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Structural biochemistry of the pentraxins

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Citation

Noone, D. P. (2024, March 12). *Structural biochemistry of the pentraxins*. Retrieved from <https://hdl.handle.net/1887/3721706>

Version: Publisher's Version

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Note: To cite this publication please use the final published version (if applicable).

8 Immunity on the mind

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Abstract

The neuronal pentraxins are a subclass of long pentraxins present in the nervous system, with the ability to bind both immune proteins and ligands associated with synaptic signalling. As such, they represent promising candidates for proteins linking seemingly disparate factors linked to neurodegeneration; the immune system and synaptic plasticity. At the time of writing, structural information relating to the NPTXs is limited to high resolution structures of individual pentraxin domains or biochemical analysis of the complexes. However, little structural information relating to the complete complexes is available. As the N-terminal regions of PTX3 has been shown to result in novel protein architecture, the same may be true for the neuronal pentraxins with ramifications for models of their function *in-situ*. In this study we being to address some of these issues using electron microscopy, biochemical analysis and structural prediction to interrogate the quaternary structure of the neuronal pentraxin, neuronal pentraxin receptor (NPTXR). This advocates a model whereby NPTXR comprises a bouquet of flowers like arrangement of trimeric bundles in a similar manner to complement related proteins C1q and the ficolins, with consequences for their biological function.

Introduction

Inflammation is linked to many diseases such as atherosclerosis (**chapter 4**)(247), with inflammation in the nervous system (neuroinflammation) also linked to pathologies of the brain such as Alzheimer's disease (AD)(248). Although the blood brain barrier limits the entry of immune cells to the central nervous system (CNS)(249), the CNS has a host of resident immune cells such as microglia which can clear cellular debris and participate in synaptic pruning(250). In a similar vein the CNS contains it's own subclass of pentraxins (**chapter 1**), the neuronal pentraxins (NPTXs); NPTX1, NPTX2 and NPTX receptor (NPTXR). The NPTXs share greater homology on a genomic sequence level to each other compared to the other pentraxins (**chapter 1**)(39), indicated the presence of a true subclass. The NPTXs are known to interact with ligands involved in synaptic activity such as AMPA receptors (AMPA)(56, 251) as well as immunological ligands such as C1(252). Furthermore, NPTX1 and NPTX2 are reported to have antagonistic functions in both synaptic plasticity and immune activation. NPTX1 appears to cause long-term depression (LTD) of synapses(253) and results in complement and microglial activation(32). Conversely, NPTX2 stimulates long-term potentiation (LTP)(251, 254, 255). and dampens the immune system(252). The NPTXs also form hexameric arrays when binding AMPAR(42, 252) suggesting nanopatterning of these pentraxins may play a similar role in the CNS as nanopatterned CRP and antibody platforms play in the immune system in the rest of the body (**chapter 3**) (8, 9, 138). However, an extra layer of complexity arises in that the NPTXs can form mixed complexes containing NPTX1,2 or NPTXR(42, 256). In fact, some reports suggest that NPTXR may act as a central hub for these pentraxin arrays (257, 258), tethering them to the membrane and allowing fine tuning of synaptic plasticity via immune mediated synaptic pruning(250). Given that a loss of synaptic plasticity is associated with neurodegeneration(259), NPTXs are potential candidates for

linking neuroinflammation to synaptic plasticity in neurodegenerative conditions. Similarly, the complement system is increasingly being linked to pathologies such as AD(60, 260) suggesting NPTXs could open unexplored avenues for therapeutics. Indeed, their therapeutic potential has been demonstrated in mice(38). However, relatively little is known about their structures, with only the pentraxin domain of NPTX1 resolved to a high resolution to date(38). Given the importance of the N-terminal regions in determining the oligomeric state of the long pentraxin PTX3 (**chapter 6**)(36) and biochemical evidence that the N-terminal regions of the NPTXs may play a similar role(42), structural information on the entire complex may reveal that the NPTXs exhibit novel structural configurations related to their function. As such, in this study we use negative stain electron microscopy (EM) of recombinantly produced NPTXR and structural prediction tools to explore the structure of NPTXR and postulate the roles of NPTXs in the CNS. This could be used as a platform for further structural studies on the NPTXs in order to obtain high resolution structures of each NPTX member, with the ultimate goal of understanding their function and the rational design of therapeutics targeting the NPTXs to treat neurodegenerative diseases.

