Intrinsic and synaptic homeostatic plasticity in motoneurons from mice with glycine receptor mutations

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1School of Biomedical Sciences and Pharmacy, Faculty of Health and Hunter Medical Research Institute, University of Newcastle, Callaghan, Australia; 2Neuroscience Research Australia and School of Medical Sciences, University of New South Wales, Randwick, Australia; and 3Department of Physiology, College of Medicine, University of Arizona, Tucson, Arizona

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Tadros MA, Farrell KE, Schofield PR, Brichta AM, Graham BA, Fuglevand AJ, Callister RJ. Intrinsic and synaptic homeostatic plasticity in motoneurons from mice with glycine receptor mutations. J Neurophysiol 111: 1487–1498, 2014. First published January 8, 2014; doi:10.1152/jn.00728.2013.—Inhibitory synaptic inputs to hypoglossal motoneurons (HMs) are important for modulating excitability in brainstem circuits. Here we ask whether reduced inhibition, as occurs in three murine mutants with distinct naturally occurring mutations in the glycine receptor (GlyR), leads to intrinsic and/or synaptic homeostatic plasticity. Whole cell recordings were obtained from HMs in transverse brainstem slices from wild-type (wt), spastic (spa), and oscillator (ot) mice (C57Bl/6, approximately postnatal day 21). Passive and action potential (AP) properties in spa and ot HMs were similar to wt. In contrast, spa HMs had lower input resistances, more depolarized resting membrane potentials, higher rheobase currents, smaller AP amplitudes, and slower afterhyperpolarization current decay times. The excitability of HMs, assessed by “gain” in injected current/firing-frequency plots, was similar in all strains whereas the incidence of rebound spiking was increased in spa. The difference between recruitment and derecruitment current (i.e., ΔI) for AP discharge during ramp current injection was more negative in spa and ot. GABA_α miniature inhibitory post synaptic current (mIPSC) amplitude was increased in spa and ot but not spa, suggesting diminished glycinergic drive leads to compensatory adjustments in the other major fast inhibitory synaptic transmitter system in these mutants. Overall, our data suggest long-term reduction in glycinergic drive to HMs results in changes in intrinsic and synaptic properties of MNs (i.e., long-term impairment in glycinergic transmission can trigger “homeostatic plasticity”) and whether this differs between the three mutants. We have examined this issue by studying a combination of intrinsic and inhibitory synaptic properties in hypoglossal motoneurons (HMs) from brainstem slices. We use HMs as our test neuron for several reasons. HMs innervate muscles of the tongue, and their excitability plays a critical role in the modulation of the startle response. The murine mutants spasmodic (spd), spastic (spa), and oscillator (ot) have naturally occurring GlyR defects that result in long-term reductions to glycinergic drive. These mutants have contributed much to our understanding of the pentameric GlyR in cell lines, at native synapses, and at the in vivo or systems level of analysis (Graham et al. 2006, 2007b; Rajendra and Schofield 1995). All three mutants exhibit an exaggerated “startle response” when disturbed by loud noises or unexpected sensory stimuli (Simon 1995). In spa, a single point mutation in the α1-subunit of the GlyR results in reduced agonist sensitivity and single channel open time (Graham et al. 2011; Plested et al. 2007; Ryan et al. 1994). The spa mutation is caused by an intrinsic insertion of a LINE 1 transposable element in the β-subunit gene, which causes exon-skipping and decreased transcriptional efficiency of β-subunit protein (Kingsmore et al. 1994; Mülhardt et al. 1994). This results in markedly reduced GlyR expression assessed in spinal cord homogenates; however, channel properties are unaffected (Graham et al. 2003, 2006). The ot mouse has a microdeletion in exon 8 of the α1-subunit, which results in almost complete absence of α1-protein and functional GlyRs in the spinal cord and brainstem (Buckwalter et al. 1994; Kling et al. 1997). Thus the ot mutation is considered a null mutation for the adult (i.e., α1/β) form of the GlyR. Within the first 2–3 wk after birth, the three mutants exhibit a similar and easily recognized “startle” phenotype, which suggests impaired control of MN excitability (Biscoe and Duchen 1986; Graham et al. 2006; Simon 1997). In spite of these defects in inhibitory synaptic transmission two of the mutants, spd and spa, survive and breed successfully. The ot mutant, however, dies ~3 wk after birth. Thus these three mouse lines provide an opportunity to ask if long-term impairment in glycinergic transmission can trigger changes in the intrinsic and synaptic properties of MNs (i.e., homeostatic plasticity) and whether this differs between the mutants. We have examined this issue by studying a combination of intrinsic and inhibitory synaptic properties in hypoglossal motoneurons (HMs) from brainstem slices. We use HMs as our test neuron for several reasons. HMs innervate muscles of the tongue, and their excitability plays a critical role...
in behaviors such as chewing, swallowing, suckling, vocalization, and respiration (Berger et al. 1995; Lowe 1980). In addition, their output is strongly modulated by glycinergic inhibitory synaptic transmission (Cifra et al. 2009; Singer et al. 1998; Umemiya and Berger 1995) and HMs appear to be one of the few MN populations that can be studied in both juvenile and adult animals (Callister et al. 1999; Graham et al. 2006).

