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Activation of the Anterior Cingulate Gyrus by 'Green Odor': A Positron Emission Tomography Study in the Monkey

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Abstract

The equivalent mixture of *cis*-3-hexenol and *trans*-2-hexenal (hexenol/hexenal), 'green odor', is known to have a healing effect on the psychological damage caused by stress. Behavioral studies in humans and monkeys have revealed that hexenol/hexenal prevents the prolongation of reaction time caused by fatigue. In the present study, we investigated which brain regions are activated by the odor of hexenol/hexenal using positron emission tomography with alert monkeys. Regional cerebral blood flow (rCBF) in the prepyriform area (the primary olfactory cortex) was commonly increased by the passive application of odor: acetic acid, isoamylacetate or hexenol/hexenal. We observed rCBF increases in the orbitofrontal cortex (the secondary olfactory cortex) by these olfactory stimuli in two of three monkeys, and found no predominance of laterality of the activated hemisphere. Furthermore, rCBF increase in the cerebellum was observed in two of three monkeys, and the odor of acetic acid increased rCBF in the substantia innominata in all monkeys. In addition to these olfactory related regions, the anterior cingulate gyrus was activated by the odor of hexenol/hexenal. These findings suggest that the increase of rCBF in the anterior cingulate gyrus by the odor of hexenol/hexenal may contribute the healing effects of this mixture observed in the monkey.

Key words: laterality, olfaction, PET, primate

Introduction

Hexenol and hexenal, which mixed together produce the so-called 'green odor', are plant origin compounds from the chloroplast membrane (Hatanaka, 1996). They have various effects on the biological function: plant-to-plant messengers in allelopathy, insect-attracting pheromone-like action and bactericidal-like action (Hatanaka, 1999). Recently, green odor has been found to have various physiological actions on the mammalian brain. Stress-induced hyperthermia was attenuated by the green odor in rats (Akutsu *et al.*, 2002). Sano *et al.* (2002) reported that the mixture of hexenol and hexenal was accepted as a pleasant odor, and it decreased the amplitude of an event-related potential (P300). However, the neural mechanisms of the effect of hexenol and hexenal are almost completely unknown.

Positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) are non-invasive functional brain-imaging techniques, which have been used to elucidate the neural mechanisms of olfaction in the human (reviewed by Zald and Pardo, 2000; Savic, 2002). By using PET, Zatorre *et al.* (1992) first reported that olfactory stimuli increase regional cerebral blood flow (rCBF) in the bilateral pyriform cortex and right orbitofrontal cortex in

the human. The finding of activation in the pyriform and orbitofrontal cortices has been replicated in other PET (Zald and Pardo, 1997; Qureshy *et al.*, 2000; Savic *et al.*, 2000, 2002) and fMRI studies (Yousem *et al.*, 1997; Sobel *et al.*, 1998a; Francis *et al.*, 1999; Poellinger *et al.*, 2001). The results of these imaging studies are consistent with the clinical findings that patients with temporal lobe epilepsy and patients that underwent temporal or frontal lobectomy have difficulty in discriminating and detecting odors (Abraham and Mathai, 1983; Eskenazi *et al.*, 1986; Jones-Gotman and Zatorre, 1988; Zatorre and Jones-Gotman, 1991). Imaging studies have also demonstrated that other regions considered to be irrelevant to olfactory processing, such as the cerebellum, claustrum, anterior cingulate gyrus and visual cortex, are activated by odors using PET (Small *et al.*, 1997; Zald and Pardo, 1997; Qureshy *et al.*, 2000; Savic *et al.*, 2000, 2002; Royet *et al.*, 2001) and fMRI (Yousem *et al.*, 1997; Sobel *et al.*, 1998b; Francis *et al.*, 1999; Poellinger *et al.*, 2001).

