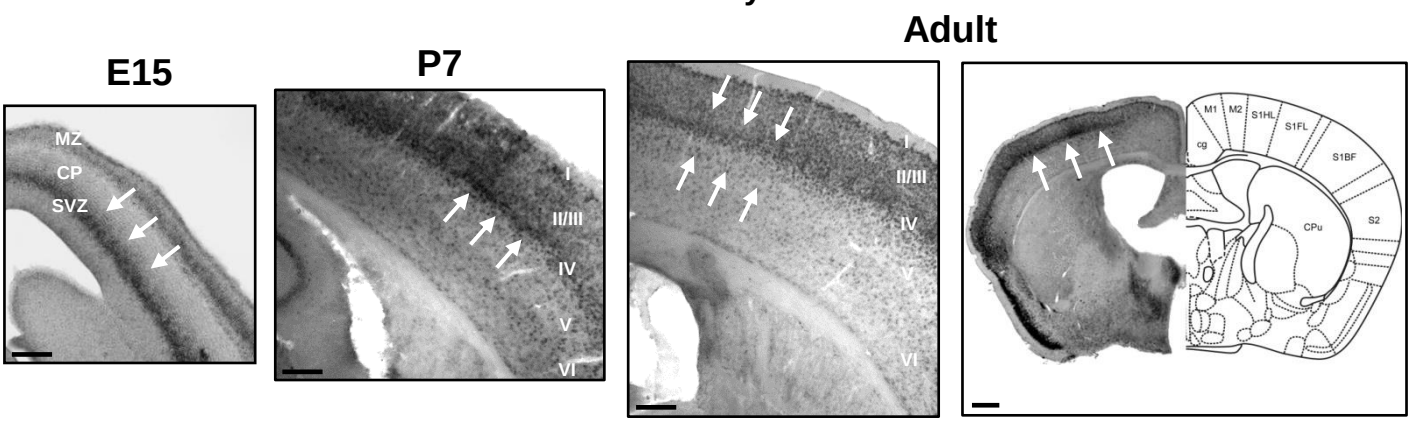
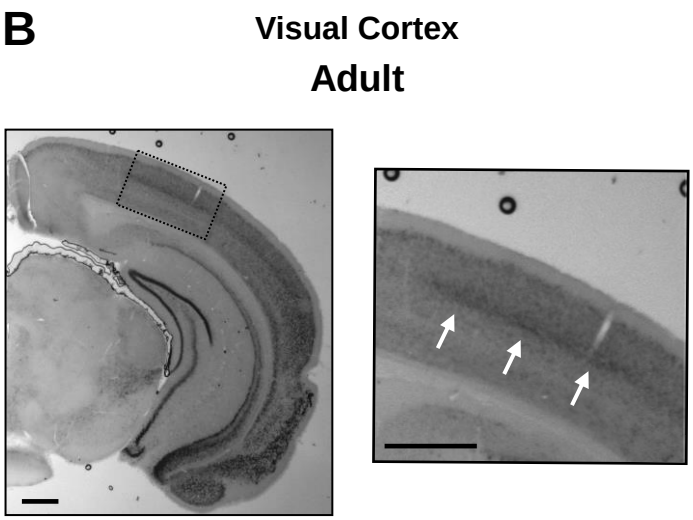
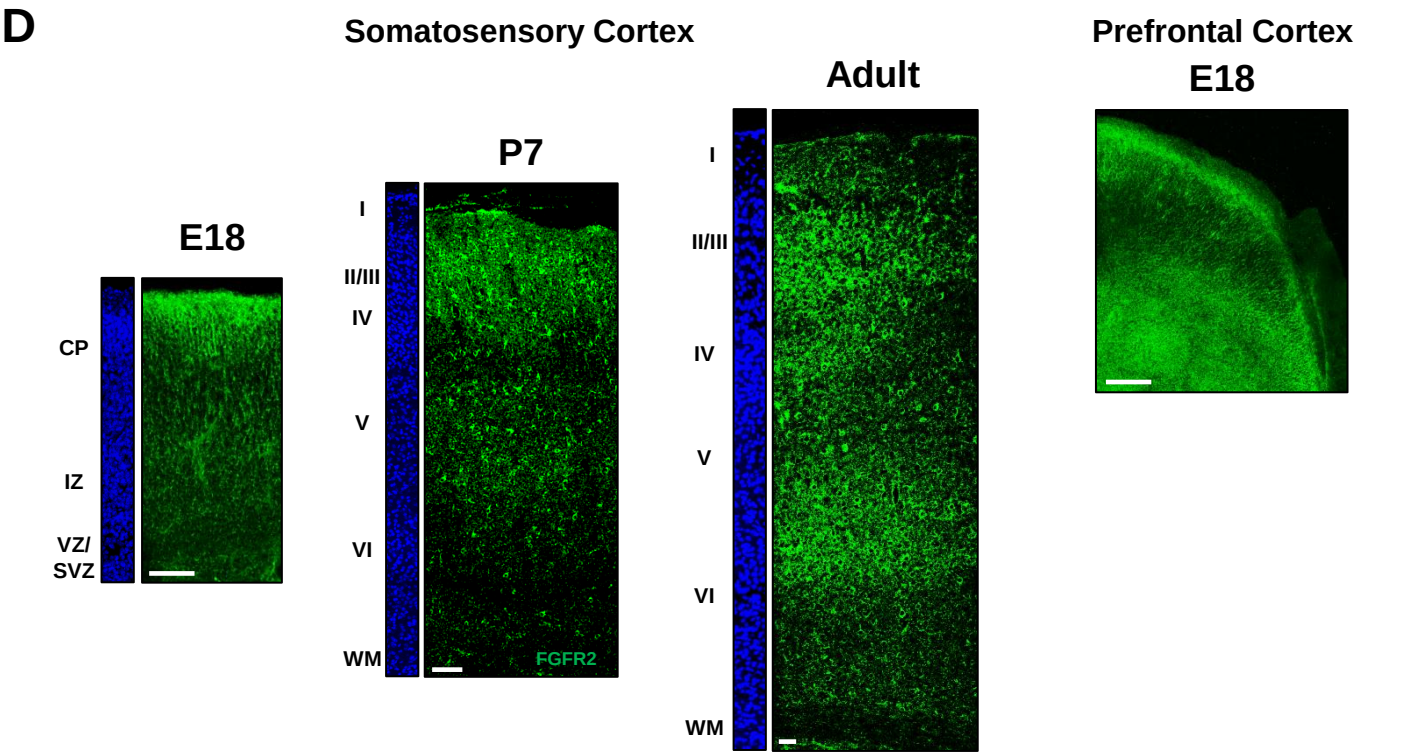


