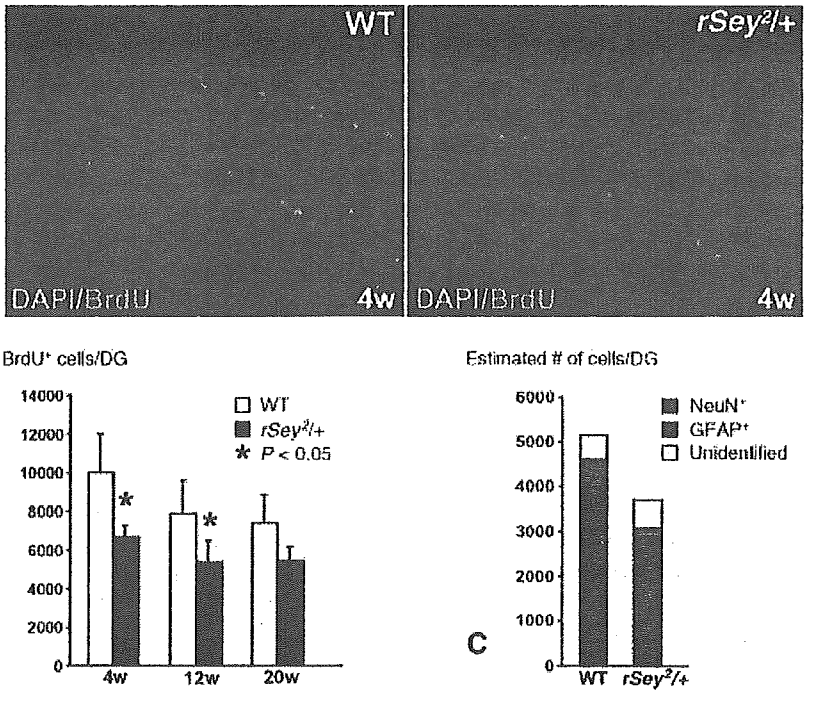


D

	BrdU⁺ cells			Developmental rate	
	12 week $n = 6$	12 + 4week $n = 3$	Survived	NeuN⁺ $n = 4$	GFAP⁺ $n = 4$
Wild type	7853 ± 1663	5168 ± 120.6	65.8 ± 0.1%	76.8 ± 8.2%	12.6 ± 1.3%
rSey²/+	5370 ± 1130	3712 ± 159.0	69.1 ± 0.1%	77.7 ± 6.9%	5.3 ± 1.0%

Table. Survival rate and developmental fates of BrdU⁺ cells in the dentate gyrus

Figure 3 Reduced cell proliferation and production of new neurons and astrocytes in the dentate gyrus of *rSey²*/+ rats at postnatal stages. (A) The number of BrdU-positive cells (green) is decreased in the DG of *rSey²*/+ rats after 3-day BrdU incorporation at 4 weeks. (B) The numbers of BrdU-positive cells within the DG are 33.3% ($P = 0.046$), 31.6% ($P = 0.021$), and 26.2% ($P = 0.051$) lower in the *rSey²*/+ rats than in the wild-type at 4, 12, and 20 weeks, respectively. (C) Estimated numbers of cells double-positive for BrdU/NeuN (new neurons) and for BrdU/GFAP (new astroglia) based on the survival rate and the developmental fates shown in (D). Formation of new neurons and astroglia is markedly decreased in *rSey²*/+ rats from 12 to 16 weeks. (D) Calculated survival rate and developmental fates of BrdU-positive cells in the DG 4 weeks after BrdU-injection at 12 weeks of age. The survival rate is not changed between WT and *rSey²*/+. In contrast, frequency of GFAP$^+$ in total BrdU$^+$ cells is less in *rSey²*/+ at 4 weeks after BrdU injection, although that of frequency of NeuN$^+$ in total BrdU$^+$ cells is similar between WT and *rSey²*/+ at 4 weeks after BrdU injection.

Decreased cell proliferation in the DG of *rSey²*/+ rats

To address the question whether postnatal neurogenesis is affected in the SGZ of *Pax6*-deficient rats, we first compared the total number of BrdU$^+$ cells in the DG between the wild type and *rSey²*/+ at 4, 12, and 20 weeks. Rats were intraperitoneally injected with BrdU three times a day for 3 days and sacrificed at 24 h after the last BrdU injection (Kempermann & Gage 1999). In the DG of the wild type, the total number of BrdU-labeled cells in the SGZ per hemisphere decreased as the stage proceeded (Fig. 3A,B). Interestingly, a significant decrease in the total number of BrdU-labeled cells was observed in *rSey²*/+ at 4 weeks (33.3% decrease), 12 weeks (31.6%), and 20 weeks (26.2%) (Fig. 3B). These data clearly indicate that the number of proliferating progenitor cells is considerably reduced in the SGZ of *Pax6*-deficient rat.

Next, we examined the fate of newborn cells in the DG. The wild type and *rSey²*/+ rats were injected with BrdU three times a day for 3 days at 12 weeks and examined 4 weeks later based on the previous protocol (Kempermann & Gage 1999). Although the proliferating rate was much decreased in the DG of *rSey²*/+ (Fig. 3B), there were no differences in the survival rate of newborn cells between the wild type and *rSey²*/+ (Fig. 3D). The percentage of NeuN$^+$ cells in total BrdU$^+$ cells (new neurons) was also unchanged in *rSey²*/+ (Fig. 3D). Contrastingly, the percentage of GFAP$^+$ cells in total BrdU$^+$ cells decreased to less than half in *rSey²*/+ (Fig. 3D). This is superficially considered to be a reduction of newborn astrocytes. However, it is now widely accepted that GFAP$^+$ astrocytes can serve as neural stem cells in the hippocampus (Seri *et al.* 2001). Eventually, the estimated total numbers of newly generated neurons and astrocytes/progenitor cells were dramatically reduced in the DG of *Pax6*-deficient rat (Fig. 3C).

In a BrdU labeling study for a longer period (2 weeks), the ratio of BrdU$^+$/Pax6$^+$ cells in the wild type was increased up to fivefold (35%) comparing with the samples labeled for a short period (30 min) (7.7% at 4 week; 6.3% at 6 week). This may imply that a population of cells expressing Pax6 contain neural stem cells (or quiescent GFAP$^+$ early progenitor cells) whose cell cycle is longer than that of GFAP$^+$ early progenitor cells. We also found

that the number of Pax6$^+$/GFAP$^+$ double-positive cells in *rSey*^{2}/+ was 22% less than that in the WT at 4 week (WT, 482 578 ± 33 757 cells/mm^3; *rSey*^{2}/+, 374 981 ± 21 527 cells/mm^3; $n = 4$, $P < 0.01$). Furthermore, the number of BrdU$^+$/Pax6$^+$ double-positive cells was 27% decreased in *rSey*^{2}/+ rats (WT, 238 208 ± 27 545 cells/mm^3; *rSey*^{2}/+, 174 921 ± 13 478 cells/mm^3; $n = 4$, $P < 0.01$) in BrdU labeling study for a longer period (2 weeks). All these results consistently suggest that Pax6 is essential for proliferation of neural progenitor cells, thereby keeping the size of the progenitor pool.

The number of GFAP$^+$ early progenitors decreased in the SGZ of *rSey*^{2}/+

To further elucidate the role of Pax6 in hippocampal neurognesis, we investigated at which step a transition of neurogenesis is impaired by detailed BrdU pulse/chase experiments combined with immunostaining with progenitor markers at 4 weeks. At the beginning, we re-examined the character of Pax6$^+$ cells in combination with BrdU labeling. Remarkably, more than 90% of total BrdU-incorporated cells in the SGZ expressed Pax6 at 30 min after BrdU injection (Fig. 4A,B). This result strongly suggests that Pax6 is vital for the cell proliferation in postnatal hippocampal neurogenesis. The fact that Pax6$^+$ cells are highly proliferative may also explain why they were often seen in clusters in the SGZ (Fig. 1A,C).

Then we investigated the cell-type transition of BrdU-incorporated cells in the SGZ of the wild-type and *Pax6*-deficient 4-week-old rats at 30 min, 24 h and 72 h after BrdU injection. In the wild type, the percentage of Pax6$^+$ cells in total BrdU$^+$ cells became markedly reduced from 30 min to 72 h, but 58% of BrdU$^+$ cells still expressed Pax6 at 72 h (Fig. 4B). The frequency of GFAP$^+$ in total BrdU$^+$ cells decreased between 30 min and 24 h after BrdU injection (Fig. 4C). Contrastingly, the ratio of PSA-NCAM$^+$ in total BrdU$^+$ cells increased between 30 min and 24 h after BrdU injection (Fig. 4D). These data are basically consistent with the results obtained in the mouse (Seri *et al.* 2001; Fukuda *et al.* 2003).

The same analyses were then performed on the DG of the *rSey*^{2}/+ rat. The percentage of Pax6$^+$ cells in total BrdU$^+$ cells slightly decreased but was statistically unchanged between the wild type and *rSey*^{2}/+ (Fig. 4B). In contrast, we found a significant decrease (38.4% decrease at 24 h; 49.6% decrease at 72 h, $P < 0.03$) in the frequency of GFAP$^+$ cells in total BrdU$^+$ cells (Fig. 4C) and an

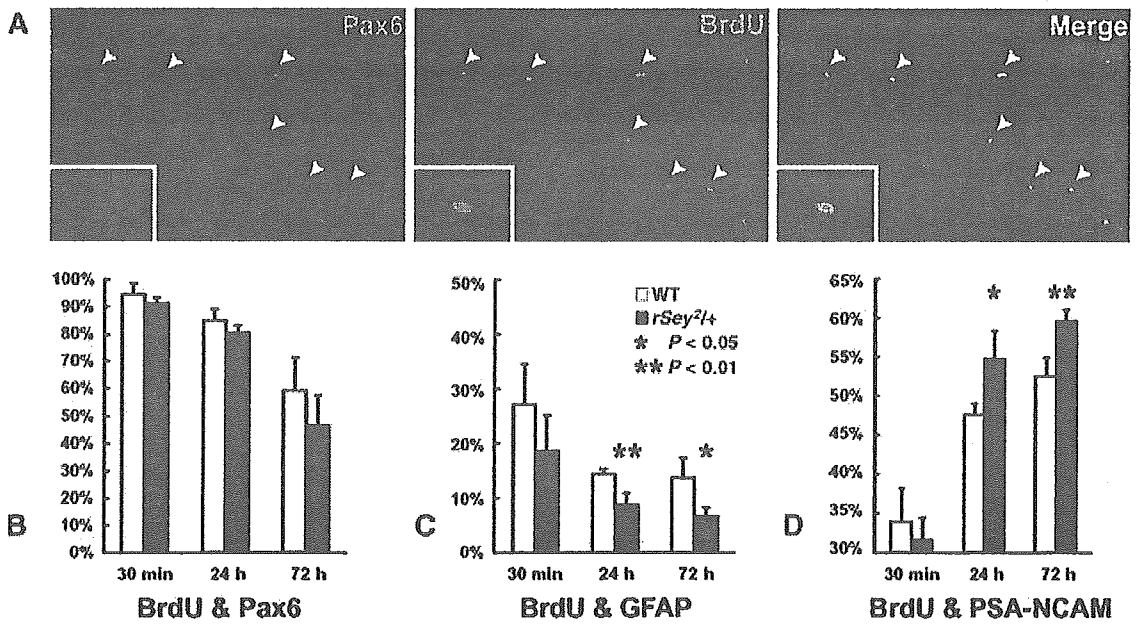


Figure 4 BrdU pulse/chase labeling assay in the DG of the 4-week-old wild type (WT) and Pax6 deficient (*rSey*^{2}/+) rats. (A) Confocal micrographs of BrdU-labeled Pax6$^+$ cells 30 min after BrdU injection. Most of BrdU labeled cells co-express Pax6 (arrows). In *rSey*^{2}/+, the expression level of Pax6 protein is reduced and the number of Pax6$^+$ cells is less than in the wild type. (B–D) Percentage of BrdU-labeled cells in the SGZ at 30 min, 24 h, and 72 h after BrdU injection. (B) At 30 min, more than 90% of BrdU$^+$ cells co-express Pax6. From 30 min to 72 h, the number of Pax6$^+$ cells becomes markedly reduced in WT and *rSey*^{2}/+. (C) Frequency of GFAP$^+$ in total BrdU$^+$ cells is less in *rSey*^{2}/+ at 24 h and 72 h after BrdU injection. (D) Contrastingly, more PSA-NCAM$^+$ cells are observed in total BrdU$^+$ cells in *rSey*^{2}/+ at 24 h and 72 h after BrdU injection.

opposite increase (15.1% increase at 24 h; 13.5% increase at 72 h, $P < 0.03$) in PSA-NCAM$^+$ cells (Fig. 4D). These data clearly indicate that maintenance of GFAP$^+$ early progenitor cells is extremely impaired in the DG of *rSey*^{2}/+.

