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Perinatal Epidermal Growth Factor Signal Perturbation Results in the Series of Abnormal Auditory Oscillations and Responses Relevant to Schizophrenia

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Auditory neurophysiological responses, such as steady-state responses, event-related potential P300/P3, and phase-amplitude coupling, are promising translational biomarkers for schizophrenia, but their molecular underpinning is poorly understood. Focusing on ErbB receptor signals that are implicated in both schizophrenia and auditory processing/cognition, we explored the causal biological links between ErbB signals and these auditory traits with an experimental intervention into rats. We peripherally challenged rat pups with one of the amniotic ErbB ligands, epidermal growth factor (EGF), and characterized its consequence on the series of these auditory electrocorticographic measures. Auditory brainstem responses (ABRs) and cortical ON responses were also assessed under anesthesia to estimate the influence of higher brain regions. An auditory steady-state paradigm revealed attenuation of spectral power and phase synchrony to 40-Hz stimuli in EGF-challenged rats. We observed a reduction in duration mismatch negativity-like potentials and a delay of P3a responses, all of which are relevant to the reported auditory pathophysiological traits of patients with schizophrenia. Moreover, the perinatal EGF challenges resulted in enhanced theta-alpha/beta and theta-gamma coupling within the auditory cortex and changes in ABRs. However, the EGF challenges retained the normal ranges of cortical ON responses, potentially ruling out their fundamental auditory deficits. Perinatal exposure of an ErbB ligand to rats strikingly reproduced the whole series of aberrant auditory responses and oscillations previously reported in patients with schizophrenia. Accordingly, these findings suggest that developmental deficits in ErbB/EGF signaling might be involved in the auditory pathophysiology associated with schizophrenia.

Key words: ASSR/EGFR/MMN/PAC/ABR

Introduction

Auditory electroencephalographic (EEG) impairments are often observed in patients with schizophrenia, such as auditory steady-state responses (ASSRs), mismatch negativity (MMN), an attention-triggered event-related potential (ERP) component P300/P3a, and phase-amplitude coupling (PAC), and some of these are expected to be clinical and translational biomarkers for schizophrenia.[1–8] Similarly, the threshold and latency of the auditory brainstem response (ABR) have been reported to be elevated and delayed, respectively, in schizophrenia.[9–11] These deficits might be associated with auditory hallucinations[6,10] or abnormal verbal recognition[12] of patients although this notion is controversial.[13] Thus, what induces the multiple sets of audio-pathophysiological impairments from the brainstem to the integrative higher brain remains a challenging question in schizophrenia research.

Epidermal growth factor (EGF) and neuregulin-1 are ligands for ErbB receptors and both implicated in the genetic association with[14–16] and the neuropathology of schizophrenia.[17,18] The ErbB members of ErbB1–4 are expressed in various types of cells in the brain,[19–21] and their abnormal expressions and genetic associates are

also reported in schizophrenia.[22,23] These receptors also interact with other ligands of the EGF family, such as transforming growth factor (TGF)α, heparin-binding-EGF, and neuregulin-3,[19,20] which exhibit genetic or neuropathologic association with schizophrenia.[24–26] Despite accumulating evidence for the phenotypic and genetic association of ErbB ligands with schizophrenia, their impact on the associated audio-pathophysiological deficits has not been fully characterized.

Maternal infections and obstetric complications induce the expression of ErbB ligands (EGF, TGFα, and neuregulin-1) in fetal amniotic fluids or the neonatal brain.[27–29] These molecules transmit peripheral signals through the developing blood-brain barrier, disrupting the structural and functional development of the brain, as proposed in the neurodevelopmental hypothesis for schizophrenia.[30,31] In agreement with this hypothesis, rodents perinatally challenged with EGF, neuregulin-1, or other ErbB ligands exhibit behavioral endophenotypes that are relevant to schizophrenia, such as prepulse inhibition of sound startles.[32–34] In addition, our pilot study on frequency MMN of the EGF-challenged rats has raised the hypothesis that the developmental deficits in ErbB signaling might contribute to the whole series of audio-pathophysiological deficits found in this disorder, but the experimental evidence of this hypothesis is limited.[35–39]

In the present study, therefore, we comprehensively evaluated the developmental impact of hyper-ErbB/EGF signaling on various audio-pathophysiological traits associated with schizophrenia. In the place of the frequency MMN we previously examined,[39] we newly adopt the duration MMN (dMMN) paradigm that involves distinct brain circuits or functions.[40,41] For these purposes, we challenged rat pups with the ErbB ligand, EGF, and characterized its consequences on these auditory responses. In addition, we compared the antipsychotic responses of the present rat model with those reported in patients with schizophrenia.

Methods

Full methods and resource information are provided in the supplementary Methods.

Animals

Newborn male Sprague-Dawley rats were obtained from a local vendor (SLC). After weaning, 2–3 male rats per cage were housed under a reversed 12-hour light/dark cycle (8:00 AM OFF and 20:00 PM ON). After surgery, rats were given foamed soft baits (CMF sprouts) to assist their normal body weight gain.[41] All physiological measurements were performed in the dark cycle, following the intensive handling of rats. All animal experiments were approved by the Animal Care and Use Committee of Niigata University and performed in accordance with the Guide for the Care and Use of Laboratory Animals of the Japan Neuroscience Society.

