A Physiological Role for Amyloid Beta Protein:

Enhancement of Learning and Memory

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Abstract

Amyloid beta protein (Aβ) is well recognized as having a significant role in the pathogenesis of Alzheimer's disease (AD). The reason for the presence of Aβ and its physiological role in non-disease states is not clear. In these studies, low doses of Aβ enhanced memory retention in two memory tasks and enhanced acetylcholine production in the hippocampus in vivo. We then tested whether endogenous Aβ has a role in learning and memory in young, cognitively intact mice by blocking endogenous Aβ in healthy 2-month-old CD-1 mice. Blocking Aβ with antibody to Aβ or DFFVG (which blocks Aβ binding) or decreasing Aβ expression with an antisense directed at the Aβ precursor APP all resulted in impaired learning in T-maze foot-shock avoidance. Finally, Aβ1-42 facilitated induction and maintenance of long term potentiation in hippocampal slices, whereas antibodies to Aβ inhibited hippocampal LTP. These results indicate that in normal healthy young animals the presence of Aβ is important for learning and memory.

Introduction

Alzheimer's disease is widely believed to be mediated by an excess of amyloid-beta peptide (Aβ). Aβ has been shown to impair learning and memory *in vivo* (1,2,3,4,5,6,7). Transgenic mice that overproduce amyloid precursor protein have decreased memory (8,9). Despite a large literature on the pathology of Aß, its physiological role remains unclear. Recent studies have suggested that Aβ, which is secreted by neurons during excitatory neuronal activity (10), down regulates excitatory synaptic transmission (11). The negative feedback loop thus formed would provide a homeostatic mechanism by which Aβ could maintain appropriate levels of neuronal activity. This suggests that whereas excess Aβ suppresses memory, appropriate levels of

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Aß support mnemonic processes. This would be consistent with the general principle that excess of mnemonics results in cognitive impairments (12).

Long term potentiation (LTP) is believed to be the synaptic correlate of memory formation. Most studies have shown that amyloid-beta peptide inhibits LTP (13,14), though a few have shown that amyloid-beta peptide facilitates LTP (15,16,17). Further support for the concept that amyloid-beta may stimulate LTP comes from amyloid precursor protein (APP) null mice, which have reduced synapses, impaired LTP, and perform poorly on spatial memory tasks (18,19). Furthermore, presenilin-1-deficient mice have a reduced level of Aß and impaired LTP (20). Whereas antibodies against Aβ improve memory in the SAMP8 mouse, a strain which overexpressses Aß (21), APP antibodies impair performance of passive avoidance tasks in rats (22) and chicks (23) that are not overexpressers. In the studies reported here, we demonstrated in young mice that inhibition of Aß with antibodies, inhibition of amyloid-beta expression with antisense, or blocking Aß with a putative inhibitor all impaired learning. In addition, we found that low doses of Aß enhanced memory in young mice and increased hippocampal acetylcholine secretion. Finally, we demonstrated that Aβ-1-42 facilitated induction and maintenance of long term potentiation and that antibodies to Aß inhibits it.

Aβ 12-28 and 1-42 Improved Retention of T-maze Foot-shock Avoidance

The 12-28 region of the Aβ peptide has been shown to be the region important for learning and memory (3). Administering high doses of Aß 12-28 produces impaired learning and memory (3). Here, we determined whether low doses of Aß12-28 can improve retention when administered by intracerebroventricular (ICV) injection

immediately after a training session in the active avoidance T-maze. The one-way ANOVA with trials to criterion as the independent variable showed a significant treatment effect ($F_{(4,42)}=14.90$, $P<0.0001$ (**Figure 1a**). Dunnett's post hoc analysis indicated that the mice that received 20 ng of Aß12-28 took significantly fewer trials to reach criterion than the mice that received saline, thus demonstrating enhanced memory.

Aβ 1-42 is considered by many to be the peptide that produces learning and memory impairments in AD. Here, we injected Aβ 1-42 ICV immediately after training in T-maze foot-shock avoidance. The one-way ANOVA with trials to criterion as the independent variable showed a significant treatment effect ($F_{(2,27)}=12.82$, $P<0.0001$ (**Figure 1b**). Dunnett's post hoc test indicated that the mice which received 8.7 ng of Aβ 1-42 took significantly fewer trials to reach criterion on the retention than the mice which received saline.

