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In order to understand the formation and evolution of the brain, we are studying the genes that are expressed in specific areas of the neocortex.

I. Genes expressed in specific areas and layers of the neocortex

The neocortex emerged in mammals and evolved most remarkably in the primate. It is puzzling, however, that during mammalian evolution the neocortex was markedly expanded while the total number of genes in the mammal was little changed. In order to understand this puzzle, we studied gene expression patterns within different areas of the neocortex. In the last ten years, we reported the following findings, which are schematically illustrated in Figure 1.

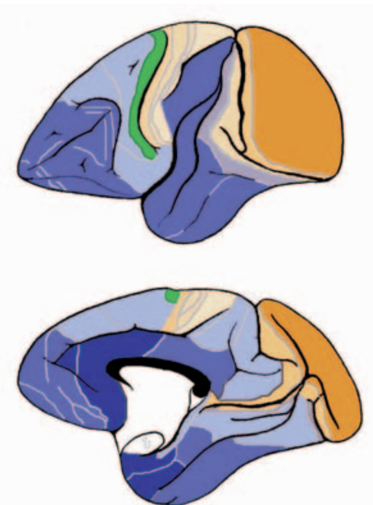

Figure 1. The expression of *occ1* (orange color) m *Rbp* (blue) and *gdf7* (green) are schematicaly illustrated in Brodmann's area figure in the guenon monkey. Top and bottom views are medial and lateral surfaces, respectively. (The figure is cited from Yamamori & Rockland, Neurosci. Res., *55*, 11-27, 2006).

By differential display methods, we found three area-specific expression genes in the primate neocortex.

Firstly, *occ1* is specifically expressed in the occipital cortex in the primate brain. Secondly, the other gene that showed marked difference within the neocortex is *gdf7*, a member of BMP/TGF-β family, which is specifically expressed in the motor cortex of the African green monkey (Watakabe *et al*., J. Neurochem., *76*, 1455-1464, 2001). Thirdly, *Rbp* (retinol-binding protein) is preferentially expressed in association and higher areas in the neocortex (Komatsu *et al*., Cerebral Cortex, *15*, 96-108, 2005).

To screen area-specific molecules systematically in the monkey neocortex, we carried out a new round of screening using the RLCS method (Suzuki et al. 1996; Shintani et al. 2004). In this analysis, mRNAs were purified from 4 distinct cortical areas, converted to cDNA by reverse transcription and digested with a pair of restriction enzymes for 2-dimensional analysis. Among the spots that showed area difference, we cloned a gene that is specifically expressed in the visual area, which turned out to be the 5-HT1B receptor gene. (Watakabe et al., Cerebral Cortex, published online on December 4, 2008).

The expression of 5-HT1B revealed by in situ hybridization was strikingly high in V1 and the lateral geniculate nucleus (LGN) (Figure 2C, E). Because the mRNA expression was low in the extrastriate cortex, the abrupt change in the intensity of mRNA staining was observed at the border between V1 and V2 (Figure 2F).

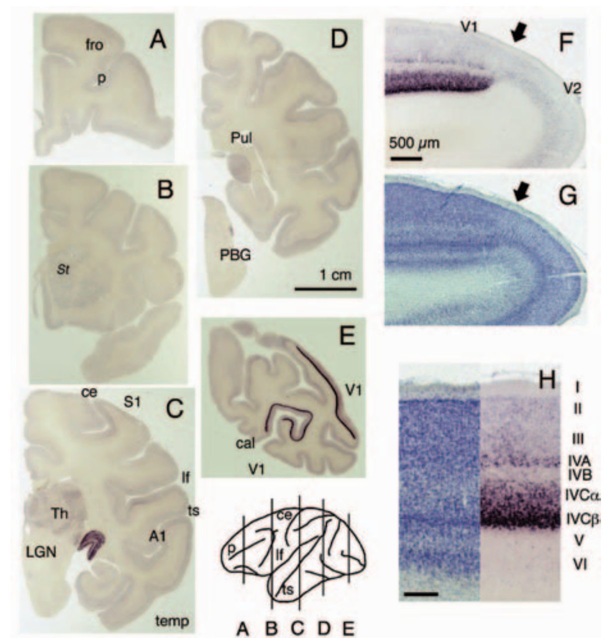

Figure 2. ISH analysis of 5-HT1B receptor mRNA. (A-E) Coronal sections of an adult monkey brain were prepared from the positions as depicted. Bar: 1 cm. Several areas are shown: fro, frontal area; temp, temporal area; S1, primary somatosensory area; A1, primary auditory area. PBG, parabigeminal nucleus; Pul, pulvinar nucleus; St, striatum. Major sulci are represented by lowercase letters: p, principal sulcus; cal, calcarin sulcus; ce, central sulcus; lf, lateral fissure; ts, superior temporal sulcus. (F) 5-HT1B receptor mRNA expression at the V1/V2 border (shown by the black arrow). Bar: 500 μm. (G) The adjacent Nissl-stained section. (H) 5-HT1B receptor mRNA expression in V1. Bar: 200 μm. (Watakabe et al., Cerebral Cortex, 2008)

Within V1, the expression of 5-HT1B receptor mRNA was mostly confined to layers IVA and IVC, the major geniculocortical input layers, and was particularly strong in the lower part of layer IVC beta (Figure 2H). In addition, the expression was also observed at lower intensity in layers II/III and VI of V1, which also receive geniculocortical inputs to a lesser extent. In the LGN, the strong mRNA expression was observed in all 6 layers, and there was no significant difference in staining intensity between the magnocellular and parvocellular layers. The 5-HT1B receptor mRNA expression was by far the most conspicuous in V1, followed by that in the LGN.

