Notes

Antibodies directed to the phospho-tau peptide (residues 111-137) dissociate tau oligomers and reduce the spatial memory deficits in non-transgenic tauopathy model rats

Sarada Subramanian* & Ganesh Savanur

Department of Neurochemistry, National Institute of Mental Health & Neurosciences, Bangalore - 560 029, Karnataka, India

In dementia, Alzheimer's disease (AD) is the most common type, characterized by the deposits of neurofibrillary tangles and senile plaques with concomitant deterioration in spatial memory and other cognitive functions. Till date, although no cure is available for AD, a few treatment options offer help in reducing the symptoms. In the present study, the sequence 111-137 in the distal N-terminal charge transition region of tau, harbouring the pathologically relevant phospho-serine (pSer 113) and phospho-threonine (pThr 123) ([111]TPpSLEDEAAGHVpTQARMVSKSKDGTGS[137]) was selected as a potential immunotherapeutic peptide. Polyclonal anti-peptide antibodies raised in rabbits effectively dissociated the oligomers/aggregates of recombinant human tau *in vitro*. Administration of affinity purified anti-peptide antibodies to the okadaic acid induced tauopathy model rats resulted in a significant progress in spatial memory functions in Barnes maze task with concomitant reduction in p-tau levels in the hippocampal homogenates. Thus, targeting the phospho-residue sequence 111-137 in tau may be therapeutically relevant for AD and other related tauopathies. These antibodies may also have a clinical value in terms of immunosassay development for quantitation of pathology associated pSer113 and pThr 123 in AD samples.

Keywords: Alzheimer disease, Barnes maze, Dementia, Okadaic acid

Alzheimer's disease (AD), the most common form of dementia, is characterized by gradual deterioration of cognitive functions due to diminished synaptic functions and loss of synapses with accumulation of insoluble amyloid ß (Aß) aggregates[1] and hyperphosphorylated tau in the form of neurofibrillary tangles (NFT)[2] in the hippocampus and neocortex of the affected brain. Apart from AD, NFT are also seen in several other neurodegenerative disorders collectively referred to as tauopathies. No effective treatment is yet available for AD and related tauopathies. Till date, the available treatments include cholinesterase inhibitors and NMDA receptor blockers, which provide symptomatic relief. In this context, Aß based immunotherapy as a disease-modifying approach for AD is being extensively pursued, albeit with limited success[3]. This is partly because some of the approaches which have targeted the most toxic oligomeric/fibrillar species resulted in affecting the relevant physiological forms and, thus, caused toxicity[4]. This has necessitated the need for alternative targets of AD research. In this regard, since NFT pathology correlates better with the degree of dementia than Aß plaques[5], targeting tau pathology might be more effective than Aβ-directed therapy for AD. In fact, therapeutic approaches that influence tau hyperphosphorylation, aggregation or propagation are being actively pursued[6-9]. A few mAbs recognising the epitopes on the N-terminus of tau protein[10] and/or all six tau isoforms and active immunization with tau are being tested in clinical trials[11,12].

Earlier, we demonstrated that passive immunization of okadaic acid (OA) induced AD model rats with antibodies to the phospho-peptide sequence in the N-terminal region (residues 50-71) containing the pathologically relevant phospho-amino acids (serine 68, threonine 69 and threonine 71) significantly improved the performance of these rats in terms of reduction in latency with reduced number of errors in Barnes maze task, with a reduction in phospho-tau (p-tau) levels in these experimental model rats[13]. In the present study, we focussed on less explored region in the distal N-terminus of tau spanning the residues 111-137 for the following reasons: (i) the chosen sequence possesses two distinct clusters of negatively charged residues followed by positively charged residues[14]; (ii) harbours Ser-113 and Thr-123 implicated to be phosphorylated in AD[15]; (iii) high predictive score for B-, T- and MHC-II epitopes within the sequence[16].

Material and Methods

Animals

Okadaic induced tauopathy model rats, earlier developed and characterized from this laboratory[13]

*Correspondence:
Phone: +91 80 2699 5165
E-mail: sarada@nimhans.ac.in

were made available for this study. This study has been approved by the Institutional Animal Ethics Committee (IAEC number: AEC/ 51/ 319/ N.C.).

Materials

The 27-amino acid long phospho-peptide of tau ([111]TPpSLEDEAAGHVpTQARMVSKSKDGTGS[137]) custom synthesized using Fmoc chemistry was purchased from Genpro Biotech Pvt Ltd (Gautam Budh Nagar, India). Purity (>90%) of the synthetic peptide was ascertained by HPLC analysis. Polyclonal anti-phospho-peptide antibodies were raised in rabbits following the immunization protocol described earlier[13]. Recognition of the full length tau by these peptide antibodies was ascertained by ELISA. Affinity purification and preparation of Fab fragments of these antibodies were done following the procedures described earlier[13]. ELISA kits for quantitation of total tau (t-tau) (# MBS009429) and phospho tau (p-tau) (# MBS1600387) in rat hippocampal homogenates were obtained from My Bio Source (USA). Recombinantly expressed full length human tau used for aggregation studies[13] was available in the laboratory. All other reagents used were purchased from Sigma Aldrich (Bangalore, India).

