Serotonin receptor 3A controls interneuron migration into the neocortex

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Neuronal excitability has been shown to control the migration and cortical integration of reelin-expressing cortical interneurons (INs) arising from the caudal ganglionic eminence (CGE), supporting the possibility that neurotransmitters could regulate this process. Here we show that the ionotropic serotonin receptor 3A (5-HT3AR) is specifically expressed in CGE-derived migrating interneurons and upregulated while they invade the developing cortex. Functional investigations using calcium imaging, electrophysiological recordings and migration assays indicate that CGE-derived INs increase their response to 5-HT3AR activation during the late phase of cortical plate invasion. Using genetic loss-of-function approaches and in vivo grafts, we further demonstrate that the 5-HT3AR is cell autonomously required for the migration and proper positioning of reelin-expressing CGE-derived INs in the neocortex. Our findings reveal a requirement for a serotonin receptor in controlling the migration and laminar positioning of a specific subtype of cortical IN.
Cortical interneurons (INs) are key cellular components involved in the assembly and function of cortical circuits. Alterations in their developmental and functional integration are thought to play a critical role in the emergence of psychiatric disorders. In mice, cortical INs mainly originate (about 70%) from the medial ganglionic eminence (MGE), which generates parvalbumin (PV)-expressing basket cells and chandelier cells or somatostatin (SST)-expressing Martinotti cells. In addition to the MGE, the caudal ganglionic eminence (CGE) and to a smaller extent the preoptic area contribute to the remaining 30% of cortical INs. CGE-derived interneurons (cINs) are comprised of diverse IN subtypes divided into two main classes: bipolar or double-bouquet INs that express the vaso-intestinal peptide (VIP) and neurogliofibrillary in multiple INs that express reelin and/or neuropeptide Y (NPY).

Recent in vivo data have demonstrated that neuronal excitability controls the laminar positioning of reelin-expressing CGE-derived INs. These data support the possibility that cell-extrinsic factors such as neurotransmitters could regulate early activity of migrating cINs and control their final laminar positioning. Interestingly, cINs express the serotonin receptor 3A (5-HT3AR) during the process of neuronal migration. The 5-HT3AR is the only known ionotropic serotonergic receptor and is expressed exclusively in inhibitory GABAergic INs in the adult cortex. Serotonin is detected as early as E10.5 in the embryonic forebrain and has been shown to regulate a variety of developmental processes involved in the construction of cortical circuits, including neuronal migration and thalamo-cortical pathfinding. In addition, early-life serotonin dysregulation plays a key role in vulnerability to psychiatric disorders. Here we determined whether 5-HT3AR-dependent signalling controls the migration of cINs.

The migration of INs is a multistep process regulated by the combinatorial expression of transcription factors and by a variety of cell-extrinsic factors including the extracellular matrix and guidance cues. To populate the neocortex, INs exit the subpallium and migrate tangentially through migratory streams mainly located in the marginal zone (MZ), subplate and the subventricular zone (SVZ) during the process of tangential migration, INs migrate radially and progressively invade the cortical plate (CP).

To investigate the molecular mechanisms regulating different steps in the migration of cINs, we performed a microarray gene expression analysis on INs preferentially derived from the CGE at three distinct developmental stages of the migratory process. Using this approach, we find that the Htr3a is upregulated in INs during the process of CP invasion. Calcium imaging and electrophysiological recordings indicate that the 5-HT3AR is functional in INs during CP invasion and that 5-HT3AR activation increases their migratory speed during this late phase of migration. Genetic loss-of-function experiments combined to time-lapse imaging and in vivo grafts reveal that the 5-HT3AR regulates the entry of cINs into the CP in a cell-autonomous manner. Finally, we find that genetic deletion of the 5-HT3AR led to the persistent laminar mispositioning of reelin-expressing CGE-derived INs.

Results

Migrating cINs specifically express functional 5-HT3AR. To study the migration of CGE-derived INs, we used GAD65-GFP (green fluorescent protein) mice that preferentially label CGE-derived INs and rarely MGE-derived INs expressing PV and SST (<5% at P21). To identify time-specific genes regulating the phase of cortical invasion, microarray-based gene expression analysis of GAD65-GFP+ INs was performed at three different developmental time points corresponding to distinct steps of the migratory process (Fig. 1a). GAD65-GFP+ cells in the process of tangential migration were obtained by microdissection of the E14.5 caudal pallium containing the SVZ migratory stream. GAD65-GFP+ cells invading the cortex were obtained by microdissection of the developing cortex at E18.5 (excluding the SVZ), whereas INs terminating migration were obtained by microdissection of the cortex at P2.5 (excluding the SVZ). GAD65-GFP+ INs were then isolated using fluorescent-assisted cell sorting (FACS). Genetic fate mapping indicated that cINs expressing the 5-HT3AR do not originate from MGE-derived progenitors that specifically express the transcription factor Nkx2.1.

To investigate the molecular mechanisms regulating different steps in the migration of cINs, we isolated the CGE from E14.5 brains at E17.5 and revealed strong expression in the CGE but not MGE at E14.5 (Supplementary Fig. 2f).

