Blocking Early GABA Depolarization with Bumetanide Results in Permanent Alterations in Cortical Circuits and Sensorimotor Gating Deficits

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A high incidence of seizures occurs during the neonatal period when immature networks are hyperexcitable and susceptible to hyper-synchronous activity. During development, γ-aminobutyric acid (GABA), the primary inhibitory neurotransmitter in adults, typically excites neurons due to high expression of the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter (NKCC1). NKCC1 facilitates seizures because it renders GABA activity excitatory through intracellular Cl\(^-\) accumulation, while blocking NKCC1 with bumetanide suppresses seizures. Bumetanide is currently being tested in clinical trials for treatment of neonatal seizures. By blocking NKCC1 with bumetanide during cortical development, we found a critical period for the development of α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate synapses. Disruption of GABA signaling during this window resulted in permanent decreases in excitatory synaptic transmission and sensorimotor gating deficits, a common feature in schizophrenia. Our study identifies an essential role for GABA-mediated depolarization in regulating the balance between cortical excitation and inhibition during a critical period and suggests a cautionary approach for using bumetanide in treating neonatal seizures.

Introduction

Neonatal seizures are among the most common pathological manifestations seen in the developing brain, affecting 1.5 to 5.5 per 1000 newborns each year (Lanska et al. 1995; Ronen et al. 1999; Saliba et al. 1999). Seizures occur more often in the neonatal period than any other time in life because immature networks have a propensity for generating synchronized activity. Unlike seizures in adults, seizures in neonates respond poorly to anticonvulsants that work by high expression of the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter (NKCC1). NKCC1 facilitates seizures because it renders GABA activity excitatory through intracellular Cl\(^-\) accumulation, while blocking NKCC1 with bumetanide suppresses seizures. Bumetanide is currently being tested in clinical trials for treatment of neonatal seizures. By blocking NKCC1 with bumetanide during cortical development, we found a critical period for the development of α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate synapses. Disruption of GABA signaling during this window resulted in permanent decreases in excitatory synaptic transmission and sensorimotor gating deficits, a common feature in schizophrenia. Our study identifies an essential role for GABA-mediated depolarization in regulating the balance between cortical excitation and inhibition during a critical period and suggests a cautionary approach for using bumetanide in treating neonatal seizures.

Materials and Methods

Animals

Timed-pregnant Swiss-Webster dams were obtained from Simonsen Laboratories, Gilroy, CA. All manipulations were performed in accordance with the guidelines of the UCSF Institutional Animal Care and Use Committee.

Drug Injections

Intraperitoneal injections were given to pregnant dams prenatally and their pups postnatally. Injections were placed into the lower abdomen with slight retraction of the injection needle to ensure that the drugs
are not directly delivered to the embryos. The following drugs were dissolved in phosphate-buffered saline (PBS) and injected at the following doses: bumetanide (Sigma) 0.2 mg/kg and hydrochlorothiazide (HCTZ, Sigma) 4 mg/kg. Drugs were diluted to concentrations so that the volume of injection (mL) = weight in kg × 10.

**Electrophysiology**

Drug-treated postnatal mice at different ages were anesthetized and processed for slice preparation as previously described (Owens et al. 1996). Neonatal brains were quickly removed into ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM) 124 NaCl, 5 KCl, 1.25 NaH2PO4, 1 MgSO4, 2 CaCl2, 26 NaHCO3, and 10 glucose oxygenated with 95% O2–5% CO2 (pH 7.4). Neonatal brains were embedded in 4% low melting point agarose in ACSF, hardened on ice, and cut into coronal slices (300 μm thick) using a vibratome (Leica VT1000s). Older animals were anesthetized with averitin (0.5–0.75 mg/g) and perfused with ice-cold solution containing (in mM) 248 Sucrose, 5 KCl, 1.25 NaH2PO4, 1 MgSO4, 2 CaCl2, and 26 NaHCO3 prior to brain removal in ACSF. Brains from older animals were cut directly on the vibratome, and sections are confined to the dorsal regions of the cortex. Electrophysiological recordings were obtained at room temperature (RT) from sections continuously perfused with oxygenated ACSF. For recordings at 32 °C, an inline heater was used to control the temperature of the bath solution (Warner 9C-20). Pyramidal neurons were visualized by microscopy under differential interference contrast and identified by their morphology, location within layer II/III of cortex, and current profile in response to 20-mV voltage steps (Noctor et al. 2004; Wang and Kriegstein 2008). Microelectrodes (6–8 MΩ) were pulled from borosilicate glass capillaries (Cornig 7056 Thin Wall) using a 2-step pipette puller (Narishige) and filled with the following solution (in mM): 130 KCl, 0.4 CaCl2, 1 MgCl2, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.3), 11 ethylene glycol tetraacetic acid, and 0.1% Lucifer yellow for majority of the recordings. For perforated patch recordings, the granicidin stock (10 mg/mL in dimethyl sulfoxide) was diluted in the internal pipette solution above to a final concentration of 25 μg/mL. Tip of the pipette was filled with the gramicidin-containing solution and the rest with normal internal pipette solution. Gigahm seals were made, and cells were applied voltage ramps from -140 to +100 mV every 20 s to monitor current changes. After ramp currents stabilized, 3 s focal application of 100 μM GABA (Sigma) was applied using the DAD superperfusion (pressure drug application) system (ALA Scientific) to activate GABA-A receptors on neurons. Voltage ramps from -140 to +100 mV were applied before and after GABA application. The resulting currents were then subtracted, and the difference plotted as I-V curves. To characterize miniature post synaptic currents (mPSCs), 5–20 min of whole-cell recordings were made using a Double Patch-clamp EPC9/2 (HEKA) with 10 kHz data acquisition frequency. The series resistances were monitored and only those that changed less than 20% during experiments were used for data analysis. Traces were analyzed using MiniAnalysis (Synaptosoft) program. Each synaptic event was manually selected based on rise time, amplitude, and decay properties. Additional drugs were added as indicated with the following estimated final concentrations: bicuculline methiodide (100 μM, Sigma), DNQX (20 μM, Tocris), tetrodotoxin (TTX, 0.5 μM, CalBiochem). To record N-methyl-D-aspartic acid (NMDA) channel currents, recordings were performed in ACSF containing TTX (0.5 μM), bicuculline methiodide (100 μM), 6,7-Dinitroquinoxaline-2,3-dione (DNQX) (20 μM), and Glycine (40 μM, Sigma). Unless otherwise noted, the holding potential for voltage clamp recordings was -70 mV.

