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Anatabine Attenuates Tau Phosphorylation and Oligomerization in P301S Tau Transgenic Mice

Daniel Paris[1*], David Beaulieu-Abdelahad[1], Ghania Ait-Ghezala[1], Venkat Mathura[1], Megha Verma[1], Alex E Roher[3], Jon Reed[1],Fiona Crawford[1] and Michael Mullan[1,2]

[1]*Roskamp Institute, Sarasota, Florida, USA*
[2]*Rock Creek Pharmaceuticals, Gloucester, Massachusetts, USA*
[3]*The Longtine Center for Neurodegenerative Biochemistry, Banner Sun Health Research Institute, Sun City, Arizona, USA*

Abstract

We have previously shown that the natural alkaloid anatabine displays some anti-inflammatory and Alzheimer amyloid (Aβ) lowering properties in the central nervous system associated with reduced STAT3 and NFkB activation. We investigated here the impact of a chronic oral treatment with anatabine in a model of tauopathy. We found that anatabine reduces the incidence of paralysis and abnormal hind limb extension reflex while improving rotarod performances in P301S mutant human Tau transgenic mice (Tg Tau P301S) suggesting that anatabine delays the disease progression in this model of tauopathy. Analyzes of brain and spinal cord homogenates reveal that anatabine reduces tau phosphorylation at multiple pertinent Alzheimer's disease (AD) epitopes and decreases the levels of pathological tau conformers/oligomers in both detergent soluble and insoluble fractions. Pathological tau species reduction induced by anatabine was accompanied by decreased Iba1 expression suggesting a diminution of microgliosis in the brain and spinal cord of Tg Tau P301S mice. In addition, we found that anatabine administration increases phosphorylation of the inhibitory residue (Ser9) of glycogen synthase kinase-3β, a primary tau kinase, associated with AD pathology, providing a possible mechanism for the observed reduction of tau phosphorylation. These data support further exploration of anatabine as a possible disease modifying agent for neurodegenerative tauopathies and, in particular AD, since anatabine also displays Aβ lowering properties.

Keywords

Tauopathy; Alzheimer; Tau; Phosphorylation; oligomer; Microgliosis; Anatabine; Glycogen synthase kinase; Protein kinase B; AKT

Introduction

Alzheimer's disease (AD), the most prevalent form of dementia in the elderly, is a progressive neurodegenerative disorder characterized by a loss of cognitive functions and by diverse psychiatric symptoms leading to confusion, agitation and aggression which constitute a major cause of patient distress. AD pathology is characterized by the deposition of extracellular Aβ peptides and by intraneuronal accumulation of hyperphosphorylated and aggregated tau protein [1]. Beside the pathological accumulation of intraneuronal neurofibrillary tangles and extracellular Aβ deposits, neurodegeneration and neuroinflammation associated with microgliosis and astrogliosis are prevalent in AD brains. AD neurodegeneration is widespread and affects many brain areas. Cholinergic dysfunction in the basal and rostral forebrain correlates with early cognitive dysfunctions in AD and is well documented [2]. In addition to the loss of cholinergic neurons, there is also a significant loss of noradrenergic neurons in the locus coeruleus (LC) which may contribute to cognitive impairment and depression in AD [3]. Norepinephrine mainly originates from the LC in the central nervous system and exerts an anti-inflammatory activity via activation of glial adrenoreceptors (AR), therefore LC neurodegeneration may also contribute to neuroinflammation in AD [4]. AR have also been implicated in Aβ formation and post-receptor components including the G protein coupled receptor kinase 2 (GRK2) (responsible for the desensitization of AR) is altered by Aβ and may also mediate Aβ induced AR dysfunction in neurons [4]. Moreover, GRK2 has been shown to result in tau hyperphosphorylation and is co-localized with neurofibrillary tangles in AD [5] suggesting that it may also contribute to tau pathology in AD highlighting AR signaling and GRK2 modulation as valuable therapeutic targets for AD [4].

The transcription factors STAT3 and NFkB are activated in AD brains and play a key role in AD neuroinflammation by driving the expression of a large array of pro-inflammatory genes including cytokines, cyclooxygenase-2 and the inducible nitric oxide synthase [6,7]. Interestingly, Aβ has been shown to stimulate STAT3 phosphorylation resulting in microglial activation [8] and neuronal death [6,9] further highlighting the importance of STAT3 in the pathophysiology of AD. Rare familial forms of AD are caused by mutations in the amyloid precursor protein (APP) or presenilin genes resulting in an increased production and accumulation of Aβ peptides [10]. Aβ peptides have therefore been considered as a major target for AD drug discovery. However, to date, Aβ targeted therapies have failed to show efficacy in late stage clinical trials suggesting that Aβ may not be a sufficient target for AD therapeutic development. Pathological tau aggregation and accumulation is highly correlated with AD disease progression whereas a poor correlation has been observed between AD and Aβ deposition [11,12] suggesting that tau could represent an alternative target for AD drug development.

Although no mutation in the tau gene have been linked to AD, several mutations causing frontotemporal dementia and parkinsonism have been indentified [13] showing that abnormal forms of tau can directly cause neurodegeneration and memory loss. Also, a tau haplotype responsible for higher tau expression [14] and GAB2

***Corresponding author:** Daniel Paris, Roskamp Institute, Sarasota, Florida, USA,Tel: 941-752-2949; Fax: 941-752-2948; E-mail: dparis@rfdn.org

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alleles leading to increased tau phosphorylation appear to augment AD risk [15] suggesting an influential role of tau in AD. Abnormal tau phosphorylation in AD is believed to induce tau dissociation from microtubules, allowing tau to oligomerize and aggregate into neurofibrillary tangles, resulting in neurodegeneration. Multiple kinases responsible for the phosphorylation of tau at AD-pertinent epitopes have been identified and could constitute attractive targets for AD [16]. In particular, glycogen synthase kinase 3 (GSK3) is an ubiquitously-expressed serine/threonine kinase which phosphorylates tau at most serine and threonine residues in hyperphosphorylated neurofibrillary tangles, also contributes to Aβ production [17], and is therefore considered to be a prime target for the treatment of AD.

Several lines of transgenic mice overexpressing human tau mutations have been developed to mimic neurodegenerative tauopathies. These animal models have revealed that soluble tau oligomers rather than larger insoluble tau aggregates present in neurofilbrillary tangles are responsible for behavioral deficits and neurodegeneration [18,19]. In particular, mice overexpressing the human tau gene with the P301S mutation (Tg Tau P301S) have been produced [20]. These mice develop motor impairments, synaptic deficits, microglial activation and neurodegeneration before tangle formation [20] and constitute an interesting model to investigate tau hyperphosphorylation and oligomerization in the brain and the spinal cord.

We have previously identified anatabine, a natural alkaloid present in plants of the Solanacea family, as a novel anti-inflammatory compound antagonizing both STAT3 and NFkB activation [21-23]. We have shown that anatabine lowers Aβ production and neuroinflammation both in vitro and in vivo suggesting it could be a useful therapeutic compound against AD, particularly as it readily crosses the blood-brain barrier [21-23]. Other studies have revealed that anatabine suppresses the induction of cycloxygenase-2 and inducible nitric oxide synthase in macrophage and reduces the incidence of tyroiditis in mice [24] further supporting an anti-inflammatory activity of anatabine in vivo. A randomized controlled clinical trial has shown that anatabine supplementation can reduce thyroglobulin antibodies in patients with Hashimoto's thyroiditis [25] whereas a survey of people using a dietary supplementation of anatabine has shown a beneficial effect on joint pain and stiffness [26] suggesting that anatabine exerts an anti-inflammatory activity also in humans. We investigated here the effect of anatabine on tau phosphorylation and oligomerization in a pure tauopathy model (Tg Tau P301S) [20]. Our data show that anatabine reduces motor impairments as well as tau phosphorylation and oligomerization in the brain and the spinal cord of Tg Tau P301S mice suggesting that anatabine should be further explored for the treatment of tauopathies and AD in particular.

