GABAergic malfunction underlying maternal immune activation-induced social deficits

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23 **Abstract**

24 Social deficits are one of the major symptoms of psychiatric disorders, including autism
 25 spectrum disorders (ASDs) and schizophrenia. However, the underlying mechanism
 26 remains ill-defined. Here, we focused on the anterior cingulate cortex (ACC), a brain
 27 region that is related to social behaviors, of mice that received poly(I:C)-induced
 28 maternal immune activation. Using whole-cell patch clamp recordings, we found that
 29 layer 2/3 pyramidal cells were hyperactive in acute ACC slices prepared from
 30 poly(I:C)-treated mice compared to those from saline-treated mice. The hyperexcitation
 31 was associated with a reduction in inhibitory synapse activity. Local injection of the
 32 GABA_A receptor enhancer clonazepam into the ACC of poly(I:C)-treated mice restored
 33 the social behaviors of the mice. These results suggest that the balanced excitability of
 34 ACC neurons is essential for social ability.

35

36 **1. Introduction**

37 Social deficits are observed in many developmental neuropsychiatric disorders,
 38 including autism spectrum disorder (ASD) and schizophrenia. Although the risk factors
 39 associated with these disorders are not yet strictly identified, genetic and environmental
 40 factors that are likely involved in the etiology of the disorders are often associated with
 41 synapse formation. Indeed, disruption of the excitatory and inhibitory balance (E/I
 42 balance) of synaptic activity is suggested to underlie the symptoms of psychiatric
 43 disorders (Gogolla et al., 2009). For instance, neuroimaging studies demonstrated
 44 hyperexcitability of the neocortex of ASD patients in a resting state (Agam et al., 2010,
 45 Assaf et al., 2010). Furthermore, hyperexcitation in the sensory cortex impairs sensory
 46 input processes (Gogolla et al., 2014) and induces social deficits (Yim et al., 2017).
 47 Thus, cortical hyperexcitation likely contributes to the symptoms of psychiatric
 48 disorders (Yim et al., 2017).

49 Previous studies suggest that a disrupted E/I balance leads to deficits in social
 50 interactions (Han et al., 2014, Yizhar et al., 2011). However, neither the responsible
 51 brain region nor the underlying synaptic mechanism is fully understood. Here, we
 52 focused on the anterior cingulate cortex (ACC), a brain region that is associated with
 53 social behaviors (Behrens et al., 2009, Singer et al., 2004). In rodents, inactivation of
 54 ACC neurons reduces empathetic behaviors (Kim et al., 2012).

55 In the present study, we employed a maternal immune activation (MIA) mouse
 56 model to investigate the relationships between ACC neuronal activity and social deficits.
 57 MIA is one of the major environmental factors causing psychiatric disorders in offspring
 58 (Atladdottir et al., 2010, Patel et al., 2017), and social deficits are observed in
 59 MIA-model mice (Choi et al., 2016, Hsiao et al., 2013, Malkova et al., 2012). Using

whole-cell patch clamp recordings, we found hyperexcitation of layer 2/3 pyramidal cells in acute ACC slices prepared from poly(I:C)-treated mice. In addition, local injection of the GABA_A receptor enhancer clonazepam into the ACC of poly(I:C)-treated mice restored the social behaviors of the mice. These results suggest that balanced excitation in ACC neurons is critical for social behaviors and that disruption of this balance leads to social disability.

2. Materials and methods

2.1. Animal experiment ethics

Experiments were performed with the approval of the animal experiment ethics committee at the University of Tokyo (approval no. P24-8 and P24-70) and according to the University of Tokyo guidelines for the care and use of laboratory animals. C57BL/6J mice (SLC, male) were housed in cages under standard laboratory conditions (12-h light/dark cycle, ad libitum access to food and water). All efforts were made to minimize the animals' suffering and the number of animals used.

