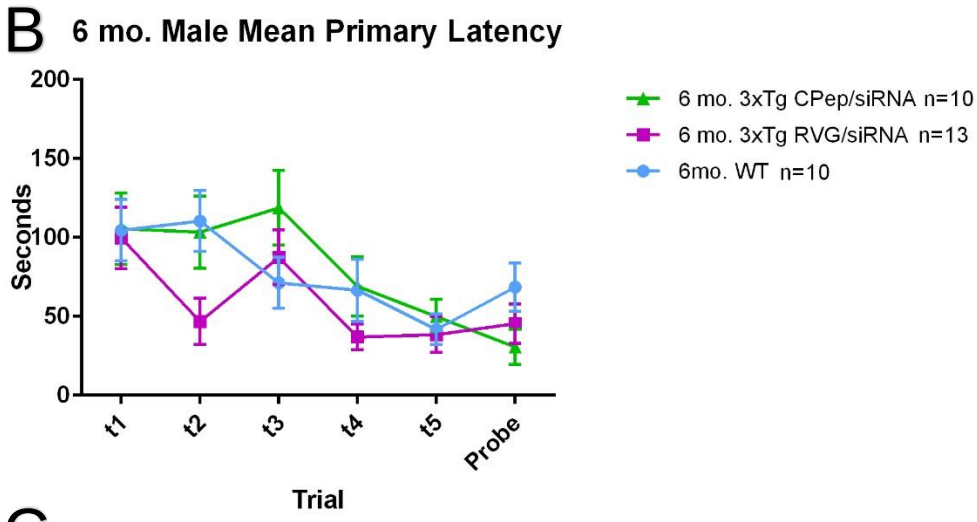
When 6 month old males are analyzed the same general trend of decreasing mean primary latency is observed (Fig. 14B). At trial 1, all three male experimental groups had approximately the same mean primary latency: WT=104.7 ±19.44 sec; 3xTg-AD RVG-9R/siRNA=99.8 ± 19.54 sec; 3xTg-AD CPep/siRNA=150.6 ± 20.55 sec. At the probe phase both 6 month male 3xTg-AD groups had shorter mean primary latencies than the 6 month male WT group, which was not expected. In the probe phase

Figure 14: Primary latencies for 6 month old mice. (A) Mean primary latency decreased over the course of the training trials for all three treatment groups. A sharp decline in mean primary latency can be seen at the start of the second training day (t4). (B) Mean primary latency for all three 6 month old male treatment groups decreased over the course of the Barnes maze protocol. However, at the probe phase both 3xTg treated groups had shorter mean primary latencies than the wild type group, although these differences are not statistically significant. (C) The mean primary latency decreased over time for all three 6 month old female treatment groups. The 3xTg RVG/siRNA consistently had a longer mean primary latency compared to both the wild type and 3xTg control peptide/siRNA treated groups. All data displayed as mean ± standard error of the mean.

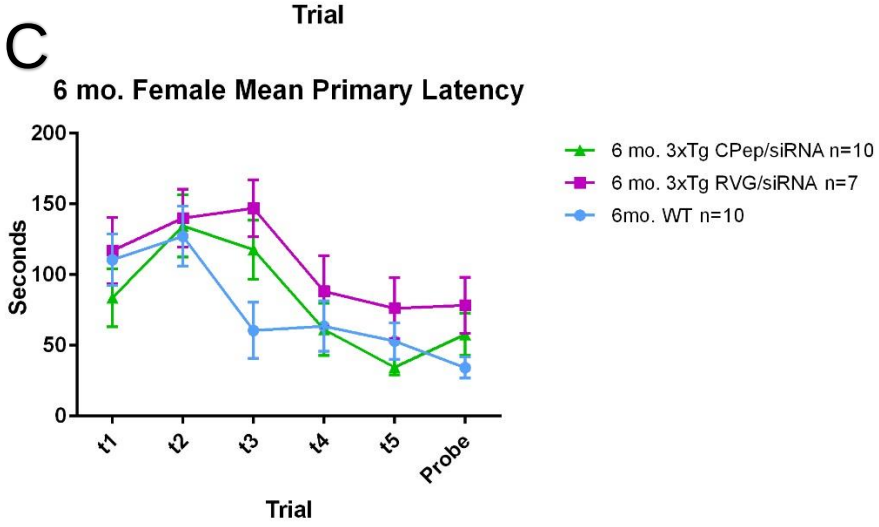
A


6 mo. Mean Primary Latency
200
150
100
50
0
Seconds
t1
t2
t3
t4
t5
Probe
Trial
6 mo. 3xTg CPep/siRNA n=20
6 mo. 3xTg RVG/siRNA n=20
6 mo. WT n=20

B


6 mo. Male Mean Primary Latency
200
150
100
50
0
Seconds
t1
t2
t3
t4
t5
Probe
Trial
6 mo. 3xTg CPep/siRNA n=10
6 mo. 3xTg RVG/siRNA n=13
6mo. WT n=10

C


6 mo. Female Mean Primary Latency
200
150
100
50
0
Seconds
t1
t2
t3
t4
t5
Probe
Trial
6 mo. 3xTg CPep/siRNA n=10
6 mo. 3xTg RVG/siRNA n=7
6mo. WT n=10

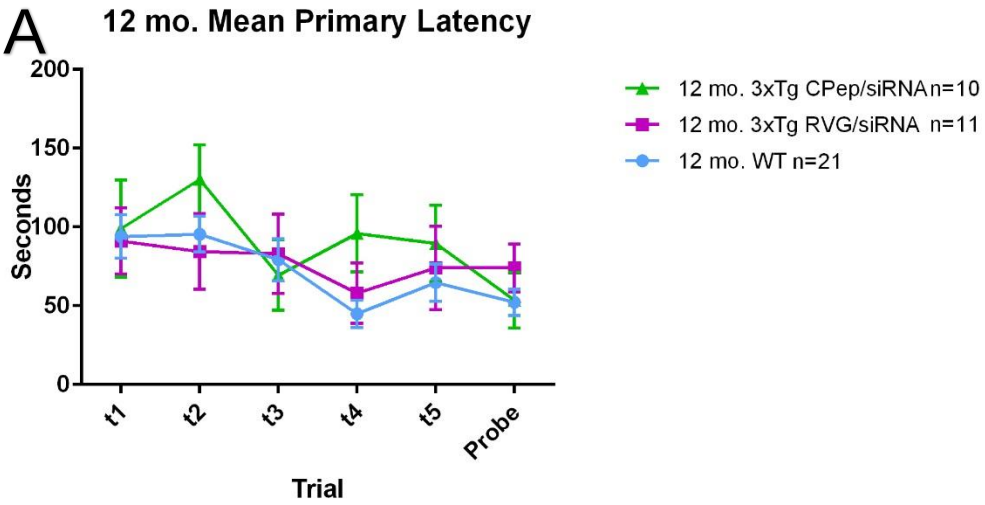
6 month male WT mice had a mean primary latency of 68.5 ± 15.40 sec, 6 month male 3xTg-AD RVG-9R/siRNA treated mice had a mean primary latency of 45.5 ± 12.52 sec, and 6 month male 3xTg-AD CPep/siRNA treated mice had a mean primary latency of 30.6 ± 11.26 sec.

Compared to 6 month old male mice, 6 month old female mice tended to have longer primary latencies (Fig. 14C). All three 6 month old female treatment groups showed decreases in mean primary latency from trial 1 to the probe phase. Six month old female WT mice had a mean primary latency of 110.6 ± 18.16 sec in trial 1 that decreased to 34.3 ± 7.54 sec in the probe phase. Six month old 3xTg-AD RVG-9R/siRNA treated female mice showed a slightly less dramatic increase in mean primary latency from trial 1, 117.0 ± 23.46 sec, to probe phase, 78.4 ± 19.87 sec. The 6 month old female 3xTg-AD CPep/siRNA treated mice had the lowest mean primary latency in trial 1 at 83.7 ± 20.58 sec. At the probe phase this group had a mean primary latency of 57.8 ± 15.00 sec. Unlike the 6 month old male mice, in the probe phase both 6 month old female 3xTg-AD groups had longer primary latencies than the 6 month old female WT group as was expected.

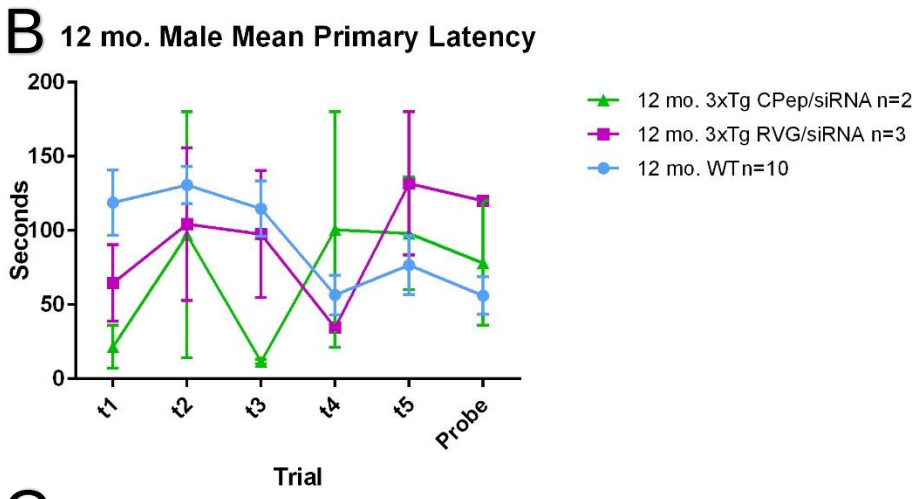
Mean primary latency for 12 month old mice also tended to decrease over the course of the Barnes maze training and probe phase (Fig. 15A). In trial 1, all three 12 month old groups had approximately the same mean primary latency: WT=93.9 ± 13.84 sec; 3xTg-AD RVG-9R/siRNA=91.1 ± 21.04 sec; and 3xTg-AD CPep/siRNA=99.0 ± 30.75 sec. The 12 month old WT mice and 12 month old 3xTg-AD RVG-9R/siRNA mice followed very similar patterns in decreasing mean primary latency. The 12 month old

Figure 15: Primary latencies for 12 month old mice. (A) Mean primary latency decreased over the course of the Barnes maze protocol for all three treatment groups. Both 3xTg treatment groups tended to have longer mean primary latencies compared to the wild type groups across the various training trials. (B) Mean primary latency for the wild type group tended to decrease over the course of the Barnes maze protocol. Small sample sizes for both 3xTg treatment groups makes interpretation of mean primary latency trends difficult. (C) Mean primary latency decreased as the Barnes maze protocol progressed for all three treatment groups. The 3xTg RVG/siRNA treated group tended to have mean primary latencies between those of the wild type group and the 3xTg control peptide/siRNA treated group. At the probe phase, all three treatment groups had approximately the same mean primary latency. All data displayed as mean ± standard error of the mean.

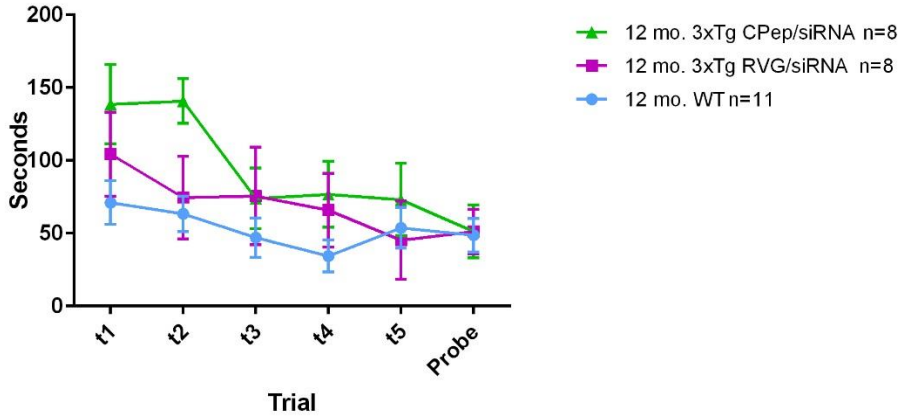
A


12 mo. Mean Primary Latency
200
150
100
50
0
Seconds
t1
t2
t3
t4
t5
Probe
Trial
12 mo. 3xTg CPep/siRNA n=10
12 mo. 3xTg RVG/siRNA n=11
12 mo. WT n=21

B


12 mo. Male Mean Primary Latency
200
150
100
50
0
Seconds
t1
t2
t3
t4
t5
Probe
Trial
12 mo. 3xTg CPep/siRNA n=2
12 mo. 3xTg RVG/siRNA n=3
12 mo. WT n=10

C


12 mo. Female Mean Primary Latency
200
150
100
50
0
Seconds
t1
t2
t3
t4
t5
Probe
Trial
12 mo. 3xTg CPep/siRNA n=8
12 mo. 3xTg RVG/siRNA n=8
12 mo. WT n=11

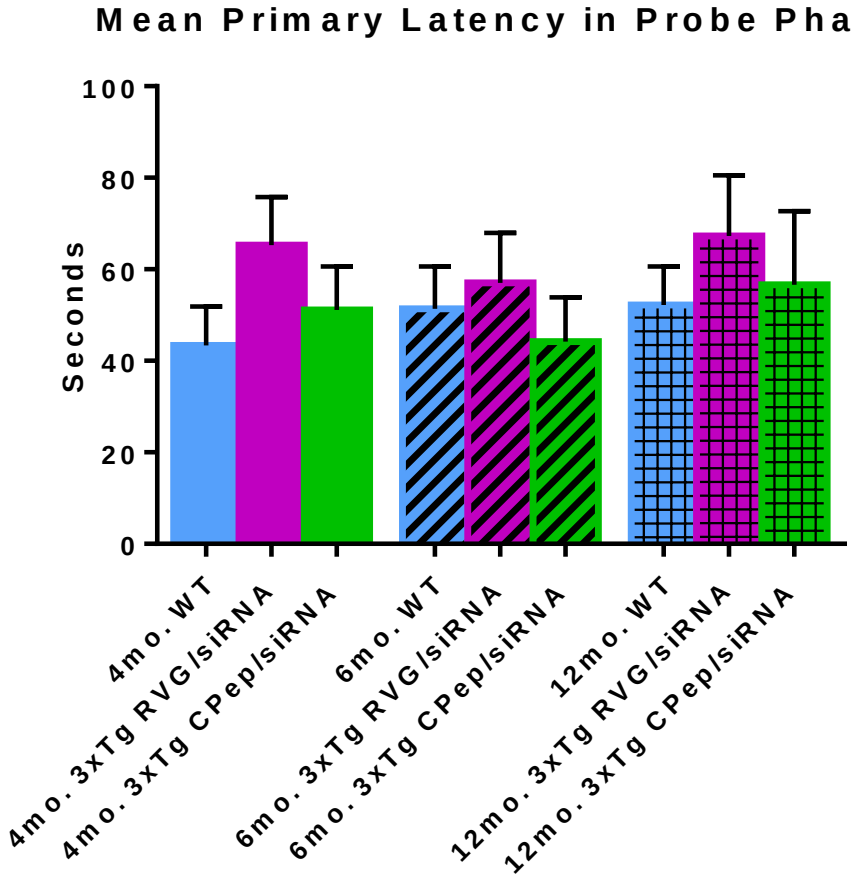
3xTg-AD CPep/siRNA had a much more variable pattern in mean primary latency over the course of the training trials compared to the other two groups. At the probe phase the 12 month old 3xTg-AD CPep/siRNA treated mice had a mean primary latency of 53.5 ± 17.79 sec.

