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Reduction in hippocampal GABAergic transmission in a low birth weight rat model of depression

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Original Article

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Abstract

Prenatal stress is believed to increase the risk of developing neuropsychiatric disorders, including major depression. Adverse genetic and environmental impacts during early development, such as glucocorticoid hyper-exposure, can lead to changes in the foetal brain, linked to mental illnesses developed in later life. Dysfunction in the GABAergic inhibitory system is associated with depressive disorders. However, the pathophysiology of GABAergic signalling is poorly understood in mood disorders. Here, we investigated GABAergic neurotransmission in the low birth weight (LBW) rat model of depression. Pregnant rats, exposed to dexamethasone, a synthetic glucocorticoid, during the last week of gestation, yielded LBW offspring showing anxiety- and depressive-like behaviour in adulthood. Patch-clamp recordings from dentate gyrus granule cells in brain slices were used to examine phasic and tonic $GABA_A$ receptor-mediated currents. The transcriptional levels of selected genes associated with synaptic vesicle proteins and GABAergic neurotransmission were investigated. The frequency of spontaneous inhibitory postsynaptic currents (sIPSC) was similar in control and LBW rats. Using a paired-pulse protocol to stimulate GABAergic fibres impinging onto granule cells, we found indications of decreased probability of GABA release in LBW rats. However, tonic GABAergic currents and miniature IPSCs, reflecting quantal vesicle release, appeared normal. Additionally, we found elevated expression levels of two presynaptic proteins, *Snap-25* and *Scamp2*, components of the vesicle release machinery. The results suggest that altered GABA release may be an essential feature in the depressive-like phenotype of LBW rats.

Significant outcomes

- Outcome 1: We find a neurodevelopmental increase in synaptic activity in hippocampal GABA transmission in both control rats and the LBW rat model of depression.
- Outcome 2: Altered paired-pulse behaviour of evoked GABA release indicates lowered GABA vesicle release probability in the LBW model.
- Outcome 3: Defective synaptic GABA release in the LBW model is accompanied by increases in mRNA encoding for distinct presynaptic proteins, including *Snap-25* and *Scamp2*, all pointing to a GABAergic synaptopathy in the LBW rat model of depression.

Limitations

- Exogenous stress hormone was administered in a rodent animal model, and tissues were analysed *ex vivo*.

Introduction

Major depressive disorder (MDD) is one of the most common neuropsychiatric diseases with the core symptoms of depressed mood and anhedonia. MDD also presents with a broad spectrum of other symptoms, including cognitive deficits and sleep disturbances, and has a high rate of comorbidity with anxiety (Fava *et al.*, 2000; Mineur *et al.*, 2013). MDD is influenced by genetic background but is also associated with environmental factors, such as adverse life events. Stress represents the most significant vulnerability factor in the development of depressive

disorders (Silva *et al.*, 2021). Moreover, an increasing number of studies suggest a neurodevelopmental origin of the disease (Markham & Koenig, 2011; Nugent *et al.*, 2011; Van den Bergh *et al.*, 2020). During foetal or early life, stress exposure can lead to adverse changes in the developing brain both structurally and functionally, which may increase the risk of MDD in adulthood (Matthews, 2000). The underlying pathophysiology of MDD is still poorly understood, but existing models and hypotheses suggest dysregulation in several neurotransmitter systems (Krishnan & Nestler, 2008), including the GABAergic inhibitory system (Luscher *et al.*, 2011; Zhang *et al.*, 2021).

GABA is the major inhibitory neurotransmitter in the mammalian brain and is released by interneurons and acts on ionotropic $GABA_A$ and metabotropic $GABA_B$ receptors, thereby exerting inhibitory control of neuronal excitability. Synaptic $GABA_A$ receptors ($GABA_A$Rs) mediate phasic inhibition, a rapid form of neurotransmission, while extrasynaptic $GABA_A$Rs are coupled to a more persistent tonic inhibition (Farrant & Nusser, 2005). It is hypothesised that GABAergic deficits are closely linked to mood disorders (Luscher *et al.*, 2011). Clinical studies showed reduced GABA levels in plasma (Petty & Schlesser, 1981) and cerebrospinal fluid (Gerner & Hare, 1981) of depressed individuals. Altered $GABA_A$R subunit mRNA expression was shown in different brain regions of humans (Merali *et al.*, 2004), suggesting both genetic and epigenetic impact in MDD. Moreover, experimental studies showed $GABA_A$R alteration in rodents with anxiety-like behaviour induced by early life stress exposure (Caldji *et al.*, 2000, 2003), suggesting GABAergic involvement in the neurodevelopmental origin of mental illnesses. Previously, our group demonstrated that a chronic mild stress (CMS) rat model of depression shows a functional deficit in GABA release, which could be reversed by antidepressant treatment (Holm *et al.*, 2011; Nieto-Gonzalez *et al.*, 2015).

Here, we examined GABAergic neurotransmission in the low birth weight (LBW) rat model of depression (Hougaard *et al.*, 2005). In this model, animals are exposed to the synthetic glucocorticoid dexamethasone (DEX) during foetal life, mimicking elevated maternal stress hormone levels during pregnancy (Conti *et al.*, 2017; Spulber *et al.*, 2015). Compared to control offspring, LBW rats show several deficits. Of relevance for depression, administration of DEX during the last week of gestation significantly increased immobility in the forced swim test and reduced sucrose preference (Abildgaard *et al.*, 2014; Wu *et al.*, 2019). Furthermore, prenatal exposure to DEX increased the susceptibility to CMS and hence the propensity to express depressive-like behaviour phenotypes (Oliveira *et al.*, 2006). Other deficits include altered hypothalamic–pituitary–adrenal axis (HPA) function and expression of corticotropin-releasing hormone (CRH) and CRH receptor type 1 (CRHR1) in the hippocampus, reduced mobility, and altered startle behaviour (Hougaard *et al.*, 2005, 2011; Kjaer *et al.*, 2010; Xu *et al.*, 2018). Additionally, LBW rats show increased anxiety-like behaviours in adulthood (Oliveira *et al.*, 2006; Nagano *et al.*, 2008).

Here, we examined postsynaptic $GABA_A$R-mediated inhibitory signalling onto granule cells in the dentate gyrus using whole-cell patch-clamp recordings in brain slices from LBW rats. In addition, quantitative real-time polymerase chain reaction (real-time qPCR) was used to analyse the levels of presynaptic protein transcripts in the hippocampus. In comparison to control offspring, we found in LBW rats a dysfunction in the GABAergic synaptic plasticity and elevated expression levels of two genes, *Snap-25* and *Scamp2*, of which the corresponding proteins are elements of the vesicle release machinery (Brand *et al.*, 1991; Söllner *et al.*, 1993). These results suggest that altered GABA release may be an essential feature of depressive disorders.

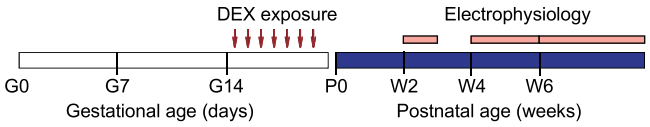

Fig. 1. DEX protocol in LBW rats. Female rats were exposed daily to dexamethasone (150 μg/kg) during the last week of gestation. Electrophysiological measurements were carried out on brain slices of male offspring at the age of 2, 4–5, and 6–8 weeks, respectively.

Material and methods

Ethics statement, animals, and dexamethasone exposure

Forty time-mated young adult rats (Wistars, HanTaC:WH, SPF) arrived at gestational day (GD) 3 at the Danish National Research Centre for the Working Environment, Copenhagen, Denmark. The rats were randomly distributed pairwise to white plastic cages (Eurostandard III, Scanbur, Denmark) with bedding of pine wood shavings and nesting material (Enviro-Dri, Brogaarden, Denmark). Environmental conditions were automatically controlled with a 12-h light–dark cycle with lights off at 06.00 am. Food (Altromin Standard Diet 1324) and tap water were provided *ad libitum*.

