Developmental Changes in the Fidelity and Short-Term Plasticity of GABAergic Synapses in the Neonatal Rat Dorsal Horn

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Submitted 12 December 2007; accepted in final form 8 April 2008

Ingram RA, Fitzgerald M, Baccei ML. Developmental changes in the fidelity and short-term plasticity of GABAergic synapses in the neonatal rat dorsal horn. J Neurophysiol 99: 3144–3150, 2008. First published April 9, 2008; doi:10.1152/jn.01342.2007. The present study demonstrates for the first time that immature GABAergic synapses in the dorsal horn exhibit a lower fidelity of transmission and slower recovery from short-term depression (STD) following repetitive activation, which are

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INTRODUCTION

Immature dorsal horn cells in the neonatal rat spinal cord are, in many ways, more excitable than those of the adult. Cutaneous receptive fields are larger, mechanical thresholds are lower, and repetitive afferent stimulation causes a greater spike afterdischarge in the neonate (Fitzgerald 1985; Fitzgerald and Gibson 1984; Holmberg and Schouenborg 1996; Torsney and Fitzgerald 2002). Since the intrinsic excitability of superficial dorsal horn neurons remains stable during early postnatal development (Baccei and Fitzgerald 2005), we have hypothesized that the differences in cutaneous responsiveness in younger and older animals arise from immature inhibitory dorsal horn networks (Baccei and Fitzgerald 2006; Fitzgerald 2005). This idea is supported by the increase in frequency and amplitude of spontaneous and evoked inhibitory currents, respectively, over the postnatal period and the minimal endogenous glycinegic inhibition in the dorsal horn until the second week of life (Baccei and Fitzgerald 2004). As in other areas of the CNS (Akerman and Cline 2006; Ben Ari et al. 1989; Rivera et al. 1999), immature neurons in the dorsal horn can be depolarized rather than hyperpolarized by γ-aminobutyric acid type A receptor (GABA_A) activation due to elevated intracellular chloride levels (Baccei and Fitzgerald 2004). However, unlike the immature hippocampus, where there is a prominent excitatory GABAergic drive, GABA_A-mediated depolarizations do not reach action potential threshold in the neonatal dorsal horn in vitro (Baccei and Fitzgerald 2004). These depolarizations may still have facilitatory effects but they will be limited by the temporal and spatial organization of other inputs in the network (Gulledge and Stuart 2003; Jean-Xavier et al. 2007). When examined in vivo, GABA_A-mediated baseline activity is clearly inhibitory from birth, as shown by the increase in dorsal horn activity and decreased reflex thresholds following neonatal spinal application of GABA_A antagonists (Bremner et al. 2006; Hathway et al. 2006). Although a simple developmental switch in GABAergic function is an unlikely explanation for the baseline cutaneous excitability in the immature spinal cord, the reduced chloride extrusion capacity of immature neurons may lead to Cl\(^{-}\) accumulation during high-frequency trains of stimuli. This may cause a transient reversal in the direction of the chloride current, thus compromising the strength of GABAergic inhibition in young animals when faced with prolonged or intense stimulation (Cordero-Erausquin et al. 2005).

Alterations in the strength of dorsal horn GABAergic synapses during repetitive stimulation are likely to depend on presynaptic factors (Jensen et al. 1999a,b; Kirischuk et al. 2002) as well as postsynaptic mechanisms such as intracellular chloride accumulation. However, to date no studies have examined the functional properties of short-term plasticity at GABAergic synapses in the developing dorsal horn under experimental conditions that minimize any shift in postsynaptic chloride levels during repetitive stimulation. In addition, although a lower fidelity of transmitter release has been reported for excitatory synapses in the immature brain stem (Chuhma and Ohmori 1998), it is still unknown whether the reliability of GABA_A-mediated inhibition is lower in the neonatal dorsal horn, which could lead to greater fluctuations in inhibitory efficacy at early postnatal ages.

