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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
Author(s)	Matsushima, Toshiya; Toji, Noriyuki; Wada, Kazuhiro et al.
Citation	Cerebral Cortex, 35(2), bhaf044 https://doi.org/10.1093/cercor/bhaf044
Issue Date	2025-03-02
Doc URL	https://hdl.handle.net/2115/97429
Rights	This is a pre-copyedited, author-produced version of an article accepted for publication in Cerebral Cortex following peer review. The version of record Risa Toshiya Matsushima, Noriyuki Toji, Kazuhiro Wada, Hiroki Shikanai, and Takeshi Izumi, 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, Cerebral Cortex, Volume 35, Issue 2, February 2025, bhaf044 is available online at: https://doi.org/10.1093/cercor/bhaf044 .
Type	journal article
File Information	cerebral cortex 2025.pdf



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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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Abstract

Embryonic exposure to valproic acid (VPA) and imidacloprid (IMI, a neonicotinoid insecticide) impairs filial imprinting in hatchlings, and the deteriorating effects of VPA are mitigated by post-hatch injection of bumetanide, a blocker of the chloride intruder NKCC1. Here, we report that these exposures depolarized the reversal potential of local GABAergic transmission in the neurons of the intermediate medial mesopallium (IMM), the pallial region critical for imprinting. Furthermore, exposure increased field excitatory post-synaptic potentials in pre-tetanus recordings (fEPSPs) and impaired long-term potentiation by low-frequency tetanic stimulation (LTP). Bath-applied bumetanide rescued the impaired LTP in the VPA slices, whereas VU0463271, a blocker of the chloride extruder KCC2, suppressed LTP in the control slices, suggesting that hyperpolarizing GABA action is necessary for the potentiation of excitatory synaptic transmission. Whereas a steep increase in the gene expression of KCC2 appeared compared to NKCC1 during the peri-hatch development, significant differences were not found between VPA and control post-hatch chicks in these genes. Instead, both VPA and IMI downregulated several transcriptional regulators (FOS, NR4A1, and NR4A2) and upregulated the RNA component of signal recognition particles (RN7SL1). Despite different chemical actions, VPA and IMI could cause common neuronal effects that lead to impaired imprinting.

Key words:

autism spectrum disorder; hyperpolarizing GABA shift; long-term potentiation; neonicotinoid; valproic acid

Introduction

Chemicals such as valproic acid (VPA) and neonicotinoid insecticides have been studied as potential environmental risk factors for developmental disorders, including autism spectrum disorder (ASD) when exposed during pregnancy (Moore et al. 2000, Rasalam et al. 2005, Bromley et al. 2013, Christensen et al. 2013 for VPA; Keil et al. 2014; Gunier et al. 2017; von Ehrenstein et al. 2019; Ongono et al. 2020 for neonicotinoids). Accordingly, in model animals, embryonic exposure to these agents impairs post-natal development of social behaviors in rodents (Meador and Loring 2013, Mabunga et al. 2015, Ergaz et al. 2016, Sano et al. 2016, Nicolini and Fahnstock 2018, Chaliha et al. 2020) and domestic chicks (Nishigori et al. 2013, Sgadò et al. 2018, Lorenzi et al. 2019, Adiletta et al. 2021, Matsushima et al. 2022). VPA acts as an inhibitor of histone deacetylase (Phiel et al. 2001), whereas neonicotinoids such as imidacloprid (IMI) are potent agonists of the nicotinic acetylcholine receptor (nAChR) (Costas-Ferreira and Faro 2021). How do these agents with different chemical actions cause similar behavioral maldevelopment?

Despite diverse and heterogeneous genetic and environmental risk factors, ASD phenotypes can arise by an excessive neuronal excitation (increased ratio of excitation / inhibition, or E-I imbalance) in cortical and subcortical networks for processing perception, memory, and emotions (Rubenstein and Merzenich 2003, Nelson and Valakh 2015). Bumetanide, a blocker of NKCC1 (Na-K-2Cl cotransporter 1) appeared to produce positive outcomes through neuroprotective GABA shift in ASD cases (Lemonnier et al. 2012, 2017, Hadjikhani et al. 2018, Fernell et al. 2021, Wang et al. 2021; for reviews see Ben-Ari 2017, Savardi et al. 2021, Delpire and Ben-Ari 2022). Although some phase-2 trials failed to support this efficiency (Sprengers et al. 2021, van Andel et al., 2023), a recent meta-analysis by Xiao et

al. (2024) indicates that bumetanide had significant positive outcomes in specific subgroups composed of younger age (3-6 years old) measured by CARS (Childhood Autism Rating Scale; Schopler et al. 1980). The possible efficacy of bumetanide treatment has also been suggested in a mouse model of fetal alcohol spectrum disorders (FASD; Skorpud et al. 2019). It is to be noticed that bumetanide also rescued pathological deficits in the *APOE4*-related Alzheimer model mouse (Taubes et al. 2021), where transcriptomic signature of disease was reversed by chronic application, although significant changes were not found in the gene expression of these chloride cotransporters. Bumetanide could thus cause beneficial effects via other mechanisms than the canonically assumed direct effects on the neuronal GABA transmission (Löscher & Kaila 2022).

E-I imbalance can produce behavioral impairments also in hatchlings of precocial birds. Specifically, when applied to newly hatched chicks, bumetanide rescued imprinting deterioration caused by embryonic exposure to VPA (Matsushima et al. 2022). Newly hatched chicks could be a valid animal model to study ASD, because they are innately predisposed to pay visual attention to animate objects and face-like configuration (Vallortigara et al. 2005, Miura and Matsushima 2012, 2016, Miura et al. 2020 for biological motion; Rosa-Salva et al. 2010, 2011, 2019 for face-like configuration) as of human newborns (Johnson 1992, Johnson 2005, Simion et al. 2008, Pavlova 2012, Pavlova et al. 2017); for recent reviews, see Matsushima et al. 2024, Sgadò et al. 2024, Kobylkov and Vallortigara 2024. Successful filial imprinting is associated with an enhanced inflow of thyroid hormones (triiodothyronine, T_3) into the dorsal pallial network via expression of Dio2 enzyme in epithelial cells of capillaries, thus elongating the sensitive period of imprinting (Yamaguchi et al. 2012). Furthermore, the higher the T_3 level, the more disposed the chicks are to social objects bearing vivid motion animacy (Takemura et al. 2018, Miura et al. 2018, Lorenzi et al. 2021). Finally, bath-applied T_3 enhances GABAergic transmission in *in vitro* slice preparations of the hatchling's telencephalon (Saheki et al. 2022), suggesting a critical role of the synaptic inhibition for social attachment formation. However, because of the unfavorable CNS pharmacokinetics (Löscher and Kaila 2022) of bumetanide as well as the abundance of non-neuronal targets within and outside the brain (Kurki et al. 2023), systemically applied bumetanide might cause positive behavioral effects indirectly, acting on peripheral hormonal tissues and the brain capillaries. It is therefore important to examine if bumetanide directly acts on CNS neurons.