Results and Discussions

NPTXR forms a large disulfide linked multimer

NPTXR was expressed and purified as previously described for PTX3(36). However, the transmembrane domain of NPTXR was replaced with a His₆-tag to circumvent the technical challenges associated with membrane protein purification, whilst allowing linkage to lipid membranes via Ni-NTA conjugated lipids(**chapter 7**). We then used negative stain EM to interrogate the structure of NPTXR. Tomograms of NPTXR revealed the presence of a bouquet of flowers like structure, reminiscent of the hexadecameric C1q, which was also imaged in parallel for comparison (**Fig. 1A&B**). In contrast to C1q, NPTXR appeared to form larger complexes and contain a greater variation in the number of head domains (median 6 ± 2.4 , **Fig. 1C&D**). This is reminiscent of the ficolins of the lectin pathway, as they also form variable higher order oligomers out of constituent trimeric building blocks(261). In support of this, the electrophoretic profile of NPTXR indicates that it forms large complexes (>200 kDa), which collapse in size when reduced, suggesting the complexes are joined together by disulfide bonds, as has been reported for PTX3 (36, 40) and NPTX1/2(42) (**Fig. 1E**). Additionally, the complex appeared glycosylated as the mass of both reduced and non-reduced NPTXR decreased after treatment with PNGase F (**Fig. 1E**), reminiscent of the electrophoretic profile of PTX3(36, 50).

