Prolonged Period of Cortical Plasticity upon Redox Dysregulation in Fast-Spiking Interneurons

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ABSTRACT

BACKGROUND: Oxidative stress and the specific impairment of perisomatic gamma-aminobutyric acid circuits are hallmarks of the schizophrenic brain and its animal models. Proper maturation of these fast-spiking inhibitory interneurons normally defines critical periods of experience-dependent cortical plasticity.

METHODS: Here, we linked these processes by genetically inducing a redox dysregulation restricted to such parvalbumin-positive cells and examined the impact on critical period plasticity using the visual system as a model (3–6 mice/group).

RESULTS: Oxidative stress was accompanied by a significant loss of perineuronal nets, which normally enwrap mature fast-spiking cells to limit adult plasticity. Accordingly, the neocortex remained plastic even beyond the peak of its natural critical period. These effects were not seen when redox dysregulation was targeted in excitatory principal cells.

CONCLUSIONS: A cell-specific regulation of redox state thus balances plasticity and stability of cortical networks. Mistimed developmental trajectories of brain plasticity may underlie, in part, the pathophysiology of mental illness. Such prolonged developmental plasticity may, in turn, offer a therapeutic opportunity for cognitive interventions targeting brain plasticity in schizophrenia.

Keywords: GABA, Oxidative stress, Parvalbumin, Perineuronal net, Schizophrenia, Visual cortex

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Schizophrenia is increasingly recognized as a neurodevelopmental disorder that involves alterations in brain circuits, including dysfunction of parvalbumin (PV)-positive, fast-spiking gamma-aminobutyric acid (GABA) neurons (1,2). Mounting evidence also indicates that redox dysregulation, an imbalance between oxidative stress and antioxidant defense systems, may play a role in the pathogenesis [reviewed in (3)]. Reduced antioxidant enzymes are reported in schizophrenic patients (4–8), and several divergent genetic (e.g., Gclc, Gclm, DISC1, PRODH, G72, NRG1) (9–17) or external factors (e.g., stress, social isolation, ketamine, neonatal ventral hippocampal lesion) (18–24) collectively lead to redox dysregulation. Animal models of globally disrupted redox state exhibit defects in fast-spiking interneurons (16,23,25) consistent with physiological consequences on excitatory-inhibitory circuit balance during neurodevelopment. Notably, the postnatal maturation of PV-positive GABA neurons has been found to define critical periods of experience-dependent cortical plasticity (26). These windows in infancy and early childhood reflect heightened circuit rewiring to adaptively match sensory maps and complex behaviors to the surrounding environment and are then gradually consolidated into adulthood. Accordingly, disruption of excitatory-inhibitory balance powerfully shifts the timing and quality of critical period development, as seen in the primary visual cortex (27–29). High metabolic demands render fast-spiking interneurons particularly vulnerable to oxidative stress (23). Here, we examined whether late developing redox dysregulation within PV cells alone would directly impact the profile of cortical plasticity.

METHODS AND MATERIALS

Animals

Wild-type mice (C57BL/6J; Charles River, Wilmington, Massachusetts) and CamK2-Cre transgenic (c/o A. Nagy, Ph.D., University of Toronto, Ontario, Canada) (30), PV-ires-Cre (c/o S. Arber, Ph.D., Friedrich Miescher Institute Basel, Basel, Switzerland) (31), or Gclc-f/f mice (32) were crossed to generate CamK2-Gclc or PV-Gclc knockout (KO)/wild-type (WT) mice raised from birth on a 12-hour light/dark cycle to various postnatal ages. To rule out potential ectopic effects of Cre expression (33,34), we were careful to compare only with homozygous Cre+/+ animals as control animals.

In Situ Hybridization

Probes for mouse Gclc and Cre were synthesized using T3/T7 RNA polymerase (Roche, Indianapolis, Indiana) labeled with digoxigenin or fluorescein and hybridized to frozen sections.

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Gccl probe was generated against a pCMV-SPORT6 plasmid containing the full-length mouse Gccl complementary DNA sequence between Not1 and Sal1 restriction enzyme sites (clone ID: 4193582, Invitrogen, Life Technologies, Grand Island, New York). Cre probe was generated using the template pBluescriptSK(-) plasmid including the full sequence of Cre subcloned from pCAG-Cre (Addgene plasmid 13775: between EcoR1 and Cla1 restriction enzyme sites). TSA-Plus DNP System (PerkinElmer Life Sciences, PerkinElmer, Branford, Connecticut) in combination with fast red staining was used to amplify the signal for double fluorescent in situ hybridization.