Materials and Methods

Mice

All the experimentations involving mice were approved by the Institutional Animal Care and Use Committee of the Roskamp Institute (IACUC protocol #44) and were performed in the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) accredited vivarium of the Roskamp Institute. Tg Tau P301S mice (line PS19) [20] were purchased from the Jackson laboratory (ME, USA). Animals were singly housed in a ventilated rack during the duration of the study. Initially, 12 Tg Tau P301S were utilized in the placebo group and 11 Tg Tau P301S were included in the anatabine treatment group. Anatabine was provided by Rock Creek Pharmaceuticals (MA, USA). Mice were treated for 40 days with anatabine at a dosage of 20 mg/Kg of body weight per 24 hours supplemented in their drinking water or received regular drinking water (placebo group) as previously described [23]. The anatabine treatment was initiated in 28 week-old Tg Tau P301S mice. During the duration of the treatment (40 days), 3 Tg Tau P301S mice in the placebo group became paralyzed and one of them died prematurely before the completion of the study and was not included for the biochemical analyzes of the brain and spinal cord. None of the mice showing paralysis were included in the behavioral analyzes. Animals were humanely euthanatized after 40 days of anatabine treatment and their brains (without cerebellum) and spinal cord were snap frozen in liquid nitrogen for biochemical analyzes.

Hind-limb extension test

After 39 days of treatment with anatabine, Tg Tau P301S mice were suspended by the tail and the degree of hind-limb extension was observed during 15 seconds. Animals showing an extension reflex in both hind-limbs were considered normal whereas mice displaying an extension reflex in only one hind-limb with partial retraction of the other hind-limb or an absence of extension reflex in both hind-limb (clasping behavior) were considered to have an abnormal extension reflex as previously described [20,27].

Accelerating rotarod test

Motor coordination was assessed with an accelerating rotarod apparatus (the apparatus was set to accelerating mode from 5 to 40 rpm in 60 seconds). On day 39 of the anatabine treatment, mice were trained on the rotarod and it was verified that each mouse was able to maintain its balance on the rod. Approximately one hour after this training session, mice were tested on the accelerating rotarod and the latency until a fall occurred recorded for each mouse.

Elevated Plus Maze

Anxious behavior was assessed using an elevated plus maze composed of two opposite open arms and two opposite closed arms separated by a central platform at 50 cm above the floor as previously described [28]. Animals were placed in the center of the maze facing an open arm and were allowed to explore the maze for 10 minutes. The time spent by the animals in the different arms of the maze was quantified using the EthoVision XT 8.0 video tracking software (Noldus Information Technology). Anxious behavior was assessed in Tg Tau P301S after 21 days of treatment with anatabine.

Dot blot analyzes

Mice (7.8 month-old) were humanely euthanatized after 40 days of treatment with anatabine and their brain and spinal cord were dissected out and snap frozen in liquid nitrogen. Brain (without cerebellum) and spinal cord samples from Tg Tau P301S mice were homogenized in 4 volumes of ice cold M-PER reagent (Pierce Biotechnology, IL, USA) containing 1X Halt protease and phosphatase inhibitor cocktail (Thermo Scientific, IL, USA). Samples were centrifuged at 20,000 g for 30 minutes at 4°C, the supernanatant was collected (M-PER soluble) and the insoluble pellet was resuspended in 2 volumes of MPER buffer containing proteases and phosphatases inhibitor by vigorous vortexing. 5 µl of each sample was spotted directly onto nitrocellulose membranes (Biorad, CA, USA). Membranes were blocked for 1 hour at room temperature in 5% non-fat dry milk dissolved in Tris buffer saline (Biorad, CA, USA) and incubated with the primary antibodies overnight at 4°C. A different nitrocellulose membrane was used for each of the primary antibody tested. Membranes were hybridized with

the antibodies CP13 (pSer202) (1/1000 dilution), PHF-1(pSer396/Ser404) (1/1000 dilution), RZ-3 (pThr231) (1/1000 dilution) to quantify tau phosphorylation at multiple AD relevant epitopes [29]. Pathological tau conformers and oligomers were detected using the conformational antibodies MC1 (1/1000 dilution) [29-31] and TOC1 (1/5000 dilution) [32-33]. Microgliosis was detected with the antibody Iba1 (1/1000 dilution) (Millipore Corporation, MA, USA). After the hybridization, membranes were rinsed in distilled water for 30 minutes and incubated for 2 hours at room temperature with secondary anti-mouse or anti-rabbit antibodies conjugated with peroxidase (Cell Signaling technology, MA, USA). Signal detection and quantification were performed with a 2 fold dilution of the SuperSignal west fento maximum sensitivity chemiluminescent substrate (Thermo Scientific, IL, USA) by chemiluminescence imaging with the ChemiDocTM XRS (Bio-Rad, CA, USA). After hybridization with the primary antibodies, all membranes were stripped for one hour at room temperature with the RestoreTM western blot stripping buffer (Thermo Scientific, IL, USA) and blocked with 5% non-fat dry milk. Membranes were then hybridized overnight at 4°C with a 1/1000 dilution of a GAPDH-HRP conjugated antibody (Santacruz, CA, USA) which was used as a reference antibody to adjust for variation in protein amounts spotted onto nitrocellulose membranes. All results were expressed as a ratio of test antibody/GAPDH chemoluminescent signals.

Human brain samples

For validation studies 10 non-demented (ND) control individuals (average age 80.7 years; range: 70-96 years) and 14 AD subjects (average age 80.3 years; range: 61-91 years) were selected. Brain samples were obtained from The Brain Donation Program at Banner Sun Health Research Institute. The operations of the Brain Donation Program and therefore the Neuropathology Core, including the autopsy consent form, have been subjected to and have been approved by the Institutional Review Board of Sun Health Corporation. Donors have all volunteered specifically for the program and all enrolled subjects or legal representatives have signed an Institutional Review Board-approved informed consent form.

All specimens had short postmortem delay times (NDC mean 3.6 h and AD 2.5 h). All AD cases fulfilled Consortium to Establish a Registry for AD (CERAD criteria) for definite and probable AD. One-hundred mg of gray matter from the frontal cortex was homogenized in 1 ml of RIPA buffer (Sigma, St. Louis, MO, USA) containing a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) with an Omni TH electric grinder (Kennesaw, GA, USA). The homogenates were centrifuged for 20 min at 14,000 x g (Beckman 22R centrifuge, Fullerton, CA, USA) and the supernatants collected for analysis. Tau phosphorylation and tau oligomerization were assessed by dot-blots as described above.

