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J. U. Sommer

Central Institute of Mental Health

A. Schmitt

University of Gottingen

M. Heck

Central Institute of Mental Health

E. L. Schaeffer

University of Sao Paulo

M. Fendt

University of Tübingen

See next page for additional authors

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Authors

J. U. Sommer, A. Schmitt, M. Heck, E. L. Schaeffer, M. Fendt, M. Zink, K. Nieselt, S. Symons, G. Petroianu, A. Lex, M. Herrera-Marschitz, R. Spanagel, P. Falkai, and P. J. Gebicke-Haerter

Differential expression of presynaptic genes in a rat model of postnatal hypoxia: relevance to schizophrenia

J. U. Sommer · A. Schmitt · M. Heck · E. L. Schaeffer · M. Fendt ·
M. Zink · K. Nieselt · S. Symons · G. Petroianu · A. Lex · M. Herrera-Marschitz ·
R. Spanagel · P. Falkai · P. J. Gebicke-Haerter

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Abstract Obstetric complications play a role in the pathophysiology of schizophrenia. However, the biological consequences during neurodevelopment until adulthood are unknown. Microarrays have been used for expression profiling in four brain regions of a rat model of neonatal hypoxia as a common factor of obstetric complications. Animals were repeatedly exposed to chronic hypoxia from postnatal (PD) day 4 through day 8 and killed at the age of

150 days. Additional groups of rats were treated with clozapine from PD 120–150. Self-spotted chips containing 340 cDNAs related to the glutamate system (“glutamate chips”) were used. The data show differential (up and down) regulations of numerous genes in frontal (FR), temporal (TE) and parietal cortex (PAR), and in caudate putamen (CPU), but evidently many more genes are upregulated in frontal and temporal cortex, whereas in parietal cortex the majority of genes are downregulated. Because of their primary presynaptic occurrence, five differentially expressed genes (CPX1, NPY, NRXN1, SNAP-25, and STX1A) have been selected for comparisons with clozapine-treated animals by qRT-PCR. Complexin 1 is upregulated in FR and TE cortex but unchanged in PAR by

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J. U. Sommer · M. Heck · M. Zink · R. Spanagel ·
P. J. Gebicke-Haerter (✉)
Department of Psychopharmacology,
Central Institute of Mental Health,
J5, 68159 Mannheim, Germany
e-mail: peter.gebicke@zi-mannheim.de

A. Schmitt · P. Falkai
Department of Psychiatry, University of Göttingen,
von-Siebold-Str. 5, 37075 Göttingen, Germany

A. Schmitt · E. L. Schaeffer
Laboratory of Neuroscience (LIM27), Institute of Psychiatry,
University of Sao Paulo, Rua Dr. Ovidio Pires de Campos 785,
05453-010 São Paulo, SP, Brazil

M. Fendt
Animal Physiology Zoological Institute, Faculty of Biology,
University of Tübingen, Auf der Morgenstelle 28,
72076 Tübingen, Germany

K. Nieselt · S. Symons
Department of Information and Cognitive Sciences,
Center for Bioinformatics Tübingen, University of Tübingen,
Tübingen, Germany

G. Petroianu
Department of Cellular Biology and Pharmacology,
Florida International University, 11200 SW 8th Street,
GL 495E, Miami, FL 33199, USA

A. Lex
Animal Physiology, Biological Institute, University of Stuttgart,
Pfaffenwaldring 57, 70550 Stuttgart, Germany

M. Herrera-Marschitz
Programme of Molecular and Clinical Pharmacology,
ICBM, Medical Faculty, University of Chile,
Av. Independencia 1027, Santiago 7, Chile

Present Address:

J. U. Sommer
Department of Otorhinolaryngology, Head and Neck Surgery,
Klinikum Mannheim, University of Heidelberg,
Mannheim, Germany

Present Address:

M. Heck
Department of Dermatology, Klinikum Mannheim,
University of Heidelberg, Mannheim, Germany

hypoxic treatment. Clozapine downregulates it in FR but upregulates it in PAR cortex. Similarly, syntaxin 1A was upregulated in FR, but downregulated in TE and unchanged in PAR cortex, whereas clozapine downregulated it in FR but upregulated it in PAR cortex. Hence, hypoxia alters gene expression regionally specific, which is in agreement with reports on differentially expressed presynaptic genes in schizophrenia. Chronic clozapine treatment may contribute to normalize synaptic connectivity.