Given the critical importance of GABA transmission during the early stages of development (Ben-Ari et al. 2012, Ben-Ari 2017), how does this appear throughout postnatal life? Compensatory mechanisms have been assumed to contribute to the reorganization of neural circuits (Nelson and Valakh 2015, Gilbert and Man 2017), yet it remains unknown why these mechanisms fail in ASD cases. To address this issue, we examined how embryonic exposure to these neurodevelopmental toxicants alters synaptic function in the intermediate medial mesopallium (IMM), which is the brain region responsible for imprinting (Horn 1985, Horn 1998, Horn 2004). In addition to the biochemical, morphological, and neurophysiological changes in the IMM associated with imprinting (Horn et al., 1985, McCabe and Horn 1994, Horn et al. 2001, Solomon et al. 2003), local excitatory synaptic transmission shows long-term potentiation (LTP) by low-frequency tetanic stimulation (Bradley et al. 1991, Bradley et al. 1999). Notably, LTP is suppressed when GABAergic transmission is blocked by bicuculline (Yanagihara et al. 1998), suggesting that plastic changes in excitatory synapses require GABA action. However, when the post-synaptic neuron is hyperpolarized, the same tetanic stimulation

instead induces a long-term depression (Matsushima and Aoki 1995), indicating bidirectional Hebbian nature of the plastic changes. Here, we report a strong association among the hyperpolarizing GABA shift, synaptic excitation, and LTP in the emergence of ASD-like phenotypes following embryonic exposure to environmental toxicants in domestic chicks. In particular, we examined the effects of blockers of chloride cotransporters (bumetanide for NKCC1 and VU0643271 for KCC2, K-Cl cotransporter 2 as chloride extruder) on LTP. Although the subcellular mechanisms are yet to be elucidated, the hyperpolarizing GABA response could be critical for the synaptic plasticity.

Materials and Methods

Compliance with ethical standards

The animal experiments were conducted according to the guidelines of the Committee on Animal Experiments of the Health Science University of Hokkaido (approval number #24-042). These guidelines are based on the national regulations for animal welfare in Japan (Law for Humane Treatment and Management of Animals after Partial Amendment No. 68, 2005).

Animals

Domestic white chickens of a White Leghorn strain (*Gallus gallus domesticus*, locally referred to as “Julia Light”) were used. Fertilized eggs were purchased from Iwamura Point Co. (Yuni, Hokkaido, Japan). Eggs were incubated in the laboratory using type P-008B incubators (Showa Furanki Co., Saitama, Japan) at controlled temperature (37.7 °C) and humidity (ca. 80%); inside of the incubator was kept in complete darkness until hatch. Chicks usually hatch after 21 days of incubation, and the hatching day (E21, embryonic 21 days) is denoted as P0 (post-hatch 0 day) in this report. All subjects were sexed except those used before hatching.

Embryonic exposures to risk chemicals

On the embryonic 14 days (E14), eggs received a single injection of 200 µL aqueous solution of chemicals to the air sac chamber through a hole on the round edge of the shell. For experimental treatments, eggs received valproic acid (VPA, 1.16 to 5.82 mg, or 7 to 35 µmole), imidacloprid (IMI, 50 to 250 µg, or 0.2 to 1.0 µmole), or D-tubocurarine chloride pentahydrate (tubocurarine, 0.2 mg, or 0.26 µmole) per egg weighing ca. 50 g. The dosages adopted in the present study were reported to be effective in behavioral analyses (Matsushima et al. 2022). Chemicals were purchased from FUJIFILM Wako Pure Chemical Co. Control eggs received a 200 µL distilled water or no injection.

Slice preparation

The chicks were housed individually in a dark incubator without light exposure until the experiment. Under anesthesia by intramuscular injection of 0.4 mL of a 1:1 mixture of ketamine (10 mg/mL, Daiichi-Sankyo Pharma Co., Tokyo, Japan) and xylazine (2 mg/mL, Sigma-Aldrich Co., St Louis, USA), the left hemisphere was dissected and immersed in ice-chilled Krebs solution composed of (in mM); NaCl 113.0, NaHCO₃ 25.0, KCl 4.7, KH₂PO₄ 1.2, CaCl₂ 2.5, MgSO₄ 1.2, glucose 11.1; pH was adjusted to 7.2-7.4 by bubbling with 95% O₂ - 5% CO₂. The left IMM was analyzed because of the functional and morphological asymmetry involved in imprinting memory formation (Horn 1985); specifically, successful training significantly increased the length of the post-synaptic density in spine synapses only in the left IMM (Horn et

al. 1985). By using a vibrating micro-slicer (type ZERO-1, Dosaka EM Co., Kyoto, Japan), four 500 μm thick consecutive slices were cut on pseudo-frontal plane roughly corresponding to A9.8 to A8.0 of the chick brain atlas by Kuenzel and Masson (1988) (Fig. 4A). Slices were stored in an interface-type recovery chamber at room temperature (26-27 $^{\circ}\text{C}$) for 1-6 hours. For recording, the slice was placed in a submersion-type chamber (volume ca. 1 mL) that was continuously perfused with Krebs solution held at 29-31 $^{\circ}\text{C}$ at a flow rate of 1.5-2.0 mL per min. The following drugs were applied to the perfusing solution: 6,7-dinitroquinoxaline-2,3-dione (DNQX, 10 μM , Tocris Co.), 1(S),9(R)-(-)-bicuculline methiodide (bicuculline, 10-20 μM , Sigma-Aldrich Co.), VU0463271 (1-10 μM , Tocris Co.), bumetanide (20 $\mu\text{g/mL}$ or 55 μM , Tocris Co., in vehicle containing 0.005N NaOH).

Electrical stimulation and whole-cell intracellular recording

Electrical stimulation was delivered via a concentric metal micro-electrode (O.D. 100 μm , USK-10, Unique Medical Co., Tokyo, Japan) inserted into the slice tissue, with the cathodal center. Constant current (60-200 μA , duration = 100-400 μsec) was supplied by an isolation unit and pulse-generator (SS-203J and SEN-8203, Nihon Kodon Co., Tokyo, Japan) at constant intervals (2 or 10 sec). Intracellular whole-cell recordings were performed using nystatin perforation patches. Patch glass pipettes were filled with a solution containing (in mM): K-gluconate 123.0, KCl 18.0, NaCl 9.0, MgCl_2 1.0, EGTA 0.2, and HEPES 10.0; the pH was adjusted to 7.3 with KOH. DC-resistance ranged in 5-8 M Ω . Nystatin (Sigma-Aldrich Co., St Louis, USA) was dissolved in DMSO at 50 mg/mL, and 1-5 μL was added to 1 mL of the pipette solution just before backfilling the glass capillaries.

Two types of amplifiers were used. A single-electrode discontinuous voltage clamp amplifier (SEVC, CEZ-3100, Nihon Kodon Co., Tokyo, Japan; switching at 20 kHz) and a conventional voltage clamp amplifier (VC, Axopatch 200B, Molecular Devices Co., California, USA). For SEVC recordings, the bridge balance and electrode capacitance compensation were adjusted. For the VC recordings, capacitance and series resistance compensation were not necessary for recording synaptic currents, thus were turned off. The data obtained by these two methods were merged because the similar signals were recorded except for the noise level (VC yielded lower noise than SEVC). The voltage and current records were low-pass filtered at 2 kHz, digitized, and stored using the CED Power 1401 interface and Signal ver.6.02 (digitized at 20 kHz for 25-50 msec per trace; Cambridge Electronic Design Ltd., Cambridge, UK) on a Windows PC. Under microscopic observation, the glass electrodes were inserted at ca. 250 μm ventral to the stimulation electrode (**Fig. 1A**), not necessarily targeting excitatory neurons. The patch recordings were made in a blinded manner, and the recorded neuron types were not identified.

Pilot experiment revealed stable recordings with no detectable changes in GABA reversal potentials up to approximately 30 min after the formation of a giga-ohm seal in control slices, indicating that the present protocol using nystatin perforation did not cause artificial changes in intracellular chloride concentration. A high-chloride pipette solution reversed the GABA current (Sakehi et al. 2022), but this method was not used in this study. In addition, as shown in the vehicle control data in **Fig. 2C** (left), no significant changes were observed in the VPA slices. Data were obtained from excitable neurons (inducible overshooting full action potentials) with initial resting membrane potentials below -30 mV at 3 min after the formation of giga-ohm seal. We discarded the initial records obtained during 10-20 min after the formation of the whole-cell patch. When the input resistance changed by more than 20% of the initial value, we discarded

all subsequently obtained data.

