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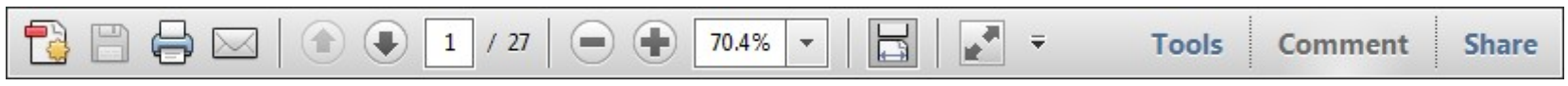

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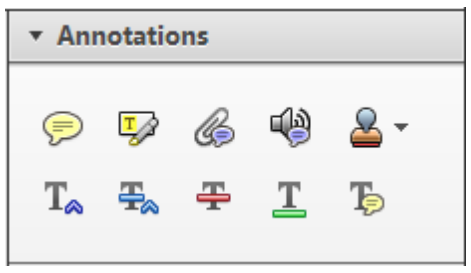

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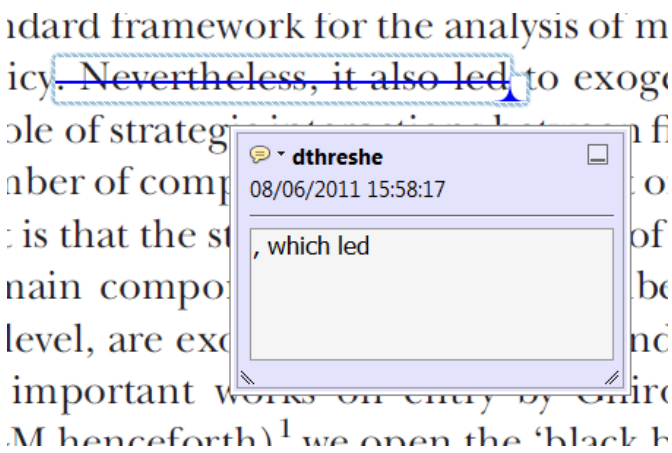

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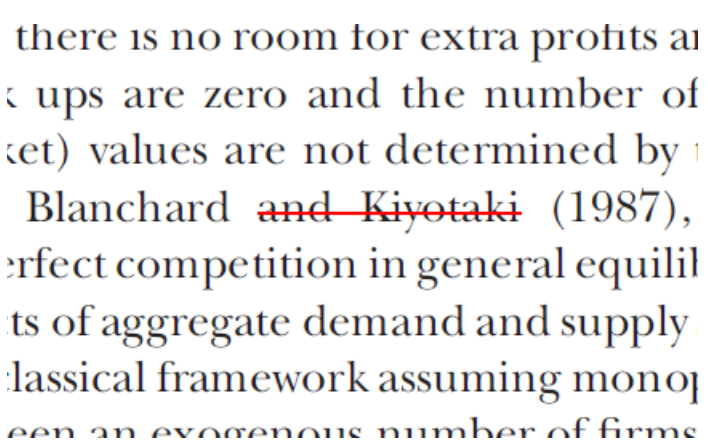

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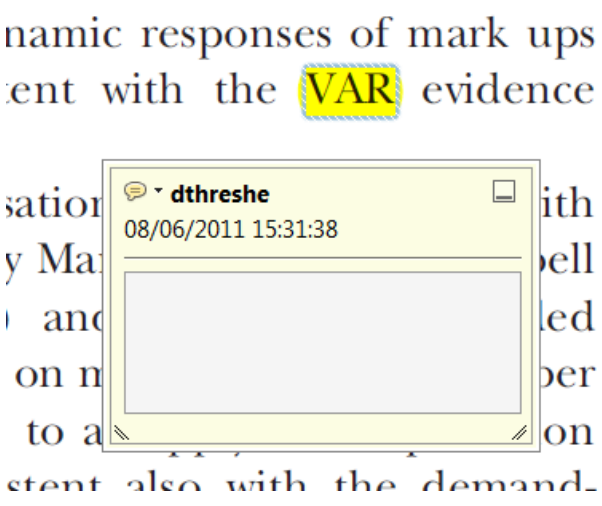

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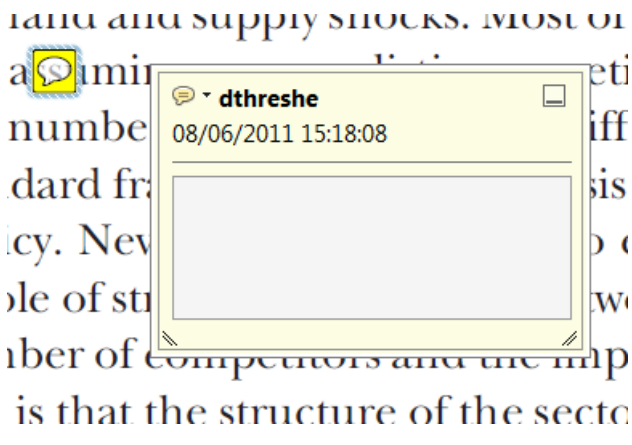

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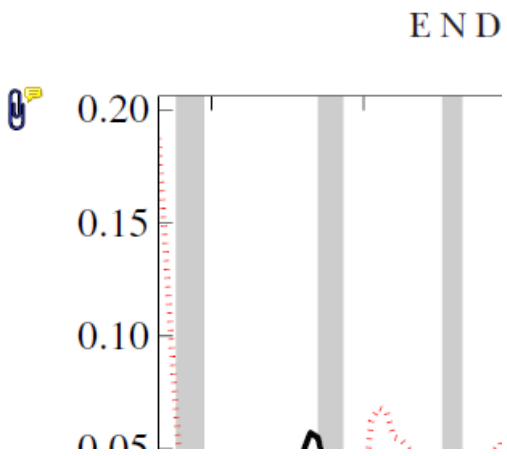

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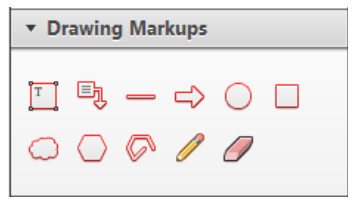

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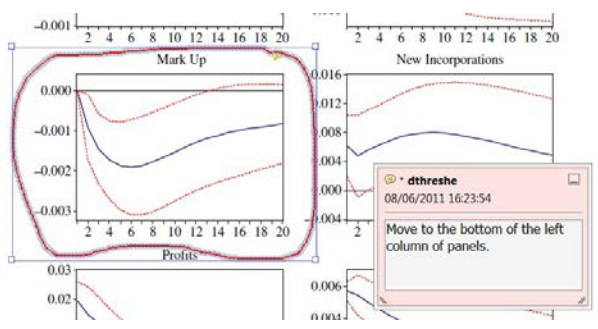

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Cerebrolysin Prevents Deficits in Social Behavior, Repetitive Conduct, and Synaptic Inhibition in a Rat Model of Autism

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Abstract: Autism spectrum disorder (ASD) is a syndrome of diverse neuropsychiatric diseases of growing incidence characterized by repetitive conduct and impaired social behavior and communication for which effective pharmacological treatment is still unavailable. While the mechanisms and etiology of ASD are still unknown, a consensus is emerging about the synaptic nature of the syndrome, suggesting a possible avenue for pharmacological treatment with synaptogenic compounds.

The peptidic mixture cerebrolysin (CBL) has been successfully used during the last three decades in the treatment of stroke and neurodegenerative disease. Animal experiments indicate that at least one possible mechanism of action of CBL is through neuroprotection and/or synaptogenesis.

In the present study, we tested the effect of CBL treatment (daily injection of 2.5 mL/Kg i.p. during 15 days) on a rat model of ASD. This was based on the offspring (43 male and 51 female pups) of a pregnant female rat injected with valproic acid (VPA, 600 mg/Kg) at the embryonic day 12.5, which previous work has shown to display extensive behavioral, as well as synaptic, impairment.

Comparison between saline vs. CBL-injected VPA animals shows that CBL treatment improves behavioral as well as synaptic impairments, measured by behavioral performance (social interaction, Y-maze, plus-maze), maximal response of inhibitory γ-amino butyric acid type A receptor ($GABA_AR$)-mediated synaptic currents, as well as their kinetic properties and adrenergic and muscarinic modulation. We speculate that CBL might be a viable and effective candidate for pharmacological treatment or co-treatment of ASD patients.

