Deletion of Dlx1 results in reduced glutamatergic input to hippocampal interneurons

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Abstract

Dlx transcription factors are important in the differentiation of GABAergic interneurons. In mice lacking Dlx1, early steps in interneuron development appear normal. Beginning around one month of age, primarily dendrite-innervating interneuron subtypes begin to undergo apoptosis in Dlx1<sup>−/−</sup> mice; this is accompanied by a reduction in GABAergic transmission and late-onset epilepsy. The reported reduction of synaptic inhibition is greater than might be expected given that interneuron loss is relatively modest in Dlx1<sup>−/−</sup> mice. Here we report that voltage-clamp recordings of CA1 interneurons in hippocampal slices prepared from Dlx1<sup>−/−</sup> animals older than postnatal day 30 (>P30) revealed a significant reduction in excitatory postsynaptic current (EPSC) amplitude. No changes in EPSCs onto interneurons were observed in cells recorded from younger animals (P9-P12). Current-clamp recordings from interneurons at these early postnatal ages showed that interneurons in Dlx1<sup>−/−</sup> mutants were immature and more excitable, although membrane properties normalized by P30. TUNEL, caspase-3, and NeuN staining did not reveal frank cell damage or loss in area CA3 of hippocampal sections from adult Dlx1<sup>−/−</sup> mice. Delayed interneuron maturation may lead to interneuron hyperexcitability, followed by a compensatory reduction in the strength of excitatory transmission onto interneurons. This reduced excitation onto surviving interneurons, coupled with the loss of a significant fraction of GABAergic inputs to excitatory neurons starting at P30, may underlie cortical dysrhythmia and seizures previously observed in adult Dlx1<sup>−/−</sup> mice.
Introduction

The mechanisms leading to generation of spontaneous epileptic seizures have been the focus of intense study for nearly 100 years. Although many possible mechanisms have emerged, impairment of GABA-mediated inhibition is likely a critical cause of seizure activity in many forms of epilepsy. Several observations support this suggestion. First, subpopulations of GABAergic interneurons are vulnerable to seizure-induced damage in experimental models of epilepsy and humans with temporal lobe epilepsy (de Lanerolle et al. 1989; de Lanerolle et al. 2003). Second, altered inhibitory drive has been reported in animal models of cortical malformation and epilepsy (Zhu and Roper 2000; Trotter et al. 2006; Jones and Baraban 2007; Jones and Baraban 2009). Third, epilepsy-associated changes in the expression and function of postsynaptic GABA_A receptors are commonly observed (Brooks-Kayal et al. 1998; Loup et al. 2000; Coulter 2000; Crino et al. 2001). Fourth, changes in the function and expression of GABA reuptake transporters have been described in neurons in an animal model of epilepsy (Calcagnotto et al. 2002). Fifth, reductions in action potential number, frequency and amplitude were observed in cultured hippocampal interneurons (but not pyramidal cells) from a mouse model of severe myoclonic epilepsy in childhood (SMEI) (Yu et al. 2006).

These results provide strong support for the conclusion that reduced GABA signaling plays a central role in epilepsy and seizure generation. However, this is largely based on data from chemoconvulsant models of acquired epilepsy and in utero (or early postnatal) injury in normal rodents or examination of tissue samples from patients with medically intractable epilepsy. Recent observations from genetically altered mice based on manipulation of factors necessary
for interneuron development demonstrate that selective interneuron reduction (i.e., “interneuronopathy”) can lead to reduced inhibition, spontaneous recurrent seizures, or both (Powell et al. 2003; Cobos et al. 2005; Marsh et al. 2009). Homozygous $Dlx1$ mutant mice ($Dlx1^{-/-}$) have an age-dependent loss of approximately 50% of calretinin (CR), 35% of neuropeptide Y (NPY) and 35% of somatostatin (SOM)-positive interneurons in the hippocampus of young adult animals (Cobos et al. 2005). Analysis of GABA-mediated postsynaptic currents in pyramidal neurons at a postnatal age when interneuron loss and seizures are observed (i.e., >P30) revealed a nearly 50% reduction in inhibition. Given that soma-targeting, basket-type, parvalbumin-positive interneurons are preserved in these mice, and given the relatively modest loss of somatostatin and calretinin-positive interneurons, this reduction in IPSC amplitude and frequency was greater than might be expected.

