Supplementary information

Supplementary Figure 1.

Schematic drawings of labeling of history of neuronal activity and its significance (**a**-**h**). Neuronal activation induces IEG expression. The IEG promoter converts neuronal activity into Venus expression. The membrane localization sequence localizes Venus to the neuronal surface, where it traces neuronal processes (**a**). Because of its slow turnover, Venus remains on the neuronal surface and accumulates with neuronal activity after the activation stops; thus, it labels neuronal activity history (**b**). In **c**–**h**, the figures show two mice that look similar, but differ in their past experiences. Over the course of several days, one mouse was busy (**c**); its neuronal circuit was highly activated (**d**) and labeled with Venus (**e**). The other mouse was relaxed (**f**); its circuit was less active (**g**) and was not labeled with Venus (**h**). Therefore, the history of neuronal activity reflects individual experiences, and the labeling enables us to distinguish between these two mice. A transgenic construct is shown in (**i**) for labeling a facet of neuronal activity history.

The labeling in PC12 cells is shown in response to no stimulation (**j**), and single and repetitive stimulation with nerve growth factor (NGF) (**k** and **l**, respectively). PC12 cells (1×10^5) were cultured on poly-D-lysine coated coverslips in a 35-mm dish with serum-free Dulbecco's modified Eagle's medium (DMEM). To examine accumulation of the labeling *in vitro*, the construct shown above was transfected into PC12 cells (1 μg DNA/dish) with Lipofectamine (Invitrogen). To estimate transfection efficiency, the gene construct with *Venus* under the control of the cytomegalovirus promoter was transfected.

The cells were cultured with serum-free DMEM. On the second, fourth, or sixth day *in vitro*,

a 20-μL nerve growth factor (NGF) solution (5 μg/μL in phosphate-buffered saline [PBS]) or PBS was added to the culture dish. Cells were fixed on the third or seventh day after the addition of NGF. Five fields were randomly chosen in the culture dish, and the percentage of Venus-positive cells in the dish was obtained. Average percentages of positive cells were estimated and compared between the single, repetitive, and no stimulation conditions. Induction and accumulation are compared as the percentage of Venus-positive cells between under the control of cytomegalovirus promoter (*CMV*) and under the control of *zif268/egr1* promoter (*zif268/egr1*) without stimulation (*PBS*), and with single and repetitive stimulation (*NGF×1* and *NGF×3*, respectively) (**m**). Accumulation efficiencies between the repetitive and no stimulation conditions are indicated above each bar.

To examine the decay of labeling, the percentage of Venus-positive cells is shown after single stimulation with NGF (*NGF×1*) along sequential days (**n**). The construct was transfected into PC12 cells (1 μg DNA/dish) with Lipofectamine (Invitrogen). After rinsing the cells with DMEM, a 20-μL NGF solution (5 μg/μL in PBS) or PBS was added to the culture dish and incubated for 1 h; then, the cultures were rinsed and further cultured in DMEM with serum. Because the cells did not survive in serum-free DMEM without NGF for 7 d, we used DMEM with serum to estimate the labeling decay with NGF stimulation against that without stimulation. On each sequential day of culture, five fields were randomly chosen, and percentages of Venus-positive cells in the dish were obtained. Average percentages of positive cells with NGF stimulation were estimated in 10 independent experiments and compared to the percentage of cells without stimulation. The number of positive cells declines gradually and becomes comparable to that of no stimulation (*PBS*) on day 5 after stimulation

(** $p < 0.01$, * $p < 0.05$, unpaired *t*-test). Scale bar, 100 μm.

Supplementary Figure 2.

ZIF268/EGR1-immunopositive cells and the labeling in the cortex. A coronal section of the parietal cortex at P13 of the transgenic mouse is shown. ZIF268/EGR1-immunopositive cells are distributed densely in layer IV, moderately in layers II–III and VI, and sparsely in layer V (**a**). The labeling of cortical barrels in layer IV is intense. The labeling is moderate in layers I, II–III, and VI, and weak in layer V (**b**). The ZIF268/EGR1-immunopositive cells were distributed similarly to the labeling, but were not observed in the thalamus (data not shown). These results suggest that the labeling reflects *zif268/egr1* expression in cortical neurons. Similar labeling is observed in the three independently generated transgenic lines, which is thought to reflect expression controlled by the *zif268/egr1* promoter; however, the labeling is not caused by artificial expression such as the location of the transgene insertion site in the genome. *I*, *II–III*, *IV*, *V*, and *VI* indicate the cortical layers. Scale bar, 300 μm.