We performed BrdU pulse labeling study with a short survival period (5 days) to examine whether Pax6 accelerates the neuronal differentiation from dividing PSA-NCAM$^+$ late progenitor cells to NeuN expressing immature neurons. The ratio of NeuN$^+$/BrdU$^+$ cells in this study was unchanged between WT and *rSey*^{2}/+ (4w WT, 63.4%; 4w *rSey*^{2}/+, 66.6%; $n = 4$, $P = 0.36$). Therefore, Pax6 may not be involved in neuronal differentiation but in maintenance of the GFAP$^+$ early progenitor cells by regulating their proliferation.

We further examined how the character of neural progenitors in the DG was different between the wild type and *rSey*^{2}/+ at 4 weeks. As described previously, 31.5% of Pax6$^+$ cells co-expressed a marker for the late progenitor, PSA-NCAM (Figs 1B and 5C). Quite interestingly, Pax6$^+$/PSA-NCAM$^+$ cells dramatically increased up to 55.5% in *rSey*^{2}/+ rats (45/81 cells; 76% increase than that of wild type; Fig. 5B,C). Moreover, GFAP and PSA-NCAM double-positive cells were scarcely detected in the DG of WT rats, while such GFAP$^+$/PSA-NCAM$^+$ cells were quite often observed in the DG of *rSey*^{2}/+ rats (Fig. 5D). These results may imply that premature neuronal differentiation occurs in the DG of the *rSey*^{2}/+. As seen in 16-week rats (Fig. 2C,C′), the number of GFAP$^+$ cells was much less in the SGZ and hilus, and GFAP$^+$ cells have thin and underdeveloped processes in *rSey*^{2}/+ rats (Fig. 5A,E). Quantitatively, the level of GFAP expression was 16% less in the mutant hippocampus as judged from real-time polymerase chain reaction (PCR) (data not shown). These results suggest that hippocampal neurogenesis is quite abnormal in *Pax6*-deficient rat. All the findings consistently suggest a pivotal role of Pax6 in maintenance of the GFAP$^+$ early progenitor cells in the postnatal hippocampus.

Wnt signaling is impaired in the DG of *rSey*^{2}/+

What kinds of molecules then regulate cell proliferation under the control of Pax6 transcription factor? Among various candidate factors, we focused on Wnt signaling molecules because their expressions are reported in the postnatal DG (Shimogori *et al.* 2004) and also because we ourselves have shown down-regulation of a Wnt ligand expression in *rSey*^{2}/*rSey*^{2} rat embryos (Osumi *et al.* 1997; Takahashi *et al.* 2002).

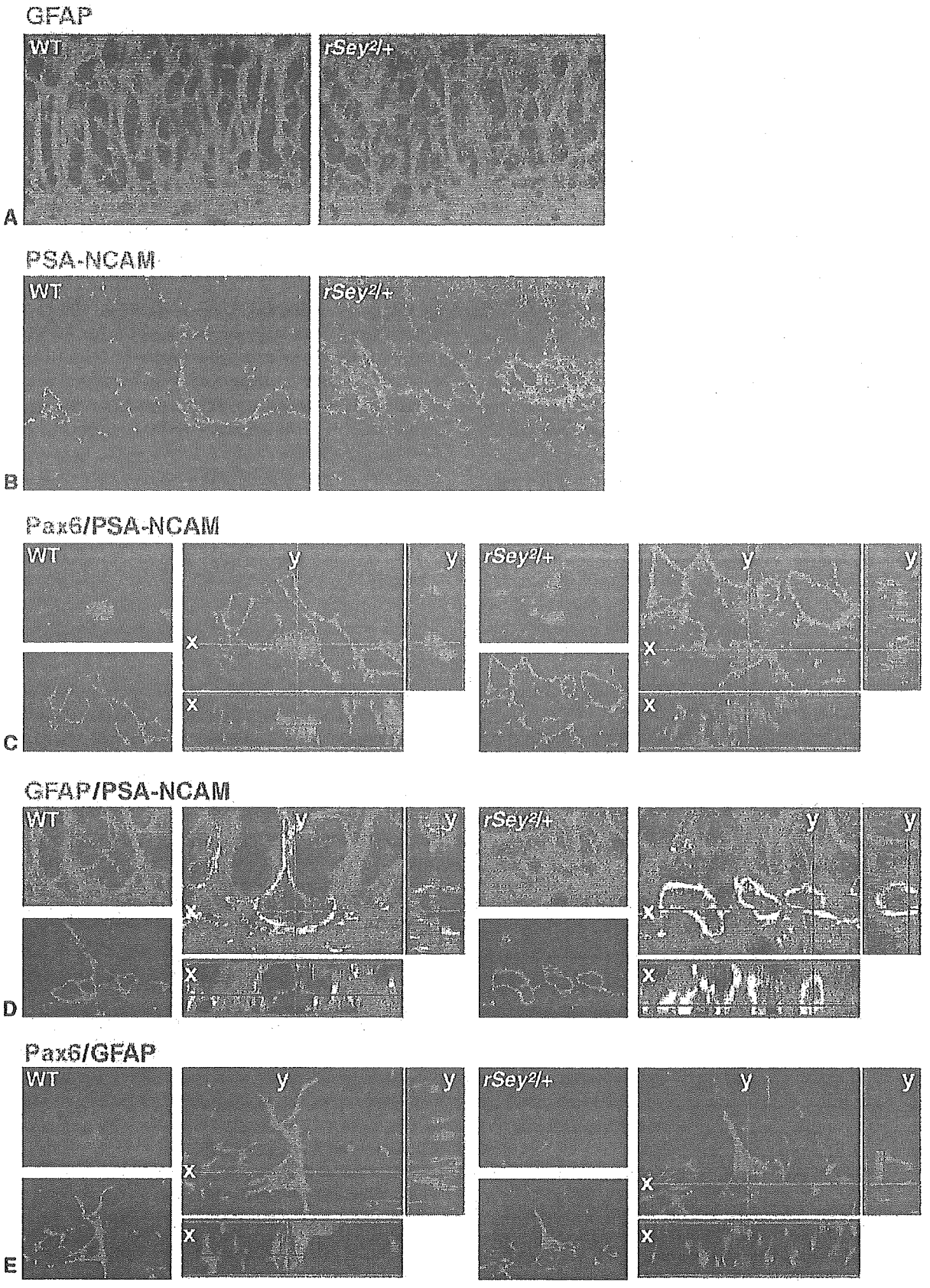
We first searched expression patterns of various Wnt signaling molecules by performing *in situ* hybridization of genes encoding Wnt ligands, Frizzled receptors, and a downstream molecule Dvl1. Among them, *Wnt7a*, *Wnt7b*, *Fz3*, and *Dvl1* showed interesting expression patterns in the DG for 3–4 weeks (Fig. 6). *Wnt7a*, a Wnt ligand, was preferentially expressed in the hilus and along the SGZ of the blades of the DG. Another Wnt ligand, *Wnt7b*, was detected in the SGZ and the GCL, but *Wnt7b*-expressing cells did not morphologically seem to be granule cells in the GCL. Weak expression of *Wnt3a* was also detected in the SGZ at 2 weeks, but almost diminished by 4 weeks in the rat (data not shown). These expression patterns of Wnt ligands hint us to imagine that they are expressed in the progenitors themselves or other cells that may constitute a niche for keeping the undifferentiated state of the progenitor cells. In the DG of *rSey*^{2}/+, the number of *Wnt7a*-expressing cells significantly decreased (687 cells in the wild type; 576 cells in *rSey*^{2}/+; 16% decrease), and the number of *Wnt7b*-expressing cells also decreased (607 in the wild type; 544 in *rSey*^{2}/+; 11% decrease). We could not observe any difference in expression of *Wnt3a* in the DG of *rSey*^{2}/+. Expression of a Wnt receptor *Fz3* was detected mainly in the GCL, and unchanged in the DG of *rSey*^{2}/+. Contrastingly, the expression level of *Dvl1* was increased in the DG of *rSey*^{2}/+ (Fig. 6). Taken altogether, Wnt signaling is impaired in the DG under the Pax6 deficient condition, which may result in reduced proliferation of GFAP$^+$ early progenitor cells in *rSey*^{2}/+.

Discussion

Pax6 expressed in GFAP$^+$ early progenitor cells in hippocampal neurogenesis

Previous papers suggest that adult hippocampal neurogenesis originates from precursor cells in the DG and results in new granule neurons through multiple steps from the GFAP$^+$ early progenitor cells to the PSA-NCAM$^+$ late progenitor cells (Seri *et al.* 2001; Fukuda *et al.* 2003; Kempermann *et al.* 2004). In the present study, we revealed that Pax6$^+$ cells frequently co-expressed GFAP and Musashi1, sometimes expressed nestin and PSA-NCAM, but scarcely co-expressed NeuN in the SGZ of the postnatal DG (Fig. 1B). That is, Pax6 is considered to be expressed mostly in the GFAP$^+$ early progenitor cells.

