Early-Life Insults Impair Parvalbumin Interneurons via Oxidative Stress: Reversal by *N*-Acetylcysteine

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Background: A hallmark of the pathophysiology of schizophrenia is a dysfunction of parvalbumin-expressing fast-spiking interneurons, which are essential for the coordination of neuronal synchrony during sensory and cognitive processing. Oxidative stress as observed in schizophrenia affects parvalbumin interneurons. However, it is unknown whether the deleterious effect of oxidative stress is particularly prevalent during specific developmental time windows.

Methods: We used mice with impaired synthesis of glutathione (*Gclm* knockout [KO] mice) to investigate the effect of redox dysregulation and additional insults applied at various periods of postnatal development on maturation and long-term integrity of parvalbumin interneurons in the anterior cingulate cortex.

Results: A redox dysregulation, as in *Gclm* KO mice, renders parvalbumin interneurons but not calbindin or calretinin interneurons vulnerable and prone to exhibit oxidative stress. A glutathione deficit delays maturation of parvalbumin interneurons, including their perineuronal net. Moreover, an additional oxidative challenge in preweaning or pubertal but not in young adult *Gclm* KO mice reduces the number of parvalbumin-immunoreactive interneurons. This effect persists into adulthood and can be prevented with the antioxidant *N*-acetylcysteine.

Conclusions: In *Gclm* KO mice, early-life insults inducing oxidative stress are detrimental to immature parvalbumin interneurons and have long-term consequences. In analogy, individuals carrying genetic risks to redox dysregulation would be potentially vulnerable to early-life environmental insults, during the maturation of parvalbumin interneurons. Our data support the need to develop novel therapeutic approaches based on antioxidant and redox regulator compounds such as *N*-acetylcysteine, which could be used preventively in young at-risk subjects.

Key Words: Anterior cingulate cortex, gene-environment interactions, glutamate cysteine ligase, glutathione, parvalbumin, schizophrenia

Converging evidence suggests that a redox dysregulation due to genetic and/or environmental factors might contribute to schizophrenia pathophysiology. Oxidative stress and abnormal levels of antioxidants, including reduced levels of glutathione (GSH), a major antioxidant and redox regulator, are observed in peripheral tissue and central nervous system of patients (1,2). The implication of redox dysregulation in the pathology is supported by the beneficial effect of the redox regulator and GSH precursor *N*-acetylcysteine (NAC) on schizophrenia patients (3–5). This redox dysregulation can have a genetic origin. Impaired upregulation of GSH synthesis is observed in patients carrying risk variants in the gene for the catalytic subunit of glutamate cysteine ligase (GCL), the rate-limiting enzyme of GSH synthesis (6). Moreover, abnormal function of proteins encoded by well-replicated susceptibility genes (*PRODH, DISC1, G72, DTNBP1, NRG1*) also causes oxidative stress and/or hypersensitivity to oxidative stress (7–11). Thus, a genetic susceptibility to redox dysregulation could render individuals vulnerable to the impact of additional environmental insults, known to generate oxidative stress and affect antioxidant systems (1). We hypothesize, on the basis of the concept that schizophrenia has a neurodevelopmental component (12), that a redox dysregulation caused by genetic susceptibilities and environmental insults during development could contribute to the emergence of the disease.

Among well-replicated observations on the pathophysiology of schizophrenia are anomalies in parvalbumin-expressing fast-spiking interneurons (PVI) and their synaptic connections to pyramidal neurons (13). Fast-spiking interneurons control the output of principal neurons and are necessary for γ neuronal synchrony, facilitating information processing during sensory perception and cognitive tasks (14–16). Abnormal γ oscillations during cognitive and sensory tasks in patients further support a dysfunction of these interneurons in schizophrenia (17–20). Studies in rodents show that PVI are sensitive to severe psychosocial stress such as maternal separation (21) and social isolation (22,23). The PVI impairment after these environmental trauma could be mediated by oxidative stress. Indeed, social isolation and ketamine administration affect PVI via superoxide overproduction (22,24). Interestingly, decreased GSH levels (25,26), sign of oxidative stress (27), reduced parvalbumin (PV) expression (28), and PVI number (29) are reported in prefrontal cortex of patients. These suggest that a redox dysregulation might contribute to PVI defects. Presently unknown, however, is whether PVI impairment results from early-life disturbances or arises later on onset or in the course of the illness. In this context, it was important to assess PVI vulnerability to oxidative stress along various stages of their maturation. In rodent models, PVI start to express PV after the first postnatal week (30,31). While they develop chemical and electrical connections, they acquire fast-spiking properties after 2–3 weeks (32). They undergo further developmental changes at adolescence (second month), including changes in dopamine modulation of their

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excitability (33) and in expression of *N*-methyl-D-aspartate and calcium ion-permeable α-Amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (34). During this maturation process, a specialized extracellular matrix, the perineuronal net (PNN), also forms around these interneurons (35) to promote maturation and synaptic and network stability (36).

To study the existence of vulnerability period(s) to redox dysregulation in PVI, we investigated these interneurons in mice with a genetically impaired GSH synthesis (*Gclm* knockout [KO] mice). These mice have 60%–70% decreased brain GSH contents (37,38), because they do not express GCLM, the modulatory subunit of GCL. This is a valid animal model, because deficit in GSH (25,26) and genetic association between *GCLM* and schizophrenia (39) have been reported. We investigated whether impaired GSH synthesis affected PVI maturation in the anterior cingulate cortex (ACC) and whether additional exogenous stress applied during specific developmental periods had deleterious and long-term effects on these interneurons. The ACC is indeed sensitive to early-life stress (40–43) and is affected in schizophrenia patients (44). Moreover, oxidative stress (27) and anomalies of non-pyramidal neurons are reported in ACC of patients (45). Finally, we explored whether PVI could be protected by an antioxidant.

