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## The multi-functional drug tropisetron binds APP and normalizes cognition in a murine Alzheimer's model

Patricia Spilman<sup>1</sup>, Olivier Descamps<sup>1</sup>, Olivia Gorostiza<sup>1</sup>, Clare Peters-Libeu<sup>1</sup>, Karen S. Poksay<sup>1</sup>, Alexander Matalis<sup>1</sup>, Alexander Patent<sup>1</sup>, Rammohan Rao<sup>1</sup>, Varghese John<sup>1,2,\*</sup>, and Dale E. Bredeesen<sup>1,3,\*</sup>

<sup>1</sup>Buck Institute for Research on Aging, 8001 Redwood Blvd., Novato, California 94945, USA

<sup>2</sup>Dominican University of California, San Rafael, CA 94901

<sup>3</sup>Department of Neurology, University of California, San Francisco, CA 94143

### Abstract

Tropisetron was identified in a screen for candidates that increase the ratio of the trophic, neurite-extending peptide sAPP $\alpha$  to the anti-trophic, neurite-retractive peptide A $\beta$ , thus reversing this imbalance in Alzheimer's disease (AD). We describe a hierarchical screening approach to identify such drug candidates, moving from cell lines to hippocampal neuronal cultures to *in vivo* studies. By screening a clinical compound library in the primary assay using CHO-7W cells stably transfected with human APPwt, we identified tropisetron as a candidate that consistently increased sAPP $\alpha$ . Secondary assay testing in neuronal cultures from J20 (PDAPP, huAPP<sup>Swe/Ind</sup>) mice showed that tropisetron consistently increased the sAPP $\alpha$ /A $\beta$  1-42 ratio. In *in vivo* studies in J20 mice, tropisetron improved the sAPP $\alpha$ /A $\beta$  ratio along with spatial and working memory in mice, and was effective both during the symptomatic, pre-plaque phase (5-6 months) and in the late plaque phase (14 months). This ameliorative effect occurred at a dose of 0.5 mg/kg/d (mkd), translating to a human-equivalent dose of 5 mg/day, the current dose for treatment of postoperative nausea and vomiting (PONV). Although tropisetron is a 5-HT<sub>3</sub> antagonist and an  $\alpha$ <sub>7</sub>nAChR partial agonist, we found that it also binds to the ectodomain of APP. Direct comparison of tropisetron to the current AD therapeutics memantine (Namenda) and donepezil (Aricept), using similar doses for each, revealed that tropisetron induced greater improvements in memory and sAPP $\alpha$ /A $\beta$ 1-42. The improvements observed with tropisetron in the J20 AD mouse model, and its known safety profile, suggest that it may be suitable for transition to human trials as

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Corresponding author: Dale Bredeesen, Buck Institute for Research on Aging, 8001 Redwood Blvd., Novato, CA 94945, USA  
415-209-2000 dbredeesen@buckinstitute.org.

\* Co-senior authors

**Author Contributions:** D.B. and V.J. conceptualized the study. O.D. performed HTS; D.B., V.J., and P.S. designed experiments. P.S., O.G., A.P., R.R., and K.P. performed experiments. P.S. analyzed data. P.S., V.J. and D.B. wrote the manuscript. Phone: +1 415-209-2000

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a candidate therapeutic for mild cognitive impairment (MCI) and AD, and therefore it has been approved for testing in clinical trials to begin in 2014.

## Keywords

Multi-functional drug; Improvement in sAPP $\alpha$ /A $\beta$ 42 biomarker ratio; Memory; Improvement; Orally effective; Alzheimer's disease; Candidate therapeutic

## 1. Introduction

In the absence of development of new therapeutics, the number of cases of Alzheimer's disease (AD) – estimated to be 5.4 million currently – will rapidly increase in the coming decades. Current treatments provide, at best, only modest and temporary symptomatic relief, without altering the underlying mechanisms that led to the onset, and mediate the progression of the disease. Alzheimer's is characterized by two pathologies: amyloid- $\beta$  (A $\beta$ ) plaques and tau neurofibrillary tangles. A $\beta$  accumulation is the initiating factor that leads to AD, while tangles play a critical role in neuronal death and AD progression (Holtzman et al., 2011; Klunk et al., 2006). Early in disease pathogenesis, A $\beta$  monomers aggregate into soluble A $\beta$  oligomers and insoluble A $\beta$  plaques, both of which can alter synaptic transmission and destroy neurons under certain conditions (Cheng et al., 2007). AD pathogenesis begins 10-15 years before the first memory and cognitive deficits of AD are apparent (Mintun et al., 2006; Morris and Price, 2001). By the time of diagnosis, substantial pathology is present and cellular damage has already occurred. Consequently, it is likely that anti-AD therapy will require initiation prior to symptom onset in order to prevent or delay the disease, which means it may have to be administered for a decade or more prior to onset. This chronic, pre-symptomatic treatment will make drug safety imperative. While it is possible that currently proposed anti-A $\beta$  interventions, such as A $\beta$  immunization or use of BACE or  $\gamma$ -secretase inhibitors, may ultimately be successful, in clinical trials thus far these approaches have shown substantial side effects and toxicities, and have not ameliorated disease symptoms (Holmes et al., 2008; Orgogozo et al., 2003; Serrano-Pozo et al., 2010). Disappointing clinical trial results with promising preclinical candidates such as Flurizan, Semagacestat, and Dimebon increase the critical need for new approaches to identify and develop AD therapeutics.

Here we utilized a straightforward approach to identify novel AD therapeutics based on their ability to increase the sAPP $\alpha$  to A $\beta$  ratio in *in vitro* and *in vivo* screens. APP may be cleaved to produce four peptides—sAPP $\beta$ , A $\beta$ , Jcasp, and C31—that mediate neurite retraction, synaptic loss, caspase activation, and ultimately programmed cell death; or, alternatively, to produce two peptides—sAPP $\alpha$ , and  $\alpha$ CTF—that inhibit neurite retraction, caspase activation, and cell death, and mediate neurite extension (Bredesen, 2009) (Fig. 1). Thus the ratio of these peptide APP derivatives may be a physiological determinant of plasticity, and drugs that alter this ratio represent candidate AD therapeutics. In support of this notion, maintaining adequate levels of sAPP $\alpha$  has been shown to be vitally important to memory, supporting maintenance of synaptic connections (Fig. 1B) (Claassen et al., 2009). We therefore hypothesized that molecules that effect an increase in the sAPP $\alpha$ /A $\beta$ 1-42 ratio may be beneficial in ameliorating the AD phenotype.

Such agents would not necessarily target secretases involved in the proteolytic processing of APP to A $\beta$  or sAPP $\alpha$  directly, but could be pleiotropic in their mechanisms of action. To identify such compounds we screened a variety of small molecule libraries, including a clinical compound library comprised of molecules already FDA-approved for other indications. For our screen, we used a single readout to identify a 'hit': an increase in sAPP $\alpha$  over control. The 'hits' then went through secondary assay screening using primary hippocampal neurons from transgenic (Tg) J20 mice (APP<sup>Swe, Ind</sup>) mouse embryos. In the primary cultures, both sAPP $\alpha$  and A $\beta$ 1–42 were measured, in order to identify candidates that increased the sAPP $\alpha$ /A $\beta$  ratio. Compounds that increased the ratio in primary cultures went into pharmacokinetic analysis for determination of brain uptake, and this was followed by pharmacodynamic analysis of selected candidates in mouse models of AD. Tropisetron ((1R, 5S)-8-methyl-8-azabicyclo [3.2.1] octan-3-yl 1methyl-indole-3-carboxylate) was a consistently successful candidate compound in this screen. It underwent initial pilot testing for effects on sAPP $\alpha$  and A $\beta$  in the J20 mouse model. As J20 mice express human APP with two mutations - Swedish (K670N, M671L) and Indiana (V717F) - we also wished to determine whether similar biochemical alterations in sAPP $\alpha$  and A $\beta$  could be seen upon treatment in a model expressing wildtype human APP (the I5 model), since the vast majority of human patients diagnosed with AD will have the wildtype sequence. (In this model tropisetron did not decrease the already very low A $\beta$  levels, but did increase sAPP $\alpha$ .) Subsequent to this, further long-term testing in J20 mice was performed, using mice between 4.5-6 months of age, the time during which A $\beta$  production is amplified and behavioral abnormalities are present, but prior to overt plaque formation. *De novo* A $\beta$  production is more readily discernible from pre-existing plaque-bound A $\beta$  at this stage. The A $\beta$  pathology is in some aspects akin to the MCI stage of the disease in humans at this point, although it should be noted that many other AD-like symptoms (e.g., cognitive impairment) and pathological hallmarks (e.g., increased tau phosphorylation, reduction in calbindin, cFos, and synapse load) precede plaque formation in these mice (Hsia et al., 1999; Mucke et al., 2000; Palop et al., 2003). In these mice, short-term working memory and spatial memory improvements were determined using the Novel Object Recognition (NOR) and the Morris Water Maze (MWM) tests, respectively. The former became our testing paradigm of choice as it was found to be a more versatile, rapid, and useful method for pharmacological development, as has been noted previously (Alkam et al., 2011; Dere et al., 2007; Zhang et al., 2012).

To compare the efficacy of tropisetron to existing therapeutics for AD directly, additional testing was done in head-to-head comparisons with the NMDA receptor antagonist memantine (Namenda) and the acetylcholinesterase inhibitor donepezil (Aricept). Tropisetron, memantine, and donepezil all improved the sAPP $\alpha$ /A $\beta$ 1-42 ratio at similar doses, but only tropisetron improved working memory. These were subchronic studies using relatively low doses, and only served to show that under the conditions of these experiments, tropisetron exerted superior effects on memory.

Tropisetron is a dual-receptor binding molecule, and our studies herein (Fig. 2) showed binding to the 5-HT<sub>3</sub> receptor (K<sub>i</sub> ~ 3nM) and  $\alpha$ <sub>7</sub>-nicotinic acetylcholine receptor ( $\alpha$ <sub>7</sub>nAChR) (K<sub>i</sub> ~ 470nM). Tropisetron increased the trophic APP peptide ratio upon

treatment in our AD mouse model at 0.5 mpk per day. In functional studies, tropisetron has been shown to be a 5-HT<sub>3</sub> antagonist and a partial  $\alpha_7$ -nicotinic receptor agonist, with low affinity for other nicotinic receptor subtypes (Macor et al., 2001). In addition to these interactions, we determined that tropisetron binds to the ectodomain of APP (K<sub>d</sub> ~ 0.9 $\mu$ M, Fig. 3).

Both  $\alpha_7$ nAChRs and the 5-HT<sub>3</sub> serotonin receptors belong to the Cys-loop ligand-gated ion channel receptor superfamily; other members of this receptor family include GABA<sub>A</sub>, GABA<sub>C</sub>, and glycine receptors. The  $\alpha_7$ nAChRs and 5-HT<sub>3</sub> receptors are highly expressed in the frontal cortex and hippocampus, and are believed to play important roles in cognition and schizophrenia (Barnes and Sharp, 1999). Agonists of  $\alpha_7$ nAChRs have been shown to exert neuroprotective and neurotrophic actions, while A $\beta$  binding has been reported to inhibit the activity of these receptors (Nagele et al., 2002). The 5-HT<sub>3</sub> receptor is unique within the serotonin family of receptors, since all other 5-HT receptors are serpentine G-protein coupled receptors. Our *in vivo* screening revealed that a potent 5-HT<sub>3</sub> antagonist, such as tropisetron, that is also an  $\alpha_7$ nAChR partial agonist, can reverse the AD phenotype in the mouse model, suggesting a therapeutic role for both of these receptors in the disease. Nicotinic activity is not general for this class of 5-HT<sub>3</sub> receptor antagonists, as two other high-affinity 5-HT<sub>3</sub> receptor antagonists - ondansetron and dolasetron - have no  $\alpha_7$ nAChR agonist activity (Toyohara and Hashimoto, 2010). Furthermore, as described below, we also discovered that tropisetron interacts directly with APP, with a sub-micromolar affinity. Thus, tropisetron interacts with three receptor targets that potentially modulate the AD phenotype, and this multi-functional mechanism makes it a unique candidate to develop as a potential therapeutic for AD and MCI (mild cognitive impairment).

Tropisetron is currently used primarily in the treatment of patients with chemotherapy-induced or post-operative nausea and vomiting (PONV) (Morrow et al., 1995). Treatment generally lasts for one week. Tropisetron has an excellent safety profile, is almost completely absorbed from the gastrointestinal tract after oral dosing, and is highly brain permeable. After the discovery of tropisetron in our *in vitro* screens, we hypothesized that treatment of our AD model mice with a human equivalent dose of tropisetron would be sufficient to induce both an improvement in memory and normalization of the aberrant APP processing resulting from the Swedish and Indiana mutations. The results of our studies presented here support our hypothesis, and suggest that tropisetron is an excellent candidate for clinical testing in AD and MCI. The data also suggest that other molecules with similar multifunctional properties (5-HT<sub>3</sub>R antagonist,  $\alpha_7$ nAChR agonist, and APP interactor) could be novel candidates as AD therapeutics.

## 2. Results

### 2.1 Tropisetron

Tropisetron is a tropinol ester: (1*R*, 5*S*)-8-methyl-8-azabicyclo [3.2.1] octan-3-yl 1*methyl*-indole-3-carboxylate hydrochloride (C<sub>17</sub>H<sub>20</sub>N<sub>2</sub>O<sub>2</sub>) with a molecular weight of 320.82 g/mol (as the HCl salt form). The chemical structure is shown in Fig. 2A and a molecular model in Fig. 2B.

## 2.2 Tropisetron receptor binding studies

Tropisetron displaced binding of the  $\alpha_7$ nAChR antagonist [ $^{125}$ I]- $\alpha$ -bungarotoxin ( $\alpha$ -bgt), with a  $K_i$  value of 470 nM ( $n = 3$ ) in SH-SY5Y cells. Our studies also demonstrated selectivity for  $\alpha_7$ nAChRs, as it binds  $\alpha_4$ nACh receptors with a  $K_i \sim 20\mu\text{M}$  ( $n=3$ ) and the muscle-type nicotinic ( $\alpha 1\beta 1\gamma\delta$  nACh) receptor  $K_i \sim 15\mu\text{M}$  ( $n=3$ ) similar to that reported previously (Macor et al., 2001). In a binding assay using human recombinant 5-HT<sub>3</sub>R expressed in CHO cells, tropisetron was shown to displace [ $^3$ H]- BRL-43694, a 5-HT<sub>3</sub>R selective antagonist. In this study, tropisetron showed high affinity for 5-HT<sub>3</sub>Rs with a  $K_i$  value of 3 nM ( $n=3$ ) (Fig. 2C).