To determine whether the 5-HT3AR is functional in migrating cINs, we isolated the CGE from E14.5 Htr3a-GFP+ slices and platted cINs in vitro in the presence of dissociated and isochronic cortical cells in order to allow Htr3a-GFP+ INs to migrate on a homogeneous substrate (Supplementary Fig. 3a). Calcium imaging revealed that application of the 5-HT3AR agonists 8-OH-DPAT and 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydro-7H-pyrrolo[2,3-d]pyrimidin-7-one (SBT) reporter mice, we observed that Htr3a-GFP+ INs only rarely overlapped with TOM+ cells at E17.5, P0.5, P21 and preferentially populated superficial cortical layers (Fig. 1c, Supplementary Fig. 2c,d). In line with these results, we observed that at E14.5, Htr3a-GFP+ cells were mainly found populating the CGE and not the MGE (Fig. 1d, e, Supplementary Fig. 2f). In the CGE, the majority of Htr3a-GFP+ cells expressed COUP-TFI and/or SP8 (Fig. 1d), two transcription factors preferentially expressed in this region during the embryonic period and only very rarely NKX2.1 (Supplementary Fig. 2e). Htr3a-GFP+ INs were observed at E14.5 exiting the subpallium by forming a tangential stream of migratory INs located in the pallial SVZ and invading the CP by E17.5 (Fig. 1e).

In situ hybridization targeting the Htr3a mRNA confirmed the labelling pattern observed in Htr3a-GFP+ brains at E17.5 (Fig. 1f) and revealed strong expression in the CGE but not MGE at E14.5 (Supplementary Fig. 2f).

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Interestingly, 5-HT3AR-mediated inward currents were detected in Htr3a-GFP⁺ INs migrating in the E18-E19 CP but not in cINs migrating tangentially in the SVZ at E18-E19 or IZ/SVZ at E14-E15 (Supplementary Fig. 4 and Supplementary Table 2), indicating that Htr3a-GFP⁺ INs migrating in the CP become functionally responsive to 5-HT3AR activation. At P2.5, 5-HT3AR activation reliably induced currents in Htr3a-GFP⁺ INs located in the CP, suggesting that although Htr3a mRNA is downregulated in cINs from E18.5 to P2.5 targeting of the 5-HT3AR to the cell membrane may increase through post-translational modifications. Taken together, these data confirm previous findings, indicating that functional 5-HT3AR is specifically expressed in CGE-derived but not MGE-derived INs. Furthermore, functional upregulation of 5-HT3AR in cINs during the process of cortical invasion suggests that 5-HT3AR could regulate this migratory phase.

5-HT3AR activation stimulates the migration of cINs. To determine whether 5-HT3AR activation could modulate the migratory behaviour of cINs, GAD65-GFP⁺ INs in the E14.5
SVZ/IZ stream or invading the E17.5 cortex were isolated through microdissection and time-lapse imaging of INs was performed in cortical cultures at E14.5 (+DIV1) and E17.5 (+DIV1) in a serotonin-free medium26 (Fig. 2a,b). This in vitro strategy has successfully been used to unmask the effects of GABA-A stimulation on cortical IN migration35. Furthermore, it has the advantage to allow the recordings of INs in a serotonin-free environment, thus removing the potential confounding effects of endogenous levels of serotonin contained in acute slices. Indeed, it has been shown that it is not possible in cortical slices to unmask the role of 5-HT3AR on the migration of cINs25. Using this in vitro strategy, time-lapse recordings revealed that the 5-HT3AR agonist SR57227 but not vehicle exposure significantly increased the mean migratory speed and significantly decreased the pausing time of GAD65-GFP+ INs at E14.5 and growth cone imaging of cINs and mINs was simultaneously performed in the CP at E17.5 (+DIV3) in serotonin-free medium (Fig. 3a–c). Using this paradigm, we found that the size and growth cone extension speed of cINs were specifically increased after mCPBG exposure, whereas no effect of the drug was observed in mINs (Fig. 3d,e, Supplementary Movies 3 and 4). To confirm the specificity of the 5-HT3AR agonist, similar experiments were performed on the growth cones of Htr3a-ko; GAD65-GFP+ INs. Quantification revealed that 5-HT3AR activation failed to increase the GC speed and size of Htr3a-ko; GAD65-GFP+ INs (Supplementary Fig. 5g,h). Taken together, these data indicate that the 5-HT3AR specifically regulates the growth cone dynamics of cINs but not mINs during CP invasion.