Tau oligomer dissociation studies

Aggregates of tau were prepared using the recombinant tau expressed and purified in our laboratory[13] and their formation was confirmed by thioflavin S binding. An aliquot of the tau aggregate (0.3 mg/mL) was incubated with Fab fragments of the anti–phospho-peptide antibodies or pre-immune IgG (1.0 mg/mL) for 24 h with agitation at 37°C and analyzed by 10% SDS-PAGE.

Studies with experimental rats

The passive immunization studies were followed according to the earlier described procedures[13]. Briefly, 6-month-old female rats were first treated with a single dose of okadaic acid (OA) (500 ng/kg body w.) through intranasal route followed by 6 doses of affinity purified anti-phospho-peptide IgG (1.0 mg/ animal/i.p.) on alternate days, starting from second day till day 12 after OA administration. While the antibody treatment was in progress, the animals were subjected to behavioural assessment by Barnes maze as described earlier[17]. Briefly, the acquisition phase included a total of 15 trials (3 trials/day), starting from day 6 till day 10 of the experimental procedure. The duration of each trial was restricted to a maximum of 2 min. In case the rat did not find the escape box within 2 min, it was gently

guided to it. Latency (time taken by the rat to find the escape box) and errors (nose pokes into wrong holes) were recorded. After a gap of 5 days, on day 15, three trials were conducted and the retention was evaluated. The mean values of the three trials were calculated and the data presented.

By using commercially available quantitative sandwich ELISA, total tau (t-tau) and phospho tau (p-tau) levels in the hippocampal homogenate were measured. The rat tau protein ELISA kit used in the present study was specific to rat species with assay sensitivity of 2 pg/mL and detection range of 15.6-500 pg/mL. The rat phospho tau protein ELISA kit was specific to rat species with assay sensitivity of 0.56 pg/mL and detection range of 1-400 pg/mL.

Statistical analysis

The data were expressed as mean ± S.E.M. and differences between groups were analyzed by ANOVA, followed by Tukey's multiple comparisons using GraphPad Version 3 (Prizm; GraphPad Software Inc, San Diego, California, USA). $P < 0.05$ was considered statistically significant.

Results and Discussion

Tau, a heat stable protein is highly enriched in neuronal cells and plays an important physiological role in several cellular functions including the microtubule stabilization, axonal transport, nuclear functions, synaptic function and neuronal signalling pathways. In the CNS, tau can exist as six isoforms arising from alternative mRNA splicing, ranging in length between 351 and 441 amino acids. Tau undergoes several post-translational modifications including phosphorylation, glycosylation, methylation, acetylation, prolyl-isomerization, nitration, sumoylation and ubiquitylation during both the physiological and pathological conditions[18]. Among them, phosphorylation is the most studied phenomenon of tau[19]. In AD, hyperphosphorylation of tau results in destabilization and dissociation from microtubules, and accumulation as neurofibrillary tangles. Since NFTs and synaptic loss strongly correlate with cognitive impairment in AD patients, attention is being focussed on targeting the phosphorylated tau for immunomodulation in AD. Till date, although the Tau based immunotherapeutic approaches have focussed on all parts of the tau molecule, particularly those antibodies targeted against the N-terminus, microtubule binding region and the C-terminus are

being progressed into clinical trials (URL: https://clinicaltrials.gov).

The structure-function relationship studies of tau revealed that the microtubule binding region is located in the C-terminal half of the protein[20]. The N-terminal portion extends from the microtubule region and interacts with cytoskeletal elements and the plasma membrane[21]. Oligomerization of the charge transition region (106-144 amino acids) within the N-terminal region of tau has been demonstrated earlier by ion mobility-coupled mass spectrometry, which has implications for normal and pathological tau action[14].

Selection of the epitope peptide

The N-terminal region (residues 106-144) is involved in tau-dimer assisted microtubule growth for normal tau action[14]. N-terminal tau fragments accumulate in tauopathy conditions, and are the candidates for trans-synaptic pathological tau transfer in spreading the neurodegeneration. Herein, we scanned this sequence using the online resource (URL: www.iedb.org) for immunologically relevant phospho-peptide epitope, to select for immunoneutralization studies in the experimental AD model rats. Our search resulted in selection of the 27-amino acid long peptide ([111]TPpSLEDEAAGHVpTQARMVSKSKDGTGS[137]) with a high predictive score for immunogenicity index within the peptide sequence. More importantly, the selected sequence has two pathologically associated phosphorylation sites at 113 (Serine) and 123 (Threonine)[15].

