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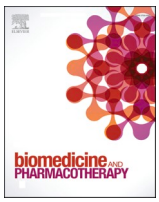

Antagonising a novel toxin "T14" in Alzheimer's disease: Comparison of receptor blocker versus antibody effects in vitro

Sibah Hasan *, Mehreen Ahmed, Sara Garcia-Ratés, Susan Greenfield

Neuro-Bio Ltd, Building F5, Culham Science Centre, Abingdon OX14 3DB, UK

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ABSTRACT

A 14mer peptide, T14, is a possible signaling molecule driving neurodegeneration. Its levels are doubled in the Alzheimer brain, but its effects can be blocked at the target alpha-7 receptor by a cyclised variant, 'NBP14′, which has beneficial effects, in a transgenic mouse model, on the behavioral and histochemical profile. Since the antagonism of T14 has evident therapeutic potential, we explore here an alternative method of preventing its action by comparing the efficacy of NBP14 with a proprietorial polyclonal antibody against T14, 'Ab-19′, at inhibiting three distinct effects of the peptide in PC12 cells: calcium influx, cell viability and compensatory acetylcholinesterase (AChE) release. None of these three parameters was affected by either blocking agent when applied alone. However, both NBP14 and the Ab-19 exhibited a dose-dependent profile against the actions of T14 in all three scenarios: the least sensitive effect observed was in the lower dose range, for both the antibody and the receptor blocker, in antagonizing T14-triggered release of AChE: this parameter is interpreted as indirect compensation for the T14-induced compromise of cell viability, triggered by the enhanced influx of calcium through the initial binding of the peptide to an allosteric site on the alpha-7 receptor. As such, it is the most delayed and indirect index of T14 action and thus the least relatively impacted by lowest doses of either NBP14 or Ab-19. In all three scenarios however the effects of T14 are successfully offset by either agent and thus offer two potentially very different therapies against Alzheimer's disease.

1. Introduction

A novel 14mer peptide (T14), has a trophic action in early development but can turn toxic if aberrantly triggered in Alzheimer's disease (AD), see [1]. T14 is cleaved at the C-terminus from its parent molecule, the enzyme acetylcholinesterase (AChE), which features various trypsin-like cleavage points, inter alia allowing for the cleavage of a 30mer, T30. Within this longer sequence T14 can be further isolated, leaving a non-active 15mer, T15 at its C-terminal (T15) [2,3]. The 30mer peptide T30 has also proven to be bioactive and has been used, as here, as a control against possible aggregation of the smaller peptide [2].

The three cell-based parameters established for demonstrating the efficacy of T14/T30 [4] have been: first, enhanced calcium influx via the peptide binding to an allosteric site on the alpha-7 receptor [5]; second, subsequent reduction in cell viability due to the ensuing excitotoxicity, [6–8] and third, an increased compensatory release in AChE [4]. Here we have compared T14 and T30 in in vitro preparations used previously [4] and assessed the two peptides in the three different cell-based parameters for the first time in a range of doses.

T14/T30 action at the alpha-7 receptor is blocked by displacement of the linear compound with a cyclical variant of T14, NBP14 [4,9]. NBP14 reverses [10,11] the toxic mechanisms of its endogenous counterpart and blocks [4] the peptide in a dose-dependent manner [11,12]: moreover, the drug has beneficial behavioral and histochemical effects when administered chronically in the 5XFAD mouse model of AD [12].

However, receptor blockade of T14 binding might not be the most effective eventual therapy and/or NBP14 might prove to have unacceptable side effects in subsequent trials: it is important therefore to consider other ways of intercepting this potentially toxic signaling molecule. Antibodies to putative key toxins have been developed for a range of conditions [13] as well as for AD itself [14]. An anti-T14 antibody has been raised against the T14 peptide (the active part of the C-terminal AChE). This antibody was tested against T14 and a range of additional compounds serving as controls: amyloid-β, the full length

Abbreviations: 5XFAD, 5 x Familiar Alzheimer Disease; AB19, antibody 19; ACh, acetylcholine; AChE, acetylcholinesterase; AD, Alzheimer's disease; APP, amyloid precursor protein; NBP14, Neuro-Bio peptide 14; PC12, pheochromocytoma cell line 12.

* Corresponding author.
E-mail address: sibah.hasan@neuro-bio.com (S. Hasan).

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T30 sequence, a peptide (T15) consisting of the inert C-terminal 15 amino acid residues of T30 and AChE. Whilst the antibody shows a strong affinity for T14, it does not recognize amyloid-β, T30, T15 or AChE [4,12,15]. Such a comparison between NBP14 and anti-T14 could indicate the optimal therapeutic approach, as well as providing further proof of concept on the bioactivity of T14. We therefore compared the efficacy of a polyclonal anti-T14 antibody, Ab-19, that will target T14 itself with the receptor blocker NBP14.