Keywords Hypoxia · Microarray · Presynaptic genes · Clozapine · PPI · Animal model

Introduction

In addition to genetic predisposition, the neurodevelopmental hypothesis of schizophrenia includes a number of environmental factors like obstetric and birth complications with ensuing consequences on the pathophysiology of the disease. Hence, the risk of schizophrenia increases with the number and severity of hypoxia-associated obstetric complications [5, 6, 22]. However, the pathogenetic implications of obstetric complications are unclear. The neurodevelopmental hypothesis of schizophrenia suggests that the onset of abnormal brain development and neuropathology occurs perinatally, whereas symptoms of the disease appear in early adulthood [32].

Schizophrenia patients show deficits in attention and processing of sensory information. A reliable test of sensorimotor gating in vertebrates is prepulse inhibition (PPI) of the acoustic startle reflex, which is disrupted in schizophrenia patients and restored by antipsychotics, mainly clozapine [2]. Several cortical brain regions and striatum are involved in PPI-modulating circuitry [30]. We previously reported on a PPI deficit in rats exposed to neonatal hypoxia appearing only during adulthood. This deficit was prevented and restored after chronic clozapine treatment [12].

In our animal model of chronic neonatal hypoxia, rats exhibited increased gene expression of the N-methyl-D-aspartate (NMDA) receptor subunit NR1 in hippocampal and cortical subregions [28], which suggests an involvement of the NMDA receptor in the pathophysiology of hypoxia-induced alterations. In schizophrenia, there is indeed strong support for disturbances of the glutamatergic and gamma-aminobutyric acid (GABA)ergic microcircuitry, mainly affecting the expression of presynaptic vesicle proteins including synaptosome-associated protein 25 (SNAP-25) and syntaxin, which form the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex [9, 10, 15, 20, 29]. Complexin (CPX) 1 and complexin 2 operate in synaptic vesicle

exocytosis by stabilizing the SNARE complex [4]. Neuropeptide Y (NPY) and neurexins (NRXN) play a role in cell adhesion and are hypothesized to play a role in the pathophysiology of schizophrenia [14, 20, 23, 26].

There is only little information about the influence of antipsychotic treatment on gene expression, as has been shown for NPY and CPX in normal rats [8, 16]. Thus, the present study investigates on the impact of neonatal hypoxia and long-term treatment with the antipsychotic clozapine on the expression of presynaptic genes in different cortical and subcortical brain regions in an animal model. Furthermore, correlations between differentially expressed genes and PPI deficits have been evaluated.

Materials and methods

Animals

A total of 12 Sprague–Dawley rats (Animal Facility, Department of Animal Physiology, University of Tübingen) were used for the experiments. The rats were housed in groups of 3 animals under a 12-h light/dark cycle with food and drinking water available ad libitum. The experiments were carried out in accordance with the international ethical guidelines for the care and use of laboratory animals for experiments (declaration of Helsinki, NIH guidelines) and were approved by the local animal care committee (Regierungspräsidium Tübingen, ZP 3/02).

Neonatal hypoxia

Chronic hypoxia (11% O₂, 89% N₂) was imposed from postnatal day (PD) 4–8 on six animal pups and their mothers by placing them in an air-tight plastic chamber for a period of 6 h per day. The six other pups were subjected to identical handling conditions and were placed in identical chambers but with regular oxygen concentrations (21% O₂ = normoxia) [12, 28].