**5-HT3AR specifically controls the migration of cINs.** To study the role of the 5-HT3AR on the migration of cINs during cortical invasion, time-lapse imaging was performed on E17.5 cortical slices during 10–12 h and single-cell tracking was performed on GAD65-GFP+ INs and Htr3a-ko; GAD65-GFP+ INs. Quantification revealed that the proportion of Htr3a-ko; GAD65-GFP+ INs migrating from the IZ into the cortex was significantly decreased versus GAD65-GFP+ INs (Fig. 4a, Supplementary Movies 5 and 6). In addition, the migratory speed of Htr3a-ko; GAD65-GFP+ INs invading the cortex from the IZ was significantly decreased and their pausing time significantly increased compared with GAD65-GFP+ INs (Fig. 4a). To determine whether decreased cortical invasion of Htr3a-ko; GAD65-GFP+ INs resulted in early positioning defects in the developing cortex, the distribution of GAD65-GFP+ INs was analysed at birth. Given that CGE-derived INs preferentially target superficial cortical layers (L1–4) in contrast to deep layers L5/6 (Fig. 1c, Supplementary Movies 5 and 6). In addition, the migratory speed of Htr3a-ko; GAD65-GFP+ INs invading the cortex from the IZ was significantly decreased and their pausing time significantly increased compared with GAD65-GFP+ INs (Fig. 4a). To determine whether decreased cortical invasion of Htr3a-ko; GAD65-GFP+ INs resulted in early positioning defects in the developing cortex, the distribution of GAD65-GFP+ INs was analysed at birth. Given that CGE-derived INs preferentially target superficial cortical layers (L1–4) in contrast to deep layers L5/6 (Fig. 1c, Supplementary Movies 5 and 6).
Figure 3 | The 5-HT3AR specifically regulates growth cone dynamics of interneurons (INs) derived from the CGE but not the MGE. (a) Schema illustrating that MGE-derived INs (mINs) were labelled using focal electroporation of a tdTOM-expressing plasmid in the MGE, whereas CGE-derived INs (cINs) were labelled using Htr3a-GFP+ slices. At E14.5 (+ DIV3), confocal time-lapse imaging of the growth cones (GCs) of cINs and mINs was performed in the cortical plate (dotted area) and GC dynamics was assessed at baseline, during and after 5-HT3AR activation with m-chlorophenylbiguanide (mCPBG 100 μM). (b) Growth cone dynamics was assessed by tracing the GC area in sequential time-lapse images and by calculating the speed of progression of the growth cone. (c) Time-lapse sequence illustrating that 5-HT3AR activation induces a delayed increase in the GC size of Htr3a-GFP+ cINs (green arrowheads) but not TOM+ mINs (white arrowheads). (d) Illustrative tracing of growth cone areas of Htr3a-GFP+ cINs and TOM+ mINs during the baseline period, during exposure to mCPBG and during the two wash periods. Note that GC size of cINs increases during the second wash period but not the GC of mIN. (e) Quantification revealed that the GC area and speed of cINs (n = 13 GC in 10 slices) but not of mINs (n = 12 GC in 10 slices) significantly increases after 5-HT3AR activation (***P<0.001, *P<0.05, two-way analysis of variance with Bonferroni’s test). Error bars are means ± s.e.m.

Supplementary Fig. 2), the distribution of GAD65-GFP+ INs and Htr3a-ko; GAD65-GFP+ INs were analysed in prospective superficial cortical layers (L1 and L2-4) and deep cortical layers (L5/6) of the developing cortex. Quantification indicated that the percentage of Htr3a-ko; GAD65-GFP+ INs located in superficial layers 2–4 at P0.5 was significantly decreased compared with GAD65-GFP+ INs (Fig. 4b) and no changes were observed in other compartments, suggesting that the 5-HT3AR regulates the positioning of CGE-derived INs in cortical layers to which they are preferentially targeted. The 5-HT3AR specifically controlled the migration of cINs, as the distribution of mINs expressing the MGE-specific transcription factor Lhx6 was unchanged across different cortical layers (Fig. 4c). The distribution of Lhx6+ and GAD65-GFP+ INs in layers 2–4, layer 5/6 and intermediate zone were significantly different at P0.5, indicating that mINs and cINs are in the process of being differentially allocated to specific cortical layers already at birth (Supplementary Fig. 5i). Isochronic graft experiments indicated that 5-HT3AR regulates cortical invasion in a cell-autonomous manner. Indeed, at E19, the cortical distribution of E14.5 CGE-isolated Htr3a-ko; GAD65-GFP+ INs grafted into the CGE of wild-type E14.5 embryos was significantly altered in comparison with grafted GAD65-GFP+ INs (Fig. 4d). Taken together, these results indicate that the 5-HT3AR controls the migration and positioning of cINs into the developing cortex.

