Spontaneous Release Regulates Synaptic Scaling in the Embryonic Spinal Network In Vivo

Miguel Angel Garcia-Bereguain, Carlos Gonzalez-Islas, Casie Lindsly, and Peter Wenner

Homeostatic plasticity mechanisms maintain cellular or network spiking activity within a physiologically functional range through compensatory changes in synaptic strength or intrinsic cellular excitability. Synaptic scaling is one form of homeostatic plasticity that is triggered after blockade of spiking or neurotransmission in which the strengths of all synaptic inputs to a cell are multiplicatively scaled upward or downward in a compensatory fashion. We have shown previously that synaptic upscaling could be triggered in chick embryo spinal motoneurons by complete blockade of spiking or GABA<sub>A</sub> receptor (GABA<sub>A</sub>R) activation for 2 d in vivo. Here, we alter GABA<sub>A</sub>R activation in a more physiologically relevant manner by chronically adjusting presynaptic GABA release using nicotinic modulators or an mGluR2 agonist. Manipulating GABA<sub>A</sub>R activation in this way triggered scaling in a mechanistically similar manner to scaling induced by complete blockade of GABA<sub>A</sub>Rs. Remarkably, we find that altering action-potential (AP)-independent spontaneous release was able to fully account for the observed bidirectional scaling, whereas dramatic changes in spiking activity associated with spontaneous network activity had little effect on quantal amplitude. The reliance of scaling on an AP-independent process challenges the plasticity’s relatedness to spiking in the living embryonic spinal network. Our findings have implications for the trigger and function of synaptic scaling and suggest that spontaneous release functions to regulate synaptic strength homeostatically in vivo.

Key words: chick embryo; homeostatic plasticity; nicotine; spinal cord; spontaneous release; synaptic scaling

Significance Statement
Homeostatic synaptic scaling is thought to prevent inappropriate levels of spiking activity through compensatory adjustments in the strength of synaptic inputs. Therefore, it is thought that perturbations in spike rate trigger scaling. Here, we find that dramatic changes in spiking activity in the embryonic spinal cord have little effect on synaptic scaling; conversely, alterations in GABA<sub>A</sub> receptor activation due to action-potential-independent GABA vesicle release can trigger scaling. The findings suggest that scaling in the living embryonic spinal cord functions to maintain synaptic strength and challenge the view that scaling acts to regulate spiking activity homeostatically. Finally, the results indicate that fetal exposure to drugs that influence GABA spontaneous release, such as nicotine, could profoundly affect synaptic maturation.

Introduction
Homeostatic plasticity is thought to maintain network activity levels through compensatory adjustments in cellular excitability and synaptic strength (Desai, 2003; Marder and Goaillard, 2006; Turrigiano, 2011; Davis, 2013). The most studied form of homeostatic plasticity has been called synaptic scaling, in which compensatory changes in synaptic strength are observed after perturbations in spiking activity for many hours (Turrigiano et al., 1998; Turrigiano, 2012). For instance, when spiking was blocked for 2 d in cortical neuronal cultures, AMPAergic synaptic strength increased (Turrigiano et al., 1998). In addition, blockade of glutamatergic receptor activation can also trigger compensatory increases in synaptic strength (O’Brien et al., 1998; Thiagarajan et al., 2005; Stellwagen and Malenka, 2006; Fong et al., 2015). These compensatory changes occur across the entire distribution of the cell’s miniature postsynaptic current (mPSC) amplitudes by a multiplicative scaling factor. Scaling is typically observed and studied in vitro, but has been described to a lesser extent in vivo in the spinal cord (Gonzalez-Islas and Wenner,
2006; Knogler et al., 2010; Wenner, 2014) and visual system (Desai et al., 2002; Goel et al., 2006; Whitt et al., 2014).

Studies of synaptic scaling are now focused on the molecular mechanisms that underlie the plasticity. The most critical component of the signaling cascade mediating synaptic scaling is the initiating factor or trigger for this form of plasticity (Rich and Wenner, 2007). Identification of the triggering signals will be crucial for understanding the function of scaling, but will also help us predict when compensatory changes in synaptic strength occur after changes to the network (development, as a response to injury or in disease, or after drug exposure). Unfortunately, identifying the triggers has been elusive because the kind of perturbations that are used to trigger scaling typically influence multiple factors coordinate, including spike rate, neurotransmission, and network activity and any associated signaling cascades (Rich and Wenner, 2007; Lee et al., 2014).

In the current study, we focused on identifying triggers of synaptic scaling that occur in the living embryonic spinal cord. Developing neural circuits display episodic patterned spontaneous network activity (SNA). This activity is generated by a recurrently connected network critically dependent on GABA- and glutamate-mediated neurotransmission, which are both excitatory connections (V, 50 ms, interval of 1 s) were delivered using an ECM 830 electroporator (Lindsly et al., 2014).