SDS-PAGE and Western Blot Analyzes

The expression of total tau, GSK3β (Ser9) phosphorylation and AKT (Thr308) phosphorylation were determined using SDS-polyacrylamide gel electrophoresis (PAGE) coupled with Western blot analysis of brain and spinal cord homogenates that were dissolved in Laemli buffer (Biorad, CA, USA) containing β-mercaptoethanol and boiled for 15 minutes. For the detection of tau oligomers by Western blot, brain and spinal cord homogenates were dissolved in Laemli buffer that did not contain β-mercaptoethanol (non reducing condition). Brain and spinal cord homogenates were separated on SDS 4-20% gradient polyacrylamide gels (Biorad, CA, USA) and then electrotransferred onto PVDF membranes (Biorad, CA, USA). Non-specific binding sites were blocked with 5% nonfat dry milk in Tris-buffered saline for 1 h at room temperature and then were incubated overnight at 4°C with a 1/1000 dilution of antibodies directed against either total tau (DA9) [29], pathological tau conformers (MC1), Phospho-Akt (Ser473) (D9E) XP and Phospho-GSK-3β (Ser9) (D85E12) XP antibodies (Cell Signaling technology, MA, USA). The following day, the membranes were washed 5 times with distilled water for 30 minutes and then incubated for 1 h at room temperature with a horseradish peroxidase-linked secondary anti-mouse or anti-rabbit antibody (1:1000 dilution) (Cell Signaling technology, MA, USA). The immunoreactive bands signal intensity were visualized and quantified by chemiluminescence imaging with the ChemiDocTM XRS (Bio-Rad, CA, USA). To adjust for protein loading variations from lane to lane, membranes were also immunoprobed (after stripping) with a 1/1000 dilution of a GAPDH-HRP conjugated antibody (Santacruz, CA, USA) or actin antibody (Chemicon, CA, USA) which were used as a reference antibody to quantify the amount of proteins electrotransferred. Protein sizes were estimated using proteins markers (Precision Plus Protein Standard, Biorad, CA, USA) and the ChemiDocTM XRS (Bio-Rad, CA, USA).

Statistical analyzes

Results are expressed as the mean ± SEM. Statistical analyzes were performed using SPSS V12.0.1 for Windows. Data were examined for assumption of normality using the Shapiro-Wilk statistic and for homogeneity of variance using the Levene's test. Statistical significance was determined by Student's t-test, univariate or repeated measures analysis of variance (ANOVA) where appropriate followed by post-hoc comparisons with Bonferroni corrections. For data not satisfying assumptions of normality and homogeneity of variance, a nonparametric Mann-Whitney test was used. P-values < 0.05 were considered significant.

Results

Effects of Anatabine on the behavior and motor impairments of Tg Tau P301S mice

Tg Tau P301S received anatabine in their drinking water at a dosage of 20 mg/Kg of body weight/24 hours or regular drinking water (placebo) for a period of 40 days. This dosage was selected based on our previous studies showing that at this dose anatabine displays an anti-inflammatory activity in the CNS [21-23]. During the time course of the treatment (40 days), 25% of Tg Tau P301S placebo mice became paralyzed (3 out of 12) whereas none (0 out of 11) in the anatabine treatment group developed paralysis during the study duration (Figure 1). All mice that displayed hind-limb paralysis were excluded from the behavioral studies. After 39 days of treatment with anatabine, non-paralyzed Tg Tau P301S were evaluated for clasping behavior in the hind-limb extension test and for motor coordination using the rotarod performance test. An extension reflex in the hind-limb is expected when a mouse is suspended by the tail, however partial or complete hind-limb retraction is commonly observed in mice with motor neuron disease [27]. Approximately 44 % (4 out of 9 non-paralyzed mice) of the placebo group (33 week-old mice) elicited an abnormal hind-limb extension reflex when suspended by the tail compared to 18% (2 out of 11) of Tg Tau P301S mice treated with anatabine (Figure 1). In addition, Tg Tau P301S mice treated with anatabine showed an increased latency to fall (T-test, $P<0.05$) from an accelerating rotarod apparatus compared to Tg Tau P301S mice receiving regular drinking water (Figure 2) showing improved motor coordination. After 21 days of treatment with anatabine, 31 week-old Tg Tau P301S were tested in the elevated plus maze to assess the impact of anatabine on

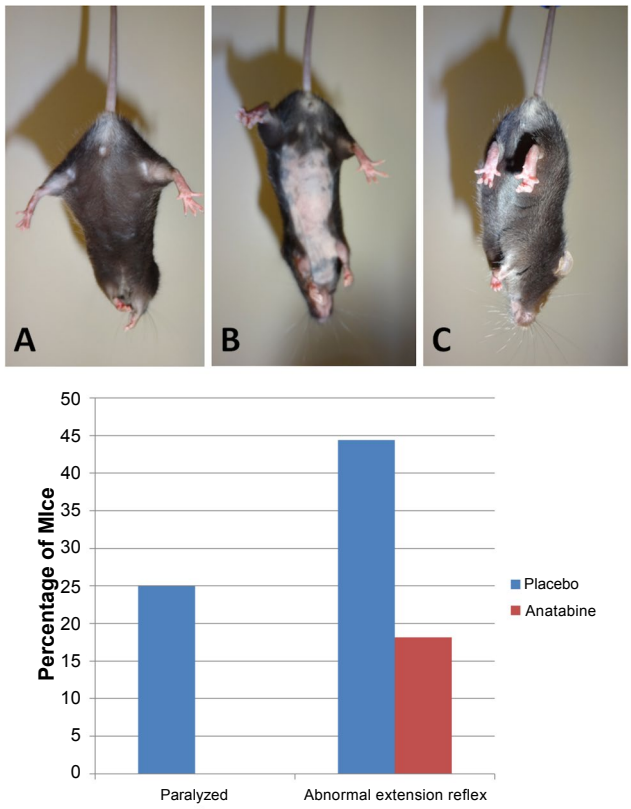

Figure 1: Impact of anatabine on the incidence of paralysis and abnormal hind-limb extension reflex in Tg Tau P301S. Representative Tg Tau P301S mice pictures showing a mouse with a normal hind-limb reflex (a) when suspended by the tail, an animal with a partial hind-limb extension reflex (b) and one mouse with a complete absence of extension reflex in both hind-limb (c). The histogram represents the proportion of Tg Tau P301S with complete posterior hind-limb paralysis and the proportion of non-paralyzed mice showing an abnormal hind-limb extension reflex in the placebo and anatabine treatment groups after 39 days of treatment.

anxiety related behavior. Tg Tau P301S mice treated with anatabine spent significantly more time (T-test, $P<0.05$) in the open arms of the elevated plus maze than placebo Tg Tau P301S mice (Figure 3). Spatial learning abilities which depend on hippocampal functions were assessed by the radial arm water maze (RAWM) [34] after 30 days of treatment with anatabine. Compared to age matched wild-type mice, 32 week-old Tg Tau P301S mice did not show spatial learning impairment in the RAWM (data not shown). A trend for a reduction in the number of errors elicited by anatabine treated Tg Tau P301S mice to locate the hidden platform in the RAWM was observed (data not shown).

Effect of anatabine treatment on tau phosphorylation and pathological tau conformers

The impact of the anatabine treatment on tau expression level was evaluated by western-blots in brain and spinal cord homogenates under denaturing and reducing conditions using the antibody DA9 which recognizes total tau [29]. No effect of the anatabine treatment was observed on tau expression (T-test, $P>0.05$) in Tg Tau P301S mice (Figure 4). To assess the effect of anatabine on tau phosphorylation in the brain and spinal cord of Tg P301S mice, we used 3 different antibodies recognizing pathological AD relevant phosphorylated epitopes (PHF-1, RZ3 and CP13). The MC1 antibody was used to detect pathological tau conformers [29-31], whereas the antibody TOC1 [33] was used to quantify tau oligomers. We employed a dot-blot approach to quantify tau phosphorylation and pathological tau conformers using non-reducing and non-denaturing conditions with detergent soluble and insoluble fractions of brain and spinal cord homogenates. We validated our dot-blot approach for the detection of pathological tau hyperphosphorylation and pathological tau conformers/oligomers by using human brain homogenates from AD and non-demented individuals. Our dot-blot approach unambiguously allowed the identification of non-demented individuals from AD patients showing that it is a suitable method for the quantification of AD hyperphosphorylated tau and AD pathological tau conformers/oligomers (Figure 5). In particular, T-test reveals statistically significant differences ($P<0.001$) for all the tau phosphorylated epitopes tested between AD and non-demented patients and a significant elevation in pathological tau conformer and tau oligomer levels($P<0.001$) in brain homogenates from AD compared to non-demented patients.

