Peptidergic influences on proliferation, migration, and placement of neural progenitors in the adult mouse forebrain

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Neural progenitor proliferation, differentiation, and migration are continually ongoing processes in the subventricular zone (SVZ) and rostral migratory stream (RMS) of the adult brain. There is evidence that peptidergic systems may be involved in the molecular cascades regulating these neurogenic processes, and we examined a possible influence of neuropeptide Y (NPY) and cholecystokinin (CCK) systems in cell proliferation and neuroblast formation in the SVZ and RMS and generation of interneurons in the olfactory bulb (OB). We show that NPY and the Y1 and Y2 receptor (R) proteins are expressed in and surrounding the SVZ and RMS and that Y1R is located on neuroblasts in the anterior RMS. Mice deficient in Y1Rs or Y2Rs have fewer Ki-67-immunoreactive (ir) proliferating precursor cells and doublecortin-ir neuroblasts in the SVZ and RMS than WT mice, and less calbindin-, calretinin-, and tyrosine hydroxylase-ir interneurons in the OB. Mice lacking CCK1Rs have fewer proliferating cells and neuroblasts than normal and a shortage of interneurons in the OB. These findings suggest that both NPY and CCK through their receptors help to regulate the proliferation of precursor cells, the amount of neuroblast cells in the SVZ and RMS, and influence the differentiation of OB interneurons.

adult neurogenesis | CCK receptors | neuropeptides | NPY receptors | rostral migratory stream

The mature mammalian nervous system arises from precisely coordinated proliferation, differentiation, and migration of precursor cells during embryonic and early postnatal development (1). In adulthood, neurogenesis is restricted to three regions: the subventricular or subependymal zone (SVZ) of the lateral ventricle, the olfactory bulb (OB), and in the subgranular zone (SGZ) of the dentate gyrus. In the adult, newborn cells in the SGZ (2) integrate into hippocampal circuitry and are associated with learning and memory formation (3), whereas neural progenitor cells in the SVZ migrate along the rostral migratory stream (RMS) to the center of the OB, where they radiate to become interneurons in granule, periglomerular, and external plexiform cell layers (4, 5).

Cascades of molecular signals that underlie fate specification and migration of adult neural progenitors have begun to be clarified (6, 7), and peptidergic systems also appear to participate in these processes. Neuropeptide Y (NPY), which is widely expressed in the central and peripheral nervous system during development and adulthood, has been implicated in proliferation of neuronal precursor cells in olfactory regions and the SGZ. Mice with targeted deletion of NPY (8) contain half as many dividing olfactory neuronal precursor cells as normal and fewer olfactory neurons by adulthood (9), likely mediated through the Y1 receptor (R) subtype (9, 10). This is supported by *in vivo* work showing that the dentate gyrus of mice lacking Y1Rs has significantly reduced cell proliferation and fewer immature neurons (11, 12). Our recent observation of strong Y2R protein expression in a tract immediately alongside the RMS (13) also suggests a possible role for this receptor.

Cholecystokinin (CCK), another member of the brain-gut peptide family, is also a candidate for regulating neurogenesis. CCK modifies the migratory abilities, proliferation, and survival of tumor astrocytes (14) and lymphocytes (15) and guides migration of gonadotropin-releasing hormone-1 (GnRH-1) neuroendocrine neurons into the brain (16). Moreover, immortalized rat brain neuroblasts express CCK1- and CCK2R mRNAs, and exposure to CCK promotes proliferation and improves viability (17).

Therefore, we investigated whether Y1, Y2, and CCK1Rs influence cell proliferation and neuroblast formation in the SVZ and RMS and affect interneuron generation in the OB of the brain of adult mice with one of these three receptors genetically deleted. We report that these mice have fewer proliferating cells, migratory neuroblasts and OB interneurons than WT mice, suggesting a role for NPY and CCK in regulating adult neurogenesis.

