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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 peri-somatic GABA 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.

Method—Here, we link 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 (3–6mice/group).

Results—Oxidative stress was accompanied by a significant loss of perineuronal nets, which normally envelop 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.

Conclusion—A cell-specific regulation of redox state thus balances plasticity and stability of cortical networks. Mis-timed 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

visual cortex; oxidative stress; parvalbumin; perineuronal net; GABA; schizophrenia

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Introduction

Schizophrenia is increasingly recognized as a neurodevelopmental disorder that involves alterations in brain circuits, including dysfunction of parvalbumin (PV)-positive, fast-spiking 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 ref. (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 (E–I) 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 E–I balance powerfully shifts the timing and quality of critical period development, as seen in the primary visual cortex (V1) (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 (C57 Bl/6J; Charles River USA) and CamK2-Cre transgenic (c/o Dr A Nagy, Univ Toronto)(30), PV-ires-Cre (c/o Dr S Arber, FMI Basel) (31), or *Gclc*-f/f mice (32) 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 to homozygous *Cre*^{+/+} animals as controls.

In situ hybridization

Probes for mouse *Gclc* and *Cre* were synthesized using T3/T7 RNA polymerase (Roche) labeled with digoxigenin or fluorescein and hybridized to frozen sections. *Gclc* probe was generated against a pCMV-SPORT6 plasmid containing the full length mouse *Gclc* cDNA sequence between Not1 and Sal1 restriction enzyme sites (clone ID: 4193582, Invitrogen). *Cre* probe was generated using the template pBluescript2SK(–) 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) in combination with fast red staining was used to amplify the signal for double FISH.

Immunohistochemistry

Mice were perfused transcardially with 0.9% saline and 4% para-formaldehyde, 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 PBS, then incubated overnight at 4°C in monoclonal antibody against Parvalbumin (Rabbit, 1:500, Swant), 8-oxodG (Mouse, 1:350, Trevigen), NeuN (Mouse, 1:400, Millipore), CD68 (rat; 1:400, Serotec), biotin-conjugated lectin *Wisteria Floribunda Agglutinin* (WFA; 1:400 in PBS, Vectorlabs), Otx2 (CD4, 1:50 gift from Dr. A. Prochiantz) and secondary antibodies (anti-IgG-Alexa-488, 594, 633/streptavidin-Alexa 488; 1:400, invitrogen). Fluoro-Jade C (Millipore) staining was performed according to previously described procedure (35).

Quantification of PV+ cells with strong 8-oxodG immunoreactivity was performed by combining threshold (between the values of 20 and 256) and particle analysis (particle size above 60 pixel²) modules of ImageJ software 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, Switzerland) as described (25). Briefly, we analyzed the amount of colocalization of immunostained voxels, using all immunostained voxels with fluorescence intensity above 0.03 (arbitrary unit, a.u.) and the Coloc module of Imaris software. Immunostained voxels with fluorescence intensity below 0.03 (a.u.) were neglected, as these were within the background fluorescence intensity level. Three to five mice were used per experimental condition.

Monocular deprivation (MD) procedure

Eyelid margins were trimmed by iris scissor and eyes sutured shut under isoflurane anesthesia. Eyes were closed for 4–5 days for short term MD from P25 (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 (V1b) from each mouse was calculated as a contralateral bias index (CBI): $[(n1-n7)+2/3(n2-n6)+1/3(n3-n5)+N]/2N$, where N=total number of cells and nx=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 peri-stimulus time histogram analysis of peak and baseline spiking activity, by alternately covering either eye. OD score was defined as $\{[\text{Peak(ipsi)} - \text{baseline(ipsi)}] - [\text{Peak(contra)} - \text{baseline(contra)}]\} / \{[\text{Peak(ipsi)} - \text{baseline(ipsi)}] + [\text{Peak(contra)} - \text{baseline(contra)}]\}$ (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 ANOVA or Student's t test.

Results

Modeling genetic redox dysregulation within PV cells in vivo

To perturb redox balance specifically within PV interneurons, we conditionally deleted the *Gclc* (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 *Gclc* promoter is associated with the illness (9). As systemic *Gclc* deletion is lethal (36), we crossed mice with the *Gclc* 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^{+/+}; *Gclc*^{f/f} (PV-*Gclc* KO) mice to homozygous PV-Cre^{+/+} (PV-*Gclc* WT) controls.

