Oxytocin-Mediated GABA Inhibition During Delivery Attenuates Autism Pathogenesis in Rodent Offspring

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Science **343**, 675 (2014);
DOI: 10.1126/science.1247190

Science (print ISSN 0036-8075; online ISSN 1095-9203) is published weekly, except the last week in December, by the American Association for the Advancement of Science, 1200 New York Avenue NW, Washington, DC 20005.

of $[A]_o$ and $[A]_i$ on local $[Cl^-]_i$, and $[Cl^-]_o$ open possibilities for developmental and experience-dependent plasticity of E_{GABA} and $E_{glycine}$ at individual synapses, so that the variance in extracellular sulfated proteoglycans composes a potential locus of analog information storage and pathologically, a rich variety of antigens. (v) Pathological conditions that alter $[A]_o$ or $[A]_i$ will have secondary effects on both cell volume and $[Cl^-]_i$. This may explain the correlation between magnetic resonance imaging evidence of cytotoxic edema after brain injury and anticonvulsant-resistant seizures, which can occur when increased $[Cl^-]_i$ compromises $GABA_AR$-mediated inhibition (*15*, *40*). Thus, the magnitude and direction of $GABA_AR$ currents at individual synapses are among the wide variety of signaling functions subserved by intra- and extracellular macromolecular networks.

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Acknowledgments: This work was supported by National Institute of Neurological Disorders and Stroke, NIH, grant NS 40109-06 the Kennedy Endowment for Child Neurology and Mental Retardation. J.G. was supported by the American Epilepsy Society postdoctoral fellowship and NIH R25. K.E. was supported by The Japan Foundation for Pediatric Research. K.T.K. was supported by the Manton Center for Orphan Disease Research and NIH R25.

Supplementary Materials

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2 September 2013; accepted 12 December 2013
10.1126/science.1245423

Oxytocin-Mediated GABA Inhibition During Delivery Attenuates Autism Pathogenesis in Rodent Offspring

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We report that the oxytocin-mediated neuroprotective γ-aminobutyric acid (GABA) excitatory-inhibitory shift during delivery is abolished in the valproate and fragile X rodent models of autism. During delivery and subsequently, hippocampal neurons in these models have elevated intracellular chloride levels, increased excitatory GABA, enhanced glutamatergic activity, and elevated gamma oscillations. Maternal pretreatment with bumetanide restored in offspring control electrophysiological and behavioral phenotypes. Conversely, blocking oxytocin signaling in naïve mothers produced offspring having electrophysiological and behavioral autistic-like features. Our results suggest a chronic deficient chloride regulation in these rodent models of autism and stress the importance of oxytocin-mediated GABAergic inhibition during the delivery process. Our data validate the amelioration observed with bumetanide and oxytocin and point to common pathways in a drug-induced and a genetic rodent model of autism.

Autism is a developmental disorder characterized by restricted interest and communication impairment generated by genetic and environmental factors. Alterations of oxytocin signals that trigger labor and are instrumental for communication, notably, parental-infant interactions, are important in autism (*1*). Here, we characterized the cellular and network alterations that occur during the transition from fetal to postnatal life and subsequently in two animal models of autism: rats exposed in utero to valproate (VPA rats) and mice carrying the fragile X mutation (FRX mice). We focused on GABAergic inhibition, as this is deficient in human and animal models of autism, which leads to an imbalance between excitation and inhibition (*2*–*4*). In addition, during development, GABAergic currents shift from excitatory to inhibitory (*5*) because of a reduction of intracellular chloride concentration ($[Cl^-]_i$) mediated by a sequential expression of the main chloride importer (Na^+-K^+-$2Cl^-$ cotransporter, NKCC1) and the main chloride exporter KCC2 (*6*). Delivery in rodents is fundamental in this sequence, with an abrupt oxytocin-mediated reduction of $[Cl^-]_i$ levels that exerts neuroprotective (*7*) and analgesic (*8*) actions on newborns. We report that this sequence is abolished in hippocampal CA3 pyramidal neurons of VPA rats and FRX mice, and its restoration by administering bumetanide to the mother rescues the GABA developmental sequence and the autistic phenotype in rodent offspring.