Aß 1-42 Improves Retention in Object Recognition

Object recognition is a non-shock episodic memory task that relies on the inclination of mice to spend more time with novel than familiar objects (24). Here, mice were administered Aβ 1-42 or saline immediately after training. Twenty-four hours later, mice were tested for recognition of the original object by determining the amount of time spent with the new versus the original object. A T-test showed that the mice which receive Aβ 1-42 spent a significantly greater amount of time with the novel object, $T_{(14)}=4.303$, $P<0.0007$ (**Figure 1c**), indicating that they had improved recognition of the original object.

Low Dose of Aß 1-42 Increases Acetylcholine in the Hippocampus

A one-way ANOVA assessing percent baseline for the 61-90 minutes post administration period showed a significant treatment effect ($F_{(2,16)} = 4.129$, $P<0.05$. Dunnett's post hoc analysis indicated that the mice which received 43 ng had significantly higher levels of acetylcholine than the mice which received saline (**Figure 1d**).

Decreasing Beta-Amyloid Prior to Training Impairs Learning of T-Maze Foot Shock Avoidance

Mice were prepared for ICV administration of an antibody to Aß1-42 (8 ng) or rabbit anti-mouse IgG (8 ng)as described below. Seventy-two hours later, mice were trained in T-maze foot-shock avoidance. A T-test indicated that the mice which received antibody to Aß prior to training took significantly longer to make criterion than the mice which received rabbit anti-mouse IgG, $T(16) = 8.102$, $P<0.0001$ (**Figure 2a**), indicating impaired learning.

In order to further verify that blocking Aβ will impair learning in CD-1 mice, we administered the Aß blocking peptide DFFVG (2 ug) or vehicle ICV 72 hrs prior to training. A T-test indicated that mice which received DFFVG took significantly longer to reach criterion than the mice which received vehicle, $T(12)$ 9.238, $P<0.0001$ (**Figure 2B**), indicating impaired learning.

Decreasing Aß with Antisense (AO) Impairs Learning of T-maze Foot Shock Avoidance

We have previously shown that in SAMP8 mice, a strain with elevated Aβ, that the administration of an antisense directed against the Aß precursor APP (AO) decreases

brain levels of APP and Aß and results in improved learning and memory (25). Here, we administered AO or a random antisense (RA) ICV to young CD-1 mice 3x (2 weeks between each administration) and trained the mice in the T-maze 2 weeks after the last injection. A T-test indicated that mice which received AO took significantly longer to reach first avoidance than the mice which receive RA, $T(10) = 4.037$, $P<0.0024$ (**Figure 2c**), demonstrating that AO produced an impairment in learning.

Aβ1-42 facilitates LTP induction and maintenance

In order to determine whether the presence of Aβ could facilitate LTP under some circumstances, we used induction stimuli that were below threshold for inducing LTP (Two TBS instead of three), and found that Aβ1-42 converted a subthreshold induction stimulus to one that facilitated LTP induction and maintenance. Successful LTP induction and maintenance (defined as at least 20% potentiation of the fEPSP 60 minutes after induction) was observed in only 1 of 9 control slices 60 minutes after two x TBS (diamonds, filled circle is the mean). In contrast, successful LTP induction and maintenance was observed in 7 of 9 slices incubated with 10ng/ml Aβ1-42 (**Figure 3a** triangles, filled circle is the mean, the dotted line indicates 20% fEPSP potentiation, p=0.015 Fisher's exact test). This result suggests that Aβ1-42 can facilitate LTP.

The effect of Aβ antibody on hippocampal LTP in CD-1 mice

The Aβ antibodies 4G8 and 6E10 (Sigma-Aldrich) have not been reported to affect LTP when applied alone, but rather they counteract the Aβ-mediated LTP inhibition *in vitro* and *in vivo* when applied with Aβ or in transgenic animals expressing human forms of Aβ associated with Alzheimer's disease and impaired LTP (26). A possible explanation is that these antibodies have much higher affinities for the human or

oligometric forms of Aβ, and thereby have greater effects on Aβ-mediated LTP inhibition. If this is true, then higher concentrations of these antibodies may inhibit LTP by themselves in wild type hippocampal slices. There are different commercially available antibodies that recognize different segments of Aβ. If we can reduce Aβ by application of these antibodies, then we would predict that we would inhibit induction and maintenance of LTP. Different Aβ antibodies producing the same result woudl be strong evidence that this approach supports a role for Aβ in normal cognition.