**Immunohistochemistry**

Mice were perfused transcardially with .9% saline and 4% paraformaldehyde, then brains were removed into 30% sucrose and transferred to ethylene glycol solution for cryoprotection. Brains were cut in coronal section (30 or 40 μm) on a freezing microtome. Sections were rinsed in phosphate buffered saline, then incubated overnight at 4 °C in monoclonal antibody against parvalbumin (rabbit, 1:500, Swant, Marly, Switzerland), 8-oxo-7,8-dihydro-20-deoxyguanine (8-oxo-dG) (mouse, 1:350, Trevigen, Gaithersburg, Maryland), NeuN (mouse, 1:400, EMD Millipore, Billerica, Massachusetts), CD68 (rat, 1:400, Serotec, AbD Serotec, Raleigh, North Carolina), biotin-conjugated lectin Wisteria floribunda agglutinin (WFA) (1:400 in phosphate buffered saline, Vector Laboratories, Burlingame, California), Ob2 (CD4, 1:50 gift from A. Prochiantz, Collège de France, Paris, France) and secondary antibodies (anti-IgG-Alexa-488, 594, 633/streptavidin (WFA) (1:400 in phosphate buffered saline, Vector Laboratories). Fluoro-Jade C (Millipore, EMD Millipore) staining was performed according to previously described procedure (35).

Quantification of PV+ cells with strong 8-oxo-dG immunoreactivity was performed by combining threshold (between the values of 20 and 256) and particle analysis (particle size above 60 pixel^2) modules of ImageJ software (National Institutes of Health, Bethesda, Maryland) to distinguish strongly labeled cells from background signal. PV+ / WFA+ cell counts were quantified using the spot module of Imaris 7.1 software (Bitplane AG, Zurich, Switzerland) as described previously (25). Briefly, we analyzed the amount of co-localization of immunostained voxels, using all immunostained voxels with fluorescence intensity above .03 (arbitrary unit) and the Colocal module of Imaris software. Immunostained voxels with fluorescence intensity below .03 (arbitrary unit) were neglected, as these were within the background fluorescence intensity level. Three to five mice were used per experimental condition.

**Monocular Deprivation Procedure**

Eyelid margins were trimmed by iris scissors and eyes sutured shut under isoflurane anesthesia. Eyes were closed for 4 to 5 days for short-term monocular deprivation from postnatal day (P)25 (during the critical period) or after (P50).

**Extracellular Recording In Vivo**

Electrophysiological recordings were performed under Nembutal/chlorprothixene anesthesia using standard techniques in mice (28). Ocular dominance (OD) in the binocular zone from each mouse was calculated as a contralateral bias index (CBI): [(n1 – n7) + 2/(n2 – n6) + 1/(n3 – n5) + N/2N], where N = total number of cells and n = number of cells corresponding to ocular dominance score of x. The CBI approaches 1.0 as contralateral (contra) eye input dominates and is reduced when the ipsilateral (ipsi) eye becomes stronger. OD score was computed on cells with a complete peristimulus time histogram analysis of peak and baseline spiking activity, by alternately covering either eye. OD score was defined as [Peak(ipsi) – baseline(ipsi)] – [Peak(contra) – baseline(contra)] / ([Peak(ipsi) – baseline(ipsi)] + [Peak(contra) – baseline (contra)]) (29). For statistical comparison of OD distributions, normalized OD scores of individual neurons were computed and plotted as a cumulative distribution for each experimental group. CBI values across groups of mice were compared by one-way analysis of variance or Student t test.

**RESULTS**

**Modeling Genetic Redox Dysregulation Within PV Cells In Vivo**

To perturb redox balance specifically within PV interneurons, we conditionally deleted the Gccl (glutamate cysteine ligase catalytic subunit) gene—the rate-limiting enzyme that produces the primary endogenous antioxidant and redox regulator, glutathione (3) (Figure 1A). Importantly, the activity of glutathione is lower in schizophrenic patients, and the GAG-repeat of the Gccl promoter is associated with the illness (9). As systemic Gccl deletion is lethal (36), we crossed mice with the Gccl gene flanked by loxp sites (32) to animals expressing Cre recombinase under control of the PV promoter (31). To rule out potential ectopic effects of Cre expression (33,34), we were careful to compare PV-Cre+/−; Gcclfl/fl (PV-Gccl KO) mice with homozygous PV-Cre+/+ (PV-Gccl WT) control animals.