In naïve rats (Fig. 1A and table S1) [see also (*7*)] and wild-type mice (Fig. 1D and table S1), the driving force of γ-aminobutyric acid type A ($GABA_A$) receptor $GABA_AR$ (DF_{GABA}) was elevated in fetal neurons on embryonic days 20 to 21 (E20 to E21) and reduced to adult values at postnatal days 15 to 30 (P15 to P30), with an abrupt reduction restricted to the delivery period (*9*). In contrast, DF_{GABA} remained elevated in

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fetal, early postnatal stages, and P15 to P30 in VPA rats (Fig. 1A and table S1) and FRX mice (Fig. 1D and table S1). Acute applications of the specific NKCC1 chloride importer antagonist bumetanide (10 μM) or oxytocin (1 μM) significantly decreased $[Cl^-]_i$ and DF_{GABA} at P0 in neurons recorded in VPA rats and FRX mice (Fig. 1, B, C, E, and F; and table S2). Therefore, the GABA developmental sequence is abolished in two animal models of autism, with GABA exerting depolarizing actions in a bumetanide and oxytocin-sensitive manner.

The chloride exporter KCC2 is down-regulated after various insults leading to elevated $[Cl^-]_i$, hyperactivity, and more KCC2 down-regulation (*10*–*12*). KCC2 was down-regulated in the hippocampi of juvenile VPA rats and FRX mice (fig. S1, A to C, and table S3). In addition, as in epileptic neurons (*10*), there was a shift of KCC2 labeling from the membrane to the cytoplasm in neurons of VPA rats (fig. S1, D and E, and table S4). Thereby, chloride export is reduced in two animal models of autism, which supports the observed alterations of the polarity of GABA actions.

We next evaluated whether the depolarizing actions of GABA were associated with neuronal excitation. In naïve animals, the specific $GABA_AR$ agonist isoguvacine (10 μM) inhibited or did not affect spike frequency in cell-attached recordings at P0 (Fig. 1, G to J, and table S5) and P15 (fig. S2,