MATERIALS AND METHODS

Animals

Experiments were undertaken on wild-type (wt), spasmodic (spd), spastic (spa), or oscillator (ot) mice (both sexes) backcrossed onto the C57Bl/6 background. The University of Newcastle Animal Care and Ethics Committee approved all procedures. The mutant lines were originally obtained from the Jackson Laboratory (Bar Harbor, ME). Most mutant animals and all wt controls were bred at the University of Newcastle. Some additional spd animals were obtained from an identical line bred at Australian BioResources (Moss Vale, NSW, Australia). Spd, spa, and ot mice were bred by mating heterozygous (spd+/-, spa+, and ot+/+) animals, resulting in 25% of the progeny being homozygous. Animals were maintained on a 12:12-h light-dark cycle and given unlimited access to food and water. Homozygous-affected animals exhibited the exaggerated “startle” response and were easily identified ~2 wk after birth according to four criteria: 1) limb clenching when picked up by the tail, 2) an impaired righting reflex, 3) a constant tremor at rest, and 4) walking on tiptoe with an arched back (Graham et al. 2006; Simon 1997).

Tissue Preparation

Mice were anesthetized with ketamine (100 mg/kg ip) and decapitated. The brainstem was rapidly removed and immersed in ice-cold sucrose-substituted artificial cerebrospinal fluid (sACSF) containing the following (in mM): 250 sucrose, 25 NaHCO3, 10 glucose, 2.5 KCl, 1 NaH2PO4, 1 MgCl2, and 2.5 CaCl2, continuously bubbled with 95% O2-5% CO2. The brainstem was placed on a Styrofoam support block and glued rostral side down onto a cutting stage with cyanoacrylate glue (Locite 454; Locite, Caringbah, Australia). The cutting stage was placed into a cutting chamber containing ice-cold, oxygenated sACSF. Transverse slices (300–400 µm thick) were obtained from the region of the brainstem containing the hypoglossal nucleus (~0.5 mm above and below the obex) using a vibrating-blade microtome (Leica VT1200s; Leica Microsystems, Wetzlar, Germany). The three to four slices containing the hypoglossal nucleus were transferred to a humidified storage chamber containing oxygenated ACSF (118 mM NaCl substituted for sucrose in sACSF). Slices were allowed to recover for 1 h at room temperature (22–24°C) before recording commenced.

Electrophysiology

Brainstem slices were transferred to a recording chamber and held in place using nylon netting fixed to a U-shaped platinum frame. The recording chamber was continually superfused with oxygenated ACSF (4–6 bath volumes/min) and maintained at a constant temperature (23°C) using an in-line temperature-control device (TC-324B; Warner Instruments, Hamden, CT). Whole cell patch-clamp recordings were obtained from HMs, visualized using infrared differential contrast optics (IR-DIC optics) and an IR-sensitive camera (Rolera-XR; Olympus). HMs were easily identified according to their large soma size (diameter: 20–35 vs. 10–15 µm for local interneurons), high capacitance (>35 pF), and low input resistance (70–80 MΩ) (Graham et al. 2006).

Intrinsic properties of HMs. Patch pipettes (3- to 4-MΩ) resistance were prepared from thin-walled borosilicate glass (PG150T-15; Harvard Apparatus, Kent, UK) and filled with a potassium-based internal containing the following (in mM): 135 KCl, 2 MgCl2, 10 HEPES, 0.1 EGTA, 2 MgATP, and 0.3 NaGTP pH 7.3 (with KOH). Whole cell patch-clamp recordings were made using a Multiclamp 700B Amplifier (Molecular Devices, Sunnyvale, CA). The whole cell recording configuration was first established in voltage-clamp mode (holding potential: ~60 mV). Series resistance was measured from the averaged response (5 trials) to a 5-mV hyperpolarizing pulse. This was measured at the beginning and end of each recording session, and data were rejected if values changed by >20%. Input resistance was obtained by calculating the chord conductance across a minimum of four responses to incrementally increasing hyperpolarizing current injections (50-pA increments in current-clamp recording mode).

Once the whole cell recording mode was established, several stimulus protocols were applied to each HM to study intrinsic properties. First, in voltage clamp, we assessed the characteristics of the AP afterhyperpolarization (AHP) current. This was recorded at ~60 mV following the delivery of a 2-ms pulse to ~10 mV. This pulse was delivered every 4 s (10 times), and the responses were averaged for analysis. We then switched the recording mode to current clamp, and the membrane potential recorded ~15 s after this switch was taken as the resting membrane potential (RMP) of the neuron. All membrane potential values have been corrected for a calculated 10-mV liquid junction potential (Barry and Lynch 1991). To record single APs a series of short-duration depolarizing steps (20-pA increments, 2-ms duration) were applied from RMP. Several measurements were then made on these APs (see Table 2). Small bias currents (~50 pA) were then injected into the recorded HM to maintain membrane potential at ~70 mV. AP discharge was examined from this potential by applying: 1) a series of long depolarizing and hyperpolarizing current steps (50-pA increments, 1-s duration); and 2) triangular ramp currents (peak amplitude of 1 nA, 3.5-s rise and fall). Rheobase current was taken as the minimum current needed to evoke an AP during a 1-s period of depolarizing current injection.