**Inhibition-Excitation Ratio**

To calculate the frequency of excitatory post synaptic current (EPSC) and inhibitory post synaptic currents (IPSCs), cells were recorded for 1–2 min to establish the baseline mPSC frequency. Focal application of bicuculline (100 μM) was applied for 1 min to isolate the EPSC frequency, and IPSC frequency is calculated by subtracting the EPSC frequency from the baseline mPSC frequency. Whenever possible, DNQX (20 μM) was applied after washout of bicuculline to isolate the IPSC frequency. Application of both bicuculline and DNQX abolished all mPSCs in our recording conditions. To calculate the inhibition-excitation ratio, we used the ratio of IPSC to EPSC frequencies for each cell.

**In Utero Injection and Electroporation of Plasmids**

Plasmids expressing green fluorescent protein (GFP) or yellow fluorescent protein (YFP) under the phosphoglycerate kinase (PGK) promoter were introduced into the in vivo developing cortex by intraventricular injection and electroporation (Saito and Nakatsuji 2001). The PGK gene encodes for the constitutively active glycolytic enzyme PGK and its promoter is used in high-expression vectors (Graham and Chambers 1997). Intraventricular injections were carried out in E15 in timed-pregnant Swiss-Webster mice as previously described (Noctor et al. 2001). Electroporations were performed using an Electro Square Porator ECM830 (Genetronics) (5 pulses, 45 mV, 100 ms, 1-s interval). One microliter of DNA was injected per brain at a concentration of 1.5 μg/μL.

**Morphological Analysis**

Electroporated postnatal mice were perfused as previously described, and 300-μm-thick coronal sections were cut using a vibratome (Leica VT1000B) and stored in PBS. Images of GFP or YFP pyramidal neurons were acquired on a Leica TSC SP5 laser-scanning confocal microscope. For morphology analysis of the GFP- pyramidal neurons, 3D reconstructions of the cell body and dendritc processes were made from Z-series stacks of confocal images using the Imaris software. All dendrite properties were quantified using the filament function from Imaris, which constructs a 3D representation of each segment of the dendrite with a series of cylinders or truncated cones. Dendrite volume is defined as the sum of the volumes of all dendrite segments. For soma area analysis, the images were collapsed and the cell body was semiautomatically traced with NIH Imagej. Spine densities were calculated by counting the number of spines on a high magnification collapsed Z stack and dividing the total spine number by the length of dendrite. Only bright protrusions adjacent to or contiguous with the dendrites are counted as spines. Dim blebs were not counted. Spines were discriminated from one another when discrete edges are detected between the individual spines.

**Developmental Assessment**

The protocols were adapted from previous study by Scearce-Levie et al. (2008). Dams were treated with PBS or bumetanide beginning on gestational day 15 and their pups treated until postnatal day 7. Beginning on P2, pups were individually numbered to blind the experimenter and tested daily for developmental milestones appropriate for the age. Early milestone testing ranged from P2 to P9 and consisted of surface righting, grasp reflex, cliff avoidance, and negative geotaxis. Later milestone testing ranged from P10 to P17 and consisted of visual placing, bar hanging, and air righting. Pups were picked at random and tests were performed in the order stated above, from the least to most traumatic (i.e., performing tests that involve falling at the end of each session) to avoid carryover effects. Unless otherwise noted, absence of a milestone was scored if the mouse did not exhibit the behavior within 60 s on the first try. During testing, all pups were transferred to a cage filled with clean bedding. Individual pups were removed, weighted, and checked for physical abnormalities including head and body deformities. To assess surface righting, each pup was placed on its back and monitored until it successfully righted itself. To assess negative geotaxis, the pup was placed facing downward on a sheet of textured plastic inclined at a 30° angle and monitored until the pup reoriented itself with its head and forelimbs higher up the plane than its hindlimbs. To assess cliff avoidance, the pup was placed with its hindlimbs resting on a circular Styrofoam platform mounted on a 30-cm high stand. The pup was positioned so its forepaws and nose were suspended over the edge of the platform and monitored until the pup moved away from the edge. To assess grasp reflex, each forepaw was gently stroked with the wooden end of a swab. If the pup...
immediately curved its paw to grasp the swab, the grasp reflex was considered present. The date when both eyes were open was recorded. Visual placing was assessed by suspending the pup by its tail and gently lowering it toward the tabletop. If the pup raised its head and extended forelimbs toward the surface, visual placing was scored as present. To assess air righting, the pup was held with its ventral side facing upward 30 cm above a chamber filled with soft bedding. The pup was released, and air righting was considered present if the pup turned while falling so that it landed on its feet. To assess bar hanging, the pup was allowed to grasp a small wire bar and then released so that it was hanging by its forelimbs. Once the pup was able to hang suspended for 10 s, bar holding was scored as present.

