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Acoustic enrichment prevents early life stress-induced disruptions in sound azimuth processing

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Abstract

Early life stress (ELS) has been shown to disrupt cognitive and limbic functions, yet its impact on sensory systems, particularly the auditory system, remains insufficiently understood. In this study, we investigated the enduring effects of ELS induced by neonatal maternal separation (MS) on behavioral and cortical processing of sound azimuth in adult male rats. We found that MS significantly impairs sound-azimuth discrimination, paralleled by broader azimuth tuning and reduced dendritic branching and spine density in neurons within the primary auditory cortex. Notably, exposure to an enriched acoustic environment during the stress period effectively protects against these MS-induced alterations, restoring behavioral performance, cortical tuning, and dendritic spine density of neurons to levels comparable to controls. Further analyses reveal that epigenetic regulation of cortical brain-derived neurotrophic factor by histone H3 lysine 9 dimethylation may underlie the observed changes in cortical structure and function. These results underscore the profound and lasting impact of MS-induced ELS on auditory processing, particularly within cortical circuits involved in spatial processing. They suggest that sensory enrichment is a potential therapeutic strategy to ameliorate the adverse effects of ELS on sensory processing, with broader implications for understanding and treating sensory deficits in stress-related disorders.

Keywords: auditory cortex, behavioral discrimination, cortical processing, early life stress, acoustic enrichment

Significance Statement

The contribution of early life stress (ELS) to sensory deficits in stress-related disorders remains largely unexplored. Here we show that ELS induced by neonatal maternal separation (MS) disrupts behavioral and cortical processing of sound azimuth in adult rats. Moreover, pairing MS with enriched acoustic exposure during the stress period alleviates these deficits in maternally separated rats. Epigenetic modulation of brain-derived neurotrophic factor gene expression by histone H3 lysine 9 dimethylation in the cortex may underlie the MS-effects and their reversal through acoustic enrichment. These findings reveal the enduring effects of ELS on sensory processing, emphasizing its broader implications for understanding stress-related disorders. Importantly, they highlight sensory enrichment as a promising therapeutic strategy to prevent sensory deficits associated with such conditions.

Introduction

Brain function depends on maintaining internal stability despite ever-changing external environments, with stress responses representing a major disruptor of this equilibrium. Previous studies in animal models have shown that early life stress (ELS), such as that induced by neonatal maternal separation (MS), often leads to lasting impairments in anxiety regulation, learning and memory, and decision-making (Cao et al., 2014; Banqueri et al., 2021; Alves et al., 2022; Kim et al., 2023). These functional disturbances are accompanied by alterations in neurotransmitter levels, synaptic plasticity, and neuronal morphology (e.g., dendritic branching and spine density) within critical brain regions including the prefrontal cortex (Monroy et al., 2010; Muhammad et al., 2012; Chocyk et al., 2013; Farrell et al., 2016), hippocampus (Monroy et al., 2010; Cao et al., 2014; Ohta et al., 2017; Kim et al., 2023), amygdala (Manzano-Nieves et al., 2020), and nucleus accumbens (Monroy et al., 2010; Muhammad et al., 2012). These findings underscore the profound impact of MS-induced ELS on the development of cognitive and emotional functions.

The sensory systems of the mammalian brain are known to develop and mature earlier than its cognitive functions, making them particularly susceptible to environmental influences during early development. For example, rodent studies have shown that while cognitive systems continue to refine throughout the juvenile and adolescent periods (Alberini and Travaglia, 2017), the auditory system, including auditory pathways and cortical areas, undergoes rapid maturation during the early postnatal period (de Villers-Sidani et al., 2007; Insanally et al., 2009; Barkat et al., 2011; Nakamura et al., 2020).

During this critical developmental window, environmental stimuli and experiences exert significant and enduring effects on auditory processing and integration (Zhang et al., 2001; Han et al., 2007; Zhou and Merzenich, 2008; Polley et al., 2013; Suta et al., 2015; Bures et al., 2017,2021; Pysanenko et al., 2018; Svobodova Burianova and Syka, 2020; Tang et al., 2022). In addition, sensory systems are interconnected with other brain structures essential for cognitive functions via direct and indirect pathways, thereby contributing to various higher cognitive functions (Moxon et al., 1999; Mohedano-Moriano et al., 2007; Kraus and Canlon, 2012; Xiao et al., 2018; Zhao et al., 2018). The differential timing in the maturation of sensory and cognitive systems suggests that early sensory inputs can profoundly influence cognitive development, particularly if sensory functions are disrupted during the critical periods.

However, few studies to date have investigated the long-term effects of MS-induced ELS on sensory processing. In the auditory system, Ye et al. (2023) recently showed that ELS induced by a combination of MS and restraint disrupts both the behavioral detection and neural encoding of rapid sound signals in adult gerbils. Despite these significant results, the precise neural mechanisms through which ELS affects auditory function later in life remain largely unknown.

Sound localization is a fundamental task of the auditory system, with the auditory cortex playing a key role in spatial sound processing (Jenkins and Merzenich, 1984; Kavanagh and Kelly, 1987; Heffner and Heffner, 1990; Zhang et al., 2013; Cheng et al., 2017,2020). It enables efficient navigation, communication, and interaction with the environment by providing critical

spatial auditory information. In humans, spatial hearing helps distinguish between different speakers, facilitating speech comprehension in social settings and noisy places, e.g., where multiple conversations occur simultaneously (Hawley et al., 2004; Grothe et al., 2010; Zündorf et al., 2013). However, the specific effects of MS-induced ELS on the auditory cortex and its implications for sound localization have not been thoroughly investigated.

In this study, we examined physiological changes within the primary auditory cortex (A1) and their impact on spatial behavior in adult rats following neonatal MS. We also explored structural and molecular changes in the cortex and their epigenetic modifications to understand the enduring effects of MS on adult rats. Finally, we assessed whether enriched sound exposure during MS could protect against the observed alterations in behavioral and cortical processing of sound azimuth in maternally separated rats.

Materials and Methods

Pregnant Sprague-Dawley (SD) rats were purchased from Shanghai Slack Laboratory Animal Co., Ltd. (China) and housed individually in cages (41×26×20 cm) at the Animal Experiment Center of East China Normal University. Housing conditions were kept at 21±1 °C with 45-55% humidity and an automatic 12/12-h light-dark cycle. The animals had ad libitum access to food and water. All experimental procedures were approved by the Institutional Animal Care and Use Committee of East China Normal University. Every effort was made to minimize animal suffering and reduce the number of animals used.