5-HT3AR is required for the laminar positioning of cINs. To determine whether 5-HT3AR deletion leads to persistent alterations in the distribution of cINs, the laminar positioning of CGE-derived INs was analysed in the somatosensory cortex of Htr3a-ko mice at P21. Quantification revealed a significant decrease in the percentage of Htr3a-ko; GAD65-GFP+ INs at P21 in superficial layers of the somatosensory cortex compared with GAD65-GFP+ INs (Fig. 5a). To determine whether the positioning of a specific subtype of CGE-derived INs was altered in...
Figure 4 | 5-HT3AR controls the migration and positioning of CGE-derived INs in the cortical plate (CP). (a) Examples of migratory paths in E17.5 acute cortical slices showing cINs remaining in the intermediate zone (IZ; white tracks) or invading the CP (red tracks). Squares indicate start positions and circles indicate end positions of tracked cells after 8 h imaging. Quantification showing a significant reduction in the percentage of Htr3a-ko; GAD65-GFP+ INs (n = 124 cells in five slices) migrating from the IZ into the CP in comparison with GAD65-GFP+ INs (n = 106 cells in five slices). In addition, the migratory speed of Htr3a-ko; GAD65-GFP+ INs is significantly decreased, whereas their pausing time significantly increased versus GAD65-GFP+ INs (***P < 0.001, **P < 0.01, unpaired Student’s t-test). (b) In vivo quantification reveals significant decrease in the percentage of Htr3a-ko; GAD65-GFP+ INs (n = 6144 cells in four brains) in layer 2–4 versus GAD65-GFP+ INs (n = 6708 cells in four brains; **P < 0.01, unpaired Student’s t-test) in the prospective P0.5 somatosensory cortex. (c) Quantification of MGE-derived INs in the prospective P0.5 somatosensory cortex labelled by in situ hybridization for the MGE-specific transcription factor Lhx6 reveals no significant layering differences between Htr3a-ko; GAD65-GFP+ mice (n = 2,013 cells in three brains) versus control GAD65-GFP+ (n = 3,731 cells in four brains). (d) In vivo quantification in host E19.0 wild-type (WT) cortex reveals significant misdistribution of isochronic-grafted Htr3a-ko; GAD65-GFP+ INs (n = 882 cells in five brains) in the developing cortex versus GAD65-GFP+ INs (n = 736 cells in four brains; *P < 0.05, unpaired Student’s t-test). Hst, Hoechst; MZ, marginal zone. Error bars are means ± s.e.m. Scale bars: (a) 50 μm; (b–d) 100 μm.

Figure 5 | 5-HT3AR controls the laminar positioning of reelin-expressing CGE-derived INs in the cortex. (a) At P21, the positioning of Htr3a-ko; GAD65-GFP+ INs (n = 2,775 cells in six brains) was significantly altered in superficial layers compared with GAD65-GFP+ INs (n = 2,707 cells in six brains; **P < 0.001, *P < 0.05 unpaired Student’s t-test). (b) The percentage of Htr3a-ko; GAD65-GFP+ INs expressing reelin (n = 706 cells in five brains) was significantly decreased in layer 1 compared with reelin+/GAD65-GFP+ INs (n = 1,376 cells in six brains; **P < 0.01 unpaired Student’s t-test). (c,d) The laminar distribution of Htr3a-ko; GAD65-GFP+ INs expressing NPY (n = 1,093 cells in eight brains) or VIP (n = 963 cells in six brains) was not altered compared with NPY+ (n = 1,127 cells in six brains) or VIP+/GAD65-GFP+ INs (n = 1,219 cells in six brains; unpaired Student’s t-test). Error bars are means ± s.e.m. NPY, neuropeptide Y; VIP, vasointestinal peptide. Scale bar, (a) 50 μm.
Htr3a-ko mice, GAD65-GFP + sections were stained for reelin, VIP and NPY, which label different CGE-derived IN populations. Quantification revealed that 5-HT3AR deletion leads to the persistent laminar mispositioning of CGE-derived reelin − but not VIP + or NPY +/−/Htr3a-ko; GAD65-GFP + INs in superficial cortical layers as compared with GAD65-GFP + INs (Fig. 5b–d). Taken together, these data indicate that the 5-HT3AR regulates the laminar positioning of a specific subclass of reelin-expressing CGE-derived INs.

Discussion

Here we report that the 5-HT3AR, a specific marker of CGE-derived cortical INs, is functionally upregulated in cINs during the process of cortical invasion and that 5-HT3AR activation stimulates the migration of cINs during the phase of cortical invasion. Furthermore, 5-HT3AR-mediated activation specifically regulated the growth cones dynamics of cINs but not of mINs. Time-lapse imaging, graft experiments and in vivo quantification in Htr3a-ko mice demonstrated that the 5-HT3AR is cell autonomously required for positioning of cINs into the nascent cortex. Finally, alterations in the early process of CP invasion lead to a persistent mispositioning of the reelin-expressing cINs subtype in superficial cortical layers of Htr3a-ko mice.

The migration of cINs into the cortex is a multi-step process. After exiting the subpallium, INs disperse into the pallium through specific tangential migratory streams mainly located in the MZ, subplate and SVZ3–5,29. The selection of migratory streams by mINs requires responsiveness to the chemokine Cxcl12 through the G-protein-coupled receptors Cxcr4 and Cxcr7 (refs 36–39). It has been hypothesized that the loss of responsiveness to Cxcl12 could allow mINs to become responsive to a yet unknown chemoattractant located in the CP. The chemoattractant properties of the CP have not been elucidated and it is unknown whether cINs are responsive to the same set of guidance cues as mINs. The fact that the 5-HT3AR is required for proper CP invasion and that serotonin levels increase during the late phase of cortical invasion22 suggests that serotonin signalling through the 5-HT3AR could specifically regulate the responsiveness of cINs to guidance cues located in the developing cortex.