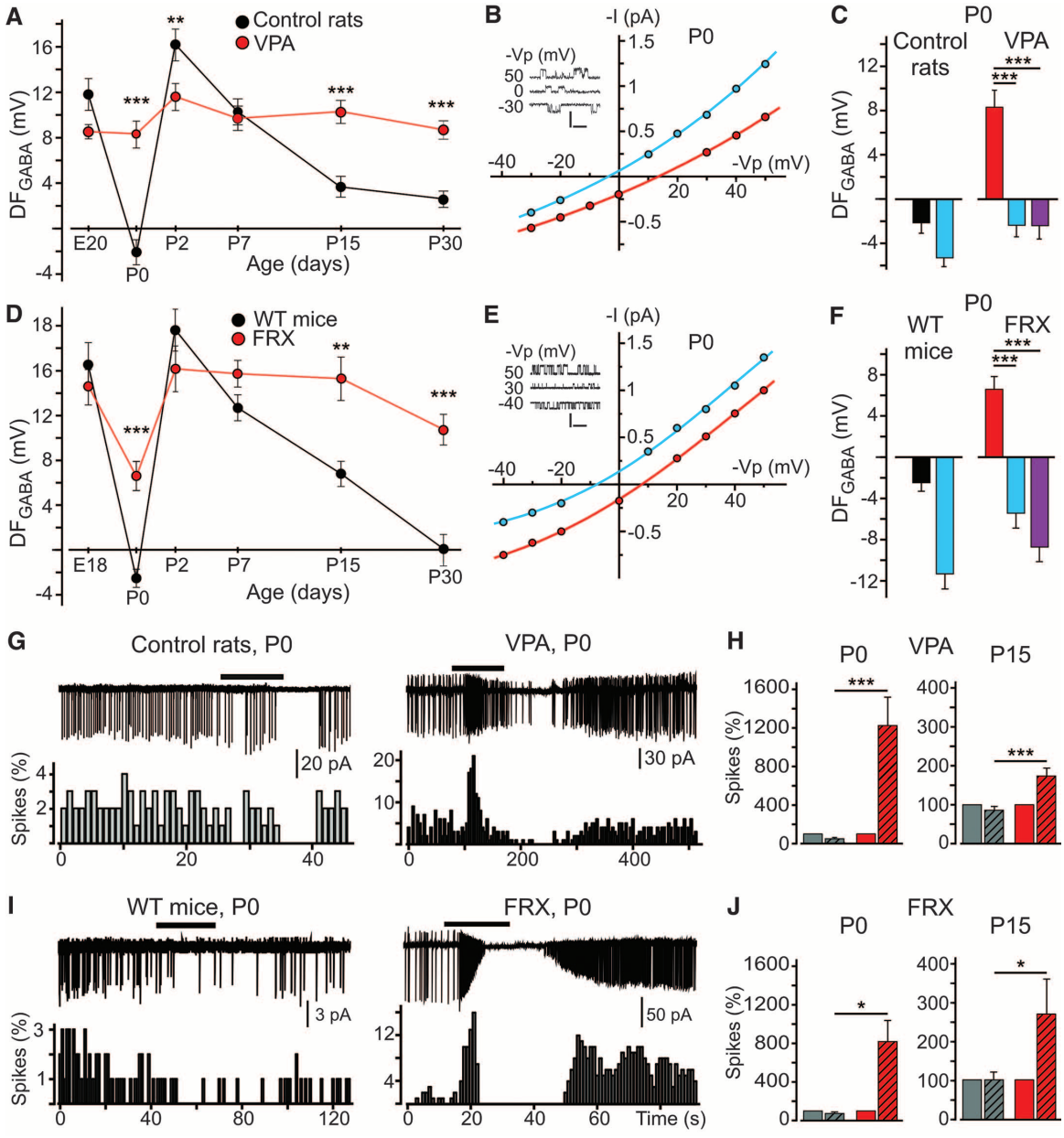

Fig. 1. Developmental excitatory-inhibitory GABA sequence is abolished in hippocampal CA3 pyramidal neurons in VPA rats and FRX mice. (**A**) Age-dependence of DF_{GABA} in control and VPA rats. (**B**) Current-voltage (*I-V*) relations of $GABA_AR$ single-channel currents at P0 in VPA rats in artificial cerebrospinal fluid (red) or bumetanide (10 μM, blue). (Inset) Single-channel openings at different holding potentials (scale bars mean 1 pA and 200 ms). (**C**) Bumetanide and oxytocin shifted DF_{GABA} from depolarizing to hyperpolarizing at P0 (control rats, black; VPA, red; bumetanide application, blue; and oxytocin application, purple). (**D** to **F**) The same as in (A) to (C) for wild-type mice (WT, black) and FRX mice (red). (**G** to **J**) Excitatory action of the $GABA_AR$ agonist isoguvacine (10 μM, black bars) on spontaneous spiking recorded in cell-attached configuration in VPA and FRX. (G) Control and VPA rats and (I) wild-type and FRX mice at P0 with and without isoguvacine. Time-course of spike frequency changes is shown under each trace. (H) Average values of normalized to control spike frequency for P0 and P15 control (gray) and VPA (red) rats and effect of isoguvacine application (hatched bars). (J) The same as in (H) but for P0 and P15 mice wild-type (gray) and FRX (red). Data are presented as means ± SEM. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