Supplementary Figure 3.

Induction of the labeling in cortical slices by electrical stimulation. Labeling under the influence of electrical stimulation is examined in parietal slices on P7. A pentobarbital solution in PBS (50 μg/g-body weight) was injected into the peritoneal cavity of P7 mice prior to decapitation. The brains were rapidly removed in dissection buffer (1 mM kynurenic acid in artificial cerebrospinal fluid [ACSF] containing: NaCl, 124; KCl, 5; NaH_2PO_4, 1.25; $MgSO_4$, 1; $CaCl_2$, 2; $NaHCO_3$, 26; and glucose, 10 [in mM]) (Kirkwood and Bear, 1994), on

ice and embedded in 2% low-melting-point agarose (Wako Chemicals, Osaka, Japan) with ACSF. They were coronally sectioned to 400-μm-thick (Vibratome) sections and collected in dissection buffer. Then, the slices were washed twice in ACSF and placed on a 1% agarose gel in ACSF. They were maintained in a CO_2 incubator (5% CO_2 at 37°C) for at least 1 h before stimulation. For electrical stimulation, a concentric electrode (300-μm diameter; Unique Medical, Tokyo, Japan) was placed at the border between layer VI and the white matter. Each pulse was 200 μs in duration and 1−30 μA in amplitude. A series of pulses was applied at 50 Hz for 1−15 min. After stimulation, the slices were maintained in a CO_2 incubator for 4 h, then fixed in 3.7% formaldehyde in PBS at room temperature, and washed in PBS. Images of the slices were obtained before stimulation, immediately after stimulation, and 4 h after stimulation under both a bright-field microscope and a fluorescence microscope (MZFLIII, Leica) with a digital camera. All images were processed with the same parameters in the ImageJ software.

After 4 h in culture, labeling is observed in layers IV and VI, which receive thalamocortical input, confirming the labeling in cortical neurons (**a** and **b**). Greater current (10 μA, 50 Hz for 15 min) induces a broader area of labeling (**c**); the labeled areas are radially arranged in columns and align at regular intervals. Fewer pulses (10 μA, 50 Hz for 1 min) cause no labeling (**d**). The labeling is dependent on past electrical stimulation; the areas and intensity of the labeling depend on the amount of current and the number of pulses. Thus, it reflected the history of electrical stimulation.

Slices are stimulated using 200-μs pulses at 50 Hz with 0 μA (**a**), 2 μA for 15 min (**b**), 10 μA for 15 min (**c**), and 10 μA for 1 min (**d**). Slices under bright-field illumination (**1**),

fluorescence illumination before stimulation (**2**), immediately after stimulation (**3**), and 4 h after stimulation (**4**). A concentric electrode is placed at the border between layer VI and the white matter (**1**–**3**), and stimulation sites are indicated by asterisks (**4**). *IV* and *VI* indicate the cortical layers. Scale bars, 300 μm.

Supplementary Figure 4.

Reduction of the labeling by unilateral whisker removal in the contralateral barrel cortex. Labeling under the influence of sensory experiences was examined *in vivo*. After the critical period of barrel formation, all whiskers on either side were removed on P5 (mice with unilateral whiskers removed [left removal, $n = 7$; right removal, $n = 9$] and control mice without removal [$n = 5$]). The mice were perfusion-fixed on P10. Their brains were coronally sectioned for immunohistochemistry. Images of ZIF268/EGR1 immunostaining in the barrel cortex of the side ipsilateral or contralateral to the whisker removal were obtained with a digital camera and were processed with the same parameters in ImageJ. The ZIF268/EGR1-immunopositive cells were counted in six serial sections of either side of the barrel cortex. To examine the effect of unilateral whisker removal, the reduction rates of the number of ZIF268/EGR1-immunopositive cells ([contra – ipsi]/ipsi) were compared between the control and experimental mice with paired *t*-tests. Images of Venus fluorescence in the barrel cortices were obtained, processed with the same parameters, and transformed into binary images in ImageJ. The labeled areas were measured. Reduction rates in the labeled area (average for [contra – ipsi]/ipsi in six serial sections per mouse) were compared between the control and experimental mice with paired *t*-tests.

