lateral spontaneous smiles were more common than bilateral smiles in neonates, but by 2 months, 80% of spontaneous smiles were bilateral. The mean duration of spontaneous laughs was longer than that of spontaneous smiles, and all spontaneous laughs were bilateral.

Both Messinger et al. (2002) and Kawakami et al. (2006) claim the necessity of longitudinal studies on spontaneous smiles to shed light on the genesis of affective expressions. This paper is the first intensive longitudinal research on spontaneous smiles and laughs. This preliminary study will explore more elaborate longitudinal research design for studying these behaviors.

Kawakami et al. (2006) concluded that "spontaneous smile" and "spontaneous laugh" might be different behaviors from the beginning. Recently, Waller and Dunber (2005) examined smiling-like display and laughing-like display in chimpanzees. To consider more detail on differences between spontaneous smiles and laughs in humans, we want to add longitudinal data.

The purposes of this study were (1) to present intensive longitudinal data of spontaneous smiles and spontaneous laughs, and (2) to further investigate the differences/similarities of spontaneous smiles and spontaneous laughs.

1. Method

1.1. Participant

One Japanese boy was observed from the day of his birth to the end of the 6th month. He had no recognized medical problems, and had experienced normal delivery. His birth weight was 2610 g. The Apgar score at the delivery was 9, and 5 min later it was 10. The gestational age was 38 weeks 6 days.

1.2. Procedure

It is difficult for researchers to record spontaneous smiles and laughs systematically because they occur unpredictably in association with irregular sleep. As adopted by Kawakami et al. (2006) and Takai (2005), we asked the mother to record spontaneous smiles and laughs by herself. The recording conditions were (1) record baby's face from near position, (2) at sleeping time, (3) on a bed if possible, (4) in silent circumstances, (5) using a tripod if possible.

Total observation days were 171 days within 181 days (6 months), and total observation time was 329 h 25 min and 35 s, 7.6% of that period of his life time. The recording strategy of the mother was "(1) all sleeping time in their home, (2) in silent circumstances, (3) when she was awake".

According to Japanese regulations, we are not required to obtain approval for this work from a Research Ethics Committee.

1.3. Definition of "spontaneous smile"

Oster (1978) used three criteria to code an infant's smile: (1) the action had to appear subjectively smile-like when viewed at normal speed; (2) there had to be more than a trace of AU12 [Action Unit in the Facial Action Coding System (FACS), Ekman & Friesen, 1978]; and (3) the AU12 component of the smile had to be visible for at least 1 s. AU12 (lip corner raising) is recognized as the basis of all smiles by other researchers (Messinger et al., 2002). Also, "lip corner raising" is an important criterion in other facial coding systems [e.g., Code 52 in The Maximally Discriminative Facial Movement Coding System (MAX), Izard, 1983].

We used strict criteria for identifying spontaneous smiles as follows: (1) lip corner raising (AU12 in FACS and Code 52 in MAX); (2) during irregular sleep, drowsiness; (3) without known external or systematically demonstrable internal causes (Wolff, 1961); (4) continuing more than 1 s; (5) smiles continued within 1/6 s are combined; (6) smiles with vocal sounds are defined as spontaneous laughs. The second criterion was used because we cannot discriminate between spontaneous and elicited smiles during an alert inactivity state.

First, we checked all tapes recorded by the mother. Second, the onset and offset of smiles and laughs were determined as follows. Our digital video camera recorder had a button to move a video sequentially by 1/30 s. When we found a smile or laugh, we moved the video back sequentially to the onset frame (immediately prior to which there were no facial movements). And from the onset, we moved the video forward sequentially to the offset (immediately following which there were no facial movements).

1.4. Coding

Two coders independently identified spontaneous smiles and laughs using the Digital Video Camera Recorder (SONY DCR-PC110). Only spontaneous smiles and laughs identified by both coders were included in the subsequent analysis. The percentage of intercoder agreement was 91.67%. Correlation of the event durations recorded by the two coders was $r = .79$ ($p < .01$).

2. Results

2.1. Spontaneous smiles

From the 9th day (no spontaneous smile was recorded until the 9th day) to 181-day-old, 565 spontaneous smiles were observed. The durations of spontaneous smiles were determined by averaging the durations recorded by the two coders. The mean duration was 2.57 s (S.D. = 1.28).

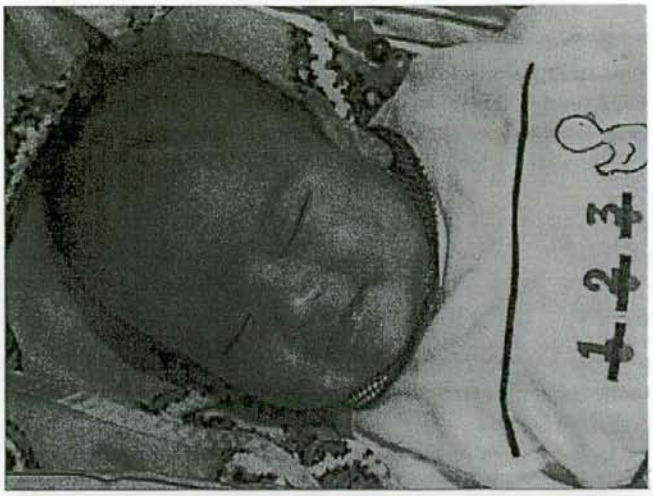
The second column of Table 1 shows the means of durations of spontaneous smiles per week. We can observe spontaneous smiles as late as 6 months of age. The fourth column shows the frequencies of spontaneous smile. By the methods adopted by this study, the comparisons of frequencies are not valuable.

At ages 4–6 months, the baby sometimes slept prone. Sixty-three spontaneous smiles were observed in this position. It is very difficult to determine the lateralities of smiles in these cases. And two spontaneous smiles were observed when he was held by the mother. Five hundred spontaneous smiles were analyzed and the lateralities noted.

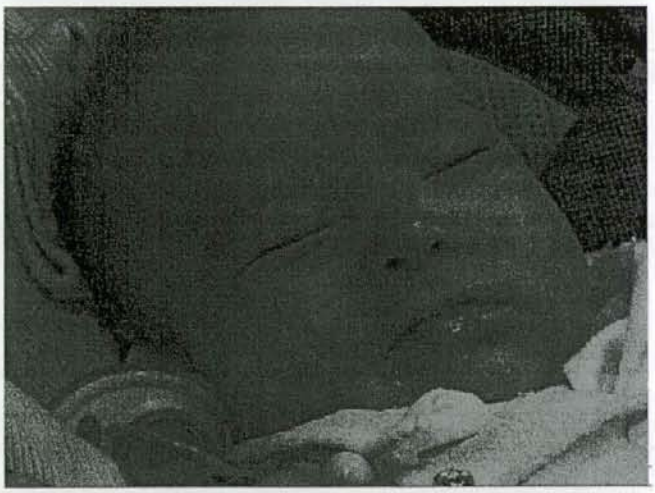
Three hundred and fifty-eight were bilateral (see Fig. 1A), and 142 were unilateral. The percentage of bilateral smiles among all smiles increases from the 2nd month as shown in Fig. 2. The number of unilateral spontaneous smiles on the right side of his face (see Fig. 1B) were the same as the number on the left side (see Fig. 1C; 71, respectively). When lying on one side, unilateral spontaneous smiles were more frequently observed on the side of the face away from the surface of the bed [top side: 112, bottom side: 7; χ^2 (1) = 92.6, $p < .01$].