Clozapine treatment

Between PD 120 and PD 150, half of the rats were being orally treated over a period of 4 weeks with 45 mg/kg/day clozapine (Novartis Pharma AG, Basel, Switzerland) [12].

Test on prepulse inhibition and tissue preparation

The startle magnitude was measured as described in [12]. Since a deficit in prepulse inhibition had been shown to surface on postnatal days 120 and 150, animals were killed at postnatal day 150. Brains were removed and immersed in −78°C cold isopentane and stored at −80°C until further

use. Slices of 120 μm thickness were cut on a cryostat and used to excise respective brain regions according to the atlas of Paxinos and Watson [25] using a micropunching tool. Resultant, cylindrical tissue blocks were collected on dry ice and stored at -80°C until further processing.

Molecular investigations

RNA isolation and reverse transcription

One milliliter of Trizol was added to excised tissue, and tissue was homogenized by 30 strokes of trituration. After addition of 200 μl chloroform, the solution was transferred to Phase-Lock tubes (Eppendorf) for phase separation. Aqueous supernatants were purified and concentrated by RNeasy mini columns (Qiagen). Quality control was performed by OD measurements in a NanoDrop ND-1000 spectrophotometer (260/280 nm >1.8) and by electrophoretic separation using an Agilent 2100 Bioanalyzer (R.I.N. values >7.0).

Reverse transcription of purified total RNA was carried out using oligo-dT primers with T7 promoter sequence and Superscript II (Invitrogen).

In vitro transcription and labeling

Reverse-transcribed cDNAs were treated with RNase and with DNA polymerase to obtain double-stranded DNA. Purified DNA was used for amplification reactions using RNAMaxx High Yield Transcription Kit (Stratagene) with T7 polymerase. Typically, this *in vitro* transcription resulted in 40- to 50-fold amplification of mRNA/cDNA.

Subsequently, cRNA was reverse-transcribed. Labeled DNA was purified and dried.

Preparation of DNA (“glutamate”) Chips

The chips represent a hypothesis driven selection of 340 cDNA sequences of genes related to glutamatergic and GABAergic neurotransmission and synaptic vesicle proteins (Online Resource 1a–f), obtained from clones purchased from Deutschen Ressourcenzentrum für Genomforschung (RZPD, Berlin). The cDNA-inserts of bacterial clones were PCR-amplified directly from aliquots of bacterial cultures grown overnight (LB medium, containing antibiotic selection). After electrophoretic quality control, PCR mixtures were dried, resuspended in betaine-containing spotting buffer [7], and spotted on amino silane slides (Nexterion, PeqLab), using a MicroGrid II Arrayer. Each DNA was double-spotted, and each array was spotted three times on the same slide. The following control DNAs were used: salmon sperm DNA, human COT-1 DNA, 4 *Arabidopsis thaliana* DNAs (for spike-ins), spotting buffer,

water, and a series of DNAs derived from artificial genes (library of random external controls = LOREC) [19].

Altogether, a chip contained 4,608 spots, including markers at the array corners to facilitate finding the correct orientation after scanning.

After spotting, DNAs were UV-cross-linked with a radiation of $250 \text{ mJ}/\text{cm}^2$. Then, they were baked at 80°C for 2 h and stored at RT.

Hybridization and scanning of slides

Spotted slides were *sequentially* washed in 1% SDS solution in distilled water, immersed in 95°C distilled water, and *incubated* in 55°C prehybridization solution for 45 min. Then, they were washed in distilled water, in isopropanol (20 s, each), and dried by a stream of nitrogen or air. Hybridizations were done in *SlideHyb Glass Array Hybridization buffer #1* (Ambion) overnight at 56°C . All subsequent washing steps at increasing stringency were performed in the dark.

Scanning was carried out in a Packard ScanArray 5,000 scanner. Each slide was scanned twice at laser strengths of 90 and 100% to obtain a larger spectrum of fluorescence intensities.

