Early depolarizing GABA controls critical-period plasticity in the rat visual cortex

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Hyperpolarizing and inhibitory GABA regulates critical periods for plasticity in sensory cortices. Here we examine the role of early, depolarizing GABA in the control of plasticity mechanisms. We report that brief interference with depolarizing GABA during early development prolonged critical-period plasticity in visual cortical circuits without affecting the overall development of the visual system. The effects on plasticity were accompanied by dampened inhibitory neurotransmission, downregulation of brain-derived neurotrophic factor (BDNF) expression and reduced density of extracellular matrix perineuronal nets. Early interference with depolarizing GABA decreased perinatal BDNF signaling, and a pharmacological increase of BDNF signaling during GABA interference rescued the effects on plasticity and its regulators later in life. We conclude that depolarizing GABA exerts a long-lasting, selective modulation of plasticity of cortical circuits by a strong crosstalk with BDNF.

RESULTS

Early depolarizing GABA controls CP plasticity

To hamper depolarizing GABA A signaling, we treated rat pups with the NKCC1 inhibitor bumetanide (intraperitoneal (i.p.) administration, 0.2 mg/kg body weight twice a day because of its pharmacokinetics11) from postnatal day (P) 3 to P8 (Fig. 1a), when NKCC1 is the main K⁺-Cl⁻ co-transporter in the cerebral cortex (Supplementary Fig. 1)8–10. Indeed, bumetanide crosses the brain-blood barrier11 and effectively shifts the reversal potential of GABA (E GABA), converting GABA A signaling to hyperpolarizing in young rats10,12,13.

To assess whether perinatal interference with depolarizing GABA affects the time course of the CP for visual cortex plasticity, we used a classical paradigm of experience-dependent plasticity (i.e., 3 days of monocular deprivation (MD)) in bumetanide- and vehicle-treated rats (Fig. 1a). When performed during the CP (but not in adults), brief MD results in a marked shift of the ocular dominance (OD) of cortical neurons in favor of the nondeprived eye14. To define the time course of the sensitive period for plasticity, we performed MD experiments at different ages spanning the normal opening and closing of the CP15.

To quantify the OD shift, we performed in vivo electrophysiological recordings and measured the contralateral-to-ipsilateral (C/I) visual-evoked potential (VEP) ratio (i.e., the ratio of VEP amplitudes recorded by stimulating the contralateral or ipsilateral eye) in bumetanide- and vehicle-treated animals either with MD or left undeprived (nonMD). Baseline binocularity was comparable between nonMD bumetanide- and vehicle-treated animals at all tested ages (C/I VEP ratio of 1.5–2 for both groups; Fig. 1c–h), indicating no specific effect of early bumetanide treatment on the general development of binocularity. The MD experiments indicated that although the onset of CP plasticity was unaffected by bumetanide treatment, its closure was significantly...

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delayed (Fig. 1b). Indeed, OD plasticity rose similarly from P17 to P20 and was maximal at P26 in both bumetanide- and vehicle-treated rats (Fig. 1c–e). Conversely, at P35, consistent and significant OD plasticity could be induced in bumetanide-treated animals only (Fig. 1f). Notably, the shift in eye preference of bumetanide-treated animals after MD at P35 involved a depression of responses driven by the deprived eye, an effect that is typical of juvenile-like plasticity 16 (Supplementary Fig. 2). The CP was over in both groups by P45, as expected (Fig. 1g,h). Altogether, these results indicate that interfering with depolarizing GABA during early development significantly extends the sensitive period for activity-dependent rearrangements of cortical circuits later in life.

**Early bumetanide treatment does not affect overall anatomy**

To exclude the possibility that the effect on plasticity was due to defects in the anatomical development of the visual system 10,17, we investigated the segregation of eye-specific inputs in the dorsal lateral geniculate nucleus (dLGN) and the migration and morphological maturation in the visual cortex of animals perinatally treated with bumetanide or vehicle, as these processes occur early in rat perinatal life 17,18. To visualize retinal afferents from both eyes, we performed intravitreal injections of cholera toxin beta (CTB) fragment conjugates of different colors and analyzed coronal dLGN sections at P35 (Fig. 3a). We found clear segregation of fibers from the two eyes in both bumetanide- and vehicle-treated rats (Fig. 3b), indicating normal development of retinogeniculate projections.

To investigate the structural development of the visual cortex, we performed in utero electroporation of progenitors of excitatory neurons committed to layer II/III with a plasmid encoding for enhanced GFP (eGFP) at embryonic day (E) 17.5 (Fig. 3c). This allowed for visualization of neuronal migration and morphological maturation of the neuronal progeny of transfected cells by eGFP fluorescence imaging.
Figure 2 Early GABAergic interference prolongs critical-period plasticity in the visual cortex in vitro. (a) Average level of plasticity (calculated as the peak amplitude response normalized to the averaged baseline) ± s.e.m. of the amplitude of layer II/III field synaptic potentials after TBS of the WM from experiments performed at different ages (shown in b–g). The level of plasticity was higher in bumetanide-treated animals than in vehicle-treated control animals at P35 (post two-way ANOVA on ranks Holm-Sidak test, ***P < 0.001) but was similar between the two groups at the other time points (P = 0.969 (P17), P = 0.387 (P20), P = 0.704 (P26), P = 0.860 (P45), P = 0.599 (P75)). (b–g) Average time course of the increase in amplitude of field potentials in vehicle- and bumetanide-treated animals at the ages indicated. Insets show an average of ten traces recorded from a slice of a control vehicle- or a bumetanide-treated animal before stimulation. Scale bars, 0.2 mV (vertical); 80 ms (horizontal). Numbers in parentheses are the number of slices processed, number of animals. Error bars represent the s.e.m.

We found that all eGFP-positive neurons reached layer II/III in both bumetanide- and vehicle-treated animals at P35 (Fig. 3d). Moreover, morphological reconstruction of eGFP-positive neurons did not reveal any significant difference in morphological maturation between the two groups (total branch number, P = 0.502; total branch length, P = 0.646; Fig. 3d and Supplementary Fig. 3). Next, to assess the development of GABAergic interneurons, we examined their laminar distribution by immunostaining for the GABA biosynthetic enzyme GAD67 in cortical sections from bumetanide- and vehicle-treated rats at P35. We found no significant differences in the distribution of interneurons between the two groups (Fig. 3e,f).

