

Research Article

Recombinant Hc-TeTx modulates TrkA-related signaling and preserves cholinergic integrity in the nucleus basalis magnocellularis after A β (25–35) injury

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ABSTRACT

The recombinant C-terminal domain of tetanus toxin (Hc-TeTx) has been reported to exert neurotrophic-like effects in experimental models of neurodegeneration, potentially through activation of signaling pathways associated with tropomyosin receptor kinase A (TrkA). However, its effects on basal forebrain cholinergic dysfunction induced by amyloid- β remain incompletely characterized. The present study evaluated whether administration of Hc-TeTx in the nucleus basalis magnocellularis (NBM) is associated with attenuation of Amyloid beta peptide 25–35 (A β _(25–35))-induced alterations in cognitive function, cholinergic markers, and related signaling pathways. Adult rats received bilateral intracranial injections of Hc-TeTx (50 ng per hemisphere) followed by A β _(25–35) (1 μ g per hemisphere) into the NBM. Spatial learning and memory were assessed using the eight-arm radial maze. Biochemical analyses included acetylcholinesterase (AChE) activity, vesicular acetylcholine transporter (VACHT) expression, and phosphorylation of TrkA, Akt, and GSK-3 β . Astrocytic reactivity was evaluated by GFAP immunoreactivity in the NBM, frontal cortex, and temporal cortex. A β _(25–35) administration impaired spatial learning and memory, reduced AChE activity and VACHT expression, altered TrkA/Akt/GSK-3 β signaling, and increased GFAP immunoreactivity. In contrast, Hc-TeTx treatment was associated with improved behavioral performance, preservation of cholinergic markers, modulation of TrkA-related signaling, and reduced astrocytic reactivity. These findings indicate that Hc-TeTx administration in the NBM is associated with attenuation of A β _(25–35)-induced cholinergic dysfunction and related molecular alterations. However, the underlying mechanisms remain to be fully established, and the present results should be interpreted as associative rather than demonstrating a direct causal role of TrkA signaling.

Abbreviations: A β , Amyloid-beta; A β (25–35), Amyloid-beta peptide fragment 25–35; A β (35–25), Reverse amyloid-beta peptide fragment (control); ACh, Acetylcholine; AChE, Acetylcholinesterase; Akt, Protein kinase B; ANOVA, Analysis of variance; BBB, Blood–brain barrier; DTNB, 5,5′-Dithio-bis-(2-nitrobenzoic acid); EDTA, Ethylenediaminetetraacetic acid; EGTA, Ethylene glycol tetraacetic acid; GFAP, Glial fibrillary acidic protein; GFAP-ir, GFAP immunoreactivity; GSK-3 β , Glycogen synthase kinase 3 beta; Hc-TeTx, C-terminal fragment of tetanus toxin; i.p., Intraperitoneal; i.m., Intramuscular; ISS, Isotonic saline solution; MAPK, Mitogen-activated protein kinase; NBM, Nucleus basalis magnocellularis; NGF, Nerve growth factor; PBS, Phosphate-buffered saline; PI3K, Phosphoinositide 3-kinase; PMSF, Phenylmethylsulfonyl fluoride; PVDF, Polyvinylidene difluoride; RIPA, Radioimmunoprecipitation assay buffer; SDS, Sodium dodecyl sulfate; SEM, Standard error of the mean; TBS, Tris-buffered saline; TRIS, Tris(hydroxymethyl)aminomethane; VACHT, Vesicular acetylcholine transporter.

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Introduction

Alzheimer's disease (AD) is the most common form of dementia, accounting for approximately 60–70% of cases worldwide (World Health Organization, 2025). It is characterized by progressive cognitive decline associated with extracellular amyloid- β ($A\beta$) deposition, intracellular tau pathology, and a marked loss of cholinergic innervation, particularly in the basal forebrain (DeTure and Dickson, 2019; Maccioni et al., 2001; Selkoe, 1991).

Among basal forebrain structures, the nucleus basalis magnocellularis (NBM) plays a critical role in cognitive function by providing widespread cholinergic projections to the frontal cortex and temporal cortex (Bierer et al., 1995; Chudasama et al., 2004; Croxson et al., 2011). Degeneration of NBM cholinergic neurons leads to impaired cortical cholinergic transmission and contributes to deficits in learning and memory (Bartus et al., 1985; Mandel et al., 1989). Experimental models based on Amyloid beta peptide 25–35 ($A\beta_{(25-35)}$) administration reproduce key aspects of cholinergic dysfunction, including reduced levels of AChE, VAcHT, and other cholinergic markers, as well as neuro-inflammatory responses and neuronal damage (Diaz et al., 2012; Harkany et al., 2000; Harkany et al., 1999; Harkany et al., 1998; Limón et al., 2009; Ortega et al., 2014; Patricio-Martínez et al., 2016).

Basal forebrain cholinergic neurons are highly dependent on nerve growth factor (NGF) signaling through the TrkA receptor, which activates multiple intracellular pathways, including PI3K/Akt and MAPK, that regulate neuronal survival and differentiation (Datta et al., 1997; Kaplan and Miller, 2000; Sofroniew et al., 2001). Dysregulation of the Akt/GSK-3 β pathway has been associated with neurodegenerative processes, including tau phosphorylation and neural vulnerability (Baki et al., 2004; De Simone et al., 2021; Takashima, 2006). Although NGF has been proposed as a therapeutic strategy, its clinical application is limited by poor blood–brain barrier (BBB) permeability and the need for invasive delivery (Weissmiller and Wu, 2012).

The non-toxic C-terminal fragment of tetanus toxin (Hc-TeTx) has emerged as a molecule with neurotrophic-like properties. Previous studies have shown that Hc-TeTx can be retrogradely transported within the central nervous system and may interact with neurotrophin-related signaling pathways (Deinhardt et al., 2006; Gil et al., 2003; Gil et al., 2000). Moreover, Hc-TeTx has been reported to exert neuroprotective effects in multiple experimental models, including excitotoxicity (Chaïb-Oukadour et al., 2004; Chaïb-Oukadour et al., 2009; Herrando-Grabulosa et al., 2013; Mendieta et al., 2009), amyotrophic lateral sclerosis and ischemia (Moreno-Igoa et al., 2010; Moreno-Martínez et al., 2020; Netzahualcoyotzi and Tapia, 2018; Radenovic et al., 2014), and neurodegenerative conditions affecting dopaminergic (Mendieta et al., 2012; Mendieta et al., 2016; Patricio et al., 2022; Patricio et al., 2019; Sánchez-González et al., 2014) and cholinergic systems (Patricio-Martínez et al., 2016).

Despite this evidence, the effects of Hc-TeTx in models of $A\beta$ -induced cholinergic dysfunction specifically targeting the NBM and its cortical projections remain insufficiently characterized. In particular, it is unclear whether Hc-TeTx is associated with preservation of cholinergic markers and modulation of TrkA-related signaling pathways in this context. Although $A\beta_{(25-35)}$ models reproduce key aspects of cholinergic dysfunction, they represent an acute and focal injury paradigm and do not capture the progressive and multifactorial nature of Alzheimer's disease. Therefore, the present study aimed to evaluate whether administration of Hc-TeTx in the NBM is associated with attenuation of $A\beta_{(25-35)}$ -induced alterations in cognitive performance, cholinergic markers acetylcholinesterase (AChE) and vesicular acetylcholine transporter (VAcHT), astrocytic reactivity, and components of the TrkA/Akt/GSK-3 β signaling pathway.

Materials and methods

Animals

Adult male Wistar rats (250–300 g; total $n = 66$) were obtained from the Claude Bernard animal facilities at the Benemérita Universidad Autónoma de Puebla (BUAP or Meritorious Autonomous University of Puebla). Animals were housed individually in groups of 4–6 per cage under constant temperature conditions ($22 \pm 2^\circ\text{C}$; 12 h light/dark cycle, lights on at 08:00 h), with *ad libitum* access to food and water. All experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and the Mexican Official Standard NOM-062-ZOO-1999. The study protocol was approved by the Institutional Animal Care and Use Committee of BUAP (approval number: 100125355-UALVIEP-22/2).

Hc-TeTx and $A\beta_{25-35}$ preparation

The recombinant C-terminal domain of tetanus toxin (Hc-TeTx, ~50 kDa) was produced as previously described (Herrando-Grabulosa et al., 2013), lyophilized, and stored at -20°C until use. The fragment exhibited a purity of 90–95% and corresponded to amino acids 864 to 1315 of the native tetanus toxin. $A\beta_{(25-35)}$ and the reverse control peptide $A\beta_{(35-25)}$ (Sigma-Aldrich Ltd., St. Louis, MO, USA) were dissolved in sterile isotonic saline solution (ISS) to a final concentration of $1\text{ }\mu\text{g}/\mu\text{L}$. To induce aggregation, peptide solutions were incubated at 37°C for 36 h prior to administration.