These findings led us to hypothesize that reduced excitation onto surviving interneurons could further contribute to inhibition loss in $Dlx1$ knockout mice. To explore this possibility, we analyzed spontaneous and miniature excitatory postsynaptic currents (sEPSCs and mEPSCs) onto CA1 interneurons, as well as intrinsic membrane properties of interneurons, in hippocampal slices prepared from wild-type and $Dlx1^{-/-}$ littermates at two ages: (i) P9-11, before interneuron death and cell loss, and (ii) >P30, after significant interneuron death and reduced synaptic inhibition. Anatomical studies designed to assess cell loss or damage in stratum pyramidale of area CA3 (the primary source of excitatory input to CA1 interneurons) were also performed.
Materials and Methods

Slice preparation

All animal work and experimental protocols were carried out in accordance with guidelines defined by the relevant national and local animal welfare bodies. All animal work was approved by the University of California – San Francisco IACUC (approval number AN078649-03A). Mice were anesthetized and decapitated, and the brain was quickly removed and placed into oxygenated, ice-cold, high sucrose artificial cerebrospinal fluid (sACSF), containing (in mM): 150 sucrose, 50 NaCl, 25 NaHCO₃, 10 dextrose, 2.5 KCl, 1 NaH₂PO₄-H₂O, 0.5 CaCl₂, 7 MgCl₂. After 2 min in sACSF, the brain was blocked, glued to the stage of a vibratome (Leica VTS1000, Bannockburn, IL), and cut into 300 μm horizontal brain slices containing hippocampus. Slices were then placed in a holding chamber containing ACSF (in mM, 124 NaCl, 3 KCl, 1.25 NaH₂PO₄-H₂O, 2 MgSO₄-7H₂O, 26 NaHCO₃, 10 dextrose, and 2 CaCl₂). After a 40 min incubation period at 35°C, slices were maintained at room temperature (6-8 hr).

Electrophysiology

For recording, an individual slice was placed in a submerged recording chamber (Warner Instruments, Hamden, CT) with oxygenated ACSF heated to ~32°C and flowing at 2-4 ml/min. Interneurons were identified in area CA1 under infrared differential interference contrast (IR-DIC) optics, and were initially distinguished based on soma shape and location outside the principal cell layer. A micropipette puller (Sutter Instrument Company, Novato, CA) was used to pull patch pipettes of between 3-7 MΩ from 1.5 mm outer diameter borosilicate glass (World Precision Instruments, Sarasota, FL). Data were obtained using an Axopatch 1D amplifier.
(Axon Instruments, Foster City, CA); data were digitized at 10 kHz and recorded using a Digidata 1320A and pClamp 8.2 software (Molecular Devices, Sunnyvale, CA). For all experiments except miniature EPSC (mEPSC) recordings, internal recording solution (pH 7.20-7.25, 285-295 mOsm, liquid junction potential -13 mV) contained, in mM, 120 K-gluconate, 10 KCl, 1 MgCl₂, 0.025 CaCl₂, 0.2 EGTA, 2 Na₂ATP, 0.2 Na₂GTP, and 10 HEPES. In mEPSC experiments, to enhance event amplitude and increase signal-to-noise ratio, internal recording solution contained, in mM, 140 CsCl, 1 MgCl₂, 10 HEPES, 11 EGTA, 2 Na₂ATP, 0.5 Na₂GTP and 1.25 QX-314.

After obtaining a stable voltage-clamp recording from a CA1 interneuron, the recording was switched to current-clamp mode and injected steps of hyperpolarizing and depolarizing current were used to determine the class of cell being recorded using published firing patterns and action potential properties (Butt et al. 2005; Flames and Marin 2005). The recording was subsequently switched back to voltage-clamp mode (holding potential, -60 mV) for recording of sEPSCs or mEPSCs. Membrane potentials were not corrected for liquid junction potential. Spontaneous EPSCs were pharmacologically isolated by perfusion of the slice with ACSF containing 5 μM bicuculline (a GABA_A receptor blocker; Sigma, St. Louis, MO). At the end of some experiments, 3 mM kynurenic acid (a non-specific glutamate receptor antagonist; Tocris, Ellisville, MO) was added to confirm that EPSCs were successfully isolated. All recordings were obtained at ~32° C. Voltage-clamp recordings were low-pass filtered at 1 kHz, and band-pass filtered at 60 Hz (Hum Bug, AutoMate Scientific, Berkeley, CA). Whole cell access resistance and holding current were continuously monitored to confirm that recordings were stable.
Spontaneous EPSCs were analyzed using Mini Analysis Program 5.2.5 (Synaptosoft, Decatur, GA), selecting each event by hand, and analyzing 300 events per cell. Because of the low frequency of mEPSCs, we analyzed at least 25 events or at least five minutes of recording time per cell. Histograms of sEPSCs were constructed using the first 100 events per cell. For decay time calculations, decay time of individual events was defined as the time to 67% of peak amplitude. All data are presented as mean ± SEM, and we determined statistical significance of results using unpaired, two-tailed Student’s *t*-tests, with *p* = 0.05 as the threshold for statistical significance. Where appropriate, the χ² test or ANOVA was used to determine statistical significance.

