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Supplemental Information

Neurotransmitter Switching Regulated by miRNAs Controls Changes in Social Preference

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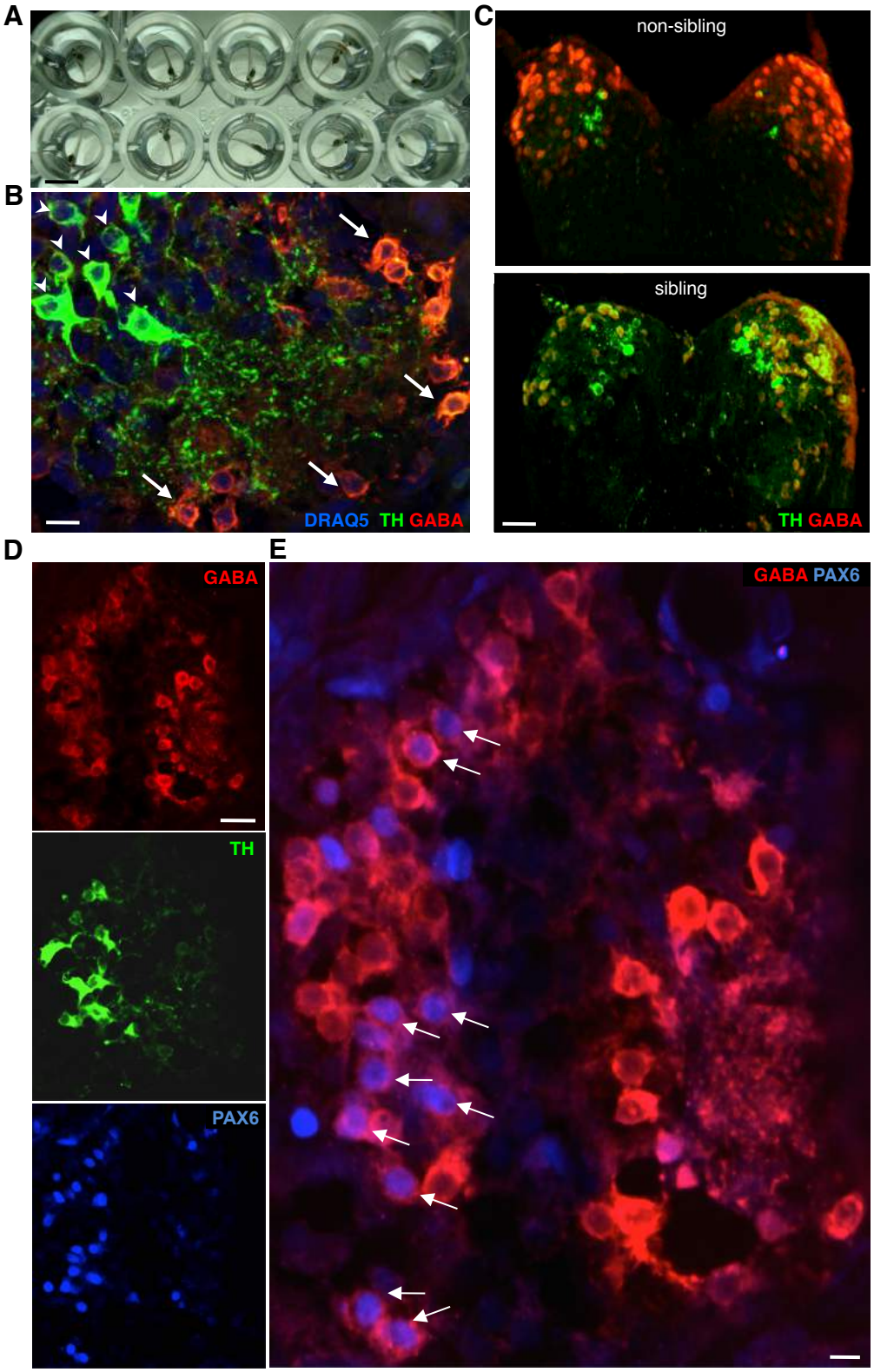
A
B
C
non-sibling
sibling
DRAQ5 TH GABA
TH GABA
D
GABA
TH
PAX6
E
GABA PAX6

Figure S1

Figure S1. Kinship odorant exposure, transmitter respecification and Pax6 localization. Related to Figure 1.

(A) Stage 39 embryos were single-housed in 48-well plates in 200 µl of solution conditioned by kin or non-kin embryos. After 12 hr of odorant exposure, kin or non-kin odorant solution was replaced with solution freshly conditioned by the same class of odorants until larvae had developed to stage 44 (one day later) and were ready for immunocytochemical examination or behavioral testing. Scale bar, 5 mm.

(B) Dopaminergic and GABAergic periglomerular (arrows) and granule (arrowheads) neurons visualized in a horizontal section of the AOB (stage 44) triple-labeled for TH, GABA, and DRAQ5. Scale bar, 20 µm.

(C) Changes in neurotransmitter expression when larvae were raised in non-sibling versus sibling conditions. Whole mount preparations of dissected AOBs (stage 44) double-labeled for TH and GABA. Scale bar, 50 µm.