Early bumetanide treatment does not affect visual function

Next we investigated whether bumetanide treatment could affect the overall development of the visual system at the functional level. In general, bumetanide-treated animals developed normally, as their gain of body weight and timing of eye opening were comparable to those of controls (Fig. 4a and Supplementary Fig. 4). Moreover, bumetanide- and vehicle-injected animals performed equally in learning a visual discrimination task (Pruisky visual water box; Fig. 4b).

Using the same test, we measured visual acuity longitudinally starting from P24 and found no deficits in its maturation in bumetanide-treated animals (Fig. 4c). We then measured basic physiological parameters in the primary visual cortex using VEP recordings at P35 (i.e., when these parameters reach adult levels in naive animals)19. We found that spatial resolution (visual acuity), contrast threshold and VEP amplitude and latency in bumetanide-treated rats were within the normal range and did not differ from those of controls (Fig. 4d–g). Thus, briefly interfering with depolarizing GABA during early development prolongs the CP for OD plasticity and WM-LTP into adulthood without affecting the basic anatomical and physiological development of the visual system.

Bumetanide effects are not due to regulation of osmolarity
To ensure that the effects of bumetanide treatment on visual cortical plasticity were due specifically to regulation of NKCC1 rather than to side effects of pharmacological treatment, we treated rat pups perinatally (P3–P8) with another NKCC1 inhibitor (furosemide; i.p., 20 mg/kg body weight, twice a day) and assessed the level of plasticity at P35. In fact, furosemide blocks both NKCC1 and KCC2; nevertheless, because of low KCC2 expression at the age of treatment (Supplementary Fig. 1), furosemide treatment mimics the effect of the NKCC1-specific blocker bumetanide on chloride regulation under our experimental conditions. Accordingly, we found that furosemide-treated animals displayed higher levels of plasticity than vehicle-treated controls at P35, as they responded to brief MD with a robust OD shift and displayed significant WM-LTP in slices (Fig. 5a,b). These plastic changes were exactly comparable to those observed in bumetanide-injected rats (Fig. 5a,b), indicating the involvement of early chloride homeostasis in regulating plasticity later in life.

Nevertheless, direct regulation of NKCC1 also results in neuronal shrinkage, as bumetanide and furosemide are diuretics approved by the US Food and Drug Administration that regulate blood osmolarity (Fig. 5c). Thus, we verified that the effects of bumetanide and furosemide on cortical plasticity are not simply due to an alteration in the volume of the extracellular space. To this aim, we treated rat pups perinatally (P3–P8) with the osmotic diuretic mannitol (i.p., 7.5 g/kg body weight)20,22, which regulates the volume of the extracellular space without affecting NKCC1 activity20,22. Mannitol-treated animals displayed the same levels of plasticity as vehicle-treated rats.
controls, both in vivo and in vitro (Fig. 5a,b), indicating that bumetanide and furosemide regulate cortical plasticity through modulation of Cl− homeostasis but not cell volume.

Variations in CP regulators accompany bumetanide’s effects

Next we investigated the possible mechanisms responsible for the higher levels of plasticity at P35 in bumetanide-treated animals. First, as inhibitory GABAergic transmission is a major regulator of closure of the CP,5,7 we used whole-cell patch-clamp recordings to examine basal GABAergic transmission in visual cortical slices from P35 animals that had been treated perinatally with bumetanide or vehicle. Although the basic membrane properties (membrane resistance, membrane capacitance and membrane resting potential) of layer II/III visual cortical neurons were comparable in the two experimental groups (Supplementary Fig. 5a–c), the frequency of miniature inhibitory postsynaptic currents (mIPSCs) was drastically reduced in the bumetanide-treated animals (bumetanide, 0.26 ± 0.05 (mean ± s.e.m.) Hz; vehicle, 0.62 ± 0.16 Hz; Fig. 6a). Conversely, there was no difference in the amplitude (Fig. 6b) or kinetics (τ and rise time; Supplementary Fig. 5d,e) of these events. Notably, we found no significant effect of perinatal bumetanide treatment on the frequency of miniature excitatory AMPA postsynaptic currents at P35 (bumetanide, 1.40 ± 0.37 Hz, n = 10 cells; vehicle, 1.79 ± 0.41 Hz, n = 9 cells; Student’s t test, P = 0.501). Thus we investigated whether we could rescue the persistent OD plasticity of bumetanide-treated animals at P35 by enhancing GABAergic transmission with i.p. injection of diazepam23,24 during the period of MD in a group of rats treated perinatally with bumetanide (Supplementary Fig. 6a). We found that the OD shift was significantly (P = 0.025) mitigated by diazepam infusion (Supplementary Fig. 6b), indicating that a reduced inhibitory tone accounts in part for the effect of bumetanide on plasticity at P35 and that other regulators of plasticity may also have a role.

Thus, as BDNF controls the closure of the CP in the visual system by influencing inhibitory circuits5,14, we investigated its expression in bumetanide- and vehicle-treated animals at P35. Using western blot experiments, we found significantly lower levels of BDNF in the visual cortices of bumetanide-treated animals (Fig. 6c). Components of the extracellular matrix, such as chondroitin sulfate proteoglycans (CSPGs), also limit experience-dependent plasticity by condensing mostly around parvalbumin (PV)-positive interneurons in the form of perineuronal nets (PNNs)14,19,25. Therefore, we investigated whether early bumetanide treatment affects PNN development
around PV-positive cells by performing immunostaining using Wisteria floribunda lectin (WFA; to label CSPG glycosaminoglycan chains) and an antibody to PV at P35 (Fig. 6d). Visual cortical slices derived from bumetanide-treated animals showed a lower PNN density compared to slices from controls (Fig. 6d,e). Notably, there was no significant difference in the absolute number of PV-positive cells (Fig. 6e) or in the density of PV-positive boutons onto pyramidal neurons between bumetanide-and vehicle-treated rats (Supplementary Fig. 7). Nevertheless, double labeling for WFA and PV confirmed that a lower proportion of PV-positive interneurons were surrounded by PNNs in bumetanide-treated animals (Fig. 6d,e).