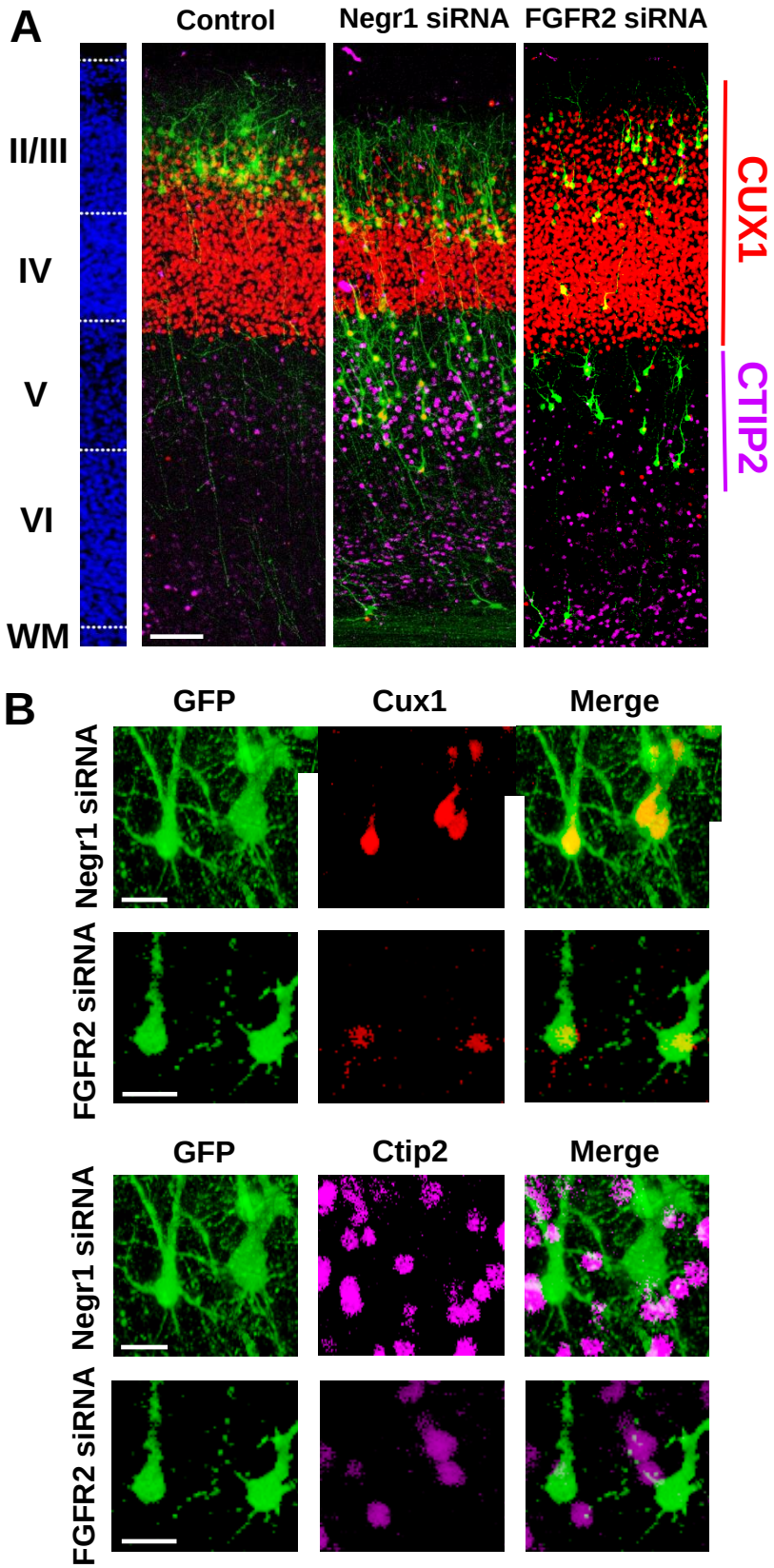




Suppl. Figure 1

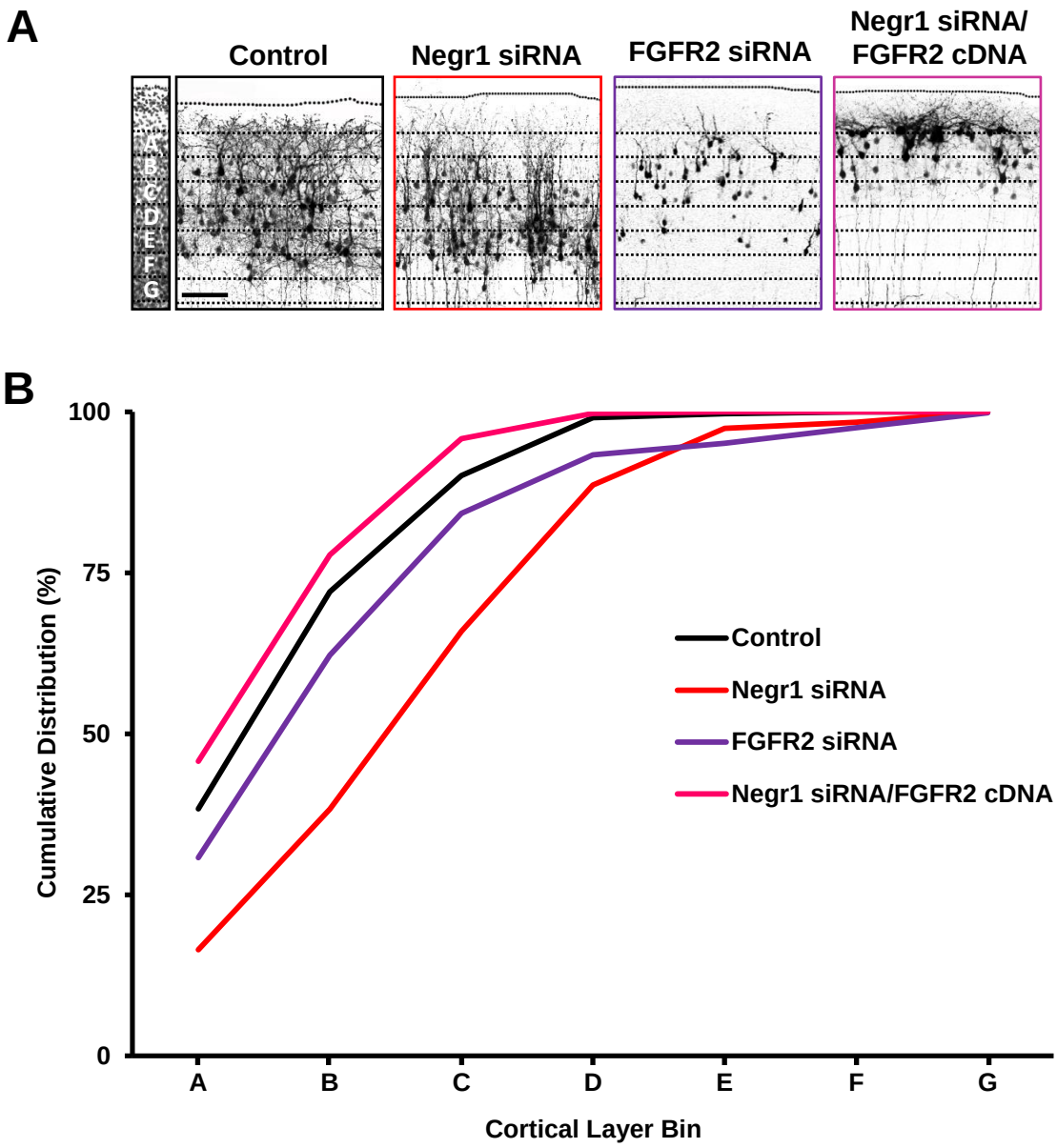
Suppl. Figure 1. Negr1 mRNA is highly expressed in the somatosensory cortex of the developing and adult mouse brain.

(A) *In situ* hybridization of coronal sections of mouse cerebral cortex at different ages with an antisense probe to mouse Negr1. Arrows: high expression of Negr1 in neuronal progenitors of LII/III neurons and marginal zone (MZ)/CP neurons (E15,5) , LII-IV neurons (P7) and LIV neurons (adult). SSC: Somatosensory cortex. Scale bars, 200µm. Atlas image from (Paxinos G, 2008)
(B) *Left*: *In situ* hybridization of a coronal section of mouse cerebral cortex with an antisense probe to mouse Negr1. Rectangle: primary visual cortex. *Right*: Blown up image of the rectangle on left image. Arrows: high expression of Negr1 in the upper layers of the visual cortex. Scale bars, 200µm.
(C) *In situ* hybridization of coronal sections of a mouse cerebral cortex with an antisense probe to mouse Negr1. Arrows: low expression Negr1 in the prospective prefrontal cortex (E15.5). Scale bars, 200µm
(D) Confocal images of FGFR2 immunostaining in the somatosensory cortex of WT mice at different ages. *Left of each image*: DAPI counterstaining. Scale bars, 100µm. VZ = ventricular zone, IZ = intermediate zone, CP = cortical plate; WM = white matter.



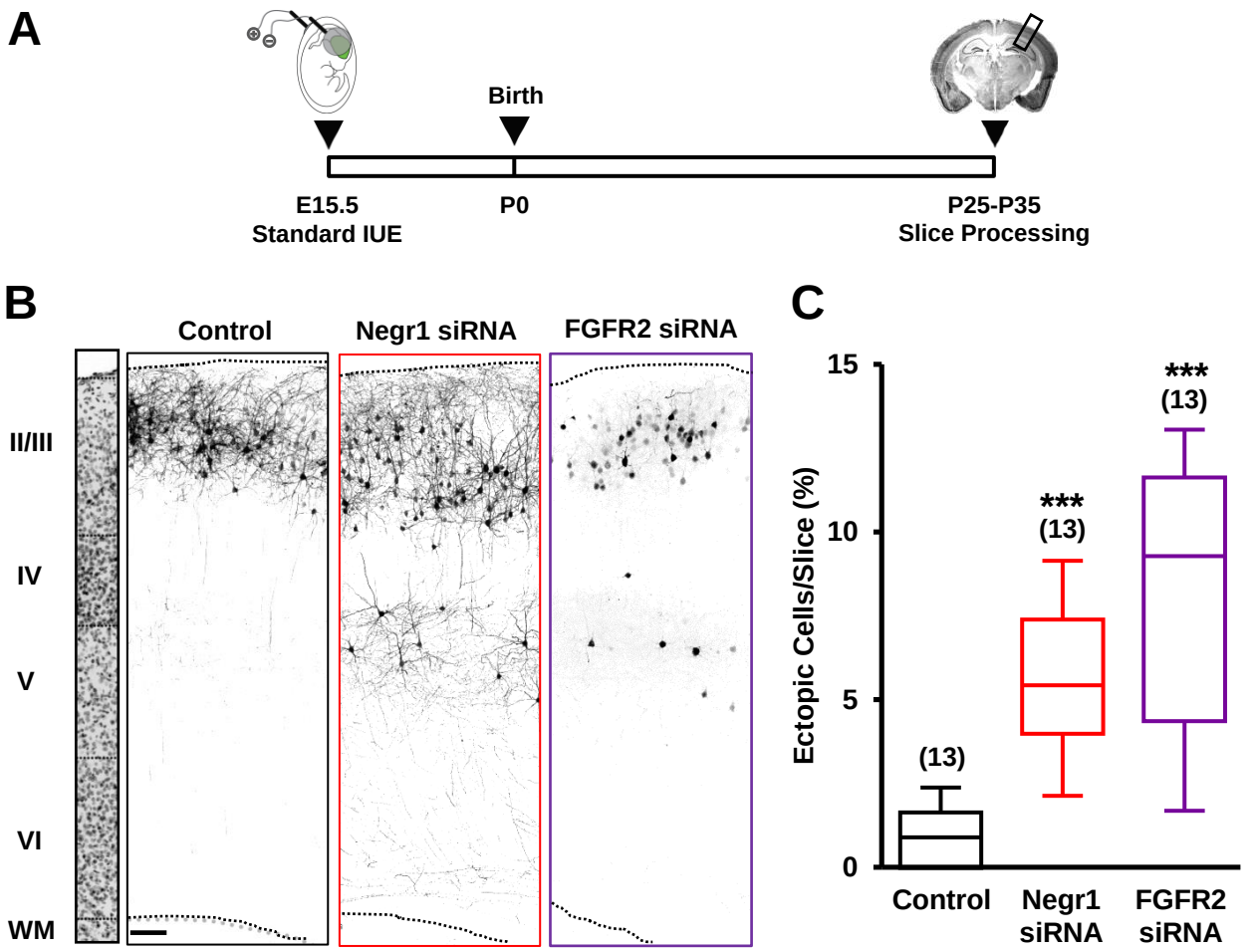
Suppl. Figure 2. Ectopic neurons retain their LII/III identity.