**Histology**

Adult Dlx1−/− and WT littermate mice were deeply anesthetized with Avertin (Sigma; 0.2 ml/10 g body weight) and perfused intracardially with 4% paraformaldehyde (PFA) in phosphate-buffered saline solution (PBS 0.1 M, pH 7.4). The brains were removed and postfixed overnight in the same fixative. Brain sections were prepared at a thickness of 50 μm on a vibratome and used for free-floating immunohistochemistry or mounted on Fisher Superfrost/Plus slides for the TUNEL assay. For immunohistochemistry, the slices were washed in phosphate-buffered saline solution, incubated in blocking solution (0.5% Triton X-100, 10% normal goat serum, 2% non-fat milk, 0.2% gelatin in PBS) for 1 hour, and incubated 1 day at 4°C in the primary antibody diluted in 0.5% Triton X-100, 3% normal goat serum, 0.2% gelatin in PBS. The antibodies used were as follows: cleaved caspase-3 polyclonal antibodies (1:500, Cell Signaling Technology) and NeuN monoclonal antibodies (1:500, Chemicon). Immunoreactivity was detected with
appropriate Alexa-488 or Alexa-594 (1:300; Molecular Probes) conjugated secondary antibodies.

TUNEL apoptotic cell detection was carried out following the manufacturer’s protocol (Apotag Red In Situ Apoptosis Detection Kit, Chemicon).

**Cell Counting**

Quantification of TUNEL⁺, cleaved caspase-3⁺, and NeuN⁺ cells in the CA3 region of the hippocampus was determined in coronal sections from 3 mice of each genotype. Cell counts were performed on digitized images obtained with a CoolSNAP EZ Turbo 1394 digital camera (Photometric, Tucson, AZ) on a Nikon ECLIPSE 80i microscope (Nikon Instruments Inc., Melville, NY) using a 10X objective. The numbers of positive cells were assessed in a 10,000 µm² area of the CA3 pyramidal layer of the hippocampus. Statistical differences between experimental groups were determined with the Student’s *t*-test. Results are presented as mean ± SEM.
**Results**

*Glutamate-mediated excitation of interneurons in Dlx1<sup>−/−</sup> mice*

Decreased excitatory input to surviving interneurons in adult *Dlx1<sup>−/−</sup>* mice might contribute to the larger-than-expected loss of inhibition previously reported in these animals (Cobos et al. 2005). Here we recorded sEPSCs from 37 visually identified CA1 interneurons in hippocampal slices prepared from >P30 *Dlx1<sup>−/−</sup>* mice and WT littermates (Fig. 1). Spontaneous EPSCs are mediated by glutamatergic input to these cells and are abolished by application of 3 mM kynurenic acid, a glutamate receptor antagonist (data not shown). For all sEPSC experiments, we obtained voltage-clamp recordings at a holding potential of -60 mV; representative sEPSC recordings for both genotypes are shown in Figs. 1A and B (upper panels). Following sEPSC data collection, cells were analyzed in current-clamp mode for classification as interneurons; sample current-clamp traces (lower panels, Fig. 1A and B) are from the same neuron as voltage-clamp traces. In the “surviving” interneuron population, mean sEPSC amplitude was reduced by approximately 25% in *Dlx1<sup>−/−</sup>* mutants as compared to age-matched littermate controls (WT: 16.87 ± 1.45 pA; *Dlx1<sup>−/−</sup>*: 12.80 ± 1.18 pA; *p* < 0.05; Fig. 1E). Other sEPSC parameters, including frequency (WT: 11.35 ± 2.23 Hz; *Dlx1<sup>−/−</sup>*: 10.77 ± 3.46 Hz; *p* = 0.89; Fig. 1C) and decay times (WT: 0.86 ± 0.08 ms; *Dlx1<sup>−/−</sup>*: 0.94 ± 0.08 ms; *p* = 0.49; Fig. 1D), were unchanged. Construction of an amplitude histogram showed that small-amplitude events predominated in mice lacking *Dlx1*, while in WT mice, large-amplitude events were more frequent (Fig. 1F). In an additional set of mEPSC recordings from 27 interneurons in >P30 *Dlx1<sup>−/−</sup>* mice and WT littermates, mean mEPSC amplitude was significantly reduced in mice lacking *Dlx1* (WT: 21.07 ± 0.83 pA; *Dlx1<sup>−/−</sup>*: 17.79...
± 1.18 pA; \( p < 0.03 \)), while no significant differences were observed in mEPSC frequency or
decay kinetics (data not shown).