Among the 13 genes of serotonin receptors, 5-HT2A receptor mRNA exhibited area and lamina preferences similar to those of 5-HT1B receptor mRNA, although its expression was moderate across all areas. Both mRNA species were highly concentrated in the geniculorecipient layers IVA and IVC, where they were coexpressed in the same neurons. Monocular inactivation by tetrodotoxin injection resulted in a strong and rapid (<3 h) downregulation of these mRNAs, suggesting the retinal activity dependency of their expression.

In collaboration with Professor Hiromichi Sato's laboratory (Osaka University), we examined the roles of the two serotonin receptors. The activation of 5-HT1B receptors in V1 by specific agonist generally facilitates visual responses but tends to suppress weak responses. Our analysis suggests that, in geniculocortical transmission, nonsychronized spontaneous activity (noise) from the LGN neurons would be reduced by the suppressive effect of 5-HT1B receptors, but the visually evoked synchronized signals would be preserved or efficiently transferred to V1, thus, enhancing the S/N ratio in input-output relationship. On the other hand, neurons in the input layers of V1 which abundantly express the 5-HT2A receptor may act as a gain controller by enhancing weak signal response and suppressing excessive response. We therefore suggest that serotonin release in V1 exerts coordinated modulatory effects through 5-HT1B and 5-HT2A receptors on the V1 neurons. It is therefore possible that the serotonin system has contributed to the evolution of the elaborated function of the primate visual system.

The other gene is testican-1, which is specifically expressed in the primate visual cortex. Since testican-1 is a member of a testican family gene which also includes occ1, we were particularly interested in examining all the known members of the testican family (testican-1, testican-2, testican-3, SPARC, SC1) other than occ1 (Takahata et al., Cerebral Cortex, published online on December 10, 2008).

Interestingly, the in situ hybridization revealed that there are three groups of expression pattern among the occ1-related (testican) family members. The expression patterns of testican-1 and testican-2 are similar to that of occ-1, starting high level in V1, progressively decreasing along the ventral visual pathway, and ending low level in the temporal areas. Complementary to them, the neuronal expression of *SPARC* mRNA is abundant in the association areas and scarce in V1. Therefore, the expression pattern of *SPARC* is similar to that of *Rbp*. In addition, whereas *occ1*, *testican-1*, and *testican-2* mRNAs are preferentially distributed in thalamorecipient layers including "blobs," *SPARC* mRNA expression avoids these layers. Neither *SC1* nor *testican-3* mRNA expression is selective to particular areas, but *SC1* mRNA is abundantly observed in blobs.

Lines of evidence suggest that ECM considered to modulate neuronal development and plastic changes. Several groups have proposed that ECM limits plasticity in the rodent neocortex and ECM degeneration is required to implement ocular dominance plasticity (Pizzorusso et al. 2002; Oray et al. 2004). Secreted glycoproteins, such as Reelin, regulate both neuronal positioning in the developing nervous system and synaptic plasticity in the adult (Bock and Herz 2003; Dityatev and Schachner 2006). The area selectivity and activity dependence in expression of occ1-related genes, secreted glycoproteins, raise the possibility that OCC1-related proteins modulate synaptic plasticity in the adult cerebral cortex, some of which mechanisms are specific in primates.

Ⅱ. Gene expression under multisensory enhancement

We have been collaborating with professor Yoshio Sakurai (Kyoto University) and developed an audio-visual discrimination task (AVD-task) system, placing auditory and visual stimuli in the same position. We use nose-poking to measure the reaction time in which a rat responds to stimuli. Using this behavioral system, we were able to confirm amodal recognition of space, which means that a rat can respond to a different modality (visual or auditory) if the stimuli are in the same position, as previously reported in other systems. We also confirmed multisensory enhancement is indeed observed in rats. These results suggest that this new modified AVD system can be used to explore the molecular and cellular mechanisms underlying multisensory processing in rats (Sakata *et al*. Exp. Brain Res., *159*, 409-417, 2004).

We studied multisensory processing further using a c-Fos mapping technique. We first developed a method to standardize the cortex to quantitatively evaluate c-Fos expression by an automatic image analyzing system (Figure 3). Combining this system with our newly developed "Cortical Box Methods" (Figure 3), we found the secondary visual cortex (V2L) in rats is specifically activated under audiovisual multisensory stimulation. Injecting muscimol into V1, V2, V2L and superior colliculus (SC), we found that V2L is specifically involved in the stimulation of multisensory reaction (Hirokawa *et al*., 2008).

