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TPM3 and TPM4 gene products segregate to the postsynaptic region of central nervous system synapses

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Synaptic function in the central nervous system (CNS) is highly dependent on a dynamic actin cytoskeleton in both the pre- and the postsynaptic compartment. Remodelling of the actin cytoskeleton is controlled by tropomyosins, a family of actin-associated proteins which define distinct actin filament populations. Here we show that TPM3 and TPM4 gene products localize to the postsynaptic region in mouse hippocampal neurons. Furthermore our data confirm previous findings of isoform segregation to the pre- and postsynaptic compartments at CNS synapses. These data provide fundamental insights in the formation of functionally distinct actin filament populations at the pre- and post-synapse.

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Introduction

The actin cytoskeleton is the predominant cytoskeletal structure in the postsynaptic compartment of excitatory synapses in the mammalian brain. The majority of postsynaptic compartments are formed at the distal ends of dendritic spines,[1] small protrusions that form along the length of dendrites. Constant remodelling of actin filaments is crucial to support the structural and functional changes that occur at CNS synapses during memory and learning. On the molecular level, changes of the structure and dynamics of actin filaments is required for the trafficking and recycling of neurotransmitter receptors such as 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid (AMPA) and *N*-Methyl-D-aspartic acid or *N*-Methyl-D-aspartate (NMDA) receptors (AMPAR and NMDAR respectively). These neurotransmitter receptors are integrated in the spine head and linked to actin filaments by a number of scaffolding proteins. Recent studies have shown the presence of distinct actin filament populations in dendritic spines which are characterized by different structural and dynamic properties.[2,3] On the structural level (for a review see ref. 3), a meshwork made of short, straight actin filaments has been observed. These filaments have a diameter of 4–6 nm and a length of 20 nm, are part of the post-synaptic density (PSD) and extend into the spine head. Throughout the spine head an additional network of actin filaments is present of which only few are making contact with the PSD. A third population of filaments with a diameter of 5–7 nm has been localized in the neck of the spine. Analysis of actin dynamics at the post-synapse has identified three types of actin filament pools, namely a dynamic pool, an enlargement pool and a stable pool.[2] The dynamic pool is thought to allow for glutamate sensitivity and the regulation of spine volume while the enlargement pool is required for processes of long-term potentiation involving long-term enlargement of the spines. The stable pool on the other hand is thought to be more involved in giving the spines stability.

Previous functional studies on the actin filament system at CNS synapses have focused on the role of known actin filament regulators that have been characterized in other cell types and subcellular compartments. These include the actin filament nucleator Arp2/3,[4,5] the filament severing protein cofilin[6,7] and actin-associated motor proteins such as myosin IIb.[8] In contrast, little is known about tropomyosins, key regulators of actin filament function at synapses.[9] Tropomyosins have been suggested to play a role at CNS synapses based on their identification in synaptosome preparations and immunogold electon microscopy of rat brain tissue showing an isoform specific segregation of tropomyosins at synapses in the cerebellum.[10-12] However the question of whether different tropomyosin isoforms co-exist in the same sub-cellular compartment at synapses has so far not been addressed.

Tropomyosin is a coiled-coil dimer which forms a head-to-tail polymer lying in the major groove of the actin-filament. Over 40 isoforms of tropomyosin are generated in mammals by alternative splicing from four different genes (TPM1–4).[9] This diversity is reflected in remarkable spatial segregation of isoforms[13] and their capacity to regulate actin filament function in an isoform specific manner.[9,14]

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Tropomyosin expression is developmentally regulated in both a tissue and cell type specific manner.[9,15] In neuronal tissue products from three of the four Tm genes have been found: TmBr1, TmBr2 and TmBr3 (TPM1); Tm5NM1–11 (TPM3); Tm4 (TPM4).[16]

Tropomyosins regulate actin filament function at least in part by controlling the access and recruitment of other actin binding proteins to the filaments thereby differentially slowing the 'off-rate' of actin monomers from actin filament ends and increasing actin filament stiffness.[17] Previous studies have demonstrated that tropomyosins can protect filaments from the severing action of ADF/cofilin[18,19] and influence myosin mechanochemistry.[20] A number of actin filament-associated proteins that have been shown to interact with actin filaments in a tropomyosin dependent way are enriched at the postsynapse of excitatory synapses in the CNS. These include the actin motor protein myosin IIb which is important in regulating actin dynamics during synapse function.[21] We have previously shown that in B35 neuroblastoma cells the TPM1 product Tm5NM1 is able to recruit myosin IIb to actin filaments.[22] The localization of Tm5NM1 in mature neuronal networks has not yet been addressed.

