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Supplementary materials

Title:
Embryonic exposure to valproic acid and neonicotinoid deteriorates the hyperpolarizing GABA shift and impairs long-term potentiation of excitatory transmission in the local circuit of intermediate medial mesopallium of chick telencephalon

Running title:
Environmental toxicants impair hyperpolarizing GABA shift and LTP in chick pallium

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Dataset
The dataset (raw data in excel format and the RStudio codes in html files) are available at the repository site of the Hokkaido University (HUSCAP)
http://hdl.handle.net/2115/93040

RNA-seq data generated in this study have been deposited to DDBJ Sequence Read Archive (BioProject accession # PRJDB18759) and are publicly available as of the date of publication.

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Statistical computations of the data shown in text figures

Fig. 1C DFgaba (driving force of GABA, V_{rev} - V_{rest})

ANOVA (E18, E20 and P1)

	Sum Sq	Df	F value	Pr(>F)
Treatment	219.3	2	6.7583	0.002491 **
Residuals	4600.6	51		

Multiple comparisons by Dunnett (control, VPA, IMI and tubocurarine)

Fit: aov(formula = DFgaba ~ treatment, data = dataset_GABA_reversal_merged_P1), Linear Hypotheses

	Estimate	Std. Error	t value	Pr(>|t|)
1_VPA - 02_control_P1 == 0	6.180	2.002	3.086	0.00723 **
2_IMI - 02_control_P1 == 0	5.432	2.412	2.252	0.06912 .
3_tubocurarine - 02_control_P1 == 0	-2.600	2.752	-0.945	0.67318

Fig. 2C Effects of bumetanide and VU0463271

Left: one-sample t-test on ΔVrev

control + VU0426371 (data: delta_Vrev_VU)
t = 6.4293 df = 6 p-value = 0.0006692
VPA+vehicle (data: delta_Vrev_vehicle)
t = -0.27735 df = 7 p-value = 0.7895
VPA+bumetanide (data: delta_Vrev_bumetanide)
t = -3.9659 df = 9 p-value = 0.003275

Right: one-sample t-test on ΔVrest

control + VU0426371 (data: delta_Vrest_VU)
t = -0.52759 df = 6 p-value = 0.6167
VPA+vehicle (data: delta_Vrest_vehicle)
t = -0.74067 df = 7 p-value = 0.483
VPA+bumetanide (data: delta_Vrest_bumetanide)
t = -0.22423 df = 9 p-value = 0.8276

Fig. 3 Field potential recordings of LTP and fEPSP/presynaptic volley ratio

Fig.3D LTP is explained by fEPSP/presynaptic volley ratio and treatments

Anova Table (Type II tests)

Response: LTP	Sum Sq	Df	F value	Pr(>F)
group	0.07890	3	1.4954	0.2312995
fEPSP	0.18719	1	10.6436	0.0023378 **
group:fEPSP	0.47743	3	9.0488	0.0001193 ***
Residuals	0.66831	38		

Linear regression with interactions between treatment and fEPSP/presynaptic volley ratio

Call: lm(formula = LTP ~ treatment * fEPSP, data = dataset_LTP_fEPSP_2)

Coefficients:	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	1.70645	0.13302	12.828	2.20e-15 ***
VPA	-0.58511	0.19940	-2.934	0.005641 **
IMI	-0.74597	0.17426	-4.281	0.000122 ***
tubocurarine	0.15785	0.22502	0.701	0.487271
fEPSP	2.1619	0.48941	-4.417	8.03e-05 ***
VPA:fEPSP	1.95353	0.56788	3.440	0.001427 **
IMI:fEPSP	2.16731	0.54708	3.962	0.000316 ***
tubocurarine:fEPSP	0.04122	0.70205	0.059	0.953484

Fig.3E Multiple comparisons of LTP by Dunnett

Fit: aov(formula = LTP ~ treatment, data = dataset_LTP_2)

Linear Hypotheses:	Estimate	Std. Error	t value	Pr(>|t|)
tubocurarine_control	0.02685	0.07130	-0.377	0.965
VPA - control	-0.12620	0.07130	-1.770	0.199
IMI – control	-0.18862	0.07130	-2.646	0.030 *