## 2.3 Tropisetron binding to APP

Surface plasmon resonance (SPR) showed that tropisetron bound to APP. Fig. 3A shows example sensograms obtained with Trx-eAPP<sub>575-624</sub> before subtraction of the 0 $\mu\text{M}$  sensogram. Fig. 3B compares the response for all three eAPP fragments. The different maximal responses reflect the varying amounts of protein cross-linked to the CM5 chip. TRX-eAPP<sub>575-624</sub> was the densest at 11,700 RU, while eAPP<sub>230-624</sub> was the least dense. Comparison of the curves obtained for the three fragments was performed with PRISM (GraphPad Inc., [www.graphpad.com](http://www.graphpad.com)). The curves were fitted to a single-site saturation binding model in which the background and the non-significant binding contributions were constrained to be the same for all three proteins. The calculated  $K_D$  was not significantly different between the proteins, suggesting that the binding site of ADDN-FO3 is most likely to be between residues 575-624 of the ectodomain of APP. Constraining the  $K_D$  to be the same for all three proteins resulted in a value of  $0.9\mu\text{M} \pm 0.1 \mu\text{M}$  with  $R^2=0.99$ .

## 2.4 Primary high-throughput screening

We used an AlphaLISA-based HTS formatable primary screening assay where we detected increases in sAPP $\alpha$  induced by a hit candidate in the cell media. The AlphaLISA-based primary assay works well with either CHO-7W cells or B103 APP cells stably transfected with wildtype human APP (APP 770), as well as SH-SY5Y neuroblastoma cells that express APP endogenously. For the assay that led to the identification of tropisetron, we used CHO-7W cells. As described in Methods, media from cells treated for 24 hours with compounds from an FDA-approved clinical compound library were assayed for sAPP $\alpha$  using a custom AlphaLISA assay and any compound that caused at least a 20% increase in sAPP $\alpha$  was considered a hit. All compounds were tested in triplicate with a reproducibility  $z$ -value  $\sim 0.6$ . The screen had a good signal over background ( $s/b$ ) ratio of  $\sim 3.0$ . This screen yielded several initial 'hits,' with a hit-rate  $\sim 0.5\%$ . Tropisetron increased sAPP $\alpha$  in media from 7W cells by  $\sim 30\%$ , and was therefore tested in primary neuronal culture.

## 2.5 Secondary screening in primary hippocampal neuronal culture

As part of our iterative screening approach, hits identified in the primary assay were next evaluated in the secondary assay using primary neuronal assay. In the J20 model of AD, the hippocampus (Hip) and entorhinal cortex (ECx) show the most obvious pathological changes, and therefore hippocampal neurons were used for this secondary screen. As

described in Methods, in neurons treated with tropisetron for 5 days, sAPP $\alpha$  was significantly increased (Fig. 4A) and A $\beta$ 1-42 (Fig. 4B) was significantly decreased.

## 2.6 Tropisetron increases sAPP $\alpha$ in the presence of ApoE $\epsilon$ 4

Humans carrying one or more copies of the ApoE  $\epsilon$ 4 allele of apolipoprotein E are at higher risk for AD (Strittmatter and Roses, 1996). Co-transfection of huAPPwt and ApoE  $\epsilon$ 4 has been shown to significantly decrease sAPP $\alpha$  secretion, and reduce the sAPP $\alpha$ /sAPP $\beta$  or sAPP $\alpha$ /A $\beta$ -1-40 ratio in several cell lines, and tropisetron – identified as “F03” – has been shown to reverse these reductions (Theendakara, 2013). Here we also show that tropisetron at 1 $\mu$ M significantly increased cell survival of both ApoE  $\epsilon$ 3- and ApoE  $\epsilon$ 4-transfected cells (Fig. 5).

## 2.7 Tropisetron exhibits excellent brain penetration

To determine the blood-brain barrier penetration of tropisetron, a pharmacokinetic (PK) study was performed as described in Methods. Tropisetron appeared in brain tissue at high levels at the first time point measured (1 hour), the drug concentration maximum ( $C_{max}$ ) was seen at 2 hours, dissipating by 6 hours post-injection. The brain/plasma ratio at  $C_{max}$  was ~2.5. In contrast, MEM-3454 (M3454, RO5313534) a quinuclidinyl indazole that is currently in Phase II clinical trials that was also tested in our brain uptake studies, showed a brain/plasma ratio of only 0.1, which is consistent with previously reported brain permeability values (Wallace et al., 2011) (Fig. 6).

## 2.8 Meta analysis reveals significant increases in the sAPP $\alpha$ /A $\beta$ 1-42 ratio

Tropisetron effects were ascertained in ten *in vivo* studies, including several pilot studies (see Suppl. Methods). Meta analysis is a powerful way to determine the effect of sample size rather than an exact effect seen in smaller groups under a single set of conditions. Meta analysis of sAPP $\alpha$  and A $\beta$ 1-42 levels in hippocampi and entorhinal cortices of individual mice was performed by conversion of all data in individual studies to percentage of control for those studies. Analysis of all such data combined revealed tropisetron’s consistent ability to increase sAPP $\alpha$  and decrease A $\beta$ 1-42, even in short studies and at a variety of doses. These sAPP $\alpha$  increases (Fig. 7A) were highly significant ( $P = 0.0003$ ), as were the A $\beta$ 1-42 decreases (0.0099; Fig. 7B); therefore the increase in the sAPP $\alpha$ /A $\beta$ 1-42 ratio was highly ( $p = 0.0001$ ) significant (Fig. 7C). The J20 mice used in the studies contributing to meta analysis were all between 4.5 and 6 months of age, when brain A $\beta$  concentration increases exponentially, resulting in plaque formation in almost all J20 mice by 7 months of age. The mouse-to-mouse variability in A $\beta$  is high during this period, but tropisetron nonetheless effected a highly statistically significant reduction.

## 2.9 Tropisetron improves performance in the Morris Water Maze (MWM)

Alzet pumps containing either tropisetron to deliver 0.5 mg/kg/day or vehicle only were implanted subcutaneously into 16-19 week-old J20 mice as described in Methods. During the last 2 weeks of the treatment period, mice performed the Morris Water Maze spatial memory task. Tropisetron-treated J20 MWM performance was intermediate between that of the NTg mice and vehicle-treated J20 mice for almost all parameters measured. In training,

J20 mice treated with tropisetron found the hidden platform more readily than the vehicle-treated J20 mice (Fig. 8A), but not as quickly as NTg mice. In the hidden probe trials, tropisetron-treated J20s were intermediate again for time spent in the platform quadrant (Fig. 8B), crosses (Fig. 8C), and latency (Fig. 8D). J20 mice can manifest anxiety and helplessness in the MWM, as reflected by floating instead of active swimming. Tropisetron-treated J20s floated far less than vehicle-treated J20s (Fig. 8E). In the reversal, the platform is moved and the mice re-trained. Tropisetron-treated J20s were intermediate again for latency on the last day of training (Fig. 9A), time spent in platform quadrant in the probe (Fig. 9B), and crosses (Fig. 9C), but notably, performed significantly better in the key parameter of reversal probe latency (Fig. 9D).

### 2.10 sAPP $\alpha$ and A $\beta$ also improved after 8-week tropisetron treatment

In hippocampi/entorhinal cortices, sAPP $\alpha$  was significantly higher in mice treated with tropisetron (Fig. 10A), and both A $\beta$ 1-40 and 1-42 were lower (Fig. 10B, C).

### 2.11 Tropisetron treatment improves A $\beta$ similarly to memantine, but results in greater cognitive improvement

The starting human dose used for memantine in AD is 5 mgs per day with a maximum dose of 20 mgs per day, while the dose used for tropisetron in PONV is 5 mg per day (Garbe et al., 1994). In our pharmacokinetic studies, memantine brain levels (comparing C<sub>max</sub> values) were more than 5-fold higher (Fig. 11A) than tropisetron, and this was seen again after four-week treatment at the human equivalent doses of 0.4 mkd and 0.5 mkd for memantine and tropisetron, respectively (Fig. 11B). The Novel Object Recognition (NOR) task paradigm was used to quantify working object memory in this and further studies as it is a more facile method to discern differences as a result of pharmacological treatment of J20 mice, and furthermore allows determination of effects on the hyperactivity, which is part of the J20 phenotype. Neither compound significantly reduced hyperactivity in this study, although there was a trend toward lower activity in treated mice for both compounds (Fig. 12A). Only tropisetron improved working object short-term memory (Fig. 12B). sAPP $\alpha$  was unchanged with either compound (Fig. 13A), but both A $\beta$ 1-40 and 1-42 were lowered similarly (Fig. 13B, C). This indicates that, while both compounds have very similar effects on APP-generated cleavage products at equivalent doses, tropisetron confers an additional benefit of improvement in cognitive performance, possibly due to its impact on the cholinergic system. There may be a number of reasons for lack of improvement in cognition by memantine in our mouse model including less than optimal brain levels achieved at the dose we used. The final brain levels of memantine were ~ 0.16  $\mu$ M at the 2h time point - which is below the range for optimal binding to the NMDA receptor and producing partial antagonism (K<sub>i</sub> ~0.3 -1 $\mu$ M) (Parsons et al., 1999). In addition, memantine is known to antagonize  $\alpha$ <sub>7</sub>nAChRs (K<sub>i</sub> ~ 1 $\mu$ M; (Parsons et al., 1999), and may initially impair cognitive performance (Aracava et al., 2005). While further studies using a variety of dosages and duration of treatment could be run to further elucidate any differences between these compounds, this initial study indicates that, at least under the conditions used here, tropisetron may be more effective in improving cognition.

## 2.12 Tropisetron improves memory and lowers A $\beta$ 1-42 more effectively than donepezil at human equivalent doses

The human dose for donepezil in AD is 5-10 mg per day (Doody et al., 2008), while for tropisetron for PONV, it is 5 mg per day (Garbe et al., 1994). After 21 days of treatment at a human equivalent dose of 0.5 mkd, tropisetron improved short-term memory (Fig. 14B), decreased A $\beta$ 1-42 (Fig. 15C), and increased sAPP $\alpha$  (Fig. 15A), all significantly; therefore the sAPP $\alpha$ /A $\beta$ 1-42 ratio was markedly improved (Fig. 15D). At the human equivalent dose of 1 mkd for the same length of time, donepezil neither improved short-term object memory nor significantly lowered A $\beta$ 1-42, but did increase sAPP $\alpha$  significantly, and therefore the ratio. The brain levels of donepezil at the end of the study were ~70 ng/g or 0.2  $\mu$ M and therefore in the range of the IC50 (G1 form = 340nM and G4 form = 200nM) for inhibition of AChE (Rakonczay, 2003). Brain levels of tropisetron were 0.34  $\mu$ M (80 ng/g), well above the Ki of 5HT $_3$ , but slightly less than the Ki for  $\alpha_7$ nAChR agonism. Previous reports have shown cognitive improvement in other murine AD models with donepezil doses similar to that we used here (Dong et al., 2005; Van Dam et al., 2008; Zhang et al., 2012) after longer treatment. The efficacy reported in these studies with donepezil is seen despite high protein binding which has been reported to be ~96% (Tiseo et al., 1998), in comparison the protein binding for tropisetron is reported to be 71% (Simpson et al., 2000).

While results of comparison of tropisetron to existing AD therapeutics under limited conditions and in a single mouse model are potentially informative, we recognize the limitation that these studies may not be a predictor of comparative efficacy in human patients.

## 2.13 Tropisetron is highly effective by oral delivery

We compared the effects of tropisetron on J20 mice when administered orally vs. subcutaneously. Both vehicle-treated J20s and J20s treated with tropisetron by pump had significantly higher activity than NTg mice (Fig. 16A); there was no significant difference in activity between NTg and J20 mice treated with tropisetron orally. In contrast, J20s treated with tropisetron orally had significantly reduced hyperactivity as compared to vehicle-treated J20s. Vehicle-treated J20s had significantly lower novelty preference as compared to NTg, but there was no significant difference between NTg and J20 mice treated with tropisetron either by pump or orally. Only J20 mice treated with tropisetron by pump had significantly greater novelty preference than vehicle-treated J20s, although there was a similar increase in novelty preference in mice treated orally with tropisetron, as well as greater individual variation (Fig. 16B).

Only J20 mice treated orally with tropisetron had significantly greater sAPP $\alpha$  (Fig. 17A). J20 mice treated with tropisetron either by pump or orally had significantly lower sAPP $\beta$  than vehicle-treated mice (Fig. 17B). J20 mice treated with tropisetron either by pump or orally had lower A $\beta$ 1-40 and A $\beta$ 1-42 (Fig. 17C-D). The sAPP $\alpha$ /sAPP $\beta$  ratio was significantly increased for both pump and orally tropisetron-treated J20s, but the increase was greater for orally-treated mice (Fig. 17E). The sAPP $\alpha$ /A $\beta$ 1-42 ratio was higher for orally-treated mice (Fig. 17F), but not significantly so.



Brain levels were similar after pump and oral delivery (Fig. 17G). However, it is noteworthy that pump delivery is continuous, whereas the oral delivery was daily, therefore total exposure to compound would be less for oral delivery. Nonetheless it was more effective at increasing sAPP $\alpha$ .

#### 2.14 NTg mice treated with tropisetron show improved memory

As a tropisetron-treated group of NTg mice was not originally included in the MWM studies, an additional study was run looking at performance in NOR. NTg mice receiving tropisetron showed reductions in activity (Fig. 18A) and increases in novelty preference (Fig. 18B). We speculate that tropisetron may improve behavior in the NOR in NTg mice either by altering processing of endogenous mouse APP and increasing sAPP $\alpha$ , by increasing acetylcholine as a result of 5-HT $_3$  receptor antagonism, or both (see discussion). Endogenous murine sAPP $\alpha$  levels were below the level of detection in our assays; in ongoing studies, acetylcholine levels are being determined.

#### 2.15 sAPP $\alpha$ increased in I5 mice

As it was important to determine if tropisetron's sAPP $\alpha$  increasing and/or A $\beta$  lowering effects could be seen in mice without APP mutations, and because sAPP $\alpha$  could not be determined by these methods in NTg mice, a single study of using I5 huAPPwt mice was performed using adult I5 mice. Hippocampi and entorhinal cortices were dissected separately rather than combined as we had determined A $\beta$  was below the level of detection in entorhinal cortex from I5 mice in our ELISA. sAPP $\alpha$ , however, could be determined in entorhinal cortices in I5 mice, and so analysis of that region was included. Tropisetron-treated I5 mice showed increases sAPP $\alpha$  (Fig. 19A) in both hippocampi and entorhinal cortices; but the already very low A $\beta$ 1-42 in hippocampi was unchanged (Fig. 19B). This study did not include behavioral analysis as these mice did not show clear differences from NTg in NOR.