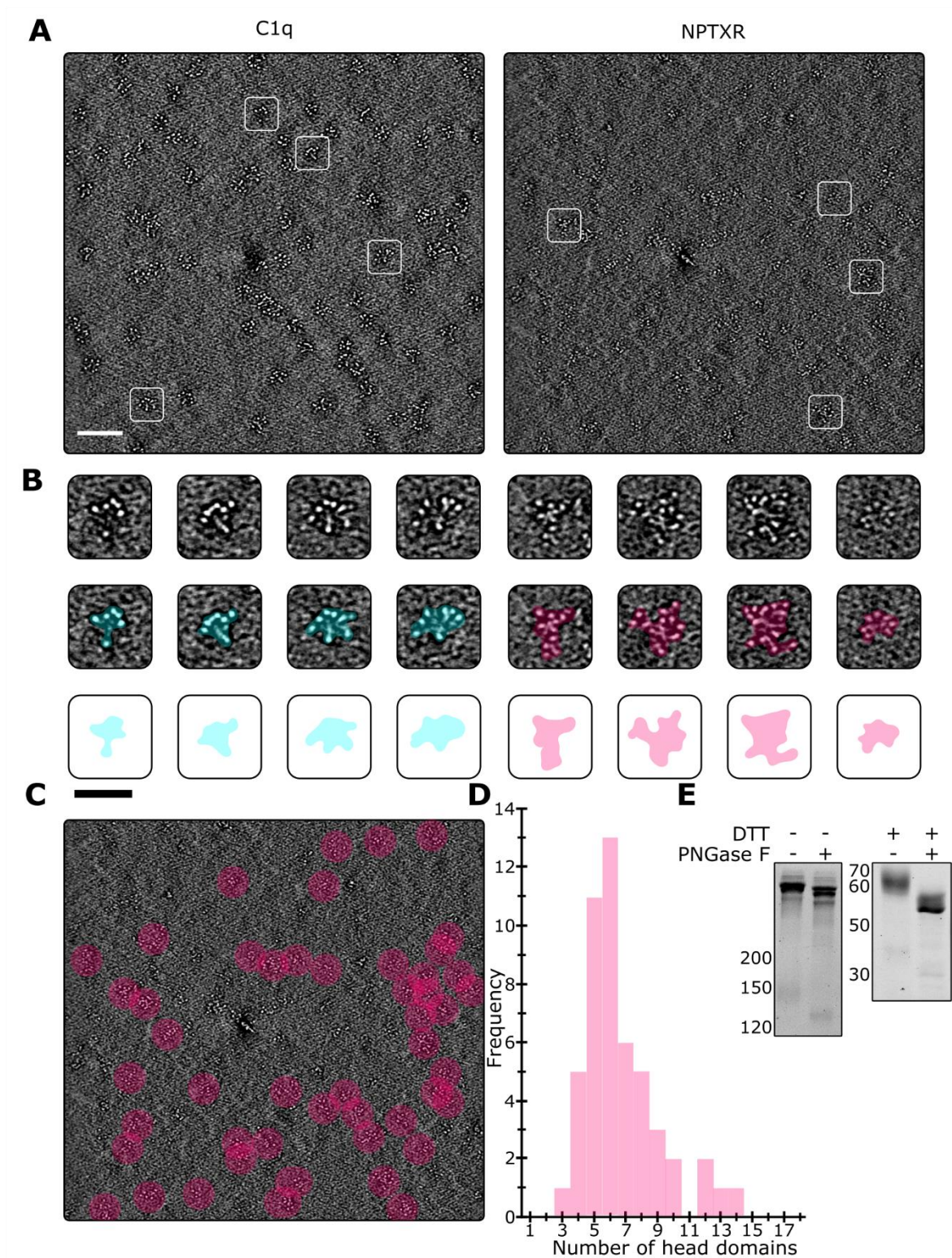


Figure 1: NPTXR Forms large bouquet of flower like homocomplexes. (A) Tomograms of C1q and NPTXR representing slices 10 nm thick. (B) Boxed particles from A enlarged (top), with protein complexes highlighted (middle) and the shapes of the complexes subtracted from background (bottom). (C) Tomogram of NPTXR from A with picked particles highlighted by pink spheres. (D) Picked particles were then used to count the number of head domains per complex, represented as a histogram. (E) gel electrophoretic profiles after TCE staining of

*NPTXR with or without DTT or PNGase F. Arrows indicate standard protein markers of the indicated molecular weight. Scale bar in **A** and **B** represent 100 and 50 nm respectively.*

Structural prediction reveals the Janus nature of NPTXR

The NPTXs share high sequence similarities with each other compared to the other pentraxins (**chapter 1**)(39), suggesting a subclass within the long pentraxin family. To explore the quaternary structure of NPTXR and the other members of the NPTX subfamily further LOGICOIL(207) was utilized to predict the oligomeric states of coiled coil N-terminal regions of each family member. All NPTXs were predicted to have some degree of trimeric nature in their N-terminal regions, suggesting a common structural motif may be present in the NPTXs (**Table 1**). NPTXs form heterocomplexes(42, 256, 257, 262) and thus could form mixed trimeric assemblies, though structural studies would be required to validate this.

To gain further insights from the LOGICOIL predictions, the sequence of the pentraxin domain of NPTXR was submitted to AlphaFold(203) as a trimer (**Fig. 2A**). This revealed a different protein architecture compared to PTX3(36), CRP(34) and SAP(37), though structural methods like cryoEM or X-ray crystallography would be needed to verify that this hypothesized model is correct. Moreover, superimposition of the CRP pentraxin domain (PDB:7PKE) showed that this arrangement would produce an A-face and B-face in the trimer, as residues homologous to the C1-binding sites and calcium coordinating residues would be on opposing sides of the trimeric assembly (**Fig. 2B**). This model implies that N-terminal regions would protrude from the A-face ultimately anchoring the complex to the cell membrane, with the B-face left unobstructed protruding into the synaptic space (**Fig. 2C**). As the B-face appears to mediate interactions with other proteins in many pentraxins resolved to date(35-37), perhaps this interface would regulate binding to ligands, whilst leaving the A-face free to interact with Fcγ receptors or the C1 complex. Indeed, the pentraxin domains of the NPTXs are known to interact with AMPAR(42, 258) with NPTX1 and 2 being shown to also interact with C1 (32). As such, heterocomplexes of NPTXR, 1 and 2 could concurrently bind to synaptic receptors and C1 linking synaptic pruning with synaptic activity.