2.2. MIA model

Maternal viral infection was imitated using poly(I:C), a synthetic analogue of double-stranded RNA (Sigma) or saline (control), which was intraperitoneally injected at doses of 0.25 and 0.125 U/g on embryonic days 12.5 and 17.5, respectively (Naviaux et al., 2013). Pups were removed from their dams at 3 postnatal weeks and housed in cages of 2–4 animals until 4–6 postnatal weeks.

84 *2.3. Three-chamber test*

85 The testing apparatus measured $51 \times 25 \times 25$ cm and was divided into three chambers
 86 ($17 \times 25 \times 25$ cm) with transparent plastic walls; the mice had free access to all the
 87 chambers through open doors. A transparent plastic cage was located in the corner of
 88 each side of the chamber. A mouse was placed in the apparatus, was allowed to explore
 89 these chambers for 10 min, and was then moved to a resting cage. Another unfamiliar
 90 mouse was placed in one of the corner plastic cages, whereas the other cage was kept
 91 vacant. In the test session, the subject mouse was returned to the testing apparatus and
 92 allowed to explore the chambers for 10 min. The testing apparatus was video-monitored.
 93 The difference between the times spent in the chamber with an unfamiliar mouse and in
 94 the vacant chamber was used as an index for social interaction.

96 *2.4. Acute slice preparation*

97 Acute slices were prepared from the ACCs of male mice (4-6 postnatal weeks). Mice
 98 were anesthetized with isoflurane and then decapitated. The brains were removed and
 99 placed in an ice-cold oxygenated solution consisting of (in mM) 222.1 sucrose, 27
 100 NaHCO_3 , 1.4 NaH_2PO_4 , 2.5 KCl, 1 CaCl_2 , 7 MgSO_4 , and 0.5 ascorbic acid. The brains
 101 were sliced at a thickness of 400 μm coronally for the ACC using a VT1200S vibratome
 102 (Leica). Slices were allowed to recover at room temperature for at least 1.5 h while
 103 submerged in a chamber filled with oxygenated aCSF consisting of (in mM) 127 NaCl,
 104 26 NaHCO_3 , 1.6 KCl, 1.24 KH_2PO_4 , 1.3 MgSO_4 , 2.4 CaCl_2 , and 10 glucose.

106 *2.5. In vitro electrophysiology*

107 All recordings were performed at 32–33°C. Whole-cell recordings were performed

108 using ACC layer 2/3 pyramidal cells using a MultiClamp 700B amplifier and a Digidata
 109 1550 digitizer controlled by pCLAMP10.5 software (Molecular Devices). Borosilicate
 110 glass pipettes (3–6 M Ω) were filled with a solution containing (in mM) 130 CsMeSO₄,
 111 10 CsCl, 10 HEPES, 10 phosphocreatine, 4 MgATP, 0.3 NaGTP, and 10 QX-314. Paired
 112 whole-cell recordings were performed in two neurons located less than 100 μ m apart;
 113 one glass pipette contained 200 μ M picrotoxin. Liquid junction potentials were not
 114 corrected. The signals were gained 5–10-fold, low-pass-filtered at 1 kHz and digitized
 115 at 20 kHz.

116

117 *2.6. Drug application*

118 Male mice (6 postnatal weeks) were anesthetized with xylazine (10 mg/kg, i.p.) and
 119 pentobarbital (2.5 mg/kg, i.p.). Stainless-steel cannulas (Plastic One) were bilaterally
 120 implanted and aimed at the ACC (AP: +1.0 mm, LM: $\pm$ 0.3 mm, DV: -2.0 mm). The
 121 cannulas were chronically secured to the skull using a mixture of acrylic and dental
 122 cement. Dummy cannulas (33-gauge) were inserted into each guide cannula to prevent
 123 clogging. Mice were allowed to recover for at least 7 days. Before testing, mice
 124 received an intra-ACC infusion (0.5 μ l per side) of clonazepam (500 nM, Wako). The
 125 clonazepam was dissolved in DMSO and was diluted in phosphate-based saline (PBS)
 126 containing 0.1 mM SR101 so that the final concentration of DMSO was 0.001%.
 127 Infusions were performed slowly over more than 2 min using 33-gauge cannulas.
 128 Twenty minutes after infusion, the mice were subjected to the three-chamber test. After
 129 the test, the infused regions were confirmed by the track of the cannulas and the
 130 fluorescence of SR101.