This yielded a progressive *Gclc* deletion in cortical PV-cells from 34% at postnatal day P20 to 70% in adulthood (>P50), as evaluated by double *in situ* hybridization for *Gclc* and *Cre* mRNA (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-7,8-dihydro-20-deoxyguanine (8-oxo-dG) (Figure 2A,B). This allowed us to further test the impact of PV cell-specific oxidative stress on post-adolescent cortical plasticity. Note that we cannot rule out the possibility of mild non-cell 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 enveloping PV-cells

As PV-cells mature through their critical period, they are increasingly enveloped 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-*Gclc* KO mice, we observed significantly fewer PV-immunoreactive cells and PNNs, as revealed by WFA staining (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 enveloped 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 enveloped with PNNs (Supplemental Figure S3).

PV-*Gclc* KO mice further exhibited a significant loss of perisomatic PV+ puncta surrounding their target pyramidal cell bodies in layer 2/3 of adult visual cortex (Supplemental Figure S1). 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 (Supplemental Figure S2). PNN loss might allow the diffusion of excessive reactive oxygen species outside the 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 (Supplemental Figure S2) was observed, indicating that this is a robust, nonspecific 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 (Supplemental Figure S4 and S5). 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 classical loss of input from an eye briefly deprived of vision in the binocular zone of primary visual cortex is typically constrained before P50 (*reviewed in ref.* (46, 47)) (Fig 4A). When the majority of PV-cells lacked *Gclc* gene expression by this age in our conditional mutant mice (Figure 1A), extracellular single-unit recording continued to yield a significant shift away from the deprived, contralateral eye following short-term (4 day) monocular deprivation (Figure 4B,C,E). The contralateral bias index (CBI) differed significantly between genotypes (0.57 in KO vs. 0.68 in WT; age range between P54 and P63, $p < 0.001$). This enhanced plasticity in PV-*Gclc* KO mice past the peak of the critical period was not corrected by concurrent administration of the benzodiazepine Diazepam (Supplemental Figure S6), suggesting an earlier window may correct the plasticity phenotype (18).

Consistent with a limited *Gclc* deletion in younger KO mice (Figure 1C) during the natural critical period, there was no difference from WT plasticity levels at that age (Supplemental Figure S7; CBI after 4 day MD = 0.49 in PV-*Gclc* KO vs. 0.51 in WT; $p > 0.2$, $n = 3$ mice each). Moreover, in CamK2-*Gclc* KO mice, carrying a more severe redox dysregulation restricted to pyramidal cells, no extended plasticity was seen as in typical adult WT mice (Figure 4D,E; CBI = 0.69 and 0.67, respectively; $p > 0.8$). Given the inverse relationship of microglial activation and the level of cortical plasticity in adult PV- and CamK2-*Gclc* KO mice, the contribution of microglial response to cortical plasticity may be more subtle than expected or reflect an optimal range of activation.

Discussion

Our results reveal a prolonged period of brain plasticity – or a failure to stabilize cortical circuits – in young adult animals under conditions of PV-cell specific glutathione dysregulation *in vivo*. Mis-timed developmental trajectories of brain plasticity may therefore contribute in part to the etiology of mental illnesses involving oxidative stress, such as schizophrenia (2, 3, 18). This reflects a redox-sensitive failure to maintain PV-cells with fully enwrapped PNNs, which normally balance plasticity and stability of cortical circuits across development in a twofold manner (38, 39).

First, intact PNNs may structurally limit the robust synaptic plasticity directly upon PV-cells (48–50). Second, PNN disruption prevents the persistent uptake of Otx2 homeoprotein into the enwrapped PV-cells required for their maintenance (27, 40, 41) (Fig. S3). Subsequent inability to engage downstream molecular pathways by Otx2 within the PV-cell would retain a juvenile plastic state (41). Notably, the major endogenous source of this Otx2 has recently been identified as the choroid plexus (51), which lines the ventricles that are commonly found to be enlarged in schizophrenia.

Two of the primary PNN components, chondroitin sulfate and hyaluronan, are sensitive to excessive free radicals (52), and the relatively late timing of *Gclc* deletion here overlaps with their usual condensation into tight nets around PV-cells. Our recent study revealed a protective function of these PNNs as a redox sensor (53). The high metabolic requirements of the fast-spiking PV-interneuron (23) may readily generate reactive oxygen species when their antioxidant systems are impaired, leading to the breakdown of PNNs themselves (Figure 2C,D) (53). Our study suggests that cell-autonomous redox dysregulation within PV-cells is the primary contributor to a reduction of PNNs observed across mouse models and human patients sharing global redox dysregulation.

Paradoxically, redox dysregulation in schizophrenia may then act in part by transiently prolonging or reopening critical periods of developmental plasticity. An excessively plastic status may precede the progressive degenerative process as in other brain disorders (29, 54). In chronic schizophrenia patients, deficits in visual cortex and long-term potentiation-like plasticity are reported when probed by transcranial direct current stimulation (55–58). First onset patients instead do not differ significantly from healthy controls with a trend toward increased plasticity (59). Individuals at high risk show greater cognitive gains relative to adults with chronic schizophrenia in response to computer-based cognitive training (60). Our study points to the importance of studying the *developmental trajectory* of cortical plasticity in schizophrenia patients to understand the relation to disease progression, remission, and treatment response (61).