The ratio of nestin-positive cells among BrdU$^+$ was less than that in a previous study using nestin-EGFP reporter mice (Filippov *et al.* 2003; Fukuda *et al.* 2003). This may be due to difference in sensitivity of anti-nestin antibody and duration of nestin-promoter-driven EGFP. More importantly, a large number of Pax6$^+$/GFAP$^+$ cells had early progenitor-like morphology with a long radial



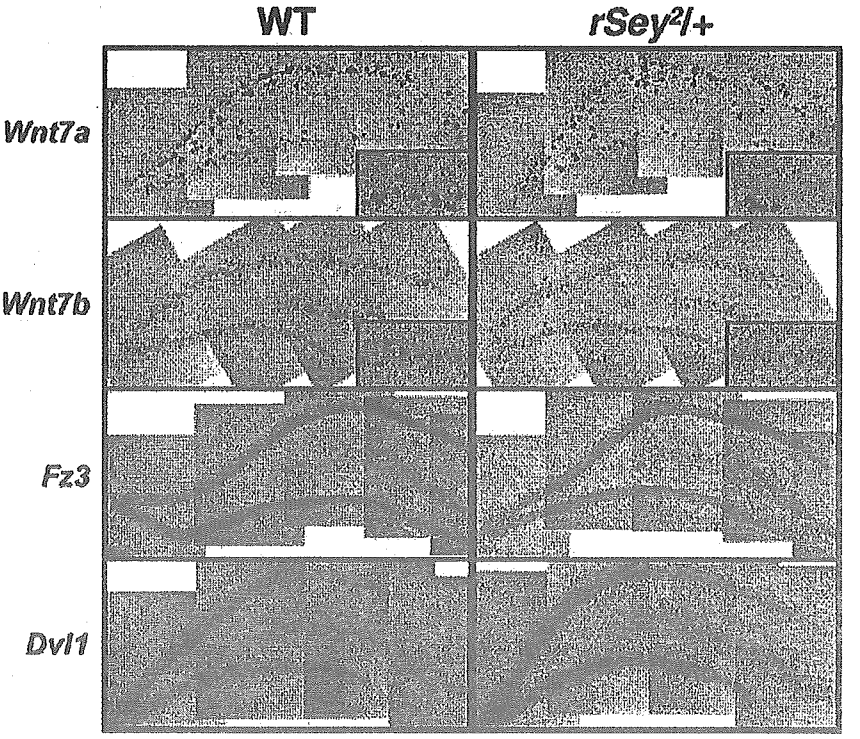


Figure 6 Altered expressions of Wnt-signaling molecules in the DG of *rSey²/+*. In the hippocampus of wild-type, *Wnt7a* is expressed in the SGZ and hilus, while *Wnt7b*, *Fz3*, and *Dvl1* are expressed in the GL and SGZ. In the hippocampus of *rSey²/+*, the number of *Wnt7a*- and *Wnt7b*-expressing cells was decreased, and the expression level of *Wnt7b* was down-regulated. Contrastingly, the expression level of *Dvl1* was increased in the DG of *rSey²/+*. No difference between the wild-type and *rSey²/+* was observed in the expression level of *Fz3*.

process (Fig. 1C′), and Pax6$^+$/nestin$^+$ mostly showed a GFAP$^+$ early progenitor shape (Fig. 1B) and sometimes a PSA-NCAM$^+$ late progenitor cell shape. Pax6$^+$ cells sometimes co-expressed PSA-NCAM, but such Pax6$^+$/PSA-NCAM$^+$ cells always exhibited the late progenitor cell-like morphology. From immuno-electron microscopy, we found that the majority of Pax6$^+$ cells showed features corresponding to type B cells, and that a small number of Pax6$^+$ cells had characters corresponding to type D cells, while Pax6$^+$ cells never showed phenotypes of granule cells (Fig. 1D,D′, E,E′). Therefore, it is concluded that more than half of Pax6$^+$ cells have the character of the GFAP$^+$ early progenitor cells in the SGZ of the hippocampus (Fig. 7).

At present there are no good markers that properly distinguish quiescent neural stem cells from the progenitor cells in the hippocampal neurogenesis. However, we found that the ratio of BrdU$^+$ cells in Pax6$^+$ cells increased fivefold in a 2-week BrdU labeling compared to that in a 30-min labeling. It is thus likely that a population of Pax6-expressing cells may include the quiescent neural stem/progenitor cells in the hippocampus.

Figure 5 Abnormal PSA-NCAM$^+$ cells and GFAP$^+$ cells in *rSey²/+* rats at 4 weeks. (A) In *rSey²/+* rats, there are fewer GFAP$^+$ cells whose processes are thin and underdeveloped. (B) PSA-NCAM$^+$ cells are increased in number and abnormally colonized at the SGZ in *rSey²/+* rats. (C) In the wild-type (WT) rat, PSA-NCAM$^+$ cells scarcely co-express Pax6. Contrastingly, many Pax6$^+$ cells co-express PSA-NCAM in *rSey²/+* rat. In *rSey²/+* rat, Pax6 expression is down-regulated comparing with WT. (D) In the WT rat, PSA-NCAM$^+$ cells scarcely co-express GFAP. Contrastingly, many PSA-NCAM$^+$ cells co-express GFAP in *rSey²/+* rat. (E) In the WT and *rSey²/+* rats, a GFAP$^+$ radial glial cell co-expresses Pax6. In the *rSey²/+* rat, a process of the GFAP+ radial glial cell is thin and undeveloped, and the expression of Pax6 is reduced.

The role of Pax6 in postnatal hippocampus

As discussed above, Pax6$^+$ cells have the character of neural stem cells and GFAP$^+$ early progenitor cells in the DG of postnatal hippocampus. It is thus expected that Pax6 is involved in cell proliferation and/or cell differentiation in hippocampal neurogenesis.

There are some papers where Pax6 is involved in cell proliferation in developing cortex (Warren *et al.* 1999). We found from BrdU labeling analyses that cell proliferation was dramatically reduced in the *Pax6*-deficient DG (Fig. 2A). In addition, more than 90% of total BrdU$^+$ cells were Pax6-positive at 30 min after BrdU injection in the SGZ of adult hippocampus (Fig. 4A,B). These data

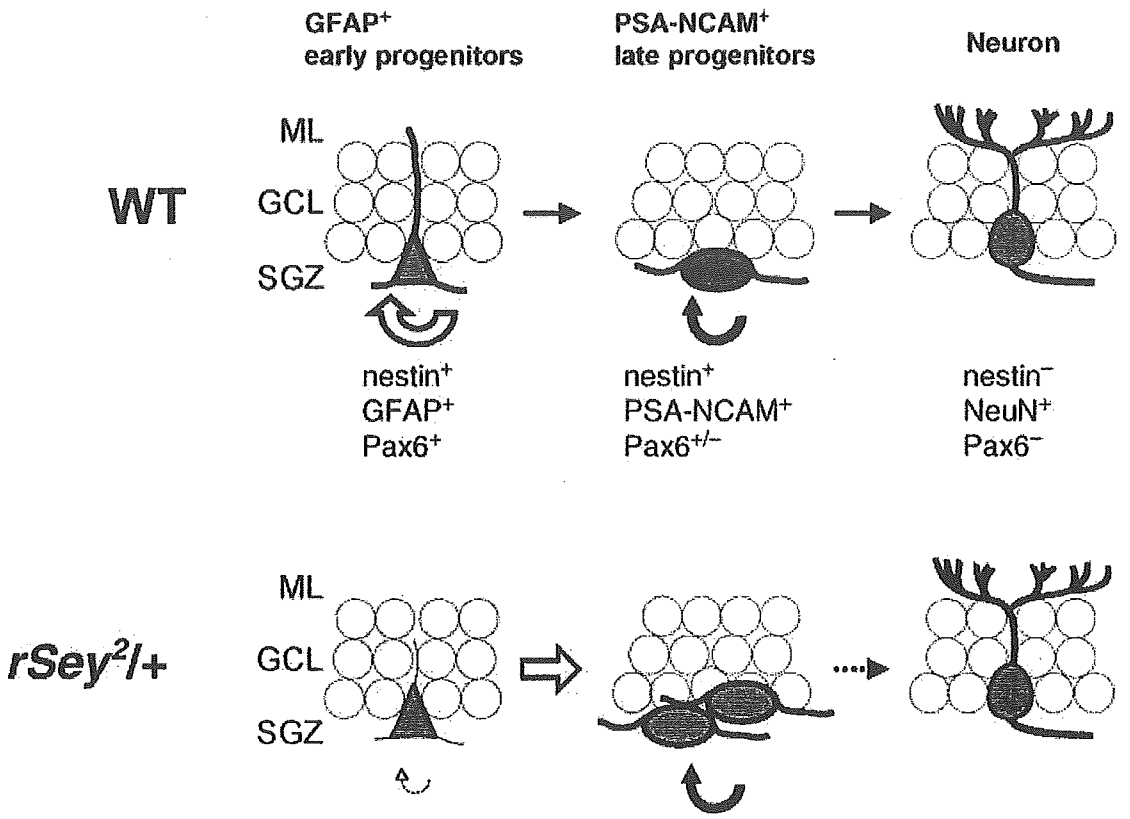


Figure 7 The role of Pax6 in adult hippocampal neurognesis. Distinct progenitor cells are identified on the basis of morphology, proliferative activity, and marker expressions. In the wild type (WT), GFAP$^+$ early progenitors have a radial glial appearance with the cell body in the subgranular zone (SGZ) of the dentate gyrus, and also express nestin and Pax6. PSA-NCAM$^+$ late progenitors have plump short processes that are oriented tangentially, and they are nestin$^{+/-}$, GFAP-, PSA-NAM$^+$ and Pax6$^{+/-}$. Mature neurons in the granule cell layer (GCL) retain a vertical morphology with a rounded or slightly triangular nucleus and clearly visible apical dendrites, and they are nestin$^-$, Pax6$^-$, and NeuN$^+$. In *Pax6* deficient condition (*rSey*^{2}/+), GFAP$^+$ early progenitors have a thinner and undeveloped radial process and are fewer in number than in the WT. There is a more rapid shift from the GFAP$^+$ early progenitor cells to the PSA-NCAM$^+$ late progenitor cells in *rSey*^{2}/+ (big arrow). These PSA-NCAM$^+$ late progenitors show abnormal morphology, ectopic location, and altered molecular character (i.e. increased PSA-NCAM$^+$/Pax6$^+$ and PSA-NCAM$^+$/GFAP$^+$ double positive cells). That is, production of the early progenitor cells is impaired in *Pax6* deficient condition, thereby generating fewer neurons (dotted arrow).

strongly suggest that Pax6 is vital for the cell proliferation in the hippocampal neurogenesis. Then, for which steps of the neurogenesis is Pax6 required in cell proliferation?

Previous papers report that both GFAP$^+$ early progenitor cells and PSA-NCAM$^+$ late progenitor cells are transit amplifying cells. Rapid transition from GFAP$^+$ early progenitors/type B cells to PSA-NCAM$^+$ late progenitors/type D cells occurs between 2 and 24 h after the BrdU single injection (Seri *et al.* 2001; Fukuda *et al.* 2003). In the similar experiment, we found dramatic decrease in the number of GFAP$^+$/BrdU$^+$ cells and an inverse increase in the number of PSA-NCAM$^+$/BrdU$^+$ cells in the SGZ of the *rSey*^{2}/+ (Fig. 4C,D). Moreover, the morphology of GFAP$^+$ cells was altered in *rSey*^{2}/+ (Figs 2C′, 5A, 5E). Our findings suggest that the maintenance of the GFAP$^+$ early progenitor cells is perturbed in the DG of the *rSey*^{2}/+, presumably resulting in a more rapid shift from the GFAP$^+$ early progenitors to the PSA-NCAM$^+$ late progenitors (Fig. 7).