In both the detergent soluble and insoluble fractions of the brain

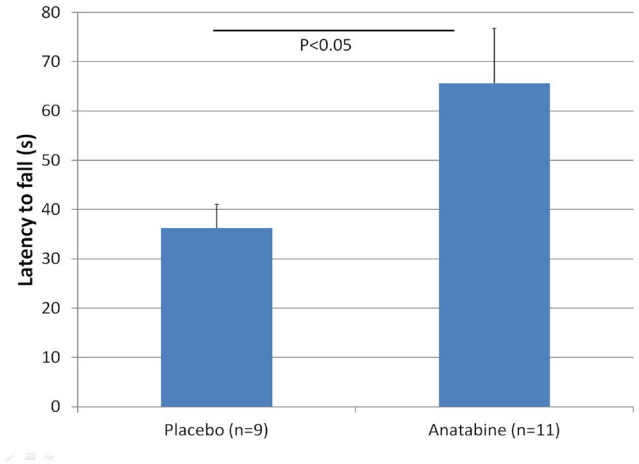

Figure 2: Chronic Treatment With Anatabine Improves Motor Performance In Tg Tau P301s Mice. The Histogram Depicts The Effect Of Anatabine On The Average Latency To Fall From An Accelerating Rotarod Apparatus After A Treatment Duration Of 39 Days.

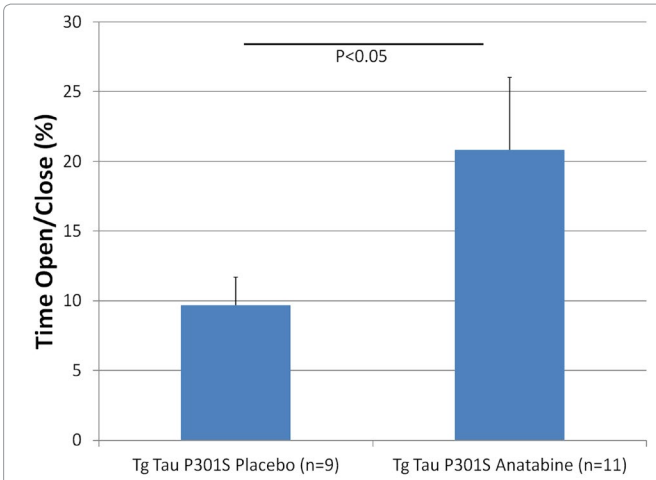

Figure 3: Effect of anatabine administration on anxiety assessed in the elevated plus maze. The histogram represents the average amount of time spent by Tg Tau P301S in the open arms of the elevated plus maze after a treatment duration of 21 days with anatabine.

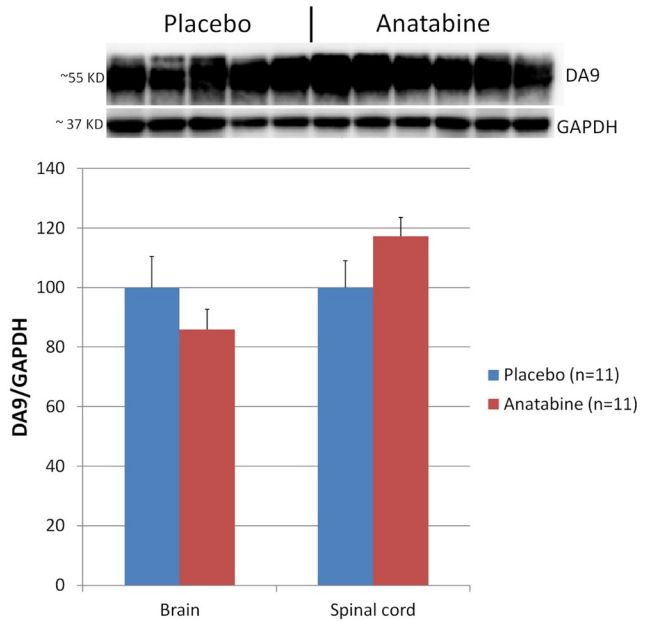

Figure 4: Impact of anatabine administration on total tau expression in the brain and spinal cord of Tg Tau P301S. A representative western-blot depicting the amount of total tau (detected with the antibody DA9) in brain homogenates of placebo and anatabine treated Tg Tau P301S mice is shown. GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) was used as a reference protein to correct for possible unequal loading and electrotransfer from lane to lane. The histogram represents the average amount of total tau (DA9/GAPDH chemiluminescent signals) quantified in the brain and spinal cord homogenates of placebo and anatabine treated Tg P301S mice.

and spinal cord homogenates of Tg Tau P301S mice, we found that tau phosphorylation was significantly reduced (T-tests, $P<0.05$) by the anatabine treatment for all the AD phosphorylated epitopes tested (Figure 6). Moreover, a significant reduction in MC1-immunoreactivity ($P<0.05$) was observed in the detergent soluble and insoluble fractions of brain and spinal cord homogenates from Tg Tau P301S mice treated with anatabine showing that anatabine prevents the formation of pathological tau conformers (Figure 7). Additionally, a significant reduction ($P<0.05$) in tau oligomers levels (TOC1 immunopositive) was detected both in the brain and the spinal cord of Tg Tau P301S treated with anatabine using dot-blots (Figure 8).

We also subjected the detergent soluble fractions of brain and spinal cord homogenates to western-blotting to detect tau oligomers using non-reducing conditions as tau oligomer formation involves disulfide cross-linking between tau molecules [30,35]. MC1 immunopositive tau oligomers were identified in brain and spinal cord homogenates from Tg Tau P301S mice by western-blotting whereas tau monomers (~50-55 KD) were only weakly immunoreactive to the conformational antibody MC1 (Figure 9) under non-reducing condition. The most abundant tau oligomeric species immunopositive for MC1 detected in brain homogenates of Tg Tau P301S mice appear to migrate at an approximate size of 290 and 420 KDa. When the western-blots were overexposed additional MC1 immunopositive materials of an approximate size of 590, 160 and 100 KD were also revealed (data not shown). We observed that anatabine significantly prevented the formation of MC1-positive tau oligomers (Figure 9) by western-blots in both the brain and the spinal cord of Tg Tau P301S mice (T-tests, $P<0.05$) further confirming the data obtained with the dot-blots. We evaluated the amount of Iba1 immunoreactivity by dot-blotting in brain and spinal cord homogenates as a marker of microgliosis [36-38]. A significant reduction in Iba1 immunoreactivity was observed both in the brain (T-test, $P<0.01$) and spinal cord (T-test, $P<0.01$) homogenates of Tg Tau P301S mice treated with anatabine. Figure 10.

