Activity-dependent secretion of progranulin from synapses

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Summary

The secreted growth factor progranulin (PGRN) has been shown to be important for regulating neuronal survival and outgrowth, as well as synapse formation and function. Mutations in the PGRN gene that result in PGRN haploinsufficiency have been identified as a major cause of frontotemporal dementia (FTD). Here we demonstrate that PGRN is colocalized with dense-core vesicle markers and is co-transported with brain-derived neurotrophic factor (BDNF) within axons and dendrites of cultured hippocampal neurons in both anterograde and retrograde directions. We also show that PGRN is secreted in an activity-dependent manner from synaptic and extrasynaptic sites, and that the temporal profiles of secretion are distinct in axons and dendrites. Neuronal activity is also shown to increase the recruitment of PGRN to synapses and to enhance the density of PGRN clusters along axons. Finally, treatment of neurons with recombinant PGRN is shown to increase synapse density, while decreasing the size of the presynaptic compartment and specifically the number of synaptic vesicles per synapse. Together, this indicates that activity-dependent secretion of PGRN can regulate synapse number and structure.

Key words: Progranulin, Synapses, Activity, Secretion, Hippocampal culture, Protein trafficking, Frontotemporal dementia

Introduction

Progranulin (PGRN) is a multi-functional, secreted growth factor expressed in a variety of tissues throughout the body (Bateman and Bennett, 2009). In the adult brain, PGRN is expressed in microglia, in pyramidal neurons of the cortex and hippocampus, as well as in cerebellar Purkinje cells and spinal motor neurons (Petkau et al., 2010). PGRN has been shown to play a role in promoting neuronal survival, enhancing neurite outgrowth and regulating inflammation in the central nervous system (CNS) (Guo et al., 2010; Ryan et al., 2009; Tang et al., 2011; Van Damme et al., 2008; Xu et al., 2011). Interest in the regulation and function of PGRN in the brain has significantly increased following the discovery that mutations in the progranulin (also known as granulin) gene (GRN) are the major cause of autosomal dominant frontotemporal dementia (FTD) with tau-negative inclusions (Baker et al., 2006; Bronner et al., 2007; Cruts et al., 2006; Gass et al., 2006; Mukherjee et al., 2006; Pickering-Brown et al., 2006; van der Zee et al., 2007).

Most of the pathogenic mutations result in null alleles, and it is believed that frontotemporal dementia results from PGRN haploinsufficiency (Baker et al., 2006; Cruts et al., 2006). However, a number of FTD-linked mutations that specifically lead to deficiencies in PGRN secretion have also been identified (Mukherjee et al., 2008; Shankaran et al., 2008; Wang et al., 2010). A further understanding of the regulation of PGRN secretion is therefore warranted.

Previous work from our lab has demonstrated that knocking down PGRN levels in rat primary hippocampal cultures reduces neuronal arborization and the density of synapses, but enhances the size of presynaptic compartments and the frequency of mini excitatory postsynaptic currents (mEPSCs) of the remaining synapses (Tapia et al., 2011). In addition, PGRN knockout mice display altered synaptic connectivity, impaired synaptic plasticity and abnormal neuronal morphology (Petkau et al., 2012). Taken together, these data suggest PGRN has neurotrophic properties and plays an important role in regulating neuronal morphology and connectivity. Despite the recent interest in how PGRN functions in the brain, the regulation of PGRN transport and secretion have not yet been characterized in neurons.

We demonstrate that PGRN is localized to a subset of synapses in both axons and dendrites and is transported bi-directionally with transport characteristics similar to those of dense-core vesicles. In addition, we demonstrate that PGRN is highly colocalized and co-transported with a well-characterized neurotrophin, brain-derived neurotrophic factor (BDNF), and is further recruited to synapses following neuronal activity. Similar to that previously shown for BDNF, PGRN is secreted from axons and dendrites in an activity-dependent manner with different temporal profiles of secretion. This secretion is dependent on the activation of voltage-gated calcium channels (VGCC) and can be blocked in Ca2+–free media or in the presence of VGCC blockers. Treatment of cultured hippocampal
neurons with recombinant PGRN results in increased synapse density. These results suggest that PGRN secretion may play a key role in regulating activity-dependent changes in neuronal connectivity.

Results
Expression of endogenous and fluorescently tagged PGRN

Immunostaining of cultured hippocampal neurons at 14 days in vitro (DIV) demonstrated that endogenous PGRN is distributed in a punctate pattern in both dendrites and axons, identified as thick MAP2-positive and thin MAP2-negative processes, respectively (Fig. 1A). To determine whether the punctate distribution of PGRN represents PGRN at synapses, we co-immunostained for the excitatory pre-synaptic marker, VGlut-1, and the excitatory post-synaptic marker, PSD-95 (Fig. 1C). Synapses were defined as points of colocalization between VGlut-1 and PSD-95. We found that 16±1.1% of endogenous PGRN puncta were localized to synapses (n=35 neurons, three cultures), indicating that the majority of PGRN is localized at extrasynaptic sites.