This yielded a progressive Gccl deletion in cortical PV cells from 34% at P20 to 70% in adulthood (>P50), as evaluated by double in situ hybridization for Gccl and Cre messenger RNA (Figure 1B,C). The cell-specific manipulation was subtle and did not cause myelin deficits (Figure 1D,E) typically observed upon global redox dysregulation (3,37). Instead, a cell-autonomous enhancement of oxidative stress was observed in the majority (69%) of PV cells when staining for a product of mitochondrial DNA oxidation, 8-oxo-dG (Figure 2A,B). This allowed us to further test the impact of PV cell-specific oxidative stress on postadolescent cortical plasticity. Note that we cannot rule out the possibility of mild noncell autonomous increase of oxidative stress below detection of our 8-oxo-dG staining threshold set to capture only those cells with enhanced oxidative stress beyond the basal levels of physiological stress.

**Redox Dysregulation Decreases Perineuronal Nets Enwrapping PV Cells**

As PV cells mature through their critical period, they are increasingly enwrapped by a perineuronal net (PNN) of extracellular matrix. Enzymatic removal of PNNs can reopen plasticity in adulthood (38,39). In the primary visual cortex of PV-Gccl KO mice, we observed significantly fewer PV-immunoreactive cells and PNNs, as revealed by WFA staining.
Extended Critical Period Plasticity in PV-Gclc KO Mice

PV cell, which might signal microglial cells. In fact, a mild activation of microglia was observed by CD68 staining (Figure 3A,B).

For comparison, we also examined the brains of mice carrying a Gclc gene deletion from principal neurons, which express the calcium/calmodulin-dependent protein kinase type 2 (CaMK2) not expressed in PV cells (27) (Figure 3B). Consistent with a more widespread redox dysregulation across the pyramidal cell population, a massive activation of microglia (Figure 3C) and neurodegeneration (Figure S2 in Supplement 1) was observed, indicating that this is a robust, non-specific response to cellular stress of any origin. In contrast, immunoreactivity for PV and PNNs remained intact in the adult visual cortex of CaMK2-Gclc KO mice (Figures S4 and S5 in Supplement 1). Microglia have recently been implicated as active participants in brain plasticity per se (42–45). We next examined the status of brain plasticity in PV-Gclc KO mice, given their PNN loss—a major brake on adult cortical rewiring (38).

Prolonged Critical Period Plasticity upon PV Cell-Specific Redox Dysregulation

The classic loss of input from an eye briefly deprived of vision in the binocular zone of primary visual cortex is typically

**Figure 1.** Modeling genetic redox dysregulation within parvalbumin (PV) cells in vivo. (A) Gclc normally forms a complex with Gclm as the rate-limiting enzyme for producing the major brain antioxidant and redox regulator, glutathione. Deletion of the Gclc gene induces redox dysregulation by lowering glutathione levels. (B) Double in situ hybridization for Gclc (green) and Cre (red) recombinase in mature primary visual cortex (V1) (> postnatal day [P]50) of wild-type (WT) (left) and PV-Gclc knockout (KO) (right) mice. (C) Quantification of co-localization reveals progressive developmental loss of Gclc expression in PV-Cre interneurons from 34% (P20) to 70% (P60) in PV-Gclc KO mice (dark gray bars) (one-way analysis of variance: p < .05), as compared with WT mice in which almost 100% of Cre-positive cells constantly express Gclc. (D) Immunohistochemical visualization of myelin basic protein (MBP) density in mouse V1 (> P50) of WT (left) and PV-Gclc KO (right) mice. (E) Quantification of mean MBP intensity shows no significant difference between genotypes (p > .3, t test). Scale bar, 150 μm. AU, arbitrary unit; GCL, glutamate-cysteine ligase; mRNA, messenger RNA; ns, non-significant; WM, white matter.

(Figure 2). The loss of PNNs was quite pronounced (Figure 2C), and the percentage of PV cells bearing PNNs was also reduced (Figure 2D). Compromised PNNs prevent the persistent uptake of Otx2 homeoprotein into the enwrapped PV cells needed for their maturation (including PNN maintenance itself) (27,40,41). Consistent with this, the percentage of Otx2+/PV+ cells was significantly reduced in PV-Gclc KO mice, especially in those cells enwrapped with PNNs (Figure S3 in Supplement 1).