Key words: GABA; VPA; behavior; patch-clamp; synapses

SIGNIFICANCE
Autism spectrum disorders (ASDs) are neurodevelopmental illnesses for which effective treatment is not yet available. Cerebrolysin (CBL) is an amino acid and peptide mixture already used in the treatment of neurological conditions like cerebral stroke, ischemia, and Alzheimer disease, substantially void of side effects. We tested the effectiveness of CBL as a treatment in an ASD animal model, finding that CBL prevents numerous behavioral as well as physiological alterations associated with ASD. We conclude that CBL has a potential as effective, low-cost treatment of ASDs.

This work has been conducted in part with funds from Mexican Consejo Nacional de Ciencia y Tecnologia, CONACyT CB-2013-01 221653 to MA and CB-2015-01 252808 to GF, and PROMEP 103.5/1365/75 to MA.

Additional supporting information may be found in the online version of this article.

Contract grant sponsor: Mexican Consejo Nacional de Ciencia y Tecnologia; Contract grant numbers: CONACyT CB-2013-01 221653; CB-2015-01 252808; PROMEP 103.5/1365/75

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Received 28 September 2016; Revised 2 April 2017; Accepted 5 April 2017

Published online 00 Month 2017 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/jnr.24072

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INTRODUCTION

Autism spectrum disorder (ASD) is a heterogeneous group of neurodevelopmental conditions characterized by repetitive or obsessive behavior, and impaired communication and social conduct (Frances, et al., 2000). Whether because of an improvement in diagnostic tools or an actual increase in occurrence, in the last decade its incidence increased from 0.7% (~ 1/150) to 1.5% (~ 1/68; CDC, 2016). ASD can be extremely incapacitating, exerting a heavy burden on the patients, their families, and the society at large.

Since ASD etiology is still largely unknown, and the syndrome can only be diagnosed in the second-third postnatal year, it is of paramount importance to identify effective and accessible treatment to relieve families and patients alike from at least part of its incapacitating effects. Progress in ASD early detection have made possible the use of cognitive behavioral therapy (CBT) for the partial recovery of ASD symptoms (Maddox et al., 2016). Yet, in many circumstances, alternative or additional therapy may be desirable for ASD patients.

It is unfortunate that there are no drugs currently available that target the specific needs of ASD patients. Among the drugs used for treating ASD symptoms are second generation antipsychotics (Downs et al., 2015), antidepressants, and drugs approved for the treatment of attention deficit disorders (Logan et al., 2015). Prescription of these drugs is typically meant for symptomatic and acute treatment of ASD symptoms, without actually treating the underlying disorder. Moreover, their prolonged use often comes with a barrage of disturbing side-effects which severely limits their clinical efficacy (Matson et al., 2011; Yalcin et al., 2016). For all these reasons there is a strong unmet need for effective pharmacological treatment of ASD.

Converging evidence from clinics (Santini and Klann, 2014; Gao and Penzes, 2015; Thomas et al., 2016) as well as animal models (Giovedí et al., 2014; Santini and Klann, 2014), suggests that ASD is associated with a variety of synaptic alterations of either or both the excitatory of the inhibitory system in critical areas of the brain controlling communication and social behavior (Codagnone et al., 2015), as well as movement (Fuccillo, 2016).

Work from several groups, including our own, suggests that inhibitory synapses mediated by the neurotransmitter γ-amino butyric acid (GABA) are severely impaired in human ASD patients as well as in the VPA and other rodent models (Schmitz et al., 2005; Yip et al., 2009; Lawrence et al., 2010; Oblak et al., 2011; Banerjee et al., 2013; Cellot and Cherubini, 2014).

Cerebrolysin (CBL) is an amino acid/peptide mixture (Gevaert et al., 2015) that has been successfully used to promote synaptic growth in the treatment of cerebral stroke (Bornstein and Poon, 2012), Alzheimer's disease (Allegri and Guekht, 2012), traumatic brain injury (Bornstein and Poon, 2012), and neurodevelopmental disorders, including schizophrenia (Flores and Atzori, 2014). In particular, CBL has been shown to potentiate $GABA_A$-receptor-mediated responses in mouse hippocampal cell cultures (Zemkova et al., 1995).

For all these reasons, based on the prenatal administration of valproate salts (VPA), we tested the efficacy of CBL in relieving ASD symptoms in a rodent model of ASD (Schneider and Przewlocki, 2005; Schneider et al., 2006). Previous studies have shown that the offspring of mothers injected with VPA during pregnancy display a set of symptoms analogous to human ASD, including impairments in communication and social behavior and increased repetitive conduct. Our results suggest that early CBL treatment improves—at least in part—the behavioral deficits and prevents the impairment of GABAergic synapses of VPA offspring.

MATERIALS AND METHODS

Experimental Animals

Experimental animals were purchased from Harlan, fed ad-libitum with Chow 5005 pellets (Nutrimix), kept in standard rat polycarbonate cages and bred at the Center of Bioscience under inverted light-darkness cycle (12 h darkness, 8:30 am–8:30 pm; 12 h light, 8:30 pm–8:30 am), hosted at a temperature between 19 and 23 °C, with humidity 25–35%, and ad-libitum feeding (Chow, 5005) and drinking. Twelve female Sprague-Dawley rats were used to produce an offspring of 94 (51 females, 43 males) rats that were used for the experiments. In order to determine precisely the first day of pregnancy, paired female rats in fertile age were examined daily, 7–9 am. Animal pregnancy was determined by the presence of a vaginal plug, which was designated as the first day of gestation. Pregnant female rats received a single intraperitoneal injection of VPA (600 mg/kg in 0.9% saline) on day 12.5 of gestation while control rats received the same volume of saline as described previously (Schneider and Przewlocki, 2005; Bringas et al., 2013). About half of the offspring of VPA-injected mothers (28 males and 24 females) displayed a variety of physical abnormalities including short, bent, or double-flexure tail, and/or syndactyly. Both female and male animals were used for this study, including an approximate number of each sex for each group and subgroups. Females were housed individually and were allowed to raise their own litters. The offspring were weaned on postnatal day (PD) 21 and rats of either sex were housed separately in cages with no more than 3 animals of the same sex. The offspring of each group was split evenly and randomly in two subgroups each, one subgroup was given chronic injections of CBL (2.5 ml/kg) and the other was treated with saline for 2 weeks. An earlier CBL administration time was selected for the electrophysiological tests, to compare the results of this study with our previous results (Banerjee et al., 2013), while behavioral tests were conducted at an older age, at which animals are more active and display a less erratic behavior.

CBL or saline was administered from PD 10-24 or from PD 45-60, before electrophysiological and behavioral experiments, respectively (Fig. 1). Three animals presented gross anatomical defects (2 presented hydrocephalus) or their health was compromised (1 was found bleeding) and they were removed from the study. All methods and procedures were in accordance with guidelines set by the National Institutes of Health for

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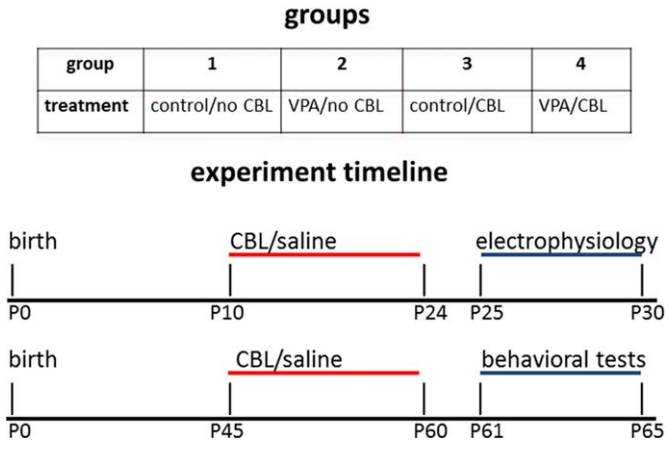

Fig. 1. Experimental groups and timeline. Four experimental groups were used for each series of experiments: group 1, offspring of saline-injected (non-VPA) mothers; group 2, offspring of VPA-injected mothers; group 3, offspring of saline-injected (non-VPA) mothers treated with CBL; group 4, offspring of VPA-injected mothers treated with CBL. For graphical purpose, in the following figures data were presented in the group order 1, 2, 4, 3. Experimental animals were injected at a different time point for behavioral (P45-P60) or electrophysiological experiments (P10-P24).