Fig.3E Pairwise comparison of LTP between control vs control+VU0463271

F test to compare two variances, data: LTP by treatment

F = 12.514	num df = 11	denom df = 10	p-value = 0.0003962

Welch Two Sample t-test, data: LTP by treatment

t = 1.4853	df = 12.894	p-value = 0.1615

Fig.3E Pairwise comparison of LTP between VPA vs VPA+bumetanide

F test to compare two variances, data: LTP by treatment

F = 0.43281	num df = 11	denom df = 10	p-value = 0.1857

Welch Two Sample t-test, data: LTP by treatment

t = -2.1297	df = 17.067	p-value = 0.04804

Fig.3F Multiple comparisons of fEPSP/presynaptic volley ratio by Dunett

Fit: aov(formula = fEPSP ~ treatment, data = dataset_LTP_fEPSP_2)

Linear Hypotheses:	Estimate	Std. Error	t value	Pr(>|t|)
VPA_control	0.23799	0.05075	4.689	< 0.001 ***
IMI_control	0.16699	0.05323	3.137	0.00867 **
tubocurarine_control	0.09215	0.05075	1.816	0.18501

Fig.3F Pairwise comparison of fEPSP/presynaptic volley ratio between control vs control+VU0463271

F test to compare two variances, data: fEPSP by treatment

F = 0.32145	num df = 11	denom df = 10	p-value = 0.07601

Welch Two Sample t-test, data: fEPSP by treatment

t = -4.1093	df = 15.535	p-value = 0.0008679

Fig.3F Pairwise comparison of fEPSP/presynaptic volley ratio between VPA vs VPA+bumetanide

F test to compare two variances, data: fEPSP by treatment

F = 1.177	num df = 11	denom df = 10	p-value = 0.8048

Welch Two Sample t-test, data: fEPSP by treatment

t = 2.6578	df = 20.998	p-value = 0.01472

Fig. 4 Gene expressions

Fig. 4B Comparisons of slc12a2/GAPDH and slc12a5/GAPDH among 3 developmental stages in 3 brain regions
Left: slc12a2/GAPDH

Anova Table (Type II tests)

	Sum Sq	Df	F value	Pr(>F)
stage	1.5124e-04	2	12.8600	6.106e-05 ***
region	1.6317e-04	2	13.8742	3.412e-05 ***
stage:region	6.4428e-05	4	2.7391	0.04354 *
Residuals	2.1169e-04	36		

Right: slc12a5/GAPDH

Anova Table (Type II tests)

	Sum Sq	Df	F value	Pr(>F)
stage	0.0077430	2	70.584	3.488e-13 ***
region	0.0019333	2	17.624	4.609e-06 ***
stage:region	0.0009829	4	4.480	0.004852 **
Residuals	0.0019746	36		

Fig. 4C Comparisons of slc12a2/GAPDH and slc12a5/GAPDH between 2 groups (VPA and control) in 3 brain regions
Left: slc12a2/GAPDH

Anova Table (Type II tests)

	Sum Sq	Df	F value	Pr(>F)
group	0.000980	1	1.7837	0.1917
region	0.041992	2	38.2346	5.604e-09 ***
group:region	0.001063	2	0.9683	0.3913
Residuals	0.016474	30		

Right: slc12a5/GAPDH

Anova Table (Type II tests)