Altogether, these findings indicate that the increased plasticity in bumetanide-treated animals at P35 is accompanied by modulation of well-known plasticity brakes, such as GABAergic inhibitory neurotransmission, BDNF and PNNs.

If release of the plasticity brakes were responsible for the higher degree of plasticity in bumetanide-treated animals at P35, then one would expect no difference in the levels of these same plasticity brakes at ages when the potential for plasticity is comparable between bumetanide- and vehicle-treated animals. Therefore, we assessed mIPSCs and BDNF levels at P26 (the peak of plasticity for both bumetanide- and vehicle-treated animals) and mIPSCs, PNNs and BDNF levels at P75 (when the CP is over in both groups). Recordings at P26 and P75 indeed showed no differences in either the frequency or amplitude of mIPSCs between bumetanide- and vehicle-treated animals (Supplementary Figs. 8a,b and 9a,b). The frequency of miniature excitatory AMPA postsynaptic currents was also superimposable between the groups (P26, bumetanide, 1.22 ± 0.26 Hz, n = 6 cells; vehicle, 1.36 ± 0.20 Hz, n = 9 cells; Student’s t test, P = 0.671; P75, bumetanide, 0.87 ± 0.22 Hz, n = 7 cells; vehicle, 1.05 ± 0.29 Hz, n = 9 cells; Student’s t test, P = 0.644). In keeping with the data on mIPSCs, we found comparable levels of BDNF at P26 and P75 in bumetanide- and vehicle-treated animals (Supplementary Figs. 8c and 9c). We assessed the density of PNNs at P75 and again

Figure 4 Early GABAergic interference does not alter the overall functional development of the visual cortex. (a) Average percentage (± s.e.m.) of vehicle- and bumetanide-treated pups that opened their eyes at the ages indicated. The time of eye opening was similar for three independent litters (χ² test, P = 0.199). The numbers in parenthesis are the numbers of animals analyzed. (b) Performance in learning the Prusky visual water discrimination task (P19–P24) expressed as the percentage of correct choices ± s.e.m. The learning curves revealed equivalent visual performances for bumetanide- and vehicle-treated animals (two-way repeated measures ANOVA, P = 0.968). (c) Development of behavioral visual acuity assessed with the Prusky visual water discrimination task. Two-way repeated measures ANOVA demonstrated similar age-dependent maturation of visual acuity in bumetanide- and vehicle-treated rats (P = 0.150). The graph depicts the average ± s.e.m. The numbers in parentheses are the numbers of animals tested. c/deg, cycles per degree. (d–g) In vivo electrophysiological recordings in the primary visual cortex showing that bumetanide treatment did not affect the development of basal visual properties such as visual acuity (d), contrast threshold (e), VEP amplitude of binocular (bin), contralateral (contra) and ipsilateral (ipsi) responses (f) and VEP latency of binocular responses (g) (Student’s t test; P = 0.466 (d); P = 0.356 (e); P = 0.385, P = 0.545, P = 0.757 (f, left to right); P = 0.993 (g)). The histograms depict the average ± s.e.m. The numbers in parentheses indicate the numbers of animals recorded.

Figure 5 The effect of early bumetanide treatment on visual cortical plasticity is due to regulation of cation–Cl⁻ co-transporters rather than the regulation of osmolarity. (a) Average ± s.e.m. of the level of OD plasticity in vehicle-, bumetanide-, furosemide- and mannitol-treated rats at P35. Higher levels of OD plasticity were found in bumetanide- and furosemide-treated animals (post ANOVA Holm-Sidak test: vehicle compared to bumetanide, P < 0.001; vehicle compared to furosemide, P = 0.004) but not in mannitol-injected rats (vehicle compared to mannitol, P = 0.958; bumetanide compared to mannitol, P = 0.009; furosemide compared to mannitol, P = 0.028). The numbers in parentheses are the numbers of animals recorded (nonMD and MD). (b) Average ± s.e.m. and single-slice values (circles) of the level of LTP 30 min after TBS in slices from animals treated as in a. Plasticity was greater in bumetanide- and furosemide-treated animals (post ANOVA Duncan’s test: vehicle compared to bumetanide, P = 0.001; vehicle compared to furosemide, P = 0.011) than in vehicle- or mannitol-injected rats (vehicle compared to mannitol, P = 0.784; bumetanide compared to mannitol, P = 0.004; furosemide compared to mannitol, P = 0.039). (c) Average serum osmolarity ± s.e.m. measured 10 min after acute injection of bumetanide at P8. Osmolarity increased in bumetanide-treated animals in comparison to vehicle controls (Student’s t test, F = 0.024). The histogram depicts the average ± s.e.m., and circles represent data from single animals. *P < 0.05, **P < 0.01, ***P ≤ 0.001.
found no significant changes in the bumetanide-treated animals (P = 0.766; Supplementary Fig. 9d).

Early DHF co-treatment rescues bumetanide’s effects at P35

What are the possible mechanisms that link the effect of early bumetanide treatment with the prolongation of CP plasticity? Depolarizing GABA and BDNF are involved in a positive-feedback reciprocal regulation during early development26,27. In particular, activation of GABA receptors triggers augmented release of BDNF and enhanced phosphorylation of its receptor TrkB (pTrkB), which in turn reduces the internalization of GABA receptors from the plasma membrane28. Therefore, we hypothesized that perinatal bumetanide treatment may disrupt the positive loop described above, thus reducing BDNF signaling in response to activation of the GABA receptor. To test this hypothesis, we collected visual cortex samples from pups treated with bumetanide or vehicle perinatally (P3–P8) and systematically challenged at P8 by acute injection of a GABA receptor agonist (midazolam; i.p., 5 mg/kg body weight; Fig. 7a). Using quantitative RT-PCR, we found a significant upregulation of Bdnf mRNA expression in vehicle- but not bumetanide-treated animals injected with midazolam (Fig. 7b).