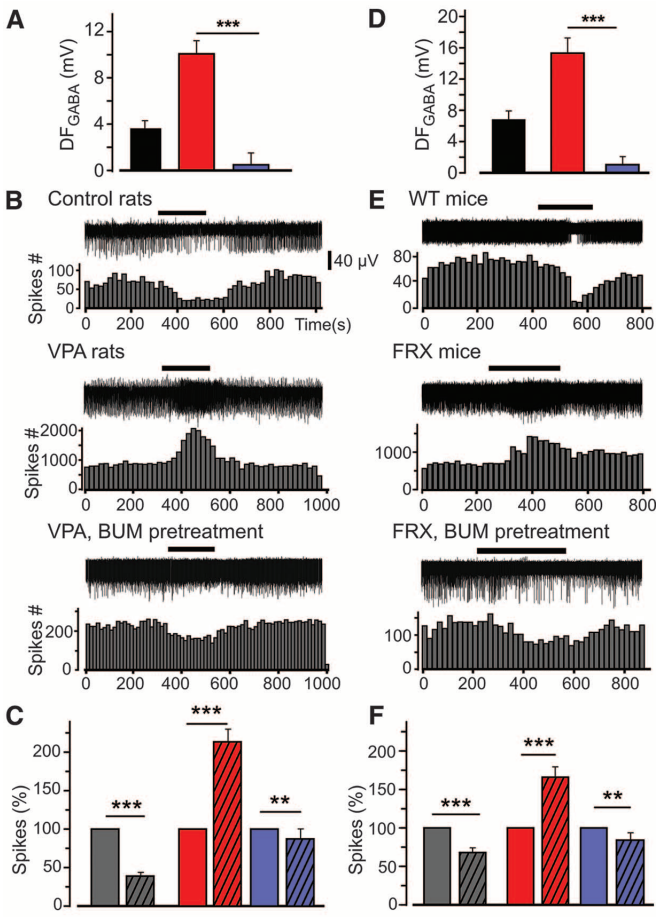

Fig. 2. Maternal pretreatment with bumetanide before delivery switches the action of GABA from excitatory to inhibitory in offspring in VPA and FRX rodents at P15. (**A**) Average values of DF_{GABA} measured in hippocampal CA3 pyramidal neurons at P15 in control (black), VPA (red), and VPA rats pretreated with bumetanide (blue). Note that pretreatment with bumetanide shifts DF_{GABA} from depolarizing to almost isoelectric level. (**B**) Effects of isoguvacine (10 µM; black bars) in rats: Representative traces of spontaneous extracellular field potentials recorded in hippocampal slices at P15 in control, VPA, and VPA rats pretreated with bumetanide (BUM). Corresponding time courses of spike frequency changes are shown under each trace. (**C**) Average histograms of normalized spike frequency in rats. Isoguvacine (hatched bars) decreased the spikes frequency in control rats (to 38.9 ± 5.1%; gray); increased it in VPA rats (to 213.5 ± 16.3%; red); and decreased it in VPA rats pretreated with bumetanide (to 82.8 ± 10.7%; blue). (**D**) The same as in (A) for mice. Wild-type mice (WT, black), FRX mice (red), and FRX mice pretreated with bumetanide (blue). (**E**) The same as in (B) for FRX mice. (**F**) The same as in (C) for FRX mice. Wild-type mice (decreased to 67.9 ± 6.1%; gray); FRX mice (increased to 165.8 ± 13.5%; red); FRX mice pretreated with bumetanide (decreased to 80.8 ± 8.2%; blue). Data are presented as means ± SEM. $^{**}P < 0.01$; $^{***}P < 0.001$.