Stereotaxic surgery

Each animal was anesthetized with ketamine-xylazine (75/10 mg/kg, i.p.) and then randomly assigned to the experimental groups: Intact (no surgery), $A\beta_{(35-25)}$ (control peptide), Hc-TeTx, $A\beta_{(25-35)}$, and Hc-TeTx + $A\beta_{(25-35)}$ all of which were administered in the NBM ($n = 12$ –14 per group). Each animal was placed in a stereotaxic apparatus (Stoelting Co., Wood Dale, IL, USA) for the administration of the $A\beta_{(35-25)}$, $A\beta_{(25-35)}$, or Hc-TeTx fragment. Each solution was injected using a $10\text{ }\mu\text{L}$ Hamilton syringe with each $1\text{ }\mu\text{L}$ infused for 300 s. Fifty ng of Hc-TeTx was dissolved in $1\text{ }\mu\text{L}$ of vehicle, followed by $1\text{ }\mu\text{g}/\mu\text{L}$ of $A\beta_{(25-35)}$ peptide, with the solution obtained then bilaterally injected into the NBM, with a 10-min interval applied between the injection of each agent at the following coordinates, according to (Paxinos and Watson, 1998): AP -0.6 mm from the bregma; ML 2.7 mm from midline; and DV, -6.5 below the dura. Postoperative care included administration of tramadol (12.5 mg/kg , s.c.) and enrofloxacin (10 mg/kg , i.m.) immediately after surgery and for the following two days. Animals were monitored daily for recovery, wound healing, and signs of distress.

Behavioral testing

The temporal sequence of the experimental procedures is illustrated in Fig. 1A. To evaluate the learning and memory process, the eight-arm radial maze was used, according to the protocol previously reported by (Patricio-Martínez et al., 2016; Patricio-Martínez et al., 2019). Briefly, beginning on day 10 post-surgery, animals were food-restricted over a 10-day period to maintain their body weight at approximately 85% of their free-feeding weight throughout the behavioral testing phase. Prior to training, all animals were habituated to the apparatus over the course of one day without any reward bait. The 15-min habituation period was repeated three times a day, at intervals of more than 1 h. Spatial learning was evaluated on days 20–21 post- $A\beta_{(25-35)}$ injection. Each animal underwent 20 trials distributed over two consecutive days (10 trials/day). At the beginning of each trial, rats were placed on the central platform and allowed to freely explore the maze until either the maximum number of arm entries was reached or 200 s had elapsed. Three of the eight arms (arms 2, 5, and 6) were consistently baited with food pellets

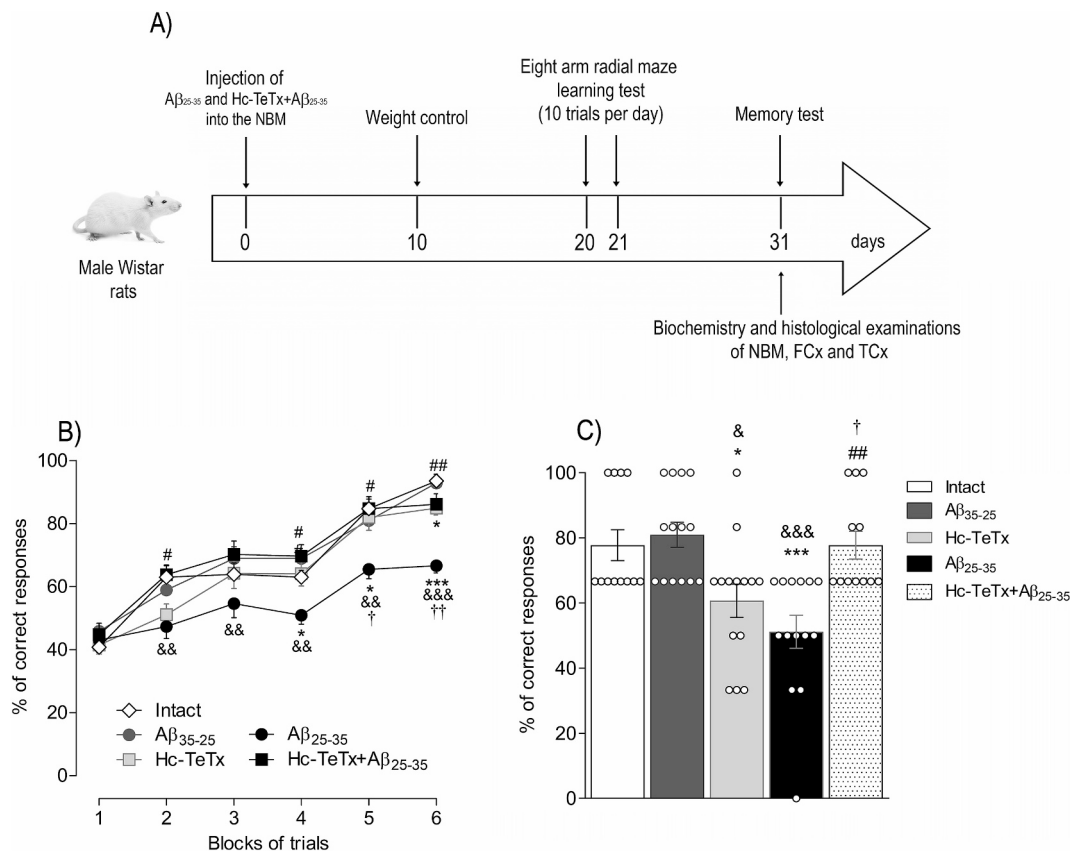


Fig. 1. Behavioral assessment and experimental design. (A) Experimental timeline and treatment groups: Intact, Aβ_(35–25), Hc-TeTx, Aβ_(25–35), and Hc-TeTx + Aβ_(25–35). Spatial learning was evaluated on days 20–21, and memory was assessed on day 31 using the eight-arm radial maze. After behavioral testing, biochemical and histological analyses were performed in the nucleus basalis magnocellularis (NBM), frontal cortex, and temporal cortex. (B) Spatial learning performance expressed as the percentage of correct responses across six acquisition trial blocks in the eight-arm radial maze. Data are presented as mean ± SEM (n = 12–14 animals per group). Statistical analysis was performed using a mixed-effects model (REML), with trial block as the repeated-measures factor and treatment as the between-subject factor, followed by Tukey's multiple comparisons test. *P < 0.05 and ***P < 0.001 vs intact group; &P < 0.01 and &&P < 0.001 vs Aβ_(35–25) group; †P < 0.05 vs Hc-TeTx group; #P < 0.05 and ##P < 0.01 vs Aβ_(25–35) group. (C) Spatial memory performance expressed as percentage of correct responses. Data are presented as mean ± SEM (n = 12–14 animals per group). Statistical analysis was performed using one-way ANOVA followed by Newman-Keuls test. ***P < 0.001, *P < 0.05 vs intact group; &&&P < 0.001, &P < 0.05 vs Aβ_(35–25) group; †††P < 0.001, †P < 0.05 vs Hc-TeTx group; ###P < 0.001 vs Aβ_(25–35) group.

throughout the experiment. During the initial trials (trials 1–3), animals were allowed to explore all eight arms. In subsequent trials, the number of allowed arm choices was progressively restricted, such that by the second day, animals were limited to three arm entries per trial. Performance was analyzed in blocks of trials to evaluate learning progression: Block 1 (trials 1–4), Block 2 (5–7), Block 3 (8–10), Block 4 (11–14), Block 5 (15–17), and Block 6 (18–20). Spatial learning and memory performance were quantified as the percentage of correct choices, defined as entries into baited arms without revisiting previously explored arms, as previously reported (Limón et al., 2011; Patricio-Martínez et al., 2016; Patricio-Martínez et al., 2019).

Ellman's cholinesterase assay

For AChE activity was determined 31 days after surgery. Animals (n = 4–6 per group) were euthanized by decapitation, and the brains were rapidly removed and dissected on ice to isolate the NBM, frontal cortex, and temporal cortex. The tissue obtained was weighed and homogenized in ice-cold phosphate-buffered saline (PBS, 0.1 M, pH 8.0). AChE activity was measured spectrophotometrically according to the method of (Ellman et al., 1961). The assay is based on the hydrolysis of acetylthiocholine iodide, which produces thiocholine. Thiocholine reacts with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) to yield the yellow chromophore 5-thio-2-nitrobenzoate, which was quantified at 412 nm. For the assay, with 0.4 mL of each homogenate taken and placed in a cell

containing 2.6 mL PBS and 100 µL DTNB. After baseline stabilization, the reaction was initiated by adding 20 µL acetylthiocholine iodide. The change in absorbance was recorded at 412 nm for 10 min at 1-min intervals using a spectrophotometer.