Ethical Treatment of Animals and the of the Guide for Care and Use of Laboratory Animals of the Mexican Council for Animal Care (Norma Official Mexicana NOM-062-ZOO-1999), and received the approval of the University Committee on Animal Research at the University of Texas at Dallas and of the Committee for Ethics, Investigation and Teaching (CEID2015045) of the Universidad Autónoma de San Luis Potosi.

Drugs

CBL was purchased from Ebewe Pharma and all other reagents were purchased from Sigma. Pilots experiments were conducted showing that a dose of 2.5 mL/Kg of CBL (half the dose used on a rat adult cohort in a different study (Vazquez-Roque et al., 2012) induced significant improvements of social behavior on VPA-offspring. The same concentration was used throughout the study.

Stock solutions of all drugs were prepared in water except for oxotremorine, for which stock solution (10 mM) was prepared in ethanol. For non-aqueous solutions, the final concentration of the solvent (10 μM ethanol) was added to the recording control solution. After recording an initial baseline for 7–10 min, drugs were bath-applied for 5 min or longer, until reaching a stable condition.

Electrophysiology

Thirty animals (15 male and 15 female) were processed for electrophysiology within 6 days after the last CBL or saline injection (P25-P30). Forane saturation anesthesia was performed prior to animal euthanasia. Once the skull was severed and opened, the brain was removed, coronal slices (270 μm) from the temporal cortex were cut and incubated in ACSF containing (in mM) 126 NaCl, 3.5 KCl, 10 glucose, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 1.5 $CaCl_2$, 1.5 $MgCl_2$, and 0.2 ascorbic acid at a pH of 7.4, It was then saturated with a mixture of 95% O_2 and 5% CO_2, at 32 °C, before being placed in the recording chamber, where 10 μM 6,7-dinitroquinoxaline-2,3-dione and 2 mM kynurenic acid were added to block α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA)-receptor-dependent glutamate channels, respectively. Miniature IPSCs (mIPSCs) were recorded in the presence of the Na^+-channel blocker tetrodotoxin (TTX, 1 μM). The recording area was selected dorsal to the rhinal fissure corresponding to the primary auditory cortex (Rutkowski et al., 2003). All recordings were performed at room temperature (22–23 °C), from layer 2/3 triangular-shaped cell body neurons, most of which were presumably pyramidal cells. IPSCs were recorded in whole-cell configuration, in voltage clamp mode, holding the membrane potential at a holding voltage V_h = -60 mV, with 3–6 MΩ electrodes filled with a solution containing (mM): 100 CsCl, 5 1,2-bis(2-aminophenoxy) ethane-N,N,N,N-tetraacetic acid K–BAPTA-, 1 lidocaine N-ethyl bromide, 1 $MgCl_2$, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid–HEPES-, 4 glutathione, 1.5 $ATPMg_2$, 0.3 GTP-Na_2, and 20 phosphocreatine. The holding voltage was not corrected for the junction potential (< 4 mV). The rise-time (10–90%) and the decay-time (single exponential) were calculated using the Mini Analysis software. Electrically-evoked IPSCs (eIPSCs) were measured by delivering electric stimuli (90–180 μs, 10–50 mA) every 20 s with an isolation unit, through a glass stimulation using a monopolar electrode filled with ACSF, and placed 150–200 μm away from the recording electrode. For allowing the comparison between the results with a previous study (Banerjee et al., 2012) in which inhibitory transmission in the VPA-treated offspring was detected, a paired-pulse protocol was used for the electrical stimulation. Since that study did not reveal any significant differences in paired-pulse between VPA offspring and controls, paired-pulse data were not analyzed in the present study. A 2-mV voltage step was applied at the beginning of every episode to monitor the quality of the recording. Recordings where series resistance changed by > 20% were discarded from the analysis. Recorded data were analyzed using Clampfit software. Sample size for number of animals and recordings for each experiment are reported in the corresponding tables (Tables IV and V), and/or in the text. T4 T5

Behavioral Testing Battery

Sixty-eight animals (32 male, 36 female) were used for the following behavioral studies. The details of size and sex for the behavioral groups are reported in the statistical tables corresponding to each experiment (Tables (I–V)). T1 T3

Social interaction test. Animals were processed for behavior within one week after the last CBL or saline injection (P61-P67). Rats were selected in a randomized order for group and tested for social interaction in an open field measuring 12 in x 24 in x 15 in. For all behavioral experiments and to avoid environmental novelty bias, animals received a 10-min-long acclimation session on the day prior to the actual experiment. On the day of the experiment, animals were isolated 3.5 h before starting the experiment and were matched for age

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TABLE I. Analysis of Variance of Open Field Social Behavior

Groups		1: control		2: VPA	3: CBL only		4: VPA + CBL
n		11 (6 m, 5 f)		13 (6m, 7f)	12 (6 m, 6 f)		13 (7 m; 6 f)
Tukey test (m = group mean)			*p*	two-way ANOVA	dfTOT = 48		
Sniffing							
one-way ANOVA	F = 36.32	m1 vs. m2	< 0.01		F		*p*
	p < 0.0001	m1 vs. m3	n.s.		row	20.01	<0.0001
	df = 3,45	m1 vs. m4	n.s.		column	55.99	<0.0001
		m2 vs. m3	< .01		r x c	32.95	<0.0001
		m2 vs. m4	< .01				
		m3 vs. m4	n.s				
Crawling/Mounting							
one-way ANOVA	F = 72.41	m1 vs. m2	< 0.01	two-way ANOVA	F		*p*
	p < 0.0001	m1 vs. m3	< 0.01		row	186.51	< 0.0001
	df = 3,45	m1 vs. m4	< 0.01		column	0.24	0.627
		m2 vs. m3	< 0.01		r x c	30.24	<0.0001
		m2 vs. m4	< 0.01				
		m3 vs. m4	< 0.01				
Following							
one-way ANOVA	F = 58.45	m1 vs. m2	< 0.01	two-way ANOVA	F	*p*	
	p < 0.0001	m1 vs. m3	< 0.01		row	163.99	< 0.0001
	df = 3,45	m1 vs. m4	< 0.01		column	2.21	0.144
		m2 vs. m3	< 0.01		r x c	9.15	0.0041
		m2 vs. m4	< 0.01				
		m3 vs. m4	< 0.01				
Sniffing Ano-genital Area							
one-way ANOVA	F = 12.07	m1 vs. m2	<0.05	two-way ANOVA	F	*p*	
	p < 0.0001	m1 vs. m3	n.s.		row	27.44	<0.0001
	df = 3,45	m1 vs. m4	n.s.		column	7.43	0.091
		m2 vs. m3	< 0.01		r x c	1.34	0.25
		m2 vs. m4	< 0.01				
		m3 vs. m4	n.s.				

TABLE II. Y-maze: Repetition Index

groups		1: control		2: VPA	3: CBL only		4: VPA + CBL
n		9 (3 m; 4 f)		9 (3 m; 4 f)	7 (3 m; 4 f)		8 (4 m; 4 f)
Tukey test (m = group mean)			*p*	two-way ANOVA	dfTOT = 32		
one-way ANOVA	F = 6.5	m1 vs. m2	< 0.05		F		p
	p = 0.00168	m1 vs. m3	n.s.		row	9.67	0.0042
	df = 3,29	m1 vs. m4	n.s.		column	5.09	0.0318
		m2 vs. m3	< 0.01		r x c	4.74	0.0378
		m2 vs. m4	< 0.01				
		m3 vs. m4	n.s				

TABLE III. Plus Maze

Groups		1: control		2: VPA	3: CBL only		4: VPA + CBL
n		22 (10m; 12f)		14 (7m; 7f)	16 (7m; 9 f)		16 (8m; 8f)
Tukey test (m = group mean)			p	two-way ANOVA	dfTOT = 67		
		% open arm entries					
one-way ANOVA	F = 4.67	m1 vs. m2	< 0.05		F		*p*
	p = .00517	m1 vs. m3	n.s.		row	7.38	0.0085
	df = 3,64	m1 vs. m4	n.s.		column	0.98	0.3259
		m2 vs. m3	< 0.05		r x c	5.64	0.0206
		m2 vs. m4	< 0.01				
		m3 vs. m4	n.s				
% time in open arm							
one-way ANOVA	F = 8.18	m1 vs. m2	< 0.05	two-way ANOVA	F		*p*