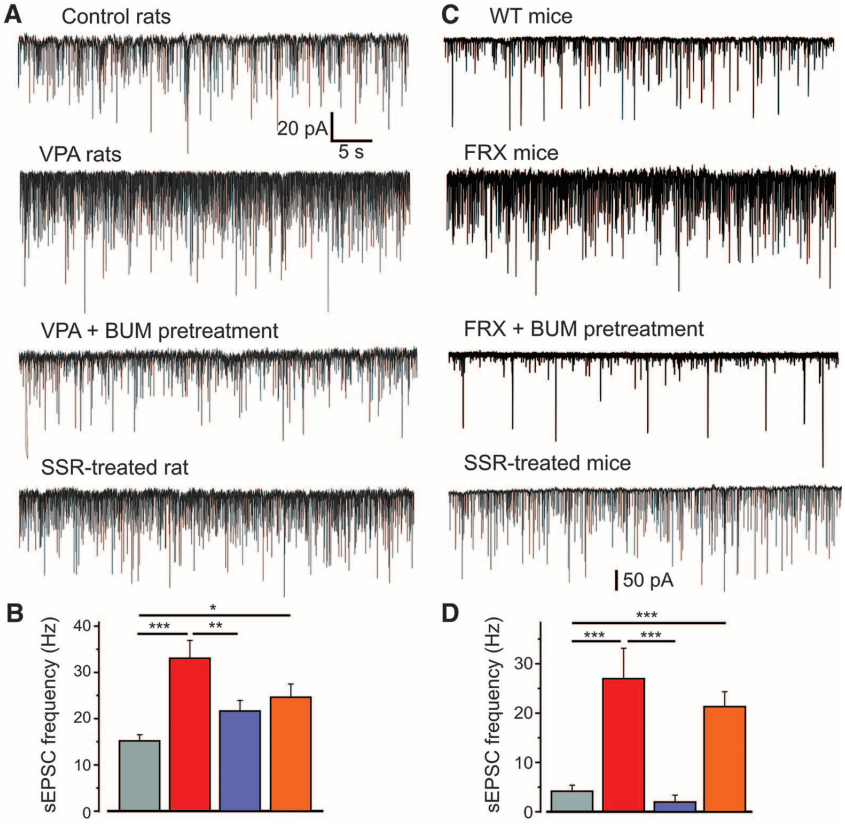

Fig. 3. Spontaneous activity is increased in VPA and FRX rodents at P15 and restored to control values by maternal pretreatment with bumetanide. Whole-cell voltage clamp recordings of sEPSCs at −70 mV from individual hippocampal CA3 pyramidal neurons in acute brain slices from P15 VPA rats or FRX mice and respective control and bumetanide or SSR126768A pretreated animals. (**A** and **C**) Representative traces of sEPSCs recorded from rats (A) and mice (C). Note that maternal pretreatment of animals with bumetanide decreases sEPSCs frequency in both models, whereas treatment with SSR126768A increases spontaneous activity of neuronal networks in rats and mice. (**B**) Average values of sEPSCs frequencies in rats: Control rats (gray) and VPA rats (red), VPA rats with maternal pretreatment with bumetanide (blue) and SSR126768A-treated rats (orange). (**D**) The same as (B) for mice. Wild-type mice (gray), FRX mice (red), FRX mice with maternal pretreatment with bumetanide (blue), and SSR126768A-treated mice (orange). One-way analysis of variance (ANOVA) Fisher's least significant difference as a post hoc test. Data are presented as means ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

A and C, and table S5) and in field-potential recordings at P15 (Fig. 2, B and E, and table S11). In contrast, isoguvacine increased spike frequency in neurons of VPA rats and FRX mice in cell-attached recordings at P0 (Fig. 1, G to J) and P15 (fig. S2, B and D, and Fig. 1, H and J) and in field-potential recordings at P15 (Fig. 2, B, C, E, and F). Hence, GABA excites newborn and juvenile neurons recorded in VPA rats and FRX mice.