Protein	N-terminal coiled coil region		Most likely	Second most likely
NPTX1	Sequence	DMCAASVAAGGAEE LRSSVLQLRETVL	Trimer	Tetramer
	Register	defgabcdefgabcdefgabcdefgab		
	Sequence	QQKETILSQKETIRELTAKLGRCESQSTL	Trimer	Tetramer
	Register	cdefgabcdefgabcdefgabcdefgab		
	Sequence	LSRTPAAETLSQLGQTLQSLKTRLENLEQYSRLNSSS	Trimer	Tetramer
	Register	fgabcdefgabcdefgabcdefgabcdefgabefgab		
	Sequence	QTNSLKDLLQSKIDELERQVLSRVNTLEEGKGGPRNDT	Trimer	Tetramer
	Register	cdefgabcdefgabcdefgabcdefgabcdefgabcde		
NPTX2	Sequence	EERVKIETALTSLHQRISELEKGQKDNRP	Trimer	Tetramer
	Register	fgabcdefgabcdefgabcdefgabcdef		
NPTX2	Sequence	QGGAQSP EEELRAAVLQLRETVVQQKETL	Trimer	Dimer
	Register	abcdefgabcdefgabcdefgabcdefga		
	Sequence	GAQREAI RELTGKLARCEGLAGGKAR	Trimer	Dimer
	Register	bcdefgabcdefgabcdefgabcdef		
	Sequence	DPGHVVEQLSRSLQTLKDRLESLEHQLRANVSNAGLP	Tetramer	Dimer
	Register	cdefgabcdefgabcdefgabcdefgabcdefgabcd		
	Sequence	GDFREVLQQRLGELERQLLRKVAELEDEKSLLHNETS	Tetramer	Dimer
	Register	efgabcdefgabcdefcdefgabcdefgabcdefgab		
NPTXR	Sequence	AHRQKTESTLNALLQRVTELERGNSAFKS	Tetramer	Dimer
	Register	cdefgabcdefgabcgabcdefgabcdef		
NPTXR	Sequence	AQQGDAAGAAPGEREELLLLQSTAEQLRQTAL	Trimer	Tetramer
	Register	fgabcdefgabcdefgabcdefgabcdefgab		
	Sequence	QQEARIRADQDTIRELTGKLGRCESGLPR	Trimer	Tetramer
	Register	cdefgabcdefgabcdefgabcdefgab		
	Sequence	DSPALILELEDAVRALRDRIDRLEQELPARVNLSA	Trimer	Dimer
	Register	cdefgabcdefgabcdefgabcdefgabcdefgab		
	Sequence	GLHSKMDQLEGQLLAQVLALEKERVASH	Trimer	Tetramer
	Register	cdefgabcdabcgabcdefgabcdefgab		
NPTXR	Sequence	SSRRQRQEVEKELDVLQGRVAELEHGSSAYS	Trimer	Tetramer
	Register	cdefgabcdefgabcdefgabcdefgabcde		

Table 1: NPTXs and their LOGICOIL predictions for N-terminal coiled coil regions and their oligomeric states. Sequences of predicted coiled coil-regions and assigned heptad register shown, with the most likely and second most likely oligomeric state also shown. Sequences are shown from N-terminal to C-terminal.