#### 2.16 Improved cognition and sAPP $\alpha$ /A $\beta$ ratio in plaque-bearing mice

In the majority of *in vivo* studies presented here, the J20 mice were in the symptomatic but pre-plaque stage. As we were interested in determining if tropisetron could reverse long-term cognitive deficits and lower A $\beta$  even in the presence of plaques, a pilot study was performed using very old mice (12-15 m.o.); at this age, J20 mice have had plaques for approximately half of their lives. In these mice, after 14 days treatment by pump at 1 mkd, followed by an additional 21 days of oral treatment at 2 mkd, cognition was not only improved as compared to age-matched vehicle-only old J20 mice, it was not significantly different from that of younger (6 m.o.) NTg mice. Many old NTg mice showed cognitive impairment, and many old vehicle-only J20 mice were neophobic by the end of treatment (Fig. S3). Tropisetron-treated old J20 mice had higher sAPP $\alpha$  (Fig. S4A) and significantly lower sAPP $\beta$  (Fig. S4B), and therefore a significantly higher sAPP $\alpha$  to sAPP $\beta$  ratio (S4C). Measurement of sAPP $\beta$  was utilized in this study since sAPP $\beta$  is reflective of the amount of BACE cleavage; *de novo* A $\beta$  generation is more difficult to determine by A $\beta$  ELISA only in plaque-bearing mice. Both A $\beta$ 1-40 (Fig. S4D) and 1-42 (Fig. S4E) were lower, and the sAPP $\alpha$  to A $\beta$  1-42 ratio higher (Fig. S4F), but not significantly so.

### 3. Discussion

Using an iterative screening approach, we have identified a molecule—tropisetron—already approved for clinical use (for short-term use as an anti-emetic, outside the U.S.) that, at a human equivalent dose, reversed the phenotype in the PDAPP mouse model of AD, thus making it a promising therapeutic candidate for Alzheimer's disease. Tropisetron increased the sAPP $\alpha$ /A $\beta$ 42 ratios *in vitro* in APP-expressing cells, in primary neuronal cultures, and *in vivo* in the J20 and I5 mouse models. This drug is a potent 5-HT $_3$  receptor antagonist and partial  $\alpha_7$ nAChR agonist, and we have shown that it also interacts directly with APP. In our tests, tropisetron competitively displaced specific binding of a radioligand with a  $K_i$  of 3nM for the 5-HT $_3$  receptor and of 470nM for the  $\alpha_7$ nAChR (Fig. 2C). Tropisetron selectively activates the  $\alpha_7$ nAChRs without affecting other nicotinic receptors, and has a greater-than 100-fold selectivity for  $\alpha_7$  versus other nicotinic receptor subtypes. Our data suggest that both of these members of the Cys-loop family of receptors (Sine and Engel, 2006) play important roles in Alzheimer's disease, and that molecules having such dual-receptor binding may be beneficial both as symptomatic and as disease modifying agents in the treatment of AD. Furthermore, as described above, we also discovered that tropisetron interacts directly with APP, with a sub-micromolar affinity. Thus tropisetron interacts with three receptors, and this multi-functional mechanism makes it a unique candidate to develop as a potential therapeutic for AD and MCI (mild cognitive impairment).

The two major subtypes of nicotinic receptors expressed in the brain are the  $\alpha_4\beta_2$  nicotinic receptor and the  $\alpha_7$ nAChR. The former provides high-affinity binding sites for nicotine in the brain (Flores et al., 1992). The  $\alpha_7$ nAChRs have been shown to bind to A $\beta$  with high affinity, as well, resulting in increased tau phosphorylation (Wang et al., 2003), cholinergic neurotransmission defects (Lee and Wang, 2003), and neuronal cell death (Wang et al., 2000). While a prominent loss of cholinergic neurons in the basal forebrain and cerebral cortex is seen in AD, the involvement of  $\alpha_7$ nAChRs in this loss is not yet completely defined (Sugaya et al., 1990). A number of nicotinic receptor agonists or partial agonists such as A582941 have been shown to improve cognitive function (Tietje et al., 2008), provide neuroprotection (TC-1698) (Marrero et al., 2004), or lower A $\beta$  (GTS-21) (Shimohama and Kihara, 2001). Some of these ligands are being tested in the clinic as possible AD therapeutics; these include RO5313534 (MEM-3454), reported to bind  $\alpha_7$ nAChRs with a  $K_i$  of 6nM. It is also a potent antagonist of the 5-HT $_3$  receptor. However, as noted above, unlike tropisetron, MEM-3454 shows poor brain permeability and does not interact with APP. Nonetheless, this compound has reportedly shown beneficial effects in a Phase IIa study in mild to moderate AD patients (Sabbagh, 2009). Similarly, another potent  $\alpha_7$ nAChR partial agonist, EVP-6124, is reported to be in Phase II clinical testing for AD (Prickaerts et al., 2012).

Tropisetron is a potent antagonist of the 5-HT $_3$  receptor with moderate  $\alpha_7$ nAChR binding and APP binding. As noted above, the 5-HT $_3$  receptor is a member of the Cys-loop ligand-gated ion channel receptors, a family of receptors that also includes the nAChRs to which it is closely related by homology (Thompson and Lummis, 2007). 5-HT $_3$  receptor antagonists have been used primarily as anti-emetics but have also been shown in preclinical testing to be of potential benefit for a number of CNS disorders including anxiety, cognitive

dysfunction, and psychosis (Barnes and Sharp, 1999). Although it is not clear how 5-HT<sub>3</sub> receptor antagonists alter cognition, antagonists such as ondansetron have been reported to improve water maze learning in aged rats (Pitsikas et al., 1993) and in rats with neurotoxic lesions of the basal forebrain (Hodges et al., 1996). Mechanistically, the 5-HT<sub>3</sub> receptor antagonists have been implicated in enhancing the release of acetylcholine from cholinergic neurons, and 5-HT<sub>3</sub> receptor antagonism by tropisetron has been shown to prevent the suppression of acetylcholine (ACh) release by 2-methyl-5-HT, a selective 5-HT<sub>3</sub> agonist (Bianchi et al., 1990). 5-HT<sub>3</sub> receptor antagonists also improve long-term potentiation (LTP) in the hippocampus through activation of GABAergic interneurons (Kawa, 1994; Morales et al., 1996; Morales and Bloom, 1997; Pigué and Galvan, 1994; Ropert and Guy, 1991; Tecott et al., 1993). In addition, 5-HT<sub>3</sub> antagonists have been reported to reduce A $\beta$  neurotoxicity (Ju Yeon and Yeon Hee, 2005).

One currently available AD therapeutic, memantine (Namenda), also interacts with the 5-HT<sub>3</sub> receptor, as a non-competitive antagonist (Rammes et al., 2001), and has also been reported to be an antagonist at the  $\alpha$ <sub>7</sub>nChR (Aracava et al., 2005). In our studies, at a dose similar to that found to be efficacious for tropisetron, memantine failed to improve memory. Similarly, donepezil, an acetylcholinesterase inhibitor, at human-equivalent dosage failed to improve memory in the PDAPP mice, at least under the conditions of the study performed here.

The lack of improvement in cognition with either donepezil or memantine in our studies could be due to a variety of factors, as noted above. In another study using the Tg2576 mouse AD model, donepezil did induce cognitive improvements when used at the dose used here, but the study was longer (6 weeks). Therefore, while it is possible that longer donepezil treatment may have resulted in cognitive benefits in J20 mice, our purpose here was to observe not only the comparative benefits, but also the speed at which they would be manifest. It is possible that the cognitive benefit from tropisetron was due to its multi-receptor binding activities, which offer the potential to enhance cholinergic function and APP non-amyloidogenic cleavage. Furthermore, the moderate  $\alpha$ <sub>7</sub>nAChR activity of tropisetron may be useful in preventing desensitization of the receptor, a phenomenon seen with sustained exposure to a potent  $\alpha$ <sub>7</sub>nAChR agonist (Revah et al., 1991; Seguela et al., 1993), while maintaining its pro-cognitive benefits. Overall, tropisetron proved superior to both donepezil and memantine in the studies performed here.

We chose sAPP $\alpha$  level as an initial readout for compound candidacy, rather than reduction of A $\beta$  alone, as decreases in sAPP $\alpha$  are also associated with AD pathology. A recent publication has shown that in AD, there is an increased association of ADAM10—the  $\alpha$ -secretase responsible for sAPP $\alpha$  production—and the protein AP2, resulting in increased removal of ADAM10 from synapses (Marcello et al., 2013). This removal of ADAM10 decreases sAPP $\alpha$ ; therefore any compound that increases sAPP $\alpha$  may mitigate this aspect of the pathology underlying AD.

Reduction of A $\beta$  in mice of 4.5 to 6 months of age reflects the ability of tropisetron to reduce net production of A $\beta$  during a period of rapid amplification in this mouse model. Based on the studies presented here, tropisetron may have the effects in MCI; nonetheless,

the reduction of A $\beta$  production should also have benefits in AD. Pilot studies have already been performed using very old (12-15 m.o.) J20 mice, and not only were A $\beta$  and sAPP $\beta$  reduced, but behavior in NOR was improved from pre-treatment baseline (Supplementary Figs. S3 and S4). Future studies include treatment for a longer duration to be able to quantify plaque reduction, as well as complete extensive pathological analysis including calbindin expression, synaptic load, and level of tau phosphorylation.

In summary, our studies show that tropisetron has excellent oral bioavailability, brain penetration, cognitive effects, and biomarker effects at currently used human equivalent doses, offering strong support for further evaluation of tropisetron in clinical trials in MCI and AD patients. Based on these results we have received approval to proceed with clinical testing of tropisetron in MCI patients. Tropisetron could potentially be useful as a single agent or in combination with complementary therapeutics for the treatment of Alzheimer's disease and MCI.

## Materials and Methods

### Drugs

Tropisetron hydrochloride was obtained from Tocris Biosciences (cat# 2459) and stored with moisture absorbent drierite at 4°C. For the *in vitro* studies tropisetron was prepared as a 1mM stock solution in dimethylsulfoxide (DMSO, SigmaAldrich). For *in vivo* testing using subcutaneous injection, tropisetron hydrochloride was dissolved in 100% DMSO to generate a 5mg/ml stock solution. For Alzet pump studies, tropisetron was dissolved in 25% DMSO and 75% physiological saline (0.9%, pH 7.8) to give 5mg/ml. MEM3454 was obtained from Aquila Pharmatech (cat #: 411104). Memantine and donepezil were both purchased from Sigma-Aldrich (D6821 and M9292, respectively) and were prepared in the same way as tropisetron.

### 4.1 *In Vitro* testing

**4.1.1 Receptor saturation and competition binding studies**—Saturation binding analysis of the  $\alpha_7$ nAChR was carried out using endogenously expressed  $\alpha_7$ nAChRs in SH-SY5Y cells with increasing concentrations of the classical antagonist [<sup>125</sup>I] $\alpha$ -bungarotoxin ( $\alpha$ -Bgt, 0.05nM). The competition assay was performed with 0.1nM-10uM tropisetron. The non-specific binding was defined by 1uM  $\alpha$ -Bgt. Receptor binding for 5-HT<sub>3</sub>R was assessed using human recombinant 5-HT<sub>3</sub> receptors expressed in CHO cells. Tropisetron was tested at 0.1nM-10uM to displace [<sup>3</sup>H]-BRL-43694 (Granisetron; 2 nM), a 5-HT<sub>3</sub>R selective antagonist. The non-specific binding was assessed with MDL-72222, a selective 5-HT<sub>3</sub>R antagonist and was defined as 10uM. Additional binding studies with  $\alpha_4$ nACh receptors and muscle-type nicotinic receptors (( $\alpha_1\beta_1\gamma\delta$  nACh) were also performed. All of the *in vitro* pharmacology studies with tropisetron were done at CEREP (Celle l'Eves cault, France).

**4.1.2 Surface plasmon resonance (SPR)**—SPR data were obtained with a Biacore T100 (GE Healthcare); the surfaces of all four flow cells (FC1, FC2, FC3, FC4) of a carboxymethylated-dextran (CM-5) chip were washed sequentially with 50mM NaOH, 1mM HCl, 0.05% H<sub>3</sub>PO<sub>4</sub>, and 20mM sodium phosphate pH 7.4, 125 mM sodium chloride

in parallel using a flow rate of 30 $\mu$ l/min for 1 min. Three fusion proteins were immobilized via amine coupling using 20mM phosphate, 125mM sodium chloride pH 7.4. The three proteins were MBP-eAPP<sub>230-624</sub> - a fusion protein containing maltose binding protein (MBP) and residues 230-624 of the ectodomain of APP (90-kDa) (FC4), eAPP<sub>230-624</sub> - a protein that contains only residues 230-624 (45-kDa)(FC2), and TRX-eAPP<sub>575-624</sub> - a fusion protein containing thioredoxin (TRX) and residues 575-624 of the ectodomain (20-kDa) (FC3). The proteins were produced as described in (Libeu et al., 2011). The proteins were concentrated to 2mg/ml in 20mM phosphate pH 6.5, 125mM sodium chloride, and then dissolved to a concentration of 50 $\mu$ g per ml in 20mM sodium acetate pH 5.0. FC1 served as a reference cell following a mock immobilization with buffer alone. For all cells, the flow rate was 10 $\mu$ l per min. The chip was blocked with 1M ethanolamine (pH 8.5). The final RU values were 11711 for TRX-eAPP<sub>575-624</sub>, 2156 for eAPP<sub>230-624</sub> and 10275 for MBP-eAPP<sub>230-624</sub>.

Compounds were diluted from 10mM solutions in DMSO to 50 $\mu$ M in 1% DMSO, 20mM sodium phosphate pH 7.4, 125mM sodium chloride, 0.05% Tween, and then serially diluted by 1.5 for 10 steps. Binding traces were recorded for each dilution with a binding phase of 60 seconds and a dissociation phase of 240 seconds. Each cycle was performed at 20°C with a constant flow rate of 20 $\mu$ l/min. An additional 240 seconds of buffer flow at 60 $\mu$ l per min across the cells was applied as a regeneration phase to facilitate complete dissociation of the compound from the protein. The sensograms were obtained by subtraction of the reference and buffer signals using the Biacore T100 Evaluation software. The binding curves were modeled with the PRISM (Graphpad, Inc).

**4.1.3 Primary screening assay**—CHO-7W cells stable transfected with huAPP<sup>w</sup>t were used for these studies. Cells in 50 $\mu$ l of Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin, were seeded at a density of  $5 \times 10^4$  cells/well in 96 well plates overnight. The cells were then incubated for twenty-four hours with compounds from the clinical library prepared from a 10mM DMSO stock solution to achieve a final concentration in each well of 1 $\mu$ M. Cell media were then removed, Complete Protease Inhibitor (Roche cat# 1183617) added, and 2 $\mu$ l of the media obtained after a 500-fold dilution was made to the levels of sAPP $\alpha$ . This was done using a custom AlphaLISA assay (Perkin-Elmer), comprising acceptor beads that bind to the C-terminus of sAPP $\alpha$  (cat# AL231C Perkin-Elmer) and donor beads that bind to the N-terminus of biotinylated APP antibody (R&D cat# AF1168). After incubation in the dark for 1h, the AlphaLISA signal was measured using a Perkin-Elmer (PE) Enspire Multi-mode plate reader. Any compound that induced a 20% increase in sAPP $\alpha$  was considered a potential "hit", and hits were re-tested to confirm initial findings. Tropicsetron increased sAPP $\alpha$  levels consistently in the CHO-7W cells by ~ 30%.