These results raised the question of whether or not reduced excitatory postsynaptic current onto
interneurons in \( Dlx1^{-/-} \) mice would also be present at ages before interneuron subpopulations
began to undergo apoptosis. Interneurons in mice lacking \( Dlx1 \) could receive less excitation
throughout development, or the decreased EPSC amplitude in \( Dlx1^{-/-} \) mice could reflect
remodeling of neural circuits that takes place at later ages. To shed light on this question, we
recorded sEPSCs from 46 visually identified CA1 interneurons in P9-12 WT and \( Dlx1^{-/-} \) mice.
Again, sample voltage-clamp recordings of EPSCs and current-clamp recordings of firing
properties are shown for each genotype (Fig. 2). At this early postnatal age, we observed no
significant differences in sEPSC amplitude (WT: 16.89 ± 2.01 pA; \( Dlx1^{-/-} \): 15.35 ± 1.57 pA; \( p =
0.55 \), Fig. 2E), frequency (WT: 2.94 ± 0.69 Hz; \( Dlx1^{-/-} \): 3.25 ± 0.81 Hz; \( p = 0.77 \), Fig. 2C), or
decay time (WT: 1.12 ± 0.08 ms; \( Dlx1^{-/-} \): 1.09 ± 0.07 ms; \( p = 0.78 \), Fig. 2D).

Interneuron firing properties in immature \( Dlx1 \) knockout mice

Interneuron numbers, as quantified using immunohistochemical approaches, appear normal in
\( Dlx1^{-/-} \) mice during the first three postnatal weeks (Cobos et al. 2005). However, it remains
unclear whether loss of \( Dlx1 \) leads to alterations in interneuron function at younger ages. To
determine whether interneurons in early postnatal \( Dlx1^{-/-} \) mice exhibit normal membrane
properties, we obtained current-clamp recordings in area CA1 (WT: \( n = 37 \) WT; \( Dlx1^{-/-} \): \( n = 35 \))
interneurons. We injected steps of depolarizing and hyperpolarizing current to classify
interneurons by firing properties as follows: fast-spiking (FS), regular-spiking nonpyramidal
(RSNP), and burst-spiking (BST). We also used these recordings to determine spike width, input resistance (R_m), resting membrane potential (V_m), and fast afterhyperpolarization amplitude (fAHP) for each interneuron. A combination of these firing property parameters and laminar location was used to classify interneurons into previously described sub-populations (Butt et al. 2005).

The relative proportions of interneuron subtypes that could be sampled with a patch-clamp recording electrode, which is largely limited to a relatively small population of cells near the surface in an acute slice preparation, were comparable between slices from WT and Dlx1^-/- mice at P9-12 (Fig. 3C). Based on our criteria, in WT controls, 11% of interneurons recorded were classified as FS cells (n = 4), 84% were RSNP cells (n = 31), and 5% were BST cells (n = 2). In Dlx1 mutants, 11% of interneurons recorded were classified as FS cells (n = 4), 83% were RSNP cells (n = 29), and 6% were BST cells (n = 2). We also found that the laminar distributions of interneuron cell bodies were comparable between WT and Dlx1 knockout mice at this age (Fig. 3D, E). All FS cells in WT and knockout mice were located in stratum oriens and alveus (O/A). In addition, most RSNP interneurons were located in stratum radiatum and stratum lacunosum-moleculare (R/LM; WT: 29% in O/A, 71% in R/LM; Dlx1^-/-: 28% in O/A, 72% in R/LM). We did not encounter sufficient numbers of BST interneurons to make a valid comparison of their distribution across hippocampal layers.