Table 1
Development of spontaneous smile

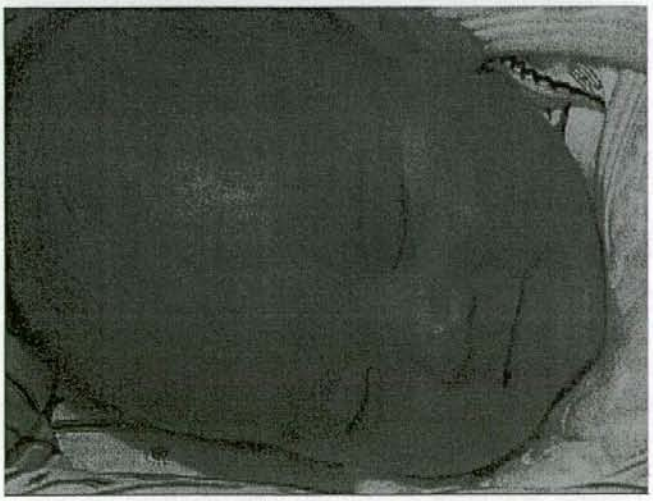
Weeks	*M* duration (s)	S.D.	Frequencies
2	2.64	1.09	21
3	2.96	1.78	19
4	2.72	.9	18
5	2.09	.77	11
6	2.48	1.4	16
7	1.79	.44	8
8	1.83	.32	4
9	2.43	1.39	14
10	2.38	.61	10
11	2.05	.89	44
12	2.66	1.18	47
13	2.43	.96	19
14	2.41	1.7	16
15	2.48	1.14	36
16	2.59	1.13	18
17	2.78	1.4	27
18	3.01	1.43	18
19	2.72	1.64	36
20	2.27	1.03	56
21	2.93	1.59	28
22	2.75	1.07	18
23	3.03	1.66	18
24	2.37	1.05	31
25	3.1	2.1	8
26	3.01	1.44	24



A (76 days-old)



B (79 days-old)



C (87 days-old)

Fig. 1. (A–C) Bilaretal and unilateral spontaneous smiles.

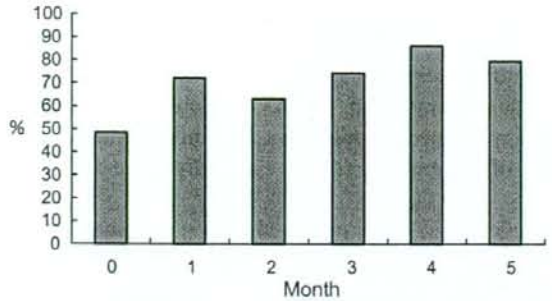


Fig. 2. Percentages of bilateral spontaneous smiles by month.

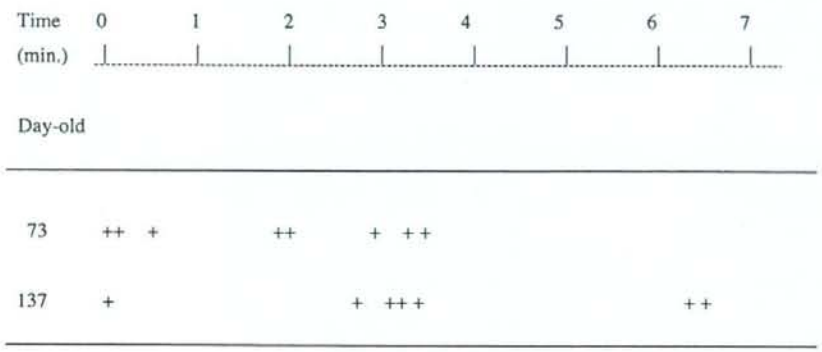


Fig. 3. Smiles bursts.

2.2. *Spontaneous laugh*

Fifteen spontaneous laughs, from 11- to 181-day-old, were observed. The mean duration of spontaneous laughs was 4.37 s (S.D. = 1.89). The mean duration of spontaneous laughs was longer than that of spontaneous smiles [$F(1, 578) = 28.34$, $p < .001$]. There was no developmental change in the duration of spontaneous laugh.

The laterality of one spontaneous laugh could not be determined because he was sleeping on his stomach. Thirteen out of 14 spontaneous laughs were bilateral. One unilateral spontaneous laugh (left side of the face) was observed at 133-day-old.

2.3. *Smile bursts*

Takai (2005) defined the bursts of spontaneous smiles as a "period of more than 7 spontaneous smiles in 7 min". In the longitudinal study on one male infant's first 6-months, Takai (2005) found seven smile bursts.

Fig. 3 shows the smile bursts of this study. Two smile bursts, at 73 and 137-day-old, were found.

3. Discussion

First, we should recognize that we observe spontaneous smiles even in the 6th month (mothers reported to us that they observed them after the 12th month). Kagan and Fox (2006) noted: "... the 1st year consists of two important transitions. One occurs at 2–3 months, and the second at 7–12 months of age. The first transition is accompanied by disappearance of newborn reflexes, endogenous smiling, ... (p. 169)". The results of this study prove that the description on spontaneous smiles in the influential handbook should be changed. At 2 months, infants show socially elicited smiles (Rochat, 2001). Spontaneous smiles and social smiles coexist during infant periods. Spontaneous smiles

do not transform into social smiles. These might be the most important results of this longitudinal study. Our positive emotions may have several roots.

From Fig. 2, the rise of bilateral smiling began at the 2nd month. Kawakami et al. (2006) discussed that developmental changes in the brain might cause this phenomenon. By the development of cerebral control, unilateral spontaneous smiles may be changed to bilateral spontaneous smiles. For this child, dramatic changes appeared at the 2nd month. By analyses of the brain at the time of spontaneous smiles, especially at the time of "smile bursts", we will learn some phases of developmental changes.

The asymmetrical tonic neck reflex (ATNR) is observed during the first 2 or 3 months of life, and it is usually integrated by 6 or 7 months (Snow, 1989). Can we relate the results of Fig. 1 to ATNR? Both Hauser (1993)'s rhesus monkeys and Holowka and Petitto (2002)s' human infants showed right hemisphere dominance for the production of facial expression. There was no dominance of lateralities in this study. This is a case study, so we need to study more cases to generalize the results.

Waller and Dunber (2005) observed silent bared teeth display (smiling-like) and relaxed open mouth display (laughing-like) in chimpanzees, and they discussed the differences of the two displays. Their morphological definitions of "smiling-like" and "laughing-like" displays are a little different from ours. The results of this study show that the durations of spontaneous laughs were longer than those of spontaneous smiles, and almost all of spontaneous laughs were bilateral as shown by Kawakami et al. (2006). Only one spontaneous laugh was unilateral in this case. This unilateral spontaneous laugh was observed at 133-day-old. Kawakami et al. (2006) claimed that "Spontaneous smile" and "Spontaneous laugh" might be different behaviors from the beginning. We confirmed this claim by the results of this study. To study the origins of spontaneous smiles and spontaneous laughs, it might be necessary to observe premature babies and to watch fetuses using a three-dimensional ultrasound scope.

To compare the frequencies of spontaneous smiles by weeks/months, fixed schedules of observation may be better using many participants.

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Development of the Laryngeal Air Sac in Chimpanzees

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Abstract Though many nonhuman primates possess a laryngeal sac, the great apes are unique in their great size. Though an enlarged sac probably arose in their common ancestor, its functional adaptations remain a matter of debate. Its development in extant great apes is likely to provide valuable information to clarify the issue. We used magnetic resonance imaging to examine the development of the laryngeal sac in 3 living chimpanzees, age 4 mo–5 yr, and identified 2 distinct growth phases of the sac. A gradual growth of the sac in early infancy results in a configuration so that it occupies the ventral region of the neck; many adult non-hominoid primates having a sac show the configuration. The subsequent rapid expansion of the sac in late infancy causes the final configuration in chimpanzees, wherein the sac expands into the pectoral, clavicular, and axillary regions. The latter phase possibly arose at latest in the last common ancestor of extant great apes and contributed to the evolution of the enlarged sac, despite the later evolutionary diversification in adult sac anatomy and growth. As many studies have advocated, the enlarged sac probably plays a role in vocalization in adults. However, physiological modifications in the laryngeal region during infancy are likely to provide valuable information to evaluate the functional adaptations of the enlarged sac in the great apes.