Data processing

Data quality was assessed by a number of procedures, including visual inspection of intensities, MA-plots, and correlation analysis of replicates. Within-array normalization was performed according to locally weighted scatter-plot smoothing (LOESS) method [34]. Only rank-invariant probes were used for model generation during this procedure [27]. For between-array normalization, scaling [34] was used. After assessing normal distribution of replicate spots using the Kolmogorov–Smirnov test, replicates were pooled by their arithmetic means. To identify differentially expressed genes, two-tailed *t*-tests and the RankProduct (RP) [3] method were used. The false discovery rate (FDR) method was employed to avoid accumulation of alpha-error. Corrected *P*-values <0.05 were considered significant. To limit the number of differentially expressed genes, only those were used which were found by both *t*-test and RP methods. Finally, to profile overall regulation, only genes that were differentially regulated in at least two regions were selected for further analyses.

Quantitative real-time PCR (qRT-PCR)

Quantitative RT-PCR was carried out in 384-well plates in an ABI Prism 7900 HT Fast Real-Time PCR cycler (Applied Biosystems, Foster City, Ca., USA). The primer pairs used for the respective genes are listed in Table 1. Beta actin was used as a reference (“house-keeping”) gene.

Table 1 Primers used in qRT-PCR

Gene	Sequences reading from 5' -end	
	Forward primer	Reverse primer
CPLX1	GCAGGGCATACGAGATAAGT	TGGTGGGGTCAGTGATGGCAGTA
NPY	CGCTCTGCGACACTACAT	CTCAGGGCTGGATCTCTTG
NRXN1	AGGGCGTCAGCTCACAATCTTCAA	TCTGCCGAGCCTGGGTATGGT
SNAP25	AGTGGCGTTTGCTGAATGAC	TGCTGGTATGACTTAATCTTGACA
STX1A	TGTCCCGAAAGTTTGTG	GCGTTCTCGGTAGTCT

Results

Microarray experiments

After statistical evaluations of the 768 genes spotted on the glass slides by Students *t* tests, many of them turned out to be significantly regulated.

Table 2 provides an overview of differentially expressed genes in frontal (FR), parietal (PAR), and temporal (TE) cortex as well as in caudate putamen (CPU). At first sight, there are apparently large regional differences in terms of up- or downregulation, i.e. many more genes are upregulated in FR [44 vs. 4 (ratio 11)] and in TE [51 vs. 22 (ratio 2.32)] compared with PAR [10 vs. 33 (ratio 0.3)]. A more balanced situation seems to occur in CPU.

Clozapine treatment and qRT-PCR results

To obtain a clearer picture, the focus was laid upon genes primarily being expressed presynaptically. Those were further investigated by qRT-PCR in the four brain regions in normoxic and hypoxic animals treated with clozapine (Table 3).

Evidently, CPX1 is upregulated in frontal and temporal cortex as well as in caudate putamen, but not in parietal cortex in hypoxic animals (Table 2). Treatment with clozapine resulted in an upregulation also in parietal cortex. NPY was upregulated by hypoxia in frontal but downregulated in temporal cortex. Neurexin1 was upregulated in both frontal cortex and CPU in hypoxic animals. SNAP25 was also upregulated in frontal cortex but downregulated in CPU. Additionally, it was upregulated in temporal cortex. Another counterregulation was observed with syntaxin1A in frontal and temporal cortex (Table 2). Interestingly, in parietal cortex, where hypoxia had no effect, STX1A was upregulated by clozapine (Table 3).

Discussion

Complexin1

Upregulation of CPX1 upon postnatal hypoxia in frontal, temporal cortex, and CPU has not been reported yet

(Fig. 1). In the light that clozapine additionally upregulated the gene in parietal cortex, it may be assumed that hypoxia-induced upregulation may be a compensatory mechanism to ensure proper vesicular transmitter release. Associations of complexins with schizophrenia have been repeatedly reported [9, 10, 13]. A recent report comes to the conclusion that complexins facilitate neurotransmitter release [33]. Specifically, it has been suggested that CPX1 downregulation during perinatal hypoxia in GABAergic neurons facilitates glutamate release and loss of overstimulated neurons—which subsequently results in reduced glutamatergic projections [24]. The upregulation of CPX1 in parietal cortex by clozapine is supported by the findings of Eastwood [8]. Our results showing the very strong correlation of CPX1 with PPI in hypoxia-treated animals (Table 4) support the view that this gene/protein is a key player in the development of Schizophrenia-like symptoms.

