## Thrombospondin 1 accelerates synaptogenesis in hippocampal neurons through neuroligin 1

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In cultured rat hippocampal neurons, we found that thrombospondin 1 (TSP1) increased the speed of synapse formation in young neurons, but not the final density of synapses in mature neurons. TSP1 interacted with neuroligin 1 (NL1) and application of the NL1 extracellular domain blocked TSP1-induced synaptogenesis. Furthermore, knocking down endogenous NL1 inhibited TSP1's effect. Our results indicate that TSP1 accelerates the speed of synaptogenesis through NL1 in hippocampal neurons.

The process of synapse formation, or synaptogenesis, is tightly regulated to ensure correct connections between neurons. Abnormalities in synaptogenesis are believed to be responsible for brain disorders such as autism and mental retardation<sup>1,2</sup>. The molecular mechanisms underlying synaptogenesis are not yet fully understood. Several proteins, including thrombospondin (TSP) and the neuroligin-neurexin complex, have been found to be important for this process<sup>3–5</sup>. Thrombospondins are multimeric extracellular-matrix glycoproteins secreted by a number of cells, including astrocytes in the brain<sup>6</sup>. A recent study found that thrombospondins promote synaptogenesis<sup>7</sup>. Applying TSP1 to cultured retinal ganglion cells (RGCs) led to a several-fold increase in the number of excitatory synapses. These newly formed synapses were postsynaptically silent, suggesting that they lacked functional AMPA receptors. Whether TSP1 induces synaptogenesis in neurons from brain

Figure 1 TSP1 accelerates the speed of synaptogenesis, but does not increase the final density of synapses in mature neurons. Neurons were treated with purified TSP1 protein at different stages and immunostained for postsynaptic marker PSD-95 and presynaptic marker synapsin 1 at the time indicated. (a-c) Representative figures for 5-10 (a), 5-8 (b) and 14–17 (c) DIV incubations are shown. Scale bars represent 10  $\mu$ m. (d) Quantification of the densities of PSD-95- and synapsin 1-positive puncta. After TSP1 incubation, the densities of PSD-95 were 147.8  $\pm$ 6.2%,  $123.8 \pm 3.8\%$ ,  $90.0 \pm 2.2\%$  and  $99.1 \pm 5.1\%$  of the control level at 5-8, 5-10, 5-14 and 14-17 DIV, respectively. The densities of synapsin 1 were  $127.5 \pm 3.8\%$ ,  $106.5 \pm 7.0\%$ ,  $87.2 \pm 11.7\%$  and  $96.4 \pm 6.0\%$ of control level at 5-8, 5-10, 5-14 and 14-17 DIV, respectively. (e) Quantification results of TSP1's effect on synapses (puncta with both PSD-95 and synapsin 1). The synapse densities were 136.2  $\pm$  5.5%, 116.5  $\pm$  13.7%, 87.6  $\pm$  4.1% and 96.9  $\pm$  5.4% of control level at 5–8, 5–10, 5–14 and 14–17 DIV, respectively. (\*\*\* P < 0.001, # P < 0.01; Student's *t* test, n = 3-4 individual experiments; error bars represent s.e.m.).

has not yet been determined, nor has the mechanism that mediates the synaptogenic effect of TSP1 been elucidated.

To test whether TSPs induce synaptogenesis in the neurons of the brain, we applied TSP1 (5  $\mu$ g ml<sup>-1</sup>) to cultured hippocampal neurons from 5 to 10 d *in vitro* (DIV), the same protocol that was used to induce synaptogenesis on RGCs<sup>7</sup> (**Supplementary Methods**). At the end of the incubation period, the neurons were immunostained with an antibody to PSD-95, an excitatory synapse marker (**Fig. 1**). This procedure led to a 24% increase in the density of PSD-95 puncta (**Fig. 1a,d**). We also stained for synapsin 1, a marker of the presynaptic terminal, but did not observe a substantial increase in the density of synapsin 1 puncta



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after TSP1 treatment (**Fig. 1a,d**). The density of full synapses, which were defined as puncta containing both PSD-95 and synapsin 1, slightly increased with TSP1 treatment (**Fig. 1a,e**). The increases in PSD-95 and in the synapse density, however, were rather small compared with what was observed in RGCs, in which the TSP1 treatment increased synapse number several fold<sup>7</sup>.

We suspected that this discrepancy might be a result of the different speeds of synaptogenesis between RGCs and hippocampal neurons and that DIV 5–10 may not be the best time to examine TSP1's synaptogenic effects in hippocampal neurons. To determine the optimal time frame, we monitored the synapse development profile in hippocampal neurons from 3–20 DIV and found that the maximal synapse growth occurs between 5 and 8 DIV, with about half of the synapses being formed in this period (**Supplementary Fig. 1**). We then applied TSP1 to the cultured neurons at 5 DIV and assessed synapse density at 8 DIV. We found that incubation of TSP1 during 5–8 DIV led to a 48% increase in the density of PSD-95–positive puncta (**Fig. 1b,d**). We also stained for synapsin 1 and found that addition of TSP1 from 5–8 DIV significantly increased the density of synapsin 1 puncta (28%, P < 0.001) and synapse density (36%, P < 0.001) (**Fig. 1b,d**e).