Keywords magnetic resonance imaging · *Pan troglodytes* · ventricular sac · vocalization

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Introduction

Many species of nonhuman primates have a laryngeal air sac, an accessory mucosal membrane pouch growing out from the laryngeal region (Hayama 1970; Hewitt *et al.* 2002; Negus 1949; Starck and Schneider 1960). There are 5 forms among nonhuman primates, based on the anatomy of the opening to the laryngeal region (Hayama 1970). Though there are controversies regarding the distributions of forms of air sacs among primates (Hewitt *et al.* 2002), all great apes and siamang definitely share 1 type: a ventricular sac that extends ventrally from bilateral laryngeal ventricles to fuse in front (Hayama 1970; Hewitt *et al.* 2002; Negus 1949; Starck and Schneider 1960). By contrast, other gibbons and humans have bilateral laryngeal ventricles, but no true laryngeal sac (Fitch 2000; Hayama 1970, 1996; Hewitt *et al.* 2002; Némai and Kelemen 1933; Negus 1949; Starck and Schneider 1960). The great apes are unique in having an enlarged sac extending into the pectoral and axillary regions (Avril 1963; Brandes 1932; Hayama 1970; Hewitt *et al.* 2002; Kleinschmidt 1938; Negus 1949; Raven 1950; Starck and Schneider 1960), though many other primates have a smaller sac, at the largest extending to the ventral region of the neck (Hayama 1970; Hewitt *et al.* 2002; Negus 1949; Starck and Schneider 1960).

In chimpanzees, the bilateral sacs fuse with each other in front of the region between the hyoid bone and the thyroid cartilage (Avril 1963). The fused sac expands superiorly to form an unpaired recess at the dorsal aspect of the hyoid body (hereafter, the hyoidal recess). Inferiorly, it forms a long unpaired recess along the ventral aspect of the laryngeal cartilages and trachea to reach the sternum (hereafter, suprasternal recess). From here, the sac expands further to form an unpaired recess at the ventral aspect of the pectoral region (hereafter, presternal recess) and bifurcates to form bilateral recesses extending into the infraaxillary regions (hereafter, axillary recesses).

The exact functions and evolutionary adaptations of the enlarged sac in the great apes remain matters of debate (Fitch and Hauser 2003; Hewitt *et al.* 2002;). The enlarged sac likely arose at the latest in the last common ancestor of the extant great apes, an evolutionary step that possibly involved changes in the rate or timing of existing developmental processes or novel growth processes (Gould 1977; McKinney and McNamara 1991; Rice 2000). Therefore, the changes in physiology that accompany distinct developmental events contributing to the formation of the enlarged sac in extant subjects are likely to shed light on its original functions. Unfortunately, there is little information on the growth of the laryngeal air sacs in great apes (Table I; Avril 1963; Brandes 1932; Huber 1931; Kleinschmidt 1938). We used magnetic resonance imaging (MRI) to examine the growth of the laryngeal air sac in 3 living chimpanzees age 4 mo–5 yr.

Methods

We studied 3 chimpanzees: Ayumu (male), Cleo (female), and Pal (female). They were born in 2000, and the biological mothers reared them in the Primate Research Institute, Kyoto University (PRI; Matsuzawa 2003). We took sagittal tomographic

images of their necks at PRI via a General Electrics Signa Profile MRI scanner (.2 Tesla), at scheduled intervals from 4 mo to 5 yr of age (Table II). The MRI protocol and experimental procedure in this study are per Nishimura *et al.* (2003, 2006). We anesthetized the subjects intramuscularly with a mixed solution containing 3.5 mg of ketamine hydrochloride (Sankyo, Tokyo) and .035 mg of medetomidine hydrochloride (Meiji Seika Kaisha, Tokyo) per kg of body mass, but we sedated subjects>4 yr orally with 3.75 mg of droperidol (in 1.5 ml) before anesthetization. We scanned them, placing them supine with their heads fixed to the coil with belts. All imaging sequences are sagittal spin echo series with fields of view of 18–28 cm, with 2.7 mm or 3 mm slice thicknesses and .8 mm or .5 mm gaps between slices (Table II). The matrix of all MR images is 256×256 pixels, and image resolutions ranged from .7×.7 to 1.09×1.09 mm/pixel. Care and use of the subjects adhered to the guidelines of the PRI (1986, 2002), and the Ethics Panel of the PRI approved the MRI examination protocol.

We measured the linear dimensions of the laryngeal air sac on MR images transferred to a personal computer via ImageJ software (W. Rasband, National Institutes of Mental Health, Bethesda, MD; http://rsb.info.nih.gov/ij/). Standard planes on the midsagittal plane were as follows: VLT, ventral line of the trachea; ACG, the level of the anterior commissure of the glottis; SSt, superiormost level of the sternum. Definitions and illustrations are in Table III and Fig. 1, respectively. The measurements included L_H, the length of the hyoidal recess, parallel to VLT from the superiormost to the inferiormost points of a growing sac before it reached the ACG, or its length to the ACG after the sac had already grown below it; L_S, the

Table I Morphological studies on the laryngeal sir sac in great apes

Species	Studies	Sex	n[a]	Developmental stages (sample size)[b]
Pongo pygnaeus	Brandes (1932)	Male	14	Adult (10), adolescence (1), juvenile (1), neonate (1), 5 yr (1)
		Female	6	Adult (4), adolescence (1), juvenile (1)
		?	1	2 mo (1)
	Avril (1960)	Female	1	10 yr (1)
	Hayama (1970)	Male	2	Adolescence (2)
		Female	3	Adult (1), adolescence (2)
Gorilla gorilla	Kleinschmidt (1938)	Male	1	10 yr (1)
	Raven (1950)	Male	1	Adult (1)
	Hayama (1970)	Male	1	Adult (1), adolescence (1)
		Female	1	Adult (1), adolescence (1)
Pan troglodytes	Avril (1963)	Male	3	Infant (1), juvenile (1), 15 yr (1)
		Female	4	Fetus (1), infant (1), 6 yr (1), 9 yr (1)
		?	3	Juvenile (1), probably infant (2)
	Hayama (1970)	Male	1	Adolescence (1)
		Female	3	Adult (1), adolescence (2)
	present study	Male	1	4 mo–5 yr[c] (1)
		Female	2	4 mo–5 yr[c] (2)

2?=unknown

[a] Total sample size for each study.

[b] Developmental stages of the subjects and sample size at each stage. If known, chronological age of each subject at the time of study.

[c] We examined the subjects longitudinally, as in Table II.