Serotonin has been reported to regulate the migration of different non-neuronal cell types including eosinophils, pulmonary artery smooth muscle cells, aortic endothelial cells and cranial neural crest cells50–44. Depending on the concentration of serotonin, cell types, experimental paradigms and embryonic age of the cells, serotonin was shown to either stimulate or inhibit migration. For example, differential effects of serotonin on the migration of neural crest cells were observed depending on the concentration of serotonin and embryonic age of the cells45. Here we report that serotonin levels at the nanomolar range increase their responsiveness to serotonin signalling through 5-HT3AR upregulation in order to respond to guidance cues located in the developing cortex. In a related developmental process, that is, embryonic thalamo-cortical wiring, a role for serotonin in modulating growth cone responsiveness to the guidance cue netrin1 has been described26. In migrating cINs, we find that the effect of 5-HT3AR activation on growth cones dynamics is delayed, suggesting that 5-HT3AR activation may have indirect effects on growth cone dynamics by modulating responsiveness to guidance cues. Although the guidance cues regulating the migration of cINs into the CP remains to be discovered, the identification of genes specifically upregulated in cINs during CP invasion provides a set of candidate genes that may specifically regulate this process. Among candidate genes from our microarray, PlexinA4 displayed a time-specific twofold upregulation during CP invasion, suggesting that this receptor could play a role in regulating CP invasion of cINs. Interestingly, PlexinA4/Semaphorin6A signalling was shown to regulate the migration of oligodendrocyte precursor cells in the developing cortex48. Further studies are required to establish whether PlexinA4 expression is regulated by 5-HT3AR signalling and regulates the migration of cINs.

The 5-HT3AR is a cation-selective ligand-gated ion channel that mediates neuronal depolarization and excitation50. Previous work in the field has demonstrated that neuronal excitability is required for the proper positioning of CGE-derived INs in superficial cortical layers18, supporting the possibility that neurotransmitters such as serotonin could regulate the migration of CGE-derived INs through 5-HT3AR-dependent signalling. Here we show that 5-HT3AR activation enhances the migratory speed and growth cone dynamics of cINs during the phase of CP invasion. The 5-HT3AR is closely related to the anion selective GABAA receptor. In conditions of low-expression levels of the potassium-chloride co-transporter KCC2, GABAA stimulation induces an increase in calcium transients and promotes the migration of cINs and their entry into the cortex35,49. In addition, other neurotransmitter systems have been shown to regulate the migration of cortical INs such as AMPA and NMDA receptors50–52. Overall, in vitro data obtained in cINs but also in other neuronal cell types such as cerebellar granule cells indicate that neuronal migration involves the modulation of calcium transients through the opening of NMDA and voltage-gated calcium channels53,55. It is thus hypothesized that early activity in neuronal networks generates ambient levels of neurotransmitters that regulate IN migration by modulating their intracellular calcium transients. Our data add to this model the possibility that ambient levels of serotonin could specifically regulate the migration of cINs into the CP through the regulation of 5-HT3AR-dependent calcium transients.

Developmental insults including alterations in the migration and maturation of cINs contribute to the emergence of psychiatric-relevant functional alterations later in life2. Early-life serotonin dysregulation in rodents following genetic deletion or pharmacological blockade of the serotonin transporter by antidepressants leads to long-term psychiatric-related phenotypes29. In this perspective, the 5HT3AR represents a novel developmental target that regulates several cellular events involved in neural circuit formation. Here we reveal that the 5-HT3AR is required to control the migration and positioning of cINs into the CP. In addition to its importance in regulating cIN subtypes, the 5HT3AR is expressed in Cajal-Retzius cells and regulates the apical dendritic growth of pyramidal neurons in the cortex through a reelin-dependent mechanism25. Taken together, our study provides further insights on the importance of the 5-HT3AR in controlling developmental processes involved in the formation of cortical circuits. It proposes that 5-HT3AR-mediated signalling constitutes a molecular mechanism that mediates neuronal excitability and that 5-HT3AR activation derived cortical INs, is functionally upregulated in cINs during the process of cortical invasion and that serotonin levels increase during the late phase of cortical invasion22 suggests that serotonin signalling promotes the migration of cINs and their entry into the superficial cortical layers of Htr3a-ko mice.

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The 5-HT3AR is a cation-selective ligand-gated ion channel that mediates neuronal depolarization and excitation50. Previous work in the field has demonstrated that neuronal excitability is required for the proper positioning of CGE-derived INs in superficial cortical layers18, supporting the possibility that neurotransmitters such as serotonin could regulate the migration of CGE-derived INs through 5-HT3AR-dependent signalling. Here we show that 5-HT3AR activation enhances the migratory speed and growth cone dynamics of cINs during the phase of CP invasion. The 5-HT3AR is closely related to the anion selective GABAA receptor. In conditions of low-expression levels of the potassium-chloride co-transporter KCC2, GABAA stimulation induces an increase in calcium transients and promotes the migration of cINs and their entry into the cortex35,49. In addition, other neurotransmitter systems have been shown to regulate the migration of cortical INs such as AMPA and NMDA receptors50–52. Overall, in vitro data obtained in cINs but also in other neuronal cell types such as cerebellar granule cells indicate that neuronal migration involves the modulation of calcium transients through the opening of NMDA and voltage-gated calcium channels53,55. It is thus hypothesized that early activity in neuronal networks generates ambient levels of neurotransmitters that regulate IN migration by modulating their intracellular calcium transients. Our data add to this model the possibility that ambient levels of serotonin could specifically regulate the migration of cINs into the CP through the regulation of 5-HT3AR-dependent calcium transients.