(A) Fluorescence images of somatosensory-cortex slices from P7 animals transfected *in utero* at E15.5, stained for Cux1 (red) and CTIP2 (magenta), and counterstained with DAPI (blue, left). Scale bar, 100 µm.
(B) Magnifications of neurons located at the border between LIV and LV from experiments in A. Scale bar, 10 µm.



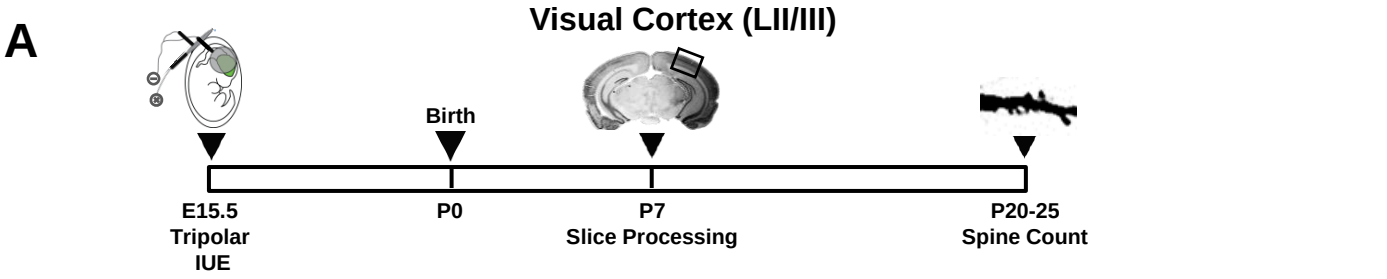
Suppl. Figure 3. Negr1 or FGFR2 downregulation causes impairment in the final positioning of neurons within layer II/III *in vivo*.

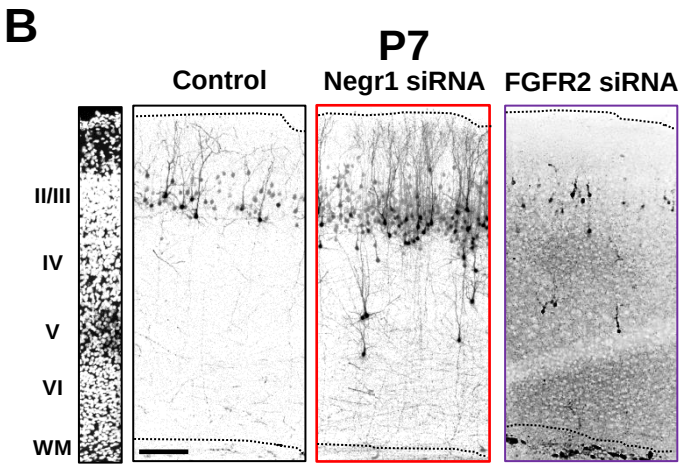
(A) GFP fluorescence in coronal sections of somatosensory cortices at P7 after *in utero* transfection (at E15.5) with scramble siRNA (control), Negr1 siRNA, FGFR2 siRNA or Negr1 siRNA together with FGFR2 cDNA divided into 7 bins from A-G. Left: DAPI counterstaining. Scale bar, 100 µm.
(B) Quantification of the total number of transfected neurons located within each of the cortical bins in experiments as in A. Data are expressed as average cumulative distribution of the total number of fluorescent cells in the bin. Negr1 siRNA and FGFR2 siRNA distribution is significantly different when compared to Control. Negr1 siRNA/FGFR2 cDNA rescued the phenotype. (Chi-Squared test, pairwise comparisons with Control (see Material and Methods for further information): Negr1 siRNA $\chi^2 = 154.4$ $p<0.0001$, FGFR2 $\chi^2 = 20.6$ $p=0.0009$, Negr1 siRNA/FGFR2 cDNA $\chi^2 = 4.858$ $p=0.433$).

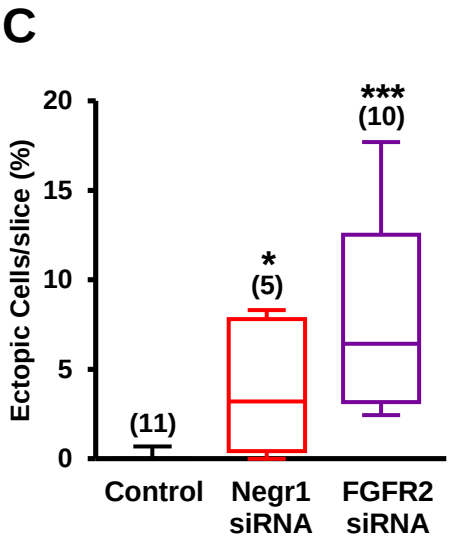


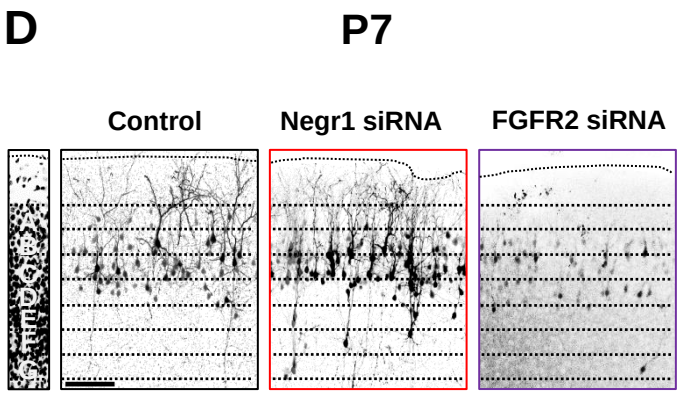
Suppl. Figure 4. The effect of Negr1 and FGFR2 downregulation on neuronal migration is long-lasting.

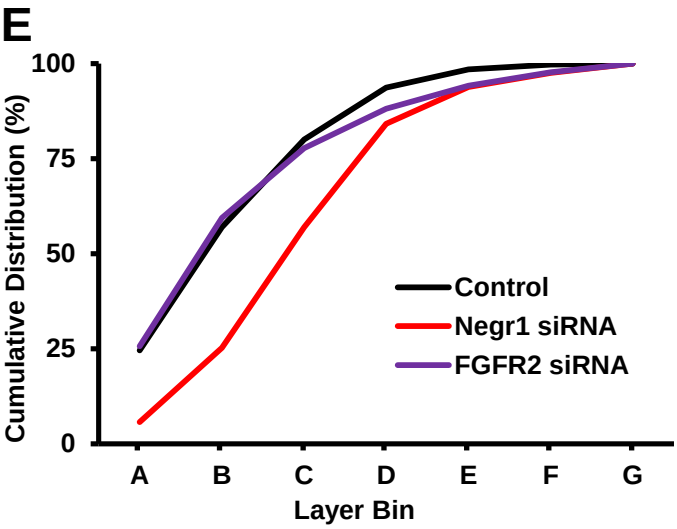
(A) Experimental protocol.
(B) GFP fluorescence in coronal sections of somatosensory cortices of animals at P25-35 after *in utero* transfection at E15.5. Scale bar, 50µm.
(C) Quantification of the number of transfected neurons that did not complete their migration in experiments as in B. Data are expressed as average percentage of the total number of fluorescent cells in the section. Asterisks: statistically significant difference (Kruskal-Wallis test against control, *post-hoc* Dunn's: *** $p < 0.001$). Numbers in parenthesis: total number of processed animals (1 slice/animal).