LTP was induced by 3 applications of theta burst stimulation (figure 3b, TBS, arrow). Non-specific IgG antibody at a similar concentration was used as control. Slices incubated in ACSF (squares, n=9 slices) or IgG (circles, n=5 slices) exhibited sustained LTP, but slices incubated with 1:100 dilution of the DAKO anti-Aβ antibody (triangles, n=8 slices) had prolonged synaptic depression after TBS, and did not exhibit post-TBS potentiation or LTP 55-60 minutes after induction (p=0.001, ANOVA **Figure 3b**).

The Aβ blocking peptide DFFVG inhibits hippocampal LTP

The blocking peptide DFFVG that impaired T-maze foot shock learning (Figure 2b) was applied to hippocampal slices to determine if it would inhibit TBS-induced LTP. DFFVG (1 μM) did not affect the baseline slope of the fEPSP. However, in the presence of DFFVG TBS failed to induce post TBS potentiation or LTP **(Figure 3c).**

Discussion

We have provided here multiple lines of evidence that Aβ's physiological role is to enhance learning and memory retention. This suggests that it is an excess of Aβ that inhibits learning and memory (27). This is not surprising in view of the fact that

numerous memory enhancers have been shown to follow the law of hormesis, with low doses enhancing and high doses inhibiting memory retention (12).

The strengths of the study are that we have shown that Aβ enhances memory in two totally different forms of memory testing, via the aversive T maze and the non-aversive object recognition test. Recently, there has been increasing interest in the use of object recognition as a memory test. Studies show that this task involves the hippocampal formation (entorhinal cortex, dentate gyrus, CA1-4 and subiculum), amygdala and parahippocampal cortices all of which comprise the declarative memory system (24). The aversive T-maze has been shown to be a hippocampal dependent task (28). We also demonstrated that not only increasing Aβ at low levels enhanced memory, but that antibodies to Aβ and an antisense to amyloid precursor protein inhibited learning in young mice. DFFVG has previously been shown to block the memory inhibiting effects of Aβ in large doses by binding to its receptor site (29). Here, we found that it inhibited learning in young mice. Previously, we have found that an antibody directed against Aβ increases acetylcholine production in the hippocampus of the SAMP8, an animal model of Alzheimer's disease with learning and memory deficits caused by an overexpression of APP (30). This is consistent with high doses of Aß suppressing acetylcholine production. Here, we showed that low doses of Aβ enhanced acetylcholine production in the hippocampus *in vivo*. This is consistent with our hypothesis that physiologic or near physiologic levels of Aß support acetylcholine production. Finally, we have shown that induction of Schaffer collateral pathway LTP can be facilitated by a low concentration of Aβ and blocked by Aβ antibodies. Schaffer collateral LTP is thought to underlie hippocampal-dependent spatial learning and memory (31).

Overall, we believe these studies strongly suggest that the physiological role of Aβ is to enhance learning and memory. Only when there is excess production does Aβ result in memory deficits. These findings are important in understanding the optimal design of drugs to treat Alzheimer's disease.

Methods Summary

All studies were conducted in CD-1 mice. Memory testing in CD-1 male mice, 8 weeks old, was done using the aversive T-maze and object recognition tasks. Aβ was administered by intracerebroventricular (ICV) injection, as were the antagonists – Aβ antibodies, DFFVG and antisense to the Aβ portion of APP. Acetylcholine levels were measured by microdialysis in response to Aβ1-42. The effects of Aβ1-42, Aβ antibodies and DFFVG were studied in long term potentiation.

Figure Legends

Figure 1a.   Low doses of Aß12-28 administered to CD-1 mice ICV immediately after training improves retention in T-maze foot-shock avoidance. The ** indicates P<0.01.