(D) PAX6 expression visualized in a horizontal section of the AOB (stage 44) triple labeled for GABA, TH, and PAX6. Scale bar, 30 µm.

(E) Superimposed image of the same GABA and PAX6 channels in (D) shown at higher magnification identifies a pool of Pax6+GABA+ granule neurons (arrows). Scale bar, 10 µm.

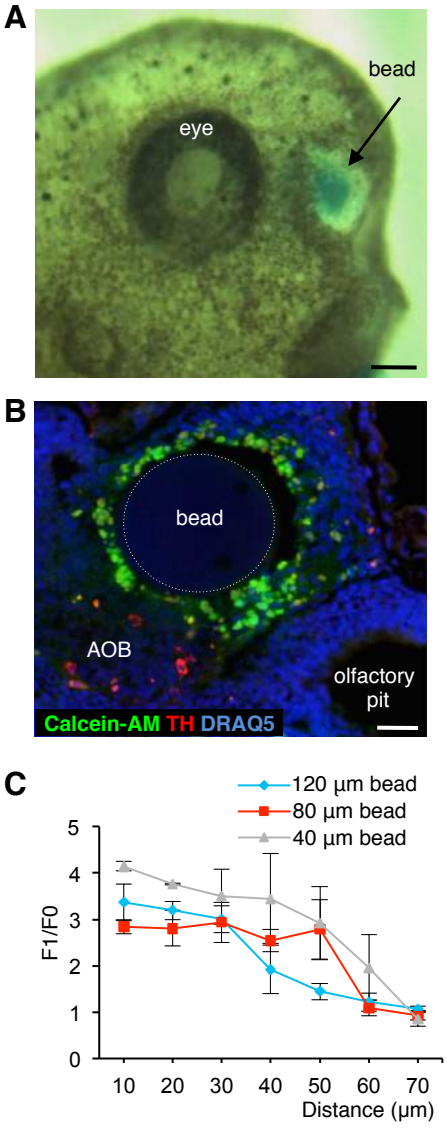
A
bead
eye
B
bead
AOB
olfactory
pit
Calcein-AM TH DRAQ5
C
120 µm bead
80 µm bead
40 µm bead
F1/F0
0
1
2
3
4
5
10
20
30
40
50
60
70
Distance (µm)

Figure S2

Figure S2. Local delivery of drugs. Related to Figure 1.

Bright field image of a stage 39 larva shows the site of incision in the olfactory pit where an agarose bead was inserted (top, arrow). Image of a horizontal section through the AOB and olfactory pit (stage 44) triple labeled for calcein-AM, TH and DRAQ5, shows fluorescent calcein-AM diffusion from the bead 24 hr after implantation (middle, dotted circle). Calcein-AM diffusion from the site of implantation for three different bead diameters (bottom) shows that layers of cells that were 70 μm from the bead had a level of fluorescence (F1) equal to background (F0). Bead size did not affect diffusion. Scale bars, 100 μm (top) and 30 μm (middle). Graph shows the mean ± SD. N=10 larvae.

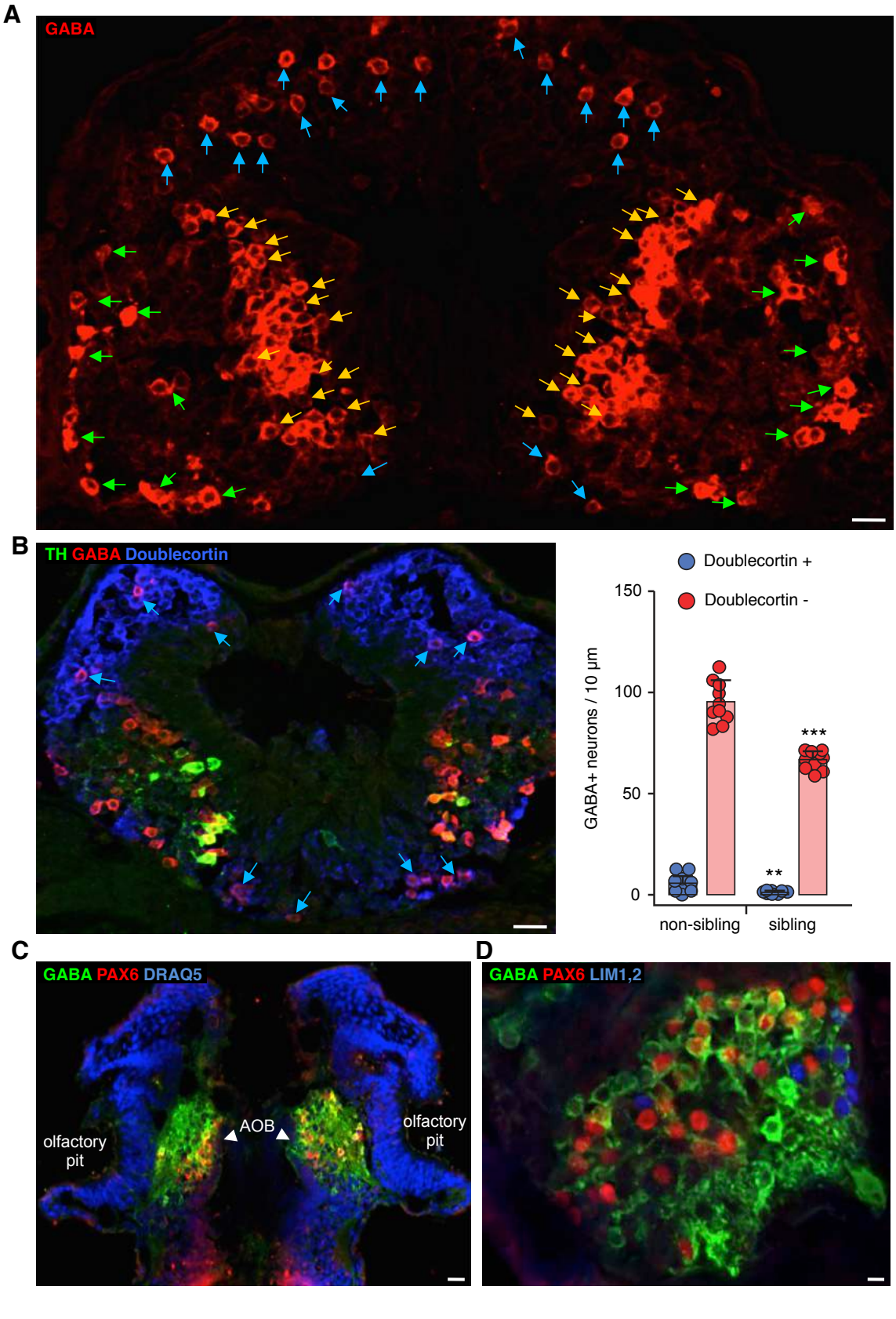
A
GABA
B
TH GABA Doublecortin
Doublecortin +
Doublecortin -
150
100
50
0
GABA+ neurons / 10 µm
**
***
non-sibling
sibling
C
GABA PAX6 DRAQ5
olfactory pit
AOB
olfactory pit
D
GABA PAX6 LIM1,2