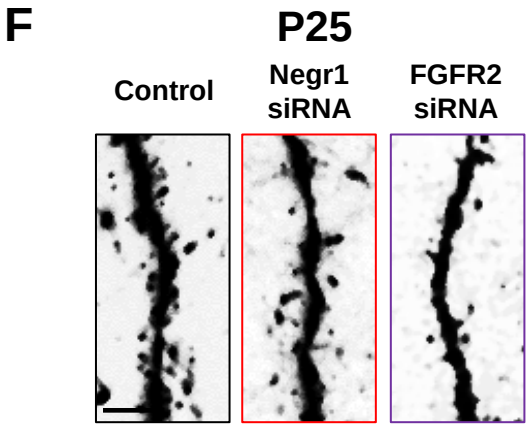


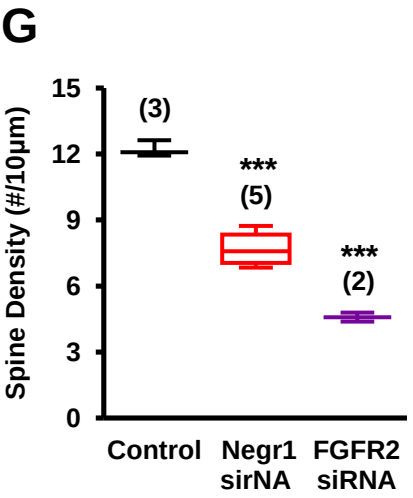








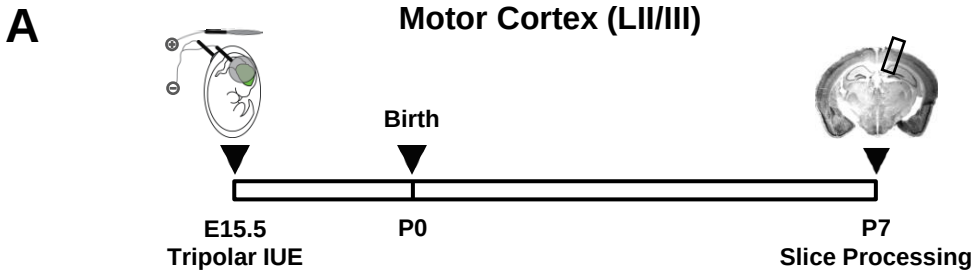


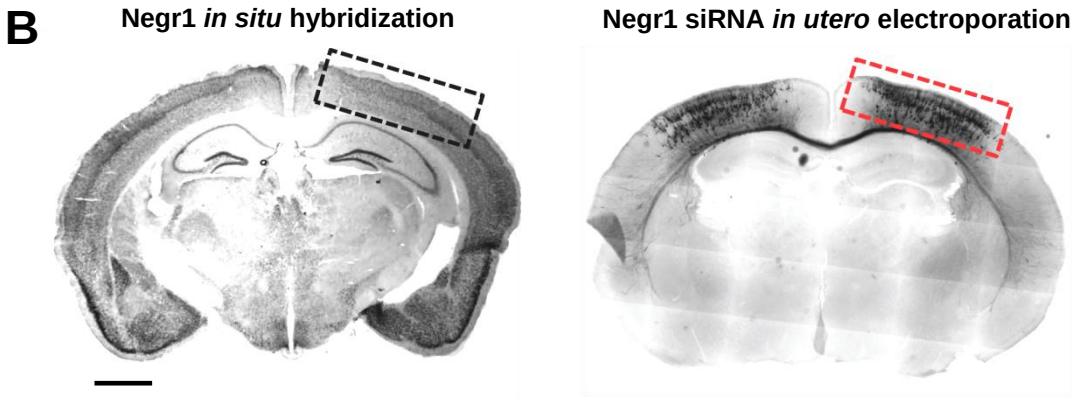


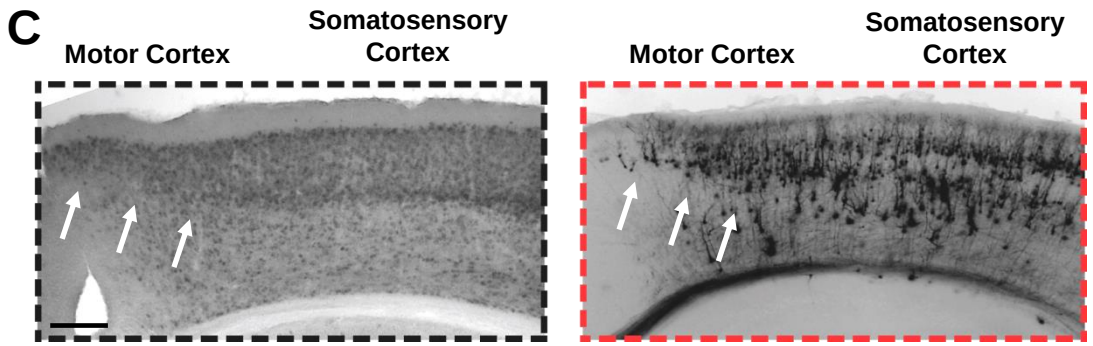
Suppl. Figure 5

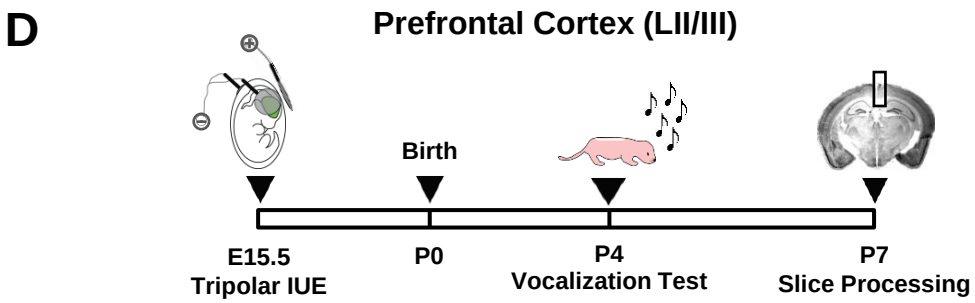
Suppl. Figure 5. Negr1 or FGFR2 downregulation affects late-born neuron migration and spine density *in vivo* in the primary visual cortex.

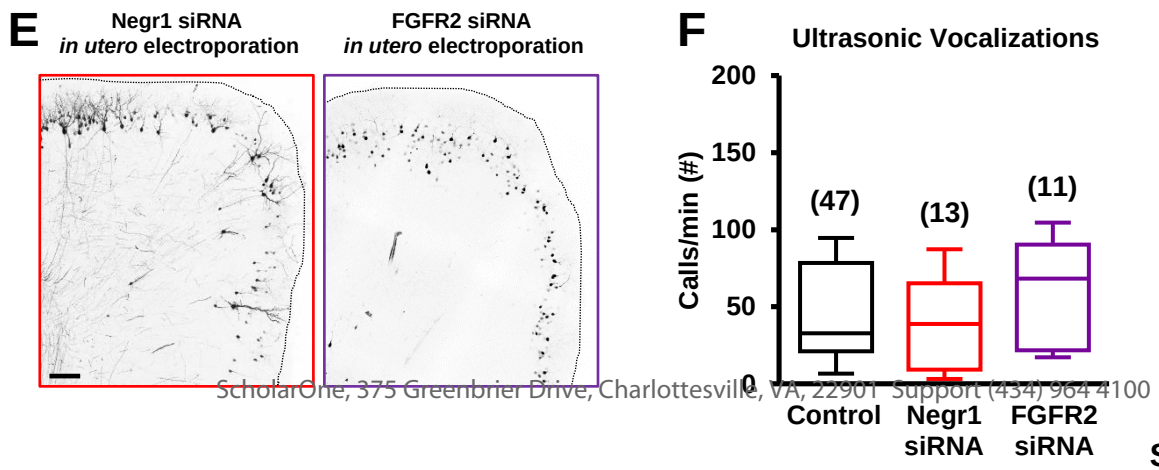
(A) Experimental protocol with tripolar *in utero* electroporation for targeting LII/III of the mouse visual cortex.
(B) GFP fluorescence in coronal sections of visual cortices of transfected animals at P7. *Left*: DAPI counterstaining. Scale bar, 100µm. Dotted lines: slice border. WM = white matter.
(C) Quantification of the number of transfected neurons that did not complete their migration in experiments as in B. Data are expressed as average percentage of the total number of fluorescent cells in the section. Asterisks: statistically significant difference (Kruskal-Wallis test against control; Dunn's *post-hoc*: *p<0.05, ***p < 0.001). Numbers in parenthesis: total number of processed animals (1 slice/animal).
(D) GFP fluorescence in coronal sections of visual cortices at P7 after *in utero* transfection (at E15.5) with scramble siRNA (control), Negr1 siRNA or FGFR2 siRNA divided into 7 bins from A-G. Left: DAPI counterstaining. Scale bar, 100 µm.
(E) Quantification of the total number of transfected neurons located within each of the cortical bins in experiments as in D. Data are expressed as average cumulative distribution of the total number of fluorescent cells in the bin. Negr1 siRNA distribution is significantly different when compared to Control whereas FGFR2 siRNA distribution does not reach statistical significance (Chi-Squared test, pairwise comparisons with Control (see Material and Methods for further information): Negr1 siRNA $\chi^2 = 86.106$ $p<0.0001$, FGFR2 $\chi^2 = 5.778$ $p=0.4486$).
(F) GFP fluorescence in dendrites of somatosensory cortical neurons of P25 transfected mice. Scale bar, 5µm.
(G) Quantification of the spine density in experiments as in E (P25 mice). Data are expressed as average. Asterisks: statistically significant difference (One-Way ANOVA against control; Holm-Sidak *post-hoc*: *** p<0.001. Numbers in parenthesis: total number of processed animals (1-3 dendrites/slice, 1-3 slices/animal).