To validate the use of PGRN tagged with either GFP or its pH-sensitive variant, super ecliptic pHluorin (SEP) for studying activity-induced PGRN secretion, we examined the distribution of PGRN–GFP. This distribution was similar to that of endogenous PGRN, with a punctate pattern of PGRN–GFP in dendrites and axons (Fig. 1B). Moreover, the distribution of PGRN–GFP at excitatory synapses was similar to that of endogenous PGRN with 11.2±1.4% of PGRN–GFP puncta localized to synapses (Fig. 1D; n=30 neurons, three cultures). Using immunocytochemistry, cells transfected with PGRN–GFP were shown to express 2.3±0.35-fold more PGRN than non-transfected cells (n=6 neurons, two cultures; supplementary material Fig. S1). Together, these data demonstrate that fluorescently tagged PGRN is a faithful marker for endogenous PGRN, similar to that shown for many neuronal proteins.

Colocalization and co-transport of PGRN and BDNF

Progranulin is a secreted protein exhibiting neurotrophic functions including regulation of neuronal survival and neurite outgrowth (Ryan et al., 2009; Tapia et al., 2011; Van Damme et al., 2008). We next determined the relationship between PGRN and brain-derived neurotrophic factor (BDNF), another secreted trophic factor expressed in both axons and dendrites (Adachi et al., 2005; Jakawich et al., 2010; Matsuda et al., 2009), but see also Dieni et al. (Dieni et al., 2012). Cells expressing PGRN–GFP and BDNF–RFP were imaged at 8–10 DIV (Fig. 2A). The density of PGRN puncta in dendrites and axons was 82.4±5.9 and 52.2±2.6 puncta per 100 µm, respectively, and the density of BDNF puncta in dendrites and axons was 82.5±5.2 and 53.7±1.9 puncta per 100 µm, respectively (n=10 neurons, three cultures). There was a high degree of colocalization between PGRN and BDNF, with 84±2.4% of PGRN–GFP colocalizing with BDNF–RFP in dendrites and 91.7±2.6% in axons (Fig. 2A,B). Moreover, 82.4±2.9% of BDNF–RFP colocalized with PGRN–GFP in dendrites and 88.2±3.0% of BDNF–RFP colocalized with PGRN–GFP in axons (n=10 neurons, three cultures; Fig. 2A,B).

To examine the transport characteristics of PGRN, neurons were transfected with PGRN–GFP and imaged live using wide-field fluorescence microscopy. PGRN–GFP puncta were transported both anterogradely and retrogradely as evidenced by kymographs with positive and negative slopes, respectively, indicating that PGRN–GFP is trafficked bidirectionally in neurons (Fig. 2C). Quantification of the kymographs indicates that PGRN–GFP has transport characteristics consistent with that of microtubule-based dense-core vesicle (DCV) transport (Table 1) (Barkus et al., 2008; Kwinter et al., 2009). To determine whether PGRN and BDNF are transported together, neurons were co-transfected with PGRN–GFP and BDNF–RFP at 9 DIV and imaged live between 18 and 24 hours after transfection. We observed a substantial overlap in the movement of the two constructs in the axons of hippocampal neurons in anterograde and retrograde directions. The degree of co-transport was quantified and found to be close to 80% in both anterograde and retrograde directions (Fig. 2B). These values are comparable to the degree of overlap reported for other colocalization studies for DCVs (de Jong et al., 2008). In support of the idea that PGRN is transported in DCVs, we observed substantial colocalization between PGRN–GFP and the dense-core vesicle cargo protein, chromogranin A (ChromA–RFP; supplementary material Fig. S2A), as well as colocalization between endogenous and GFP-tagged PGRN, and the dense-core vesicle cargo protein, secretogranin II (SGII; supplementary material Fig. S2B,C). Although this data strongly suggests that

![Fig. 1. PGRN is localized to a subset of synapses in axons and dendrites.](Image)
PGRN is transported in DCVs, immunoelectron microscopy is required to unequivocally confirm these findings.

Activity-induced translocation of PGRN

Previous work has demonstrated that activity enhances the recruitment of BDNF to synapses (Dean et al., 2012). To determine whether PGRN is also recruited to synapses following enhanced neuronal activity, we co-transfected neurons with PGRN-GFP and the pre-synaptic marker synaptophysin-RFP (synRFP) at 10 DIV and imaged neurons at 13 DIV. To enhance neuronal activity, cells were treated with 0.5 mM 4-aminopyridine (4-AP) plus 10 μM bicuculline and imaged 0, 5 and 10 minutes after treatment. 4-AP is a K+ channel blocker, and enhances neuronal activity by increasing the amplitude and duration of action potentials (Alkadhi and Hogan, 1989). Bicuculline acts as a GABAA receptor antagonist, thus blocking the inhibitory input to the target neuron (Curtis et al., 1970). These compounds have been utilized together in a large number of studies to enhance neural activity (Hardingham et al., 2002; Hoey et al., 2009; Kuriu et al., 2006). We observed a significant (P<0.05) increase in the density of PGRN–GFP puncta 10 minutes after 4-AP and bicuculline treatment (labeled as ‘4-AP’), indicating that PGRN is recruited into axons following enhanced neural activity (Fig. 3A,C,D). In contrast, the density of synRFP remained unchanged providing a control for the specificity of activity-induced recruitment of PGRN into axons (Fig. 3A,C).