Neurexin1

Neurexins and neuroligins are emerging as central organizing molecules for excitatory glutamatergic and inhibitory GABAergic synapses. They function as cell adhesion molecules, bridging the synaptic cleft. Neurexin 1beta and neuroligin normally form trans-synaptic complexes [1]. Finally, there is a recent report showing that disruption of the neurexin 1 gene is associated with schizophrenia [26].

Preconditioning ischemia can downregulate the increased neurexin–neuroligin1–PSD-95 interaction induced by ischemia injury and exerts a neuroprotective effect. These results indicate that the neurexin-neuroligin1 is an important signaling module in hypoxic injury and a novel possible target in therapeutics of brain ischemia [21].

SNAP25

Recent genetic studies implicate alterations in SNAP-25 gene structure, expression, and/or function in contributing directly to neuropsychiatric and neurological disorders. Protein-by-protein analyses indicated a significant reduction in SNAP-25 immunoreactivity in the schizophrenia non-suicide group [11, 15].

Table 2 Differentially expressed genes in some regions of normoxic and hypoxic rat brains

CPU		FR		PAR		TE	
Genelist <i>t</i> -test fdr correction		Genelist <i>t</i> -test fdr correction		Genelist <i>t</i> -test fdr correction		Genelist <i>t</i> -test fdr correction	
The factor “hypoxia” regulated the following genes in comparison with “normoxia”		The factor “hypoxia” regulated the following genes in comparison with “normoxia”		The factor “hypoxia” regulated the following genes in comparison with “normoxia”		The factor “hypoxia” regulated the following genes in comparison with “normoxia”	
UP	DOWN	UP	DOWN	UP	DOWN	UP	DOWN
	ATF2	ATF2			CAPN2	ADCY3	
	ATF4		AURKB		CAPON	ADCY6	
	ATP2A2	CALM2		CASK		ATP2A2	
CPX1		CAP2			EAAT3		BDNF
CPX2		CAPN8			GABBR1		BNPI
	GAP43	CPX1			GAPDH	CAPN2	
		DEAD BOX		LOC294679	GNA12	CAPN6	
GAPDH	GATA4	DLGAP2				CASQ2	
	GRM2	DUSP1			LOC362704	CPX1	
	ITPR1	FYN			MAPK3	DAT1	
	MARK1	GAPDH			PIPS	EAAT3	
		GNAO			PLD2	ERK7	
MPPS7		MAPK14			PLEKHC1	GABRG3	
MYD116	NM_017261	MAPK3		POU3F4		GAD67	
NOS2		MRPS7			PPP1R14A	GADD45A	
NRXN1		NELL2			PPP2R2C		GAP43
	PKN1	NPY			PPP5C	GATA4	
	PLD1		NOS2-1149		PREFOLDIN5		GNAO
	PLEKHC1	NRXN1			PRKCZ	GPSM1	
	PPM1B	PACSIN2			PTPRA	GRM8	
	PPP1CC	PIM3			PTPRR		ID3
	PPKCC	PLAT			RAB14	ITPR3	
	PTP4A1		PPP1R14B		RAB3A		LOC294679
PTPRA		PPP1R14C			RAB3B	LOC362704	
PTPRN		PTP4A2		RN.28284	RN.25045		MGC105762
	PTPRN2	PTPRD					NPY
	RAB10	PTPRN			SHANK1		NR2F2
	RN.28284	PTPRN2			SLC2A1		NRXN3
	SACMIL	RAB3A			SLC6A12	NUP54	
	SLC1A3	SLC1A3			SLK	PIPS	