Next we assessed TrkB activation by western blot analysis with an antibody to pTrkB (Y816) in bumetanide- and vehicle-treated pups after acute midazolam injection. In keeping with the RT-PCR data, we found that GABA receptor activation enhanced TrkB phosphorylation in vehicle- but not bumetanide-treated pups (Fig. 7c). Together these results suggest that early interference with depolarizing GABA by bumetanide treatment impairs both the synthesis and release of BDNF.

To investigate whether the perinatal decrease in BDNF signaling mediated the effects of early bumetanide treatment on visual cortical plasticity later in life, we performed a rescue experiment by increasing BDNF signaling concomitant to bumetanide treatment. In particular, we injected bumetanide together with the BDNF mimetic drug 7,8-dihydroxylavone (DHF; i.p., 5 mg/kg body weight28) from P3 to P8; (Fig. 7d), which induces TrkB phosphorylation in the cortex in vivo28. Infusion of DHF and bumetanide together during early development normalized the level of plasticity at P35 both in vivo (Fig. 7e,f) and in vitro (Fig. 7g,h). Notably, DHF treatment per se did not interfere with plasticity, as WM-LTP induction at P26 in rats treated perinatally with DHF or vehicle showed comparable levels of plasticity (Supplementary Fig. 10). Notably, treatment of DHF together with bumetanide also rescued inhibitory GABAergic transmission (Fig. 8a), BDNF expression (Fig. 8b) and PNNs (Fig. 8c). Thus, disruption of positive feedback regulation between depolarizing GABA and BDNF during early development accounts for the effects of early bumetanide treatment on the plasticity of the visual system later in life.

DISCUSSION

Depolarizing GABA also controls CP plasticity

Hyperpolarizing and inhibitory GABA has a major role in regulating CP plasticity in the visual cortex5,7,14. In particular, opening of the CP requires reaching a threshold level in the development of GABAergic inhibition8, whereas complete development of GABAergic inhibition leads to the closure of the time window of maximal plasticity29. Unlike in adults, GABA depolarizes neuronal cells during early development, representing the main excitatory drive for immature cortical networks8. Consequently, depolarizing GABA has been implicated in a series of developmental processes, including neuronal migration and morphological differentiation, as well as synapse maturation in the cortex5,9. To our knowledge, we report for the first time a key role for early depolarizing GABA in the long-lasting control of the plasticity of neuronal circuits.

To interfere with early GABA action, we used systemic administration of the NKCC1 inhibitor bumetanide, with timing and doses that are effective in abolishing GABA-induced depolarization10. We evaluated the plasticity of cortical circuits using two independent measures,
Figure 7  DHF treatment during early GABAergic interference rescues the bumetanide-induced effect on plasticity. (a) Cartoon of the experimental protocol. (b) Bdnf mRNA expression in P8 vehicle- and bumetanide-treated pups in basal conditions or after induction by i.p. injection of the GABA_A receptor agonist midazolam (Mid). Gapdh was used as the internal standard. Two-way ANOVA showed that bumetanide treatment blocked (post Tukey test, *P = 0.004) the midazolam-induced increase (post Tukey test, *P = 0.003) of Bdnf mRNA expression. The histogram depicts the average ± s.e.m., and circles indicate single-animal data (analyzed in triplicate). (c) Ratio of pTrkB to TrkB protein expression in P8 vehicle- and bumetanide-treated pups in basal conditions or after induction by midazolam. Two-way ANOVA showed that bumetanide treatment blocked (post Tukey test, *P = 0.029) the midazolam-induced increase (post Tukey test, **P = 0.006) of pTrkB/TrkB protein expression. The histogram depicts the average ± s.e.m., and circles indicate single-animal data. (d) Cartoon of the experimental protocol. Bum, bumetanide. (e) C/I VEP ratios in P35 animals treated perinatally with vehicle, bumetanide or bumetanide + DHF in either nonMD or MD conditions. Two-way ANOVA revealed that plasticity in animals treated with bumetanide + DHF was negligible (P = 0.505). ***P < 0.001. The histogram depicts the average ± s.e.m., and circles indicate data from single animals. (f) Average degree of OD plasticity ± s.e.m. for the data in e (Student’s t test, *P = 0.030). The dashed orange line represents the average level of plasticity in vehicle-treated rats. The numbers in parentheses are the numbers of animals recorded (nonMD, MD). (g) Average time course of WM-LTP in P35 animals treated with vehicle, bumetanide or bumetanide + DHF. Error bars represent the s.e.m. The insets show the average of ten traces recorded from animals treated with vehicle, bumetanide or bumetanide + DHF before (continuous line) and 30 min after (dashed line) TBS. Scale bars, 0.2 mV (vertical); 2 ms (horizontal). (h) Data from the single slices (circles) recorded in g and the average ± s.e.m. of the plasticity level (normalized to controls, dashed orange line; Student’s t test, **P = 0.005).
window for plasticity while leaving the overall anatomical and physiological maturation of the visual system unaffected. We documented clear segregation of retinogeniculate fibers in the dLG, no alteration in migration or morphological maturation of layer II/III pyramidal neurons and normal laminar distribution of interneurons in the developing visual cortex. Visual acuity, binocularity, contrast threshold, VEP amplitude and latency were within the normal range at P35, the same age at which consistent and significant plasticity could be induced in bumetanide-treated animals only.

The combined regulation of CP plasticity and overall visual system development found in previous studies (i.e., dark rearing from birth15, BDNF overexpression in transgenic mice5,29, environmental enrichment from birth3 or IGF-1 administration after weaning30) may depend on the fact that these long-lasting experimental protocols set in motion a number of different pathways acting in parallel to regulate physiological maturation and simultaneously delimit experience-dependent plasticity. Conversely, we found that early and selective NKCC1 inhibition left a specific mark on developing cortical circuits, affecting only the duration of the plasticity window.