Interestingly, in these recordings, we found that interneurons in slices from Dlx1^-/- mice (P9-12) exhibited what would be considered immature intrinsic membrane properties (Fig. 3A, B and Table 1) (Okaty et al. 2009). Specifically, in Dlx1 mutants, interneurons exhibit a high input
resistance, as evident in the hyperpolarizing steps shown in Figs. 3A and B. High input resistance suggests that interneurons lacking \textit{Dlx1} are functionally immature as compared to age-matched WT interneurons (Fig. 3F; WT \textit{R}_{in}: 291.35 \pm 18.13 \text{ M\Omega}; \textit{Dlx1}^{+/−} \textit{R}_{in}: 359.16 \pm 27.99 \text{ M\Omega}; \textit{p} < 0.05). Consistent with functional immaturity, interneurons at this age also exhibit a depolarized resting membrane potential (Fig. 3G; WT \textit{V}_{m}: -56.6 \pm 1.0 \text{ mV}; \textit{Dlx1}^{−/−} \textit{V}_{m}: -51.6 \pm 1.3 \text{ mV}; \textit{p} < 0.05). Evaluation of action potential width and other standard parameters failed to uncover additional differences between WT and \textit{Dlx1}^{−/−} interneurons (Table 1). We also sorted these data by interneuron subtype and found that, in \textit{Dlx1}^{−/−} RSNP cells, \textit{V}_{m} was significantly depolarized (WT \textit{V}_{m}: -55.9 \pm 1.0 \text{ mV}; \textit{Dlx1}^{−/−} \textit{V}_{m}: -51.8 \pm 1.4 \text{ mV}; \textit{p} < 0.03). Finally, we generated a plot of firing frequency versus current step size to examine excitability of interneurons; to ensure that similar populations of cells were being compared across genotypes, we limited this analysis to RSNP interneurons located in R/LM. We found that interneurons lacking \textit{Dlx1} exhibited higher firing rates in response to a given current injection than did WT interneurons (\textit{p} < 0.05, one-way ANOVA; Fig. 3H), consistent with the hypothesis that interneurons in early postnatal mice lacking \textit{Dlx1} are hyperexcitable.

\textit{Interneuron function in adult Dlx1 knockout mice}

We repeated these current-clamp experiments in mice older than P30 (Fig. 4), when significant interneuron loss was observed in the SOM- and CR-expressing subpopulations of \textit{Dlx1}^{−/−} mice (Cobos et al. 2005). At this age, interneuron input resistance was comparable between WT and \textit{Dlx1}^{−/−} mice (WT \textit{R}_{in}: 187.44 \pm 13.25 \text{ M\Omega}; \textit{Dlx1}^{−/−} \textit{R}_{in}: 212.34 \pm 16.47 \text{ M\Omega}; \textit{p} = 0.24), as was resting membrane potential (WT \textit{V}_{m}: -58.5 \pm 1.2 \text{ mV}; \textit{Dlx1}^{−/−} \textit{V}_{m}: -56.8 \pm 1.4 \text{ mV}; \textit{p} = 0.35). All other membrane and firing properties were also normal in interneurons from \textit{Dlx1} mutant mice at
Sorting interneurons by subtype also failed to reveal differences in intrinsic properties at this age (data not shown). The overall laminar distribution of the surviving interneuron subtypes was similar between WT and knockout animals at this age (Fig. 4D, E). FS cells were primarily located in O/A in both WT and knockout animals (WT: 87.5% in O/A, 12.5% in R/LM; Dlx1\(^{-/-}\): 83% in OA, 17% in R/LM); in addition, most RSNP cells were located in R/LM in both WT and knockout animals (WT: 17% in O/A, 83% in R/LM; Dlx1\(^{-/-}\): 25% in O/A, 75% in R/LM). Although the overall proportions of interneurons subtypes encountered were largely comparable between WT and knockout animals, the proportion of BST interneurons encountered in Dlx1\(^{-/-}\) mice (7%) was half of the proportion observed in WT mice (14%; Fig. 4C).

CA3 pyramidal neurons are unaffected in mice lacking Dlx1

Cell death or damage to presynaptic CA3 pyramidal neurons, which provide excitatory input to CA1 interneurons, could contribute to the circuit alterations we observed. To exclude this possibility, we performed a series of histology studies on hippocampal sections from adult WT and Dlx1\(^{-/-}\) littermates (Fig. 5). We directly assessed apoptosis in Dlx1 mutants by TUNEL staining and cleaved caspase-3 immunohistochemistry. Compared to controls, we observed no change in the numbers of TUNEL\(^{+}\) (Fig. 5A-B') or activated caspase-3\(^{+}\) (Fig. 5C-D') CA3 pyramidal neurons in Dlx1 mutant mice. Furthermore, immunohistochemistry for NeuN, a postmitotic neuronal marker, was used to determine the numbers of neurons within the CA3 pyramidal region of the hippocampus. Quantification revealed no significant differences in the numbers of NeuN\(^{+}\) neurons in WT and Dlx1\(^{-/-}\) mice (Fig. 5E-G).
Discussion

A homeostatic balance between excitation and inhibition is critical for proper brain function. In adult mice lacking the transcription factor *Dlx1*, we previously observed apoptotic loss of a subset of the cortical and hippocampal interneuron population, with concomitant reductions in GABA-mediated synaptic transmission, cortical dysrhythmia, and generalized seizures (Cobos et al. 2005). Here we provide additional evidence that *Dlx1* may be important in the functional maturation of hippocampal interneurons. We also describe a significant alteration in the excitatory synaptic innervation of surviving interneurons in hippocampal slices from adult *Dlx1*--/-- mice.