The present study demonstrates for the first time that immature GABAergic synapses in the dorsal horn exhibit a lower fidelity of transmission and slower recovery from short-term depression (STD) following repetitive activation, which are

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likely to hinder the consistency of inhibitory control in the neonatal spinal cord.

**Methods**

Patch-clamp recordings in spinal cord slices

All experiments were conducted in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986. Neonatal Sprague–Dawley pups (P3–P21) were anesthetized with sodium pentobarbital (30 mg/kg, administered intraperitoneally) and then perfused transcardially with ice-cold dissection solution consisting of (in mM): 250 sucrose, 2.5 KCl, 25 NaHCO3, 1.0 NaH2PO4, 6 MgCl2, 0.5 CaCl2, and 25 glucose, which had been continuously bubbled with 95% O2-5% CO2. Following perfusion the vertebral column was rapidly removed and placed in a bath filled with dissection solution. The lumbar spinal cord was isolated and immersed in low-melting-point agarose (3% in above solution; GibcoBRL, Paisley, UK) and parasagittal slices (350–400 μm) were cut using a Vibrorslice tissue slicer (HA-752; Campden Instruments; Leicester, UK). The slices were placed in a chamber filled with oxygenated dissection solution for 30 min then allowed to recover for a minimum of 1 h at room temperature in an oxygenated artificial cerebrospinal fluid (aCSF) solution containing (in mM): 125 NaCl, 2.5 KCl, 25 NaHCO3, 1.0 NaH2PO4, 1.0 MgCl2, 2.0 CaCl2, and 25 glucose.

Spinal cords were transferred to a submersion-type recording chamber (RC-22; Warner Instruments) and mounted on the stage of an upright microscope (Zeiss Axioskop 2; Welwyn Garden City, UK). Dorsal horn neurons were visualized with infrared differential interference contrast, and whole cell patch-clamp recordings were obtained as described previously (Baccei and Fitzgerald 2004). The tissue was continually perfused at room temperature with oxygenated aCSF as described previously (Baccei and Fitzgerald 2004). The tissue was continuously perfused at room temperature with oxygenated aCSF solution at a rate of 1–3 ml/min. Glass pipette microelectrodes, with a resistance of 5–7 MΩ, were filled with a solution consisting of (in mM): 130 CsCl, 2.5 MgCl2, 10 HEPES, 2.0 Na2ATP, 0.4 Na3GTP, at pH 7.2 (270 mOsm). The local anesthetic agent N-(2,6-dimethylphenyl) carbamoylmethyl)trihydrammonium bromide (QX-314, 5 mM) was added to the pipette solution to block sodium channels. Synaptic responses were evoked extracellularly from a holding potential of −70 mV with a glass pipette microelectrode placed nearby in the superficial dorsal horn (50–100 μm from the recorded cell). The electrode was filled with aCSF solution and connected via silver wire to a constant-current stimulator (NeuroLog system; Digitimer, Hertfordshire, UK) controlled via a PC running Clampex software (Axon Instruments, Union City, CA). Monosynaptic GABAergic inhibitory postsynaptic currents (IPSCs) were pharmacologically isolated by bath application of antagonists to glycinegic and glutamatergic transmission (0.5 μM strychnine, 20 μM D-2-amino-5-phosphonopentanoic acid (D-AP5), and 10 μM 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo[f]quinoxaline-2,3-dione (NBQX)).

Off-line analysis of IPSC amplitudes was conducted using Clampfit software (Axon Instruments) and a test stimulus of the same intensity. Recovery was calculated as: mean z = mean y/ (mean x – mean y), where z is the amplitude of the response to the test stimulus and x and y are as described earlier.

Data are expressed as means ± SE and were tested for statistical significance (set at P < 0.05) using two-way repeated-measures (RM) ANOVA unless otherwise stated.

**Results**

Whole cell patch-clamp recordings were obtained from 128 neurons in the neonatal rat dorsal horn from the following three age groups: P3 (n = 41), P10 (n = 48), and P21 (n = 39). All neurons were located in the superficial dorsal horn (laminae I–II) and, as such, were found within 150 μm of the dorsal white matter.