Figure S3

Figure S3. Neurotransmitter switching does not occur in newborn but in Pax6+ neurons. Related to Figure 2.

(A) Location of different classes of GABAergic neurons in the AOB. Periglomerular neurons (green arrows), granule cells (yellow arrows), and dorso-ventral interneurons are located outside the developing glomeruli (blue arrows). Horizontal section, stage 44. Scale bar, 20 μm.

(B) Differentiating cells (blue arrows) lie outside the AOB. Horizontal section (stage 44) triple labeled for TH, GABA, and doublecortin. Scale bar, 30 μm. Quantification of the number of GABAergic neurons / 10 μm section that are either doublecortin- negative or positive across non-sibling and sibling raising conditions. The lack of doublecortin expression displayed by TH+ and GABA+ neurons in the AOB indicates that this form of experience-dependent NT respecification is not occurring in newborn differentiating neurons. Graphs show data points (circles) and the mean ± SD. N = 10 larvae from 3 independent experiments. Unpaired Student's t-test. **p ≤ 0.01, ***p ≤ 0.001

(C) AOB (arrows) and olfactory pit triple labeled for GABA, PAX6, and DRAQ5. Horizontal section, stage 44. Scale bar, 50 μm.

(D) AOB triple labeled for GABA and transcription factors PAX6 and LIM1 shows a pool of Pax6+GABA+ differentiated neurons and the absence of GABA+ cells expressing Lim1,2. Horizontal section, stage 44. Scale bar, 10 μm.