Results

NPY, Y1R, and Y2R Proteins Are Expressed in the Mouse SVZ and RMS. The NPY system is expressed in the SVZ and throughout the RMS of adult mice [Fig. 1 *A–R* and supporting information (SI) Fig. 5]. Although limited to few interspersed fibres within the SVZ and RMS, dense NPY-immunoreactive (ir) fiber networks surrounded these regions (Fig. 1 *A*, *D*, *G*, and *P*). At the level of the SVZ, dense Y1R-ir patches were observed in the striatum along the wall of the lateral ventricle, with lower levels on the septal side of the ventricular wall (Fig. 1*B*). Y1R-like immunoreactivity (LI) in the anterior olfactory nucleus surrounded doublecortin (DCx) migrating cells in the RMS (Fig. 1*E*). DCx-ir cells were only found to coexpress Y1R protein in the anterior RMS of the OB (Fig. 1 *H* and *J–L*). Y2R-LI was expressed in a similar pattern (Fig. 1 *C*, *F*, *I*, *N*, and *R*) (13); however, throughout the RMS, in contrast to the Y1R, Y2R-LI surrounded and intermingled with DCx-ir cell bodies and processes, but no certain coexpression was observed (Fig. 1 *M–O*). GFAP-ir astrocytes did not colocalize with NPY, Y1R, or Y2R at the level of the SVZ or RMS (Fig. 1 *P–R*).

Fewer Proliferating Cells, Neuroblasts, And Interneurons in $Y1^{-/-}$ and $Y2^{-/-}$ Mice. Ki-67 is a marker for proliferating cells in the adult brain (18). When compared with normal, the number of Ki67-ir cells in the SVZ was reduced by 50 ± 9% (mean ± SE) in mice lacking Y1Rs ($Y1^{-/-}$), and by 39 ± 12% in those without Y2Rs

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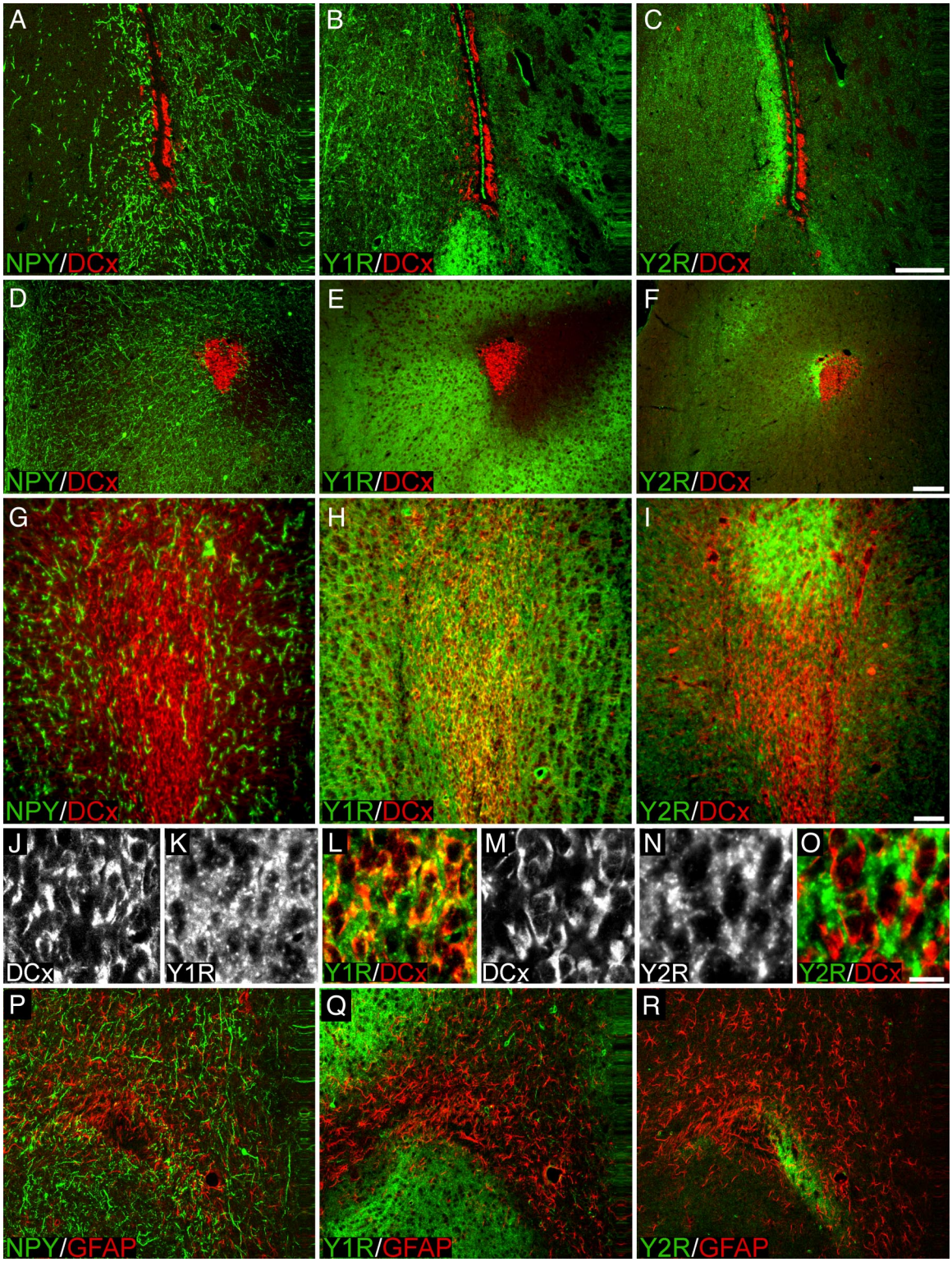