Developmental insults including alterations in the migration and maturation of cINs contribute to the emergence of psychiatric-relevant functional alterations later in life2. Early-life serotonin dysregulation in rodents following genetic deletion or pharmacological blockade of the serotonin transporter by antidepressants leads to long-term psychiatric-related phenotypes29. In this perspective, the 5HT3AR represents a novel developmental target that regulates several cellular events involved in neural circuit formation. Here we reveal that the 5-HT3AR is required to control the migration and positioning of cINs into the CP. In addition to its importance in regulating cIN subtypes, the 5HT3AR is expressed in Cajal-Retzius cells and regulates the apical dendritic growth of pyramidal neurons in the cortex through a reelin-dependent mechanism25. Taken together, our study provides further insights on the importance of the 5-HT3AR in controlling developmental processes involved in the formation of cortical circuits. It proposes that 5-HT3AR-mediated signalling constitutes a molecular
mechanism controlling the migration and laminar positioning of a reelin-expressing CGE-derived IN subtype and thus further supports the important role of early activity in the development of reelin-expressing CGE-derived INS18.

Methods

Mice. Animal experiments were conducted according to the Swiss and international guidelines and approved by the local Geneva animal care committee. Adult timed pregnant mice were obtained by overnight (ON) mating and the following morning was counted as embryonic day (E) 0.5. To study CGE-derived INs, we used transgenic mice expressing GFP under the control of the reelin-expressing CGE-derived INs18.

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Tissue processing and immunohistochemistry (IHC). Pregnant females were euthanized by lethal intraperitoneal (i.p.) injection of pentobarbital (50 mg kg–1). Embryos were collected in Hanks’ balanced salt solution (HBSS), manually triturated and dissociated in trypsin at 37 °C. Glutamine, 1% penicillin–streptomycin, 2 mM N-acetyl-cysteine and 1 mM sodium butyrate were added to the dissociation solution. Cells were then centrifuged for 5 min at 1,200 r.p.m. and pellets were resuspended in Neurobasal medium (NBM; Invitrogen) supplemented with 2% B27 (Gibco), 2 mM glutamine, 1% penicillin–streptomycin, 2 mM sodium pyruvate, 10 μM ketamine, 10 mM HEPES, 200 mM L-glutamine, 10 μM ascorbic acid, and 5 μg ml–1 heparin. Cells were counted as embryonic day (E) E0.5. To study CGE-derived INs, we used transgenic mice expressing GFP under the control of the reelin regulatory sequences (Htr3a-GFP) provided by the GENSAT Consortium16. Both strains were maintained on a C57Bl/6 background. C57Bl/6 wild-type mice were used for in vivo graft experiments. The Nkx2.1-Cre mice have been previously described25 and crossed to R26R-tdTomR Donation reporter mice were obtained from Jackson Laboratory. Htr3a-GFP mice were crossed to R26R-tdTomR mice to obtain Htr3a-GFP; R26R-tdTomR mice. Htr3a-ko mice have been previously described25 and crossed to GAD65-GFP mice to obtain Htr3a-ko; GAD65-GFP animals.

In situ hybridization. Sections were hybridized as described previously25 with the respective DIG-labelled RNA probes. The Htr3a placid probe26 was linearized with HindIII-HF for RNA probe synthesis by T7 polymerase (kind gift from Dr B. Emerit). The Lhx6 placid probe27 (kind gift from Dr M. Denaxa) was linearized with NotI for antisense RNA probe synthesis by T3 polymerase. The unbound probe was washed and slices were incubated with alkaline phosphatase-conjugated anti-DIG antibody (1:2,000; Roche) ON at 4 °C. NBT/BCIP (Roche) was used as a colourless phosphatase substrate to reveal the hybridized probe. Fast Red (Roche) was used as an alkaline phosphatase fluorescent substrate.

Preparation of dissociated cultures and acute slices. For migration studies, E14.5 and E17.5 cortices from GAD65-GFP+ coronal sections were isolated under a fluorescence scope (Leica, M165 FC) through microdissection. For calcium recordings, microdissection of the CGE of E14.5 Htr3a-GFP caudal cortical slices and cortices of E14.5 wild-type coronal slices was performed. Isolated tissue was collected in Hank’s balanced salt solution (HBSS), manually triturated and dissociated in trypsin at 37 °C for 15 min and blocked in fetal calf serum. Dissociated cells were then centrifuged for 5 min at 1,200 r.p.m. and pellets were resuspended in neurobasal medium (NBM; Invitrogen) supplemented with 2% B27 (Gibco), 2 mM glutamine, 1% penicillin–streptomycin, 2 mM N-acetyl-cysteine and 1 mM sodium pyruvate. For cell density assays, cells were plated in 96-well microplates (Costar 3720) coated with laminin (10 μg ml–1; Invitrogen) and poly-D-lysine (0.1 mg ml–1; Sigma). Acute brain slices were prepared as described24 from Htr3a-GFP+; GAD65-GFP+ and Htr3a-ko; GAD65-GFP+ embryos. Briefly, brains were extracted in HBSS with 3% ultra-pure low-melting point agarose (Invtrogen, Roth) and 250-μm-thick slices were cut on a Vibratome (VT1000S; Leica) in cold oxygenated (95% O2,5% CO2) artificial cerebrospinal fluid (ACSF) containing in mM: NaCl (125), KCl (3), CaCl2 (1.6), MgCl2 (1.5), Na2HPO4 (1.25), NaHCO3 (26), d-glucose (10) Na2HPO4, pH 7.6.