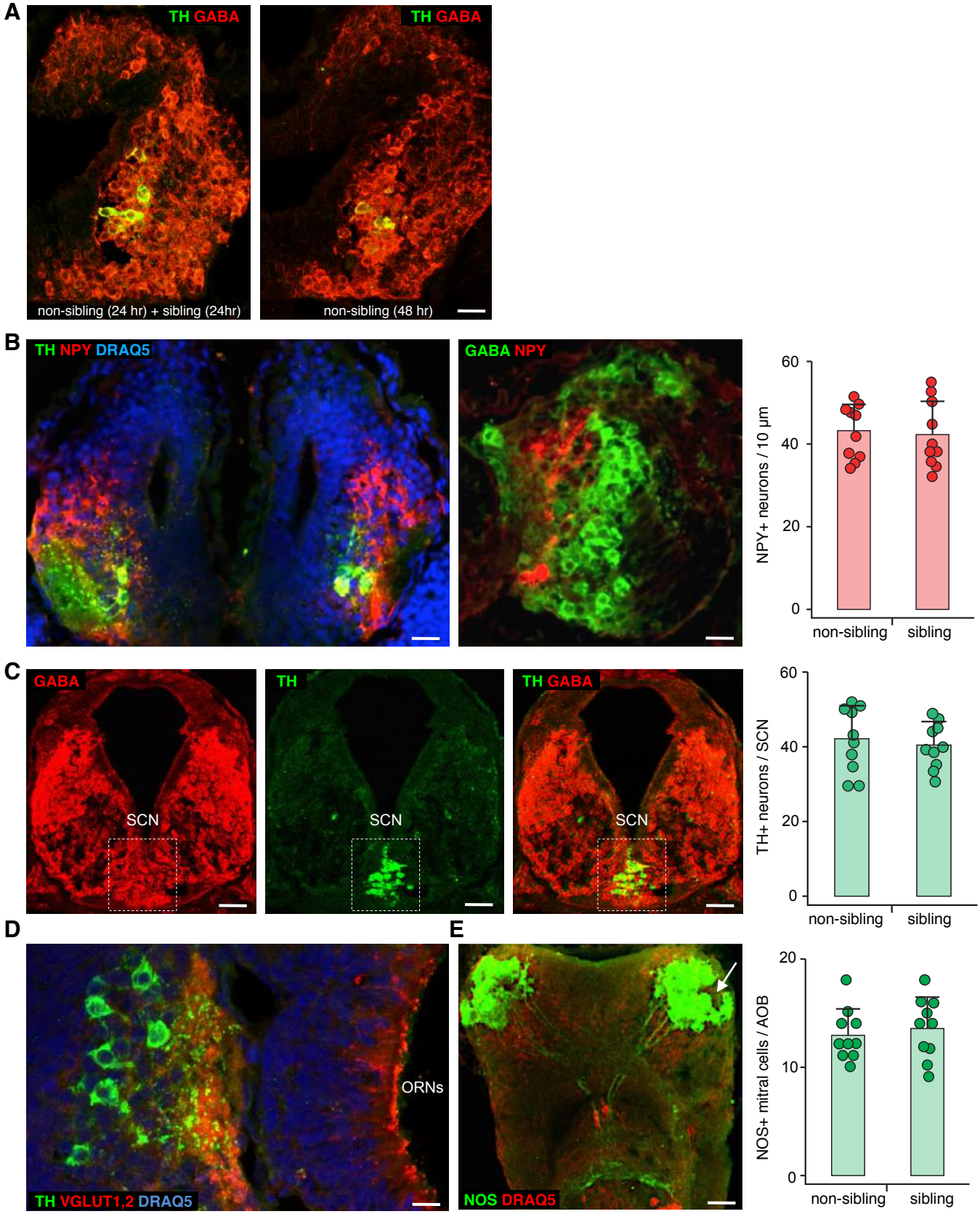
A
TH GABA
non-sibling (24 hr) + sibling (24hr)
TH GABA
non-sibling (48 hr)
B
TH NPY DRAQ5
GABA NPY
NPY+ neurons / 10 µm
60
40
20
0
non-sibling
sibling
C
GABA
SCN
TH
SCN
TH GABA
SCN
TH+ neurons / SCN
60
40
20
0
non-sibling
sibling
D
ORNs
TH VGLUT1,2 DRAQ5
E
NOS DRAQ5
NOS+ mitral cells / AOB
20
10
0
non-sibling
sibling

Figure S4

Figure S4. Dopamine/GABA switching is partially reversible and specific. Related to Figure 2.

(A) Partial reversibility of dopaminergic plasticity in the AOB double labeled for TH and GABA. Larvae were exposed to the non-sibling raising condition for 24 hr (from stage 39 to stage 42) then exposed to either sibling or non-sibling raising condition for another 24 hr (stage 42 to stage 44). Horizontal sections. Scale bar, 40 μm.

(B) No change in number of NPY+ neurons / 10 μm in the AOB (right) across non-sibling and sibling raising conditions. Triple labeling for TH, NPY, and DRAQ5 (left) and double labeling for GABA and NPY (middle). Horizontal sections, stage 44. Scale bars, 30 μm.

(C) No change in the number of TH+ neurons / 10 μm of the suprachiasmatic nucleus (SCN) across non-sibling and sibling raising conditions. Horizontal sections, stage 44, double labeled for TH and GABA. Scale bar, 100 μm .

(D) VNO sensory input (glutamatergic olfactory receptor neurons, ORNs) in the AOB and olfactory pit, triple labeled for TH, VGLUT1,2 and DRAQ5. Horizontal sections, stage 44. Scale bar, 20 μm.

(E) No change in the number of NOS+ mitral cells (arrow) in the AOB across non-sibling and sibling raising conditions. Whole mount preparation of dissected brain (stage 44) double labeled for neuronal nitric oxide synthase (NOS) and DRAQ5. Scale bar, 50 μm.

(B, C, E) Graphs show data points (circles) and the mean ± SD. N = 10 larvae from 3 independent experiments.