We next determined whether excitatory GABA is associated with enhanced network activity. In hippocampal slices of VPA rats and FRX mice, there was bursting activity at P0 and a fourfold increase of the frequency of glutamatergic spontaneous excitatory postsynaptic currents (sEPSCs) at P0 (fig. S3, A to C and F to H; and tables S6 to S8) and P15 (fig. S3, D, E, I, and J; and table S9). Application of bumetanide restored control glutamatergic sEPSC frequency at P0 (fig. S3, B and G, and table S7) and P15 (fig. S3, E and J, and table S9). Therefore, developing networks of VPA rats and FRX mice are hyperactive with bumetanide-sensitive enhanced glutamatergic activity likely due to GABAergic excitation impinging on principal cells.

We then tested the hypothesis that restoring low $[Cl^-]_i$ and inhibitory GABA actions during delivery rescues naïve electrophysiological features in juvenile offspring. We treated pregnant VPA rats and FRX mice females orally 1 day before delivery with bumetanide (2 to 2.5 mg/kg in drinking water) and recorded neuronal activity in offspring at P15. Bumetanide pretreatment restored control DF_{GABA} values (Fig. 2, A and D, and table S10), suppressed the excitatory actions of isoguvacine (Fig. 2, B, C, E, and F; fig. S4; and tables S11 and S12), and significantly reduced ongoing activity and frequency of glutamatergic sEPSCs (Fig. 3 and table S9). Thus, elevated $[Cl^-]_i$ and excitatory GABA actions at birth produce long-term effects in juvenile VPA rats and FRX mice that can be restored by maternal pretreatment with bumetanide.

As the perinatal excitatory-to-inhibitory shift of GABA is mediated by oxytocin receptors (*7*), we tested the effect of a selective oxytocin receptor antagonist SSR126768A in naïve rodents. Pretreatment of naïve mothers one day before delivery with SSR126768A in drinking water produced in juvenile rats elevated DF_{GABA} (fig. S5A and table S13), excitatory GABA actions (fig. S5B and table S14), and exacerbated glutamatergic activity (Fig. 3 and table S9). Therefore, blocking oxytocin signals during delivery in naïve animals produces actions similar to those observed in the VPA rats and FRX mice and stresses the importance of the oxytocin-GABA link.

We then used behavioral tests to determine whether treatment of mothers with bumetanide shortly before delivery prevents autistic-like behaviors in offspring. The isolation-induced ultrasonic vocalizations that pups emit when separated from their mothers (*9*, *13*) were reduced in P4 VPA rats with fewer calls and shorter total call durations than age-matched control rats. In addition, FRX mice (P8) had a higher probability of emitting downward and chevron calls than age-matched wild-type mice (Fig. 4A and table S16). Maternal pretreatment with bumetanide rescued this