Recording of extracellular field potential

Extracellular field potentials were recorded through a pair of insulated nichrome microwire (O.D. 100 μm). One of the exposed tips (anode) was inserted in the slice tissue, and another (cathode) was submerged in the perfusion solution, and the electrodes were connected to a differential amplifier (MEG-5100, Nihon Koden Co., Tokyo, Japan; gain 0.1 mV/V, band-pass filter 15 Hz - 3 kHz). The cathodal wire was inserted at ca. 250 μm ventral to the stimulation electrode; when accompanied by intracellular recordings, the wire was instead placed at ca. 500 μm ventral (**Fig. 1A, 3A**). As described above, the recorded signals were digitized and stored using the CED Power 1401 and Signal software. Recordings during the initial 10-30 min were discarded until waveform drift ceased. When the amplitude of the pre-synaptic fiber volley changed by more than 10% between the pre-and post-tetanic periods, we discarded all the data obtained from the slice, including the data before the changes.

RNA-seq and qPCR analysis

Total RNA was extracted from the punched tissue using the FastGene RNA Premium Kit (FG-81050, NIPPON Genetics Co., Tokyo, Japan) and reverse-transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (4368814, Thermo Fisher Scientific Inc., Massachusetts, USA). The expression levels of individual genes were quantified by absolute quantification using KOD SYBR qPCR Mix (QKD-201, TOYOBO Inc., Tokyo, Japan). Standard curves were generated using pGEM-T Easy plasmids containing the amplified target regions. The primers used for standard plasmid construction and qPCR are listed in supplementary **Table S1**. Gene expression levels were normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which served as the internal control.

Total RNA for RNA sequencing was extracted from the punched tissue using a Monarch Total RNA Miniprep Kit (T2010S, New England Biolabs, Inc., Massachusetts, USA), and sequencing was outsourced (NIPPON Genetics Co., Tokyo, Japan). Sequencing reads were trimmed using fastp v0.20.1 and subsequently mapped to the chicken reference genome (GCF_016699485.2) using the STAR v2.3.7a-RSEM v1.3.3 pipeline for expression quantification. Differential expression analyses were performed using the edgeR package in R.

Statistical analyses

Graphics and statistical computations were performed using RStudio version 3.6.3 (2020-02-29). Conventional parametric tests were conducted. See the Results and Supplementary Materials for details on each data set.

Results

Effects on the hyperpolarizing GABA shift

Whole-cell patch recordings were successfully obtained in 164 neurons from 41 chicks (post-hatch 1 day, P1; 12-36 hours post-hatch; 15 males and 26 females) and 8 embryos (embryonic 18 and 20 days, E18 and E20; unsexed); 79 neurons by single-electrode discontinuous voltage clamp (SEVC) and 85 neurons by conventional voltage clamp (VC). As chicks usually hatch on E21 (or P0), peri-hatch recordings were made at 2 days intervals. In 143 of the 164 neurons, current responses showed a clear dependence on the holding membrane

potential (V_{holding}) around the resting membrane potential (V_{rest}), and the reversal potential (V_{rev}) was reliably determined at a precision of 1-3 mV by systematically changing V_{holding} . In the other 21 neurons, V_{rev} was not measurable either because the neuron was directly excited to fire by a low-intensity stimulation subthreshold for post-synaptic responses, or because the response was not dependent on V_{holding} .

The traces in **Fig. 1B** show examples recorded from a control P1 neuron. In normal Krebs solution (**Fig. 1Ba**), the synaptic currents were composed of an initial inward current followed by a net outward current when V_{holding} was set above V_{rest} . The outward component (presumptive GABA component; the onset indicated by vertical green lines) was reversed to a net inward component at hyperpolarized V_{holding} . The blockade of fast excitatory synaptic transmission by DNQX in the bath (**Fig. 1Bb**) unmasked the GABAergic late component with clear V_{holding} dependence. The reversal potential ($V_{\text{rev}} = -65$ mV) was determined as the V_{holding} where the earliest component of the GABA current was nullified ($I_{\text{ipsc}} = 0$ pA; horizontal red lines). We compared the difference ($V_{\text{rev}} - V_{\text{rest}}$) to represent the driving force of GABA (DFgaba, mV). Comparisons among 3 groups of unexposed control (E18, E20, and P1) revealed a hyperpolarizing shift of DFgaba; changes in V_{rev} and V_{rest} are shown in the supplementary **Fig. S1Aa,b** and statistical examinations.

These effects paralleled the behavioral effects on imprinting reported previously (Matsushima et al. 2022), namely, impairment by VPA and IMI but not by tubocurarine. DFgaba was more depolarized than control when chicks had been exposed to VPA and IMI on embryonic day 14 (E14) but not tubocurarine (**Fig. 1C**). We examined the effects of VPA and IMI in two doses (7 and 35 μmole for VPA, and 50 and 250 μg for IMI per egg); data obtained from these two dose levels were merged because of considerable overlaps (supplementary **Fig. S1B**). Multiple comparisons using Dunnett’s method revealed differences in VPA and IMI compared to the P1 control group on V_{rev} , whereas no significant effects were found on V_{rest} .

Bath applied bumetanide acutely hyperpolarized V_{rev} without changes in V_{rest} in VPA slices; **Fig. 2A** shows an example. With DNQX in the bath, V_{rev} was around V_{rest} (-43mV). When bumetanide (blocker of NKCC1; 20 $\mu\text{g/mL}$ or 55 μM) was added to the DNQX perfusate, V_{rev} was hyperpolarized by 10 mV in 15-20 min (**Fig. 2Ab**). The current component was GABAergic as it was blocked by bicuculline (**Fig. 2Ac**). **Fig. 2C** shows the population data. Changes in V_{rev} and V_{rest} (ΔV_{rev} and ΔV_{rest}) recorded at 15-25 min after the onset of bumetanide perfusion are plotted and tested by one-sample t-test against the null hypothesis of $\Delta V = 0$ mV. Note that vehicle of the bumetanide solution (pH = ca. 8.2 due to addition of NaOH) had no significant effects. Therefore, IMM neurons are candidate targets of bumetanide for the acute rescue of imprinting in the VPA exposed chicks (Matsushima et al. 2022). On the other hand, VU0463271 (blocker of KCC2; 1 μM) hyperpolarized V_{rev} in control slices, again without significant changes in V_{rest} ; **Fig. 2B** shows example traces, and **Fig. 2C** the population data. Therefore, we examined the effects of bumetanide and VU0463271, applied under the same conditions (concentration and duration of treatment), on synaptic plasticity measured by field potential recording.

Effects on the excitatory synaptic transmission and its LTP

Extracellular field potential recordings were successfully obtained from 70 slice preparations from 32 chicks (P1: 14 males and 18 males). Presynaptic fiber volleys were reliably recorded in 68 slices; in the other two slices (exposed to IMI), large stimulus artifacts obscured the pre-

synaptic volleys.

Fig. 3B illustrates how presynaptic volleys and fEPSP were measured. The stimulating and recording electrodes were placed such that a biphasic compound spike (presynaptic fiber volley) was followed by a small positive deflection (shoulder) on the decaying slope (pre-tetanus phase, 60 superimposed traces for 10 min). Low-frequency tetanic stimulation (5 Hz for 60 s, 300 pulses) was applied to induce LTP as reported previously (Yanagihara et al. 1998). Post-tetanus recordings lasted for 30 min at 10 s intervals; the superimposed traces obtained in the last 10 min (60 traces) of the post-tetanus phase are shown. The slice was subsequently perfused with the DNQX Krebs to reveal the onset (blue dashed vertical lines) and amplitude of the monosynaptic component of EPSP (red dashed vertical lines), and the base of the DNQX sensitive component (DNQX base; dashed horizontal line) was determined; average traces were enlarged and superimposed on the right. In the slice shown here, the fEPSP onset occurred 2.3 msec after the stimulus, and its amplitude was measured at 3.1 msec. The fEPSP was normalized to the pre-synaptic volley recorded during the pre-tetanus phase. The fEPSP onset ranged from 1.3 to 3.1 msec (2.26 ± 0.04 msec, mean $\pm$ s.e.m., $n=70$), and its amplitude was measured at the latency ranging from 0.3 to 1.7 msec (0.97 ± 0.03 msec, mean $\pm$ s.e.m., $n=70$).