Activity also enhanced the recruitment of PGRN to synapses. Indeed, 26.3±3% of synRFP puncta that did not contain PGRN–GFP prior to stimulation, were associated with PGRN–GFP 10 minutes after stimulation (n=11 cells, three experiments). Moreover, we observed a significant (P<0.01) increase in the integrated density (product of the mean gray value and area) of PGRN–GFP puncta that were already at synapses, 5 and 10 minutes after stimulation (Fig. 3B–D). The increased integrated density of PGRN–GFP at synapses, and localization of PGRN–GFP to synRFP clusters not initially associated with PGRN, together demonstrate an activity-dependent recruitment of PGRN to synapses (Fig. 3C,D).

Activity enhances PGRN secretion

Previous work has demonstrated that activity enhances the secretion of BDNF from axons and dendrites (Dean et al., 2009; Lessmann and Brigadski, 2009; Matsuda et al., 2009). To determine whether PGRN is similarly secreted from cultured hippocampal neurons, we transfected neurons with a vector expressing PGRN fused to SEP, a pH-sensitive marker for vesicular exocytosis (Gandhi and Stevens, 2003; Miesenböck et al., 1998; Sankaranarayanan et al., 2000). The fluorescence of SEP is quenched at pH, 6 and therefore not visible while inside the acidic lumen of a secretory vesicle, which has a pH of ~5.5 (Mellman et al., 1986; Njus et al., 1986). When the acidified vesicle fuses with the plasma membrane, the vesicle lumen undergoes a rapid increase in pH to ~7.4 resulting in the unquenching of SEP fluorescence (Miesenböck et al., 1998). PGRN–SEP puncta were sparsely distributed in both dendrites and axons (supplementary material Fig. S3A,B). To demonstrate that these few PGRN–SEP puncta represent unquenched PGRN–SEP at the cell surface, cultures were treated for 1 minute with an acidic solution containing 2-(N-morpholino)ethanesulfonic acid (MES), pH 5.2. Following MES treatment, we observed a rapid decrease in PGRN–SEP fluorescence in both dendrites and axons (supplementary material Fig. S3A,B). To demonstrate that PGRN–SEP fluorescence was quenched in the majority of vesicles, cells were treated with a solution of 50 mM NH4Cl (pH 7.4), which has been demonstrated to rapidly diffuse across cell membranes and neutralize the acidic vesicular lumen (Miesenböck et al., 1998). Following a 1 minute application of
NH₄Cl, we observed an increase in the number of fluorescent PGRN–SEP puncta in axons and dendrites at both synapses and extrasynaptic sites, suggesting that the majority of PGRN is localized intracellularly (supplementary material Fig. S3A,B).

To determine whether PGRN secretion is regulated by neuronal activity, the change in SEP fluorescence following enhanced activity was monitored using time-lapse confocal imaging. Specifically, cells were transfected with superecliptic pHluorin-tagged PGRN (PGRN-SEP) to visualize exocytotic events and either synRFP or PSD-95–RFP to demarcate synapses in axons and dendrites, respectively. Cells were imaged for 5 minutes in a low KCl solution to obtain baseline levels of PGRN secretion (see Materials and Methods) and then switched to a solution containing 70 mM KCl, or 0.5 mM 4-AP and 10 μM bicuculline and imaged every 12 seconds for 10 minutes. The average density of PGRN–SEP fluorescent puncta rapidly increased in axons following treatment with 4-AP and bicuculline, and was maintained throughout the imaging period.

### Table 1. Transport characteristics of PGRN-GFP in axons and dendrites of 14 DIV hippocampal neurons

<table>
<thead>
<tr>
<th></th>
<th>All events (n=67)</th>
<th>Anterograde events (n=43)</th>
<th>Retrograde events (n=24)</th>
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<tbody>
<tr>
<td><strong>Axon</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flux (per minute)</td>
<td>4.95±0.66</td>
<td>2.68±0.43</td>
<td>2.27±0.27</td>
</tr>
<tr>
<td>Velocity (μm/s)</td>
<td>1.73±0.06</td>
<td>1.70±0.07</td>
<td>1.73±0.07</td>
</tr>
<tr>
<td>Run length</td>
<td>6.19±0.32</td>
<td>6.61±0.51</td>
<td>5.73±0.24</td>
</tr>
<tr>
<td><strong>Dendrite</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flux (per minute)</td>
<td>2.46±0.60</td>
<td>1.61±0.53</td>
<td>0.86±0.19</td>
</tr>
<tr>
<td>Velocity (μm/s)</td>
<td>1.06±0.11</td>
<td>1.03±0.13</td>
<td>1.03±0.10</td>
</tr>
<tr>
<td>Run length</td>
<td>4.71±0.63</td>
<td>4.44±0.68</td>
<td>4.48±0.64</td>
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Values are from 11 kymographs and 11 cells (means ± s.e.m.).