ASSR Recording

Rats (9–11 wk old) were anesthetized with the medetomidine-based mixture during surgery.[41,42] Four stainless-steel bolts were screwed to the skull to contact the dura mater at the right primary auditory cortex, the right frontal cortex, the frontal sinus (reference), and the cerebellum (ground) as described previously.[41,42] The maximum sound pressure level (SPL) was set to 80 dB at 5 cm above the center of the chamber floor. The stimuli comprised 1000-ms click trains composed of 1-ms DC pulses presented at 20, 40, 60, and 80 Hz.[43] The order of the stimulation frequencies was pseudorandomly selected. For data analyses, see supplementary Methods.

MMN and P3a Recording

The stimuli were 12-kHz sine tones with the maximum SPL set to 85 dB.[42] Tones were presented using a duration oddball paradigm with a short- vs long-deviant conditions. Standard tones (50-ms duration for the long-deviant condition and 150-ms duration for the short-deviant condition; 90% probability) and deviant tones (the opposite allocation, 10% probability) were presented a total of 2000 times with a stimulus onset asynchrony of 500 ms.

Cortical ON Response and ABR Recording

Rats (12–17 wk old) were anesthetized with chloral hydrate (400 mg/kg, ip). We monitored auditory ON response from electrocorticography (ECoG) electrode on the auditory cortex. The 100-ms sine tones of 2, 8, 16, and 20 kHz were delivered in pseudorandom order. Stimulus sounds at 50 dB SPL were initially provided and thereafter increased in 10-dB steps. Only in the ABR test, we placed subcutaneous electrodes on the parietal cortex and in the pinnae and neck. The 2-ms tone bursts of 2, 8, 16, 24, and 32 kHz were produced by a tweeter, and their responses were amplified 1000-fold, bandpass filtered (0.3–2 kHz), digitized, and averaged across 500 trials.

Drug Treatments

To activate ErbB signaling perinatally, recombinant human EGF (0.875 µg/g/d; Higeta Shoyu) was subcutaneously administered to male rat pups repeatedly on postnatal days 2–10.[41] Control littermates received saline injections. After growth, risperidone (RIS; Janssen Pharmaceuticals) was administered for at least 2 weeks in drinking water (60 mg/L) with a target dose of 3 mg/kg/day. The final doses, which were estimated with the amount of consumed water, were indistinguishable between groups; 3.47 ± 0.11 mg/kg/day for control rats

and 3.68 ± 0.12 mg/kg/day for EGF rats (their difference $P = .22$, Student's t-test).

Statistical Analysis

Statistical details are shown in supplementary table 1. All data in each group were initially subjected to the Shapiro-Wilk test and Levene's test to test their normality and homogeneity, respectively. Non-normally distributed data were analyzed using the Mann-Whitney U-test. Normally distributed data were analyzed using Student's t-test or Welch's t-test. Time series data were analyzed by 2-way repeated ANOVA with Greenhouse-Geisser correction followed by Shaffer's modified post hoc tests. A P-value less than .05 was significant.

Results

Impaired ASSRs in EGF-Challenged Rats

EGF is known to penetrate the blood-brain barrier and activate ErbB1 and ErbB2 receptors in the neonatal brain.[17] Here, we investigated whether the ErbB receptor activation later influences ASSRs in the rat auditory cortex using ECoG. Click stimulation at various frequencies produced distinct responses in grand average event-related spectral perturbations (ERSPs) (figure 1A) and intertrial phase coherence (ITPC) (figure 1B). ERSPs in EGF-challenged rats were significantly lower at 40 Hz but higher at 20 Hz compared with those of the controls ($P < .05$, $n = 14$–18 each, Student's or Welch's t-test) (figure 1C). The ITPC in EGF-challenged rats displayed similar alterations ($P < .01$, Student's or Welch's t-test). These effects were largely stable throughout the stimulus presentation period (supplementary figure 1A). Additionally, only control rats tended to show 40-Hz harmonic activity in response to the 20-Hz stimuli (supplementary figure 2). There was no difference in the baseline power between rat groups, however (supplementary figure 1B). These results indicate that perinatal EGF challenges result in abnormal phase synchronization in the auditory cortex.

Impairments in dMMN- and P3a-Like Responses

Although MMN-like responses of the same EGF-challenged rats were assessed with the frequency deviation,[41] it has been proposed that reduced/delayed dMMN would be a better biomarker for schizophrenia.[44–46] We employed the oddball paradigms with 50-ms and 150-ms sine tones (both 12 kHz) in their short- and long-deviant conditions and measured dMMN-like potentials from the auditory cortex electrode as well as P3a-like potentials from the frontal cortex electrode (figure 2A). In the long-deviant condition, the deviant stimulus produced more negative ERPs than the standard stimulus in both groups (both $P < .05$, $n = 10$ each, Shaffer's test; figure 2B), whereas no ERP differences by the stimulus type were observed in the short-deviant condition (supplementary figures 3A and 3B). Therefore, the long-deviant condition only resulted in dMMN-like waveforms (deviant ERP minus standard ERP; figure 2C). The peak amplitude of the dMMN-like potential was lower in EGF-challenged rats than that in control rats ($P < .05$, Welch's t-test; figure 2D). However, the peak latency did not differ between rat groups.