When examining the 12 month old male mean primary latency data, it is difficult to interpret trends in the 3xTg-AD groups due to such small sample sizes (3xTg-AD RVG-9R/siRNA n=3; 3xTg-AD CPep/siRNA n=2) (Fig. 15B). Twelve month old male WT mice showed a decrease in mean primary latency from 118.9 ± 22.00 sec in trial 1 to 56.1 ± 12.69 sec in the probe phase.

Twelve month old female mice also showed a decrease in mean primary latency (Fig. 15C). At trial 1, 12 month old female WT mice had the shortest primary latency at 71.1 ± 15.06 sec, followed by the 3xTg-AD RVG-9R/siRNA treated group at 104.3 ± 26.69 sec, and the 12 month female 3xTg-AD CPep/siRNA treated group had the longest primary latency at 138.6 ± 27.09 sec. Throughout the course of the training trials the 12 month old female 3xTg-AD CPep/siRNA treated group tended to perform worse than the other two experimental groups, as was expected. However, at the probe phase the mean primary latencies for all three groups converged: WT=48.7 ± 11.52 sec; 3xTg-AD RVG-9R/siRNA=51.2 ± 15.50 sec; 3xTg-AD CPep/siRNA=51.25 ± 18.24 sec.

Primary latency in the probe phase slightly increased with age in the WT groups, however this was not the case with the 3xTg-AD groups (Fig. 16). At all three ages, 3xTg-AD RVG-9R/siRNA treated mice had the longest primary latencies. Among all ages and treatment groups, 4 month old WT mice had the shortest mean primary latency (43.4 ± 9.42 sec), as was expected.

Figure 16: Mean primary latency in the probe phase. Mean primary latency during the probe phase is used as a measure of learning. Shorter primary latency during the probe phase suggests better learning. The mean primary latency during the probe phase of the Barnes maze slightly increased with age for the wild type mice, as is expected. 3xTg mice treated with RVG/siRNA consistently displayed higher mean primary latencies compared to wild type controls across all three age groups. All data displayed as mean ± standard error of the mean.



4mo. WT n=20; 4mo. 3xTg RVG/siRNA n=20; 4mo. 3xTg CPep/siRNA n=20

6mo. WT n=20; 6mo. 3xTg RVG/siRNA n=20; 6mo. 3xTg CPep/siRNA n=20

12mo. WT n=21; 12mo. 3xTg RVG/siRNA n=11; 12mo. 3xTg CPep/siRNA n=10

Total errors are any nose pokes a mouse makes in a hole that is not an escape hole at any time during the trial. Total errors should decrease with learning in trials 1-5. However, at the probe phase, it is likely to see a rise in total errors due to the fact the escape box is not present.

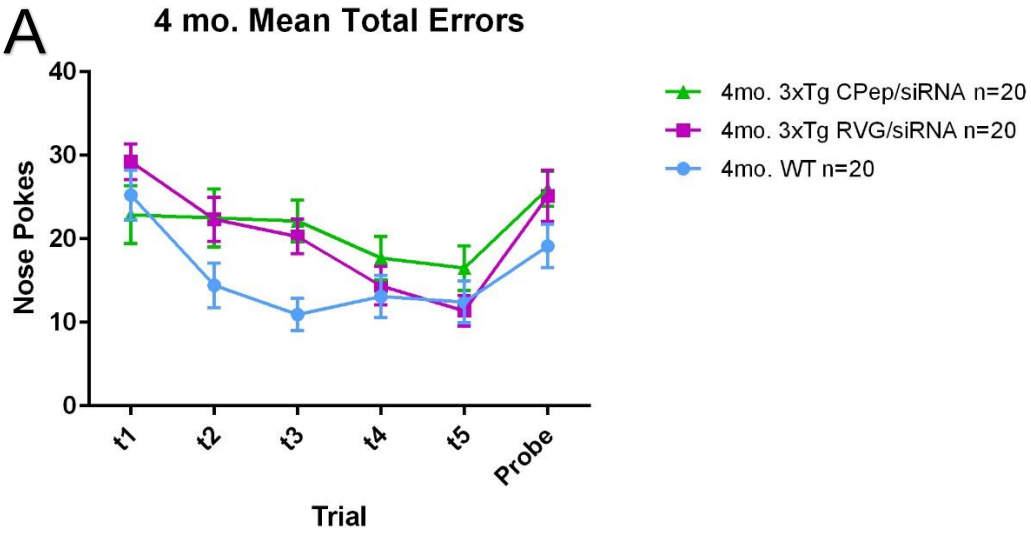
Four month old mice showed a decrease in total errors in all three treatment groups over the five training trials (Fig. 17A). In the probe phase, 4 month old WT mice had the fewest total errors, 19.2 ± 3.19, as was expected. The 4 month old 3xTg-AD RVG-9R/siRNA treated and 3xTg-AD CPep/siRNA treated groups had approximately the same number of total errors in the probe phase, 25.2 ± 3.34 and 26.0 ± 2.46, respectively. The total errors for the 4 month WT (t1=25.3 ± 3.38) and 4 month 3xTg-AD RVG-9R/siRNA treated (t1=29.3 ± 2.48) decreased slightly from trial 1. However, the total errors for the 3xTg-AD CPep/siRNA treated group increase in the probe phase from 22.9 ± 3.72 in trial 1.

In the 4 month old male experimental groups, both 3xTg-AD groups had more total errors than the WT group (Fig.17B). In the probe phase, the 4 month old male WT mice had the fewest total errors (13.1 ± 0.98). The 3xTg-AD RVG-9R/siRNA treated mice had the highest mean total errors in the probe phase with 31.8 ± 4.20. The 4 month old male 3xTg-AD CPep/siRNA had 23.9 ± 3.52 mean total errors in the probe phase.

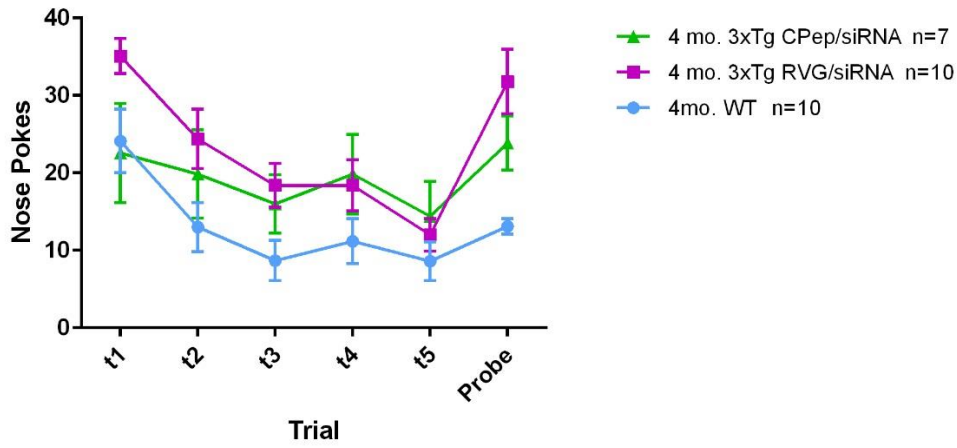
Four month old female WT mice displayed nearly the same mean total errors in trial 1, 26.3 ± 4.44, as in the probe phase, 25.2 ± 4.45 (Fig. 17C). Four month old female 3xTg-AD RVG-9R/siRNA treated mice continued to show a decrease in mean total errors from trial 1, 23.4 ± 2.56, through the probe phase, 18.5 ± 3.47. Although, the

Figure 17: Total errors for 4 month old mice. (A) Mean total errors of all 4 month old mice. Total errors follow a similar trend as primary errors, decreasing over time. (B) Total errors for 4 month old male mice tended to decrease over the course of the training trials, but increased when mice were tested during the probe phase. (C) Total errors generally decreased during training and rose for all three treatment groups during the probe phase. All data displayed as mean ± standard error of the mean.

A


4 mo. Mean Total Errors
Nose Pokes
40
30
20
10
0
t1
t2
t3
t4
t5
Probe
Trial
4mo. 3xTg CPep/siRNA n=20
4mo. 3xTg RVG/siRNA n=20
4mo. WT n=20

B


4 mo. Male Total Errors
Nose Pokes
40
30
20
10
0
t1
t2
t3
t4
t5
Probe
Trial
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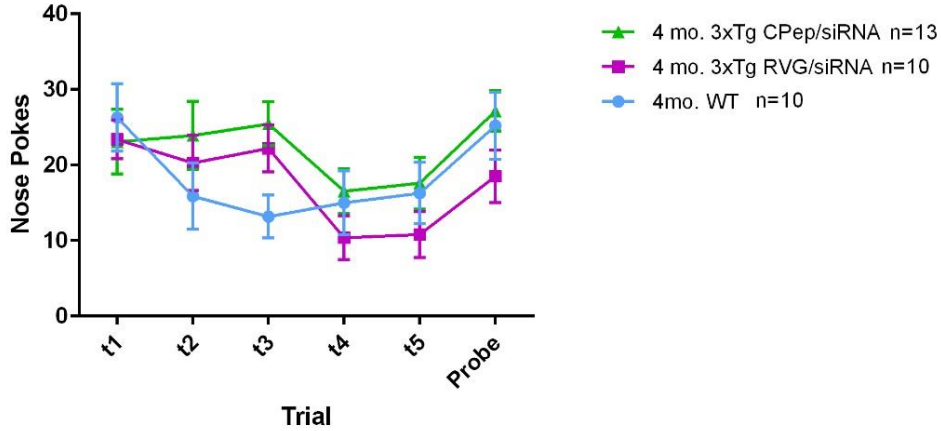
4 mo. 3xTg RVG/siRNA n=10
4mo. WT n=10

C


4 mo. Female Mean Total Errors
Nose Pokes
40
30
20
10
0
t1
t2
t3
t4
t5
Probe
Trial
4 mo. 3xTg CPep/siRNA n=13
4 mo. 3xTg RVG/siRNA n=10
4mo. WT n=10

mean total errors for this group still rose from trial 5 to the probe phase. The 4 month old 3xTg-AD CPep/siRNA treated mice had the greatest mean total errors in the probe phase, 27.2 ± 3.21, as was expected.

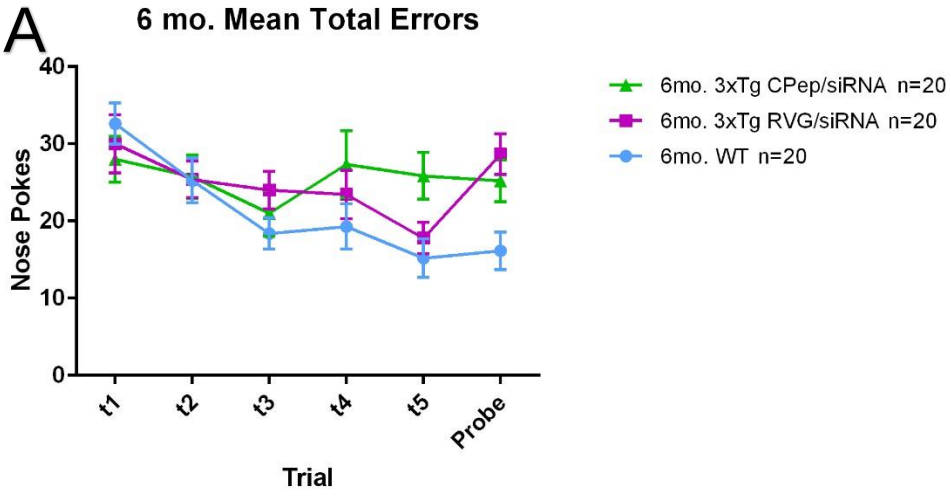
Compared to the 4 month old group, 6 month old mice had slightly more mean total errors in trial 1 (Fig. 18A). All three 6 month old treatment groups showed a decline in mean total errors over the course of the five training trials. In the probe phase, both 6 month old 3xTg-AD groups had more mean total errors (3xTg-AD RVG-9R/siRNA=28.7 ± 2.84; 3xTg-AD CPep/siRNA=25.2 ± 2.98) than the 6 month old WT group (16.2 ± 2.79).

Six month old WT males saw a steady decline in total errors over the course of trials 1-4, before plateauing from trial 4 through the probe phase (Fig. 18B). At the probe phase, 6 month old WT males had 10.9 ± 2.40 mean total errors. The two 6 month old male 3xTg-AD groups made more total errors in the probe phase than their WT counterparts. Six month old male 3xTg-AD RVG-9R/siRNA treated mice made the most mean total errors in the probe phase with 33.2 ± 3.29. The 6 month old male 3xTg-AD CPep/siRNA treated mice had a mean total errors in the probe phase, 25.5 ± 3.90, that fell between the other two treatment groups.