Properties of GABAergic quantal currents (miniature inhibitory postsynaptic currents) in HMs. We have shown previously in spinal cord dorsal horn neurons that reduced glycinergic drive in spa is accompanied by compensatory upregulation in GABAergic neurotransmission (Graham et al. 2003). Therefore, in a separate series of experiments we recorded GABA_A-mediated miniature inhibitory post synaptic currents (mIPSCs) from HMs in each genotype. All experiments used patch pipettes filled with a caesium chloride-based internal solution containing the following (in mM): 140 CsCl, 10 HEPES, 0.1 EGTA, 2 ATP, and 0.3 GTP (pH adjusted to 7.3 with 1 M CsOH, 21–23°C). Holding potential was set at ~70 mV, and GABA_Aergic mIPSCs were pharmacologically isolated by application of 6-cyano-7-nitroquinazoline-2,3-dione (CNQX; 10 µM), strychnine (1 µM), and tetrodotoxin (TTX; 1 µM) to the bath solution. Drugs were applied to the slice for a minimum of 3 min before data capture commenced. GABA_Aergic currents were recorded for 4–6 min. Application of bicuculline (10 µM) blocked all synaptic activity, indicating that the mIPSCs recorded were mediated via activation of GABA_A receptors (n = 3 for each genotype).

Data Capture and Analysis

Intrinsic properties of HMs. Data were digitized online (sampled at 20 kHz, filtered at 6 kHz) via an ITC-16 computer interface (Instrutech, Long Island, NY) and stored on a Macintosh computer using Axograph X software (Molecular Devices, Sunnyvale, CA). All data were analyzed offline using the Axograph software. The AHP current recorded in voltage clamp was averaged (10 trials), and the amplitude and latency of the maximum outward current after the large inward
current (or action current) were measured (Callister et al. 1997). An exponential was fit to the decay phase of the response to calculate the AHP current decay time constant.

Onsets of APs were identified using the derivative-threshold method to detect the inflection point, with dV/dt set at 20 mV/ms. AP properties were measured for rheobase APs, generated by short-duration depolarizing steps (20-pA increments, 2-ms duration). The rheobase current was taken as the minimum current step that would evoke one AP. The difference between AP threshold and the maximum positive peak was taken as AP amplitude. AHP current amplitude was measured as the difference between AP threshold and the maximum negative peak. Finally, AP half-width was measured at 50% of the AP maximum positive peak.

The derivative threshold method was also used to detect APs evoked during injection of long-duration depolarizing steps and slow triangular current ramps. We calculated the instantaneous frequency as the reciprocal of the interspike interval. Mean AP frequency was taken as the average of all instantaneous frequencies for APs evoked by a single current step. Frequency/current plots (F/I plots) were generated using mean frequency (per current step) and the corresponding current amplitude.

For the APs discharged during triangular current injection trials, instantaneous AP frequency was calculated as above and the currents associated with onset (recruitment current) and cessation of spiking (derecruitment current) were measured. The difference between these two values (i.e., derecruitment current minus recruitment current), referred to as ΔI (Bennett et al. 2001b) was calculated. Positive ΔI values occur when neurons stop firing at injected current values larger than that at which they were recruited and are consistent with a dominant role of active conductances underlying firing rate adaptation. Negative ΔI values, on the other hand, occur when the injected current associated with derecruitment is less than the amount needed for recruitment. This likely indicates activation of persistent inward currents (Bennett et al. 2001b, 2006; Pambo-Pambo et al. 2009; Turkin et al. 2010).

**Properties of GABAergic mIPSCs in HMs.** GABAergic mIPSCs were filtered at 2 kHz, recorded onto videotape (A. R. Vetter, Rebersburg, PA), and either simultaneously or subsequently digitized (sampled at 10 kHz) using WCP software (kindly provided by J. Dempster, Strathclyde Electrophysiology Software, Glasgow, UK). This software enabled the detection and analysis of mIPSC properties for each recorded cell. The detection threshold for synaptic events was set just above background noise levels (~5 pA). A plot of mIPSC amplitude vs. record number was constructed to ensure there were no changes (i.e., rundown) in recording conditions during each experiment. If an obvious trend in mIPSC amplitude was detected, data were rejected. Mean values for mIPSC rise time (calculated over 10–90% of peak amplitude), peak amplitude, and decay time constant (calculated over 20–80% of the decay phase) were obtained for each cell using automated procedures within the WCP program. Mean mIPSC frequency was obtained by dividing the number of captured events by the recording time in seconds.

**Statistics**

All analysis was undertaken using SPSS software (SPSS v.10, SPSS, Chicago, IL). ANOVA was used to compare variables across genotypes. Scheffé’s post hoc tests were used to determine which genotypes differed. Data that failed Levene’s test for homogeneity of variance were compared using the nonparametric Kruskal-Wallis test, followed by Tamhane’s T2 post hoc test. G-tests, with Williams’ correction, were used to determine if the incidence of rebound spiking differed between genotypes. Statistical significance was set at P < 0.05, and all data are presented as means ± SE.

**Drugs**

TTX was obtained from Alomone Laboratories (Jerusalem, Israel). CNQX and strychnine were purchased from Sigma (St. Louis, MO).