Fig. 3. Activity increases the density of PGRN–GFP puncta at axons and enhances the recruitment of PGRN–GFP to synapses. (A) The density of PGRN–GFP puncta in axons is increased at 10 minutes after treatment with 4-AP and bicuculline (labeled as ‘4-AP’; normalized to controls). Conversely, the density of synRFP puncta is unchanged (n=8–14 neurons per condition, three cultures). (B) The integrated density of PGRN–GFP puncta that are specifically colocalized with synRFP puncta is increased at 5 minutes and 10 minutes following 4-AP and bicuculline treatment (normalized to controls; n=8–14 neurons per condition, three cultures). *P<0.05, **P<0.01, Student’s t-test. (C) Confocal images of 14 DIV hippocampal neurons co-transfected with PGRN-GFP and synRFP, before treatment and 10 minutes following treatment with 4-AP and bicuculline. White arrowheads indicate existing PGRN–GFP puncta colocalized with synRFP that increase in integrated density by 10 minutes after activity induction. Black arrowheads indicate PGRN–GFP puncta that are newly recruited to synRFP puncta. Scale bar: 5 μm. (D) Fluorescence distribution histogram depicting mean gray value of PGRN–GFP and synRFP puncta along a length of axon (from inset in C).
There was also a dramatic increase in the average density of PGRN–SEP fluorescent puncta in axons following stimulation with 70 mM KCl, which was similar in magnitude and time course of response to that observed following 4-AP and bicuculline stimulation (Fig. 4C).

To examine the role of Ca\(^{2+}\) in activity-mediated exocytosis of PGRN-containing vesicles, we stimulated the cells in Ca\(^{2+}\)-free medium and examined the average density of PGRN–SEP fluorescent puncta. Stimulating cells with 4-AP and bicuculline or 70 mM KCl in Ca\(^{2+}\)-free medium abolished activity-mediated increase in PGRN–SEP fluorescent events (Fig. 4B,C). In addition, blocking voltage-gated calcium channels (VGCCs) with CdCl\(_2\) similarly abolished activity-mediated increase in the density of PGRN–SEP fluorescence events (Fig. 4B,C). Despite the minimal number of secretion events under basal conditions (0.155±0.006 events per 100 \(\mu m\) in a 10 minute period, \(n=33\) cells), this was further reduced in the absence of Ca\(^{2+}\) (0.099±0.008 events per 100 \(\mu m\) in a 10 minute period; \(n=22\) cells; \(P<0.005\) Student’s \(t\)-test). These data suggest that Ca\(^{2+}\) influx through the VGCC is necessary for activity-dependent fusion of PGRN–SEP-containing vesicles.

To determine whether PGRN is preferentially secreted at postsynaptic sites following activity, we analyzed the number of PGRN–SEP events at PSD-95–RFP puncta. Similar to that observed in axons, we observed no significant difference in the proportion of PGRN–SEP events at PSD-95 clusters (Fig. 5C), indicating that PGRN is not preferentially secreted from synapses following activity. Interestingly, PGRN secretion at extrasynaptic sites did not appear to mediate the recruitment of synaptic markers to sites of PGRN secretion. We therefore do not believe that PGRN secretion acts as a ‘tag’ for the formation of new synapses.

Recombinant PGRN treatment increases synapse density but decreases the size of presynaptic compartments

To examine the effects of secreted PGRN on synapse density and morphology, we treated hippocampal cultures with different concentrations of recombinant PGRN (rPGRN). Cells were treated with rPGRN at 0, 3, 7 and 10 DIV, transfected with...
our findings demonstrate that PGRN is co-transported with BDNF in axons and dendrites, and appear to be similarly recruited to synapses following enhanced neuronal activity. Disruptions in BDNF transport have been reported in a number of disease states including Huntington’s disease, focal ischemia, glaucoma, Alzheimer’s disease and Rett syndrome (Gauthier et al., 2004; Kokaia et al., 1998; Pease et al., 2000; Santi et al., 2006). Finally, we have demonstrated that treatment of neurons with recombinant PGRN results in an increase in synapse density and a decrease in the size of presynaptic compartments, complementing our previous findings in PGRN knockdown cells (Tapia et al., 2011).

Neuropeptides are packaged in the Golgi apparatus and then trafficked to sites of release. We find that BDNF and PGRN are co-transported in both axons and dendrites, and appear to be similarly recruited to synapses following enhanced neuronal activity. Disruptions in BDNF transport have been reported in a number of disease states including Huntington’s disease, focal ischemia, glaucoma, Alzheimer’s disease and Rett syndrome (Gauthier et al., 2004; Kokaia et al., 1998; Pease et al., 2000; Poon et al., 2011; Roux et al., 2012). As PGRN and BDNF are co-transported in both axons and dendrites, and appear to be similarly recruited to synapses following enhanced neuronal activity, activity also induces the secretion of PGRN from both axons and dendrites and at both synaptic and extrasynaptic sites. Despite the fact that axons and dendrites exhibit distinct temporal profiles of activity-induced PGRN secretion, secretion from both compartments requires activation of VGCCs and an increase in intracellular Ca²⁺. This is consistent with that reported for regulated BDNF secretion (Balkowiec and Katz, 2002; Goodman et al., 1996; Hartmann et al., 2001; Santi et al., 2006). Finally, we have demonstrated that treatment of neurons with recombinant PGRN results in an increase in synapse density and a decrease in the size of presynaptic compartments, complementing our previous findings in PGRN knockdown cells (Tapia et al., 2011).