Figure 1. Bumetanide hyperpolarizes GABA reversal potential in immature neurons (A) and (B) voltage-ramps obtained from P0 cortical neurons of pups treated with intraperitoneal injections of either saline or bumetanide under gramicidin-perforated patch-clamp conditions. $E_{\text{GABA}}$ reversed at more hyperpolarized potentials in bumetanide-treated mice than in controls (as indicated by the $x$-intercept). (C) Blocking NKCC1 with bumetanide effectively changed the chloride gradient in newborn cortical neurons, making GABA hyperpolarizing to the resting potential instead of depolarizing as in control cells. Bar graphs indicate mean ± SEM (number of recorded cells are indicated in parenthesis in bar graphs, $n = 3$ animals per condition, ***$P < 0.0001$, t-test). (D) Comparison of $E_{\text{GABA}}$ values for control- and bumetanide-treated cells recorded at RT and at 32 °C. Increasing the recording temperature did not significantly alter the $E_{\text{GABA}}$. Bar graphs indicate mean ± SEM (number of recorded cells are indicated in parenthesis in bar graphs, $n = 2$ animals at 32 °C, ***$P < 0.0001$, t-test). (E) Timeline of different drugs treatments. Black line represents length of control (PBS) treatment, and colored lines represent different bumetanide (Bum) exposure windows.
Social Preference
The protocols were adapted from a previously studied construct (Scearce-Levie et al. 2008). Mice were tested in a 3-chambered box with small openings in the dividing walls allowing access from the center chamber into left and right chambers. Each chamber was cleaned and fresh bedding added between trials. The paradigm consisted of a 3-stage procedure. Stage 1: habituation; test mouse was first placed in the center chamber and allowed to explore all 3 chambers of the apparatus for 5 min. Stage 2: Sociability; an unfamiliar mouse (male C57BL6 mouse) was placed in either the left or right chamber enclosed in a small internal wire cage; placement of Stranger 1 in the left or right chamber alternated between trials, with an empty but otherwise identical wire cage in the opposite chamber. Following placement of Stranger 1 into the left or right chamber, the test mouse was allowed to leave the center chamber and explore all 3 chambers of the apparatus for 10 min, and time spent in each compartment was recorded. Stage 3: Novelty; with the initial stranger (now familiar) retained in its original chamber, a second, unfamiliar mouse (novel, male C57BL6 mouse) was placed in the previously empty wire cage in the opposite chamber. The test mouse was allowed to leave the center chamber and explore all 3 chambers of the apparatus for a second period of 10 min, and time spent in each compartment was recorded.

Open Field
Testing was conducted as previously described, and experimenter was blinded to the condition (Cheng et al. 2007). Activity in the open field was tested with the automated Flex-Field/Open Field Photobeam Activity System (PAS; San Diego Instruments). The system consisted of 2 identical clear plastic chambers (41 x 41 x 38 cm), a PAS control box, a PC interface board, and a microcomputer for recording and analysis of data. Two sensor frames, each consisting of a 16 x 16 photobeam array at 1.5 and 6 cm above the bottom of the cage, were used to detect movements in the horizontal and vertical planes. The test was initiated by placing the mouse in the center of the arena. Horizontal beam breaks (ambulatory moves) in the arena were counted more than 15 min. The arena was cleaned and dried after each test. The illumination level of the box was 271 lux.

Elevated Plus Maze
Testing was conducted as previously described, and experimenter was blinded to the condition (Cheng et al. 2007). The elevated plus-shaped maze consisted of 2 open arms and 2 closed arms equipped with rows of infrared photobeams (Hamilton-Kinder). Mice were habituated to dim lighting in the testing room for 30 min and then were placed individually at the center of the apparatus and allowed to explore for 10 min. The time spent and distance traveled in each of the arms were recorded by infrared beam breaks. After each mouse was tested, the apparatus was thoroughly cleaned.

Startle Reactivity and Prepulse Inhibition
Acoustic startle reactivity and prepulse inhibition (PPI) were measured as described, and experimenter was blinded to the condition (Esposito et al. 2006). Acoustic startle reactivity was measured with 2 identical startle chambers (Hamilton-Kinder) containing a transparent non-restrictive plexiglas box resting on a platform inside a soundproof ventilated box. An acoustic speaker generating broadband bursts was mounted 15 cm above the box produced all the acoustic stimulus. Mouse movements were detected and transduced by a piezoelectric accelerometer mounted under each cylinder. Movements were digitized and stored by a computer and interface assembly. Movements were monitored for 65 ms after the onset of each stimulus, and the maximum amplitude response (Newtons) was used to determine the startle response. All tests were performed during the same phase of the light cycle, between the 12-h light cycles from 8 AM to 8 PM.

For testing, mice were placed inside the soundproof chamber. After a 5-min acclimation period, each session consisted of 80 trials of 5 types: 24 trials of a 40 ms, 120 dB (sound pressure level scale) startle stimulus alone (to measure maximum acoustic startle amplitude); 14 trials without startle stimulus (to measure baseline movements in the chamber); and 14 trials each of a 40-ms stimulus at 4, 8, or 16 dB above the 65 dB background (prepulse), followed by a 100-ms interval and a 40 ms 120-dB startle stimulus. In the first 5 and the last 5 trials of each session, the startle stimulus alone was presented to determine the degree of habituation to the startle stimulus. The other trials of startle stimulus alone and prepulse plus startle stimulus were presented in pseudorandom order with an average intertrial interval of 15 s (range: 7-25 s). The percentage of PPI of the startle response was calculated as follows: 100 - ([average response to prepulse plus startle stimulus/average response to startle stimulus alone] x 100). Thus, a high value indicates high PPI reflected by a large reduction in startle response when the prepulse preceded the startle stimulus.