According to the previous P3a protocol for conscious rats,[47] we measured grand average ERPs from the frontal cortex electrode (figure 2E and supplementary figure 3C). The deviant stimulus produced significantly more positive ERPs than the standard stimulus in both groups (both $P < .05$, Shaffer's test; figure 2E). The peak latency for the deviant stimulus appeared to be delayed in the EGF group. In the short-deviant condition, however, no significant difference by stimulus type was observed in either group (supplementary figure 3D). Figure 2F shows the P3a-like waveforms in the long-deviant condition, which were calculated from the ERP difference between the stimulus types. The peak amplitude was not different between the groups ($P = .40$, Student's t-test; figure 2G), although the peak latency was significantly longer in EGF-challenged rats ($P < .05$, Student's t-test).

Of note, the magnitude of the dMMN-like potentials in control rats depended on the number of standards preceding the deviant, whereas, in EGF-challenged rats, this dependence was disrupted, as in schizophrenia (supplementary figures 4 and 5).[48,49] These results indicate that perinatal ErbB1/EGF hypersignals in rats disrupted their auditory dMMN- and P3a-like responses.

Abnormal Resting-State PAC Within the Auditory Cortex

In the resting and listening states of the above experiments, we calculated the cross-frequency PAC within or between the auditory and frontal ECoG data. In the resting states, theta-alpha/beta coupling and theta-gamma coupling were significantly elevated within the auditory cortex of EGF-challenged rats ($P < .05$, $n = 29$ and 30, Mann-Whitney U-test; figure 3A). However, there was no significant difference in cross-frequency coupling within the frontal cortex or between brain regions (figures 3B–D). In contrast, the listening-state PAC did not differ between any groups (supplementary figures 6A–D). There was no difference in the ECoG power spectra between groups (figure 3E and supplementary figure 6E). These results indicate that the increased PAC of EGF-challenged rats was limited to their resting states and within the auditory cortex.