AChE activity was calculated from the linear rate of absorbance change (ΔA/min) using the molar extinction coefficient for DTNB ($\epsilon = 13,600 \text{ M}^{-1} \text{ cm}^{-1}$) and expressed as µmol of acetylthiocholine hydrolyzed per minute per mg of tissue.

Tissue preparation and protein extraction

For the Western analysis, (n = 4 per group) were euthanized by decapitation, and the brains were rapidly removed and dissected on ice to isolate the NBM, frontal cortex, and temporal cortex. Tissue samples were homogenized in ice-cold RIPA lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), and 0.5% sodium deoxycholate, supplemented with 1 mM sodium orthovanadate (Na₃VO₄), 10 mM sodium fluoride (NaF), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM EDTA, 1 mM EGTA, and a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA).

Homogenates were centrifuged at (12,500 rpm for 30 min at 4 °C), and the supernatants were collected for protein analysis. Total protein concentration was determined using the Bradford assay (Bradford, 1976). For electrophoresis, protein samples were diluted in Laemmli sample buffer (1:1), denatured at 95 °C for 5 min, and immediately

cooled on ice. Protein extracts were stored at 4 °C until use. Equal amounts of protein were loaded per lane (10 µg for NBM samples and 20 µg for cortical samples).

Western blot

Protein samples were separated by SDS-PAGE using 10–12% polyacrylamide gels and electrophoresed at 100 V for approximately 2 h. Proteins were subsequently transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore) using a wet transfer system. Membranes were blocked for 1 h at room temperature in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) supplemented with 5% non-fat dry milk. Subsequently, membranes were incubated overnight at 4 °C with the following primary antibodies: rabbit anti-VACHT (1:200; Santa Cruz Biotechnology, sc-15315), rabbit anti-TrkA (1:200; Santa Cruz Biotechnology, sc-118), rabbit anti-phospho-TrkA (Tyr674/675; Cell Signaling Technology, C50F3), mouse anti-GSK-3β (1:200; Santa Cruz Biotechnology, sc-377213), rabbit anti-phospho-GSK-3β (Tyr216; 1:200; Santa Cruz Biotechnology, sc-135653), rabbit anti-Akt (1:1000; Cell Signaling Technology, C67E7), rabbit anti-phospho-Akt (Ser473; 1:2000; Cell Signaling Technology, D9E), and mouse anti-β-actin (1:1000; Millipore, MAB1501). After three washes in TBS-T, membranes were incubated for 1 h at room temperature with HRP-conjugated secondary antibodies, either goat anti-rabbit IgG or goat anti-mouse IgG (1:3000; Millipore or USBiological). Immunoreactive bands were detected using an enhanced chemiluminescence (ECL) detection system (Millipore).

Densitometric analysis was performed using integrated band density values. For phosphorylated proteins, phosphorylation levels were calculated as the ratio of phosphorylated to total protein expression: pTrkA/TrkA, pAkt/Akt, and pGSK-3β/GSK-3β. These ratios were then expressed as percentages relative to the corresponding control group. β-actin was used as a loading control to verify protein loading consistency. VACHT immunoreactivity was quantified by integrated density analysis and expressed as a percentage relative to the control group.

Immunohistochemistry for GFAP

Once the behavioral experiments had been conducted, all animals were euthanized ($n = 4$ per group) using pentobarbital overdose (100 mg/Kg, i.p.) and transcardially perfused with 200–250 mL 4% paraformaldehyde in PBS (pH = 7.4). The brains were removed and stored in a 30% sucrose solution, after which 50 µm coronal sections taken from the NBM, frontal cortex, and temporal cortex were obtained using a vibratome (Leica VT1000S). Sections corresponding to stereotaxic coordinates from +3.7 mm to −2.12 mm relative to bregma were selected according to (Paxinos and Watson, 1998).

Free-floating sections were rinsed in PBS and incubated overnight (48 h) at 4 °C with a polyclonal rabbit anti-GFAP antibody (1:500; Dako, Denmark). After washing in PBS, sections were incubated using a biotin-streptavidin detection system (LSAB + Kit, Dako), followed by visualization with 3,3'-diaminobenzidine (DAB; 50 mg / 100 mL) in the presence of hydrogen peroxide (H_2O_2 ; 3%). Sections were mounted on gelatin-coated slides, dehydrated through graded alcohols, cleared, and coverslipped. Images were acquired using a Leica DM1000 LED microscope (40× objective) coupled to a ProgRes C14plus camera and analyzed using PROGRES GRYPHAX software. Image acquisition was performed under identical conditions for all groups. Regions of interest (ROIs) were defined based on anatomical landmarks according to Paxinos and Watson (1998).

Quantification of GFAP immunoreactivity was performed by measuring the percentage of stained area relative to the total area of the field. A fixed threshold was established based on the intact group and applied uniformly across all experimental groups to ensure consistency. For each animal, 8–10 sections per region were analyzed, and measurements were averaged to obtain a single value per animal.

Quantification was performed using a fixed threshold applied across all groups. All image analyses were conducted by an investigator blinded to the experimental group allocation.

Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism software 8.0 (GraphPad Software, San Diego, CA, USA). Data distribution was assessed for normality using the Shapiro-Wilk test. Spatial learning acquisition data from the eight-arm radial maze, which included repeated measurements across trial blocks, were analyzed using a mixed-effects model with restricted maximum likelihood estimation (REML), considering trial block as the repeated-measures factor and treatment as the between-subject factor. When significant effects were detected, post hoc multiple comparisons were performed using Tukey's test. Spatial memory performance, AChE activity, Western blot data, and GFAP immunoreactivity in the NBM, frontal cortex, and temporal cortex were analyzed using one-way analysis of variance (ANOVA). When appropriate, pairwise comparisons between groups were performed using the Newman–Keuls post hoc test to identify specific group differences. A significance level of $P < 0.05$ was considered statistically significant.

Results

The recombinant Hc-TeTx prevents $A\beta_{(25-35)}$ -induced cognitive impairment following NBM administration

Spatial learning and memory were evaluated using the eight-arm radial maze (Fig. 1A). During the acquisition phase, learning was assessed across six trial blocks to monitor the progression of task acquisition. A mixed-effects model (REML) revealed significant effects of time, treatment, and the time × treatment interaction, indicating that learning progression differed among the experimental groups.

No significant differences were observed during the first or third trial blocks. In the second block, the $A\beta_{(25-35)}$ group exhibited an 18% reduction in correct responses compared with the $A\beta_{(35-25)}$ group ($P < 0.01$), whereas the Hc-TeTx + $A\beta_{(25-35)}$ group showed a 26% improvement relative to the $A\beta_{(25-35)}$ group ($P < 0.05$). By the fourth block, the $A\beta_{(25-35)}$ group displayed impaired task acquisition, with 19% and 29% fewer correct responses than the intact and $A\beta_{(35-25)}$ groups, respectively ($P < 0.05$; $P < 0.01$). This deficit was partially reversed by Hc-TeTx treatment, as the Hc-TeTx + $A\beta_{(25-35)}$ group performed approximately 30% better than the $A\beta_{(25-35)}$ group ($P < 0.01$).

The impairment persisted during the fifth block, with the $A\beta_{(25-35)}$ group exhibiting reductions of 22%, 18%, and 20% relative to the intact, $A\beta_{(35-25)}$, and Hc-TeTx groups, respectively ($P < 0.05$, $P < 0.01$). In contrast, the Hc-TeTx + $A\beta_{(25-35)}$ group showed a 23% improvement compared with the $A\beta_{(25-35)}$ group ($P < 0.05$). By the sixth and final acquisition block, the intact and $A\beta_{(35-25)}$ groups reached comparable performance levels (94% and 93% correct responses, respectively), whereas the $A\beta_{(25-35)}$ group remained significantly impaired, showing 29%, 28%, and 20% fewer correct responses than the intact, $A\beta_{(35-25)}$, and Hc-TeTx groups, respectively ($P < 0.001$, $P < 0.01$). The Hc-TeTx group also exhibited a modest but significant 9% reduction compared with the intact group ($P < 0.05$). Importantly, Hc-TeTx treatment improved the performance of $A\beta_{(25-35)}$ -treated animals by 21%, partially restoring learning deficits induced by $A\beta_{(25-35)}$ ($P < 0.01$) (Fig. 1B).

During the memory phase, the $A\beta_{(25-35)}$ group showed a marked impairment, with 51% correct responses, significantly lower than the intact group (78%; $P < 0.001$). The $A\beta_{(35-25)}$ group (81%) did not differ from the intact group. Notably, the Hc-TeTx-alone group showed a moderate reduction in memory performance (61%), which was significantly different from both the intact and $A\beta_{(35-25)}$ groups ($P < 0.05$). In contrast, the Hc-TeTx plus $A\beta_{(25-35)}$ group showed restored performance

(78%), reaching levels comparable to the intact group and significantly higher than the $A\beta_{(25-35)}$ group ($P < 0.01$) (Fig. 1C).