To investigate precise mechanisms of olfactory function, we need to bridge the gap between human PET studies described above and electrophysiological studies in the monkey (Tanabe *et al.*, 1975a,b; Rolls and Baylis, 1994). Recently, we have developed a system for imaging the monkey brain using PET (Onoe *et al.*, 1994, 2001; Takechi *et al.*, 1994; Kondoh *et al.*, 2002), and elucidated the mechanisms of olfactory processing in the alert monkey (Kobayashi *et al.*, 2002). Although there are not a few advantages of fMRI over PET, we chose the latter because of: (i) easier presentation of odors and ventilation to the alert monkey; (ii) clear visualization of the skull base areas especially the prepyriform area and orbitofrontal cortex; and (iii) no dose limitation of radioisotope. In the present study we used the PET systems in alert monkeys to address the question: which brain regions are activated by passive odor application of hexenol and hexenal? We compared the activated brain regions by hexenol and hexenal with those activated by acetic acid or isoamylacetate. We found that rCBF in the prepyriform area is commonly increased by these odors, and rCBF in the anterior cingulate gyrus was increased by hexenol and hexenal.

Materials and methods

Animals

Three male adult rhesus monkeys (*Macaca mulatta*), weighing 4.8, 6.5 and 9.0 kg, were used for this experiment. The monkeys were maintained and handled in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved by the animal research committee of Hamamatsu Photonics and BF Research Institute. The surgical protocol for implantation of a head holder is the same as reported previously (Kobayashi *et al.*, 2002). Briefly, after initial anesthesia with ketamine–HCl (10 mg/kg i.m.; Sankyo, Japan) and atropine (0.05 mg/kg; Tanabe Seiyaku, Japan), pentobarbital (20 mg/kg; Dainippon Pharmaceutical, Japan) was administered to maintain the animal at a deep surgical level of anesthesia. During surgery pentobarbital was injected intravenously as needed to maintain a deep surgical level of anesthesia. A custom-made stainless steel head holder was implanted to which the positioner could be fitted during PET imaging. Antibiotics were administered for a week after surgery.

Olfactory stimulation

All monkeys were trained to sit on a monkey chair that was equipped with the instrument for olfactory stimulation. During the PET scan, the unanesthetized monkey sat on the chair, and its head was fixed in the center of the PET scanner gantry. A bundle of tubes for each odor stimulus was set just upon the nostrils. Tubes were connected to closed glass bottles which contained the following solutions: propylene glycol solvent, 5% acetic acid, 98% isoamylacetate, and an equivalent mixture of 0.03% *cis*-3-hexenol and 0.03% *trans*-2-hexenal (hexenol/hexenal; Soda Aromatic, Japan) diluted with triethyl citrate. Acetic acid and isoamylacetate have a vinegar-like and a banana-like smell, respectively. As control, we applied pure air (80% nitrogen and 20% oxygen) at 540 ml/min through propylene glycol solvent to remove odor. We applied an odor by aerating the solution by pure air at 540 ml/min. The intensity of the odor was chosen to be easily identifiable by humans. To prevent the odor from contaminating the scanner room, an outlet was set behind the monkey and connected to the outlet of the room fan. Breathing was recorded with the aid of a thermo-probe in front of the nostril.

PET scan

The rCBF was measured by the bolus injection of [^{15}O]water with PET (Raichle *et al.*, 1983). PET Scanners (EXACT HR, Siemens, Germany, for monkey A; or SHR7700, Hamamatsu Photonics KK, Japan, for monkeys B and C) were used. A transaxial resolution of EXACT HR in high sensitivity three-dimensional mode and SHR7700 in enhanced two-dimensional mode was 3.8 mm full width at half maximum and 2.6 mm, respectively. The effects of radiation self-attenuation were corrected by an initial transmission scan for 30 min using an external positron emitting isotope (^{68}Ge). The [^{15}O]water (5 mCi in 2.5 ml saline in EXACT HR and 60 mCi in SHR7700) was injected through a venous cannula placed into a sural vein at 0.2 ml/s, and the emission scan started 30 s after the injection, when the radioactivity reached the brain. An odor stimulus was delivered for 30 s just after the scan started. Three scans with 30 s time frames for 90 s were used for analysis. We performed 20 randomized trials in a day, and the interscan was set at 10–15 min. The number of PET scans for each odor stimulus is 10 in monkey A, 14 in monkey B and 16 in monkey C. The