Statistical Analysis
Group measures are expressed as mean ± standard error of the mean (SEM); error bars also indicate SEM. Prism software (version 4.0) was used for statistical analysis. We assessed the statistical significance or differences between control and experimental conditions with a 2-tailed Student’s t-test, a one-way analysis of variance (ANOVA) for 3 or more groups and a 2-way repeated measure ANOVA with posttest for the PPI test. For developmental milestone tests, we used logrank test to assess for statistical significance. Experimenter were blinded during testing. Statistical significance was set at P < 0.05.

Results
Bumetanide Negatively Shifts GABA Reversal Potential in Newborn Neurons
To test the effect of bumetanide on the GABA reversal potential (E>GABA), we gave daily intraperitoneal injections of either bumetanide (0.2 mg/kg) (Dzhala et al. 2005) or PBS to pregnant mice beginning at gestational day 15 (E15) until birth (P0). We performed gramicidin-perforated patch recordings of P0 cortical neurons in acute brain slices from either bumetanide- or saline-treated pups to confirm that bumetanide can shift E>GABA toward a hyperpolarizing potential. Perforated patch recording allowed us to measure the intracellular Cl concentration without perturbing the Cl gradient. At room temperature, E>GABA was significantly more negative in neurons of bumetanide-injected animals than in controls (t-test, control vs. bumetanide: −40.9 ± 2.8 mV vs. −65.8 ± 3.4 mV; P < 0.0001; Fig. 1A–C). With the unaltered resting membrane potential of cortical neurons at P0 (PBS: −50.9 ± 5.1 mV, bumetanide: −51.1 ± 4.4 mV; P > 0.05), GABA hyperpolarized the neonatal cortical neurons of bumetanide-treated mice, thus validating the effectiveness of bumetanide in abolishing GABA-induced depolarization (Fig. 1C). Because temperature can affect transporter kinetics, we repeated our recordings at more physiological temperatures. The E>GABA values from recordings performed at 32 °C were not significantly different from those recorded at room temperature of 25 °C, which confirms the efficacy of bumetanide to block NKCC1 in the brain (t-test, control RT vs. 32 °C: −40.9 ± 2.8 mV vs. −43.7 ± 0.7 mV; P > 0.05; bumetanide RT vs. 32 °C: −65.8 ± 3.4 mV vs. −68.1 ± 0.9 mV; P >0.05; Fig. 1D).

Blocking NKCC1 with Bumetanide during a Critical Period Leads to Lasting Changes in Cortical Excitatory Transmission
A recurrent theme in neocortical development is the idea that changes to sensory afferents during a critical period in
development will result in lasting alterations in cortical organization. The formation of ocular dominance columns in the visual cortex and barrels in the somatosensory cortex are well-known examples of such critical periods (Hensch 2005; Hensch and Fagiolini 2005). Because GABAergic signaling provides the main excitatory drive during corticogenesis (until the first postnatal week in rodents), the period of GABA depolarization may provide a window for initial circuit formation in the neocortex (Ben-Ari 2006).

To test the effect of bumetanide in cortical circuit formation, we exposed pregnant mice and their pups to bumetanide at different developmental windows (Fig. 1E). We used PBS treatment from E15 to P7 and bumetanide treatment from P7 to 14 (after GABA becomes hyperpolarizing, (Rivera et al. 1999) as controls. To measure synaptic connectivity of cortical neurons, we recorded mPSCs in layer II/III pyramidal neurons at a holding potential of −70 mV in the presence of TTX (0.5 μM) to block action potentials. Even though pyramidal neurons in layer II/III may not homogenously, we studied this population because they are the main source of intracortical synaptic connections, and our previous study showed that depolarizing GABA is important for circuit formation of these upper layer cortical neurons (Wang and Kriegstein 2008). To isolate the α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA) and GABAergic mPSCs, we bath applied the GABAA receptor antagonist bicuculline methiodide (100 μM) or the non-NMDA glutamate receptor antagonist DNQX (20 μM), respectively (Fig. 2AB). In 4-week-old control animals, the pyramidal neurons exhibited robust AMPA and GABA synaptic innervation with approximately equal numbers of excitatory and inhibitory mPSCs (PBS: AMPA mPSCs: 2.0 ± 0.2 Hz, GABA mPSCs: 2.3 ± 0.3 Hz, n = 19, 3 animals; Fig. 2C). By contrast, bumetanide treatment during the entire period when GABA is normally depolarizing (E15–P7) resulted in a drastic 10-fold reduction in the frequency of AMPA mPSCs (PBS vs. bumetanide: 2.0 ± 0.2 Hz vs. 0.22 ± 0.07 Hz, bumetanide n = 16, 3 animals, P < 0.0001), while GABA mPSC frequency was unchanged (PBS vs. bumetanide 2.3 ± 0.3 Hz vs. 2.4 ± 0.4 Hz, bumetanide n = 16, 3 animals, P > 0.05; Fig. 2C). Calculating the ratio of inhibitory to excitatory synaptic events at 4 weeks revealed that bumetanide-treated mice had a significantly larger ratio of inhibitory mPSCs compared with the controls (PBS vs. bumetanide E15–P7, 1.2 ± 0.2 vs. 10.6 ± 3.9, P = 0.0113; Fig. 2C). This defect in forming excitatory synaptic inputs persisted to adulthood and resulted in a large ratio of GABA to AMPA mPSCs (PBS vs. bumetanide E15–P7, 0.98 ± 0.22 vs. 2.68 ± 0.17, P < 0.0001; Fig. 2D).