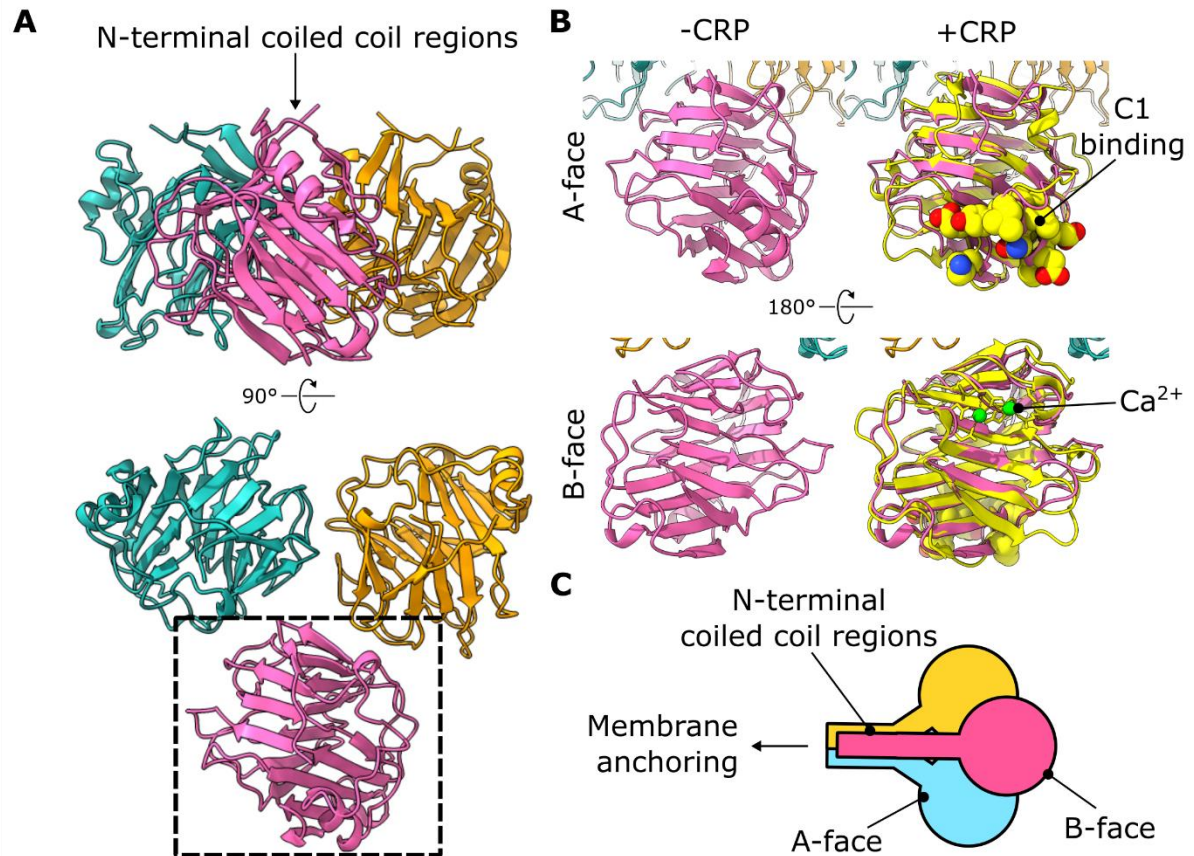


Figure 2: AlphaFold prediction of a trimeric pentraxin domain of NPTXR. (A) Resulting trimeric pentraxin domain complex from AlphaFold prediction. (B) Enlarged monomer boxed off in A with and without CRP superimposed. On CRP the C1 binding site and Ca²⁺ are highlighted as yellow and green spheres respectively. (C) Schematic of the structure showing how it would relate to the locations of the A-face, B-face, N-terminal coiled coil regions and membrane.

Alignment of the primary sequences of the N-terminal domains of NPTXR with C1qC revealed similarities between the two (**Table 2**). Firstly, both sequences contain an abundance of glycine and proline residues, consistent with the collagen-like region of C1q containing characteristic P-O-G motifs(263). Homology at this region may suggest that NPTXR also multimerizes with other trimeric assemblies of NPTXR, leading to the bunching of trimeric building blocks in a similar manner to C1q(9) and the ficolins(261). Additionally, the ficolins and C1q contain an N-terminal cysteine which participates in inter subunit disulfide bonding in order to multimerize(261, 264). Similarly, NPTXR contains a cysteine in a similar region (**Table. 2**), which could suggest that this cysteine is mediating intersubunit disulfide bonds between NPTX N-terminal domains (**Fig. 1E**). Indeed, the electrophoretic profile of NPTXR supports disulfide bonding mediating higher order structures arrangements of NPTXR (**Fig. 1E**). Furthermore, using the NPTXR construct used in this study, trimers alone would results in complexes smaller than those seen via gel electrophoresis (≈ 162 kDa, **Fig. 1**). Indeed EM analysis also suggested larger complexes, similar to scale to C1q (**Fig. 1**). Taken together this data implies that NPTXR forms large bouquet of flower like arrangements much in a similar way to C1q and the ficolins.

C1qC	LKLLLLLLLLL---PLRGQANTG	CYGIP----	GMPGLPGAPG----	KDG	49
NPTXR	MKFLAVLLAAGMLAFLGAVI	CIIASVPLAASPARALPGGADNASVASG			48
	: :*	: *	. :*	***	*
C1qC	YDGLPGPKGEPGIPAI	PGIRGPKGQKGEPLPGH	PGKNGPMGPPGMP		96
NPTXR	AAASPGPQR-----	SLSALHGAGGSAGPPALPG	AAPAAHPLPPGPL		90
	***:	:: :*	* * * * *	***	
C1qC	---GVPGPM-----	G-----	IPGEPGEEGRYKQK		120
NPTXR	FSRFLCTPLAAACPSGA	QQGDAAGAAPGEREELL-L			125
	:	*:	*	***	

Table 2: Sequence alignment of the multimerization domain of C1qC with NPTXR. Conserved and conservative residues are respectively represented by “*” and “:”. N-terminal cysteine is highlighted in yellow.