TABLE IV. Input/Output (I/O) Curves Parameters

Groups		1: control	2: VPA	3: CBL only	4: VPA + CBL		
n animals		6 (3m, 3f)	6 (3m, 3f)	8 (4m, 4f)	10 (5m, 5f)		
I/O threshold							
recordings		10 (5m, 5f)	10(5m, 5f)	7 (3m, 4f)	11 (6m, 5f)		
Tukey test (m = group mean)			p	two-ways ANOVA	dfTOT = 37		
one-ways ANOVA	F = 1.98	m1 vs. m2	n.s.		F		*p*
	p = 0.134	m1 vs. m3	n.s.		row	0	1
	df = 3,34	m1 vs. m4	n.s.		column	5.99	0.019
		m2 vs. m3	n.s.		r x c	0	1
		m2 vs. m4	n.s.				
		m3 vs. m4	n.s				
I/O slope							
	recordings	10(5m, 5f)	10(5m, 5f)	7 (3m, 4f)	11 (6m, 5f)		
Tukey test			P	two-ways ANOVA	dfTOT = 37		
one-ways ANOVA	F = 3.54	m1 vs. m2	< 0.05		F	*p*	
	p = 0.0258	m1 vs. m3	n.s.		row	1.3	0.262
	df = 3,34	m1 vs. m4	n.s.		column	6.36	0.0165
		m2 vs. m3	n.s.		r x c	0.4	0.53
		m2 vs. m4	n.s.				
		m3 vs. m4	n.s				
I/O saturation current							
	recordings	9(4m, 5f)	9(4m, 5f)	6(3m, 3f)	9(5m, 4f)		
Tukey test			*P*	two-ways ANOVA	dfTOT = 32		
one-ways ANOVA	F = 10.43	m1 vs. m2	< 0.01		F	*p*	
	p < 0.0001	m1 vs. m3	n.s.		row	17.88	0.0002
	df = 3,31	m1 vs. m4	n.s.		column	12.33	0.0013
		m2 vs. m3	< 0.01		r x c	1.09	0.3038
		m2 vs. m4	< 0.01				
		m3 vs. m4	n.s.				

TABLE V. Modulation

groups		1: control	2: VPA	3: CBL only	4: VPA + CBL	
Norepinephrine-induced evoked IPSCurrent increase (%)						
n animals		3 (2m;1f)	3 (2m;1f)	3 (2m;1f)	3 (2m;1f)	
n recordings		9 (5m; 4f)	9 (4m; 5f)	6 (3m; 3f)	9 (5m; 4f)	
Tukey test (m = group mean)			p	two-way ANOVA	dfTOT = 32	
one-way ANOVA	F = 6.19	m1 vs. m2	n.s.		F	*p*
	p = 0.00221	m1 vs. m3	n.s.		row	15.6	0.005
	df = 3,29	m1 vs. m4	n.s.		column	3.77	0.062
		m2 vs. m3	< 0.01		r x c	0	1
		m2 vs. m4	< 0.05				
		m3 vs. m4	n.s				
Oxotremorine-induced evoked IPSC decrease (%)						
n animals		3 (2m;1f)	3 (2m;1f)	3 (2m;1f)	3 (2m;1f)	
n recordings		9 (5m; 4f)	7 (4m; 3f)	9 (4m; 5f)	6 (5m; 1f)	
Tukey test			*p*	two-way ANOVA	dfTOT = 30	
one-way ANOVA	F = 3.41	m1 vs. m2	<0.05		F	*p*	
	p = 0.0316	m1 vs. m3	n.s.		row	0	1
	df = 3,27	m1 vs. m4	n.s.		column	2.36	0.1361
		m2 vs. m3	n.s.		r x c	0	0.0092
		m2 vs. m4	n.s.				
		m3 vs. m4	n.s				

(within 1 week), sex, and weight (within 10 %). Behavior was assessed for 15 mins for sniffing or licking any body part other than the anogenital area, crawling/climbing, following or approaching a partner with the same experimental condition and treatment during the activity phase of the animals, and recorded on a PC with an infrared sensitive camera under conditions of dim illumination (about 5 lux, used throughout the behavioral experiments). Anogenital inspection was assessed separately by counting when the animal actively (rather than randomly) pursued and/or touched the anogenital area of the

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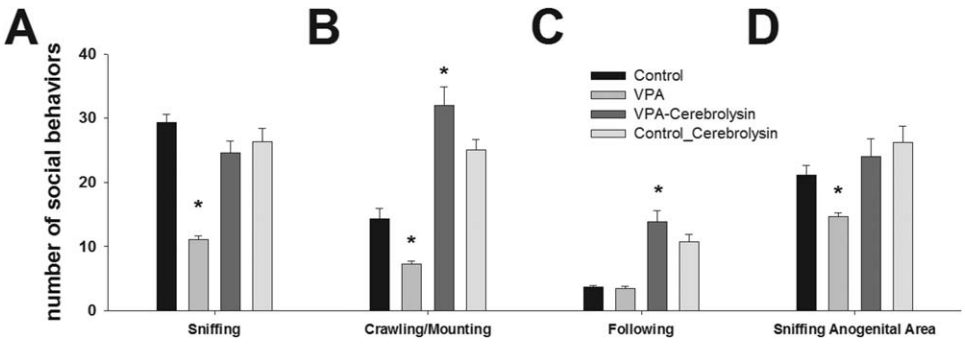

Fig. 2. CBL I improves social behavioral patterns. We measured 4 types of social behaviors: A) sniffing, B) crawling/mounting, C) following, and D) sniffing anogenital area (for definition see: Material and Methods). Of the four types of social behavior, three of them (sniffing, crawling/mounting and sniffing anogenital area) were impaired in VPA offspring. CBL treatment improved all three impaired behavior and increase the following behavior (same meaning of the symbols, see ANOVA plus *post-hoc* Tukey test on Table I). CBL treatment on control (non-VPA) offspring did not change sniffing behavior nor sniffing anogenital area, but did increase Crawling/Mounting and Following behaviors above the control level (ANOVA plus *post-hoc* Tukey test).

partner with its nose. These behaviors were analyzed separately for each rat. The open field chamber was cleaned with a 70% alcohol solution between each trial. All experiments were performed at room temperature (22–23 °C).

Y-maze. Rats were assessed for repetitive behavior on a Y-maze (Merali et al., 2014; Kumar and Sharma, 2016). Each arm of the maze was 16 inches long, 4.5 inches wide, and enclosed by walls 6 inches high. Each rat was placed at the end of one arm and allowed to move freely in the maze. The series of arm entries was recorded for 10 min with a video camera. The spontaneous alteration performance was used to assess their repetitive behavior and was defined by an entry into all three arms on consecutive occasions. The number of maximum alternations was the total number of arm entries minus two, and the percentage of alternation was calculated as (actual alternations/maximum alternations) x 100. The Y-maze was wiped clean between trials with a 70% alcohol solution.

Since no overall statistical differences were detected in the social behavior tests between male and female rats either in the offspring treated prenatally with VPA or in the untreated control we pooled animals from both sexes in the subsequent statistical analysis.

Elevated plus maze. Rats were analyzed for anxiety on an elevated plus-maze with the two opposite open arms measuring 20 inches x 14 inches and two opposite arms enclosed with a wall 16 inches high. The plus-maze was elevated from the floor at a height of 21.5 inches. The trials were started by placing the rats in the center of the maze. Rats were tracked for 5 min with a video camera, and then returned to their home cage. The number of open and closed arm entries, as well as the total time spent in open and closed arms, were measured. The plus maze was wiped clean between trials with a 70% alcohol solution.

Statistical Analysis

To eliminate any possible bias, experimental procedures as well as data analysis were performed in a blind fashion: the experimenter did not know which group the subject belonged to, and the student analyzing the data did not know animal group.

Results are presented as mean ± SEM and analyzed for statistical significance with one- and two-way analysis of variance (ANOVA) along with sample size for behavioral and electrophysiological data. Statistical difference with the corresponding control group was assessed with Tukey tests and is indicated in the tables, except for miniature synaptic currents, for which the Kolmogorov-Smirnnov test was used. Outliers were excluded from the statistics only when they differed from the mean by more than 3 standard deviations. An asterisk (*) on top of the corresponding bar indicated statistically significant differences ($p < 0.05$). All other comparisons should be considered as non-significant (n.s.). The sample size for each experiment is shown in the corresponding tables for male (m) and female (f) test animals used. For the electrophysiology experiment the number of recordings is available along with the number of animals used.

