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Early-life experiences altered maturation of lateral habenula in mice models, resulting in behavioral disorders in adulthood

Supplementary methods

Animals

Wild-type male and female C57BL/6J mice (B6/J) were purchased from Japan SLC Inc. (Hamamatsu, Japan). Heterozygous GAD1(GAD67)-GFP knock-in mice were provided by RIKEN Bio Resource Center (Tamamaki et al., 2003; RBRC03674, Ibaraki, Japan). To drive the expression of mCherry in GAD2 (GAD65) neurons (GAD2-mCherry mice), GAD2-IRES-Cre female mice (Taniguchi et al., 2011; Stock No. 010802, The Jackson Laboratory, Bar Harbor, USA) were crossed with R26R-H2B-mCherry male mice (Abe et al., 2011; CDB0204K, RIKEN CLST, Wako, Osaka, Japan). Adult mice were maintained in single-sex groups with up to five littermates per cage in a temperature-controlled environment (22-25℃) with a 12/12-h light/dark cycle (lights were turned on at 5:00, and off at 17:00). Pups were maintained in a cage with a dam. Food and water were supplied ad libitum. Results were similar in cases where males and females were analyzed separately; therefore, we presented pooled data of males and females. All experimental groups were avoided to configure from only one littermate.

Immunohistochemistry and lectin histochemistry procedure

Mice were brought out from the breeding room and were transcardially perfused with phosphate-buffered saline (PBS), subsequently with 3.7% formaldehyde (Wako, Osaka, Japan) in PBS under deep anesthesia with pentobarbital (50 mg/kg body weight, i.p.;

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Kyoritsu Seiyaku, Tokyo, Japan). The perfusion procedures were completed in 30 min before the expression of Zif268/Egr1 started; thus, influences of general stress during experimental procedures were excluded. Brains were dissected out and kept in the same fixative at 4℃ overnight. After washing in PBS, they were embedded in gelatin (16.7% gelatin and 16.7% glycerol in PBS) and postfixed in the same fixative for 4 days at 4℃. Coronal sections of 70-μm thickness were cut using Vibratome (VT1000S, Leica Microsystems, Wetzlar, Germany). Along the length of the LHb from anterior (at -1.22 from bregma) to posterior (at -2.06 from bregma), 14 serial sections were collected (Paxinos and Franklin., 2004) and stored in PBS containing 0.02% sodium azide at 4℃ until staining.

For immuno- or lectin-enzyme-histochemistry, the alternative sections were incubated with primary antibodies in 0.5% Triton X-100 in PBS (PBST) with 5% normal goat serum (16210064, Thermo Fisher Scientific, Waltham, USA; PBSTN) or biotinylated WFA lectin (B-1355, Vector, Burlingame, USA) in PBST for 3 days. The sections were incubated in the appropriate biotinylated secondary antibody for 2 hours: anti-rabbit IgG (1:300; BA-1000, Vector) and anti-mouse IgG (1:300; BA-2000, Vector). After washing, they were incubated for 2 hours in ABC reagent (PK-6100, Vector). The reactive signals were visualized with Metal Enhanced DAB Substrate Kit (34065, Thermo Fisher Scientific).

For double fluorescence immunohistochemistry, the alternative sections were incubated for 6 days with primary antibody in 3% bovine serum albumin (9048-46-8, Wako) in PBST. They were incubated with appropriate secondary antibodies coupled with Alexa 488, 594, or 647 (1:200, Invitrogen, Carlsbad, USA; Jackson Immuno

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Research Labs, West Grove, USA) or streptavidin with Alexa 594 for WFA lectin staining (S11227, Thermo Fisher Scientific), counterstained with 4',6-diamidino-2-phenylindole (DAPI; 1:10000; D9542, Sigma-Aldrich, St Louis, USA), and mounted on glass slides with Mowiol 4-88 (Merck Millipore, Burlington, USA). Images were obtained with a confocal laser scanning microscope (LSM780, Zeiss, Jena, Germany), equipped with a 20× fluorescence objective lens. Series of confocal z-stack images were recorded with 2 μm interval. Their X/Y resolution was 708,49 × 708,49 μm (1024 × 1024 pixel). In all slices optically sectioned, the number of double positive cells was analyzed by using ImageJ cell counter plug-in (National Institutes of Health, Bethesda, MD; http://rwsbweb.nih.gov/ij/) (https://imagej.nih.gov/ij/plugins/cell-counter.html).