Towards a structural and functional model of the NPTXs

The low-resolution structural information coupled with the structural predictions and analysis presented in this chapter, can be viewed in the context of the wider literature on the NPTXs to begin to build a model of the role of NPTXs in the nervous system. This in turn may have implications for neuroinflammation and diseases such as AD. NPTX1 can form heterocomplexes with NPTXR on the presynaptic membrane to trans-synaptically cluster AMPAR via interactions with the N-terminal domains of AMPAR (256). Similarly NPTX2 is also known to bind to the presynaptic membrane in order to cluster AMPAR on the postsynaptic membrane to aid glutamatergic signaling(251, 254, 265). In fact, NPTXR may be the central hub of this system given that overexpression of NPTXR recruits NPTX1 and NPTX2 to the cell surface(257) and that overexpression of NPTXR also increases levels of membrane bound NPTX1 and 2(258). Together with the data above, this suggests a model whereby presynaptic NPTXR is anchored to the membrane via its transmembrane domain, forming trimeric coiled coils with other NPTXR or soluble NPTX1 and NPTX2 proteins, thereby mooring them to the presynaptic membrane (**Fig. 3A-D**). Given the homology of the N-terminal domain of NPTXR to the multimerizing N-terminal of C1q (**Table 2**) and the imaging of large disulfide linked complexes of similar scale to C1q (**Fig. 1**), we postulate that trimeric bundles of NPTXs further oligomerize to form higher order structural arrays. We have presented this as multimerization mediated via the membrane proximal portion of NPTXR N-terminal domains, though additional multimerization via the transmembrane domains of distinct NPTXR molecules cannot be ruled out without further structural studies. High order NPTX structures would in turn would enable the organization of postsynaptic ligands such as AMPAR, facilitating the transmission of information across the synapse (**Fig. 3A-D**). However, there is likely a degree of redundancy in this system, given that triple NPTX knockout mice are still viable and showed no obvious cerebral defects(266), and indeed this system appears to interact with other synaptic organizers, such as the C1q-like proteins that interact with postsynaptic G protein-coupled receptors(267). On the other hand, the system does appear to have some therapeutic utility, as NPTX1 has been employed to create a chimeric synthetic synaptic organizer that improves symptoms in mouse models of AD(38), suggesting manipulation of the NPTXs may lead to new therapeutic avenues.

Interestingly, outside of embryonic development in the adult brain, NPTX1 and NPTX2 appear to have opposing functions. NPTX1 appears to be associated with low neuronal activity, LTD and synapse elimination, whereas NPTX2 appears to be associated with high neuronal activity, strengthening of the synapse and LTP. For example, lowering of NPTX1 levels has been shown to lead to an increase in the number of excitatory synapses and enhances LTP(253). Additionally, NPTX1 has been shown to accumulate at the mitochondria, colocalizing with the apoptotic regulator BAX at the outer membrane, in response to low activity inducing apoptotic pathways(268). Furthermore, given the ability of NPTX1 to bind to C1, and reports that it colocalizes with C1 and C4 at phagocytosed synapses in microglia(32), NPTX1 may also participate in synaptic pruning using a complement-dependent mechanism. This would support previous reports that C1q and C3 are needed for synaptic pruning in mouse models(269). Conversely, NPTX2 is synaptogenic(251, 254) and production of NPTX2 is induced after LTP(255). NPTX2 is also better at clustering AMPAR receptors than NPTX1, a property which appears to be determined by the N-terminal domains of NPTX1 and NPTX2, even though this is not the domain that binds to the AMPARs(42). In fact, the ratio of NPTX1 and 2 within NPTX heterocomplexes is dependent on the activity of the neuron, with increased activity leading to a greater proportion of NPTX2 and more AMPAR clustering(42). Together this shows that heterocomplexes vary their compositions depending on the activity of the neuron. As such, central NPTXR hubs anchoring these dynamically shifting proportions of NPTX1 and NPTX2 regulate AMPAR clustering at excitatory synapses, ultimately leading to LTP or LTD forming the molecular basis for synaptic plasticity, learning and memory (**Fig. 3E**).