The recombinant Hc-TeTx modulates cholinergic markers altered by $A\beta_{(25-35)}$ in the NBM and cortical regions

AChE activity

AChE activity was measured in the frontal and temporal cortices 31 days after $A\beta_{(25-35)}$ administration (Fig. 2). In the frontal cortex, the $A\beta_{(25-35)}$ group showed a significant reduction in AChE activity (1.9 $\mu\text{mol}/\text{min}/\text{mg}$ of tissue) compared with the $A\beta_{(35-25)}$ control group (3.0 $\mu\text{mol}/\text{min}/\text{mg}$ of tissue; $P < 0.05$) (Fig. 2A). No significant differences were observed between the intact group (mean 2.2 $\mu\text{mol}/\text{min}/\text{mg}$ of tissue), and Hc-TeTx-alone group (2.6 $\mu\text{mol}/\text{min}/\text{mg}$ of tissue). In contrast, animals treated with Hc-TeTx plus $A\beta_{(25-35)}$ showed significantly higher AChE activity (3.3 $\mu\text{mol}/\text{min}/\text{mg}$ of tissue) than the $A\beta_{(25-35)}$ group (Fig. 2B).

In the temporal cortex, $A\beta_{(25-35)}$ injection resulted in a marked decrease in AChE activity (1.4 $\mu\text{mol}/\text{min}/\text{mg}$ of tissue), with both the intact group (2.6 $\mu\text{mol}/\text{min}/\text{mg}$ of tissue; $P < 0.001$) and the $A\beta_{(35-25)}$ group (2.0 $\mu\text{mol}/\text{min}/\text{mg}$ of tissue; $P < 0.05$). Interestingly, the Hc-TeTx-alone group also showed reduced AChE activity (1.9 $\mu\text{mol}/\text{min}/\text{mg}$ of tissue) compared to the intact group ($P < 0.01$), suggesting that Hc-TeTx may modulate cholinergic activity in a context-dependent manner. In contrast, the Hc-TeTx + $A\beta_{(25-35)}$ group showed higher AChE activity than the $A\beta_{(25-35)}$ group ($P < 0.05$) indicating partial preservation of cholinergic function under $A\beta$ -induced injury conditions (Fig. 2B).

VAcHT expression

VAcHT protein levels were analyzed as a marker of cholinergic neurons in the NBM, frontal cortex, and temporal cortex (Fig. 3). In the NBM, $A\beta_{(25-35)}$ significantly reduced VAcHT levels to 57.0% of the intact group ($P < 0.01$). In contrast, the Hc-TeTx plus $A\beta_{(25-35)}$ group presented higher VAcHT levels (85.7%) compared to the $A\beta_{(25-35)}$ group ($P < 0.05$). No significant differences were observed in the Hc-TeTx-alone or $A\beta_{(35-25)}$ groups (83.2% and 86.0%, respectively) relative to the intact animals (Fig. 3A).

In the frontal cortex, $A\beta_{(25-35)}$ markedly decreased VAcHT expression to 32.4% of intact values ($P < 0.001$). This reduction was attenuated in the Hc-TeTx plus $A\beta_{(25-35)}$ group (97%; $P < 0.001$ vs $A\beta_{(35-25)}$). The $A\beta_{(35-25)}$ and Hc-TeTx groups did not differ significantly from the intact group (Fig. 3B). Similarly, in the temporal cortex, $A\beta_{(25-35)}$

reduced VAcHT levels (50.3% of intact; $P < 0.01$), while the Hc-TeTx + $A\beta_{(25-35)}$ group showed higher values (83.9%; $P < 0.05$ vs. $A\beta_{(25-35)}$). No significant differences were observed in the $A\beta_{(35-25)}$ or Hc-TeTx-alone groups (Fig. 3C).

The recombinant Hc-TeTx modulates Trk-A receptor phosphorylation and downstream signaling pathways altered by $A\beta_{(25-35)}$

TrkA phosphorylation

Phosphorylation of the Trk-A receptor was evaluated in the NBM, frontal cortex, and temporal cortex (Fig. 4). In the NBM, $A\beta_{(25-35)}$ administration reduced Trk-A receptor phosphorylation to 66.9% of control levels. In contrast, the intact and $A\beta_{(35-25)}$ groups showed values of 99.9% and 95.3%, respectively. The Hc-TeTx-alone group presented levels comparable to controls (102.1%). Notably, the Hc-TeTx + $A\beta_{(25-35)}$ group showed higher TrkA phosphorylation (93.2%) compared to the $A\beta_{(25-35)}$ group (Fig. 4A). In the frontal cortex no significant difference in TrkA phosphorylation were observed among experimental groups (Fig. 4B). In the temporal cortex, a significant difference was detected between the $A\beta_{(25-35)}$ group (86.8%) and the $A\beta_{(35-25)}$ group (119.7%) (Fig. 4C).

Akt phosphorylation

Phosphorylation of Akt (Ser473) was assessed in the same regions (Fig. 5). In the NBM, the $A\beta_{(25-35)}$ group showed a marked reduction in Akt phosphorylation (33.9%) compared to the intact (100%) and $A\beta_{(35-25)}$ groups (93.4%). The Hc-TeTx-alone group showed intermediate values (68.5%), while the Hc-TeTx plus $A\beta_{(25-35)}$ group exhibited higher phosphorylation levels (87.6%) compared to the $A\beta_{(25-35)}$ group (Fig. 5A). In the frontal cortex, $A\beta_{(25-35)}$ markedly reduced Akt phosphorylation (14.7%) compared to the intact and $A\beta_{(35-25)}$ groups (100% and 59.5%, respectively). The Hc-TeTx-alone group showed increased phosphorylation (77.7%) and the Hc-TeTx plus $A\beta_{(25-35)}$ group showed further increased levels (89.4%) compared to the $A\beta_{(25-35)}$ group (Fig. 5 B). No significant difference was observed in Akt phosphorylation in the temporal cortex among experimental groups (Fig. 5 C).

GSK-3 β phosphorylation

Phosphorylation of GSK-3 β (Tyr 216) was also analyzed (Fig. 6). In the NBM, $A\beta_{(25-35)}$ significantly increased GSK-3 β phosphorylation (281.5%) compared to intact (100%) and $A\beta_{(35-25)}$ groups (87.1%) ($P < 0.05$). The Hc-TeTx-alone group showed levels similar to controls (92.1%), while the Hc-TeTx plus $A\beta_{(25-35)}$ -treated group exhibited lower

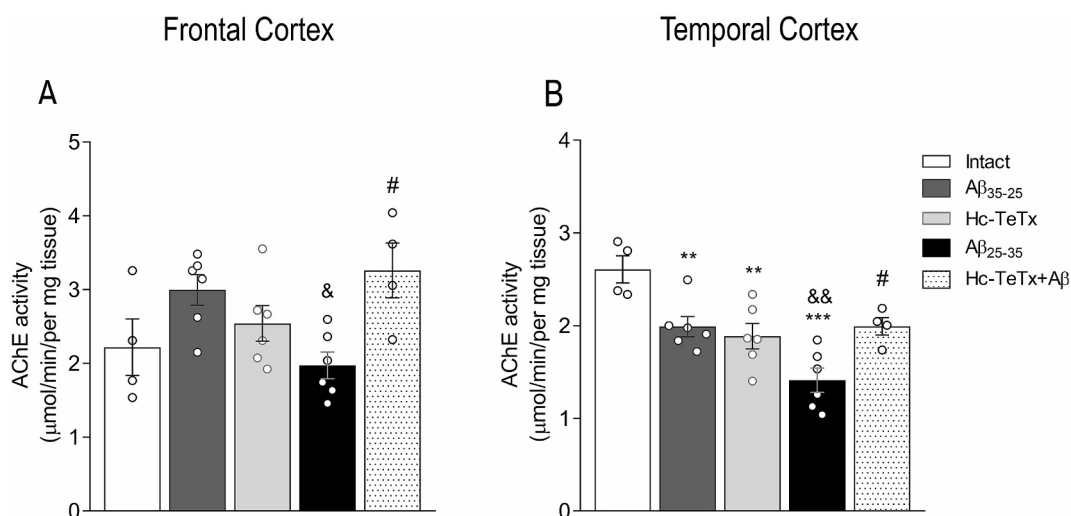


Fig. 2. AChE activity in cortical regions. Acetylcholinesterase (AChE) activity was measured in the frontal cortex (A) and temporal cortex (B) 31 days after treatment. Data are expressed as mean $\pm$ SEM ($n = 4-6$ animals per group) and presented as $\mu\text{mol}/\text{min}/\text{mg}$ tissue. Statistical analysis was performed using one-way ANOVA followed by Newman-Keuls multiple comparison test. *** $P < 0.001$, ** $P < 0.01$ vs intact group; & $P < 0.05$, && $P < 0.01$ vs $A\beta_{(35-25)}$ group; # $P < 0.05$ vs $A\beta_{(25-35)}$ group.