GABA immunohistochemistry

STAINperfect Immunostaining Kit was used for GABA immunohistochemistry (ImmuSmol, Bordeaux, France). Under deep anesthesia, the mice were transcardially perfused with a fixation solution in the kit. Their brains were postfixed in the same fixative for 2.5 hours at 4℃, embedded in gelatin (16.7% gelatin, 16.7% glycerol in PBS), and postfixed again in 4% paraformaldehyde (26126-25, Nacalai tesque, Kyoto, Japan) in PBS at 4℃ overnight. Coronal sections of 70-μm thickness were cut using Vibratome. Immunofluorescence staining for GABA was performed according to the manufacture's protocol.

Primary antibodies and lectin

For immuno- or lectin-enzyme-histochemistry, the following primary antibodies or lectin

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were used: an anti-aggrecan rabbit antibody (1:4500 for DAB staining; 1:500 for fluorescence staining; AB1031, Merck Millipore), an anti-GABA chicken antibody (1:200; IS1036, ImmuSmol), an anti-NeuN mouse monoclonal antibody (1:1500, MAB377, Merck Millipore), an anti-Parvalbumin goat antibody (1:500 for fluorescence staining with NeuN; AB_2571614, FRONTIER INSTITUTE, Sapporo, Japan), an anti-Parvalbumin mouse monoclonal antibody (1:90000 for DAB and fluorescence staining; P3088, Sigma-Aldrich), biotinylated Wisteria Floribunda lectin (1:900 for DAB and fluorescence staining; B-1355, Vector), an anti-Zif268/Egr1 rabbit antibody (1:1500 for DAB staining; 1:300 for fluorescence staining; C-19, Santa Cruz Biotechnology, Dallas, USA), and anti-mCherry animal antibody (1:1000 for fluorescence staining; Novus, Centennial, USA).

Densities of PV neurons and percentages of PNNs area in LHb from early-life to adulthood

To quantify densities of PV cells and Zif268/Egr1 immunopositive cells (Zif268/Egr1 cells), immunohistochemical staining of PV and Zif268/Egr1 were analyzed. To quantify percentages of PNNs area, immunohistochemical staining of aggrecan and lectin-histochemical staining of WFA were analyzed. The samples were observed under a microscope (DMRE, Leica) equipped with a 10× objective lens, in bright field. By changing focal planes with moving the stage 5 μm interval in z-axis, series of z-stack images were obtained with a digital camera (Ds-Ri1, Nikon, Tokyo, Japan). The X/Y resolutions of the images were 1393.02 × 1114.42 μm (1280 × 1024 pixels). These data were processed extended depth of field plugin (http://bigwww.epfl.ch/demo/edf/) of

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ImageJ software for converting z-stack images to an omnifocal image.

To count the cell number, the PV neurons were marked in all sections by using the ImageJ's cell counter. The number of Zif268/Egr1 cells was analyzed by using ImageJ's threshold (130) after unifying grayscale of background and analyze particle tools after processing watersheds. To obtain the total PNNs area in an individual, pixels positive for WFA or aggrecan were counted in all sections by using ImageJ (https://imagej.net/Auto_Threshold), and were converted to area (mm^2). These did not affect statistical significances between control and repeated maternal deprivation$_{P10\text{-}20}$ at P20; thus, the measurements were reliable but not under the influences of background signals.

Light/dark box test

To adjust the brightness of the light compartment to 200 lux, the apparatus was put in a big black box (L 67 cm × D 67 cm × H 97 cm) with five LEDs inside (Y07CLL40W01CHJ, Yazawa, Tokyo, Japan). The apparatus was wiped with 70% ethanol and air-dried before the trials. After placing the mice in the center of the dark compartment, mice in the light compartment were recorded for 5 min with a digital video camera (c920, Logitech, Lausanne, Switzerland) at P70-77. The time spent in the dark compartment was calculated by subtracting the time spent in the light compartment from the 5 min recording time.