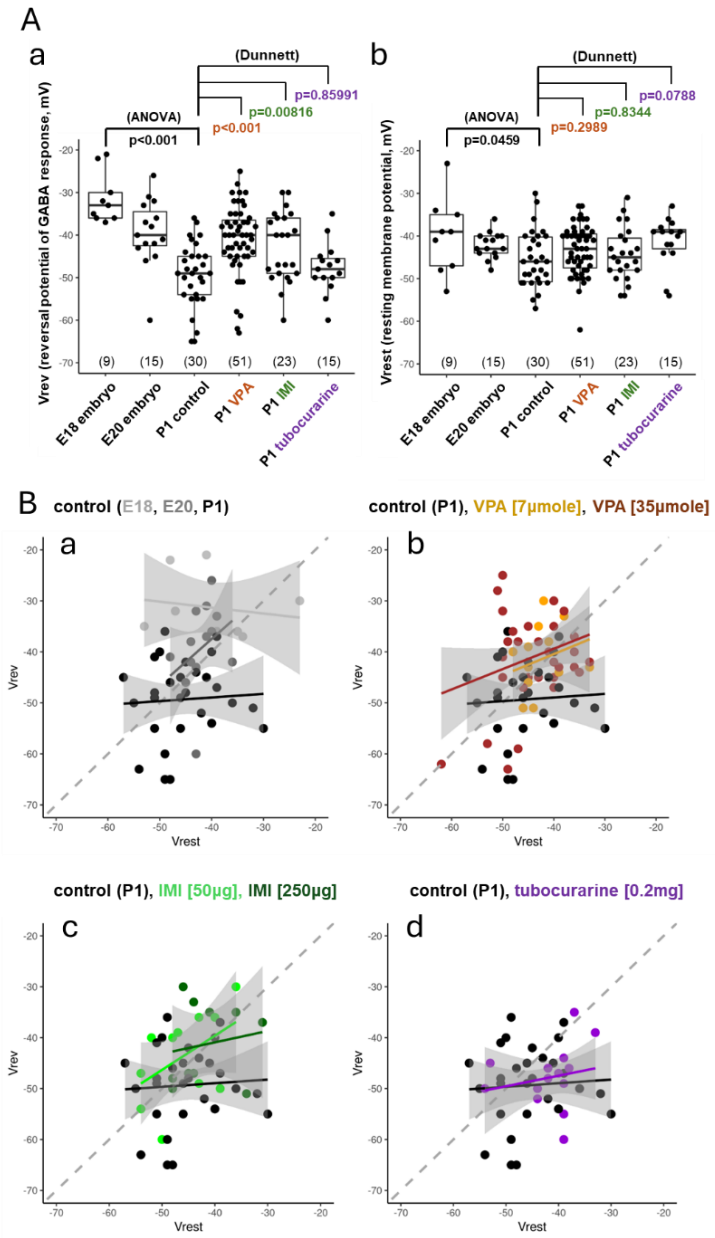
	Sum Sq	Df	F value	Pr(>F)
group	0.001481	1	2.6375	0.1148
region	0.043029	2	38.3219	5.467e-09 ***
groupt:region	0.002099	2	1.8691	0.1718
Residuals	0.016843	30		

Supplementary figures and tables

Fig. S1

A: Comparisons of V_{rev} (GABA reversal potential) **(a)** and V_{rest} (resting membrane potential) **(b)** among groups. A rapid hyperpolarizing shift occurred in V_{rev} during four days of the pre-/neonatal period. One-way ANOVA was applied to the three control groups (E18, E20, and P1), and multiple comparisons using Dunnett's method to the P1 groups (VPA, IMI, and tubocurarine against control as the reference). Numbers in parentheses indicate the number of recorded neurons.

B: V_{rev} was plotted against V_{rest} for the developmental changes (**a**; E18, E20 and P1), the effect of VPA of two doses (**b**; 7 and 35 µmole per egg), IMI of two doses (**c**; 50 and 250 µg per egg), tubocurarine (**d**; tubocurarine 0.2mg per egg). Same set of the control P1 data were duplicated in **a**-**d**. V_{rest} values considerably overlapped among groups. On V_{rev}, linear fitting analyses using *lm()* function was performed as shown in the following page.



Aa One-way ANOVA on Vrev (control; E18, E20 and P1)

Anova Table (Type II tests)

Response: Vrev	Sum Sq	Df	F value	Pr(>F)
treatment	2596.6	2	23.181	6.904e-08 ***
Residuals	2856.4	51		

Aa Dunnett multiple comparisons on Vrev (control, VPA, IMI and tubocurarine)

Fit: aov(formula = Vrev ~ treatment, data = dataset_GABA_reversal_merged_P1)