2. Materials and methods

2.1. PC12 cell culture

PC12 cells are a cloned, pheochromocytoma cell line derived from the adrenal medulla [16]. They are easily cultured and readily accessible to experimental manipulations. Wild-type PC12 were purchased from Sigma (Merck KGaA, Darmstadt, Germany, 88022401). The culture was routinely plated in 100 mm dishes (Corning) coated with collagen (2 mg/cm^2) and maintained in growth medium with Dulbecco's Modified Eagle's Medium - high glucose (DMEM) supplemented with heat-inactivated 10% horse serum (HS) and 5% fetal bovine serum (FBS), 10 mM 4-(2-hydroxyethyl)- 1-piperazineethanesulfonic acid (HEPES), 2 mM Glutamax, 1:100 Penicillin/streptomycin and 2.5 ug/ml Amphotericin B, making a complete DMEM solution. Cells were maintained at 37 °C in a humidified atmosphere 5% CO2 and the medium was replaced every 2–3 days. For splitting, cells were dislodged from the dish using a pipette with medium, with a portion of these replated onto new culture dishes. Cells were used between passages 14 and 26.

2.2. Calcium fluorometry

PC12 cells were plated in 100 μl of complete DMEM as described above, 2 days before the experiment in 96 well plates. On the day of the experiment, the Fluo-8 solution (Abcam) was prepared as described by the provider by adding 10 μl of Fluo-8 in the assay buffer that contains 9 ml of Hank's Balanced Salt Solution (HBSS) and 1 ml of pluronic F127 Plus. Subsequently, 100 μl of growth medium was removed and 70 μl of Fluo-8 solution were added. Treatments with T30, T14 and/or NBP14, Ab-19 were added together, when appropriate, and incubated for 30 min in the incubator and 90 min room temperature. After 2 h, the plate was placed in the fluorescence plate reader (Fluostar, Optima, BMG Labtech, Ortenberg, Germany). Before reading the fluorescence, acetylcholine (Ach; stock solution of 128 μM), an agonist (and orthosteric ligand) of the nicotinic receptors, was prepared and placed in the Fluostar injector. For each well, the reading was formed by a basal fluorescence reading followed by acetylcholine injection that induced an increase of calcium via nicotinic receptors, at a concentration of 53.3 μM.

2.3. Cell viability assay

A Cell Counting Kit-8 (CCK-8) was used. By utilizing the highly water-soluble tetrazolium salt (WST-8), CCK-8 produces a water-soluble formazan dye upon reduction in the presence of an electron carrier. WST-8 is reduced by dehydrogenases in cells to give a yellow-colored product (formazan), which is soluble in the tissue culture medium. The amount of the formazan dye generated by the activity of dehydrogenases in cells is directly proportional to the number of living cells. PC12 cells were plated in 100 μl of complete DMEM the day before the experiment in 96 well plates. Treatments with T30, T14 and/or NBP14, Ab-19 at different concentrations (in a volume of 20 μl complete DMEM) were added and incubated for 2 h in the incubator. The medium in which the PC12 cells are, during the incubation, contains Ach from the FBS [17] and HS [18]. Subsequently, 12 μl of CCK-8 solution (10% of total volume) were added. The plate was incubated for 2 h in the incubator and then placed in the absorbance plate reader. The absorbance was measured at 450 nm in a Vmax plate reader (Molecular Devices, Wokingham, UK).

2.4. AChE activity assay

AChE activity was measured using the MAK119 AChE Assay Kit from Sigma-Aldrich (St Louis, MO), which is an advance essay of the Ellman essay (Ellman et al., 1961). Thiocholine, produced by AChE reacts with 2-nitrobenzoic acid forming a colorimetric product, which is proportional to the AChE activity. PC12 cells were plated in 100 μl of complete DMEM 2 days before the experiment in 96 well plates. On the day of the experiment, after removal of the 100 μl complete DMEM medium, cells were treated with T30, T14 and/or NBP14, Ab-19 (added to HBSS) at different concentrations and incubated for 1 h 45 in the incubator. During this incubation, Ach was still available from the PC12 cells themselves as they contain and secrete this nicotinic agonist [19]. After the incubation, absorbance measurements were taken for an interval of 60 min across experiments at 405 nm in a Vmax plate reader (Molecular devices, Wokingham, UK).

2.5. Statistical analyses

At least three independent experiments were performed for all PC12 assays. Data for the three cell-based parameters were expressed as percentage of untreated control cells. Statistical analysis of the Log-transformed data was performed with GraphPAD Prism 9 software using 1-way ANOVA followed by Dunnett's post-hoc tests. Statistical significance was taken at a p value < 0.05. All results in the graphs are presented as mean ± SEM.