Forced swim test

On 10 days after the light/dark box test, a cylinder (22-cm diameter × 22-cm height) was

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filled with water up to a height of 15 cm and placed at 25-28°C in 200 lux. A mouse was placed in the cylinder, and its activities were recorded using the overhead digital video camera for 10 min. Its positions were traced using Motr (Ohayon et al., 2013), which was analyzed with a custom-written MATLAB script. To evaluate its general locomotor activity, the total trace length of its body center was measured. Immobilization was defined as satisfying all of the following conditions. First, acceleration was maintained below 0 for more than 3 sec, which indicated moving at a constant speed, reducing speed, or stopping. Second, speed was maintained at less than 3 cm/sec for more than 3 sec; the speed did not drop to 0 by an acting inertial force. Third, change in the distance between the nose and the tail root was less than 0.4 cm/sec for more than 3 sec. Immobilization time was analyzed from 5 to 10 min. After the test, the mouse was dried with a paper towel and returned to the cage. The cylinder was washed with water and dried between trials.

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Supplementary Results

Aggrecan positive PNN at P20 in the repeated maternal deprivation$_{P10\text{-}20}$ group

In the course of LHb maturation at P20, the percentages of aggrecan positive PNN area were not significantly different between the control and repeated maternal deprivation$_{P10\text{-}20}$ groups (t_{11} = 1.63, p = 0.16 in Student's t-test, p = 0.13 in F-test; Fig. S6I-K). In addition, the densities of aggrecan positive cells were not significantly different between the groups (t_{11} = 1.40, p = 0.18 in Student 's t-test; Fig. S6L). These results showed that the repeated maternal deprivation$_{P10\text{-}20}$ had no effect on maturation of aggrecan positive PNNs.