Fig. 1. Double immunolabeling of NPY, Y1R, or Y2R with DCx or GFAP in WT mouse SVZ, RMS, and OB. (*A–C*) SVZ. (*D–F*) RMS. (*G–I*) OB. (*A*, *D*, and *G*) NPY (green) and DCx (red). (*B*, *E*, and *H*) Y1R (green) and DCx (red). (*C*, *F*, and *I*) Y2R (green) and DCx (Red). (*J–L*) Confocal images of colocalization (*L*) between DCx (*J*) and Y1R (*K*) in RMS at level of OB. (*M–O*) RMS showing no certain colocalization (*O*) between DCx (*M*) and Y2R (*N*). (*P–R*) RMS. (*P*) NPY (green) and GFAP (red). (*Q*) Y1R (green) and GFAP (red). (*R*) Y2R (green) and GFAP (red). [Scale bars: *C*, 100 μm (applies to *A–C*, and *P–R*); *F*, 100 μm (applies to *D–F*); *I*, 50 μm (applies to *G–I*); *O*, 10 μm (applies to *J–O*)].

($Y2^{-/-}$) (Fig. 2 *A* and *B*, Table 1, and SI Fig. 6). The reduction in proliferating cells led to a decrease in neuroblasts migrating through the SVZ and RMS seen by using DCx as a marker for neuroblasts (19). Thus, the number of DCx-ir cells in the SVZ and RMS of $Y1^{-/-}$ mice was reduced by 57 ± 3%, and 24 ± 4% fewer DCx-ir cells were found in $Y2^{-/-}$ mice (Fig. 2 *E*, *F*, *I*, *J*, *M*,