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Previous work by Dean and colleagues showed that BDNF is recruited to synapses following increased neuronal activity (Dean et al., 2012), which is similar to our findings for PGRN. These observations suggest that in addition to increasing protein synthesis and trafficking of neurotrophins to distal sites, activity can capture vesicles at active synapses, where it may act to rapidly modulate synaptic strength. This phenomenon has also been observed at the *Drosophila* neuromuscular junction, where increased neuronal activity results in the recruitment of peptidergic vesicles to synaptic boutons (Shakiryanova et al., 2006). In addition, it has been previously demonstrated that stimulating hippocampal neurons with KCl results in increased dendritic localization of BDNF mRNA (Tongiorgi et al., 1997).
Similar to what we have shown for PGRN, BDNF is secreted from axons and dendrites in an activity-dependent manner, and this is dependent on the activation of VGCC and an increase in intracellular Ca\(^{2+}\) concentration (Balkowiec and Katz, 2002; Goodman et al., 1996; Hartmann et al., 2001; Santi et al., 2006).

We have demonstrated that PGRN is released with different temporal profiles of secretion from axons and dendrites. Although activity results in a rapid increase in PGRN–SEP fluorescent events in both axons and dendrites, in axons the number of PGRN–SEP events is maintained for the duration of 4AP and bicuculline treatment, whereas in dendrites the number of PGRN–SEP fluorescent events decreases sharply over time. Previous studies have also reported different modes of activity-mediated BDNF release in axons and dendrites. BDNF secretion events in dendrites have been shown to be larger and faster than in axons, indicating full fusion of BDNF-containing vesicles (Dean et al., 2009; Matsuda et al., 2009). In contrast, the characteristics of axonal BDNF release events following activity correspond to partial fusion of BDNF-containing vesicles, indicating the kiss-and-run mode of BDNF release (Dean et al., 2009; Matsuda et al., 2009). Indeed, the recycling of vesicles following multiple kiss-and-run events at the axon may explain the sustained levels of PGRN–SEP fluorescent events in the axon following stimulation. Conversely, complete fusion of PGRN–SEP-containing vesicles in the dendrites may account for the decay in the average density of PGRN–SEP fluorescent events after the initial activity-induced increase.

Axonal and dendritic PGRN vesicles may in fact belong to two distinct populations, which may also contribute to different activity-induced PGRN release profiles in the axonal and dendritic compartments. Accordingly, it has been previously shown that distinct subsets of BDNF-containing vesicles are differentially sorted to axons and dendrites (Dean et al., 2012).
It has been previously suggested that presynaptic and postsynaptic BDNF release may differentially affect synaptic function. Indeed, presynaptic BDNF secretion increases only presynaptic strength, whereas postsynaptic BDNF secretion increases both postsynaptic and presynaptic strength (Dean et al., 2009). It is possible that the different modes of activity-mediated secretion of PGRN from the axon and dendrites may also have distinct effects on synaptic function; however, this has yet to be tested.

In addition to distinct methods of BDNF sorting and release in axons and dendrites, there is evidence that BDNF can be secreted constitutively as well as its well-described regulated release (Lessmann and Brigadski, 2009; Lu et al., 2005). Indeed, a recent study has demonstrated that acute and gradual increases in BDNF concentration activate different intracellular cascades, leading to differences in spine morphology (Ji et al., 2010). These results suggest that it is not only the concentration of secreted BDNF that is important in the regulation of neuronal function and morphology, but also the manner in which it is secreted: constitutively or in response to neuronal activity. It is possible that constitutive and regulated secretion of PGRN also differentially regulates synapse structure and function.

PGRN has an important role in regulating neurite outgrowth (Gao et al., 2010; Laird et al., 2010; Ryan et al., 2009; Van Damme et al., 2008), and neuronal activity might be a key factor in modulating the manner in which PGRN exerts its effects on neuronal arborization. For instance, acute increases in BDNF concentration and resulting transient activation of TrkB enhances neurite elongation and spine head enlargement (Ji et al., 2010). Conversely, gradual increases in BDNF concentration and sustained TrkB activation promote neurite branching and spine neck elongation (Ji et al., 2010). In addition, local acute applications of BDNF to the neurites of cultured hippocampal neurons have been demonstrated to induce axon differentiation (Shelly et al., 2007). It is yet unknown how changes in the concentration of PGRN and the location of PGRN release impact neuronal outgrowth and synapse morphology.