Figure 1b.   Low doses of Aβ 1-42 administered to CD-1 mice ICV immediately after training improves retention in T-maze foot-shock avoidance. The ** indicates P<0.01.

Figure 1c.   Low dose of the Aβ 1-42 administered to CD-1 immediately after training improves retention of object recognition 24 hours later. The ** indicates P<0.01.

Figure 1d.   Administration of Aβ 1-42 increased acetylcholine levels in the hippocampus of CD-1 mice during the 61-90 minute time period of collection. The * indicates P<0.05.

Figure 2a.   Antibody to Aβ (DAKO) administered 72 hours prior to training to CD-1 mice impaired acquisition of T-maze foot-shock avoidance. The ** indicates P<0.01.

Figure 2b.   DFFVG administered 72 hours prior to training impaired T-maze acquisition in CD-1 mice. The ** indicates P<0.01.

Figure 2c.   Antisense directed at the Aß region of the APP peptide impairs acquisition of T-maze acquisition in CD-1 mice. The ** indicates P<0.01.

Figure 3a.   Aβ 1-42 is important for hippocampal LTP. A, Two theta-burst stimuli

(TBS) applied to the Schaffer collateral pathway does not induce long-term synaptic

potentiation (LTP) at CA1 synapses. Each symbol represents one hippocampal slice, and

the mean is depicted by a filled circle. The dotted line indicates 20% increase in the slope

of the fEPSP, which is the threshold used to define successful induction/maintenance of

LTP. Only 1/9 slices under control conditions (open diamonds); in contrast 7/9 slices in

the presence of Aβ (10 ng/ml) exhibit LTP (p=0.015 Fisher's exact test).

Figure 3b.   B, Three TBS applied to the Schaffer collateral pathway after obtaining

baseline responses (arrow) induces LTP in control buffer (filled squares) and in the

presence of a nonspecific IgG (open circles), whereas in the presence of an Aβ antibody

(DAKO, open triangles) there is post TBS synaptic depression, and no LTP.

Figure 3c.   C, As in B, three TBS (arrow) induces LTP in control buffer (filled

squares), but application of the putative Aß inhibitor peptide DFFVG, blocks LTP

without affecting the slope of the baseline fEPSP (open triangles). DFFVG blocked

TBS-LTP in hippocampal slices of CD-1 mice compared to vehicle. The TBS-LTP

consisted of a train of 5 pulses of 100Hz applied at 200ms intervals 10 times.

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Methods

Mice

CD-1 male mice from an in house colony, 8 weeks of age, served as test subjects. This colony has been maintained as an outbred strain obtained from Charles Rivers Breeding Laboratories of Wilmington, MA. The mice are tested regularly to ensure that they are virus and pathogen free. All subjects were experimentally naïve. Mice were on a 12 h light:12 h dark cycle with lights on at 0600. Water and food (PMI Nutrition LabDiet 5001) were available *ad libitum*. All experiments were conducted after institutional approval of the animal use subcommittee, which subscribes to the NIH Guide for Care and Use of Laboratory Animals.

Drugs

Amyloid beta protein 1-42 (Aβ) was purchased from American Peptide Co. (Sunnyvale, CA). Aβ 12-28 was purchased from Phoenix Pharmaceuticals, Inc. (Belmont CA). Antisense oligonucleotide (AO) and random antisense were purchased from Midland Certified Reagent Co. (Midland, TX). Antibody to Aβ was purchased from DAKO Corporation (Carpinteria, CA). DFFVG was obtained from Sigma-Genosys (The Woodlands, TX). All drugs were dissolved in saline.

Surgery and Drug Administration

Forty-eight hours prior to training, mice were anesthetized with tribromoethylene, placed in a stereotaxic instrument, the scalp was deflected and a hole drilled through the skull over the injection site. The injection coordinates for the ICV injections is 0.5 mm posterior to the bregma and 1.0 mm to the right and left of the sagittal suture. The

injection depth was 2.0 mm. In the acquisition studies mice were injected immediately after the hole was drilled and trained 72 hours later. In the retention studies, mice were trained 48 hr after surgery. Immediately after training, mice were again placed in the stereotaxic apparatus under light isoflurane anesthesia. Within three minutes after training, a 0.5 ml solution of saline or drug solution was injected into each injection site over 60 s through a 30 gauge needle, which was attached to a 10 μl syringe. After ICV injection, the scalp was closed and the mice were returned to their cages.