RESULTS

Cerebrolysin Prevents Behavioral Deficits

In order to confirm the validity of our animal model we performed a series of tests to ascertain whether the VPA offspring consistently reproduced ASD features. We used four stereotyped social behavioral patterns from an open-field system, a Y-maze to evaluate repetitive behavior, and a plus-maze to measure anxiety. The social behavior patterns—which analyzed interactions of each experimental animal with a second animal of the same type—consisted in: sniffing, crawling mounting, following, and sniffing the anogenital area (see the methods section). As expected, VPA offspring presented significant deficit in almost all the analyzed behavioral patterns.

Social behavior. VPA animals showed fewer social interactions in the open-field arena as sniffing and crawling/mounting (approximately 50% reduction in VPA [Fig. 2A and B, first and second column]; n = 11 and n = 13, respectively), and sniffing the anogenital area F2

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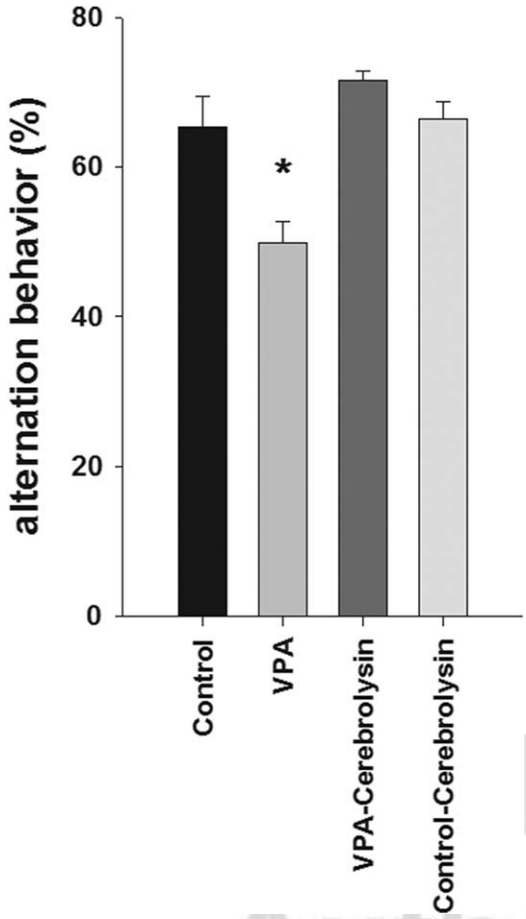

Fig. 3. CBL enhances alternation behavior in the Y-maze. Alternation behavior is decreased in VPA offspring compared to control, and CBL treatment maintains it at the control levels. CBL treatment by itself (4th bar) did not induce any change compared to untreated controls (non VPA offspring, see ANOVA plus *post-hoc* Tukey test on Table II).

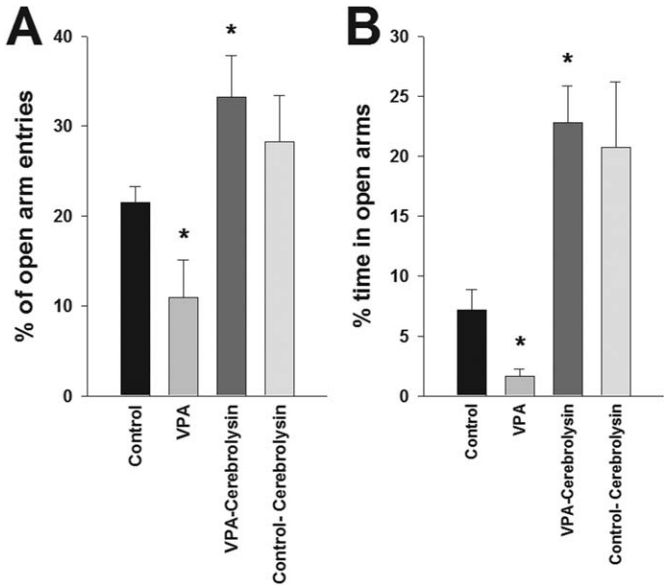

Fig. 4. CBL prevents anxiety behavior. The bars in each graph refer to the untreated control group (first bar), untreated VPA offspring (second bar), CBL-treated VPA offspring (third bar), and CBL-treated control (non-VPA) offspring (fourth bar). A: Percentage of open arm entries is decreased in VPA animals compared to non-VPA offspring. CBL treatment prevents the decrease to levels significantly higher than control in both VPA and non-VPA offspring. The asterisk (*) indicates significant decrease with respect to control ($p < 0.05$). B: Percentage of time in the open arms is decreased in VPA animals compared to non-VPA offspring. CBL treatment prevents the decrease to levels significantly higher than control in both VPA and non-VPA offspring. The asterisk (*) indicates significant decrease with respect to control.

(more than 20% decrease, Fig. 2D; same sample), but not in following (Fig. 2C).

Compared to saline-treated animals, CBL treatment prevented the VPA-induced deficits in sniffing, crawling/mounting, and sniffing the anogenital area (third bars in Fig. 2A and B, 2D; n = 13). CBL treatment by itself (on non-VPA animals; fourth bars in each graph of Fig. 2; n = 12) improved the crawling/mounting (Fig. 2B) and following (Fig. 2C) in the open field test. Statistical analysis (Table I) of sniffing, crawling/mounting, and sniffing the anogenital area suggested a group effect of CBL to increase all of the social behavior measured with the open field (F_r significant, $p < 0.01$ for all four behaviors).

Repetitive behavior and anxiety. VPA animals displayed significantly less alternation behavior in the Y-maze (Fig. 3, first two bars; n = 9 each group). CBL treatment prevented the deficits in alternation behavior (Fig. 3, third bar; n = 8) without changing the baseline behavior in the control (non-VPA) offspring (n = 7). The percentage of open-arm entries and the percentage of time in the open arms of the plus-maze were, respectively, about 50% and 80% smaller in VPA offspring compared to controls (offspring of saline-injected mothers [Fig. 4A and B] first two bars; n = 14 and 22, respectively). CBL treatment prevented the VPA-induced decrease in percentage (%) of open arm entries and % time spent in the open arm of the plus maze (Fig. 4A and B, third bar in each graph; n = 16) compared to the saline-treated animals. CBL treatment by itself (on non-VPA animals; fourth bars in Fig. 4A and B) improved the % of open arm entries and of time in the open arms in the plus maze (n = 16).

In agreement with the results obtained for social behaviors, statistical analysis (Tables II and III) suggested that CBL decreases repetition behavior and anxiety (F_r significant, $p < 0.01$), greatly increasing the percentage of time in the plus-maze open arms ($p < 0.0001$).

Altogether, these results suggest that CBL not only prevents at least some behavioral deficits in VPA animals, but also that CBL treatment by itself may promote social and alternation behavior, and decrease anxiety, even in the control (non-VPA) group.