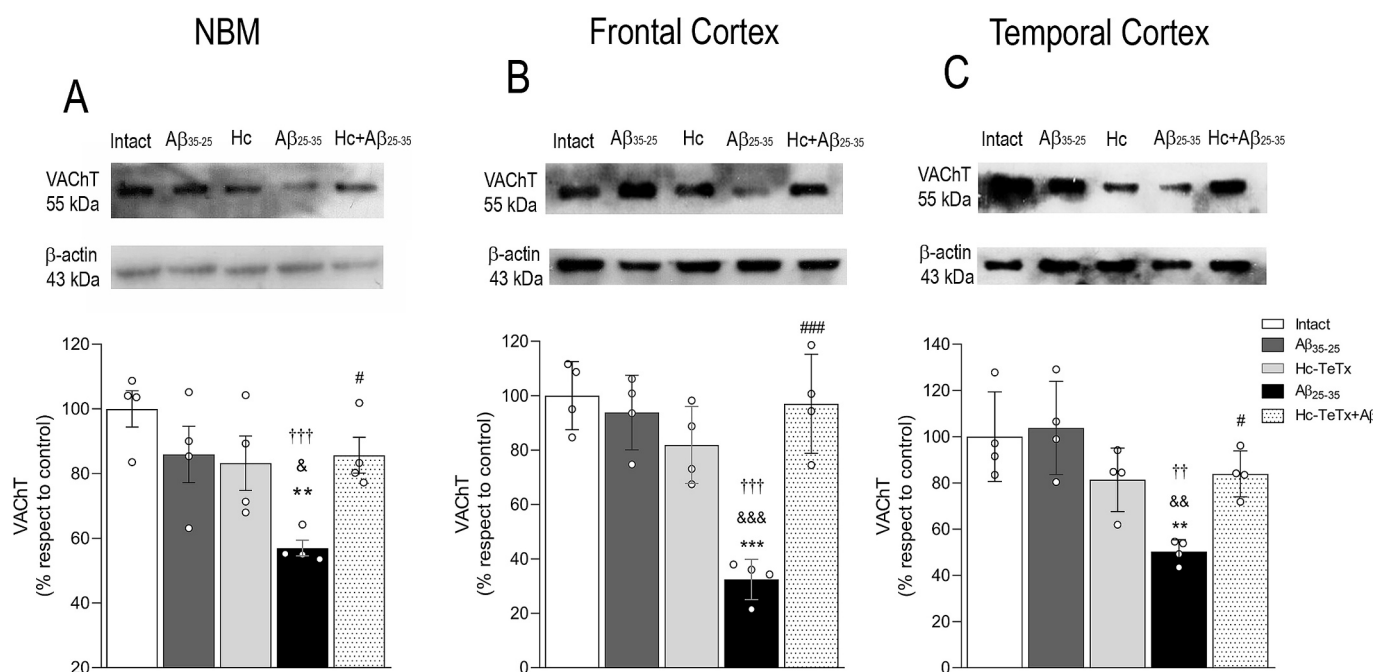


Fig. 3. VACHT expression in the NBM and cortical regions. Vesicular acetylcholine transporter (VACHT) expression was evaluated in the nucleus basalis magnocellularis (NBM) (A), frontal cortex (B), and temporal cortex (C). Data are expressed as percentage relative to the intact group (set as 100%) and presented as mean $\pm$ SEM ($n = 4$ animals per group). Statistical analysis was performed using one-way ANOVA followed by Newman-Keuls multiple comparison test. $**P < 0.01$, $***P < 0.001$ vs intact group; $&P < 0.05$, $&&P < 0.01$, $&&&P < 0.001$ vs Aβ₍₃₅₋₂₅₎ group; $††P < 0.01$, $†††P < 0.001$ vs Hc-TeTx group; $#P < 0.05$, $###P < 0.001$ vs Aβ₍₂₅₋₃₅₎ group.

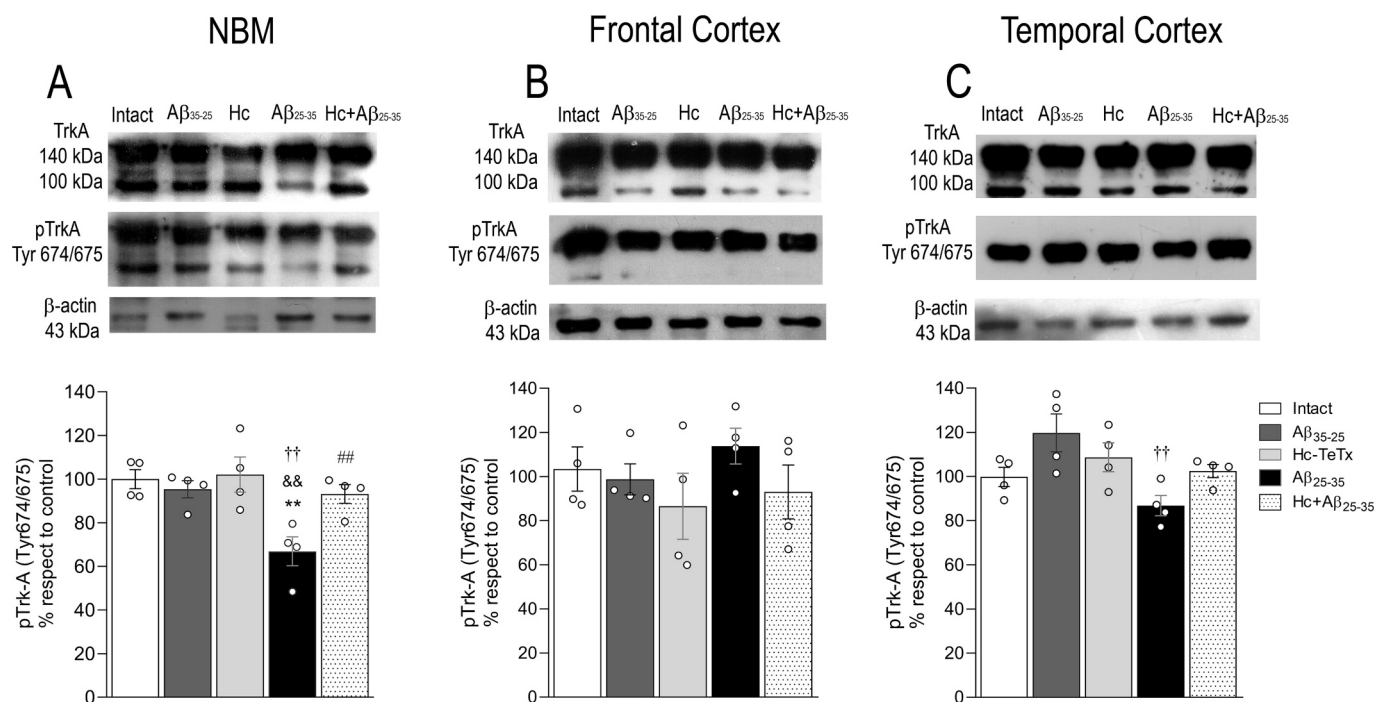


Fig. 4. TrkA phosphorylation in the NBM and cortical regions. Phosphorylated TrkA (pTrkA) levels were evaluated in the nucleus basalis magnocellularis (NBM) (A), frontal cortex (B), and temporal cortex (C). Phosphorylation levels were calculated as the pTrkA/TrkA ratio and expressed as a percentage of the control group, which was set at 100%. Data are presented as mean $\pm$ SEM ($n = 4$ animals per group). Statistical analysis was performed using one-way ANOVA followed by Newman-Keuls multiple comparison test. $**P < 0.01$ vs intact group; $&P < 0.01$ vs Aβ₍₃₅₋₂₅₎ group; $††P < 0.01$ vs Hc-TeTx group; $##P < 0.01$ vs Aβ₍₂₅₋₃₅₎ group.

phosphorylation levels (131.9%; $P < 0.05$ vs. Aβ₍₂₅₋₃₅₎) (Fig. 6 A). In the frontal cortex Aβ₍₂₅₋₃₅₎ increased GSK-3β phosphorylation to 155.6%, compared with 72.6% in the intact group and 86.8% in the Aβ₍₃₅₋₂₅₎ group ($P < 0.05$). Similarly, in the temporal cortex, Aβ₍₂₅₋₃₅₎ increased

phosphorylation (223.3%) compared to intact (99.9%) and Aβ₍₃₅₋₂₅₎ groups (107.3%) ($P < 0.05$). In both regions, the Hc-TeTx plus Aβ₍₂₅₋₃₅₎ group showed reduced phosphorylation levels (98.7% and 104.8%, respectively) compared to the Aβ₍₂₅₋₃₅₎ group (Fig. 6B-C).