**RESULTS**

Figure 1 and Table 1 summarize the effect of each GlyR mutation on glycineric mIPSCs in HMs. The data for homozygote mutant animals have been reported previously (Graham et al. 2006). Here we compare these data from the different mutant genotypes to wt HMs, rather than between hetero- and homozygotes, as in our previous article. Each mutation dramatically reduced glycineric mIPSC amplitude compared with wt values in the order spd > spa > ot (Fig. 1D).
Table 1. Properties of glycnergic mIPSCs in wild-type and GlyR-mutant HMs

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Rise Time, ms</th>
<th>Amplitude, pA</th>
<th>Decay Time, ms</th>
<th>Frequency, Hz</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt (n = 23)</td>
<td>0.9 ± 0.1</td>
<td>71.7 ± 3.5</td>
<td>4.9 ± 0.2</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>spd (n = 20)</td>
<td>0.7 ± 0.1*</td>
<td>290 ± 3.0*</td>
<td>2.7 ± 0.2*</td>
<td>1.0 ± 0.3*</td>
</tr>
<tr>
<td>spa (n = 19)</td>
<td>1.1 ± 0.1</td>
<td>210 ± 2.1*</td>
<td>4.6 ± 0.3</td>
<td>0.5 ± 0.1*</td>
</tr>
<tr>
<td>ot (n = 15)</td>
<td>1.7 ± 0.1*</td>
<td>194 ± 1.8*</td>
<td>12.4 ± 1.2*</td>
<td>0.2 ± 0.1*</td>
</tr>
</tbody>
</table>

Values are means ± SE. Data are from Graham et al. 2006. mIPSCs, miniature inhibitory postsynaptic currents; GlyR, glycine receptor; HMs, hypoglossal motoneurons; wt, wild type; spd, spasmodic; spa, spastic; ot, oscillator. *Differs from wt.

Table 2. Intrinsic properties of wild type and GlyR mutant HMs

<table>
<thead>
<tr>
<th>Animal age, days</th>
<th>Number of cells</th>
<th>Input resistance, MΩ</th>
<th>RMP, mV</th>
<th>AP threshold, mV</th>
<th>AP amplitude, mV</th>
<th>AP half-width, ms</th>
<th>AHP amplitude, mV</th>
<th>AHP decay time, ms</th>
<th>FI ratio</th>
<th>FI minimum rate, Hz</th>
<th>FI gain, Hz/pA</th>
<th>Sag ratio</th>
<th>Rebound spiking, %</th>
<th>Δτ, pA</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt (n = 16)</td>
<td>64</td>
<td>81.3 ± 4.4</td>
<td>-74.0 ± 0.7</td>
<td>-55.6 ± 0.5</td>
<td>76.5 ± 1.0</td>
<td>0.63 ± 0.02</td>
<td>-27.0 ± 0.6</td>
<td>39.0 ± 1.1</td>
<td>182 ± 15</td>
<td>3.9 ± 0.4</td>
<td>0.11 ± 0.01</td>
<td>1.77 ± 0.04</td>
<td>54.7</td>
<td>+273 ± 10.0</td>
</tr>
<tr>
<td>spd (n = 17)</td>
<td>48</td>
<td>75.6 ± 5.1</td>
<td>-74.0 ± 1.0</td>
<td>-55.3 ± 0.7</td>
<td>72.8 ± 1.3</td>
<td>0.67 ± 0.02</td>
<td>-27.9 ± 0.9</td>
<td>40.7 ± 1.8</td>
<td>176 ± 15</td>
<td>4.1 ± 0.4</td>
<td>0.08 ± 0.01</td>
<td>1.75 ± 0.04</td>
<td>75.5* (vs. wt)</td>
<td>+144 ± 9.1</td>
</tr>
<tr>
<td>spa (n = 12)</td>
<td>61</td>
<td>68.4 ± 3.9* (vs. wt)</td>
<td>-71.0 ± 1.1* (vs. ot)</td>
<td>-51.9 ± 0.7* (vs. wt, spd)</td>
<td>68.3 ± 1.5* (vs. wt, ot)</td>
<td>0.70 ± 0.03</td>
<td>-26.5 ± 0.9</td>
<td>47.5 ± 2.5* (vs. wt)</td>
<td>279 ± 25* (vs. wt, spd)</td>
<td>3.8 ± 0.4</td>
<td>0.09 ± 0.01</td>
<td>1.77 ± 0.05</td>
<td>-4.7 ± 8.4* (vs. wt)</td>
<td></td>
</tr>
<tr>
<td>ot (n = 12)</td>
<td>57</td>
<td>75.4 ± 4.1</td>
<td>-74.5 ± 0.8</td>
<td>-52.9 ± 0.6* (vs. wt)</td>
<td>73.8 ± 1.2</td>
<td>0.65 ± 0.02</td>
<td>-29.5 ± 0.8</td>
<td>37.5 ± 1.4</td>
<td>245 ± 23</td>
<td>3.8 ± 0.4</td>
<td>0.09 ± 0.01</td>
<td>1.70 ± 0.04</td>
<td>48.3</td>
<td></td>
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Values are means ± SE. RMP, resting membrane potential; AP, action potential; AHP, afterhyperpolarization; FI, frequency/current. *Different from data for the genotype indicated in parentheses. AP properties (rows 5–9) are based on APs generated in response to 2-ms step.