Neonatal MS

Male offspring from timed-pregnant SD rats were cross-fostered to form letters of 10 per each at postnatal day (PND) 1 (birth was designated as PND0). The pups were then randomly assigned to either MS or control group, with 5 pups per group. The MS protocol was based on early studies with minor modifications (Shin et al., 2019; Talani et al., 2023). Briefly, five pups were gently placed into a clean cage, transferred to a different room, and kept in a temperature-controlled incubator (26±2 °C, 45-55% humidity) for 3 h daily (10:00 to 13:00) from PND2 to 20. After the 3-h separation, the pups were returned to their home cage with their dams. Control pups were handled twice daily at 10:00 and 13:00, being moved from one side of the cage to the other, but remained with their dams throughout.

At weaning (PND21), all pups were separated from their dams and housed in groups of four per cage until PND56, during which various experimental procedures were conducted.

Enriched sound exposure

The MS pups were exposed to an enriched acoustic environment (EAE) during their daily 3-h MS period in the incubator from PND2 to 20. Acoustic stimuli consisted of pulse trains with a duration of 1 s, containing 2, 5, 10, or 15 tone pips (50 ms duration with 5 ms ramps). The frequency of each tone pip in the pulse train was set at 1.5, 2.3, 3.5, 5.3, 8.1, 12.3, 19, or 29 kHz. These pulse trains with different frequencies and repetition rates were randomly delivered at ~65 dB sound pressure level (SPL) as measured at the center of the incubator. To minimize adaptation, a silent interval (randomly set

at either 0.5 or 1 s) was introduced between pulse trains. After weaning at PND21, pups were housed in groups of four per cage until PND56 for experiments.

Open field test

The open field test was conducted in a rectangular box (42×42×37 cm). During the test, each rat was placed in the center of the box, facing the wall, and allowed to explore freely for 15 min. The total distance traveled was recorded and analyzed using the True-Scan System (Coulbourn Instruments, USA). The box was cleaned with 75% ethanol between tests.

Elevated zero-maze test

The elevated zero-maze consisted of a circular platform (5.5 cm wide) with an outer diameter of 92 cm, featuring two open arms and two closed arms positioned opposite each other. The closed arms had 20 cm high walls, while the open arms had no barriers. The apparatus was raised 50 cm above the floor. During testing, each rat was placed in one of the open arms, facing a closed arm, and allowed to explore freely for 5 min. Behavior was recorded and analyzed using the ANY-maze system (Stoelting, USA). Between tests, the maze was cleaned with 75% ethanol.

Sound-azimuth discrimination task

A wooden semicircular apparatus with a 150 cm radius was used for the sound-azimuth discrimination task. At the center, a starting box (20×7×8 cm) allowed the rats to initiate the task by running forward. Speakers were positioned at 10° intervals along the curved wall, each with a waterspout beneath. Water from these spouts was delivered via an automatic lick-

detection system. Each trial consisted of six bursts of white noise (50 ms duration, 5 ms rise-decay, ~65 dB SPL) randomly emitted at 2 pulses per second (pps) from one of the speakers. Rats were trained to identify the sound azimuth and lick the corresponding waterspout for a water reward.

Rats had ad libitum access to food but were water-restricted prior to the task (Rutkowski and Weinberger, 2005; Zhang et al., 2013; Cheng et al., 2020). The task was conducted in a soundproof, double-walled room. The behavioral task consisted of two phases: pre-training and training. In the five-day pre-training phase, rats learned to exit the starting box, approach a waterspout, and lick it to receive water in response to auditory cues from a fixed angle (0º). In the training phase, rats were placed in the starting box facing forward. They left the starting box after random sound bursts were played from one of the speakers. The trial concluded when rats licked a spout. If they approached and licked the spout under the correct speaker, they received 2-3 drops of water as a reward, marking the trial as successful. If they licked an incorrect spout, the trial was recorded as an error. The rats returned to the starting box after each trial.

Each rat participated in ~35 trials per day, with the percentage of correct responses calculated at the end of each day. The task was considered complete once a rat achieved ≥70% correct responses on 2 of 3 consecutive training days.

Auditory brainstem response (ABR) and cortical recording

Recordings were conducted in a soundproof, double-walled room. As described previously (Zhang et al., 2013; Liu et al., 2019; Cheng et al.,

2020,2023), rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg per kg body weight). Areflexia was maintained with supplemental doses of 8 mg/ml dilute pentobarbital during surgery and recording sessions. Body temperature was continuously monitored using a rectal probe and maintained at ~37 °C with a feedback-controlled heating pad.

ABRs were recorded using three subdermal electrodes placed at the scalp midline, posterior to the stimulated ear, and on the midline of the back 1-2 cm posterior to the neck. Tone pips (3, 10, 15, or 20 kHz) at different intensities were generated using TDT System III (Tucker-Davis Technologies, USA) and delivered through a calibrated earphone with a sound tube inserted into the external auditory meatus. ABR signals were acquired, filtered, amplified, and analyzed using equipments and software manufactured by Tucker-Davis Technologies (USA). The ABR threshold was defined as the lowest sound intensity capable of eliciting a characteristic response pattern.

For cortical recording, a 2 cm flat-headed nail was adhered to the exposed skull with acrylic glue and dental cement. The rat's head was secured in a head-holder, with the eye-snout line aligned to 0º azimuth and 0º elevation in the frontal auditory field. After reflecting the temporalis muscle, the auditory cortex was exposed, and the dura was carefully removed. Parylene-coated tungsten microelectrodes (1-2 MΩ at 1 kHz; FHC, USA), which were inserted perpendicularly into the cortex at a depth of ~450-550 μm (layer IV; Games and Winer, 1988; Roger and Arnault, 1989), were used to record the spike activity of individual neurons or small neuron clusters. The A1 location was identified according to the Paxinos and Watson atlas (Paxinos and Watson, 2005) and other studies (Polley et al., 2006,2007; Rutkowski et

al., 2003). Neurons in A1 typically exhibit short-latency responses to tone pips at specific frequencies.

Acoustic stimuli (tone pips of 50 ms duration, 5 ms rise-decay) were played from a speaker placed 34 cm away from the rat's head. The speaker was moved to different azimuths in the frontal auditory space using a remote-controlled system operated by electric motors. Recordings began with stimuli emitted from a speaker positioned 30º contralateral to the recording site (c30º). The cortical responses evoked were recorded and frequency tuning curves were constructed by presenting pure tones at 50 different frequencies (1-30 kHz) across eight sound intensities (ranging from 0 to 70 dB SPL in 10-dB steps) in a randomized, interleaved manner at a rate of 2 pps. Characteristic frequencies (CFs) were defined for neurons at each cortical site as the frequency that elicited a reliable response at the lowest threshold (i.e., minimum threshold, MT). After determining the CF and MT at each recording site, the number of responses elicited by CF stimuli (20 dB above the MT) was recorded at 10° intervals, from 90° contralateral (c90°) to 90° ipsilateral (i90°) relative to the recording site. These stimuli were presented 32 times at each azimuth, and the azimuth-selectivity curve was plotted by correlating the total spike count (adjusted for spontaneous activity) with the azimuth angle. Software programs (SigCal, SigGen, and Brainware; Tucker-Davis Technology, USA) were used for speaker calibration, stimulus generation, online monitoring of cortical responses, and data storage for later analysis.