Impact Of Anatabine On Brain Akt And Gsk3β Phosphorylation

Glycogen synthase 3β (GSK3β) is one of the main serine-threonine kinase responsible for tau phosphorylation and has been shown to affect tau phosphorylation at multiple AD relevant epitopes including Ser396, Ser404, Thr231 and Ser202 [39-40]. GSK3β is inactivated

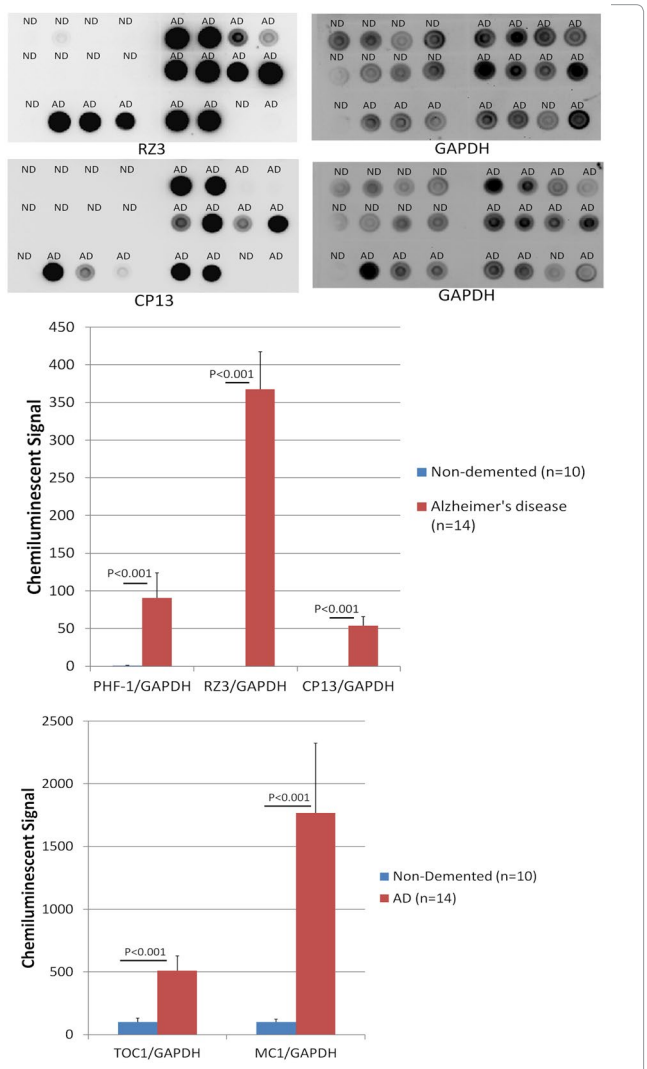

Figure 5: Quantification of tau hyperphosphorylation, pathological tau conformers and oligomers in brain homogenates from non-demented and AD patients by dot-blots. Representative dot-blots revealing the amount of CP13 (Ser202) and RZ3 (Thr231) tau phosphorylation in brain homogenates from non-demented (ND) and AD patients (AD). Protein content in each sample was normalized using GAPDH as a reference protein. The histogram represent the average CP13/GAPDH, PHF-1 (Ser396/Ser404)/GAPDH, RZ3/GAPDH chemiluminescent signals quantified in brain homogenates from AD and non-demented patients. Pathological tau conformer and tau oligomer levels were quantified with the antibodies MC1 and TOC1 respectively. The histogram represents the average MC1/GAPDH and TOC1/GAPDH chemiluminescent signals quantified in brain homogenates from AD and non-demented patients.

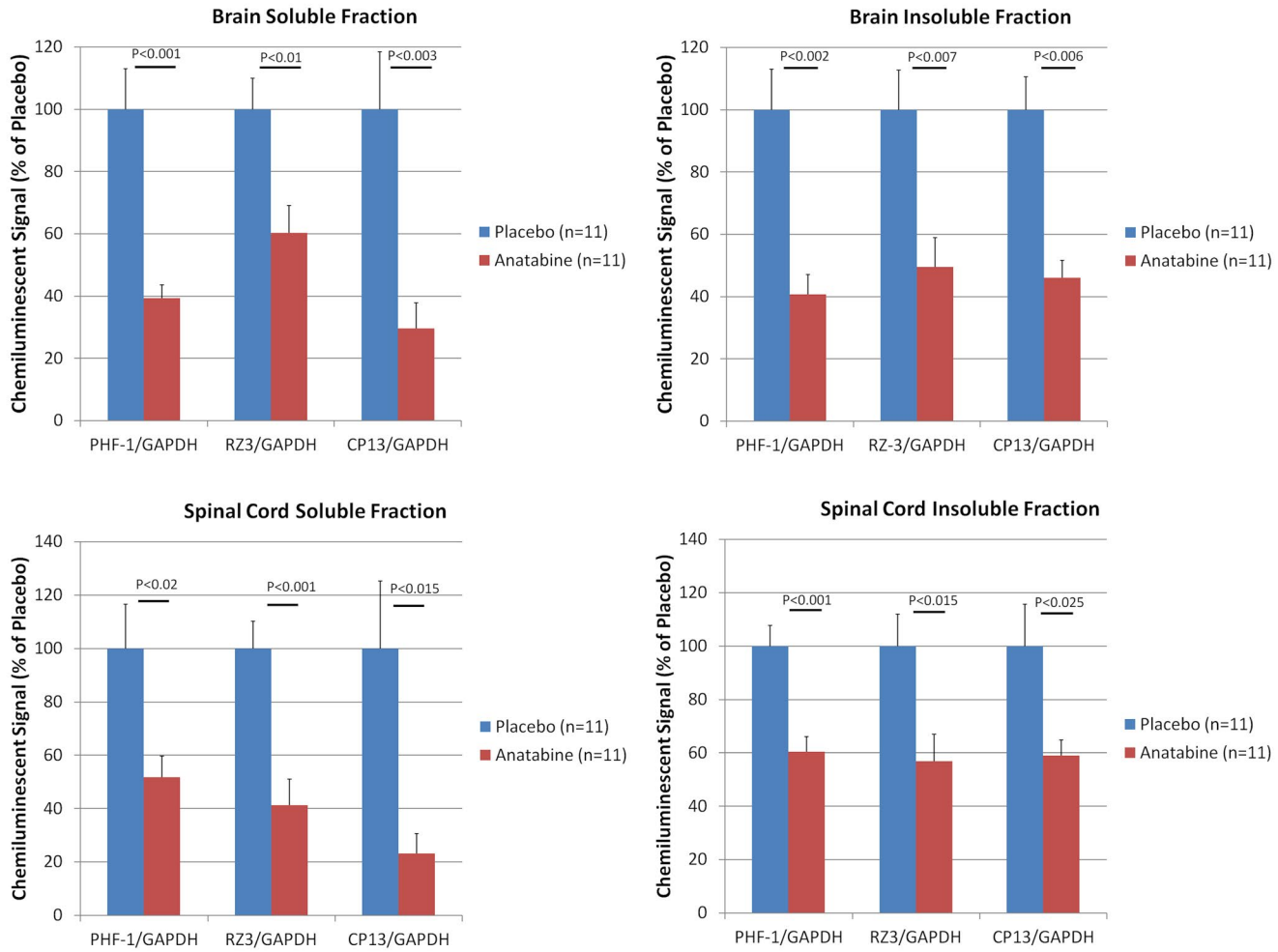

Figure 6: Effect of anatabine administration on the level of tau hyperphosphorylation in detergent soluble and insoluble fractions of the brain and spinal cord of Tg Tau P301S mice. The histograms represent the average values of PHF-1/GAPDH, RZ3/GAPDH and CP13/GAPDH chemiluminescent signals quantified by dot-blots in brain and spinal cord detergent soluble and detergent insoluble fractions.

upon phosphorylation of Ser9 by protein kinase B (AKT) [41] whereas AKT phosphorylation at Ser473 results in AKT activation [42]. We therefore evaluated the possible impact of the anatabine treatment on GSK3βSer9 and AKT Ser473 phosphorylation. We found a significant increase in brain AKT Ser473 phosphorylation (T-test, P<0.001) and GSK3β Ser9 phosphorylation (T-test, P<0.001) in Tg Tau P301S mice treated with anatabine. Figure 11.