Table II Ages of the subjects at the times of scans, parameters of MRI scanning, and measurements of dimensions

Subjects	Age (mo)	Pixel sizes[a] (mm/pixel)	FOV (mm)	Thickness (inter-slice gap)[b] (mm)	L_H (mm)	L_S (mm)
Ayumu	4	.74	190	2.7 (.8)	8.29	absent
	6	.70	180	3.0 (.5)	9.90	.49
	9	.74	190	2.7 (.8)	12.34	1.48
	12	.70	180	3.0 (.5)	15.34	2.33
	18	.70	180	3.0 (.5)	15.85	3.12
	22	.90	230	2.7 (.8)	15.74	4.36
	25	.86	220	3.0 (.5)	17.58	3.04
	30	.98	250	3.0 (.5)	18.48	8.15
	36	1.09	280	3.0 (.5)	21.42	14.32
	42	.98	250	3.0 (.5)	2.89	54.22[d]
	48	.98	250	3.0 (.5)	21.50	53.55[d]
	52	.98	250	3.0 (.5)	19.30	53.82[d]
	54	.98	250	2.7 (.8)	26.77	—
	60	.98	250	3.0 (.5)	28.05	—
Cleo	4	.70	180	3.0 (.5)	7.04	1.12
	6	.70	180	3.0 (.5)	1.25	.43
	9	.70	180	3.0 (.5)	8.58	1.37
	12	.74	190	2.7 (.8)	1.36	2.18
	18	.70	180	3.0 (.5)	1.86	1.96
	25	.86	220	3.0 (.5)	14.37	9.86
	31	.98	250	3.0 (.5)	18.50	38.84[d]
	36	.98	250	3.0 (.5)	18.71	39.90[d]
	43	.98	250	3.0 (.5)	21.33	—
	48	.98	250	3.0 (.5)	25.87	54.71[d]
	54	.98	250	3.0 (.5)	27.86	—
	60	.98	250	3.0 (.5)	29.69	—
Pal	4	.70	180	3.0 (.5)	9.21	absent
	6	.74	190	2.7 (.8)	1.44	.23
	9	.70	180	3.0 (.5)	8.44	2.88
	12	.70	180	3.0 (.5)	11.45	6.99
	18	.70	180	3.0 (.5)	13.78	9.67
	24	.86	220	3.0 (.5)	16.16	42.03[c]
	30	.98	250	3.0 (.5)	15.32	—
	36	.98	250	3.0 (.5)	18.97	48.82[d]
	42	.98	250	3.0 (.5)	17.84	5.82[d]
	48	.98	250	3.0 (.5)	22.23	—
	54	.98	250	3.0 (.5)	22.00	61.63[d]
	60	.98	250	3.0 (.5)	25.48	—

L_H=length of the hyoidal recess of the laryngeal sac; L_S=length of suprasternal recess of the laryngeal sac; absent=the suprasternal recess had not developed; —=no measurements because the landmarks were outside the image field.

[a] The values varied irregularly during infancy, but had little influence on the accuracy of measurements.

[b] This means that the slice interval of all scans was 3.5 mm, regardless of differences in slice thickness and interslice gap.

[c] The sac almost reached the superior edge.

[d] The sac had already expanded beyond the superior edge of the sternum.

length of the suprasternal recess, parallel to VLT, from the ACG to the inferiormost point of the growing sac before it reached the SSt, or the length to the SSt after the sac grew beyond that level (Fig. 1).

Table III Definitions for the standard planes used

Landmarks and planes	Abbr.	Definition
Level of the anterior commissure of the glottis	ACG	The line perpendicular to the VLT from the anterior commissure of the glottis at the base of the epiglottis
Superiormost level of the sternum	SSt	The line perpendicular to the VLT and tangential to superior surface of the sternum
Ventral line of the trachea	VLT	The most ventral straight line of the trachea

Results

We obtained good MR images showing the longitudinal growth in the laryngeal air sac for each subject (Fig. 2), though for Ayumu at 4 and 6 mo, the images are slightly obscure because of motion artifacts. Nevertheless, all the images were adequate for the evaluation of anatomical development (Table II; Fig. 3).

We identified a small pouch at the dorsal aspect of the body of the hyoid at 4 mo for all 3 subjects (Figs. 2a, 3). The sac expanded superiorly in the dorsal area of the hyoidal body and inferiorly to the level of the anterior commissure of the glottis by 6 mo at the latest, for all subjects (Fig. 3). The air sac continued to expand superiorly to occupy the entire dorsal area of the hyoidal body in the first year of life (Figs. 2b, 3). It gradually expanded inferiorly to sit along the ventral aspect of the laryngeal cartilages in early infancy, despite variations in growth rates (Figs. 2b, 3).

In all subjects, in late infancy the sac grew rapidly below the ventral aspect of the trachea to reach and extend beyond the superior edge of the sternum (Figs. 2c, 3): after 1.5 yrs for female Pal, after 2.5 yr for female Cleo, and after 3.5 yr for male Ayumu (Figs. 2c, 3). The supra-and presternal recesses of the sac then widened greatly (Fig. 2d). We did not evaluate further expansions of the sac into the pectoral, clavicular, or axillary regions, which we did not image.

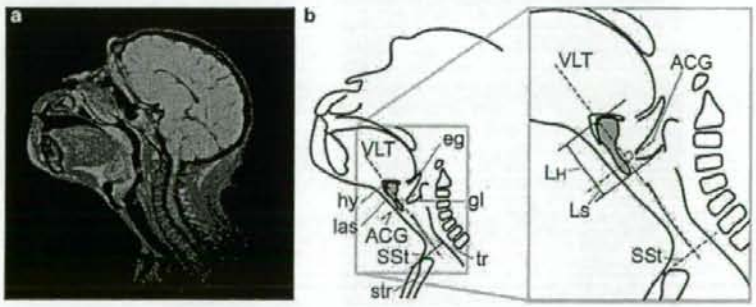

Fig. 1 Diagram of measurements of the laryngeal air sac. (**a**) Magnetic resonance (MR) image. (**b**) Lengths of the hyoidal recess (L_H) and the suprasternal recess (L_S) of the laryngeal sir sac (las). ACG=level of the anterior commissure of the glottis; SSt=superior-most level of the sternum; VLT=ventral line of the trachea (see also definitions in Table III); eg=epiglottis; gl=glottis; hy=hyoid bone; str=sternum; tr=trachea.

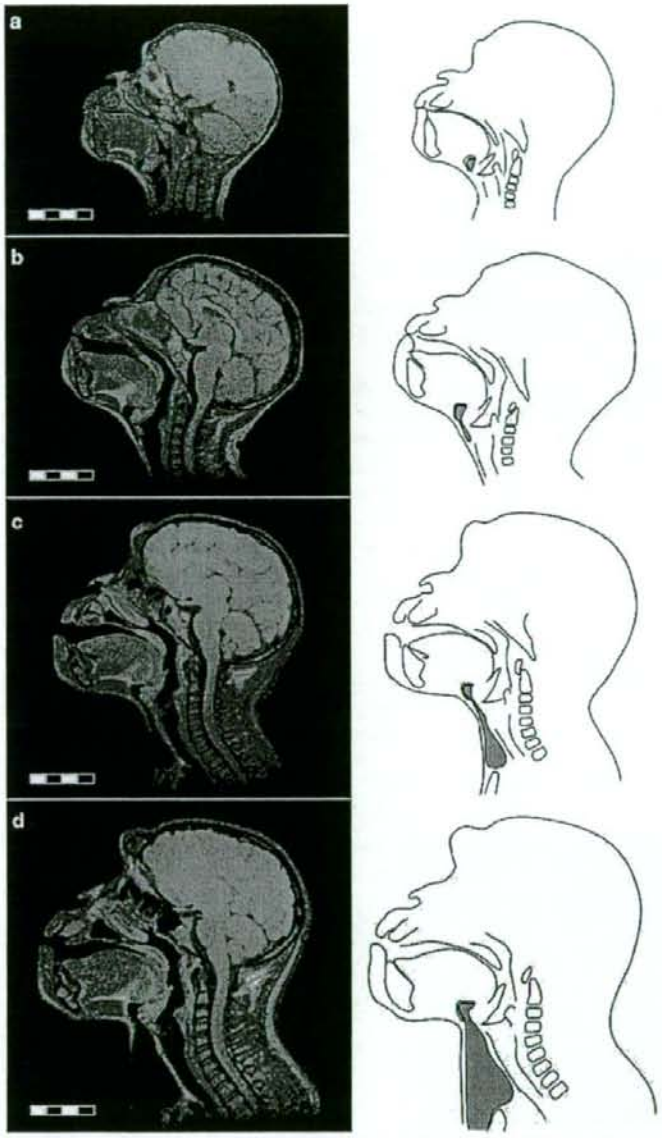

Fig. 2 MR images of the laryngeal air sac for the same female chimpanzee (Pal). (a) At 4 mo, a small pouch had formed in the dorsal area of the hyoid bone. (b) At 18 mo, the sac occupied the entire area dorsal to the hyoid bone and inferior to the ventral aspect of the laryngeal cartilages. (c) At 24 mo, the sac had expanded to reach the sternum. (d) At 48 mo, the sac had expanded inferiorly into the ventral aspect of the pectoral regions and had widened at the level of the trachea. Scale in cm.