From GD 14 to 21, dams were injected subcutaneously in the nape of the neck with DEX (Sigma-Aldrich, Denmark) (150 μg/kg) once daily (LBW group; Fig. 1). Vehicle control animals were injected with 4% ethanol/isotonic saline. Injections were given during the dark phase of the light–dark cycle. For the study of 4- to 5- and 6- to 8-week-old rats, a maximum of 1 male per litter was selected randomly at weaning on postnatal day 21 (P21) and housed in groups of four with cage-mates of similar prenatal exposure until transfer to Translational Neuropsychiatry Unit, Department of Clinical Medicine, Aarhus University. Here, they were housed in clear Type 3 Makrolon cages and kept as described above. Offspring for the study of 2-week-old rats were also generated here, using similar procedures as for the older age groups.

All efforts were made to minimise animal suffering and to reduce the number of animals used. The Danish National Committee for Ethics in Animal Experimentation, appointed by the Danish Ministry of Justice, granted ethical permission for the studies. The Laboratory Animal Welfare Committee at the National Research Centre for the Working Environment reviewed the LBW model of depression and found it in compliance with the ethics of the NRCWE regarding experiments of laboratory animals. All procedures were carried out in compliance with the EC Directive 86/609/EEC and Danish law regulating experiments on animals (permission 2007-561/1378 and 2007-561/1396). Two, 4- to 5-, and 6- to 8-week-old male offspring were used for electrophysiological studies.

Quantitative real-time polymerase chain reaction

Hippocampi were dissected (left and right) from 8-week-old LBW and control vehicle animals. Dissection, tissue homogenisation, RNA extraction, RNA characterisation, and cDNA synthesis were

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Table 1. Characteristics of gene-specific real-time qPCR primers – Reference genes

Gene symbol	Gene name	Accession No.*	Primer sequence	Amplicon size†
Reference genes				
18s rRNA	18s subunit ribosomal RNA	M11188	(+) acggaccagagcgaaagcat (–) tgtcaatcctgtccgtgtcc	310
ActB	Beta-actin	NM_031144	(+) tgtcaccaactgggacgata (–) ggggtgttgaaggtctcaaa	165
CycA	Cyclophilin A	XM_345810	(+) agcactggggagaaaggatt (–) agccactcagtcttggcagt	248
Gapd	Glyceraldehyde-3-phosphate dehydrogenase		(+) tcaccaccatggagaaggc (-) gctaagcagttggtggtgca	168
Hmbs	Hydroxymethylbilane synthase	NM_013168	(+) tcctggctttaccattggag (–) tgaattccaggtgagggaac	176
Hprt1	Hypoxanthine-guanine phosphoribosyltransferase 1	NM_012583	(+) gcagactttgctttccttgg (–) cgagaggtccttttcaccag	81
Rpl13A	Ribosomal protein L13A	NM_173340	(+) acaagaaaaagcggatggtg (–) ttccggtaatggatctttgc	167
Ywhaz	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta	BC094305	(+) ttgagcagaagacggaaggt (–) gaagcattggggatcaagaa	136

*GenBank accession number of cDNA and corresponding gene, available at http://www.ncbi.nlm.nih.gov/.
†Amplicon length in base pairs.

carried out as described previously (Elfving *et al.*, 2008). cDNA was stored undiluted at −80°C until use. The cDNA samples were diluted 1:30 with DEPC water before being used as a real-time qPCR template.

Real-time qPCR reactions were carried out in 96-well PCR plates using the Mx3000P (Stratagene, USA) and SYBR Green. The gene expression of 8 reference genes (*18sRNA, ActB, CycA, Gapd, Hmbs, Hprt1, Rpl13A, Ywhaz*), 14 genes encoding for synaptic proteins (*Scamp2, Snap-25b* (the protein encoded by the *Snap-25b* isoform will be referred to as *Snap-25* hereafter), *Snap-29, Snapin, Syntaxin 1A, Synapsin I-III, Synaptophysin, Synaptotagmin I-III, Vamp1, and Vamp2*), and 7 other genes (*Gr, Mr, Munc13, Munc18, Rims1, Scna, and Vgat*) were investigated as previously described (Bonefeld *et al.*, 2008; Elfving *et al.*, 2008). Briefly, each SYBR Green reaction (10 µl total volume) contained 1x SYBR Green master mix (BIORAD, CA, USA), 0.5 µM primer pairs, and 3 µl of diluted cDNA. All samples were run in duplicate. A standard curve, performed in duplicate, was generated on each plate. Essential gene-specific data about primer sequence and amplicon sizes are given in Tables 1 and 2. Primers were obtained from DNA Technology A/S and Sigma-Aldrich, Denmark.

For data normalisation, we first measured mRNA levels for the reference genes. Stability comparison of the expression of the eight reference genes was conducted with the Normfinder software (www.moma.dk/normfinder-software) (Andersen *et al.*, 2004), and the best combination of two was selected. Values for each individual were normalised with the geometric mean of the reference genes *CycA* and *ActB* in the hippocampus.

Brain slice preparation

Rats were anaesthetised with isoflurane and decapitated. The brain was dissected and placed in ice-cold artificial cerebrospinal fluid (ACSF) composed of (in mM): 126 NaCl, 2.5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 D-glucose (osmolality 305–315 mOsmol/kg), 2 kynurenic acid, pH 7.4 when bubbled with carbogen (5% CO_2 – 95% O_2). 350-µm-thick coronal slices were prepared with Leica Microtome VT1200S (Leica Biosystems, Germany) in ice-cold bubbled ACSF. Slices rested in a holding chamber for at least 1 h in bubbled ACSF at room temperature (22–25°C). 0.2 mM ascorbic acid and 0.2 mM pyruvic acid were added to the ACSF during slicing and storage to improve slice quality.

In vitro *electrophysiology*

For whole-cell patch-clamp recordings, slices were transferred into a recording chamber perfused with 33 ± 1°C bubbled ACSF at 2–3 ml/min. Neurons were visualised using a custom-built infrared microscope with a × 40 water-immersion objective (Olympus, Ballerup, Denmark) and a CCD100 camera (DAGE-MTI, Michigan City, IN, USA). Recordings were carried out using a MultiClamp 700B amplifier (Axon Instruments, Union City, CA, USA). Patch-pipettes were pulled from borosilicate glass (OD = 1.5 mm, ID = 0.8 mm; Garner Glass Company, Claremont, CA, USA) using a DMZ Universal Puller (Zeitz Instruments, Munich, Germany). After filling the pipettes with intracellular solution containing (in mM): 140 CsCl, 2 $MgCl_2$, 0.05 EGTA, 10 HEPES, adjusted to pH 7.2 with CsOH (280–290 mOsmol/kg), their resistances were 3–5 MΩ. Giga seals (>1 GΩ) were obtained before break-in, and cells were held in voltage-clamp at a holding potential (V_{hold}) of -70 mV, while resistance was compensated by around 70% (lag 10 µs). During the experiments, whole-cell capacitance and series resistance were monitored. The observed range of the series resistance was 5.7 MΩ to 19.8 MΩ. The average series resistance change was 1.9 ± 0.19 MΩ, in percentage 19.5% ± 1.9% (n = 107). In some experiments, where the exact changes in resistances were not recorded, the increases were in the order of 2–3

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Table 2. Characteristics of gene-specific real-time qPCR primers – Target genes