We demonstrate that treatment of neurons with recombinant PGRN increases synapse density but decreases the integrated density of the presynaptic protein, VGlut-1 and the number of synaptic vesicles per synapse. It is possible that the effects of exogenous PRGON on presynapse size and synapse number might be direct or secondary to changes in neuronal or synaptic activity. It is important to note that although treatment of cells with recombinant PGRN decreases the size of VGlut-1 puncta, and viral-mediated knockdown of PGRN in >90% of cells results in an increase in size of VGlut-1 puncta (Tapia et al., 2011), transient transfection of cells with PGRN-GFP (~1% of cells transfected) did not significantly change the size of VGlut-1 puncta (Tapia et al., 2011). This suggests that PGRN-mediated changes in VGlut-1 size are not cell autonomous and are probably regulated by levels of postsynaptic PGRN. This is in accordance with previous evidence demonstrating an important role for PGRN in regulating neuronal connectivity and synaptic plasticity (Petkau et al., 2012; Tapia et al., 2011). We postulate that activity-mediated secretion of PGRN regulates the number and morphology of synaptic connections. However, many important questions remain unanswered. Does PGRN exert different effects when secreted synaptically or extrasynaptically? Does PGRN act locally or globally once secreted in response to neuronal activity? Does PGRN exert differential effects when secreted presynaptically or postsynaptically, and do the time course and pattern of secretion impact its actions? Answers to these questions will provide further understanding of the basic biology of PGRN, which is greatly needed to further our understanding of the pathophysiology of FTD.

### Materials and Methods

#### DNA constructs

**PGRN-GFP**

The human PGRN cDNA (accession no. NM_000208) was a kind gift from Max Cynader (University of British Columbia, BC, Canada). The PGRN–GFP fusion protein was generated by amplifying the PGRN coding sequence without the stop codon for cloning into JPA7-GFP (Sampo et al., 2003). The forward primer included a HindIII restriction site (underlined): 5′-TCTAACGGTGCGCC- AAAGGTGGAGCCTG-3′. The reverse primer incorporated an AgeI site (underlined) in addition to changing the STOP codon to a glycine (in bold): 5′- CGACCGTATTCCTCCAGACGTGTC-3′. Plasmid composition was confirmed by sequencing.

**PGRN-superflourescent pHluorin (PGRN–SEP)**

Superflourescent pHluorin (SEP; accession no. AY533296; a kind gift from Michael Ehlers, Duke University, Durham, NC) was PCR amplified: forward, 5′- ACCGGTCATGAGTAAAGGAG-3′; reverse, 5′-TCTAGAATTATTTGTAGTT- TTCA-3′. It was then subcloned into the pJP7 vector replacing GFP to produce PGRN-SEP.

#### Other constructs

BDNF-RFP was a kind gift from Gary Banker (Oregon Health and Science University, Portland, OR) chromograninA-RFP was a kind gift from Laurent Cynader (University of British Columbia, BC, Canada). The PGRN–GFP fusion DNA constructs

#### Hippocampal culture and transfection

Primary hippocampal cultures were prepared from embryonic day 18 (E18) Sprague-Dawley rats as previously described (Xu et al., 2000) or from E16 C57Bl/6 mice also as previously described (Kacch and Banker, 2006). Neurons were plated at a density of 197 cells/mm² and 46 cells/mm², respectively, and maintained in Neurobasal/B27 (Invitrogen, Carlsbad, CA). Neurons were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) at 9–10 days in vitro (DIV) according to the manufacturer’s protocol and imaged at 10–11 or 13–16 DIV.

#### Activity treatments

High K⁺ (51.5 mM NaCl, 70 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 25 mM HEPES and 30 mM glucose, pH 7.4); 4-aminopyridine (4-AP) and bicuculline (119 mM NaCl, 2.5 mM KCl, 0.5 mM CaCl₂, 3.5 mM MgCl₂, 25 mM HEPES, 30 mM glucose, 0.5 mM 4-AP and 10 µM bicuculline, pH 7.4); CdCl₂ treatment (119 mM NaCl, 2.5 mM KCl, 0.5 mM CaCl₂, 3.5 mM MgCl₂, 25 mM HEPES, 30 mM glucose and 100 µM CdCl₂, pH 7.4); high K⁺ in calcium-free medium (119 mM NaCl, 2.5 mM KCl, 7 mM MgCl₂, 25 mM HEPES, 30 mM glucose and 100 µM EDTA, pH 7.4); high K⁺ and CdCl₂ treatment (51.5 mM NaCl, 70 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 25 mM HEPES, 30 mM glucose and 100 µM EDTA, pH 7.4); high K⁺ and CdCl₂ treatment (119 mM NaCl, 2.5 mM KCl, 0.5 mM CaCl₂, 3.5 mM MgCl₂, 25 mM HEPES, 30 mM glucose, 0.5 mM 4-AP, 10 µM bicuculline and 100 µM CdCl₂, pH 7.4); 4-AP and bicuculline in calcium-free medium (119 mM NaCl, 2.5 mM KCl, 7 mM MgCl₂, 25 mM HEPES, 30 mM glucose, 0.5 mM 4-AP, 10 µM bicuculline and 100 µM CdCl₂, pH 7.4).