Monosynaptic GABA<sub>R</sub>-mediated IPSCs are smaller and more variable in immature dorsal horn neurons

To minimize changes in E<sub>C</sub> during repetitive stimulation, we used an intracellular solution with a cesium chloride base. Under these conditions (in the presence of antagonists to glutamatergic and glycinegic transmission), monosynaptic GABA<sub>R</sub>-mediated IPSCs appeared as inward currents from a holding potential of −70 mV and exhibited a reversal potential of about 0 mV (Fig. 1, A and B). These currents were abolished by the addition of the selective GABA<sub>R</sub> antagonist gabazine (10 μM), confirming that they were GABAergic in origin (Fig. 1C).

To examine the size and variability of IPSC amplitudes at different ages, multiple IPSCs were evoked with focal stimulation (at 0.03 Hz). A stimulus width of 100–200 μs was used and the threshold at which an IPSC could be discerned above baseline noise (10 pA) in 50% of trials was determined by slowly increasing the stimulus amplitude in 1-μA increments. All further stimulation was conducted at an intensity of twice the threshold amplitude (unless otherwise stated) and the total charge applied did not differ significantly between age groups (P3 = 6,272 ± 627 μA·μs, P10 = 8,655 ± 1,095 μA·μs, P21 = 5,950 ± 982 μA·μs; one-way ANOVA, P > 0.05). However, the mean evoked IPSC amplitude increased significantly by almost twofold between postnatal days 3 (P3) and P21 (Fig. 1, D and E; P3, n = 27; P10, n = 21; P21, n = 30).

An examination of the IPSC decay kinetics in a subset of cells revealed no significant changes over the period of postnatal development studied (P3: τ = 63.6 ± 2.4 ms, n = 10; P10: τ = 57.7 ± 1.45 ms, n = 10; P21: τ = 56.7 ± 2.0 ms, n = 10; one-way ANOVA, P = 0.55). For each cell the coefficient of variation (CV) of IPSC amplitude was calculated as an absolute value and the mean of these values was compared across age groups (Fig. 1, D and E). A significant developmental decrease in the CV of IPSCs was seen between 3 and 21 days after birth (P3: 0.47 ± 0.04, n = 11; P10 = 0.42 ± 0.06, n = 11; P21 = 0.30 ± 0.03, n = 10; one-way ANOVA with post hoc Tukey test, P < 0.05).

Paired-pulse ratios of IPSCs are similar at all postnatal ages

To establish whether the efficacy of GABAergic synapses altered during repetitive activation and whether this was affected by postnatal age, we first examined the effect of paired-pulse stimulation on IPSC amplitude. A variety of interstimulus intervals (25 ms to 5 s) were applied and the resulting ratios (mean IPSC2/mean IPSC1) were compared between age groups. Moderate paired-pulse facilitation was observed at all
Interestingly, significant frequency-dependent IPSC (Fig. 2) size of IPSCs at the end of the train compared with the first stimulus train, we calculated the MPA to reflect the average measure of short-term plasticity occurring during a given run of three cells per age where 1.5-fold threshold was used. As intensity applied was twice threshold, except for that in a pilot run of three cells per age where 1.5-fold threshold was used. 

A range of frequencies (1, 5, 10, and 20 Hz). The stimulus prolonged stimulation, we applied trains of 40 focal stimuli at a frequency of about 70% seen at 20 Hz. The STD (measured during 10-Hz trains at P10) was found to be sensitive to [Ca]ext because lowering [Ca]ext from 2 to 0.8 mM significantly increased the MPA from 0.36 ± 0.09 to 1.13 ± 0.13 (P < 0.01, one-way ANOVA, n = 3). The abolition of STD by decreasing [Ca]ext demonstrates its sensitivity to changes in the probability of transmitter release and thus presynaptic origin. In addition, the first and last IPSCs in the train exhibited a similar ECl, suggesting that postsynaptic chloride homeostasis was maintained during the stimulation protocol (IPSC1 ECl 8.6 ± 3.3 mV, IPSC40 ECl 14.8 ± 3.1 mV; Student's t-test, P > 0.05, n = 4).