Traditionally, multisensory integration was thought to occur in higher neocortical areas through a merging of different modalities of primary sensory information. Our results suggest that the multisensory integration may in fact occur at a relatively "early sensory" area such as V2. Previous elecrophysiological studies also show that there exist multisensory areas in the secondary visual area and in the boundary areas between two modal areas (Toldi *et al*., 1986; Barth *et al*., 1995; Wallace *et al*., 2004). This observation is consistent with our findings using the newly developed behavioral system and c-Fos analyzing system.

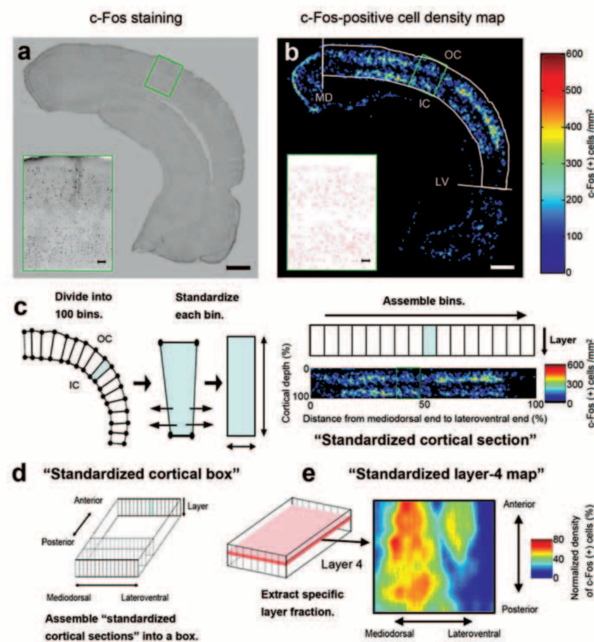

Figure 3 (a) Representative photomicrograph of cortical section stained by antibody against c-Fos protein. (b) The local density of c-Fos-positive cells was computed and pseudocolored. The mediodorsal end (MD), lateroventral end (LV), inner contour (IC) and outer contour (OC) were manually chosen to extract part of the cortex. Scale bars, 1 mm (a, b) and 100 mm (insets in a, b). (c) The extracted cortex was divided into 100 bins (left), and each bin was converted into a standard rectangle (left to center as shown by thick arrows). The standardized rectangular bins were reassembled into a stripe to form a standardized cortical section (right). (d) Standardized cortical sections were assembled from the posterior section to the anterior section to form a standardized cortical box. (e) A specific layer fraction (layer 4, for example) from a standardized cortical box was extracted to construct a standardized layer map (Shown in Hirokawa et al., 2008).

Ⅲ. Neocortical areas revealed by layer specific gene expression in rats.

To investigate area differences in rodent neocortex in terms of gene expressions, we chose three genes, *RORbeta, ER81* and *Nurr1*, whose mRNAs are mainly expressed in layers 4, 5 and 6, respectively. To reveal their complex spatial distribution patterns, we used double in situ hybridization histochemistry (ISH) and cortical box method for the analyses, as mentioned above (Hirokawa et al., 2008).

Double ISH revealed the large area differences in the relative abundance and extent of intermixing of the expressions of these mRNAs. Based on this finding, we quantitatively analyzed the ISH patterns of the three genes by cortical box method as described above. We made three major discoveries. Firstly, the three genes showed unique area distribution patterns that were mostly complementary to one another. Secondly, the cortical areas defined on the basis of the expression patterns of the three genes matched well with the cytoarchitectonic areas defined by Nissl staining. Finally, principal component analysis results suggested that the expressions of these three genes may be dictated by common rules that are tightly associated with the function and topology of the cortical areas.

The expression of the three layer specific genes showed tight relationships with the functional areas. This not only provides insight into the cortical area architecture underlining the complex spatial expression patterns of these genes, but also may serve as a framework for investigating gene expression regulation within the neocortex for future researchers.

Publication List

〔Original papers〕

- Hirokawa, J., Watakabe, A., Ohsawa, S., and Yamamori, T. (2008). Analysis of Area-Specific Expression Patterns of RORbeta, ER81 and Nurr1 mRNAs in Rat Neocortex by Double In Situ Hybridization and Cortical Box Method. PLoS ONE *3*, e3266.
- Lyckman, A.W., Horng, S., Leamey, C.A., Tropea, D., Watakabe, A., Van Wart, A., McCurry, C., Yamamori, T., and Sur, M. (2008). Gene expression patterns in visual cortex during the critical period: Synaptic stabilization and reversal by visual deprivation. Proc. Natl. Acad. Sci. USA *105*, 9409-9414.
- Hirokawa, J., Bosch, M., Sakata, S., Sakurai, Y., and Yamamori, T. (2008). Functional role of the secondary visual cortex in multisensory facilitation in rats. Neuroscience *153*, 1402-1417.
- Takahata, T., Hashikawa, T., Higo, N., Tochitani, S., and Yamamori, T. (2008). Difference in sensory dependence of occ1/Follistatin-related protein expression between macaques and mice. J. Chem. Neuroanat. *35*, 146-157.