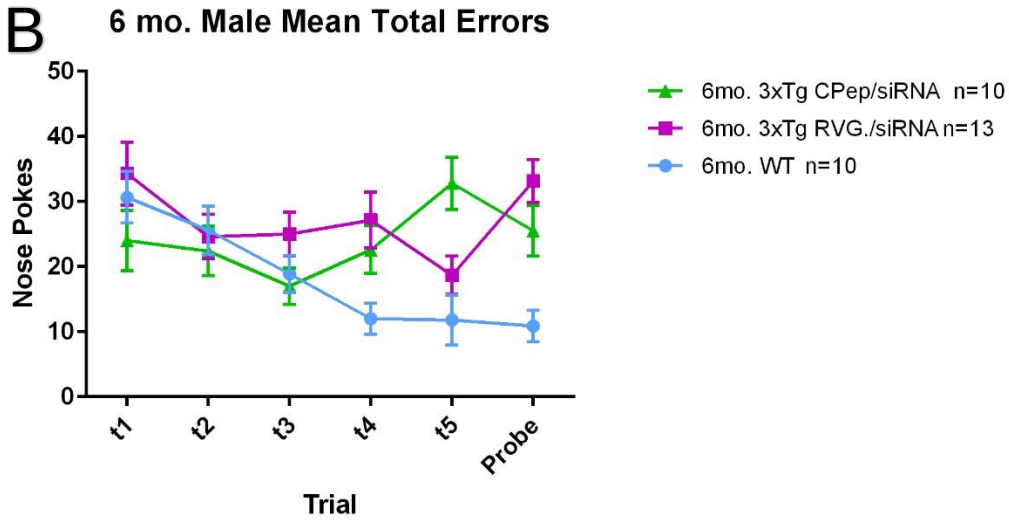
Six month old female mice showed less dramatic decline in mean total errors compared to the 6 month old males (Fig. 18C). Six month old WT females saw a decline in mean total errors from 34.4 ± 3.70 in trial 1 to 21.4 ± 3.59 in the probe phase. Six month old female 3xTg-AD RVG-9R/siRNA treated mice showed a very slight decline in mean total errors from 22.0 ± 4.82 in trial 1 to 20.3 ± 2.33 in the probe phase. The six month old female 3xTg-AD CPep/siRNA treated mice tended to have the greatest mean

Figure 18: Total errors for 6 month old mice. (A) In general, mean total errors for 6 month old mice decreased over the course of the training trials. No statistically significant differences were found among the three treatment groups. (B) Mean total errors for the wild type group consistently decreased over the training trials and probe phase, as was expected. The 3xTg RVG/siRNA treated group had approximately the same mean total errors at training trial 1 as at the probe phase. Mean total errors for the 3xTg control peptide/siRNA treated group decreased on the first training day (trials 1-3), however mean total errors increased on both training trials on the second training day (trials 4-5). (C) Mean total errors for the three treatment groups slightly decreased over the course of the training trials and the probe phase. At the probe phase, all three treatment groups had approximately the same mean total errors. All data displayed as mean ± standard error of the mean.

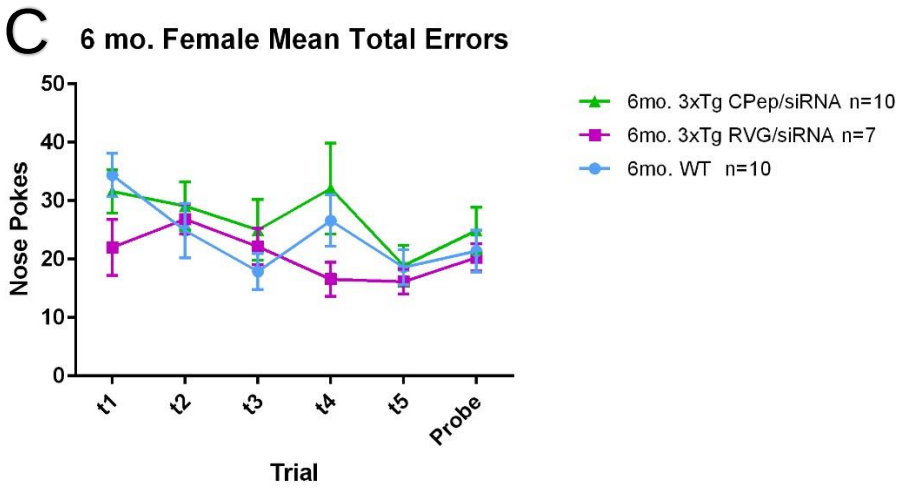
A


6 mo. Mean Total Errors
Nose Pokes
0
10
20
30
40
t1
t2
t3
t4
t5
Probe
Trial
6mo. 3xTg CPep/siRNA n=20
6mo. 3xTg RVG/siRNA n=20
6mo. WT n=20

B


6 mo. Male Mean Total Errors
Nose Pokes
0
10
20
30
40
50
t1
t2
t3
t4
t5
Probe
Trial
6mo. 3xTg CPep/siRNA n=10
6mo. 3xTg RVG./siRNA n=13
6mo. WT n=10

C


6 mo. Female Mean Total Errors
Nose Pokes
0
10
20
30
40
50
t1
t2
t3
t4
t5
Probe
Trial
6mo. 3xTg CPep/siRNA n=10
6mo. 3xTg RVG/siRNA n=7
6mo. WT n=10

total errors over the course of the training trials. In the probe phase, this group had 24.9 ± 3.72 mean total errors, down from 31.6 ± 3.83 in trial 1.

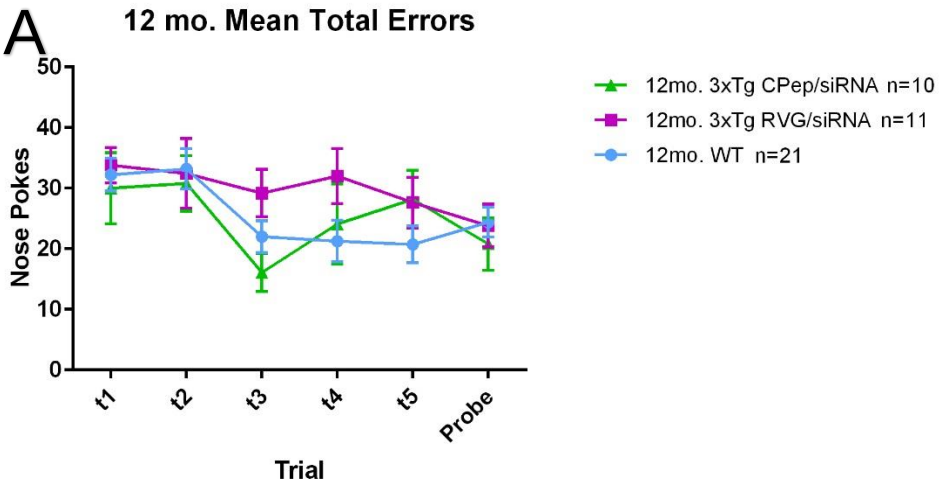
Twelve month old groups also showed a decline in mean total errors over the course of the Barnes maze protocol (Fig. 19A). Twelve month old WT mice had a decrease from 32.2 ± 2.72 mean total errors in trial 1 to 24.4 ± 2.46 mean total errors in the probe phase. Both the 12 month old 3xTg-AD RVG-9R/siRNA treated mice and 3xTg-AD CPep/siRNA treated mice showed similar declines as the WT group, declining from 33.8 ± 2.91 and 30.0 ± 3.54 mean total errors to 23.8 ± 5.90 and 20.8 ± 4.32 mean total errors in the probe phase, respectively.

Twelve month old male WT mice had a decline in mean total errors from 29.9 ± 4.80 in trial 1 to 25.1 ± 3.24 in the probe phase (Fig. 19B). The unusually large increases and decreases in mean total errors over the course of the training trials for the two 3xTg-AD groups is likely due to the small sample sizes. However, strictly looking at the change from trial 1 to the probe phase, both 12 month old male 3xTg-AD groups showed a decline in mean total errors. Twelve month old male 3xTg-AD RVG-9R/siRNA treated mice had 30.7 ± 3.28 mean total errors in trial 1 and 19.7 ± 4.18 in the probe phase. The 12 month old male 3xTg-AD CPep/siRNA treated mice showed a decline in mean total errors from 39.5 ± 8.50 in trial 1 to 15.5 ± 9.5 in the probe phase.

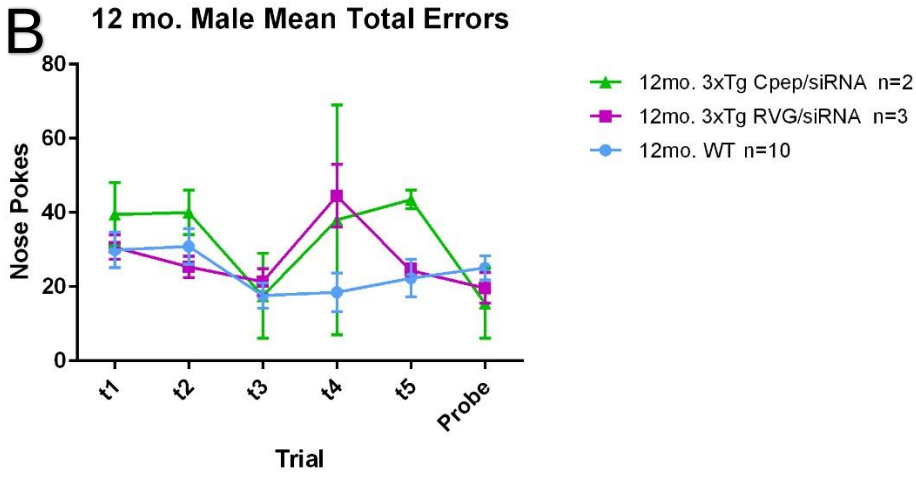
Twelve month old female WT mice tended to have more mean total errors than the 12 month old male WT mice (Fig. 19C). In trial 1, 12 month old female WT mice had 34.4 ± 2.93 mean total errors which declined to 23.7 ± 3.80 in the probe phase. The 12 month old female 3xTg-AD RVG-9R/siRNA treated group tended to have more mean

Figure 19: Total errors for 12 month old mice. (A) The mean total errors for all three treatment groups also tended to decreased over the course of the five training trials and probe phase. As with the mean primary errors, at the probe phase, all three treatment groups had approximately the same mean total errors. (B) Mean total errors for the 12 month old male wild type group tended to be lower than both 3xTg groups. (C) Mean total errors decreased over time for all three treatment groups. Interestingly, the 3xTg control peptide/siRNA treated group had the lowest mean total errors at every training trial except for trial 5. All data displayed as mean ± standard error of the mean.

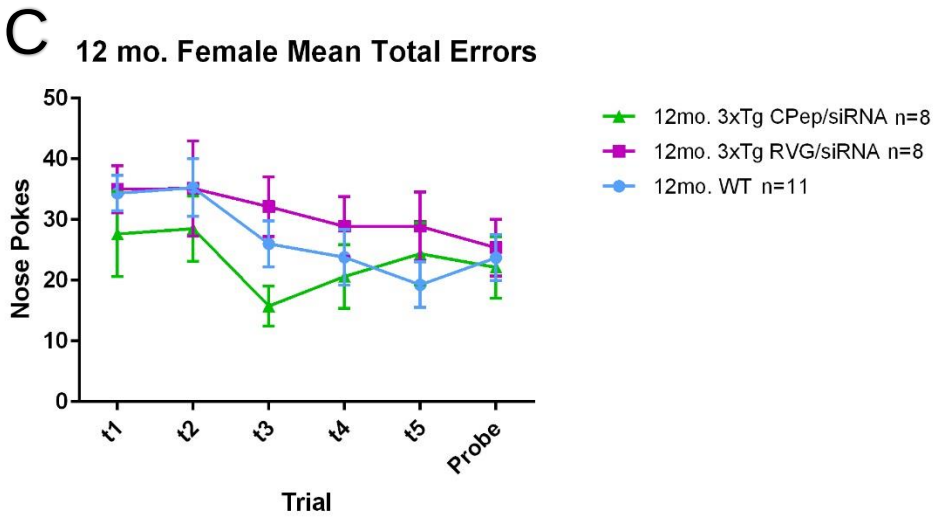
A


12 mo. Mean Total Errors
Nose Pokes
50
40
30
20
10
0
t1
t2
t3
t4
t5
Probe
Trial
12mo. 3xTg CPep/siRNA n=10
12mo. 3xTg RVG/siRNA n=11
12mo. WT n=21

B


12 mo. Male Mean Total Errors
Nose Pokes
80
60
40
20
0
t1
t2
t3
t4
t5
Probe
Trial
12mo. 3xTg Cpep/siRNA n=2
12mo. 3xTg RVG/siRNA n=3
12mo. WT n=10

C


12 mo. Female Mean Total Errors
Nose Pokes
50
40
30
20
10
0
t1
t2
t3
t4
t5
Probe
Trial
12mo. 3xTg CPep/siRNA n=8
12mo. 3xTg RVG/siRNA n=8
12mo. WT n=11

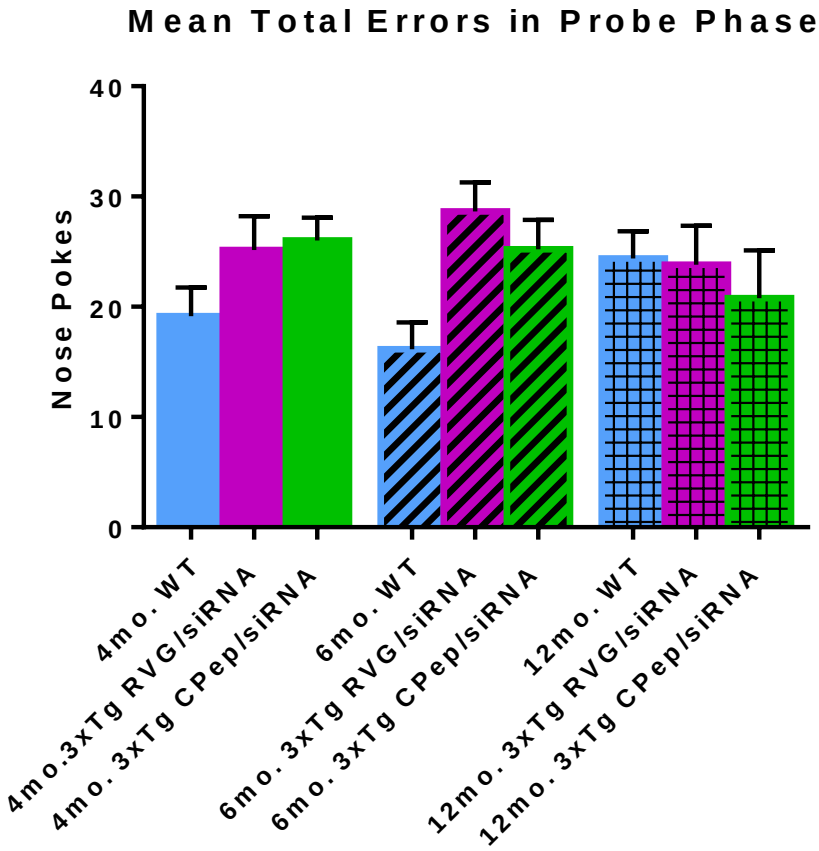
total errors than the other two treatment groups. In trial 1, the 3xTg-AD RVG-9R/siRNA treated group had 35.0 ± 3.85 mean total errors and by the probe phase this was down to 25.4 ± 4.64. The 12 month old female 3xTg-AD CPep/siRNA treated group tended to have the fewest mean total errors compared to the other two treatment groups. In trial 1, the 3xTg-AD CPep/siRNA treated group had 27.6 ± 7.02 mean total errors which decreased to 22.1 ± 5.05 in the probe phase.

Mean total errors in the probe phase show no consistent trends across the three age groups tested in this experiment (Fig. 20). In 4 month old mice and 6 month old mice, the 3xTg-AD groups had higher mean total errors compared to their age matched WT controls. However, this was not the case in the 12 month treatment groups. In the 12 month treatment groups, the 3xTg-AD mice had slightly lower mean total errors than the age matched WT control group.