3. Results

3.1. Dose dependant changes induced by T14 and T30 in cell-based assays

For the calcium assays, T30 and T14 showed a proportional dose-dependent increase of calcium influx, and a gradual decrease for doses > 5 μM (Fig. 1; left panel; 1-way ANOVA, factor "dose"; T30: $P < 0.0001$, $F_{10,\ 28} = 17.27$, $R^2 = 0.8605$; T14: $P < 0.0001$, $F_{7,\ 20} = 20.82$, $R^2 = 0.7491$). T14 induces a calcium increase of 32.38% from 2.5 nM whereas T30 only showed a significant increase of 24.05% from 250 nM. T14 is more potent than T30, as the increase in calcium influx starts at a lower dose, i.e. 2.5 nM. T14 no longer induce an increase in calcium at 20 μM, whereas T30 still induce a significant increase up to 20 μM.

For the cell viability parameter, T14 in the range of 2.5 nM-10 μM reduced cell counting in a dose-dependent manner (Fig. 1; middle panel; 1-way ANOVA, factor "dose"; $P < 0.0001$, $F_{7,30} = 14.09$, $R^2 = 0.7668$), due to its calcium-related toxicity at longer term. T14 at lower doses (<2.5 nM) was inefficient in inducing toxicity. T14 at higher dose (20 μM) did not reduce cell viability, as there were no toxic effects seen with calcium at that dose. However, T30 was able to reduce cell viability from 250 nM to 10 μM and in the higher range of 30–40 μM (1-way ANOVA; $P < 0.0001$, $F_{8,33} = 6.745$, $R^2 = 0.6205$). This effect at the higher range is likely to be cause by non-specific effect, as there was no increase of calcium at 30–40 μM for T30, therefore no calcium-related toxicity. The AChE release confirmed the different profile between of T14 and T30 (Fig. 1; right panel; 1-way ANOVA, factor "dose"; T30: $P < 0.0001$, $F_{10,30} = 21.31$, $R^2 = 0.8766$; T14: $P < 0.0001$, $F_{9,\ 27} = 17.20$, $R^2 = 0.8515$), as seen in cell viability. T14 was more effective at the dose of 250 nM in releasing the compensatory release of AChE, with an increase of 43.12% (vs. 26.65% for T30).

3.2. Dose dependant effects of NBP-14 and Ab-19 on their own to discard any unwanted toxic effects in PC12 cells

When either NBP14 or Ab-19 were applied alone, none of the parameters studied (calcium influx, cell viability, and AChE release) were