**4.1.4 Primary hippocampal neuronal cultures**—Hippocampal neuronal cultures were generated from E18 embryos resulting from a J20 male, J20 female crosses. Such mating of heterozygous J20s typically results in 75% of the embryos, and therefore the cells in culture, carrying the transgene. Briefly, hippocampi were dissected from embryos using 12-22 embryos, and the dissected tissue combined in ice-cold Hank's Balanced Salt Solution

(HBSS, Invitrogen). The pooled hippocampi then underwent trypsinization and DNAase 1 (both Sigma-Aldrich, St. Louis MO) treatment to generate a single-cell suspension. Cells were plated onto poly-L-lysine (Sigma) coated 48-well plates at a density of  $5 \times 10^5$  cells/well. The medium was Neurobasal with B27, glutamax and pen-strep (all Invitrogen, [www.invitrogen.com](http://www.invitrogen.com)). The cells were allowed to mature for four to five days before the addition of compound at 1 $\mu$ M final. Cells were treated once a day every day for four days, and both media and cells collected two hours after the last addition of compound on the 5<sup>th</sup> day. Partial media changes were timed so that the last day of treatment was the 3<sup>rd</sup> day after media change, when APP biochemical readouts were the highest. sAPP $\alpha$  was determined from media using the Perkin-Elmer AlphaLISA and A $\beta$ 1-42 was determined from media and cells using Invitrogen's sensitive ELISA kit for A $\beta$ 1-42.

**4.1.5 Apolipoprotein 4 (ApoE  $\epsilon$ 4) transfection**—Human ApoE cDNA constructs were generously provided by Dr. Yadong Huang (Gladstone Institute of Neurological Disease, University of California, San Francisco, CA 94143). A172 human glioblastoma cells that endogenously express APP were cultured in DMEM containing 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were transfected with human ApoE  $\epsilon$ 4 or ApoE  $\epsilon$ 3 using XtremeGENE HP (Roche, [www.roche-applied-science.com](http://www.roche-applied-science.com)) in OptiMEM I Reduced Serum Media (Invitrogen) according to the manufacturer's instructions. Twenty-four hours later, culture medium was replaced, and varying concentrations of the drug were added to the cells. Following an additional twenty-four hours, cell survival was determined by the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (M6494, [www.invitrogen.com](http://www.invitrogen.com)).

## 4.2 *In vivo* testing

**4.2.1 Pharmacokinetic (PK) studies**—Five adult (>3 months of age) NTg mice were used for pharmacokinetic analysis to determine brain and plasma levels of compounds. All were injected subcutaneously with 50 $\mu$ l of 5mg/ml tropisetron, MEM3454 (M3454), or memantine (Mem) in DMSO, and individual mice were anesthetized by ketamine/xylazine injection and blood collected via cardiac puncture followed by saline perfusion 1, 2, 4, 6, and 8 hours after injection. Brains were halved and frozen. Plasma was collected after centrifugation of whole blood at 5,000 rpm for 10 min. Plasma and hemi-brains were sent to IAS (Integrated Analytical Systems, Berkeley, CA) for compound level analysis with an LC-MS/MS approach.

**4.2.2 Animals**—The derivation and characterization of PDAPP huAPP<sup>Swe/Ind</sup> (J20) and huAPP<sup>wt</sup> (I5) mice have been described elsewhere (Hsia et al., 1999; Mucke et al., 2000). PDAPP mice were maintained by heterozygous crosses with C57BL/6J mice (Jackson Laboratories, [jaxmice.jax.org](http://jaxmice.jax.org)). PDAPP mice were heterozygous with respect to the transgene. Non-transgenic littermates were used as controls. Mice were maintained in temperature controlled rooms with 12-hour light-dark cycle and all procedures were in compliance with the Buck Institute Animal Care and Use Committee and AAALAC guidelines.

**4.2.3 I5 study**—Adult I5 mice were injected subcutaneously with 50 $\mu$ l of a 5mg/ml stock solution of tropisetron in DMSO (10 mkd) every day for five days. Two hours after the last injection on Day 5, mice were anesthetized by ketamine/xylazine injection, blood collected, brain tissue saline perfused and hippocampi and entorhinal cortices dissected separately and snap frozen for biochemical and compound level analysis. Plasma collection and analysis were performed as in the PK studies. Experimental groups for I5 studies were n = 3 for both vehicle control and tropisetron; mice were males 4-5 months of age and were sibling-matched in each group. I5 mice do not develop plaques, but mice of the ages similar to those of J20 study mice were used for consistency. I5 mice do not show memory impairment in the testing paradigms used here, so this study was for analysis of sAPP $\alpha$  and A $\beta$  1-42 only.

**4.2.4 Eight-week MWM study**—Mice were implanted with subcutaneous Alzet pumps and the pumps were replaced at 28 days. All groups started with 12-13 mice; some mice died during the course of the study or were eliminated from analysis due to thigmotaxis (circling the perimeter of the pool) or excessive floating, therefore for MWM analysis non-transgenic (NTg) vehicle control n = 13, J20 vehicle control n = 8, and J20 tropisetron-treated n = 8. For biochemistry, J20 vehicle control was n = 7 (one mouse died after MWM was completed). All were 4-5 months old at the start of treatment and therefore 6-7 months old at the end, both males and females were used, and siblings sorted into each treatment group.

**4.2.5 Morris Water Maze (MWM)**—The overall health of all mice was assessed on the first day of the MWM (Morris, 1984) study with special attention to vision and ability to swim. On the second day the mice were trained to swim to a 12  $\times$  12 cm platform submerged 1.5 cm below the water surface in a rectangular enclosed pool (15  $\times$  122 cm). The water maze itself consisted of a round pool (122 cm diameter) filled with water (21 $\pm$ 1 $^{\circ}$ C) opacified with non-toxic white tempera paint powder. The pool was surrounded by distinct extra-maze cues (graphic patterns). The first 3 days of training comprised the “Visual” test. In this task, the platform was marked with a visible cue (15 cm tall, black and white striped pole placed on top of the platform). Mice were separated into 2 groups and each group underwent four trials per day, either in the morning or afternoon, with the order of the groups alternating daily. The platform location was changed each day, as were the four locations mice were lowered into the water (“drops”). Trials were aborted after 60 s. Hidden platform training was performed in the following week. For this training, the platform was again submerged 1.5 cm in the same location throughout hidden platform training, but the drop location varied semi-randomly between trials. Mice received one training session per day for 5 consecutive days comprised of 4 trials. Mice that did not find the platform within 60 s were guided to it and allowed to sit on it for 10 s. Hidden probe trials were run on Day 4 and Day 5. For the probe trials, the platform was removed, and mice were allowed to swim for 60 s before they were removed. The drop location was 180 $^{\circ}$  from where the platform was placed during hidden platform training. The same drop location was used for all hidden probe trials. The following week, the mice underwent additional hidden platform training, but with the platform in a new location (“Reversal”). Reversal training and probe trials were just as described for the initial hidden training, but took place for only 3 consecutive days and with only one probe trial. Behavior was recorded

with the Ethovision video tracking system (Noldus, [www.noldus.com](http://www.noldus.com)). Escape latencies, distance traveled, swim paths, swim speeds, percentage time spent in each quadrant, and platform crossings were recorded for subsequent analysis.

**4.2.6 Biochemistry**—In all *in vivo* studies with the exception of the I5 study, a single sample of combined hippocampus and entorhinal cortex from individual mice was used for biochemical analyses. In the I5 study, hippocampi and entorhinal cortices were dissected and analyzed separately. These brain regions were used because the pathological and biochemical alterations are the most pronounced in these areas in the J20. A $\beta$  was only measurable in hippocampus in the I5 mouse. All tissues were weighed and sonicated in freshly prepared 5M Guanidine (Gdn)-HCl in 50mM Tris-NCl pH 8.0 at 20% weight/volume. Samples were rotated for 2-3 hours at room temperature after sonication and typically stored frozen before use. Invitrogen ELISA kits for A $\beta$ 1-40 and 1-42 were used according to the manufacturer's instructions. For sAPP $\alpha$  level analysis, protein from 100 $\mu$ l of the Gdn sonicate was precipitated with 400 $\mu$ l of ice-cold methanol overnight at -20°C. The samples were centrifuged at 10,000 rpm for 15 minutes at 4°C to pellet the protein, and after all of the methanol was removed, the protein was resuspended in 100 $\mu$ l 1X AlphaLISA Hi-Block buffer (Perkin-Elmer) and sonicated on ice until the pellet was well-resuspended by microscopic inspection. The AlphaLISA for sAPP $\alpha$  described above was used according to the manufacturer's instructions. In addition, Immuno-Biological Laboratories (IBL, [www.ibl-america.com](http://www.ibl-america.com)) ELISA for sAPP $\alpha$  was used to confirm AlphaLISA results in most studies, and for this assay, the Gdn sonicate was used.

**4.2.7 Comparison to memantine**—Mice received vehicle control, 0.5 mkd tropisetron or 0.4 mkd memantine in 28-day Alzet pumps implanted subcutaneously. The memantine dose was chosen to be similar to the tropisetron dose, which was a human-equivalent dose. Memantine is currently used at oral dose of 5-20 mgs per day for treatment of AD while tropisetron is used at oral dose of 5 mgs per day for PONV. Given memantine's relatively high brain levels, the dose chosen was slightly lower than that for tropisetron. Each group started with 7-8 J20 4-5 m.o. mice. Mice underwent working object memory testing at 26 days using the NOR task at the end of the study, vehicle control n = 8, tropisetron n = 7, memantine n = 6. For biochemistry, vehicle control n = 8, tropisetron n = 6, memantine n = 5. Plasma and brain levels at the end of the study were determined by IAS using plasma collected post-centrifugation of whole blood and post-saline perfused brain tissue. Hippocampi and entorhinal cortices were combined from individuals for analysis.

**4.2.8 Novel Object Recognition (NOR) Task**—For most behavioral testing presented here, the NOR task, rather than MWM, was used to determine improvements in memory. A pilot study had shown that tropisetron treatment increased J20 performance in NOR, and the task had far fewer drawbacks than the MWM. Mice, and J20 mice in particular, are not good swimmers and often manifest anxiety in the form of floating or thigmotaxis (circling the perimeter of the pool) and many such mice have to be eliminated from data analysis. Great individual variation is seen in MWM and therefore large numbers of mice need to be tested to identify differences between groups. Others have also found that NOR may be a better testing paradigm for compound screening (Alkam et al., 2011; Dere et al., 2007; Zhang et



al., 2012), particularly when the large animals numbers needed for MWM are not available, or shorter treatment periods are used. In addition, NOR allows for a determination of the activity level, and J20 mice are hyperactive compared to NTg. The NOR protocol used was based on that of Bevins and Besheer (Bevins and Besheer, 2006). Mice have a natural curiosity for the novel, and typically spend more time with a new object than one with which they have become familiar, provided that their memories for what is novel and what is familiar remain intact. Thus mice with impaired memories will often spend approximately equal time with the new and old objects. In addition, the J20 are hyperactive (Chen et al., 2000; Palop et al., 2003), and therefore often travel farther during the task. Individual mice were allowed to become acclimated to a 20 × 30cm black opaque box (“arena”) and the operator for 20 minutes per day for 2 days prior to testing. During testing, there was an “acquisition” phase wherein the mouse spent 10 minutes with two identical objects. Both the attention paid to each object (touching or coming within 2 cm of the object with the nose, designated as an interaction) and the distance the mouse traveled during the 10 minutes were recorded. After a 1-hour rest, the mouse was returned to the arena for short-term memory assessment; for this, one of the objects had been changed. Novelty preference (NP) was calculated as percentage of total interactions with both objects with 50% - or an equal number of interactions with each object – as zero preference. Mice were euthanized 24 hours after NOR testing.

**4.2.9 Comparison to donepezil**—Mice received vehicle, 0.5 mkd tropisetron, or 1 mkd donepezil in 14-day model 1002 Alzet pumps; at the end of the 14-day period, they received daily subcutaneous injections at those dosages for an additional 9 days. While the dose of donepezil was chosen to be similar to that of tropisetron, it is also equivalent to the human starting dose of 5 mg per day. The human equivalent dose was calculated through normalization of body-to-surface area using the formula:

$$HED (mg/kg) = mouse\ dose (mg/kg) \times Mouse\ Km / Human\ Km$$

where the mouse Km is 3 (and human Km is 37) (Reagan-Shaw et al., 2008) This dose also should be adequate to inhibit AChE (Rakonczay, 2003). Mice underwent short-term memory testing using the NOR task at 14 days (at which time no improvement was seen with donepezil, and so treatment was extended) and at 22 days. Injections were used for extended treatment as humans receive donepezil once a day rather than by continuous pump delivery, and we thought this may alter responses to treatment. Plasma and brain levels at the end of the study were determined as described above. Biochemical markers were assayed as described above from combined hippocampus and entorhinal cortex.

**4.2.10 Comparison of oral and pump delivery**—Mice received either 1mkd tropisetron by 28-day Alzet pump, or 4mkd tropisetron by a single oral dose. The oral dosing was performed by mixing 5µl of an appropriate stock solution of tropisetron with 5µl of liquid syrup to make the dose palatable. The mice were fed the 10µl total from the tip of a pipette, rather than gavage. As comparison of pharmacokinetics after oral and subcutaneous injection showed brain levels are 4-5 fold lower after oral dosing, the oral dose in this study

was 4-fold higher. Mice underwent NOR testing at 21 days of treatment and were euthanized 24 hours later.

**4.2.11 Meta-analysis of all *in vivo* studies**—A total of ten *in vivo* experiments wherein J20 mice were treated with tropisetron were included in meta-analysis. For sAPP $\alpha$ , A $\beta$ 1-42, and the ratio, all values for individuals were converted to percentage of the average vehicle control value in each separate experiment, and then all of these converted values were analyzed together (N = 71 control, N = 72 tropisetron). All statistics used student's t-test, unpaired, and two-tailed analyses unless otherwise specified.

**4.2.12 Tropisetron treatment of NTg mice**—NTg mice were treated with 0.5 mkd tropisetron for 28 days by Alzet pump as described above in the memantine study. At 27 days, mice performed the NOR task, and were euthanized 24 hours later.

### 4.3 Supplementary methods

**4.3.1 Pilot studies**—In addition to the *in vivo* studies presented in the body of the manuscript, five additional studies were run and included in meta-analysis. These included: 1) 4-day subcutaneous injection of 4-5 m.o. J20 males at 10 mkd, control n = 3, tropisetron n = 1 (all studies began with 3 or more mice per group, but some mice are not available for biochemical analysis); 2) 5-day BID subcutaneous injection at 10 mk (20 mkd) of 4-5 m.o. J20 females, control n = 4, tropisetron n = 4; 3) 12-day subcutaneous injection at 10 mkd of 4-5 m.o. J20 males, control n = 4, tropisetron n = 4; 4) 5-day subcutaneous injection at 0.3 mkd of 4-5 m.o. J20 males, control n = 2, tropisetron n = 4; and 5) 28-day pump delivery of 0.5 mkd to 12-14 m.o. J20 males and females, control n = 2, tropisetron n = 2. All biochemical analysis was done using microdissected hippocampus and entorhinal cortex.