Postnatal *Dlx1* expression appears to be restricted to dendrite-innervating interneurons (Cobos et al. 2005; Cobos et al. 2006); these are the same cells that undergo apoptosis in adult *Dlx1*--/-- mice (Cobos et al. 2005). However, the magnitude and dynamics of the observed change in synaptic inhibition suggest that inhibition from soma-targeting interneurons, which are present in normal numbers, may also be altered (Cobos et al. 2005). Furthermore, interneurons from *Dlx1*--/-- mice show reduced dendrite branching and length (Cobos et al. 2005), suggesting that the dendritic space available for receiving excitatory synapses may be reduced. These lines of evidence led us to hypothesize that surviving interneurons in mice lacking *Dlx1* receive reduced excitatory input, contributing to a larger-than-expected loss of inhibition in these animals. Here we report a significant decrease in the amplitude of glutamatergic postsynaptic currents onto surviving interneurons in >P30 mice lacking *Dlx1*. One functional consequence of *Dlx1*--/-- interneurons receiving less excitation is that these inhibitory cells would be less active, contributing to a larger...
network reduction in GABA release. This conclusion is consistent with our previous findings (Cobos et al. 2005), a role for reduced inhibition in epilepsy (de Lanerolle et al. 1989; de Lanerolle et al. 2003) and was also reported in a rat model of cortical dysplasia and epilepsy (Xiang et al. 2006). In support of this conclusion, a recent examination of interneuron firing rates in a model of cortical dysplasia reported that reductions in glutamatergic drive of interneurons were correlated with reduced spontaneous firing rates of these cells (Zhou and Roper, 2010).

Our results also suggest that a novel circuit rearrangement occurs in adult Dlx1 mutant mice. A reduction in excitation of CA1 interneurons could be explained by a simple reduction in the number of excitatory synapses on surviving interneurons, possibly associated with damage or loss of the presynaptic glutamatergic CA3 pyramidal neurons innervating these cells (Katz 1962; Cormier and Kelly 1996). However, EPSC frequency was unchanged in our recordings and we failed to detect any CA3 cell damage or loss using TUNEL, caspase or NeuN staining, suggesting that glutamatergic input and release probability are normal (Manabe et al. 1992). The reduced EPSC amplitude that we observed suggests that fewer functional glutamate receptors are present at excitatory synapses onto CA1 interneurons in adult Dlx1 mutant mice. As our recordings were obtained at a holding potential of -60 mV, virtually all EPSCs recorded are likely to be mediated by AMPA receptors. Therefore, our results predict reduced AMPA receptor number at glutamatergic synapses on interneurons in mice lacking Dlx1, an interesting possibility that remains to be explored in future studies.

Intrinsic properties of interneurons lacking Dlx1
Surviving interneurons in adult $Dlx1^{-/-}$ mice could have intrinsic membrane properties that could also lead them to release less GABA onto pyramidal neurons. For instance, these interneurons could have a more hyperpolarized resting membrane potential or lower threshold for spike generation, meaning that a given excitatory input would be less likely to lead to an action potential, and thus GABA release, from the interneuron. However, current-clamp recordings from interneurons in $Dlx1$ knockout mice older than P30 demonstrated that intrinsic firing properties are comparable to those of WT interneurons, arguing against this possibility. In P9-12 $Dlx1^{-/-}$ mice, however, CA1 interneurons exhibited a depolarized resting membrane potential and higher input resistance as compared to WT interneurons, suggesting that interneurons lacking $Dlx1$ may undergo delayed maturation (Okaty et al. 2009).

How loss of $Dlx1$ might affect intrinsic membrane properties of young interneurons, and ion channel expression in particular, is unclear. Prenatally, $Dlx1$ (in conjunction with other $Dlx$ genes) controls neurite extension of young neurons through regulation of cytoskeleton-associated factors (Cobos et al. 2007). In adult interneurons, $Dlx1$ promotes dendrite length and branch number development (Cobos et al. 2005). Cell size is one determinant of input resistance; if interneurons lacking $Dlx1$ are smaller at P9-12, this could contribute to the increased input resistance observed. However, measurements of capacitance in these neurons were not different between $Dlx1^{-/-}$ mice and WT littermates (data not shown), arguing against this possibility.