The outcome of bumetanide treatment on AMPA and GABA synaptic transmission remained the same in recordings performed at 32 °C, suggesting that the effects occur at physiological temperatures (Supplementary Fig. 1AB). Furthermore, bumetanide treatment had no effect on the amplitude and kinetics of the AMPA and GABA mPSCs, suggesting that the decrease in AMPA frequency likely reflects the decrease in synaptic input rather than changes in AMPA receptor signaling (Supplementary Fig. 1C–F). The decrease in AMPAergic transmission was not accompanied by defects in NMDA receptor signaling, as NMDA mPSCs isolated by recording in 0 Mg2+ bath solution containing bicuculline and DNQX revealed unaltered frequency and amplitudes (Supplementary Fig. 2A–E).

To determine the minimal window during which bumetanide treatment would result in permanent changes in cortical excitatory synaptic transmission, we exposed mice to bumetanide at different developmental windows (E15–E19, E15–P5, E17–P7, P0–P7, and P7–P14; Fig. 1E). Neither prenatal treatment (E15–E19) nor postnatal treatment (P0–P7 and P7–P14) with bumetanide resulted in significant changes in AMPA or GABA mPSC frequencies (Fig. 2C,D). However, bumetanide injections from E17 to P7 produced similar effects as treatment from E15 to P7 (Fig. 2C,D). Interestingly, it appears that bumetanide treatment from E17 to P7 resulted in a transient decrease in GABA mPSC frequency at 4 weeks (PBS vs. E17–P7: 2.3 ± 0.3 Hz vs. 0.9 ± 0.4 Hz, E17–P7: n = 7, 2 animals; P = 0.0480). However, it is unclear whether this decrease in GABA mPSC was meaningful as this difference barely reached statistical significance and disappeared by adulthood. When examining the ratio of GABAergic to AMPA mPSCs, the control conditions had a near 1:1 ratio of inhibitory to excitatory synapses, but bumetanide exposure from E15 to P7 and E17 to P7 significantly increased the ratio both at 4 weeks and in adults (4 weeks: PBS vs. E17–P7: 1.16 ± 0.19 vs. 2.46 ± 0.66, P = 0.0247; adult: PBS vs. E17–P7: 0.98 ± 0.23 vs. 1.78 ± 0.17, P = 0.0064; Fig. 2C,D). Together these data suggest that systemic blockade of NKCC1 with bumetanide during the period of GABA depolarization permanently disrupts excitatory synapse formation in the cortex, resulting in an abnormal balance between excitatory and inhibitory inputs in the adult brain.

**Bumetanide Disrupts Morphology of Cortical Neurons**

Disruptions in synaptic connections are often reflected by changes in neuron morphology. Previous studies have shown that AMPA receptor trafficking into developing synapses is required to stabilize new dendritic branches (Rajan and Cline 1998; Wu and Cline 1998; Shi et al. 1999; Haas et al. 2006). To examine the effect of bumetanide treatment on the morphology of cortical neurons, we performed in utero injection and electroporation of plasmids expressing either GFP or YFP into E15 mice embryos during the period of generation of upper layer cortical neurons. We injected the animals with either PBS or bumetanide from E15 to P7 and fixed and sectioned the brain at 4 weeks to take confocal images of labeled neurons. At 4 weeks postnatal, GFP neurons from PBS- and bumetanide-injected animals had typical pyramidal neuron morphology and indistinguishable soma sizes (Fig. 3A–C). However, neurons from bumetanide-treated animals exhibited fewer primary and secondary dendrites compared with control cells (t-test, primary: PBS vs. bumetanide, 6.9 ± 0.2 vs. 5.3 ± 0.2, P < 0.0001; secondary: PBS vs. bumetanide, 11.9 ± 0.5 vs. 8.1 ± 0.3, P < 0.0001; PBS: n = 16, 3 animals, bumetanide n = 48, 4 animals; Fig. 3D,E). We semiautomatically traced the neurons to further evaluate the effect of bumetanide treatment on morphology. We found that compared with controls, bumetanide-treated cells had significantly decreased dendrite length, dendrite volume, branch levels, branch points, number of dendrite segments, and terminal points (PBS: n = 17, 3 animals, bumetanide n = 18, 4 animals; t-tests, PBS vs. bumetanide, P < 0.0001 for all parameters; Fig. 3F, Supplementary Fig. 3C–F). This extensive impairment of dendrite formation in bumetanide-treated cells is consistent with recent evidence showing that membrane depolarization by GABA is critical for the morphological maturation of cortical neurons (Cancedda et al. 2007; Wang and Kriegstein 2008).
To further elucidate the effect of systemic bumetanide treatment on synapse formation, we measured dendritic spines, which are postsynaptic sites of excitatory synapses. We used confocal microscopy to analyze the mean spine density on secondary dendrites of GFP+ or YFP+ cells from animals treated with PBS or bumetanide from E15 to P7. We found that the mean spine density in PBS-treated animals was 0.41 ± 0.01 spines/µm, whereas in the bumetanide-treated
animals, the density was significantly decreased to 0.34 ± 0.01 spines/μm (PBS: n = 56, 3 animals; bumetanide n = 47, 4 animals; t-test, P < 0.001; Fig. 4A-C). The decreased spine density in bumetanide-injected animals reflected the overall decrease in presynaptic release events, as evidenced by the decreased in AMPA mPSCs, confirming that blocking GABA-mediated depolarization during a critical period disrupts cortical synaptogenesis.