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Supplementary Figures

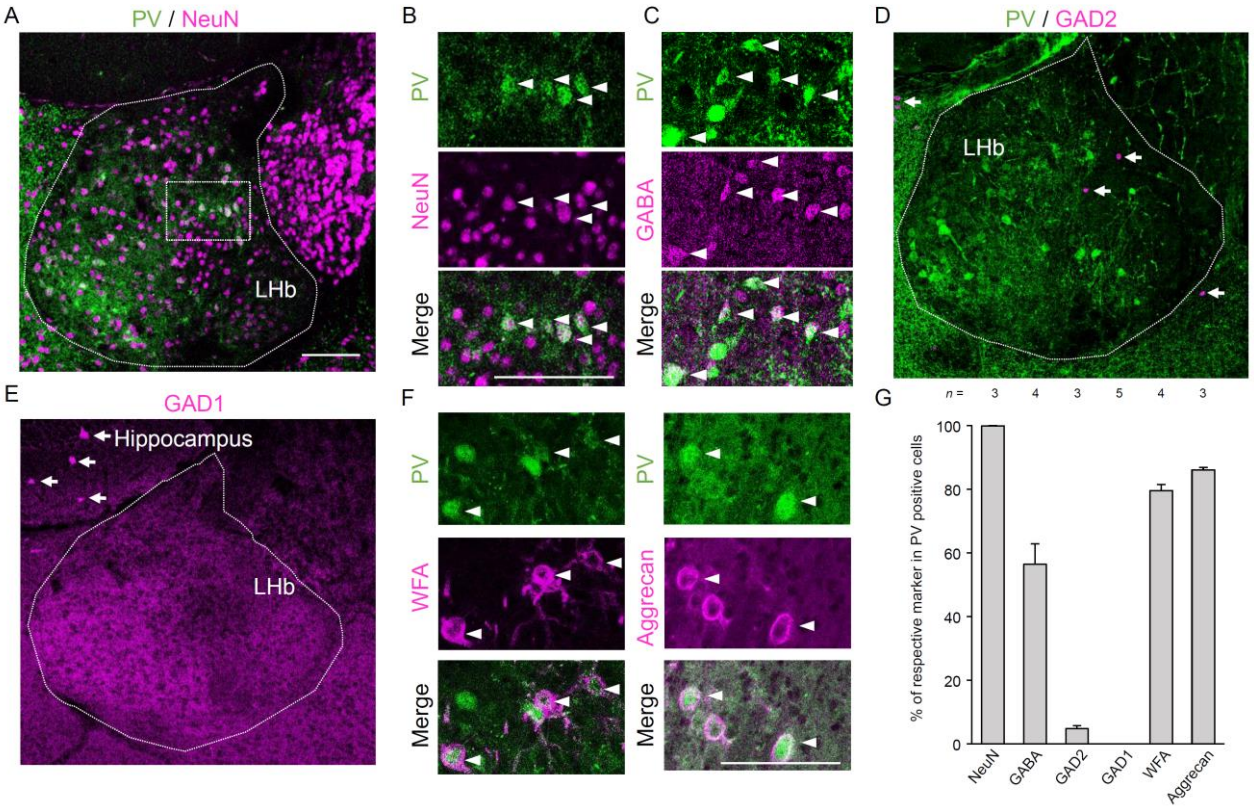

Fig. S1: PV neurons in adult LHb contain GABA; double immunofluorescence staining of PV, NeuN, GABA, and PNNs.

Double immunofluorescence staining of PV (green) and NeuN (magenta) is shown in low magnification (**A**). High-magnification image of the dotted rectangle area in (A) showing PV positive cells are doubly stained with NeuN (arrowheads) (**B**). PV neurons are positive for GABA immunoreactivity (arrowheads) (**C**). A few GAD2 reporter positive cells are found in the LHb (magenta, arrows in GAD2-mCherry mice); however, PV neurons (green) are negative for the GAD2 reporter (magenta) (**D**). GAD1 reporter positive cells are not found in the LHb but seen in the hippocampus (magenta, arrows in

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GAD1-GFP knock-in mice) (**E**). PV neurons (green) are surrounded by PNNs, as determined using WFA lectin staining and aggrecan (magenta) (**F**). Percentages of PV neurons that were positive for the respective markers (**G**). Figs. A, B, C, and F are photomicrographs of a single optical section. Figs. D and E show omnifocal images with 10 optical sections. Arrowheads indicate double fluorescence positive cells. LHb, lateral habenular nucleus; PV, parvalbumin; GAD, glutamic acid decarboxylase; WFA, wisteria floribunda agglutinin. The position of the images was near 1.92 mm posterior from bregma. Scale bars = 100 μm.

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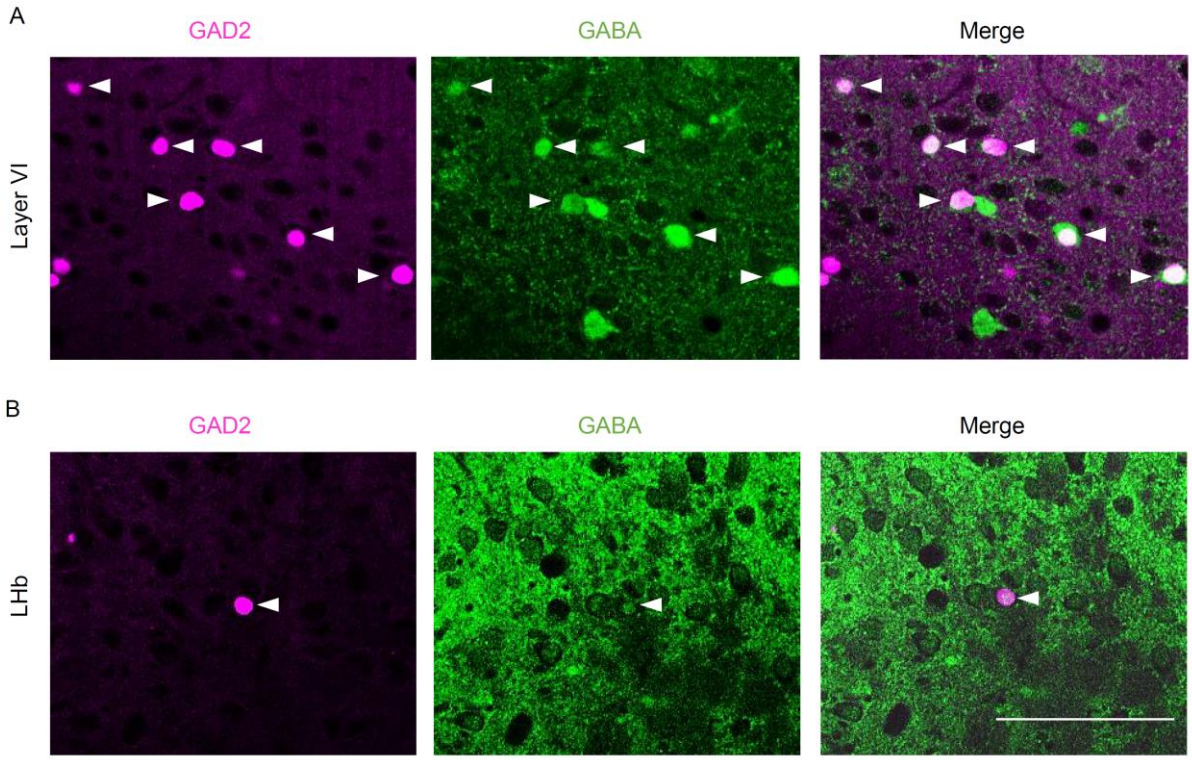