Our finding that the shorter incubation of TSP1 from 5–8 DIV produced a larger increase in synapse density than a longer incubation from 5–10 DIV surprised us. This result suggests that TSP1 may initially speed up the synaptogenesis process, but it may not increase synapse density in the end. To test this hypothesis, we treated cultured neurons with TSP1 for a longer period, from 5–14 DIV, when the neurons were mature and their synaptogenesis had reached a plateau. TSP1 incubation from 5–14 DIV did not increase synapse density; instead, synapse density was slightly reduced (**Fig. 1d,e**). We also treated neurons with TSP1 from 14–17 DIV and did not find much change (**Fig. 1c–e**). These results support the hypothesis that TSP1 accelerates the speed of synaptogenesis, but does not increase the final density of synapses in mature neurons.

The majority of fast excitatory synaptic transmission in the brain is mediated by AMPA-type glutamate receptors. Synapses lacking AMPA receptors are considered to be functionally silent<sup>8</sup>. To test whether TSP1-induced synapses have AMPA receptors, we stained cultured hippocampal neurons with an antibody to the AMPA receptor subunit GluR1. Although incubation of TSP1 with cultured neurons from 5–8 DIV significantly increased (P < 0.001) the number of PSD-95 puncta present, it did not increase the density of GluR1 clusters (**Supplementary Fig. 2**). Similarly, TSP1 increased the number of puncta that had NMDA receptor subunit NR1, but not of GluR1, indicating that TSP1-induced synapses are mainly silent synapses (**Supplementary Fig. 3**).

The effect of TSP1 on synapse formation is similar to the effect of neurexins, which induce the formation of synapses lacking AMPA receptors via neuroligin<sup>9,10</sup>. The similarities between TSP1 and neurexin-neuroligin–induced synaptogenesis suggest that the two processes may share a common mechanism. To examine the relationship between TSP1 and neuroligin, we applied the extracellular domain of neuroligin 1 (NL1-ECD)<sup>11</sup> to cultured hippocampal neurons, alone or with TSP1. When applied alone, NL1-ECD caused a small increase in synapse density (**Fig. 2a,b**). Notably, when we incubated NL1-ECD with TSP1, the synaptogenic effect of TSP1 was mostly blocked (**Fig. 2a,b**). A similar effect was also observed when we stained for NR1 (**Supplementary Fig. 3**). These results suggest that NL1 mediates the synaptogenic effect of TSP1 on excitatory synapses.

The finding that NL1-ECD blocked TSP1's synaptogenic effect suggests that they may interact with each other. To test this, we carried out co-immunoprecipitation experiments and found that NL1 could be efficiently co-immunoprecipitated with TSP1 when co-transfected



Figure 2 Neuroligin 1 extracellular domain blocks TSP1's synaptogenic effect and TSP1 interacts with neuroligins. (a) The neurons were incubated with media alone (control), TSP1, soluble NL1-ECD or TSP1 with NL1-ECD as indicated and stained with antibody to PSD-95 and synapsin 1. The arrows indicate synapses with both PSD-95 and synapsin 1. Scale bars represent  $5 \,\mu\text{m}$ . (b) Quantification of the synapse densities. TSP1 significantly increased the synapse density, whereas application of both NL1-ECD and TSP1 greatly reduced TSP1's synaptogenic effect. The synapse densities for NL1-ECD-, TSP1- and TSP1+NL1-ECD-treated neurons were  $107.2 \pm 4.2\%$ ,  $135.8 \pm 7.5\%$  and  $116.7 \pm 5.0\%$  of the control level, respectively (\*\*\* P < 0.001, \*\* P < 0.01; Student's *t* test, n = 3). (c) Co-immunoprecipitation of TSP1 and YFP-NL1, YFP-NL2, YFP-NL3, YFP and CFP-NRX1. The neuroligins only co-immunoprecipitated with TSP1. IB, immunoblot; IP, immunoprecipitation. (d) A saturation binding curve of TSP1 to NL1-ECD and VLDLR-N revealed that the binding affinity of TSP1 to NL1 was comparable to that of VLDLR. hTSP1, human TSP1 protein; VLDLR-N, N-terminal repeat 1-8 fragment of VLDLR.