Figure 3. Bumetanide treatment alters morphology of cortical neurons. (A) and (B) 3D reconstructions of 2 cortical neurons from 4-week-old control or bumetanide-treated (E15-P7) mice. Animals were electroporated with GFP plasmids at E15 for visualization of the cells. Photographs on the left show flattened confocal stacks of neurons that were semi-automatically traced to produce dendritic diagrams on the right. Multiple rotational views of the same 2 neurons are shown on the right. Quantification of the soma size (C), number of primary dendrites (D), number of secondary dendrites (E), and total branching points (F) of traced cortical neurons. Compared with control, bumetanide-treated pyramidal neurons have less developed dendritic morphology. Scale bar, 20 μm. Bar graphs indicate mean ± SEM (PBS: n = 3 animals, bumetanide n = 4 animals; ***P < 0.0001, t-test).
Bumetanide Treatment Results in Developmental Delay

Abnormal cortical circuits have been implicated in many neurological and psychiatric disorders including epilepsy, obsessive-compulsive disorder, autism, and schizophrenia (Treiman 2001; Nordstrom and Burton 2002; Casanova et al. 2003; Lisman et al. 2008). To assess the functional consequences of bumetanide treatment, we conducted a broad screen for differences in various fundamental behavioral domains. We began our analysis in control or bumetanide-treated (E15–P7) young pups to assess their ability to reach developmental milestones. On average, bumetanide-treated pups weighed less than their age-matched controls during the treatment period, as expected due to this drug’s diuretic effects on the kidney (Fig. 5A). Bumetanide did not affect the age at which the pups attained the grasp reflex, surface righting, pinnae detachment, air righting, and cliff avoidance reflexes (Supplementary Fig. 3A–E). However, it did delay the pups’ negative geotaxis, bar holding, and visual placing abilities (logrank test: negative geotaxis: $P < 0.05$; bar holding: $P < 0.05$; visual placing: $P < 0.05$; $n = 30$ animals per condition Fig. 5C–E). Furthermore, pups treated with bumetanide were less active compared with controls (Fig. 5B). As a control for reduced weight from diuresis, we treated mice with high doses of the diuretic HCTZ from E15 to P7 and assessed their development. As an additional control for the systemic effects of bumetanide, we gave daily bumetanide injections to pups from P7 to P14 (bumetanide P7 to P14), during a period when GABA is no longer depolarizing. HCTZ-treated mice weighed less than their age-matched controls until P7 when the injections stopped, and similarly, bumetanide treatment from P7 to P14 decreased the weight of the pups during that period (Supplementary Fig. 4A). However, neither HCTZ or bumetanide treatment from P7 to P14 resulted in any signs of the developmental delays seen in bumetanide-treated mice (Supplementary Fig. 4B–J). These observations suggest that reduced weight due to bumetanide treatment is unlikely to have caused the behavioral abnormalities seen and that bumetanide treatment during the perinatal period results in decreased strength and motor coordination.

Bumetanide Treatment Decreases Anxiety-Related Behavior and Impairs PPI of the Startle Reflex

We performed a battery of behavioral tests to assess possible differences between control and bumetanide-treated (E15–P7) adult mice (8–12 weeks old). We began with general neurological tests and found no significant difference between control versus bumetanide-treated animals’ weights, activity levels, activity intervals, and latency to stop twisting.

Figure 4. Bumetanide treatment decreases spine density of cortical pyramidal neurons. (A) and (B) images of secondary dendrites from 4-week-old cortical pyramidal neurons from control or bumetanide-treated mice electroporated with GFP at E15. (C) Quantification of the average spine density shows that neurons from bumetanide-treated animals exhibited a significant spine decrease compared with controls. Scale bar, 20 μm. Bar graphs indicate mean ± SEM (PBS: $n = 56$, 3 animals; bumetanide $n = 47$, 4 animals; ***$P < 0.0001$, t-test). Quantification of reconstructed cells demonstrated that total dendrite length (D), total dendrite volume (E), total number of dendrite segments (F), number of dendrite terminal points (G), and full branch level (H) are all significantly decreased in bumetanide-treated pyramidal neurons compared with control. Bar graphs indicate mean ± SEM (PBS: $n = 17$ cells, 3 animals, bumetanide $n = 18$ cells, 4 animals, ***$P < 0.0001$, t-test).
The developmental delays seen in negative geotaxis and bar holding were not present in adulthood as both groups performed comparably in these tasks (Supplementary Fig. 5A). To test for activity and anxiety-related behavior, we performed the open field test and found that bumetanide treatment did not change the total movements made in the center or periphery of the field (Supplementary Fig. 5B). To test for social behavior, we placed the animals in the center chamber of a 3-chambered cage. The test occurred in 3 phases: the habituation phase (right and left chambers were empty), the socialization phase (empty side and social side with a bait mouse), and novelty phase (familiar mouse on one side and novel mouse in the other). In these assays, animals with autism-like behaviors would spend less time socializing and have problems recognizing novel objects. However, we did not see any significant differences between control and bumetanide-treated animals, though it took a longer time for bumetanide-treated animals to initiate socialization with both the familiar and novel mice in the cage (Supplementary Fig. 5C).

To further test for anxiety-related behaviors, we performed the elevated plus maze, where a tendency to spend more time in the open arms is thought to be an operational measure of decreased anxiety or emotionality (Dawson and Tricklebank 1995). Bumetanide-treated animals showed significantly increased entries into the open arms and trended toward spending more time and traveling longer distances than controls in the open arms (PBS vs. bumetanide open entries: 8.1 ± 1.0 vs. 11.4 ± 1.0; n = 30 animals per condition, t-test, P < 0.05; Fig. 6A). There was no significant difference between the 2 treatment groups in closed arm time, distance, and entries (Fig. 6B).