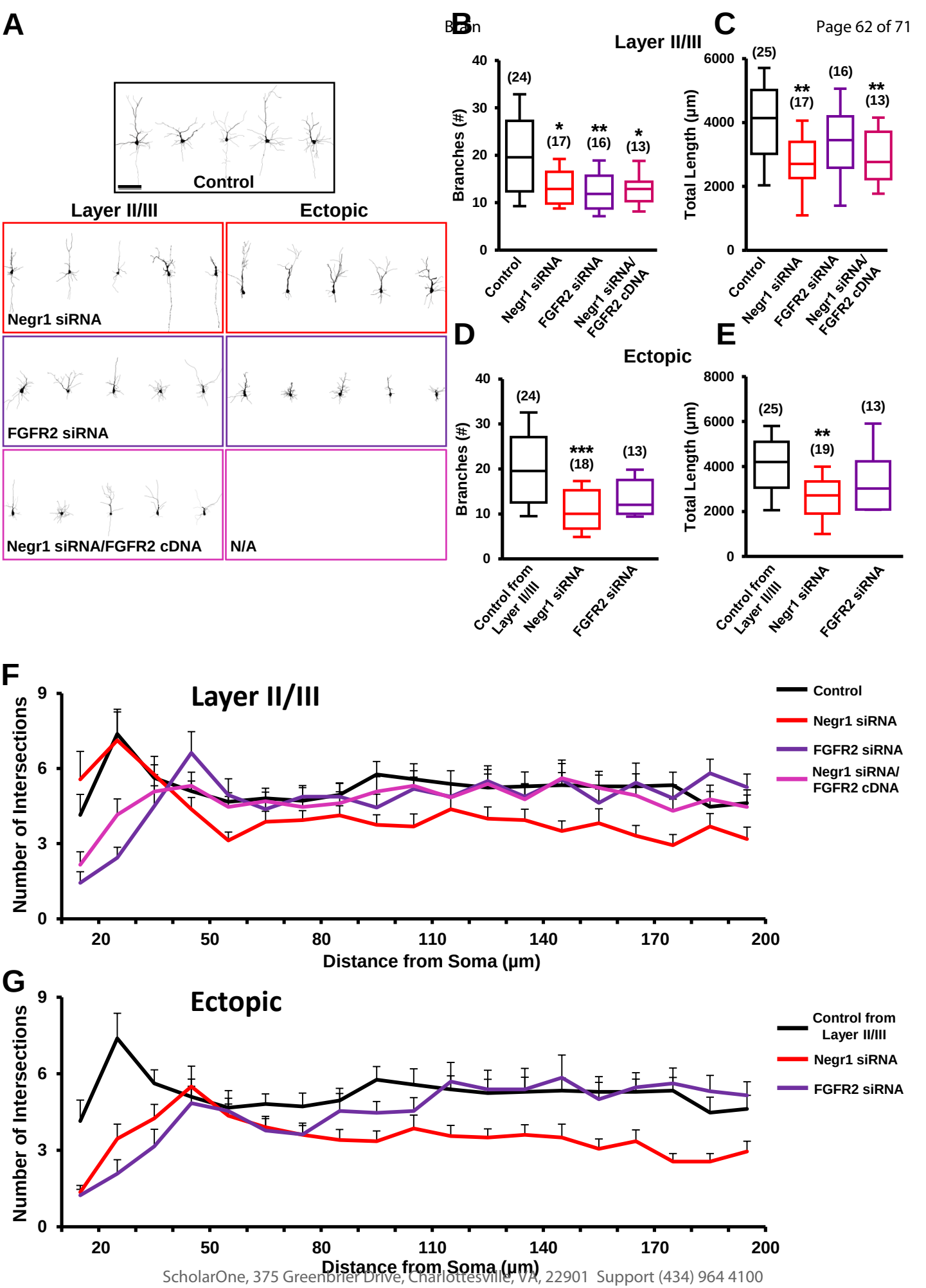




Suppl. Figure 6

Suppl. Figure 6. Negr1 or FGFR2 downregulation does not cause migration defects in the motor and prefrontal cortices.

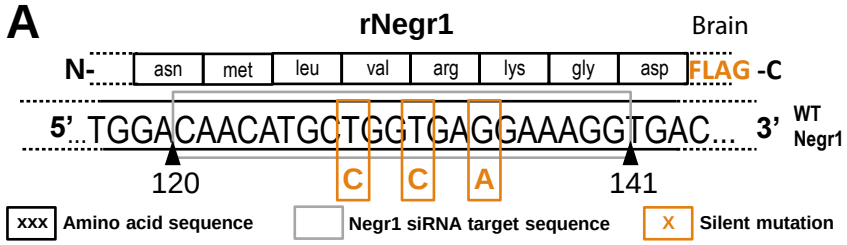
(A) Experimental protocol with tripolar *in utero* electroporation for targeting LII/III of the motor cortex.
(B) Coronal brain sections from WT animals after *in situ* hybridization against Negr1 (left) or from animals electroporated *in utero* with Negr1 siRNA in the motor/somatosensory cortex (right). Scale bar, 1mm.
(C) Higher magnification images from squared panels in B. Arrows point to the fading of the migration phenotype in correspondence (right) of the fading of the high expression of Negr1 mRNA (left). Scale bar, 200µm.
(D) Experimental protocol with tripolar *in utero* electroporation for targeting LII/III of the prefrontal cortex.
(E) GFP fluorescence of brain sections from animals electroporated *in utero* with Negr1 siRNA (left) or FGFR2 siRNA (right) in the prefrontal cortex. Scale bars, 100µm.
(F) Quantification of the number of ultrasonic vocalizations in transfected pups upon isolation from their dam and littermates at P4. Data are expressed as the average of the total number of emitted calls/min (One-Way ANOVA test: *N.S.*). Numbers in parenthesis: total number of analyzed animals, 3-6 litters per experimental case.

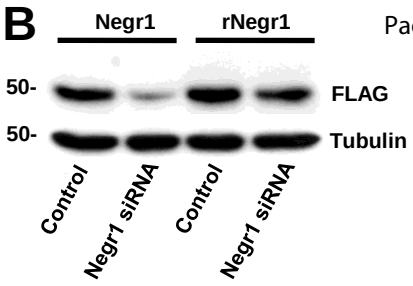


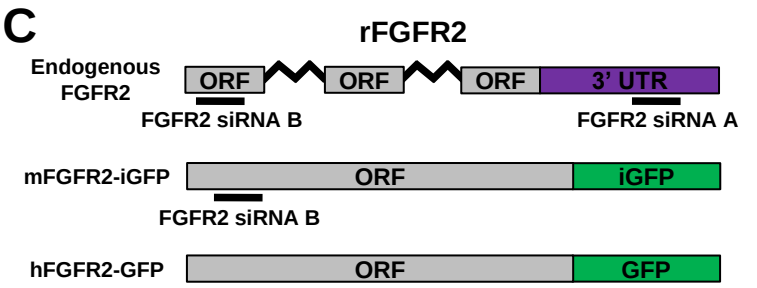
Suppl. Figure 7

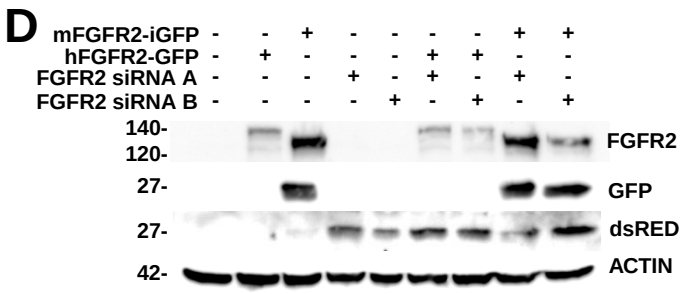
Suppl. Figure 7. Negr1 and FGFR2 downregulation differently affect morphological maturation of pyramidal neurons *in vivo.*

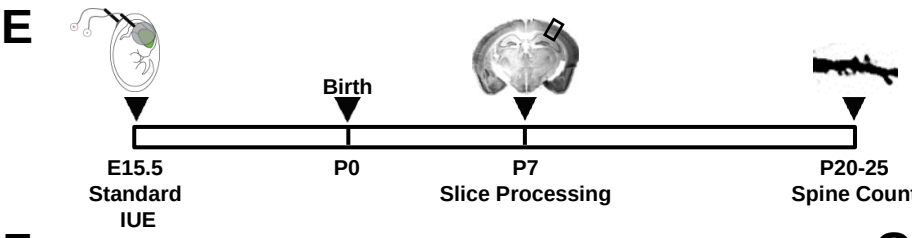
(A) Examples of neuronal reconstructions at P7 from Negr1, FGFR2 downregulated animals or animals co-transfected with Negr1 siRNA and FGFR2 cDNA (*in utero*) at E15.5. Scale bar, 20μm.
(B) and (C) Histograms showing average number and total length of dendritic branches in transfected neurons that reached layer II/III. Asterisks: statistically significant difference (Kruskal-Wallis test against control, Dunn's *post-hoc* for graph B; One-Way ANOVA test against control, Holm-sidak *post-hoc* for graph C: *p<0.05, **p < 0.01). Numbers in parenthesis: total number of processed cells, 4-7 animals per experimental case.
(D) and (E) Histograms showing average number and total length of dendritic branches in transfected neurons arrested ectopically in the layer V. Asterisks: statistically significant difference (Kruskal-Wallis test against control, Dunn's *post-hoc*: **p < 0.01; ***p < 0.001). Numbers in parenthesis: total number of processed cells, 4-7 animals per experimental case.
(F) Sholl analysis of the dendritic arborization of transfected neurons in layer II/III. Data are expressed as average of the total number of analyzed cells. N = 13-21 cells, 4-7 animals per experimental case.
(G) Sholl analysis of dendritic arborization of transfected ectopic neurons. Data are expressed as average of the total number of analyzed cells. N = 13-21 cells, 4-7 animals per experimental case.