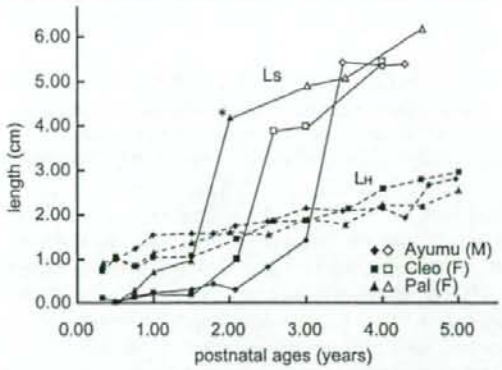

Fig. 3 Growth of the laryngeal sac in 3 chimpanzees: Ayumu (male, diamonds); Cleo (female, triangles), and Pal (female, squares). L_H=length of the hyoidal recess; L_S=length of the suprasternal recess of the sac. We could not measure L_S at some points because the landmarks were out of the image. Open symbols mean that the sac had already expanded beyond the superior edge of the sternum. The sac reached almost the superior edge of the sternoid at 2 yr of in Pal (*; Fig. 2c). In early infancy, the laryngeal sac grows gradually to form the configuration that most adult primate species having a sac show. After late infancy, the sac expands rapidly in the great apes. See also the definitions in Methods.

Discussion

MRI showed 2 distinct phases in the growth of the laryngeal air sac in chimpanzees. In early infancy, the laryngeal sac grows gradually to expand superiorly to the dorsal area of the body of the hyoid and then to extend inferiorly along the ventral aspect of the laryngeal cartilages. Many nonhominoid primates show such a configuration, despite differences in the forms of the sac. Despite controversies regarding the distributions of each type, depending on species, they have a subhyoid sac extending from a ventral midsagittal opening just above the glottis, an infraglottal sac forming from a ventral midsagittal opening between the thyroid and cricoid cartilages, a dorsal sac from a dorsal infraglottal opening between the cricoid cartilage and the first tracheal ring, or they may show bilateral supraventricular sacs arising from bilateral fissures above the ventricles (Hayama 1970; Hewitt *et al.* 2002; Negus 1949; Starck and Schneider 1960). The sac expands within the laryngeal in most species, and at its largest, it expands within the neck (Geist 1965; Hayama 1970; Hewitt *et al.* 2002; Hill and Booth 1957; Negus 1949; Starck and Schneider 1960; Tucker and Tucker 1975). By contrast, from late infancy, the chimpanzees showed a rapid expansion of the sac along the trachea and beyond the neck, despite the slight differences in the timing between subjects. Though we did not evaluate it, the growth phase possibly continues quite early in development to form a sac expanding into the pectoral, clavicular, and axillary regions. Siamang (*Synphalangus syndactylus*) have a ventricular sac, as in great apes, and their sac expands in part to reach the sternum (Hayama 1970; Marler and Tenaza 1977). A ventricular sac may therefore have an advantage over the other forms in enlargement of the laryngeal sac. However, siamang probably do not share the rapid growth phase, so their sac shows no large expansion. Thus, the rapid growth phase in late infancy is

likely a derived phase that contributes principally to the formation of the enlarged sac in chimpanzees.

We found that the rapid growth phase of the laryngeal sac started in the 2 female subjects earlier than in the male, but it remains unclear whether this depends on the sexual dimorphism of the sac. In all great apes and siamang, both males and females have a similar configuration of the sac (Hayama 1970). However, all great apes probably show sexual dimorphism in the volume of the sac, which in turn depends on sexual dimorphism in body size. They also show sex-related differences in behavior patterns involving the laryngeal sac; e.g., inflation of the sac for resonance of thoracic percussion during drumming in male gorillas, and for size exaggeration displays in male orangutans (Marler and Tenaza, 1977; Tuttle 1986). Such differences might depend on sexual dimorphism in volume, but there is little information on the issue. Future studies using large longitudinal samples are necessary to determine whether sexual differences in developmental patterns might contribute to adult sexual dimorphism in sac volumes.

The sacs reached the dorsal region of the hyoidal body by 4 mo in all subjects, but we could not evaluate the time of fusion of the bilateral growing sacs. Avril (1963), using 6 chimpanzee cadavers ranging from the fetal to the subadult stage (Table I), found that the unilateral sac expands greatly and that another sac extends ventrally to fuse with it in the late juvenile period, suggesting that a unilateral sac would have expanded rapidly in late infancy in the subjects examined here. If this is true, fusion of the bilateral sacs by itself can have no influence on the rapid expansion of a sac in chimpanzees. However, the fusion in juvenile or adult periods may modify the functions of a unilateral large sac that has already expanded.

There is little information on the growth patterns of the large sac for other great apes (Table I). Adult gorillas have a configuration of the sac that is almost the same as that in chimpanzees (Avril 1963; Kleinschmidt 1938; Raven 1950). By contrast, orangutans have a different configuration, in which bilateral sacs extend inferiorly from the ventricles to the neck region and fuse in the pectoral region (Avril 1963; Brandes 1932; Huber 1931). However, in orangutans the unilateral sac expands greatly, and another sac expands inferiorly to fuse with it in the late juvenile period, as with chimpanzees (Brandes 1932; Huber 1931). Thus, though there are slight differences in the sac configuration, all the great apes possibly share a rapid expansion of the unilateral sac in late infancy.

The functions of the laryngeal sac in primates are still a matter of debate. Suggested functions include storage of expired air to increase oxygen uptake (Negus 1949) or reduction of the hyperventilation caused by a long sequence of repetitive loud calls (Hewitt *et al.* 2002), generating another sound source in the laryngeal ventricles (Brandes 1932; Huber 1931; Fitch and Hauser 2003; Kelemen 1948), resonating the laryngeal voice source to help produce loud and long calls (Gautier 1971; Fitch and Hauser 2003; Marler and Tenaza 1977; Napier and Napier 1985; Schön 1971; Schön Ybarra 1995), or buffering against the pressure induced by intensive expiratory airflow following air trapping during 3-dimensional arboreal locomotion (Hayama 1970; 1996). These functions excluding the first and last are relevant to vocalization. Though current analysis techniques and acoustic theory need to address such hypotheses (Lieberman 2006; Riede *et al.* 2006), vocal behavior patterns in great apes have attracted particular attention to the major

functions of the enlarged sac in adults (Brandes 1932; Fitch and Hauser 2003; Hewitt *et al.* 2002; Huber 1931; Kelemen 1948; Marler and Tenaza 1977). However, an enlarged sac may not necessarily have evolved to be advantageous for an aspect of vocalization. Though the mature sac probably serves some of the aforementioned functions in adults, separate functions could have arisen-or disappeared-with each developmental event of the sac; e.g., gradual growth in early infancy, rapid expansion in late infancy, or fusion of the bilateral sacs in the late juvenile period. Thus, we suggest that, among the phases, physiological changes accompanying the rapid expansion of the sac in late infancy are likely to shed light on the original functional adaptations of the enlarged sac in the common ancestor of the extant great apes.