Gene symbol	Gene name	Accession No.*	Primer sequence	Amplicon size†
Synaptic proteins				
Scamp2	Secretory carrier membrane protein 2	NM_023955	(+) tggctgagttcaatcccttc (–) agctcagcagctttcctgtc	196
Snap-25b	Synaptosomal associated protein 25 kDa	NM_030991	(+) ctggcatcaggactttggtt (–) attattgccccaggcttttt	200
Snap-29	Synaptosomal associated protein 29 kDa	NM_053810	(+) acacggagaagatggtggac (–) tggcttggtacttgctttcc	219
Snapin		NM_001025648	(+) tggatctggacccctatgtt (–) tttgcttggagaaccaggag	182
Synapsin I		NM_019133	(+) caccaggatgaagacaagca (–) gtcgttgttgagcaggaggt	184
Synapsin II		NM_001034020	(+) catgggtgtttgctcagatg (–) accacgacaggaaacgtagg	127
Synapsin III		NM_017109	(+) cacagcaagaatggcagaga (–) ttagtctgtggaccccaagg	182
Synaptophysin		NM_012664	(+) cagtgggtctttgccatctt (–) ttcagccgacgaggagtagt	222
Synaptotagmin I		NM_001033680	(+) cttctccaagcacgacatca (–) ccacccacatccatcttctt	219
Synaptotagmin II		NM_012665	(+) aggtgaaagtgcccatgaac (–) ctcttgccattctgcatcaa	241
Synaptotagmin III		NM_019122	(+) ggactccaatgggttctcag (–) agcaggttgtccaaaaccac	234
Syntaxin 1A		NM_053788	(+) accgcttcatggatgagttc (–) gagctcctccagttcctcct	155
Vamp1	Vesicle-associated membrane protein 1	NM_013090	(+) gtgctgccaagctaaaaagg (–) actaccacgattgatggcaca	88
Vamp2	Vesicle-associated membrane protein 2	NM_012663	(+) ctgcacctcctccaaatctt (–) cttggctgcacttgtttcaa	191
Other proteins				
Gr	Glucocorticoid receptor	NM_012576	(+) cacccatgaccctgtcagtg (–) aaagcctccctctgctaacc	156
Mr	Mineralocorticoid receptor	NM_013131	(+) taagtttccccacgtggttc (–) atccacgtctcatggctttc	148
Munc13-1		NM_022861	(+) cacggtcgcctcgttcagcaa (–) atggcagggtggagtgacgc	245
Munc18-1		NM_013038	(+) atgcgactcaccctgctgctg (–) cggaggaggggcaggggct	85
Rims1	Regulating synaptic membrane exocytosis 1	NM_52829	(+) gtgcctccagtgggttat (–) gggttggagaacgtgac	214
Snca	Synuclein, α	NM_019169	(+) aagcctagagagccgtgtggagc (–) gcctctgccacaccctgcttg	127
Vgat	Vesicular GABA transporter	NM_031782	(+) ggggagttcgggggtcacga (–) cccagcacgaacatgccctga	92

*GenBank accession number of cDNA and corresponding gene, available at http://www.ncbi.nlm.nih.gov/.
†Amplicon length in base pairs.

MΩ. In all experiments, the neurons were discarded if resistance increased more than 50% or exceeded 20 MΩ.

Data acquisition and analysis

Currents were low-pass filtered (8-pole Bessel) at 3 kHz before being digitised at 20 kHz using a DA converter (BNC-2110), a PCI acquisition board (PCI-6014, National Instruments, Austin, TX), and a custom-written LabView 6.1 (National Instruments)-based software containing an acquisition interface and analysis module (EVAN v. 1.4, courtesy of Istvan Mody). This software was also used to detect and analyse spontaneous and miniature inhibitory postsynaptic currents (IPSCs) with a 6–8 pA amplitude detection threshold. All events were visually inspected before making an average of around 50 spontaneous inhibitory postsynaptic currents (sIPSCs). Event amplitude, 10–90% rise time, and frequency were measured.

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Table 3. Parameters of spontaneous GABAergic inhibitory postsynaptic currents (sIPSCs) in dentate granule cells of untreated control and control vehicle rats

	Amplitude (pA)	Frequency (Hz)	Rise time (10–90%, µs)	Decay (ms)
sIPSCs				
Young (2 weeks)				
Untreated control (n = 5)	37.9 ± 2.0	2.27 ± 0.98	691 ± 24	8.1 ± 1.2
Control vehicle (n = 4)	41.3 ± 11.0	2.06 ± 0.69	730 ± 27	7.9 ± 0.3
Adolescent (4–5 weeks)				
Untreated control (n = 20)	58.6 ± 6.4	3.35 ± 0.46	329 ± 21	6.2 ± 0.3
Control vehicle (n = 6)	66.0 ± 12.2	3.81 ± 0.64	280 ± 22	6.2 ± 0.6
Young Adult (6–8 weeks)				
Untreated control (n = 8)	56.9 ± 5.8	3.46 ± 0.67	319 ± 44	6.2 ± 0.4
Control vehicle (n = 5)	62.3 ± 12.7	2.75 ± 1.16	316 ± 22	5.8 ± 0.3

No significant changes found.

sIPSC weighted decay time constant (τ_w) was calculated using double-exponential fits:

$$I(t) = A_1 * \exp\left(-\frac{t}{\tau_1}\right) + A_2 * \exp\left(-\frac{t}{\tau_2}\right)$$

where $I(t)$ is the current as a function of time (t), A_1 and A_2 are amplitude constants, and τ_1 and τ_2 are the two decay time constants. The goodness of fit was determined by visual inspection of the residuals.

IPSCs were also electrically evoked by paired-pulse stimulation every 10 s with an inter-event interval of 50 ms using a bipolar matrix microelectrode (FHC Inc, ME, USA) placed in the granule cell layer 200–300 µm from the recorded cell. The stimulation intensity was kept constant at 20–40% above the threshold for evoking single IPSCs. The paired-pulse ratio of average IPSC amplitudes was measured from averages of 10–20 sweeps.

Tonic $GABA_A$ receptor-mediated currents were assessed by a high concentration of the $GABA_A$ receptor antagonist 2-(3-carboxypropyl)-3-amino-6-methoxyphenyl-pyridazinium bromide (SR95531 > 100 µM), which produced an outward shift in the holding current. For quantification, 5 ms long samples were taken from the recording every 100 ms and plotted against time, omitting baseline points falling onto the decay of IPSCs (Drasbek & Jensen, 2006). Mean currents were calculated in 4 s long segments at three time points (denoted *a*, *b* and *c*): just before SR95531 application (*b*), 20 s before (*a*) and after (*c*). The tonic current was calculated as *c* – *b*. If the baseline variation (*b* – *a*), which defines the stability of the recording, was more than 6 pA, recordings were discarded.

Statistical analysis

For electrophysiological sIPSC and paired-pulse data, two-way ANOVA for group comparison was followed by post hoc Bonferroni tests to determine effects of age and treatment (control vs. LBW). For mIPSCs and tonic GABA currents consisting of one age group, unpaired Student's t-tests were used. Data are presented as means ± SEM, with *n* indicating the number of neurons. Real-time qPCR data were analysed using unpaired Student's t-test followed by Holm–Sidak correcting for multiple comparisons. Statistical analyses were performed with GraphPad Prism version 8.00/5.00 for Windows (GraphPad Software, San Diego, CA, USA).

Compounds

Kynurenic acid, ascorbic acid, SR95531, tetrodotoxin, and dexamethasone were obtained from Sigma-Aldrich (Denmark), while pyruvic acid was purchased from MP Biomedicals (Irvine CA, USA). SR95531 (5 mM) was dissolved in 50% DMSO (dimethyl sulfoxide) and 50% dH_2O (distilled water), while dexamethasone was dissolved in 4% ethanol/isotonic saline. Compounds, except dexamethasone, were stored at – 20°C until use.