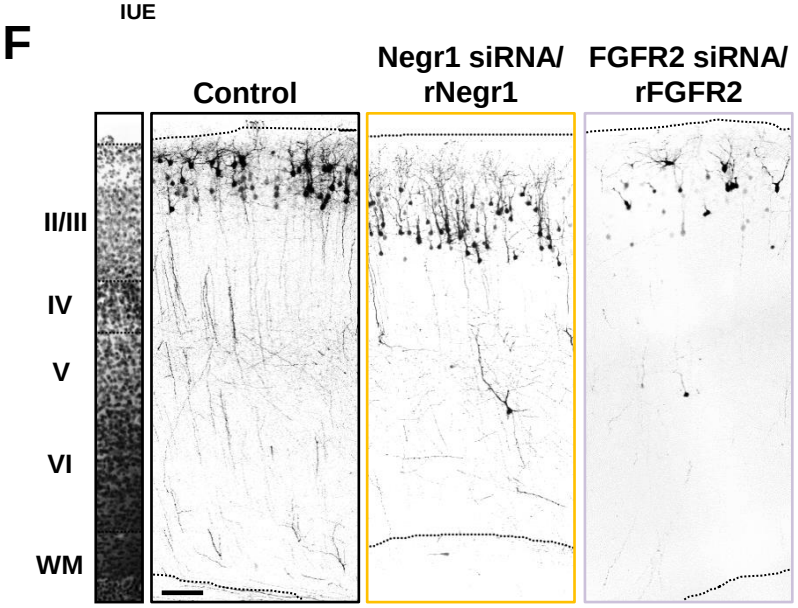


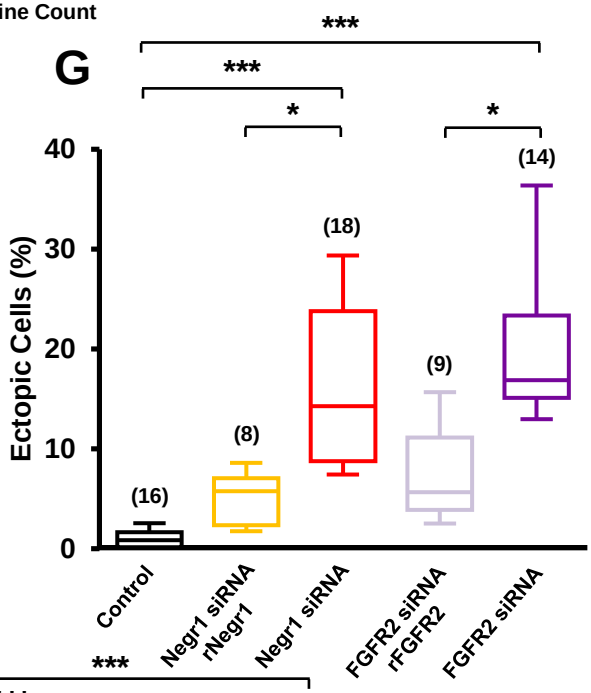


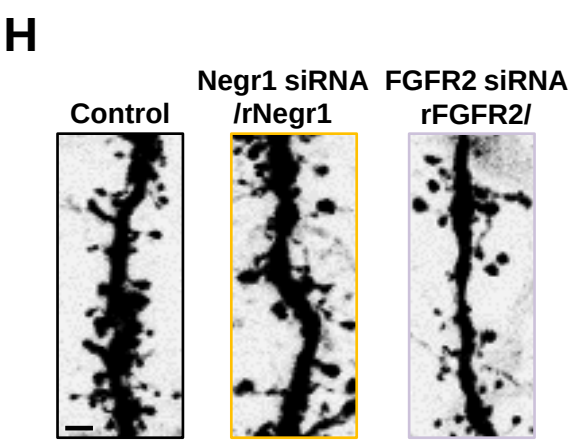


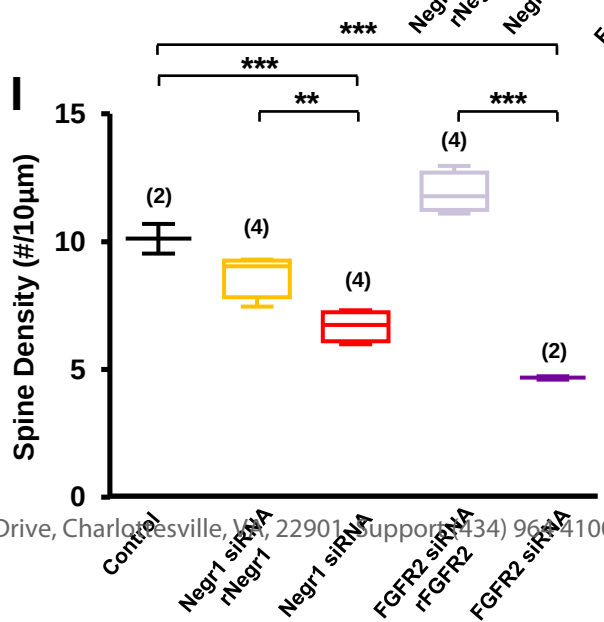








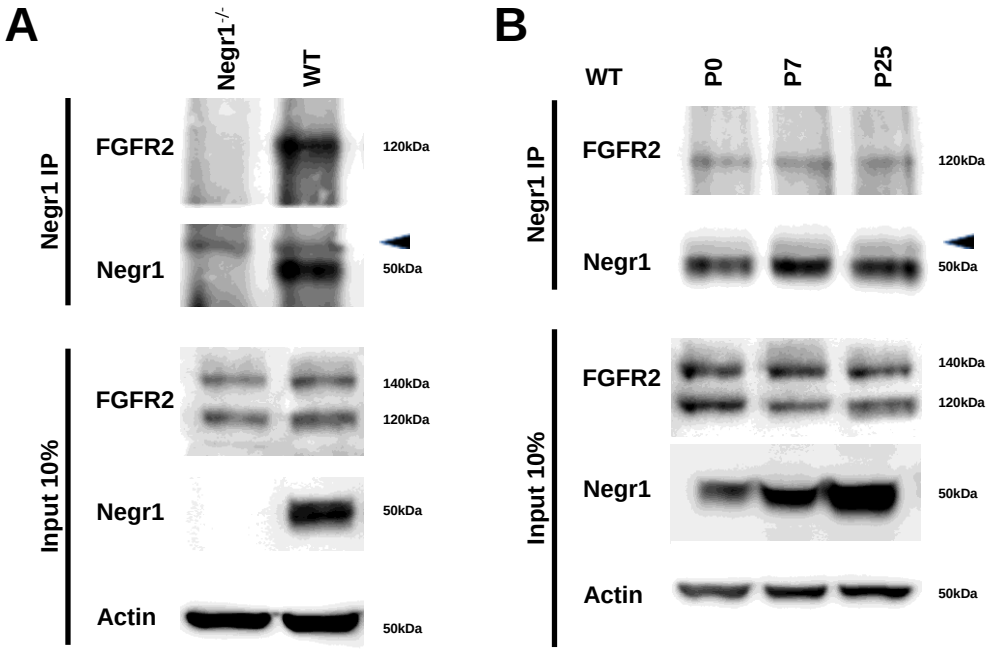


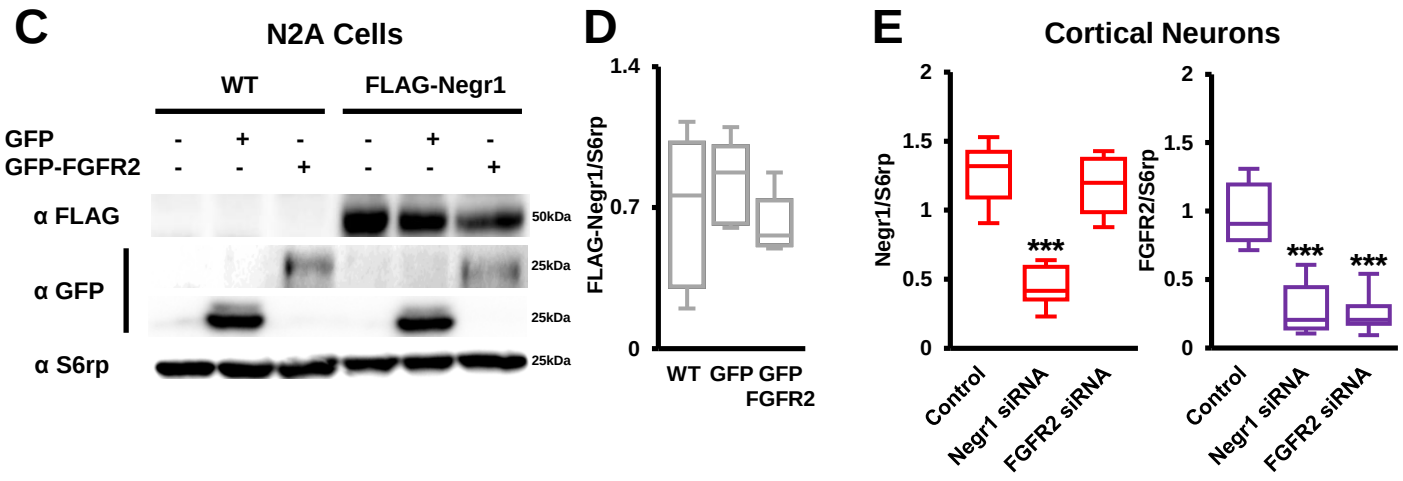


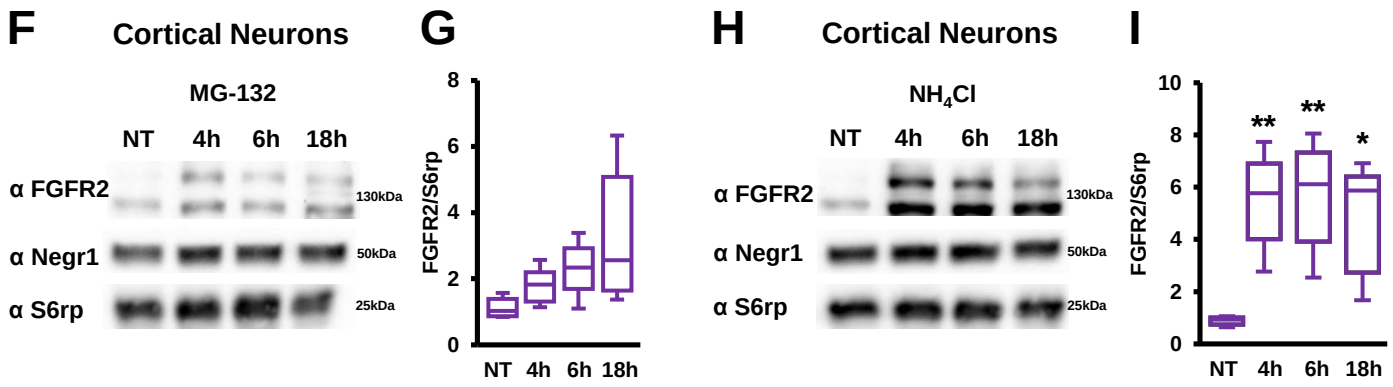
Suppl. Figure 8

Suppl. Figure 8. Expression of siRNA-resistant Negr1 or siRNA-resistant FGFR2 rescues the impairment in neuronal migration and spine density caused by Negr1 or FGFR2 siRNA, respectively.

(A) Cartoon showing the site-directed mutagenesis performed to obtain a siRNA-resistant variant of Negr1 expressing at the C-terminus FLAG tag (rNegr1).
(B) Western blot showing the downregulation of WT Negr1-FLAG exogenously expressed in neurons compared to insensitivity of rNegr1 when transfected with Negr1 siRNA.
(C) Cartoon showing the site of action of the two siRNAs developed against FGFR2. FGFR2 siRNA A targets the 3'UTR of the endogenous mouse and human FGFR2 genes, whereas FGFR2 siRNA B targets an open reading (ORF) frame specific to mouse. Both mouse and human FGFR2 cDNAs (mFGFR2-iGFP and hFGFR2-GFP) are resistant to FGFR2 siRNA A, since they lack the 3'UTR of the endogenous gene. The hFGFR2 cDNA is resistant also to FGFR2 siRNA B, since the latter is specific for the mouse FGFR2. iGFP stands for IRES::GFP.
(D) Representative immunoblots on protein extracts from HEK293 cells transfected with hFGFR2-GFP, mFGFR2-iGFP, DsRED-FGFR2siRNA A and Ds-RED-FGFR2 siRNA B. The co-transfection of FGFR2 siRNA A with either mFGFR2-iGFP or hFGFR2-GFP (ratio 3:1) did not reduce FGFR2 expression level. FGFR2 siRNA B affected the expression of only mFGFR2-iGFP.