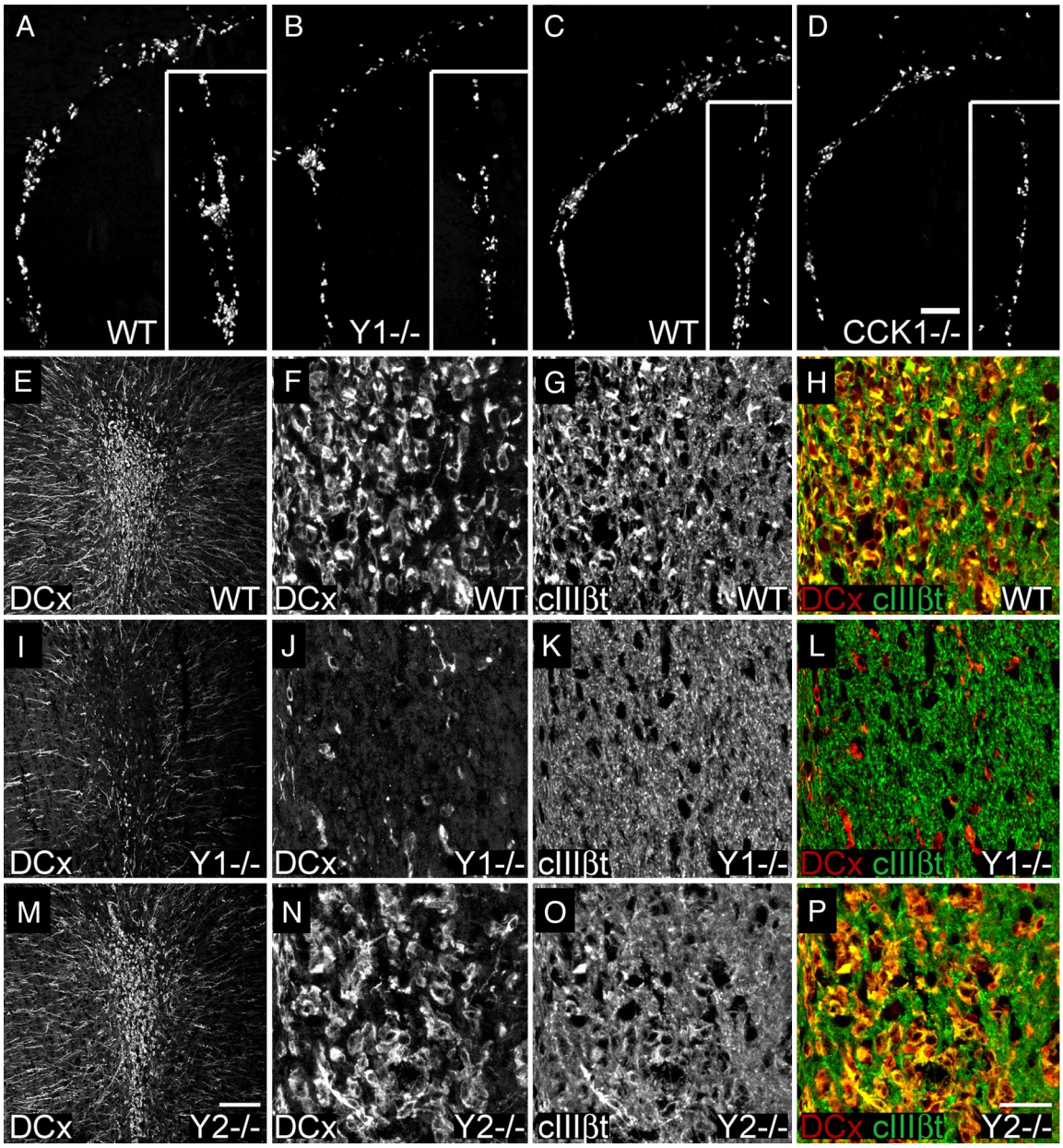

Fig. 2. Reduced numbers of Ki67-, DCx-, and βIII-tub (cIIIβt)-ir cells in the SVZ and RMS of $Y1^{-/-}$, $Y2^{-/-}$, and $CCK1^{-/-}$ mice. (*A–D*) Ki67-LI in SVZ of WT (*A* and *C*), $Y1^{-/-}$ (*B*), and $CCK1^{-/-}$ (*D*) mice. (*E*, *F*, *I*, *J*, *M*, and *N*) Confocal images of DCx-LI in RMS of WT (*E*), $Y1^{-/-}$ (*I*), and $Y2^{-/-}$ (*M*) mice and at higher magnification in *F*, *J*, and *N*, respectively. (*G*, *H*, *L*, *K*, *O*, and *P*) βIII-tub-LI in RMS of WT (*G*), $Y1^{-/-}$ (*K*), and $Y2^{-/-}$ (*O*) mice and double-immunolabeling with DCx in *H*, *L*, and *P*, respectively. [Scale bars: *D*, 100 μm (applies to *A–D*); *M*, 100 μm (applies to *E*, *I*, and *M*); and *P*, 20 μm (applies to *F–H*, *J–L*, *N*, *O*, and *P*)].

and *N*, Table 1, and SI Fig. 7). Also, the assembly of neuroblasts in $Y1^{-/-}$ mice appeared disrupted compared with the chain-like organization characteristic of normal mice (Fig. 2 *E*, *F*, *I*, and *J* and SI Fig. 7). Similar reductions in the number of neuron-specific βIII-tubulin (βIII-tub)-ir neuroblasts migrating through the RMS were observed in $Y1^{-/-}$ and $Y2^{-/-}$ mice (Fig. 2 *G*, *K*, *O*), and these cells were often found to colocalize with DCx-ir cells (Fig. 2 *H*, *L*, and *P*). No obvious differences in the expression of GFAP-ir astrocytes were observed in $Y1^{-/-}$ or $Y2^{-/-}$ mice (SI Fig. 8).