T-Maze training and testing procedures

The T-maze is a working memory learning task and reference memory task. The T-maze consisted of a black plastic alley with a start box at one end and two goal boxes at the other. The start box was separated from the alley by a plastic guillotine door, which prevented movement down the alley until training began. An electrifiable stainless steel rod floor ran throughout the maze to deliver scrambled foot-shock.

Mice were not permitted to explore the maze prior to training. A block of training trials began when a mouse was placed into the start box. The guillotine door was raised and a buzzer sounded simultaneously; 5 sec later foot-shock was applied. The goal box entered on the first trial was designated "incorrect" and the foot-shock was continued until the mouse entered the other goal box, which in all subsequent trials was designated as "correct" for the particular mouse. At the end of each trial, the mouse was returned to its home care until the next trial.

Mice in the pretraining injection groups were trained until they made 1 avoidance. The parameters for post-training were set so that the control groups would have poor retention (mean trials to criterion between 9 and 10) so that drug-induced improvement of

retention could be detected. Training used an intertrial interval of 35 sec, the buzzer was

of the door-bell type, sounded at 55 dB and shock was set at 0.35 mA (Coulbourn

Instruments scrambled grid floor shocker model E13-08). Retention was tested one week

later by continuing training until mice reach a criterion (5 avoidences in 6 consecutive

trials). The results were reported as the number of trials to criterion for the retention test.

Object Recognition

Object recognition is an episodic memory task that involves the hippocampus. In

this task, an animal was exposed to two similar objects which it was allowed to explore

for 5 minutes. Twenty-four hours later, the mouse was exposed to one of the same objects

and a new novel object. The mouse was injected ICV after the training day where it is

exposed to the two similar objects. The underlying concept of the task is the animal

spends more time exploring the new novel object versus the old object. Thus, the greater

the retention/memory at 24 hour, the less time spent with the old object. However, if the

animal spends an equal or greater amount of time exploring the old object, then the

weaker the memory of the original object. Mice were habituated to an empty apparatus

for 5 minutes a day for 3 days prior to entry of the objects. On the first day, two similar

objects were placed in the maze. Mice are placed in the maze and allowed to explore the

objects for 5 minutes. During the one day retention test, one of the same objects was

placed in the maze as well as a new object in a new location. The percent time spent

exploring the new versus the old object was recorded.

Cannula Implantation and Microdialysis

Mice were anesthetized with tribromoethylene and a guide cannula was

sterotaxically implanted into the right hippocampus (2.6 mm dorsal and 3.5 mm to the

right of the bregma and 2.6 mm below the skull surface). A second cannula was

implanted into the ventricle (0.5 mm anterior and 1.0 mm to the left of the bregma and

1.0 mm below the surface of the skull). The guide cannulas were fixed to the skull and

sealed until probe insertion. The mice were allowed to recover from surgery for 2 days

and were again lightly anesthetized with isoflurane for insertion of the CMA/7

Microdialysis Probe 2 mm (CMA/Microdialysis, 73 Princeton St, North Chelmsford,

MA) through the guide cannula. Each probe was tested for percent recovery prior to use

by placing it in artificial cerebral spinal fluid with a known amount of acetylcholine.

Mice were individually placed into a lidless round cage and connected to a wire extended

from a swivel (Instech, Swivel Model 375/22QE). Artificial cerebropinal fluid

containing 10 μM physostigmine to block acetylcholinesterase activity was perfused at a

flow rate of 1.0 μl/min with a Sage Syringe Pump (Model 341A). After a 1 hr

equilibration period, dialysates were collected in micro test tubes. Sampling was done

every 30 min for two hours to establish baseline. At the end of 2 hrs, an injection cannula

was placed inside the guide cannula and Aβ 1-42 or saline was infused into the ventricle.

Samples were collected every 30 min for 2 hrs post injection. The brains were then

removed to ascertain the location of the dialysis probe and injection cannula.