Results

Earlier studies suggested that hippocampal $GABA_A$ receptor-mediated signalling is disturbed in depressive phenotypes observed after exposure of rodents to CMS (Holm *et al.*, 2011; Nieto-Gonzalez *et al.*, 2015). To further investigate the GABAergic involvement in models of depressive disorders and gain insight into GABAergic malfunction during development, we studied LBW rats that develop a depressive phenotype due to prenatal stress exposure.

Control rats show developmental changes in phasic $GABA_A$ receptor-mediated signalling

Hippocampal GABAergic interneurons and their synapses show morphological development from embryonic day 13 (E13) (Amaral & Kurz, 1985) until adolescence (P28–P55) (Corbin & Butt, 2011) in rats. The maturation of GABAergic neurons involves changes in their intrinsic properties and a shift from slow to fast postsynaptic signalling (Hollrigel & Soltesz, 1997; Banks *et al.*, 2002; Doischer *et al.*, 2008). The development of electrophysiological properties of inhibitory neurons is not complete in early adolescence in the visual cortex, where it reaches a plateau around 8 weeks of age (Jang *et al.*, 2010). Therefore, to investigate the postnatal development of GABAergic inhibition in the dentate gyrus, we studied three groups, aged 2 (developing), 4–5 (young adolescence) and 6–8 (later stage of adolescence – early adulthood) weeks, respectively. We recorded $GABA_A$ receptor-mediated IPSCs using whole-cell patch-clamp recordings from dentate gyrus granule cells. In the presence of kynurenic acid to block ionotropic glutamate receptors at a holding potential of –70 mV, IPSCs appeared as fast inward currents due to the CsCl-based pipette solution (E_{Cl} ~ 0 mV). sIPSC parameters of untreated control and

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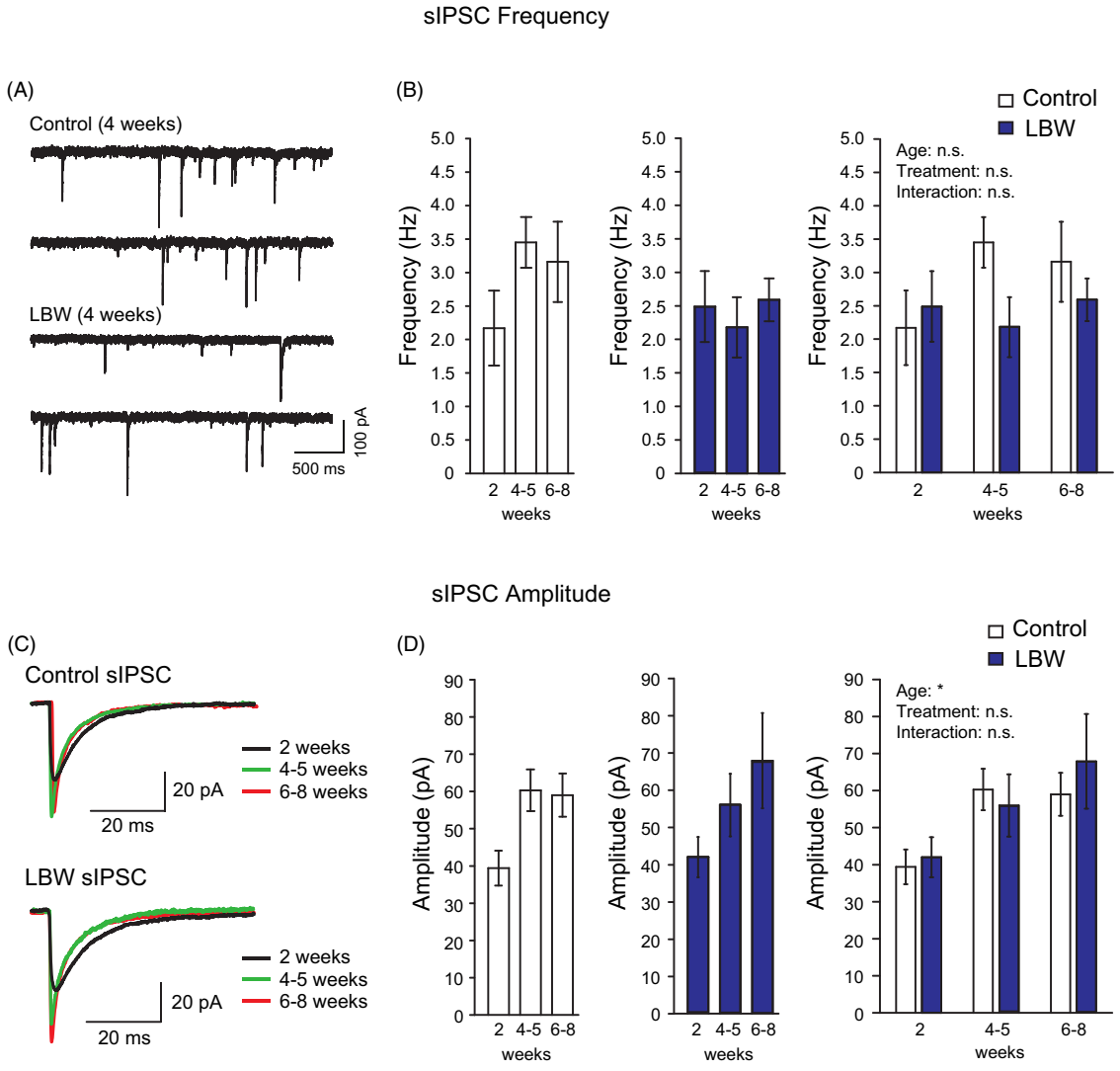

Fig. 2. Postnatal maturation of GABAergic activity in the dentate gyrus of control and LBW rats. (*A*) Representative traces showing whole-cell recordings from dentate gyrus granule cells in brain slices from control and LBW rats 4 weeks postnatally. sIPSCs occurred at 4.0 Hz in control and at 2.5 Hz in the LBW slice. (*B*) sIPSC frequency in control and LBW animals in three age groups (2, 4–5, and 6–8 weeks old). Average sIPSC frequencies were not significantly different, as examined by two-way ANOVA indicated to the right (n.s.). In LBW rats, sIPSC frequencies showed similar values throughout development. (*C*) Averaged sIPSCs from the three age groups are superimposed, showing the increase in the amplitude during development in control and LBW rats. (*D*) sIPSC amplitudes in control and LBW rats in different age groups. Amplitudes increased significantly from 39.4 ± 4.7 (2 weeks; $n = 9$) to 60.3 ± 5.6 (4–5 weeks; $n = 26$) and 59.0 ± 5.8 (6–7 weeks; $n = 13$) in control rats. A similar pattern was seen in LBW rats through development, with no significant differences compared to control.

control vehicle groups showed no statistically significant differences (Table 3; n.s.). Therefore, these data were pooled and referred to as controls. In 2-week-old control offspring, the amplitude of sIPSCs was 39.4 ± 4.7 pA ($n = 9$). In 4- to 5-week-old control offspring, this had increased to 60.3 ± 5.6 pA ($n = 26$), while no further increase was observed at 6–8 weeks of age, yielding 59 ± 5.8 pA ($n = 13$; Fig. 2C, D). A two-way ANOVA confirmed that age did have a statistically significant effect on sIPSC amplitudes ($F = 3.299$; $p = 0.0421$). The frequency of sIPSCs did not change significantly with increasing age (Fig. 2A, B). These results suggest that phasic inhibition increases during postnatal development and, most prominently, between 2 and 4–5 weeks of age in our experiments.