In the probe phase, the escape box is no longer present. The number of nose pokes in the escape hole, which led to the escape box during the training trials, were counted and recorded for each mouse during the probe phase. The greater the number of nose pokes in the escape hole the better the mouse learned the location of the escape box during the five training trials.

Mean escape hole pokes declined with age regardless of treatment as was expected (Fig. 21). Four month old WT and 4 month old 3xTg-AD CPep/siRNA treated mice had nearly the same number of nose pokes in the escape hole during the probe phase (4.0 ± 0.69 and 4.1 ± 0.77, respectively). The 4 month old 3xTg-AD RVG-9R/siRNA group had slightly fewer nose pokes in the escape hole, 3.1 ± 0.92, compared to the other two 4 month old groups. The 6 month old groups show a trend in

Figure 20: Mean total errors in the probe phase for all mice. Mean total errors in the probe phase tended to increase with age for the wild type groups. Both 3xTg-AD groups had higher mean total errors at four months and six months of age compared to wild type control groups. All data displayed as mean ± standard error of the mean.

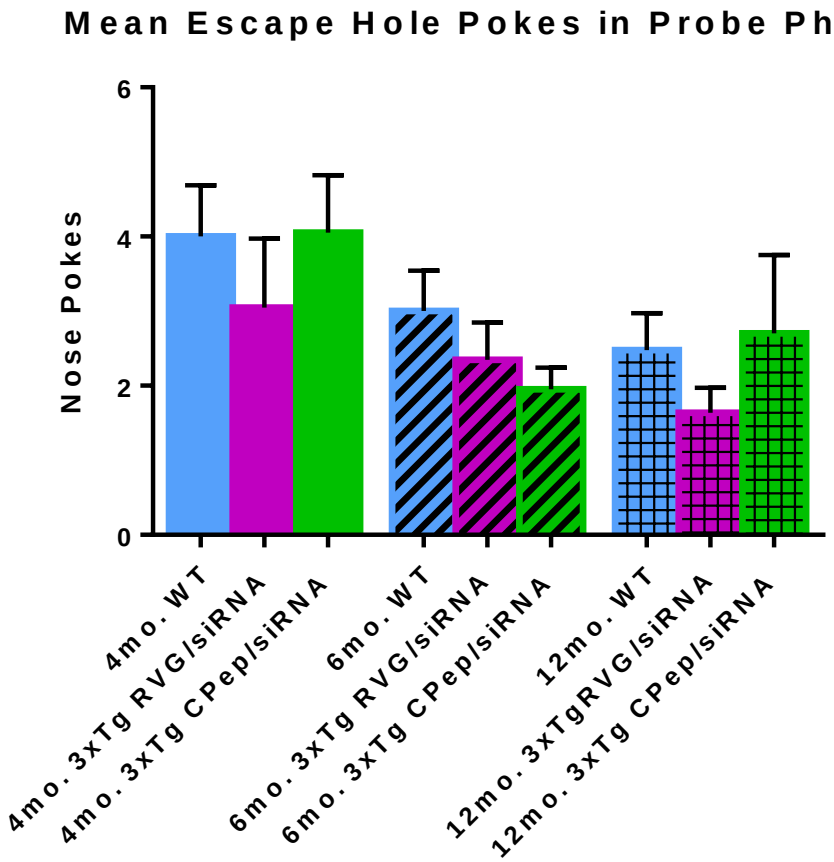


4mo. WT n=20; 4mo. 3xTg RVG/siRNA n=20; 4mo. 3xTg CPep/siRNA n=20

6mo. WT n=20; 6mo. 3xTg RVG/siRNA n=20; 6mo. 3xTg CPep/siRNA n=20

12mo. WT n=21; 12mo. 3xTg RVG/siRNA n=11; 12mo. 3xTg CPep/siRNA n=10

Figure 21: Mean escape hole pokes in the probe phase for all mice. During the probe phase of the Barnes maze protocol, each time a mouse pokes its nose into the hole where the escape box was located during the training trials is recorded. The mean number of nose pokes into the escape hole decreases with age regardless of strain or treatment, as is expected. 3xTg-AD RVG-9R/siRNA consistently had lower mean escape hole nose pokes compared to wild type controls across all ages. The six month old groups display a trend supporting our hypothesis. The six month old 3xTg-AD mice had fewer mean nose pokes in the escape hole than the six month wild type mice, but more mean nose pokes in the escape hole than the 3xTg-AD control peptide/siRNA treated mice. All data displayed as mean ± standard error of the mean.



4mo. WT n=20; 4mo. 3xTg RVG/siRNA n=20; 4mo. 3xTg CPep/siRNA n=20

6mo. WT n=20; 6mo. 3xTg RVG/siRNA n=20; 6mo. 3xTg CPep/siRNA n=20

12mo. WT n=21; 12mo. 3xTg RVG/siRNA n=11; 12mo. 3xTg CPep/siRNA n=10

escape hole nose pokes supporting the hypothesis. The 6 month old WT group had the greatest escape hole pokes (3.0 ± 0.54), followed by the 3xTg-AD RVG-9R/siRNA group (2.35 ± 0.50), and the 3xTg-AD CPep/siRNA group had the fewest escape hole pokes (1.95 ± 0.29). Compared to the 4 month old cohort and the 6 month old cohort, the 12 month old treatment groups had the fewest escape hole pokes in the probe phase. Twelve month old WT mice had 2.5 ± 0.50 mean escape hole nose pokes in the probe phase. Twelve month old 3xTg-AD RVG-9R/siRNA treated mice had the fewest mean escape hole nose pokes of any treatment group at any age with 1.6 ± 0.34. The 12 month old 3xTg-AD CPep/siRNA treated mice surprisingly had more mean nose pokes in the escape hole, 2.7 ± 1.06, than the 12 month old WT mice.

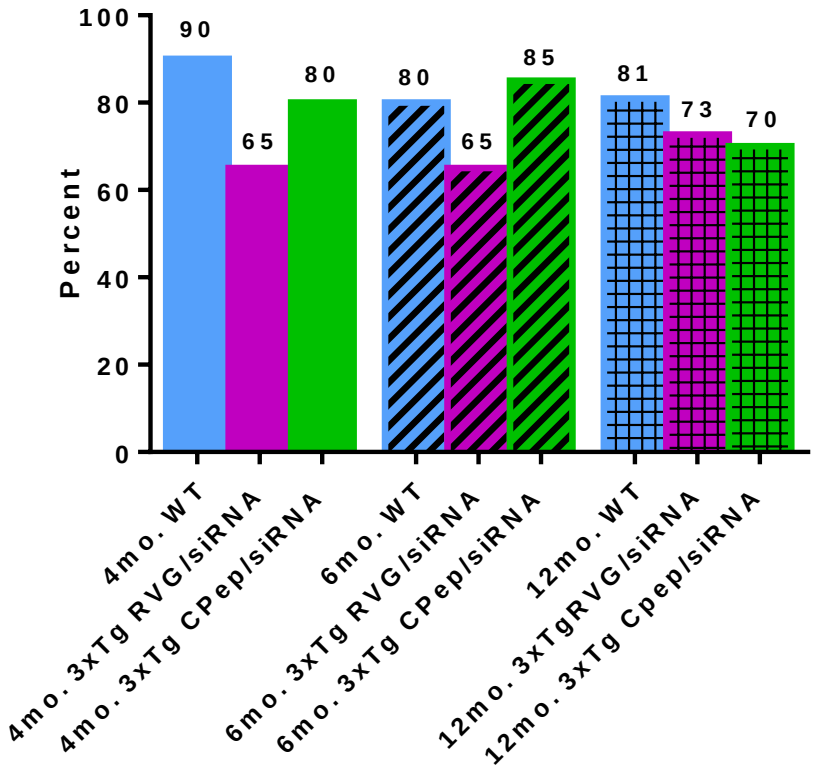
For each treatment group at each age the percent of mice finding the escape hole in the probe phase was calculated (Fig. 22). The 4 month old WT mice were most successful in finding the escape hole during the probe phase, as was expected. It would be expected that the group with fewest mice finding the escape hole during the probe phase would be the 12 month old 3xTg-AD CPep/siRNA treated group, however, this was not the case. The 4 month old 3xTg-AD RVG-9R/siRNA and 6 month old 3xTg-AD RVG-9R/siRNA groups had the lowest percentage of mice finding the escape hole during the probe phase compared to all other ages and treatments. 65% of mice from these two groups were able to find the escape hole during the probe phase.

Immunofluorescence

The number of synapses in all three 4 month old treatment groups were approximately the same (Fig. 23A). In 4 month old male mice, both the 3xTg-AD RVG-9R/siRNA and 3xTg-AD CPep/siRNA groups had significantly fewer synapses than the

Figure 22: Percent of mice finding the escape hole in the probe phase. The percent of mice able to find the escape hole during the probe phase tended to decrease with age, as was expected. The four month wild type mice were most successful in find the escape hole during the probe phase (90%). The percent of wild type mice finding the escape hole decreased slightly at 6 months and 12 months of age. All 3xTg groups tended to be less successful at finding the escape hole during the probe phase compared to the wild type controls, with the exception of the 6 month old 3xTg control peptide/siRNA treated mice. The 12 month age group displays a trend similar to what was hypothesized. The 12 month wild type group was the most successful at finding the escape hole of the three treatment groups and the 12 month old 3xTg control peptide/siRNA treated group was the least successful. The 12 month 3xTg RVG/siRNA treated group's success rate fell between that of the 12 month wild type mice and the 3xTg control peptide/siRNA treated group.

Percent of Mice Finding Escape Hole in Probe Phase



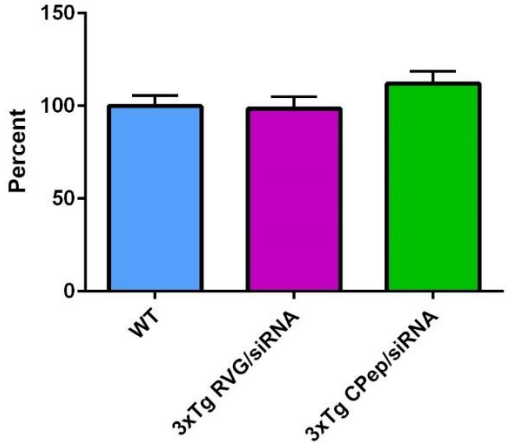
4mo. WT n=20; 4mo. 3xTg RVG/siRNA n=20; 4mo. 3xTg CPep/siRNA n=20

6mo. WT n=20; 6mo. 3xTg RVG/siRNA n=20; 6mo. 3xTg CPep/siRNA n=20

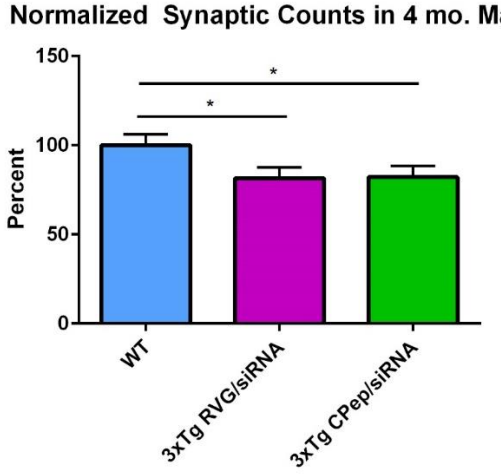
12mo. WT n=21; 12mo. 3xTg RVG/siRNA n=11; 12mo. 3xTg CPep/siRNA n=10

Figure 23: Synapses in 4 month old mice. (A) All three 4 experimental groups had approximately the same number of synapses. (B) When 4 month old males are analyzed separately, both 3xTg-AD groups had significantly fewer synapses than the 4 month old male WT group. (C) In 4 month old females, the 3xTg-AD CPep/siRNA treated group had significantly more synapses than the 4 month old female WT group. All data displayed as mean ± standard error of the mean, *p<0.05, **p<0.01, ***p<0.001 ****p<0.0001.

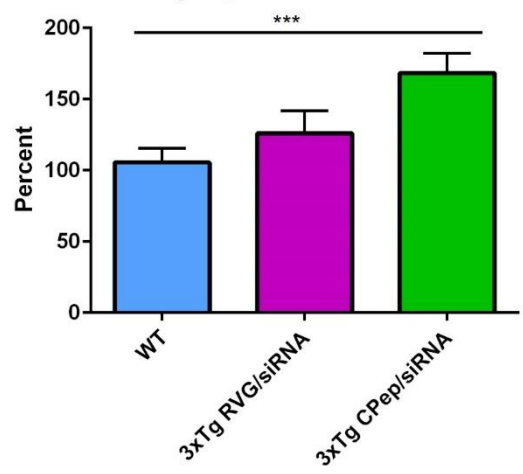
A


Normalized Synaptic Counts for 4 mo. Mice
Percent
150
100
50
0
WT
3xTg RVG/siRNA
3xTg CPep/siRNA

B


Normalized Synaptic Counts in 4 mo. Males
Percent
150
100
50
0
*
*
WT
3xTg RVG/siRNA
3xTg CPep/siRNA

C


Normalized Synaptic Counts in 4 mo. Females
Percent
200
150
100
50
0
***
WT
3xTg RVG/siRNA
3xTg CPep/siRNA

4 month old male WT mice (Fig. 23B). Four month old female 3xTg-AD CPep/siRNA mice had significantly more synapses than the 4 month old female WT mice (Fig. 23C).

The 6 month old 3xTg-AD CPep/siRNA treated mice had significantly fewer synapses than both the WT and 3xTg-AD RVG-9R/siRNA groups (Fig. 24A). This is also true when 6 month old females are analyzed separately (Fig. 24C). In 6 month old male mice, the 3xTg-AD CPep/siRNA treated mice also had fewer synapses than the WT and 3xTg-AD RVG-9R/siRNA groups, however the difference is not statistically significant (Fig. 24B).

In 12 month old mice, the 3xTg-AD RVG-9R/siRNA and 3xTg-AD CPep/siRNA groups both had significantly fewer synapses than the 12 month old WT group (Fig. 25A). Twelve month old male 3xTg-AD RVG-9R/siRNA and 3xTg-AD CPep/siRNA treated mice had fewer synapses than the 12 month old WT male mice, however these differences are not statistically significant (Fig. 25B). In 12 month old female mice, the 3xTg-AD CPep/siRNA group had fewer synapses than the 3xTg-AD RVG-9R/siRNA group. Both of the 3xTg-AD groups had fewer synapses than the 12 month old WT females, however these differences are not statistically significant (Fig. 25C).

Western Blot

Four month old mice display trends in Kremen1 protein expression supporting the hypothesis, however no significant differences were found (Fig. 26A). When male and female 4 month old mice are analyzed separately, WT mice showed the lowest Kremen1 expression followed by the 3xTg-AD RVG-9R/siRNA treated group. The 3xTg-AD CPep/siRNA treated mice displayed the highest levels of Kremen1 (Fig. 26B,C).