Isolated E10 spinal cords were placed ventral side down in the recording chamber of an Olympus IX70 inverted microscope and continuously perfused with Tyrode’s solution. The solution was heated to 27°C and transfected neurons were imaged through the ventral white matter using a 10X objective. Clomeleon is a fusion protein containing 2 fluorophores, the Cl−-insensitive cyan fluorescent protein (CFP) and the Cl−-sensitive yellow fluorescent protein (YFP) (Kuner and Augustine, 2000). Illumination results in excitation of CFP (430 – 450 nm), producing emission at 485 nm, which excites the YFP fluorophore through fluorescence resonance energy transfer (FRET). Therefore, emitted light was passed through a dichroic mirror with a 460 nm cutoff and then filtered through emission filters for CFP (485 ± 15 nm) or YFP (530 ± 15 nm). To limit differential photobleaching of the Clomeleon fluorophores, we used neutral density filters so that only 1.5% of light reached the specimen. In addition, we limited exposure time (200 ms) using a uniblitz shutter (Vincent Associates). The emission for each fluorophore was then captured onto an intensified CCD camera (Stanford Photonics) and images were recorded using SimplePCI software (Hamamatsu) as a 20-frame average. Images were then processed in SimplePCI by measuring the mean intensity of a region of interest (ROI) drawn around a cell body and subtracting the mean intensity for a background ROI located in a nonlabeled part of the cord. This process was conducted for both CFP and YFP images and the resulting values were expressed as a ratio (YFP/CFP) for each cell. Because Clomeleon is sensitive to internal pH, we monitored and maintained the pH of the bath at 7.2–7.3 during recordings.

Clomeleon ratios were calibrated based on our previous study (for a complete description, see Lindsly et al., 2014). We measured the FRET ratios in the following solutions: 150 mM Cl−, 75 mM Cl−, 50 mM Cl−, 30 mM Cl− (chloride and gluconate concentrations summed to 150 mM). All calibration solutions contained 10 µM nigericin (K+ /H+ antiporter) and 100 µM tributyltin chloride (Cl− /OH− antiporter) to remove transmembrane H+ /OH− and Cl− gradients (Berglund et al., 2006). The dissociation constant (Kd) and Rmax (value for Clomeleon completely unbound by Cl−) were determined from a nonlinear regression of the average ratios to Cl− using the following equation: r = [(Kd * Rmax) + [(Cl−] * Rmin)] /[(Cl−] + Rmax) (Dzhala et al., 2012). Rmax could not be evaluated from a 0 mM Cl− calibration solution as ratio values dropped and did not recover in other calibration solutions. The Rmin (value for Clomeleon completely bound by Cl−) was determined from ratios measured in a KF solution as F− is known to saturate the YFP moiety. As described previously (Lindsly et al., 2014), in 5 spinal cords we found, Rmax = 0.53 ± 0.01, Kp = 91.6 ± 27.0, and Rmin = 2.7 ± 0.28. These values were used for conversion of ratios to Cl− using the following formula: Cl− = Kd / Rmin – Rmin − Rmax) (Berglund et al., 2006; Pond et al., 2006; Dzhala et al., 2012).

Electrophysiology. Experiments were performed on E8–E10 white leghorn chicken embryos. Tight-fitting glass suction electrodes were used to record from the ventrolateral funiculus (VLF). For monitoring SNA, VLF signals were amplified (1000X), filtered (0.1 Hz to 1 kHz) by an extracellular amplifier (A-M Systems), and acquired using PClamp 10 (Molecular Devices). Analyses of the data were performed offline.

Materials and Methods

Dissection. Eggs (chick embryos of either sex) were placed in a 1550 Digital Hatcher incubator (GQF Manufacturing) for 8–10 d (embryonic days 8–10 (E8–E10), stages 34–36 (Hamburger and Hamilton, 1951)) at 38°C. The lumbarosacral spinal cord with attached spinal nerves was isolated under cooled (15°C) oxygenated Tyrode’s solution containing the following (in mM): 139 NaCl, 12 glucose, 17 NaHCO3, 3 KCl, 1 MgCl2, and 3 CaCl2. Tyrode’s solution was constantly bubbled with a mixture of 95% O2/5% CO2 to maintain pH at ~7.3. After dissection, the cord was allowed to recover for at least 6 h in Tyrode’s solution at 18°C. The cord was then transferred to a recording chamber and continuously perfused with oxygenated Tyrode’s solution that was slowly heated to recording temperature (28°C). Stable episodes of SNA (constant burst frequency, every 8–10 min) were established before starting the experiments.