Table 2 continued

CPU	FR		PAR		TE	
Genelist <i>t</i> -test fdr correction	Genelist <i>t</i> -test fdr correction		Genelist <i>t</i> -test fdr correction		Genelist <i>t</i> -test fdr correction	
The factor “hypoxia” regulated the following genes in comparison with “normoxia”	The factor “hypoxia” regulated the following genes in comparison with “normoxia”		The factor “hypoxia” regulated the following genes in comparison with “normoxia”		The factor “hypoxia” regulated the following genes in comparison with “normoxia”	
UP	DOWN	UP	DOWN	UP	DOWN	UP
	SLC1A3	SLC6A12			PLD2	
	SLC1A6	SNAP25		SRC	PNOC	
SLC32A1		SOD1	SYT1-1511		PPM1G	
	SNAP25	SQSTM1		TRANSC.LOCUS	PPP1R10	
	STAT3	SRC				PPP2R2C
SYN1		STK39			PPP2R5B	
VAMP2		STX1A			PPP4R1	
		TRANSC.LOCUS			PPP5C	
					PRKCH	
					PRKCZ	
					RAB10	
					RGS3	
					RN.137813	
					RN.15926	
					RN.7389	
					SLC1A6	
					SLC32A1	
					SLC6A12	
					SMAGP	
					SNAP25	
					SP1	
					SQSTM1	
					STAT3	
					STK6	
						STX1A
						SYN1
						SYN2
					TCF12	
					VAMP1	

Many more genes are upregulated in frontal (FR) and temporal (TE) cortex compared with parietal (PAR) cortex

Table 3 “Clozapine”-induced effects in normoxic and hypoxic animals (empty = no effect)

Normoxic				Hypoxic			
Region	Gen	Reg.-Dir.	P-value	Region	Gen	Reg.-Dir.	P-value
CPU	CPX1			CPU	CPX1	up	0.026
FR	CPX1	down	0.043	FR	CPX1		
PAR	CPX1			PAR	CPX1	up	0.012
TE	CPX1			TE	CPX1		
CPU	NPY			CPU	NPY		
FR	NPY			FR	NPY		
TE	NPY			TE	NPY		
CPU	NRXN1			CPU	NPY		
FR	NRXN1			FR	NRXN1		
PAR	NRXN1			PAR	NRXN1		
TE	NRXN1			TE	NRXN1		
CPU	SNAP25			CPU	NRXN1		
FR	SNAP25			FR	SNAP25		
PAR	SNAP25			PAR	SNAP25		
TE	SNAP25			TE	SNAP25		
FR	STX1A	down	0.015	FR	SNAP25		
PAR	STX1A	up	0.048	PAR	STX1A	up	0.002
TE	STX1A			TE	STX1A		

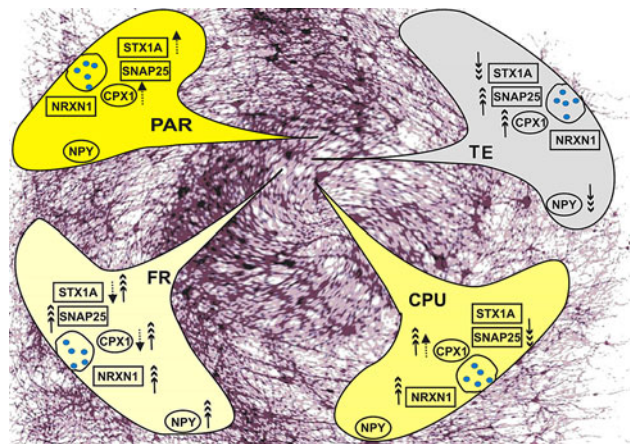


Fig. 1 Hypoxia- and clozapine-mediated differential regulation of presynaptic transcripts in some rat brain regions. Triple arrows : *hypoxia* : chip results; dotted arrows : *clozapine* : qRT-PCR results

In another study, however, there were no differences in the immunoreactivity of proteins measured in schizophrenic patients. Apparently prefrontal cortical dysfunction in schizophrenia may exist without gross changes in the abundance of many synaptic proteins [13].