There are some reports that Pax6 promotes neuronal differentiation in the developing cortex and adult SVZ (Heins *et al.* 2002; Hack *et al.* 2004, 2005). To test the possibility that Pax6 is involved in neuronal differentiation in the postnatal hippocampus, we examined the ratio of NeuN$^+$/total BrdU$^+$ cells at 5 days time point after the BrdU injection in *rSey*^{2}/+. The ratio of NeuN$^+$/BrdU$^+$ cells in *rSey*^{2}/+ was not different from that in the WT, even though there is a more rapid shift from the

GFAP$^+$ early progenitor cells to the PSA-NCAM$^+$ late progenitor cells in *rSey²/+*. Therefore, such abnormally differentiated PSA-NCAM$^+$ late progenitor cells did not effectively contribute to produce neurons. In fact, these PSA-NCAM$^+$ late progenitor cells exhibited abnormal characters; they often retained GFAP expression, which is hardly observed in the WT, and did not line up at the SGZ but colonized in disorganized positions (Fig. 5B,D). Since we observed increased cell death in *rSey²/+* (M.M. and N.O., unpublished observation), such functionally abnormal PSA-NCAM$^+$ late progenitor cells may eventually die off in the SGZ of the *rSey²/+*. Taken altogether, Pax6 functions in cell proliferation rather than differentiation in the DG.

We found a marked decrease of the percentage of GFAP$^+$ in BrdU$^+$ cells in *rSey²/+* compared with the wild type, while no significant difference was detected in the percentage of NeuN$^+$ in all BrdU$^+$ cells at 4 weeks after the BrdU injection (Fig. 3C,D). However, since the total number of BrdU$^+$ cells dramatically decreased in *rSey²/+*, newly generated granule cells were markedly reduced in *rSey²/+* rat. Eventually, at 16 weeks, the GCL became thinner in the DG of *rSey²/+* rats than that of the wild type (Fig. 2B). Therefore, it is suggested that Pax6 primarily functions to maintain the progenitor pool in the hippocampus; if the size of the progenitor pool is reduced by Pax6 haplo-insufficiency, the subsequent production of neurons is severely impaired.

The number of Pax6$^+$/GFAP$^+$ cells, BrdU$^+$ cells (BrdU labeling three times a day for 3 days) and BrdU$^+$/Pax6$^+$ cells (BrdU labeling two times a day for 2 weeks) were already more reduced in *rSey²/+* rats than in WT rats at the earliest time point we observed (4 weeks). Therefore, it is possible that Pax6 is necessary for the production of GFAP$^+$ early progenitor cells during the initial formation of the hippcampus. This is quite reasonable because we observed that extremely less GFAP$^+$ cells (including not only those mature astrocytes but also neural stem/progenitor cells) were produced in the DG of *rSey²/+* rats (Fig. 3C,D). Taken all together, it is concluded that Pax6 is necessary for keeping a good balance between cell proliferation and differentiation in the hippocampal neurogenesis (Fig. 7).

Pax6–Wnt pathway in hippocampal neurogenesis

Because Pax6 is a transcription factor, its influence on the production of GFAP$^+$ early progenitor cells is naturally brought by transcriptional regulation of other genes. Although several secreted molecules such as EGF, FGF2, BDNF, and Shh have been known to regulate adult neurogenesis (Craig *et al.* 1996; Kuhn *et al.* 1997; Tropepe *et al.* 1997; Zigova *et al.* 1998; Machold *et al.* 2003), we could not find any difference in expression of these molecules in the DG of *rSey²/+* rats (not shown). However, we have found dramatically different expression patterns of genes involved in Wnt signaling pathway.

Wnt genes encode secreted proteins that regulate fate decisions of various cells depending on the context. The functions of Wnt signaling are studied intensively in many aspects of embryogenesis such as anterior–posterior axis formation, cell type specification, cell proliferation, and axonal growth (Patapoutian & Reichardt 2000; Wang & Wynshaw-Boris 2004; Zou 2004). Although Wnt signaling in the postnatal brain has been comparatively little investigated, a recent report describes remarkably patterned gene expressions of Wnt signaling components in the postnatal mouse brain including the hippocampus (Shimogori *et al.* 2004). In addition, Wnt signaling has already been reported to be altered in embryonic brains of *Pax6* mutant mice and rats (Grindley *et al.* 1997; Osumi *et al.* 1997; Warren & Price 1997; Kim *et al.* 2001; Takahashi *et al.* 2002). We found specific expression of *Wnt7a* and *Wnt7b* in the wild-type SGZ, and marked reduction of *Wnt7a* and *Wnt7b* expressions in the DG of the *rSey²/+*. Conversely, *Dv1* was up-regulated in the DG of the *rSey²/+* (Fig. 6). Therefore, Wnt signaling is altered in the *Pax6*-deficient DG.

During cortical development, Wnt signaling has multiple and stage-specific roles. In early embryonic stages, Wnt7a, 7b, and stabilized β-catenin promote self-renewal of neural precursor cells and suppress neural differentiation (Chenn & Walsh 2002; Viti *et al.* 2003). On the other hand, it is reported that the Wnt/β-catenin pathway directs neuronal differentiation of the cortical precursor cells at later developmental stages (Hirabayashi *et al.* 2004). Curiously, in adult hippocampus, lithium facilitates proliferation and differentiation of progenitor cells to a specific neural cell type by perturbing functions of GSK3β, a pivotal player not only in the PI3 kinase pathway but also in Wnt/β-catenin pathway (Chen *et al.* 2000; Kim *et al.* 2004). In the present study, we found that specific expressions of Wnt ligands in the SGZ and that Wnt signaling is altered in the DG of *Pax6*-deficient rat (Fig. 6) suggest an intriguing possibility that impaired Wnt signaling may perturb the production of GFAP$^+$ early progenitor cells in postnatal hippocampus. Expression patterns of *Wnt7a* and *7b* may also support the idea that cells expressing Wnt ligands constitute an environment as a stem cell niche to maintain neural progenitor cells. It would be important to elucidate how Pax6-Wnt signaling coordinately regulates proliferation of neural stem cells/GFAP$^+$ early progenitor cells in the SGZ.

Experimental procedures

Animals

Large colonies of heterozygous *Pax6* mutant rats (*rSey²*/+) and wild-type Sprague–Dawley (SD) rats (littermates of *rSey²*/+ rats) were maintained at Tohoku University School of Medicine and National Institute of Neuroscience. The genotype of *rSey²*/+ rats was distinguishable based on the presence of eye defects. All animal experiments were carried out in accordance with the National Institute of Health guidelines for the care and use of laboratory animals and were approved by the Committee for Animal Experiments in the aforementioned organizations.

Tissue preparation

Rats were deeply anesthetized with diethyl ether or pentobarbital sodium before sacrifice. Brains were perfused transcardially with 4% paraformaldehyde (PFA) in 0.01 M PBS (sodium phosphate buffer, pH 7.4) or 4% PFA and 0.5% picric acid in 0.01 M PBS, or 2% PFA and 2.5% glutaraldehyde in 0.01 M PBS, for immunohistochemistry, immuno-electron microscopic analysis, and conventional electron microscopic analysis, respectively. The brains were incubated in the same fixative for 2 h at 4 °C and cut into 70 μm coronal sections with a vibratome (Leica) or cut by a cryostat (Leica) into 14 μm sagittal sections.

Immunohistochemistry

Procedures were basically according to the previous reports (Osumi *et al.* 1997; Fukuda *et al.* 2003). Detailed information will be provided on request. Antibodies against Musashi, nestin, and PSA-NCAM are kind gifts from Drs Hideyuki Okano, Masaharu Ogawa, and Tatsunori Seki (Miyata & Ogawa 1994; Seki & Arai 1999; Kaneko *et al.* 2000). Fluorescent signals were detected using a confocal laser-scanning microscope (Leica) or a fluorescent microscope (Axioplan-2, Zeiss).

BrdU labeling analyses

Four-week-old rats received single intraperitoneal injections of 5-bromo-2-deoxyuridine (BrdU) (Sigma, St. Louis, MO) at 50 μg/kg body weight (10 mg/mL stock, dissolved in 0.9% saline), and were sacrificed at 30 min, 24 h, and 72 h after the injection (Seri *et al.* 2001; Kempermann *et al.* 2004). For cell fate analyses, 4-, 12- or 20-week-old rats received similar injections of BrdU three times a day for 3 days, and were sacrificed at day 1 or 4 weeks later (Kempermann & Gage 1999). For quiescent stem cell analysis, 4-week-old rats received injections of BrdU twice a day for 14 days, and were sacrificed 1 day later (Magavi *et al.* 2000). Seventy micrometers free-floating sections were cut and incubated in 2 N HCl for 1 h at room temperature, and washed in 0.01 M PBS (Saegusa *et al.* 2004). Otherwise, 14 μm frozen sections were boiled in 0.01 M citric acid and incubated in 2 N HCl for 10 min at 37 °C, and washed in 0.01 M PBS.

Quantification

For BrdU pulse/chase examination, percentages of $Pax6^+$, $GFAP^+$, or PSA-$NCAM^+$ in total $BrdU^+$ cells were calculated in three sections per hemisphere. For quantification analysis, sampling of BrdU-positive cells was performed throughout the DG in its rostrocaudal extension. Every sixth section (14 μm) was used for counting, and the total number was obtained by multiplying the value by 6 (Kempermann & Gage 1999). For the fate analysis, $BrdU^+$/$NeuN^+$ in total $BrdU^+$ cells and $BrdU^+$/$GFAP^+$ in total $BrdU^+$ cells were counted in three adjacent sections in the same rostrocaudal regions of a DG (Kempermann & Gage 1999). For the quantification of the number of $GFAP^+$/$Pax6^+$ double-positive cells and $Pax6^+$/$BrdU^+$ double-positive cells, we counted these cells within the limited range in six adjacent sections and calculated the density. The number of these cells was counted in the blind manner.

Electron microscopy

Procedures were basically according to the previous reports (Yuasa *et al.* 1996; Saegusa *et al.* 2004). Detailed information will be provided on request. These ultrathin sections were stained with lead citrate and uranyl acetate, and observed under a Hitachi H-7000 electron microscope.

In situ hybridization

Procedures were basically according to the previous reports (Osumi *et al.* 1997; Takahashi *et al.* 2002). *Wnt7a*- and *Wnt7b*-expressing cells were counted on three adjacent sections in the same rostrocaudal region of a DG.

Statistical analysis

Statistical analyses were performed with Microsoft Excel (Office 98), and ANOVA or two-sided *t*-test was applied when appropriate.

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References

Altman, J. & Das, G.D. (1965) Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. *J. Comp. Neurol.* **124**, 319–335.

Alvarez-Buylla, A., Seri, B. & Doetsch, F. (2002) Identification of neural stem cells in the adult vertebrate brain. *Brain Res. Bull.* **57**, 751–758.

Chen, G., Rajkowska, G., Du, F., Seraji-Bozorgzad, N. & Manji, H.K. (2000) Enhancement of hippocampal neurogenesis by lithium. *J. Neurochem.* **75**, 1729–1734.

Chenn, A. & Walsh, C.A. (2002) Regulation of cerebral cortical size by control of cell cycle exit in neural precursors. *Science* **297**, 365–369.

Craig, C.G., Tropepe, V., Morshead, C.M., *et al.* (1996) *In vivo* growth factor expansion of endogenous subependymal neural precursor cell population in the adult mouse brain. *J. Neurosci.* **16**, 2649–2658.

Doetsch, F., Caille, I., Lim, D.A., Garcia-Verdugo, J.M. & Alvarez-Buylla, A. (1999) Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell* **97**, 703–716.

Doetsch, F., Garcia-Verdugo, J.M. & Alvarez-Buylla, A. (1997) Cellular composition and three-dimensional organization of the subventricular germinal zone in the adult mammalian brain. *J. Neurosci.* **17**, 5046–5061.