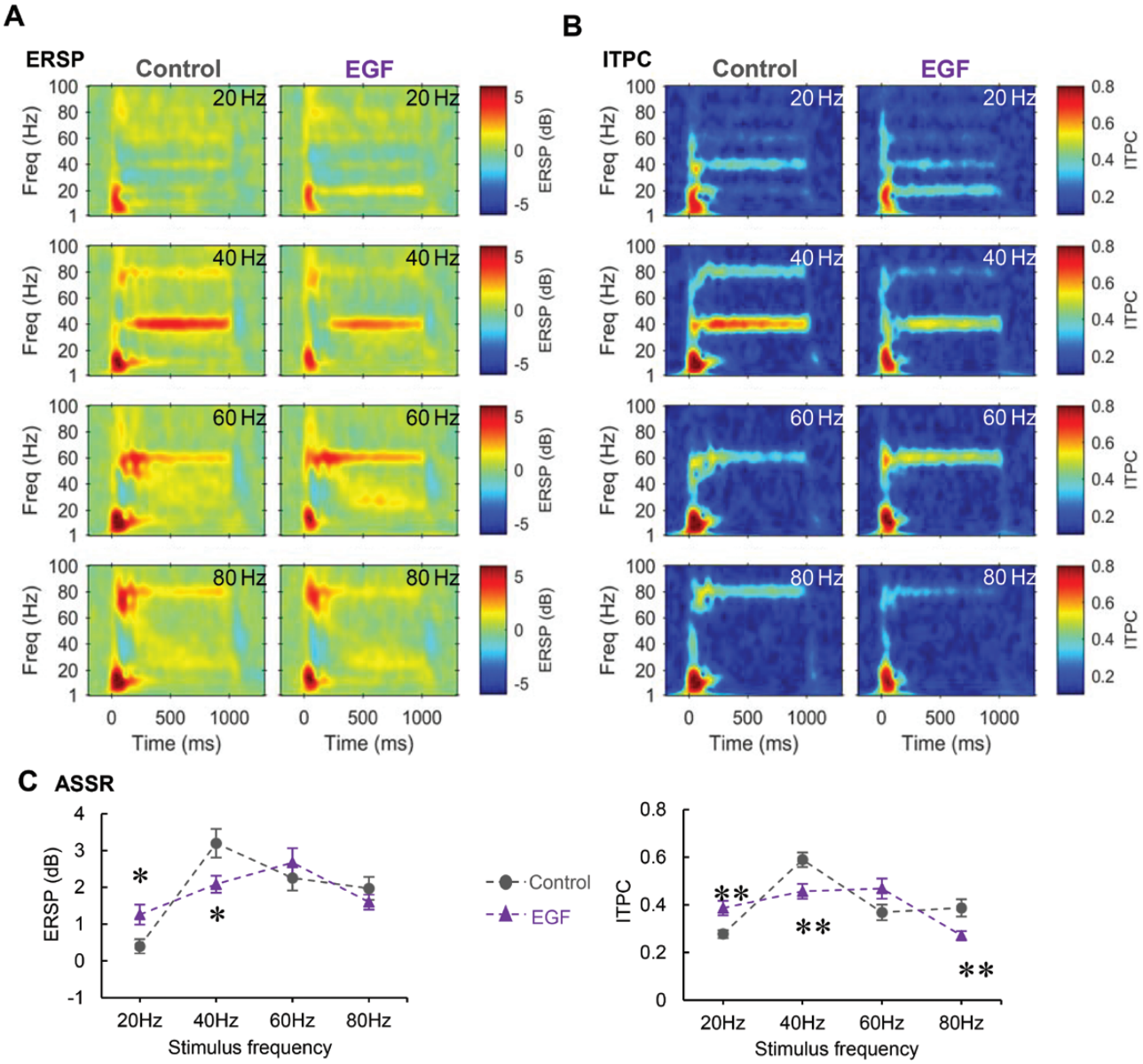

Fig. 1. Electrocorticogram responses to auditory steady-state stimuli. (A) Grand average event-related spectral perturbations (ERSPs) and (B) intertrial phase coherence (ITPC) for 20-, 40-, 60-, and 80-Hz auditory stimuli were recorded from the auditory cortex of awake control and EGF-challenged rats (n = 14–18, each). (C) ERSPs and ITPC of the auditory steady-state response (ASSR; 0–1000 ms) in the stimulus frequency ±3-Hz range at each stimulus frequency. Error bars denote standard error of the mean; *P < .05, **P < .01 between rat groups.

Cortical ON Response and ABR Thresholds Under Anesthesia

Does the above auditory pathophysiology involve principal deficits in their audibility? To address this question, we performed cortical ERP and ABR tests under anesthesia (figure 4 and supplementary figure 7). The anesthetic condition presumably minimized the central influences of sensory filtering or attention deficits[50,51] as well as those of ascending nerve efferents.[52,53] We measured the peak amplitudes of ON responses of the auditory ERPs (figure 4A). At the higher frequency ranges, the amplitudes were indistinguishable across the given tone intensities between rat groups. At the lower frequency ranges, their responses to 50- and 60-dB stimuli modestly differed, although the responses to 70 dB-stimuli produced marked differences in their amplitudes: higher at 2 kHz but lower at 8 kHz in the EGF group (P < .01, n = 5 and 6, Student's t- or Mann-Whitney U-test). In contrast, the ABR test produced controversial results (figure 4B). ABR thresholds in EGF-challenged rats were markedly elevated in the high frequency range (24, 32 kHz, P < .001; 16 kHz, P < .01; n = 10 each, Student t-, Welch t-, or Mann-Whitney U-test).