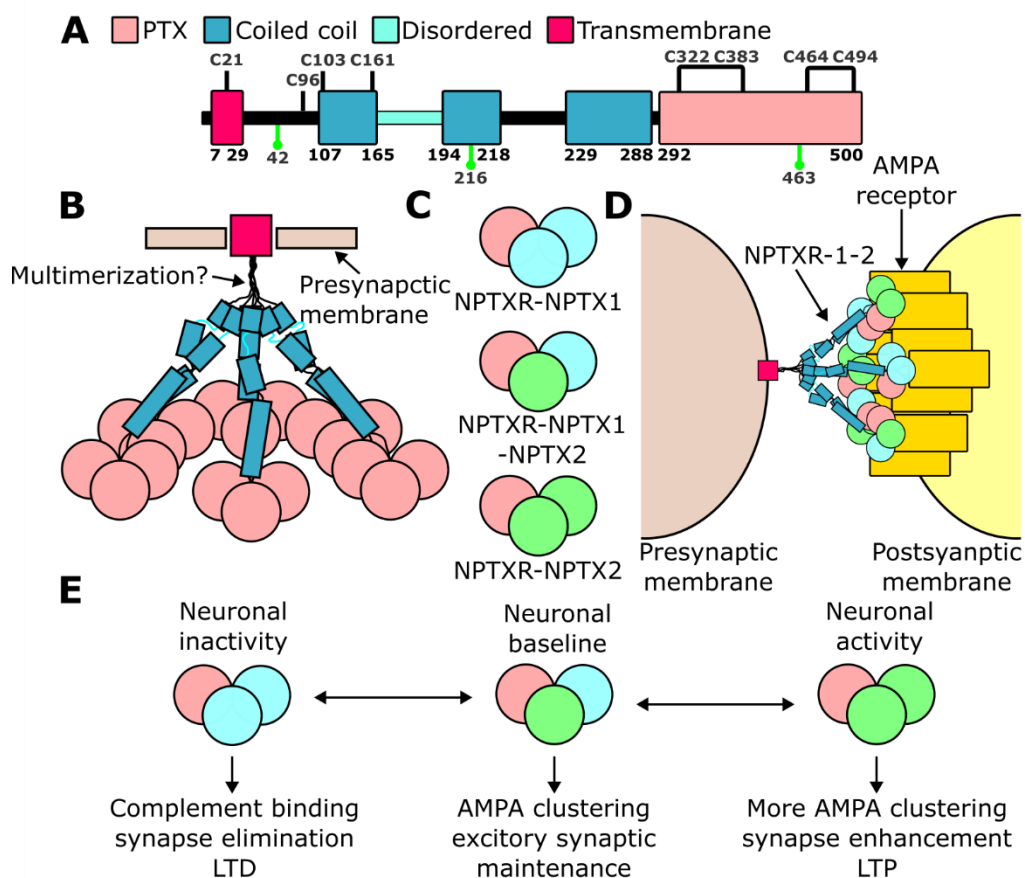


Figure 3: Hypothesized model of NPTXR quaternary structure and function. (A) Schematic of the primary sequence of NPTXR. Residues numbers are shown to show the borders of domains in black and in grey preceded by a C for the locations of intra-molecular disulfide bonds (closed black line) and inter-molecular disulfide bonds (black lines). Glycosylation is shown as green lines with a circular tip. (B) Model of trimeric NPTXR associated with the presynaptic membrane. (C) Possible heterocomplexes of NPTX pentraxin domains. (D) Model of NPTXR function at synapses, namely reaching across the synaptic cleft to cluster, pattern and stabilise postsynaptic ligands such as AMPA receptors, in a hexamer of trimers. (E) Proposed model of NPTXs in synaptic plasticity.

Pentraxins & complement in neurodegeneration

Loss of synapses and dysfunctional synaptic plasticity occur in the early stages of neurodegenerative diseases such as AD(270). As the NPTXs are linked to synaptic plasticity, they could also play a role in these disorders. Indeed, Amyloid- β increases NPTX1 levels and NPTX1 is found in damaged neurites of AD patients(271). Moreover, NPTX1 is increased in the serum of patients with early stage AD and is secreted presynaptically in response to Amyloid- β (272). Conversely, levels of NPTXR and NPTX2 are reduced in AD patients(273, 274), with dual NPTX2 knockout and Amyloid- β containing mouse models indicating that these two factors are synergistic in the progression of AD(275). Again, it appears that NPTX1 has adverse effect on synaptic health, contributing to both LTD and neurodegeneration, whereas NPTX2 appears to strengthen synapses and protect them from neurodegeneration.

Furthermore, these properties appear to be linked with the ability of NPTX1 and NPTX2 to associate with the classical complement cascade(32). Here again the two have opposing roles with NPTX2 implicated in dampening the complement system and NPTX1 with complement activation. For instance, NPTX2 knockout mice having greater complement activation and less excitatory synapses, with the latter being rescued by complement inhibitors(252). On the other hand, NPTX1 may facilitate the complement dependent phagocytosis of synapses(32). Microglia, the phagocytes of the central nervous system, appear to be crucial in homeostatic synaptic pruning via interactions with complement proteins(276), with the knockout of NPTX2 disrupting this and causing aberrant microglia responses. Together this implies NPTX2 has a dampening effect on the complement cascade(277)and that the NPTXs may form a link between dysregulated complement activation, synapse elimination and neurodegeneration.

Complement has long been associated with neurodegenerative pathologies. For instance, in AD mouse models C1q associates with synapses and activates complement prior to plaque formation(60), suggesting it could play a causative role in AD. Similarly, mouse models that lack the ability to activate the complement system, display less neurodegeneration(260) and indeed complement inhibitors prevent early synapse loss in AD mouse models(60). Taken together, complement and NPTXs clearly play a role in synaptic plasticity and neurodegeneration. As such, greater knowledge of their structures and biochemistry may enable the rationale design of therapies much as been posited above for CRP and has been already reported for an NPTX1 chimeric protein(38).