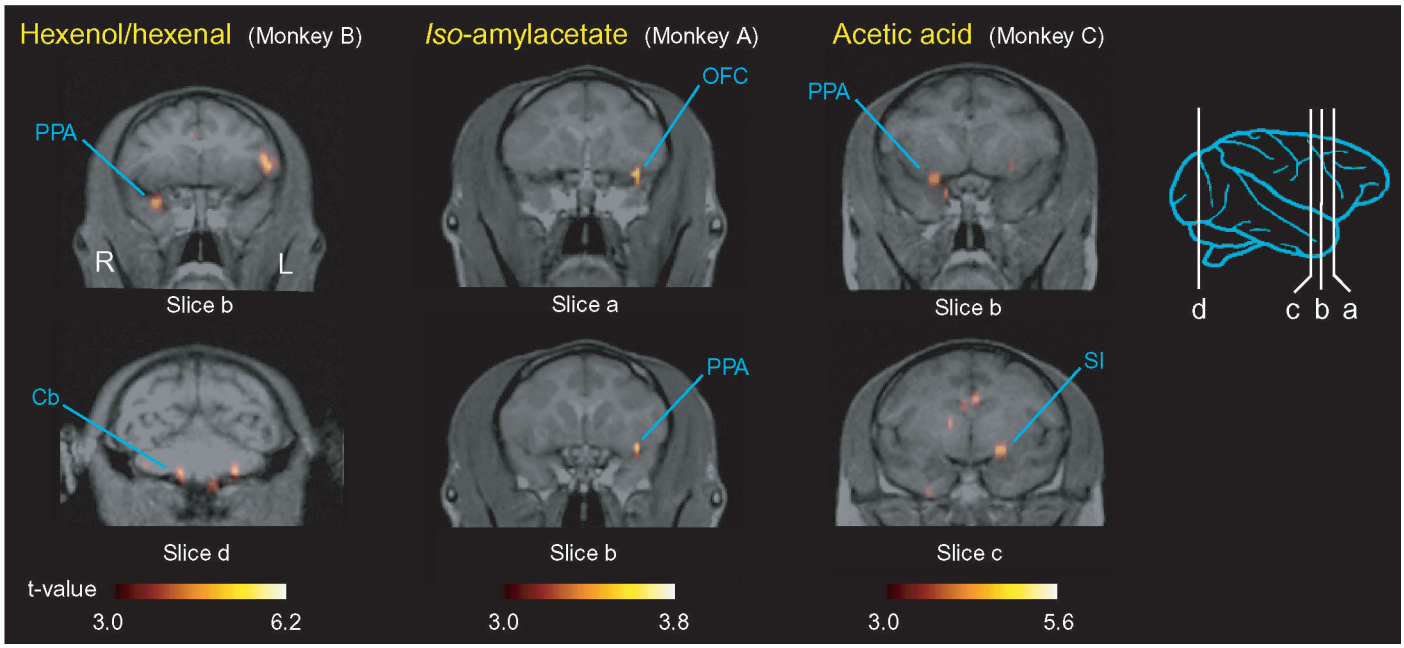

Figure 1 Activated areas in response to odor stimuli superimposed upon MRI slices. Hexenol/hexenal, isoamylacetate, and acetic acid commonly increased rCBF in the prepyriform area (the primary olfactory cortex) in Monkey A, B and C. In addition, rCBF in the substantia innominata, orbitofrontal cortex (the secondary olfactory cortex) and cerebellum were occasionally increased. The right scheme is a lateral view of monkey brain indicating coronal slice locations. The color scales indicate the range of *t*-values. Cb, cerebellum; L, left; OFC, orbitofrontal cortex; PPA, prepyriform area; R, right; SI, substantia innominata.

number of control scans was 30 in monkeys A and B, and 32 in monkey C. Scans were obtained within a 5-week period.