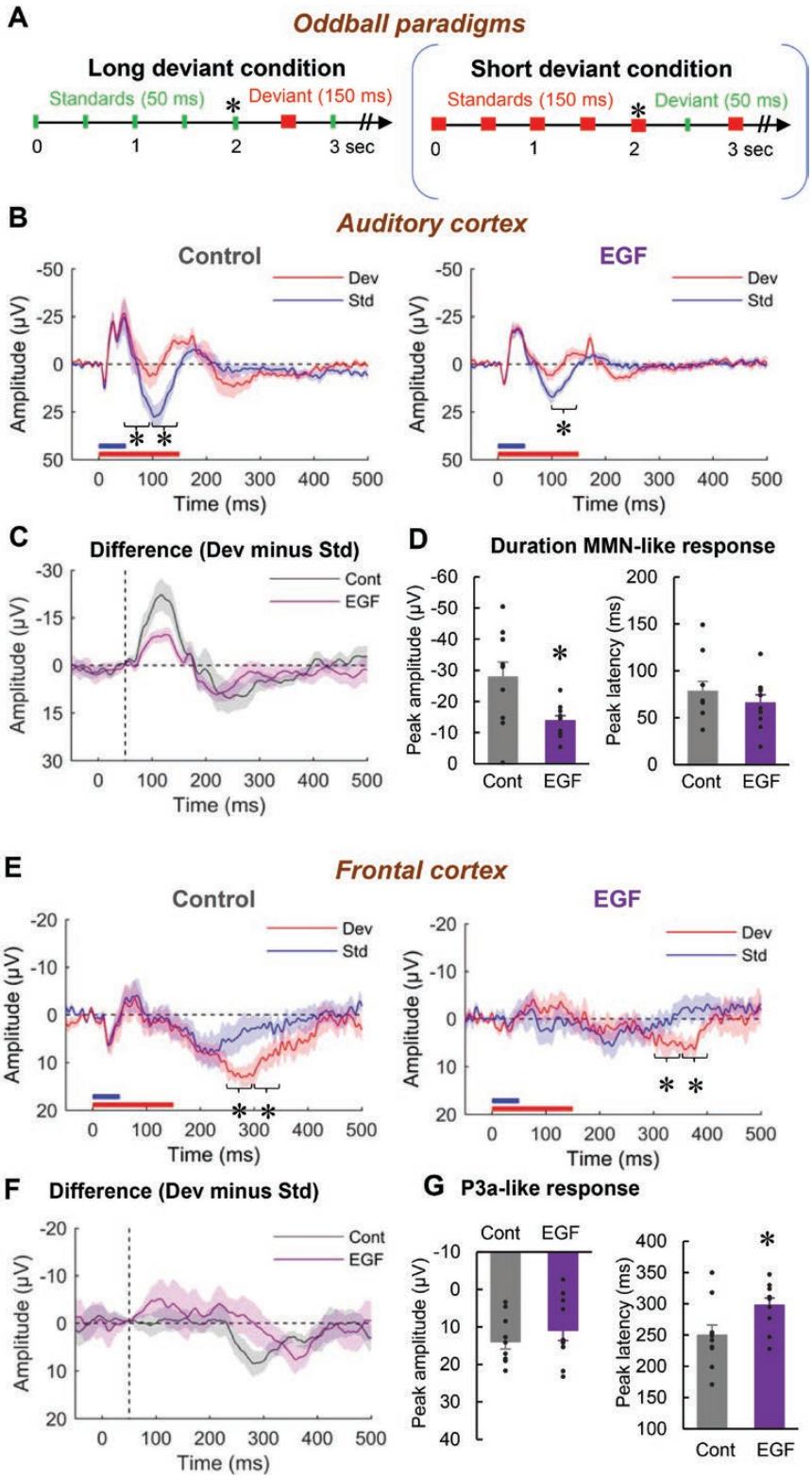

Fig. 2. Mismatch negativity (MMN)-like and P3a-like responses with the long-deviant condition. (A) Duration oddball sequences for short- and long-deviant conditions. Epochs were extracted for each deviant and the standard (*) immediately preceding it. (B) Grand average event-related potentials (ERPs) were recorded from the auditory cortex of awake control and epidermal growth factor (EGF)-challenged rats (n = 10, each). (C) Difference waveforms were calculated by subtracting the ERP for the standard stimulus from that of the deviant stimulus. (D) The peak amplitudes and their latencies of duration MMN-like responses. (E) Grand average ERPs were recorded from the frontal cortex of the above rats. (F) Difference waveforms were calculated by subtracting the ERP for the standard stimulus from that of the deviant stimulus. (G) The peak amplitudes and their latencies of P3a-like responses. Shaded areas denote standard error of the mean, $*P < .05$ between stimulus types or rat groups.

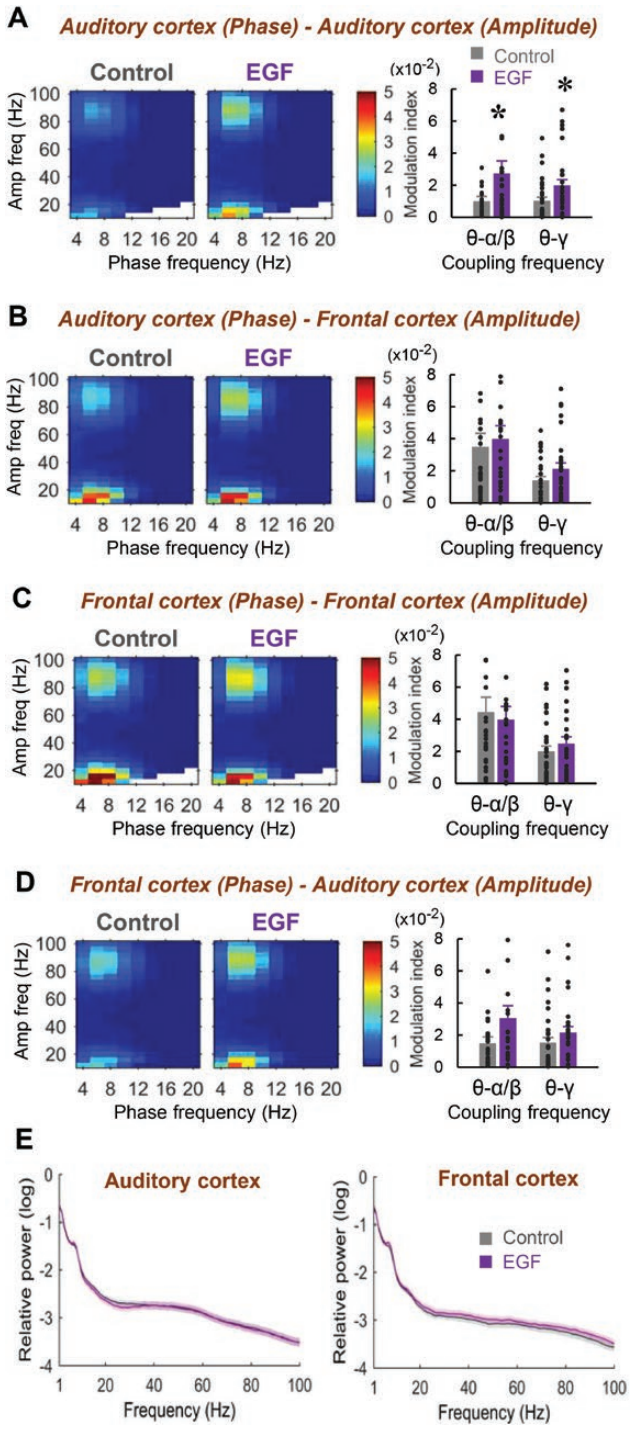

Fig. 3. Resting-state phase-amplitude coupling (PAC) and power spectral densities. (A–D) Grand average PAC modulation indices were calculated within the auditory (A) or the frontal cortex (C), or between the auditory and frontal cortices ([B] *Phase* vs *Amplitude* and [D] *Amplitude* vs *Phase*, respectively) of control ($n = 30$) and epidermal growth factor (EGF)-challenged rats ($n = 29$) for the acclimation (resting) period of the above auditory steady-state response (ASSR) and mismatch negativity (MMN) tests. The mean strength of theta-alpha/beta and theta-gamma coupling was compared between rat groups. (E) Power spectral densities at the resting-state condition. $^{*}P < .05$ between rat groups.