Interestingly, immature membrane properties of interneurons in young $Dlx1$ knockout mice might lead, in a homeostatic manner, to the reduction in interneuron excitation observed later in development. A depolarized resting membrane potential and increased input resistance at young
ages could combine to enhance excitability of interneurons. Consistent with this prediction, our results show that interneurons lacking \textit{Dlx1}, when recorded at P9-12, fire more frequently in response to current steps of a given size than do interneurons from WT littermates. In turn, this increased excitability could lead to the removal of AMPA receptors from glutamatergic synapses on these immature interneurons in \textit{Dlx1}^{-/-} mice as a compensatory response (Turrigiano et al. 1998), in an attempt to prevent excessive GABA release during the first few postnatal weeks, a time when GABA is depolarizing (Ben-Ari et al. 2007).

Conclusion and Perspective

Here we describe a functional reorganization of excitatory circuits in the hippocampus of mice lacking \textit{Dlx1}. We also report a novel role for \textit{Dlx1} in the electrophysiological maturation of interneurons. Alterations in glutamatergic input to hippocampal interneurons are likely to be compensatory responses to reduced inhibition secondary to interneuron loss (Cobos et al. 2005) and delayed maturation of interneurons in young (P9-12) \textit{Dlx1}^{-/-} mice.

These findings confirm the hypothesis that loss of \textit{Dlx1} results in an imbalance between hippocampal excitation and inhibition that cannot be explained solely by the loss of a number of SOM, NPY and CR interneurons. These findings have implications not only for epilepsy, but also for neuropsychiatric disorders, in which alterations in interneuron function have been postulated to underlie circuit dysfunction (Rubenstein and Merzenich 2003; Lewis et al. 2005; Gogolla et al. 2009). For instance, reduced interneuron expression of GAD1 is a consistent phenotype in schizophrenia (Lewis et al. 2005; Lisman et al. 2008). It is hypothesized that reduced excitatory drive onto interneurons, through NMDA receptors, contributes to homeostatic
reductions in GAD1 and GABA, and thereby a reduction in inhibition (Lisman et al. 2008).

Thus, studies of homeostatic regulation of circuit function may shed light on system
deregulation in human neurological and neuropsychiatric disorders.

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Figure Legends

Figure 1. Excitatory drive of interneurons is decreased in >P30 *Dlx1*<sup>−/−</sup> mice.

A, Upper trace: representative voltage-clamp recording from a WT RSNP interneuron showing sEPSCs (downward deflections). Lower trace: the same cell recorded in current-clamp mode, confirming its identity as a RSNP interneuron. B, Upper trace: representative sEPSC recording from a *Dlx1*<sup>−/−</sup> RSNP interneuron demonstrating reduced excitatory input. Lower trace: the same cell recorded in current-clamp mode. C, Mean sEPSC frequency is comparable between WT and *Dlx1*<sup>−/−</sup> mice. D, sEPSC decay time is also comparable. E, sEPSC amplitude is significantly decreased (*p* < 0.05, Student’s t-test) in *Dlx1*<sup>−/−</sup> mice as compared to WT mice. F, Amplitude histogram of sEPSCs shows a shift toward smaller events in *Dlx1*<sup>−/−</sup> mice. Black bars, WT. Gray bars, *Dlx1*<sup>−/−</sup>.

Figure 2. Excitatory drive of interneurons is normal in P9-12 *Dlx1*<sup>−/−</sup> mice.

A, Upper trace: representative voltage-clamp recording from a WT RSNP interneuron. Lower trace: the same cell recorded in current-clamp mode, confirming its identity as a RSNP interneuron. B, Upper trace: representative sEPSC recording from a *Dlx1*<sup>−/−</sup> RSNP interneuron. Lower trace: the same cell recorded in current-clamp mode. C, Recordings of sEPSCs in P9-12 WT and *Dlx1*<sup>−/−</sup> interneurons show that mean sEPSC frequency is unchanged in young *Dlx1* mutant mice. D, Mean sEPSC decay time is also similar between WT and mutant mice. E, Mean sEPSC amplitude is unchanged. Black bars, WT. Gray bars, *Dlx1*<sup>−/−</sup>.
Figure 3. Young $Dlx1^{-/-}$ interneurons exhibit immature membrane properties compared to young WT interneurons.