Membrane and Action Potential Properties

Results for membrane and action potential properties of HMs across genotypes are shown in Table 2. Input resistance (RIN) and RMP were similar in wt, spd, and ot HMs. In contrast, spa HMs had lower input resistance and a more depolarized RMP compared with both wt data and the other mutants. Furthermore, AP threshold was more depolarized in spa and ot HMs compared with wt. As might be expected, based on their lower input resistance and higher AP threshold, rheobase current was higher in spa HMs. In addition, spa HMs had smaller AP amplitudes compared with wt HMs. Collectively, these results indicate spa HMs exhibit a reduced intrinsic excitability.

Properties of Action Potential AHP

We recorded the outward AHP current in HMs by injecting a large depolarizing pulse (to −10 mV from a holding potential of −60 mV, 2-ms duration) and compared the properties of the AHP current across genotypes (Fig. 2). No significant differences were observed in the amplitude of the AHP current (Fig. 2B); however, the decay time constant of the AHP current was longer in spa HMs compared with wt (Fig. 2C), suggesting that potassium conductances underlying the AHP current are prolonged in this mutant.

Responses to Depolarizing Current Injection

Injection of square current steps of increasing amplitude (50-pA increments, from a membrane potential of −70 mV, 1-s duration) resulted in tonic AP discharge in HMs from all four genotypes. Example responses to three levels of current injection in a wt HM are shown in Fig. 3A with plots of the associated instantaneous frequency during the current steps shown in Fig. 3B. As shown previously for spinal MNs in cats and Table 1). This was most notable in the spa and ot mutants. The effect of each mutation on GlyR kinetics varied: mIPSC decay time was unaltered in spa, decreased in spd, and increased in ot (Table 1). Together, these data show the three mutations dramatically effect GlyR-mediated inhibition but via different physiological mechanisms.

The presence of any GlyR-mediated mIPSCs in the ot animals is worthy of comment, as this mouse is considered a null mutation for the adult form of the GlyR (Buckwalter et al. 1994; Kling et al. 1997). In Graham et al. 2006, we suggested that there are only a few GlyR clusters (i.e., synapses) in the ot animals (Table 1). Together, these data show that there are only a few GlyR clusters (i.e., synapses) in the ot animals.

Table 1. Properties of glycnergic mIPSCs in wild-type and GlyR-mutant HMs

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and mature HM in rats (Kernell 1965; Sawczuk et al. 1995; Viana et al. 1995), firing frequency declined markedly during the current step and the magnitude of the decline was greater for larger current steps. Such spike-frequency adaptation was a feature of all HMs examined from all genotypes.

To characterize the steady-state F/I relationship for each neuron, mean frequency was measured for each level of injected current. To facilitate averaging among neurons within a genotype, current values were normalized to each rheobase current for that genotype. Such normalized F/I data indicate the change in discharge frequency with increased current (in pA) above rheobase. Figure 3C shows the averaged F/I relation for each genotype with each curve offset along the horizontal axis by the average value of the rheobase current for that genotype. From these F/I relationships, we compared three features that represent different aspects of intrinsic excitability across genotypes: 1) rheobase current indicating how readily a neuron can be brought to threshold for repetitive discharge, 2) discharge frequency at rheobase indicating the minimal frequency upon which discharge rate can be modulated, and 3) gain (average slope of F/I relation) indicating the efficacy by which increases in depolarizing current are transformed into augmented spike-frequency output. A fourth feature, namely maximal firing frequency, was not measured because we did not deliver the high levels of current required to reach depolarization blockade of APs, associated with maximal firing rates (e.g., Pilarski et al. 2011). Of the above features only rheobase current differed: specifically it was increased in spa (Table 2). Thus spa HMs appear less susceptible to being brought to spiking threshold; however, once activated HMs in all strains respond similarly to depolarizing current modulation.

Responses to Hyperpolarizing Current Injection

We next examined the response of HMs to hyperpolarizing current injection, as this is often examined in MNs to assess the properties of the hyperpolarization-activated mixed cationic inward current (Ih). This current is highly expressed in adult HMs (Bayliss et al. 1994) and spinal MNs (Takahashi 1990) and plays a role in regulating AP discharge by modulating the amplitude and time course of synaptic inputs (Reyes 2001). The response of HMs to hyperpolarization was assessed via injection of hyperpolarizing current steps (−50-pA increments, 1-s duration) until peak membrane hyperpolarization reached −110 mV. Depolarizing “sag” was used as an index of Ih magnitude and was quantified as the ratio of the peak hyperpolarized membrane potential at the outset of the hyperpolarizing current pulse to that at the termination of the pulse (Fig. 4A, solid and open arrowheads). Sag ratios did not differ across the four genotypes; however, more current was required to hyperpolarize spa HMs to −110 mV compared with wt (−664 ± 22 vs. 594 ± 15 pA), consistent with their lower input resistance (Table 2).

Upon removal of the hyperpolarizing pulses, all HMs exhibited some degree of rebound depolarization. This was of sufficient magnitude in some neurons to elicit spiking (Fig. 4A, left). The proportion of neurons exhibiting rebound spiking was greater in spd compared with wt animals (Fig. 4B). These data suggest there is a selective change in the currents activated by the release from hyperpolarization in spd HMs.