Materials and methods

NPTXR expression and purification

Sequence of the codon optimised His₆-tagged NPTXR construct used in this study.

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AAGCTTGCCGCCACCATGGAATTTGGCCTGAGCTGGGTTTTCTGGTGGCTCTGCTGAGAGGCGT
GCAGTGTCACCACCACCATCACCACGAGAACCTGTACTTTCAAGGCGCCTCTGTGCCTCTGGCCG
CTTCTCCTGCTAGAGCACTTCTGGCGGAGCCGATAATGCTTCTGTTGCTTCTGGCGCTGCTGCT
AGCCCTGGACCTCAAAGATCTCTGTCTGCACCTTACGGCGCTGGCGGATCTGCTGGACCTCCAGC
ATTGCTGGTGTCTCCAGCTGCTTCTGCTCATCCTCTTCCACCTGGGCTCTGTTTACGAGATTCC
TGTGTACACCTCTGGCTGCCGCTTGTCCATCTGGTGCTCAACAAGGGGATGCTGCTGGCGCAGCT
CCTGGCGAAAGAGAAGAACTGCTGCTGCTCCAGTCTACCGCCGAGCAGCTTAGACAGACAGCCCT
GCAGCAAGAGGCCAGAATCAGAGCCGATCAGGACACCATCAGAGAGCTGACAGGCAAGCTGGGCA
GATGTGAATCTGGCCTGCCTAGAGGATTGCAAGGCGCCGACCTAGAAGAGACACAATGGCCGAT
GGACCTGGGATAGCCCTGCTCTGATCCTGGAAGTGAAGATGCCGTGCGGGCCCTGAGAGACAG
AATCGACAGACTGGAACAAGAGCTGCCCCGCCAGAGTGAACCTTTCTGCAGCTCCAGCTCCTGTGT
CTGCCGTGCCTACAGGACTGCACAGCAAGATGGATCAGCTGGAAGGACAGCTGCTGGCTCAGGTG
CTGGCCCTGGAAAAAGAAAGAGTGGCCCTGAGCCACTCCAGCAGAAGGCAGAGACAAGAGGTGCA
GAAAGAACTGGACGTCCTGCAGGGCAGAGTGGCCGAACCTTGAACACGGCAGCTCTGCCTATTCTC
CACCTGACGCCTTCAAGATCAGCATCCCTATCCGGAACAACCTATATGTACGCTAGAGTGCCGAAG
GCCCTGCCAGAGCTGTATGCCTTTACAGCCTGCATGTGGCTGAGAAGCAGATCTTCTGGCACAGG
CCAGGGCACCCCTTTTAGCTATTCTGTGCCTGGACAGGCCAACGAGATCGTGCTGCTGGAAGCCG
GACACGAGCCTATGGAAGTGTGATCAACGACAAGGTGGCCAGCTGCCTCTGAGCCTGAAGGAT
AATGGCTGGCACCACATCTGTATCGCCTGGACCACAAGAGATGGCCTTTGGAGCGCCTATCAGGA
TGGCGAACTGCAAGGCTCTGGCGAAAATCTGGCTGCTTGGCACCCCTATCAAGCCTCACGGAATCC
TGATCCTCGGCCAAGAGCAGGATACCCCTCGGCGGCAGATTTGATGCCACACAGGCCCTTCGTGGGC
GACATTGCCCAGTTCAATCTGTGGGATCACGCCCTGACACCAGCACAGGTTCTGGGAATCGCCAA
TTGCACAGCCCCCTCTGCTGGGAAATGTGCTGCCCTGGGAAGATAAGCTGGTGGGAAGCCCTTTGGCG
GAGCTACCAAGGCCGCCCTTCGATGTGTGCAAAGGCAGAGCCAAAGCCTGAGAATTC
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Cloning, expression and purification was performed as previously described for PTX3 (*chapter 6*)(36).

Electron microscopy

Negatively stained samples and electron microscopy was performed as previously described for PTX3 (*chapter 6*)(36).

SDS-PAGE and glycan analysis

This was performed as previously described for PTX3 (*chapter 6*)(36).

Acknowledgements: This research was supported by the following grants to THS: European Research Council Grant 759517; The Netherlands Organization for Scientific Research Grants OCENW.KLEIN.291 and VI.Vidi.193.014.

Author Contributions: T.T. vd K carried out NPTXR experiments under the supervision of DPN. T.T. vd K and DPN analysed the data in communication with THS. AlphaFold structures and sequence analysis was performed by DPN.