131

132 2.7. Histology

133 Layer 2/3 pyramidal neurons in the ACC were intracellularly perfused with 2 mM
134 biocytin through whole-cell recordings. After the recordings, the slices were fixed for 20
135 h at 25°C in 0.1 M Na₃PO₄, pH 7.4, containing 3% (w/v) formaldehyde and 0.01%
136 (w/v) glutaraldehyde. All subsequent incubations were performed at room temperature
137 and were followed by three 30-min rinses with PBS containing 0.3% (v/v) Triton X-100
138 (PBS-X). After being blocked with PBS-X containing 1% (v/v) goat serum and 0.12%
139 (w/v) λ-carrageenan (PBS-XCG) for 1 h, the slices were incubated for 48 h with
140 1/1,000-diluted mouse anti-gephyrin antibody (147021; Synaptic Systems) in PBS-XCG.
141 The slices were further incubated for 24 h with a mixture of 5 µg/mL Alexa Fluor
142 488-conjugated streptavidin (S11223; Life Technologies) and 10 µg/mL Alexa Fluor
143 647-conjugated goat antibody to mouse IgG (A21236; Life Technologies) in PBS-XCG.
144 The sections were mounted onto gelatinized glass slides and cover-slipped with 50%
145 (v/v) glycerol and 2.5% (w/v) triethylenediamine (antifading reagent) in 20 mM
146 Tris-HCl (pH 7.6). The slices were observed under a Leica TCS SP8 confocal
147 laser-scanning microscope (Leica Microsystems) with a water-immersion objective lens
148 (25×, 0.95 numerical aperture, Leica) and HyD detectors (Leica). We acquired image
149 stacks at depths ranging from 5 to 25 µm relative to the surface with the pinhole at 1.0
150 airy disk unit and zoom factor at 1. The image stacks were saved as 8-bit TIFF files. The
151 image size was 4,096 × 4,096 pixels, corresponding to a pixel dimension of 114 nm in
152 both the X and Y directions. Alexa Fluor 488 and Alexa Fluor 647 were excited with
153 488-nm and 638-nm laser beams and observed through 500–550 and 650–800 nm
154 emission prism windows, respectively. Up to 20 images were acquired at a Z-interval of
155 1 µm per stack and were deconvoluted using Huygens Essential software (version 3.7;

156 Scientific Volume Imaging).

157

158

159 **3. Results**

160 *3.1. Poly(I:C)-treated mice are less social*

161 We treated pregnant mice with poly(I:C), a synthetic analogue of double-stranded RNA
 162 that mimics a molecular pattern in viral infection, and used the offspring of these mice
 163 as the MIA model (Naviaux et al., 2013). We first monitored the exploratory behavior of
 164 these mice in a three-chamber maze in which one chamber contained an unfamiliar
 165 mouse in the corner box and nothing in the other box (Fig. 1A). Control mice whose
 166 mothers had been treated with saline during their embryonic periods preferentially
 167 explored the chamber with the unfamiliar mouse, but mice whose mothers had been
 168 treated with poly(I:C) (hereafter termed poly(I:C)-treated mice) spent less time in the
 169 chamber containing the mouse (Fig. 1B, $P = 0.016$, $D_{19} = 0.44$, Kolmogorov-Smirnov
 170 test, $n = 11$ control and 10 poly(I:C) mice). The total time spent exploring both the
 171 mouse-containing and vacant chambers did not differ between these two groups (Fig.
 172 1B inset; $P = 0.87$, $t_{19} = 0.19$, Student's t -test), meaning the spatial exploration
 173 motivation was intact in poly(I:C)-treated mice.

