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LETTER TO THE EDITOR

Intracellular TG2 Activity Increases Microtubule Stability but is not Sufficient to Prompt Neurite Growth

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Dear Editor,

Microtubules (MTs) are the major cytoskeletal filaments in living cells. They are highly dynamic structures, and their assembly and disassembly are tightly regulated to achieve specific cellular functions. For example, MTs in the cell body are sufficiently dynamic to permit the cell to quickly rearrange its cytoskeleton for morphogenesis or migration. On the other hand, highly-differentiated neurons contain stable subcellular structures such as axons, which often extend for a long distance and are stable for many years. MTs in axons are known to be significantly more stable than their counterparts in non-neuronal cells. A significant fraction of MTs extracted from the brain is stable upon treatment with Ca^{2+} and cold, a condition known to cause the complete disassembly of many other non-neuronal MTs [1]. The extraordinary stability of neuronal MTs is attributed to post-translational modifications including detyrosination, acetylation, polyglutamylation, and polyglycylation [2]. However, none of these modifications are sufficient to confer the changes in MT stability *in vitro* [3, 4]. A recent study by Song *et al.* provided fundamentally new insight into our understanding of MT stability [4]. Their studies showed that cold- and Ca^{2+}-stable MTs contain chemically distinct tubulins that are post-translationally modified by polyamination. Polyamination refers to the covalent cross-linkage of polyamines to the carboxamide groups of protein-bound glutamine residues *via* isopeptide bonds. This process is catalyzed by transglutaminases (TGs) [4]. Song *et al.* showed that tubulin is a substrate of transglutaminase 2 (TG2) and that TG2 inhibition decreases MT stability *in vitro* and *in vivo*. They also showed that TG2 inhibition suppresses neurite outgrowth in SY2H cells, suggesting a positive correlation between MT stability and neurite outgrowth. However, whether increasing the MT stability by polyamination helps neurite growth is not clear. We therefore hypothesized that increased TG2 activity promotes neurite outgrowth by stabilizing axonal microtubules *via* polyamination.

In order to test whether TG2 activity is correlated with neurite outgrowth, we first determined whether differentiation increases TG2 activity in PC12 cells, a neuronal cell line that differentiates to grow neurites upon stimulation with nerve growth factor (NGF) [5]. To probe TG2 activity, we used 5-(biotinamido)pentylamine (5-BP, Fig. S1A), a TG substrate that is covalently crosslinked to substrate proteins by active TGs [6]. Therefore, proteins conjugated to 5-BP can be detected by HRP-streptavidin in Western blots, and the 5-BP signal strength is indicative of TG activity. To our surprise, TG activity in PC12 cells was dramatically downregulated in differentiation medium (Fig.1A, lane 2) as compared to complete medium (lane 1). In complete medium, where PC12 cells proliferate and do not grow neurites, we saw several bands with a strong 5-BP signal, and the dominant (strongest) band appeared at ~65 kDa, followed by a strong signal at ~50–55 kDa, and several bands between 35 and 50 kDa. In differentiation