Coronal sections of the barrel cortex at P10 with bilateral whiskers intact (*Intact*: **a**, **b**), right whiskers removed (*Right Removed*: **c**, **d**), and left whiskers removed (*Left Removed*: **e**, **f**). Cortical layers (*IV* and *VI*) are indicated. Scale bar, 300 μm. Reduction rates for the number of ZIF268/EGR1-immunopositive cells in the contralateral barrel cortex are shown in (**g**) with whiskers intact (*Ctrl*: $n = 5$) and with whiskers unilaterally removed (*Unilateral removal*: $n = 16$). Labeling of the density reduction rates in the contralateral barrel cortex are shown in (**h**) with whiskers intact (*Ctrl*: $n = 5$) and with whiskers unilaterally removed (*Unilateral removal*: $n = 16$). * and ** indicate significant reductions in contralateral labeling compared to the ipsilateral side (paired *t*-tests: $p < 0.05$ and $p < 0.01$, respectively).

In the side contralateral to whisker removal, the ZIF268/EGR1-immunopositive cells was significantly less (12%) than in the ipsilateral side (paired *t*-test: $t = -2.457$, $p = 0.0266$). The immunopositive cells were not significantly different between sides in animals with intact whiskers (paired *t*-test: $t = -0.228$, $p = 0.8308$) (**g**). The results confirm that unilateral whisker removal reduces neuronal activity in the contralateral barrel cortex. Furthermore, the labeled area on the side contralateral to whisker removal is significantly smaller (31%) than that on the ipsilateral side (paired *t*-test: $t = -3.494$, $p = 0.0033$), although the labeled areas are similar on both sides in animals with intact whiskers (paired *t*-test: $t = 0.736$, $p = 0.5025$) (**h**).

Unilateral whisker removal slightly, but significantly, reduced the number of ZIF268/EGR1-immunopositive cells in the contralateral barrel cortex, indicating reduced neuronal activity. In contrast, the labeling was reduced significantly and remarkably in the contralateral barrel cortex, suggesting that the labeling can change a small difference in

neuronal activity into a distinct difference. Thus, it is thought that a facet of neuronal activity history is labeled and that the distribution and intensity of the labeling indicates the quality and quantity of the experience, respectively. The results indicate that the labeling reflects the sensory experiences of whiskers and the related neuronal activity in the barrel cortex.

Supplementary figure 5.

Unilateral labeling in the FR by lateralized expression of Venus in the LHb. The FR was segmentally labeled in all cases but a case shown below in (**a**), which represents unilateral labeling from the LHb to the FR in the consecutive sections. Ventrally, the FR is intensely labeled; dorsally, it is moderately labeled. Weak labeling is observed in the LHb. (**b**) Higher magnification of the label in the LHb is shown. *Arrowheads* indicate the label in the FR. *Arrows* indicate the label in the LHb.

The FR is segmentally labeled; the lengths of the segments vary among the cases. The graph represents the relative dorsoventral positions along the FR (P13, 11 cases) (**c**). The vertical axis indicates relative dorsoventral position as %DV; the dorsal and ventral end of the section is 0% and 100%DV, respectively. The FR is situated in the range of 10-90%DV. *Squares* and *diamonds* indicate the dorsal and ventral end of the label, respectively. The lines between the square and diamond indicate the dorsoventral ranges of the labeled segments. The line colors indicated the sides of the label; *black* and *blue* indicate the left and right, respectively.

Venus mRNA in the LHb is expressed in the same side of the FR labeling. Comparing to the right LHb (**d**), many *Venus* mRNA positive cells are observed in the left

LHb (*arrow* in **e**) in the sane side of the FR labeling (*arrowhead* in **f**).

MHb, medial habenular nucleus. *LHb*, lateral habenular nucleus. *R* and *L* indicate the right and left, respectively. Scale bars, 500 μm (**a**), 200 μm (**b**) and 100 μm (**d**-**f**).

Supplementary Figure 6.

Unilateral Venus labeling of the FR in the independent transgenic lines. (**a**) In the second transgenic line (*zsgv-b*), the left FR is weakly labeled (*arrow*), which is possibly due to the lower copy number of the transgene. (**b**) In the third transgenic line (*zsgv-c*), the dorsal and medial MHb axons are labeled in the FR core (*arrowheads*), which is thought to be due to ectopic expression depending on the location of the transgenic insertion in the genome. Its details are described (Ichijo and Toyama, 2014). (**c**) In another case of *zsgv-c*, sheath of the left FR is labeled in addition to the FR core; thus, the labeled area in the left (*arrow*) is larger than the right (*arrowhead*), indicating that the LHb axons are unilaterally labeled. Unilateral FR labeling is observed in the three independently generated transgenic lines; thus, it is thought to be controlled by *zif268/egr1* promoter. *FR*, fasciculus retrofleus. *R* and *L* indicate the right and left, respectively. Scale bars, 100 μm.