Azimuth-selectivity curves were classified into four categories based on their shapes: azimuth-selective, hemifield, multipeak, or nonselective. Azimuth-selective curves featured a prominent peak at a specific angle that is

at least 50% higher than the minimum at both lateral angles. Hemifield curves started at an ipsilateral angle, increased by more than 50%, and then either plateaued or decreased by less than 50% across a broad range of contralateral angles. Multipeak curves displayed two distinct peaks, each exceeding the trough and lateral angles by at least 50%. Lastly, nonselective curves lacked a clear peak, with spike counts across all angles varying by less than 50%.

Corticosterone analysis

Rats were anesthetized with pentobarbital (50 mg/kg body weight), and blood samples were collected and stored at 4 °C overnight to allow clotting. The following day, the samples were centrifuged at 3000 rpm for 10 min to separate the serum. Serum corticosterone levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Shanghai Xinyu Biotechnology, China) following the manufacturer's instructions.

Morphological analysis of neurons

Four-week-old rats were anesthetized with pentobarbital (50 mg/kg body weight) and secured in a stereotaxic apparatus (Stoelting, USA). After exposing the skull, small holes were drilled above both hemispheres of the auditory cortex. A total volume of 200 nl of recombinant adeno-associated virus (AAV-sparse-NCSP-YFP-2E5; Braincase, China) was bilaterally injected into the A1 at coordinates (AP: -4.5 mm, ML: ±6.5 mm, DV: -4.5 mm; Paxinos and Watson, 2005) using a syringe. The virus was delivered at 20 nl/min using a micro-infusion pump (RWD, China). After injection, the needle remained in place for 10 min to allow diffusion, and the scalp was sutured. The animals

were returned to their home cages for recovery after waking from anesthesia. A four-week recovery period ensured full healing and optimal viral expression.

After the four-week recovery, rats were deeply anesthetized with pentobarbital (80 mg/kg body weight) and perfused intracardially with saline followed by 4% paraformaldehyde in 0.1 M potassium phosphate-buffered saline (pH 7.2). The brains were extracted and immersed in the same fixative with 20% sucrose for 12-24 h. Brain tissue was sectioned coronally at a thickness of 100 μm using a freezing microtome (Leica CM3050S, Germany). Sections were rinsed with phosphate-buffered saline (PBS) and mounted onto slides. Images of layer IV pyramidal neurons in A1 were captured using single-photon confocal microscopy (Leica SP8, Germany).

For spine density analysis, the acquired images were processed in Fiji software (NIH, USA), and dendritic spines were traced and categorized into stubby, mushroom, or thin subtypes (Yang et al., 2014; Tang et al., 2022). Stubby spines were defined as having a large head closely attached to the dendritic shaft without a distinct neck, mushroom spines had a large head and narrow neck, and thin spines had a small head and elongated thin neck. Spine density was calculated as the average number of spines per 10 μm segment of tertiary apical or basal dendrites.

In addition, dendrites were traced and cortical neuron morphology was reconstructed using the NeuronJ plug-in in Fiji. Sholl analysis quantified dendritic complexity by counting intersections of dendrites with concentric circles, incrementally spaced 10 μm apart from the soma.

Western blotting

Rats were deeply anesthetized with pentobarbital (80 mg/kg body weight). Following decapitation, the brains were quickly removed, and the A1 (Paxinos and Watson, 2005) were dissected. The tissue was homogenized in RIPA buffer (50 mmol/L Tris pH 7.5, 150 mmol/L NaCl, 1% Triton X-100, 0.1% SDS, 2 mmol/L EDTA, 0.5% sodium deoxycholate) containing protease inhibitor cocktail, as previously described (Liu et al., 2019; Tang et al., 2022). Equal amounts of protein from each sample were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, USA). The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline (TBS) (150 mM NaCl, 20 mM Tris-HCl, pH 7.4) for 1.5 h at room temperature (RT), followed by overnight incubation at 4 °C with primary antibodies. After three washes with TBST (containing 0.1% Tween 20 in TBS), the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at RT. Following additional washes, protein bands were visualized using the ChemiDocTM XRS+ System (Bio-Rad, USA), and the grayscale values of the bands were quantified using ImageJ software (NIH, USA). The primary antibodies used in this study were: rabbit anti-H3K9me2 (1:1000; Cell Signaling Technology, USA), rabbit anti-BDNF (1:800; Proteintech, USA), and rabbit anti-β-actin (1:1000; Proteintech, USA).

Experimental design and statistical analysis

Body weight, locomotor activity, anxiety-related behavior, and serum corticosterone levels were assessed as described above and compared across rat groups to confirm the induction of ELS by MS. In addition, ABR parameters, cortical tuning, dendritic spine density, and molecular expression

were analyzed to investigate the effects of MS-induced ELS on sound azimuth processing. Finally, the combination of MS and enriched acoustic exposure was explored to evaluate its potential to protect against alterations in sound azimuth processing in maternally separated rats.

Statistical comparisons were conducted using either an unpaired Student's t-test, Mann-Whitney test, or one- or two-way Analysis of Variance (ANOVA), depending on the experimental design. Prior to analysis, the Kolmogorov-Smirnov test was used to evaluate the normality of the data distribution. If significant effects were detected in the ANOVA, post hoc tests were performed for multiple comparisons. In addition, cumulative data distributions were compared across groups using the Kolmogorov-Smirnov test and proportion data were analyzed using the χ^2 test. Differences were considered statistically significant at $p<0.05$.

Results

Fig. 1 outlines the experimental timelines and procedures for the study. To confirm that ELS was induced by MS, we assessed several stress indicators, including body weight, locomotor activity, anxiety-related behavior, and serum corticosterone levels, in MS rats and age-matched controls at specific PNDs. On PND56, the animals were subjected to a sound-azimuth discrimination task, ABR recordings, cortical recordings, dendritic spine assessment, and molecular expression analysis to evaluate the lasting effects of MS on young adult rats.

Signs of ELS following MS

The body weights of both MS and control groups on different PNDs are presented in **Fig. 2A** and **B**. Statistical analysis revealed that the MS group had a consistently lower body weight than the control group (two-way ANOVA, $F(1,483)=28.00$, $p<0.001$; post hoc Student-Newman-Keuls test $p<0.001$-0.007 on PND35, 42, 49, and 56 but $p>0.31$ at other time points). Thus, early MS resulted in a moderate, lasting reduction in body weight, persisting at least until PND56.