Figure 24: Synapses in 6 month old mice. (A) In 6 month old mice, the 3xTg-AD CPep/siRNA treated mice had significantly fewer synapses than both the 6 month old WT and 6 month old 3xTg-AD RVG-9R/siRNA treated mice. (B) When 6 month old males are analyzed separately, the WT and 3xTg-AD RVG-9R/siRNA mice had approximately the same number of synapses. The 6 month old male 3xTg-AD CPep/siRNA treated mice had fewer synapses than both other treatment groups, however these differences were not statistically significant. (C) In 6 month old female mice, the 3xTg-AD CPep/siRNA treated mice had significantly fewer synapses than both the WT and 3xTg-AD RVG-9R/siRNA treated mice. All data displayed as mean ± standard error of the mean, *p<0.05, **p<0.01, ***p<0.001 ****p<0.0001.

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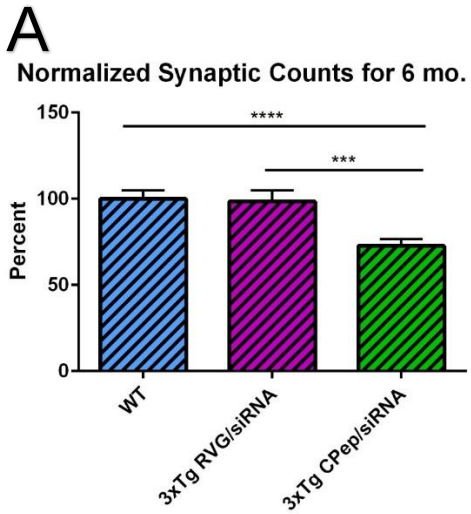
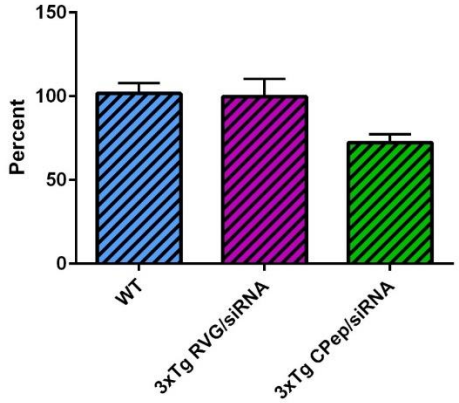
A


Normalized Synaptic Counts for 6 mo. Mice
Percent
150
100
50
0
****
***
WT
3xTg RVG/siRNA
3xTg CPep/siRNA

B


Normalized Synaptic Counts in 6 mo. Males
Percent
150
100
50
0
WT
3xTg RVG/siRNA
3xTg CPep/siRNA

C

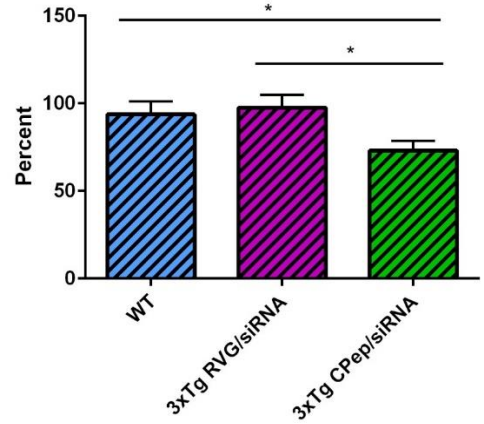

Normalized Synaptic Counts in 6 mo. Females
Percent
150
100
50
0
*
*
WT
3xTg RVG/siRNA
3xTg CPep/siRNA

Figure 25: Synapses in 12 month old mice. (A) Twelve month old WT mice had significantly more synapses than both 3xTg-AD treatment groups. Both 3xTg-AD treatment groups had approximately the same number of synapses. (B) In 12 month old males, both 3xTg-AD treatment groups had fewer synapses than the WT group, however, no statistically significant differences were found. (C) Twelve month old female 3xTg-AD CPep/siRNA treated mice had approximately 10% fewer synapses than the 3xTg-AD RVG-9R/siRNA treated mice and 20% fewer synapses than the WT mice. All data displayed as mean ± standard error of the mean, *$p<0.05$, **$p<0.01$, ***$p<0.001$ ****$p<0.0001$.

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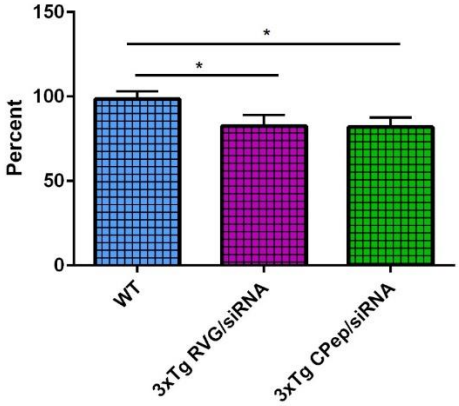
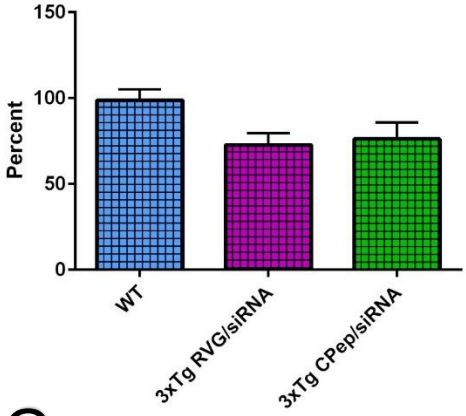
A


Normalized Synaptic Counts for 12 mo. Mice
150
100
50
0
Percent
*
*
WT
3xTg RVG/siRNA
3xTg CPep/siRNA

B


Normalized Synaptic Counts in 12 mo. Males
150
100
50
0
Percent
WT
3xTg RVG/siRNA
3xTg CPep/siRNA

C

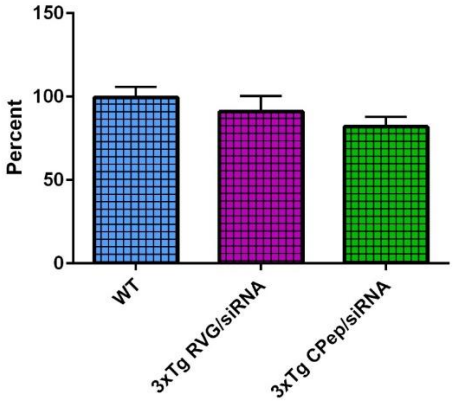
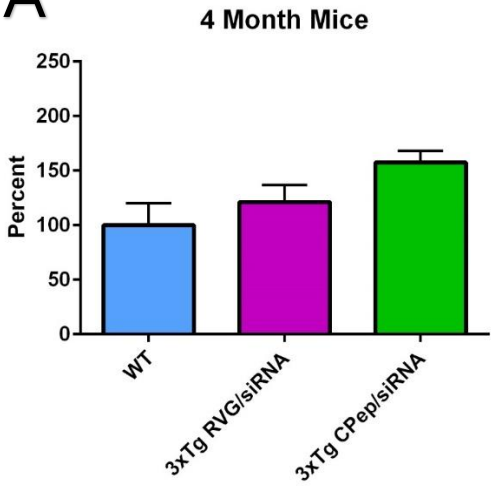
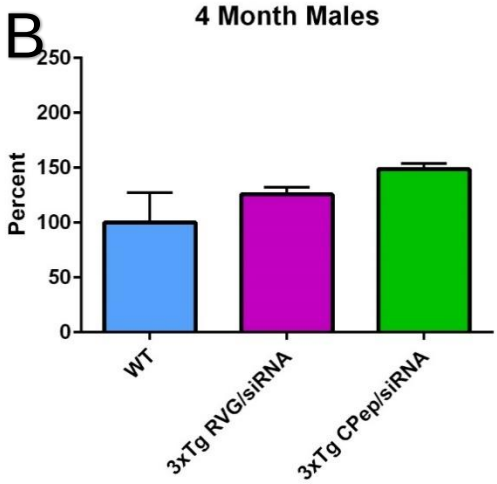

Normalized Synaptic Counts in 12 mo. Females
150
100
50
0
Percent
WT
3xTg RVG/siRNA
3xTg CPep/siRNA

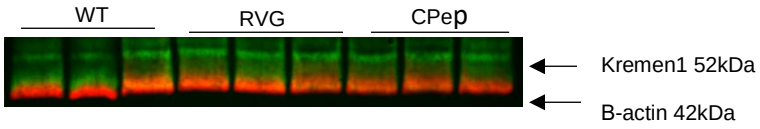
Figure 26: Kremen1 protein expression levels in 4 month old mice. (A) Kremen1 protein expression levels were lowest in 4 month old WT mice and highest in 3xTg-AD CPep/siRNA treated mice. Kremen1 protein levels in 3xTg-AD RVG/siRNA treated mice fell between the other two groups. These trends hold true for 4 month old male (B) and 4 month old female (C) mice. All data displayed as mean ± standard error of the mean.

A

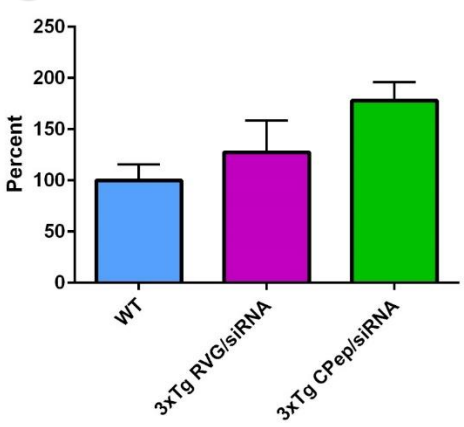

4 Month Mice
Percent
250
200
150
100
50
0
WT
3xTg RVG/siRNA
3xTg CPep/siRNA

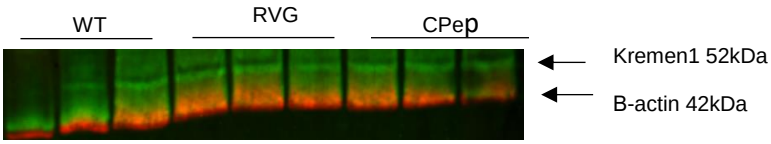
B


4 Month Males
Percent
250
200
150
100
50
0
WT
3xTg RVG/siRNA
3xTg CPep/siRNA


WT
RVG
CPep
Kremen1 52kDa
B-actin 42kDa

C


4 Month Females
Percent
250
200
150
100
50
0
WT
3xTg RVG/siRNA
3xTg CPep/siRNA


WT
RVG
CPep
Kremen1 52kDa
B-actin 42kDa

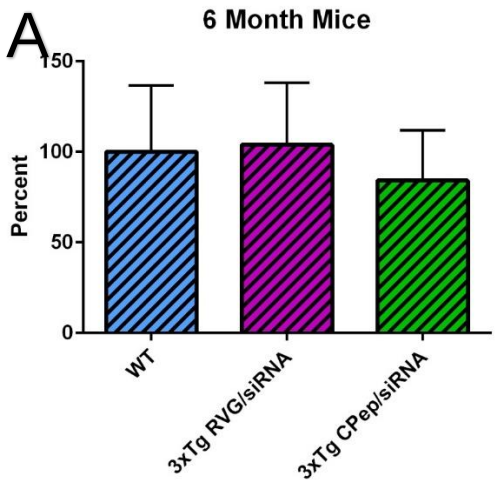
Six month old WT and 3xTg-AD RVG-9R/siRNA treated mice had approximately the same levels of Kremen1 protein expression (Fig. 27). Interestingly, 6 month old 3xTg- AD CPep/siRNA treated mice showed the lowest levels of Kremen1 protein expression. These trends hold true when 6 month old female mice are analyzed separately (Fig. 27B,C). When 6 month old males are analyzed separately, WT and 3xTg-AD CPep/siRNA treated mice had approximately the same level of Kremen1 expression (Fig. 27B).

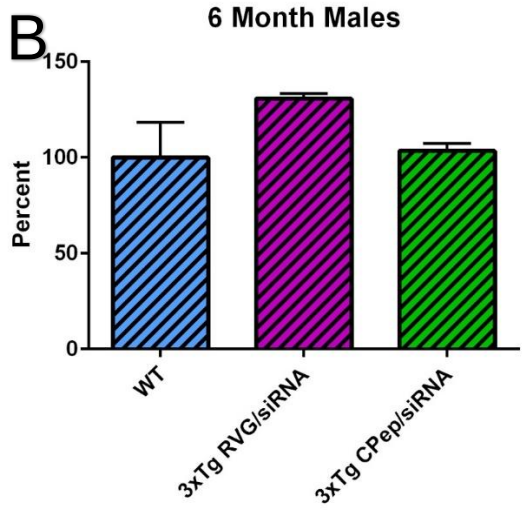
In 12 month old mice WT and 3xTg-AD RVG-9R/siRNA treated mice had approximately the same levels of Kremen1 protein expression (Fig. 28A). CPep/siRNA treated 12 month old mice show higher levels of Kremen1. These trends hold true when 12 month old males and females are analyzed separately (Fig. 28B,C).

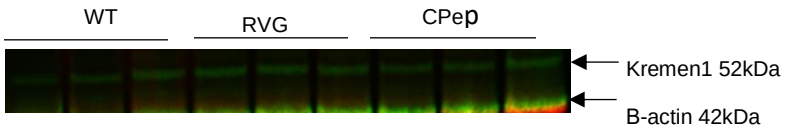
q-PCR

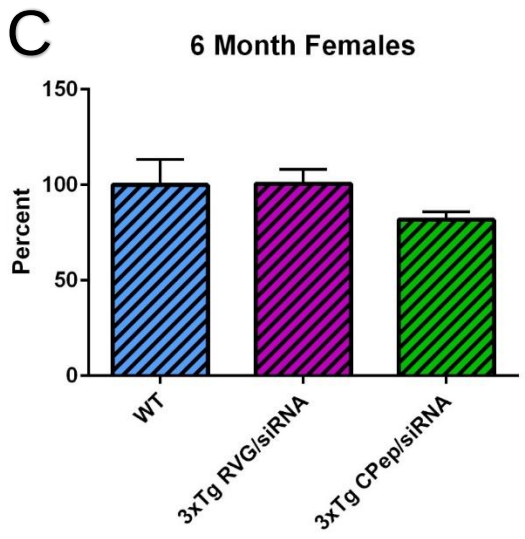
Kremen1 mRNA expression in 4 month old mice follows a trend supporting our hypothesis (Fig. 29A). Four month old WT mice showed the lowest level of Kremen1 mRNA expression. Both 4 month old 3xTg-AD groups had Kremen1 mRNA expression levels higher than the 4 month old WT group. The 3xTg-AD CPep/siRNA treated group had the highest Kremen1 mRNA expression of all three 4 month old groups. These trends are also true for the 4 month old male mice (Fig.29B). However, data gathered from 4 month old female mice show a slightly different trend (Fig. 29C). Four month old female 3xTg-AD RVG-9R/siRNA treated mice had the highest Kremen1 mRNA expression compared to the other two 4 month old female groups. Four month old female WT and 3xTg-AD CPep/siRNA treated mice had very similar levels of Kremen1 mRNA expression.