One of the widely used cross-species behavioral paradigms to measure sensorimotor gating, commonly altered in schizophrenia patients, is the PPI of the startle response (Swerdlow et al. 2000; Braff et al. 2001). PPI occurs when a low-intensity prepulse precedes a startle stimulus, resulting in a reduced startle response. To assess whether bumetanide resulted in sensory or startle disturbance, we first tested the animals’ maximum startle response. We found that bumetanide-treated animals showed a significantly higher startle amplitude compared with PBS-treated controls with the 120-dB stimulus without a prepulse (PBS vs. bumetanide 120-dB stimulus: 2.32 ± 0.21 N vs. 2.95 ± 0.11 N, n = 29 per condition, t-test, P < 0.05; Fig. 6C). This indicates that bumetanide does not impair, but rather, may increase the animal’s sensory response.
reactivity. Importantly, when we analyzed control and bumetanide groups for their maximal PPI baseline, we found that PPI was significantly impaired in mice treated with bumetanide compared with PBS treatment (2-way ANOVA, \(n = 29\) per condition, \(P < 0.0001\); Fig. 6D). Because the differences in initial startle response might confound the effects of PPI, we performed a post hoc analysis of animals with similar startle response. We selected animals that responded to the 120-dB stimulus with >1.5 N for both control (\(n = 21\)) and bumetanide-treated (\(n = 29\)) groups. In our post hoc analysis, the decrease
in PPI is still significant between the treatment groups despite having no significant differences in their startle response, confirming our finding of impaired sensorimotor gating seen with bumetanide treatment (2-way ANOVA of PPI, \(P < 0.0001\); Fig. 6E,F). To further ensure that the impairment in PPI is specific to perinatal bumetanide treatment, we repeated these tests with another cohort of animals treated with PBS, HCTZ, or bumetanide from P7–P14. While animals in the bumetanide P7–P14 group did show a significant increase in their startle response to the 120-dB stimulus without the prepulse compared with the PBS-treated controls (PBS vs. bumetanide P7–P14 120-dB stimulus: 1.27 ± 0.16 N vs. 2.08 ± 0.16 N, \(n = 12\) per condition, \(t\)-test, \(P < 0.05\); Supplementary Fig. 6A), neither HCTZ or bumetanide P7–P14 demonstrated the decreases in PPI seen in animals treated perinatally with bumetanide (2-way ANOVA, \(P > 0.05\); Supplementary Fig. 6B). These data suggest that blocking NKCC1 activity, and thus abolishing the excitatory effects of GABA, during a critical period results in functional impairments in sensorimotor gating functions in the adult mice.

### Discussion

A precise balance of excitatory and inhibitory synaptic inputs is essential for the proper function of mammalian cortical circuits. Disruptions in this balance can lead to severe neurological and psychological disorders. We now provide novel evidence that systemic blockade of early GABA-mediated depolarization leads to lasting disruption in AMPA receptor-mediated glutamatergic transmission in the adult cortex. First, using electrophysiological recording, we show that abolishing GABA-mediated excitation in vivo during a critical period significantly shifts the balance between excitation and inhibition in the adult cortical circuit. Second, we show through an array of morphological analyses that dendritic arbors as well as spine density are reduced when GABA excitation is blocked. Finally, we show that treating animals with bumetanide during the perinatal period results in developmental delay and impairment in sensorimotor gating. These findings support a cautious approach to the clinical use of bumetanide in infants since cortical circuits are still maturing at this stage.

A variety of studies have shown the importance of GABA-mediated depolarization in excitatory synapse development. In *Xenopus* tectal neurons, a premature hyperpolarizing shift of \(E_{\text{GABA}}\) increased the ratio of inhibitory to excitatory synapses (Akerman and Cline 2006). In the mouse neocortex, altering the chloride gradient of immature cortical neurons in vivo also disrupted AMPA synapse development and altered the balance between inhibitory and excitatory synapses (Wang and Kriegstein 2008). Here, we not only show that this disruption in excitatory synaptic transmission is long lasting but also that there is a critical period for excitation-inhibition matching that occurs around E17–P7 in mice, a period that corresponds to robust cortical synaptogenesis and overlies the developmental window when GABA is depolarizing (Owens et al. 1999; Ben-Ari 2006). Interestingly, blocking GABA-mediated depolarization during either the prenatal period (E15–E19) or postnatal period (P0–P7) did not produce a persistent effect on AMPA transmission. This suggests that the window from E17 to P7 is a sensitive period during which immature cortical neurons are primed to receive glutamatergic inputs. Any moderate level of depolarizing activity during this period would allow the neurons to develop appropriate levels of AMPAergic inputs. Therefore, only inhibiting NKCC1 activity during this entire period would be able to produce a lasting disruption in excitatory synapse development. In addition, the normal amplitudes and kinetics of the AMPA and GABA mPSCs suggest that the decrease in excitatory mPSC frequency is likely due to an overall decrease in the number of functional excitatory inputs rather than postsynaptic scaling.

The morphological changes in cortical neurons that result from bumetanide treatment confirm 2 previous reports that examined the consequences of abolishing the excitatory effects of GABA in development. These previous studies used a single-cell genetic approach to alter \(E_{\text{GABA}}\) and produced cortical neurons with fewer and shorter dendrites as well as decreased spine density (Cancedda et al. 2007; Wang and Kriegstein 2008). The anatomical disruption resulting from bumetanide treatment is correlated with the physiological changes seen since dendritic spines constitute major postsynaptic sites for excitatory synaptic transmission. Our data seem to contradict the results of a recent study whereby morphology and synaptic density of hippocampal CA1 pyramidal neurons are unaffected in *Nkcc1*-null mice (Pfeffer et al. 2009). This discrepancy may be partially attributed to compensation that occurred in their genetic model as GABA remains slightly depolarizing in *Nkcc1*-knockout mice. Our short-term pharmacological manipulation bypasses this caveat the hyperpolarization caused by GABA may explain the morphological changes seen in our study. More interestingly, these types of morphological abnormalities have been linked to a variety of neurological and psychiatric disorders including schizophrenia. For instance, mice with a microdeletion of 22q11.2 orperturbation in the neuregulin-1 signaling cascade, both of which are highly associated with schizophrenia, show decreased spine and glutamatergic synapse density, as well as impaired dendrite growth (Li et al. 2007; Mukai et al. 2008).