Responses to Triangular Current Ramps

We also measured the response to triangular ramp current injections (1-nA peak with 3.5-s rise and fall; holding potential of −70 mV), as this procedure has been used to investigate and test for engagement of active conductances in MNs (Bennett et al. 2001b; ElBasiouny et al. 2010; Theiss et al. 2007) and dorsal horn neurons (Reali et al. 2011). Figure 5A shows an example response in a wt HM to triangular current injection, and Fig. 5B shows the associated F/I plot for the rising and falling phases of the current injection. As outlined in MATERIALS AND METHODS, ΔI (current at cessation of spiking minus current at onset of spiking) was calculated for each neuron to provide an indirect assessment of the influence of two competing forms of active conductances: those related to spike-frequency adaptation (giving rise to positive ΔI values) and those associated with persistent inward currents (PICs; which yield negative ΔI values).

Figures 5, C–F, shows histograms of ΔI values obtained for each of the four strains. Mean ΔI values (Fig. 5G and Table 2) were all near zero pA although slightly positive for wt and modestly negative for ot. A Kruskal-Wallis test indicated a significant effect of genotype on ΔI, and post hoc testing indicated a significant difference in ΔI values between wt and both spa and ot. When normalized to rheobase current, the average ΔI represents, for example, ~0.08 × rheobase for ot mutants. This implies that PICs provided only ~8% of the
current needed to recruit and sustain in vitro activity in HMs in the ot mouse. This stands in contrast to the highly enhanced PICs (50% of rheobase) observed in sacral MNs of adult rats following chronic spinal cord injury (Bennett et al. 2001a). As such, there appears to be relatively modest adaptations in the active conductances underlying firing rate adaptation or PICs as a consequence of GlyR mutations and these are confined to the spa and ot mutants.

Properties of GABAergic mIPSCs

GABAergic mIPSC properties were investigated in a separate series of experiments because intrinsic properties and
inhibitory synaptic currents cannot be studied with the same internal pipette solution. mIPSC data were obtained from 58 HMs from 21 animals (wt = 7; spd = 5; spa = 5; and ot = 4). As in our experiments on intrinsic membrane properties every effort was made to age match animals; however, spd animals were slightly older than wt animals (27.3 ± 0.7 vs. 20.0 ± 0.2 days).

The properties of GABAergic mIPSCs in the four strains are summarized in Table 3 and Fig. 6. The rise and decay times were similar for mIPSCs in all strains (~2.0 and 15 ms, respectively; Table 3). Although mIPSC frequency varied considerably it did not differ among strains. Finally, mIPSC amplitude was unchanged in spd but significantly greater in the spa and ot mutants compared with wt (35.0 ± 1.9 and 38.5 ± 4.0 pA vs. 26.5 ± 1.3 pA, respectively). Together, these data suggest GABA_A receptor channel kinetics and release probability in GABAergic terminals on HMs are not altered in any of the mutants. In contrast, and consistent with our recordings from spinal dorsal horn neurons from spa, the number of synaptically located GABA_A receptors is increased in spa and ot HMs.

DISCUSSION

GlyR mutations in the spd, spa, and ot mice result in decreased levels of glycine-mediated inhibitory synaptic transmission and severe motor disturbances (Graham et al. 2006;

Table 3. Properties of GABAergic mIPSCs in wild-type and GlyR-mutant HMs

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Rise Time, ms</th>
<th>Amplitude, pA</th>
<th>Decay Time, ms</th>
<th>Frequency, Hz</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt (n = 22)</td>
<td>2.0 ± 0.1</td>
<td>26.5 ± 1.3</td>
<td>14.7 ± 1.0</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>spd (n = 12)</td>
<td>2.3 ± 0.1</td>
<td>21.1 ± 2.4</td>
<td>16.4 ± 1.9</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>spa (n = 13)</td>
<td>2.1 ± 0.2</td>
<td>35.0 ± 1.9</td>
<td>13.8 ± 0.8</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>ot (n = 11)</td>
<td>2.1 ± 0.1</td>
<td>38.5 ± 4.0*</td>
<td>16.3 ± 1.0</td>
<td>0.5 ± 0.1</td>
</tr>
</tbody>
</table>

Values are means ± SE. *Differs from wt.
Differences in Intrinsic Properties in GlyR Mutants

One of the major findings of our study was that the intrinsic properties of HMs differ in the three GlyR mutants. In spa, numerous intrinsic properties were altered whereas changes in spd and ot animals were more limited. Some explanation for

GABAergic mIPSC amplitude observed in spa and ot HMs suggests some sort of synaptic “compensation” has occurred in an attempt to maintain an appropriate level of inhibitory drive that contributes to the survival of spa animals but fails in the lethal ot mutant. Our major findings are summarized in Fig. 7 and suggest, barring the changes we observed in Δf, that the altered intrinsic and synaptic properties in the spa and ot mutants are consistent with some form of homeostatic plasticity in response to reduced glycinergic inhibition. In contrast, the increased rebound spiking observed in the spd mutant is not consistent with homeostatic plasticity.