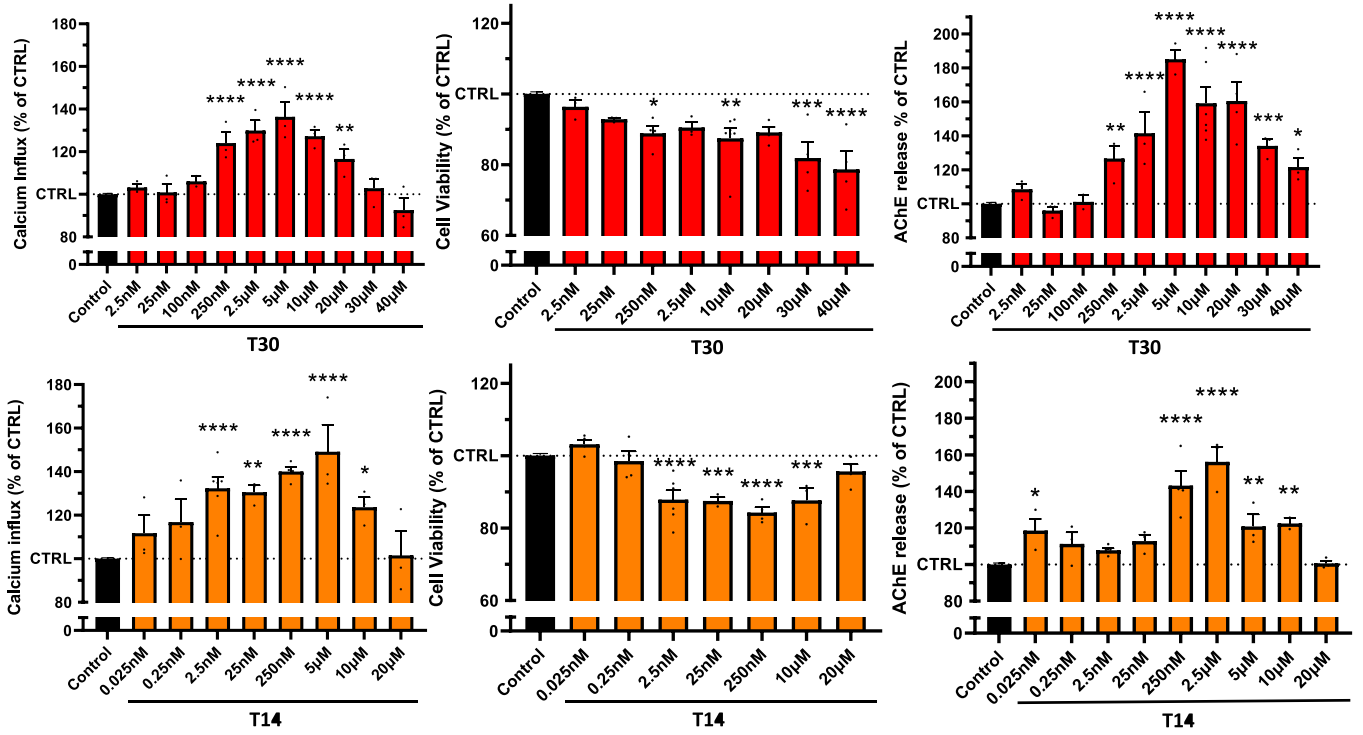

Fig. 1. The effects of the T30 and T14 peptide at different doses on calcium influx (left panel), cell viability (middle panel) and AChE release (right panel) in PC12 cells. For each dose, 3–10 replicates of PC12 cells were cultivated and used to assess the drug response in each assay (mean ± SEM). These three cell-based parameters are expressed as % of untreated control cells. Comparisons between multiple-dose treatments (n = 3–10) were performed by one-way analysis of variance (ANOVA, factor "dose") followed by Dunnett post-hoc tests to determine the significance of each dose in comparison to its control (=100%). *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; ****$P < 0.0001$.

different compared to their respective controls, for all doses tested from 1 nM to 50 nM (Fig. 2). The calcium influx showed a variation between 96.6% and 104% (Fig. 2; left panel) with anti-T14 showing a non-significant dose-dependent reduction from 104% to 96.6 in the doses ranging from 1 nM to 50 nM (Fig. 2; left panel; 1-way ANOVA, factor "dose"; AB19: $P = 0.118$, $F_{4,26} = 2.041$, $R^2 = 0.2389$; NBP14: $P = 0.766$, $F_{4,25} = 0.4576$, $R^2 = 0.06822$). The cell viability showed a variation between 98.1% and 106.3% (Fig. 2; middle panel; 1-way ANOVA; AB19: $P = 0.9919$, $F_{4,25} = 0.06418$, $R^2 = 0.01016$; NBP14: $P = 0.2702$, $F_{4,25} = 1.377$, $R^2 = 0.1806$). The AChE release parameter showed a variation between 98.4% and 106.7% (Fig. 2; right panel; 1-way ANOVA; AB19: $P = 0.5916$, $F_{4,25} = 0.7118$, $R^2 = 0.1022$; NBP14: $P = 0.8459$, $F_{4,25} = 0.3436$, $R^2 = 0.1022$).

3.3. Reversal of T14 toxicity by Ab-19 and NBP14 in a dose-dependent manner

Since it was the more potent under these particular experimental conditions, T14 was chosen over T30 as the test toxin to compare the efficacy of Ab-19 and NBP14. Based on the T14 dose response in Fig. 1, the optimized conditions with the most significant difference between T14 and control was at the dose of 250 nM (and was sufficient to induce an increase of 40%): with $P < 0.01$ for Calcium, $P < 0.001$ for Cell viability, and $P < 0.0001$ for AChE). Therefore, the Antibody and NBP14 efficiencies were tested against T14 at 250 nM. Here, we compared the efficacy of Ab-19 with NBP14. PC12 cells treated with T14 alone showed an increase in calcium influx (129 ± 5.19%) relative to the control. The cytotoxic effect of T14 was diminished when applied alongside either NBP14 or Ab-19 as seen in the Fig. 3B. A dose-dependent decrease in calcium influx can be seen as the dose increases from 5 nM to 50 nM, for