At the functional level, the dissociation between CP plasticity and overall visual system development may be convenient, as cortical networks may remain customizable by experience even at the completion of the period of physiological maturation. This idea is consistent with the robust plasticity described in fully mature mice after prolonged MD14.

The absence of gross neuroanatomical defects in bumetanide-treated animals is seemingly in contrast with the reported alteration in cortical neuron morphology after treatment with bumetanide starting from the embryonic stage to the first postnatal week10. This discrepancy may be due to a longer time period of the previous treatment (E15–P7) in comparison to our manipulation (P3–P8). Moreover, treatment from E15 to P7 includes the time of delivery. As delivery is associated with a marked shift of GABA action from depolarizing to hyperpolarizing33,4, part of the discrepancy with our results could depend on the fact that manipulation from E15 to P7 includes a time period in which GABA is not depolarizing.

Early depolarizing GABA and BDNF exert long-term control of CP regulators

Critical-period plasticity in the visual cortex is controlled by fundamental regulators such as inhibitory GABAergic transmission, BDNF and the extracellular matrix14. In particular, GABAergic inhibition and PNNs are considered to be classical plasticity brakes because their downregulation in adulthood effectively restores the sensitivity of cortical networks to MD and WM-LTP27,25,35. In keeping with this idea, the high plasticity of bumetanide-treated rats at P35 was accompanied by a decrease in inhibitory neurotransmission, the number of PNNs and BDNF levels. Notably, we found that the GABA inhibitory tone was involved in the higher OD plasticity of bumetanide-treated rats at P35, as enhancing GABAergic transmission with diazepam significantly dampened the response to MD. We also found no difference in the expression of these brakes at ages when plasticity was the same between bumetanide- and vehicle-treated animals (P26, peak of the CP; and P75, adulthood). The amplitude and frequency of GABAergic events that we recorded at all ages were lower than those reported in other studies using access resistances lower than ours36–39, although the frequency that we report here is in agreement with a recent study using an access resistance similar to ours32.

Besides their coordinated effect on visual cortex plasticity, GABAergic transmission, BDNF and the extracellular matrix also interact with one another in the control of visual cortex development5,25,40. For example, activation of GABA_A receptors by depolarizing GABA during early development triggers the synthesis and release of BDNF36,27. The consequent activation of TrkB, in turn, promotes cell-surface expression of GABA_A receptors, leading to a positive feedback loop36. We found that disruption of this positive loop was a key event in the plasticizing effects of bumetanide treatment. Indeed, BDNF signaling downstream of GABA_A receptor activation was significantly impaired in bumetanide-treated pups, and the long-term consequences of NKCC1 inhibition (i.e., increased plasticity at P35) were counteracted by TrkB activation through DHF treatment. Notably, restoration of a normal closure of the CP by early treatment with DHF and bumetanide together was also accompanied by recovery of the normal levels of GABAergic inhibition, PNNs and BDNF.
confirming the tight correlation between functional plasticity and these plasticity brakes. However, DHF treatment per se did not alter cortical plasticity. Altogether, these findings demonstrate that the loop between depolarizing GABA and BDNF during early development regulates the widely described interaction between hyperpolarizing GABA, BDNF and PNNs, which are major regulators of CP plasticity later in life.

Mechanisms of CP regulation by early depolarizing GABA

Our data demonstrate that BDNF signaling is a crucial mediator of the effects of early depolarizing GABA on cortical inhibition and plasticity later in development. But what are the mechanisms that may account for the long-term, plasticizing effects of transient interference with depolarizing GABA?

At least three different mechanisms (working alone or in combination) can be hypothesized. First, depolarizing GABA signaling might directly 41 or indirectly (through BDNF 26, 27) promote the subsequent maturation of inhibitory circuitry necessary for the closure of the CP 5, 7. Nevertheless, this hypothesis seems unlikely. As GABAergic inhibition is also necessary for the opening of the CP, if the above hypothesis were true, one would expect a temporal shift of the whole CP rather than its mere prolongation, as we found in the current study.

Second, spontaneous activity (before the onset of visual experience) and visually evoked discharges represent a continuum that acts to determine and instruct visual circuit development and plasticity 8, 42. By definition, the CP is a phase of heightened sensitivity to environmental stimuli during which cortical networks are customized by visual experience 14. When network activity is reduced, such as in dark rearing, the visual system reacts with an extension of the CP to prolong the effective time window during which visual experience can instruct circuit refinement 15. In this context, a precocious reduction of GABA-mediated excitation during early development may trigger extended plasticity of the visual cortex to compensate for the early loss of wiring instructions coded by the spontaneous activity patterns. The specific effect of early bumetanide treatment on the closure (and not the opening) of the CP, together with the retention of a juvenile-like plasticity at P35, would favor this hypothesis.

A third possible explanation for the long-term effects of early GABA interference resides in epigenetic mechanisms 43, 44. Classic experiments have shown that early environmental experiences (such as levels of early maternal care) influence adult behavior by modifications in chromatin structure 45. Thus, we speculate that transient interference with GABA-mediated excitation may leave persistent marks on specific classes of cortical neurons, reverberating on altered function and structure (i.e., reduced inhibitory transmission and reduced PNN) later in life. These epigenetic changes could display their effects at specific developmental stages (i.e., P35) with no apparent impact on the initial, default developmental trajectory of plasticity and its regulators.

Therapeutic perspective

Bumetanide is a drug approved by the US Food and Drug Administration that has been used extensively in the past as a loop diuretic and is used currently to treat heart failure and respiratory disorders in infancy 50. Notably, because of its ability to inhibit the brain-specific transporter NKCC1, bumetanide has been proposed as a promising candidate to treat brain diseases 46 on the basis of studies in animal models 12, 34, 46 and recent clinical trials 46– 48. However, the use of this drug in neonates has been partially questioned 10, 49, 50 because of the key role of depolarizing GABA during early development 8. Our data similarly suggest possible long-term consequences of NKCC1 inhibition during early development. Our study also sheds light on the existence of therapeutic windows for pharmacological interventions aimed at prolonging CP plasticity without affecting overall neuronal circuit development 46.