Linear Hypotheses:	Estimate	Std. Error	t value	Pr(>|t|)	
1_VPA – 02_control_P1 == 0	8.314	1.797		4.628	< 0.001 ***
2_IMI – 02_control_P1 == 0	6.594	2.164		3.047	0.00816 **
3_tubocurarine - 00_control_P1 == 0	1.600	2.469		0.648	0.85991

Ab On-way ANOVA on Vrest (control; E18, E20 and P1)

Anova Table (Type II tests)

Response: Vrest	Sum Sq	Df	F value	Pr(>F)
Treatment	257.84	2	3.2731	0.04599 *
Residuals	2008.76	51		

Ab Dunnett multiple comparisons on Vrest (control, VPA, IMI and tubocurarine)

Fit: aov(formula = Vrest ~ treatment, data = dataset_GABA_reversal_merged_P1)

Linear Hypotheses:	Estimate	Std. Error	t value	Pr(>|t|)	
1_VPA - 02_control_P1 == 0	2.133	1.390		1.535	0.2989
2_IMI - 02_control_P1 == 0	1.162	1.674		0.694	0.8344
3_tubocurarine - 00_control_P1 == 0	4.200	1.910		2.199	0.0788

Ba: control developmental changes

Call: lm(formula = Vrev ~ treatment, data = dataset_GABA_reversal_control)

Coefficients:	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-49.333	1.366	-36.106	< 2e-16 ***
treatment01_control_E20	9.867	2.367	4.169	0.000119 ***
treatment02_control_E18	18.000	2.844	6.328	6.25e-08 ***

Bb: VPA dose-response

Call: lm(formula = Vrev ~ treatment, data = dataset_GABA_reversal_VPA)

Coefficients:	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-49.333	1.467	-33.621	< 2e-16 ***
treatment1_VPA_7umole	7.619	2.601	2.929	0.00446 **
treatment2_VPA_35umole	8.577	1.975	4.344	4.17e-05 ***

Bc: IMI dose-response

Call: lm(formula = Vrev ~ treatment, data = dataset_GABA_reversal_IMI)

Coefficients:	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-49.333	1.437	-34.336	< 2e-16 ***
treatment3_IMI_1ppm	5.487	2.613	2.100	0.04080 *
treatment4_IMI_5ppm	8.033	2.874	2.796	0.00733 **

Bd: tubocurarine

Call: lm(formula = Vrev ~ treatment, data = dataset_GABA_reversal_tubocurarine)

Coefficients:	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-49.333	1.300	-37.953	<2e-16 ***
treatment5_tubocurarine	1.600	2.251	0.711	0.481

Fig. S2 Acute effects of blockers of chloride cotransporters, VU0463271 for KCC2 and bumetanide for NKCC1. Field EPSP responses. Pre-tetanus recording started after the acute effects of the blockers were stabilized, i.e., at 20 min or longer after the onset of infusion. The fEPSP / presynaptic volley ratio recorded in pre-tetanus (x-axis) and the subsequent changes in the fEPSP (26-30 min post-tetanus, y-axis) were plotted. Control and VPA data are reproduced from **Fig. 3D**. Note that larger fEPSP appeared after VU463271, whereas fEPSP was smaller after VPA.