Transfections (in ovo electroporations) and Clomeleon imaging. On E3 (stages 16–18; Hamburger and Hamilton, 1951), a small window was cut into the shell of white leghorn chicken eggs (Hy-line) and plasmids coding for the Clomeleon protein under control of a CMV promoter (kind gift of George Augustine) were injected into the central canal of the neural tube. Two electrodes spaced 4 mm apart were lowered onto the chorioallantoic membrane on either side of the embryo and 5 pulses (25 V, 30 ms, interval of 1 s) were delivered using an ECM 830 electroporator (Lindsly et al., 2014).
Whole-cell patch-clamp recordings were made from motoneurons localized between lumbosacral segments 1 and 3 and were identified by their lateral position in the ventral cord. Motoneurons were held at \(-70\) mV to acquire mPSCs (TTX, 0.5 \(\mu\)M added to bath). Tyrode’s solution was used as the extracellular recording solution and mPSC patch solution contained the following (in mM): 5 NaCl, 100 K-gluconate, 36 KCl, 10 HEPES, 1.1 EGTA, 1 MgCl\(_2\), 0.1 CaCl\(_2\), 1 Na\(_2\)ATP, and 0.1 MgGTP; pH adjusted to 7.3 with KOH. Patch-clamp tight seals (\(>2\) GQ) were obtained using electrodes pulled from thin-walled glass (World Precision Instruments) using a micropipette puller (Sutter Instruments) to obtain resistances between 5 and 10 M\(\Omega\). Recordings were terminated whenever significant increases in series resistance (\(>20\%\)) occurred. AMPA and GABA mPSCs were separated by their decay kinetics (AMPA \(\tau \approx 6.0\) ms, GABA \(\tau \approx 7.0\) ms, values between 6.0 and 7.0 ms were not included in the analysis) as described previously (Gonzalez-Islas and Wenner, 2006). The mPSCs were acquired on an Axopatch 200B patch clamp amplifier (Molecular Devices), digitized online using PClamp 10 (Molecular Devices), and analyzed using Minianalysis software (Synaptosoft). Bar charts and associated average values were obtained by determining an average mPSC amplitude for each cell (variable number of mPSCs/cell, 5 pA cutoff) and then calculating the average of all cells. To approximate whether mPSC cumulative amplitude distributions from treated embryos were multiplicatively related to control distributions (“scaled”), it was necessary to account for mPSC amplitudes that fell into the noise after different treatments (particularly AMPA mPSCs; Garcia-Bereguiaín et al., 2013). Therefore, the following steps were taken: cumulative probability distributions were obtained by combining mPSC amplitudes in control or different treatments (30 mPSCs per cell or, in some cases, 50 mPSCs per cell as in Figs. 3, 9). We multiplied the distribution with the largest amplitudes by a factor of \(<1\), then removed values that fell below the 5 pA cutoff and compared the distributions using a Kolmogorov–Smirnov test. For example, all GABA mPSC amplitudes from DhβE-treated motoneurons were multiplied by 0.65, values that fell below 5 pA were removed, and the downscaled distribution was compared with the distribution of GABA mPSC amplitudes from control motoneurons using the Kolmogorov–Smirnov test.

In ovo drug application and movement counts. Windows in the shell of the egg were opened at E8 and lidocaine hydrochloride aqueous solution (35 mg/ml plus 10 ms HEPES, pH 7.2) was continuously infused onto the choroidallantoic membrane of the embryos at a rate of 13.5 \(\mu\)l/h, as described previously (Gonzalez-Islas and Wenner, 2006). For other drug applications, a single bolus of the drug was delivered onto the choroidallantoic membrane of the embryo at a rate of 13.5 \(\mu\)l/h, as described previously (Gonzalez-Islas and Wenner, 2006). For other drug applications, a single bolus of the drug was delivered onto the choroidallantoic membrane of the embryo at a rate of 13.5 \(\mu\)l/h, as described previously (Gonzalez-Islas and Wenner, 2006). We assessed SNA (E8–E10) by determining the frequency of inspiratory and expiratory events (Fig. 2A). We injected DhβE (5 \(\mu\)M), and recovered 4 h after adding the drug and largely followed control levels throughout the experiment.

To assess the effect of nicotinic modulators on the excitability of the spinal cord after chronic drug treatment (E8–E10), spinal cords were isolated and maintained in recirculating Tyrode’s solution in the absence of nicotine receptor modulators. As shown in Figure 1B, the frequency of SNA was no different in spinal cords isolated from control embryos, nicotine-treated embryos, or DHβE-treated embryos. These results suggest that chronic nicotinic modulator treatment did not produce sustained changes in spinal excitability.

Nicotinic modulators induced synaptic scaling in vivo

We have shown previously that complete blockade of GABA\(_{R}\) activation triggers upsampling of AMPA and GABA mPSCs (Willik, 2008). To determine whether scaling could be triggered by simply altering GABA vesicle release presynaptically, we treated embryos from E8 to E10 with nicotinic modulators as described above. We tested whether decreasing GABA\(_{R}\) activation by chronic DHβE treatment would cause an upsampling. After 2 d of treatments (E8–E10), we isolated the spinal cord and made whole-cell measurements of mPSCs from spinal motoneurons. AMPA and GABA mPSCs were kinetically isolated from the same set of motoneurons (Gonzalez-Islas and Wenner, 2006). We found that 2 d of DHβE treatment resulted in an increase in both AMPAergic and GABAergic mPSC amplitude compared with controls (Fig. 2, Table 1). Moreover, mPSC amplitudes from DHβE-treated embryos demonstrated the characteristic scaling profile, such that amplitudes were upscaled across their distribution by a multiplicative factor (Fig. 2C, D).

Because decreasing nAChR activation caused upsampling, we hypothesized that increasing nAChR activation and therefore GABA release could cause downsampling by chronically increasing GABA\(_{R}\) activation. We treated embryos with nicotine to increase GABA release from E8 to E10 and measured mPSCs at E10. We found that chronic nicotine treatment caused a decrease of
AMPARergic and GABAergic mPSC amplitude (Fig. 3, Table 1). Further, AMPARergic and GABAergic mPSCs were multiplicatively scaled downward (Fig. 3C,D). No significant differences were found in the frequency of mPSCs for any of the treatments (Table 1). The results suggested the possibility that inhibiting or enhancing nAChR activation could trigger upscaling or downscaling, respectively.