Our present study reports for the first time the presence of different tropomyosin gene products from the TPM3 and TPM4 genes within the same synaptic compartment at CNS synapses. Furthermore our data obtained using dissociated cultures of mouse hippocampal neurons confirm previous findings of the segregation of TPM1 and TPM4 products to the pre- and postsynaptic compartment respectively. Taken together with data on the functional diversity of tropomyosins we can conclude that tropomyosins are instrumental in the formation of actin filament populations with different functional properties at the pre- and postsynapse. This will have important implications for future studies that aim to understand actin filament dependent mechanisms at CNS synapses.

Results

Previous studies using immunogold electron microscopy of cerebellar tissue from rats revealed the localization of TPM1 products to the pre- and TPM4 products to the postsynaptic compartment. For our study we used primary neuronal cultures from mouse embryonal hippocampi, harvested at 16.5 d after gestation as previously described.[23] Immunocytochemical staining using an antibody directed against exon 9c of the TPM1 gene shows the localization of TmBr1/3 at the presynapse as determined by co-localization with the synaptic marker protein synapsin (**Fig. 1**). Using an antibody directed against exon 1b of the TPM3 gene, detecting all non-muscle products of the TPM3 gene we demonstrate the postsynaptic localization of these products as indicated by the close proximity but lack of overlap with the presynaptic marker synapsin (**Fig. 2A and B**). Similarly we show the localization of Tm4 to the postsynaptic compartment with only little immunostaining of the presynaptic compartment which is detected using an antibody against the presynaptic marker synaptophysin (**Fig. 2C and D**). To further confirm the localization of Tm4 at the postsynapse we decided to use a non-antibody dependent approach. We tested the localization of exogenously expressed EYFP-Tm4 in mature cultures of hippocampal neurons using lentivirus-mediated gene-transfer techniques which allows the identification of individual dendritic trees in a neuronal network. Analysis of infected cells demonstrated the presence of EYFP-tagged Tm4 in dendritic spines (**Fig. 2E and F**) supporting a postsynaptic localization of TPM4 gene products.

Discussion

The cytoskeletal architecture at the post-synapse of synaptic connections in the CNS plays a pivotal role in supporting and mediating processes of memory and learning. To understand the mechanisms that control the actin cytoskeleton at the CNS synapse we studied the segregation of TPM1, 3 and 4 gene products to the pre- and postsynaptic compartments of cultured mouse hippocampal neurons. Our data show for the first time the localization of products from the TPM3 gene at synapses of CNS neurons with an enrichment at the post-synaptic site. The localization of TPM1 and TPM4 products at the pre- and post-synapse of CNS neurons is consistent with previous findings by Had et al. which showed an enrichment of TmBr1/3 at the presynaptic terminals of parallel fibers in the cerebellum and an enrichment of Tm4 at the post-synapse of synapses in the molecular layer.[11]

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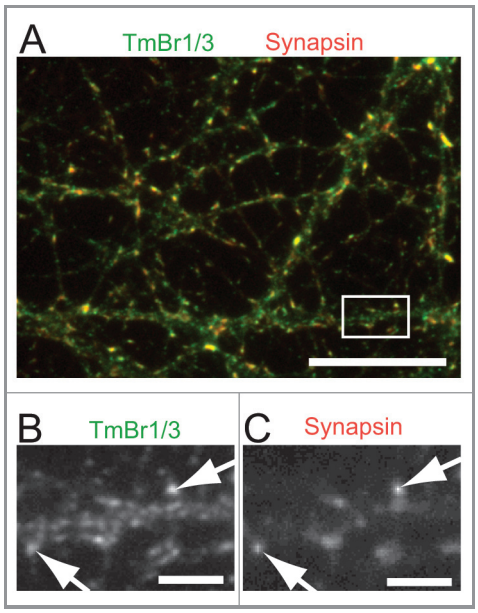

Figure 1. Localization of TPM1 gene products at the post-synapse of hippocampal neurons using immmunocytochemistry. Hippocampal neurons were co-stained using an antibody detecting exon 9c of TPM1 gene products (TmBr1,3)[24] (A, B) together with anti-synapsin (A, C) to identify the pre-synaptic compartment. Note the co-localization of TmB1/3 with the presynaptic marker synapsin (arrows). Scale Bars = 25 μm (A), 5 μm (B, C).