Finally, we investigated whether the rate at which immature GABAergic synapses recover from STD differed from their mature counterparts. A train of 40 stimuli at 20 Hz was applied followed 1 to 8 s later by a single test stimulus (Fig. 3A) to measure recovery of IPSC amplitude (see METHODS for details). Postnatal age had a significant effect on the rate of recovery from depression (P3, n = 12; P10, n = 10; P21, n = 9; P < 0.05), with P3 neurons exhibiting a delayed recovery compared with those from P21 rats (Fig. 3B). The time required for IPSCs to recover to 50% of the baseline amplitude was 6.82 s at P3, 2.64 s at P10, and 0.84 s at P21.

Recovery of GABAergic transmission from STD is significantly slower in immature dorsal horn neurons. To examine the function of GABAergic synapses during prolonged stimulation, we applied trains of 40 focal stimuli at a frequency of 20 Hz. The stimulus intensity applied was twice threshold, except for that in a pilot run of three cells per age where 1.5-fold threshold was used. As a measure of short-term plasticity occurring during a given stimulus train, we calculated the MPA to reflect the average size of IPSCs at the end of the train compared with the first IPSC (Fig. 2C). Interestingly, significant frequency-dependent STD of IPSC amplitude was seen at all ages (P3, n = 11; P10, n = 11; P21, n = 10; P < 0.0001), and the extent of STD (as assessed by MPA) did not differ significantly between age groups (P = 0.85; Fig. 2, D and E) with a maximal reduction of about 70% seen at 20 Hz. The STD (measured during 10-Hz trains at P10) was found to be sensitive to [Ca]ext because lowering [Ca]ext from 2 to 0.8 mM significantly increased the MPA from 0.36 ± 0.09 to 1.13 ± 0.13 (P < 0.01, one-way ANOVA, n = 3). The abolition of STD by decreasing [Ca]ext demonstrates its sensitivity to changes in the probability of transmitter release and thus presynaptic origin. In addition, the first and last IPSCs in the train exhibited a similar ECl, suggesting that postsynaptic chloride homeostasis was maintained during the stimulation protocol (IPSC1 ECl 8.6 ± 3.3 mV, IPSC40 ECl 14.8 ± 3.1 mV; Student's t-test, P > 0.05, n = 4).

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FIG. 2. Paired-pulse facilitation and short-term depression (STD) of GABAergic transmission occur to a similar extent at all postnatal ages examined. 
A: sample traces demonstrating paired-pulse facilitation of GABAergic IPSCs at both P3 and P21. 
B: the paired-pulse ratio (mean amplitude IPSC2/mean amplitude IPSC1) did not differ significantly across postnatal development (one-way ANOVA; \( P > 0.05 \)). 
C: mean plateau amplitudes were calculated as mean \( y/\text{mean } x \) after a train of 40 stimuli at 1–20 Hz. 
D: the magnitude of STD was dependent on frequency (two-way repeated-measures [RM] ANOVA, \( P < 0.0001 \)) but not postnatal age (\( P = 0.85 \)). 
E: population data for STD seen over 40 stimuli at 10 Hz. No statistical difference was found between age groups (two-way RM ANOVA, \( P = 0.76 \)).
DISCUSSION

These results demonstrate for the first time that GABAergic synapses in the superficial dorsal horn undergo age-dependent changes in the strength and reliability of transmission. In addition, although paired stimuli lead to a facilitation of inhibitory strength, prolonged stimulation produces short-term depression (STD) of GABAergic signaling at all postnatal ages. Finally, the data clearly demonstrate that immature GABAergic synapses recover more slowly from STD.