Estivill-Torrus, G., Pearson, H., van Heyningen, V., Price, D.J. & Rashbass, P. (2002) Pax6 is required to regulate the cell cycle and the rate of progression from symmetrical to asymmetrical division in mammalian cortical progenitors. *Development* **129**, 455–466.

Filippov, V., Kronenberg, G., Pivneva, T., *et al.* (2003) Subpopulation of nestin-expressing progenitor cells in the adult murine hippocampus shows electrophysiological and morphological characteristics of astrocytes. *Mol. Cell. Neurosci.* **23**, 373–382.

Forster, E., Tielsch, A., Saum, B., *et al.* (2002) Reelin, Disabled 1, and beta 1 integrins are required for the formation of the radial glial scaffold in the hippocampus. *Proc. Natl. Acad. Sci. USA* **99**, 13178–13183.

Fujita, S. (2003) The discovery of the matrix cell, the identification of the multipotent neural stem cell and the development of the central nervous system. *Cell Struct. Funct.* **28**, 205–228.

Fukuda, S., Kato, F., Tozuka, Y., *et al.* (2003) Two distinct subpopulations of nestin-positive cells in adult mouse dentate gyrus. *J. Neurosci.* **23**, 9357–9366.

Fukuda, T., Kawano, H., Osumi, N., Eto, K. & Kawamura, K. (2000) Histogenesis of the cerebral cortex in rat fetuses with a mutation in the Pax-6 gene. *Brain Res. Dev. Brain Res.* **120**, 65–75.

Gage, F.H. (2000) Mammalian neural stem cells. *Science* **287**, 1433–1438.

Gotz, M. (2003) Glial cells generate neurons—master control within CNS regions: developmental perspectives on neural stem cells. *Neuroscientist* **9**, 379–397.

Gotz, M., Stoykova, A. & Gruss, P. (1998) Pax6 controls radial glia differentiation in the cerebral cortex. *Neuron* **21**, 1031–1044.

Grindley, J.C., Hargett, L.K., Hill, R.E., Ross, A. & Hogan, B.L. (1997) Disruption of PAX6 function in mice homozygous for the Pax6Sey-1Neu mutation produces abnormalities in the early development and regionalization of the diencephalon. *Mech. Dev.* **64**, 111–126.

Hack, M.A., Saghatelyan, A., de Chevigny, A., *et al.* (2005) Neuronal fate determinants of adult olfactory bulb neurogenesis. *Nature Neurosci.*

Hack, M.A., Sugimori, M., Lundberg, C., Nakafuku, M. & Gotz, M. (2004) Regionalization and fate specification in neurospheres: the role of Olig2 and Pax6. *Mol. Cell. Neurosci.* **25**, 664–678.

Heins, N., Malatesta, P., Cecconi, F., *et al.* (2002) Glial cells generate neurons: the role of the transcription factor Pax6. *Nature Neurosci.* **5**, 308–315.

Hirabayashi, Y., Itoh, Y., Tabata, H., *et al.* (2004) The Wnt/beta-catenin pathway directs neuronal differentiation of cortical neural precursor cells. *Development* **131**, 2791–2801.

Kaneko, Y., Sakakibara, S., Imai, T., *et al.* (2000) Musashi1: an evolutionally conserved marker for CNS progenitor cells including neural stem cells. *Dev. Neurosci.* **22**, 139–153.

Kempermann, G. & Gage, F.H. (1999) Experience-dependent regulation of adult hippocampal neurogenesis: effects of long-term stimulation and stimulus withdrawal. *Hippocampus* **9**, 321–332.

Kempermann, G., Gast, D., Kronenberg, G., Yamaguchi, M. & Gage, F.H. (2003) Early determination and long-term persistence of adult-generated new neurons in the hippocampus of mice. *Development* **130**, 391–399.

Kempermann, G., Jessberger, S., Steiner, B. & Kronenberg, G. (2004) Milestones of neuronal development in the adult hippocampus. *Trends Neurosci.* **27**, 447–452.

Kim, A.S., Anderson, S.A., Rubenstein, J.L., Lowenstein, D.H. & Pleasure, S.J. (2001) Pax-6 regulates expression of SFRP-2 and Wnt-7b in the developing CNS. *J. Neurosci.* **21**, RC132.

Kim, J.S., Chang, M.Y., Yu, I.T., *et al.* (2004) Lithium selectively increases neuronal differentiation of hippocampal neural progenitor cells both in vitro and in vivo. *J. Neurochem.* **89**, 324–336.

Kuhn, H.G., Dickinson-Anson, H. & Gage, F.H. (1996) Neurognesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation. *J. Neurosci.* **16**, 2027–2033.

Kuhn, H.G., Winkler, J., Kempermann, G., Thal, L.J. & Gage, F.H. (1997) Epidermal growth factor and fibroblast growth factor-2 have different effects on neural progenitors in the adult rat brain. *J. Neurosci.* **17**, 5820–5829.

Machold, R., Hayashi, S., Rutlin, M., *et al.* (2003) Sonic hedgehog is required for progenitor cell maintenance in telencephalic stem cell niches. *Neuron* **39**, 937–950.

Magavi, S.S., Leavitt, B.R. & Macklis, J.D. (2000) Induction of neurogenesis in the neocortex of adult mice. *Nature* **405**, 951–955.

Miyata, T. & Ogawa, M. (1994) Developmental potentials of early telencephalic neuroepithelial cells: a study with microexplant culture. *Dev. Growth Differ.* **36**, 319–331.

Nakatomi, H., Kuriu, T., Okabe, S., *et al.* (2002) Regeneration of hippocampal pyramidal neurons after ischemic brain injury by recruitment of endogenous neural progenitors. *Cell* **110**, 429–441.

Osumi, N. (2001) The role of Pax6 in brain patterning. *Tohoku J. Exp. Med.* **193**, 163–174.

Osumi, N., Hirota, A., Ohuchi, H., *et al.* (1997) Pax-6 is involved in the specification of hindbrain motor neuron subtype. *Development* **124**, 2961–2972.

Patapoutian, A. & Reichardt, L.F. (2000) Roles of Wnt proteins in neural development and maintenance. *Curr. Opin. Neurociol.* **10**, 392–399.

Saegusa, T., Mine, S., Iwasa, H., *et al.* (2004) Involvement of highly polysialylated neural cell adhesion molecule (PSA-NCAM)-positive granule cells in the amygdaloid-kindling-induced sprouting of a hippocampal mossy fiber trajectory. *Neurosci. Res.* **48**, 185–194.

Seki, T. & Arai, Y. (1993) Highly polysialylated neural cell adhesion molecule (NCAM-H) is expressed by newly generated granule cells in the dentate gyrus of the adult rat. *J. Neurosci.* **13**, 2351–2358.

Seki, T. & Arai, Y. (1999) Different polysialic acid-neural cell adhesion molecule expression patterns in distinct types of mossy fiber boutons in the adult hippocampus. *J. Comp. Neurol.* **19**, 115–125.

Seri, B., Garcia-Verdugo, J.M., McEwen, B.S. & Alvarez-Buylla, A. (2001) Astrocytes give rise to new neurons in the adult mammalian hippocampus. *J. Neurosci.* **21**, 7153–7160.

Shimogori, T., VanSant, J., Paik, E. & Grove, E.A. (2004) Members of the Wnt, Fz, and Frp gene families expressed in postnatal mouse cerebral cortex. *J. Comp. Neurol.* **473**, 496–510.

Simpson, T.I. & Price, D.J. (2002) Pax6; a pleiotropic player in development. *Bioessays* **24**, 1041–1051.

Stoykova, A., Gotz, M., Gruss, P. & Price, J. (1997) Pax6-dependent regulation of adhesive patterning, R-cadherin expression and boundary formation in developing forebrain. *Development* **124**, 3765–3777.

Stoykova, A. & Gruss, P. (1994) Roles of Pax-genes in developing and adult brain as suggested by expression patterns. *J. Neurosci.* **14**, 1395–1412.

Takahashi, M., Sato, K., Nomura, T. & Osumi, N. (2002) Manipulating gene expressions by electroporation in the developing brain of mammalian embryos. *Differentiation* **70**, 155–162.

Tramontin, A.D., Garcia-Verdugo, J.M., Lim, D.A. & Alvarez-Buylla, A. (2003) Postnatal development of radial glia and the ventricular zone (VZ): a continuum of the neural stem cell compartment. *Cereb. Cortex.* **13**, 580–587.

Tropepe, V., Craig, C.G., Morshead, C.M. & van der Kooy, D. (1997) Transforming growth factor-alpha null and senescent mice show decreased neural progenitor cell proliferation in the forebrain subependyma. *J. Neurosci.* **17**, 7850–7859.

Viti, J., Gulacsi, A. & Lillien, L. (2003) Wnt regulation of progenitor maturation in the cortex depends on Shh or fibroblast growth factor 2. *J. Neurosci.* **23**, 5919–5927.

Wang, J. & Wynshaw-Boris, A. (2004) The canonical Wnt pathway in early mammalian embryogenesis and stem cell maintenance/differentiation. *Curr. Opin. Genet. Dev.* **14**, 533–539.

Warren, N., Caric, D., Pratt, T., *et al.* (1999) The transcription factor, Pax6, is required for cell proliferation and differentiation in the developing cerebral cortex. *Cereb. Cortex.* **9**, 627–635.

Warren, N. & Price, D.J. (1997) Roles of Pax-6 in murine diencephalic development. *Development* **124**, 1573–1582.

Yuasa, S., Kawamura, K., Kuwano, R. & Ono, K. (1996) Neuron-glia interrelations during migration of Purkinje cells in the mouse embryonic cerebellum. *Int. J. Dev. Neurosci.* **14**, 429–438.

Zigova, T., Pencea, V., Wiegand, S.J. & Luskin, M.B. (1998) Intraventricular administration of BDNF increase the number of newly generated neurons in the adult olfactory bulb. *Mol. Cell. Neurosci.* **11**, 234–245.

Zou, Y. (2004) Wnt signaling in axon guidance. *Trends Neurosci.* **27**, 528–532.