Despite differences in effect, the functions principally depend on some physiological modifications in the laryngeal region, including activities of related musculature and manipulation of the airflow. Unfortunately, few studies have evaluated growth-related changes in physiology in the laryngeal region in chimpanzees, probably because of technical limitations. Such studies on infants, not on juveniles and adults, promise to provide valuable insight into the original functional adaptations of the enlarged sac in the great apes and its apparent evolutionary loss in humans.

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Research report

Involvement of glial cell line-derived neurotrophic factor in inhibitory effects of a hydrophobic dipeptide Leu-Ile on morphine-induced sensitization and rewarding effects

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Abstract

There are few efficacious medications for drug dependence at present. We have previously demonstrated that Leu-Ile, which induces the expression of not only tumor necrosis factor-α (TNF-α) but also glial cell line-derived neurotrophic factor (GDNF), inhibits methamphetamine (METH) and morphine (MOR)-induced sensitization and rewarding effects by regulating extracellular dopamine levels *via* the induction of TNF-α expression, and indicated the potential of Leu-Ile as a novel therapeutic agent for METH and MOR-induced dependence. In the present study, we investigated the involvement of GDNF in inhibitory effects of Leu-Ile on MOR-induced sensitization and rewarding effects. Repeated treatment with MOR for 9 days, which results in an enhancement of the locomotor-stimulating effects (sensitization) of MOR, increased GDNF levels in the nucleus accumbens compared with those in saline-treated mice. Repeated pre-treatment with Leu-Ile for 9 days potentiated the MOR-induced increase in GDNF levels. MOR at a low dose (3 mg/kg) produced place preference in GDNF heterozygous knockout (GDNF-(+/−)) mice, but not in littermate GDNF-(+/+) mice. No inhibitory effect of Leu-Ile on MOR-induced place preference was observed in GDNF-(+/−) mice. These results suggest that GDNF is involved in the inhibitory effects of Leu-Ile on MOR-induced sensitization and rewarding effects.

Keywords: Morphine (MOR); Glial cell line-derived neurotrophic factor (GDNF); Sensitization; Rewarding effects; Leu-Ile; Mice

1. Introduction

Drugs of abuse are able to elicit compulsive drug-seeking behaviors upon repeated administration, which ultimately leads to the phenomenon of addiction. Evidence indicates that the susceptibility to develop addiction is influenced by sources of reinforcement, variable neuroadaptive mechanisms, and neurochemical changes that together lead to altered homeostasis of the brain reward system [7].

Neurotrophic factors and cytokines, which are known to influence synaptic transmission and neuronal morphology [1,2,12], may be involved in alterations of the morphology of dendrites and dendritic spines in the nucleus accumbens (NAc) and prefrontal cortex after repeated injections of psychostimulants [18,19]. Glial cell line-derived neurotrophic factor (GDNF) inhibits the cocaine-induced upregulation of tyrosine hydroxylase (TH) activity in the ventral tegmental area (VTA) and blocks behavioral responses to cocaine [10]. GDNF would be a candidate for therapeutic agents against drug dependence. However, there are serious obstacles to its therapeutic application: it is difficult to deliver GDNF from the periphery to the brain, since it is a macromolecule that cannot penetrate the blood–brain barrier

Abbreviations: CPP, conditioned place preference; DA, dopamine; EIA, enzyme immunoassay; GDNF, glial cell line-derived neurotrophic factor; METH, methamphetamine; MOR, morphine; NAc, nucleus accumbens; TNF-α, tumor necrosis factor-α; TH, tyrosine hydroxylase; VTA, ventral tegmental area

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[8], and is easily broken down by proteases in the blood stream. Therefore, GDNF cannot be used directly as a therapeutic tool for drug dependence. We hypothesized that a low-molecular-weight compound which induces production of GDNF in the brain could be a novel therapeutic agent for drug dependence.

Recently, we have demonstrated that Leu-Ile, which induces the expression of tumor necrosis factor-α (TNF-α) and GDNF, inhibits methamphetamine (METH)-induced sensitization and rewarding effects by negating the METH-induced inhibition of dopamine (DA) uptake as well as attenuating the METH-induced increase in extracellular DA levels in the NAc *via* the induction of TNF-α and GDNF expression [15]. Moreover, we have demonstrated that Leu-Ile inhibits MOR-induced sensitization and rewarding effects by regulating extracellular DA levels *via* the induction of TNF-α expression [14].

In the present study, to extend our findings, we examined the involvement of GDNF in addition to TNF-α in inhibitory effects of Leu-Ile on MOR-induced sensitization and rewarding effects.

2. Materials and methods

2.1. Reagents

GDNF as a standard for the enzyme immunoassay (EIA) was donated by Amgen (CA, USA). Leu-Ile was purchased from Kokusan Chemical Co., Ltd. (Tokyo, Japan). All other materials used were of reagent grade.

2.2. Animals

Animals were housed in plastic cages and kept in a temperature-, humidity-, and light-controlled room (23 ± 1 °C; 50 ± 5% humidity; 12:12 h light/dark cycle starting at 8:00 a.m.) and had free access to food and water, except during behavioral experiments. All animals' care and use were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Nagoya University School of Medicine. Animals were treated according to the Guidelines of Experimental Animal Care issued from the Office of the Prime Minister of Japan.

The wild-type C57BL/6J mice were obtained from Slc Japan (Hamamatsu, Japan).

Male C57BL/6J-GDNF heterozygous knockout (GDNF-(+/−)) mice, 8–12 weeks of age, were used in the experiments. GDNF-(+/−) were generated as described previously [17]. GDNF (−/−) homozygous knockout mice die shortly after birth (postnatal 7 days), but GDNF (+/−) mice are viable. GDNF levels in the frontal cortex, NAc, caudate putamen, and hippocampus of GDNF-(+/−) mice are 54.8, 65.4, 59.0, and 66.8%, respectively, of those in littermate GDNF-(+/+) mice [15]. Littermate GDNF-(+/+) mice were used as controls in the behavioral experiments.

2.3. Locomotor activity

Locomotor activity was measured using an infrared detector (Neuroscience Co., Ltd., Tokyo, Japan) in a plastic box (32 cm × 22 cm × 15 cm high) [11,14]. Mice were administered Leu-Ile (1.5 and 15 μmol/kg, i.p.) or vehicle, and habituated for 1 h in the box. Mice were administered MOR (10 mg/kg, s.c.) or saline 1 h after the Leu-Ile administration, and the locomotor activity was measured for 2 h immediately after the MOR or saline administration [14]. Leu-Ile and MOR were injected once a day for 9 days.

2.4. Enzyme immunoassay

GDNF levels were measured using an EIA with a minor modification [13]. Mice were administered Leu-Ile (1.5 and 15 μmol/kg, i.p.) once a day 1 h before MOR (10 mg/kg, s.c.) treatment for 9 days and decapitated 24 h after the last administration of MOR (Fig. 1A). Homogenate buffer (0.1 M Tris–HCl [pH 7.4] containing 1 M NaCl, 2% bovine serum albumin, 2 mM EDTA, and 0.2% Na_3N) was added to brain tissue at a ratio of 1 g wet weight per 19 ml of buffer, pulse-sonicated for 100 s, and centrifuged at 100,000 × *g* for 30 min. The supernatant was collected and used for the EIA.