The fEPSP amplitude was normalized by the pre-tetanus average and plotted against the time starting from -10 min (at the point after the recording was stabilized) until 30 min post-tetanus (**Fig. 3C**). The mean of six consecutive traces (at 10 s intervals, i.e., 1 min) was further averaged over the number of slice preparations (12 or 11 slices) for each time point, with s.e.m. of 72 or 66 traces as the vertical error bars. Note that inter-group differences emerged as early as 5-10 min post-tetanus and monotonically increased afterwards. If the last 5 min period (26-30 min post-tetanus) is focused, control and tubocurarine slices showed comparable LTP, whereas the slices exposed to VPA (35 μ mole per egg) and IMI (250 μ g per egg) failed to show LTP. Furthermore, LTP was resumed if the VPA slices were treated by bumetanide in the bath (55 μ M) starting at ca. 20 min before the pre-tetanus recordings (VPA+bumetanide). On the other hand, pre-treatment of control slices with VU0463271 (1 μ M) for ca. 20 min tended to suppress LTP (control+VU0463271).

The normalized amplitude of post-tetanus fEPSP during the last 5 min was averaged for each slice and compared among the groups (**Fig. 3E**). VPA and IMI slices, but not tubocurarine, failed to show LTP at the population level, although a statistically significant level appeared only in IMI according to the ANOVA test of the Dunnett's method. Direct pairwise comparisons between control vs. control+VU0463271 and VPA vs. VPA+bumetanide yielded significant differences using the F-test and t-test, respectively. It is however noticed that fEPSP was partially potentiated in control+VU0463271 (**Fig. 3C**), and the difference was detected by F-test, not by t-test of the means (**Fig. 3E**). We conclude that LTP was correlated with the GABA shift (**Fig. 1C**) at the group population level.

The fEPSP amplitude normalized by the pre-synaptic fiber volley (recorded in the pre-tetanus phase) showed a nearly negative image of LTP, where the VPA and IMI groups were higher than the control and tubocurarine groups (**Fig. 3F**). In addition, at the level of individual slices (**Fig. 3D**), a higher LTP appeared when the fEPSP/pre-synaptic volley ratio was lower, and the interaction with the group was significant. Two-way ANOVA revealed a significant main effect of fEPSP ($F=10.6436$, $Df=1$, $p=0.0002$) and interaction with the group factor ($F=9.0488$, $Df=3$, $p=0.0001$) but the main effect of the group was not significant ($F=0.14954$, $Df=3$, $p=0.2312$); for detailed statistics see supplementary materials. The VPA and IMI slices,

but not the tubocurarine slices, showed a statistically higher fEPSP/pre-synaptic volley ratio after ANOVA using Dunnett’s method (**Fig. 3F**). Direct pairwise comparisons between the control and control+VU0463271, as well as VPA vs. VPA+bumetanide, yielded significant differences by t-test. Therefore, we conclude that embryonic exposure to VPA and IMI increases DNQX-sensitive excitatory synaptic responses in a manner coupled with LTP impairment.

Effects on gene expression profiles, RNA-seq, and qPCR analyses

To search for the underlying molecular events of the deteriorated hyperpolarizing GABA shift and the LTP impairment, gene expression profiles were examined by RNA-seq using the punched tissue of the IMM studied for whole-cell recording. Slice preparations obtained from six chicks (all females, two chicks each for the control, VPA, and IMI groups) were chosen such that group-typical DF_{gaba} (V_{rest} and V_{rev}) were recorded (see the inset table in **Fig. 4A**). After whole-cell recordings were made, 1 mm-sized punched tissues were sampled around the recording sites from four consecutive slices and mixed to represent each chick. Histological reconstruction revealed that the sampling was confined mostly within the IMM (**Fig. 4A**).

Statistically different gene expressions with a false discovery rate (FDR) below 0.10 are summarized in **Fig. 4D** for VPA and IMI; full lists are shown in supplementary **Table S2**. Out of 25,467 genes (and 85,704 isoforms), small sets of 24 (for VPA) and 42 genes (for IMI) showed increased or decreased expression, respectively, compared with the control. Nine genes were overlapping between VPA and IMI. Two of these nine genes were annotated, namely upregulated RN7SL1 (RNA component of signal recognition particle 7SL1) and downregulated MHCY2B7 (major histocompatibility complex Y, class II beta 7); the rest were classified as LOC genes. Among the VPA and IMI sets, NR4A2 (VPA, down-regulated; nuclear receptor subfamily 4 group A member 2), NR4A1, and FOS (IMI, down-regulated) require particular attention, because *de novo* variants of NR4A2 are associated with several neurodevelopmental cases (Lévy et al. 2018, Singh et al. 2020, Song et al. 2022) and NR4A1 is associated with dendritic spine formation in cortical neurons (Chen et al. 2014, Li et al. 2016). However, genes coding NKCC1 and KCC2 were not found in the list of those with low FDR values.

Parallel to the developmental shift in DF_{gaba} (**Fig. 1C**), the expression of the genes encoding the chloride cotransporters (SLC12A2 for NKCC1 and SLC12A5 for KCC2 after normalization by GAPDH, **Fig. 4B**; qPCR analysis) showed a significant increase from E14 to E18 to P1. Note that the latter (KCC2) showed a much steeper increase than the former (NKCC1). Punched tissue samples were prepared from three brain regions (IMM, arcopallium, and optic tectum) without electrophysiological experiments from five chicks at each stage, and gene expression profiles were analyzed using qPCR. Two-way ANOVA revealed a significant effect of development ($p < 0.0001$) and brain region ($p < 0.0001$), with a significant interaction ($p = 0.04354$ for NKCC1 and 0.00485 for KCC2, respectively; see Supplementary for statistics).

Contrary to the developmental change, both SLC12A2 (NKCC1) and SLC12A5 (KCC2) did not significantly differ between the control and VPA groups in the qPCR dataset (**Fig. 4C**). Three brain regions were sampled, each from six control and six VPA chicks. A significant difference appeared only in the factor of brain regions in both SLC12A2 and SLC12A5 ($p < 0.0001$), but not groups nor interactions.

Interpretation of the present gene expression data needs careful consideration. Notably, the present analysis included both neurons and non-neuronal cells. NKCC1 is expressed in many cell types, whereas KCC2 is neuron-specific (Kaila et al. 2014, Watanabe and Fukuda 2015,

Löscher and Kaila 2022), so that the observed changes/differences might not be linked with the neuronal effects on DFgaba. Kurki et al. (2023) further reported that NKCC1b isoform predominates in neurons while NKCC1a in oligodendroglia and other cells in mice. Our RNA-seq analysis in chicks also revealed 4 isoforms for NKCC1 and 2 isoforms for KCC2, however, the cell-type specificity of isoforms has not been examined. Further studies are required.

Discussion

The present results revealed the cellular events associated with the perinatal development of social behavior and its disorders by environmental toxicants in domestic chicks. Embryonic exposure to VPA and IMI alters developmental landscape (Matsushima et al. 2024), causing distinct gene expression patterns in the IMM of hatchlings that are critical for imprinting. The characteristic set of events accompanied at the neuronal level, namely, deteriorated hyperpolarizing DFgaba, increased synaptic excitation, and the LTP impairment occurred in a correlated manner. In particular, pharmacological manipulation of DFgaba by bumetanide and VU0463271 appeared to regulate LTP, although the underlying causal links are not specified. The deteriorated hyperpolarizing GABA shift is supposed to reduce the efficacy of the GABA-ergic inhibitory action, thereby partly boosting the neuronal excitation. It is therefore possible that systemically applied bumetanide mitigates the impaired memory formation through direct action on the IMM neurons, while indirect action (e.g., through facilitatory thyroid hormone) cannot be disregarded. On the other hand, these events in IMM are not linked to the impaired biological motion preference caused by the IMI exposure (Matsushima et al. 2022); probably, sub-pallial visual pathways are involved.