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ストレス事象の予測に関する脳機能画像解析

Neuroimagaing studies of anticipating stressful events

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◎われわれは，しばしば予測という心理的な構えを準備することで，ストレスに対する心理的負荷を軽減していると考えられる．そこで著者らはストレス事象の予測に着目して，①ストレス事象の予測がいかなる脳部位において行われるか，②予測することによりストレス事象の認知処理過程が影響を受けるか，③ストレス関連障害の代表としてうつ病をとらえ，ストレス事象の予測に関する脳機能が健常人と異なるか，について脳機能画像解析法を用いて検討を行った．その結果，ストレス事象を予測することにより，前頭前野を含む脳内ネットワークを介して感覚野におけるストレスフルな入力を減弱させることが推測された．また，うつ病を対象とした研究から，うつ病患者ではこの脳内ネットワークが適切に活動していない可能性が推定された．

Key word：ストレス，予測，うつ病，ファンクショナルMRI(fMRI)，脳磁図(MEG)

ヒトや動物が環境との相互作用のなかで，過剰な環境の要求や苦痛な刺激にさらされたときに引き起こされるストレスの反応過程は，生理的反応とともに心理的過程を伴っている．とくにヒトのストレスの反応過程を考えるうえでは心理的な要因を抜きにしては考えにくい．ヒトのストレス反応は心理社会的ストレスからもたらされることが非常に多いが，心理社会的ストレスはそれ自体が直接的にストレス反応を引き起こすのではなく，それがストレスとなるには個人の認知的な処理過程が必要である．この認知処理過程に関して著者らは，前頭前野-辺縁系を中心とした複数の領域が活性化することを明らかにしてきた[1,2]．

一方，われわれはしばしば予測という心理的な構えを準備することで，ストレスに対する心理的負荷を軽減していると考えられる．たとえば，結果が思わしくない場合に，発表の前に結果を予測し，これから受けるストレスを軽減するといったことを行うことがある．あるいは山道を歩いているときに，この山には熊がいるという情報を受けていた場合と，そうでない場合は実際に熊が出現した場合の対応が異なる可能性がある．

そこで著者らは予測することがストレス事象への適応を強化していると考え，ストレス事象の予測に関する検討を機能的磁気共鳴画像法(functional magnetic resonance imaging：fMRI)と脳磁場計測法(magnetoencepharography：MEG)といった脳機能画像解析手法を用いて行った．まず，快あるいは不快といったストレス事象の予測がいかなる脳部位において行われているかをfMRIを用いて明らかにした．さらに，予測することによりストレス事象の感覚入力が影響を受けるかについてMEGを用いて検討した．また，うつ病をストレス事象の認知情報処理過程の障害として仮定し，ストレス事象の予測に関する脳機能に関して健常人との比較検討を行った．本稿ではこれらの研究成果を中心に紹介する．

ストレス事象の予測に関連した脳賦活課題

課題は2つ1組の刺激(警告刺激S1と標的刺

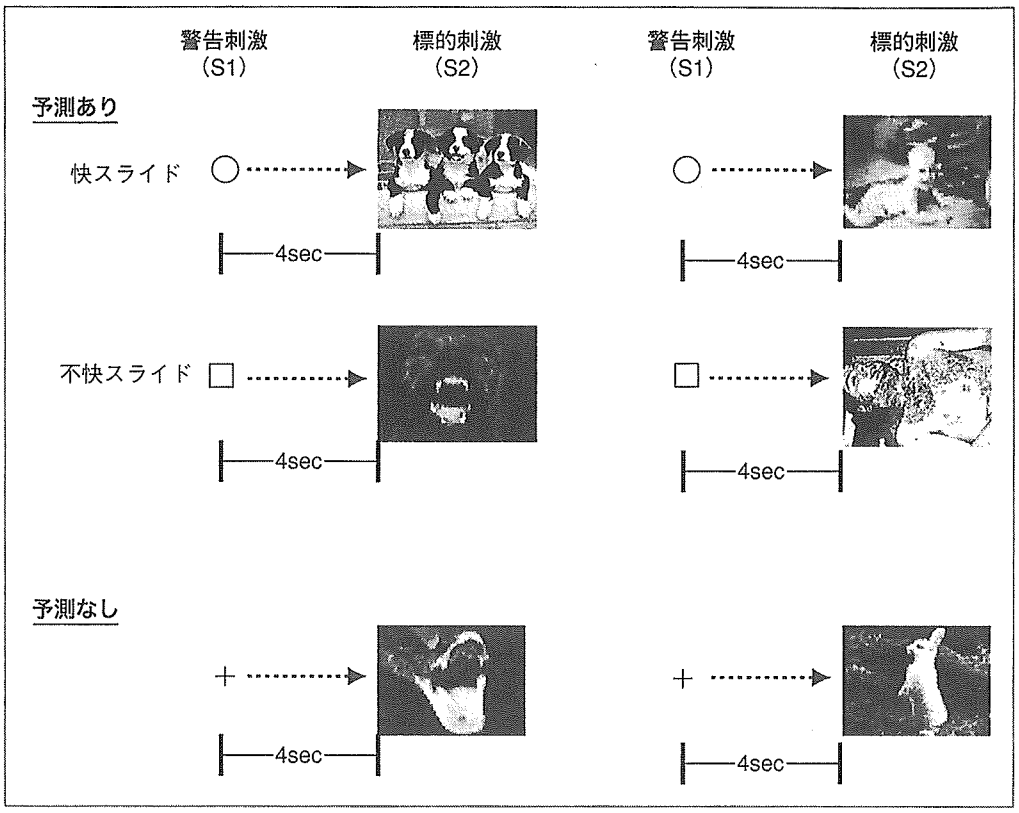

図 1　情動的ストレス事象の予測課題

激 S2)を一定の刺激間間隔(4 sec)でモニターに呈示し，S2 後にボタン押し反応をさせた．S1 刺激として○，□，＋の幾何学図形を呈示した(100 msec)．S2 刺激として，International Affective Picture System(IAPS)[3]より異なる情動価(快/不快が各 30 枚)をもつスライドを抽出し呈示した(2 sec)．被験者には，○-快，□-不快のように S1-S2 の組合せを固定した条件(予測可能条件)とつねに＋を S1 刺激としていかなる S2 が提示されるか予測できない条件(予測不可能条件)を用いた条件を作成した(図 1)．

以下の研究はこの新規に作成した課題を用いて行った．また，すべての対象者からは広島大学医学部倫理委員会の承認を受けたプロトコールに従い，書面による研究の目的と内容を説明したうえで，文書による同意を得た．

ストレス事象の予測に関連した脳機能局在

まず著者らは，快あるいは不快といったストレス事象がいかなる脳部位において行われているかを fMRI を用いて検討した．健常人 15 例を対象に，1.5T の MRI 装置(島津 Marconi 社製)を用い，ストレス事象予測課題遂行時の脳活動を測定した．解析は SPM99 を用い，予測可能条件と予測不可能条件のときの脳活動領域を比較検討した．

予測可能条件では，予測不可能条件と比較して前頭前野の領域(内側前頭前野，腹外側前頭前野，背外側前頭前野)で有意な活動上昇を認めた．とくに，快刺激を予測しているときには，左背外側前頭前野，左内側前頭前野，右小脳の活動が認められたのに対し，不快刺激を予測しているときには右腹外側前頭前野，右内側前頭前野，右扁桃体，左帯状回前部，および両側の視覚野(左右後頭葉，右楔部，左舌状回)の活動がみられた(図 2)[4]．

この結果から，将来の情動ストレス事象の予測における前頭前野の役割が示唆された．また，左前頭前野の活動は快刺激の予測と関連し，一方で，右前頭前野の活動は不快刺激の予測と関連していることが示唆された．また，不快刺激の予測に際してのみ，扁桃体，前帯状回，視覚野の活動がみられたことから，視覚野におけるネガティブな情報の入力を調節するといった相互の関連性が想定

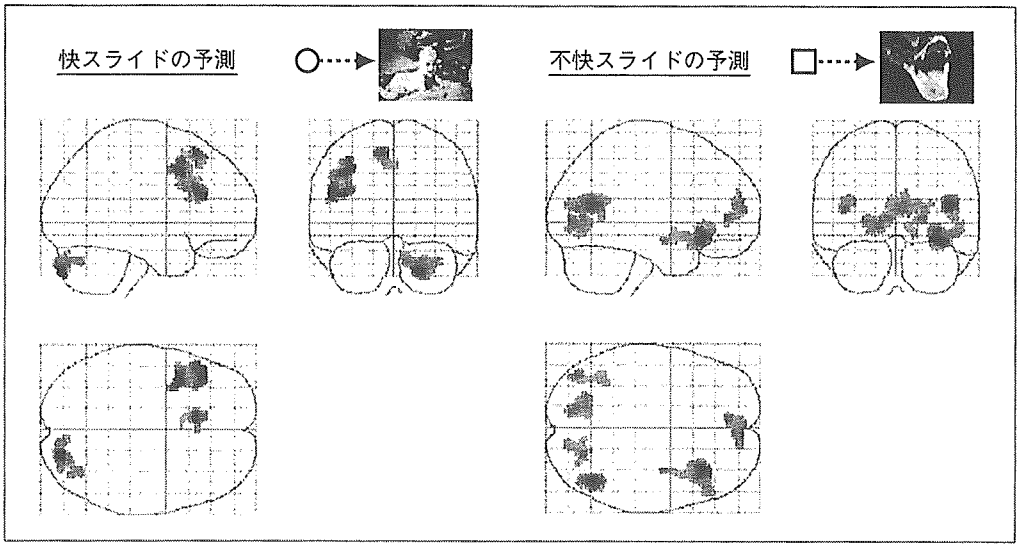

図 2　ストレス事象の予測に関連した脳機能局在(fMRI study)

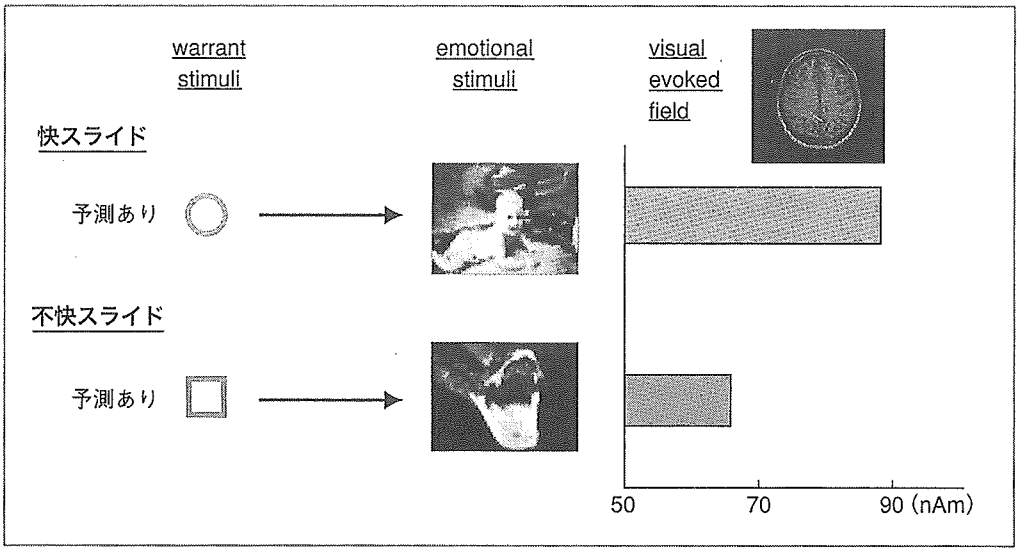

図 3　ストレス事象の予測に関連した視覚誘発反応(MEG study)

された.

予測の視覚誘発反応に与える影響

予測することによりストレス事象の感覚入力が影響を受けるかについて MEG を用いて検討した. 健常人 6 例を対象に, 全頭型 204 チャンネル脳磁図システム(Neuromag 社製)を用い, 課題遂行時の脳磁図を記録した. 被験者は, ○-快, □-不快のように S1-S2 の組合せを固定した条件で, 脳磁場データは情動価ごとに S2 呈示後 1,000 msec を加算平均し, 情動スライドに対する visual evoked field(VEF)の脳内信号源の推定を行った.

快および不快な情動スライド提示後に, 後頭葉視覚野のほぼ同じ位置に VEF を認めたが, VEF の強度は快刺激の予測と比較して不快刺激を予測したものでは小さかった(図 3).