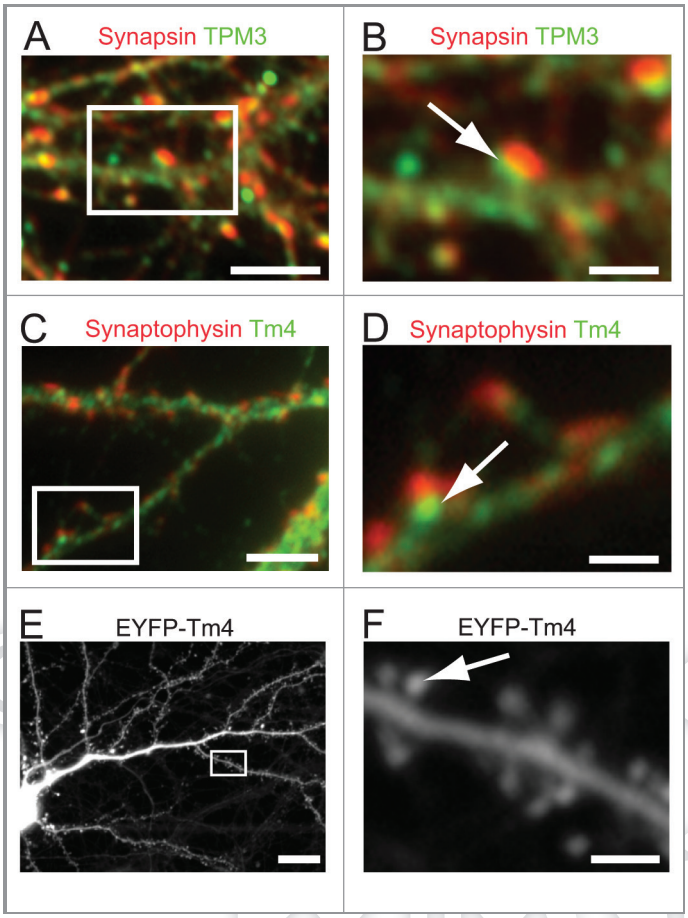

Figure 2. Localization of TPM3 and TPM4 gene products at the post-synapse of hippocampal neurons using immmunocytochemistry. Hippocampal neurons were co-stained using an antibody detecting the TPM3 gene products (A and B) or TPM4 gene products (C and D) together with anti-synapsin (A and B) or anti-synaptophysin (C and D) to identify the presynaptic compartment. Note the close localization but lack of overlap of tropomyosins (arrow) and synapsin or synaptophysin signal which demonstrates the postsynaptic enrichment of these tropomyosins. (E and F) Infection of 20 d in vitro cultured hippocampal neurons with lentiviral particles expressing EYFP-tagged Tm4. Note the localization of Tm4 at the spine heads along the dendrites of the imaged neuron. Scale Bars = 10 μm (A, C and E), 5 μm (B, D and F).

The spatial segregation of tropomyosins at the CNS synapse is intriguing due to the central role of tropomyosins in defining different actin filament populations with distinct dynamic and mechanical properties. Functional characterization of the TPM1 gene product TmBr3 and the TPM3 gene product Tm5NM1 in B35 neuroblastoma cells allowed us to formulate a current model of the formation of distinct actin filament populations. While the association of TmBr3 with actin filaments promotes the formation of shorter less stiff filaments, the association of Tm5NM1 results in longer and stiffer filaments by blocking ADF/cofilin activity and the recruitment of the actin motor protein myosin IIb.[22] The functional properties of Tm4 have not yet been fully assessed in this cellular model. However in a fluorescence recovery after photobleaching (FRAP) approach YFP-Tm4 showed a different dynamic association with actin filaments in human osteosarcoma cells (U2OS) as compared with YFP-tagged TPM3 gene products with YFP-Tm4 displaying a more rapid fluorescence recovery at actin structures.[25] These data are suggestive for specific functional roles for Tm5NM1 and Tm4 in the same subcellular compartments.

The confirmation of the segregation of tropomyosins to the the pre- and post-synapse and the finding of different tropomyosin isoforms at the post-synapse in hippocampal neurons is significant since cultures of primary hippocampal neurons are an easily accessible system for genetic manipulation and electrophysiological analyses to study the actin cytoskeleton at the CNS synapse. Elevated expression of Tm5NM1 leads to an increase in the filamentous actin pool and the size of growth cones of hippocampal and cortical neurons.[26] Changes in the size of dendritic spines are associated with long-term potentiation (LTP),[27,28] in which strengthening of individual synapses occurs through presynaptic input on depolarized postsynaptic compartments, an essential process for learning and memory. Increased dendritic spine head size as seen during LTP is also associated with an increased pool of filamentous actin in the spine heads.[29] Therefore the localization of TPM3 gene products in the post-synapse provides a regulatory component to control actin filament pool size in this compartment which eventually can control learning and memory.