Fig. S2: GAD2 positive neurons contain GABA. GAD2 reporter positive cells (magenta) are labeled with GABA immunostaining (green) in layer VI of the adult cerebral cortex (**A**). A double positive cell is shown in the LHb (**B**). Arrowheads indicate double positive cells. GAD2, glutamic acid decarboxylase 2. Scale bar = 100 µm.

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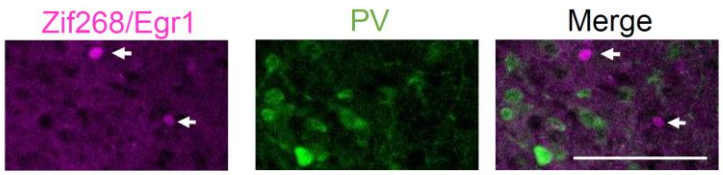

Fig. S3: Zif268/Egr1 cells were negative for PV immunoreactivity in adults. LHb was double stained with Zif268/Egr1 (magenta; arrows) and PV (green; open arrows) after the single restraint stress at P60; no cell was double stained. PV, parvalbumin; The position of the images was near 1.92 mm posterior from bregma. Scale bars = 100 µm.

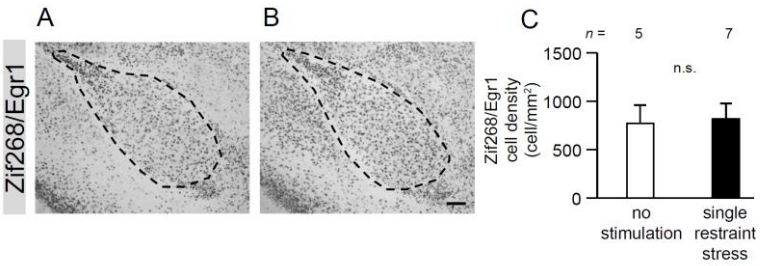

Fig. S4: No effect of single restraint stress on neuronal activity observed by the density of Zif268/Egr1 cells in adult basolateral amygdala. Zif268/Egr1 cells without stimulation are shown (**A**). Those after the single restraint stress are shown (**B**). Their densities are compared between the groups (**C**). Error bars represent SEMs. Scale bar = 100 µm.

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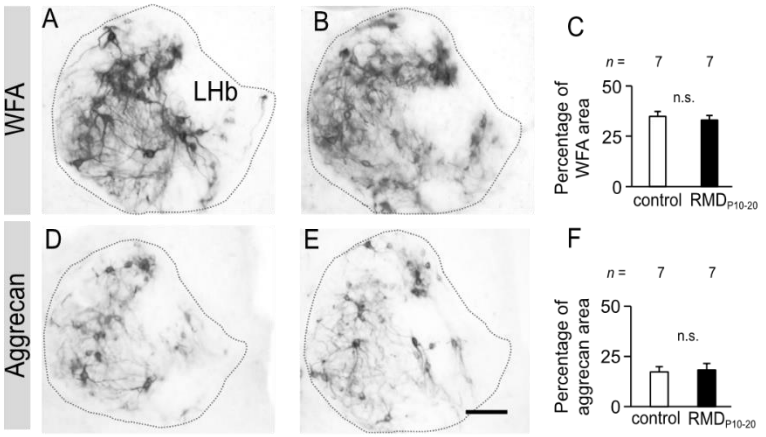