この結果から, 不快刺激が予測できる場合にはその感覚入力が抑制される可能性が推定された. 著者らはこれまでに情動ストレスにより sensory gating system の調節が起こることを明らかにしている[5]が, 今回の結果と合わせ考えると, 感覚の入力がさまざまなストレスに関連した脳内認知処理過程により影響を受ける可能性を示唆している.

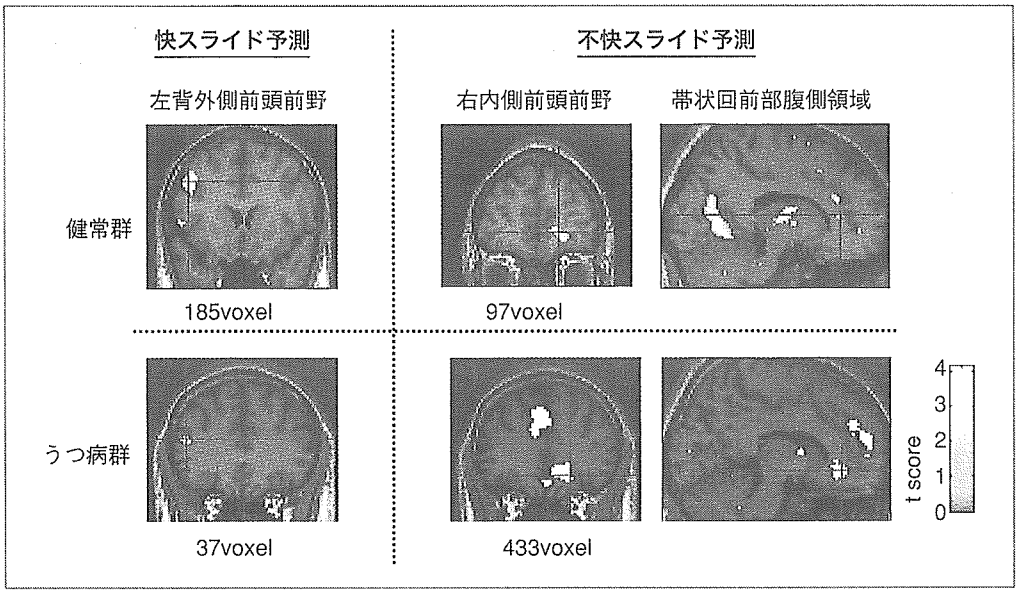

図 4　うつ病のストレス事象の予期に関連した脳活動

うつ病の将来のストレス事象の予測

うつ病患者の否定的認知の3徴として自分自身，自分の経験，自分の将来に関する認知がよく知られている．このうち，将来に対する否定的な見方について，うつ病患者は独特の見通しをもっている．すなわち，将来にわたって快い事象は起こらず，不快な事象は永続的に続くと考えている．そこで，ストレス事象の予測に関する脳機能に関してうつ病患者と健常人との比較検討をfMRIを用いて行った．対象は，DSM-IVの大うつ病性障害の診断基準を満たす広島大学病院精神科・神経科入院服薬中のうつ病患者6例，および年齢・性別・利き手をマッチングさせた健常人6例である．

健常人では快刺激を予測しているときでは左背外側前頭前野，左内側前頭前野，右小脳の活動が認められた．一方で，不快刺激を予測しているときでは右腹外側前頭前野，右内側前頭前野，右扁桃体，左帯状回前部，および両側の視覚野の活動がみられた．これに対して，うつ病患者では快刺激の予測に関連した左前頭前野の活動低下が示され，一方で，不快刺激の予測に関連した右腹外側前頭前野の活動低下，右内側前頭前野，帯状回前部腹側領域の活動亢進が示された(図4)．

すなわち，うつ病では快事象の予測は小さく，不快事象の予測では大きく脳が活動する可能性が推定され，うつ病に特徴的な否定的認知と強く関連しているものと考えられた．

おわりに

以上の健常人を対象とした研究結果から，ストレス事象を予測することにより前頭前野を含む脳内ネットワークを介して感覚野におけるストレスフルな入力を減弱させることが推測された．また，うつ病を対象とした研究から，うつ病患者ではこの脳内ネットワークが適切に活動していない可能性が推定された．今後，これらの研究結果を踏まえてストレス適応の強化や適応破綻の防止の方策だけでなく，うつ病をはじめとするストレス関連疾患の病態や臨床症状[6]を，予測といった心理的機制を切り口として脳科学的に検討することができるかもしれない．

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文献

1) Shirao, N. et al.：Gender differences in brain activity toward unpleasant linguistic stimuli concerning interpersonal relationships：an fMRI study. *Eur. Arch. Psychiatry Clin. Neurosci.*(in press)

2) Shirao, N. et al. : Gender differences in brain activity toward unpleasant word stimuli concerning body image : an fMRI study. *Br. J. Psychiatry.* (in press)
3) Lang, P. J. et al. : International Affective Picture System (IAPS) : Technical manual and affective ratings. Gaineville, University of Florida, 1999.
4) Ueda, K. et al. : Brain activity during expectancy of emotional stimuli : An fMRI study. *Neuroreport*, **14** : 51-55, 2003.
5) Yamashita, H. et al. : Visual emotional stimuli modulate auditory sensory gating : Studied by magnetic P50 suppression. *Eur. Arch. Psychiatry Clin. Neurosci.* (in press)
6) 岡本泰昌・他：情動・行動の脳内機構に関するfMRI研究—うつ病の病態解明に向けて．心身医学．(印刷中)

うつ病と前頭前野

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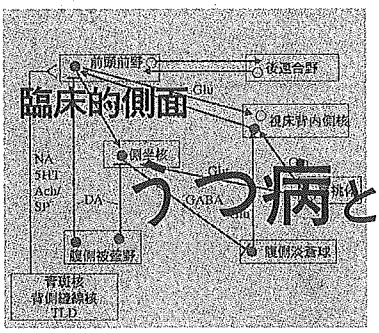

うつ病と前頭前野

岡本泰昌　山脇成人

はじめに

うつ病は，気分の変化だけでなく，認知・行動面の障害を伴い，日常生活の様々な面に影響が及ぶ．例えば，家事が困難となったり，仕事上の作業能力が低下し就業困難になったりする場合もある．日常生活の中で遭遇する様々な出来事を処理する際に，高次の認知機能が大きく役割を果たしていると考えられ，その神経基盤は前頭前野にあると想定される．

うつ病の前頭前野の機能を明らかにするために，いくつかの方法論を用いた研究が行われてきた．これまで前頭前野機能を反映することが明らかな神経心理学的検査や，安静時の脳血流・代謝を測定する脳機能画像検査が主として用いられてきた．近年両者をあわせた神経心理学的課題施行時のうつ病の脳機能測定が行われるようになってきており，うつ病の前頭前野の生理的役割の解明の一助となることが期待されている[1]．

本稿では，前頭前野の内，これまでうつ患者で比較的まとまった知見が得られている背外側前頭前野 dorsolateral prefrontal cortex (DLPFC) と，帯状回前部 anterior cingulated cortex (ACC) を中心に取り上げ，神経心理学的検査と安静時脳機能画像検査により得られた知見を順に紹介したい．さらに最近，われわれのところで行っている神経心理学的課題を施行している時のうつ病の脳機能測定の結果を提示したい．

神経心理学的研究

これまでの健常者を対象とした基礎的検討から言語流暢性課題，Wisconsin Card Sort Test (WCST)，Tower of London 課題は左の DLPFC の機能を，Stroop 課題は ACC の機能を反映していると考えられている[2]．

言語流暢性課題の成績低下が，治療中の重度なうつ病や未治療な中等度うつ病において示されている[3~7]．これに対していくつかの報告は成績低下は認めないとしている[8,9]．

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Trichard ら[10]は，課題の成績とうつ病の重症度は相関しないこと，抑うつ症状が寛解した後に課題成績は回復することを明らかにしている．複数のうつ病の WCST における成績低下が報告されている[3,6,9,11]．しかし Austin ら[8]は，この成績低下は部分的なもので，しかもメランコリー型でのみ認められたとしている．

Moritz ら[6]や Ravnkilde ら[7]は，Stroop 課題を用いてうつ病患者で干渉効果が大きいことを報告している．Schatzberg ら[12]は，非精神病性と比べて精神病性のうつ病では，特にこの干渉効果が大きいことを報告している．さらに，寛解したうつ病患者においても有意な Stroop 干渉効果は残存し，素因的な異常を指摘する報告もある[10]．これに対して，うつ病で特異的な Stroop 干渉効果は認めないとする報告も存在する[8,11]．

対象としたうつ病のサブタイプ，重症度，治療の有無，測定時期といった様々な要因もあり，一部相反する結果もあるが，うつ病において左 DLPFC や ACC の機能が低下を示唆する報告が多く占めている．

安静時脳機能画像研究

安静時に行われた脳機能画像研究は，多くの研究がうつ病患者の DLPFC，特に左半球において血流や代謝の低下を示唆している[13,14]．この DLPFC の活性の低下は，疾患の重症度と相関し[15]，精神運動遅滞[16]や認知障害[17]と関連することも指摘されている．さらに，ACC に関しても血流や代謝の低下を示唆する所見が得られているが，これは皮質の体積減少に起因するとの指摘[18]もあり，結果の解釈に注意を要する．

うつ病の安静時の脳血流・代謝に関しては，左の前頭前野や帯状回前部での低下が比較的一致した見解として得られている．

神経心理学的課題施行時の脳機能画像研究

最近，われわれのところで行っている左背外側前頭前野や帯状回前部などを賦活することが知られている言語流暢

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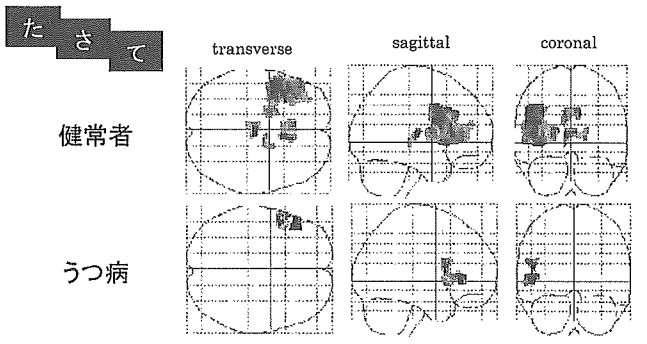

図 1　言語流暢性課題遂行中の脳活動の比較
左前頭前野(背外側)，帯状回でみられる脳活動が乏しい．「夕食の献立は」→「…」(思考抑制)

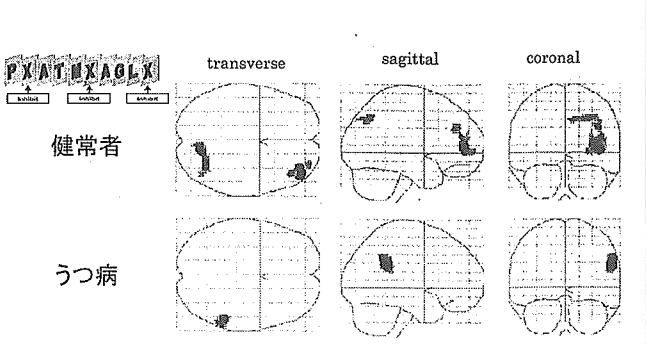

図 2　GO/NOGO 課題遂行中の脳活動の比較
右前頭前野(右上および中前頭回)の脳活動は乏しい．
「はずみで」，「魔が差した」(衝動的→自殺)