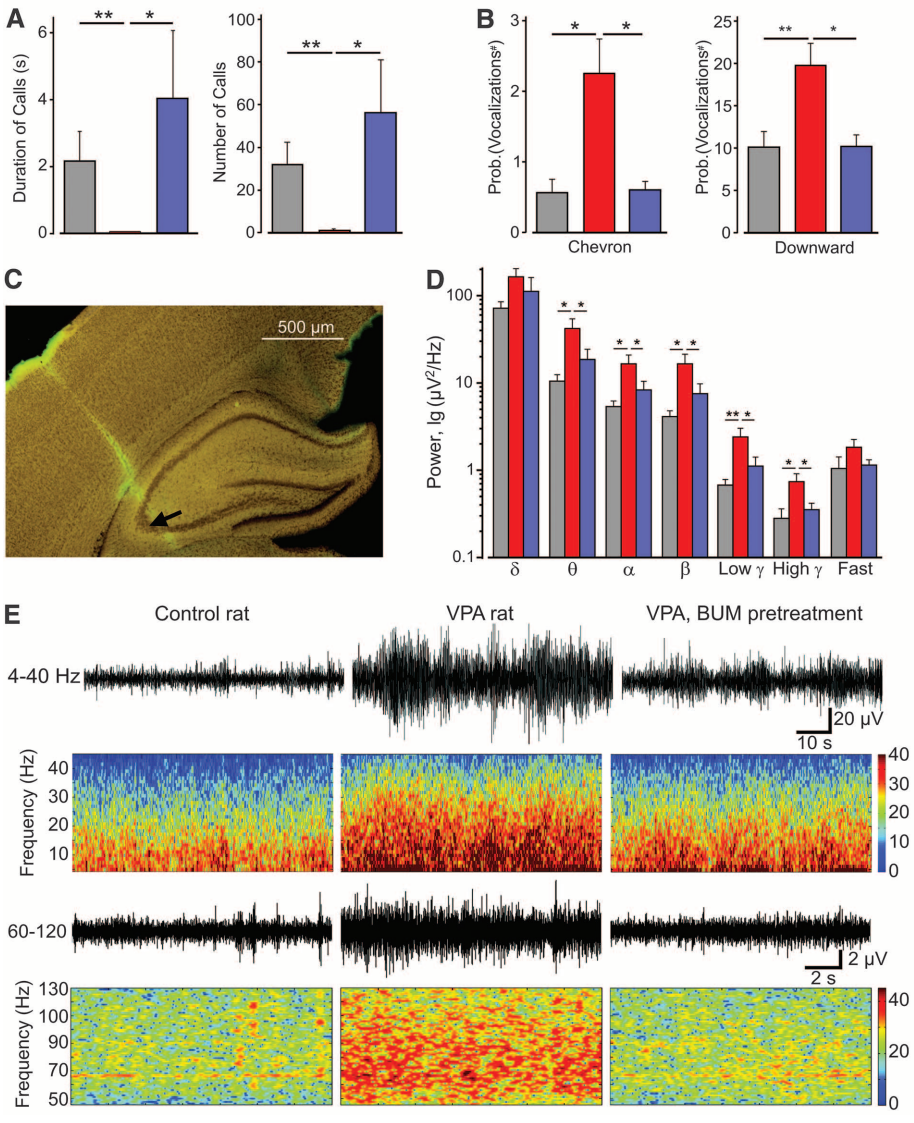

Fig. 4. Maternal pretreatment with bumetanide restores aberrant behavior and brain oscillations of animal models of autism. Isolation-induced ultrasonic vocalizations in (**A**) P4 control (gray); VPA (red, small bar in the middle); and VPA rats with maternal bumetanide pretreatment (blue); and (**B**) in P8 wild-type (gray), FRX (red), and FRX mice with maternal bumetanide pretreatment (blue). (**C** to **E**) EEG recordings in vivo were made in the CA3 area of hippocampus of head-restrained control, VPA, and VPA rats with maternal bumetanide pretreatment at the ages P13 to P15. (C) A coronal section showing the location of the DiI-labeled recording electrode (arrow). (D) Integral power of δ (0.5 to 4 Hz), θ (4 to 7 Hz), α (7 to 12 Hz), β (12 to 25 Hz), low γ (25 to 60 Hz), high γ (60 to 120 Hz) band components of EEG revealed by Fourier transform analysis. Control (gray) vs. VPA (red): for θ, α, β, and high γ, $*P < 0.05$, for low γ, $**P < 0.01$; VPA (red) versus VPA with bumetanide maternal pretreatment (blue) for θ, α, β, and high and low γ $*P < 0.05$. (E) Representative traces of CA3 pyramidal layer EEG recordings from control, VPA, and VPA rats with bumetanide maternal pretreatment after band-pass filtering at frequency ranges indicated on the left of the traces. Corresponding time-frequency representations are shown under each trace. One-way ANOVA Fisher's least significant difference post hoc test. Data are presented as means ± S.E.M.

behavioral alteration in VPA rats (Fig. 4A and table S16) and FRX mice (Fig. 4B and table S16). Furthermore, offspring of naïve mothers pretreated with SSR126768A to block oxytocin signals had an increased probability of emitting downward calls (P8, mice) and a longer latency to reach home bedding than age-matched control pups in the nest-seeking test (P9 rats) (fig. S5C and table S15). Therefore, bumetanide restores naïve behavior in VPA rats and FRX mice, and blocking oxytocin signaling produces behavioral alterations and autistic–like features.