Dissociation of Tau aggregates by polyclonal phospho-tau epitope peptide antibodies

The influence of the anti-phospho-peptide antibodies in dissociating the aggregates of tau was assessed by SDS-PAGE analysis. The results presented in Fig. 1 suggest that the Fab fragments of these antibodies effectively dissociated the oligomers/aggregates of tau *in vitro*, in particular, the higher order oligomers. The non-specific effect of dissociation by the antibodies was ruled out by conducting the experiment with Fab fragments of pre-immune IgG.

Passive immunization studies

In this study, okadaic acid (OA) induced tauopathy rats earlier characterized and available in the laboratory were chosen as the experimental models since it is well established that selective inhibition of PP2A by okadaic acid can induce an AD-like accumulation of hyperphosphorylated tau[22] and cognitive deficiency in OA-treated rats[23]. Using these OA-rats, passive immunization studies were conducted with the phospho-peptide antibodies. It is evident from the results presented in Table 1 that anti-phospho-peptide antibody treated animals have shown marked reduction (up to 50%) in the number of errors and latency in finding the escape hole in comparison to pre-immune IgG treated rats. Antibody treatment significantly improved the spatial memory functions in OA rats. More importantly, passive immunization had dramatically decreased p-tau levels in the hippocampal homogenates (Table 1) without any significant change in the t-tau levels across the groups. Based on our results, it is hypothesized that, these antibodies may
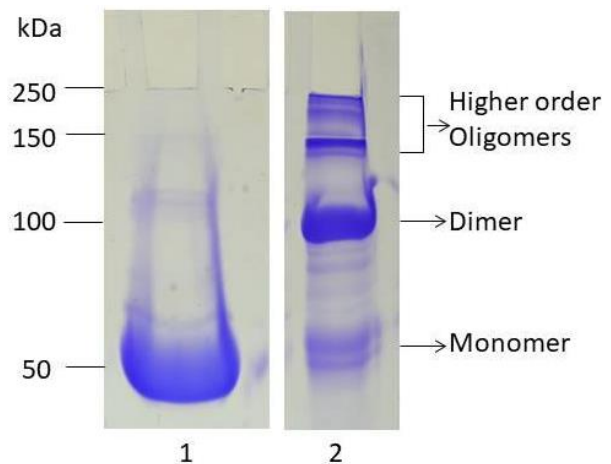

Fig. 1 — Representative SDS-PAGE (10%) images demonstrating the influence of the anti-phospho-peptide antibodies on dissociation of oligomers/aggregates of tau. [Shown are the mobility patterns of tau in the presence of Fab fragments of the anti-phospho-peptide antibodies (lane 1) or control (pre-immune) IgG (lane 2)]

Table 1 — Performance by the experimental rats in Barnes maze task during the retention phase. The corresponding p-tau levels in the hippocampal homogenates are indicated

Parameter	Ctrl	OA rats	OA rats+Pre-immune IgG	OA rats+Anti-phospho-peptide IgG
Number of errors	1.3±0.4	4.9±0.8[##]	4.4±0.9	2.6±0.6*
Latency (s)	15.4±3.7	61.4±6.5[##]	55.7±8.0	27.3±4.9*
p-tau (pg/100 mg tissue)	50±24	85±29[#]	81±20	42±19*
t-tau (pg/ 100 mg tissue)	252±34	258±49	231±44	264±37

[n=6 in each sub-group. Data are presented as mean ± S.E.M. [#]$P <0.05$, [##]$P <0.001$ compared to age matched controls. *$P <0.05$ compared to age matched OA treated rats. The data was analyzed by ANOVA followed by Tukey's multiple comparisons test]

bind to the aggregates of tau, i.e. either extracellular or intracellular pathological species and by disposing them, these antibodies may halt disease progression. Alternatively, these antibodies may halt trans-cellular spread of pathological tau[24] in the CNS. These antibodies may also have a clinical value in terms of immunosassay development for quantitation of pathology associated pSer113 and pThr 123 in AD samples.

Conclusion

Results obtained from the passive immunization studies conducted with antibodies to the phospho-peptide (111-137) in OA induced AD rats are presented. The polyclonal antibodies raised against the sequence 111-137 in the distal N-terminal charge transition region of tau, harbouring the pathologically relevant phospho-serine (pSer 113) and phospho-threonine (pThr 123) have effectively dissociated tau aggregates *in vitro*. Passive immunization with these anti-peptide antibodies have resulted in a significant progress in spatial memory functions in OA rats. A concomitant reduction in p-tau levels in the hippocampal homogenates of the anti-peptide antibody treated rats has also been recorded. Further studies are warranted to elucidate the mechanism of action of these phospho-peptide antibodies in improving the spatial memory functions and the possible therapeutic potential of the identified N-terminal phosphopeptide (residues 111-137) of Tau for AD/tauopathy vaccine development.

Conflicts of interest

Authors declare no conflict of interests.

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