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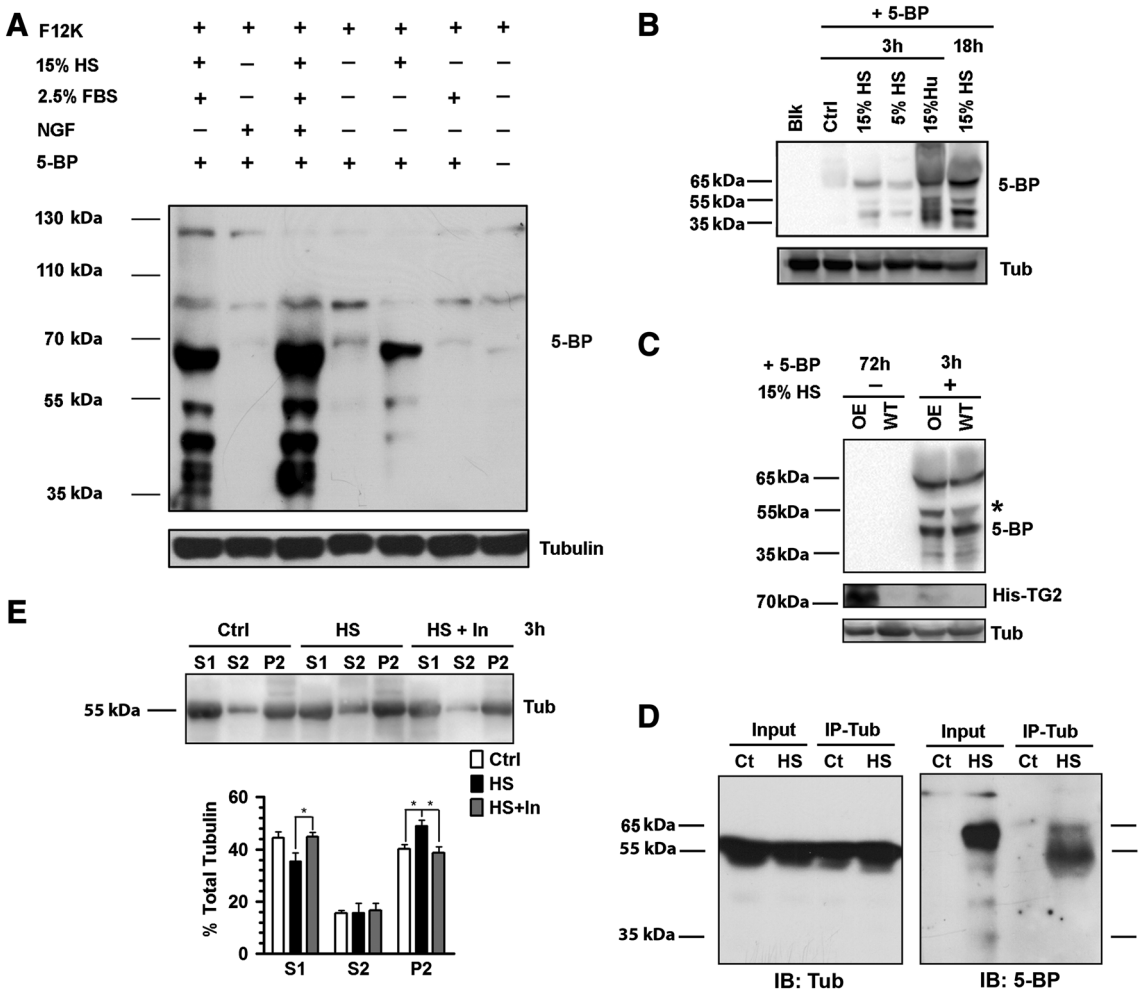

Fig. 1 Horse serum increased TG2 activity and microtubule stability in cortical neurons. **A** Horse serum was responsible for high TG activity in PC12 cells. **B** Horse serum (HS) and human serum (Hu) increased TG activity in DIV5 cortical neurons. Blk, no 5-BP or serum; Ctrl, no serum but with 5-BP for 3 h. **C** TG2-specific substrate upon horse serum treatment at close to 55 kDa (*). TG2 expression was blotted using anti-His antibody at ~78 kDa. OE, overexpression of TG2. **D** Horse serum resulted in 5-BP modification of tubulin in cortical neurons. **E** Horse serum significantly increased the cold- and Ca^{2+}-stable tubulin fraction (P2) in neurons, while TG2 inhibition reversed this effect ($n = 4$ independent experiments; $^*P < 0.05$ using Student's *t*-test). In, TG2 inhibitor. S1, unstable cold-labile tubulin. S2, cold-stable but Ca^{2+}-labile tubulin. P2, CCS-tubulin.

medium, where NGF-induced PC12 cells differentiate, the strong 5-BP signal was completely gone. In order to understand what component of the culture medium modulates TG activity, we tested culture medium with or without horse serum, with or without fetal bovine serum (FBS), and with or without NGF. Western blotting showed that the decrease of TG activity was not because of the addition of NGF (lane 3), but rather because of serum starvation (lane 4). And it was the horse serum (lane 5) but not the FBS (lane 6) that dramatically increased the TG activity. Increasing the FBS concentration to 15% did not affect the result (Fig. S2A), and the proteins in the cells rather than in the medium (e.g. serum proteins) were responsible for the 5-BP signal (Fig. S2B). The three weak bands indicated in the negative control (lane 7) might have been due to endogenous biotinylated proteins rather than the 5-BP signal.

Because horse serum appeared to be responsible for the high TG activity in PC12 cells, we tested whether horse serum increases TG activity in primary neurons maintained in serum-free medium. TG activity was hardly detectable at DIV5 (days *in vitro*) in neurons without serum exposure (Fig. 1B, Ctrl lane). In contrast, 5% horse serum treatment for 3 h induced significant TG activity, and 15% horse serum induced even higher TG activity, indicating a dose-dependent effect. Human serum (15%) also significantly enhanced TG activity after 3 h treatment (Fig. 1B), but 15% FBS did not (Fig. S2). Both horse serum and human serum are adult sera, while FBS is a fetal serum, suggesting that one or more components in adult serum but not fetal

serum could be responsible for activating TG. Prolonged treatment for 18 h with horse serum further increased the total incorporation of 5-BP, indicating a time-dependent additive effect (Fig. 1B).