In our previous study (Gonzalez-Islas et al., 2016), we had shown that nicotine acted presynaptically to enhance GABA release at E10. To confirm that nicotinic modulation was altering GABA release in the E8 embryo, we performed the following experiments. We isolated spinal cords from E8 embryos and superfused Tyrode’s solution with or without nicotine for at least 6 h. We then recorded from motoneurons and found that 6 h of nicotine treatment caused a sustained increase of GABA mPSC frequency, but not amplitude (Fig. 4A,B). In addition, we wanted to determine whether the increase in GABAergic mPSC frequency was maintained in vivo. Therefore, we treated embryos at E8 for 12 h with either saline or nicotine (10 μM). We then isolated spinal preparations and allowed them to recover overnight (nicotine-treated cords were isolated and maintained in nicotine-containing Tyrode’s solution) and made whole-cell recordings from motoneurons the next day (spinal cords from nicotine-treated embryos were recorded in the continued presence of nicotine). We found that GABAergic mPSC frequency was still increased compared with controls (Fig. 4C). Interestingly, AMPARergic mPSC amplitude decreased after just 12 h of in ovo nicotine treatment, whereas GABAergic mPSC amplitude trended downward but did not reach significance (Fig. 4D). This suggested that downscaling could occur in as little as 12 h, as opposed to upscaling, which takes >12 h of GABAAR perturbation. Together, these results suggested that nicotinic modulators altered GABA release in vivo and triggered synaptic scaling.
GABAergic scaling was mediated by changes in intracellular chloride

We demonstrated previously that GABAergic upscaling was mediated by increases in intracellular chloride (CI\textsubscript{in}, from \(\sim 50\) to \(\sim 85\) mM) after complete GABA\textsubscript{A}R blockade (gabazine injection) or spike blockade (lidocaine infusion) (Gonzalez-Islas et al., 2010; Lindsly et al., 2014). Therefore, we tested whether GABAergic upscaling occurred through the same process after Dh\textsubscript{H\beta E}-mediated reductions in GABA release. We assessed motoneuron CI\textsubscript{in} after Dh\textsubscript{H\beta E} treatment using the ratiometric chloride indicator Clomeleon, as we had done previously (Lindsly et al., 2014). Clomeleon is a fusion protein of CFP (chloride-insensitive) and YFP (chloride-sensitive) and is electroporated into spinal neurons at E3 (Fig. 5). We assessed the ratio of YFP to CFP for ROIs drawn around labeled motoneurons at E10 to approximate CI\textsubscript{in} (Fig. 5C; see Materials and Methods). After Dh\textsubscript{H\beta E} treatment from E8 to E10, motoneuron Clomeleon ratios were altered (Fig. 5D) similar to that described after spike or GABA\textsubscript{A} receptor blockade (Lindsly et al., 2014). The results support the idea that the scaling mechanisms are similar after blockade of nicotinic receptors, GABA\textsubscript{A} receptors, and spiking.

The mechanism of GABAergic downscaling is unknown. Therefore, we treated embryos with nicotine from E8 to E10 to trigger GABAergic downscaling and assessed motoneuron CI\textsubscript{in} using Clomeleon. We found that GABAergic downscaling led to changes in Clomeleon ratios that were consistent with a reduction in CI\textsubscript{in} (Fig. 5).

Synaptic scaling induced by nicotine receptor modulators were dependent on GABA\textsubscript{A} receptor activation

The above results were consistent with the idea that in ovo treatment with nicotine or DH\textsubscript{H\beta E} altered GABA vesicle release and therefore GABA\textsubscript{A}R downscaling and in this way triggered scaling. If nicotinic modulators triggered scaling through activation of the GABA\textsubscript{A}R, then blocking the GABA\textsubscript{A}R should prevent changes in mPSC amplitude triggered by manipulating nAChR function. To block GABA\textsubscript{A}R activation completely, we injected the GABA\textsubscript{A}R antagonist gabazine (10 \(\mu\)M) at E8 and recorded mPSCs from spinal motoneurons at E10. As described previously, we found
that gabazine triggered an increase in both AMPA and GABA mPSC amplitude (Fig. 6, Table 1). All of the distributions demonstrated a multiplicative scaling compared with controls (data not shown).

To test the idea that nicotinic modulator-mediated scaling is dependent on GABA<sub>R</sub> activation, we treated embryos with a combination of gabazine plus a nicotinic modulator. First, we treated embryos from E8 to E10 by injecting both gabazine and DHβE in ovo at E8. This treatment led to an upward scaling of AMPA and GABA mPSC amplitudes that was indistinguishable from that of gabazine treatment alone (Fig. 6, Table 1). This finding suggested that upscaling produced by inhibiting either nAChRs or GABA<sub>R</sub>s was not additive, consistent with the idea that both processes were acting through GABA<sub>R</sub>s. Next, we treated embryos from E8 to E10 by in ovo injections of a combination of gabazine and nicotine at E8. We found that, although nicotine alone caused a downscaling of mPSCs, nicotine and gabazine triggered an upscaling of AMPA and GABA mPSCs that was no different from gabazine treatment by itself (Fig. 6, Table 1). These results suggested that nicotinic modulators triggered bidirectional scaling through far more mild changes in GABA<sub>R</sub> activation than complete GABA<sub>R</sub> blockade.