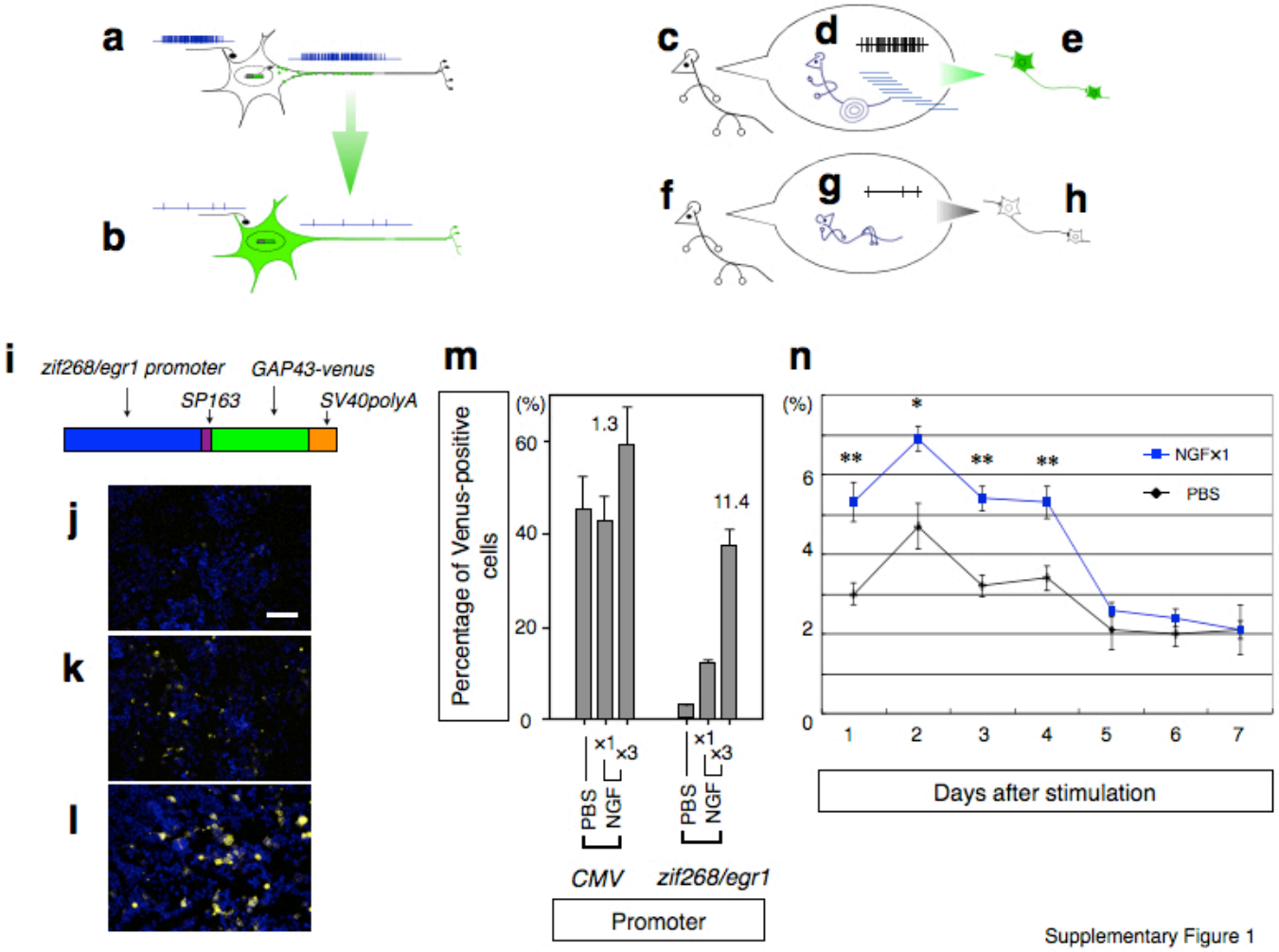
Supplementary figure 7.