METHODS

Methods and any associated references are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

G.D. performed in vitro electrophysiological recordings, western blot experiments, in utero electroporation and immunohistochemistry and wrote the manuscript. M.A. performed in vivo electrophysiological recordings, western blot experiments, immunohistochemistry, behavioral testing and retinogeniculate axon labeling and wrote the manuscript. C.C. contributed to in vivo electrophysiological recordings. G.B. and S.N. performed patch-clamp experiments. G.Z. and Y.B. performed RT-PCR and immunohistochemistry. M.C. and L.C. designed the experiments and wrote the manuscript. M.C., Y.B. and L.C. provided financial support. All authors read and revised the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Animal treatment. All procedures were approved by Istituto Italiano di Tecnologia licensing, the Italian Ministry of Health and EU guidelines (Directive 2010/63/EU). Long Evans rats were housed at 21 °C, in a 12-h light, 12-h dark cycle and given food and water ad libitum. Female and male littermates were injected i.p. twice a day, from P3 to P8, with bumetanide (0.2 mg/kg body weight; Sigma), furosemide (20 mg/kg body weight; Sigma), DHF (5 mg/kg body weight; Sigma) or vehicle (DMSO, 0.04% in physiological solution) and once a day with maninolt (7.5 g/kg body weight; Sigma). For the midazolam experiments, vehicle- and bumetanide-treated pups received one injection of midazolam at P8 (i.p., 5 mg/kg body weight; IB1). MD was performed as described19. A subset of rats treated perinatally with bumetanide were monoclonally deprived at P35 and treated daily with diazepam (2 mg/kg body weight, i.p24, kindly provided by Roche) to enhance GABAergic inhibition, starting 4 h before eyelid suture. Rat body weights were monitored twice a day.

In vivo electrophysiology. Rats were anesthetized with Hypnorm/Hypnovel (in water; 0.375 ml/kg body weight; VetaPharma, UK), and the skull overlying the binocular visual cortex was drilled on one side. A glass micropipette (2 MΩ) filled with NaCl (3 M) was mounted on a motorized micromanipulator and inserted into the binocular visual cortex. VEPS were recorded from three to four penetrations per animal, and the electrode was positioned at 100- and 400-μm depth within the cortex. Electrical signals were amplified (10,000-fold), band-pass filtered (0.3–100 Hz), digitized and averaged in synchrony with the stimulus contrast reversal. Analysis of the amplitude of VEP responses was performed blind to animal treatment. Visual stimuli were gratings of various spatial frequencies and contrast generated by a VSG2/5 card (Cambridge Research Systems, Rochester, UK) on a display (Sony Multiscan G900; mean luminance, 15 cd/m²) positioned 20–30 cm from the rat's eyes to include the central visual field (110 × 85° of visual angle).

Steady-state VEPs: visual acuity and contrast threshold. VEP recordings in steady-state mode were used to measure spatial resolution and contrast threshold. The visual response was measured as the amplitude (μV) of the second harmonic of the stimulation frequency (4 Hz), calculated after Fourier analysis of the signal. Visual acuity was assessed after presentation of gratings of variable spatial frequencies (90% contrast). Visual acuity was determined by extrapolation to 0 V of the linear regression through the four to six points closest to the noise level in a curve where VEP amplitude was plotted against the log spatial frequency. In the same temporal frequency (4 Hz), we analyzed contrast threshold in response to 0.07 cycles per degree (c/deg) gratings. Contrast threshold was taken as the lowest contrast that evoked a VEP response greater than the mean value of the noise.

Transient VEPs: C/I ratio and degree of plasticity. Transient VEPs were recorded in response to the abrupt contrast reversal (1 Hz) of a square-wave grating (spatial frequency, 0.1 c/deg; contrast, 90%). For each animal, we calculated a C/I ratio, i.e., the ratio of VEP amplitudes recorded by stimulating the contralateral and ipsilateral eye. We summarized the level of OD plasticity in vivo for each age and treatment by calculating an index (degree of plasticity). The average value of this quantity, i.e., mean, (index), was obtained by subtracting the mean value of the C/I ratio of the MD group, mean (C/I)MD, from that of the nonMD animals, mean (C/I)nonMD, of the same age and treatment:

\[
\text{mean}(\text{index}) = \text{mean}(C/I)_{\text{nonMD}} - \text{mean}(C/I)_{\text{MD}}
\]

The variance, var, of this index incorporates the uncertainties of both the nonMD and MD groups, according to the error propagation formula:

\[
\text{var}(\text{index}) = \text{var}(\text{mean}(C/I)_{\text{nonMD}}) + \text{var}(\text{mean}(C/I)_{\text{MD}})
\]

In vitro electrophysiology. For acute visual cortex slices, rats were anesthetized with isoflurane and transcardially perfused with an ice-cold cutting solution with the following composition (in mM): 0.1 MgCl2 2.5 KCl 1.25 NaH2PO4 2 MgSO4 0.1 CaCl2 26 NaHCO3 206 sucrose; and 12 n-glucose (~300 mOsm, pH 7.4, oxygenated with 95% O2 and 5% CO2). Coronal slices (350-μm thick; VT1000S, Leica Microsystems vibratome) were incubated at 35 °C for 30 min in artificial cerebrospinal fluid (ACSF) with the following composition (in mM): 124 NaCl; 2.5 KCl; 1.25 NaH2PO4; 2 MgSO4; 2 CaCl2; 2 NaHCO3; 0.02 sulfispyrazone; 1 ascorbic acid; 0.5 myo-inositol; 2 pyruvic acid; and 12 n-glucose (~310 mOsm, pH 7.4), oxygenated with 95% O2 and 5% CO2. After 1 h of recovery at room temperature, slices were perfused with ACSF (35 °C, 1.7 ml/min).