Upscaling was converted to downscaling by increasing spontaneous GABA release alone

We were surprised by the observation that simply reducing GABA vesicle release with DHβE caused an upscaling that was equivalent to completely blocking GABA<sub>R</sub> activation (Figs. 2, 6, Table 1). Further, we could produce downscaling by slight increases in GABA release. This dramatic control of scaling by nicotinic modulators was even more surprising because, in our previous study, the predominant effect of nicotine and DHβE was on AP-independent spontaneous release (Gonzalez-Islas et al., 2016). To test the idea that scaling could be triggered by alterations in GABA<sub>R</sub> activation due to spontaneous release, we blocked spike activity (SNA) by infusing a solution containing the voltage-gated Na<sup>+</sup> channel blocker lidocaine into the egg from E8 to E10 (Gonzalez-Islas and Wenner, 2006). This caused an upscaling of AMPA mPSC amplitude, as described previously (Fig. 7A–D, Table 1). We restored some of the GABA<sub>R</sub> activation that was lost due to action potential (AP) blockade (lidocaine) by also treating embryos with nicotine from E8 to E10 (lidocaine + nicotine) and increasing spontaneous GABA release. Strikingly, by simply increasing spontaneous GABA release with nicotine, we prevented the lidocaine-induced AMPAeric upscaling (Fig. 7A–D, Table 1). In fact, the distribution of AMPAeric mPSC amplitudes clearly shifted to the left of the AMPAeric mPSC amplitude that was lost due to action potential (AP) blockade (lidocaine treatment led to GABAergic upscaling and lidocaine triggered an upscaling of AMPA and GABA mPSC amplitudes that was indistinguishable from that of gabazine treatment alone (Fig. 6, Table 1). This finding suggested that upscaling produced by inhibiting either nAChRs or GABA<sub>R</sub>s was not additive, consistent with the idea that both processes were acting through GABA<sub>R</sub>s. Next, we treated embryos from E8 to E10 by in ovo injections of a combination of gabazine and nicotine at E8. We found that, although nicotine alone caused a downscaling of mPSCs, nicotine and gabazine triggered an upscaling of AMPA and GABA mPSCs that was no different from gabazine treatment by itself (Fig. 6, Table 1). These results suggested that nicotinic modulators triggered bidirectional scaling through far more mild changes in GABA<sub>R</sub> activation than complete GABA<sub>R</sub> blockade.

### Reducing spontaneous GABA release through an mGluR2 agonist triggered upscaling in vivo

To confirm the observation that altering GABAergic spontaneous release by itself was capable of triggering scaling, we sought a separate method for altering GABAergic release. Previous work had suggested that mGluR2 agonists (e.g., DCG-IV) reduced spontaneous GABAergic release in different systems including the spinal cord (Poncer et al., 1995; Doi et al., 2002; Gitsch, 2006; Zhou et al., 2011). We therefore tested and found that acute DCG-IV application (5 μM) produced a decrease in GABA, but not AMPA, mPSC frequency in E8 motoneurons (Fig. 8A, Table 1). DCG-IV’s effect on GABA mPSC frequency was observed throughout the 4–6 h recording period. Acute DCG-IV application had no effect on GABA or AMPA mPSC amplitude (Fig. 8C, Table 1), although it did alter the AMPA and GABA mPSC decay constant (Table 1). Next, we tested whether DCG-IV had effects on evoked GABAergic PSCs. We stimulated a slip of the VL containing interneurons that project directly to motoneurons while recording the motoneuron population potential from the ventral root using suction electrode recordings. We isolated the GABAergic response by blocking glutamate receptors (CNQX and APV) and measuring the earliest, likely