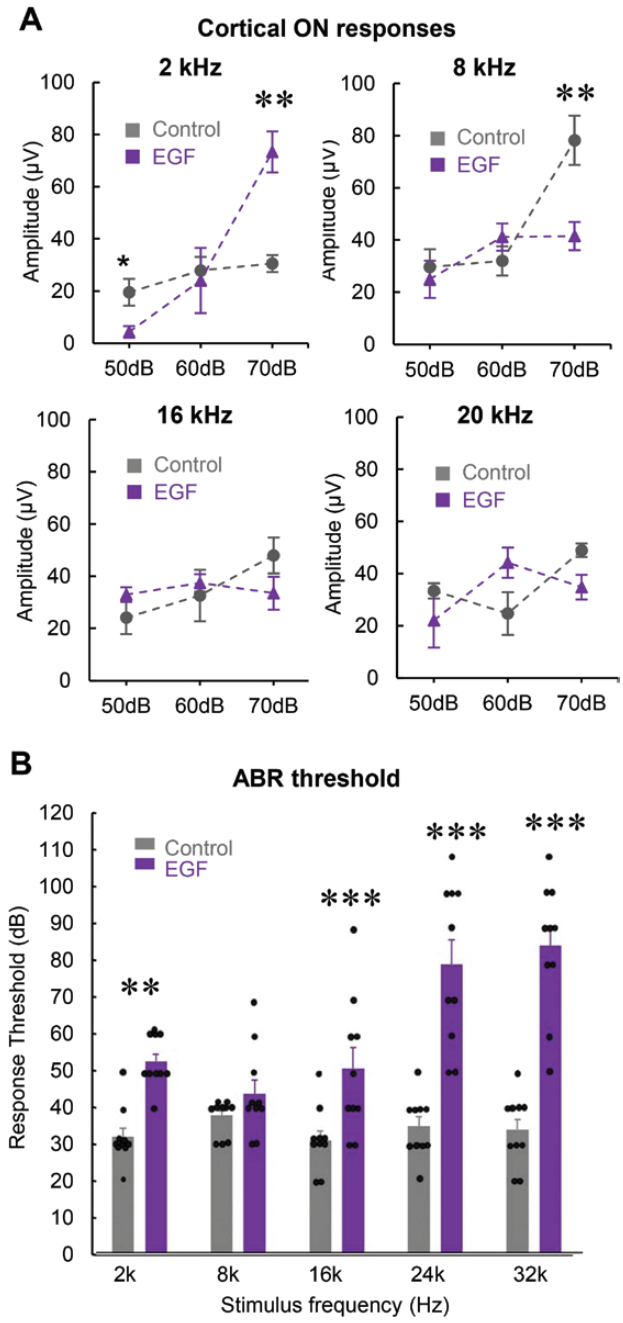

Fig. 4. Cortical ON responses and auditory brainstem responses (ABRs) at low sound pressure levels. (A) Grand average event-related potentials to various sine tones were recorded under anesthesia from the auditory cortex of control ($n = 5$) and epidermal growth factor (EGF)-challenged rats ($n = 6$). The peak amplitudes of ON responses are plotted against 3 sound pressure levels. (B) ABRs to 2–32 kHz tone bursts (20–110 dB) were monitored from control and EGF-challenged rats ($n = 10$, each). ABR thresholds were determined at each stimulus frequency. See real waveforms in supplementary figure 7. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ between rat groups.

The Antipsychotic Effects

Our previous studies have shown that subchronic treatment with antipsychotics ameliorates behavioral

and neurophysiological deficits of this rat model.[54,55] Therefore, we investigated the effects of RIS on dMMN- and P3a-like responses and resting-state PAC (figure 5). RIS treatment failed to affect the amplitude deficits of dMMN-like responses in EGF-challenged rats ($P < .05$, $n = 19$ and 22, Mann-Whitney U-test; figures 5A–C). As this treatment almost abolished the positive ERP responses, we hardly detected P3a-like potentials themselves (supplementary figure 8). The increases in the theta-alpha/beta and theta-gamma coupling were blunted within the auditory cortex of EGF-challenged rats treated with RIS (theta-alpha/beta, $P = .26$, theta-gamma, $P = .85$, both Mann–Whitney U-test) (figure 5D and supplementary figure 9). These results suggest that subchronic RIS administration could reverse the abnormally high resting-state PAC but not dMMN. RIS had no significant effects on the power spectral densities of the auditory cortex, however (figure 5E).

Discussion

Patients with schizophrenia exhibit a series of auditory physiological traits at the distinct test paradigms: dMMN, P3a, ASSR, ABR, and PAC. These audio-physiological responses are almost replicated by the present rat model, verifying their reverse translatability into rodents.[8] In particular, our findings indicate that perinatal exposure to the cytokine EGF disrupts various auditory oscillations and responses in rats, most of which are implicated in schizophrenia pathophysiology.

Accordance and Discordance of the ErbB/EGF-Induced Traits With Schizophrenia Pathophysiology

We compare the magnitude of auditory neurophysiological responses between schizophrenia patients and the present ErbB/EGF intervention in rats. Meta-analyses have reported that the effect size and disease specificity of these neurophysiological impairments are promising: 40-Hz ASSRs and MMN are reduced in schizophrenic patients with the effect sizes of 0.33–0.83 and 0.45–0.73 with a 95% confidence range, respectively.[45,56] The latency of P3a is delayed in patients with an effect size of 0.38–0.75.[57] In the present study, EGF-challenged rats attained effects sizes of 1.24, 0.85, and 1.06 in dMMN, 40-Hz ASSR, and P3a, respectively. Therefore, the neuropathological traits produced in the present study are either comparable to or more pronounced than those observed in patients with schizophrenia.