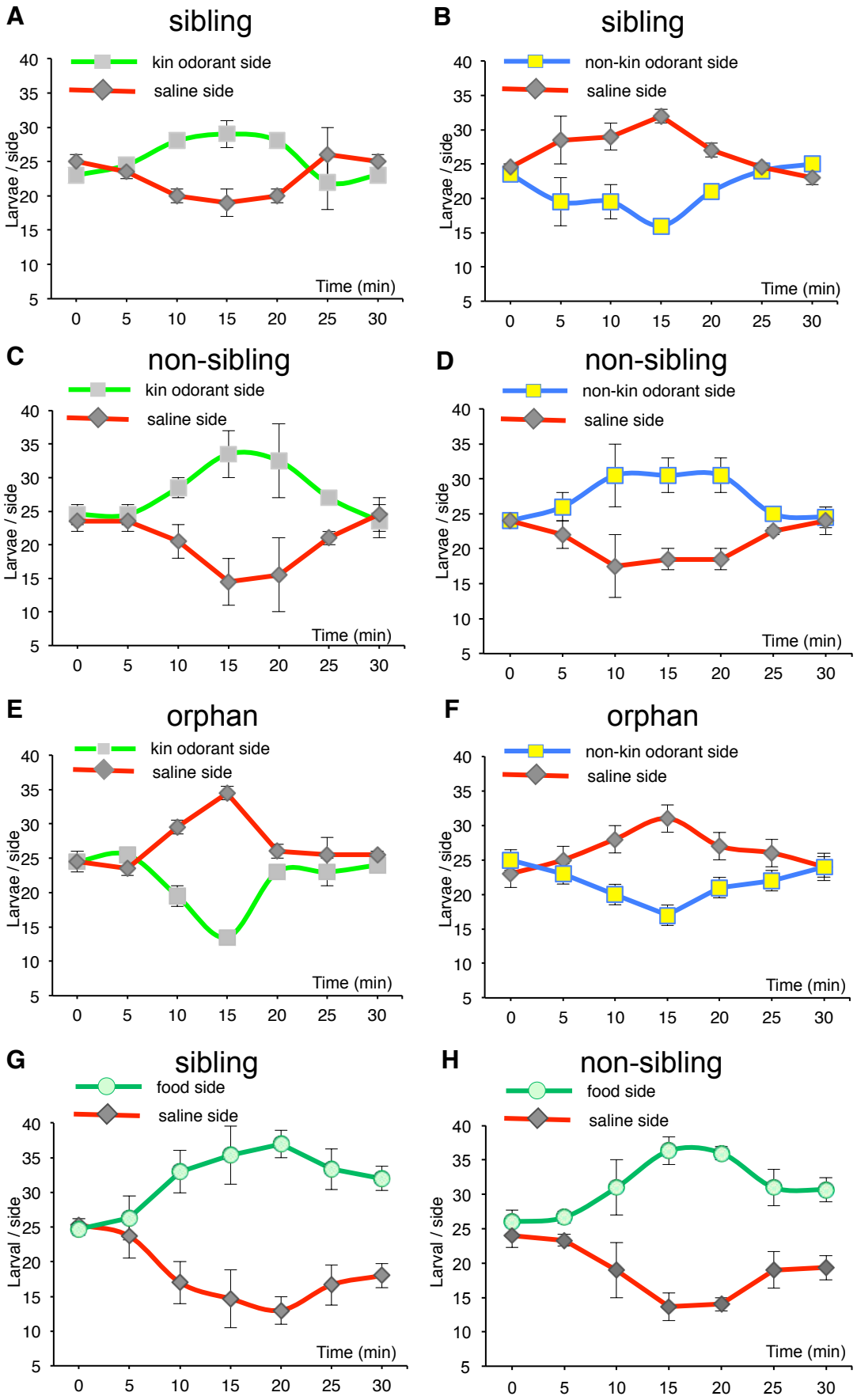
A
sibling
kin odorant side
saline side
Larvae / side
Time (min)
B
sibling
non-kin odorant side
saline side
Larvae / side
Time (min)
C
non-sibling
kin odorant side
saline side
Larvae / side
Time (min)
D
non-sibling
non-kin odorant side
saline side
Larvae / side
Time (min)
E
orphan
kin odorant side
saline side
Larvae / side
Time (min)
F
orphan
non-kin odorant side
saline side
Larvae / side
Time (min)
G
sibling
food side
saline side
Larval / side
Time (min)
H
non-sibling
food side
saline side
Larval / side
Time (min)

Figure S5

Figure S5. Larval distribution assay across raising conditions. Related to Figure 3.

(A, B) Larval distribution assay for stage 44 larvae raised in the sibling condition and tested with kin or non-kin odorants. N=3 groups of 50 larvae/test.

(C, D) Larval distribution assay for stage 44 larvae raised in the non-sibling condition and tested with kin or non-kin odorants. N=3 groups of 50 larvae/test.

(E, F) Larval distribution assay for stage 44 larvae raised in the orphan condition and tested with kin or non-kin odorants. N=3 groups of 50 larvae/test.

(G, H) Larval distribution assay for stage 44 larvae raised in the sibling or non-sibling condition and tested with food. N=3 groups of 50 larvae/test.

(A-H) Graphs show data plotted as mean ± SD.