<table>
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<tr>
<th>Condition</th>
<th>Amplitude AMPA</th>
<th>Frequency AMPA</th>
<th>Decay AMPA</th>
<th>n</th>
<th>Amplitude GABA</th>
<th>Frequency GABA</th>
<th>Decay GABA</th>
<th>n</th>
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<tr>
<td>Control E8–E10</td>
<td>8.1 ± 0.4</td>
<td>0.49 ± 0.11</td>
<td>4.3 ± 0.9</td>
<td>7</td>
<td>13.0 ± 0.6</td>
<td>0.56 ± 0.32</td>
<td>30.4 ± 10.9</td>
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<td>10.4 ± 0.6**</td>
<td>0.59 ± 0.15</td>
<td>3.9 ± 0.9</td>
<td>8</td>
<td>16.6 ± 1.3*</td>
<td>0.51 ± 0.11</td>
<td>25.5 ± 10.2</td>
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<td>Gabazine E8–E10</td>
<td>10.7 ± 1.0**</td>
<td>0.59 ± 0.17</td>
<td>4.1 ± 0.5</td>
<td>9</td>
<td>19.2 ± 2.1***</td>
<td>0.49 ± 0.11</td>
<td>26.6 ± 3.7</td>
<td>9</td>
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<tr>
<td>Gabazine DHβE E8–E10</td>
<td>10.5 ± 0.7**</td>
<td>0.44 ± 0.19</td>
<td>4.3 ± 1.1</td>
<td>12</td>
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<td>0.33 ± 0.11</td>
<td>3.9 ± 0.6</td>
<td>14</td>
<td>18.6 ± 0.7***</td>
<td>0.50 ± 0.15</td>
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<td>1.11 ± 0.37</td>
<td>4.4 ± 1.1</td>
<td>8</td>
<td>17.3 ± 2.6*</td>
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<td>0.58 ± 0.12</td>
<td>4.2 ± 1.4</td>
<td>9</td>
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<td>16.1 ± 1.7</td>
<td>0.17 ± 0.05*</td>
<td>26.1 ± 1.1*</td>
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<td>0.39 ± 0.07</td>
<td>4.1 ± 0.2</td>
<td>7</td>
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<td>DCG E8–E10</td>
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<td>0.42 ± 0.16</td>
<td>4.5 ± 0.3</td>
<td>16</td>
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Amplitude, frequency, decay constant of AMPA, and GABA mPSCs are shown for different conditions. The number of cells for each condition are also shown, along with significant differences (p < 0.05; **p < 0.01; ***p < 0.001). Values are clustered into four groups for statistical comparisons. The first group includes embryos treated with DHβE, gabazine, DHβE/gabazine, nicotine/gabazine, lidocaine, lidocaine/nicotine, or control from E8–E10 (GABA mPSC amplitude: ANOVA with post hoc Tukey test for multiple comparisons; GABA mPSC frequency and AMPA mPSC amplitude and frequency: Kruskal–Wallis nonparametric test with Conover–Iman procedure for multiple comparisons). The second group includes embryos treated with nicotine for 12 h or control (Student’s t test was used for all parameters except AMPA mPSC decay: Kruskal–Wallis nonparametric test). The third group includes embryos acutely treated with nicotine, DCG-IV, or controls at E8 (AMPA mPSC amplitude: ANOVA with post hoc Tukey test for multiple comparisons; all other parameters: Kruskal–Wallis nonparametric test with Conover–Iman procedure for multiple comparisons). The fourth group includes embryos treated with nicotine, DCG-IV, or controls from E8–E10 (all Kruskal–Wallis nonparametric test with Conover–Iman procedure for multiple comparisons).
The evoked GABAergic response was either unaffected or slightly reduced by DCG application (5 μM), but appeared to recover quickly. To determine whether DCG-IV affected SNA-driven movements, we monitored embryonic movements after E8 in ovo injection of DCG-IV (2 μM). We found that embryonic movements were initially reduced, but fully recovered 2 h after drug injection (Fig. 8E). We reasoned that, if DCG-IV reduced spontaneous GABA release and reduced GABAR activation due to spontaneous release was key to triggering upscaling, then chronic in ovo treatment with DCG-IV should trigger upscaling. Indeed, chronic DCG-IV (2 μM) triggered increased AMPAergic and GABAergic mPSC amplitudes (Fig. 9, Table 1). Further, amplitude distributions demonstrated the characteristic scaling profile (Fig. 9E,F). Together, the results show that manipulation of GABAR activation by altering spontaneous GABA release using two different techniques triggers scaling in the living embryonic spinal cord.

**Discussion**

Bidirectional synaptic scaling triggered by altering spontaneous GABA release

SNA is a ubiquitous feature of nascent circuits and, in the developing spinal cord, is important in muscle/joint development (Ruano-Gil et al., 1978; Roufa and Martonosi, 1981) and axonal pathfinding (Hanson and Landmesser, 2004). We have determined that infusing a voltage-gated Na+ channel blocker (lidocaine) into the living embryo for 2 d blocked SNA-generated embryonic movements and produced a scaling up of both AMPA and GABAergic mPSC amplitudes (Gonzalez-Islas and Wenner, 2006). Scaling is thought to be triggered by reductions in baseline spiking as a compensatory mechanism to reestablish spike rate. Although embryonic movements did not actually recover in the presence of lidocaine, we did observe a homeostatic recovery after blockade of either of the main excitatory neurotransmitter receptors. *In ovo* blockade
of GABA<sub>R</sub> or glutamatergic receptors at E8 transiently reduced embryonic movements, which then recovered to normal levels 12 h after drug injection, even though antagonists remained effective throughout this period (Wilhelm and Wenner, 2008). Because both GABA<sub>R</sub> and glutamate receptor antagonists reduced embryonic movements similarly, we might have expected that either perturbation would trigger upscaling, but only GABA<sub>R</sub> blockade induced scaling. This result was consistent with the idea that reduced activation of GABA<sub>R</sub> triggered scaling rather than SNA-dependent spiking.