Lateralized activation in the LHb induced by water-immersion restraint stress. Average number of c-Fos-immunopositive cells in both sides, R + L, are shown (**a**). Average differences in the right and left sides, R – L, are shown (**b**). Average absolute values of the differences, |R − L|, are shown (**c**). ** and * indicate significant differences from zero ($p <$

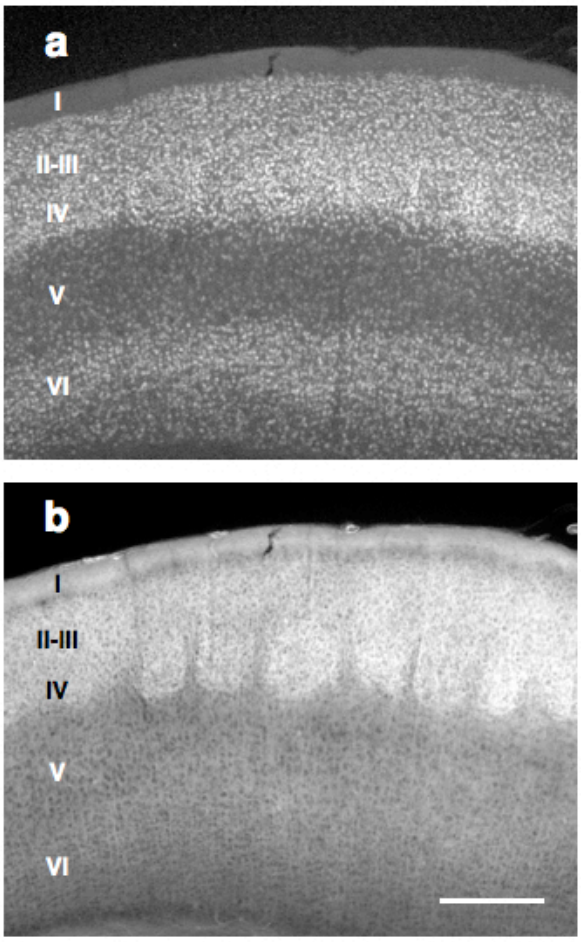
0.01 and $p < 0.05$, respectively, Wilcoxon signed-rank tests). Hours after water-immersion restraint stress are indicated.

References for supplementary information

Kirkwood, A., Bear, M.F., 1994. Hebbian synapses in visual cortex. J. Neurosci. 14, 1634-1645.



Supplementary Figure 1



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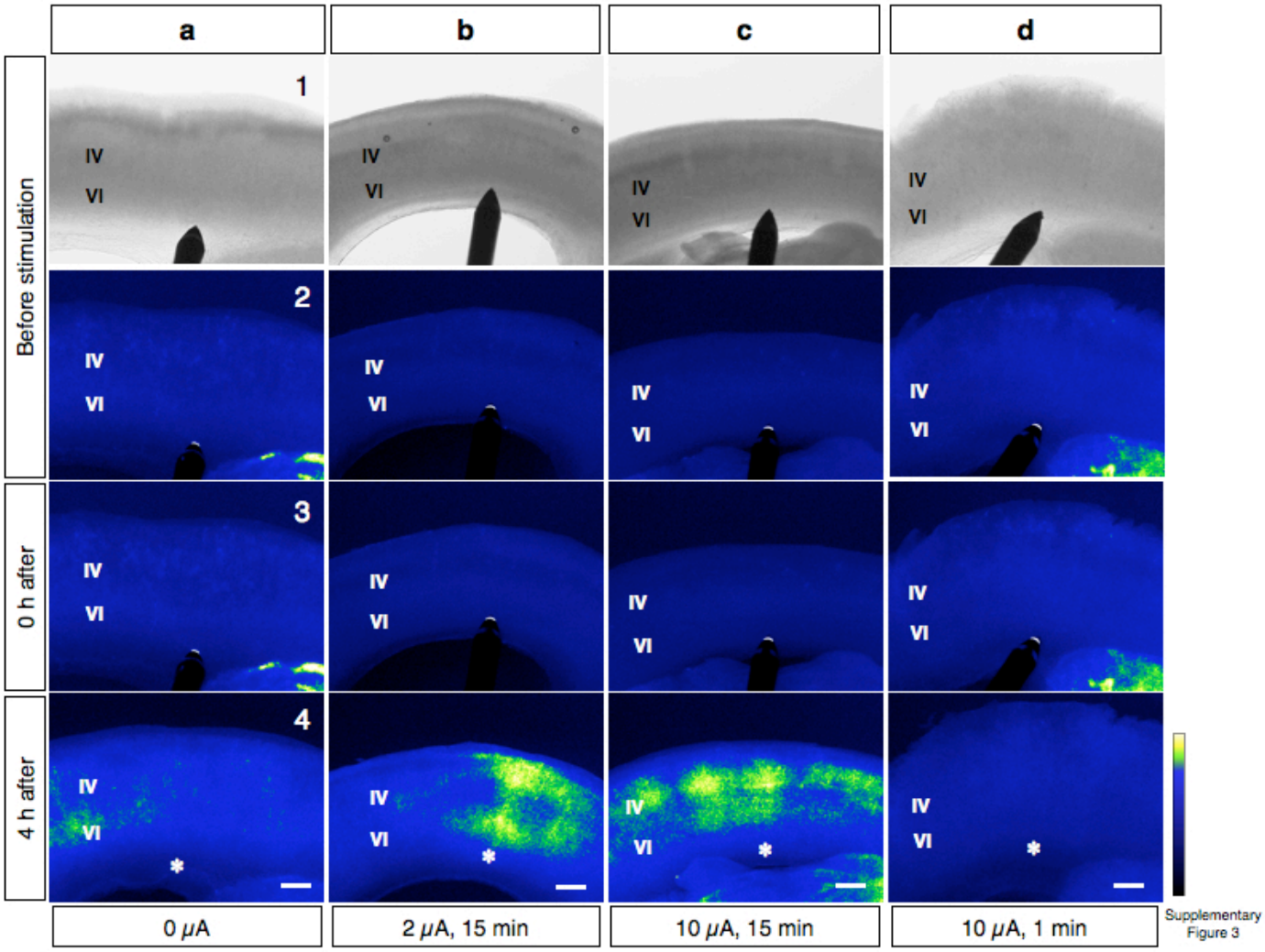