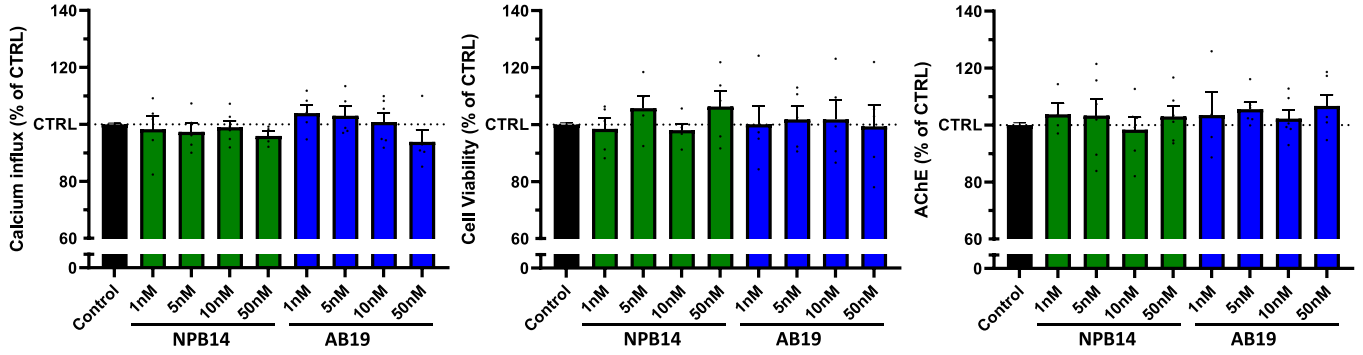

Fig. 2. The effects of the NBP14 peptide and Antibody (AB19) at different doses on calcium influx (left panel), cell Viability (middle panel) and AChE release (right panel) in 4–10 replicates of PC12 cells (mean ± SEM). These three parameters are expressed as % of untreated control cells. 1-way ANOVA ("factor" dose) tests were used for statistics. None of the doses differed significantly. calcium influx: $P \geq 0.118$; Cell Viability: $P \geq 0.2702$; AChE: $P \geq 0.5916$.