As the 5-BP probe is a general TG substrate, we set out to determine whether adult serum stimulation causes an increase in the activity of TG2, which has been reported to be responsible for the majority of TG activity in the brain [7]. Therefore, we overexpressed TG2 in cortical neurons by electroporation of a plasmid encoding human His-TG2 (Fig. 1C, OE group). Without horse serum treatment, TG2 activity was not detectable in control neurons or in neurons overexpressing TG2, even after 72 h incubation with 5-BP from DIV2 to DIV5 (5 days of overexpression), at which time a relatively high level of His-TG2 was clearly detectable (Fig. 1C). In contrast, after 3 h treatment with horse serum and 5-BP at DIV2, neurons overexpressing TG2 (2 days of overexpression) showed enhanced activity for a protein band at ~50–55 kDa (indicated by an asterisk in Fig. 1C) compared to control neurons treated with horse serum (WT). The molecular weight of this band is similar to that of tubulin, a previously reported substrate of TG2 [4].

To confirm that tubulin polyamination was indeed increased upon horse serum treatment, we immunoprecipitated β-tubulin from DIV5 cortical neurons that were treated with 5-BP along with or without horse serum overnight (Fig. 1D). Cell extracts before (Input) and after immunoprecipitation (IP-Tub) were separated and blotted against tubulin as a loading control (Fig. 1D, left blots) or streptavidin to reveal tubulin that incorporated 5-BP (Fig. 1D, right blots). In agreement with previous data, the 5-BP signal was present only in neurons treated with horse serum (right panel, HS lanes). In the Input group, the strongest 5-BP signal at ~65 kDa (HS) indicated an unknown TG substrate. In the IP-Tub group, the 5-BP signal appeared at the same molecular weight as β-tubulin (left blots), i.e. at 55 kDa, while the signal at ~65 kDa was decreased, confirming the enrichment of tubulin. These data indicated that horse serum indeed upregulates TG2 activity, which contributes to the increased polyamination of tubulin.

Next, we confirmed that increased TG2 activity enhanced tubulin stability in terms of increasing the fraction of cold- and Ca^{2+}-stable tubulin (CCS-tubulin). A previously published protocol was used for this purpose (see the Supplementary methods and Fig. S3A) [4]. Briefly, DIV5 neurons were treated with 10% horse serum and 5-BP for 3 h (Fig. 1E), with or without a TG2 inhibitor, along with a negative control (without HS treatment but with 5-BP). In the figure, S1 represents unstable cold-labile tubulin, whereas S2 represents cold-stable but Ca^{2+}-labile tubulin and P2 represents CCS-tubulin. These fractions were blotted for either 5-BP (Fig. S3B) or β-tubulin (Fig. 1E). The results showed that the CCS-tubulin in the P2 fraction was significantly increased upon serum stimulation for 3 h, while the S1 fraction decreased and the S2 fraction was unchanged. An irreversible TG2 inhibitor ZH-I-47B (Fig. S1B) [8] reversed this effect, indicating that the horse serum-induced increase of MT stability was indeed TG2-dependent. Interestingly, the 5-BP signal mostly appeared in the S1 fraction (Fig. S3B), indicating that the 5-BP-modified tubulin was not present in the CCS-tubulin fraction. Although 5-BP is a substrate of TG2, it does not contain positive charges like endogenous polyamines. Previous studies have suggested that polyamination stabilizes tubulin through the addition of positive charges. Therefore, incorporation of 5-BP is not exactly the same as the endogenous polyamine modification that confers MT stability, and the increased stabilized tubulin we found here resulted from the modification of endogenous polyamines. In any case, our results showed that the increased TG2 activity induced by horse serum increased MT stability in primary cortical neurons.

Next, we tested the hypothesis that increased tubulin stability promotes neurite growth. First, we treated DIV2-3 neurons with 10% horse serum for 24 h, and surprisingly, treated cells grew significantly shorter axons (longest neurite) than untreated cells (Fig. 2A, B). Since polyamination is a post-translational modification and only requires a short time to take effect (<3 h in our study), we measured the short-term effect of horse serum on the neurite growth rate to rule out compounding factors from long-term treatment. Therefore, we optimized a protocol to measure neurite growth rate (short-term incubation) in two periods: the first spanned from the time horse serum was added (0 h) to 3 h post-incubation; the second was from 3 to 6 h after horse serum addition, at which time our previous results showed that TG2 activity and MT stability had already increased. Growth rates were monitored every 10 min by measuring the displacement of neurite tip locations. Our measurements showed no evident difference in the growth rates in either period compared with control cultures without horse serum (Fig. 2C–E). The cumulative curve of growth rates in the 3–6 h period showed almost identical trends, indicating that increasing tubulin stability is not able to increase axonal growth rates in neurons. Therefore, we did not find a positive correlation between TG2 activity and neurite growth.