Dysregulated synaptic plasticity has been assumed to be a critical construct in human ASD pathology (Markram & Markram 2010, Gilbert and Man 2017). However, the effects of prenatal VPA on LTP has remained to be controversial in the rodent model of ASD. Rinaldi et al. (2007) reported that VPA exposure enhanced post-synaptic form of LTP and caused hyper-reactivity and hyper-connectivity in rats. On the other hand, perinatal VPA is shown to impair plastic changes in synaptic transmission (Zhang et al. 2003, Mohammadkhani et al. 2022). The present results in the chick IMM are in accord with the latter, despite the different local circuits of the avian pallium.

How do VPA and IMI impair hyperpolarizing GABA shift?

RNA-seq and qPCR analyses quantify the mRNA levels but do not address the functional amount of the protein molecules. Protein synthesis, molecular dimerization, and/or cytoplasmic trafficking to the target membrane (Agez et al. 2017) may be impaired after embryonic exposure to risky chemicals, as might be suggested by the increase in RN7SL1 expression. Although the physiological roles of the long non-coding RNA in the normal central nervous system are still largely unknown, recent molecular analyses of post-mortem brains of patients with major depression suicides (Zhou et al. 2018) and Alzheimer's disease (Liu et al. 2024) suggest their possible involvement in neuroinflammation. Alternatively, functions of the transporters might be regulated through transcription or the downstream (such as splicing, subcellular trafficking and phosphorylation; Blaesse et al. 2009). Quantitative phosphoproteomics are needed, together with specification of the responsible cell types and sub-cellular localization.

Activity-dependent regulation of cotransporter functions might also be considered. In the present study, brain slices were sampled from inexperienced newly-hatched chicks taken from

dark incubators. Normally, however, chicks would be exposed to barrages of sensory stimuli (somatosensory, visual and auditory) at hatching. Actually, WNK1 kinase is reported to be sensitive to Cl⁻, therefore neural activity dependent (Heubl et al. 2017). Hormonal controls must be considered in this context. Beside the thyroid hormone described in Introduction, social perception of chicks is subject to oxytocin and related nonapeptides (Loveland et al. 2019). As the maternal oxytocin does at delivery in rats (Tyzio et al. 2006, 2014), these hormonal actions might have effects as they vigorously moved at hatching.

Is LTP critical for memory formation or homeostatic control of synaptic excitation?

Horn (2004) argued that the biochemical, ultrastructural, and neurophysiological events of IMM are associated with filial imprinting, sharing many features with the process of memory formation in the mammalian hippocampus (Hp). It is highly plausible to assume that the observed LTP, despite being much smaller and fluctuating than that found in mammalian Hp, is critical for some aspects of memory formation. However, this does not imply that LTP represents memory traces. Rather, as for the consolidation processes of episodic memory in the Hp, LTP in the chick IMM could be responsible for the dynamic process of storage relocation from the IMM to elsewhere (Horn 1985). Alternatively, LTP could be responsible for the homeostatic control of the synaptic excitation, as suggested in ASD (Nelson and Valakh 2015).

Do domestic chicks have pathogenetic constructs of the human ASD?

Various nuclear receptors have been suggested as key molecules responsible for neurodevelopmental disorders including ASD (Kong et al. 2020). In particular, the nuclear receptor superfamily 4 group A (NR4A1 and 2) requires attention, as NR4A2 was specified as the chick ortholog of the mammalian cortical layer 5/6 specific genes (Fujita et al. 2019). In situ hybridization revealed high levels of expression in the hyperpallium, mesopallium, and arcopallium of P1 chicks, indicating cell-type homology of the cortical/pallial architectures between birds and mammals. In human cases, haploinsufficiency (Lévy et al. 2018), heterozygous truncation (Song et al. 2022) and *de novo* mutation (Singh et al. 2020, Jesús et al. 2021) of NR4A2 have been reported to be associated with intellectual disabilities, language impairment and epilepsy. Further brain-wide developmental surveys need be conducted on chicks.

Acknowledgements:

Critical comments and instructive suggestions for the manuscript by Dr. Giorge Vallortigara (University of Trento, CIMeC) are gratefully acknowledged. The valuable instructions on the toxicology of imidacloprid and its metabolites provided by Dr. Yoshinori Ikenaka (Hokkaido University, Faculty of Veterinary Medicine) are also appreciated. We appreciate the instructive comments and suggestions for revisions by the anonymous reviewers. We also thank Editage (www.editage.com) for the English language editing.

Funding information:

This study was supported by grants funded by JSPS KAKENHI (Grants-in-Aid for Scientific Research (C) #24K06614 to T.M. and T.I, Fostering Joint International Research (B) #19KK0211 to T.M.).

Contribution of the authors:

T.M. and T.I. conceived the study. T.M. and H.S. performed neurophysiological experiments

using slice preparations. N.T. and K.W. designed and performed molecular biology studies (RNA-Seq and qPCR). T.M. performed statistical analyses. T.M., N.T., K.W., and T.I. wrote the manuscript. All authors agree to publish the paper in its final form.

Declaration of the conflict of interest:

The authors declare no conflict of interest.