(E) Experimental protocol.
(F) GFP fluorescence in coronal sections of somatosensory cortices at P7 transfected with control siRNA, rNegr1 together with Negr1 siRNA (rNegr1/Negr1 siRNA), rFGFR2 siRNA together with FGFR2 siRNA (rFGFR2/FGFR2 siRNA) or FGFR2 siRNA. *Left*: DAPI counterstaining. Scale bar, 100μm.
(G) Quantification of the number of transfected neurons that did not complete their migration in experiments as in F. Data are expressed as average percentage of the total number of fluorescent cells in the section. Asterisks: statistically significant difference (Kruskal-Wallis test, Dunn's *post-hoc*: *$p<0.05$, ***$p < 0.001$). Numbers in parenthesis: total number of processed animals (1 slice/animal).
(H) GFP fluorescence in dendrites of somatosensory cortical neurons of P20-25 transfected mice. Scale bar: 5 μm.
(I) Quantification of the spine density of transfected neurons in experiments as in H. Data are expressed as average density of spines. Asterisks: statistically significant difference (One-Way ANOVA; Holm-Sidak *post-hoc*: *** $p<0.001$,**$p<0.01$. Numbers in parenthesis: total number of processed animals (1-3 dendrites/slice, 1-3 slices/animal).



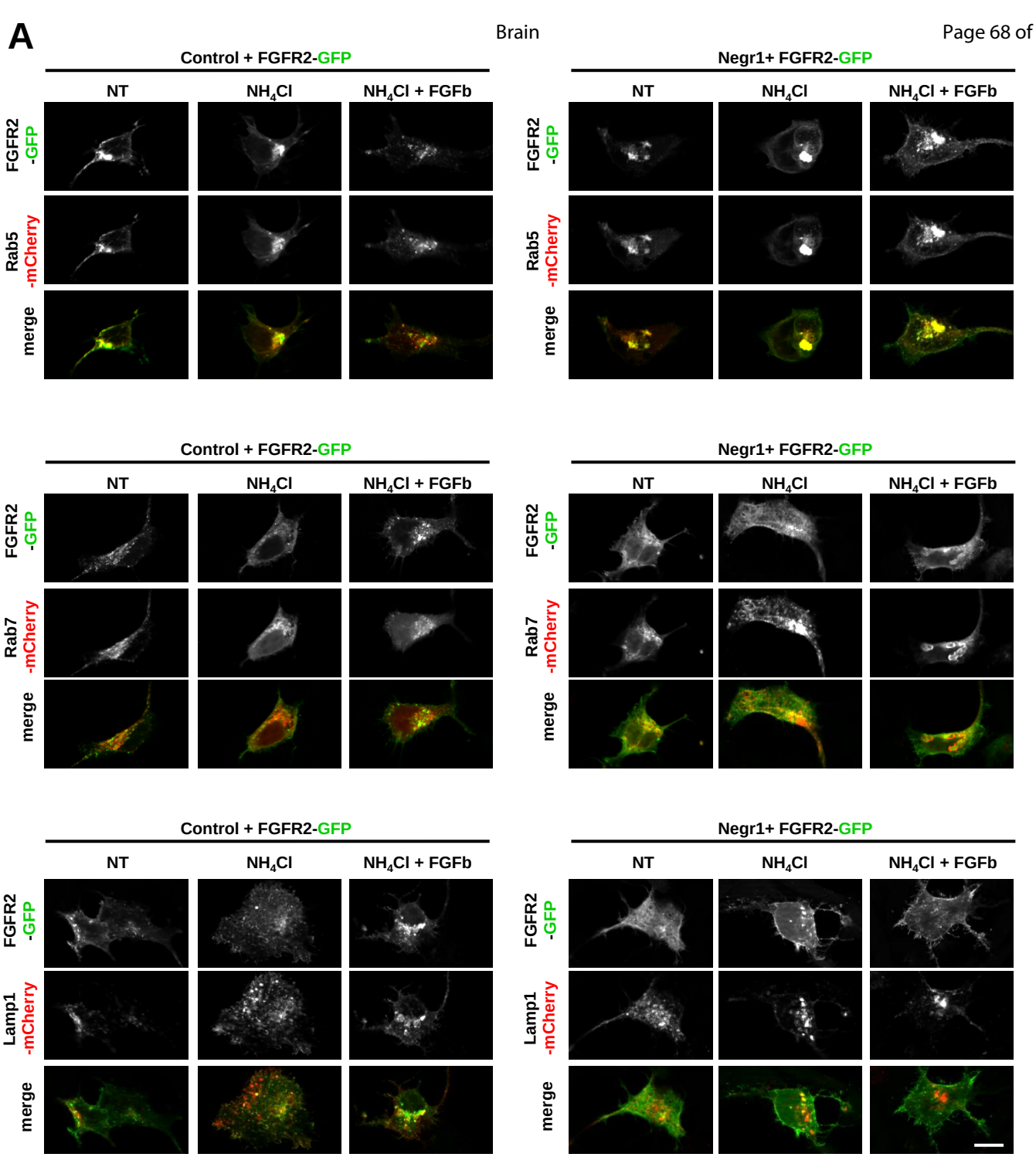




Suppl. Figure 9

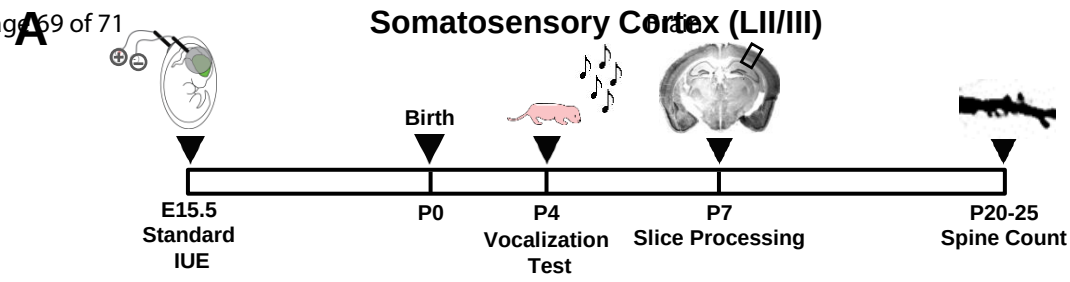
Suppl. Figure 9. Negr1 influences FGFR2 degradation and signaling