Next, we conducted an open field test on PND21 and 56 to investigate the potential impact of MS on locomotor activity (**Fig. 2C**). As shown in **Fig. 2D**, the distance traveled by MS group during the different test periods was comparable to that by control group on both PND21 and 56 (two-way ANOVA, $F(1,111)=2.81$, $p=0.10$ on PND21 and $F(1,150)=0.02$, $p=0.89$ on PND56). These data suggest that MS has little effect on locomotor activity in the rats.

We also evaluated anxiety-related behaviors using the elevated-zero maze test (**Fig. 2E**). As shown in **Fig. 2F**, the total distance traveled by the MS group was similar to that traveled by the control group on PND21 (unpaired Student's t-test, $t(34)=0.67$, $p=0.51$). However, the MS group exhibited lower open arm entries (unpaired Student's t-test, $t(34)=2.45$, $p=0.02$) and spent significantly less time in the open arms (unpaired Student's t-test, $t(34)=2.08$, $p=0.045$), indicating increased anxiety-like behavior. On PND56, although the MS group traveled a shorter total distance compared to the control group (unpaired Student's t-test, $t(24)=2.58$, $p=0.017$), no significant differences between the groups were observed regarding open arm entries (unpaired Student's t-test, $t(24)=1.10$, $p=0.28$) or time spent in the open arms (Mann-Whitney test, $p=0.57$). Therefore, early MS led to increased

anxiety-related behaviors during the juvenile period, but these behaviors appeared to normalize by adulthood.

To further assess stress levels in rats following MS, we measured serum corticosterone levels on PND21 and 56 (**Fig. 2G**). On PND21, the MS group had significantly higher corticosterone levels compared to the control group (Mann-Whitney test, $p<0.0001$), but no significant differences were found on PND56 (unpaired Student's t-test, $t(30)=1.56$, $p=0.13$). These results indicate an acute corticosterone response to MS that diminishes by adulthood.

Performance in the sound-azimuth discrimination task

To determine the effects of early MS on behavioral processing of sound azimuth in adulthood, we compared the performance of MS and control rats in a sound-azimuth discrimination task on PND56. In this task, rats were trained to localize the azimuth of a sound stimulus and then lick the associated water-spout for a reward (see also **Materials and Methods**). As shown in **Fig. 3A**, the percentage of correct trials increased gradually for both MS and control rats as training progressed (two-way ANOVA, $F(16,218)=37.02$, $p<0.001$), indicating that both groups learned to identify and selectively respond to a new, randomly set target location. However, the average correct responses by the MS group were lower than those by the control group throughout the training period (two-way ANOVA, $F(1,218)=156.79$; post hoc Student-Newman-Keuls test $p<0.001$-0.023 on training days 2-15 but $p>0.17$ on other days). Specifically, the MS group took an average of 9.3±1.0 d and 17.6±0.9 d to achieve 50% and 70% accuracy, respectively, which was significantly longer than the respective 5.4±0.5 d and 9.8±0.8 d taken by the control group

(**Fig. 3B**; unpaired Student's t-test, t(15)=3.46, p=0.003 for 50% cut-off and t(15)=6.38, p<0.001 for 70% cut-off).

We also calculated the azimuth deviation (AD) as an index of performance in the error trials. AD represents the angular difference between the spout the rat licked and the position of the target speaker. Positive or negative AD values indicate that the spout was to the right or left of the target speaker, respectively. As shown in **Fig. 3C**, the proportions of positive and negative ADs were comparable on each training day (two-way ANOVA, F(1,264)=1.92, p=0.17 for the MS group and F(1,200)=2.83, p=0.094 for the control group), indicating no azimuth preference for either group during the task. Furthermore, we compared the absolute AD values between the MS and control groups, with smaller ADs indicating higher performance accuracy. As shown in **Fig. 3D**, AD values decreased with training for both groups (two-way ANOVA, F(16,218)=15.79, p<0.001), but the average values for the MS group consistently remained larger than those for the control group throughout the training period (two-way ANOVA, F(1,218)=47.22, p<0.001; post hoc Student-Newman-Keuls test p<0.001-0.044 for training days 1,4-6,9,10,13 but p>0.05 on other days).

Taken together, these data show that the task performance of the MS group was consistently poorer than that of the control group when tested in adulthood, indicating that MS-induced ELS impairs sound-azimuth discrimination.

Thresholds and wave latencies of ABRs

To ensure that the lower performance of MS rats in the sound-azimuth discrimination task was not simply due to alterations in hearing sensitivity resulting from MS, we recorded the ABRs and compared the ABR parameters, including thresholds and wave latencies, between MS and control groups on PND56 (**Fig. 4A** and **B**). As shown in **Fig. 4C**, the ABR thresholds for the MS group were comparable to those for the control group at various frequencies (two-way ANOVA, $F(1,68)=0.022$, $p=0.88$). Furthermore, there were no significant differences in the ABR latencies of waves I and IV between the two groups (**Fig. 4D**; two-way ANOVA, $F(1,68)=0.37$, $p=0.55$ for wave I and $F(1,68)=0.43$, $p=0.51$ for wave IV). These findings suggest that neonatal MS has little impact on hearing thresholds and neural conduction velocities within the brainstem.

Sound azimuth selectivity of neurons in cortical field A1

The response selectivity and reliability of cortical neurons contribute to the accuracy of auditory-related behaviors (Zhang et al., 2013; Cheng et al., 2017,2020; Francis et al., 2018; Liu et al., 2019; Tang et al., 2022). To investigate the neural basis for behavioral changes induced by MS, we documented azimuth tuning of cortical neuron by constructing azimuth selectivity curves on PND56 and compared the findings for the MS and control rats (**Fig. 5A** and **B**).

As detailed in **Materials and Methods**, all recorded azimuth selectivity curves were categorized as being either azimuth-selective, multipeak, hemifield, or nonselective (**Fig. 5B**). The distribution analysis revealed that the MS group exhibited significantly lower percentages of azimuth-selective and

hemifield neurons but a higher percentage of multipeak neurons compared to the control group (**Fig. 5C**; χ^2 test, $p<0.001$ for azimuth-selective, $p=0.005$ for hemifield, and $p<0.001$ for multipeak). The percentages of nonselective neurons were similar for the two groups (χ^2 test, $p=0.057$).