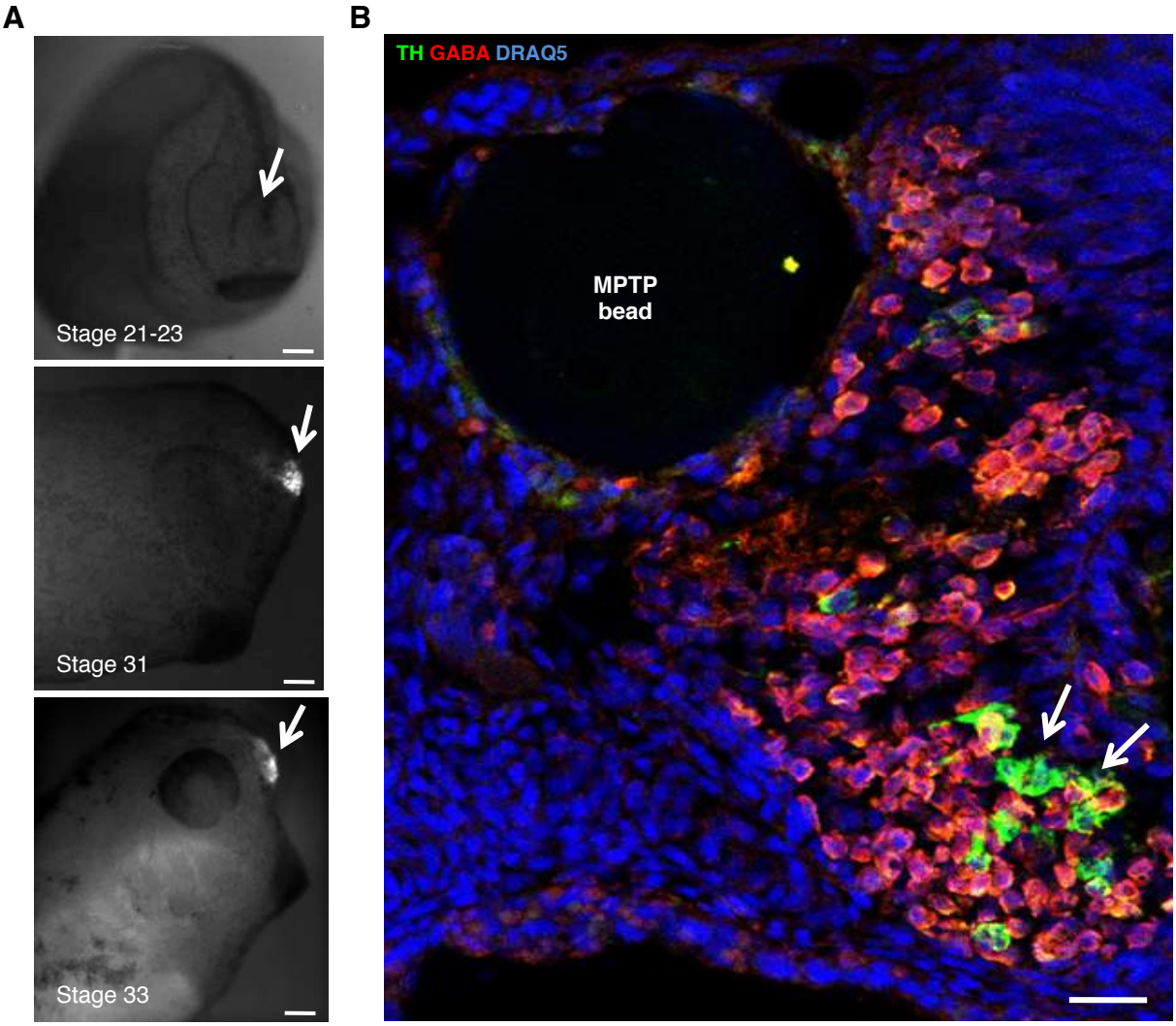

Figure S6

Figure S6. Electroporation of a.375 and a.200b and ablation of dopaminergic neurons with MPTP treatment. Related to Figure 4.

(A) Locus of electroporation (arrow) of FAM-tagged antagonists (LNA) in the AOB performed at stage 21-23 (top) and scored at stage 31 (middle) and 33 (bottom). Scale bars, 100 μm.

(B) MPTP ablation of TH+GABA- interneurons spares TH-GABA+ neurons in the AOB as well as TH+ neurons of the neighboring ventral OB (arrows). MPTP-bead implanted larva triple labeled for TH, GABA, and DRAQ5. Horizontal section, stage 44. Scale bar, 20 μm.