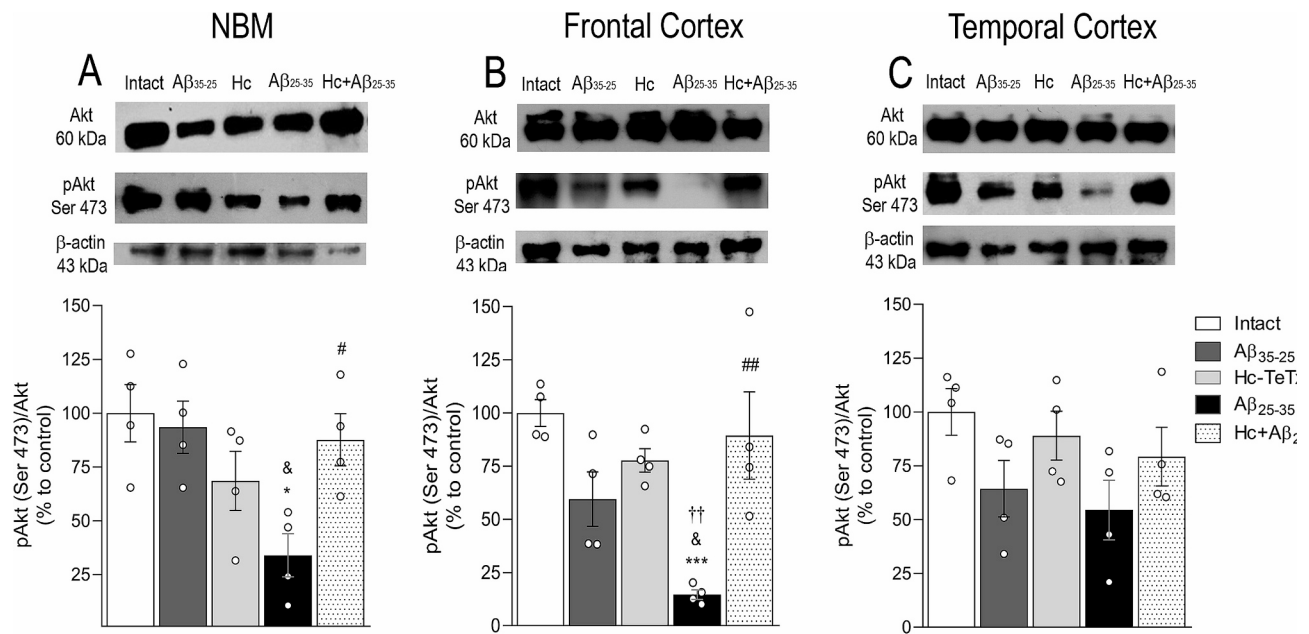


Fig. 5. Akt phosphorylation in the NBM and cortical regions. Phosphorylated Akt (pAkt, Ser473) levels were evaluated in the nucleus basalis magnocellularis (NBM) (A), frontal cortex (B), and temporal cortex (C). Phosphorylation levels were calculated as the pAkt/Akt ratio and expressed as a percentage of the control group, which was set at 100%. Data are presented as mean $\pm$ SEM ($n = 4$ animals per group). Statistical analysis was performed using one-way ANOVA followed by Newman–Keuls multiple comparison test. * $P < 0.05$, *** $P < 0.001$ vs intact group; & $P < 0.05$ vs $A\beta_{(35-25)}$ group; †† $P < 0.01$ vs Hc-TeTx group; # $P < 0.05$, ## $P < 0.01$ vs $A\beta_{(25-35)}$ group.

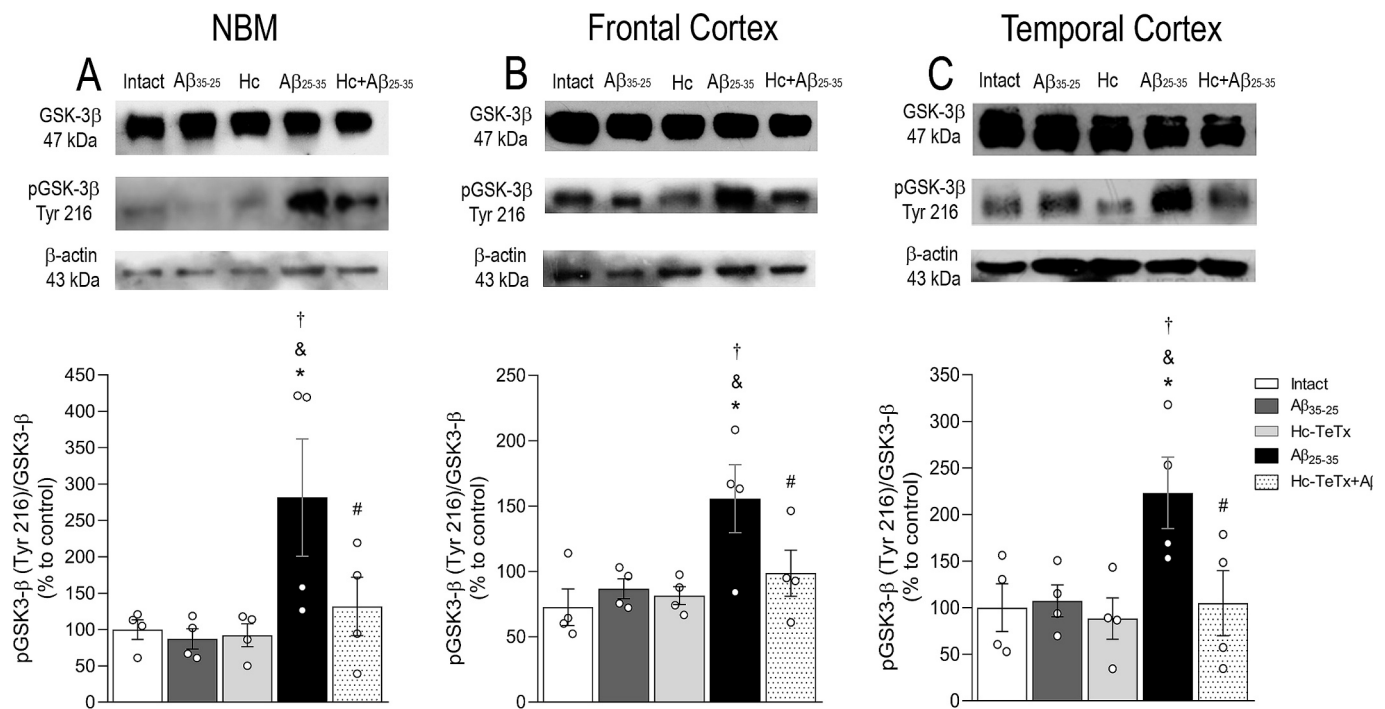


Fig. 6. GSK-3 β phosphorylation in the NBM and cortical regions. Phosphorylated GSK-3 β (pGSK-3 β , Tyr216) levels were evaluated in the nucleus basalis magnocellularis (NBM) (A), frontal cortex (B), and temporal cortex (C). Phosphorylation levels were calculated as the pGSK-3 β /GSK-3 β ratio and expressed as a percentage of the control group, which was set at 100%. Data are presented as mean $\pm$ SEM ($n = 4$ animals per group). Statistical analysis was performed using one-way ANOVA followed by Newman–Keuls multiple comparison test. * $P < 0.05$ vs intact group; & $P < 0.05$ vs $A\beta_{(35-25)}$ group; † $P < 0.05$ vs Hc-TeTx group; # $P < 0.05$ vs $A\beta_{(25-35)}$ group.