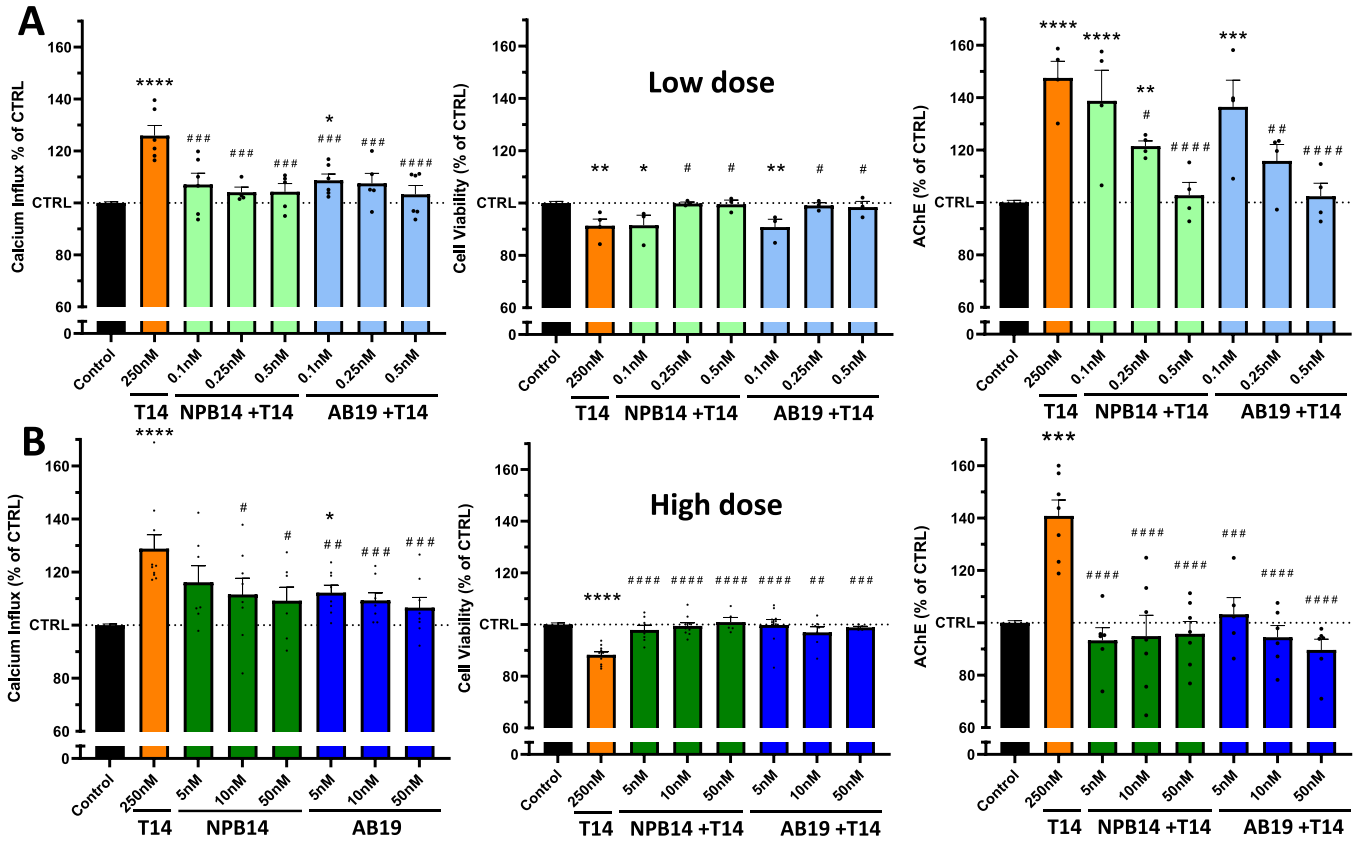

Fig. 3. The inhibiting effects of different high (A) and low (B) doses of NBP14 peptide and Antibody (AB19) on T14-induced calcium influx (left panels), T14-induced cell toxicity (middle panel) and T14-induced AChE (right panels), in 3–10 replicates of PC12 cells (mean ± SEM). These three cell-based parameters are expressed as % of untreated control cells. 1-way ANOVA ("factor" dose) tests were used for statistics followed by Dunnett post-hoc tests to determine the significance of each dose in comparison to its control (=100%). *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; ****$P < 0.0001$, and versus T14: $^{\#}$: $P < 0.05$; $^{\#\#}$: $P < 0.01$; $^{\#\#\#}$: $P < 0.001$; $^{\#\#\#\#}$: $P < 0.0001$.

both NBP14 (5 nM: 116 ± 6.23%; 10 nM: 112 ± 6.17%; 50 nM: 109 ± 5.16%; 1-way ANOVA, factor "dose"; $P = 0.0011$, $F_{4, 37} = 13.43$, $R^2 = 0.3805$) and Ab-19 (5 nM: 112 ± 2.86%; 10 nM: 109 ± 2.79%; 50 nM: 107 ± 3.88%; $P < 0.0001$, $F_{4, 39} = 12.05$, $R^2 = 0.5527$). At lower doses (<1 nM), a dose-dependent decrease in calcium influx was also seen as the dose increases from 0.1 nM to 0.5 nM (Fig. 3A; NBP14: $P < 0.0001$, $F_{4, 26} = 13.43$, $R^2 = 0.6738$; AB19: $P < 0.0001$, $F_{4, 28} = 14.05$, $R^2 = 0.6675$; post-hoc Dunnett comparisons between each dose with T14: $P < 0.05$).

The viability of PC12 cells returned to control conditions when applied with NBP14/Ab-19 in addition to T14 (Fig. 3B), as seen in NBP14 (5 nM, 97.9 ± 1,88%; 10 nM, 99.4 ± 1.19%; 50 nM, 101 ± 1.85%) and Ab-19 (5 nM, 99.8 ± 2.15%; 10 nM, 97.0 ± 2.07%; 50 nM, 98.9 ± 0.411%). There was no significant decrease (vs. control) for NBP14 and Ab-19 at 5 nM, 10 nM and 50 nM ($P \geq 0.4935$); their efficacy in terms of preventing cell death from T14 is prominent when compared to T14 alone (NBP14: $P < 0.0001$, $F_{4, 37} = 19.61$, $R^2 = 0.6795$; AB19: $P < 0.0001$, $F_{4, 37} = 11.54$, $R^2 = 0.555$; post-hoc Dunnett comparisons between each dose with T14 alone: $P < 0.05$). At lower doses (<1 nM; NBP14: $P = 0.0015$, $F_{4, 18} = 6.86$, $R^2 = 0.6039$; AB19: $P = 0.0009$, $F_{4, 18} = 7.592$, $R^2 = 0.6278$), the only doses that fail to prevent cell death from T14 was the lowest ones, i.e., 0.1 nM (Fig. 3A).

All treatments of either NBP14 or Ab-19 given in conjunction with T14 showed a reduction in AChE activity from the T14-treated cells, in Fig. 3B (NBP14: $P < 0.0001$, $F_{4, 30} = 10.81$, $R^2 = 0.5904$; AB19: $P < 0.0001$, $F_{4, 27} = 18.82$, $R^2 = 0.7360$). This is clearly demonstrated by a reduction in absorbance relative to the control as the concentration of Ab-19 increases (5 nM, 103 ± 6.34%; 10 nM, 94.5 ± 4.46%; 50 nM, 89.7 ± 4.05%). The absorbance of the NBP14, however, stays relatively stable with increasing concentration (93.3 ± 4.80%, 94.9 ± 7.94%, 95.8 ± 4.69%). There was no significant increase (vs. control) for NBP14 ($P \geq 0.7154$) and Ab-19 ($P \geq 0.1726$) at 5 nM, 10 nM and 50 nM. At lower doses (<1 nM), a clear dose-dependent decrease in AChE was seen as the dose increases from 0.1 nM to 0.5 nM (NBP14: $P < 0.0001$, $F_{4, 19} = 18.15$, $R^2 = 0.7926$; AB19: $P < 0.0001$, $F_{4, 19} = 16.10$, $R^2 = 0.7722$). There was no significant increase (vs. control) for NBP14 and Ab-19 at the doses of 0.5 nM (Fig. 3A).