Normally, migrating RMS neuroblasts differentiate into two OB interneuron subtypes: granule and periglomerular neurons (5). These cells include several subclasses expressing specific neurochemical markers, such as the dopamine synthesizing enzyme tyrosine hydroxylase (TH) (20) and the calcium binding proteins calbindin (CB) and calretinin (CR) (21). To determine whether defects in cell proliferation and migratory neuroblasts observed in the SVZ and RMS of $Y1^{-/-}$ or $Y2^{-/-}$ mice led to changes in the generation of interneurons in the OB, we quantified the number of OB interneurons in mutant and control mice.

CB serves as a marker for interneurons primarily located in the glomerular layer of the OB (21). Fewer CB-ir neurons were found in mice lacking Y1R (34 ± 1%) and Y2R (25 ± 2%) compared with WT mice (Fig. 3 *A–C* and Table 1). CR is distributed more widely throughout the layers of the OB (21). In the glomerular layer, $Y1^{-/-}$ mice had 19 ± 6% fewer CR-ir interneurons with a 34 ± 6% reduction in $Y2^{-/-}$ mice (Table 1 and SI Fig. 9). Similar reductions in CR-ir interneurons were observed in the granular cell layer, with 19 ± 4% fewer cells in $Y1^{-/-}$ mice, and 27 ± 2% less cells in $Y2^{-/-}$ mice (Fig. 3 *F–H*

Table 1. Number of cells (mean ± SE) counted in the SVZ, RMS and OB of Y1$^{-/-}$, Y2$^{-/-}$, CCK1$^{-/-}$, and WT mice and percentage change from WT

Mouse	Ki67-ir cells, no.	DCx-ir cells, no.	CB-ir cells in OB glomerular layer, no.	CR-ir cells in OB glomerular layer, no.	CR-ir cells in OB granular layer, no.	TH-ir cells in OB glomerular layer, no.
WT (C57/blb)	1,828 ± 152 (0 ± 8)	4,106 ± 200 (0 ± 5)	974 ± 42 (0 ± 4)	1,980 ± 92 (0 ± 5)	1,659 ± 78 (0 ± 5)	934 ± 41 (0 ± 4)
Y1$^{-/-}$	907 ± 152 (50 ± 9)*	1,757 ± 121 (57 ± 3)*	647 ± 12 (34 ± 1)*	1,600 ± 114 (19 ± 6)*	1,340 ± 60 (19 ± 4)*	585 ± 34 (37 ± 4)*
Y2$^{-/-}$	1,124 ± 226 (39 ± 12)*	3,128 ± 148 (24 ± 4)*	728 ± 20 (25 ± 2)*	1,311 ± 113 (34 ± 6)*	1,212 ± 39 (27 ± 2)*	623 ± 23 (33 ± 2)*
WT (129/SvEV)	1,559 ± 50 (0 ± 3)	3,007 ± 147 (0 ± 5)	643 ± 25 (0 ± 4)	1,448 ± 50 (0 ± 3)	873 ± 31 (0 ± 4)	648 ± 19 (0 ± 3)
CCK1$^{-/-}$	1,008 ± 57 (35 ± 4)*	1,503 ± 123 (50 ± 4)*	550 ± 11 (14 ± 2)*	983 ± 42 (32 ± 3)*	620 ± 20 (29 ± 2)*	423 ± 8 (34 ± 1)*

Numbers in parentheses indicate percentage of change from WT mean.
*$P < 0.05$ (ANOVA) vs. WT.

and Table 1). In the glomerular layer mice lacking Y1- or Y2Rs had, respectively, 37 ± 4% and 33 ± 2% fewer TH-ir cells than WT mice (Table 1 and SI Fig. 9). Taken together, these findings support the notion that both Y1 and Y2R signaling are involved in regulation of proliferation along the SVZ-RMS pathway and in generation of olfactory interneurons.