174

175 *3.2. Excitatory inputs to ACC pyramidal cells are increased in poly(I:C)-treated mice*

176 Hyperexcitation of synaptic transmission is implied in neuropsychiatric symptoms
 177 (Gogolla et al., 2009, Rubenstein and Merzenich, 2003). The ACC is a brain region
 178 related to social behavior (Lamm et al., 2011). Thus, we prepared acute ACC slices and
 179 recorded spontaneous EPSCs from layer 2/3 pyramidal cells (Fig. 2A). The EPSC

180 frequencies were higher in slices from poly(I:C)-treated mice than in those from control
181 mice (Fig. 2B; $P = 1.6 \times 10^{-3}$, $t_{17} = 3.76$, Student's t -test; $n = 9$ control and 10 poly(I:C)
182 cells). The EPSC amplitudes were also higher in poly(I:C)-treated mice than in control
183 mice (Fig. 2C, $P = 1.9 \times 10^{-2}$, $t_{17} = 2.60$, Student's t -test; $n = 9$ control and 10 poly(I:C)
184 cells).

185

186 *3.3. Excitatory synaptic connectivity in the ACC is intact in poly(I:C)-treated mice*

187 We next focused on excitatory synaptic connections between ACC layer 2/3 pyramidal
188 cells. We conducted double whole-cell recordings from ACC layer 2/3 pyramidal cells.
189 The synaptic connection probability did not differ between control and poly(I:C)-treated
190 mice (Fig. 3A; $P = 0.40$, Fisher's exact test). The synaptic strength between connected
191 cell pairs did not differ significantly (Fig. 3B; $P = 0.093$, $t_{12} = 0.09$, Student's t -test).
192 These results suggest that excitatory synaptic connectivity in the ACC was not affected
193 in poly(I:C)-treated mice.

194

195 *3.4. Inhibitory conductance in the ACC is reduced in poly(I:C)-treated mice*

196 We next focused on inhibitory synapses. The immunoreactivity of gephyrin, a protein
197 that anchors inhibitory receptors, was reduced in the somata and dendrites of layer 2/3
198 pyramidal cells in the ACC of poly(I:C)-treated mice (Fig. 4; Soma: $P = 5.0 \times 10^{-3}$, $U =$
199 279 ; Dendrite: $P = 0.043$, $U = 262$, Mann-Whitney's U -test). We recorded spontaneous
200 EPSCs from ACC layer 2/3 pyramidal cells in which 200 μ M picrotoxin, a GABA_A
201 receptor channel blocker, was intracellularly injected to reduce local GABAergic
202 influences (Inomata et al., 1988, Nelson et al., 1994). Note that we employed
203 intracellular picrotoxin loading instead of direct recording of inhibitory synaptic

transmission because voltage-clamp recording at 0 mV can isolate only peri-somatic inhibitory inputs (Williams and Mitchell, 2008), whereas intracellularly perfused picrotoxin can affect even GABA_A receptors located in distal dendrites. In slices from control mice, the picrotoxin-loaded neurons exhibited higher EPSC frequencies than the simultaneously recorded control neurons (Fig. 5A; $P = 6.6 \times 10^{-3}$, $t_{10} = 3.42$, paired t -test; $n = 11$ pairs). By contrast, in slices from poly(I:C)-treated mice, intracellular perfusion with picrotoxin did not further increase the EPSC frequency (Fig. 5B; $P = 0.29$, $t_{14} = 1.09$, paired t -test; $n = 15$ pairs). These results suggest malfunction of GABA_A receptors in MIA-model mice.

3.5. Clonazepam treatment in the ACC rescues social behavior

We bilaterally injected clonazepam, a pharmacological enhancer of GABA_A receptors that contain $\alpha 2/3$ - $\gamma 2$ subunits (Benke et al., 1996, McKernan et al., 1991), into the ACC of poly(I:C)-treated mice at a dose of 80 pg/site 20 min before the behavioral test (Fig. 6A). The poly(I:C) mice treated with clonazepam injection explored the mouse chamber more preferentially than vehicle-treated poly(I:C) mice (Fig. 6B,C; $P = 0.016$, $D_{20} = 0.44$, $n = 11$ poly(I:C)-treated mice), although the total exploration time was not different between the two groups (Fig. 6B inset; $P = 0.92$, $t_{20} = 0.10$, paired t -test). Thus, hyperexcitation of the ACC is responsible for social deficits in MIA-model mice.