Postnatal development of spinal inhibitory circuits

Dorsal horn neurons in immature animals have larger cutaneous receptive fields and lower mechanical thresholds than those in older animals. In addition, repeated stimulation of cutaneous A fibers leads to increased activity and a prolonged afterdischarge, not observed in older animals (Jennings and Fitzgerald 1998). The consistency of the intrinsic membrane excitability of dorsal horn neurons across postnatal development suggests that these distinct firing properties arise from differences in circuit organization or function. One possibility is that inhibitory transmission is not yet mature in the first weeks of life. The local circuit interneurons of the substantia gelatinosa are born last (Altman and Bayer 1984; Bice and Beal 1997) and, unlike projection neurons, the maturation of their dendritic morphology occurs postnatally (Bicknell Jr and Beal 1984). One aspect of inhibitory control that appears immature in early postnatal life is the glycinergic system because only very weak glycine-receptor–mediated transmission is seen in lamina II in the first week after birth (Baccei and Fitzgerald 2004). This is in contrast to GABA_ARs, which are activated in the neonatal dorsal horn and are crucial in limiting the excitability of the immature spinal cord from birth (Bremner et al. 2006; Hathway et al. 2006). Although tonic GABAergic inhibition mediated by extrasynaptic GABA_ARs has been reported in many areas of the CNS, including the adult mouse dorsal horn (Ataka and Gu 2006), recent evidence has demonstrated that lamina II neurons in P15–P21 rats lack a persistent GABA_AR-mediated conductance at physiological temperatures (Mitchell et al. 2007). Further studies are required to elucidate the precise contribution of extrasynaptic GABA_ARs to the overall inhibitory tone within the rat dorsal horn during early postnatal development.

Although GABAergic inhibition is present in early postnatal life, it may be less efficient than that in the adult. Previous reports have found that the magnitude of primary afferent-evoked GABA_AR-mediated IPSCs in the dorsal horn increases over the first 2 wk (Baccei and Fitzgerald 2004). The present data suggest that this does not result solely from a postnatal strengthening of primary afferent synapses but also reflects an age-dependent increase in the efficacy of local inhibitory synapses within the superficial dorsal horn because the amplitude of focally evoked IPSCs increases over a similar developmental period. Other more subtle differences in the properties of synaptic transmission may also have important implications for signal integration within the superficial dorsal horn. Our data indicated that the fidelity of GABAergic synapses, which is inversely related to the CV of IPSC amplitude, was significantly lower in immature neurons than that in those from older animals. Because GABA_AR activation is known to be involved in the restriction of receptive field size (Bremner et al. 2006), reduced and less reliable GABAergic transmission could contribute to the larger receptive fields seen in younger animals. The precise mechanisms underlying the age-dependent changes in the CV are not clear, although the lack of a postnatal change in the paired-pulse ratios (PPR) suggests that there is no significant change in the probability of GABA release over the age range studied. Since the CV of IPSC amplitudes is primarily

FIG. 3. The rate of recovery from STD increases with age. A: to measure recovery from depression, 20-Hz trains were applied followed by single stimuli (z) at varying intervals (Δt), as shown in the example trace. B: plotting the normalized recovery as a function of Δt demonstrates a significantly slower recovery from depression at immature GABAergic synapses (two-way RM ANOVA; P < 0.05).
determined by the number of active release sites and the release probability at these sites (Faber and Korn 1991), the decreased reliability of inhibition in immature neurons may therefore be the result of a lower number of active GABA release sites (Chuhma and Ohmori 1998). An age-dependent elevation in the number of GABA release sites could potentially explain both the observed developmental increase in the amplitude of focally evoked IPSCs (Fig. 1E) as well as previous reports that the frequency, but not amplitude, of GABAergic miniature (m)IPSCs increases during the early postnatal period (Baccei and Fitzgerald 2004).

Because extracellular stimulation was used in this study, it is possible that the observed developmental changes in the size and variability of IPSCs could instead be ascribed to differences in the response of GABAergic interneurons to electrical stimulation. This seems unlikely because the threshold for IPSC induction did not vary significantly across the three age groups studied. A previous study also identified no age-related changes in the membrane excitability of a mixed population of dorsal horn neurons (Baccei and Fitzgerald 2005), although we cannot completely discount the possibility that examining only GABAergic interneurons could have shown developmental changes in this parameter.