Reduction in Cell Proliferation, Migratory Neuroblasts, and OB Interneurons in Mice Lacking CCK1 Receptors. Mice lacking CCK1Rs (CCK1$^{-/-}$) had 35 ± 4% fewer Ki67-ir cells when compared with WT (Fig. 2 *C* and *D* and Table 1). Thus, this reduction in cell proliferation may influence the levels of migratory neuroblasts detected in the SVZ-RMS pathway of these mutant mice. The number of DCx-ir neuroblasts in the SVZ and RMS was reduced in CCK1$^{-/-}$ mice (50 ± 4%) (Fig. 4, Table 1, and SI Fig. 10). Interestingly, a reduction in the generation of OB interneurons was also observed. Fewer CB-ir neurons were found in the glomerular layer of CCK1$^{-/-}$ (14 ± 2%) mice (Fig. 3 *D* and *E* and Table 1). In the glomerular layer, CCK1$^{-/-}$ mice had 32 ± 3% fewer CR-ir interneurons (Table 1 and SI Fig. 11). A similar reduction in CR-ir interneurons was observed in the granular cell layer, with 29 ± 2% fewer cells in CCK1$^{-/-}$ mice (Fig. 3 *I* and *J* and Table 1). Quantification of TH-ir interneurons in the glomerular layer revealed that CCK1$^{-/-}$ mice had 34 ± 1% fewer TH-ir cells than WT (Table 1 and SI Fig. 11). These findings support the notion that CCK1R signaling is required for normal proliferation of SVZ-RMS neural precursor cells, influencing the number of migrating cells along this route and the generation of OB interneurons.

Discussion

Using genetically modified mice and immunohistochemistry, we provide evidence that NPY and CCK by actions mediated through, respectively, Y1, Y2, and CCK1Rs can regulate cell proliferation in the adult mouse SVZ and RMS and that mice lacking these receptors have fewer migratory neuroblasts in this pathway and less OB interneurons. Moreover, whereas Y1R protein is expressed by migrating neuroblasts in the anterior RMS, Y2R protein was only found in close proximity to and surrounding RMS neuroblasts. No corresponding information is available for the CCK1R because of lack of suitable anti-receptor antibodies.

Immunohistochemical studies revealed a dense NPY-ir fiber network surrounding Ki-67-ir proliferating cells and DCx-ir/βIII-tub-ir neuroblasts in the SVZ and RMS. Generally, the Y1R and Y2R proteins were distributed within close proximity to the SVZ and RMS and often surrounded Ki-67-ir and DCx-ir cells in these regions. However, DCx-ir neuroblasts in the RMS were only found to coexpress Y1R protein in the anterior RMS at the level of the OB but not in proliferating cells/neuroblasts in the SVZ and posterior RMS. This was surprising, because previous reports on cells cultured from the rat dentate gyrus (8–10 days old) showed that 95% of BrdU-ir cells and 80% of neuroblasts coexpressed Y1R protein (11). This may thus represent age, species, and/or regional differences. However, it is also possible that the Y1R in SVZ is expressed at levels too low to be detected with our methodology but at sufficiently high levels in the anterior RMS, indicating increasing receptor levels during neuroblast maturation. Nevertheless, our finding that Y1R protein