Methods and Materials

Animals

Gclm KO mice (B6.129-*Gclm*^{tm1Tdal}) were provided by T. Dalton (University of Cincinnati) (46). *Gclm* KO mice were backcrossed with C57BL/6J mice over >10 generations. Mice were housed under a 12-hour light-dark cycle in groups of 3–5 individuals/cage. All breeding mice were obtained from heterozygotes (HZ × HZ). Experimental *Gclm* KO mice were from breeding individuals (males KO × females HZ), and control *Gclm* WT mice were from breeding individuals (WT × WT). Experiments were performed on males and were approved by the Local Veterinary Office.

Induction of an Additional Oxidative Stress During Various Developmental Periods

A pharmacological approach was chosen to generate additional oxidative insult in regions richly innervated by dopaminergic neurons (i.e., prefrontal cortex including ACC). This was accomplished by administration of a specific inhibitor of the dopamine reuptake transporter, GBR-12909 (GBR). The GBR-12909 (BioTrend, Zurich, Switzerland) was injected (SC 5 mg/kg) daily, between postnatal days 10–20 (preweaning), or 30–40 (pubertal), or 80–90 (young adult). Phosphate buffer saline (PBS) was used for control injection.

NAC Treatment

Fresh solution (1 g/L) of NAC (Sigma-Aldrich, St. Gallen, Switzerland), a precursor of GSH with antioxidant properties (47), was provided as drinking water to pregnant females and lactating mothers and their offspring until sacrifice (at day 40). The NAC solution was renewed every other day. In addition, NAC was injected into pups (SC 1 g/kg) daily from day 5 to 10, a period in which PV expression begins.

Immunohistochemistry and Stereological Quantification of PVI

Mice were anesthetized and perfused, and their brains were fixed (48). Coronal frozen sections (40 μm) were used to investigate the ACC and primary somatosensory cortex (SM1). Brain sections were immunolabeled for PV as in Steullet *et al.* (38) and Cabungcal *et al.* (48). Cell density count of PVI was quantified in the ACC and SM1 with the StereoInvestigator 7.5 software (MBF Bioscience, Williston, Vermont) (38). Stereological counting started with low magnification (2.5× objective) to identify the boundaries of the region of interest (ROI) on 2–4 consecutive sections from each animal. The ACC (at Bregma approximately 1.3–1.4) was delineated from the boundaries of infralimbic and secondary motor cortices after the anatomical cytoarchitectonic areas given by Franklin and Paxinos (49). This included the cingulate cortex area 1 (cg1) and part of area 2 (cg2). A small intermediate zone was created between these three regions, to ensure that the ROI in the ACC did not overlap with infralimbic and secondary motor cortices. An optical dissector (counting box) within the section thickness and sampling frames adapted for the ACC and SM1 were used to analyze and count neurons (50). The optical dissector boxes (40 × 40 μm with a depth of 15 μm) were placed by the software in each sampling frame starting from a random point inside the ROI of the ACC or SM1. Counting was carried out with higher magnification (40× objective). The PVI were counted when they were in focus at the surface of the box until they were out of focus at 15-μm depth of the counting box. A 5-μm guard zone was used to avoid artifacts caused by tissue shrinkage due to the immune-preparation process. Because the ACC and SM1 differed in their size, the volume of brain sections analyzed was not equal for each region. In ACC of 10- and 20-day-old mice, 24 counting frames were used, whereas quantification in SM1 required 37 frames. The number of frames was increased to 26 in ACC of 40- and 90-day-old mice. The mean numbers of PVI/unit volume in the ACC (or SM1) were compared between genotypes or between treatments with *t* tests.

Immunofluorescence Staining, Confocal Microscopy, and Image Analysis

Oxidative stress was visualized with a mouse monoclonal primary antibody against 8-oxo-7,8-dihydro-20-deoxyguanine (8-oxo-dG), a DNA adduct formed by the reaction of hydroxyl radicals with the DNA guanine base (51). To assess 8-oxo-dG labeling in various types of interneurons, brain sections containing the ACC were incubated for 48 hours with rabbit polyclonal anti-PV, anti-calbindin-28k (anti-CB), or anti-calretinin (anti-CR) (1:2500; Swant, Marly, Fribourg, Switzerland) primary antibodies together with the mouse monoclonal anti-8-oxo-dG (1:350; AMS Biotechnology, Bioggio-Lugano, Switzerland) primary antibody. To visualize the PNN that specifically surrounds PVI, sections were incubated in a solution containing the biotin-conjugated lectin *Wisteria floribunda agglutinin* (WFA). Briefly, sections were first incubated with PBS + Triton .3% + sodium azide (1 g/L) containing 2% normal horse serum, followed by 48-hour incubation with rabbit polyclonal anti-PV (1:2500; Swant) and biotin conjugated-WFA (1:2000; Sigma). Sections were then washed, incubated with their appropriate fluorescent secondary antibodies (goat antimouse immunoglobulin G [1:300; Alexa Fluor 488; Molecular Probes, Eugene, Oregon], antirabbit immunoglobulin G [1:300; CY3; Chemicon International, Temecula, California], CY2-streptabidin [1:300]), and counterstained with 100 ng/mL DAPI (4′-6-diamidino-2-phenylindole; Vector Laboratories, Burlingame, California). Sections were visualized with a Zeiss confocal microscope with Plan-NEOFLUAR objectives. All peripherals were controlled with LSM 510 software (Carl Zeiss AG, Feldbach, Switzerland). Z stacks of nine images (with a 2.13-μm interval) were scanned (1024 × 1024 pixels) for analysis with IMARIS 7.3 software (Bitplane AG, Zurich, Switzerland). Images were filtered with a Gaussian filter to remove