WM-LS recordings. Electrical stimulation (100-μs duration) was delivered by a bipolar concentric stimulating electrode (FHC, St. Bowdoinham, ME) placed at the border of the WM and layer VI. Field potentials in layer II/III of the binocular visual cortex were recorded by a micropipette (1–3 MΩ) filled with NaCl (3 M). Baseline responses were obtained every 30 s with a stimulation intensity that yielded a half-maximal response. After 10 min of stable baseline, a TBS was delivered. The protocol of TBS stimulation consisted of two trains of TBS every 10 s. Each TBS train consisted of 12 bursts at 5 Hz. Each burst consisted of four pulses at 100 Hz. Data, filtered at 0.1 Hz and 3 kHz and sampled at 25 kHz, were acquired with a patch-clamp amplifier (Multiclamp 700, Molecular Devices) and analyzed using pClamp 10.2 software (Molecular Devices).

Patch-clamp recordings. Whole-cell patch-clamp recordings were made from layer II/III pyramidial neurons of the binocular visual cortex at room temperature. Glass micropipettes (resistance, 4–8 MΩ) were filled with an internal solution of (in mM) 120 K+ gluconate; 20 KCl; 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES); 0.1 ethylene glycol tetraacetic acid (EGTA); 2 MgCl2; 10 phosphocreatine Na2+; 2 Mg2+ATP; and 0.25 Na2GTP (~300 mOsm, pH 7.4). The membrane resting potential was measured immediately after whole-cell access at 0 current. Criteria for accepting a recording included input resistance >200 MΩ and access resistance <25 MΩ, with a maximal variation of 20%. Capacitance, input and access resistance were measured online with Clampex 10.2. For mIPSCs, the holding potential was set at ~70 mV, and gap-free recordings were performed for 5 min in tetrodotoxin (0.2 μM; Tocris) to establish the baseline of mPSC frequency. Then, 6,7-dinitroquinoxaline-2,3-dione (DNPQ) (20 μM; Tocris) was bath applied for 5 min to isolate mIPSCs. Application of both bicuculline methiodide (25 μM; Sigma) and DNPQ abolished all mIPSCs. Miniature excitatory postsynaptic current (mEPSC) frequency was calculated by subtracting the frequency of mIPSCs from the frequency of baseline mPSCs. Data, filtered at 0.1 Hz and 5 kHz and sampled at 25 kHz, were acquired with a patch-clamp Multiclamp 700 amplifier and analyzed using pClamp 10.2 software. All chemicals were purchased from Sigma, unless otherwise specified.

Labeling and analysis of retinogeniculate axons. Labeling of retinogeniculate axons was performed as described previously18,31. Rats received an intravitreal injection of CTB conjugated with Alexa Fluor 488 (10 mg/ml; Invitrogen) into the left eye and CTB–Alexa Fluor 594 (10 mg/ml; Invitrogen) into the right eye 2 d before perfusion. Animals were perfused transcardially with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer, Brain coronal sections were cut on a freezing microtome (50-μm thick) and collected in a serial order through the entire thalamus.

CTB labels were examined with a Leica confocal microscope using a 10× objective (air, numerical aperture (NA) 0.4). For each animal, we acquired the five largest sections through the middle of the dLGN18. The relative areas occupied by the ipsilateral and contralateral projections were calculated by dividing the average of the five ipsilateral or contralateral areas by the average of the five total dLGN areas. To determine the extent of overlap between ipsilateral and contralateral projections to the same dLGN, the ipsilateral and contralateral areas were measured, and their sum was subtracted from the total dLGN area and expressed as a percentage of it.

Tripolar in utero electroprotoporation of the visual cortex. Tripolar in utero electroporation of the visual cortex was described as previously52. Timed-pregnant Long Evans rats were anesthetized at E17.5 with isoflurane (induction, 3.5%; surgery, 2.5%), and uterine horns were exposed by laparotomy. Expression vectors (pCAG-IREs-eGFP; 1.5 mg/μl in water) and Fast Green dye (0.3 μg/μl; Sigma, St. Louis, MO) were injected (5–6 μl) through the uterine wall into one of the embryo's lateral ventricles by a 30-G needle. While the embryo's head was carefully held between standard forceps-type circular electrodes (10-mm diameter; positive poles; Napa Gene, Chiba, Japan), a third electrode

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Slice histology. P35 rats were transcardially perfused with 4% PFA in 0.1 M phosphate buffer (PB), pH 7.4. Brains were cut coronally (80-µm thick) with a freezing microtome. Free-floating slices were permeabilized and blocked with PBS containing 0.3% Triton X-100, 10% normal goat serum (NGS; Jackson Immunoresearch) and 0.2% bovine serum albumin (BSA) and then incubated with NeuN antibody (mouse anti-NeuN, 1:1,000, Millipore, #MAB377) followed by Alexa-568 fluorescent secondary antibody (1:600, Invitrogen, #A11031).

Confocal image acquisition and analysis. For high-magnification images of the cell morphology of eGFP-transfected neurons, 80-µm-thick z stacks were acquired with a 20× objective (oil, NA 0.7), and z series were projected to two-dimensional representations followed by neurite tracing and quantification with the Neurolucida software.

Immunohistochemistry. Animals were deeply anesthetized and perfused with 4% PFA in 0.1 M PB, pH 7.4. Brains were cut coronally with a freezing microtome. For PV immunostaining, free-floating sections were blocked for 2 h at room temperature with 10% NGS and 0.3% Triton X-100 in PBS. Slices were incubated overnight at 4 °C with parvalbumin-specific primary antibody (guinea pig polyclonal, #195004, 1:1,000, Synaptic Systems55), followed by donkey anti-guinea pig DyLight-488 (1:200, Jackson Immunoresearch). For PNN labeling, we used lectin WFA (αL1516-2mg2). The blocking solution contained 3% BSA in PBS, and biotinylated WFA (1% BSA in PBS; 1:100, Millipore) was applied overnight at 4 °C. WFA was revealed with 1 h of incubation in Cy3-conjugated ExtrAvidin (1:500, Sigma). For GAD67 immunostaining, the blocking solution contained 10% NGS, 3% BSA and 0.3% Triton X-100 in PBS. Anti-GAD67 (mouse monoclonal, #MAB5406, 1:500, Millipore) was applied overnight at 4 °C. The signal was counted blind to treatment on a fluorescence microscope using a 40× objective.