Acetylcholine Measurements

Acetylcholine in perfusate samples was measured by HPLC-electrochemical

detection (HPLC-ED) coupled to a post column solid phase reactor containing

immobilized enzymes (choline oxidase and acetylcholinesterase) which were loaded into

ESA Model 5040 analytical cell with platinum target at a potential of +0.3 V. Samples

(30 μl) were injected onto the ACH-3 column (ESA) using a mobile phase that consists

of 100 mM Na_2HPO_4, 0.5 mM TMAC1, 0.005% reagent MB, 2.0 mM OSA at pH 8.0

adjusted with H_3PO_4. The flow rate was 0.35 ml/min at a temperature of 35°C. After

separation on the analytical column, acetylcholine was detected with the use of a post-

column solid phase reactor. Acetylcholine was converted into hydrogen peroxide which

was quantified at the amperometric platinum working electrode.

TBS-LTP *(Theta burst stimuli-LTP)*

Hippocampal slices (400 mm thick) were obtained from CD-1 mice. Extracellular

recordings were made from the stratum radiatum of the CA1 region of hippocampal

slices using a glass electrode filled with 2M NaCL ($\sim$5 MΩ DC resistances). Bipolar

constant-current pulses were applied to the Schaffer collateral pathway to elicit excitatory

postsynaptic potentials (EPSPs). A theta burst stimulus consisted of a train of 5 pulses at

100 H_2 applied at 200 mg intervals EPSP ten times. LTP induction was achieved by

applying 3 theta burst stimuli (a total of 200 pulses). The stimulus intensity utilized was

that which would evoke a 50% maximal amplitude EPSP.

A

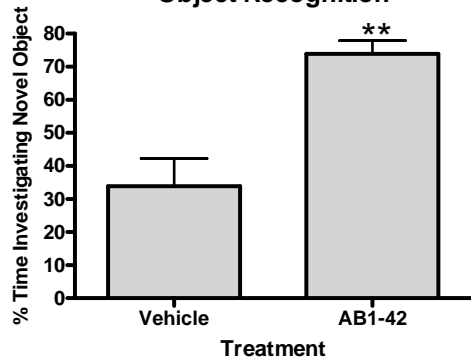Effect of Aβ12-28 on Retention of T-maze
Footshock Avoidance

B
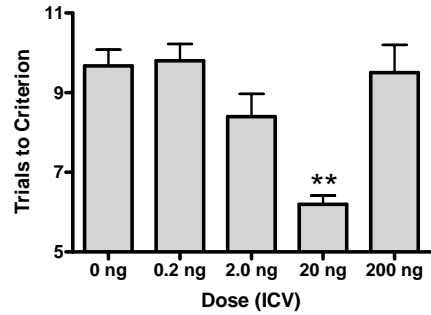
**Effect of Aβ 1-42 on Retention of T-maze
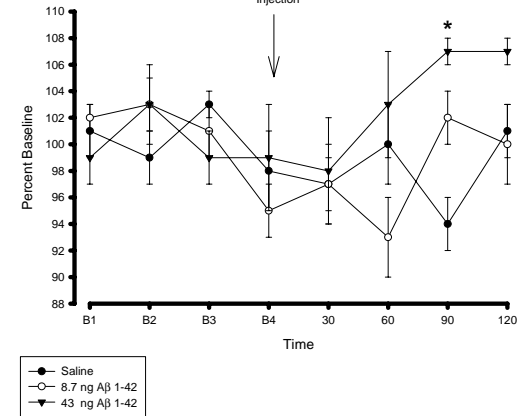Foot-shock Avoidance**