Data analysis

The data were analyzed with statistical parametric mapping (SPM99, MRC Cyclotron Unit, UK) implemented in Matlab (MathWorks, USA). Adjusted rCBF values were analyzed using statistical parametric mapping (SPM96, MRC Cyclotron Unit, UK) implemented in Medx (Sensor Systems, USA). The scans were filtered by a Gaussian filter set at 4 mm full width at half-maximum. Global activity was set as a confounding covariate. The mean global cerebral blood flow was normalized to a level of 50 ml/100 ml/min. We subtracted images of pure air from those of each odor to detect the activated region by the odor. In addition, we made the images of hexenol/hexenal subtracted by those of isoamylacetate or acetic acid. The threshold for significance was set at $t = 3.00$, corresponding to an uncorrected probability of $P < 0.0025$. To identify the anatomical localization of the activated area, T_1-weighted, high-resolution (256 × 256 matrix, 1.2 mm thickness; 60 slices) magnetic resonance image (MRI) scans were obtained using a 3 Tesla scanner (Signa VH/I, GE Medical Systems, USA) or 0.5 Tesla scanner (MRT-50A/II, Toshiba, Japan) under deep pentobarbital anesthesia before surgery. Structural MRIs from each subject were co-registered to the PET data following realignment of the PET time series using a software program (3D Brain Station, Loats Associates, USA). To identify the activated regions, we referred to stereotaxic coordinates of a monkey brain atlas (Paxions *et al.*, 2000).

To estimate rCBF increase quantitatively, we measured the relative change of rCBF in the prepyriform area and anterior cingulate gyrus. We set the region of interest (ROI; 1.2 × 1.2 × 3.6 mm) in the activated regions showing the highest *t*-value during hexenol/hexenal application, and the same ROIs were set in the other sessions. Adjusted rCBF values during control and odor application were compared by one-way ANOVA with *post-hoc* Scheffé's test for multiple comparisons. To compare rCBF values between the left and right orbitofrontal cortex, we subtracted images of pure air from those of all odors and measured adjusted rCBF values in the ROIs set in the orbitofrontal region showing the highest *t*-value. Statistical comparisons were performed by one-way ANOVA with *post-hoc* Scheffé's test for multiple comparisons. All statistical values are presented as mean ± standard deviation. The level of $P < 0.05$ was considered statistically significant in the ROI-based analysis.

Results

Detection of activated regions by odor application

As shown in Figure 1 and Table 1, the subtraction images of pure air from each odor, hexenol/hexenal, isoamylacetate and acetic acid, revealed a similar increase of rCBF in the prepyriform area in all monkeys as reported previously (Kobayashi *et al.*, 2002). In two out of three monkeys, rCBF in the cerebellum was significantly increased by hexenol/

Table 1 Common activated regions by olfactory stimulation

Area	Odor																	
	Hexenol/hexenal						Isoamylacetate						Acetic acid					
	Monkey A		Monkey B		Monkey C		Monkey A		Monkey B		Monkey C		Monkey A		Monkey B		Monkey C	
	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L
Prepyriform area	3.66	–	6.17	–	3.82	–	–	3.31	3.14	–	3.26	–	3.61	–	5.09	–	3.38	4.56
Substantia innominata	3.31	–	–	–	–	–	–	–	–	–	–	–	3.13	–	3.29	–	–	4.18
Orbitofrontal cortex	–	–	–	–	3.12	3.31	–	3.21	–	–	–	3.49	–	–	–	–	3.46	3.79
Cerebellum	–	–	3.44	3.22	–	3.15	–	–	3.22	–	–	3.41	3.73	–	5.06	3.38	3.32	–
Anterior cingulate gyrus	–	3.71	3.86	–	–	4.09	–	–	3.54		–	–	–	–	–	–	–	–

The maximum *t*-values in the areas are presented. R, right; L, left.