4. Discussion

We found that the ACC in MIA-model mice is hyperexcitable due to a reduction in inhibitory inputs and that strengthening the inhibition of the ACC of these mice restores

228 their social deficits. These results suggest that GABAergic malfunction in the ACC
229 underlies social deficits of neuropsychiatric disorders.

230 We measured the sociability of poly(I:C)-treated mice using the three-chamber
231 behavior test. For these behavioral experiments, we used juvenile mice, which are
232 younger than those generally used in the three-chamber test in previous studies. Our
233 results indicate that the MIA-model mice exhibit social deficits even at an early age.

234 Accumulating evidence has suggested that neuronal hyperexcitation caused by the
235 net imbalance between excitation and inhibition underlies nervous system disorders in
236 humans, including epilepsy, mental retardation, cognitive impairment, and ASD (Eichler
237 and Meier, 2008, Verret et al., 2012). In poly(I:C)-treated MIA-model mice, cortical
238 parvalbumin-positive interneurons are lacking, and this deficit leads to behavioral
239 abnormalities, including reversing behavior and social deficits (Yim et al., 2017). We
240 found that ACC neurons were physiologically hyperactive due to the hypofunction of
241 GABAergic synapses in poly(I:C)-treated mice. This hyperexcitation of ACC neurons
242 may cause the social deficits of poly(I:C)-treated mice because the pharmacological
243 enhancement of GABAergic transmission in the ACC alone was sufficient to attenuate
244 their social dysfunction. A recent study revealed that inhibition of the primary
245 somatosensory cortex is also sufficient to restore the social interactions (Yim et al.,
246 2017). Social behavior is complex and is unlikely generated in a single brain region.
247 Because brain regions are connected in a complex manner, it is possible that specific
248 inhibition of ACC affects the excitability of other brain regions, such as the primary
249 somatosensory cortex, and vice versa.

250 Phasic inhibition, one of the forms of inhibition produced by activation of GABA_A
251 receptors, works to efficiently modulate excitatory synaptic activity (Hayama et al.,

252 2013, Liu, 2004, Muller et al., 2012). Thus, a lack of phasic inhibition is expected to
 253 lead to neuronal hyperexcitation. The phasic inhibition is generated by synaptic GABA_A
 254 receptors that contain $\alpha 2/3$ - $\gamma 2$ subunits (Farrant and Nusser, 2005). To restore the phasic
 255 inhibition, therefore, we used clonazepam, an enhancer of GABA_A receptors that
 256 contain $\alpha 2/3$ - $\gamma 2$ subunits. Clonazepam is prescribed for epilepsy and anxiety disorders
 257 and was also suggested to ameliorate dementia (Busche et al., 2015). However, it has
 258 not been applied for human social disorders. A previous work suggested that
 259 intraperitoneal injection of clonazepam restored behavioral deficits in a mouse model of
 260 autism (Han et al., 2014). These results together with ours suggest that clonazepam may
 261 act on inhibitory transmission of the ACC and ameliorate sociability.
 262

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 354 Study at The University of Tokyo.

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357 **Figure 1 | Poly(I:C)-treated mice exhibit low social interaction.** **A**, The diagram
358 depicts the exploration tracks of control and poly(I:C)-treated mice during a
359 three-chamber test for a total of 10 min. Mice were treated twice with saline or
360 poly(I:C) on embryonic days 12.5 and 17.5. During the test, an unfamiliar mouse was
361 presented in the corner of the right chamber; the left chamber was vacant. **B**, The graph
362 plots the cumulative distributions of the difference between the times spent in the left
363 and right chambers by 11 saline-treated and 10 poly(I:C)-treated mice. The inset
364 indicates the total time spent exploring the left and right chambers.
365 Kolmogorov-Smirnov test.