We demonstrate that treatment with bumetanide during a critical early period results in developmental delay and long-term impairment in sensorimotor gating. Bumetanide-treated pups demonstrated a delay in achieving milestones associated with motor coordination and strength (negative geotaxis and bar holding), and a relative decrease in locomotor activity. Even though these delays do not persist into adulthood, they mark disruptions in normal development. These observations suggest that it may be important to monitor for developmental delay in clinical trials where bumetanide is used to treat neonatal seizures.

Even though bumetanide treatment was systemic, the altered behavioral phenotypes seen in adult mice are most likely due to the changes in cortical synaptic transmission. No evidence suggests that Swiss-Webster mice have any predisposition to neurological or behavioral phenotypes. Because the brain matures in a caudal–rostral gradient, by E15, NKCC1 expression is largely confined to the telencephalon and, by P0, largely to the cortical plate (Li et al. 2002). Therefore, our bumetanide treatment window should primarily affect cortical neurons without affecting other subcortical structures. Furthermore, bumetanide treatment from P7 to P14 (after \(E_{\text{GABA}}\) becomes hyperpolarizing) showed no disturbance in cortical circuit development or behavioral abnormalities in PPI. PPI is thought to reflect sensorimotor gating, which is a fundamental component of information processing in the brain necessary for stimulus recognition and sequential organization of stimuli.
behavior and is severely disrupted in patients with schizophrenia due to altered forebrain circuits that regulate inhibition via descending influences on the pontine tegmentum (Braff et al. 2001; Swerdlow et al. 2001; Bast and Feldon 2003). Interestingly, many lines of evidence have linked glutamate dysfunction in the cortico-limbic circuit with schizophrenia. For instance, the noncompetitive NMDA antagonist phencyclidine (PCP) can produce a drug-induced model of schizophrenia with impairments in PPI as well as other positive symptoms such as visual and auditory hallucination (Jentsch and Roth 1999; Lisman et al. 2008). Acute treatment of experimental animals with PCP produces an overall decrease in the level of AMPA glutamate receptor density especially in the hippocampus, dentate gyrus, parietal cortex, and amygdala (Zavitsanou et al. 2008). In addition, neuregulin-1 dysfunction, which has been strongly associated with schizophrenia, is thought to contribute to the disease by destabilizing synaptic AMPA receptors leading to loss of synaptic NMDA currents and reduced number of spines (Li et al. 2007). Further evidence of involvement of glutamatergic signaling in schizophrenia is demonstrated in AMPA GluR1 knockout mice, which show PPI impairments and other cognitive defects seen in the disease (Wiedholz et al. 2008).

In our model, we effectively decreased cortical AMPA synaptic transmission by blocking GABA-mediated depolarization during development. Our bumetanide-treated animals replicated several features that resemble PCP models of schizophrenia, including PPI impairment, increase in the acoustic startle reflex, and decrease in AMPA receptor function in cortical structures (Curzon and Decker 1998; Geyer et al. 2002; Mukai et al. 2008). While it is unknown if all parts of the cortico-limbic circuit are affected by bumetanide treatment, future studies might elucidate the specific alterations in this circuit.

Whether our animal model of bumetanide-treated mice is a valid model for bumetanide treatment of human neonatal seizures remains to be elucidated. In rodents, NKCC1 is expressed from mid-embryonic stage (E12) until the first postnatal week (Plotkin et al. 1997; Li et al. 2002; Wang et al. 2002; Dzhala et al. 2005). Similarly, Dzhala et al. (2005) demonstrated that in the human cortex, NKCC1 is expressed from midgestation (~gestational week 20) until the first year of life with the highest expression occurring during the perinatal period. We found that the minimal window to cause deficits in cortical synapse formation is from E17 to P7 in mice, which correlates with the period of high NKCC1 expression. While current clinical trials do not involve bumetanide treatment during the prenatal period, given the differences in the length of cortical development between human and rodents, and our limited ability to test measurable behaviors in rodents, our results still warrant a cautionary approach to using bumetanide long-term in treating neonatal seizures.

An abnormal balance between excitation and inhibition of cortical circuits has been implicated in schizophrenia (Lisman et al. 2008). Recently, a genome-wide screen found rare structural variants that disrupt multiple genes in schizophrenia, and pathways involving neuregulin and glutamate were disproportionately affected (Walsh et al. 2008). Included in these pathways are genes involved in potassium-chloride transport, SLC12A9 and SLC12A6, which affect neuronal excitability (Moser et al. 2008; Walsh et al. 2008). Our data indicate that transient early bumetanide exposure produces a persistent clear disturbance in the formation of excitatory synapses in cortical neurons and leads to deficits in sensorimotor gating. While our data suggest caution for long-term use of bumetanide to treat neonatal seizures, side effects in short-term use remain to be evaluated, and bumetanide may still be beneficial in treating certain forms of epilepsy in the adult brain where altered neuronal chloride transport might be involved (Cohen et al. 2002; Pond et al. 2006).

Supplementary Material
Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

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References
Bumetanide Alters Cortical Circuits and Sensorimotor Gating Functions