Maturation of sIPSC kinetics in control rats

To study the kinetics of $GABA_A$ receptor-mediated synaptic currents, we analysed their waveform, including rise time (10–90%) and decay of sIPSCs. Rise times decreased during postnatal development in control rats. The rise time of 708 ± 168 µs ($n = 9$) in 2-week-old rats was shortened in 4- to 5- and 6- to 8-week-old rats to 318 ± 17 µs ($n = 26$) and 318 ± 42 µs, respectively ($n = 13$; $F = 21.51$; $p < 0.0001$; Fig. 3A, B). The decay of averaged sIPSCs showed a significant acceleration from 2 to 4–5 and 6–8 weeks of age, while weighted decay time constants were 8.0 ± 0.6 ms ($n = 9$), 6.2 ± 0.2 ms ($n = 26$), and 6.1 ± 0.6 ms ($n = 13$), respectively ($F = 20.11$; $p < 0.0001$; Fig. 3C, D). A decrease in both sIPSC rise time and decay suggests an acceleration of the receptor kinetics during postnatal development in dentate granule cells, and the developmental changes in kinetics seemed to occur particularly during the first month of age.

Development of phasic inhibition in LBW rats

In LBW rats, sIPSC amplitudes also increased with age (42 ± 5.4 pA, $n = 6$; 56 ± 8.4 pA, $n = 13$; 67.9 ± 12.8 pA, $n = 17$ in 2, 4- to 5- and 6- to 8-week-old rats, Fig. 2C, D). Comparison of sIPSC

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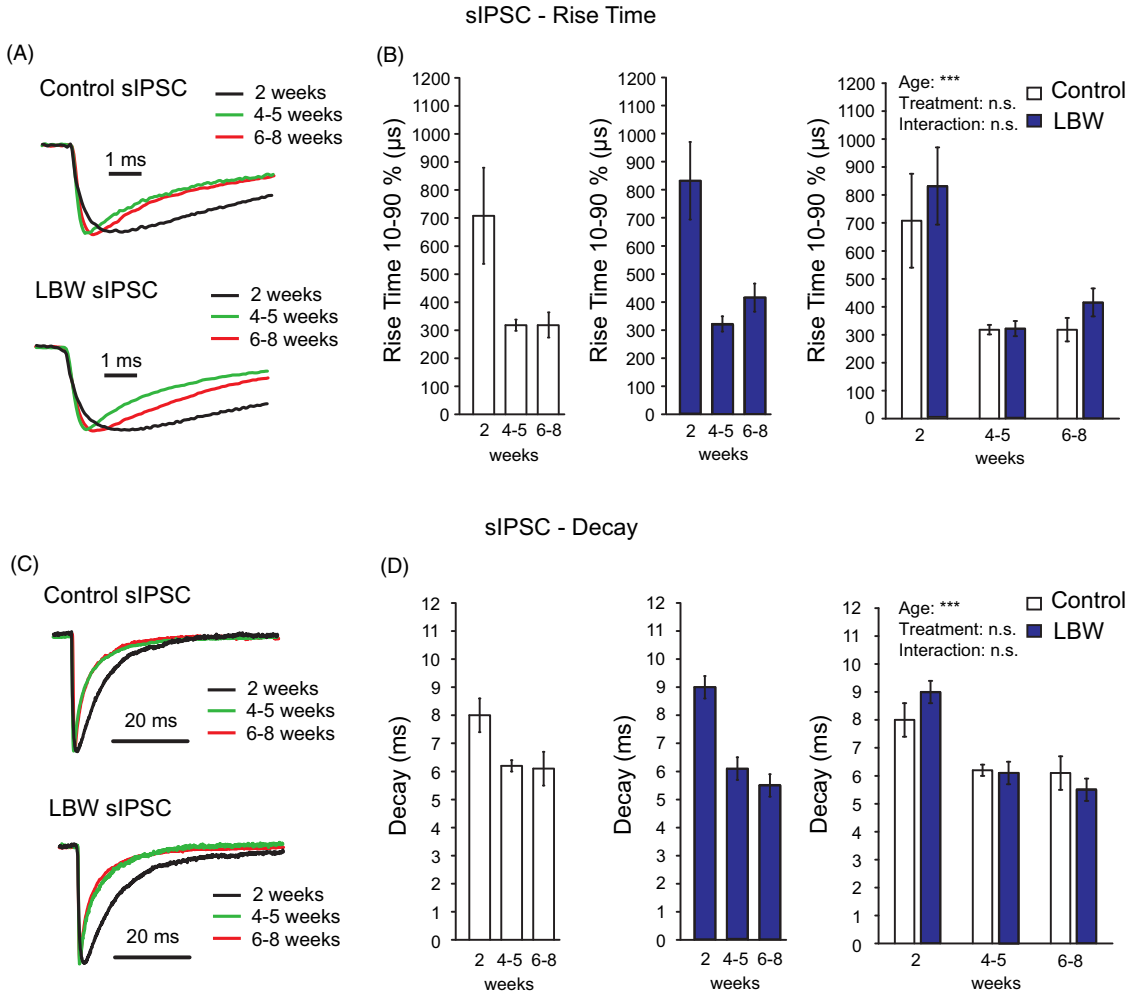

Fig. 3. Postnatal maturation of sIPSC kinetics in dentate gyrus of control and LBW rats. (*A, C*) Representative sIPSCs from different age groups superimposed after normalisation to the same peak amplitude to illustrate the developmental changes in rise time and decay in control and LBW rats. (*B*) Histograms summarising the sIPSC rise time in control and LBW animals in the same age groups. Average rise time of sIPSCs decreased significantly from 708 ± 168 (2 weeks; $n = 9$) to 318 ± 17 (4–5 weeks; $n = 26$) and 318 ± 42 µs (6–8 weeks; $n = 13$) in control. LBW rats showed a similar developmental acceleration of the rise time. Rise times in LBW rats were not different in any age group compared to the age-matched controls. (*D*) sIPSC decay in control and LBW rats in the same age groups. Average decay time constant of sIPSCs significantly decreased from 8.0 ± 0.6 (2 weeks; $n = 9$) to 6.2 ± 0.2 (4–5 weeks; $n = 26$) and to 6.1 ± 0.6 ms (6- to 8-week-old group; $n = 13$) in control. LBW rats show a similar developmental change in the decay phase of sIPSCs. sIPSC decays in LBW rats were not different in any age group compared to control. n.s.: not significant, $^{***}p < 0.001$.

amplitudes of control and LBW rats in each age group showed no significant differences (Fig. 2D). In comparison to the developmental increase in sIPSC frequencies in controls, LBW rats showed similar frequencies of 2.49 ± 0.53 Hz ($n = 6$), 2.18 ± 0.45 Hz ($n = 13$), and 2.59 ± 0.32 Hz ($n = 17$) in the three age groups (Fig. 2A, B). In addition, there was a tendency of sIPSC frequency of 4- to 5-week-old LBW rats to be lower than that of controls; however, this result was not statistically significant ($p = 0.08$, Fig. 2A, B).

In LBW rats, sIPSC rise times (10–90%) followed the same developmental pattern as in controls, yielding 832 ± 138 µs ($n = 6$), 322 ± 27 µs ($n = 13$), 416 ± 50 µs ($n = 17$; Fig. 3A, B) with increasing age. Thus, comparison of rise time in the control and LBW animals in each age group showed no significant differences (Fig. 3B). The decay of sIPSCs in LBW rats also showed a similar developmental pattern with increasing age to that of controls (9.0 ± 0.4 ms, $n = 6$; 6.1 ± 0.4 ms, $n = 13$, and 5.5 ± 0.4 ms, $n = 17$, respectively; Fig. 3D). The observed developmental patterns of both rise time and decay in LBW rats suggest a normal development of synaptic $GABA_A$ receptor kinetics in LBW rats.