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368 **Figure 2 | ACC neurons are hyperactive in poly(I:C)-treated mice. A,**

369 Representative EPSC traces in layer 2/3 pyramidal cells in acute slices prepared from

370 control and poly(I:C)-treated mice. **B,** Frequency of spontaneous EPSCs in layer 2/3

371 pyramidal cells in acute ACC slices prepared from control and poly(I:C)-treated mice.

372 The data were calculated from 9 neurons from 5 saline-treated mice and 10 neurons

373 from 10 poly(I:C)-treated mice. Student's *t*-test. **C,** The same as B but for the

374 amplitudes of spontaneous EPSCs.

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379 **Figure 3 | Excitatory connections are not changed in the ACC of poly(I:C)-treated**

380 **mice. A,** The probability of an excitatory monosynaptic connection in ACC layer 2/3

381 pyramidal cells prepared from control and poly(I:C)-treated mice. Chi-squared test. **B,**

382 The synaptic efficacy is the average of evoked monosynaptic EPSPs containing synaptic

383 failure. They were measured from 9 neurons in control mice and 5 neurons in

384 poly(I:C)-treated mice. Student's *t*-test.

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391 **Figure 4 | Gephyrin expression is reduced in the ACC of poly(I:C)-treated mice A,**

392 Representative confocal image of fluorescent immunoreactivity of gephyrin (*magenta*)

393 in the somata and dendrites of layer 2/3 pyramidal cells in mice whose mothers had

394 been treated with saline or poly(I:C) during the embryonic period. Neurons were

395 visualized with Alexa Fluor 488-conjugated streptavidin (*green*). Biocytin was injected

396 through patch-clamp pipettes into the cell. **B,** Gephyrin immunoreactivity in

397 Alexa-injected neurons was measured in the somata and dendrites of 15 and 13 neurons

398 in 3 control and 3 poly(I:C)-treated mice. Mann-Whitney *U*-test.

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404 **Figure 5 | Inhibitory conductance is decreased in poly(I:C)-treated mice.** Dual
 405 recordings of spontaneous EPSCs from ACC layer 2/3 pyramidal cells in control (A)
 406 and poly(I:C)-treated (B) mice. In each pair, one neuron was intracellularly perfused
 407 with 200 μ M picrotoxin. The data are box plots of 11 pairs from 10 saline-treated mice
 408 and 15 pairs from 10 poly(I:C)-treated mice. Paired *t*-test.

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413 **Figure 6 | GABA_A receptor activation in the ACC recovers social behavior.** **A**, *Post*
414 *hoc* histological inspection reveals successful cannula implantation in the ACC. The
415 target locations of local injection of clonazepam are indicated by the red circles ($n = 11$
416 mice). The locations were confirmed based on the track of the cannulas and the infusion
417 of 0.1 mM SR101. **B**, The same diagram as in Figure 1, depicting the exploration tracks
418 of control and poly(I:C)-treated mice during a three-chamber test for a total of 10 min,
419 but for 11 poly(I:C)-treated mice administered vehicle or clonazepam in the bilateral
420 ACC 20 min before the behavioral test. **C**, The graph plots the cumulative distributions
421 of the difference between the times spent in the left and right chambers by 11 vehicle-
422 or clonazepam-treated poly(I:C)-treated mice. The inset indicates the total time spent
423 exploring the left and right chambers. Kolmogorov-Smirnov test.

424

A

Saline

Poly(I:C)

10 pA
200 ms**B** $***P=1.6\times 10^{-3}$
 $t_{17}=3.76$ **C** $**P=1.9\times 10^{-2}$
 $t_{17}=2.60$

A

Connection probability

B

Synaptic efficacy (mV)

A Saline

Poly(I:C)

B

Gephyrin immunoreactivity (a.u.)

 $*P=5.0 \times 10^{-3}$
 $U=279$ $*P=0.0427$
 $U=262$

EPSC frequency (Hz)