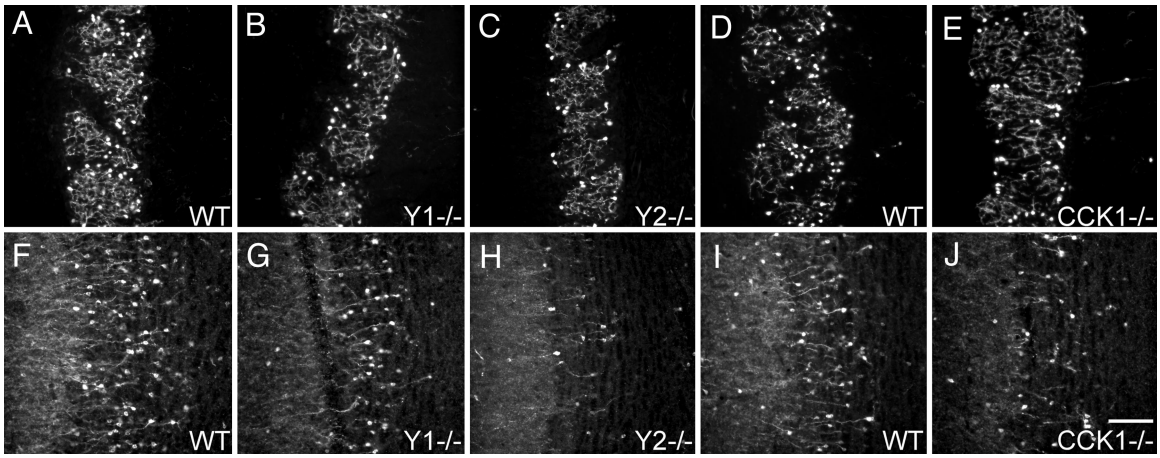

Fig. 3. Fewer CB- and CR-ir cells in the OB of Y1$^{-/-}$, Y2$^{-/-}$, and CCK1$^{-/-}$ mice. (*A–E*) CB-LI in glomerular cell layer of WT (*A* and *D*), Y1$^{-/-}$ (*B*), Y2$^{-/-}$ (*C*), and CCK1$^{-/-}$ (*E*) mice. (*F–J*) CR-LI in granular cell layer of WT (*F* and *I*), Y1$^{-/-}$ (*G*), Y2$^{-/-}$ (*H*), and CCK1$^{-/-}$ (*J*) mice. (Scale bar: 100 μm).

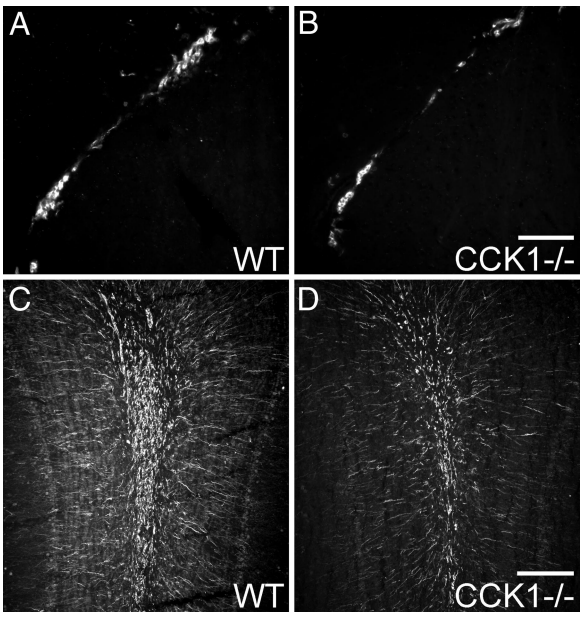

Fig. 4. Reduced DCx-ir cells in the SVZ and RMS of CCK1$^{-/-}$ mice. DCx-LI at level of SVZ (*A* and *B*) and RMS (*C* and *D*) of WT (*A* and *C*) and CCK1$^{-/-}$ (*B* and *D*) mice. [Scale bars: *B*, 100 μm (applies to *A* and *B*); *D*, 200 μm (applies to *C* and *D*)].

is expressed on DCx-ir neuroblasts provides evidence of the ability of NPY to have direct influence on this cell type.

The lack of Y2R protein expression on Ki67- or DCx-ir cells suggests that any NPY- mediated effects on these cells via this receptor are indirect. A possible target would be glial cells, which form specialized tubes through which neuroblasts migrate (4); however, we found no evidence for coexpression of Y2Rs and GFAP-ir astrocytes in the SVZ or RMS.

Our results are consistent with previous reports that NPY promotes proliferation of olfactory neuronal precursors (9) and cultured precursors derived from the postnatal hippocampus by actions mediated through Y1Rs (10–12). Y1R-mediated regulation of cell proliferation has also been reported in the dentate SGZ of 35-day-old mice lacking Y1Rs, where 40% fewer cells than normal incorporated BrdU (11, 12). Such Y1R-mediated proliferative effects of NPY require activation of the extracellular signal-regulated kinase (ERK1/2) subgroup of mitogen-activated protein kinases and protein kinase C (9, 11), which have been implicated in proliferation of neural progenitors (22).

The finding of fewer Ki-67 cells in the SVZ of Y2$^{-/-}$ mice suggests that the Y2R may also contribute to regulating the proliferation of precursor cells in the adult brain. The mechanism involved is unclear but may occur through modulation of phosphotyrosine-containing proteins (23) or via a PKC dependent pathway (24), both of which have links to Y2R-mediated effects. Involvement of other Y Rs in precursor proliferation in the adult brain warrants further investigation, considering observations of Y3R-mediated proliferative effects on rat aortic endothelial cells (25), and Y5R-mediated proliferation, migration, and differentiation of cultured human endothelial cells (26).