性課題や，右背外側前頭前野などを賦活することが知られている GO/NOGO 課題などの神経心理学的課題施行時のうつ病の脳機能測定の結果を提示する．

▣ 言語流暢性課題を用いたうつ病者の脳機能評価

対象は DSM-IV の大うつ病性障害の診断基準をみたす広島大学病院精神科・神経科入院服薬中のうつ病患者 10 例，および年齢・性別・利き手をマッチングさせた健常ボランティア 10 例である．うつ病者の Hamilton うつ病評価尺度 17 項目(HRSD)の平均総得点は 19 点(14～27 点)であった．なお，対象者からは，広島大学医学部倫理委員会の承認をうけたプロトコールに従い，書面による研究の目的と内容を説明した上で，文書による同意を得た．

言語流暢性課題は，被験者に対し 3 秒毎にひらがなの頭文字(例えば 'た')を視覚的に提示し，そのたびにその頭文字で始まる単語を声には出さず頭の中で思い浮かべるよう教示した．対照課題では，被験者に対し 3 秒毎に 'やすみ' と提示し，そのたびに 'やすみ' と頭の中で繰り返すよう教示した．

課題は，各 condition を 30 秒ごとに交互に 3 回ずつ繰り返すブロックデザインとし，この間の脳活動を 1.5 Tesla の MRI 装置(GE 社製)を用いて撮像し，SPM 99 を用いて解析を行った(random effect model)．

検査直後に実施したパフォーマンスデータは，単語の算出数は健常者群で 25.8+8.3 個，うつ病患者群で 16.4+4.8 個であった．脳賦活領域については，健常者群，うつ病患者群ともに対照課題遂行中と比較して言語流暢性課題遂行中に左前頭前野および島で有意に活動が上昇した．健常者群では，これらの領域に加えて帯状回前部や視床において有意な活動がみられたが，患者群ではみられなかった(図 1)．また，両群の直接比較においては，患者群で，健常者群と比較して左前頭前野の一部で信号上昇の程度が有意に低かった．また，回帰分析の結果からは，単語の算出数，HRSD の総得点，抗うつ薬の投与量との間に有意な相関のある領域は見られなかった[19]．

これらの結果から，うつ病患者では言語流暢性課題遂行中に賦活される左前頭前野の機能低下が示唆された．言語流暢性課題は，自らの記憶からの言葉の取り出しをみている．これは，うつ病者の症状のなかで精神抑制(制止)に関連しているものと考えられる．例えば女性うつ病患者の場合，夕食の献立が浮かばないといった訴えを聞くことがある．これらの訴えは記憶にある料理のレパートリーから，本日の献立を状況に合わせて取り出してくる作業であり，この作業は言語流暢性課題とかなり類似している．

▣ GO/NOGO 課題を用いたうつ病者の脳機能評価

対象は DSM-IV で大うつ病の診断基準を満たすうつ病患者群 5 例(男性 3 例，女性 2 例，平均年齢 47.6±12.6 歳，平均 HRSD 22.0±6.4 点)と，年齢・性別をマッチングさせた健常ボランティア 5 例(平均年齢 47.4±11.1 歳)．課題は X または Y 以外のアルファベットのみがランダムに提示される GO condition，X または Y とそれ以外のアルファベットとが 1：1 の比率でランダムに提示される NOGO condition で構成され，各 condition を 30 秒ごとに交互に 3 回ずつ繰り返すブロックデザインとした．被験者には，ランダムに提示されるアルファベットに対して，X または Y 以外のアルファベットが出た時にはできるだけ早く右手の人差し指でボタンを押し(GO)，X または Y が出た時には反応しない(NOGO)ように教示した．なお本研究は広島大学医学部倫理委員会の承認をうけ，被験者には書面によって研究の目的と内容を説明して，文書による同意を得たうえで行った．課題は，各 condition を 30 秒ごと

に交互に3回ずつ繰り返すブロックデザインとし，この間の脳活動を1.5 Teslaの MRI 装置(GE社製)を用いて撮像し，SPM 99を用いて解析をおこなった(fixed effect model)．

予備的な結果ではあるが，健常者群では response inhibition に関連した脳活動が，右中および上前頭回，右下頭頂葉および後頭葉を含む2つの領域でみられた[20]．一方，うつ病患者群では右上側頭回および下頭頂葉を含む1つの領域でみられたが，右前頭前野の有意な活性化はみられなかった(図2)．

この結果は，うつ病患者において右前頭葉の反応性が低下している可能性を示唆しており，そのため行動の抑制機構が不良となることが想定される．そのため，自殺などの衝動行為がおこりやすくなるものと考えられた．

むすび

多くの研究者が，前頭前野がうつ病の病態に大きな役割を果たしていると考えている．しかしながらうつ病の症状と脳の生理的機能の関連についてはほとんど明らかにされていないのが現状である．今回提示したような神経心理学的課題遂行時の脳機能測定は，この問題を解決するための有効な手段となるであろう．今後は，これまで得られた結果をふまえ，よりうつ病の臨床症状に特異的な神経心理課題を作成していくことが重要と考えられる[21]．さらに，そのいくつかを組み合わせていくことで，うつ病の病態に関連した前頭前野の役割を明らかにできることが期待される．

文献

1) Rogers MA, Kasai K, Matsuo K, et al. Executive and prefrontal dysfunction in unipolar depression : a review of neuropsychological and imaging evidence. Neuroscience Research. 2004 ; 50 : 1-11.
2) 鹿島晴雄，加藤元一郎．前頭葉機能検査—障害の形式と評価法．神経進歩．1993 ; 37 : 93-109.
3) Franke P, Maier W, Hardt J, et al. Assessment of frontal lobe functioning in schizophrenia and unipolar major depression. Psychopathology. 1993 ; 26 : 76-84.
4) Beats BC, Sahakian BJ, Levy R. Cognitive performance in tests sensitive to frontal lobe dysfunction in the elderly depressed. Psychol Med. 1996 ; 26 : 591-603.
5) Landro NI, Stiles TC, Sletvold H. Neuropsychological function in non-psychotic unipolar major depression. Neuropsychiatry Neuropsychol Behav Neurol. 2001 ; 14 : 233-40.
6) Moritz S, Birkner C, Kloss M, et al. Executive functioning in obsessive-compulsive disorder, unipolar depression and schizophrenia. Arch Clin Neuropsychol. 2002 ; 17 : 477-83.
7) Ravnkilde B, Videbech P, Clemmensen K, et al. Cognitive deficits in major depression. Scand J Psychol. 2002 ; 43 : 239-51.
8) Austin MP, Mitchell P, Wilhelm K, et al. Melancholic depression : a distinct pattern of frontal impairment in melancholia. Psychol Med. 1999 ; 24 : 73-85.
9) Grant MM, Thase ME, Sweeney JA. Cognitive disturbance in outpatient depressed younger adults : evidence of modest impairment. Biol Psychiatry. 2001 ; 50 : 35-43.
10) Trichard C, Martinot JL, Alagille M, et al. Time course of prefrontal lobe dysfunction in severely depressed in-patients : a longitudinal neuropsychological study. Psychol Med. 1995 ; 2 : 79-85.
11) Degl'Innocenti A, Agren H, Backman L. Executive deficits in major depression. Acta Psychiatr Scand. 1998 ; 97 : 182-8.
12) Schatzberg AF, Posener JA, De Battista C, et al. Neuropsychological deficits in psychotic versus non-psychotic major depression and no mental illness. Am J Psychiatry. 2000 ; 157 : 1095-100.
13) Bench CJ, Friston KJ, Brown RG, et al. Regional cerebral blood flow in depression : the relationship with clinical dimensions. Psychol Med. 1993 ; 23 : 579-90.
14) Rubin E, Sackeim HA, Prohovnik I, et al. Regional cerebral blood flow in mood disorders : IV. Comparison of mania and depression. Psychiatry Res Neuroimaging. 1995 ; 61 : 1-10.
15) Drevets WC. Functional neuroimaging studies of depression : the anatomy of melancholia. Annu Rev Med. 1998 ; 49 : 341-61.
16) Videbech P, Ravnkilde B, Pedersen TH, et al. The Danish PET/depression project : clinical symptoms and cerebral blood flow. A regions-of-interest analysis. Acta Psychiatr Scand. 2002 ; 106 : 35-44.
17) Bench CJ, Friston KJ, Brown RG, et al. The anatomy of melancholia : focal abnormalities of cerebral blood flow in major depression. Psychol Med. 1992 ; 22 : 607-15.
18) Drevets WC, Price JL, Simpson JR, et al. Subgenual prefrontal cortex abnormalities in mood disorders. Nature. 1997 ; 386 : 824-7.
19) Okada G, Okamoto Y, Morinobu S, et al. Attenuated left prefrontal activation during a verbal fluency task in patients with depression. Neuropsychobiology. 2003 ; 47 : 21-6.
20) Asahi S, Okamoto Y, Okada G, et al. Negative correlation between right prefrontal activity during response inhibition and impulsiveness : a fMRI study. Eur Arch Psychiatry Clin Neurosci. 2004 ; 254 : 245-51.
21) 岡本泰昌，岡田　剛，上田一貴，他．情動・行動の脳内機構に関するfMRI研究—うつ病の病態解明に向けて．心身医学．印刷中．

されるというような、能力改善ないしは問題行動改善に直結するものを追い求めがちである。保護者のニーズも多くはそこにある。軽度発達障碍の子どもの支援にしばしば見られるように、障碍の特性を理解し、その特性に応じた対応をプログラム化して、子どもが見通しを持って生活できるようにするという昨今の流れは、一見した分かりやすさにもかかわらず、本当に子どもが必要としている支援なのかどうか。そのことを子どもの心の面、主体としての育ちの面に定位して吟味し直す姿勢が、いま支援の問題を考える際に是非とも必要である。そしてそのためには、まずもって、発達の考え方を吟味し直し、さらに発達障碍という概念を考え直す必要があると思われるが、どうだろうか。

【参考文献】
鯨岡峻『両義性の発達心理学』、ミネルヴァ書房、一九九八年
鯨岡峻『〈育てられる者〉から〈育てる者〉へ』、NHKブックス、二〇〇二年
鯨岡峻（編著）『〈共に生きる場〉の発達臨床』、ミネルヴァ書房、二〇〇二年
小林隆児・鯨岡峻（編著）『自閉症の関係発達臨床』、日本評論社、二〇〇五年

特集・発達障害児の支援にむけて

発達障害の支援の向こう側

発達障害支援論序説

田中康雄

たなかやすお
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はじめに

「たなか先生。ボクは学校で友達がたくさんいるよ。感謝と笑顔を忘れないから、友達がたくさんいるよ」

小学時代から付き合っている少年のことばである。中学生になり、背も高くなり、頼もしくなった彼のことばは、笑顔とともに輝いていた。そしてしばらくこのことばが頭の片隅から離れないでいる。のどの奥に引っ掛かった小骨のように気にかかっている。

私は児童精神科医として、多くの子どもとその家族、特に養育者と出会い、さらに多くの関係者とも出会ってきた。最近特に出会う「軽度発達障害」のある方々に在る「生きにくさ」について、共に悩みながら支援策を検討し続けている。その途中で、今少しだけ迷いの森に足を踏み入れているような戸惑いと不安を抱きつつある。ここで私は足を止め、改めて「発達障害」について検討し、「支援」について考えてみたい。

発達障害について

「発達障害」という言葉は一見自明のようでありな