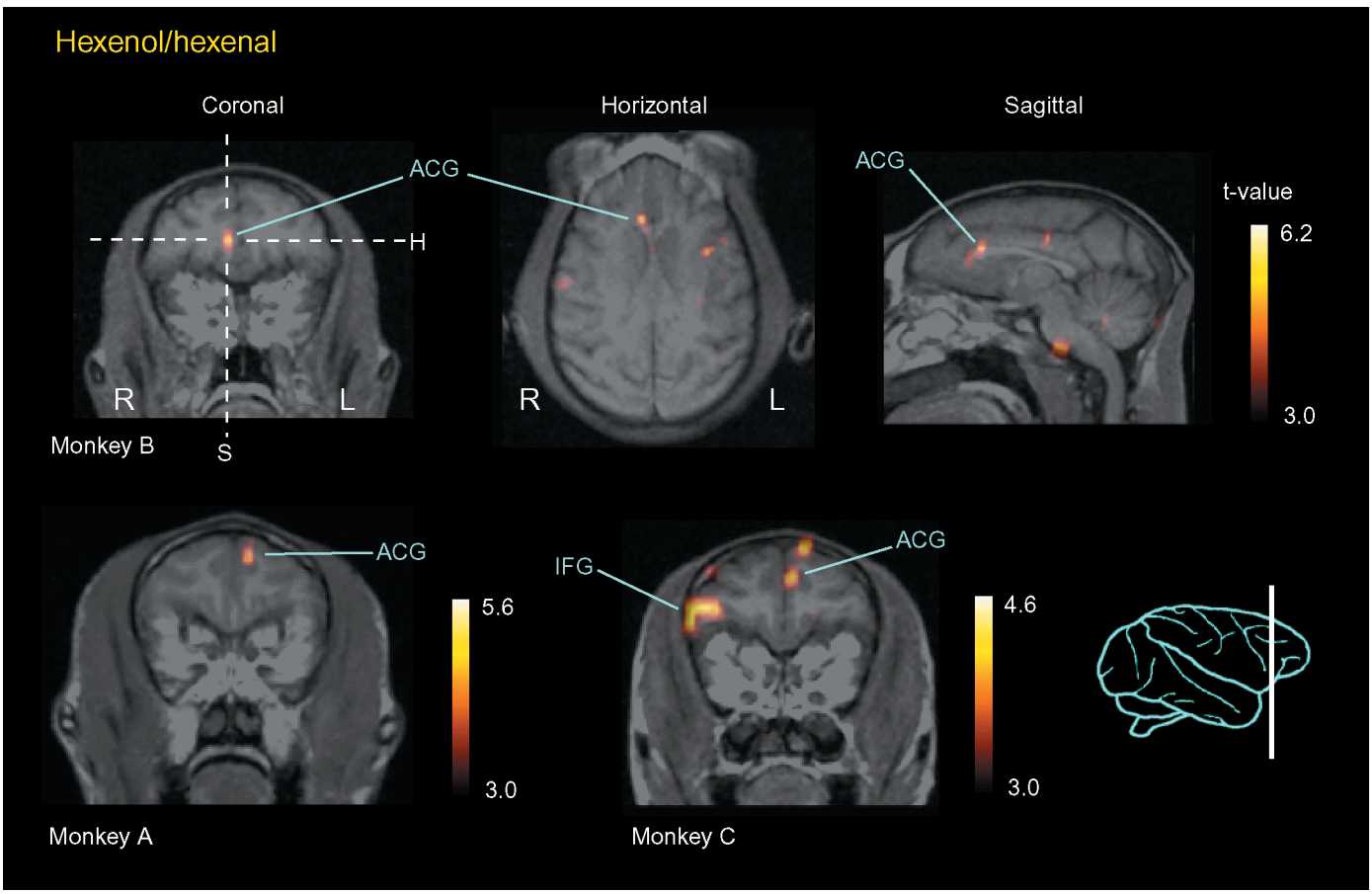

Figure 2 Areas of activation responsible for hexenol/hexenal stimuli superimposed upon MRI slices. Hexenol/hexenal commonly increased rCBF in the anterior part of cingulate gyrus. Lateral view of schematic monkey brain indicates a coronal slice location. The horizontal (H) and vertical (S) broken lines in the left top coronal slice represent the position of the horizontal and sagittal slice, respectively. The color scales indicate the range of *t*-values. ACG, anterior cingulate gyrus; IFG, inferior frontal gyrus; L, left; R, right.

hexenal, isoamylacetate and acetic acid. In one of the monkeys, rCBF in the orbitofrontal cortex was consistently increased by all odor stimuli. Application of hexenol/hexenal increased rCBF in the anterior cingulate gyrus (anterior portion) in all monkeys (Figure 2). Increase of rCBF in the anterior cingulate gyrus was also observed during isoamylacetate application in one of the three monkeys, though the activation site is posterior to those by hexenol/hexenal application. Acetic acid application did not increase rCBF in the anterior cingulate gyrus. Application