**4.3.2 Pilot study in old, plaque-bearing mice**—Old (12-15 m.o.) J20 mice received 1 mkd tropisetron (n = 8) or saline vehicle (n = 9) by 14-day Alzet pump for 13 days, at which time no improvement in memory as determined by NOR testing was seen, therefore treatment was continued for an additional 21 days by oral dosing at 2 mkd. The oral dose was higher as oral availability is lower than subcutaneous. Aged-matched NTg mice were treated similarly with vehicle only (n = 5). All mice underwent baseline NOR testing 4 days before the commencement of treatment and, as many old NTg mice performed poorly in NOR, a relatively young (6 m.o.) NTg cohort was added and treated with vehicle as above. NOR testing was also performed at 13 days of treatment and the end of the study, a total of 35 days. Biochemical analysis of sAPP $\alpha$ , A $\beta$  1-40 and 1-42 from a single sample from each mouse comprised of combined hippocampus and entorhinal cortex was performed as described above, but here, analysis of sAPP $\beta$  was added. The Perkin-Elmer AlphaLISA for sAPP $\beta$  was used and the assay performed as described above for sAPP $\alpha$ . sAPP $\beta$  is the N-terminal fragment generated as a result of BACE cleavage of APP (Fig. 1) and as BACE cleavage is the first step in A $\beta$  production it can be indicative of *de novo* A $\beta$  production. This assay was used because in very old plaque-bearing mice there is an enormous amount of pre-existing, plaque-associated A $\beta$ . To determine the effects of a compound such as tropisetron that reduces net A $\beta$  production but does not necessarily clear existing plaques, an assay of sAPP $\beta$  in addition to A $\beta$  itself was thought to provide additional information. In

our hands, measurement of a soluble versus non-soluble fraction of A $\beta$  in these very old mice did not prove to be useful, since very little soluble A $\beta$  could be detected.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## Abbreviations

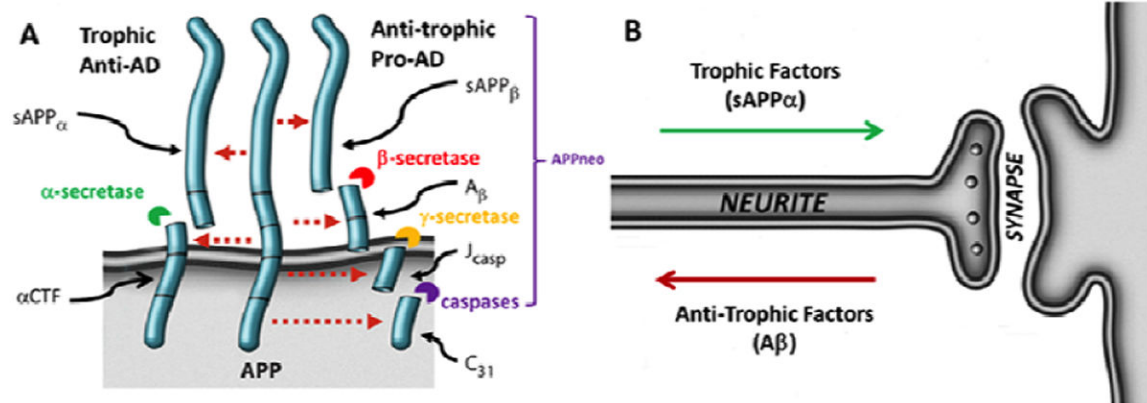
<b>AD</b>	Alzheimer's Disease
<b>APP</b>	Amyloid precursor protein
<b>A<math>\beta</math></b>	amyloid beta
<b>sAPP<math>\alpha</math></b>	soluble amyloid precursor protein alpha
<b>sAPP<math>\beta</math></b>	soluble amyloid precursor protein beta
<b>CHO 7W</b>	Chinese hamster ovary cells stably transfected with human APP wildtype

<b><math>\alpha_7</math>nAChR</b>	alpha 7 nicotinic acetylcholine receptor
<b>5-HT<sub>3</sub></b>	5-hydroxytryptophan 3 (receptor)
<b>GABA</b>	gamma-aminobutyric acid
<b>PONV</b>	post-operative nausea and vomiting
<b>MCI</b>	mild cognitive impairment
<b>BACE</b>	beta-site APP cleaving enzyme
<b>Jcasp</b>	juxtamembranar cytoplasmic domain
<b>C31</b>	c-terminal fragment of caspase-cleaved APP
<b><math>\alpha</math>CTF</b>	c-terminal fragment of ADAM10 ( $\alpha$ -secretase) cleavage of APP
<b><math>\beta</math>CTF</b>	c-terminal fragment of BACE cleavage of APP
<b>NMDA</b>	N-methyl-D-aspartate (receptor)
<b>NOR</b>	Novel object recognition
<b>HTS</b>	high-throughput screening
<b>MWM</b>	Morris water maze
<b>AChE</b>	Acetylcholine esterase
<b>ADAM10</b>	a disintegrin and a metalloprotease 10
<b>PDAPP</b>	platelet-derived growth factor promoter driven human APP gene
<b>HED</b>	human equivalent dose
<b>BID</b>	twice a day
<b>Mkd</b>	mg/kg/day

### Highlights

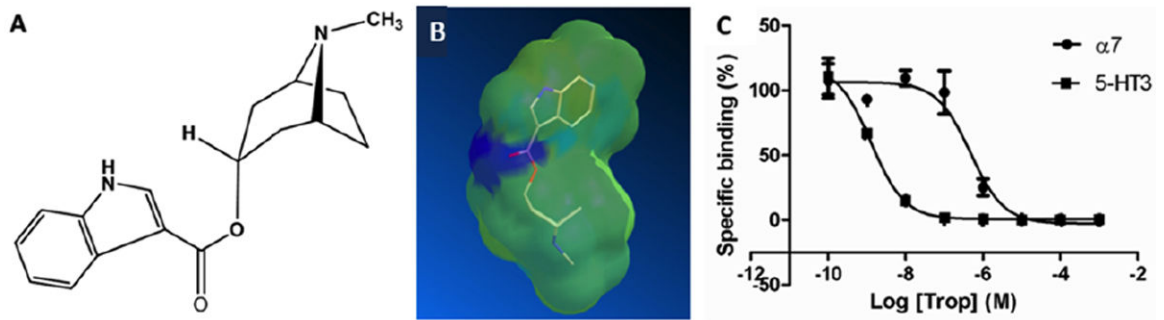
1. Dramatic improvement of memory and biomarker ratios in AD mouse model by a multi-functional drug candidate
2. Drug candidate is a potent 5HT-3 antagonist, a  $\alpha 7$  nicotinic receptor partial agonist and binds the ectodomain of APP.
3. Drug candidate has superior efficacy in the AD model in comparison to two current AD drugs
4. Suitable for human clinical trials as a candidate therapeutic for AD.





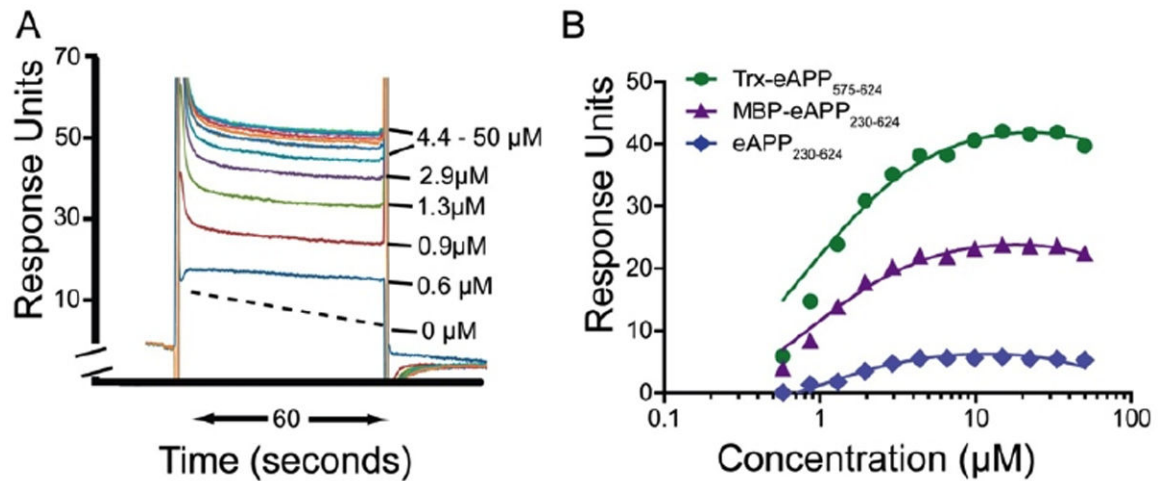
**Figure 1. Mediation of alternative plasticity states by APP**

APP undergoes cleavage via two different pathways (A). The trophic pathway includes  $\alpha$ -secretase cleavage, producing sAPP $_{\alpha}$  and  $\alpha$ CTF, which mediate neurite outgrowth, caspase inhibition, and synaptic (Syn) maintenance. Alternatively, cleavage by BACE,  $\gamma$ -secretase, and caspase produces sAPP $_{\beta}$ , A $_{\beta}$ , J $_{casp}$ , and C $_{31}$ , mediating neurite (Nt) retraction, synaptic loss, caspase activation, and potentially cell death (B) (Descamps et al JAD 2011;25:51-57).



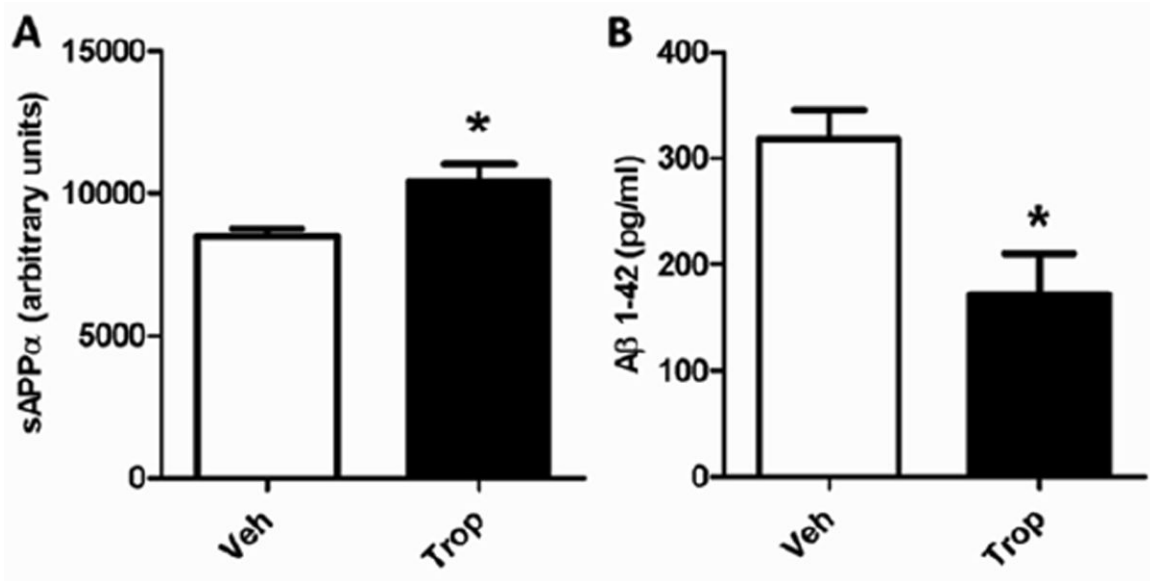
**Figure 2. Molecular structure of tropisetron and receptor binding to 5-HT<sub>3</sub> and  $\alpha_7$ nACh receptors**

The molecular structure of tropisetron is shown in A and a molecular model is shown in B. The competitive binding curves for tropisetron at both the 5-HT<sub>3</sub> and  $\alpha_7$ nACh receptors are shown in C.



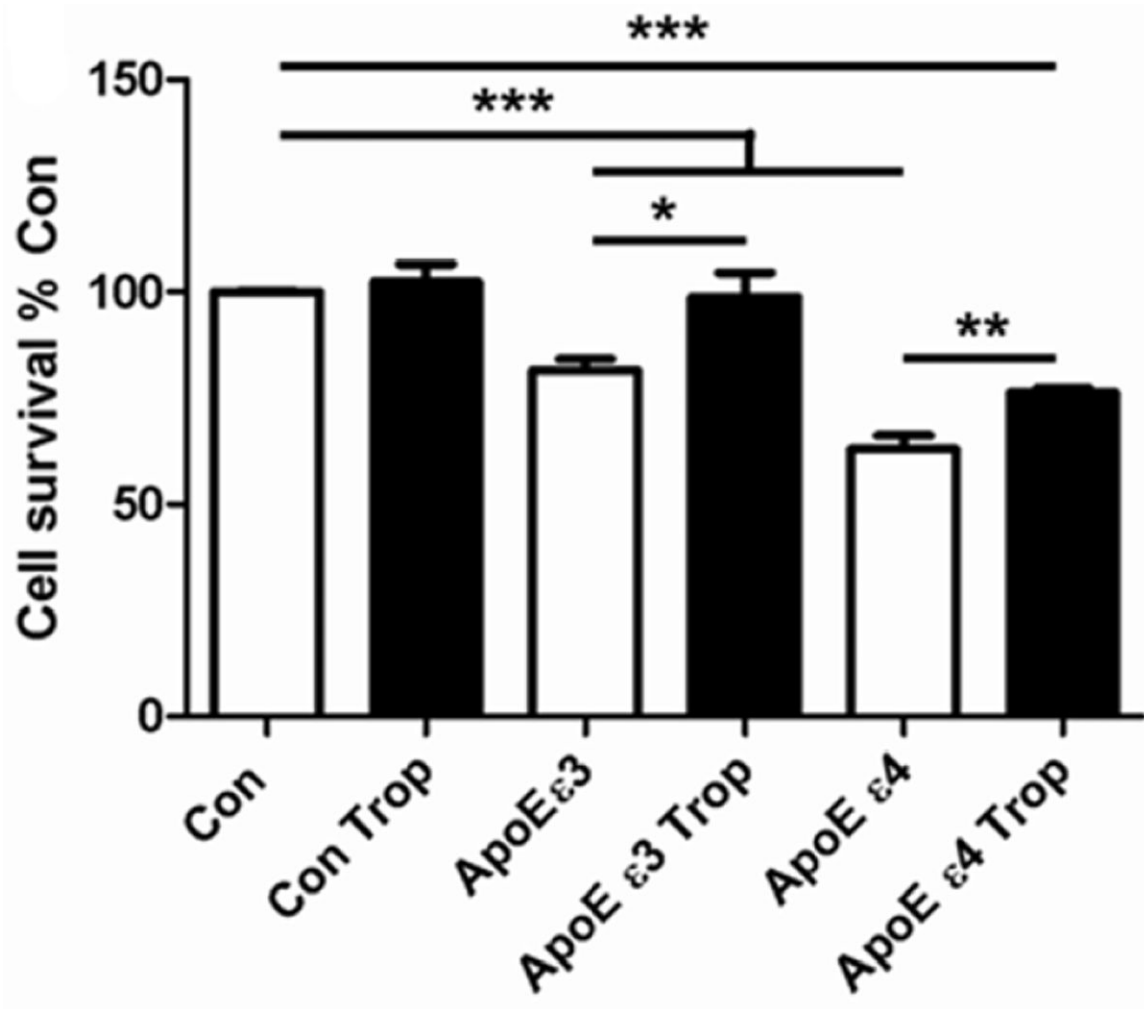
**Figure 3. Tropisetron binds directly to APP**

Surface plasmon resonance data was obtained as described in Methods. The sensorgrams obtained by flowing varying concentrations of tropisetron (0-50  $\mu\text{M}$ ) through the Trx-eAPP<sub>575-624</sub> flow cell and before subtraction of the no-drug control is shown in **A**. The binding isotherms for tropisetron to various fragments of the ectodomain of APP<sub>695</sub> are shown in **B**. The binding isotherms for tropisetron were fitted using a single binding site model. All three curves were constrained to share the same equilibrium constant with PRISM (GraphPad Inc). The resulting binding equilibrium constant was calculated to be  $0.9\mu\text{M} \pm 0.1\mu\text{M}$  with  $R^2=0.99$ .