The recombinant Hc-TeTx modulates GFAP immunoreactivity induced by $A\beta_{(25-35)}$ in the NBM and cortical regions

GFAP immunoreactivity was evaluated in the NBM, frontal cortex,

and temporal cortex (Fig. 7). In the NBM, $A\beta_{(25-35)}$ administration significantly increased GFAP-positive area (42.8%) compared to the intact (16.0%) and $A\beta_{(35-25)}$ groups (25.0%) ($P < 0.001$ and $P < 0.01$, respectively). The Hc-TeTx plus $A\beta_{(25-35)}$ group showed a reduced

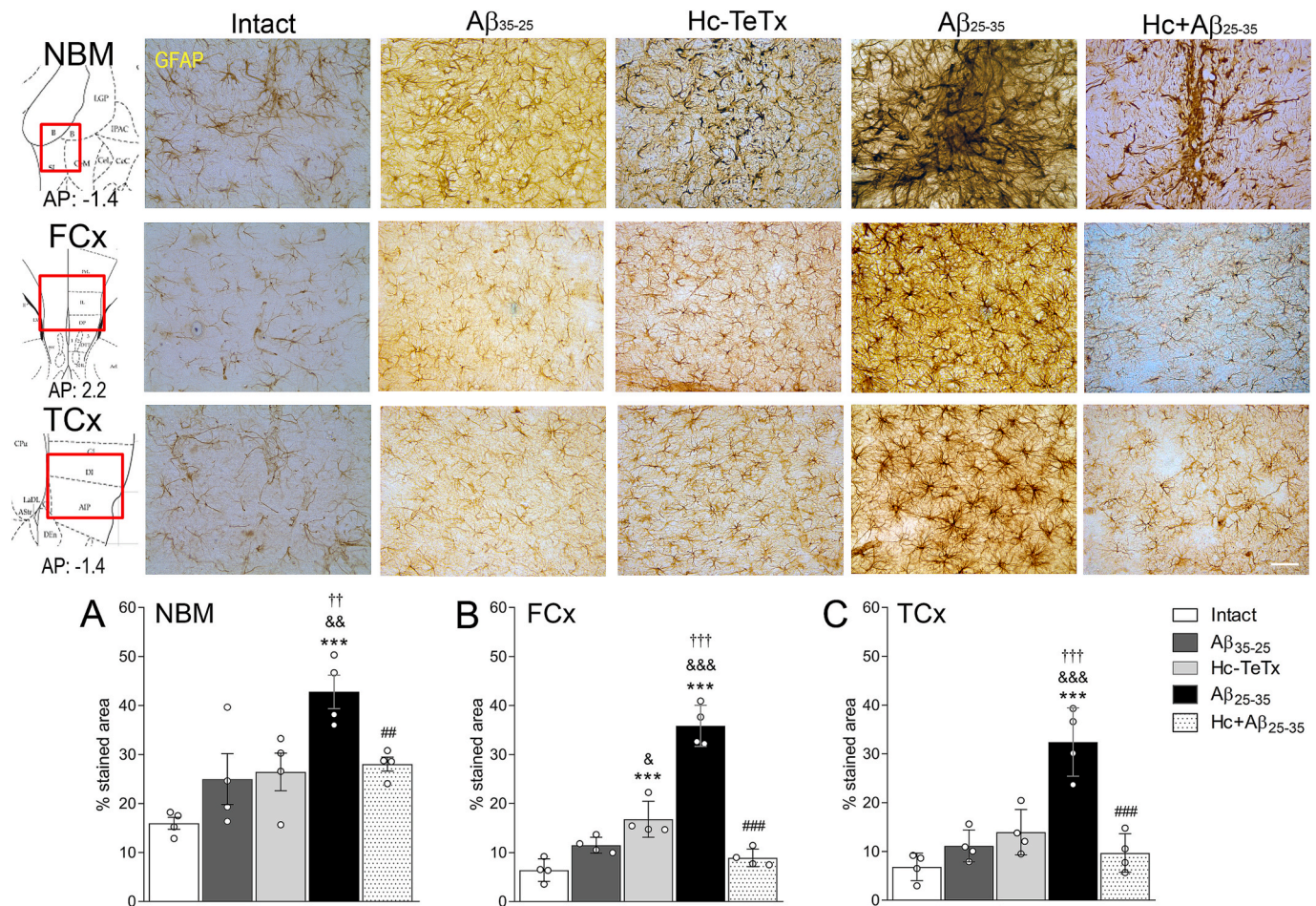


Fig. 7. GFAP immunoreactivity in the NBM and cortical regions. Representative photomicrographs of GFAP immunoreactivity (GFAP-ir) in the nucleus basalis magnocellularis (NBM), frontal cortex, and temporal cortex for each experimental group. Scale bar = 50 μ m. Quantification of GFAP immunoreactivity is shown as percentage of stained area relative to the total field in the NBM (A), frontal cortex (B), and temporal cortex (C). Data are presented as mean $\pm$ SEM ($n = 4$ animals per group). Statistical analysis was performed using one-way ANOVA followed by Newman-Keuls multiple comparison test. *** $P < 0.001$ vs intact group, & $P < 0.05$, && $P < 0.01$, &&& $P < 0.001$ vs $A\beta_{(35-25)}$ group; †† $P < 0.01$, ††† $P < 0.001$ vs Hc-TeTx group; ## $P < 0.01$, ### $P < 0.001$ vs $A\beta_{(25-35)}$ group.

GFAP-positive area (28.0%) compared to the $A\beta_{(25-35)}$ group ($P < 0.01$). The Hc-TeTx-alone group (26.4%) did not differ significantly from control groups (Fig. 7A).

In the frontal cortex $A\beta_{(25-35)}$ markedly increased GFAP immunoreactivity (35.9%) compared to intact (6.4%), $A\beta_{(35-25)}$ (11.5%), and Hc-TeTx-alone groups ($P < 0.001$). Notably, the Hc-TeTx-alone group showed higher GFAP levels than the intact and $A\beta_{(35-25)}$ groups ($P < 0.001$ and $P < 0.05$, respectively), suggesting that Hc-TeTx may induce an astrocytic response even in the absence of $A\beta$ injury. However, the Hc-TeTx plus $A\beta_{(25-35)}$ group showed reduced GFAP levels (9.0%) compared to the $A\beta_{(25-35)}$ group ($P < 0.001$) (Fig. 7B). Similarly, in the temporal cortex, $A\beta_{(25-35)}$ increased GFAP immunoreactivity (32.4%) compared to intact (6.8%), $A\beta_{(35-25)}$ (11.1%), and Hc-TeTx-alone groups ($P < 0.001$). The Hc-TeTx plus $A\beta_{(25-35)}$ group showed lower GFAP levels (9.7%) compared to the $A\beta_{(25-35)}$ group ($P < 0.001$) (Fig. 7C).

Discussion

The present study shows that bilateral administration of Hc-TeTx into the NBM attenuated the behavioral and neurochemical alterations induced by $A\beta_{(25-35)}$. These findings should be interpreted within the context of an $A\beta_{(25-35)}$ -induced focal injury model targeting the NBM, which captures key aspects of cholinergic dysfunction but does not reproduce the progressive and multifactorial nature of AD. Within this

experimental framework, Hc-TeTx treatment was associated with improved performance in the radial arm maze, higher cortical AChE activity, preservation of VAcHT expression in the NBM and its projection areas (frontal cortex and temporal cortex), partial restoration of TrkA and Akt phosphorylation, lower phosphorylation of GSK-3 β at Tyr216, and reduced GFAP immunoreactivity. Taken together, these findings support the view that Hc-TeTx counteracts several consequences of $A\beta_{(25-35)}$ -induced cholinergic injury in the basal forebrain, although the present data do not establish a definitive causal mechanism.

Acetylcholine (ACh) plays a central role in cortical and hippocampal function, and its loss is closely associated with cognitive decline (Whitehouse et al., 1981). The integrity of basal forebrain cholinergic neurons depends on NGF, which regulates cholinergic gene expression (Sofroniew et al., 2001). Given the limitations of NGF as a therapeutic agent, alternative strategies that mimic its trophic effects have been explored. In this context, Hc-TeTx has been proposed as a molecule capable of activating survival pathways with neurotrophin-like properties (Gil et al., 2001; Gil et al., 2000; Herrando-Grabulosa et al., 2013). Previous studies demonstrated that the C-terminal fragment of tetanus toxin preserves efficient neuronal uptake and retrograde axonal transport properties, supporting its capacity to access interconnected neuronal circuits (Kissa et al., 2002).

The rationale of this study was to evaluate these effects specifically in the NBM, a key cholinergic nucleus projecting to the frontal cortex and temporal cortex and critically involved in learning and memory

processes (Bartus et al., 1982; Croxson et al., 2011; Selden et al., 1998). Lesions of the NBM impair cortical cholinergic transmission and cognitive performance (Bartus et al., 1985; Mandel et al., 1989). The present findings extend previous observations in the medial septum by demonstrating that Hc-TeTx is also associated with protection in the NBM-cortical cholinergic system. Thus, the novelty of this study lies in its anatomical and functional context rather than in establishing a novel mechanistic pathway.

Behaviorally, A β _(25–35) administration in the NBM impaired learning and memory, consistent with the role of basal forebrain cholinergic neurons in cortical activation and cognitive performance. Hc-TeTx treatment was associated with improved performance in A β _(25–35)-treated animals, in agreement with previous reports indicating that Hc-TeTx can reduce excitotoxic damage and modulate neuronal survival-related pathways (Herrando-Grabulosa et al., 2013). However, these behavioral findings should be interpreted alongside the biochemical data, as the present study did not directly assess acetylcholine release, synaptic function, or neuronal survival.

An important observation is that Hc-TeTx alone was not completely neutral across all behavioral outcomes. Although animals treated with Hc-TeTx alone showed preserved learning acquisition, they exhibited reduced memory performance compared with control groups. Similar findings have been reported following administration of Hc-TeTx into the medial septum (Patricio-Martínez et al., 2016) suggesting that this effect may not be restricted to a specific brain region but could reflect a broader modulation of basal forebrain circuits. Thus, the effect of Hc-TeTx may depend on the functional state of the tissue and on the behavioral domain evaluated.