Discussion

We previously reported that anatabine lowers Aβ production both in vitro and in vivo and readily crosses the blood-brain barrier, suggesting it could represent a potential compound for the treatment of AD [22]. While searching for the mechanism of action of anatabine, we delineated the signaling pathways impacted by this compound. In particular, we identified that anatabine prevents the activation of NFkB and STAT3 signaling pathways which are known to regulate inflammatory processes as well as the expression of BACE-1, the rate limiting enzyme responsible for the production of Aβ peptides [21-23]. Late stage AD clinical trial using compounds that directly target Aβpeptides have been unsuccessful suggesting that once Aβ deposition reaches a certain level targeting it may not be helpful. As AD is defined by the accumulation of Aβ deposits and by the intraneuronal inclusion of hyperphosphorylated tau proteins, therapies targeting both Aβ and tau pathologies are expected to be superior to therapeutic interventions solely targeting Aβ. Since anatabine prevents the phosphorylation of STAT3 and NFkB [21-23], we hypothesized that anatabine likely reduces the activity of kinases upstream of these transcription factors which may also be responsible for tau phosphorylation. We therefore investigated here the impact of anatabine on tau phosphorylation in a pure model of tauopathy (Tg Tau P301S mice) to determine whether this compound is capable of reducing both Aβ and tau pathologies in preclinical models.

The anatabine treatment was initiated in 28 week-old (~6 and 1/2 months) Tg Tau P301S and lasted for 40 days. At that age, Tg Tau P301S mice (PS19 line) display hind-limb retraction when lifted by the tail which is indicative of disease progression in various mouse model of neurodegeneration [20,27]. This progresses to limb weakness, brain atrophy and eventually paralysis at 7 months of age in Tg Tau P301S mice [20]. Anatabine which is bioavailable orally was administered in the drinking water of the animals for a period of 40

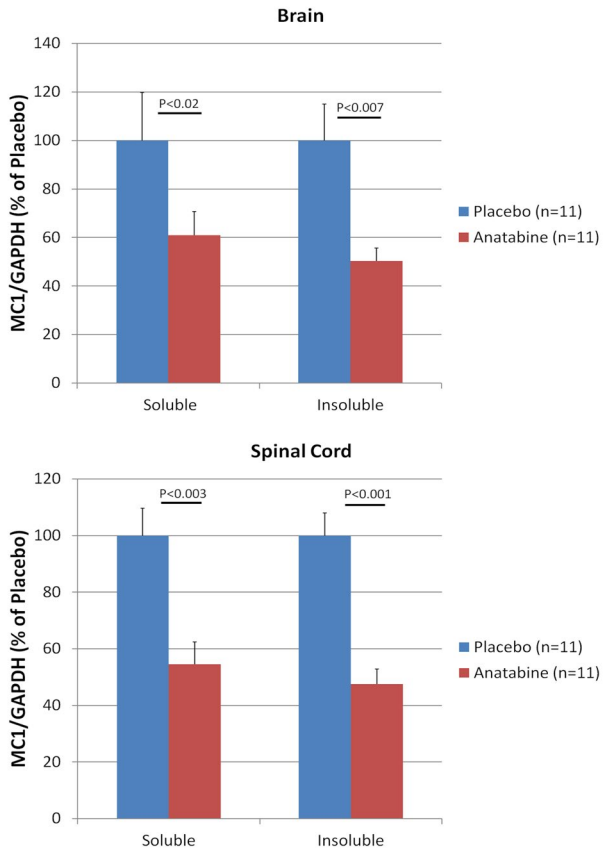

Figure 7: Impact of anatabine administration on the level of pathological tau conformers in the brain and spinal cord of Tg Tau P301S mice. The histograms represent the average pathological MC1/GAPDH chemiluminescent signal quantified in detergent soluble and insoluble fractions of brain and spinal cord homogenates.

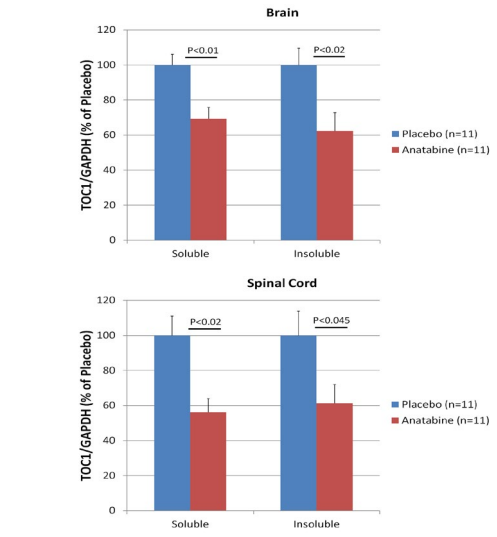

Figure 8: Effect of anatabine administration on the level of tau oligomers in the brain and spinal cord of Tg Tau P301S mice. The histogram represents the average TOC1/GAPDH chemiluminescent signal quantified in detergent soluble and insoluble fractions of brain and spinal cord homogenates .

days. During the treatment period, the behavior of Tg Tau P301S mice was evaluated using a battery of behavioral tests. Our data show that anatabine improves locomotor coordination in Tg Tau P301S mice since the latency to fall from an accelerating rotarod apparatus was significantly increased (by approximately 2 fold) in anatabine treated mice. In addition, abnormal hind-limb extension reflex and hind-limb paralysis were reduced by anatabine in Tg Tau P301S suggesting that anatabine could prevent disease progression in Tg Tau P301S mice although a larger cohort of mice will be required to confirm this observation. Examination of multiple pertinent AD phosphorylated tau epitopes show that anatabine reduces tau phosphorylation both in detergent soluble and insoluble fractions of the brain and spinal cord homogenates of Tg Tau P301S mice. We found in particular that tau phosphorylation at Ser396/Ser404 (PHF-1), Thr231 (RZ3) and Ser202 (CP13) was significantly reduced in the brain and spinal cord of Tg Tau P301S treated with anatabine. Our data are consistent with previous data showing that inhibition of tau hyperphosphorylation can prevent motor impairments and delay disease progression in transgenic mouse model of tauopathy [43] and suggest that the reduction in tau phosphorylation induced by anatabine could be responsible for the prevention of locomotor deficits in Tg Tau P301S mice. We did not find that spatial learning was impaired in Tg Tau P301S compared to wild-type mice using the Morris water maze or the radial arm water maze (RAWM) (data not shown) in agreement with previous reports showing an absence of cognitive impairment or minimal alteration of cognition despite extensive tau pathology and presynaptic pathology in hippocampal neurons in various mouse model of tauopathy [44,45]. A trend for a reduction of the number of errors to locate the hidden platform in the RAWM was observed in anatabine treated Tg Tau P301S mice suggesting that anatabine may facilitate spatial learning in Tg Tau P301S mice (data not shown). In addition, we found that anatabine prolongs the time spent by Tg Tau P301S in the open arms of the elevated plus maze suggesting that anatabine reduces anxiety-like behavior.