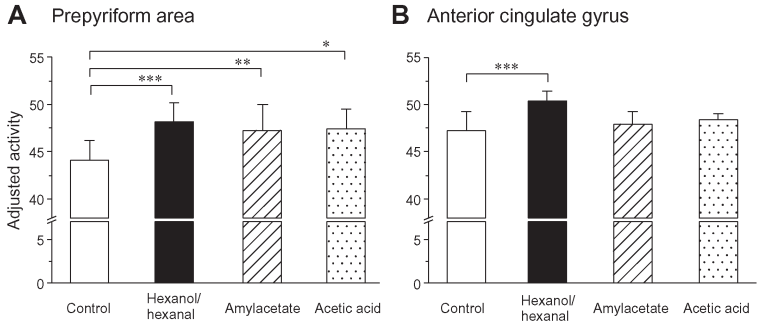

Figure 3 Adjusted rCBF values in the right prepyriform area (A) and right anterior cingulate gyrus (B) of monkey C during application of pure air (Control), hexenol/hexenal, isoamylacetate (Amylacetate), and acetic acid. Although all odors significantly increase the rCBF in the prepyriform area, hexanol/hexenal only increases the rCBF in the anterior cingulate gyrus. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

of acetic acid increased rCBF in the substantia innominata of all three monkeys (Figure 1, Table 1). There was no common activated region by isoamylacetate outside of the prepyriform area.

Subtraction of hexenol/hexenal minus isoamylacetate or acetic acid only demonstrated subthreshold rCBF increase ($t < 3.00$) in the anterior cingulate gyrus.

Quantitative analysis of rCBF increase in the prepyriform area and anterior cingulate gyrus

Figure 3 shows an example of the relative value of rCBF change (see Materials and methods) in monkey C. All odors significantly increased rCBF in the right prepyriform area, and there was no significant difference in rCBF increase among the odors (Figure 3A). Monkeys A and B also showed significant increase of rCBF in the left or right prepyriform area without a difference in rCBF increase among the odors (data not shown). On the other hand, only hexenol/hexenal caused significant increase of rCBF in the right anterior cingulate gyrus ($P < 0.001$; Figure 3B). rCBF values in the anterior cingulate gyrus of monkeys A and B were also increased by hexenol/hexenal application compared to those of control ($P < 0.001$ and $P < 0.015$, respectively).

Laterality of activation in the orbitofrontal cortex by odor stimuli

There is a hypothesis that the right orbitofrontal cortex predominantly processes the olfactory information (Zatorre *et al.*, 1992; Savic *et al.*, 2000, 2002). To test the hypothesis we measured adjusted rCBF values of the left and right orbitofrontal cortex. Figure 4 shows adjusted rCBF values in monkey C. Although rCBF values during odor application were significantly higher than those of control in both hemispheres ($P < 0.001$), rCBF value of odors in the left orbitofrontal cortex was almost similar to that in the right. Taken together with the finding that only the left orbitofrontal cortex was activated by isoamylacetate in monkey A (Table 1), we conclude that there is no obvious laterality of

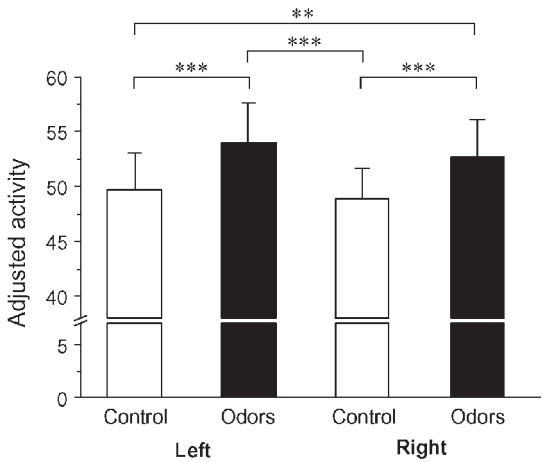

Figure 4 Adjusted rCBF values in the orbitofrontal cortex of monkey C during application of pure air (Control) and three odors (hexenol/hexenal, isoamylacetate, and acetic acid; Odors). In both left and right orbitofortnal cortex, rCBF values of Odors are significantly larger than those of Control. rCBF values of Control and Odors in the left orbitofrontal cortex are similar to those in the right. **$P < 0.01$; ***$P < 0.001$.

rCBF increase in the orbitofrontal cortex during passive olfactory stimulation.