Previous studies indicated that TG2-dependent stabilization of MTs is required for neurite growth in the neuron-like cell line SH-SY5Y [4], and this is supported by evidence that TG2 inhibitors block neurite outgrowth. Our results confirmed that elevated TG2 activity increased the fraction of CCS MTs. However, TG2 activity was minimal in conditions where cortical neurons grow long axons, and

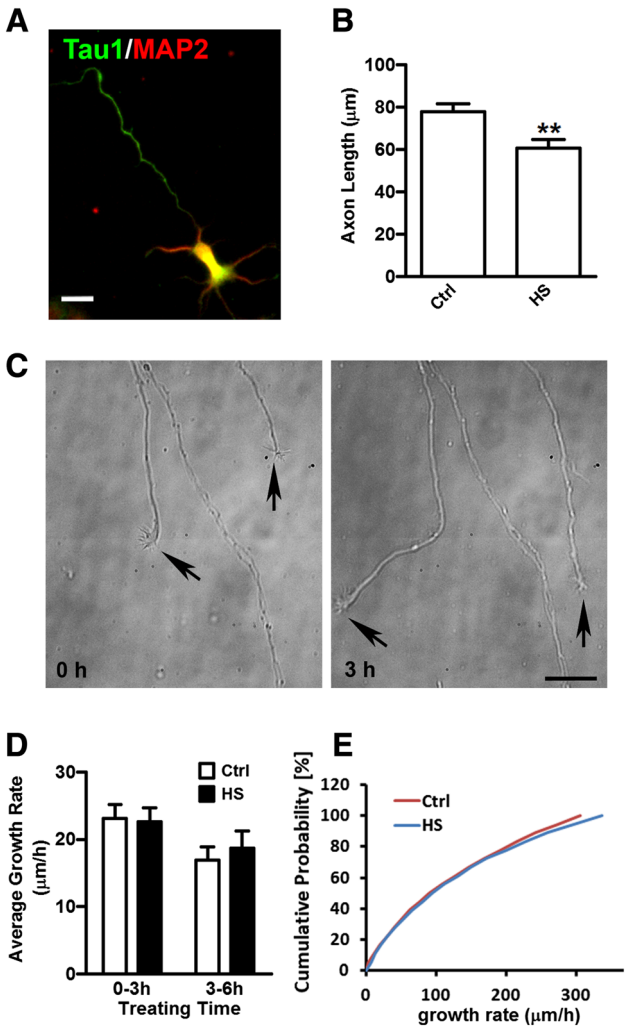

Fig. 2 Increased tubulin stability was not sufficient to increase neurite growth. **A** Representative image of an immunostained neuron at DIV3 (red, MAP2 staining; green, Tau1 staining; scale bar, 20 μm). **B** Statistical analysis of total axon length of indicated treatment groups ($n > 200$; $^{**}P < 0.01$ using Student's *t*-test). **C** Representative images of growing axons (arrows) using live imaging (scale bar, 20 μm). **D** Statistical analysis of axonal growth rate at indicated time periods (0–3 h, $n > 24$; 3–6 h, $n = 18$). **E** Cumulative probability of axonal growth rate based on data as in (**B**).

PC12 cells grow long neurites. Also, elevated TG2 activity did not increase the rate of neurite growth in cortical neurons, suggesting that increased MT stability is not sufficient to promote neurite growth. Although we found an inhibitory effect of horse serum on axonal growth after 24 h of treatment, we can not conclude that there is a negative correlation between MT stability and axonal growth, due to the protein-rich composition of horse serum, which could regulate axonal growth by mechanisms unrelated to TG2. We also note that TG2 is not just a transglutaminase, but also possesses GTPase, protein disulfide isomerase, and protein kinase activity [9]. Therefore, increasing TG2 activity in neurons could cause comprehensive changes not limited to MT stability, and this may contribute to the absence of a correlation between TG2 activity and neurite growth in our study.

This study also provides a new way to enhance intracellular TG2 activity using horse serum or adult human serum. Horse serum contains a complex mixture of components. Future studies are needed to determine which specific molecule(s) in horse serum are responsible for activating intracellular TG2; this would facilitate a clearer understanding of how TG2 activity relates to MT stability in regulating neurite growth.

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