Previous Work on Intrinsic Homeostatic Plasticity in GlyR Mutants

It is now well accepted that “homeostatic plasticity” prevents hypo- or hyperactivity in neural circuits and that such plasticity can occur via modification of the intrinsic properties of a neuron and/or its synaptic inputs (Turrigiano 1999). Biscoe and Duchen (1986) first examined the effect of naturally occurring GlyR mutations in the spa mutant. With the use of an ex vivo spinal cord preparation and sharp microelectrode recording, they showed RMP, RIN, AHP current, and response to current injection were unaltered in spa spinal MNs, even though they concluded spa spinal MNs were hyperexcitable based on enhanced responses to dorsal root stimulation. Our data, however, suggest most of the intrinsic properties of spa HMs (see Differences in Intrinsic Properties in GlyR Mutants) are altered in a manner that would make them less responsive to excitatory synaptic inputs (Fig. 7). The approaches employed in each study may explain these differences. We studied a different neuron population (HMs vs. spinal MNs), employed whole cell patch-clamp methods, and activated neurons by current injection vs. synaptic stimulation. We, and others, have shown that neurons can respond differently to current injection and synaptic stimulation (Graham et al. 2007a).

A recent study on the same three GlyR mutants found evidence for intrinsic homeostatic plasticity in medial vestibular nucleus (MVN) neurons (Camp et al. 2010). AHP amplitude was increased, and this was accompanied by reduced spontaneous firing frequencies and lower gain values (in response to current injection) in MVN neurons from all three mutants. These results differ to our study in three ways. First, the changes in intrinsic properties of HMs differed markedly across the three genotypes. Second, we found no evidence for altered gain in FI/C plots in HMs (Fig. 3C). Finally, we found the minimum current needed to elicit repetitive discharge (i.e., rheobase) shifted to higher values in spa mutants. A possible explanation for the differences in intrinsic plasticity observed in MVN neurons is that MVN neurons are spontaneously active, whereas HMs discharge in bursts (Berger 2000; Camp et al. 2006; Sekirnjak and du Lac 2002). As HMs are not spontaneously active (Berger 2000), AHP current properties may not be as important in determining neuronal excitability as in MVN neurons.

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Simon (1997). Because two of the mutants (spd and spa) survive to adulthood and reproduce, we asked whether the intrinsic and synaptic properties of HMs, which are involved in chewing, swallowing, suckling, vocalization, and respiration (Lowe 1980), exhibit homeostatic plasticity to maintain network stability in the face of reduced glycinergic drive. We find a number of intrinsic properties, which shape neuronal excitability, are altered in the three mutant strains. The changes were most notable in HMs from the spa mouse. The increased
the different forms of plasticity lies in the effect of each mutation on GlyRs and inhibitory conductances. For the lethal ot mutant, the explanation seems straightforward. The only change in intrinsic properties we observed was a slightly elevated threshold for AP generation and a more negative ΔI (Table 2 and Figs. 5 and 7); these would reduce and enhance HM excitability, respectively. Whatever the net effect of these changes, even when combined with increased GABAergic excitability, respectively. Whatever the net effect of these changes, the overall increased GABAergic input is accompanied by increased GABAergic input and changes in intrinsic properties that combine to reduce HM excitability. Together these changes are consistent with homeostatic adaptation. In ot, dramatically decreased GlyR-mediated input is accompanied by increased GABAergic input and minimal adaptation of intrinsic properties. This is consistent with homeostatic adaptation but is clearly insufficient to maintain appropriate HM output in this lethal mutation. RIN, input resistance; RMP, resting membrane potential.

In spd, the response to hyperpolarizing current injection was the only intrinsic property that differed from wt HMs (Fig. 4). Based on sag ratios (Table 2), Iₛ was similar in the four strains; however, rebound spiking at the offset of the hyperpolarization step was more prevalent in spd HMs (Fig. 4B). The increased appearance of rebound spiking in spd HMs could be caused by changes in several conductances, including low voltage-activated or T-type calcium current, decreased A current, or altered sodium current expression (Berger 2000). Regardless, they had no affect on RMP, input resistance or the gain of the F/I relationship (Fig. 3) in spd HMs. In conclusion, the increased incidence of rebound spiking we observed in spd HMs would increase excitability and is therefore not consistent with homeostatic plasticity.

In contrast to the ot and spd mutants, HMs in spa mice exhibit numerous changes in their intrinsic properties. These include lowered input resistance, increased rheobase current, more depolarized AP threshold, and slower AHP current decay time. Together, these changes would decrease the likelihood of AP discharge during periods of excitatory synaptic input and are consistent with homeostatic plasticity. The more profound intrinsic adaptations in the spa vs. spd mutant may be explained by several observations. First, the spa mutation results in a greater reduction in glycinergetic drive to HMs. This may activate homeostatic mechanisms to maintain HM output at appropriate levels. We have some evidence for such intrinsic plasticity in dorsal horn neurons. In spa neurons the A-type potassium current is increased (Graham et al. 2003, 2007b, 2011). Similarly, we interpreted this as a homeostatic adaptation that would reduce excitability and stabilize dorsal horn circuits in the face of reduced inhibitory drive.