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Figure legends

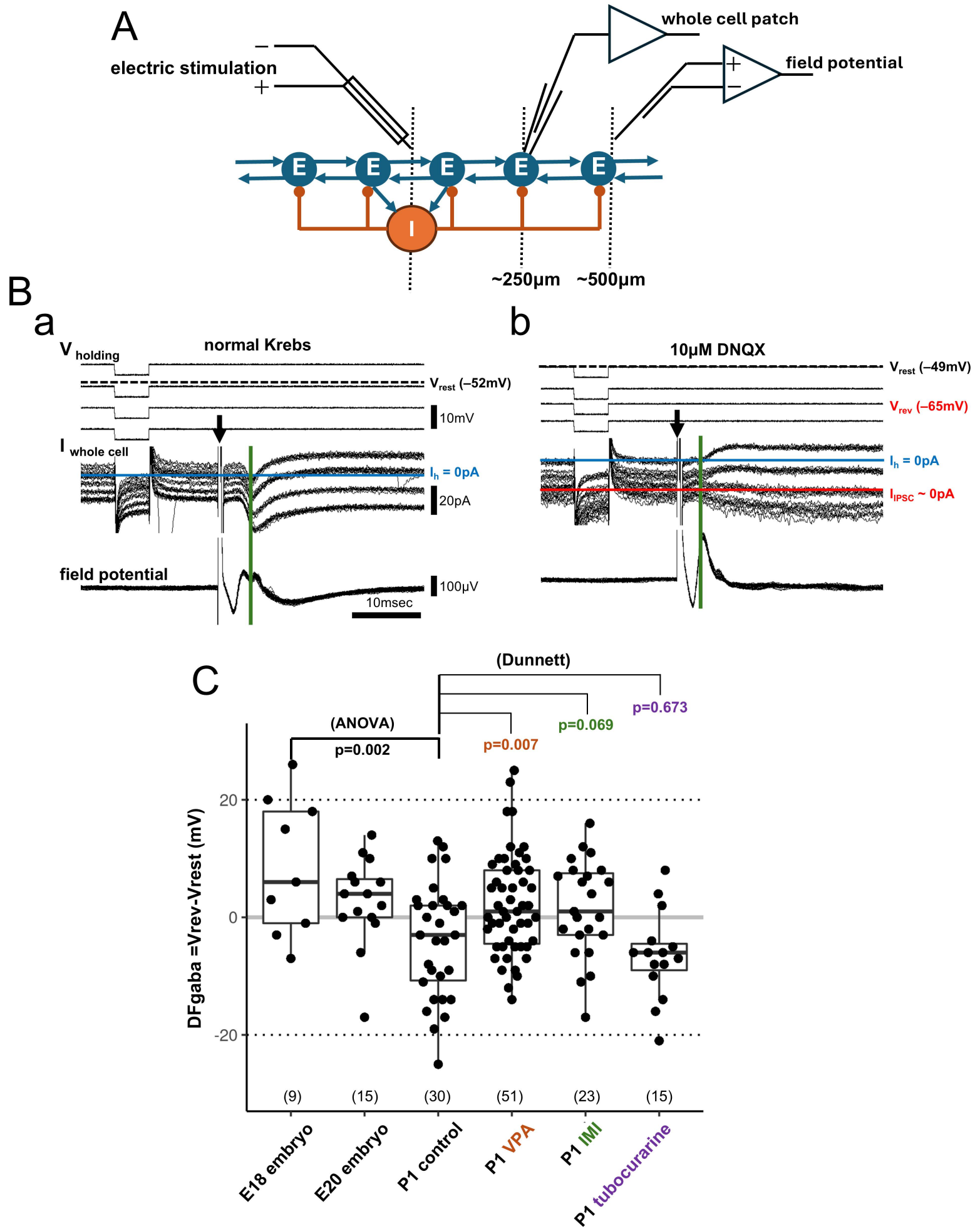
Fig. 1 Whole-cell voltage-clamp recording from intermediate medial mesopallium (IMM) neurons revealed the reversal potential of local GABA-A-ergic transmission. **A)** Schematic diagram of the estimated local network of IMM composed of excitatory and inhibitory neurons (E and I, respectively). Electrical stimulation elicits excitatory and inhibitory synaptic responses in nearby neurons. **B)** Representative recording of whole-cell current ($I_{\text{whole cell}}$) by voltage-clamp mode in normal Krebs (a) and after DNQX was added to the bath perfusate (b). Superimposed traces (>10 for each level of the holding potential V_{holding}). Stimulus intensity: 111 μA (a) and 200 μA (b), duration: 400 μsec , intervals: 2 sec. V_{rev} (reversal potential) was determined at the level of null initial synaptic current ($I_{\text{IPSC}} = 0$, red line). V_{rest} (resting potential) was determined at the level of null holding current ($I_h = 0$, blue line). **C)** Comparisons of DF_{gaba} (driving force of GABA; $V_{\text{rev}} - V_{\text{rest}}$) among groups. One-way ANOVA was applied to the three control groups (E18, E20, and P1, i.e., 2 days intervals). A rapid hyperpolarizing shift occurred during four days of the pre-/neo-natal period. Multiple comparisons were made using Dunnett’s method to the P1 groups; VPA [7 and 35 $\mu\text{mole per egg}$], IMI [50 and 250 $\mu\text{g/egg}$], and tubocurarine [0.2mg/egg] against control as the reference. Significantly depolarized DF_{gaba} was found in VPA, but it was suggestive in IMI and not significant in tubocurarine. Numbers in parentheses indicate the number of recorded neurons. See supplementary Fig. S1 for each of V_{rev} and V_{rest} measured in different doses.

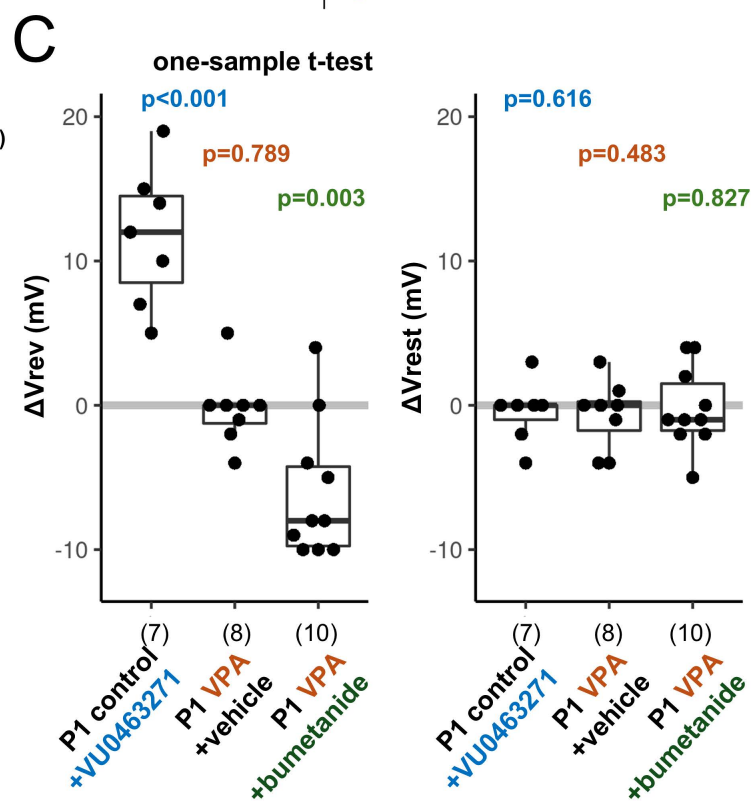
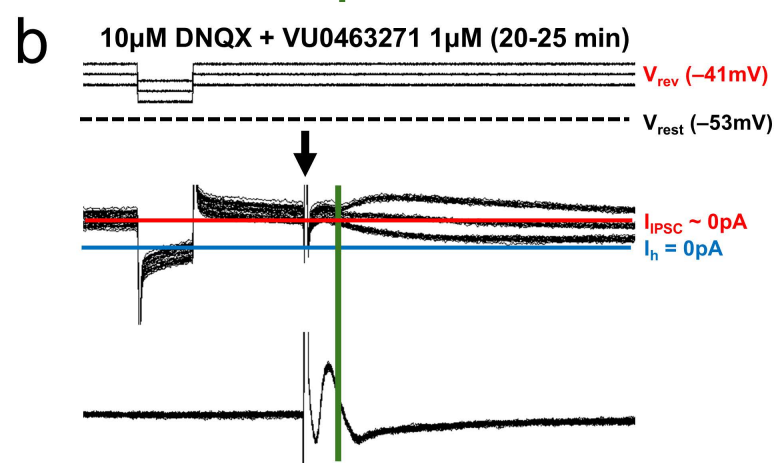
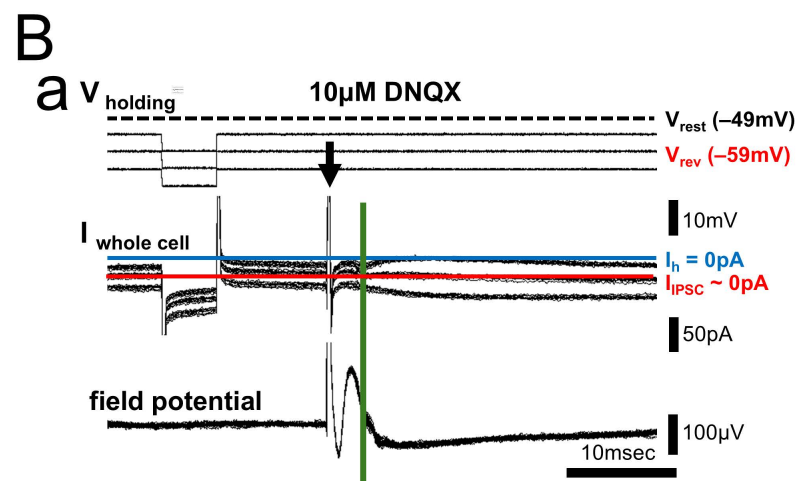
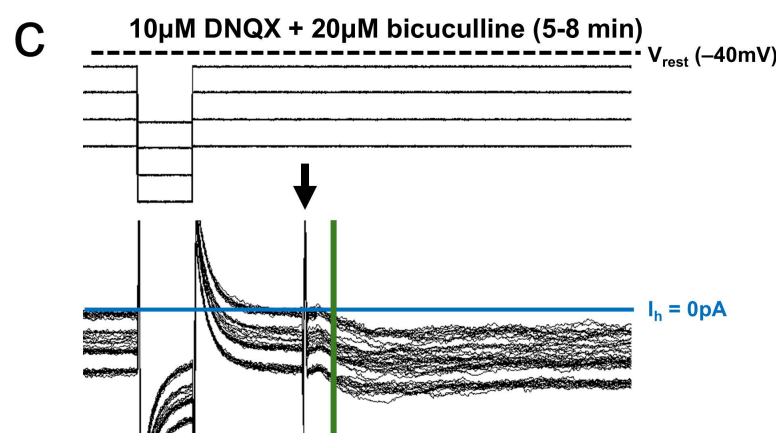
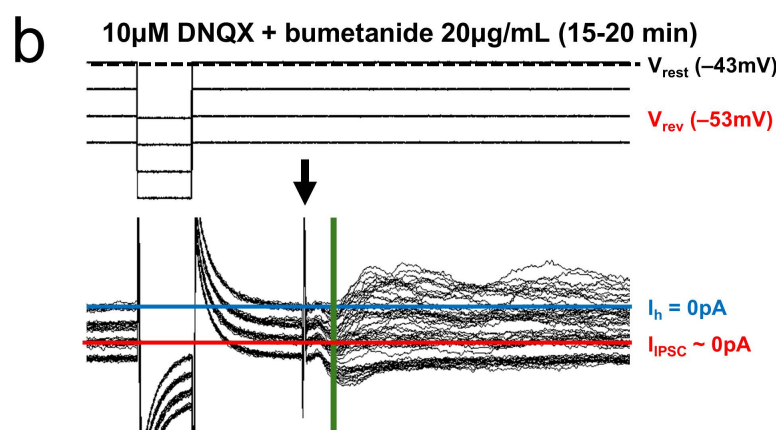
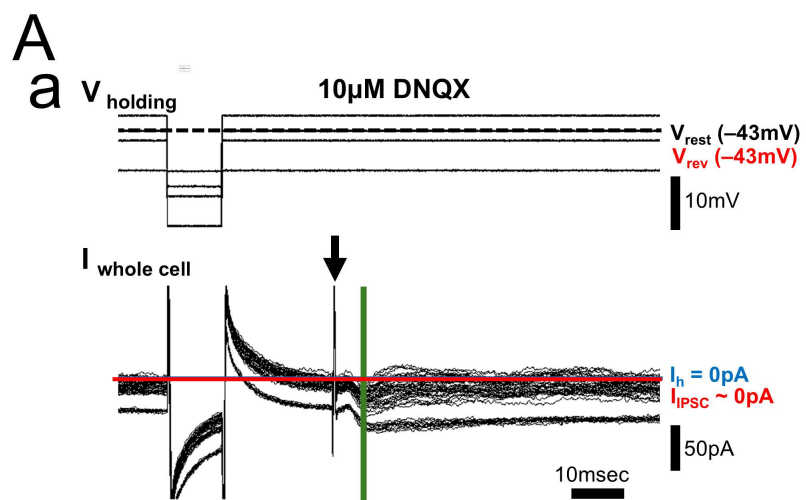
Fig. 2 Acute effects of bumetanide (NKCC1 blocker) and VU0463271 (KCC2 blocker) on V_{rev} and V_{rest} of VPA-exposed and control chicks, respectively. Representative recordings (**A** and **B**) and population data (**C**). **A)** In IMM slice obtained from a VPA-exposed chick, V_{rev} was determined in DNQX-Krebs (**Aa**), and in bumetanide added to the perfusion for 15-20 min (**Ab**); hyperpolarization shift occurred at $\Delta V_{\text{rev}} = -10\text{mV}$. Replacement of bumetanide with bicuculine completely vanished the voltage-sensitive current, leaving a lasting slow inward current (**Ac**). **B)** In the control slice, the addition of VU0463271 to the perfusate Krebs caused a depolarizing shift of V_{rev} . **C)** ΔV_{rev} and accompanying ΔV_{rest} are shown in each group; a one-sample t-test was repeated for each.