A, Sample current clamp recording from a WT RSNP interneuron. B, Sample trace from a $Dlx1^{-/-}$ RSNP interneuron. Note increased input resistance (dashed line). C, The overall proportions of interneuron subtypes encountered were comparable between WT and mutant mice. D, In both WT and $Dlx1^{-/-}$ mice, all FS cells were found in stratum oriens/alveus (O/A). E, In both WT and $Dlx1^{-/-}$ mice, most RSNP cells were recorded in stratum radiatum and stratum lacunosum-moleculare (R/LM). F, Input resistance is significantly increased in interneurons recorded from $Dlx1$ knockout mice ($p < 0.05$, Student’s t-test). G, Resting membrane potential is significantly depolarized in $Dlx1^{-/-}$ interneurons ($p < 0.05$, Student’s t-test). H, Plot of firing frequency versus current showing that interneurons from $Dlx1^{-/-}$ mice fire at modestly higher rates in response to a given step size, as compared to WT interneurons ($p < 0.05$, one-way ANOVA). Black bars: WT. Gray bars: $Dlx1^{-/-}$.

Figure 4. Adult $Dlx1^{-/-}$ interneurons are indistinguishable from WT interneurons.

A, Sample traces from WT FS, RSNP, and BST interneurons. B, Sample traces from $Dlx1^{-/-}$ FS, RSNP, and BST interneurons, showing that $Dlx1^{-/-}$ interneurons older than P30 exhibit normal membrane properties. C, The proportion of BST interneurons recorded in $Dlx1^{-/-}$ mice was approximately half of the proportion observed in WT mice (WT: 14% of interneurons recorded were BST cells; $Dlx1^{-/-}$: 7% were BST cells). D, FS
cells showed a normal laminar location profile in $Dlx1^{-/-}$ mice. E, RSNP cells are also
show a normal laminar distribution in $Dlx1^{-/-}$ mice. Black bars: WT. Gray bars: $Dlx1^{-/-}$.

**Figure 5. CA3 pyramidal neurons are unaffected in $Dlx1$ knockout mice.**

A-D', No detectable apoptosis is observed in the CA3 pyramidal region of adult control (A-A', C-
C') and $Dlx1$ mutant (B-B', D-D') hippocampi as revealed by active caspase-3 antibody (C', D')
and TUNEL (A', B') staining of the corresponding DAPI labeled (A, B, C, D) sections. E-F,
Compared to control (E), no significant change in the number of NeuN$^+$ cells was noticed within
the CA3 pyramidal layer of the $Dlx1$ mutant (F) hippocampus. G, Quantification of the number
of NeuN$^+$ cells in the CA3 pyramidal layer of the adult hippocampus. Data shown are mean ±
SEM. Scale bar, 250 µm.
Table 1. *Dlx1* knockout interneurons are functionally immature at P9-12.

<table>
<thead>
<tr>
<th></th>
<th>FF</th>
<th>Rin</th>
<th>fAHP1</th>
<th>AP</th>
<th>Vm</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>(2xT) width</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>15.08 ± 1.69</td>
<td>291.35</td>
<td>19.50 ± 0.81</td>
<td>1.54 ± 0.04</td>
<td>-56.59 ± 0.95</td>
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<tr>
<td>Dlx1 KO</td>
<td>19.51 ± 2.02</td>
<td>359.16</td>
<td>21.62 ± 1.28</td>
<td>1.67 ± 0.08</td>
<td>-51.57 ± 1.25</td>
</tr>
</tbody>
</table>

Shown are mean values ± SEM for firing frequency at twice the firing threshold, input resistance, amplitude of fast afterhyperpolarization, action potential width, and resting membrane potential. Recordings were obtained from hippocampal CA1 interneurons in P9-12 WT and mutant mice. Bold indicates p < 0.05, Student’s t-test.
Table 2. *Dlx1* knockout interneurons at >P30 exhibit normal membrane and firing properties.

<table>
<thead>
<tr>
<th>FF (2xT)</th>
<th>Rin</th>
<th>fAHP</th>
<th>AP</th>
<th>Vm</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WT</strong></td>
<td>43.08 ± 8.96</td>
<td>187.44 ± 13.25</td>
<td>25.32 ± 1.55</td>
<td>1.05 ± 0.05</td>
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<tr>
<td><strong>Dlx1 KO</strong></td>
<td>43.14 ± 11.11</td>
<td>212.34 ± 16.47</td>
<td>28.01 ± 1.26</td>
<td>1.13 ± 0.07</td>
</tr>
</tbody>
</table>

Shown are mean values ± SEM for firing frequency at twice the firing threshold, input resistance, amplitude of fast afterhyperpolarization, action potential width, and resting membrane potential. Recordings were obtained from hippocampal CA1 interneurons in >P30 WT and mutant mice.