To validate pH-dependent SEP, cells were first treated with a solution containing 80 mM NaCl, 70 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 25 mM HEPES and 30 mM glucose, pH 7.4; 4-aminopyridine (4-AP) and bicuculline (119 mM NaCl, 2.5 mM KCl, 0.5 mM CaCl₂, 3.5 mM MgCl₂, 25 mM HEPES, 30 mM glucose, 0.5 mM 4-AP and 10 µM bicuculline, pH 7.4); CdCl₂ treatment (119 mM NaCl, 2.5 mM KCl, 0.5 mM CaCl₂, 3.5 mM MgCl₂, 25 mM HEPES, 30 mM glucose and 100 µM CdCl₂, pH 7.4); high K⁺ in calcium-free medium (119 mM NaCl, 2.5 mM KCl, 7 mM MgCl₂, 25 mM HEPES, 30 mM glucose and 100 µM EDTA, pH 7.4); high K⁺ and CdCl₂ treatment (51.5 mM NaCl, 70 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 25 mM HEPES, 30 mM glucose and 100 µM EDTA, pH 7.4); high K⁺ and CdCl₂ treatment (119 mM NaCl, 2.5 mM KCl, 0.5 mM CaCl₂, 3.5 mM MgCl₂, 25 mM HEPES, 30 mM glucose, 0.5 mM 4-AP, 10 µM bicuculline and 100 µM CdCl₂, pH 7.4); 4-AP and bicuculline in calcium-free medium (119 mM NaCl, 2.5 mM KCl, 7 mM MgCl₂, 25 mM HEPES, 30 mM glucose, 0.5 mM 4-AP, 10 µM bicuculline and 100 µM CdCl₂, pH 7.4).

To validate pH-dependent SEP, cells were first treated with a solution containing NaHCO₃ (pH 7.4), and then with one containing 2-(N-morpholino)ethanesulfonic acid (MES). 50 mM NaCl was substituted for 50 mM NH₄Cl, and all other components remained the same as in the control solution, with the pH adjusted to 7.4. For the treatment with acidic MES solution, 25 mM HEPES was replaced with 25 mM MES and all other components remained unchanged; the pH was adjusted to 5.2. The average PGRN–SEP fluorescent event density was calculated by dividing the total number of PGRN–SEP fluorescent events in each frame by the total neurite length, and averaging for all the frames in the 10 minute time-lapse imaging series. The number of PGRN–SEP fluorescent events was manually counted for each of the time-lapse images. All visible puncta more than 2 pixels in size were counted. Colocalization of PGRN–SEP fluorescent puncta and synRFP or PSD-95–RFP puncta was determined by manually counting all overlapping puncta (>1 pixel overlap) in each frame.
Recombinant PGRN treatment
Neurons were treated with recombinant PGRN protein (R&D Systems, Minneapolis, MN) at concentrations of 0, 100 and 250 ng/ml on 0, 7 and 10 DIV. Synapse density and size were analyzed at 14 DIV.

Immunohistochemistry
Cultures were fixed with 4% paraformaldehyde and 4% sucrose for 10 minutes, permeabilized with 0.1% Triton-X for 10 minutes, and blocked with 10% goat serum for 1 hour at room temperature. Primary and secondary antibodies were diluted in 1% goat serum in PBS. Cells were incubated in primary antibody at 4°C overnight, and in secondary antibody for 1 hour at room temperature. Primary antibodies: rabbit anti-PGRN (Invitrogen, Carlsbad, CA), rabbit anti-MAP2 (Abcam, San Francisco, CA), guinea pig anti-VGlut-1 (Millipore, Billerica, MA), mouse anti-PSD-95 (Affinity BioReagents, Golden, CO), mouse anti-SGII (Santa Cruz Biotech, Santa Cruz, CA). Secondary antibodies: Alexa Fluor 488, Texas Red or Cy3-conjugated goat anti-rabbit, anti-mouse or anti-guinea pig (Molecular Probes, Eugene, OR).

Electron microscopy
Hippocampal cultures were plated on Aclar membrane inserts in a multiwell chamber (Costar) treated with poly-L-lysine. At 13 DIV, cells were fixed and embedded in JEMBED and Spurr’s resin. Grids were cut and stained with 2% uranyl acetate and Reynold’s lead. Over 50 separate images (magnification 50,000×) were acquired for each treatment and analyzed with the experimenter blind to the experimental condition.

Image acquisition
Imaging of PGRN–GFP and BDNF–RFP, as well as endogenous PGRN immunostaining, were done under 63× magnification on a standard wide-field fluorescence DMI 6000 B Leica microscope equipped with a cooled CCD camera and controlled by MetaMorph software as previously described (Kwinter and Silverman, 2009). Imaging for all other experiments was done using an inverted confocal Olympus FV1000 microscope (60× or 1.4 NA oil Plan-Apochromat). All images in a given experiment were captured and analyzed using the same exposure time and conditions.