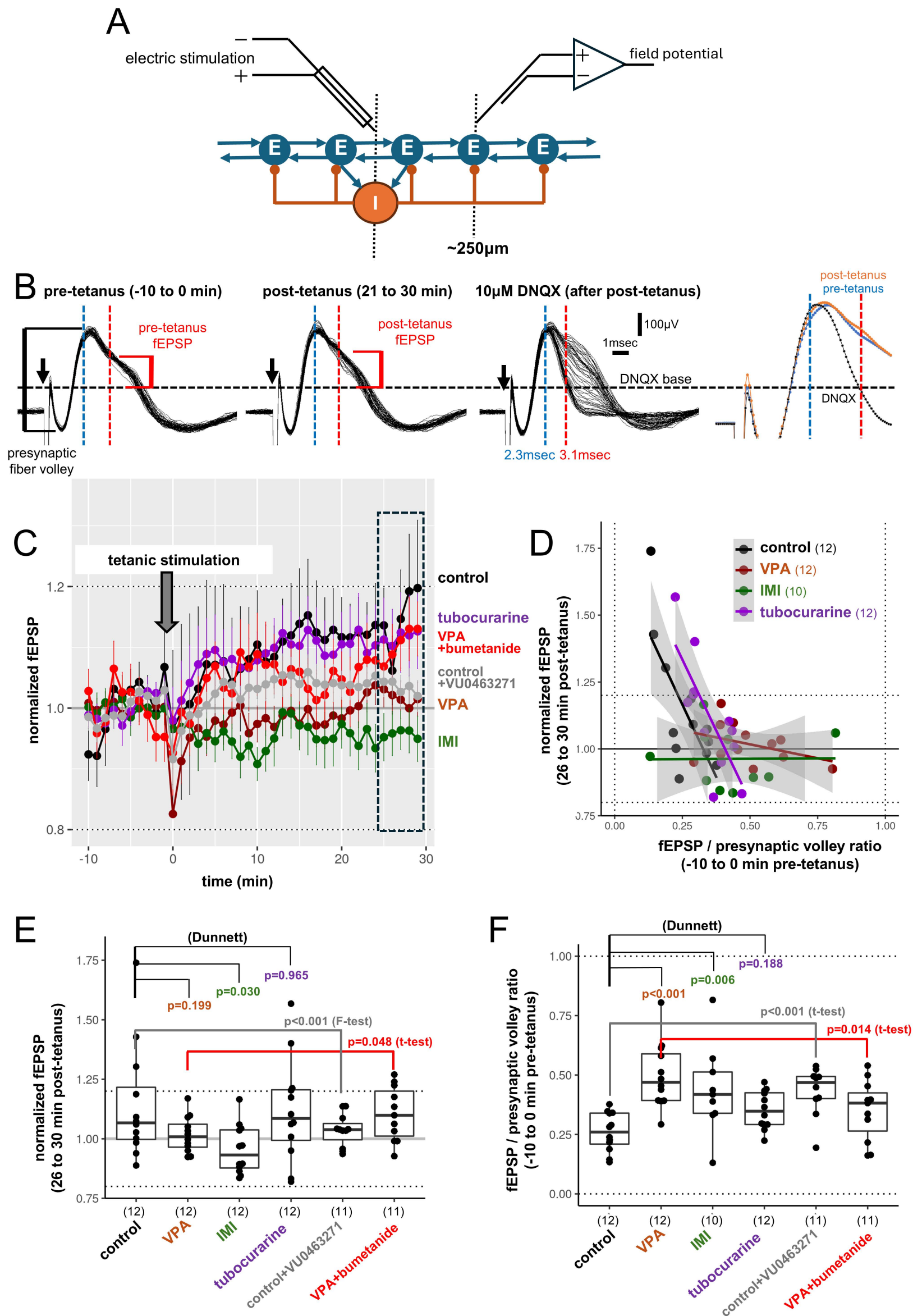
Fig. 3 Potentiation of excitatory post-synaptic responses (LTP) was examined in IMM slices. **A)** Schematic diagram. **B)** Representative recording of field EPSPs (fEPSPs); superimposed pre-tetanic traces (left, 60 traces for 10 min), post-tetanic traces (middle, 60 traces for 10 min), and traces recorded in DNQX-Krebs (right, 36 traces for 6 min). Pre-synaptic fiber volleys were measured as the amplitude of the initial bi-phasic component just after the electrical stimulation. The amplitude of the fEPSPs was measured at 0.8 msec (red dashed lines) from the onset point of the DNQX-sensitive component (blue dashed lines). The traces recorded at 5-6 min after the DNQX application yielded the base level (DNQX base, black dashed horizontal line). **C)** Time course of the fEPSPs before and after the low-frequency tetanic stimulation (5Hz x 300 pulses, downward arrow) after normalization by the pre-tetanic level. Dots and vertical lines indicate the mean and s.e.m. (6 traces x 12 or 11 experiments per min). **D)** Normalized fEPSP changes induced by tetanic stimulation (LTP, y-axis) were plotted against the fEPSP amplitude recorded pre-tetanus (x-axis) in four groups of slices. See supplementary Fig. S2 for the acute effects of bumetanide and VU0463271. **E)** Normalized fEPSPs were compared during the last 5 min (26-30 min post-tetanus, dashed box). Multiple comparison using Dunnett’s method was applied to VPA [35 $\mu\text{mole per egg}$], IMI [250 $\mu\text{g/egg}$], and tubocurarine [0.2 mg/egg] against control data. Multiple comparisons revealed significant differences only in IMI. Repeated pairwise comparisons revealed significant differences between control vs. control+VU0463271 (significant by F-test) and between VPA vs.