into HEK293T cells (**Fig. 2c**). We also tested TSP1's interaction with two other neuroligins, NL2 and NL3, and found that TSP1 bound to all three forms of neuroligins (**Fig. 2c**). In contrast, neurexin 1 (NRX1), the binding partner of neuroligins, and a YFP control did not bind to TSP1 (**Fig. 2c**), indicating that the interactions between TSP1 and neuroligins are specific. We also performed a solid phase-based binding assay to measure the relative interaction strength between TSP1 and NL1 and compared that with the known TSP1-binding protein VLDLR (very low-density lipoprotein receptor)<sup>12</sup>. We found that the binding strength of TSP1 to NL1 was comparable to that of VLDLR (**Fig. 2d**). Furthermore, we found that NL1-ECD competed with NL1 and attenuated the interaction between TSP1 and NL1 during

## **BRIEF COMMUNICATIONS**



**Figure 3** Neuroligin 1 knockdown suppresses TSP1-induced synaptogenesis. (a) Neurons were infected with GFP alone, GFP-shNL1 #1 or GFP-shNL1 mis (mismatched control) lentivirus with or without TSP1 treatment, as indicated. The GFP signal identified the infected neurons. In the presence of shNL1 #1, TSP1 no longer increased synapse density. The arrows indicate the synapses with both PSD-95 and synapsin 1. Scale bars represent 5  $\mu$ m. (b) Quantification of the synapse densities. Although TSP1 increased the synapse density in GFP-expressing control neurons, its effect was inhibited in NL1 knockdown neurons. With the synapse densities of the TSP1-treated GFP group normalized to 100%, the densities of the TSP1-treated GFP, the untreated and TSP1-treated shNL1 #1, the untreated and TSP1-treated shNL1 mismatch group neurons were 135.5  $\pm$  10.7%, 85.5  $\pm$  3.2%, 89.9  $\pm$  6.3%, 93.3  $\pm$  3.4% and 116.7  $\pm$  4.1%, respectively (\*\* *P* < 0.01 compared with the untreated group; Student's *t* test, *n* = 3–4 for each group, ns, not significant).

co-immunoprecipitation (**Supplementary Fig. 4**). This explains how NL1-ECD blocked TSP1's synaptogenic effect on neurons.

To examine whether NL1 is necessary for TSP1's synaptogenic effect, we knocked down the endogenous NL1 in cultured hippocampal neurons using lentiviral-mediated RNA interference. Two small-hairpin sequences to *Nlgn1 (neuroligin 1)*, shNL1 #1, as described previously<sup>13</sup>, and shNL1 #2, which targets to a different region of *Nlgn1*, were generated and both effectively knocked down rat NL1 (Supplementary Fig. 5). They differed, however, in their abilities to knockdown mouse NL1. shNL1 #1 knocked down both rat and mouse NL1, whereas shNL1 #2 knocked down rat NL1 but not mouse NL1 (Supplementary Fig. 5). When we introduced the shNL1 #1 to cultured hippocampal neurons, we found that, although the synapse density increased significantly in the GFP-only group (P < 0.01) and the mismatched shRNA group (P < 0.01), TSP1 failed to increase synapse density in the shNL1 #1 group (Fig. 3a,b). Similarly, when shNL1 #2 was introduced to hippocampal neurons, the synaptogenic effect of TSP1 was also blocked (Supplementary Fig. 6). We then introduced shNL1 #1 or #2 together with mouse Nlgn1 (HA-mNL1) cDNA to the hippocampal neurons and measured TSP1's synaptogenic effect. Expression of mouse NL1 successfully rescued TSP1's synaptogenic effect in the shNL1 #2 group, but not in the shNL1 #1 group, as NL1 expression could not be restored in the shNL1#1 group (Supplementary Fig. 6). These results provide further evidence that NL1 mediates the synaptogenic effect of TSP1.

In summary, our results indicate that TSP1 accelerates synaptogenesis in the early stage of cultured hippocampal neurons, but does not increase synapse density in mature neurons, and this synaptogenic effect of TSP1 is mediated by NL1. How TSP1 induces synaptogenesis through NL1 is still an open question. It has been shown that aggregation of NL1 by antibodies is sufficient to induce neuroligin-mediated formation of postsynaptic structures<sup>9</sup>. Because TSPs form multimeric protein complexes, it is possible that TSP1 may induce synaptogenesis by causing aggregation of NL1. The observation that TSP1 does not increase synapse density in mature neurons suggests that there could be a mechanism to control the total number of synapses in hippocampal neurons. In comparison with RGCs, the synaptogenic effect of TSP1 in hippocampal neurons is small and gradually disappears during maturation; suggesting that these two types of neurons may employ different mechanisms in response to TSP1.

Note: Supplementary information is available on the Nature Neuroscience website.

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## AUTHOR CONTRIBUTIONS

J. Xu designed and conducted the experiments, analyzed the data and wrote the manuscript. N. X. contributed to some of the experiments. J. Xia designed the experiments, analyzed the data and wrote the manuscript.

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