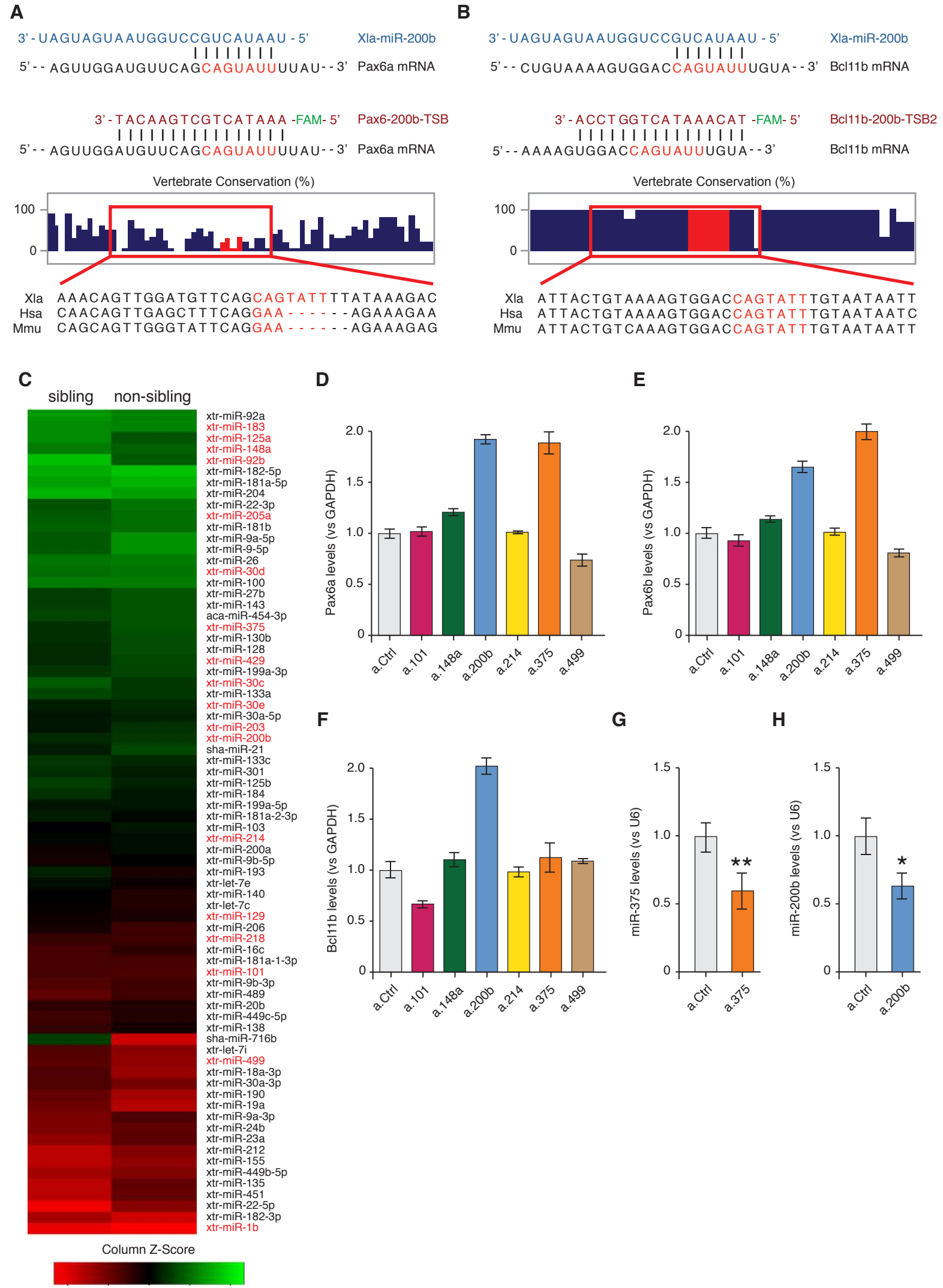
A
3' - UAGUAGUAAUGGUCCGUCAUAAU - 5'
Xla-miR-200b
5' - - AGUUGGAUGUUCAGCAGUAUUUUAU - - 3'
Pax6a mRNA
3' - TACAAGTCGTCATAAA -FAM- 5'
Pax6-200b-TSB
5' - - AGUUGGAUGUUCAGCAGUAUUUUAU - - 3'
Pax6a mRNA
Vertebrate Conservation (%)
100
0
Xla AAACAGTTGGATGTTCAGCAGTATTTTATAAAGAC
Hsa CAACAGTTGAGCTTTCAGGAA - - - - - AGAAAGAA
Mmu CAGCAGTTGGGTATTCAGGAA - - - - - AGAAAGAG
B
3' - UAGUAGUAAUGGUCCGUCAUAAU - 5'
Xla-miR-200b
5' - - CUGUAAAAGUGGACCAGUAUUUGUA - - 3'
Bcl11b mRNA
3' - ACCTGGTCATAAACAT -FAM- 5'
Bcl11b-200b-TSB2
5' - - AAAAGUGGACCAGUAUUUGUA - - 3'
Bcl11b mRNA
Vertebrate Conservation (%)
100
0
Xla ATTACTGTAAAAGTGGACCAGTATTTGTAATAATT
Hsa ATTACTGTAAAAGTGGACCAGTATTTGTAATAATC
Mmu ATTACTGTCAAAGTGGACCAGTATTTGTAATAATT
C
sibling
non-sibling
xtr-miR-92a
xtr-miR-183
xtr-miR-125a
xtr-miR-148a
xtr-miR-92b
xtr-miR-182-5p
xtr-miR-181a-5p
xtr-miR-204
xtr-miR-22-3p
xtr-miR-205a
xtr-miR-181b
xtr-miR-9a-5p
xtr-miR-9-5p
xtr-miR-26
xtr-miR-30d
xtr-miR-100
xtr-miR-27b
xtr-miR-143
aca-miR-454-3p
xtr-miR-375
xtr-miR-130b
xtr-miR-128
xtr-miR-429
xtr-miR-199a-3p
xtr-miR-30c
xtr-miR-133a
xtr-miR-30e
xtr-miR-30a-5p
xtr-miR-203
xtr-miR-200b
sha-miR-21
xtr-miR-133c
xtr-miR-301
xtr-miR-125b
xtr-miR-184
xtr-miR-199a-5p
xtr-miR-181a-2-3p
xtr-miR-103
xtr-miR-214
xtr-miR-200a
xtr-miR-9b-5p
xtr-miR-193
xtr-let-7e
xtr-miR-140
xtr-let-7c
xtr-miR-129
xtr-miR-206
xtr-miR-218
xtr-miR-16c
xtr-miR-181a-1-3p
xtr-miR-101
xtr-miR-9b-3p
xtr-miR-489
xtr-miR-20b
xtr-miR-449c-5p
xtr-miR-138
sha-miR-716b
xtr-let-7i
xtr-miR-499
xtr-miR-18a-3p
xtr-miR-30a-3p
xtr-miR-190
xtr-miR-19a
xtr-miR-9a-3p
xtr-miR-24b
xtr-miR-23a
xtr-miR-212
xtr-miR-155
xtr-miR-449b-5p
xtr-miR-135
xtr-miR-451
xtr-miR-22-5p
xtr-miR-182-3p
xtr-miR-1b
Column Z-Score
-2
-1
0
1
2
D
Pax6a levels (vs GAPDH)
2.0
1.5
1.0
0.5
0
a.Ctrl
a.101
a.148a
a.200b
a.214
a.375
a.499
E
Pax6b levels (vs GAPDH)
2.0
1.5
1.0
0.5
0
a.Ctrl
a.101
a.148a
a.200b
a.214
a.375
a.499
F
Bcl11b levels (vs GAPDH)
2.0
1.5
1.0
0.5
0
a.Ctrl
a.101
a.148a
a.200b
a.214
a.375
a.499
G
miR-375 levels (vs U6)
1.5
1.0
0.5
0
**
a.Ctrl
a.375
H
miR-200b levels (vs U6)
1.5
1.0
0.5
0
*
a.Ctrl
a.200b