Figure 27: Kremen1 protein expression in 6 month old mice. (A) Kremen1 protein levels were approximately the same in 6 month old WT and 3xTg-AD RVG-9R/siRNA treated mice. Six month old 3xTg-AD CPep/siRNA treated mice had the lowest levels of Kremen1 protein expression. (B) In 6 month old male mice, WT and 3xTg-AD CPep/siRNA had approximately the same levels of Kremen1 expression. Six month old male 3xTg-AD RVG-9R/siRNA treated mice had the highest levels of Kremen1 expression among the three male groups. (C) Six month old female WT and 3xTg-AD RVG-9R/siRNA treated mice had approximately the same level of Kremen1 protein expression. 3xTg-AD CPep/siRNA treated female mice had the lowest level of Kremen1 expression. All data displayed as mean ± standard error of the mean.


A
6 Month Mice
Percent
150
100
50
0
WT
3xTg RVG/siRNA
3xTg CPep/siRNA


B
6 Month Males
Percent
150
100
50
0
WT
3xTg RVG/siRNA
3xTg CPep/siRNA


WT
RVG
CPep
Kremen1 52kDa
B-actin 42kDa


C
6 Month Females
Percent
150
100
50
0
WT
3xTg RVG/siRNA
3xTg CPep/siRNA

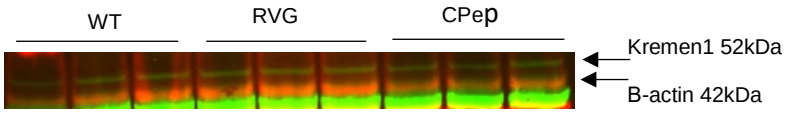
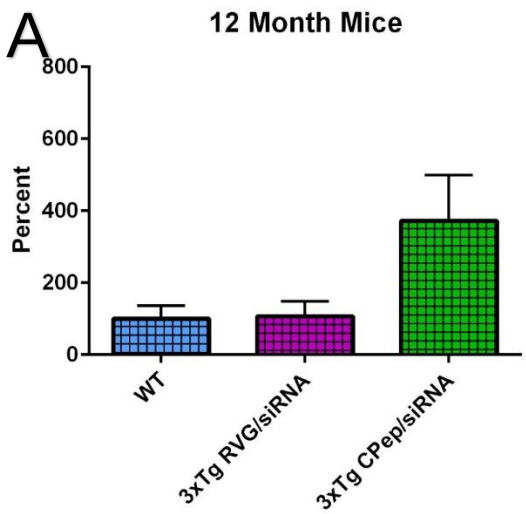
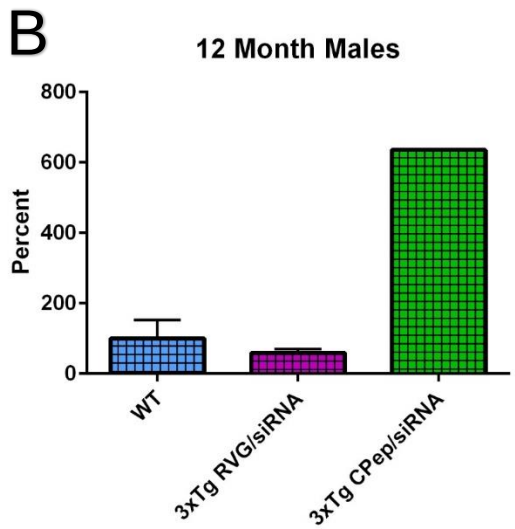
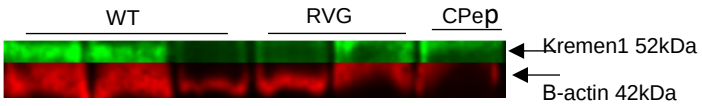
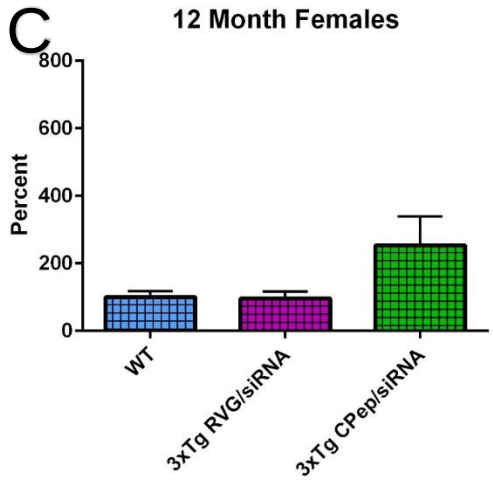

WT
RVG
CPep
Kremen1 52kDa
B-actin 42kDa

Figure 28: Kremen1 protein expression levels in 12 month old mice. (A) Kremen1 protein expression levels were approximately the same for 12 month old WT and 3xTg-AD RVG-9R/siRNA treated mice. The 12 month old 3xTg-AD CPep/siRNA treated mice had the highest levels of Kremen1 protein expression. These trends hold true when 12 month old males (B) and females (C) are analyzed separately. All data displayed as mean ± standard error of the mean.


A
12 Month Mice
Percent
800
600
400
200
0
WT
3xTg RVG/siRNA
3xTg CPep/siRNA


B
12 Month Males
Percent
800
600
400
200
0
WT
3xTg RVG/siRNA
3xTg CPep/siRNA


WT
RVG
CPep
Kremen1 52kDa
B-actin 42kDa


C
12 Month Females
Percent
800
600
400
200
0
WT
3xTg RVG/siRNA
3xTg CPep/siRNA

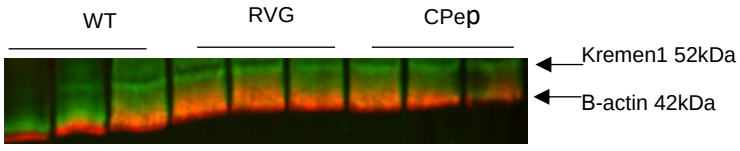
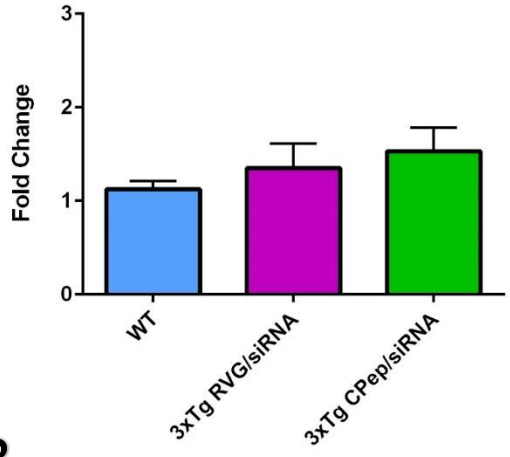

WT
RVG
CPep
Kremen1 52kDa
B-actin 42kDa

Figure 29: Kremen1 mRNA expression in 4 month old mice. (A) 4 month old PCR data follows trends similar to that of the 4 month old western blot data. The 4 month old WT mice had the lowest levels of Kremen1 mRNA expression, followed by the 4 month old 3xTg-AD RVG-9R/siRNA treated mice, and the 4 month old 3xTg-AD CPep/siRNA treated mice had the highest. These trends are also seen when 4 month old male mice are analyzed separately (B). (C) In 4 month old female mice, WT and 3xTg-AD CPep/siRNA treated mice had approximately the same levels of Kremen1 mRNA expression. The 4 month old female 3xTg-AD CPep/siRNA showed the highest levels of Kremen1 mRNA expression. All data displayed as mean ± standard error of the mean.

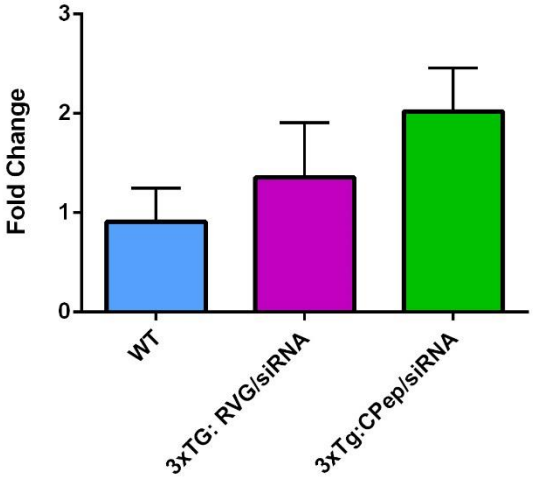
A

Fold Change in Kremen1 Expression in 4mo. Mice



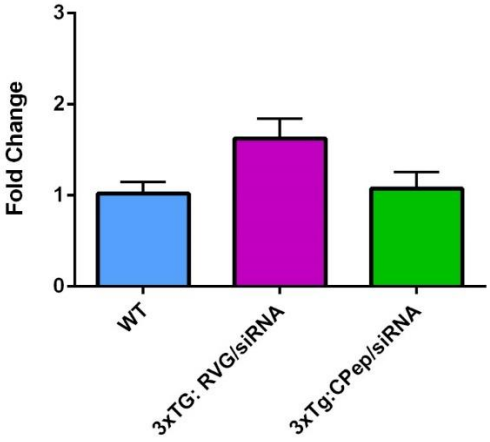
B

Fold Change in Kremen1 Expression in 4mo. Males



C

Fold Change in Kremen1 Expression in 4mo. Females

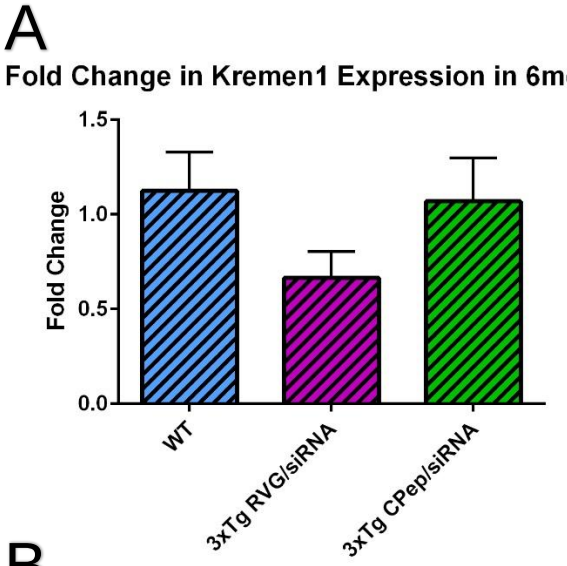


Six month old mice display a trend in Kremen1 mRNA expression that is opposite of the 4 month old mice (Fig. 30A). In 6 month old mice, the WT mice had the highest level of Kremen1 mRNA expression and the 3xTg-AD CPep/siRNA treated mice had the lowest level. The 6 month old 3xTg-AD RVG-9R/siRNA treated mice had a Kremen1 mRNA expression level that fell between the other two treatment groups. When 6 month old males and females were analyzed separately the 3xTg-AD RVG-9R/siRNA treated group had the lowest level of Kremen1 mRNA expression for both sexes (Fig. 30B, C). In both male and female 6 month old mice WT and 3xTg-AD CPep/siRNA treated mice had similar levels of Kremen1 mRNA expression.

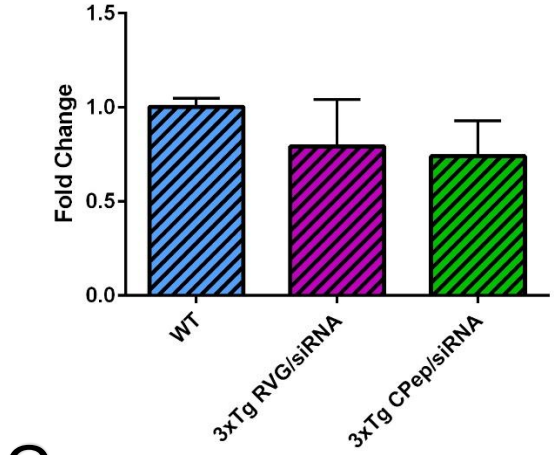
In 12 month old mice, the 3xTg-AD CPep/siRNA had the highest levels of Kremen1 mRNA compared to the other two 12 month old groups (Fig. 31A). The 12 month old 3xTg-AD RVG-9R/siRNA treated mice had the lowest levels of Kremen1 mRNA of the three 12 month old experimental groups. When 12 month old males are analyzed separately the WT and 3xTg-AD RVG-9R/siRNA groups had approximately the same levels of Kremen1 mRNA expression (Fig. 31B). Of the three 12 month old male experimental groups, the 3xTg-AD CPep/siRNA treated mice showed the highest level of Kremen1 mRNA expression. In 12 month old females the WT and 3xTg-AD CPep/siRNA groups had approximately the same level of Kremen1 mRNA expression (Fig. 31C). The 12 month old female 3xTg-AD RVG-9R/siRNA treated group had the lowest level of Kremen1 mRNA expression compared to the other two treatment groups.

Figure 30: Kremen1 mRNA expression in 6 month old mice. (A) Six month old WT mice and 3xTg-AD CPep/siRNA had approximately the same levels of Kremen1 mRNA expression. The 6 month old 3xTg-AD RVG-9R/siRNA treated mice showed the lowest levels of Kremen1 mRNA expression. These trends hold true when 6 month old female mice are analyzed separately (C). (B) In 6 month old male mice, the WT group had the highest levels of Kremen1 mRNA expression. The 6 month old male 3xTg-AD CPep/siRNA treated mice have slightly lower Kremen1 mRNA expression than the 3xTg-AD RVG-9R/siRNA treated mice. All data displayed as mean ± standard error of the mean.