Release probability of GABAergic terminals is reduced in LBW rats

We next studied the short-term plasticity of GABAergic synapses, which correlates with release probability at the presynaptic terminals. We recorded electrically evoked IPSCs by stimulating within the granule cell layer using a paired-pulse protocol in three age groups. At an inter-pulse interval of 50 ms, IPSCs in control animals showed paired-pulse depression in all three age groups, yielding 0.86 ± 0.07 ($n = 6$), 0.85 ± 0.04 ($n = 9$), and 0.62 ± 0.08 ($n = 5$), respectively (Fig. 4A, B). These results indicate a developmentally regulated short-term plasticity in control rats. Interestingly, in 2-week-old LBW rats the paired-pulse ratio was similar to that of controls (0.94 ± 0.11, $n = 6$); however, at older ages the paired-pulse ratios were increased (at 4–5 weeks: 1.17 ± 0.11, $n = 13$, $p < 0.05$; at 6–8 weeks: 1.01 ± 0.07, $n = 7$, $p = 0.10$, post hoc Bonferroni test). Thus, we observed a switch from paired-pulse depression to paired-pulse facilitation in the LBW rats, which can be interpreted as a loss of the normal developmental increase in the release probability in LBW animals.

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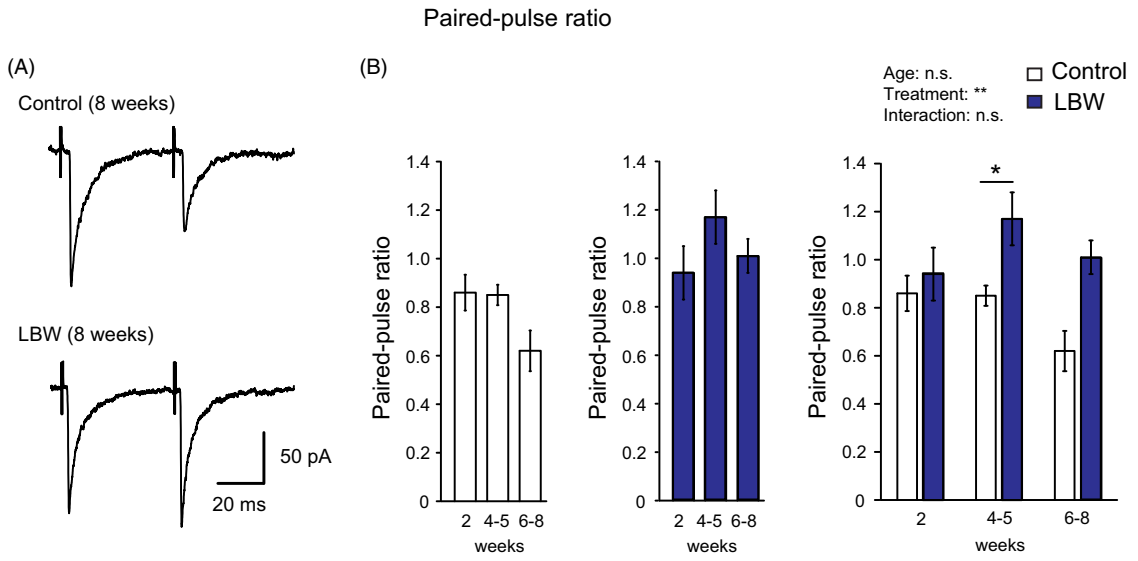

Fig. 4. Reduction in GABAergic probability of release in the LBW rats in adolescence and adulthood. (*A*) Traces showing electrically evoked $GABA_A$ receptor-mediated IPSCs in a control and a LBW rat slice. In control, the paired-pulse ratio of the IPSCs was 0.66, whereas it was 1.04 in LBW. The ratios were calculated as the second IPSC amplitude normalised to the first. These exemplar recordings were carried out 8 weeks postnatally, and the responses indicate a reduction of GABA release probability in the LBW rat exposed to dexamethasone *in utero*. (*B*) Histogram showing the paired-pulse ratio of the evoked IPSCs in three age groups (2, 4–5, and 6–8 weeks old). Paired-pulse depression was increasing during development in control rats from 0.86 ± 0.07 (2; $n = 6$) and 0.85 ± 0.04 (4–5; $n = 9$) to 0.62 ± 0.08 (6–8 weeks old; $n = 5$). In LBW rats, the paired-pulse depression seen in the 2-week-old group turned into paired-pulse facilitation at 4–5 and 6–8 weeks. Between control and LBW rats, no changes were observed at 2 and 6–8 weeks, but at 4–5 weeks postnatally, paired-pulse ratios were significantly increased in LBW rats. These data suggest a presynaptic defect in evoked GABA vesicle release in LBW animals. n.s.: not significant, $^{**}p < 0.01$.

Effect of action potential blockade on GABAergic activity

To examine the fraction of GABAergic activity that is not dependent on action potential firing, we added the Na^+ channel blocker tetrodotoxin (TTX) to the slices from 4- to 5-week-old rats. We recorded action potential-independent miniature IPSCs (mIPSC). In slices from controls, TTX (1 μM) reduced the IPSC frequency to 1.13 ± 0.29 Hz ($n = 7$), which was similar to the resulting frequency in LBW slices (1.08 ± 0.24 Hz, n.s., $n = 10$, Fig. 5A), while the fractional decrease in GABAergic frequency was more pronounced in the control slices (by 69%) than in LBW slices (by 51%; Fig. 5B). Thus, action potential-driven GABA release appeared to be reduced in LBW slices, while TTX resistant miniature release, reflecting the number of active synaptic GABAergic boutons, was similar. The amplitudes and kinetics of averaged mIPSCs showed no significant differences in LBW rats compared to untreated control rats (data not shown).

Tonic GABAergic activity is unchanged in LBW rats

Part of the postsynaptic response to GABA is mediated by peri- and extrasynaptic $GABA_A$ receptors, which respond to the low ambient GABA concentration in the extracellular space, giving rise to a persistent, tonic GABA current. To examine the tonic current in adolescent dentate granule cells, we recorded the GABA current in whole-cell configuration from 4- to 8-week-old rats. We measured the outward shift upon blockade of $GABA_A$ receptors using the competitive antagonist SR95531 (~100 μM). The average SR95531-sensitive current density of control rats was 2.2 ± 0.5 pA/pF ($n = 9$), and in LBW rats, it was 1.7 ± 0.5 pA/pF ($n = 9$, n.s.) (Fig. 5C, D). Granule cell capacitances were similar (9.7 ± 0.7 vs. 12 ± 1.4 pF), suggesting an unaltered tonic inhibition in the dentate gyrus.

Synaptic vesicle proteins SNAP-25 and SCAMP2 are upregulated in LBW rats

As we found a failure of the normal developmental increase in the release probability at GABAergic terminals, we tested whether this could be due to impaired synaptic vesicle protein expression. The mRNA levels of 8 reference genes and 14 genes of synaptic vesicle proteins were investigated in the hippocampus from 8-week-old LBW and control rats (Fig. 6A). The mRNA levels of *Snap-25* (percentage of control: 120% ± 4.7%, t = 4.301, df = 12, $p = 0.001029$) and *Scamp2* (151% ± 6.8%, t = 9.051, df = 10, $p = 0.000004$) were significantly upregulated, while mRNA levels of *Snap-29, Snapin, Synapsin I, II, III, Synaptophysin, Synaptotagmin I, II, III, Syntaxin 1A, Vamp1*, and *Vamp2* were unchanged. Additionally, 7 other genes were investigated (*Gr, Mr, Munc13, Munc18, Rims1, Scna, and Vgat*), which can be associated with GABAergic neurotransmission, but their mRNA levels were unchanged (Fig. 6B). The upregulation of *Snap-25* and *Scamp2* suggests a differential gene expression of synaptic vesicle proteins in LBW rats, which might be linked to the dysfunction in the GABAergic synaptic neurotransmission, and in particular, the apparent change in presynaptic vesicle release probability. When running multiple corrections, both *Snap-25* and *Scamp2* stayed significant ($p = 0.020386$ and $p = 0.000083$, respectively).