There were significant decreases in tissue levels of these proteins in prefrontal cortex of schizophrenic patients relative to controls, *whereas* the mRNAs encoding these proteins were not decreased [18]. Normal levels of SNAP-25

Table 4 Correlations of qRT-PCR expression data with prepulse inhibition (PPI)

Gene	Region	Entity			Normoxia			Normoxia			Hypoxia		
		N	rho	P	Without Clozapine			With Clozapine			Without Clozapine		
					N	rho	P	N	rho	P	N	rho	P
CPX1	CPU	23	0.35	0.11	12	-0.38	0.23	6	0.03	0.96	5	1	0
NPY	TE	23	0.1	0.65	12	0.000	1.000	6	-0.31	0.54	5	0.3	0.62
NRXN1	CPU	23	0.07	0.77	12	0.7	0.01	6	0.66	0.16	5	0.2	0.75
SNAP25	FR	20	0.02	0.93	9	0.02	0.97	4	-1.000	0.000	5	-0.3	0.62

Levels of gene expression in individual animals were correlated with the performance shown in the behavioral testing of prepulse inhibition (PPI). Four out of the five genes investigated turned out to show significant correlations (P -value smaller than 0.05) in specific brain regions under specific conditions. As shown in the table, in normoxic, non-medicated animals, PPI negatively correlates with complexin 1 level in CPU ($P = 0.02$) and SNAP25 ($P < 0.01$) level in the frontal cortex. A positive correlation between PPI and neuropeptide Y can be seen in the temporal cortex ($P = 0.02$). In contrast, in caudate putamen of hypoxic animals, complexin 1 shows a positive correlation with PPI. A comparable correlation is found also in CPU of clozapine-treated hypoxic animals with neurexin1 ($P = 0.01$)

are noted in schizophrenia patients in Brodman area 17, decreased levels in areas 10 and 20, and an elevated level in area 9 [31].

In comparison with our results (Fig. 1), those findings strongly support the notion that regionally distinct expressions prevail and have always to be taken into consideration.

NPY

Regionally different gene expression of NPY is observed also in our report (Fig. 1). Patients with schizophrenia showed deficits in NPY mRNAs [14, 23]. Olanzapine and clozapine administration *acutely* decreased NPY mRNA levels in the nucleus accumbens, striatum, and anterior cingulate cortex of rats [16, 20]. The proportion of NPY neurons in the upper cortical layers was low, *but it was high* in the deep white matter in the disorganized and paranoid form [17].

Syntaxin 1

This presynaptic protein is another good example for regionally different gene expression. It is upregulated in FR but downregulated in TE, whereas there is no change in CPU and PAR by hypoxia treatment. Clozapine upregulates its expression in PAR but downregulates it in FR (Fig. 1). From literature reports, we know that there were significant negative correlations between age and mRNA levels of syntaxin 1A in schizophrenic patients. They were increased in the younger (58–79 years) subgroup of schizophrenic patients—which suggests that they reflect overall neurodevelopmental abnormalities in synaptic connectivity in the temporal cortex of schizophrenic patients [29].

Conclusions

Presynaptic proteins have been reported to be differentially regulated in schizophrenia. In our animal model of perinatal hypoxia, alterations in presynaptic genes in adulthood during the time period of behavioral deficits have been shown and may be involved in the pathophysiology leading to schizophrenia symptoms. However, we focused only on genes of the glutamatergic system, and all conclusions are limited to this domain. Our results reveal that chronic clozapine treatment may differentially influence gene expression and in this way contribute to the treatment of symptoms. Animal models of risk factors of schizophrenia are warranted to elucidate disease mechanisms and specific effects of antipsychotic treatment in disease states.

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Conflict of interest The authors declare that they have no conflict of interest.

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