Both Y1$^{-/-}$ and Y2$^{-/-}$ mice had fewer migratory neuroblasts in the SVZ and RMS than WT mice, likely a consequence of the reduced number of proliferating cells observed in both mouse models. Thus, we support recent studies in Y1$^{-/-}$ mice reporting fewer neuroblasts in the SGZ (10–12). Interestingly, the assembly of neuroblasts in the RMS of Y1$^{-/-}$ mice appeared disjointed and disrupted compared with normal, where neural precursors migrate tangentially in a chain-like structure and maintain contact with other migrating cells (4). The shift from chain-like to a more individual migration may be a consequence of alterations in the extracellular matrix or adhesive properties of migrating cells. Loss of Y1R (or Y2R) function may alter signaling through other receptor systems, such as the integrins (27), or reelin, which can disturb chain-like migration of neuroblasts (28). Links between NPY and reelin have been reported in cortical neurons (29), as has an association between NPY receptors and the transmembrane-4 superfamily protein CD63 (30), the latter being part of a protein complex that binds integrin (31).

Mice lacking CCK1Rs were also found to have a lower number of Ki-67-ir proliferating precursors and DCx-ir neuroblasts in the SVZ and RMS, suggesting that CCK, through CCK1R signaling, is a regulator of cell proliferation. We also have evidence that mice lacking CCK2Rs have fewer Ki67-ir proliferating cells (data not shown), suggesting that this receptor may also be involved. In agreement, recent findings show that both CCK1- and CCK2Rs are expressed by immortalized rat brain neuroblasts (17). This may occur through CCK1- or CCK2R-mediated induction of intracellular signaling cascades that lead to tyrosine phosphorylation of paxillin and p130^{Cas} (17), which leads to formation of focal adhesion complexes, actin cytoskeleton regulation, and cellular growth (32, 33) or by activation of the ERK1/2 and the PKB/Akt intracellular pathways (17), which can influence a variety of cellular functions, including their growth, differentiation, and survival (34, 35).

Another role for NPY and CCK receptors may be to influence the differentiation and final placement of newly derived interneurons in the OB, because there was a reduced number of CB-, CR-, and TH-ir interneurons in the glomerular and granular layers of mice lacking these receptors. How these deficits in OB interneurons impact on glomerular and granular synaptic organization and resultant behavior remains unclear, but they may contribute to increased aggression and changes in motor activity, exploration, and anxiety observed in Y1$^{-/-}$ mice (36, 37) and the anxiolytic- and antidepressant-like phenotype observed in Y2$^{-/-}$ mice (38, 39). Reductions in olfactory interneurons observed in mice lacking CCK1Rs may underlie alterations in exploratory and anxiety-related behaviors observed in such mice (40, 41).

Taken together, our findings suggest that NPY, by actions mediated through Y1- and Y2Rs and CCK through CCK1R signaling, is involved in regulating the proliferation of precursors in the SVZ and RMS, influencing their differentiation into distinct interneuronal subsets in the OB. These findings may help to explain how neurogenesis is regulated in the adult brain and may lead to better ways of harvesting therapeutically useful cells for treatment of neurodegenerative illness.

Materials and Methods

Animals. One female and five male Y1$^{-/-}$ (10), two female and three male Y2$^{-/-}$ (42), and three female and three male WT mice on a C57/bl6 background and aged 4–7 months were used. Two female and two male CCK1$^{-/-}$ (43) and two female and two male WT mice on a 129/SvEv background and aged 4–6 months were analyzed. Animals were maintained under standard conditions on a 12-h day/12-h night cycle with water and food ad libitum. All procedures were approved by the local ethical committee (Stockholms Norra djurförsöksetiska nämnd 397/04).

Immunohistochemistry. Immunohistochemistry, using tyramide signal amplification (TSA+; NEN Life Science Products), was performed as described in ref. 13 (see *SI Text*).

Quantification. Cells in the SVZ and RMS were quantified in each hemisphere at 9 (DCx, SVZ/RMS), 6 (Ki67, SVZ), and 7 (CB, CR, TH, OB) rostro-caudal levels in Y1$^{-/-}$, Y2$^{-/-}$, and WT mice. For CCK1$^{-/-}$ and WT mice, 14 (DCx, SVZ/RMS), 5 (Ki67, SVZ), and 5 (CB, CR, TH, OB) levels were examined (see *SI Text*).

Statistical Analysis. Data were analyzed with Statistica (StatSoft). ANOVA and Tukey multiple comparison tests were used, with statistical differences set at $P < 0.05$.

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