4. Discussion

The PC12 cell line, derived from the adrenal medulla, is one of the most commonly used in neuroscience research, including studies on neurotoxicity, neuroprotection, neurosecretion, neuroinflammation, and synaptogenesis [20]: the cells are frequently used for an in vitro drug screening platform [21], because they can synthesize, store and release different neurotransmitters such as dopamine and acetylcholine [19]. PC12 cells offer the same features as chromaffin cells which are, in turn derived from the neural crest but located in the center of an accessible peripheral organ, the adrenal medulla: PC12 cells have therefore been described as offering a 'window' into the brain [22] and as such were deemed to offer an appropriate model for our study.

This cell line has the additional advantage of being widely used as an in vitro model for neurodegenerative disorders. Alzheimer's disease can be modeled by the exogenous administration of β-amyloid peptide [23], and Parkinson's disease by inducing cell death with 1-methyl-4-phenyl-pyridinium or 6-hydroxydopamine [24]. In addition, the adrenal medulla in Alzheimer's patients shows various pathological features comparable to those seen in the central nervous system, e.g., numerous

Lewy body like inclusions, neurofibrillary tangles and paired helical filaments, as well as expression of APP [25].

The most immediate, direct parameter assessed here was the calcium influx into cells via the alpha-7 nicotinic receptor. This effect is dependent on extracellular calcium since it is abolished in the presence of the chelator EDTA [26] and decreased by up to 5-fold in a Ca^{2+} free solution [27]. It has already been established in transfected oocytes that the peptide modulates calcium entry via an allosteric site of the alpha-7 receptor [5]: T14 induces a direct calcium increase only in the presence of the primary ligand ACh [5] and hence has been posited to act at an allosteric site to modulate influx of the ion. Although PC 12 cells possess other nicotinic receptors [28,29], the ACh-triggered calcium influx monitored here is most likely mediated exclusively via the alpha-7. The peptide fails to induce any calcium increase in oocytes transfected with α4/β2 receptors [5]. Furthermore, in *ex vivo* brain slices, T30 causes a significant attenuation of electrically evoked response selectively in the substantia nigra that contains alpha-7 receptors but not in the striatum, an area rich in nicotinic receptors, but where the alpha-7 specifically is absent [15].

The binding of both T14 and T30 to the alpha-7 receptors has already been respectively characterized in GH4-ha7 cells [2]. However here we compare for the first time, the full dose response relations of both T14 as well as T30 (Fig. 1) At higher doses), both T14 and T30 appear to block calcium influx, as demonstrated previously in alpha-7 transfected oocytes [5], most probably due to channel inactivation [30] and/or channel desensitization [31]. In this study, T14 proved the more potent when compared with T30 as it inhibits the alpha-7 receptors above 10 μM, whereas T30 inhibits the channel above 20 μM, with values back to control level. In contrast in an earlier study [2], T30 had stronger binding to the α7 receptors compared to T14 at 10 μM when measured acutely in live GH4-ha7 cells. However, this discrepancy was apparent using the distinctly different and much more indirect parameter of competitive binding between T30/T14 and the α-Bungarotoxin (α-Bgtx), which has very high affinity for the receptors. In this scenario, T14 exhibited approximately 40% efficacy in displacing α-Bgtx at 10 mM concentration, whereas the same concentration of T30 was about 65% efficacious [2]. This differential potency of T30 over T14 could be explained by the different preparation used, different cell line, and most importantly a different time course (2 h versus 1 day) most likely via the longer-term metabotropic response of the alpha-7 receptors [32] than that used in the current study.

The second cell-based parameter that we tested was cell viability. Although CCK8 was used as a parameter of cell viability but could be interpreted as changes in cell proliferation (in proliferating cells). However, this scenario can be discounted because we have showed that cell viability was also compromised using other technique, the sulforhodamine assay which stains cells density (based on protein content) that are still in good shape and attached to the bottom of the well [33]. In this case, a clear difference between the effects of T14 and T30 for cell viability was that, at higher doses the effects of T14 gradually reduced back to baseline, presumably due to inhibition of the triggering calcium influx as the ionophore becomes phosphorylated [34], whereas the effects of T30 only increased, i.e. an increasing number of cells died. The percentage of living cells is still lower than control level and can be due to non-specific toxic action of the larger peptide, perhaps due to aggregation or non-specific binding irrespective of the alpha-7 receptor, as observed previously [5].