In addition, individual pathophysiological profiles of EGF-challenged rats exhibited many similarities to those reported for patients with schizophrenia. In the ASSR test, the present ErbB/EGF intervention also replicated the power alterations in 80- and harmonic 40-Hz ASSRs as well as the changes in their phase synchronization.[58–60] Although we found a discordant increase in 20-Hz ASSRs in this rat study, a clinical study on patients with schizophrenia found a similar increase in 20-Hz response with a low SPL.[60] In addition, the enhancement in theta-alpha/beta and theta-gamma PAC is also consistent between the present results and reports on patients with schizophrenia.[4–6]

Some of our results contradict those of previous reports on schizophrenia. One of these is the antipsychotic effect on P3a; the antipsychotic medication shortens P3a latency in patients with schizophrenia,[61] whereas it suppressed P3a amplitudes in the present rat study. Another controversy is that dMMN-like potentials in the short-deviant paradigm were blunted in the present study. The result that the dMMN-like responses were sensitive to the degree of preceding stimulus regularity in control rats allows us to assume that the potential changes observed here are relevant to dMMN in humans.[48] Compared with our pilot study on frequency MMN of the same EGF-challenged rats, the electrode position detecting MMN-like potential was different; frequency MMN-like potentials were recorded from the electrode on the frontal cortex, whereas the present dMMN-like potentials were from that of the auditory cortex. Although the positional difference in MMN detection is not clear in EEG recording on the human scalp, this finding appears to agree with the literatures that the deviation detection system presumably differs between frequency and duration deviation of tone stimulus.[40,41] These MMN differences between humans and rats might originate from the differences in electrode positioning or structural differences in their auditory system as suggested in a study of rat MMN.[62]

ErbB Signaling in the Audio-Pathophysiological Traits Implicated in Schizophrenia

Previous postmortem studies have indicated an association of schizophrenia with the ErbB1/EGF system as well as with the ErbB4/neuregulin system.[17,26] Genetic studies support this association,[15,22,23] although there are several controversies.[63,64] This notion has been tested by various animal models.[65,66] ErbB1 and ErbB4 receptors are similarly distributed in various neural cells: glial cells, GABAergic neurons, and dopaminergic cells.[19–21] EGF-driven or neuregulin-evoked ErbB signals regulate their phenotypic development as well as their expression of glutamate receptors.[38,67–69] Therefore, rodents with disrupted ErbB signaling are often employed to model the GABAergic or dopaminergic deficits in schizophrenia.[19–21] Thus, it is possible that GABAergic or dopaminergic impairments may contribute to the auditory abnormalities in this rat model.

There are only a few studies on auditory neurophysiology in neurodevelopmental models of schizophrenia. Rats receiving neonatal ventral hippocampal lesions showed no reduction in the 40-Hz ASSRs in the awake