Prolonged developmental plasticity may then provide a diagnostic opportunity for cognitive interventions targeting brain plasticity in the prodromal stage (62–64). Unfortunately, the current study was limited before and after the age range we studied (P54–63), either by premature death of PV-*Gclc* KO mice by P80 (as PV is also expressed in muscle) or insufficient deletion of the *Gclc* gene at younger stages. Also, we cannot fully rule out the possibility that the prolonged plasticity in these mice is mediated by an as yet unknown consequence of *Gclc* deletion not directly related to redox dysregulation. Future studies in other animal models are required to rigorously map the trajectory of brain plasticity across the lifespan in relation to redox dysregulation and other risk factors.

Altered PNNs have been found in the human *post mortem* amygdala, 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 up-regulation (41, 51), for correcting aberrant brain plasticity in mental illness.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Modeling genetic redox dysregulation within 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 V1 (> P50) of WT (left) and PV-*Gclc* 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 grey bars) (1way ANOVA: $p < 0.05$), as compared to 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 ($p > 0.3$, t-test). Scale bar, 150 μ m.

Figure 2. Redox dysregulation and loss of perineuronal nets (PNNs) enwrapping PV-cells
(A) Double-labeling for 8-oxo-dG (green) and PV (red) in mature V1 (>P50) of WT (left) and PV-*Glc* KO (right) mice. Higher magnification (inset) confirms elevation of oxidative stress in PV cells of PV-*Glc* KO mice. **(B)** (left) Quantification (%) of PV+ cells also expressing high levels of 8-oxo-dG in PV-*Glc* mice (69.3% KO vs 7.2% WT; $P < 0.0001$, t-test). (right) Total number of PV (+) cells, $**p < 0.01$ t-test. **(C)** Double immunolabeling of PNNs with WFA (green) and PV (red) in mature V1 (>P50) of WT (left) and PV-*Glc* KO (right) mice. Scale bar, 100 μ m. **(D)** (left) Quantification of the number of WFA+ cells reveals loss of PNNs in PV-*Glc* KO mice (vs WT; $***p < 0.001$, t-test). (right) Percentage of PV-cells enwrapped by WFA+ PNN also reduced in KO mice ($**p < 0.01$, t-test).

Figure 3. Microglial activation after cell-specific redox dysregulation

(A) Immunohistochemistry for CD68, a marker of activated microglia, shows mild elevation in PV-*Glc* KO (middle) compared to WT mice (left) in V1(>P50) ($p<0.01$, t-test). (B) Marked elevation of oxidative stress labeling (8-oxo-dG) in CamK2-*Glc* KO (middle) compared to WT mice (left) ($p<0.05$, t-test) (C) Massive activation of microglia (CD68) in CamK2-*Glc* KO (middle) compared to WT mice (left) in V1 (>P50) ($p<0.01$, t-test). Scale bar, 150 μ m.

Figure 4. Prolonged cortical plasticity by PV-cell specific redox dysregulation

(A) Contralateral eye bias assessed by single-unit electrophysiology in mouse V1 after 4 days of short-term monocular deprivation (STMD) in adulthood. (B–D) Ocular dominance distribution after STMD (5 mice each) in (B) WT (127 cells), (C) PV-*Gclc* KO (121 cells) and (D) CamK2-*Gclc* KO mice (108 cells). Age range from P54 to P63. Visual responses classified on a seven-point scale from exclusively contralateral (group 1) to ipsilateral driven (group 7). PV-*Gclc* KO vs WT: *** $p < 0.0005$; PV-*Gclc* KO vs CamK2-*Gclc* KO: *** $p < 0.0001$, χ^2 -test. (E) Cumulative probability of quantified spike response after adult STMD confirms shifted ocular dominance scores for PV-*Gclc* KO (pink filled circles), compared to WT (gray filled circles) (* $p < 0.05$, Kolmogorov-Smirnov test). (F) Mean contralateral bias index (CBI), starting from the same baseline values (0.68) in non-deprived mice across genotypes, is shifted only in PV-*Gclc* KO mice by STMD (0.57; *** $p < 0.001$ vs WT, adjusted P value, Tukey's multiple comparison test). No adult plasticity in CamK2-*Gclc* KO mice (mean CBI = 0.69 vs 0.67 in WT mice; $p > 0.8$, adjusted P value, Tukey's multiple comparison test, 1 way ANOVA). Shaded area indicates CBI range of non-deprived (aplastic) mice.