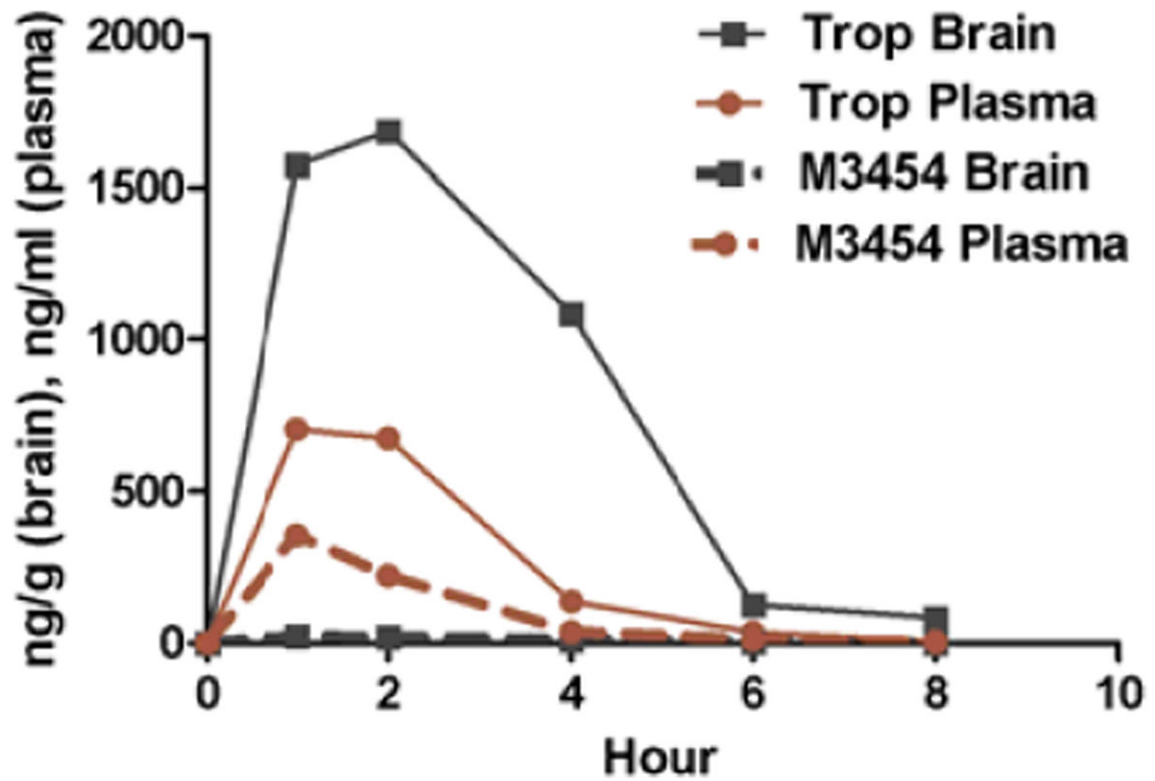
**Figure 4. Tropisetron increases sAPP $\alpha$  and decreases A $\beta$ 1-42 in primary hippocampal neuronal culture**

In primary hippocampal neuronal cultures, tropisetron at 1 $\mu$ M significantly ( $P = 0.0473$ ) increased sAPP $\alpha$  (A) and significantly ( $P = 0.0486$ ) decreased A $\beta$ 1-42 (B). Both t-test, two-tailed, unpaired statistical analyses.



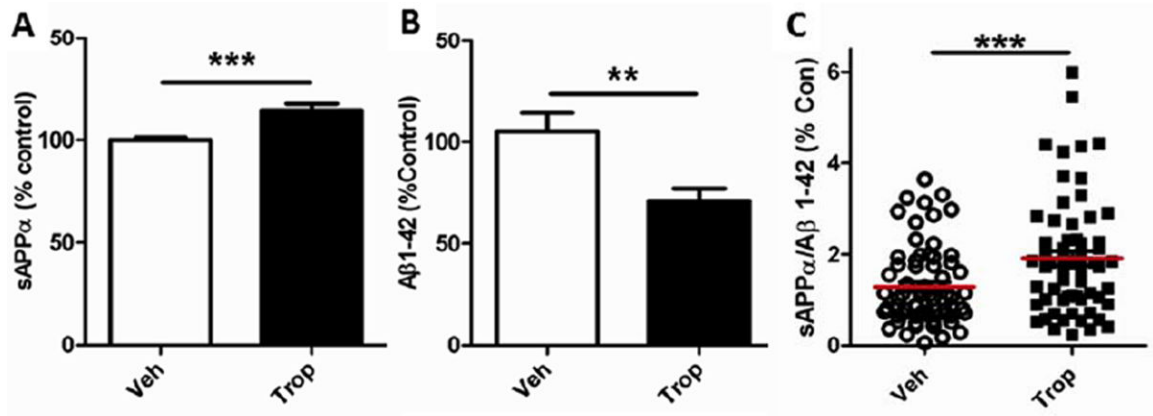
**Figure 5. Tropisetron increases cell survival after ApoE ε4 transfection**

Transfection with either ApoE ε3 or ε4 decreased cell survival ( $P = 0.0001$ ). Tropisetron treatment increased survival after transfection with either ApoE ε3 ( $P = 0.0207$ ) or ε4 ( $P = 0.003$ ); it did not change survival of untransfected cells. All t-test, two-tailed, unpaired statistical analyses.



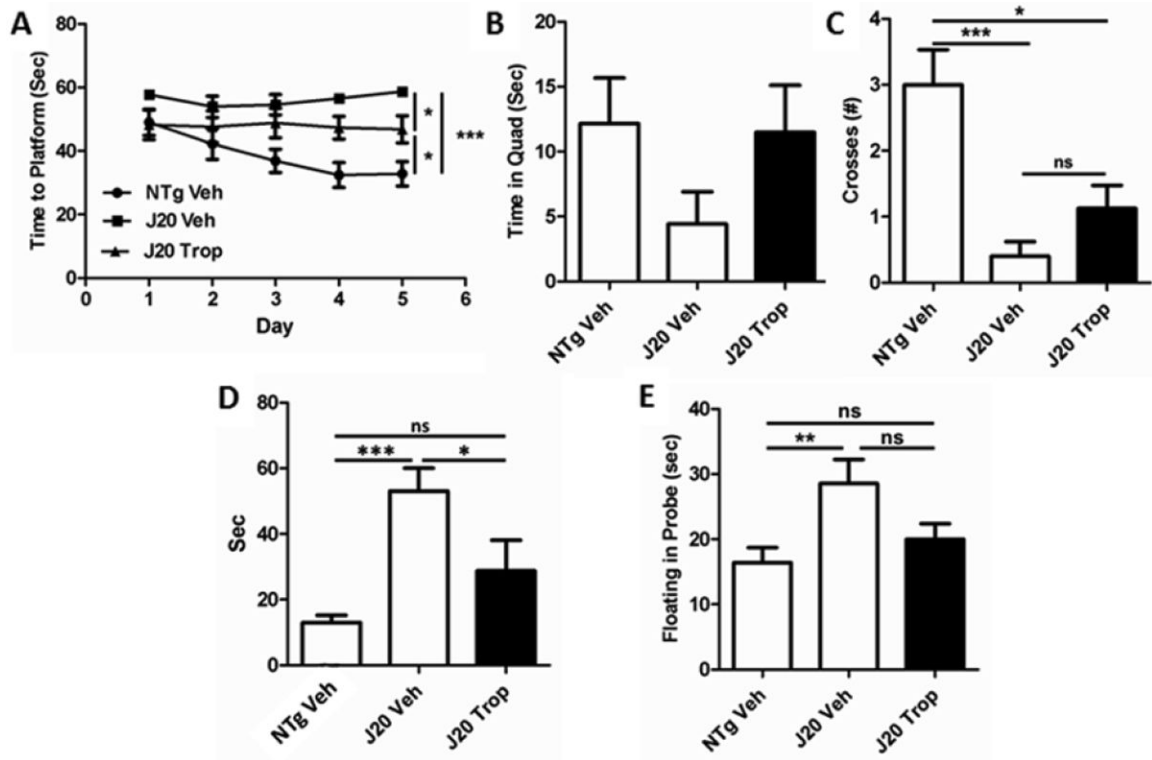
**Figure 6. Tropisetron exhibits superior brain penetration to MEM-3454**

Tropisetron injected subcutaneously into adult mice at 10 mg/kg appeared in brain tissue at high levels at the first time point measured (1 hour), and dissipated by 6 hours post-injection. The brain/plasma level was 2.5 at the peak. A compound currently in clinical trials - MEM-3454 (M3454) - that also binds  $\alpha 7nAChR$  (but not APP) shows much lower brain penetration and a lower brain/plasma ratio when given at the same dose and route.



**Figure 7. Meta-analysis of tropisetron effects on sAPP $\alpha$  and A $\beta$ 1-42 in hippocampus/entorhinal cortex**

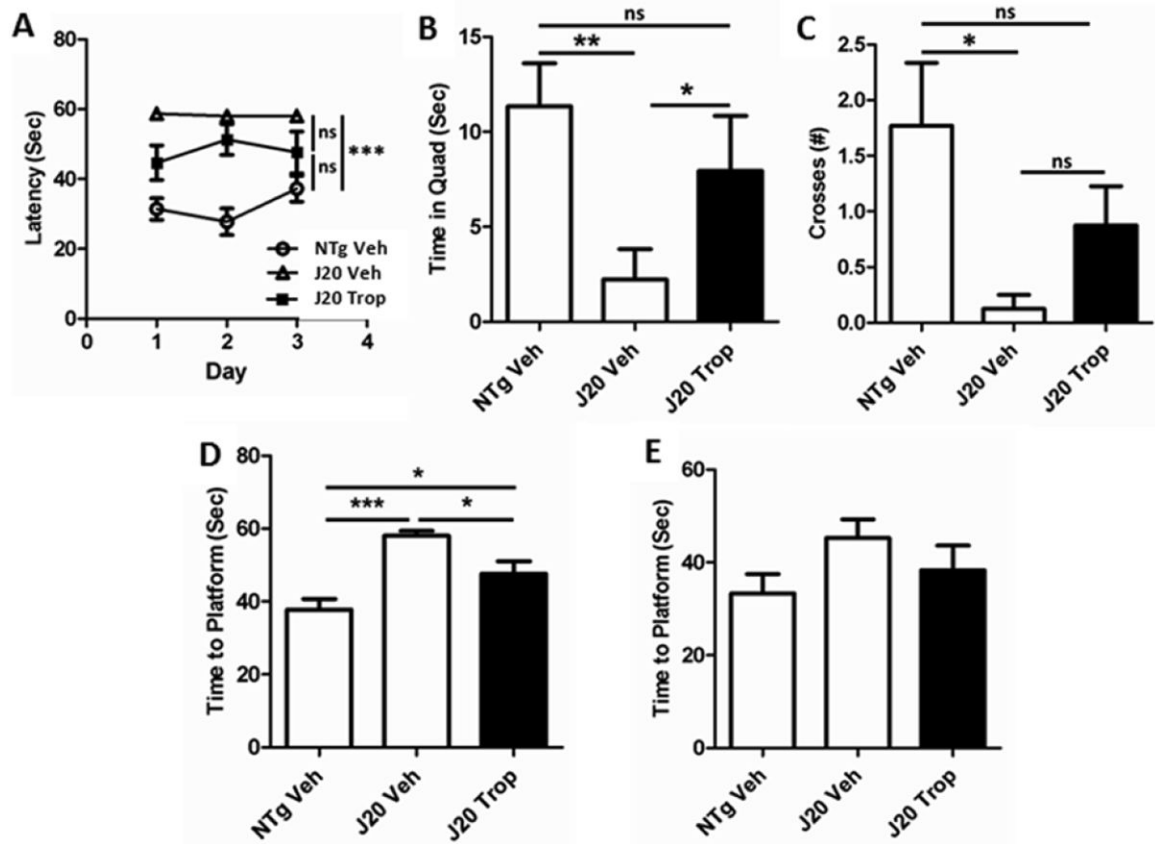
(A) When sAPP $\alpha$  values for individuals from eight separate *in vivo* experiments (vehicle n = 68, tropisetron n = 67) are converted to percentage of vehicle control for those experiments, the increase in sAPP $\alpha$  as a result of tropisetron treatment is very highly significant (P = 0.0001). (B) Decreases in A $\beta$ 1-42 are also highly significant (P = 0.0021). The sAPP $\alpha$ /A $\beta$ 1-42 is also highly significant (0.0008) (Fig. 7C). All t-test, two-tailed, unpaired statistical analyses.