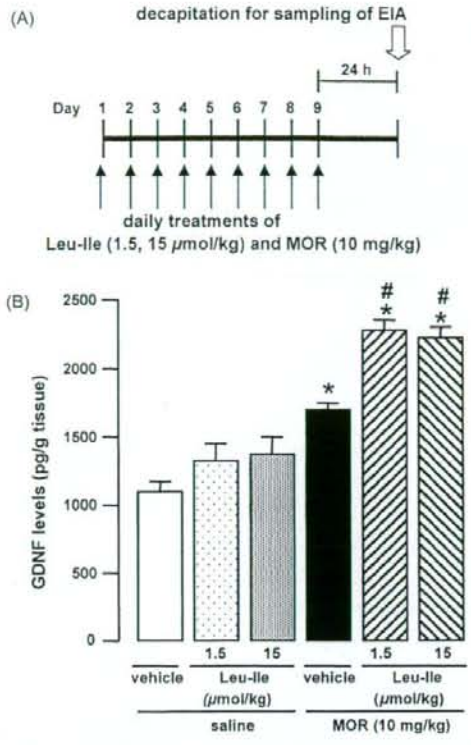

Fig. 1. Effect of Leu-Ile on morphine (MOR)-induced increase in glial cell line-derived neurotrophic factor (GDNF) levels. (A) Experimental schedule for measurement of GDNF levels using the enzyme immunoassay (EIA) method. Mice were treated with Leu-Ile (1.5 and 15 μmol/kg, i.p.) or vehicle 1 h before MOR (10 mg/kg, s.c.) or saline once a day for 9 days and decapitated 24 h after the last MOR or saline administration. (B) Change of GDNF levels in the nucleus accumbens after the administration of Leu-Ile and/or MOR. Values are mean ± S.E. ($n = 6$–8). $^{*}p < 0.05$ vs. vehicle/saline-treated mice. $^{\#}p < 0.05$ vs. vehicle/MOR-treated mice.

2.5. Conditioned place preference

The apparatus used for the place conditioning task consisted of two compartments: a transparent Plexiglas box and a black Plexiglas box (both 15 cm × 15 cm × 15 cm high). To enable mice to distinguish easily the two compartments, the floors of the transparent and black boxes were covered with white plastic mesh and black frosting Plexiglas, respectively. Each box could be divided by a sliding door (10 cm × 15 cm high). The place conditioning paradigm was performed by using a previously established procedure [14,16]. The experimental schedule for the conditioned place preference (CPP) task is shown in Fig. 2A. In the pre-conditioning test, the sliding door was opened, and the mouse was allowed to move freely between both boxes for 15 min once a day for 3 days. On the third day of the pre-conditioning test, we measured the time that the mouse spent in the black and transparent boxes by using a Scanet SV-20 LD (Melquest Co., Ltd., Toyama, Japan). The box in which the mouse spent the most time was referred to as the "preferred side", and the other box as the "non-preferred side". Conditioning was performed during six successive days.

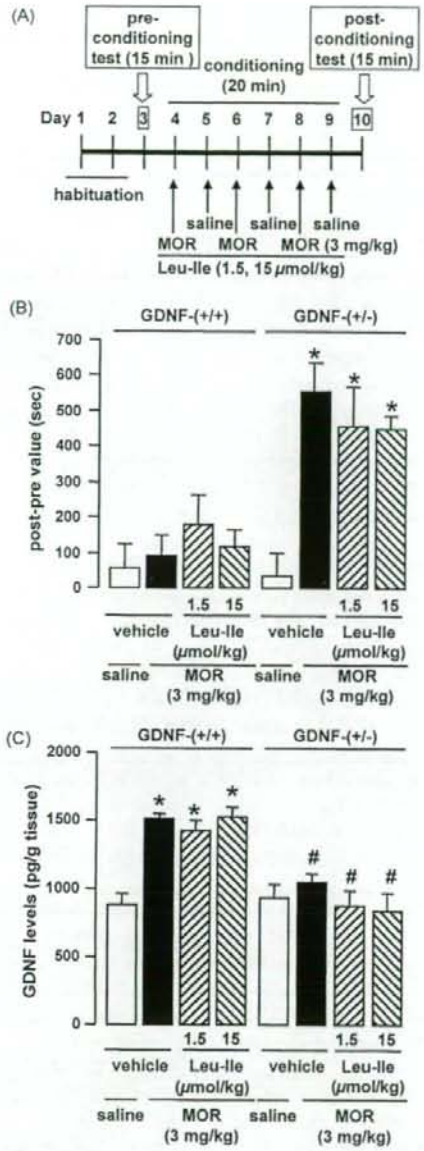

Fig. 2. Effect of Leu-Ile on MOR-induced place preference in GDNF-(+/−) mice. (A) Experimental schedule for the conditioned place preference task. On the third day of the pre-conditioning test, we measured the time that the mouse spent in the black and transparent boxes. Mice were subcutaneously given MOR (3 mg/kg, s.c.) and put in its non-preferred side for 20 min. On the next day, the mouse was given saline and placed opposite the drug conditioning site for 20 min. These treatments were repeated for three cycles (6 days). In the post-conditioning test, the sliding door was opened, and we measured the time that the mouse spent in the black and transparent boxes for 15 min. Closed arrows indicate the days of MOR or saline administration. Mice were treated with Leu-Ile (1.5 and 15 μmol/kg, i.p.) or vehicle 1 h before MOR (3 mg/kg, s.c.) or saline administration. (B) Effect of Leu-Ile treatment on MOR-induced place preference in GDNF-(+/−) mice. Mice were co-treated with Leu-Ile and MOR in the conditioning phase. Mice were treated with Leu-Ile (1.5 and 15 μmol/kg, i.p.) 1 h before MOR (3 mg/kg, s.c.) or saline administration. Values are means ± S.E. (n = 10–14). $^{*}p<0.05$ vs. vehicle/MOR-treated GDNF-(+/+) mice. (C) Change of GDNF levels in the NAc after post-conditioning test in conditioned place preference paradigm. Mice were co-treated with Leu-Ile (1.5 and 15 μmol/kg, i.p.) and MOR (3 mg/kg, s.c.) in the conditioning period and decapitated 24 h after post-conditioning test. Values are means ± S.E. (n = 5). $^{*}p<0.05$ vs. vehicle/saline-treated GDNF-(+/+) mice. $^{\#}p<0.05$ vs. vehicle/MOR-treated GDNF-(+/+) mice. Abbreviations as in Fig. 1.

Mice were given MOR or saline in the apparatus with the sliding door closed. That is, a mouse was subcutaneously given MOR and put in its non-preferred side for 20 min. On the next day, the mouse was given saline and placed opposite the drug conditioning site for 20 min. These treatments were repeated for three cycles (6 days). In the post-conditioning test, the sliding door was opened, and we measured the time that the mouse spent in the black and transparent boxes for 15 min, using the Scanet SV-20 LD. Place conditioning behavior was expressed by post–pre, which was calculated as: [(post-value) − (pre-value)], where post- and pre-values were the difference in time spent at the drug conditioning and the saline conditioning sites in the post-conditioning and pre-conditioning tests, respectively.

2.6. Statistical analysis

All data were expressed as means ± S.E. Statistical differences among more than three groups were determined using a one-way analysis of variance (ANOVA), followed by the Bonferroni multiple comparison test. $p<0.05$ was regarded as statistically significant.