We next quantified two indices for each recorded azimuth-selective curve: the angular range (AR), defined as the width at half maximum of the curve, and the slope, calculated as half the sum of the slopes for both limbs of the curve. The slope for each limb was determined by dividing the change in normalized response (10% to 90%) by the corresponding azimuth range over which this change occurred. The AR and the slope were used as measures of selectivity and sensitivity, respectively, of cortical neurons to sound azimuth variations; a smaller AR and higher slope of an azimuth-selective curve indicate sharper tuning and higher sensitivity. Our data showed a significant rightward shift in the AR distribution (i.e., larger ARs) for the MS compared to control groups (**Fig. 5D**; Kolmogorov-Smirnov test, $p<0.0001$). Further analysis confirmed that the MS group had larger average AR compared to the control group (**Fig. 5E**; Mann-Whitney test, $p=0.008$). In contrast, the slope distribution of azimuth-selective curves showed a significant leftward shift (i.e., lower slopes) for the MS compared to control groups (**Fig. 5F**; Kolmogorov-Smirnov test, $p=0.001$). Consistent with this, the average slope for the MS group was significantly lower than that for the control group (**Fig. 5G**; unpaired Student's t-test, $t(15)=3.21$, $p=0.006$). Additionally, the intensity thresholds for these neurons were significantly higher in the MS group compared to the controls (24.3±0.8 vs. 21.0±0.8 dB SPL; Mann-Whitney test, $p<0.001$), and

494 their response latencies were notably longer (12.8±0.3 vs. 11.4±0.2 ms;
495 Mann-Whitney test, $p<0.001$).

496 Collectively, these results suggest that MS significantly degrades the
497 sound azimuth tuning of cortical neurons in adult rats.

498 **Dendritic arborization and spine density of cortical neurons**

499 As described in **Materials and Methods**, above electrophysiological data
500 were primarily obtained from neurons in the granular layer (layer IV) of A1. To
501 investigate the structural basis of altered cortical spatial processing after MS,
502 we compared the dendritic arborization and spine density of pyramidal
503 neurons in this cortical layer between MS and control groups on PND56.

504 The effects of MS on dendritic arborization of cortical neurons were
505 assessed using Sholl concentric ring analysis. As illustrated in **Fig. 6A** and **B**,
506 there were significantly fewer intersections between dendritic branches and
507 Sholl circles in the proximal segments (40-80 μm from the soma) in the MS
508 compared to the control groups (two-way ANOVA, $F(1,826)=4.62$, $p=0.032$;
509 post hoc Student-Newman-Keuls test $p=0.005$-0.04). Additionally, the total
510 dendritic branch lengths in the MS group were shorter than those in the
511 control group (**Fig. 6C**; unpaired Student's t-test, $t(58)=2.01$, $p=0.049$). These
512 results indicate a reduced complexity of dendritic arborization in cortical
513 neurons following MS.

514 The comparison of dendritic spine densities between MS and control
515 groups is illustrated in **Fig. 6D** and **E**. A significantly lower density of basal
516 spines was observed in the MS compared to control groups (**Fig. 6E**, left;
517 unpaired Student's t-test, $t(62)=2.42$, $p=0.019$). Further analysis revealed that

518 the density of mushroom-shaped spines was lower in the MS compared to the
519 control groups (two-way ANOVA, F(1,186)=3.18, p=0.076; post hoc Student-
520 Newman-Keuls test p=0.043), while no significant differences were found
521 between the groups for stubby- and thin-shaped spine densities (post hoc
522 Student-Newman-Keuls test p=0.94 for stubby-shaped spines and p=0.32 for
523 thin-shaped spines). Similarly, the density of apical dendritic spines in the MS
524 group was significantly lower than that in the control group (**Fig. 6E**, right;
525 unpaired Student's t-test, t(64)=2.23, p=0.03). Further analysis showed that
526 the densities of mushroom- and thin-shaped spines in the MS group were
527 significantly lower than those in the control group (two-way ANOVA,
528 F(1,192)=8.72, p=0.004; post hoc Student-Newman-Keuls test p=0.003 for
529 mushroom-shaped spines and p=0.01 for thin-shaped spines), whereas the
530 stubby-shaped spine densities were comparable between the two groups
531 (post hoc Student-Newman-Keuls test p=0.66). Thus, MS induces segment-
532 and type-specific effects on spine protrusion in cortical neurons of adult rats.

533 **Effects of enriched acoustic exposure on MS-induced cortical changes**

534 Early acoustic inputs are crucial for the structural and functional
535 development of cortical neurons (Zhang et al., 2001; Zhou and Merzenich,
536 2008; Zhu et al., 2014; Ouda et al., 2016; Pysanenko et al., 2018; Svobodova
537 Burianova and Syka, 2020). To investigate if early sound exposure can
538 reverse MS-induced cortical changes, a group of MS rats was housed in an
539 EAE during their 3-h daily MS between PND2 and 20, referred to as MS
540 paired with EAE rats (i.e., MS+EAE rats). Serum corticosterone levels in the
541 MS+EAE group were measured on PND21, while sound-azimuth
542 discrimination performance, cortical azimuth tuning, spine density of cortical

543 neurons, and molecular expression were assessed on PND56. These results
544 were then compared with those of the age-matched MS and control groups
545 (**Fig. 7**).

546 As shown in **Fig. 8**, corticosterone levels measured on PND21 were
547 significantly higher in the MS+EAE group compared to the control group (one-
548 way ANOVA, $F(2,33)=19.61$, $p<0.001$; post hoc Student-Newman-Keuls test
549 $p=0.004$), although the values were lower than those observed in the MS
550 group (post hoc Student-Newman-Keuls test $p=0.004$).

551 During the sound-azimuth discrimination task, the MS+EAE group took an
552 average of 6.5±0.8 d and 11.0±1.4 d to achieve 50% and 70% accuracy,
553 respectively. These times were both shorter than those of the MS group (**Fig.**
554 **9A**; one-way ANOVA, $F(2,21)=6.54$, $p=0.006$, post hoc Student-Newman-
555 Keuls test $p=0.027$ for 50% cut-off; one-way ANOVA, $F(2,21)=16.82$, $p<0.001$,
556 post hoc Student-Newman-Keuls test $p<0.001$ for 70% cut-off) but
557 comparable to the control group (post hoc Student-Newman-Keuls test $p=0.34$
558 for 50% cut-off and $p=0.42$ for 70% cut-off).

559 For cortical recording, the distribution of ARs for azimuth-selective curves
560 of cortical neurons in the MS+EAE group shifted significantly to the left
561 compared to the MS group (**Fig. 9B**; Kruskal-Wallis one-way ANOVA,
562 $p<0.0001$; post hoc Dunn's test $p<0.01$), and was now comparable to that of
563 the control group (post hoc Dunn's test $p>0.05$). As expected, the average AR
564 for the MS+EAE group was significantly smaller than that for the MS group
565 (**Fig. 9C**; one-way ANOVA, $F(2,22)=7.94$, $p=0.003$; post hoc Student-
566 Newman-Keuls test $p=0.018$). Thus, the value for the MS+EAE group was

effectively not different from that for the control group (post hoc Student-Newman-Keuls test p=0.20). Additionally, the distribution of slopes for azimuth-selective curves of cortical neurons in the MS+EAE group shifted slightly to the right compared to the MS group (**Fig. 9D**; Kruskal-Wallis one-way ANOVA, $p<0.0001$; post hoc Dunn's test $p>0.05$), again being not different from the control group (post hoc Dunn's test $p>0.05$). The average slope for the MS+EAE group was also slightly higher than that for the MS group (**Fig. 9E**; one-way ANOVA, $F(2,22)=7.52$, $p=0.003$; post hoc Student-Newman-Keuls test, $p=0.35$), aligning more closely with the control group (post hoc Student-Newman-Keuls test, $p=0.012$).