In the current study, we provide even stronger support for the idea that scaling was triggered by alterations in GABA<sub>R</sub> activation. We did this by modulating presynaptic GABA release and therefore GABA<sub>R</sub> activation through nAChR modulators (Gonzalez-Islas et al., 2016) or an mGluR2 agonist. Nicotine increased and DhβE or DCG-IV decreased baseline GABA vesicle release. Here, we show for the first time that increasing GABA<sub>A</sub> receptor activation chronically in ovo with nicotine triggered downscaling and, for GABAergic mPSCs, this was mediated by reductions in Cl<sub>in</sub>. Further, chronically decreasing GABA release triggered upscaling of AMPA and GABA<sub>A</sub> mPSC amplitudes. GABAergic upscaling was mediated by an increase in Cl<sub>in</sub>, the same mechanism mediating scaling after GABAergic blockade (Lindsly et al., 2014). In addition, we showed that the ability of the nicotinic modulators to trigger bidirectional scaling was dependent on GABA<sub>A</sub> receptor activation (Fig. 6). The results show that scaling can be triggered in vivo through a physiologically relevant alteration of GABA<sub>A</sub>R activation.

Because GABA<sub>A</sub>R activation is greatest during bouts of SNA, we had assumed that scaling was triggered by alterations in GABAergic signaling associated with bursts of SNA. However, in the interburst interval, there is also spike-dependent and spike-independent GABA release that activates GABA<sub>A</sub>Rs. Strikingly, our findings strongly argue that scaling was triggered by GABA<sub>A</sub>R activation due to AP-independent spontaneous GABA release. First, downscaling was triggered after
chronically increasing spontaneous GABA release with nicotine (Figs. 3, 4). Second, nicotine was also capable of converting upscaling produced by chronic lidocaine-treatment to downscaling (lidocaine + nicotine) through a spike-independent mechanism (Fig. 7). Third, scaling triggered by nicotinic modulators was dependent on GABAAR activation (Fig. 6). Fourth, we were able to trigger upscaling with DCG-IV, which reduced spontaneous GABA release but had only minimal effects on embryonic movements or evoked GABA release (Figs. 8, 9). Together, our findings provide a strong argument that alterations in spontaneous release trigger scaling and extend similar findings in vitro to an in vivo system (see below). This result suggests that a function of spontaneous release is for the homeostatic regulation of synaptic strength.

SNA does not appear to be controlled homeostatically by scaling

Although our initial studies suggested the possibility that scaling was triggered as a means to homeostatically maintain SNA, we now think that this is unlikely. First, the actual homeostatic recovery of embryonic movements after GABAAR blockade occurs several hours before the expression of upscaling (Wilhelm and Wenner, 2008; Lindsly et al., 2014). Second, there are multiple examples in which upscaling or downscaling was expressed in embryonic motoneurons, but SNA frequency in the spinal preparation was unchanged (Fig. 1B; Lindsly et al., 2014). Similar results have been described in the embryonic zebrafish spinal cord (Knogler et al., 2010). Finally, an identical nicotine-induced downscaling was observed whether SNA-driven movements were increased (nicotine) or virtually eliminated (nicotine/lidocaine). This finding suggested that spontaneous GABAergic neurotransmission was more important in triggering scaling than GABAergic transmission driven by bursts of spiking during SNA. Therefore, the signaling cascades that mediate scaling must be sensitive to smaller GABAergic quantal currents associated with spontaneous release or single APs rather than to the strong GABAergic depo-
larizations that lead to bursts of spiking and produce large calcium transients during SNA.

**Triggering synaptic scaling**

Understanding the triggers for synaptic scaling is important for elucidating the function of this form of plasticity. Although it is clear that altering GABAAR activation triggers scaling in the embryonic spinal cord, this is the first demonstration that changes in AP-independent release trigger scaling in vivo. However, reductions in glutamate receptor activation due to spontaneous release have been shown to trigger AMPAergic upward scaling in cultured networks (Sutton et al., 2006; Aoto et al., 2008; Wang et al., 2011; Fong et al., 2015; Kavalali, 2015). It has been shown recently that upscaling can be triggered by reduced mEPSC-dependent receptor activation, which can lead to calcium entry that is amplified through calcium-induced calcium release (Reese and Kavalali, 2015). Such signaling may be triggered through GABA ARs early in development, when GABA is depolarizing; glutamatergic signaling may assume this role later in development, when GABA becomes hyperpolarizing. Blockade of spontaneous neurotransmission has also been shown to signal compensatory changes in motoneuron excitability and quantal content (Frank et al., 2006; Bichler et al., 2007).