Fig. S5: No effects of RMD_{P10-20} on perineuronal nets (PNNs) in adult LHb. The PNNs are shown with WFA lectin-staining in the groups of control (**A**) and repeated maternal deprivation during P10-20 (RMD_{P10-20}) (**B**); percentages of the WFA positive area are compared between the groups (**C**). The PNNs are shown with the aggrecan immunostaining in the control (**D**) and RMD_{P10-20} (**E**) groups; percentages of the aggrecan positive area are compared between groups (**F**). Error bars represent SEMs. LHb, lateral habenular nucleus; WFA, wisteria floribunda agglutinin; Scale bar = 100 μm.

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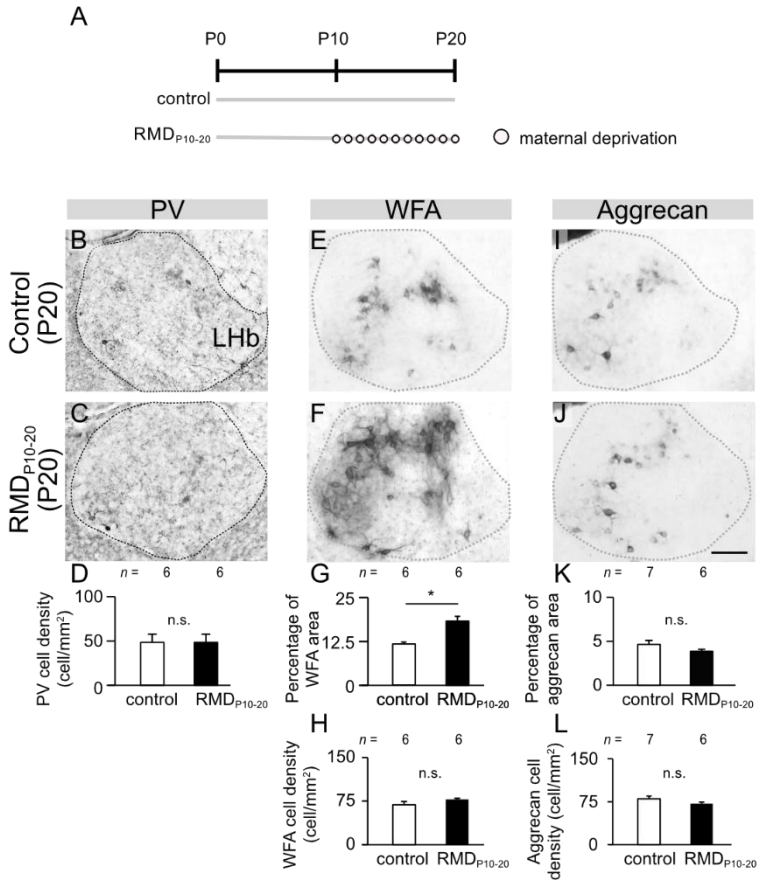

Fig. S6: The excess area of WFA positive PNN at P20 under the influence of early-life stress during P10-20. Schematic diagram of experiments. RMD_{P10-20}, repeated maternal deprivation during P10-20 (**A**). In the LHb, PV neurons at P20 are shown in the control (**B**) and RMD_{P10-20} (**C**) groups. Their densities are compared between the groups (**D**). The PNNs at P20 are shown with WFA lectin-staining in the control (**E**) and RMD_{P10-20} groups (**F**); percentages (**G**) and densities (**H**) of the WFA positive area are compared between the groups. The PNNs at P20 are shown with aggrecan immunostaining in the control (**I**) and RMD_{P10-20} groups (**J**); percentages (**K**) and densities (**L**) of the aggrecan

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positive area are compared between the groups. LHb, lateral habenular nucleus; PV, parvalbumin; PNNs, perineuronal nets, WFA, wisteria floribunda agglutinin; Scale bar = 100 µm.