The third cell-based parameter is enhanced release of AChE from extant cells as retaliation to the T-14 induced excitotoxic death of their neighboring counterparts: hence, the AChE-release indicates a compensation for the drop in T14-induced cell viability, [4]. However, following T30 at high doses, the AChE compensatory release, reduced but still significant, probably reflects the continuing cell loss whereas for T14, the effect on AChE release is back to control level, as was the effect on cell viability, hence no compensation is triggered.

Neither the receptor blocker NBP14 nor the antibody Ab-19 had significant effects when applied alone, i.e. all responses remained within 10% of the baseline control. NBP14 or Ab-19 are both themselves inert and non-toxic as shown in other preparations such as in PC12 cells [4], in *ex vivo* brain slice preparation that maintains brain functional activity with voltage-sensitive dye imaging (VSDI) [10,11] as well as in an *ex vivo* model procedure, which combines the advantages of both in vivo and in vitro preparations using artificial cerebrospinal fluids (aCSFs) [9]. The inefficacy of NBP14, and indeed the antibody seen here, suggests that, compared with *ex vivo* rat brain slices [9], there is little endogenous T14 released to exert any effect as an extracellular signaling molecule, and that such responses would need to be evoked or supplied with exogenous peptide, as here.

In the presence of exogenous T14, the induced calcium influx is minimally viable, and much reduced, already with the lowest doses (0.1 nM) of NBP14 or Ab-19. However significant reversal effects on cell viability are only observed for either agent, at the higher dose of 0.25 nM, whilst the most delayed and indirect effect of AChE release is only completely blocked at the higher dose of 0.5 nM, in a dose-dependent manner (Fig. 3A). This differential sensitivity in inhibitory effect would discount a simple one-on-one complexing of the antibody (at 0.1 nM) and T14, or indeed a simple receptor block of the initial effect by NBP14, that could then ensure the same subsequent near-complete antagonism across all the three parameters tested.

The profile of AChE release suggests that the dose of the agent is the critical factor (see Fig. 3A, low dose) rather than the duration as the short vs. longer-term responses, reflected by the different cell based-parameters, show no obvious difference between themselves, at high dose (Fig. 3B).

The parallel effects seen with NBP14 and Ab-19 suggest the final effect is dependent not on the respective mechanism of action of either agent, but on the properties of the eventual T14 target, perhaps on the long-term effects mediated via the G protein-coupled α7 nicotinic receptor [35].

5. Conclusions

This in vitro study has shown that the anti-T14 antibody Ab-19 is as potent as the receptor blocker NBP-14 in the PC12 cells. We have previously reported that T14 selectively activates metastatic breast cancer cells via the alpha-7 nicotinic receptor [36] and have suggested that Alzheimer pathology could share a similar molecular mechanism with cancer, with T14 acting as the key signaling molecule [37] driving a an aberrant recapitulation of the development (via alpha-7 receptors). Therefore, the therapeutic application of immunotherapy targeting T14 itself, rather than targeting the later stage of neuroinflammation (or amyloid), could be viewed as a potential therapy in multiple neurodegenerative disorders.

CRediT authorship contribution statement

Sibah Hasan: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Visualization, Supervision. **Mehreen Ahmed**: Formal analysis, Investigation, Data curation for data shown in Fig. 3B. **Sara Garcia-Ratés**: Conceptualization, Writing – review & editing, Supervision, Project administration. **Susan Greenfield**: Conceptualization, Writing – original draft, Supervision, Project administration, Funding acquisition.

Author information

The authors declare the following competing financial interests: Susan Greenfield is the founder of Neuro-Bio Limited (www.neuro-bio.com), a privately owned Company, and holds shares in the Company. Sibah Hasan and Sara Garcia-Ratés are employees of Neuro-Bio Ltd. Mehreen Ahmed (who has now left the company) was a full-time master

student (University of Nottingham) on industrial placement with Neuro-Bio Ltd.

Conflict of interest statement

The authors declare competing financial interests: Sibah Hasan and Sara Garcia-Ratés are currently employees of Neuro-Bio Ltd. Susan Greenfield is the founder and president of Neuro-Bio Ltd. and holds shares in the company.

Data availability

Data will be made available on request.

Acknowledgments

This project was funded by Neuro-Bio Ltd. The work described in this paper is covered by international patent applications, "WO2015/004430" (Family 1 – NBP-14) and "WO2016/156803" (Family 5 – anti-T14).

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