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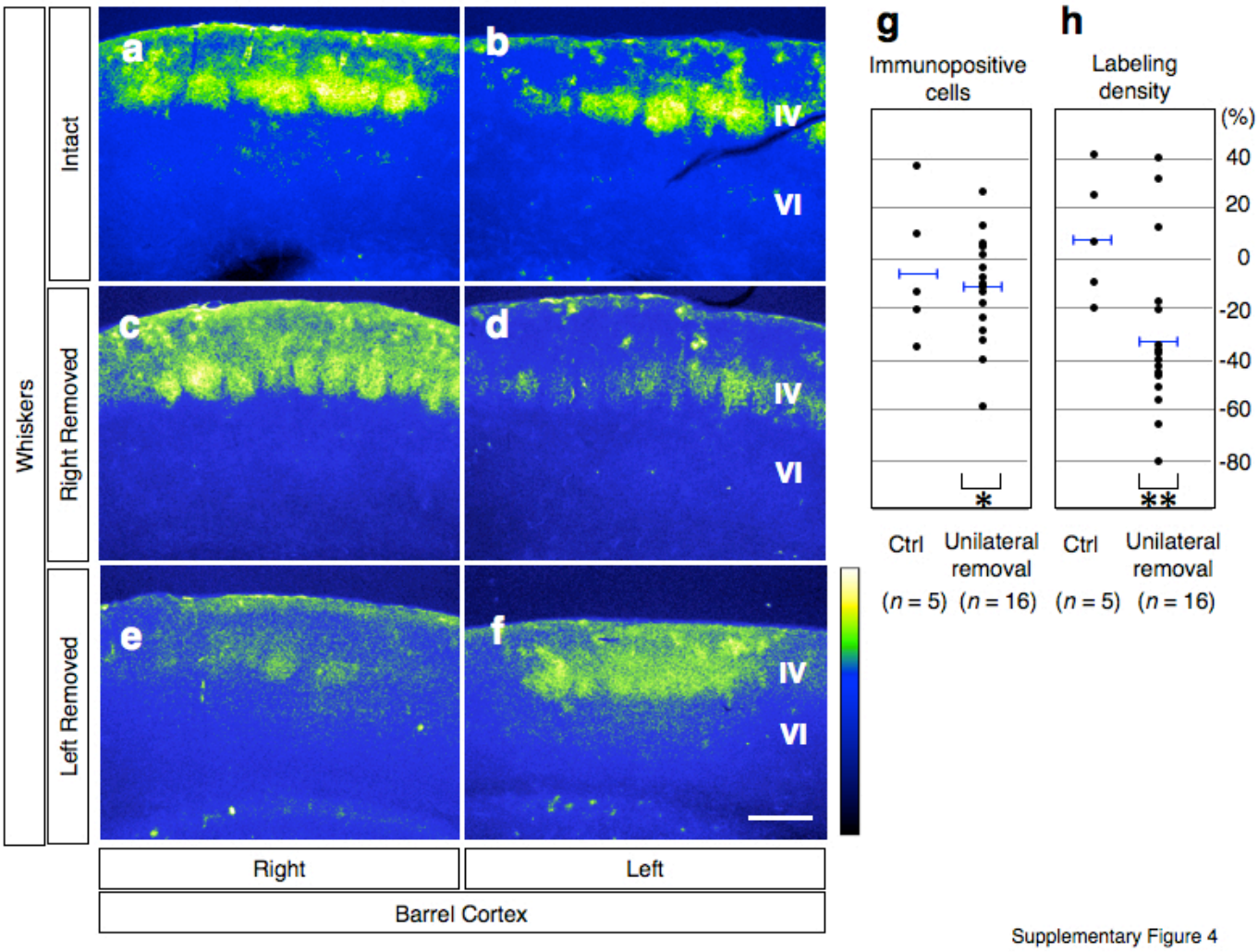