Several studies have shown that soluble oligomers of tau (rather than high molecular mass aggregates of tau present in neurofibrillary tangle) are the main tau pathological species responsible for neurodegeneration [32,43,46,47]. In particular, passive immunization using the conformational antibody MC1 appears superior at reducing tau pathology and hind-limb weakness in a mouse model of tauopathy than a high affinity pan-tau antibody [48,49] highlighting the importance of MC1 pathological tau conformers in the development of neurodegenerative tauopathy. Interestingly, MC1 recognizes pathological conformations of tau in the brain that occur at an early stage of AD preceding tau aggregation in neurofibrillary tangles [11,31,50,51]. In addition, passive immunization with a selective tau oligomer antibody has been shown to reverse taupathy phenotypes without affecting neurofibrillary tangles further emphasizing the critical role of tau oligomers in the development of tauopathy [52]. Using a selective oligomeric tau antibody (TOC1) which preferentially recognizes tau oligomers over monomers or aggregated polymers [33] , we observed a reduction in TOC1 imunoreactivity in the brain and spinal cord of Tg Tau P301S mice treated with anatabine. We also tested the impact of anatabine on the levels of MC1 pathological tau conformers in the brain and spinal cord of Tg Tau P301S mice by dot blotting. Our data show that anatabine reduces the amount of MC1 pathological tau conformers in both the brain and spinal cord of Tg Tau P301S mice. Western-blot analysis of brain and spinal cord homogenates from Tg Tau P301S under non-reducing condition reveals the presence of MC1 immunopositive tau oligomers. The

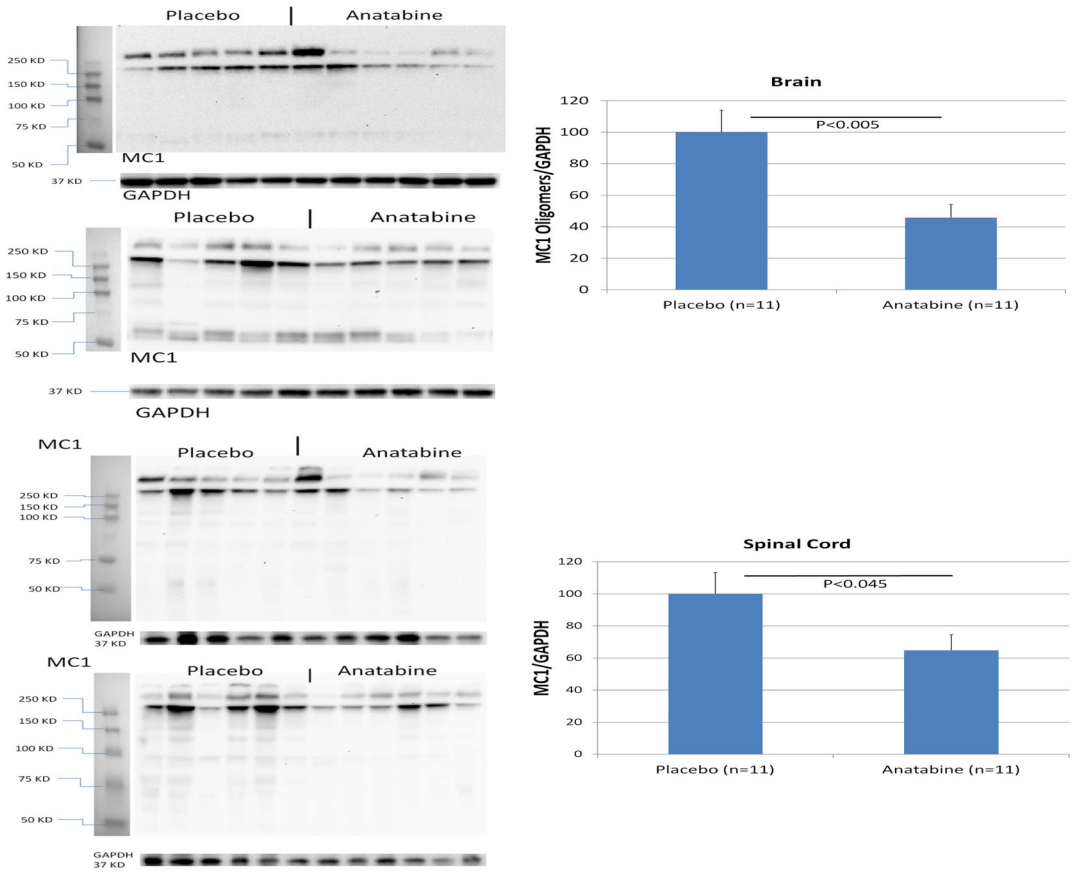

Figure 9: Quantification of MC1 immunopositive tau oligomers by western-blots in the brain and spinal cord of Tg Tau P301S. Representative western-blots revealing MC1 immunopositive tau oligomers in the brain and spinal cord (detergent soluble fraction, non-reducing conditions were used) of Tg Tau P301S of the placebo and anatabine treatment group. The histogram represents the quantification of MC1 tau oligomers in the brain and spinal cord of Tg Tau P301S mice.

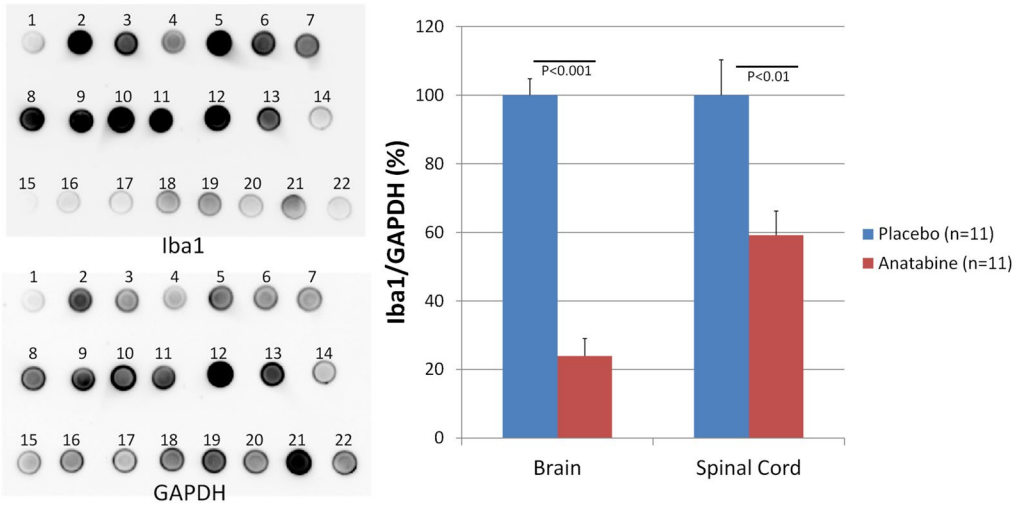

Figure 10: Effect of the anatabine treatment on brain and spinal cord Iba1 levels. A) A representative dot blot showing the level of Iba1 and GAPDH (as a reference protein) in brain homogenates from placebo Tg Tau P301S mice (1 to 11) and from Tg Tau P301S mice treated with anatabine (12 to 22) is shown. B) The histogram represents the quantification of Iba1/GAPDH chemiluminescent signal observed in the brain and spinal cord homogenates of Tg Tau P301S receiving the placebo and anatabine.

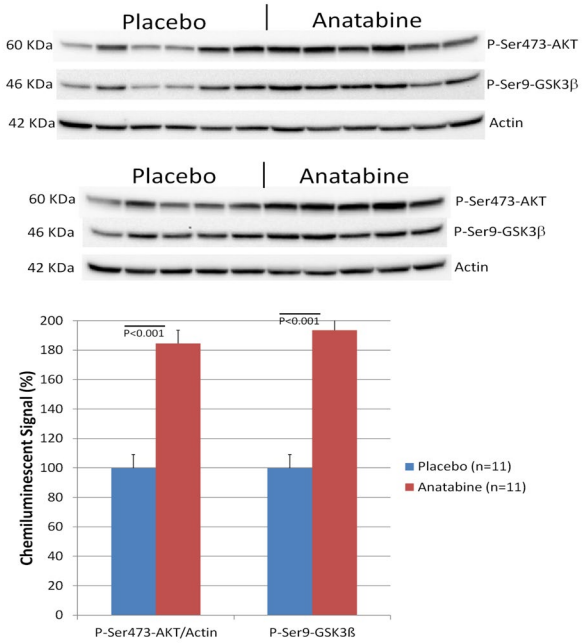

Figure 11: Effect of anatabine on the activation of AKT and GSK3β in the brain of Tg Tau P301S mice. A representative western-blot of brain homogenates from placebo and anatabine treated Tg Tau P301S mice showing phosphorylated AKT at Ser473, phosphorylated GSK3β at Ser9 and actin as a reference protein is shown. The histogram represents the average values of phosphorylated AKT/Actin and phosphorylated GSK3β/Actin for the brains of Tg Tau P301S mice of the placebo and anatabine treatment groups.

levels of these MC1 tau oligomers detected by Western-blots appear significantly decreased in the brain and spinal cord of anatabine treated Tg Tau P301S mice further confirming the data obtained with the dot blot approach showing a reduction in tau oligomers in anatabine treated mice. While anatabine reduces tau phosphorylation and tau oligomers in detergent soluble and insoluble fractions of brain homogenates, further work will be required to determine whether anatabine can also prevent the formation of neurofibrillary tangle although it is apparent that tau oligomers rather than neurofibrillary tangles plays a critical role in disease progression [52].