**Figure 8. Tropisetron-treated mice show improvements in spatial memory**

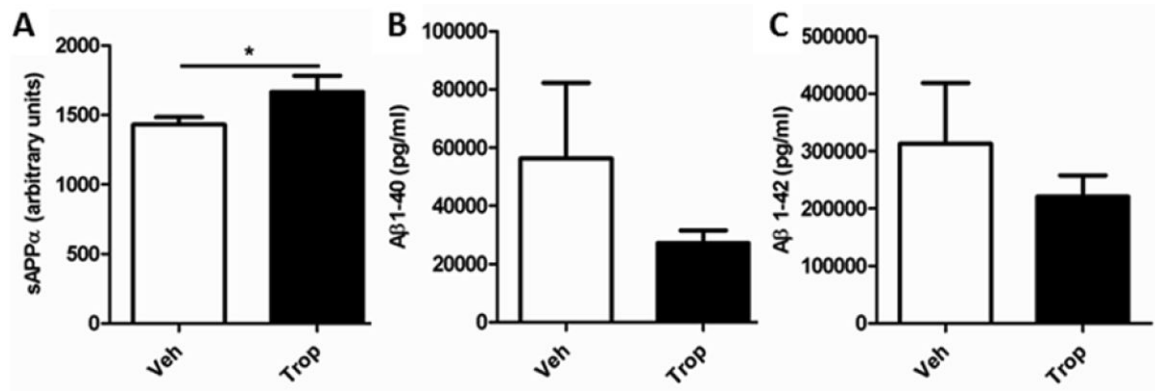
Mice were treated for 8 weeks as described in Methods. In the last 2 weeks of treatment, mice performed the Morris Water Maze (MWM) spatial memory task. (A) In training, J20 mice treated with tropisetron found the hidden platform significantly faster than vehicle treated J20s by Day 5 of training ( $P = 0.0113$ ), but did not become significantly faster with days of training. In the hidden probe, where the platform was removed and attempts of the mice to find it were recorded, while tropisetron-treated J20s spent more time in the platform quadrant (B) than vehicle-treated J20s, and crossed the platform site (C) more frequently, these increases were not significant; and while tropisetron-treated J20s crossed the platform significantly fewer times than NTg ( $P = 0.0196$ ), vehicle-treated mice crossed far less ( $P = 0.0005$ ). (D) Tropisetron-treated J20s' latency to the platform site was significantly improved over vehicle-treated J20 ( $P < 0.05$ ) and not significantly different than NTg; vehicle-treated J20s were much slower ( $P = 0.0002$ ) than NTgs (ANOVA, Bonferroni post-hoc). (E) All mice floated rather than initially swimming actively at first, with vehicle-treated J20s floating the most, significantly more than the NTgs ( $P = 0.0088$ ); tropisetron-treated mice were intermediate.





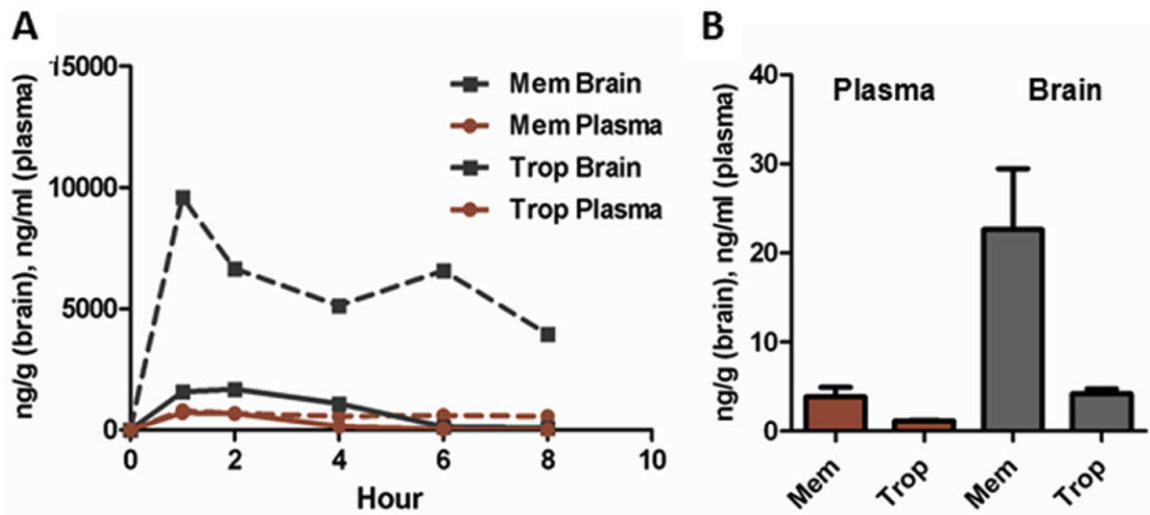
**Figure 9. Tropisetron-treated mice perform well in the MWM reversal**

In the reversal, the platform is moved and the mice re-trained. (A) On the last day of training, vehicle-treated J20s took significantly longer to find the platform than NTg mice ( $P = 0.0004$ ), tropisetron-treated J20s were neither significantly faster than vehicle-treated J20s nor significantly slower than NTgs. In the reversal probe, neither time spent in the platform quadrant (B) nor number of crosses of the platform site (C) by tropisetron-treated J20s was significantly less than that of NTg mice, but they spent significantly more time in the target quadrant ( $P < 0.05$ , ANOVA, Bonferroni post-hoc) and had a significantly shorter latency (D) than vehicle-treated J20s ( $P = 0.0061$ ), although they were not as rapid as NTg mice ( $P = 0.034$ ). (E) When the platform was visually cued, there was no significant difference in the time it took for mice to reach it, although vehicle-treated J20s floated more and therefore took slightly longer to reach the platform. All t-test, two-tailed, unpaired statistical analyses unless otherwise indicated.



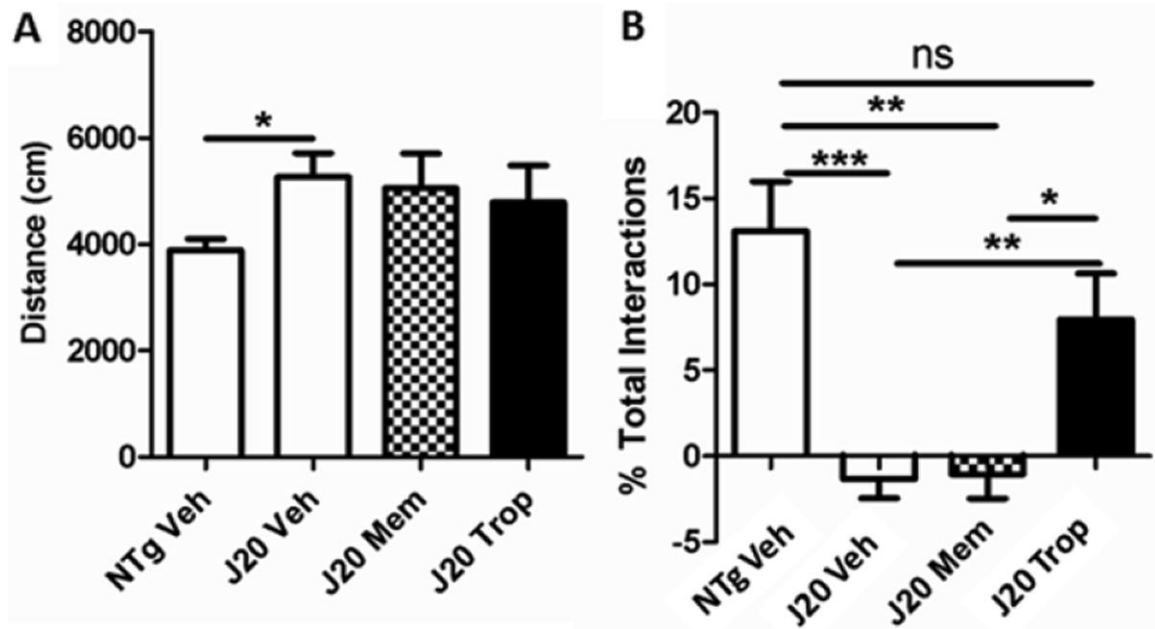
**Figure 10. sAPP $\alpha$  and Abeta improved after eight-week tropisetron treatment**

(A) sAPP $\alpha$  was significantly ( $P = 0.0425$ , t-test, one-tailed, unpaired) higher, and (B) A $\beta$ 1-40 and (C) 1-42 lower, but not significantly so in hippocampi/entorhinal cortices.



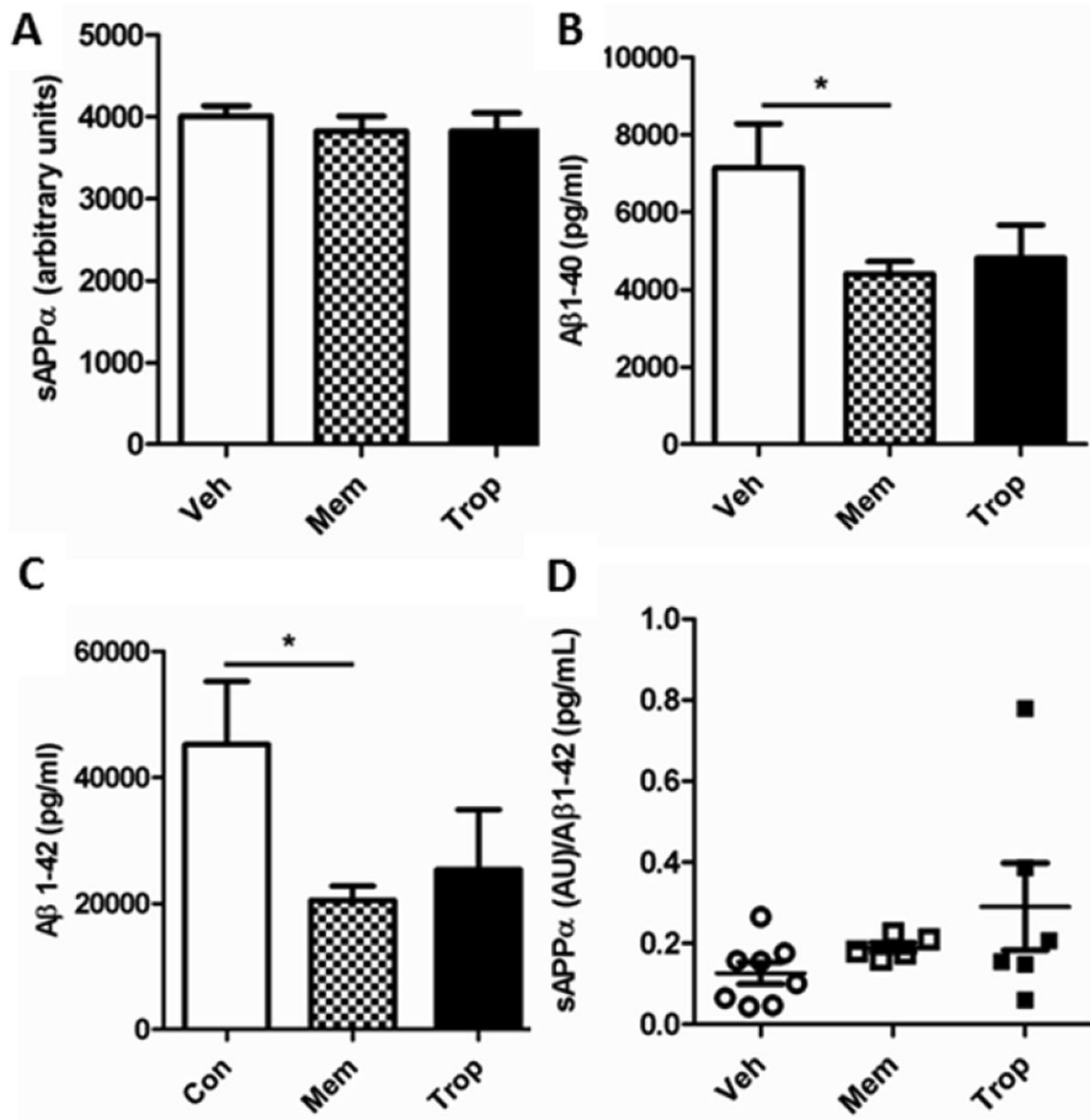
**Figure 11. Memantine is more brain-penetrant than tropisetron**

(A) After a single dose at 10mkd, memantine brain levels were more than 5-fold higher than those of tropisetron. (B) Plasma levels were higher for memantine in the comparative study and brain levels were again approximately 5-fold higher.

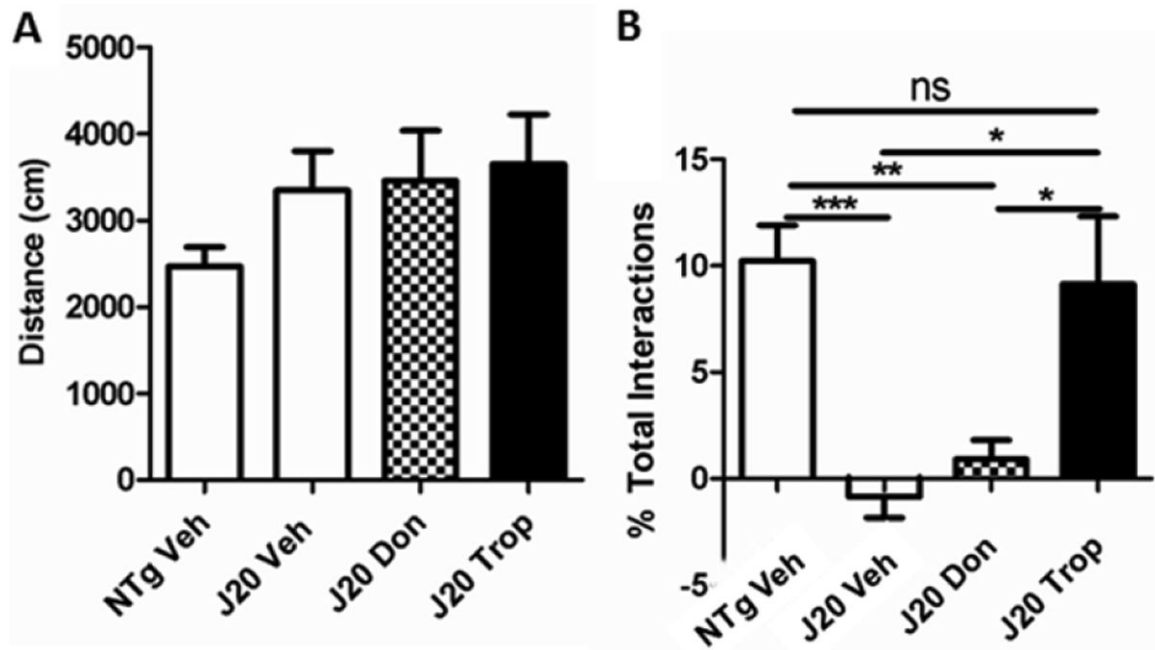


**Figure 12. Tropisetron, but not memantine, improved working memory**

(A) No treatment significantly lowered hyperactivity in this study, but there was a trend for both memantine and tropisetron to do so. (B) Tropisetron treatment significantly improved novelty preference (NP) as compared to vehicle-treated J20 controls ( $P = 0.012$ ) and memantine-treated J20s ( $P = 0.0297$ ); and this NP was not significantly different from NTg controls. Memantine did not improve NP. All t-test, two-tailed, unpaired statistical analyses.