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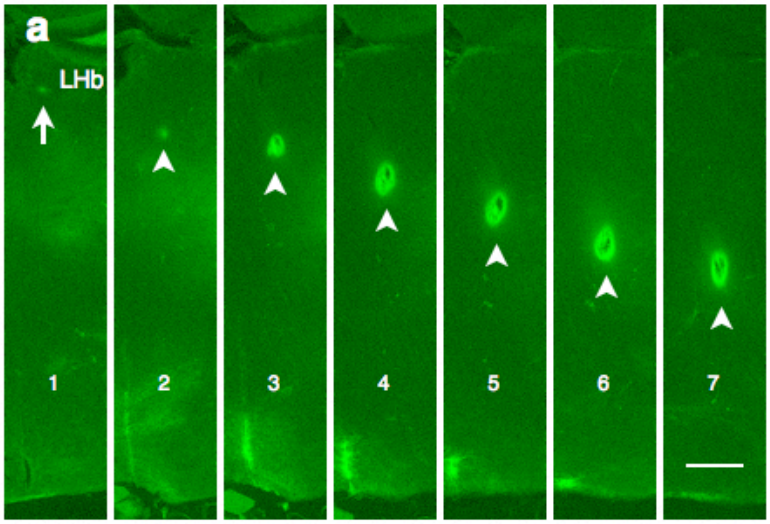
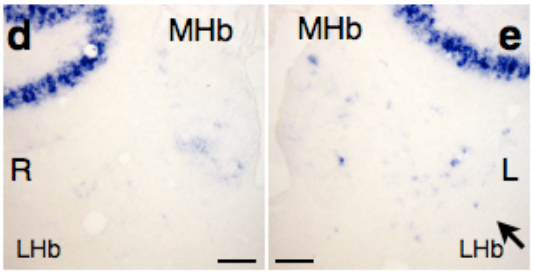
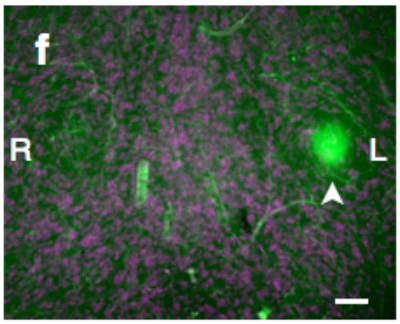
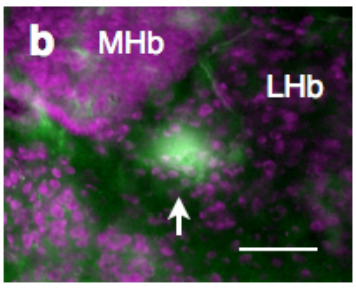
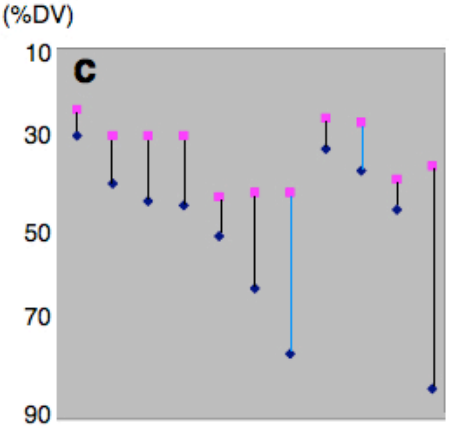
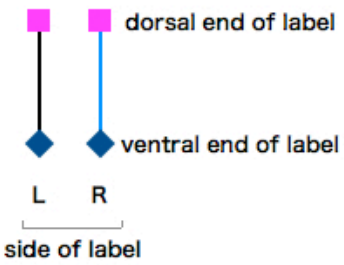