Figure 6. Synaptic scaling induced by nicotine receptor modulation is dependent on GABAAR activation. A, Representative single-cell average of AMPA and GABA mPSCs after different chronic treatments. B, C, Bar chart showing average values of AMPAergic (B) and GABAergic (C) mEPSC amplitude for motoneurons from control embryos and embryos treated with gabazine (10 μM), gabazine + DHβE (5 μM), or gabazine + nicotine (10 μM). Error bars represent SE and show a significant difference from controls (*p < 0.05, **p < 0.01, ***p < 0.001). D, Cumulative probability plot for AMPAergic mEPSC amplitudes (control embryos, n = 7; gabazine-treated embryos, n = 7; gabazine/DHβE-treated embryos, n = 10; gabazine/nicotine-treated embryos, n = 7). E, Cumulative probability plot for GABAergic mEPSC amplitudes (control embryos, n = 7; gabazine-treated embryos, n = 8; gabazine/DHβE-treated embryos, n = 9; gabazine/nicotine-treated embryos, n = 7). Controls are the same as in Figure 2.
In embryonic motoneurons, GABA_AR activation due to spontaneous release or spike-induced release of a single vesicle is likely to produce a similar response postsynaptically and activate similar signaling cascades. Therefore, it is possible that scaling could be triggered by altering the frequency of individual spikes in the interval between bursts of SNA. Consistent with this idea, 2 d of blockade of spiking with lidocaine treatment did trigger upscaling in embryonic motoneurons (Gonzalez-Islas and Wenner, 2006); however, lidocaine may reduce spontaneous release by reducing AP-dependent acetylcholine release. Although altering spontaneous release rate can trigger scaling, it is also clear that reducing spike rate in specific presynaptic inputs to a neuron in *in vitro* and *in vivo* can trigger compensatory synaptic strengthening selectively at those inhibited inputs (Webb and Cope, 1992; Hou et al., 2008; Béique et al., 2011; Deeg and Aizenman, 2011). These synapse specific compensations appear to be due to changes in postsynaptic receptor activation rather than postsynaptic spiking, which remains unperturbed in most of these studies. Therefore, local synaptic compensations due to altered presynaptic spiking and cell-wide synaptic scaling triggered by changing spontaneous release at all synaptic inputs may represent similar plasticity mechanisms that only differ by the proportion of synaptic inputs perturbed. Consistent with this idea, blocking glutamate receptor activation at all of a cultured neuron’s inputs triggers upward scaling (Sutton et al., 2006; Chen et al., 2014; Fong et al., 2015; Reese and Kavalali, 2015). Our findings are consistent with a neurobiological model in which increases in neurotransmitter release (i.e., upscaling) can lead to increases in synaptic strength, while decreases in neurotransmitter release (i.e., downscaling) can lead to decreases in synaptic strength. This model is supported by the finding that blocking neurotransmission-mediated scaling appears to be independent of changes in neurotransmitter release, whereas spike-dependent scaling is sensitive to changes in neurotransmitter release. Therefore, neurotransmission-mediated scaling appears to be dependent on retinoic acid synthesis and FMRP, whereas spike-dependent scaling is not (Soden and Chen, 2010). Further, neurotransmission-mediated scaling appears to be sensitive to additional changes in synaptic strength. For example, downsampling triggered by increasing GABAergic transmission was no different whether spiking was increased or abolished (Figs. 3, 7). Further, in cultured cortical neurons, we demonstrated that
scaling produced after 24 h of AMPAR blockade was no different whether spiking was reduced or normal (Fong et al., 2015).

It is also possible that reduced neurotransmitter activation is downstream of reductions in spike rate. This could explain why altering spiking has no effect when neurotransmission is perturbed. Reducing the frequency of isolated spikes could trigger upscaling by reducing AP-dependent transmitter release and thus receptor activation. Alternatively, recent work suggests a second possibility in which chronic spike blockade

Figure 8. DCG-IV reduced GABA mPSC frequency, but only transiently influenced evoked GABAergic responses or in ovo embryonic movements. A. AMPA and GABA mPSCs observed in a single motoneuron before (control) and 30 min after adding DCG-IV (5 μM) to the bath. B. Acute application of DCG-IV (5 μM) decreased GABA, but not AMPA, mPSC frequency in the E8 spinal preparation. Error bars represent SE and show a significant difference from controls (*p < 0.05). C. Acute application of DCG-IV had no effect on GABA or AMPA mPSC amplitude in the E8 spinal preparation. D. Acute application of DCG-IV (5 μM) had no influence or transiently reduced VLF-evoked GABAergic potentials observed in ventral root recordings. E. In ovo injection of DCG-IV (2 μM) at E8 transiently reduced embryonic movements, which were then recovered 2 h after DCG-IV injection. Error bars represent SE and show a significant difference from controls (***p < 0.01).
triggers changes in DNA methylation status; these epigenetic changes have been shown to alter the frequency of spontaneous release and thereby could trigger scaling (Nelson et al., 2008; Qiu et al., 2012; Meadows et al., 2015; Yu et al., 2015; Sweatt, 2016). In this way, spike-dependent scaling is achieved through the recruitment of transmission-mediated plasticity.

**Importance of nicotinic regulation of GABA$_A$Rs in embryonic development**

Perturbing baseline tonic nAChR activation alters GABA$_A$R activation and triggers synaptic scaling. SNA and nicotinic regulation of neurotransmitter receptor activation are widespread throughout the developing nervous system (Hellström-Lindahl et al., 1998; O’Donovan, 1999; Blanken-
ship and Feller, 2010). Therefore, nicotinic regulation of GABA release could have profound implications for fetal exposure to drugs influencing GABAAR activation, such as benzodiazepines or nicotine.

References


Wilhelm JC (2008) The GABAA receptor is a critical part of the sensing machinery that triggers homeostatic plasticity of synapti strength and intrinsic excitability.