VPA+bumetanide (t-test). **F**) The ratio of the fEPSP amplitude to the pre-synaptic fiber volley was compared among groups. Significant differences were found in VPA and IMI, but not in tubocurarine. Pairwise comparisons revealed significant differences between control vs. control+VU0463271 and between VPA vs. VPA+bumetanide.

Fig. 4 Gene expression profiles. **A**) Histological reconstruction of sampled regions for RNA-seq analysis in IMM. The inset table shows averaged DF_{gaba} , V_{rest} and V_{rev} recorded from 6 chicks: 2 controls, 2 VPA [35 μ mole per egg], and 2 IMI [250 μ g/egg]. Four pieces of punched tissue were sampled from each chick at A9.6 to A8.0; the AP level follows the chick brain atlas by Kuenzel & Masson (1988). **B, C**) Expression of SLC12A2 (NKCC1) and SLC12A5 (KCC2) normalized by GAPDH; qPCR analysis in 3 brain regions (IMM, arcopallium, and optic tectum). Developmental changes from embryonic E14, E18, to post-hatch P1; 5 chicks each (**B**). Effects of E14 exposure to VPA analyzed on P1; 6 chicks each (**C**). Inset boxes show the statistics. **D**) Expression fold changes in the genes by RNA-seq analysis with significantly different expression by FDR (false discovery rate) < 0.1 for VPA and IMI. Upregulated and downregulated levels, compared to the control, are color-coded in red and blue. See supplementary **Table S2** for the full data.

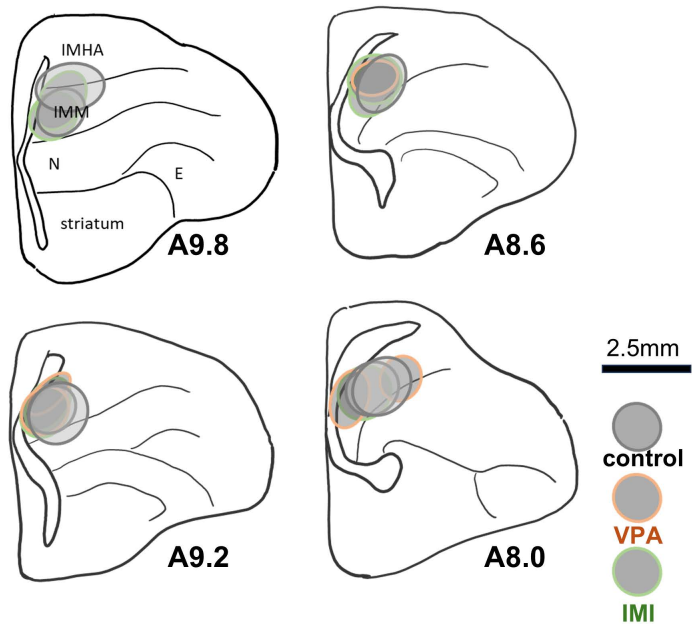




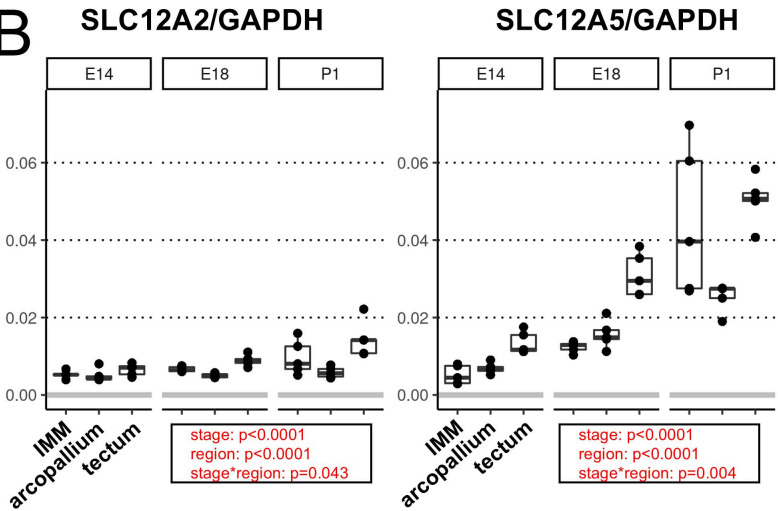


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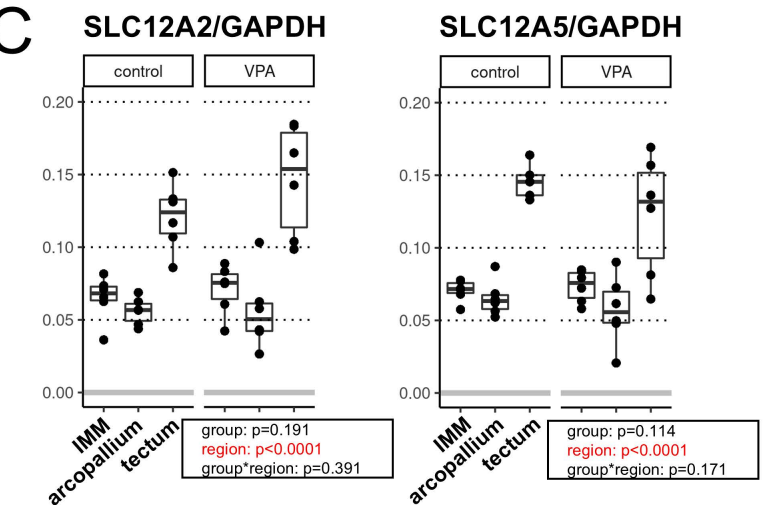
treatment	# cells	DF _{gaba}	V _{rest} (mV)	V _{rev} (mV)
control	3	-8.2	-49.8	-58.0
control	3	-3.7	-42.0	-45.7
VPA	2	+2.5	-44.0	-41.5
VPA	3	+4.0	-43.3	-39.3
IMI	3	+0.7	-39.7	-39.0
IMI	2	+3.5	-38.5	-35.0



B



C



D