Significance of Responses to Ramp Current Injection

Plasticity in PICs is known to be important in both normal and damaged MN circuits. PICs can be revealed in MNs by injecting slow depolarizing and repolarizing current ramps (Bennett et al. 2001b; Hamm et al. 2010; Hounsgaard et al. 1988; Lee and Heckman 1998; Pambo-Pambo et al. 2009; Turkin et al. 2010). They are important for normal motor behaviors in spinal MNs (Heckmann et al. 2005), underlie hyperexcitability associated with spasticity after spinal cord injury (Bennett et al. 2004; ElBasiouny et al. 2010; Gorassini et al. 2004), and contribute to calcium-mediated excitotoxicity in amyotrophic lateral sclerosis (ElBasiouny et al. 2010; Kuo et al. 2004; Pieri et al. 2009).
There were significant differences in the magnitude of $\Delta I$ between $wt$ and the $spa$ and $ot$ mutants (Fig. 5G). In spinal MNs a key indicator of PIC activation is a relatively large and negative $\Delta I$. Surprisingly, $\Delta I$ was negative in the $spa$ and $ot$ mutants and positive in $wt$ HMs. This might be interpreted as a modest increase in PIC expression in the $spa$ and $ot$ mutants. This would lead to increased excitability of $spa$ and $ot$ HMs: a finding not consistent with homeostatic plasticity. However, the influence of firing rate adaptation also needs to be considered in the overall interpretation of $\Delta I$. Firing rate adaptation, by itself, would tend to produce positive $\Delta I$s and reduce HM excitability (Bennett et al. 2001a; Turkin et al. 2010). Furthermore, the magnitude of firing rate adaptation increases when MNs are driven to discharge at higher frequencies (Kernell 1965; Kernell and Monster 1982). In the present study, all HMs were driven with the same triangular ramp current. Thus factors like the more leftward-shifted $F/I$ curve in $wt$ vs. $spa$ and $ot$ HMs (Fig. 3C) mean $wt$ HMs were driven to fire at higher frequencies during our triangular ramp current stimulus. This could lead to greater firing rate adaptation in $wt$ HMs, and contribute to their positive $\Delta I$. Future experiments, using ramps that will drive maximal AP firing, are needed to dissect out the effects of firing rate adaptation and PIC enhancement.

GABA Compensation in $spa$ and $ot$ Mice

The increased GABAergic mIPSC amplitude in the $spa$ and $ot$ mutants suggests robust GABA compensation occurs in HMs (Figs. 6 and 7, Table 3). Surprisingly, GABAergic compensation does not occur in HMs in the $spd$ mutant. This result is similar to our previous work on the three mutants in the spinal dorsal horn. In dorsal horn neurons we found that, like the present data on HMs, diminished glycineric drive was accompanied by a compensatory increase in GABAergic drive in $spa$ but not $spd$ (Graham et al. 2003, 2011). In contrast, we found no evidence for GABA compensation in the spinal dorsal horn of $ot$ animals despite the increased GABAergic mIPSC amplitude we report here for HMs.

For HMs, the differences in the extent of GABA compensation (Figs. 6 and 7) in the mutants may be explained by the nature of each mutation. Strychnine binding data suggest GlyR function in the $spa$ mutant. As noted above, there is evidence of GABA compensation in presumptive spinal MNs in the $spa$ animal. Rather, GABAergic drive was decreased, as assessed by the amplitude of evoked IPSCs (von Wegerer et al. 2003). Thus the interplay between the major inhibitory synaptic transmitter systems (glycine and GABA) is complex and maybe region specific.

GABA compensation is even greater (−40%, Table 3) in $ot$ HMs and is also consistent with homeostatic plasticity in the face of reduced GlyR function. The $ot$ mutation is, however, lethal. Perhaps the failure of GABA compensation to “rescue” the mutation is the complete lack of the adult form of the GlyR (i.e., $\alpha_i/\beta$) in $ot$ animals (Kling et al., 1997). Even though some glycineric mIPSCs can be recorded in $ot$ animals at $P19$ they are small, have slower kinetics, and are infrequent (Table 1). In short, the level of glycineric inhibition they provide is insufficient to support normal motor output in the HM brainstem circuitry.

Conclusions and Future Directions

Neural networks must maintain stability in the face of constantly changing synaptic inputs, and it is now well established that the nervous system can compensate for changes in synaptic drive to maintain appropriate AP discharge (Nelson and Turrigiano 1998). In the $spd$, $spa$ and $ot$ mutants there is a significant reduction in the level of glycineric inhibitory drive to neurons within the spinal cord and brainstem. This causes severe motor dysfunction in the form of a “startle” syndrome-like phenotype (Simon 1997). We have demonstrated that the intrinsic and synaptic properties of HMs in the $spa$ mouse undergo compensatory changes (i.e., homeostatic plasticity) that could reduce neuronal excitability to levels required for essential behaviors (like chewing and swallowing). Such homeostatic plasticity was also observed to a lesser extent in the lethal $ot$ mutant, in the form of GABA compensation, but this is insufficient to maintain HM excitability at levels compatible with life. Homeostatic plasticity, however, was not observed in the $spd$ mouse. This suggests that developmental adaptation to reduced glycineric inhibition is more complex than just homeostatic plasticity.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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