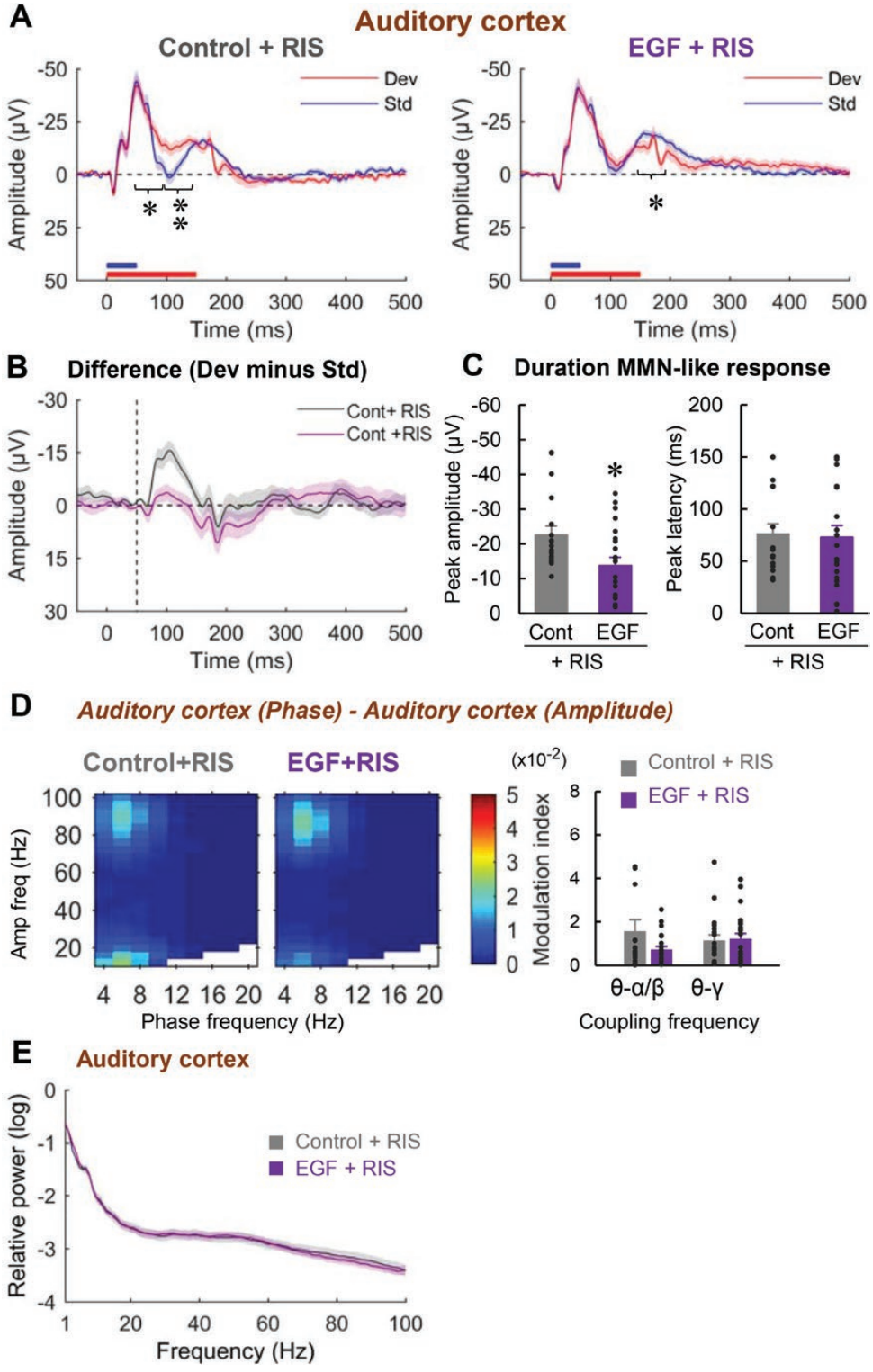

Fig. 5. Effects of risperidone (RIS) on impaired duration mismatch negativity (dMMN)-like responses and resting-state phase-amplitude coupling (PAC). (A) Event-related potentials (ERPs) from the deviant and standard tones were recorded from awake control (n = 19) and epidermal growth factor-challenged rats (n = 22), which had been orally treated with RIS for 2 weeks. (B) Difference waveforms were calculated by subtracting the ERP for standard stimulus from that of the deviant stimulus. (C) The peak amplitudes (left) and their latencies (right) of dMMN-like responses. (D) PAC modulation indices were calculated in the resting-state condition (left panels). Bars represent the mean strength of theta-alpha/beta and theta-gamma coupling (right panels). (E) Power spectral densities of the auditory cortex in the resting-state condition. *P < .05, **P < .01.

auditory cortex,[70] whereas a study using microelectrodes under urethane anesthesia reported that 40- and 80-Hz ASSRs were decreased at the posterior auditory cortex.[71] Decreased MMN-like responses have also been reported in this rat model.[72] Of note, a previous pharmacological study found a substantial deficit in ErbB1 signaling in this rat model, supporting the present conclusion.[73] Another rat model, which is established by prenatal exposure of rats to methylazoxymethanol, exhibits MMN deficits,[74] although the contribution of ErbB signaling to this model remains unknown.

In addition, there is the audio-pathophysiology of the mouse genetic mutants, which lack the genes of NMDA receptor, Src, or phospholipase C.[41,75–77] These mutant mice have shown auditory deficits in either MMN or ASSR. With respect to ErbB signals in their auditory deficits, it is of great interest that EGF-driven ErbB1 activation potently evokes those signal cascades of Src and phospholipase C to modulate the expression of AMPA and NMDA receptors.[19,43,76,77]

Limitations of Data Explanations

The ABR deficits in patients appear to match the ABR deficits of this rat model,[9,10] indicating the possibility that the effects of perinatal EGF challenge may spread to either the brainstem or the cochlear level. However, previous behavioral tests of EGF-challenged animals have not revealed any obvious decline in sound recognition or audibility.[32,78] These behavioral-level trends appear to agree with their audibility estimated by the cortical ON responses and ASSRs. The modest alteration in cortical ON responses and ASSRs cannot directly account for the ABR reduction in the high-frequency ranges of this rat model. However, the known biological role of ErbB suggests its potential influences on the lower auditory system; EGF-evoked ErbB1 signals promote the growth of supernumerary hair cells, which do not directly influence ABR thresholds,[79] whereas neuregulin-1-triggered ErbB2/B3 signals determine the survival of spiral ganglion neurons, which presumably influences ABRs.[80–83] However, we do not rule out that the present ErbB/EGF intervention produced any influences on the brainstem or cochlear functions.

Technical differences must be considered when comparing electrophysiological data between humans and rats. EEG is recorded from electrodes on the human scalp, whereas ECoG is recorded from electrodes on the rat dura mater. The other is the variation in brain size. There is another technical issue; the repeated sound stimuli over a long period might promote rats to sleep, especially during the ASSR test. As sleeping suppresses ASSR in humans,[84] we omitted the data from the analysis, which represented the sleep states. The arousal levels of rats were not further controlled for the final analysis, however. As EGF mainly activates ErbB1 (EGFR) and ErbB2 signaling, the audio-pathophysiological consequences of ErbB3/4 activation remain unknown and require future studies.[19] Thus, the present discussions must keep these limitations in mind.

Conclusions

The entire set of disease-associated auditory traits was almost reproduced by the perturbation of the single molecular cascade from ErbB1/EGF. These findings suggest that abnormal ErbB signals during development, which can be triggered by infection, inflammation, and hypoxia, might contribute to the auditory pathophysiology of patients with schizophrenia.

Supplementary Material

Supplementary data are available at *Schizophrenia Bulletin Open* online.

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