Finally, as alterations of gamma oscillations have been observed in patients with autism (*14*), we tested whether similar changes occur in vivo in VPA rats. With intracranial electroencephalographic (EEG) recordings in the hippocampal CA3 region, hyperactivity was observed in VPA (P15) but not in age-matched naïve rats. These included enhanced network oscillation power in a broad spectrum of frequencies, including gamma but excluding fast ripples and very low (δ) frequencies. Maternal pretreatment with bumetanide restored physiological values in offspring (Fig. 4, C to E, and table S17). Therefore, the polarity of GABA actions during delivery exerts long-term effects on brain oscillations in VPA rats.

During parturition, the human fetus is subjected to an important stress associated with a high surge of catecholamine levels. This adapts neonates to extrauterine life by promoting lung maturation and increased cardiovascular performance and blood flow to the brain (*15*). However, in rodents, elevated catecholamine levels produce KCC2 down-regulation, elevated $[Cl^-]_i$ levels, excitatory GABA, and neuronal hyperactivity (*16*, *17*) that are prevented during delivery by oxytocin (*7*). Similar deleterious alterations are observed in epilepsies and other pathologic conditions (*10*–*12*, *18*). It is noteworthy that complicated deliveries have elevated catecholamines in umbilical cord blood and have been associated with an increased prevalence of autism (*15*–*20*). Whether GABA exerts excitatory actions in humans with autism is not known. However, in keeping with this hypothesis, agents that act through GABA (benzodiazepines and phenobarbital) produce paradoxical effects in patients with autism (*21*) and experimental epilepsy in rodents (*10*). Note also that rodent KCC2 activity is altered by autism-linked genetic mutations. Oxytocin improves information processing by exciting GABAergic interneurons and inhibiting their target pyramidal neurons (*22*). An excitatory shift of this link will enhance glutamatergic drive in neurons and thereby affect information-processing in the developing brain.

To conclude, our observations suggest that in addition to triggering labor and inducing trust, empathy, and parental-infant relationships in humans (*23*), oxytocin signals might exert a protective action during delivery, preventing deleterious effects of enhanced activity. Further investigations are needed to better understand the links among pregnancy complications, cesarean sections, and autism (*16*, *17*). In conclusion, our results validate the clinical actions of bumetanide (*24*) and oxytocin (*25*) and emphasize the importance of investigating how and when developmental sequences are disrupted in animal models of autism in order to develop novel therapeutic avenues (*26*).

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Acknowledgments: We thank at INMED C. Rivera and C. Pellegrino for their assistance on the Western blot and for the panKCC2 antibody, R. Martinez for his technical help with the ultrasonic setup, and M. L. Scattoni and M. Wohr for their assistance with the vocalization experiments and analysis. This study was supported by INSERM (funds to INMED), Neurochlore, France's Agence Nationale de la Recherche (ANR-12-RPIB-0001-01), and the Simons Foundation (SFARI award #230267 to Y. B-A.). We also thank Sanofi-Syntelabo for the gift of SSR126768A. On 13 January 2011, Neurochlore filed a patent entitled "Compounds for the treatment of autism" (U.S. patent number 13/522372, worldwide PCT/EP2011/050394); Y. B-A. and E.L. are identified as inventors of this patent. Y. B-A. and E.L. are founders and shareholders of Neurochlore, a company focused on the treatment of developmental disorders. R.N. and D.C.F. own shares in Neurochlore.

Supplementary Materials

www.sciencemag.org/content/343/6171/675/suppl/DC1
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Figs. S1 to S5
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14 October 2013; accepted 10 December 2013
10.1126/science.1247190