FACS and microarrays. Acute cortical slices were prepared as described above and under a fluorescence dissecting scope (Leica, M165 FC), the E14.5 pallium including the SVZ stream were isolated from GAD65-GFP+; caudal cortical slices. Each tissue point was obtained in triplicate from three different litters and consisted of pooled embryos or pups (n = 4–5). Following dissociation of the tissue, GAD65-GFP+ cells were isolated using FACS with the FACS VantageSE and collected in RNA Later solution (Invtrogen). RNA from the cells was extracted using the RNaseasy Mini Kit (Qiagen) and the quality was checked on an Agilent 2100 Bioanalyzer (Agilent Technologies). A small-scale protocol from Affymetrix (High Wycombe) was used to reproducibly amplify and label total RNA. Briefly, approximately 100 ng total RNA was converted into double-stranded cDNA using a cDNA synthesis kit (SuperScript; Invtrogen) with a special oligo(dT)24 primer containing a 7′ RNA promoter site added to the 5′ poly(T) tract. After the first strand amplification biotinylated cRNA was fragmented and hybridized to Mouse Genome 430 2.0 Array (Affymetrix) following the standard protocols. GeneChips were incubated at 45 °C for 16 h with biotin-labelled cRNAs probes, and washed and stained using a streptavidin-phycocerythrin conjugate with antibody amplification using Affymetrix GeneChip Fluidics Station 450. GeneChips were scanned on a GC3000 scanner (Affymetrix). The signal intensities were analysed using the RMA (version 6.6 beta) and the data were normalized using robust multi-array average (RMA)25. The messenger RNA expression levels of genes (ProbeSet) were compared using a two-way analysis of variance model and contrasted with uncorrected P values set at <0.01 threshold.

ELISA quantification of protein expression. Microdissection of the cerebral cortex was performed at E14.5. Trypsinized dissociated cells were plated on 96-well plates and cultured in NMB supplemented with mCPTB (10 μM; Tocris) and the mitotic inhibitor abarbinol (1 μM). Quantification was performed using ELISA kit (Thermo scientific) and 5-HT2AR antibody (1:1,000, LSBio) on E14.5 day in vitro (1 + DIV1) or E14.5 (+ DIV4) cells. Absorbance levels were measured on a SpectraMax Paradigm Multimode Microplate reader ( Molecular Devices). Absorbance levels for the SHT5,ΔR were normalized to absorbance levels generated for the total number of cells via Janus Green whole-cell staining.

Focal electroporation on slices. For focal electroporation on slices, acute coronal slices of E14.5 Htr3a-GFP+ embryos were transferred on membranes (nucleopore Track-Eth; 1 μm pore size, Whatman) floating on NBM in an incubator (37 °C, 5% CO2) for at least 2 h following Vibratome sectioning. A plasmid encoding for a red fluorescent protein (pUBI-tdTOM) was focally injected in vivo at the concentration of 1 μg ml–1 through a beveled glass micropipette into the MGE using a nanoinjector (B203XXZV, WPI) and the precision of the injection site was controlled by addition of 10% Fast Green (Sigma) to the plasmid. The slices were kept moisturized in HBSS and electroporated between platinum Petri dish and commercial platinum electrodes (CUY701-PSE and CUY701-PSL; Nepa Gene) with two unipolar square pulses (ΔP: 100 V; duration: 5 ms; interval: 500 ms) generated by a CUY21-SQ electroporator (Nepa Gene). Slices were then cultured 3 days in vitro (+ DIV3) in an incubator (37 °C and 5% CO2).

Calcium imaging and analysis. Calcium experiments were performed in GCE-isolated cultures (E14.5 + DIV1–3; see above; Preparation of dissociated cultures and acute slices adhered with Fluoro2 AM (1 μM; Invitrogen) in NBM for 20 min in an incubator (37 °C, 5% CO2). Obtained Paraffin Gere done in a bath chamber (RC-26; Warner Instruments) continuously superfused with ACSF (95% O2, 5% CO2) under an inverted epifluorescence microscope (Observer Z1; Zeiss) equipped for live imaging (Life Technologies) with an oil-immersion ×40 objective (Zeiss, Plan NeoFluar ×40/1.3 oil Ph3) and CoolSNAP-HQ camera. ACSF and microscope bath chamber temperatures were kept at 37 °C. The software MetaFluor (version 7.7.0.0; Molecular Devices) was used to acquire images each second and monitor intracellular calcium changes. Calcium events were analysed in Clampfit (version 9.2.0.11; Molecular Devices). A baseline recording was obtained before challenging cells with mCPTB (1 μM) or SR57227 hydrochloride (1 μM). mCPTB-evoked specific responses to 5-HT3AR activation, experiments were performed with blockers for voltage-gated sodium channels, AMPA receptors, NMDA receptors and GABA A receptors (respectively, TTX, 200 nM; NBQX, 2 μM; D-AP5, 20 μM and gabazine, 4 μM; Tocris) superfused 5 min before and along with mCPTB. Cell viability was systemically assessed by a final optical extinction of 100 mM KC1 in ACSF. A calcium event was defined as a calcium increase two times greater than the baseline activity and lasting for more than 1 s. Htr3a-GFP+ cINs were recorded at E14.5 (+ DIV1) and E14.5 (+ DIV3) in the presence and absence of blockers.