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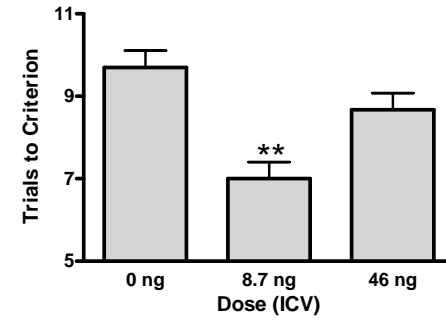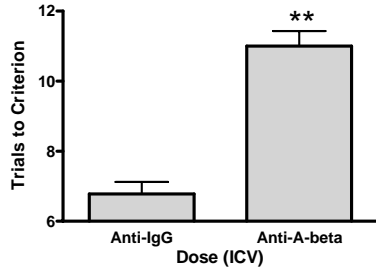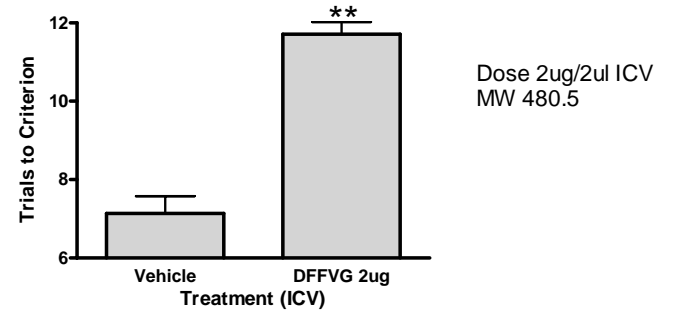
A

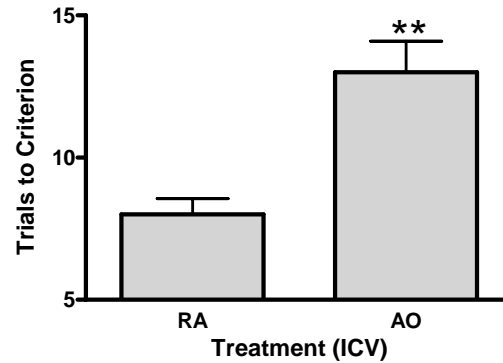**Effect of Antibody to Aβ Administered 72 hrs Prior to Training on Acquisition of T-maze Foot-shock Avoidance**

B

Effect of DFFVG 72 hours Administered 72 hrs Prior to Training on Acquisition of T-maze Foot-shock Avoidance
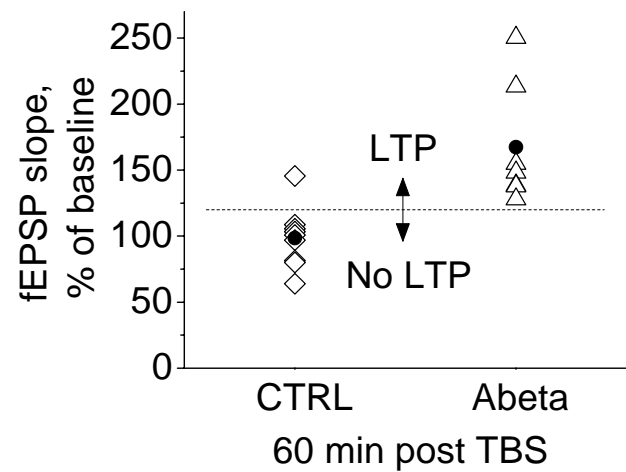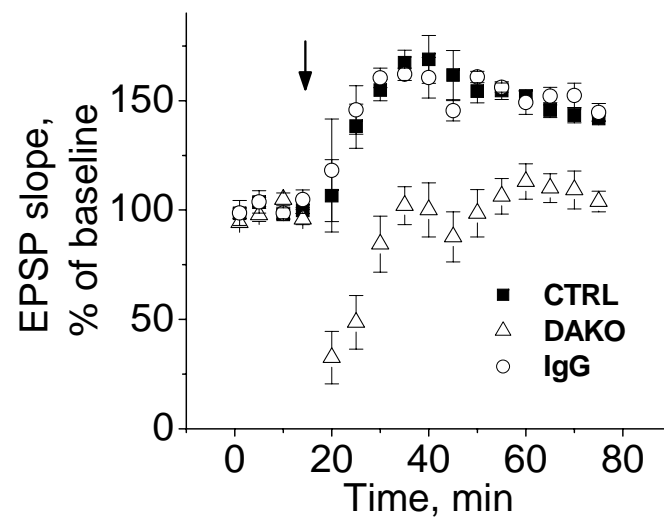

Dose 2ug/2ul ICV
MW 480.5

C

Effect of Antisense to Aβ Administered 3x Prior to Training on Acquisition of T-maze Footshock Avoidance