A


Fold Change in Kremen1 Expression in 6mo. Mice
Fold Change
1.5
1.0
0.5
0.0
WT
3xTg RVG/siRNA
3xTg CPep/siRNA

B


Fold Change in Kremen1 Expression in 6mo. Males
Fold Change
1.5
1.0
0.5
0.0
WT
3xTg RVG/siRNA
3xTg CPep/siRNA

C

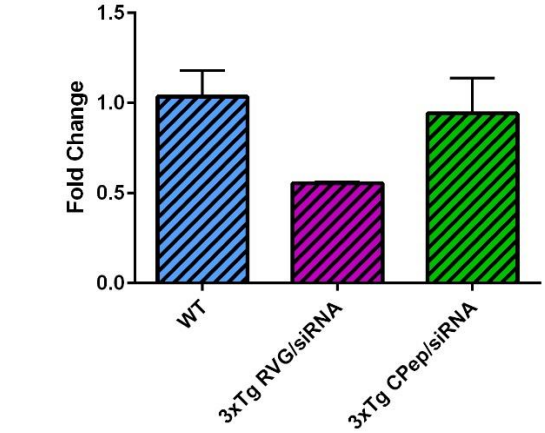
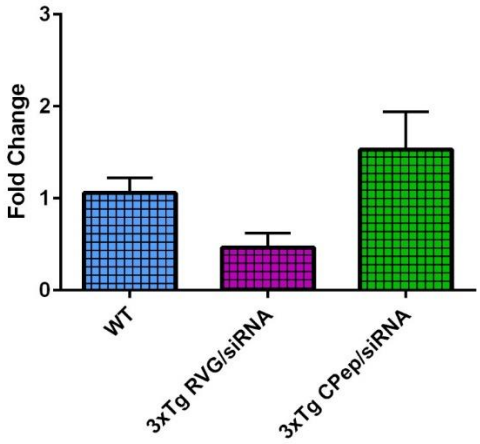

Fold Change in Kremen1 Expression in 6mo. Females
Fold Change
1.5
1.0
0.5
0.0
WT
3xTg RVG/siRNA
3xTg CPep/siRNA

Figure 31: Kremen1 mRNA expression in 12 month old mice. (A) In 12 month old mice, 3xTg-AD RVG-9R/siRNA treated mice showed lower levels of Kremen1 mRNA expression compared to the 12 month old WT group. The 12 month old 3xTg-AD CPep/siRNA treated mice had the highest levels of Kremen1 mRNA expression. (B) In 12 month old male mice, WT and 3xTg-AD RVG-9R/siRNA had approximately the same levels of Kremen1 mRNA expression. The male 3xTg-AD CPep/siRNA treated mice had the highest levels of mRNA expression, however it should be noted that this group is only n=1. (C) In 12 month old female mice, the 3xTg-AD RVG-9R/siRNA treated mice had the lowest levels of Kremen1 mRNA expression compared to the 12 month old female WT mice. All data displayed as mean ± standard error of the mean.

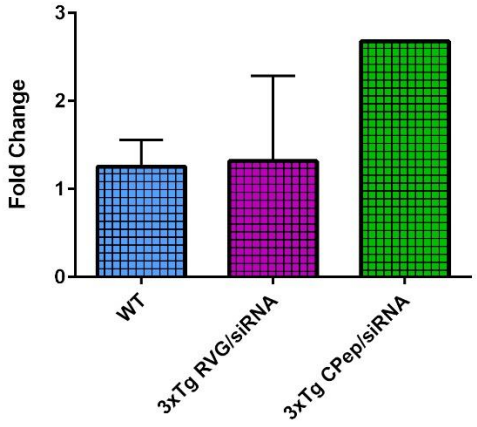
A

Fold Change in Kremen1 Expression in 12 mo. Mice



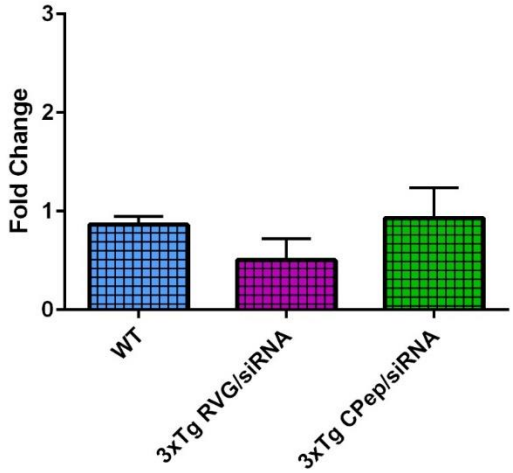
B

Fold Change in Kremen1 Expression in 12 mo. Males



C

Fold Change in Kremen1 Expression in 12 mo. Females



DISCUSSION

The goal of this study was to investigate the downregulation of Kremen1 via RVG-9R/siRNA complexes. Kremen1 downregulation was quantified at the mRNA and protein levels. Impacts of Kremen1 downregulation were evaluated in behavioral measures via the Barnes maze as well as at the synaptic level by quantifying synapses in each treatment group.

High variability in behavioral data makes it inherently difficult to interpret. The measures used in this study do not take in to account the entirety of a mouse's behavior while on the maze. For example, some mice may sit still and not walk around on the maze. So, naturally, the primary errors and total errors would be very low compared to those mice that do explore the maze. This poses a problem when interpreting the compiled data. Low primary errors and total errors may be due to fast learning or may be due to the fact that a mouse did not explore the maze. This makes it difficult to interpret true trends in the data despite having a large N.

Primary errors, total errors, primary latency, and nose pokes in the escape hole may not be sensitive enough measures to detect differences between wild type, 3xTg-AD RVG-9R treated, and 3xTg-AD CPep treated mice. Other measures that have shown differences between wild type and 3xTg-AD mice include time spent in the target quadrant and percent of holes searched in the target quadrant (the target quadrant being the quarter of the maze where the escape hole was located) (Attar et al., 2007). With these measures Attar et al. were able to detect differences between WT and 3xTg-AD mice as early as 4 months of age (2007). O'Leary et al. found errors, distance traveled on the maze, and hole deviation scores to be more sensitive indicators of

learning on the Barnes maze compared to primary latency when C57BL/6J mice were tested (2013).

The multitude of adaptations for the Barnes maze allows for many possibilities to optimize the protocol in order to detect differences between WT and 3xTg-AD mice.

The fact that there were no statistically significant differences in the number of synapses among the three treatment groups for all 4 month old mice is not surprising. At this age 3xTg-AD mice have not developed AD pathology (Oddo et al., 2003) that induces synaptic loss. That being said, it is surprising that in the 4 month old male mice, both 3xTg-AD groups had significantly fewer synapses than the WT mice. However, in 4 month old female mice, both 3xTg-AD groups had significantly more synapses than the WT group. This variability in results between the two sexes may be due to the small number of mice analyzed for synapses from each sex (n=3 per sex per treatment).

By the time 3xTg-AD mice are 6 months old Aβ deposits are present and synaptic transmission is impaired (Oddo et al., 2003). Therefore, it is expected that the 3xTg-AD mice should have fewer synapses than the WT mice at 6 months of age. In the 6 month old mice analyzed for synapses, the 3xTg-AD CPep/siRNA treated mice had significantly fewer synapses than the WT mice, as was expected. The 6 month old 3xTg-AD CPep/siRNA treated also had significantly fewer synapses than the 3xTg-AD RVG-9R/siRNA treated mice (which had approximately the same number of synapses as the WT mice) indicating that the RVG-9R/siRNA treatment may provide some protection from synaptic loss. These differences were also seen when 6 month old male and female mice were analyzed separately, however, in males the differences were not statistically significant.

At 12 months of age 3xTg-AD mice have widespread Aβ plaques, neurofibrillary tangles, and significant synaptic impairment (Oddo et al., 2003). By 12 months of age it is expected the 3xTg-AD mice will have significantly fewer synapses than WT mice of the same age. This was found to be true in the mice analyzed for synapses in this study. Both 12 month old 3xTg-AD groups had significantly fewer synapses than the 12 month old WT group. However, both 3xTg-AD groups had approximately the same number of synapses indicating there may not be a protective effect from the RVG-9R/siRNA treatment.

The 12 month old female mice show a trend in number of synapses that more closely supports our hypothesis, although the differences are not statistically significant. The 3xTg-AD CPep/siRNA treated mice had the fewest number of synapses compared to the other two treatment groups. The 12 month old female 3xTg-AD RVG-9R/siRNA treated mice had fewer synapses than the WT group but more synapses than the 3xTg-AD CPep/siRNA treated group.

Interpreting trends in the 12 month old males is difficult do to the small number of mice analyzed. Synaptic count data is only reflective of 1 mouse for both male 3xTg-AD groups. Both 3xTg-AD treatment groups had approximately the same number of synapses and fewer synapses than the WT group. This is similar to the trend seen when all 12 month mice are analyzed together.

Overall, synaptic count data from the 6 month old mice supports our hypothesis that the RVG-9R/siRNA treatment will prevent synaptic loss. The trends seen in the 4 month old mice may be due to the lack of AD pathology at this age point. Four months of age may be too early to detect a significant synaptic protective effect from the RVG-

9R/siRNA treatment. With that being said, the trends in the 12 month old mice may be due to the advanced AD pathology. The widespread Aβ plaques and tau neurofibrillary tangles at this age point may negate any potential protection from the RVG-9R/siRNA treatment.

It should be noted that only three mice of each sex from each treatment group were analyzed for number of synapses. A larger number of mice analyzed may give a clearer insight into the true effects of the siRNA treatment on number of synapses.

Data from 4 month old western blots show trends supporting our hypothesis. The 4 month old WT mice show the lowest levels of Kremen1 protein, followed by 3xTg-AD RVG-9R/siRNA treated mice, and 3xTg-AD CPep/siRNA treated mice had the highest levels of Kremen1 protein expression. These trends are consistent when 4 month males and females are analyzed separately, however, there are no statistically significant differences. These trends indicate the ability of RVG-9R/siRNA to target and downregulate Kremen1 expression on the protein level in 4 month old mice.

In 6 month old mice, WT and 3xTg-AD RVG-9R/siRNA treated mice had similar levels of Kremen1 protein expression. Interestingly, the 3xTg-AD CPep/siRNA treated mice had the lowest levels of Kremen1 protein expression. These trends were also seen when 6 month old females were analyzed separately. In 6 month old males, the 3xTg-AD had the highest levels of Kremen1 protein expression. Based on these results, there is no indication of an effect of the RVG-9R/siRNA treatment on Kremen1 protein levels in 6 month old mice.

Trends in 12 month old western blot data support our hypothesis. In all 12 month old mice, and when males and females are analyzed separately, the 3xTg-AD CPep/siRNA treated mice had the highest levels of Kremen1 protein expression. The WT and 3xTg-AD RVG-9R/siRNA treated mice had approximately the same levels of Kremen1 protein expression. Compared to the 3xTg-AD CPep/siRNA treated mice, the 3xTg-AD RVG-9R/siRNA treated mice had lower levels of Kremen1 protein expression indicating the potential for RVG-9R/siRNA to downregulate Kremen1.

q-PCR results from 4 month old mice are consistent with western blot results. Kremen1 mRNA expression was lowest in 4 month old WT mice and highest in 3xTg-AD CPep/siRNA treated mice. The Kremen1 mRNA expression 3xTg-AD RVG-9R/siRNA treated fell between those of the other two groups. These results support our hypothesis that RVG-9R/siRNA treatment will downregulate Kremen1 at the mRNA level.

Kremen1 mRNA expression in 6 month old mice is different from what was expected. In all 6 month old mice, the 3xTg-AD RVG-9R/siRNA treated mice showed the lowest levels of mRNA expression. The WT and 3xTg-AD CPep/siRNA treated mice had approximately the same levels. These same trends were seen when 6 month old females were analyzed separately. It was hypothesized that the 6 month old WT mice would have the lowest levels of Kremen1 mRNA expression. These results may indicate the ability of RVG-9R/siRNA treatment to downregulate Kremen1 on the mRNA level, however, a larger sample size is needed to confirm this trend. Kremen1 mRNA expression was approximately the same in both 3xTg-AD 6 month old male groups. However, both of these groups showed lower levels of Kremen1 expression compared

to the 6 month old male WT group. With n=3 for each of these groups, a larger sample size would provide more insight into the true effects of RVG-9R/siRNA treatment on Kremen1 mRNA expression.

Like the 6 month old mice, the 12 month old 3xTg-AD RVG-9R/siRNA treated mice had the lowest levels of Kremen1 mRNA expression of all three treatment groups. This is also seen in 12 month old females when they are analyzed separately. In 12 month old males, WT and 3xTg-AD RVG-9R/siRNA treated mice had approximately the same levels of Kremen1 mRNA expression. The 12 month old male 3xTg-AD CPep/siRNA treated mice had the highest levels of Kremen1 mRNA expression. However, in the males it should be noted that both 3xTg-AD groups have only n=1 thus making it difficult to interpret true effects of the RVG-9R/siRNA treatment. Trends in 12 month old mice indicate the potential of RVG-9R/siRNA to target and downregulate Kremen1 at the mRNA level, however, larger sample sizes are needed to make accurate conclusions.

Overall, there are trends in the molecular data that indicate the potential for RVG-9R/siRNA to target and downregulate Kremen1 *in vivo* and to provide protection from synaptic loss. However, inconsistencies in the data do not allow for a concrete conclusion to be made regarding the ability of RVG-9R/siRNA to target and downregulate Kremen1. Results from brain analysis experiments may not be reflected in the behavioral data for a variety of reasons. One dose of the RVG-9R/siRNA treatment may not be enough to make a significant impact on Kremen1 levels in the 1 week experimental timeline. Or, the measures used in the Barnes maze protocol may not be sensitive enough to detect differences in the treatment groups.

Over 5 million Americans are currently living with AD (Alzheimer's Association, 2017; Taylor et al., 2017). While there is currently no cure for AD, the role of Kremen1 as an antagonist of the canonical Wnt signaling pathway in combination with the results of this study show the potential of Kremen1 as a therapeutic target for AD. Further investigation of the role of Kremen1 in the development of AD is needed to determine if downregulating this protein will prevent synaptic loss and memory deficits.

REFERENCES

2017 alzheimer's disease facts and figures.(2017). *Alzheimer's & Dementia: The Journal of the Alzheimer's Association, 13*(4), 325-373. Retrieved from http://dx.doi.org/10.1016/j.jalz.2017.02.001

Attar, A., Liu, T., Chan, W. C., Hayes, J., Nejad, M., Lei, K., et al. (2013). A shortened barnes maze protocol reveals memory deficits at 4-months of age in the triple-transgenic mouse model of alzheimer's disease. *Plos One, 8*(11), e80355. Retrieved from https://doi.org/10.1371/journal.pone.0080355

Barnes, C. A. (1979). Memory deficits associated with senescence: A neurophysiological and behavioral study in the rat. *Journal of Comparative and Physiological Psychology, 93*(1), 74-104.

Bekris, L. M., Yu, C., Bird, T. D., & Tsuang, D. W. (2010). Genetics of alzheimer disease. *Journal of Geriatric Psychiatry and Neurology, 23*(4), 213-227. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3044597/

Brewer, G. J. (1997). Isolation and culture of adult rat hippocampal neurons. *Journal of Neuroscience Methods, 71,* 143-155.