Discussion

A shift from slow to fast GABAergic synaptic signalling of GABAergic neurons has previously been documented from birth until adolescence in different brain regions in rodents (DG: (Hollrigel & Soltesz, 1997); CA1: (Cohen *et al.*, 2000); cortex: (Doischer *et al.*, 2008); superior colliculus: (Jüttner *et al.*, 2001)). Consistent with these studies, we found that the phasic synaptic inhibitory signalling arriving at granule cells in the dentate gyrus shows an acceleration in the rising and decaying phases of sIPSCs

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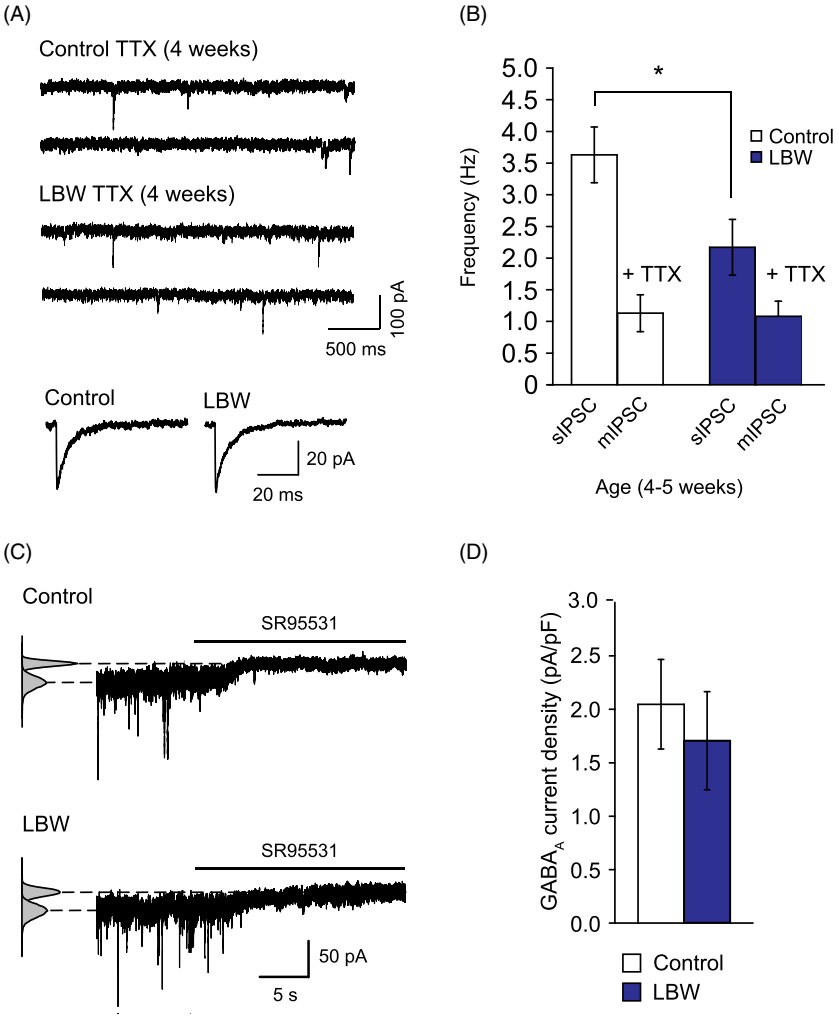

Fig. 5. Extrasynaptic and action potential-independent synaptic $GABA_A$ receptor activities are unaltered in adolescent LBW rats. (*A*) Whole-cell recordings of miniature IPSCs (mIPSCs) showed no change in frequency and amplitude of GABAergic single vesicle responses between groups. Experiments were performed in the presence of TTX, and averages of 50 mIPSCs from each group are shown. Experiments were carried out 4 weeks after birth. (*B*) The action potential blocker TTX reduced the sIPSC frequency fractionally more in LWB rats compared with controls in 4- to 5-week-old rats. The findings indicate that the action potential-dependent, TTX-sensitive component of GABA release is smaller in LBW rats than in controls. Action potential-independent GABA release was similar in both groups. (*C*) Tonic extrasynaptic $GABA_A$ receptor-mediated currents were revealed by the $GABA_A$ receptor antagonist SR95531 (~100 μM). All-points histograms (left) represent the mean currents and noise levels before and after SR95531. Tonic $GABA_A$-mediated currents were similar in this representative control (23.6 pA) and LBW rat (22.9 pA) experiment. The experiments were performed 4–8 weeks after birth. (*D*) No significant difference was found between control and LBW rats.

until postnatal weeks 4–5. The maturation seemed to stabilise at around 4–5 weeks as no further change was observed at 6–8 weeks of age. The acceleration of fast synaptic $GABA_A$ currents could arise due to several developmental events during postnatal maturation. The morphology of dendritic and axonal arbours evolves until the second and third weeks of age (Seress & Ribak, 1990). It contributes to an increase in membrane capacitance and a decrease in input resistance, increasing the speed of postsynaptic events (Doischer *et al.*, 2008). $GABA_A$ receptors with different subunit compositions show distinct kinetics (Mohler *et al.*, 1992), and $GABA_AR$ subunit expression is known to change during juvenile and adolescence in a region-dependent manner (McKernan *et al.*, 1991; Fritschy *et al.*, 1994). The $GABA_AR$ α_2-subunit mediates 10x slower kinetics over α_1-containing $GABA_ARs$ (Dixon *et al.*, 2014). Thus, developmental increases in α_1 and γ_2 and decreases in α_2 subunit expression in the hippocampus (Killisch *et al.*, 1991; Poulter *et al.*, 1992; Lopez-Tellez *et al.*, 2004) may likely cause the observed acceleration in dentate inhibitory signalling (Okaty *et al.*, 2009). However, several other factors might contribute, such as changes in the intrinsic properties of the neurons (Doischer *et al.*, 2008), postnatal maturation of the vesicle release machinery (Kirischuk & Grantyn, 2003), or changes in the firing rate of different inhibitory cell populations.

Our observation of a developmental increase in the amplitude of $GABA_AR$-mediated sIPSCs can result from the increase in number and size of GABAergic synapses seen during postnatal development (Seress & Ribak, 1990). Our results in control rats also suggest a slight increase in the sIPSC frequency between 2 and 4–5 weeks of age, although these changes were not statistically significant. sIPSC frequency represents a summated picture of spontaneous activity of the synapses and the activity rate of the circuitry. Both the synapses and the connectivity rate of the network undergo quantitative and qualitative maturation during the postnatal period (Kilb, 2012). However, the number of interneurons shows a 40% decrease due to programmed developmental cell death during the same period (Southwell *et al.*, 2012). These opposing effects might be counterbalanced, resulting in a somewhat constant spontaneous GABAergic activity in the developing dentate gyrus after postnatal week 2. Our results also point to an increase in the release probability from GABAergic terminals between postnatal weeks 2 and 4–5, consistent with earlier studies (Jüttner *et al.*, 2001; Kirischuk *et al.*, 2005), which correlates with an increase in readily releasable pool size after postnatal week 2 in hippocampal interneurons (Mozhayeva *et al.*, 2002).