Discussion

Prepyriform and orbitofrontal cortices

Olfactory information is conveyed from the olfactory bulb to the pyriform cortex, the primary olfactory cortex in the monkey (Tanabe *et al.*, 1975a). Several human PET studies are consistent with this anatomical finding (Zatorre *et al.*, 1992; Small *et al.*, 1997; Qureshy *et al.*, 2000; Savic *et al.*, 2000, 2002). However, Zald and Pardo (1997) reported only subthreshold activation is observed in the pyriform cortex by odor stimulation, and Dade *et al.* (1998) reported the absence of increased rCBF in the pyriform cortex during olfactory encoding. The lack of activation in the pyriform cortex by odor is also observed in human fMRI studies (Yousem *et al.*, 1997, 1999; Sobel *et al.*, 1998a). In terms of

the monkey, our previous (Kobayashi *et al.*, 2002) and the present studies confirmed that a passive odor stimulation increases rCBF in the prepyriform area. This finding is consistent with electrophysiological results in the alert monkey (Tanabe *et al.*, 1975a,b). On the other hand, rCBF in the orbitofrontal cortex is not constantly activated among monkeys by odor stimulation in the present study as reported previously (Kobayashi *et al.*, 2002), though most of human PET studies have reported the increase of rCBF in the orbitofrontal cortex (Zatorre *et al.*, 1992; Small *et al.*, 1997; Zald and Pardo, 1997; Qureshy *et al.*, 2000; Savic *et al.*, 2000, 2002; see the discussion in Kobayashi *et al.*, 2002).

Interestingly, Zatorre *et al.* (1992) reported that in humans the right, but not the left, orbitofrontal cortex processes the olfactory information. This hypothesis is supported by later PET studies (Savic *et al.*, 2000, 2002). However, some studies reported that rCBF increase in the left orbitoforntal cortex is predominant to the right during odor perception (Zald and Pardo, 1997; Qureshy *et al.*, 2000). Thus, dominance of hemisphere during odor processing seems to be controversial in the human. In the present study using alert monkeys, we observed rCBF increase in the bilateral orbitofrontal cortex of monkey C by hexenol/hexenal or acetic acid, and isoamylacetate increased rCBF in the left orbitofrontal cortex of monkeys A and C (Table 1). The quantitative analysis in monkey C shows no significant difference of rCBF increase between the left and right orbitofrontal cortex. Thus, there may be no apparent laterality of activation in the monkey orbitofrontal cortex by passive olfactory stimulation, rather the laterality might depend on the properties of odors and/or additional meanings of odors.

Cerebellum and substantia innominata

Previous PET and fMRI studies have revealed that there is a large difference in activated regions except for the pyriform and orbitofrontal cortices. There are several explanations for the discrepancy. First, the activation pattern in the brain by odor depends on the kind of odors (Yousem *et al.*, 1997). Second, Sobel *et al.* (1998a,b) reported that the way of sensing the odor—sniffing and smelling—is a critical factor that determines what regions are activated. Third, olfactory-related tasks are also an important factor for the pattern of activated brain regions (Royet *et al.*, 2001; Savic *et al.*, 2000). In the present study, the cerebellum and substantia innominata were activated by passive olfactory stimulation. Human PET studies have shown that complex and specific tasks using odors increased rCBF in the human cerebellum (Qureshy *et al.*, 2000; Savic *et al.*, 2000; Royet *et al.*, 2001). However, the precise role of the cerebellum during odor simulation is still unclear in the human. The present results show that the monkey may be a suitable model for elucidating the precise role of these olfactory-related regions during odor stimuli.

The activation of the substantia innominata is consistent with the finding obtained from a tracing study that used horseradish peroxidase in the monkey, which showed that the substantia innominata receives afferents from the prepyriform cortex (Naito *et al.*, 1984). However, there are few human PET studies that reported substantia innominata activation.