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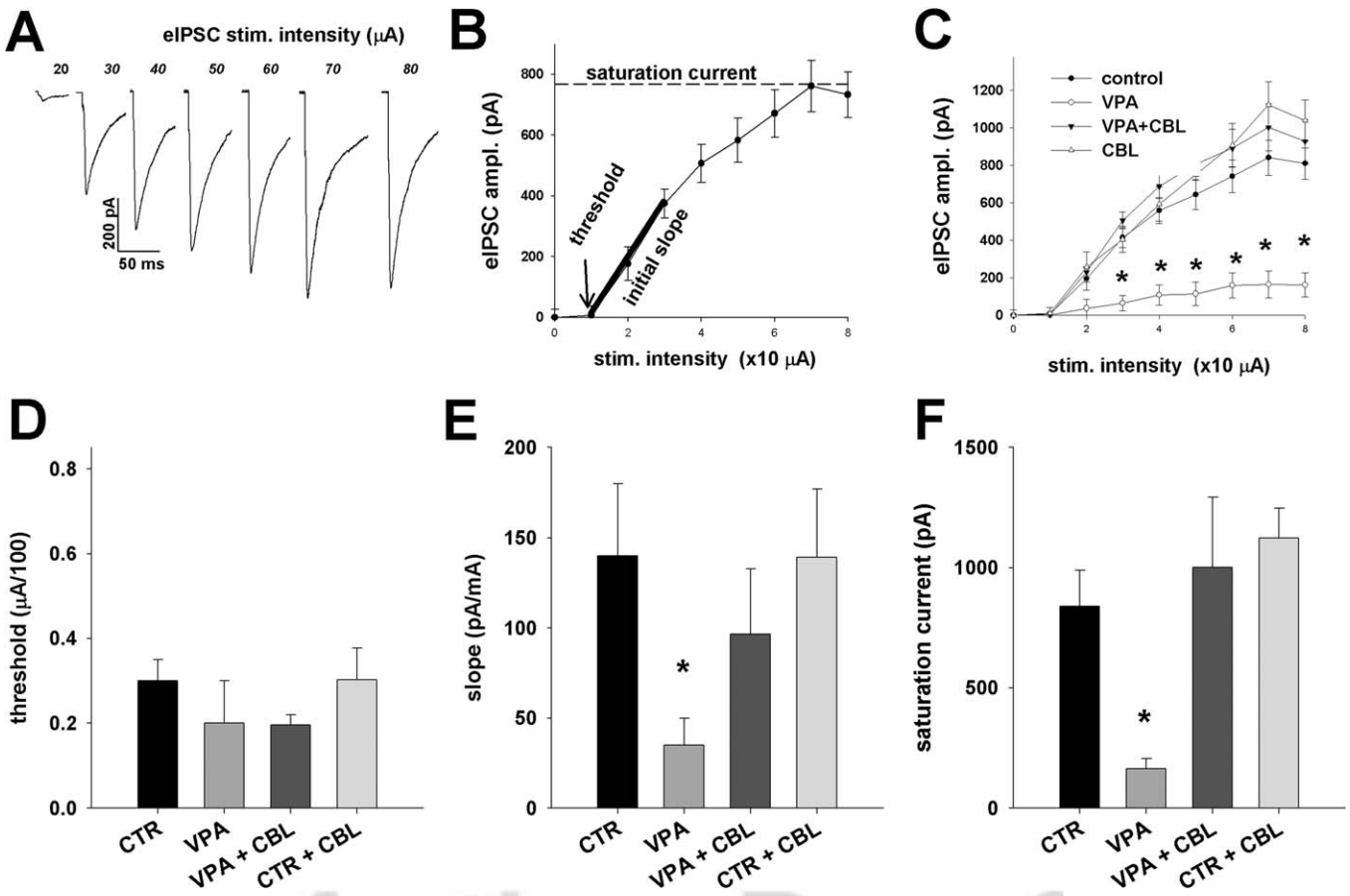

Fig. 5. CBL prevents GABAergic transmission deficits. A: Example of input/output (I/O) curve: electrophysiological recording of seven responses to electrical stimulation in layer 2/3; average of 4 eIPSCs evoked at different stimulation intensities indicated above. B: I/O curve: peak amplitudes of traces plotted as a function of stimulation intensity. C: I/O curve for control, VPA offspring, CBL-treated VPA offspring, and CBL-treated (non VPA) animals. CBL-treated (both VPA and non-VPA offspring) display normal eIPSC amplitudes. Asterisks represent significance between VPA untreated animals and CBL-treated VPA animals. D, E, and F: Effect of CBL on I/O threshold (D), slope (E), and saturation intensity of I/O curves (F). VPA offspring display lower slope and saturation current (but not different threshold) compared with non-VPA offspring. CBL treatment does not change the threshold but recovers the decrease in slope and saturation current (3rd vs. 2nd bars in graphs E and F, respectively), without affecting any of the I/O curve parameters in non-VPA offspring.

Cerebrolysin Prevents VPA-Induced Inhibitory Synaptic Impairment

Strength of synaptic inhibition: input/output curves. We previously found that VPA offspring differ in synaptic (but not extrasynaptic) transmission, mediated by γ-amino butyric acid type A receptors ($GABA_A$Rs) in the supragranular temporal cortex of the rat (Banerjee et al., 2013). In particular, we found that the saturation currents of electrically evoked GABAergic postsynaptic currents (eIPSCs) recorded in cortical layer 2/3 are greatly depressed (around 80% depression, namely down to 20% of control offspring) in VPA animals compared to controls (Banerjee et al., 2013).

In order to evaluate whether CBL treatment affected GABAergic transmission in VPA offspring we used juvenile rats in the typical age range allowing comfortable patch-clamp recording (see the methods section, same age range of a previous work; Banerjee et al., 2013) to compare activation threshold, slope, and saturation levels of electrically evoked postsynaptic currents (eIPSCs) from input/output (I/O) curves (traces in Fig. 5A, and corresponding I/O curve example in Fig. 5B for a CBL-treated VPA animal) in the same brain area of four groups of animals: untreated controls (CTR, n = 6 animals), untreated VPA offspring (VPA, n = 6), VPA offspring treated with CBL (VPA + CBL, n = 10), and (as a further control) in the control offspring treated with CBL (CTR + CBL, n = 8). F5

Examination of the I/O curves for the 4 groups (Fig. 5C) confirmed that the eIPSC threshold (Fig. 5D) was unmodified in VPA offspring (VPA vs. control offspring, first two bars), while both the I/O slope (Fig. 5D, first two bars, same sample) and the saturation current (Fig. 5F, first two bars, same sample) were significantly

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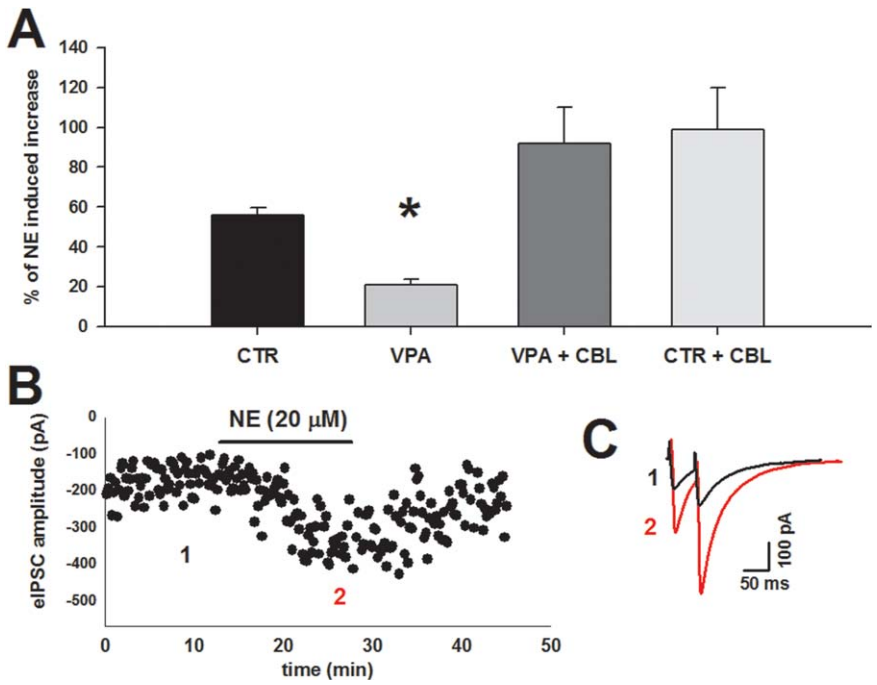

Fig. 6. Cerebrolysin treatment prevents the deficit in noradrenergic modulation of GABAergic currents. A: eIPSC amplitude increases by about 60% in the presence of norepinephrine (NE, 20 μM, first bar) in untreated control (non VPA) offspring but only by about 20% in VPA offspring. CBL treatment recovered NE modulation above the control levels in VPA animals (3rd bar) as well as in non-VPA animals (4th bar). B: Time course of the effect of NE application (horizontal bar) in a VPA, CBL treated animal. C: Average of 4 representative recordings before (black) or after (red) NE application. The numbers in B indicate approximately the time frame of the recordings.

reduced in VPA offspring. CBL treatment did not change I/O-curve threshold in either VPA offspring or controls (Fig. 5C, third and fourth bars), but did prevent the decrease in both slope and saturation current in the I/O curves in VPA offspring (third bar in Fig. 5E and 5F, respectively). I/O slope in CBL-treated control (non-VPA) offspring (fourth bar in Fig. 5E and F, respectively) were not statistically different compared with either untreated controls (first bars in the same graphs), or VPA-CBL-treated offspring (third bars in the same graphs, two-way ANOVA, $F_r = 1.3$, n.s.). On the contrary, two-way ANOVA of eISPC saturation current suggested that CBL treatment might prevent VPA-induced GABAergic deficit by increasing GABAergic transmission per-se ($F = 17.88$, $p < 0.001$). Statistical analysis is reported in table IV.