3. Results

3.1. Effect of Leu-Ile on MOR-induced increase in GDNF levels

Single MOR treatment at the dose of 10 mg/kg increases locomotor activity, and repeated administration for 9 days results in an enhancement of the locomotor-stimulating effect of MOR (sensitization) [14]. Leu-Ile (1.5 and 15 μmol/kg, i.p.) inhibits the MOR-induced hyperlocomotion and sensitization [14]. The sensitization to the locomotor-stimulating effects is argued to reflect one neuroadaptive process associated with dependence. To confirm the involvement of GDNF in the inhibitory effects of Leu-Ile on MOR-induced sensitization, GDNF levels in the NAc were determined after the co-administration of Leu-Ile and MOR using the EIA method. MOR (10 mg/kg) increased GDNF levels in the NAc compared with those in the vehicle/saline-treated mice. GDNF levels after the co-administration of Leu-Ile (1.5 and 15 μmol/kg, i.p.) and MOR (10 mg/kg) were much more increased compared with those in the vehicle/MOR-treated mice ($F_{(5,38)}=28.1$, $p<0.05$, one-way ANOVA) (Fig. 1B). These results suggest that GDNF is involved in the effects of Leu-Ile on the sensitization.

3.2. Effect of Leu-Ile on MOR-induced place preference in GDNF-(+/−) mice

We have investigated the effects of Leu-Ile on the rewarding effects of MOR in the CPP paradigm, in which animals learn the association of an environment paired with drug exposure. Therefore, CPP is considered a measure of the rewarding properties of drugs of abuse. Leu-Ile (1.5 μmol/kg, i.p.) inhibits

MOR-induced place preference in C57BL/6J mice [14]. The involvement of GDNF in the rewarding effects of MOR and the inhibitory effects of Leu-Ile on MOR-induced place preference were examined in GDNF-(+/−) mice. The experimental schedule is described in Fig. 2A. As shown in Fig. 2B, at the low dose of MOR (3 mg/kg, s.c.), GDNF-(+/−) mice developed place preference, although littermate control GDNF-(+/+) mice did not ($F_{(7,86)} = 7.6, p < 0.05$, one-way ANOVA). When Leu-Ile (1.5 and 15 μmol/kg, i.p.) was administered 1 h before MOR, it failed to exhibit a significant effect on the action of MOR in GDNF-(+/−) mice (Fig. 2B). We measured the GDNF levels in the NAc after CPP test using EIA methods. As shown in Fig. 2C, in the NAc of GDNF-(+/+) mice, administration of MOR (3 mg/kg, s.c.) during conditioning phase in CPP test increased GDNF levels compared with vehicle/saline-treated mice ($F_{(7,32)} = 12.1$, $p < 0.05$, one-way ANOVA). Moreover, Leu-Ile increased GDNF levels in combination with MOR (3 mg/kg, s.c.) in CPP paradigm compared with vehicle/saline-treated GDNF-(+/+) mice (Fig. 2C). Conversely, in the NAc of GDNF-(+/−) mice, we confirmed that administration of MOR (3 mg/kg, s.c.) during conditioning phase in CPP test, which could develop place preference, failed to increase GDNF levels. Moreover, Leu-Ile, which could not inhibit rewarding effects of MOR, also failed to increase GDNF levels in combination with MOR in CPP paradigm (Fig. 2C). These results suggest that GDNF acts to negate the rewarding effects of MOR and is involved in the effects of Leu-Ile on the rewarding effects.

4. Discussion

GDNF enhances the survival and maintains the differentiated properties of dopaminergic neurons in cell cultures. The use of GDNF appears to be a promising strategy to promote the survival and function of the nigrostriatal dopaminergic pathway damaged in Parkinson's disease [6]. Transplantation of simian virus-40 glial cells, which produces and secretes GDNF, or delivery of GDNF-conjugated nanoparticles into dorsal and ventral striatum impairs the acquisition of cocaine self-administration in rats [3,4]. The upregulation of the GDNF pathway in the midbrain, is the molecular mechanism by which the putative anti-addiction drug ibogaine mediates its desirable action of reducing ethanol consumption [5]. Infusion of GDNF into the VTA blocks certain biochemical adaptations (induction of TH, NR1 subunit of *N*-methyl D-aspartate receptors, ΔFosB and protein kinase A catalytic subunit) to chronic cocaine or MOR treatment as well as cocaine-induced place preference [10]. Conversely, responses to cocaine are enhanced in rats by intra-VTA infusion of anti-GDNF antibody and in GDNF-(+/−) mice [10]. GDNF has pronounced effects on the dopaminergic system *in vivo*, including neuroprotective effects against METH-induced neurotoxicity [20]. However, as said at the beginning of this article, GDNF cannot be used directly as a therapeutic tool for drug dependence.

Recently, we have demonstrated that Leu-Ile, which induces the expression of TNF-α and GDNF, inhibits METH and MOR-induced sensitization and rewarding effects [14,15]. In the present study, to extend our findings, we examined the involvement of GDNF in the inhibitory effects of Leu-Ile on MOR-induced sensitization and rewarding effects.

GDNF levels in the striatum are increased by the intracerebroventricular administration of Leu-Ile in rats [13]. Expression levels of GDNF mRNA are significantly elevated 24 h after Leu-Ile treatment in cultured neurons compared with the control group and GDNF levels after the co-administration of Leu-Ile and METH are significantly increased compared with those in the vehicle/METH-treated mice [15]. Leu-Ile inhibits the MOR-induced locomotor sensitization, at least in part, through the action in the NAc, since it has inhibitory effects on the repeated MOR treatment-induced increase in extracellular DA levels [14]. In the present study, GDNF levels in the NAc were determined after the co-administration of Leu-Ile and MOR using the EIA method. Leu-Ile potentiated MOR-induced increase in GDNF levels (Fig. 1) in addition to TNF-α [14] in the NAc. GDNF inhibits the drug-induced upregulation of tyrosine hydroxylase activity [10]. TNF-α activates plasmalemmal and vesicular DA transporter [11]. Thereby, we suggest that GDNF and TNF-α induced by Leu-Ile attenuate the MOR-induced increase in extracellular DA levels in the NAc and then inhibit MOR-induced sensitization. In addition, Leu-Ile treatment in combination with METH or MOR and after withdrawal from repeated treatment with METH or MOR inhibits place preference and sensitization to METH or MOR [14,15]. GDNF acts to negate the rewarding effects of MOR, since GDNF-(+/−) mice showed greater MOR-induced place preference compared with littermate control mice (Fig. 2A and B). GDNF could be involved in the inhibitory effects of Leu-Ile on the rewarding effects of MOR, since no effects of Leu-Ile were observed in the GDNF-(+/−) mice (Fig. 2A and B) and Leu-Ile failed to increase GDNF levels in combination with MOR in CPP paradigm in the NAc of GDNF-(+/−) mice (Fig. 2C). GDNF blocks the biochemical and behavioral responses to chronic cocaine or MOR exposure [10]. GDNF decreases TH levels in normal animals, suggesting an active down-regulation of the synthesis of this enzyme [9]. These results suggest that Leu-Ile plays an inhibitory role in the rewarding effects and sensitization induced by MOR in addition to METH *via* the induction of GDNF expression.

Our previous findings indicated that Leu-Ile inhibits MOR-induced sensitization and rewarding effects by attenuating the MOR-induced increase in extracellular DA levels *via* the induction of TNF-α expression [14]. In the present study, we demonstrated that GDNF is also involved in the inhibitory effects of Leu-Ile on MOR-induced sensitization and rewarding effects. Taken together, Leu-Ile inhibits MOR-induced sensitization and rewarding effects *via* the induction of not only TNF-α, but also GDNF, expression. Leu-Ile could be a novel therapeutic agent for MOR-induced dependence.

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