The presence of different tropomyosin isoforms provides a potential mechanism for the formation of the structurally and functionally distinct actin filament populations that have been observed at the postsynapse.[2,3] LTP involves the increased expression and insertion of AMPARs into the postsynaptic plasma membrane[30,31] and it is this AMPAR trafficking that is believed to be controlled by a dynamic actin cytoskeleton.[32,33] Gu et al. showed that increased ADF/cofilin activity enhanced insertion of AMPARs at the postsynaptic plasma membrane after tetraethylammonium (TEA) induced LTP.[33]

This emphasizes the importance of understanding how changes in the actin network at the post-synapse are mediated during LTP. NMDA receptor activation leads to the phosphorylation and activation of Myosin Light Chain (MLC)[21] and the phosphorylation and deactivation of ADF/cofilin.[34] A product from the TPM3 gene has been shown to direct these functional outcomes.[22] Both the phosphorylation of MLC and the phosphorylation of ADF/cofilin are essential for stable LTP.[21,32,35] Based on the role of tropomyosins in controlling access of actin-associated proteins to distinct actin filaments, we speculate that TPM3 and TPM4 may be instrumental in mediating the effect of NMDAR receptor induced changes downstream or in parallel to the phosphorylation of MLC and ADF/cofilin.

While our study established the localization of TPM3 and TPM4 gene products at the post-synapse, further studies will be required to analyze the sub-compartment specific distribution of products from these two genes. For this, recent advances in super-resolution microscopy applications such as STED, STORM and PALM microscopy will be very valuable and will help to characterize the role of these product in synapse function.[36-38]

Our findings of the presence of products from both the TPM3 and TPM4 gene (illustrated in **Fig. 3**) adds significantly to our

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current view of actin filament populations at the post-synapse. The identification of two different tropomyosins at the post-synapse provides a potential mechanism to create and regulate different actin filament populations.

Material and Methods

Hippocampal-cortical co-cultures were prepared as described previously.[23] In brief, hippocampi and cortices were dissected from embryonal day 16.5 C57Bl6 mice and triturated in plating medium containing DMEM/10% FBS (Gibco) using firepolished glass Pasteur pipettes. $1x10^3$ hippocampal cells were plated on poly D-lysine (0.1 mg/ml; Sigma)-coated 12-mm glass coverslips (Menzel) mounted in the center of a 12-well culture plate well and 1 x 10^5 cortical cells around the edge of the wells. After 2 h, the plating medium was replaced by 1 ml of Neurobasal medium containing B27 supplement and Glutamax (Gibco). For immunofluorescence staining, cells were fixed after 21 d with 4% paraformaldehyde in PBS. Cells were permeabilized with 0.1% Triton in PBS solution and stained with the following antibodies: monoclonal CG3, directed against all non-muscle TPM3 products,[24] rabbit anti Tm4,[24] anti-synapsin (AB1543, Millipore) and anti-synaptophysin (Millipore, MAB5258). All fluorophore-conjugated antibodies were obtained from Molecular Probes.