The comparison of dendritic spine densities of cortical neurons revealed that an EAE significantly impacts spine morphology and density in rats exposed to MS. The spine densities of both basal and apical dendrites in the MS+EAE group were significantly higher than those in the MS group (**Fig. 9F**; one-way ANOVA, $F(2,89)=6.00$, $p=0.004$, post hoc Student-Newman-Keuls test $p=0.003$ for basal dendrites and $F(2,92)=4.23$, $p=0.018$, post hoc Student-Newman-Keuls test $p=0.028$ for apical dendrites) and comparable to those in the control group (post hoc Student-Newman-Keuls test $p=0.16$ for basal dendrites and $p=0.76$ for apical dendrites). Additionally, the densities of mushroom-shaped spines in both basal and apical dendrites in the MS+EAE group were significantly higher than those in the MS group (two-way ANOVA, $F(2,267)=5.53$, $p=0.004$, post hoc Student-Newman-Keuls test $p<0.001$ for basal dendrites and $F(2,276)=7.05$, $p=0.001$, post hoc Student-Newman-Keuls test $p<0.001$ for apical dendrites), and aligned more closely with values of the control group (post hoc Student-Newman-Keuls test $p<0.001$ for basal

dendrites and p=0.11 for apical dendrites). No significant differences in the stubby- and thin-shaped spines were observed between the MS+EAE and MS groups (all post hoc Student-Newman-Keuls test p>0.49).

These data indicate that exposure to an EAE during the stress period broadly prevents the MS-induced alterations in sound-azimuth discrimination performance, cortical azimuth tuning, and spine density of cortical neurons in adult rats.

Epigenetic regulation of cortical brain-derived neurotrophic factor (BDNF)

BDNF plays a crucial role in dendritic spine development and structural plasticity of cortical neurons (Li et al., 2012; Lu et al., 2013; Ramnauth et al., 2022; Wang et al., 2022; Zhang et al., 2023). To begin documenting the molecular alterations accompanying MS-induced cortical changes and their potential restoration to normal values by an EAE, we quantified cortical BDNF expression using quantitative immunoblotting. Results revealed that BDNF level in the MS group was significantly lower than that for the control group (**Fig. 9G**, left panel; one-way ANOVA, F(2,24)=3.7, p=0.04; post hoc Student-Newman-Keuls test p=0.035). In contrast, BDNF expression in the MS+EAE group was not significantly different from that in the control group (post hoc Student-Newman-Keuls test p=0.082).

Given the known epigenetic regulation of BDNF, we further investigated cortical expression of histone H3 lysine 9 dimethylation (H3K9me2), a modified histone associated with gene silencing, including the repression of BDNF gene transcription (Padeken et al., 2022; Zhao et al., 2022). As shown

in the right panel of **Fig. 9G**, H3K9me2 level in the MS group was significantly higher than that in the control group (one-way ANOVA, $F(2,21)=5.32$, $p=0.014$; post hoc Student-Newman-Keuls test $p=0.02$). In contrast, H3K9me2 level in the MS+EAE group was significantly lower than that in the MS group (post hoc Student-Newman-Keuls test $p=0.016$) and comparable to that in the control group (post hoc Student-Newman-Keuls test $p=0.61$).

These data suggest that MS leads to decreased cortical BDNF expression, potentially through H3K9me2-mediated epigenetic regulation. In contrast, pairing MS with an EAE appears to substantially prevent these MS-induced molecular changes.

Discussion

In this study, we established an ELS model by separating the rat pups from their mother for 3 h daily from PND2 to 20. Consistent with earlier studies (Hsu et al., 2003; Koe et al., 2016; Cui et al., 2020; Wang et al., 2020), MS led to lower body weights, increased anxiety-like behaviors, and heightened stress responses, confirming that this neonatal MS protocol successfully induced ELS. We found that MS-induced ELS significantly reduced performance accuracy in the sound-azimuth discrimination task when tested in adulthood, which coincided with broader azimuth tuning and reductions in the dendritic branching and spine density of neurons within the cortical field A1. Our results therefore demonstrate the profound and enduring effects of MS as an ELS on behavioral and cortical processing of sound azimuth. These findings indicate potential consequences of ELS for speech processing and

language comprehension in humans, for example, because of the role of spatial hearing in maintaining speech intelligibility in noisy environments (Hawley et al., 2004; Grothe et al., 2010; Zündorf et al., 2013).

Previous studies have shown gender differences in the effects of MS-induced ELS. For example, Talani et al. (2023) reported that male, but not female, mice displayed alterations in hippocampal synaptic transmission and cognitive functions, such as spatial memory and novelty preference, following neonatal MS. Additionally, anxiety-like behaviors were observed exclusively in male mice post-MS (Romeo et al., 2003; Bailoo et al., 2014). While some studies have found contrasting results (Veenema et al., 2007; Tsuda and Ogawa, 2012; Cui et al., 2020), the effects of MS tend to be more pronounced in male animals, likely owing to differing hormonal patterns and neurotransmitter interactions between sexes. Furthermore, recent research has shown that MS similarly affects behavioral discrimination of brief gaps in sound for both male and female gerbils (Ye et al., 2023). Based on these findings, this study focused on male rats to examine the impact of MS-induced ELS on auditory processing. Future studies could explore sex-specific effects of MS on cortical processing of sound azimuth.

Earlier studies in cats, ferrets, and macaques have shown that lesions in the auditory cortex lead to significant impairments in sound localization (Jenkins and Merzenich, 1984; Kavanagh and Kelly, 1987; Heffner and Heffner, 1990). Similarly, early noise exposure reduces the sound azimuth selectivity of cortical neurons in rats, resulting in diminished performance in spatial discrimination tasks (Xu et al., 2010; Pan et al., 2011; Guo et al., 2012). Conversely, an EAE enhances azimuth tuning of cortical neurons and

improves sound localization abilities of rats (Cai et al., 2009). These findings suggest that the auditory cortex is critical for normal sound localization in mammals. It has been proposed that alterations in cortical response selectivity and reliability may affect the neural encoding of acoustic details, thereby impairing auditory-related behaviors (Zhang et al., 2013; Cheng et al., 2017,2020; Liu et al., 2019; Tang et al., 2022). Thus, post-MS changes in cortical azimuth processing may at least partially explain the decrease in discrimination acuity observed in the MS rats during behavioral tasks. Given that the sound-azimuth discrimination task used in this study involves significant learning and memory components, the impact of MS on learning ability and task performance accuracy warrants further investigation.