Figure S7

Figure S7. miR-375/miR-200b regulate Pax6 and Bcl11b levels in the AOB. Related to Figure 5.

(A) Interaction between *miR-200b* and the MRE *on Pax6a* mRNA (top) and between the target-site blocker Pax6-200b-TSB and the MRE on *Pax6a* mRNA (second from top). Vertical lines indicate base-pair matching. Vertebrate conservation of the region flanking the MRE on *Pax6a* mRNA (second from bottom), adapted from Basewise conservation by PhyloP (UCSC Genome Browser), with Multiz alignments for *Xenopus laevis* (Xla), *Homo sapiens* (Hsa), and *Mus musculus* (Mmu) (bottom). The MRE is poorly conserved.

(B) Interaction between *miR-200b* and the second MRE on *Bcl11b* mRNA (top), and between the target-site blocker Bcl11b-200b-TSB2 and the second MRE on *Bcl11b* mRNA (second from top). Vertebrate conservation of the region flanking the MRE on *Bcl11b* mRNA (second from bottom), with Multiz alignments (bottom).

(C) Heat map of Z-scores for the indicated subset of miRs showing the largest fold changes between sibling and non-sibling conditions. Red entries were further evaluated by qPCR.

(D, E, F) Preliminary screening of top miR candidates suggested that both a.200b and a.375 increased *Pax6a* and *Pax6b* levels, as measured by qPCR, while all others had no detectable effect. Only a.200b increased *Bcl11b* levels. Graphs show the mean ± SD of the 3 technical replicates. N = 5 independent experiments with 15 AOB samples per condition.

(G, H) qPCR of miR levels to test the efficacy of LNA antagonists. a.375 decreased *miR-375* levels (G), and a.200b decreased *miR-200b* levels (H) compared to a.Ctrl in the AOB *in vivo*. Graphs show the mean ± SD. N = 5 independent experiments with 15 AOB samples per condition. Unpaired Student's t-test, *$p<0.05$; **$p<0.01$.

Genes regulating dopaminergic and GABAergic signaling

Gene name	Definition	Function
TH	tyrosine hydroxylase	enzyme
DDC	dopa decarboxylase	enzyme
VMAT	vescicular monoamine transp	transporter
MAO	monoamine oxidase A	enzyme
DAT	dopamine transporter	transporter
COMT	catechol-O-methyltransferase	enzyme
DRD2	dopamine receptor D2	receptor
DRD1	dopamine receptor D1	receptor
PAX6	paired box protein 6	transcription factor
GAD1	glutamate decarboxylase	enzyme
GLSA	glutaminase	enzyme
VGAT, SLC32A	vesicular inhibit aminoacid transp	transporter
ABAT	4-aminobutyrate aminotransferase	enzyme
GABRA	GABA receptor subunit alpha	receptor
GABBR	GABA type B receptor	receptor
SLC6A1, GAT	neurotransmitter transporter, GABA	transporter
GABRR	GABA receptor subunit rho	receptor
GABARAP, ATG8	GABA(A) receptor-associated protein	protein
Bcl11b	C2H2-type zinc finger protein	transcription factor

Table S1

Table S1. Potential miR target genes. Related to Figure 5.

Genes involved in the dopaminergic (blue) or GABAergic (red) pathways including regulatory genes (i.e. transcription factors and genes that determine the NT identity of neurons) and genes involved in NT metabolism (i.e. synthetic enzymes, transporters, degradative enzymes, and receptors).