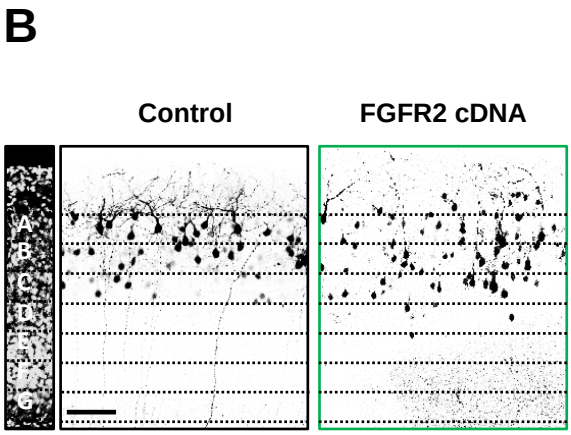
(A) Representative immunoblots on protein extracts from lysates of adult murine forebrain from Negr1$^{-/-}$ and WT animals in co-immunoprecipitation (IP) experiments with goat anti-Negr1 (Negr1 IP). INPUT: 10% of total lysate. Similar experiments were repeated 4 times. Arrowhead points to a band due to IgG heavy chain.
(B) Representative immunoblots on protein extracts from forebrain lysates prepared from P0, P7 and P25 WT mice in co-immunoprecipitation experiments with goat anti-Negr1 (Negr1 IP). INPUT: 10% of total lysate. Similar experiments were repeated 4 times. Arrowhead points to a band due to IgG heavy chain.
(C) Representative immunoblots on protein extracts from samples of WT N2A cells or N2A stably expressing FLAG-Negr1 and co-transfected with either GFP or GFP-FGFR2. Successful transfection was monitored by GFP signal, while total protein amount loading was monitored by S6rp staining.
(D) Quantification of FLAG-Negr1 protein levels normalized to S6rp in experiments as in B. Data are expressed as mean ; n=5 independent experiments.
(E) Quantification of Negr1 (*left*) and FGFR2 (*right*) protein levels normalized to S6rp in experiments as in Figure 4A. Data are expressed as mean (One-way ANOVA, *post-hoc* Bonferroni: *** $p<0.01$); n=8-9 independent experiments.
(F) Representative immunoblots on protein extracts from cortical DIV14 neurons treated with vehicle (NT) or MG-132 to prevent proteasomal degradation for the indicated time (hours).
(G) Quantification of FGFR2 protein level normalized to S6rp in experiments as in E. Data are expressed as mean of 5 independent experiments.
(H) Representative immunoblots on protein extracts from cortical neurons treated at DIV14 with vehicle (NT) or NH_4Cl to prevent lysosomal degradation for the indicated time (hours).
(I) Quantification of FGFR2 protein level normalized to S6rp in experiments as in G. Data are expressed as mean. Asterisks: statistically significant difference *vs* control NT cells (One-way ANOVA, *post-hoc* Bonferroni:* $p<0.05$, ** $p<0.01$, n=5 independent experiments).

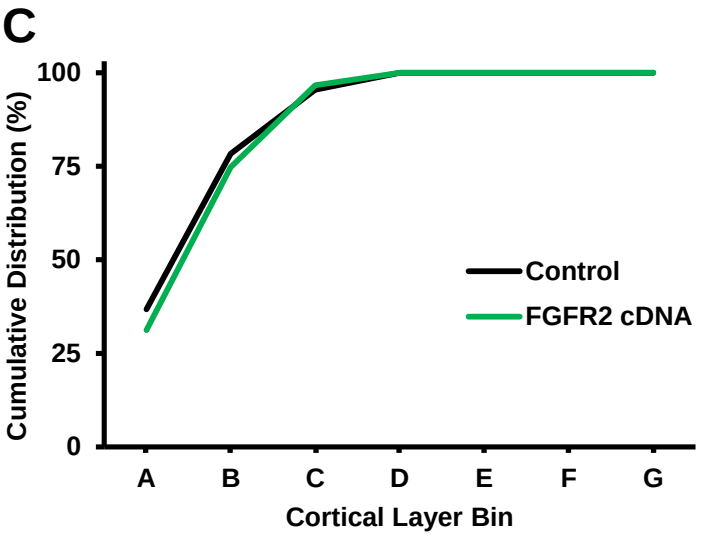


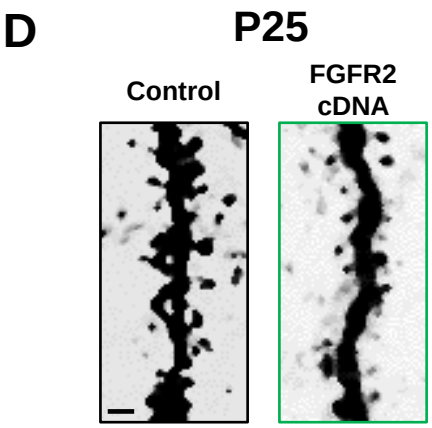
Suppl. Figure 10. Negr1 influences FGFR2 intracellular trafficking.

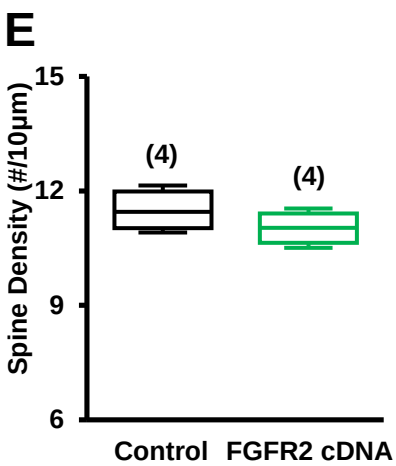
(A) Single channel representative images of mCherry (red) and GFP fluorescence (green) in WT (control) or HEK293 cells stably expressing FLAG-Negr1 and transfected with FGFR2-GFP together with mCherry-Rab5 (marker of early endosomes), mCherry-Rab7 (marker of late endosomes) or mCherry-LAMP1 (marker of lysosomes)-expressing vectors. Images from main figure 3K. Scale bar, 20μm.

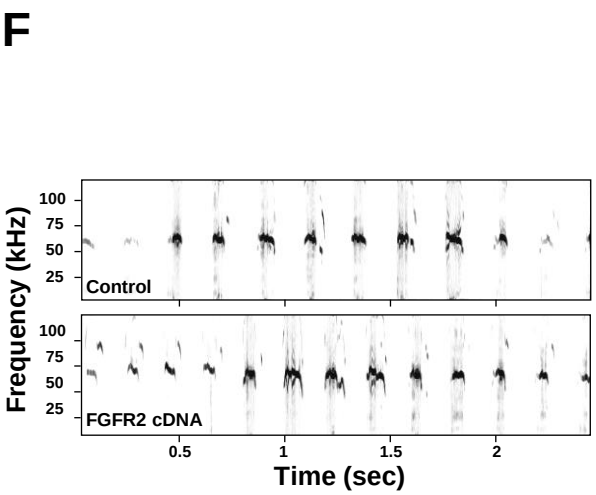


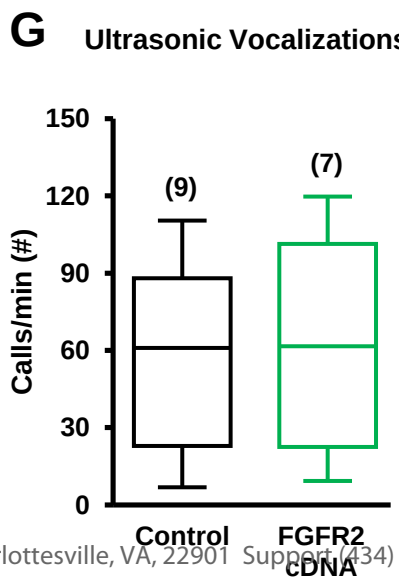












Suppl. Figure 11

Suppl. Figure 11. FGFR2 overexpression does not affect *in vivo* migration of late-born neuron, spine density and USV.

(A) Experimental protocol with standard *in utero* electroporation for targeting LII/III of the mouse somatosensory cortex.
(B) GFP fluorescence in coronal sections of visual cortices at P7 after *in utero* transfection (at E15.5) with GFP (control) or FGFR2 cDNA divided into 7 bins from A-G. Left: DAPI counterstaining. Scale bar, 100 µm.
(C) Quantification of the total number of transfected neurons located within each of the cortical bins in experiment as in D. Data are expressed as average cumulative distribution of the total number of fluorescent cells in the bin. The distributions are not statistically different. (Chi-Squared test (see Material and Methods for further information): $\chi^2 = 2.501$ p=0.4752).
(D) GFP fluorescence in dendrites of somatosensory cortical neurons of P25 transfected mice. Scale bar, 5µm.
(E) Quantification of the spine density in experiments as in E (P25 mice). Data are expressed as average. (Student's T-test: *N.S.* p=0.2123). Numbers in parenthesis: total number of processed animals (1-3 dendrites/slice, 1-3 slices/animal).
(F) Example of a fraction of the recordings of ultrasonic vocalizations emitted upon isolation from dam and littermates by P4 pups transfected *in utero*.
(G) Quantification of the number of ultrasonic vocalizations during the entire isolation in experiments as in B. Data are expressed as the average of the total number of emitted calls/min. (Student's T-test: *N.S.* p=0.98).