Image analysis
Transport of PGRN–GFP and BDNF–RFP. All DIV neurons transfected with PGRN–GFP and BDNF–RFP were imaged using time-lapse imaging at 37°C. Streamed recordings were made of transport events along a section of the axon using the cell body to determine anterograde and retrograde directions of transport. The output files were distance–time plots (kymographs) of transport events, which were manually traced on MetaMorph. Anterograde and retrograde events were traced separately. Each trace consisted of a run >2 μm in length and stopped if the vesicle remained stationary for four or more frames. Custom software was then used to calculate the parameters of vesicle flux, velocity and run length (Kwinter et al., 2009). Vesicle flux was defined as the sum of the distances traveled by all vesicles, standardized by the length and duration of each recording.

PGRN colocalization (density of colocalized clusters)
Images of PGRN–GFP and BDNF–RFP were thresholded separately by subjectively evaluating real clusters using ImageJ. The established threshold was then applied to all images across experimental groups to enable unbiased analysis. The density of puncta was calculated by dividing the total number of PGRN–GFP or BDNF–RFP puncta by the total neurite length. Points of colocalization were determined using the ImageJ colocalization plugin (http://rsb.info.nih.gov/ij/plugins/colocalization.html). Points of colocalization were defined as regions >4 pixels in size where the intensity ratio of the two channels was >50.

Percent PGRN colocalization
Images of PGRN or PGRN–GFP plus chromogranin A–RFP or SGII were analyzed as above. Percentage colocalization was quantified by dividing the total number of colocalized puncta by the number of PGRN puncta.

PGRN–GFP punctum area and integrated density
Neurons expressing PGRN–GFP and synRFP were treated with 0.5 mM 4-aminopyridine (4-AP) and 10 μM bicuculline and imaged 5 and 10 minutes after treatment. Images were thresholded using ImageJ. Puncta were thresholded subjectively, and the appropriate threshold was applied to all the images in the experiment. PGRN–GFP punctum area and integrated density (IntDen; product of the area and the mean gray value) were then determined before and after treatment of cells using ImageJ.

Analysis of PGRN–GFP at synapses
To determine the integrated density of synaptic PGRN–GFP puncta, PGRN–GFP puncta that colocalized with synRFP were identified in Adobe Photoshop and analyzed. All PGRN–GFP puncta not associated with synRFP were similarly analyzed in a separate pool.

Synapse density
Masks of GFP-transfected neurons were made using Adobe Photoshop, enabling the visualization of all VGlut-1 and PSD-95 immunolabeling associated with the transfected cell. Images of VGlut-1 and PSD-95 immunolabeling were then thresholded using ImageJ. Puncta were thresholded subjectively, but once the appropriate threshold was selected, it was applied to all the images in the experiment. Points of colocalization were analyzed using the ImageJ colocalization plugin.

Live imaging of PGRN–SEP
Neurons transfected with PGRN–SEP and synRFP or BDNF–RFP were imaged every 12 seconds at 37°C. Neurons were initially imaged for 5 minutes in a control solution (119 mM NaCl, 2.5 mM KCl, 0.5 mM CaCl2, 3.5 mM MgCl2, 25 mM HEPES and 30 mM glucose, pH 7.4). After 5 minutes of imaging, cells were switched to the various treatments and imaged for an additional 10 minutes.

Author contributions

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its pro-peptide are stored in presynaptic dense core vesicles in brain neurons. J. Cell Biol. 196, 775-788.


Fig. S1. Level of PGRN in PGRN-GFP-expressing cells. (A-C) Confocal images of 14 DIV hippocampal neurons transfected with PGRN-GFP and immunostained for PGRN. PGRN immunoreactivity is high in the cell body and proximal neurites in cells transfected with PGRN-GFP. Box in A-C demarcates higher magnification images of more distal neurites in A’-C’. Arrows denote PGRN-GFP puncta that are also immunostained with anti-PGRN. Arrowheads denote PGRN puncta in untransfected cells. Scale bar=20 μm (A-C) and 5 μm (A’-C’).
Fig. S2. PGRN colocalizes with the dense core vesicle cargo protein, Chromogranin A. Confocal images of 14 DIV hippocampal neurons expressing PGRN-RFP and ChromA-RFP (A), expressing PGRN-GFP and immunostained for secretogranin II (SGII) (B), or immunostained for both PGRN and SGII (C). Arrows denote colocalization and arrowheads points to PGRN or PGRN-GFP puncta that are not colocalized with DCV markers. The percent PGRN or PGRN-GFP puncta colocalized with DCV markers are shown in the merged images (n>5 cells per condition, 2 cultures). Scale bar=20 μm.
Fig. S3. Validation of PGRN-SEP. Confocal images of 14 DIV hippocampal neurons co-transfected with PGRN-SEP and PSD-95-RFP (A), or PGRN-SEP and synRFP (B). Treatment with NH₄Cl (pH 7.4) unquenches the SEP fluorescence and reveals PGRN-SEP within the dendrite and the axon (right panel). Treatment with MES (pH 5.2) quenches SEP fluorescence (middle panel). Scale bar=2 μm.