One possible explanation involves changes in neurotransmitter balance. Hc-TeTx has been shown to influence serotonin uptake (Insarte et al., 1999; Najib et al., 1999), and activation of 5-HT1A receptors has been associated with memory processes (Winter and Petti, 1987). In addition, the present findings revealed reduced cortical AChE activity in animals treated with Hc-TeTx alone, suggesting that alterations in cholinergic homeostasis may also contribute to the observed behavioral effects. Given the well-established interactions among neurotrophic signaling, cholinergic neurotransmission, and cognitive function, it is conceivable that Hc-TeTx modulates memory performance through changes in neurotransmitter balance rather than through overt neurotoxic effects.

Nevertheless, this interpretation remains speculative. The mechanisms underlying the memory impairment observed in Hc-TeTx-alone animals remain unclear, and further studies evaluating dose–response relationships, temporal dynamics, acetylcholine release, serotonergic markers, cholinergic neuron integrity, and neurotrophic signaling will be necessary to clarify the context-dependent effects of Hc-TeTx.

The cholinergic data support the behavioral findings. A β _(25–35) reduced AChE activity and VAcHT expression in the frontal cortex and temporal cortex, indicating disruption of cholinergic projections originating from the NBM. These findings are consistent with previous reports showing that A β peptides impair cholinergic markers and neurotransmission (Abe et al., 1994; Harkany et al., 1995; Stepanichev et al., 2014). In contrast, Hc-TeTx plus A β _(25–35) treatment was associated with higher AChE activity and preservation of VAcHT expression. Given that VAcHT is a marker of presynaptic cholinergic terminals, these results are consistent with preservation of cholinergic projections, although they do not directly demonstrate neuronal survival or neurotransmitter release. The observation that Hc-TeTx reduced AChE activity in intact animals but increased AChE activity in A β _(25–35)-treated animals suggests a context-dependent effect on cholinergic regulation. Rather than acting as a simple enhancer or suppressor of cholinergic signaling, Hc-TeTx may differentially modulate cholinergic homeostasis depending on whether the tissue is under physiological or injury conditions. This interpretation is consistent with previous evidence showing that recombinant Hc-TeTx protects against A β _(25–35)-induced cholinotoxicity in rats, as well as with reports linking neurotrophic/Akt-

related signaling to the regulation of cholinergic markers (Madziar et al., 2008; Patricio-Martínez et al., 2016). In the presence of A β -induced damage, this modulation may contribute to the preservation of cholinergic markers, whereas in intact animals it may alter the balance of cholinergic activity. However, this interpretation remains speculative, as the present study did not directly measure acetylcholine release, cholinergic neuron survival, or synaptic function. Therefore, additional studies including direct measurements of acetylcholine release are required, particularly because A β peptides have been shown to reduce cholinergic neurotransmission *in vivo* (Abe et al., 1994). Basal forebrain cholinergic neurons express high levels of TrkA receptors (Holtzman et al., 1995; Urschel and Hulsebosch, 1992; Vazquez and Ebendal, 1991), and Hc-TeTx has been shown to interact with neurotrophin signaling pathways (Gil et al., 2003; Gil et al., 2000).

Previous studies have shown that Hc-TeTx can induce phosphorylation of Trk receptors and activate downstream signaling cascades such as Akt and ERK, suggesting neurotrophic-like activity (Gil et al., 2003). In addition, structural and biochemical evidence indicates that Hc-TeTx may interact with neurotrophin receptors such as TrkB, potentially binding to extracellular domains involved in ligand recognition, although the specificity and physiological relevance of this interaction remain to be fully established (Candalija et al., 2021).

In the present study, A β _(25–35) reduced TrkA phosphorylation in the NBM, whereas Hc-TeTx treatment was associated with higher phosphorylation levels. Consistent with these observations, A β _(25–35) reduced Akt phosphorylation and increased GSK-3 β phosphorylation at Tyr216, whereas Hc-TeTx treatment partially reversed these changes. Dysregulation of the Akt/GSK-3 β pathway has been implicated in neurodegeneration (Takashima, 2006), and phosphorylation at Tyr216 is associated with increased GSK-3 β activity (Dajani et al., 2001). However, the present study did not assess tau phosphorylation, enzymatic activity of GSK-3 β , or Ser9 phosphorylation. Therefore, these findings should be interpreted as indicative of partial normalization of signaling rather than definitive mechanistic evidence. Previous studies suggesting a role of PI3K/Akt signaling in VAcHT regulation (Madziar et al., 2008) may provide a possible framework for interpreting these observations.

An important consideration is the persistence of signaling changes at 31 days post-injection. The present data do not demonstrate the continued presence of Hc-TeTx in the tissue. A more plausible interpretation is that early exposure to Hc-TeTx may trigger downstream cellular responses that persist over time, such as preservation of trophic signaling or attenuation of secondary damage. This interpretation should be considered cautiously and represents a limitation of the study.

Regarding glial responses, A β _(25–35) increased GFAP immunoreactivity in the NBM, frontal cortex, and temporal cortex, consistent with astrocyte activation in neurodegenerative conditions (Diaz et al., 2012; Drouet et al., 2000). Hc-TeTx treatment was associated with reduced GFAP levels in A β -treated animals, suggesting modulation of astrocytic reactivity. Previous studies have reported similar effects of Hc-TeTx on astrocytes (Mendieta et al., 2012; Mendieta et al., 2016; Netzhualcoyotzi and Tapia, 2018; Patricio et al., 2022; Patricio-Martínez et al., 2019). However, GFAP reflects astrocyte reactivity rather than the full neuroinflammatory profile, and microglial activation was not evaluated. Additionally, the increased GFAP immunoreactivity observed in the Hc-TeTx-alone group, particularly in the frontal cortex, should be interpreted cautiously. GFAP is a marker of astrocytic reactivity, but it does not distinguish between inflammatory, compensatory, or trophic astrocytic responses. Therefore, the increase in GFAP induced by Hc-TeTx alone should not be considered direct evidence of neuroinflammation. Instead, it may reflect an astrocytic response associated with neurotrophic support, synaptic remodeling, or tissue adaptation. Astrocytes are recognized sources of BDNF and other trophic factors that regulate neuronal survival, synaptic plasticity, and neurotransmission. In this regard, previous studies have shown that Hc-TeTx increases BDNF levels in the hippocampus and frontal cortex (Getachew et al., 2019). Therefore, the astrocytic response associated with Hc-TeTx

may contribute to the neurotrophic environment underlying its protective effects. This interpretation is also consistent with the context-dependent modulation of cholinergic markers observed in the present study, suggesting that Hc-TeTx may promote homeostatic mechanisms that support neuronal function under both physiological and pathological conditions. Nevertheless, the present data do not allow determination of the inflammatory or trophic nature of this astrocytic response, nor do they identify the cellular source of BDNF. Further studies using microglial markers, inflammatory cytokines, and cell-specific approaches, such as GFAP/BDNF co-localization analyses, will be required to clarify whether astrocytes contribute significantly to BDNF production in this model.

The present study has several limitations. It is based on a single A β _(25–35) injection model, which reproduces selected features of cholinergic dysfunction but does not fully recapitulate the progressive and multifactorial nature of Alzheimer's disease. In addition, the mechanistic interpretation relies on endpoint measurements without causal manipulation of TrkA or downstream signaling pathways; therefore, the findings should be interpreted as associative rather than definitive evidence of pathway causality. Microglial activation, tau-related pathology, synaptic function, cholinergic neuron survival, and direct neurotransmitter release were not assessed. Finally, further studies should evaluate dose–response relationships, temporal dynamics, glial phenotypes, neurotransmitter release, and the long-term safety profile of Hc-TeTx.

The present study indicates that Hc-TeTx administration in the NBM is associated with attenuation of A β _(25–35)-induced cognitive impairment and preservation of cholinergic markers in the NBM and its cortical projection areas. These effects were accompanied by modulation of TrkA-related signaling, partial normalization of the Akt/GSK-3 β pathway, and reduced astrocytic reactivity. However, the underlying mechanisms remain to be fully established, and further studies are required to determine the causal involvement of TrkA signaling and the relevance of these effects in more comprehensive models of neurodegeneration.

CRedit authorship contribution statement

Aleidy Patricio-Martínez: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation. **Felipe Patricio:** Writing – review & editing, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Ivette Espinoza Pérez:** Methodology, Investigation. **Isabel Martínez:** Writing – review & editing, Writing – original draft, Visualization, Investigation. **Victor Alemán-Alemán:** Writing – review & editing, Visualization, Investigation. **José Aguilera:** Writing – review & editing, Visualization, Investigation. **Ilhuicamina Daniel Limón:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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