Electrophysiological recordings. Coronal brain sections of 300-μm thick were obtained at E14.5–E15, E18–E19, P2/P3 using a Vibratome (Leica, VT1000S). During recording, the whole-cell patch clamp configuration was performed with an Axopatch 200B (Molecular Devices) amplifier (Axopatch 200B), pCa 11.0 (KCl (12), KC (3.5), CaCl2 (2.5), MgSO4 (1.3), NaHPO4 (1.25), NaHCO3 (25), glucose (25), continuously bubbled with 95% O2 and 5% CO2, pH 7.4. Whole-cell
voltage and current clamp recordings were made from visually identified GFP+ neurons using an EPC9 amplifier (HEKA) and an upright microscope (Zeiss). Axoskeleton (E2S) equipped with infrared differential interference contrast (IR-DIC) and standard epifluorescence. Patch pipettes had a resistance of 4–5 MΩ when filled with (in mM): 105 K-gluconate, 30 KCl, 0.5 CaCl₂, 5 EGTA, 2 Mg-ATP, 10 HEPES. Application of serotonin (5-HT, 100 μM; Tocris) or SR 57272 hydrochloride (100 μM; Tocris) was performed via pressure ejection from a second pipette connected to a picosprieter. To isolate specific responses to 5-HT, recordings at E17-E18 were performed with blockers (TX1, 1 μM; NBQX, 10 μM; D-AP5, 100 μM and gabazine, 20 μM; Tocris). Cells were voltage clamped at −60 mV and currents were evoked by series of 20 ms-duration depolarizing voltage pulses. Current clamp recordings were performed in response to (1 s) hyperpolarizing and depolarizing current injections. Signals were filtered at 1-5 kHz, sampled at 2–10 kHz and off-line analysis was performed using Igor Pro (Wavemetrics).

**Time-lapse imaging and analysis.** Time-lapse imaging of migrating GAD65-GFP+ cells in cultures was performed using an automated microscope (ImageXpress Micro XL, Molecular Devices) equipped with objective ×10 (Plan Fluor ×10/0.30 Ph1), which allows the simultaneous recording of migrating cells in multiple wells at 37 °C under 95% O₂ and 5% CO₂. Following a control time-lapse imaging period of 360 min, SR57272 hydrochloride (100 nM; Tocris), serotonin (5-HT, 100 nM; Tocris) or vehicle (NBDM) was added to the medium and recordings were performed for an additional 360 min. Using Metamorph software (version 7.4; Molecular Devices), cells were tracked at E14.5 (÷ DIV1) and at E17.5 (÷ DIV1). Time-lapse imaging of cells in the interface between the pia and cortical (50–220 μm from the pia) compartments was manually drawn in Metamorph and GFP+ INs from GAD65-GFP+ and Htr3a-ko; GAD65-GFP+ slices located in the IZ compartment at start were tracked during 8 h. The percentage of GAD65-GFP+ INs entering the cortex and not going back into the IZ or further migrating in the MZ during the 8 h time period was calculated. Migration speed was calculated as the total distance travelled by GAD65-GFP+ INs divided by total imaging time excluding the pausing time. The percentage pausing time was calculated as the fraction of time the cell nucleus was stationary during the imaging period. For simultaneous imaging of growth cones of CGE INs entering the cortex and not going back into the IZ, a continuous superfusion of oxygenized ACSF (95% O₂/5% C₂) was used. Images were acquired using an automated microscope (ImageXpress Micro XL, Molecular Devices) equipped with a 60×/1.4 oil-immersion objective (Plan Apo VC H 60/1.4, Nikon) and ×200/0.75, Nikon) and oil-immersion ×40 and ×60 objectives (CFI Plan Fluor ×40/1.3 and CFI Plan Apo VC H ×60/1.4, Nikon). Quantification of the distribution of IN subtypes in the cortex was done by apointing a 10-bin grid at the level of the primary somatosensory cortex and bins corresponding to cortical layers were pooled.

**Statistical analysis.** No statistics were used to determine optimal group sample size; however, sample sizes were similar to those used in previous publications from our group and others. No samples were excluded from statistical analysis. Statistical analyses (GraphPad Prism software, version 6.0) were performed using paired or unpaired Student’s t-test, one-way or two-way analysis of variance with Tukey’s or Bonferroni’s multiple comparisons test.

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