Caricasole, A., Copani, A., Caraci, F., Aronica, E., Rozemuller, A. J., Caruso, A., et al. (2004). Induction of dickkopf-1, a negative modulator of the wnt pathway, is associated with neuronal degeneration in alzheimer's brain. *The Journal of Neuroscience, 24*(26), 6021-6027. doi:10.1523/JNEUROSCI.1381-04.2004

Cerpa, W., Gambrill, A., Inestrosa, N. C., & Barria, A. (2011). Regulation of NMDA-receptor synaptic transmission by wnt signaling. *The Journal of Neuroscience, 31*(26), 9466-9471. doi:10.1523/JNEUROSCI.6311-10.2011

Chen, J., Park, C. S., & Tang, S. (2006). Activity-dependent synaptic wnt release regulates hippocampal long term potentiation. *Journal of Biological Chemistry, 281*(17), 11910-11916. doi:10.1074/jbc.M511920200

Chishti, M. A., Yang, D., Janus, C., Phinney, A. L., Horne, P., Pearson, J., et al. (2001). Early-onset amyloid deposition and cognitive deficits in transgenic mice expressing a double mutant form of amyloid precursor protein 695. *Journal of Biological Chemistry, 276*(24), 21562-21570. doi:10.1074/jbc.M100710200

Ciani, L., Boyle, K. A., Dickins, E., Sahores, M., Anane, D., & Lopes, D. M. … Salinas, P. C. (2011). Wnt7a signaling promotes dendritic spine growth and synaptic strength through Ca2+/Calmodulin-dependent protein kinase II. *Proceedings of the National Academy of Sciences of the United States of America, 108*(26), 10732-10737.

Ciani, L., & Salinas, P. C. (2005). WNTS in the vertebrate nervous system: From patterning to neuronal connectivity. *Nature Reviews. Neuroscience, 6*(5), 351-362. Retrieved from http://dx.doi.org/10.1038/nrn1665

Clevers, H., & Nusse, R. (2012). Wnt/beta-catenin signaling and disease. *Cell, 149*(6), 1192-1205.

Davidson G, Mao B, Del Barco Barrantes I, Niehrs C. (2002). Kremen proteins interact with Dickkopf1 to regulate anteroposterior CNS patterning. *Development, 129*, 5587–96

Davidson, G., Wu, W., Shen, J., Bilic, J., Fenger, U., Stannek, P., et al. (2005). Casein kinase 1 gamma couples wnt receptor activation to cytoplasmic signal transduction. *Nature, 438*, 867-872.

De Ferrari and, G. V., & Inestrosa, N. C. (2000). Wnt signaling function in Alzheimer's disease. *Brain Research Reviews, 33*(1), 1-12. doi:https://doi.org/10.1016/S0165-0173(00)00021-7

De Ferrari, G. V., Chacon, M. A., Barria, M. I., Garrido, J. L., Godoy, J. A., Olivares, G., et al. (2003). Activation of wnt signaling rescues neurodegeneration and behavioral impairments induced by beta-amyloid fibrils. *Molecular Psychiatry, 8*, 195-208.

Dickins, E. M., & Salinas, P. C. (2013). Wnts in action: From synapse formation to synaptic maintenance. *Frontiers in Cellular Neruoscience, 7* doi:doi: 10.3389/fncel.2013.00162

Flood, D.G.,Reaume,A.G.,Dorfman,K.S.,Lin,Y.G.,Lang,D.M.,Trusko,S. P.,etal.(2002).FADmutantPS-1gene-targetedmice:increasedAbeta42and A beta deposition without APP overproduction. *Neurobiol.Aging* 23, 335–348. doi: 10.1016/S0197-4580(01)00330-X

Games, D.,Adams,D.,Alessandrini,R.,Barbour,R.,Berthelette,P.,Blackwell, C., etal.(1995). Alzheimer-type neuropathology in transgenic mice over expressing V717F beta-amyloid precursor protein. *Nature* 373, 523–527.doi: 10.1038/373523a0

Garrido, J. L., Godoy, J., Alvarez, A., Bronfman, M., & Inestrosa, N. C. (2002). Protein kinase C inhibits amyloid b-peptide neurotoxicity by acting on members of the wnt pathway. *The FASEB Journal,* doi:10.1096/fj.02-0327fje

Gogolla, N., Galimberti, I., Deguchi, Y., & Caroni, P. (2009). Wnt signaling mediates experience related regulation of synapse numbers and mossy fiber connectivities in the adult hippocampus. *Neuron, 28*, 510-525.

Gordon, M. D., & Nusse, R. (2006). Wnt signaling: Multiple pathways, multiple receptors, and multiple transcription factors. *Journal of Biological Chemistry, 281*(32), 22429-22433. doi:10.1074/jbc.R600015200

Gotz, J., & Ittner, L. M. (2008). Animal models of alzheimer's disease and frontotemporal dementia. *Nature Reviews. Neuroscience, 9*(7), 532-544. Retrieved from http://dx.doi.org/10.1038/nrn2420

Hebert, L. E., Weuve, J., Scherr, P. A., & Evans, D. A. (2013). Alzheimer disease in the united states (2010-2050) estimated using the 2010 census. *Neurology, 80*(19), 1778-1783.

Holcomb,L.,Gordon,M.N.,Mcgowan,E.,Yu,X.,Benkovic,S.,Jantzen,P.,etal. (1998). Accelerated Alzheimer-type phenotype in transgenic mice carrying both mutant amyloid precursor protein and presenilin1 transgenes. *Nat.Med.* 4, 97–100. doi:10.1038/nm0198-097

Hsiao,K.,Chapman,P.,Nilsen,S.,Eckman,C.,Harigaya,Y.,Younkin,S.,etal. (1996). Correlative memory deficits, Abeta elevation, and amyloid plaques in transgenic mice. *Science* 274, 99–102.doi:10.1126/science.274.5284.99

Huang Y, Wang X, Huang W, Cheng Q, Zheng S, Guo S, Cao H, Liang XJ, Du Q, Liang Z. (2015). Systemic Administration of siRNA via cRGD-containing Peptide. *Scientific Reports 5,*12458. doi: 10.1038/srep12458

Huey, R., Hawthorne, S., McCarron P. (2016). The potential use of rabies virus glycoprotein-derived peptides to facilitate drug delivery into the central nervous system: a mini review. *Journal of Drug Targeting 25*(7), 379-385.

Inestrosa, N. C., Alvarez, A., Godoy, J., Reyes, A., & De Ferrari, G. V. (2000). Acetylcholinesterase-amyloid-beta-peptide interaction and wnt signaling involvement in abeta neurotoxicity. *Acta Neurol Scand Suppl., 176*, 53-59.

Inestrosa, N. C., De Ferrari, G. V., Garrido, J. L., Alvarez, A., Olivares, G. H., Barría, M. I., et al. (2002). Wnt signaling involvement in β-amyloid-dependent neurodegeneration. *Neurochemistry International, 41*(5), 341-344. doi:https://doi.org/10.1016/S0197-0186(02)00056-6

Inestrosa, N. C., Montecinos-Oliva, C., & Fuenzalida, M. (2012). Wnt signaling: Role in alzheimer disease and schizophrenia. *Journal of Neuroimmune Pharmacology, 7*(4), 788-807. Retrieved from http://dx.doi.org/10.1007/s11481-012-9417-5

Inestrosa, N. C., & Toledo, E. M. (2008; 2008; 2008; 2008). The role of wnt signaling in neuronal dysfunction in alzheimer's disease. *Molecular Neurodegeneration, 3*, 9-9. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2515306/

Jankowsky,J.L.,Slunt,H.H.,Ratovitski,T.,Jenkins,N.A.,Copeland,N.G., and Borchelt,D.R.(2001).Co-expression of multiple transgenes in mouse CNS: a comparison of strategies. *Biomol. Eng.* 17, 157–165.doi:10.1016/S1389-0344(01)00067-3

Killick, R., Ribe, E. M., Al-Shawi, R., Malik, B., Hooper, C., Fernandes, C., et al. (2014). Clusterin regulates β-amyloid toxicity via dickkopf-1-driven induction of the wnt–PCP–JNK pathway. *Molecular Psychiatry, 19*(1), 88-98.

Kohn, A. D., & Moon, R. T. (2005). Wnt and calcium signaling: Î²-catenin-independent pathways. *Cell Calcium, 38*(3), 439-446. Retrieved from http://www.sciencedirect.com/science/article/pii/S0143416005001090

Kumar, P., Wu, H., McBride, J. L., Jung, K., Hee Kim, M., Davidson, B. L., et al. (2007). Transvascular delivery of small interfering RNA to the central nervous system. *Nature, 448*(7149), 39-43. Retrieved from http://dx.doi.org/10.1038/nature05901

Lentz, T. L., Burrage, T. G., Smith, A. L., Crick, J., & Tignor, G. H. (1982). Is the acetylcholine receptor a rabies virus receptor? *Science, 215*, 182-184.

Mao, B., Wu, W., Davidson, G., Marhold, J., Li, M., Mechler, B. M., et al. (2002). Kremen proteins are dickkopf receptors that regulate wnt/[beta]-catenin signalling. *Nature, 417*(6889), 664-667. Retrieved from http://dx.doi.org/10.1038/nature756

Montcouqiol, M., Crenshaw, E. B. 3., & Kelley, M. W. (2006). Noncanonical wnt signaling and neural polarity. *Annual Review of Neuroscience, 29*, 363-386.

Moon, R. T., Kohn, A. D., Ferrari, G. V. D., & Kaykas, A. (2004). WNT and [beta]-catenin signalling: Diseases and therapies. *Nature Reviews. Genetics, 5*(9), 691-701. Retrieved from http://dx.doi.org/10.1038/nrg1427

Mucke,L.,Masliah,E.,Yu,G.Q.,Mallory,M.,Rockenstein,E.M.,Tatsuno, G., et al.(2000).High-level neuronal expression of abeta1-42 in wild- type human amyloid protein precursor transgenic mice: synaptotoxicity without plaque formation. *J. Neurosci.* 20, 4050–4058.Availableonlineat: http://www.jneurosci.org/content/20/11/4050.long

Niehrs, C. (2006). Function and biological roles of the dickkopf family of wnt modulators. *Oncogene, 25*(57), 7469-7481.

Oakley,H.,Cole,S.L.,Logan,S.,Maus,E.,Shao,P.,Craft,J.,etal.(2006). Intraneuronal beta-amyloid aggregates, neurodegeneration, and neuron loss in transgenic mice with five familial Alzheimer's disease mutations: potential factors in amyloid plaque formation. *J. Neurosci.* 26, 10129–10140.doi: 10.1523/JNEUROSCI.1202-06.2006

Oddo, S., Caccamo, A., Shepherd, J. D., Murphy, M. P., Golde, T. E., Kayed, R., et al. (2003). Triple-transgenic model of Alzheimer's disease with plaques and tangles: intracellular Abeta and synaptic dysfunction. Neuron 39, 409-421. doi: 10.1016/S0896-6273(03)00434-3

O'Leary, T. P., & Brown, R. E. Optimization of apparatus design and behavioral measures for the assessment of visuo-spatial learning and memory of mice on the barnes maze. *Learning and Memory, 20*, 85-96. doi:doi/10.1101/lm.028076.112.

Osada, M., Ito, E., Fermin, H. A., Vazquez-Cintron, E., Venkatesh, T., Friedel, R. H., & Pezzano, M. (2006). The Wnt Signaling Antagonist Kremen1 is Required for Development of Thymic Architecture . *Clinical and Developmental Immunology*, *13*(2-4), 299–319. http://doi.org/10.1080/17402520600935097

Purro, S. A., Dickins, E. M., & Salinas, P. C. (2012). The secreted wnt antagonist dickkopf-1 is required for amyloid β-mediated synaptic loss. *The Journal of Neuroscience, 32*(10), 3492-3498. doi:10.1523/JNEUROSCI.4562-11.2012

Seib, D. R., Corsini, N. S., Ellwanger, K., Plaas, C., Mateos, A., Pitzer, C., et al. (2013). Loss of dickkopf-1 restores neurogenesis in old age and counteracts cognitive decline. *Cell Stem Cell, 12*(2), 204-214.

Selkoe, D. J. (2002). Alzheimer's disease is a synaptic failure. *Science, 298*, 789-791.

Spires-Jones, T., & Hyman, B. (2017). The intersection of amyloid beta and tau at synapses in Alzheimer's disease. *Neuron, 82*(4), 756-771. Retrieved from http://dx.doi.org/10.1016/j.neuron.2014.05.004

Sturchler-Pierrat,C.,Abramowski,D.,Duke,M.,Wiederhold,K.H.,Mistl,C., Rothacher,S.,etal.(1997).Two amyloid precursor protein transgenic mouse models with Alzheimer disease-like pathology. *Proc.Natl.Acad.Sci.U.S.A.* 94, 13287–13292. doi:10.1073/pnas.94.24.13287

Tamai, K., Semenov, M., Kato, Y., Spokony, R., Liu, C., Katsuyama, Y., et al. (2000). LDL-receptor-related proteins in wnt signal transduction. *Nature, 407*, 530-535.

Taylor, C. A., Greenlund, S. F., McGuire, L. C., Lu, H., & Croft, J. B. (2017). Deaths from alzheimer's disease -- united states, 1999-2014. *MMWR Morb Mortal Wkly Rep 2017, 66*, 521-526.

Ward A, Crean S, Mercaldi CJ, Collins JM, Boyd D, Cook MN, et al. (2012) Prevalence of apolipoprotein e4 genotype and homozygotes (APOE e4/4) among patients diagnosed with Alzheimer's disease: A systematic review and meta-analysis. Neuroepidemiology 38, 1–17.

Webster, S. J., Bachstetter, A. D., Nelson, P. T., Schmitt, F. A., & Van Eldik, L. J. (2014; 2014; 2014; 2014). Using mice to model alzheimer's dementia: An overview of the clinical disease and the preclinical behavioral changes in 10 mouse models. *Frontiers in Genetics, 5*, 88. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4005958/

Wilson, R. S., Segawa, E., Boyle, P. A., Anagnos, S. E., Hizel, L. P., & Bennett, D. A. (2012; 2012). The natural history of cognitive decline in alzheimer's disease. *Psychology and Aging, 27*(4), 1008-1017. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3534850/

Wu, D., & Murashov, A. K. (2013; 2013; 2013; 2013). MicroRNA-431 regulates axon regeneration in mature sensory neurons by targeting the wnt antagonist Kremen1. *Frontiers in Molecular Neuroscience, 6*, 35. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3807041/

Zeng, X., Tamai, K., Doble, B., Li, S., Huang, H., Habas, R., et al. (2005). A dual-kinase mechanism for wnt co-receptor phosphorylation and activation. *Nature, 438*, 873-877.

APPENDIX: ANIMAL CARE AND USE COMMITTEE APPROVAL LETTER

East Carolina University

Animal Care and
se Commitee
212 Ed Warren Life
Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

April 12, 2016

Alex Murashov, MD
Department of Physiology
Brody 6N-98
ECU Brody School of Medicine

Dear Dr. Murashov:

Your Animal Use Protocol entitled, "Prevention of Memory Deficits in Alzheimer Mouse with siRNAs" (AUP #Q340) was reviewed by this institution's Animal Care and Use Committee on April 12, 2016. The following action was taken by the Committee:

"Approved as submitted"

Please contact Dale Aycock at 744-2997 prior to hazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP and are familiar with its contents.**

Sincerely yours,

Eddie Johnson/jd

Eddie Johnson
Interim Chair, Animal Care and Use Committee

EJ/jd

Enclosure