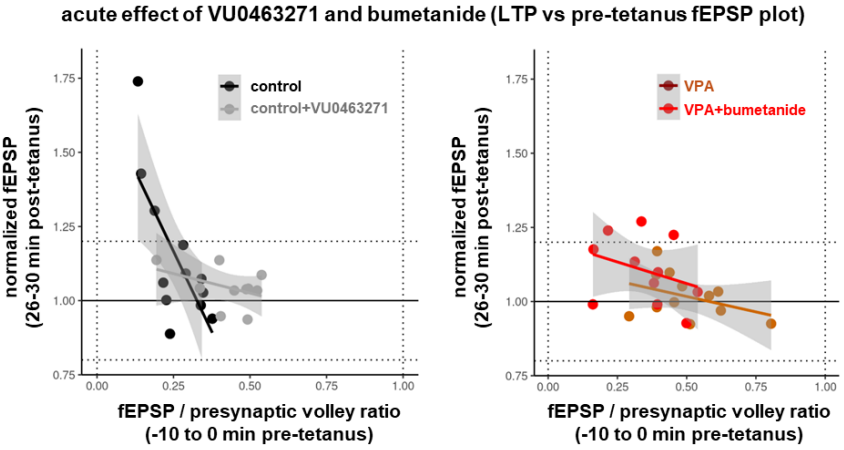


Table S1

gene	accession No.	forward	reverse
SLC12A2	XM_040655729.1	CCAGATGTGAACTGGGGATCA	TTGCCTCCGAGGACCCATAT
SLC12A5	XM_040688297.1	GGTCTTCCTTTGCTGCTCCT	GGGAAGATGTAGGCCAGCAG
GAPDH	NM_204305.2	GCAGATGCAGGTGCTGAGTA	GACACCCATCACAAACATGG

Table S2 RNA-seq data of IMM after embryonic exposure to VPA and IMI. Those genes with FDR (false discovery rate) <0.1 were shown. Those genes indicated in red (and blue) were similarly upregulated (and downregulated) in both of the VPA and IMI. Genes are ordered according to the ratio to the control. Genes labelled as LOCxxxx represent uncharacterized genes.

gene name	NCBI symbol	VPA	IMI
WIPF3-like	LOC107055396	7.47	10.09
LOC124417499	LOC124417499	7.41	9.03
ERVK-10-like	LOC121107320	7.38	7.02
RN7SL1	RN7SL1	5.37	4.44
LOC121113304	LOC121113304	2.53	3.29
SNORA73	LOC112530237	6.79	0.00
KCNQ1	KCNQ1	6.64	0.00
LOC124418341	LOC124418341	6.50	0.00
LOC101748814	LOC101748814	4.64	0.00
QRFPR	QRFPR	4.04	0.00
TMEM233	TMEM233	3.97	0.00
HBA1	HBA1	1.69	0.00
LOC121106571	LOC121106571	0.00	6.78
PHF7-like	LOC112533357	0.00	6.58
CDCA8-like1	LOC107057183	0.00	5.99
LOC121111083	LOC121111083	0.00	5.97
LOC121113405	LOC121113405	0.00	5.92
LOC124417295	LOC124417295	0.00	5.03
LOC770574	LOC770574	0.00	4.03
LOC107054402	LOC107054402	0.00	3.47
ODF3-like	LOC107050025	0.00	2.98
LOC121109595	LOC121109595	0.00	2.85
LOC107053944	LOC107053944	0.00	2.67
LOC107052086	LOC107052086	0.00	2.60
LOC771161	LOC771161	0.00	2.29
LOC107049018	LOC107049018	0.00	2.20
LOC107049870	LOC107049870	0.00	2.17

AASDH	AASDH	0.00	1.83
OLIG2	OLIG2	0.00	-1.59
CEBPD	CEBPD	0.00	-1.61
RASL11A	RASL11A	0.00	-1.98
CYR61	CYR61	0.00	-2.01
DOK1	DOK1	0.00	-2.07
NR4A1	NR4A1	0.00	-2.19
FOS	FOS	0.00	-2.26
DDIT4	DDIT4	0.00	-2.30
IL1B	IL1B	0.00	-2.40
TAC1	TAC1	0.00	-2.56
THRSPB	THRSPB	0.00	-2.86
MHCYL	MHCYL	0.00	-4.47
CLDN1	CLDN1	0.00	-6.68
LOC107054815	LOC107054815	0.00	-6.71
CDCA8	LOC121107214	0.00	-8.40
LOC112532278	LOC112532278	0.00	-8.95
NR4A2	NR4A2	-1.75	0.00
CDCA8-like2	LOC107053791	-2.24	0.00
DDC	DDC	-3.40	0.00
CAPG	CAPG	-4.86	0.00
LOC121111356	LOC121111356	-6.90	0.00
CDCA8-like3	LOC107057197	-8.35	0.00
PPP1RL	PPP1RL	-11.25	0.00
MHCY2B7	MHCY2B7	-7.82	-5.03
LOC121106939	LOC121106939	-5.42	-8.03
LOC107049675	LOC107049675	-8.50	-8.48
LOC124417862	LOC124417862	-11.1	-11.05