Early auditory inputs are crucial for the structural and functional maturation of neurons in the auditory cortex (Sanes and Woolley, 2011; Kral 2013; Chang and Kanold, 2021). Manipulation of auditory inputs during the critical period of cortical development profoundly alters the tuning properties of these neurons, subsequently influencing auditory perception and behavior (Zhang et al., 2001; Han et al., 2007; Xu et al., 2007; Zhou and Merzenich, 2008,2009; Popescu and Polley, 2010; Polley et al., 2013; Pysanenko et al., 2018; Bures et al., 2021; Tang et al., 2022). Dendritic spines, which are small membranous protrusions along neuronal dendrites, serve as the primary sites for excitatory synaptic connections (Harris and Kater, 1994; Nimchinsky et al., 2002; Moyer and Zuo, 2018; Akhgari et al., 2024). Therefore, alterations in the dendritic morphology and spine density of cortical neurons can disrupt auditory inputs, leading to changes in neuronal tuning properties (Ouda et al., 2016; Svobodova Burianova and Syka, 2020; Cheng et al., 2022; Tang et al.,

690 2022). In this study, we hypothesized that MS-induced changes in cortical
691 tuning of sound azimuth are associated with modifications in the dendritic
692 morphology and spine density of neurons. Our findings are consistent with this
693 hypothesis: adult MS rats exhibited significantly lower dendritic complexity and
694 dendritic spine density of cortical neurons compared to the controls, at least in
695 the middle cortical layer where physiological recordings were conducted.
696 These changes in dendritic morphology and spine density likely disrupt
697 auditory inputs during cortical development, leading to altered neuronal tuning
698 and behavioral processing. Importantly, the observed morphological
699 alterations may underlie more general changes in auditory processing rather
700 than being limited to spatial processing. Our ongoing research is investigating
701 the post-MS effects on spectral tuning of cortical neurons and frequency
702 discrimination performance to explore this possibility.
703 We further determined whether an EAE could renormalize disrupted
704 dendritic spine development and degraded cortical azimuth tuning caused by
705 MS, thereby restoring behavioral performance. Our results revealed significant
706 changes in dendritic spine density within the cortex of MS+EAE rats,
707 approaching control levels. Furthermore, cortical tuning and behavioral
708 performance in these rats also became nearly indistinguishable from those of
709 the controls. Thus, pairing MS with an EAE generally protects against the
710 changes in cortical spine density observed in adult MS rats, leading to the
711 recovery of cortical azimuth tuning and behavioral outcomes. Since EAE
712 exposure only partially mitigates the elevated corticosterone levels induced by
713 MS, it is conceivable that the beneficial effects of EAE on MS were primarily
714 attributed to its impact on cortical development rather than through a reduction

715 in stress itself. These findings suggest that enriched sensory exposure can
716 serve as an effective strategy to counteract the effects of ELS on sensory
717 function.

718 The molecular mechanisms through which ELS affects the dendritic
719 structure of cortical neurons remain incompletely understood. Previous
720 studies revealed that ELS impacts synaptic structure and connections within
721 cognitive and limbic systems through the epigenetic regulation of BDNF gene
722 expression, since BDNF is crucial for dendrite and spine development
723 (Tenkumo et al., 2020; Jiang et al., 2021; Sun et al., 2021). In addition,
724 epigenetic mechanisms, such as DNA methylation, have been shown to
725 modulate the plasticity of auditory cortex in mice (Schwartz et al., 2020). We
726 thus examined cortical expression of BDNF and its key epigenetic regulator,
727 H3K9me2, in rats exposed to MS. Our findings show that MS significantly
728 downregulated BDNF while upregulating H3K9me2, suggesting that ELS may
729 influence the dendritic structure and spines of cortical neurons through the
730 epigenetic modulation of BDNF gene expression by H3K9me2, thereby
731 influencing the structural and functional changes in neurons within the
732 auditory cortex. These results confirm that this epigenetic regulatory
733 mechanism extends to the sensory cortex, where it appears to play a critical
734 role in mediating the effects of ELS on neuronal structure and function.
735 Interestingly, for rats in the MS+EAE group, cortical levels of BDNF and
736 H3K9me2 were similar to their respective levels in the control group. This
737 finding indicates that exposure to an EAE during MS can potentially prevent
738 ELS-induced changes in neuronal structure and function via a similar
739 regulatory mechanism. It should be noted that methylation pathways, such as

740 those yielding histone H3 lysine 4 trimethylation (H3K4me3) or histone H3
741 lysine 9 trimethylation (H3K9me3), may also influence BDNF gene
742 expression, potentially counteracting or coordinating the effects of H3K9me2
743 (Gupta et al., 2010; Snigdha et al., 2016; Ell et al., 2024). The antagonistic
744 interaction between histone modifications and their impacts on BDNF could
745 represent a dynamic regulatory response to ELS. Therefore, further research
746 is needed to elucidate the detailed molecular mechanisms and explore the
747 roles of additional potential signaling pathways, particularly those involving
748 H3K4me3 and H3K9me3, in the effects of MS-induced ELS on auditory
749 processing.

750 One remaining question is the origin of the cortical changes observed
751 after MS. Here we demonstrated an MS-induced degradation in sound
752 azimuth tuning of cortical neurons. The distinct changes in response
753 characteristics recorded at the cortical level suggest that at least some of
754 these effects may stem from intrinsic cortical remodeling following MS. This
755 idea is further supported by evidence of altered cortical BDNF expression and
756 its epigenetic regulation, as well as changes in the dendritic branching and
757 spine density of cortical neurons, in rats exposed to MS. Additionally, our ABR
758 recordings suggest that MS has little impact on hearing thresholds and neural
759 conduction velocities within the auditory brainstem. However, we cannot
760 exclude the possibility that the observed post-MS changes are partially due to
761 feed-forward responses reflecting altered subcortical processing. Indeed,
762 recent studies on temporal responses of gerbils to sound gaps have reported
763 impairments within the auditory pathway, including the auditory cortex,
764 brainstem, and periphery, following MS (Ye et al., 2023). Therefore, the

765 effects of MS-induced ELS on sound azimuth processing in the subcortical
766 pathway, particularly in the auditory thalamus and inferior colliculus, clearly
767 warrant further investigation.

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Figure legends

Figure 1. Experimental timelines and procedures for assessing the effects of maternal separation (MS). The timelines illustrate the procedures performed on different postnatal days (PNDs) in MS and age-matched control rats. MS was conducted from PND2 to 20 (orange bar). Body weights were measured at various intervals between PND2 and 56. Corticosterone (cort) levels and behavioral assessments, including open field and elevated zero-maze tests, were conducted on PND21 and 56. Recombinant adeno-associated virus was bilaterally injected into the primary auditory cortex (A1) on PND28 for subsequent evaluation of dendritic spines of neurons. On PND56, animals underwent a sound-azimuth discrimination task, auditory brainstem response (ABR) recordings, cortical recordings, dendritic spine assessment, and molecular expression analysis.