Cerebrolysin prevents adrenergic and cholinergic modulation of GABAergic fibers. In our previous study (Banerjee et al., 2013), we found that not only slope and saturation currents of $GABA_AR$-mediated neurotransmission I/O curves in VPA-animals were deficient, but also that GABAergic presynaptic modulation by adrenergics and cholinomimetics was impaired, as VPA offspring do not possess the adrenergic-induced increase in eIPSC following bath application of norepinephrine (NE) shown in healthy controls (Salgado et al., 2011, 2012), and shows a limited decrease in eIPSCs amplitude induced by the muscarinic agonist oxotremorine also shown in healthy controls (Salgado et al., 2007).

In the present study we confirmed those results and showed that CBL treatment prevents the unresponsiveness to NE (20 μM, Fig. 6A, representative time course and average of ten traces in Fig. 6B and C, respectively), and (at least in part) enhances the responsiveness to oxotremorine (10 μM, Fig. 7A, representative time course and average of ten traces in Fig. 7B and C, respectively, n = 3 animals each groups, number of recordings for each experiment shown in table V). F6 F7

As a further control, we also determined the effect of CBL treatment on the modulation of GABAergic activity in control (non-VPA) offspring. CBL treatment significantly increased eIPSC NE sensitivity (Fig. 6A, fourth bar) with respect to untreated non-VPA offspring, but did not alter the sensitivity to the muscarinic agonist oxotremorine (Fig. 7B, fourth bar, n = 3 animals each groups, number of recordings for each experiment shown in table V). Two-way ANOVA corroborated the hypothesis that CBL enhances NE modulation ($F_r = 15.6$, $p < 0.01$) regardless of VPA or saline pre-treatment. Statistical analysis is reported in table V.

Effect of CBL on miniature IPSCs. Analysis of the miniature postsynaptic inhibitory currents (mIPSCs)

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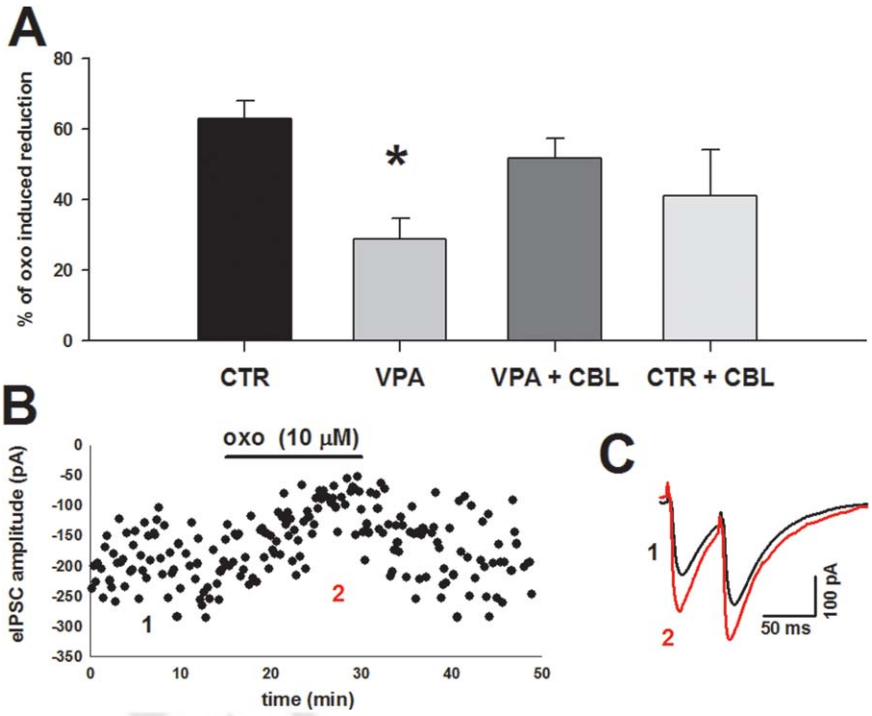

Fig. 7. Cerebrolysin treatment prevents the deficit in muscarinic modulation of GABAergic currents. A: eIPSC amplitude decreases by about 60% in the presence of oxotremorine (oxo, 10 μM, first bar) in untreated control (non-VPA) offspring but only by about 30% in VPA offspring. CBL treatment recovered oxotremorine modulation in VPA animals (3rd bar) and does not affect oxotremorine modulation in non-VPA animals (4th bar). B: timecourse of the effect of oxotremorine application (horizontal bar) in a VPA, CBL treated animal. C: Average of 4 representative recordings before (black) or after (red) oxotremorine application.

showed a similar mean mIPSC amplitude but a lower frequency and an increased rise-time and decay-time in VPA offspring compared to offspring from saline injected mothers (shown as a fraction of the control value in the first bars of Fig. 8A, B, C, and D, untreated VPA offspring, n = 8 recordings from 3 animals; untreated saline-injected offspring, n =10 recordings, respectively, from 3 animals each) consistent with our previous data (Banerjee et al., 2013). CBL-treatment did not change mIPSC amplitude (Fig. 8A), neither prevented the VPA-induced decrease in mIPSC frequency (Fig. 8B), but partially prevented the increase in rise-time (Fig. 8C) as well as in decay-time, $p < 0.05$ each, same sample (representative traces shown in Fig. 8E; CBL-treated VPA offspring: n = 7 recordings from 3 animals; CBL-treated saline-injected offspring: n = 6 recordings from 3 animals). Each trace displayed is the average of 10 traces aligned at the onset, in the peak amplitude range between 40 and 50 pA for each example, the two curves shown represent the best fit with a single exponential for untreated VPA (dashed-line) vs. CBL-treated VPA (solid line).

Altogether these data suggest that CBL treatment normalizes most (but not all) the parameters of GABAergic synaptic transmission altered in VPA offspring, perhaps by enhancing basal GABAergic transmission (regardless of VPA treatment).

DISCUSSION

Autism Spectrum Disorder is a poorly understood psychiatric disease of large prevalence whose increasing cohort is in dire need of effective treatment. Our study showed that CBL treatment substantially prevents—sometimes even above the control levels—well-established behavioral deficits associated with the VPA ASD model, including social behaviors measured in the open-field (sniffing, crawling/mounting and sniffing anogenital area), alternation behavior (Y-maze), and anxiety (percentage in open arm entries and time in open arms in a plus maze protocol). Since CBL did not affect animal mobility (see supplementary Fig.1), in agreement with previous work (Gandal et al., 2010; Vazquez-Roque et al., 2012), these results were not associated to change in locomotion. Interestingly, while both sniffing and sniffing/anogenital area in the CBL-treated control (non-VPA) cohort was similar to the control (untreated non-VPA sample), for most of the other behaviors tested (crawling/mounting, following, and plus-maze tests), the performance of the CBL-treated control (non-VPA) was above the untreated control levels, suggesting that CBL treatment by itself may both enhance the development or performance of brain circuits for the control of social interaction and anxiety regardless of the ASD impairment.

While these data leave no doubt on the specific beneficial effects of the CBL treatment in our ASD model,