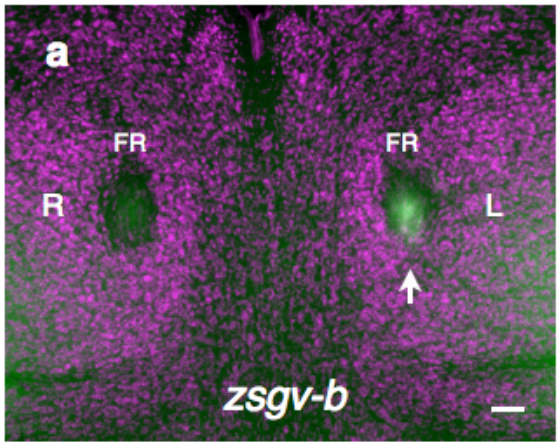


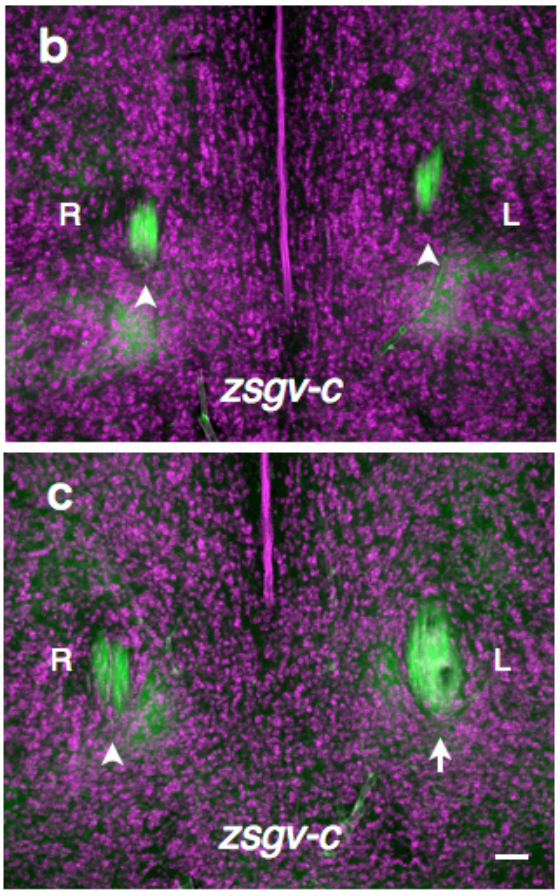




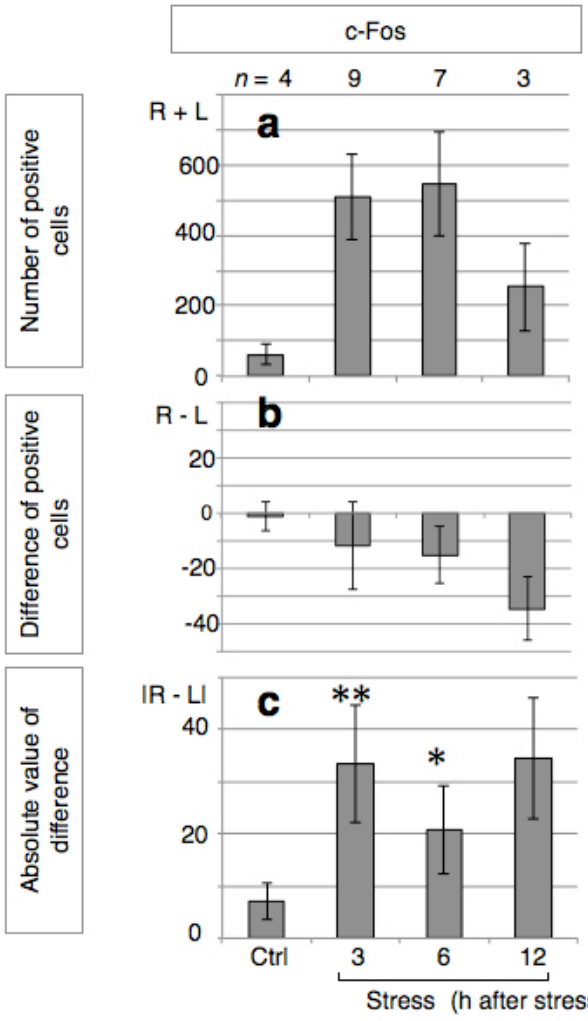


Supplementary Figure 5





Supplementary Figure 6



Supplementary Figure 7