Anterior cingulate gyrus

Subtraction images of pure air from each odor demonstrate that isoamylacetate and acetic acid stimuli activated no common brain region except for the prepyriform area, but that hexenol/hexenal application increased rCBF in the anterior cingulate gyrus in all monkeys (Figure 2, Table 1). However, the subtraction images of hexenol/hexenal minus isoamylacetate or acetic acid only revealed the subthreshold increase of rCBF in the anterior cingulate gyrus, and the ROI-based analysis showed no significant difference of rCBF increase between hexenol/hexenal and other odors. The reason for this discrepancy is that isoamylacetate or acetic acid also increased rCBF in the anterior cingulate gyrus though it did not reach the threshold level (Figure 3). Then one question arises: why does hexenol/hexenal increase rCBF more than the other odors in the anterior cingulate gyrus?

In the monkey brain, the anterior cingulate gyrus (area 24) receives projections from the orbitofrontal cortex (area 13) (Vogt and Pandya, 1987), which receives afferents from the primary olfactory cortex (Barbas, 1993; Carmichael *et al.*, 1994). These anatomical findings suggest that olfactory stimulation may influence the neural activity in the anterior cingulate gyrus. Human imaging studies have revealed controversial results concerned with the activation in the anterior cingulate gyrus by olfactory stimulation. Although several human imaging studies using PET (Francis *et al.*, 1999; Savic *et al.*, 2000, 2002; Royet *et al.*, 2001) and fMRI (Sobel *et al.*, 1998a,b; Poellinger *et al.*, 2001) have revealed that passive olfactory stimuli activate the anterior cingulate gyrus, Zatorre *et al.* (1992) and Small *et al.* (1997) have not reported the activation of the anterior cingulate gyrus by odor stimuli. The anterior cingulate gyrus is involved in the limbic circuit (Martin, 1989), which is considered to process emotion or affective behavior (reviewed by Bush *et al.*, 2000; Cardinal *et al.*, 2002). A recent imaging study using PET reveals a decrease of rCBF in the anterior cingulate gyrus in major depressive patients (Bench *et al.*, 1992; Chua *et al.*, 1996; Kennedy *et al.*, 1997). In addition, an electrophysiological study in the alert monkey showed that reward expectancy related activities were observed for several neurons in the anterior cingulate gyrus (Shidara and Richmond, 2002). These findings suggest that the anterior cingulate gyrus may play a key role for motivation for tasks, and might be activated by odors that stimulate the limbic system. Taken together with the findings that hexenol/hexenal causes pleasantness (Sano *et al.*, 2002), hexenol/hexenal may simultaneously activate both the olfactory and limbic systems, which may activate the anterior cingulate gyrus effectively.

Recent studies using hexenol/hexenal have revealed various biological functions in mammals. Akutsu *et al.* (2002) reported that hexenol/hexenal attenuates hyperthermia caused by autonomic stress response to novel environment in the rat. In addition, there is a report that a low concentration (0.03%) of hexenol/hexenal was accepted as a pleasant odor by humans, and decreased the amplitude of an event-related potential (P300) (Sano *et al.*, 2002). Our behavioral study in the alert monkey also revealed the improvement of delay time observed during a long-lasting performance of a continuous task by a passive application of hexenol/hexenal (Onoe *et al.*, 2003). In addition, hexenol/hexenal prevented the prolongation of response time caused by fatigue in the mental loaded task, the advanced trail making test, which can estimate the function of the frontal cortex in the human (unpublished observation). Therefore, the activation in the anterior cingulate gyrus by the odor of hexenol/hexenal may correlate with the improvement of response time during a long continuous task.

In summary, we investigated the brain regions activated by the odor of hexenol/hexenal in alert rhesus monkeys. In addition to the olfactory-related regions (prepyriform area, substantia innominata and orbitofrontal cortex), odor application of hexenol/hexenal activated the anterior cingulate gyrus, which was not commonly activated by odors of isoamylacetate or aceteic acid. This action of hexenol/hexenal may contribute to its physiological effect of stress release.

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