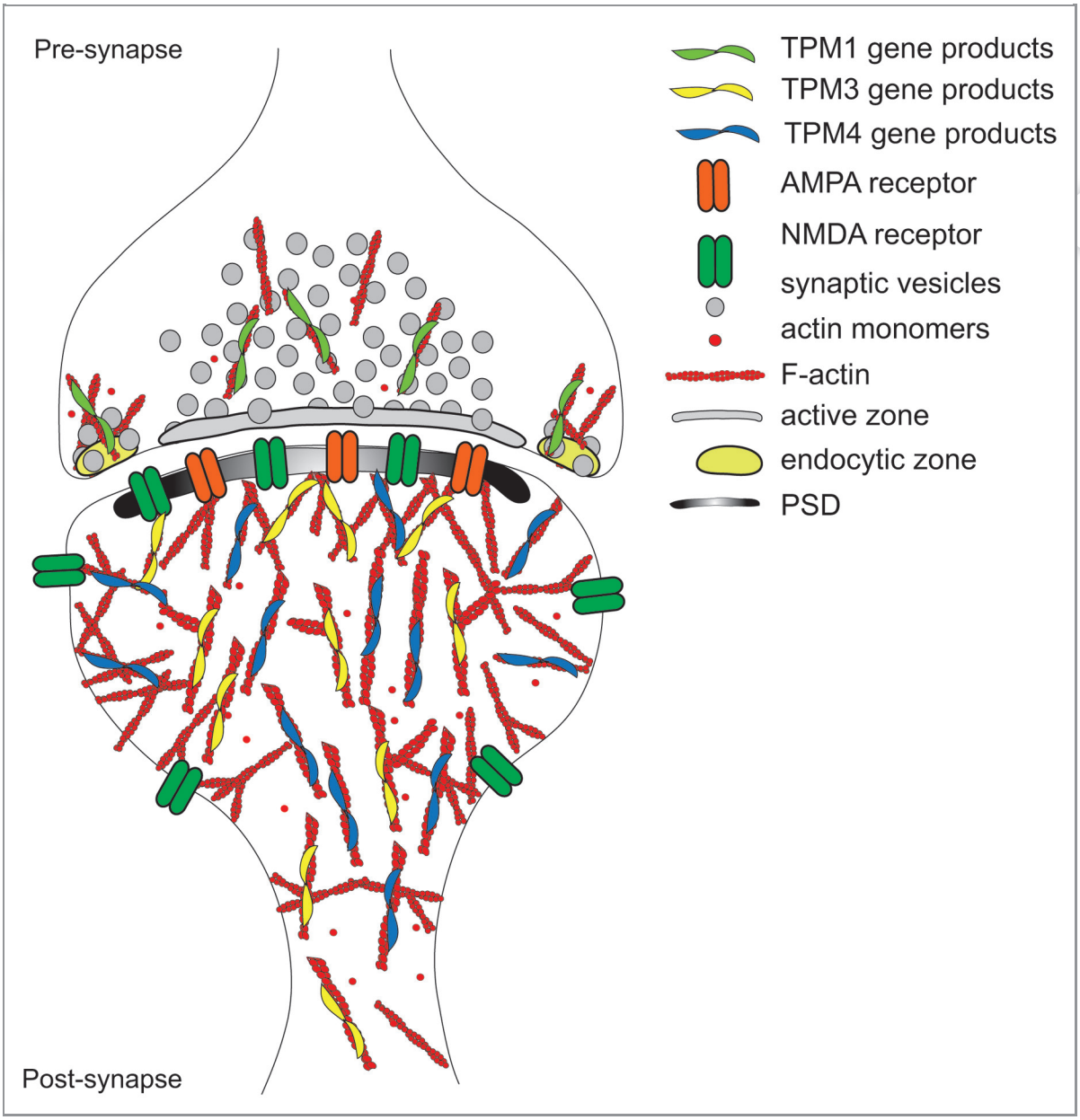

Figure 3. Schematic of an excitatory synapse in the CNS. The actin filament organization and relative localization of Tropomyosins at the CNS synapse are depicted. Note the localization of TPM1 gene products at the presynapse while TPM3 and TPM4 gene products localize to the postsynapse.

Catalogue numbers are given in brackets. The following secondary antibodies were used: Alexa 647-conjugated donkey anti-rabbit (A31573), Alexa 555-conjugated donkey anti-rabbit (A31572), Alexa 488 donkey anti-mouse (A21202), Alexa 555 donkey anti-mouse (A31570). Images of neuronal cultures were taken on an IX81 (Olympus) and an Axioskop 40 (Carl Zeiss) microscope.

A EYFP-Tm4 expression construct was prepared by sub-cloning of EYFP-Tm4 from pEYPF-Tm4[25] into a pRRLsin18. PPT.CMV.Wpre[39] resulting in the EYFP-Tm4 expressing vector pCMV-EYFP-Tm4. For the preparation of Lenti-viral particles 293 cells were plated in T75 flasks at a density of 4×10^6 cells per flask. Cells were co-transfection with pCMV-YFP-Tm4, pREV, pRRE and pVSVg using $CaCl_2$ transfection as previously described.[40] The transfected cells were incubated at 37°C and 5% CO_2 in DMEM, 10% FBS, the medium was changed for fresh medium and incubated for 28–32 h. After the incubation, supernatant was collected and the titer determined using a FACSCalibur flow cytometer (BD Biosciences).

For infection, hippocampal neurons were plated as described above. Lenti-viral particle stocks were diluted in neurobasal medium to achieve a concentration of 3×10^5 particles per 10 µl. Infected cultures were fixed at two days post infection by applying equal volume of 8% PFA in PBS. After fixation, coverslips were washed with PBS and mounted on glass slides for imaging.

Ethics Statement

All procedures with animals were performed to avoid unnecessary discomfort, pain or injury to the animals and have been approved by the Animal Care and Ethics Committee of the Children's Medical Research Institute and Children's Hospital at Westmead, Australia.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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