Functional implications of GABAergic short-term plasticity for spinal nociceptive processing

Repetitive stimulation of afferent inputs leads to increased excitability in the spinal cord (termed central sensitization), which may include long-term potentiation (LTP) of excitatory synapses onto lamina I projection neurons (Ikeda et al. 2003). These output cells of the superficial dorsal horn are under tight GABAergic control and removal of this inhibition unmasks new excitatory input that may contribute to spinal LTP (Torsney and MacDermott 2006). Thus GABA$_A$R activation during conditioning trains may modulate the threshold for induction of LTP, as has been shown in other areas of the CNS (Grover and Yan 1999). Indeed, enhancing spinal GABA$_A$R function in vivo by applying the benzodiazepine diazepam abolishes LTP of C-fiber–evoked field potentials in the dorsal horn (Hu et al. 2006). However, few studies to date have examined the properties of short-term plasticity at GABAergic synapses in the dorsal horn. The present results suggest that although short bursts of activity could lead to the potentiation of GABA$_A$R transmission (as seen in paired-pulse experiments), prolonged activation of GABAergic synapses results in frequency-dependent STD that occurred with a similar magnitude at all postnatal ages studied. This depression of inhibitory strength can act as a low-pass filter on GABAergic signaling (Baimoukhametova et al. 2004), resulting in a reduction of the output of inhibitory neurons during periods of high-frequency firing, which may encourage activity-dependent plasticity such as LTP and central sensitization.

The time needed for GABAergic synapses to recover from STD creates an additional “window” of disinhibition during which GABAergic signaling will not function at full efficacy. This may facilitate excitatory postsynaptic potential summation and subsequent action potential discharge (Thompson et al. 1993) in the period following the initial stimulus, and may thus contribute to the afterdischarge observed in dorsal horn neurons in vivo. Since immature GABAergic synapses exhibited a significantly slower recovery from STD, this temporal window of reduced inhibition will be longer in the neonatal dorsal horn. During this window, any further activation of the synapse could lead to a cumulative decrease in GABAergic strength. As a result, the efficacy of immature GABAergic synapses may be progressively decreased during the repetitive stimulation of sensory inputs to the spinal cord. This could explain the previous observation that repetitive activation of primary afferents in vivo leads to a significant increase in the afterdischarge in neonatal dorsal horn neurons, which disappears by 21 days after birth (Jennings and Fitzgerald 1998).

The efficacy of GABAergic inhibition onto immature dorsal horn neurons during high-frequency stimulation may also be compromised by postsynaptic shifts in the chloride equilibrium potential ($E_{Cl}$), which can be caused by the reduced chloride extrusion capacity seen at early postnatal ages (Cordero-Erausquin et al. 2005). This mechanism does not underlie the STD seen in the present study because we used a high-chloride intracellular solution that maintained a stable $E_{Cl}$ during the stimulus trains. Instead, the STD observed under our experimental conditions is likely to be presynaptically mediated, given that the magnitude of depression was significantly influenced by levels of extracellular calcium. Possible underlying mechanisms include depletion of the releasable pool of vesicles (Dobrunz and Stevens 1997), inactivation of calcium channels and/or the release machinery (Forsythe et al. 1998; Zucker and Regehr 2002), or the activation of presynaptic autoreceptors (Cui et al. 2000; Zucker and Regehr 2002). Overall, it appears that both pre- and postsynaptic factors can contribute to a reduction in the efficacy of GABAergic inhibition during prolonged and intense activity.

In conclusion, we have described a postnatal increase in both the strength and fidelity of GABAergic signaling in the superficial dorsal horn and the rate of recovery of inhibitory strength following repetitive stimulation. This result has important implications for the spatial and temporal processing of sensory information in young animals and may contribute to their enhanced responsiveness to cutaneous stimulation.

GRANTS

This work was funded by a grant from the Medical Research Council.

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