Prenatal stress has been found to cause several changes in brain structures during embryonic development (Weinstock, 2011; Franke *et al.*, 2020), changes that must be long-lasting to cause neuropsychiatric disorders later in life. This raises the question, how stress *in utero* affects the postnatal maturation of hippocampal GABAergic signalling. In 2-week-old (juvenile) LBW rats,

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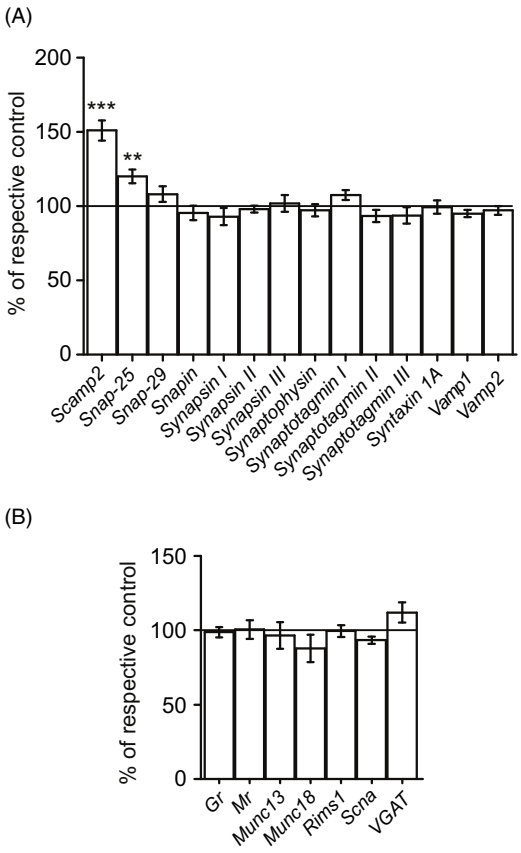

Fig. 6. Increase in the mRNA expression levels of presynaptic proteins, *Snap-25* and *Scamp2* in 8-week-old LBW rats compared to vehicle controls. (*A*) Real-time qPCR was used to quantify mRNA expression levels of synaptic vesicle proteins in the hippocampus of the LBW rats. The normalised values are plotted as mean group values ± SEM and expressed as per cent of respective control. Control: $n = 10$, LBW: $n = 7$. Unpaired t-test, $^{**}p < 0.01$, $^{***}p < 0.001$. After Holm–Sidak multiple corrections, values were $p = 0.020386$ for *Snap-25* and $p = 0.000083$ for *Scamp2*, respectively. (*B*) Real-time qPCR was also used to quantify mRNA expression levels of seven selected genes in the hippocampus in the LBW rat model. No significant changes were observed. Control: $n = 10$, LBW: $n = 7$.

GABAergic signalling was comparable to that of control animals. This suggests that GABAergic malfunction due to prenatal stress will manifest later in life, as we observed pronounced differences in 4- to 5-week-old animals. However, abnormal development of GABAergic neurons has been observed already at birth in a mouse model of prenatal stress (Stevens *et al.*, 2013). In the offspring of mice subjected to acute bright light stress, a delay in the interneuron progenitor migration was demonstrated (Stevens *et al.*, 2013). This is indicative of a delay in developmental processes.

The probability of GABA release decreased during postnatal development in LBW rats. This is consistent with earlier findings in our laboratory, showing decreased release probability of dentate gyrus GABAergic terminals in the CMS model of depression in adulthood (Holm *et al.*, 2011). These similarities across different depression models suggest that malfunction in GABA release might be central to the underlying pathomechanisms of depressive disorders.

Our data showed a tendency of the frequency of spontaneous IPSCs to be downregulated (although this was not statistically significant), whereas the action potential-independent miniature IPSCs showed no difference compared to controls. The tendency of a decreased frequency of sIPSCs could reflect alterations in the action potential-driven GABA release from the presynaptic terminals of interneurons. This could occur due to a decrease in the number of presynaptic interneurons following the delay in interneuron progenitor migration reported by (Stevens *et al.*, 2013). Our finding of unchanged mIPSC frequency does, however, not support this notion. Alternatively, a decreased frequency in sIPSCs could occur due to a decrease in the probability of GABA release from nerve terminals. Our findings of changes in the paired-pulse ratio of evoked synaptic responses further support this idea of decreased probability of GABA release (Dobrunz & Stevens, 1997).

Decreased GABA concentration is a common feature in brain tissues of depressed human patients (Sanacora *et al.*, 1999; Bhagwagar *et al.*, 2007), which might influence the basal tonic GABA signalling in the hippocampus. However, when testing the extrasynaptic $GABA_A$ receptor-mediated tonic current without exogenous agonists in the dentate gyrus of the LBW rats, we found no abnormality compared to control rats, consistent with earlier findings in the CMS model of depression (Holm *et al.*, 2011).

Testing across several presynaptic proteins, we showed that *Snap-25* mRNA is upregulated in the LBW rats. This corresponds well with the increased expression in SNAP-25 protein levels in the hippocampus and prefrontal cortex in rats whose mothers underwent restraint three times daily during the last week of gestation (Cao *et al.*, 2018).

Mechanistically, SNAP-25 regulates synaptic strength (Bark *et al.*, 2004; Scullin *et al.*, 2012) via the downregulation of voltage-gated Ca^{2+} channels (Condliffe *et al.*, 2010). Antonucci and colleagues showed a stronger paired-pulse depression at GABAergic synapses in SNAP-25 heterozygous (SNAP-$25^{+/-}$) cell cultures, where the expression level of SNAP-25 is expected to be half of that in control cells (Antonucci *et al.*, 2013). This effect might be due to the decreased inhibitory effect of SNAP-25 on Ca^{2+} channels (Condliffe *et al.*, 2010), leading to an increased vesicle release probability at GABAergic terminals (Antonucci *et al.*, 2013; Kochlamazashvili & Haucke, 2013). However, it is essential to note that SNAP-25 is also involved in glutamate release (Antonucci *et al.*, 2013). We showed an increased expression level of *Snap-25* mRNA in the hippocampus of adult LBW rats, which may cause an opposite effect in the GABAergic synapses, leading to a decrease in the vesicle release probability through downregulation of Ca^{2+} channel activity. Thus, if an increased *Snap-25* mRNA is translated into protein, this presents one possible explanation of the observed decrease in release probability of GABAergic terminals in adult LBW rats. Since the latter was already prominent in adolescence, this raises the idea that upregulation of SNAP-25 might be a characteristic feature already in developing LBW rats, which may be a topic for future investigations.

Finally, *Scamp2* was also upregulated in adult LBW rats. SCAMP2 is involved in both exocytic and endocytic secretory pathways (Brand & Castle, 1993), and overexpression of SCAMP2 in neuroendocrine cells inhibits both exo- and endocytosis of secretory granules (Liu *et al.*, 2002), while knockdown of SCAMP2 also leads to decreased exocytosis (Liao *et al.*, 2008). SCAMP2 brain expression is involved in the regulation of different monoamine transporters (Müller *et al.*, 2006; Fjorback *et al.*, 2011). However, its precise role in the brain has yet to be elucidated. Furthermore, in humans, a correlation was found between single-nucleotide polymorphism of SCAMP2 and neuroticism (Luciano *et al.*, 2012). Thus, the increased expression level of SCAMP2 found in the hippocampus of LBW rats may alter

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neurotransmission in the monoaminergic neurotransmitter systems of the brain, although its connections to GABA release in this animal model are not clear at the moment.

In conclusion, we demonstrated that prenatal stress exposure leads to decreased GABAergic signalling in the dentate gyrus in the LBW rat model of depression. Although this will require further investigations, we propose that increased expression of *Snap-25* in the hippocampus is linked to the reduction in both spontaneous and evoked GABA release onto dentate granule cells. Our results further support the GABAergic hypothesis of depressive disorders, and thus, the multifaceted mechanisms of the GABAergic system might be a putative target for clinical treatment.

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Author contributions. ZD and JLNG performed electrophysiological experiments and analyses. BE carried out the molecular biology experiments, and KSH and GW generated the animals. BE, MMH, KJ, and GW designed and supervised the research. All authors contributed to the writing of the manuscript and the preparation of figures.

Conflict of interest. GW is the Editor-in-Chief of Acta Neuropsychiatrica, but actively withdrew and was not involved during the review or decision process of this manuscript.

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