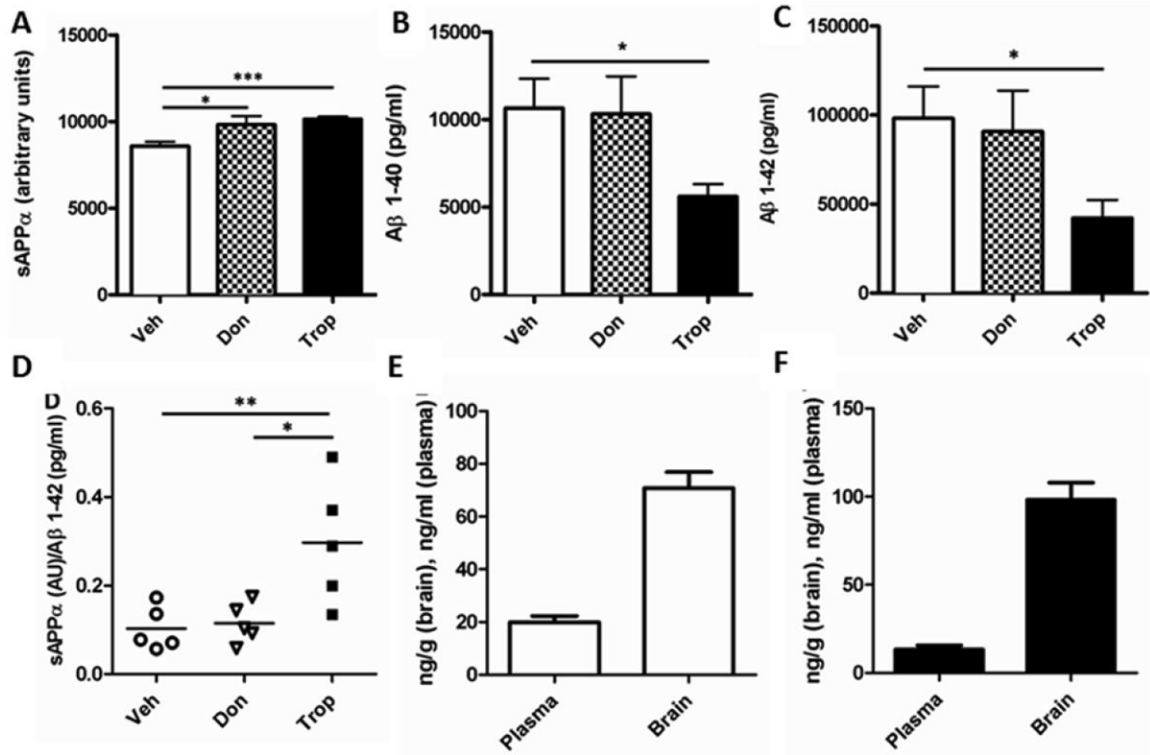


**Figure 13. Tropisetron and memantine have similar effects on Aβ in hippocampus/entorhinal cortex**  
 There was no increase in sAPPα as a result of treatment with either compound. Both Aβ1-40 (A) and Aβ1-42 (B) were lowered similarly by each compound, significantly so for memantine ( $P = 0.0475$ ,  $P = 0.0415$ , respectively). All t-test, one-tailed, unpaired analyses. (C) Ratios for memantine and tropisetron. (D-E) Both plasma and brain levels of memantine were higher than tropisetron, as expected.



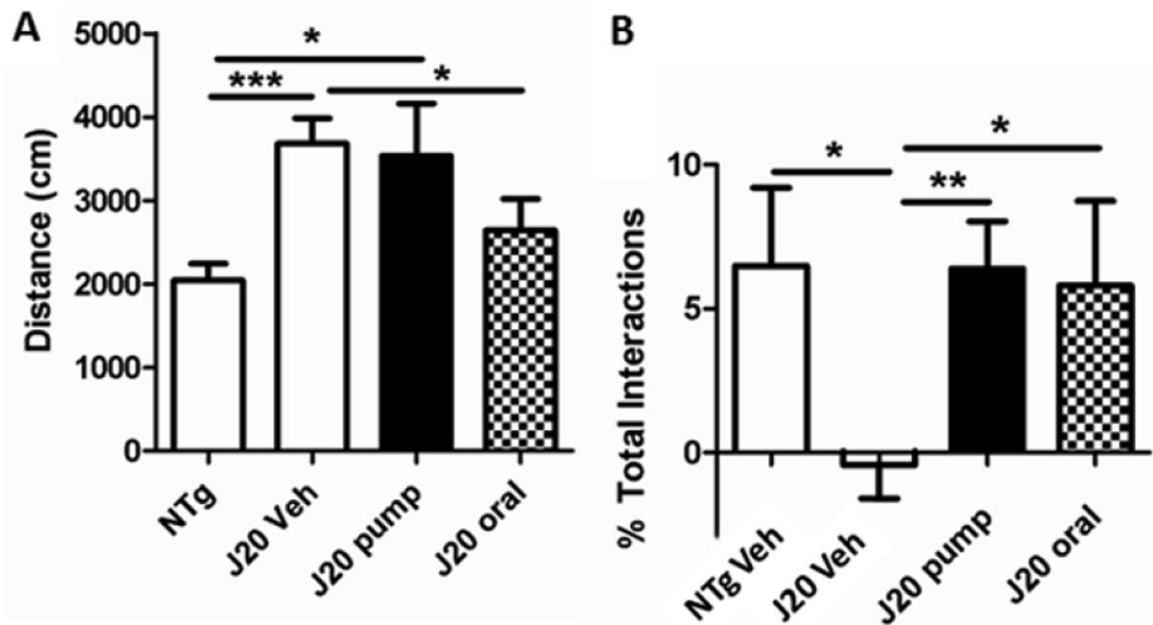
**Figure 14. Tropisetron, but not donepezil, improved working memory at a human equivalent dose**

(A) There was no reduction in hyperactivity by any treatment in this study. (B) Only tropisetron significantly ( $P = 0.0258$ ) improved novelty preference as compared to vehicle-treated J20s controls.



**Figure 15. Tropisetron and donepezil effects on sAPP $\alpha$  and A $\beta$ 1-42**

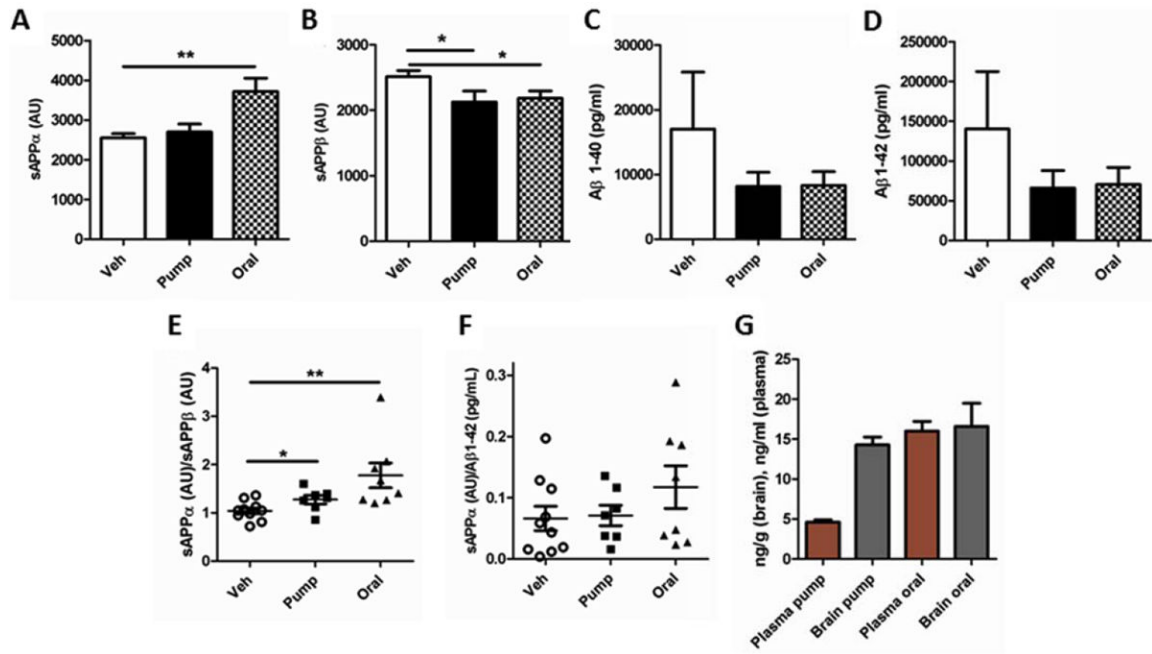
(A) Both tropisetron and donepezil increased sAPP $\alpha$  significantly ( $P = 0.0004$ ,  $P = 0.0301$ , respectively). (B-C) Tropisetron-treated mice had significantly lower A $\beta$ 1-40 ( $P = 0.0353$ ) and A $\beta$ 1-42 ( $P = 0.0446$ ) than donepezil-treated J20s (t-test, one-tailed, unpaired). Hippocampi and entorhinal cortices from individual mice were used for analysis. There was no statistical difference between donepezil- and vehicle-treated mice. (D) The sAPP $\alpha$ /A $\beta$ 1-42 ratio was significantly higher for tropisetron-treated than both vehicle- ( $P = 0.0096$ ) and donepezil-treated ( $P = 0.0122$ ) mice. Plasma and brain levels of donepezil (E) and tropisetron (F) were similar. All t-test, one-tailed, unpaired statistical analyses.



**Figure 16. Oral delivery of tropisetron is as effective as pump delivery for improving working memory**

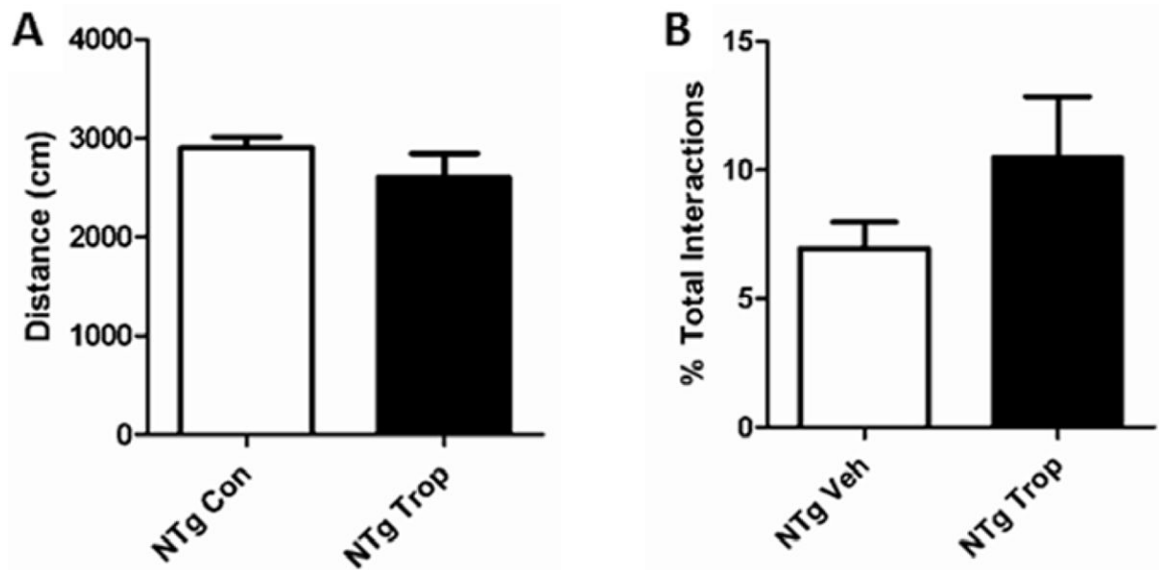
(A) J20 mice receiving either vehicle or tropisetron by pump were still hyperactive as compared to NTg mice ( $P = 0.0004$  and  $P = 0.0303$ , respectively), but hyperactivity was reversed by tropisetron orally ( $P = 0.0461$ ; not significantly different than control NTg). (B) Compared to NTg mice, vehicle-treated J20s had significantly ( $P = 0.0409$ ) lower novelty preference (NP). There was no significant difference in NP between NTg and either tropisetron-treated group, but only mice treated with tropisetron by pump had significantly ( $P = 0.0075$ ) greater NP than vehicle-treated J20s.



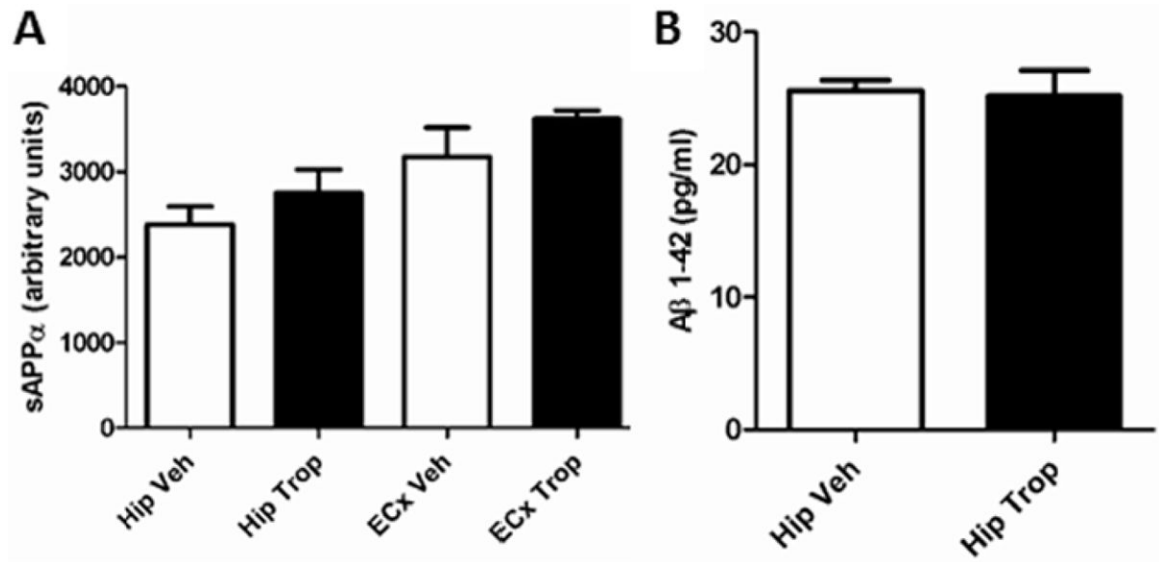


**Figure 17. Tropisetron oral delivery effects on sAPP $\alpha$**

(A) Oral tropisetron treatment significantly ( $P = 0.0016$ ) increased sAPP $\alpha$ . (B) sAPP $\beta$  was significantly lower with both pump and oral delivery ( $p = 0.0118$ ,  $p = 0.012$ , respectively). (C, D) A $\beta$ 1-40 and A $\beta$ 1-42 were lower after either oral or pump delivery. (E) The sAPP $\alpha$ /sAPP $\beta$  ratio was significantly increased with either pump or oral delivery ( $p = 0.0435$ ,  $p = 0.0072$ , respectively), but was greater for orally-treated mice. (F) The sAPP $\alpha$ /A $\beta$ 1-42 ratio was higher for orally-treated mice. All samples hippocampus/entorhinal cortex.



**Figure 18. Tropisetron improves working memory in NTg mice**  
(A) Tropisetron reduced activity and (B) increased NP in NTg mice.



**Figure 19. Tropisetron increased sAPP $\alpha$  in I5 mice**  
It increased sAPP $\alpha$  (C) but did not change A $\beta$ 1-42 (D) in hippocampi from I5 mice.