Figure 2. Signs of stress induced by MS. **A**, Body weight trajectory of MS rats and age-matched controls on the different PNDs. n=30 on PND2-49 and n=23 on PND56 for the MS group; n=27 on PND2-49 and n=22 on PND56 for the control group. Error bars represent SEM. +, $p<0.01$; #, $p<0.001$. **B**, Individual body weights on PND35, 42, 49, and 56, showing significant differences between MS and control groups at each time point. **C**, Representative movement traces of MS and control groups in the open field test on PND21 (top) and 56 (bottom). **D**, Comparison of distances traveled by MS and control groups in the open field test in three time ranges (0-5, 5-10, and 10-15 min) on PND21 (top; n=20 for the MS group and n=19 for the control group) and 56

(bottom; n=30 for the MS group and n=22 for the control group). **E**, Representative movement traces for MS and control groups in the elevated zero-maze test on PND21 (top) and 56 (bottom). **F**, Comparison of distances traveled, open arm entries, and time spent in the open arms by MS and control groups in the elevated zero-maze test on PND21 (top; n=18 for both MS and control groups) and 56 (bottom; n=13 for both MS and control groups). *, $p<0.05$. **G**, Serum corticosterone levels measured on PND21 (left; n=12 for both MS and control groups) and 56 (right; n=16 for both MS and control groups). Values (mean±SEM) are normalized to the age-matched control group.

Figure 3. Effects of MS on sound-azimuth discrimination performance. **A**, Percent correct on the sound-azimuth discrimination task on different training days for each of MS (n=8) and control (n=9) rats (left) and average percent correct for both groups (right). The dashed lines show 50% and 70% of the maximal scores. Error bars represent SEM. *, $p<0.05$; +, $p<0.01$; #, $p<0.001$. **B**, Training days to achieve 50% and 70% performance on the task for both groups. **C**, Percent distribution of azimuth deviations (ADs) on each day, with positive and negative ADs indicating deviations to the right or left of the target speaker, respectively, for MS (left) and control (right) groups. **D**, Average ADs on different training days for both groups.

Figure 4. Thresholds and wave latencies of ABRs determined using tone pips of different frequencies. **A**, Schematic of the experimental setup for ABR

recording. Acoustic stimuli were delivered through a calibrated earphone with a sound tube positioned inside the external auditory meatus. ABRs were recorded by placing electrodes (indicated by the arrows) subdermally at the scalp midline, posterior to the stimulated ear, and on the midline of the back 1-2 cm posterior to the neck of the animal. **B**, ABR patterns of MS and control rats in response to tone pips of 10 kHz. The ABR threshold was defined as the lowest sound intensity capable of eliciting a response pattern characteristic of that observed at higher intensities (arrow). **C**, ABR thresholds at different frequencies for MS (n=9) and control (n=10) groups. Error bars represent SEM. **D**, Latencies of waves I and IV (measured at 70 dB SPL of acoustic stimuli) for both groups.

Figure 5. Degraded cortical azimuth tuning following MS. **A**, Schematic of the setup for electrophysiological recording in A1. During recording, acoustic stimuli were delivered from a speaker positioned 34 cm from the head. The speaker could be placed at any specific azimuth in the frontal auditory space (0° elevation) using a remote-control system. c or i, contralateral or ipsilateral relative to the recording site (see also **Materials and Methods**). **B**, Representative azimuth selectivity curves recorded for control rats, categorized as azimuth-selective, multipeak, hemifield, or nonselective. **C**, Percent distribution of cortical azimuth selectivity curves recorded for MS and control groups. +, $p<0.01$; #, $p<0.001$. **D**, Cumulative frequency histograms of angular ranges (ARs) for MS (n=79) and control (n=74) groups. **E**, Average ARs for MS (n=9) and control (n=8) groups, using the number of animals as

the sample size. Error bars represent SEM. **F**, Cumulative frequency histograms of slopes for both groups. **G**, Average slopes for both groups.

Figure 6. Dendritic architecture and spine density alterations following MS. **A**, Representative tracings of dendritic arbors from cortical neurons in MS and control rats, overlaid with concentric Sholl analysis circles to evaluate dendritic complexity. Scale bar=100 µm. **B**, Sholl analysis showing the number of dendritic intersections as a function of distance from the soma in cortical neurons for MS (n=28) and control (n=26) groups. Error bars represent SEM. *, $p<0.05$; +, $p<0.01$. **C**, Total dendritic length of cortical neurons in MS (n=33) and control (n=27) groups. **D**, Representative images of dendritic segments from basal (left) and apical (right) dendrites in MS and control groups. Scale bar=5 µm. **E**, Quantification of dendritic spine density in basal (left; n=28 for the MS group and n=36 for the control group) and apical (right; n=36 for the MS group and n=30 for the control group) dendrites. The inset shows a schematic representation of spine morphologies.

Figure 7. Experimental timelines and procedures for assessing the effects of an enriched acoustic environment (EAE) on MS-induced changes. Note that the MS+EAE rats were housed in an EAE during their 3-h daily MS between PND2 and 20 (gray bar). Corticosterone levels were measured for the MS+EAE, MS, and control groups on PND21 and sound-azimuth discrimination performance, cortical recordings, dendritic spine assessment, and molecular expression analyses were performed on PND56.

Figure 8. Serum corticosterone levels measured on PND21 for the MS+EAE (n=12), MS, and control groups. Values (mean±SEM) are normalized to the age-matched control group. +, $p<0.01$; #, $p<0.001$.

Figure 9. Effects of an EAE on MS-induced changes. **A**, Training days to achieve 50% and 70% performance on the sound-azimuth discrimination task for the MS+EAE (n=7), MS, and control groups. Error bars represent SEM. *, $p<0.05$; +, $p<0.01$; #, $p<0.001$. **B**, Cumulative frequency histograms of ARs. n=71 for the MS+EAE group. **C**, Average AR for each group, using the number of animals as the sample size. n=8 for the MS+EAE group. **D**, Cumulative frequency histograms of slopes. **E**, Average slope for each group, using animals as the sample size. **F**, Quantification of dendritic spine density in basal (top; n=28 for the MS+EAE group) and apical (bottom; n=29 for the MS+EAE group) dendrites. The inset shows a schematic representation of spine morphologies. **G**, Cortical expression levels of brain-derived neurotrophic factor (BDNF, left panel; n=9 for different groups) and histone H3 lysine 9 dimethylation (H3K9me2, right panel; n=8 for different groups). The insets show representative Western blots.