PV-Gclc KO mice further exhibited a significant loss of persomatic PV+ puncta surrounding their target pyramidal cell bodies in layer 2/3 of adult visual cortex (Figure S1 in Supplement 1). Instead, there was no indication of neurodegeneration by Fluoro Jade C staining, suggesting that PV cells did not die in PV-Gclc KO mice but were rather impaired (Figure S2 in Supplement 1). PNN loss might allow the diffusion of excessive reactive oxygen species outside the

**Figure 2.** Redox dysregulation and loss of perineuronal nets (PNNs) enwrapping parvalbumin (PV) cells. (A) Double-labeling for 8-oxo-7,8-dihydro-20-deoxyguanine (8-oxo-dG) and PV (red) in mature primary visual cortex (V1) postnatal day [P]50 of wild-type (WT) (left) and PV-Gclc knockout (KO) (right) mice. Higher magnification (inset) confirms elevation of oxidative stress in PV cells of PV-Gclc KO mice. (B) Left: Quantification (% of PV+ cells also expressing high levels of 8-oxo-dG in PV-Gclc mice [69.3% KO versus 7.2% WT; ***p < .001 t test). Right: Total number of PV+ cells, *p < .01 t test. (C) Double immunolabeling of PNNs with Wisteria floribunda agglutinin (WFA) (green) and PV (red) in mature V1 (P>50) of WT (left) and PV-Gclc KO (right) mice. Scale bar, 100 μm. (D) Left: Quantification of the number of WFA+ cells reveals loss of PNNs in PV-Gclc KO mice (versus WT; ***p < .001, t test). Right: Percentage of PV cells enwrapped by WFA1 PNN also reduced in KO mice (**p < .01, t test).
Immunohistochemistry for CD68, a marker of activated microglia, shows marked elevation of oxidative stress labeling (8-dihydro-2-deoxyguanine [8-oxo-dG]) in calcium/calmodulin-dependent protein kinase type 2 (CaMK2)-Gclc knockout (KO) (middle) compared with wild-type (WT) mice (left) in primary visual cortex (V1) (postnatal day [P]50) (p < .01, t test). (B) Marked elevation of oxidative stress labeling (8-oxo-dG) in calcium/calmodulin-dependent protein kinase type 2 (CaMK2)-Gclc KO (middle) compared with WT mice (left) (p < .05, t test). (C) Massive activation of microglia (CD68) in CaMK2-Gclc KO (middle) compared with WT mice (left) in V1 (P50) (p < .01, t test). Scale bar, 150 μm. AU, arbitrary unit; WM, white matter.

**Figure 3.** Microglial activation after cell-specific redox dysregulation. (A) Immunohistochemistry for CD68, a marker of activated microglia, shows mild elevation in parvalbumin (PV)-Gclc knockout (KO) (middle) compared with wild-type (WT) mice (left) in primary visual cortex (V1) (postnatal day [P]50) (p < .01, t test). (B) Marked elevation of oxidative stress labeling (8-dihydro-2-deoxyguanine [8-oxo-dG]) in calcium/calmodulin-dependent protein kinase type 2 (CaMK2)-Gclc KO (middle) compared with WT mice (left) (p < .05, t test). (C) Massive activation of microglia (CD68) in CaMK2-Gclc KO (middle) compared with WT mice (left) in V1 (P50) (p < .01, t test). Scale bar, 150 μm. AU, arbitrary unit; WM, white matter.
Altered PNNs have been found in the human postmortem amygdala and entorhinal and prefrontal cortices of patients (65–67), and GABA-related functional deficits are seen even in their visual cortex (68–71). Pathophysiological changes (1–3) are consistent with dematuration or a removal of brakes on plasticity (72,73). Several molecular brakes of relevance to schizophrenia (other than PNNs) have been identified to limit critical period plasticity, including myelin signaling through a NgR-PirB complex (2,74,75) or dampened nicotinic receptor signaling by Lynx1 (29,76). Curiously, all brakes to date converge on PV cell function as a hub of vulnerability (77).

This may offer novel strategies or drug targets, in addition to antioxidants (18,25) or Otx2 upregulation (41,51), for correcting aberrant brain plasticity in mental illness.

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