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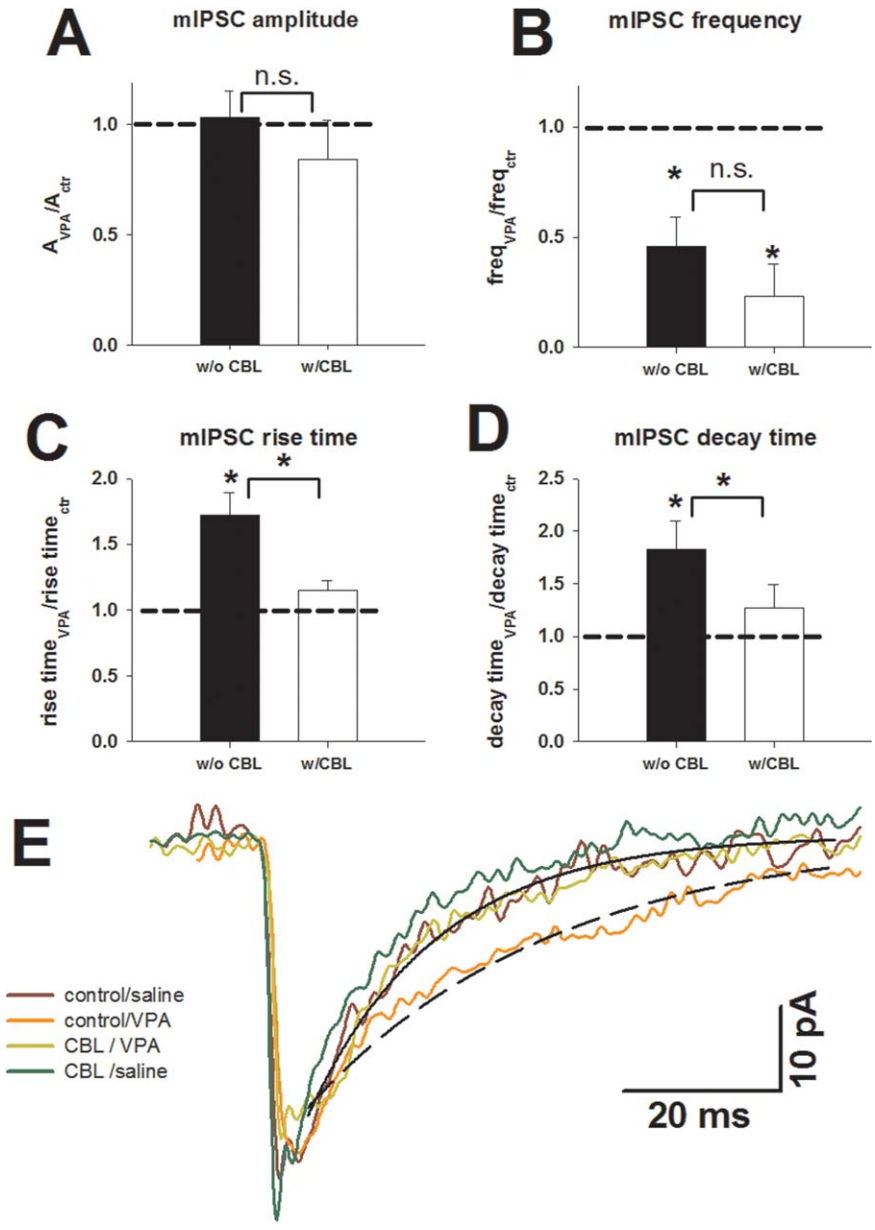

Fig. 8. Effect of CBL on action potential-independent GABA release. A: VPA offspring displays similar mIPSC amplitude as control, and CBL does not change their ratio (A_{VPA}/A_{ctr}). B: VPA offspring has a lower mIPSC frequency compared to control. CBL treatment does not change their ratio ($freq_{VPA}/freq_{ctr}$). C: VPA offspring has slower mIPSC rise-time and (D) decay-time. CBL treatment recovers both. Legend: w/o CBL: untreated animals; w/CBL: CBL-treated animals. The asterisk (*) or "n.s." on top of the bars indicates a statistical difference (or no difference, respectively) between VPA vs. saline offspring. The same symbols on the line between the bars within each graph indicate a difference (or no difference, respectively) between CBL-treated vs. untreated animals. E: Cerebrolysin recovers mIPSC kinetics: the 4 traces are the average of 10 mIPSC recorded in the presence of TTX (1 μM) in each of the conditions indicated in the captions (saline-control, saline-VPA, CBL-control, CBL-VPA). CBL treatment prevents the increase in rise- and decay-times present in VPA offspring.

they provide no explanation on the cellular mechanisms involved in the amelioration of the ASD symptoms. In spite of our lack of knowledge on the details of the neuroanatomy and pathophysiology of neural circuits involved in ASD, cellular advances have emerged in the last few decades from ASD clinics and animal models, indicating that the basic synaptic mechanisms, including the control of the inhibitory/excitatory synaptic homeostasis/equilibrium, are likely to be altered in ASD. Among such studies (including our own), previous animal-model work has shown severe impairment in GABAergic function of ASD patients and ASD models.

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In particular, our previous study (Banerjee et al., 2013) suggests that VPA offspring GABAergic synapses are deficient both presynaptically (VPA animals show impaired presynaptic modulation by NE and by the muscarinic agonist oxotremorine and a lower mIPSC frequency) and postsynaptically (VPA animals have greatly reduced I-O GABAergic saturation currents and longer rise- and decay-times).

In the present study we showed that CBL treatment prevents almost all of the previously identified GABAergic deficits in VPA offspring, including the decrease in saturation and slope of I/O curves—which is a measure of the overall effectiveness of GABAergic transmission—as well as the adrenergic and muscarinic modulation and the slowing of the kinetics of mIPSCs. These data suggest that CBL treatment acts both presynaptically (preventing the failure of presynaptic modulation) as well as postsynaptically (preventing the alteration in saturation current and rise and decay times) on inhibitory transmission, at least in the rat VPA ASD model. The failure of CBL treatment to prevent the mIPSC frequency decrease indicates that CBL reverts some but not all VPA-induced detrimental synaptic effects.

An open question is that of the mechanisms of action of the treatment. CBL is an extract from the porcine brain that has been recently characterized biochemically as mixture of aminoacids and peptides including a number of 7-15 residue-long peptides originating from myelin basic protein, a kinase catalytic unit, tubulin, and actin segments, as well as from other uncharacterized cytosolic proteins (Gevaert et al., 2015). The mixture has been successfully used in Europe over the last 30 years for the treatment of a variety of neurological disorders with a strong component of synaptic impairment like cerebral stroke (Ziganshina et al., 2010; Ziganshina and Abakumova, 2015), traumatic brain injury (Alvarez et al., 2003; Bornstein and Poon, 2012) and Alzheimer disease (Plosker and Gauthier, 2009, 2010; Allegri and Guekht, 2012; Vazquez-Roque et al., 2012). While our study does not explain the molecular details and cellular mechanisms of action of the CBL treatment, in view of the effects of CBL revealed by many decades of its clinical use, the beneficial effects of CBL treatment are not completely surprising.

CBL induces synaptic growth in animal models (Juárez et al., 2011; Vazquez-Roque et al., 2012) and has been proposed to induce synaptic (re-)growth (Flores and Atzori, 2014). It is possible that at least one component of CBL crosses the blood brain barrier preventing cellular and behavioral deficits by acting at the synaptic level. As alternative or additional hypotheses on how CBL produces its beneficial effects, we speculate that CBL may change the permeability of the blood brain barrier allowing one or more beneficial endogenous molecules to reach a central target unreachable before CBL treatment, or that the peptide mixture may exert an anti-inflammatory effect that, directly or indirectly, recovers the out-of-balance factor critical in the etiology of the ASD symptoms.

CONCLUSION

Given the virtual absence of side effects in the 30 years of clinical use of CBL in Europe, and that in spite of the lack of a thorough knowledge of CBL mechanism of action only a handful of drugs are effective in the neuropsychiatric pharmacopeia function through well-understood cellular and molecular mechanisms, our results suggest that CBL may be a promising pharmacological tool for the treatment of ASD patients. Further studies are still needed to determine the precise mechanisms of action and duration of CBL therapeutic effects in the treatment of neurodevelopmental psychiatric illness. AQ2

ACKNOWLEDGEMENTS

Associate Editor: Dr. Cristina Ghiani, Developmental Neuroscience and Dev. Disorders.

CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest.

ROLE OF AUTHORS

Roberto Cuevas-Olguin and Swagata Roychowdhury contributed equally to this work. All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: M.A., G.F. and M.K., acquisition of behavioral data and their analysis: R.C.O., S.R., A.B., F.G.O., M.E.B., electrophysiology experiments and analysis: R.C.O., S.R., A.B., F.G.O., E.E.R. All authors contributed to data analysis and interpretation. Drafting of the manuscript, critical revision of the manuscript for important intellectual content: M.A., G.F., and M.P.K. Statistical analysis: R.C.O., M.A., S.R. Study supervision and funding: M.A., G.F., and M.P.K.

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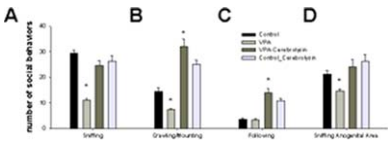

Cerebrolysin is a peptidic mixture successfully used worldwide during the last 30 years in the treatment of acute neurological damage. Administration of cerebrolysin to a rodent model of autistic spectrum disorder improves behavioral symptoms (in the graphs) as well as several physiological deficits associated with the autism model.

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