Microglial activation is one of the earliest brain pathologies in Tg Tau P301S and has been shown to correlate with hipocampal atrophy in this tauopathy model [20]. Interestingly, inhibition of microglial activation using an immunosuppressant prevented hipocampal atrophy, neuronal loss as well as tau hyperphosphorylation in Tg Tau P301S mice [20] suggesting a mechanistic link between microglial activation and the progression of tau pathologies. We therefore evaluated Iba1 level in brain and spinal cord homogenates of Tg Tau P301S mice as Iba1 expression is increased in activated microglia [36]. We observed that anatabine significantly suppressed Iba1 expression in the brain and spinal cord of Tg Tau P301S suggesting that anatabine is opposing microglial activation in Tg Tau P301S mice. The reduction in microgliosis induced by anatabine in Tg P301S mice may be consequential to the reduction of tau phosphorylation and oligomerization. We have shown however, that in a mouse model of multiple sclerosis, anatabine can mitigate microgliosis in the brain and the spinal cord [23] which could suggest that anatabine can directly oppose microgliosis in Tg Tau P301S mice. Microgliosis occurs prior to any significant tau pathology in Tg Tau P301S mice and also contributes to tau phosphorylation and oligomerization [20]. The anti-inflammatory activity of anatabine may therefore also play an important role in the mitigation of tau phosphorylation and oligomerization observed after anatabine treatment in Tg Tau P301S mice.

Anatabine displays a chemical structure closely related to nicotine and is therefore anticipated to act as an agonist of nicotinic acethylcholine receptors (nAChR). Our previous data showing that anatabine inhibits both STAT3 and NFkB phosphorylation [21-23] support this contention as nAChR agonists typically elicit an anti-inflammatory activity by blunting both STAT3 and NFkB activation [53-55]. Recent data have shown that anatabine is a preferential α7 nAChR agonist and a weaker or partial α4β2 nAChR agonist [56]. Interestingly, selective α7 nAChR agonists have been shown to prevent tau phosphorylation both in vitro and in vivo by stimulating protein kinase B (AKT) resulting in the inactivation of glycogen synthase kinase3β, one of the main kinase responsible for tau hyperphosphorylation [57-59]. We therefore investigated the possible impact of anatabine on AKT and GSK3β activation in the brain of Tg Tau P301S mice. We observed an activation of AKT (increased phosphorylation at Ser473) as well as an increased in GSK3β phosphorylation at Ser9 (indicative of GSK3β

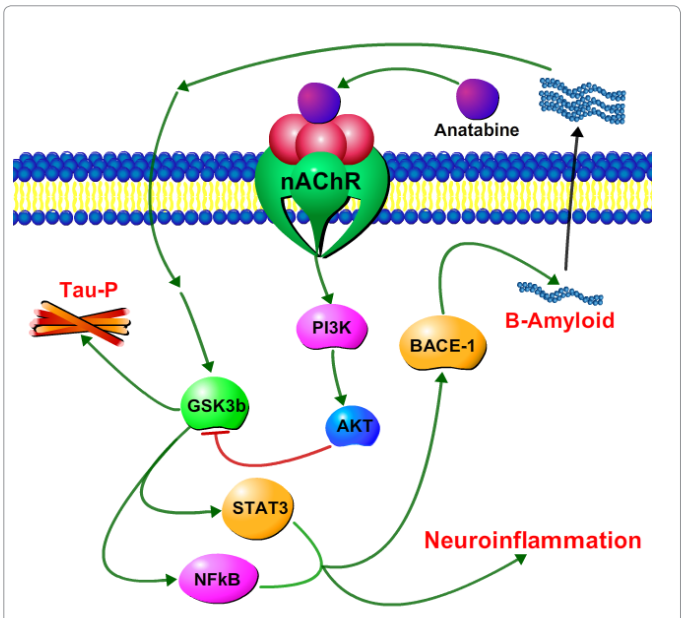

Figure 12: Proposed hypothesis for the mechanism of action of anatabine in AD. In AD, excessive accumulation of Aβ peptides results in GSK3β activation and tau hyperphosphorylation, followed by tau oligomerization and neurodegeneration. Increased GSK3β activity in AD will also induce the activation of the transcription factors STAT3 and NFκB which have central roles in inflammatory reactions, regulating many proteins involved in neuroinflammation including cytokines. Additionally, STAT3 and NFκB activation will induce BACE-1 expression (the rate limiting enzyme responsible for Aβ production), possibly resulting in a self propagating pathway contributing to both Aβ production/accumulation, neuroinflammation and tau hyperphosphorylation. Upon binding of anatabine to nicotinic acetylcholine receptors (nAChR) and stimulation, the survival signal transduction pathway PI3K and AKT is activated, leading to an increased AKT dependent Ser9 phosphorylation of GSK3β which dominantly inhibits its activity, resulting in decreased tau phosphorylation at multiple serine and threonine residues. Following the inactivation of GSK3β by anatabine, a decreased activation of STAT3 and NFκB will occur, resulting in decreased neuroinflammation, reduced BACE-1 expression and Aβ production. It is therefore anticipated that anatabine has the potential to mitigate the three main pathologies associated with AD: tau hyperphosphorylation, Aβ accumulation and neuroinflammation.

inactivation) in the brain of Tg Tau P301S mice treated with anatabine which could explain the reduction in tau phosphorylation observed in anatabine treated mice and provide support for a mechanism involving a stimulation of α7 nAChR. A 50% increase in the phosphorylation of the inhibitory residue (Ser9) of GSK3β was also observed in the spinal cord of Tg Tau P301S mice treated with anatabine (data not shown). We have shown that anatabine opposes neuroinflammation by preventing the activation of NFkB and STAT3 [21-23]. It is likely that the inhibition of STAT3 and NFkB observed is consequent to the inactivation of GSK3β by anatabine, as both STAT3 and NFkB activation are highly dependent on GSK3β activity [60,61]. We have summarized on Figure 12 a possible mechanism of action for anatabine that highlights its potential therapeutical application in AD. Further mechanistic work will be required to precisely identify the mode of action of anatabine responsible for the prevention of tau phosphorylation and determine whether it is mainly mediated via a reduction of microgliosis/neuroinflammation, an inhibition of the kinases responsible for tau phosphorylation or a combination of both.

Our data provide in vivo evidence that a chronic oral treatment with anatabine reduces pathological tau hyperphosphorylation and the formation of pathological tau conformers/oligomers leading to a reduction of tau-related functional deficits in Tg Tau P301S mice. These data support further exploration of anatabine as a possible disease modifying agent for tau-associated neurodegenerative conditions and, in particular AD, since we have shown that anatabine also displays Aβ lowering properties [22].

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