For immunohistochemistry quantifications, PNNs and PV-positive cells were counted blind to treatment on a fluorescence microscope using a 40× objective (oil, NA 1.25) and Stereoinvestigator software (Microbrightfield). Cells were counted in vertical columns (250-µm width) spanning the entire thickness of the primary visual cortex. Six to eight sections were analyzed for each experimental animal. For the colocalization analysis, we calculated the percentage of PV-positive cells surrounded by PNNs. Counting of GAD67-positive cells was performed on three sections for each experimental animal. For each section, images of the visual cortex were acquired with a 20× objective (air, NA 0.5) by a Zeiss Axioscop Observer z1 microscope with a motorized stage. Acquisitions were automatically performed using the MosaX and Z-Stack modules of the Zeiss Axiovision software (v4.3.1). Five z series for each image were projected to two-dimensional representations. Cells were separately counted in layers II/III, IV and V/VI of the primary visual cortex with Adobe Photoshop software (200 µm × 600 µm counting frame). Only cells larger than 5 µm with a clearly visible nucleus were counted. For the counting of PV-positive boutons, confocal images from layer II/III of the visual cortex were acquired with a 60× objective (oil, NA 1.4) using a confocal microscope (A1, Nikon). Two fields for each animal were analyzed. Perisomatic PV-positive boutons around putative cell somas were counted using NIS-Elements AR software and are expressed as the number of boutons per cell soma in a single (largest) foci section, as described previously56.

For each animal, bouton densities from at least 15 cell somas were averaged. For all experiments, data acquisition and quantification were performed blind to the treatment.

Eye-opening observations. From P11, rat pups were inspected for eye opening twice daily. Eye opening was defined as the initial break in the membrane sealing the lids of both eyes.

Prusky water maze. Rats were tested blind to treatment in the visual water task as described previously3,19. The apparatus consisted of a trapezoidal-shaped pool filled with opaque water and partially divided at one end into two arms by a divider. Visual stimuli consisted of gratings or gray fields generated with two computer monitors and placed at the end of each arm. A hidden platform was placed below the grating. During the learning phase (P17 or P19 to P23 or P24), visual stimuli consisted of gratings of low spatial frequency (0.1 cycles/degree). Animals were trained to associate the stimulus grating with the submerged platform. We report the performance in the learning task as the percentage of correct choices over the training sessions.

Behavioral assessment of visual acuity was performed from P24 to P32. The limit of discrimination was estimated by increasing the spatial frequency of the grating until performance fell below 70% accuracy. For each rat, distinct sessions of visual acuity estimation were run three times a day every other day. A frequency-of-seeing curve was constructed from the total data, and the spatial frequency corresponding to 70% accuracy was taken as the acuity value41.

Quantitative RT-PCR. Visual cortices were dissected 3 h after midazolam treatment in P8 rat pups. Quantitative RT-PCR was performed as described previously54. Total RNAs were extracted (TRizol reagent, Invitrogen), treated with DNase and purified with the RNaseasy Mini Kit (Qiagen, USA). cDNA was synthesized from RNAs (2 μg) using SuperScript VILO (Invitrogen Life Technologies, USA). Quantitative real-time PCR was performed using a CFX 384 thermal cycler with real-time detection of fluorescence (Bio-Rad). Individual PCR reactions were conducted using the MESA GREEN quantitative PCR (qPCR) kit (Eurogentec SA, Belgium). Primers (Sigma) were designed on different exons to avoid amplification of genomic DNA. Glyceraldehyde 3-phosphate dehydrogenase (G3PDH) was used as a standard for quantification. The primer sequences used were as follows: Bdf1 exon 9a, forward AGTGGGCCCATATCGTGTCCCGC, reverse TTTCTGTCCTATCCAGCAGCAG; Gadph, forward ACTAACCCTTGGTCCCAAATCT, reverse CTCTTGTCTCTTCAGTATCCTT. Each PCR cycle consisted of denaturation for 10 s at 94 °C, annealing for 20 s at 60 °C and extension for 30 s at 72 °C.
The fluorescence intensity of SYBR green was read and acquired at 72 °C after completion of the extension step of each cycle. The PCR conditions for the individual primer sets were optimized by varying the template cDNA and primer concentration to obtain a single PCR product and an amplification efficiency $>90\%^{54}$. Relative expression values were calculated using the $\Delta$Ct method. Mean cycle threshold values from triplicate experiments (Ct) were calculated for Bdnf exon 9a and Gapdh and were corrected for PCR efficiency and inter-run calibration. Bdnf:Gapdh ratios were then calculated for each sample.

**Statistical analysis.** Statistical analysis was performed with SigmaPlot 11.0. Sample size was calculated by the sample-size calculator in Sigma-plot 11.0 (power $>0.05$; $\alpha = 0.05$). All data were run for a normality test by default in SigmaPlot 11.0 before any statistical comparison tests. Data that were normally distributed are summarized by the mean ± s.e.m., whereas data non-normally distributed are summarized with percentiles and a box chart. Pairwise comparisons of quantitative phenotypes between rats of different groups were assessed by two-tailed Student's $t$ test. When more than two groups or factors were analyzed, one- or two-way ANOVA followed by a Holm-Sidak, Tukey or Duncan test or a one-way ANOVA on ranks with Dunn's post hoc test was used for data normally or not normally distributed, respectively. For the analysis of learning in the Prusky water box and the longitudinal measurements of behavioral visual acuity, we ran a two-way repeated-measures ANOVA. Eye-opening observations were compared with a $\chi^2$ test. A Grubbs' test (extreme Studentized deviate) was performed to verify the presence of outliers in the data sets. The level of significance used was $P < 0.05$.

For image acquisition at the confocal microscope, all slides were acquired in a random order and in a single session for each litter of animals to minimize errors caused by fluctuation in laser output and degradation of fluorescence. For electrophysiological recordings, care was used to record from one bumetanide- and one vehicle-injected animal on alternating days. For the LTP, MD and behavioral experiments, the animals treated with bumetanide or vehicle were littermates.

Where not otherwise specified, data collection and analysis were not performed blind to the conditions of the experiments.

A **Supplementary Methods** checklist is available.

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